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THESE

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Etude moléculaire du recrutement du facteur de transcription/réparation TFIIH sur les sites d'ADN endommagé

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Résume de la thèse de doctorat

Etude moléculaire du recrutement du facteur de transcription/réparation TFIIH sur les sites d'ADN endommagé

Le génome eucaryote est constamment soumis à l'effet de différents agents endommageant l'ADN. Il existe plusieurs voies de réparation de l'ADN qui protègent les cellules de la mutagenèse induite par ces agents. Une de ces voies est le mécanisme de réparation par excision de nucléotides (NER). La NER élimine une grande variété de lésions de l'ADN, y compris les dimères de cyclobutane pyrimidine (CPD) et les photoproduits 6-4 pyrimidinepyrimidone (6-4PP), tout deux produits par les rayons ultraviolets solaires. La NER peut emprunter deux sous voies pour réparer une lésion. Tout d'abord, la réparation globale du génome (GGR), qui élimine les dommages de l'ADN présents dans l'ensemble du génome. Ensuite, la réparation couplée à la transcription (TCR), qui corrige les lésions situées sur des gènes activement transcrits (Hanawalt, 2002). Le modèle général de NER comprend la détection de la lésion soit par la protéine XPC en GGR ou par l'ARN polymérase II, CSA et CSB en TCR. Ensuite, les deux voies utilisent un processus commun qui commence par l'ouverture de l'ADN par le facteur de réparation et de transcription TFIIH qui comprend les protéines XPB et XPD pourvues de domaines ATPase et hélicase (Zurita et Merino, 2003). Ensuite, les facteurs XPA et RPA sont recrutés au complexe de réparation, et l'ouverture de l'ADN est amplifiée autour de la lésion (Evans et al., 1997). Plus tard, les endonucléases XPG et XPF génèrent l'incision du simple brin d'ADN endommagé d'une longueur de 30 nucléotides (O'Donovan et al., 1994; Sijbers et al., 1996; Staresincic et al., 2009). La NER se termine lorsque l'ADN polymérase comble le trou dans l'ADN provoqué par l'excision du fragment endommagé (Shivji et al., 1995).

TFIIH est un complexe multiprotéique constitué de dix sous-unités. XPB/p89, XPD/p80, p62, p52, p44, p34, et TTD-A/p8 forment le cœur de TFIIH, alors que Cdk7, cyclin H et MAT1 forment le sous complexe CAK (Cycline-dependent kinase Activating Kinase). Le CAK et le cœur de TFIIH sont liés par l'hélicase XPD (Rossignol et al., 1999). TFIIH est impliqué dans l'initiation de la transcription des ARNm et des ARNr

(Gerard et al., 1991, Iben et al., 2002) et est également un facteur clé de la NER (Schaeffer et al., 1993).

Des mutations dans trois des dix sous-unités de TFIIH sont associées à plusieurs maladies génétiques (Lehmann, 2003). Des mutations dans l'hélicase XPB conduisent à la trichothiodystrophy (TTD) ou au xeroderma pigmentosum (XP) associé au syndrome de Cockayne (CS). Des mutations dans XPD sont associées au XP, à la TTD, au XP/CS, ainsi qu'au syndrome COFS (Cérébro-Oculo-Facio Skeletal) (Graham et al., 2001). Des mutations dans la sous-unité TTD-A/p8 sont associées à la TTD (Giglia-Mari et al., 2004).

Cdk7 (dans TFIIH) est la kinase responsable de la phosphorylation de la sérine 5 présente dans le domaine carboxyl-terminal de l'ARN polymérase II. Plusieurs facteurs régulent l'activité de Cdk7. Parmi eux, la cycline H, MAT1, TFIIE, le médiateur (Svejstrup et al., 1996), XPD (Keriel et al., 2002) et des petits ARN nucléaires U1 (O'Gorman et al., 2005). La phosphorylation de la sérine 5 conduit au recrutement de facteurs de capping en 5' de l'ARN messager (Cho et al., 1997). Elle régule également l'échappée du promoteur en initiant la transition entre les étapes d'initiation et d'élongation de la transcription

TFIIH; un facteur à la composition dynamique

L'une des questions pertinentes concernant TFIIH est de savoir comment un seul complexe peut participer à des processus aussi divers que la transcription et la réparation de l'ADN. Un de mes projets fût d'étudier le rôle du sous complexe CAK de TFIIH dans la réparation par excision de nucléotides. Nous avons utilisé une technique d'immunoprécipitation de chromatine (ChIP) suivie de la détection des complexes protéiques par Western-Blot pour étudier la composition de TFIIH dans des cellules humaines au cours du temps, après une irradiation UV. Nous avons montré que le complexe CAK se dissociait du cœur de TFIIH après le recrutement de ce facteur dans le complexe de réparation sur l'ADN (Coin et al., 2008). Dans le même temps, le cœur TFIIH s'associe avec des facteurs NER spécifiques, y compris XPA. C'est l'association avec XPA qui catalyse le détachement du CAK du cœur TFIIH, et qui déclenche

l'incision/excision de l'oligonucléotide endommagé. Lorsque la réparation de l'ADN est terminée, nous montons que les facteurs de réparation sont libérés du cœur TFIIH, et que le CAK est de nouveau détecté au sein de TFIIH, sur la chromatine. Ces résultats montrent que la composition du facteur TFIIH est dynamique afin d'adapter l'engagement de ce complexe dans des processus cellulaires différents

Role des sous-unités XPB et XPD de TFIIH dans la NER

Deux des sous-unités de TFIIH, XPB et XPD, possèdent des motifs ATPase et hélicase. Les hélicases à ADN sont des protéines qui séparent les fragments double-brin d'ADN en utilisant l'hydrolyse des nucléotides triphosphate comme source d'énergie. Les hélicases ont sept motifs conservés et ouvrent l'ADN en de déplaçant de 3' en 5' ou de 5' en 3' en fonction de leur spécificité (Tuteja et Tuteja, 2004). XPB(3'-5') et XPD(5'-3') sont deux hélicases de sens opposés. Les modèles prédisent donc que la fonction de XPB et XPD dans la NER serait d'ouvrir l'ADN endommagé en 5' pour XPB et en 3' pour XPD, par rapport à la lésion (Bootsma et Hoeijmakers, 1993). Les études génétiques confirment ces modèles puisque des mutations dans le site de liaison de l'ATP dans ces protéines inhibent la NER *in vitro* et *in vivo* en raison d'un déficit d'ouverture de l'ADN endommagé (Coin et al., 2006). Cependant, ces études portaient sur le motif I responsable de l'activité ATPasique et ne s'étaient pas étendues à d'autres motifs (II à VI), responsables de l'activité hélicase à proprement parler. Nous avons donc introduit des mutations dans les motifs III et VI de XPB qui inhibaient spécifiquement son activité hélicase, tout en laissant intact son activité ATPase. En étudiant l'effet de ces mutations sur les activités de TFIIH, nous avons constaté que l'ouverture de l'ADN autour de la lésion ne nécessitait pas l'activité hélicase de XPB et était uniquement dépendante de son activité ATPase. Contrairement à XPB, l'ouverture de l'ADN autour de la lésion nécessite l'activité hélicase de XPD (Coin et al., 2007). Enfin, nous avons démontré que la sous-unité p52 de TFIIH régulait positivement l'activité ATPase de XPB via une interaction directe entre ces deux protéines. Cette interaction est altérée par des mutations retrouvées chez les patients XP-B, expliquant ainsi pourquoi leur TFIIH n'est pas en mesure de provoquer l'ouverture de l'ADN autour de la lésion, et sa réparation.

Sachant que l'activité ATPase d'XPB est nécessaire à la NER, mais pas son activité hélicase, nous nous sommes penchés sur le rôle de cette activité dans la NER. Pour ce faire, nous avons analysé le recrutement de TFIIH au site de l'ADN endommagé *in vivo* et avons constaté que le complexe n'était pas recruté sur ses sites en absence de l'activité ATPase d'XPB. A l'opposé, l'activité ATPase d'XPD n'est pas requise pour le recrutement de TFIIH sur les lésions (Oksenych et al. Soumis à EMBOj). Le recrutement de TFIIH aux sites endommagés de l'ADN ne dépend pas de l'activité helicase d'XPB, mais nécessite deux nouveaux domaines récemment identifiés; il s'agit d'un motif conservé de séquence R-E-D et d'un motif dit « Thumb like » du fait de sa forme en pouce (Fan et al., 2006). Ces motifs sont à la fois importants pour le recrutement de TFIIH à l'ADN endommagé en NER mais également aux promoteurs en transcription. Nous avons ensuite montré que les motifs R-E-D et Thumb permettaient à XPB de développer une activité ATPase optimum en présence d'ADN.

L'ensemble de ces résultats nous a permis de redéfinir le rôle des hélicases XPB et XPD dans la NER et de proposer un nouveau modèle où l'activité ATPase d'XPB permettait la stabilisation de TFIIH sur l'ADN endommagé alors que l'activité hélicase XPD permet l'ouverture de l'ADN autour de la lésion

La restauration de la transcription après inhibition due aux lésions; un phénomène complexe

Les modifications post-traductionnelles des histones qui composent la chromatine sont à l'origine de la régulation de nombreux processus cellulaire. Il est connu que la méthylation du résidu K79 de l'histone H3 (H3K79) est important pour la réponse à l'irradiation UV et la survie des cellules de levure *Saccharomyces cerevisiae* (Game et al., 2005; Evans et al., 2008). Cette méthylation est assurée par l'histone méthyltransferase DOT1 (Feng et al., 2002), initialement décrit comme inhibiteur du silencing télomérique (Singer et al., 1998). Toutefois, il n'existe pas d'informations claires sur le mécanisme par lequel DOT1 et la méthylation de l'histone H3 K79 sont importants pour la survie des cellules après UV.

Récemment, j'ai mis l'accent sur le rôle des orthologues de DOT1 chez les mammifères (DOT1L chez l'humain et mDOT1 chez la souris) dans la réponse à

l'irradiation UV. Tout d'abord, nous avons observé le recrutement de cette histone méthyltransferase localement dans les noyaux de cellules irradiées. Ensuite, nous avons montré que mDOT1 était importante pour le retour de la transcription après une attaque génotoxique ainsi que pour la trans-activation de gènes UV-induits. L'utilisation de la technique de ChIP nous a permis de découvrir qu'en l'absence de DOT1 la machinerie de transcription de base ne se trouve plus présent sur le promoteur des gènes actifs après irradiation UV (Oksenych et al., En préparation). Au total, ces données nous conduisent à penser que le recrutement de DOT1 et la méthylation de l'histone H3 K79 sont importantes pour relancer le processus de transcription inhibée par des agents génotoxiques

Conclusion

En conclusion générale, mon travail a contribué à la compréhension du mécanisme de réparation par excision de nucléotides, ainsi que des phénotypes de patients XP-B. Nous avons fourni de nouvelles données qui révèlent sous un nouveau jour le processus de recrutement de TFIIH aux sites d'ADN lésé. Nous avons montré, qu'une fois recrutée, la composition du facteur TFIIH changeais, rompant ainsi avec le dogme d'un facteur à la composition statique et unique. Enfin, nous avons initié un travail qui va permet d'élargir nos connaissances concernant le lien entre la chromatine et la restauration de la transcription inhibée par des agents génotoxiques

Références

Bootsma D., and Hoeijmakers JH. (1993). DNA repair. Engagement with transcription. Nature *363*, 114-5

Cho EJ., Takagi T., Moore CR., and Buratowski S. (1997). mRNA capping enzyme is recruited to the transcription complex by phosphorylation of the RNA polymerase II carboxi-terminal domain. Genes Dev. *11*, 3319-26

Coin F., Proietti di Santis L., Nardo T., Zlobinskaya O., Stefanini M., and Egly JM. (2006). p8/ TTD-A as a repair-specific TFIIH subunit. Mol Cell. *21*, 215-26

Coin F., Oksenych V., and Egly JM. (2007). Distinct roles for the XPB/p52 and XPD/p44 subcomplexes of TFIIH in damaged DNA opening during nucleotide excision repair. Mol Cell. *26*, 245-56

Coin F., Oksenych V., Mocquet V., Groh S., Blattner C., and Egly JM. (2008). Nucleotide excision repair driven by the dissociation of CAK from TFIIH. Mol Cell. *31*, 9-20

Evans E., Moggs JG., Hwang JR., Egly JM., and Wood RD. (1997). Mechanism of open complex and dual incision formation by human nucleotide excision repair factors. EMBO J. *16*, 6559-73

Evans ML., Bostelman LJ., Albrecht AM., Keller AM., Strande NT., and Thompson JS. (2008). UV sensitive mutations in histone H3 in *Saccharomyces cerevisiae* that alter specific K79 methylation states genetically act through distinct DNA repair pathways. Curr Genet. *53*, 259-74

Fan L., Arvai AS., Cooper PK., Iwai S., Hanaoka F., and Tainer JA. (2006). Conserved XPB core structure and motifs for DNA unwinding: implications for pathway selection of transcription or excision repair. Mol Cell. *22*, 27-37

Feng Q., Wang H., Ng HH., Erdjument-Bromage H., Tempst P., Struhl K., and Zhang Y. (2002). Methylation of H3-lysine 79 is mediated by a new family of HMTases without a SET domain. Curr Biol. *12*, 1052-8

Game JC., Williamson MS., and Baccari C. (2005). X-ray survival characteristics and genetic analysis for nine Saccharomyces deletion mutants that show altered radiation sensitivity. Genetics *169*, 51-63

Gerard M., Fischer L., Moncollin V., Chipoulet JM., Chambon P., and Egly JM. (1991). Purification and interaction properties of the human RNA polymerase B(II) general transcription factor BTF2. J Biol Chem. *266*, 20940-5

Giglia-Mari G., Coin F., Ranish JA., Hoogstraten D., Theil A., Wijgers N., Jaspers NG., Raams A., Argentini M., van der Shpek PJ., Botta E., Stefanini M., Egly JM., Aebersold R., Hoeijmakers JH., and Vermeulen W. (2004). A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A. Nat Genet. *36*, 714-9

Graham JM. Jr., Anyane-Yeboa K., Raams A., Appeldoorn E., Kleijer WJ., Garritsen VH., Busch D., Edersheim TG., and Jaspers NG. (2001). Cerebro-oculo-facio-skeletal syndrome with a nucleotide excision-repair defect and a mutated XPD gene, with prenatal diagnosis in a triplet pregnancy. Am J Hum Genet. *69*, 291-300

Hanawalt PC. (2002). Subpathways of nucleotide excision repair and their regulation. Oncogene *58*, 8949-56

Iben S., Tschochner H., Bier M., Hoogstraten D., Hozak P., Egly JM, and Grummt I. (2002). TFIIH plays an essential role in RNA polymerase I transcription. Cell *109*, 297-306

Keriel A., Stary A., Sarasin A., Rochette-Egly C., and Egly JM. (2002). XPD mutations prevent TFIIH-dependent transactivation by nuclear receptors and phosphorylation of RARalpha. Cell *109*, 125-35

Lehmann AR. (2003). DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy. Biochimie *85*, 1101-11

O'Donovan A., Davies AA., Moggs JG., West SC., and Wood RD. (1994). XPG endonuclease makes the 3' incision in human nucleotide excision repair. Nature *371*, 432-5

O'Gorman W., Thomas B., Kwek KY., Furger A., and Akoulitchev A. (2005). Analysis of U1 small nuclear RNA interaction with cyclin H. J Biol Chem. *280*, 36920-5

Oksenych V., Bernardes de Jesus B., Zhovmer A., Egly JM., and Coin F. (Submitted). Molecular insights into the recruitment of TFIIH to sites of DNA damage.

Rossignol M., Kolb-Cheynel I., and Egly JM. (1997). Substrate specificity of the cdk-activating kinase (CAK) is altered upon association with TFIIH. EMBO J. *16*, 1628-37

Shaeffer L., Roy R., Humbert S., Moncollin V., Vermeulen W., Hoeijmakers JH., Chambon P., and Egly JM. (1993). DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor. Science *260*, 58-63

Shivji MK., Podust VN., Hubscher U., and Wood RD. (1995). Nucleotide excision repair DNA synthesis by DNA polymerase epsilon in the presence of PCNA, RFC, and RPA. Biochemistry *34*, 5011-7

Sijbers AM., de Laat WL., Ariza RR., Biggerstaff M., Wei YF., Moggs JG., Carter KC., Shell BK., Evans E., de Jong MC., Rademakers S., de Rooij J., Jaspers NG., Hoeijmakers JH., and Wood RD. (1996). Xeroderma pigmentosum group F caused by a defect in a structure-specific DNA repair endonuclease. Cell *86*, 811-22

Singer MS., Kahana A., Wolf AJ., Meisinger LL., Peterson SE., Goggin C., Mahowald M., and Gottschling DE. (1998). Identification of high-copy disruptors of telomeric silencing in Saccharomyces cerevisiae. Genetics *150*, 613-32

Staresincic L., Fagbemi AF., Enzlin JH., Gourdin AM., Wijgers N., Dunand-Sauthier I., Giglia-Mari G., Clarkson SG., Vermeulen W., and Scharer OD. (2009). Coordination of dual incision and repair synthesis in human nucleotide excision repair. EMBO J. *28*, 1111-20

Svejstrup JQ., Vichi P., and Egly JM. (1996). The multiple roles of transcription/repair factor TFIIH. Trends Biochem Sc. *21*, 346-50

Tuteja N., and Tuteja R. (2004). Unraveling DNA helicases. Motif, structure, mechanism and function. Eur J Biochem. *271*, 1849-63

Zurita M., and Merino C. (2003). The transcriptional complexity of the TFIIH. Trends Genet *19*, 578-84

Abbreviations

6-4PP	Pyrimidine (6-4) pyrimidone photoproducts
9-1-1	Rad9-Rad1-Hus1
AAG	Alkyladenine DNA glycosylase
AP	Apurinic/apyrimidinic
APE	AP-endonuclease; apurinic/apyrimidinic endonuclease
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia and Rad3 related
ATRIP	ATR interacting protein
BER	Base excision repair
BRCA	Breast cancer protein
BTF2	Basal transcription factor 2
CAK	Cdk activating kinase
CDC	Cell division cycle
Cdk	Cyclin-dependent kinase
Chk	Checkpoint (protein)
CHO	Chinese hamster ovary
COFS	Cerebro-oculo-facio-skeletal syndrome
CPD	Cyclobutane-pyrimidine dimers
CS	Cockayne's Syndrome
CSN	COP9 signalosome (Constitutive photomorphogenic 9 signalosome)
CTD	C-terminal domain
DDB	DNA damage binding (protein)
DDR	DNA damage response
DNA-PK	DNA-dependent protein kinase
DOT1	Disruptor of telomere silencing 1
DSB	Double-strand breaks
ERCC	Excision repair cross-complementing rodent repair deficiency
FA	Fanconi anemia
FANC	Fanconi anemia complementation group
GFP	Green fluorescent protein
GGR	Global genome (DNA) repair

GHKL	Gyrase/Hsp90/histidine-kinase/MutL
GTF2H	General transcription factor IIH (polypeptides 1-5)
HAT	Histone acetyltransferase
HD	Helicase domain
HMGN	High-mobility group nucleosome-binding domain-containing protein
HR	Homologous recombination
HSP	Heat shock protein
IDL	Insertion/deletion loops
KMT4	Lysine methyltransferase 4
LIG	DNA ligase
LP	Long-patch (BER)
LPO	Lipids peroxidation
MAT1	Ménage à trois (Tfb3)
MDC	Mediator of DNA damage checkpoint
MLH	MutL homologues
MMR	Mismatch repair
MMS	Methyl methanesulfonate
Mre11	Member of the Rad52 epistasis group (protein 11)
MRN	Mre11/Rad50/NBS1 DNA damage repair complex
MSH	MutS homologues
MSI	Micro-satellite instability
Mut	"Mutator"
NBS	Nijmegen breakage syndrome
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NMR	Nuclear magnetic resonance
OGG	8-oxoguanine DNA N-glycosilase
ORF	Open reading frame
PARP	Poly (ADP-ribose) polymerase
PCNA	Proliferating cell nuclear antigen
PHD	Pleckstrin homology domain
PIKK	Phosphatidylinositol-3-kinase related kinases
Pleckstrin	Platelet and leukocyte C kinase substrate, and KSTR amino acids
PMS	Post-meiotic segregation (protein)

PNK	Polynucleotide kinase
PTB	Phosphotyrosine binding N-terminal domain
RFC	Replication factor C
RING	Really interesting new gene (a type of zinc finger motif)
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPA	Replication protein A
SAM	S-Adenosyl methionine
SCID	Severe combined immunodeficiency
ssDNA	single-strand DNA
SOD	Superoxide dismutase
SMA	Spinal muscular atrophies
SMC	Structural maintenance of chromosomes
SNP	Single nucleotide polymorphism
SP	Short-patch (BER)
SSB	Single-strand breaks
TBP	TATA-box binding protein
TCR	Transcription-coupled (DNA) repair
TFIIH	Transcription factor II H
Tfb	Transcription factor b
TLS	Trans-lesion synthesis
TTD	Trichothiodystrophy
UDG	Uracil DNA glycosylase
UV	Ultra violet (100nm – 400nm)
VSG	Variant surface glycoproteins
WRN	Werner syndrome protein
XAB	XPA-binding (protein)
XP	Xeroderma pigmentosum
XRCC	X-ray repair cross complementing

List of figures and tables

Success in the medicine of the second half of last (19th) century provided a hope of the better future. Human existence, which depends on the human nature, can be modified, if this nature would be modified. Human life went mad, and our ageing is a kind of disease, which has to be treated as any other. When ageing will be treated and became physiological, it will lead us to the real natural end, with its deep logical sence.

At this point of view, our life is not absurdity any more. It gets sence and goal, for which human must work. Only science can solve the question of human existence, and it has to get the widest field to work in this direction. I know that my ideas are quite hypothetical, but all the positive results are obtained through hypotheses. Younger forces will check and develop them.

"Study of human nature"

Ilya Ilyich Mechnikov (Élie Metchnikoff)

(1845, Kharkiv Province, Russian Empire – 1916, Paris, France)

Introduction

DNA repair

Living organisms are continuously exposed to damaging agents both from the environment and from endogenous metabolic processes, whose action results in modification of proteins, lipids, carbohydrates and nucleic acids. DNA modifications occur after radiation (ionizing radiation, X-rays, UV light), hydrolysis, exposure to reactive oxygen or nitrogen species, alkylating agents and lipid peroxidation products (Lindahl, 1993; Tudek, 2007). To protect the genome stability, there are several DNA repair pathways, organized in different sub-pathways. The cells can directly reverse the base modification process in the nucleotides, to use DNA demethylation, as well as excision of bases and nucleotides. Mismatch repair (MMR) pathway is used for excision of misincorporated bases in the newly replicated DNA strand. Excision of bulky damage from both DNA strands or from the transcribed strand is performed by nucleotide excision repair (NER) sub-pathways, global genome repair (GGR) and transcription-coupled repair (TCR), respectively. In addition, there can be excision of oxidized, methylated and misincorporated bases from DNA by base excision repair (BER). DNA strand breaks, both single strand breaks (SSB) and double strand breaks (DSB), have to be repaired as well. For the DSB repair there are two pathways, depending on the cell cycle. First, non-homologous end-joining (NHEJ) in the G1 phase, and, second, homologous recombination (HR), predominantly in the S phase, with the appearance of the sister chromatid template.

Despite the protection provided by these mechanisms some of the damage escapes repair. Unrepaired DNA damage may block replication and engage alternative DNA polymerases in the process of so-called translesion synthesis (TLS) to by-pass the lesion in an error-free or error-prone fashion. Damaged DNA leads to replication and transcription errors and in consequence to mutagenesis, aging and various diseases, including carcinogenesis and neurodegeneration.

DNA base modifications

DNA base modifications are formed by both exogenous (environmental) and endogenous factors, and the types of lesions produced are identical for both groups. Endogenous DNA damage occurs at a high frequency compared with exogenous-induced modifications (Jackson and Loeb, 2001). It has been estimated that the DNA damage from endogenous sources gives rise to 20000 lesions per mammalian cell per day. Most of these lesions are deaminations, spontaneous hydrolysis of the N-glycosidic bond, alkylations, and damage by reactive oxygen or nitrogen species and lipid peroxidation products (Lindahl, 1993; Drablos, 2004). Lesions are also caused by errors in the DNA metabolism, including the formation of SSB and DSB from the collapse of replication forks and the introduction of modified nucleic acid bases during DNA replication.

Deamination of DNA bases

DNA bases containing an exocyclic amino group, namely adenine (A), guanine (G), cytosine (C), and 5-methylcytosine (5-meC) are susceptible to spontaneous hydrolytic deamination to hypoxanthine (Hyx), xanthine (X), uracil (U), and thymine (T), respectively. Deamination occurs more frequently in single-stranded than in double-stranded DNA, where the amino groups are protected by participating in hydrogen bonds (Lindahl, 1993). Spontaneous deamination is rather slow, but it can be significantly accelerated *in vivo* by nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3) formed during inflammation, as well as by UV- or γ-irradiation (Kalvi et al. 2007).

Loss of DNA bases by N-glycosidic bond hydrolysis

The N-glycosidic bond between base and deoxyribose in DNA can be hydrolyzed spontaneously or by DNA N-glycosylases during removal of damaged or incorrect bases from DNA by BER. This process leads to formation of an apurinic/apyrimidinic (AP) site. Additionally, reactive oxygen species (ROS) and alkylating agents promote the release of bases, often by introducing lesions that destabilize the N-glycosidic bond (Lindahl, 1993; Guillet and Boitex, 2003). AP-sites are among the most frequent endogenous lesions found in DNA and about 10000 lesions are formed per human cell per day. Purines are lost at a rate 500 times higher than pyrimidines, and the depurination

rates of A and G are comparable (Loeb and Preston, 1986). AP-sites are highly damaging lesions, can block replication and are both cytotoxic and mutagenic (Loeb and Preston, 1986; Guillet and Boiteux, 2003). Unrepaired AP-sites may rearrange to generate single strand breaks (Lindahl, 1993).

Alkylation of DNA bases

Alkylating agents can react with 12 different positions of DNA bases, including all exocyclic oxygens and most of ring nitrogens, and can modify oxygen atoms in the phosphate groups of the sugar-phosphate backbone. Depending on the mode of action, alkylating agents are divided into two types: S_N1-type agents (N-methyl-N-nitrosourea, MNU) alkylate both oxigens and nitrogens in nucleic acids, and S_N2-type agents (methyl methanesulfonate, MMS) alkylate mainly nitrogens. Ring nitrogen atoms engaged in hydrogen bonding are almost non-reactive in double-stranded DNA, but can be more readily alkylated in single-stranded DNA or RNA (N3 of cytosine and N1 of adenine) (Drablos et al., 2004). The major product of DNA base methylation is N7-methylguanine, a rather non-mutagenic and non replication-blocking lesion. Hovewer, destabilization of the N-glycosidic bond due to N7-substitution of guanine results in the formation of AP sites or imidazole ring opening to yield very mutagenic lesion 7me-FapyG (Tudek et al., 1992). The second most common DNA base methylation is N3-methyladenine (3-meA), which is a potent replication-blocking lesion and is perhaps the most toxic adduct produced by alkylating agents, resulting in TP53 induction, S-phase arrest, chromosomal aberrations and apoptosis (Engelward et al., 1998). In contrast to the limited miscoding potential of N-purines, O^6-methylguanine (O^6-meG) and, to a lesser extent, O^4-methylthymine (O^4-meT) are major contributors to mutagenicity induced by alkylating agents. Endogenous factors may alkylate DNA bases, and among them the best known is S-Adenosylmethionine (SAM) (Rydberg and Lindahl, 1982). SAM is a methyl donor in many biochemical reactions. It participates in enzymatic methylation of DNA cytosines at C5 position, which regulates gene expression in eukaryotes.

Oxidation-induced DNA damage

Reactive oxygen species (ROS), together with reactive nitrogen species (RNS) are known to induce both deleterious and beneficial effects. They can be induced by exogenous or environmental factors such as UV light, X-rays or γ-rays (which produce hydroxyl radical $^{*}OH$ by radiolysis of water), xenobiotics, cigarette smoke. Endogenously, they are formed as by-products of the respiratory electron transport chain, cytochrome P450 and xanthine oxidase metabolism, by microsomes and peroxisomes, and are produced by neutrophils, eosinophils and macrophages during inflammation and in various metal-catalyzed reactions (Valko et al., 2006). Aerobically growing cells depend on energy formed by reduction of atmospheric oxygen to water by the respiratory electron transport chain (Babcock and Wikstrom, 1992). The main product of mitochondrial respiration is superoxid anione radical ($O2^{*-}$), which shows limited reactivity, but upon escape from the respiratory electron transport chain induces side effects by further conversion to H_2O_2 by superoxide dismutase (SOD), and then to hydroxyl radical *OH. The reactivity of this radical is so high that it can diffuse no further than one or two molecular diameters before reacting with a cellular component. So, it must be generated close to the DNA molecule to be able to oxidize it (Michiels et al., 1994).

Oxidation is the major contributor to baseline DNA damage. The production of oxidized bases is about 10000 lesions per cell per day. Nearly 100 different free radical DNA modifications have been identified. They are classified as base- or deoxyribose lesions, strand breaks and cross-links (Halliwell and Aruoma, 1991; Dizdaroglu, 1992). Free radicals attack thymine at two principal sites, the 5,6-double bond and 5-methyl group. The following oxidized thymines have been detected in DNA: thymine glycole (Tg), 5,6-dihydrothymine (diHT), 5-hydroxy-5,6-dihydrothymine (5-OH-diHT) and 6-hydroxy-5,6-dihydrothymine (6-OH-diHT), 5-hydroxy-5-methylhydantoin (hmh), and others. Cytosine is oxidized at the 5,6-double bond, which results in changing the planar aromatic ring structure into a non-planar non-aromatic structure, similarly as in the case of thymine. The main oxidative cytosine modifications found in DNA are 5,6-dihydroxy-5,6-dihydrocytosine (cytosine glycol, Cg), its deamination and dehydration products 5,6-dihydroxy-5,6-dihydrouracil (uracil glycol, Ug) and 5-hydroxycytosine (5-OH-C),

respectively. 5-hydroxyuracil (5-OH-U) is formed from Ug by dehydratation or from 5-OH-C by deamination, and others (Kreutzer and Essigman, 1998; Purmal et al., 1998).

Hydroxyl radicals react with Carbon 8 of guanine, yielding C8-OH adduct radical. The C8-OH is oxidized to 7,8-dihydro-8-oxodeoxyguanine (8-oxoG) or is reduced to 2,6-diamino-4-hydroxy-5-formamidopirymidine (FapyG). 8-oxoG and FapyG are the major oxidative damages of guanine, and 8-oxoG is regarded as the most abundant oxidative DNA damage. It is often used as the marker of cellular oxidative stress. Roughly 80 8-oxoG are generated per human cell per day (Lindahl, 1993; Halliwell, 1999).

Peroxinitrite and hydroxyl radicals can cause damage to DNA both by direct attack on the bases or sugar moieties or indirect, through cell membrane lipids peroxidation (LPO). LPO products interact with DNA resulting in generation of adducts to bases, abasic sites, single or double strand breaks and chromosomal aberrations. Polyunsaturated fatty acids (PUFA) are constituents of phospholipid membranes, with the most abundant linoleic and arachidonic acids. Attack of ROS and RNS on polyunsaturated fatty acids causes formation of radicals and breaking of double bonds, which leads to lipid molecules fragmentation with generation of aldehydes and epoxides. Lipid peroxidation compounds react with exocyclic DNA adducts, and indirectly are contributing to oxidative DNA damage (Burcham, 1998).

UV-induced DNA modifications

UV radiation induces two of the most abundant mutagenic and cytotoxic DNA lesions such as cyclobutane-pyrimidine dimers (CPD, Figure 1) and 6-4 photoproducts (6-4PP) with their Dewar isomers (Figure 2, reviewed in Sinha and Hader, 2002).

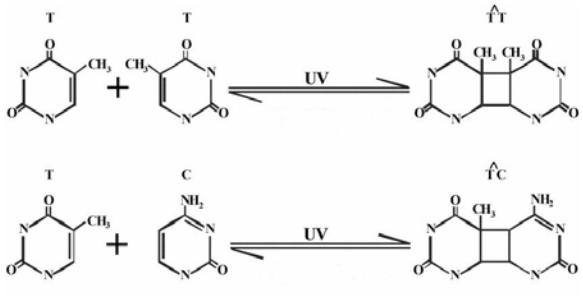

Figure 1. **Formation of the CPD lesion**.
At the top: thymine-thymine dimer. At the bottom: thymine-cytosine dimmer. (Sinha and Hader, 2001)

After UV irradiation, the CPD is the most abundant and most cytotoxic lesions; the 6-4PP may have more serios mutagenic effect. Dewar isomers are formed by the photoisomerization of 6-4PP by wavelengths longer than 290 nm

(Matsunaga et al., 1993). UV sources containing a higher proportion of radiation bordering between UV-B (280nm – 320nm) and UV-A (320nm – 380nm), such as solar UV irradiation, should produce a higher proportion of Dewar isomers since the photoisomerization is most efficient around 320nm, which corresponds to the UV absorption maximum of 6-4PP (UV-C corresponds to 100nm – 280nm and is widly used at the laboratory experiments). Consequently, it was suggested that all 6-4PP should be converted into Dewar isomers upon exposure to sunlight (Taylor et al., 1990). The CPD and 6-4PP make up around 75% and 25%, respectively, of the UV-induced DNA damage products. Both classes of lesions distort the DNA helix. CPD and 6-4PP induce a bend or kink of 7-9° and 44°, respectively (Kim et al., 1995; Wang and Taylor, 1991). The ability of UV radiation to damage a given base is determined by the flexibility of the DNA; the nature of the bases plays a major role since the distribution of the dimeric photoproducts strongly depends on the pyrimidine bases involved. Sequences that facilitate bending and unwinding are favorable sites for damage formation. CPD forms at higher yields in single-stranded DNA and at the flexible end of poly(dA)-(dT) tracts, but not in their rigid center. CPD formation is less frequent when there is bending of the DNA towards the minor groove. One of the transcription factors having a direct effect on DNA damage formation and repair is the TATA-box binding protein (TBP). It induces the selective formation of 6-4PP in the TATA-box, where the DNA is bent, but CPD are formed at the edge of TATA-box and outside, where the DNA is not bent (Aboussekhra and Thoma, 1999). CPD have been reported to be formed preferentially at the major p53 mutational hotspot in UV-B induced mouse skin tumors. The biological effects of CPD have been extensively studied in

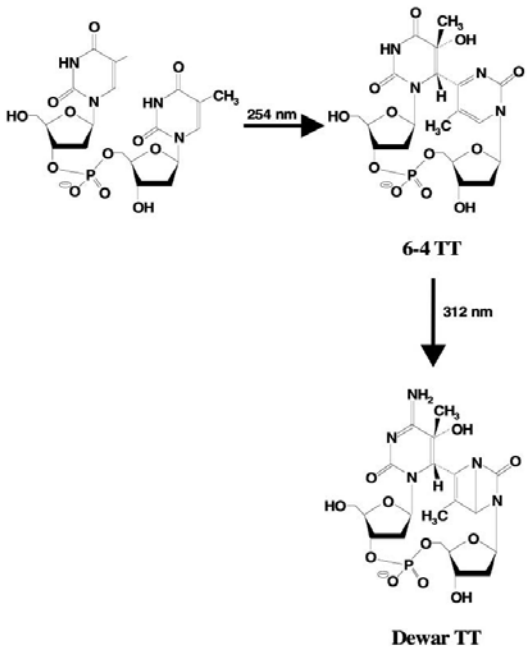

Figure 2. **Formation of UV-induced 6-4 photoproducts and Dewar isomers**.
Adapted from (Sinha and Hader, 2001)

microbes and mammals. CPD have been reported to inhibit the progress of DNA polymerases. Mammalian RNA polymerase II has been reported to stall at both CPD and 6-4PP. If unrepaired, a single CPD is sufficient to completely eliminate expression of a transcriptional unit (reviewed in Sinha and Hader, 2002). There is evidence that the stalled RNA polymerase II remains bond to the site of obstruction. Persisting lesion may thus not only reduce the overall concentration of free RNA polymerase but also eliminate transcription of the gene in which they are located. Every CPD acts as a block to transcription and replication, and only a small fraction of dimmers results in a mutation. Therefore, these DNA lesions may interfere with DNA transcription and replication and can lead to misreading of the genetic code causing mutations.

Base excision DNA repair (BER)

The base excision DNA repair pathway is responsible for removal of more that 10000 DNA lesions daily in each human cell. In addition, lesions targeted by the BER pathway are relatively small, causing little DNA helix distortion. Many of these lesions have been shown not to inhibit elongation by some DNA and RNA polymerases both *in vivo* and *in vitro* (Doetsch, 2002). BER is the major repair pathway involved in the removal of DNA damage involving structurally non-distorting and non-bulky lesions, oxidized or ring-saturated bases, alkylated and deaminated bases, as well as apurinic/apyrimidinic sites and mismatches (Lindahl et al., 1997). Proteins engaged in BER are conserved from bacteria to eukaryotes. BER is initiated by a damage-specific DNA N-glycosylase that recognizes and removes the modified or mismatched base by hydrolysis of the N-glycosidic bond between a 2'-deoxyribose and the base, or by non-enzymatic hydrolytic depurination leading to base loss, as well as by single strand break with ends other than 3'-OH and 5'-P (Figure 3).

DNA damage recognition by DNA N-glycosylases

Twelve genes with their splicing variants encoding various glycosylases have been found in mammalian cells. These proteins have different substrate specificities and modes of action. Glycosylases effectively ensure repair of the majority of endogenous and exogenous DNA base lesions. They often contain a conserved motif of helix-hairpin-

helix (HhH) in the active site, which enables them to bind DNA and an ion of metal. Four structural superfamilies of DNA glycosylases have been identified. UDG superfamily-1 is based on structural similarity to uracil DNA glycosylase UDG. Enzymes in this family are active against uracil in ssDNA and dsDNA, and recognize uracil explicity in an extrahelical conformation through a combination of protein and bound-water interactions. Some of these enzymes are mismatch-specific and explicity recognizes the widowed guanine on the complementary strand rather than the extrahelical scissile pyrimidine. AAG superfamily-2 is based on structural similarity to human alkyladenine DNA glycosylase AAG. Members of the UDG and AAG superfamilies are compact single-domain enzymes with relatively small DNA-interaction surface. MutM/Fpg superfamily-3 is based on structural similarity to bacterial 8-oxoguanine DNA glycosylase Fpg. All known members have the unique feature of using their N-terminal proline residue as the key catalytic nucleophile. HhH-GPG superfamily-4 is named for the characteristic active site borne by family members comprising a helix-hairpin-helix followed by a Glycine-Proline-rich loop and catalytic aspartate residue (Pearl, 2000; Fromme et al., 2004).

Glycosylases are generally divided into two types. First group: *E. coli* Tag, AlkA, UDG, Mug, MutY, and human ANPG, hUNG1, hUNG2, hSMUG1, hTDG, hUDG, MUTYH, and hMBD4 monofunctional DNA N-glycosylases that remove a deaminated, alkylated, or mismatched baseleaving an AP-site. Second group consists of *E. coli* Fpg, Nth, Nei, as well as human hOGG1, hOGG2, hNTH1, hNEIL1, hNEIL2 and hNEIL3 bifunctional DNA N-glycosylases/AP-lyases, that remove oxidized or ring-saturated bases and additionally to the glycosylase activity have a 3'-AP-lyase activity which incises the phosphodiester bond at the 3' side of the deoxyribose through β-elimination leaving a single strand break. *E. coli* Fpg and Nei or mammalian NEIL1 and NEIL2 bifunctional glycosylases additionally carry out δ-elimination reaction with removal of the deoxyribose residue end generation of 3'-phosphate termini.

Role of end processors in DNA repair

The AP sites or DNA ends generated after lesion excision or excision-incision by mono- or bifunctional glycosylases, respectively, are not suitable for the next repair steps. These repair intermediates are very mutagenic (Simonelli et al., 2005). DNA ends

containing modified 3' and 5' ends may arise because of direct chemical modification during SSB formation through the action of ROS (Demple and DeMott, 2002). Ionizing radiation is a major contributor to the formation of damaged 3' ends. Anti-tumor drugs, such as bleomycin and neocarzinostatin, can also generate SSB (Dedon and Goldberg, 1992). Blocked 3' ends in human cells may arise because of abortive DNA topoisomerase I (TOP1) activity (Leppard and Champoux, 2005).

Several enzymes are known to restore 3'-OH and 5'-P ends of broken DNA to allow gap filling and DNA ligation. DNA free ends processing is quite diverse enzymatic step due to variety of termini that can arise. AP-endonuclease (APE) is the main enzyme responsible for processing of the BER-intermediates. APE generates 3'-OH termini either by cleaving the phosphodiester bond at 5' side of the intact AP site by its 5'-AP-lyase activity, or by removal of 3'-P by its 3'-phosphoesterase and 3'-phosphatase activities. Mammalian APE1 (termed also APEX, HAP1, Ref-1) and APE2 have strong AP-lyase activity and 3'-phosphoesterase activities, but quite weak 3'-phosphatase activity. Xth, APE1 and APE2 possess 3'–>5' exonuclease activity. They can remove more that one nucleotide and be used as proofreading activities in BER (Hadi et al., 2002). 3'-phosphate can be also removed by mammalian polynucleotide kinase, PNK, which forms APE-independent BER pathway in the combination with NEIL1/NEIL2 glycosylases (Wiederhold et al., 2004). Human PNK is the major DNA 5'-kinase and 3'-phosphatase. It is able to phosphorylate the 5' end of SSB and removes blocking phosphate lesions from the 3' end (Dianov and Parsons, 2007). Human MN23/NDP kinase was identified as tumor suppressor, and is associated with tumor metastasis. Its reduced expression is related to an increased metastatic potential in most cancer cell types. NM23/NDK kinase was shown to activate transcription and to have a nuclease activity (Postel et al., 2000).

There are two alternative sub-pathways of BER: short-patch (SP) replaces one nucleotide, and long-patch (LP), which is replacing several nucleotides (often 6 to 13).

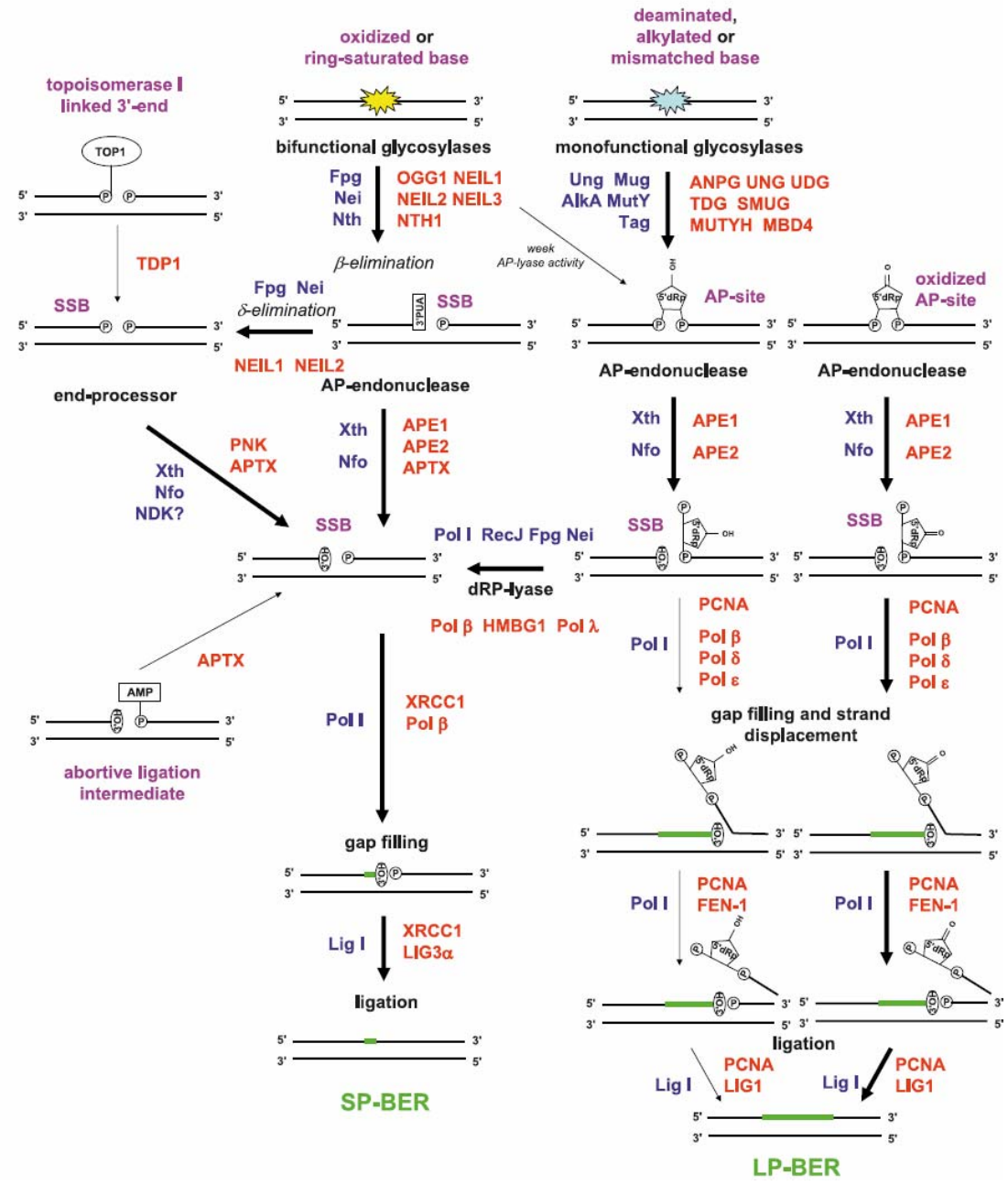

Figure 3. **Model for BER and SSBR subpathways**. **P**, phosphate; **OH**, hydroxyl group; **3' PUA**, 3' unsaturated aldehyde; **5' dRp**, 5' deoxyribose phosphate; **AMP**, adenylate group; **TOP1**, Topoisomerase I linked 3' end. *Escherichia coli* enzymes are on the left (blue), human enzymes are on the right (red). Adapted from Krwawicz et al. (2007).

Short-patch Base excision repair (SP-BER)

Oxidized and ring-saturated bases are recognized and removed from DNA by the bifunctional DNA N-glycosylases/AP-lyases. APE removes 3'-modifications, including 3'-phosphates (3'-P), leaving 3'-OH and 5'-P ends suitable for filling by mammalian

DNA polymerase β and for end-sealing by LIG3α (Figure 3). Alkylated and deaminated bases as well as some types of mismatches are recognized and removed from DNA by the monofunctional DNA N-glycosylases. In this process the N-glycosydic bond connecting the aberrant base to the sugar-phosphate backbone is cleaved and an AP site is created (Krokan et al., 1997). The AP site is recognized and processed by the APE that hydrolyzes the phosphodiester DNA backbone at the 5' side of the AP site, leaving 3'-OH and 5'-desoxyribosephosphate ends flanking the gap. The choice of the pathway depends on the ability of the enzymes to remove the 5'-sugar phosphate. In mammalian cells, both pathways are initiated by DNA polymerase β, which inserts one nucleotide into the repair gap. In SP-BER DNA polymerase β also removes 5'-desoxyribosephosphate (5'-dRP) by its 5'-dRPase activity, and DNA LIG3α-XRCC1 complex seals the ends. DNA polymerase λ may partially backup polymerase β, since it also has a 5'-dRPase activity. XRCC1 is a platform protein. It is interacting with DNA polymerase β, LIG3α, PNK, APE1, and PARP-1. The lesions removed by bifunctional DNA glycosylases are processed mainly by SP-BER, since the 3'-OH and 5'-P ends may be readily filled in by DNA polymerase β.

Long-patch Base excision repair (LP-BER)

In human cells modification of the 5'-dRP moiety by oxidation or reduction prevents its excision by DNA polymerase β, and the lesion is further processed by LP-BER (Figure 3). DNA polymerase β leaves the damaged place, and replication sliding clamp PCNA is recruited together with DNA polymerase δ or ε. The polymerase adds few nucleotides to the 3'-OH end and generates a flap containing the 5'-dRP, which is removed by FEN-1. The ends are sealed by DNA ligase I (LIG1). PCNA interacts not only with the polymerase, but also with FEN-1 and LIG1. Replication protein A, RPA, interacts with MUTYH and UNG2 glycosylases. It is required by DNA polymerases δ and ε for DNA synthesis, and may stimulate LP-BER. DNA polymerase δ requires also replication factor C (RF-C), which loans the PCNA on the DNA double helix. DNA polymerase ε is highly processive in the absence of PCNA (Sung and Demple, 2006).

In mammalian cells 8-oxoG is repaired through a 2-6 nucleotide patch, since the hOGG1 3'-AP-lyase activity is ten times lower than its N-glycosydase activity, and repair

proceeds through LP-BER (Sattler et al., 2003). APE1 was shown to increase human hOGG1 turnover on damaged DNA and stimulate its excision activity (Hill et al., 2001). BER is further complicated by other proteins. PARP-1 binds to DNA single strand breaks immediately after its formation, and dissociates after self-poly-ADP-ribosylation. PARP-1 has been proposed to prevent cleavage of the strand break ends by nucleases; It also stimulates LP-BER strand displacement synthesis by DNA polymerase β (Parsons et al., 2005). Werner syndrome protein, WRN, stimulates DNA synthesis and provides proofreading of 3'-mismatches through its 3'–>5' exonuclease activity (Harrigan et al., 2006). Cockayne syndrome group B (CSB) protein functions in the catalysis of 8-oxoG excision by BER and in the maintenance of efficient hOGG1 expression (Tuo et al., 2002).

Mismatch DNA repair (MMR)

Maintaining low mutation rates is essential for the cell stability. Natural isolates of *Escherichia coli* have been found to have elevated mutation rates and strains showing this phenotype are termed "mutators". Although the mutator phenotype may have some beneficial effects allowing better adaptation to environment conditions, it also generates many deleterious and lethal mutations (Funchain et al., 2000).

The first described *E. coli* mutator gene is mutT1, which specifically increases 100 to 100,000 folds the occurrence of AT-CG transversions. MutT pyrophosphohydrolase is specifically acting on 8-oxodGTP and preventing incorporation of this mutagenic substrate into DNA. Later, others *E. coli* mutators were described (MutM/Fpg, MutY, MutH, MutS, MutL, and MutU/UvrD as well as respectively weak mutators ung, sodA, dam, oxyR, and polA). Mutations may also appear as result of mutated tRNAs (mutA and mutC), and proofreading ε subunit of DNA polymerase III holoenzyme (mutD/dnaQ).

Counterparts of bacterial DNA repair proteins have been found in eukaryotic organisms, including humans. It was shown that DNA repair deficiency results in accumulation of DNA damage, which may lead to aging and development of human diseases, including cancer and neurological disorders (reviewed in Arczewska and Kusmierek, 2007).

Loss of MMR brings a mutator phenotype, which causes predisposition to cancer. MMR status also affects meiotic and mitotic recombination, DNA damage signaling, apoptosis and cell-type specific processes such as class-switch recombination, somatic hyper-mutation, and triplet-repeats expansion (reviewed in Jiricny, 2006).

Over the past decade, a significant proportion of cancers of the colon, endometrium and other organs were found to exhibit a phenotype known as microsatellite instability. Microsatellites are repeated-sequence motifs, such as $[A]_n$ or $[CA]_n$, which are present in our genome in large numbers. During DNA synthesis, the primer and template strands in a microsatellite can occasionally dissociate and re-anneal incorrectly (Kunkel, 1993). This gives rise to heteroduplex DNA molecules, in which the number of microsatellite-repeat units in the template and in the newly synthesized strand differs. These heterogeneities, in which the partnerless nucleotide is partially extrahelical, are known as insertion/deletion loops (IDL). Together with base-base mismatches, which are cased by errors of DNA polymerases that escape their proof-reading functions, IDL are addressed by the MMR system, which degrades the error-containing section of the newly synthesized strand and therefore provides the DNA polymerase with another chance to generate an error-free copy of the template sequence. In the absence of MMR, IDL and base-base mismatches remain uncorrected which results in a mutator phenotype that is accompanied by microsatellite instability and cancer.

Much of our understanding of mammalian MMR has come from the study of *Escherichia coli* and *Saccharomyces cerevisiae*. However, the importance of MMR malfunction for human disease can only be appreciated in mammalian systems. These studies revealed that MMR proteins are implicated in other DNA-metabolic pathways, ranging from DNA-damage signaling to recombinogenic and mutagenic processes that are limited to multicellular organisms.

The MMR machinery has to satisfy two criteria: it must efficiently recognize base-base mismatches insertion/deletion loops, and it must direct the repair machinery to the newly synthesized DNA strand, which carries the erroneous genetic information. How these tasks are fulfilled was first elucidated in *E. coli* with reconstitution of MMR system *in vitro* (Lahue et al., 1989). That system included purified MutH, MutL and MutS proteins, DNA helicase II, single-strand DNA binding protein, DNA polymerase III

holoenzyme, exonuclease I, DNA ligase, along with ATP (adenosine triphosphate) and the four deoxynucleoside triphosphates.

In *E. coli*, the recognition of biosynthetic errors is mediated by a MutS homodimer, which then recruits the homodimer MutL. The ATP-dependent formation of this ternary complex activates the endonuclease activity of MutH, which is bound to a hemi-methylated GATC site. These sites are normally methylated on adenines. However, because the modifying enzyme deoxyadenine methylase lags behind the replication fork by about two minutes, and the newly synthesized strand is transiently unmethylated. MutS-MutL-activated MutH uses this time window to incise the unmethylated strand. The UvrD helicase unwinds the ends of the nicked error-containing strand from the template. This enables one of several exonucleases to digest the unwound DNA, either in the 5'–>3' direction when the nearest hemi-methylated GATC site lies 5' from the mismatch, or in the 3'–>5' direction if it lies 3' from the mismatch. The exonucleolytic degradation stops once the mismatch was removed. After, DNA polymerase III fills the gap and the repair is completed when DNA ligase seales the remaining nick.

Although the MutS and MutL proteins are highly conserved, MutH is found only in Gram-negative bacteria and no functional homologue has been identified in other organisms. This led to the suggestion that the processing of mismatches, arising during replication, could be directed by strand breaks such as the 5' and 3' termini of Okazaki fragments in the lagging strand, or the 3' terminus of the leading strand. The MMR during recombination could be directed to the invading strand by its 3' terminus. This hypothesis was supported experimentally. *E. coli* strain without MutH process mismatches *in vivo* and *in vitro* (Lahue et al. 1989), the substrate contains the strand breaks in the vicinity to the mispair. Similar observations were made in the human MMR *in vitro* systems (Holmes et al., 1990; Fang et al., 1993).

Mismatch recognition in mammalian cells

In human cells (see Figure 4 for MMR), five MutS homologues (MSH) have been identified. MSH2, MSH3 and MSH6 participate in MMR in the form of heterodimers (Kunkel and Erie, 2005). The most abundant mismatch-binding factor is composed of MSH2 and MSH6. This factor, which is often referred to as MutSα, initiates the repair of

base-base mismatches and insertion/deletion loops, of one or two extrahelical nucleotides, whereas MutSβ, a heterodimer of MSH2 and MSH3, initiates the repair of larger IDL.

The partial redundancy between MutSα and MutSβ helps to explain the different tumor phenotypes of *Msh2*-, *Msh3*- and *Msh6*-null mice. As *Msh2*$^{-/-}$ animals lack all mismatch recognition functions, they have the most severe phenotype. The lack of MSH6

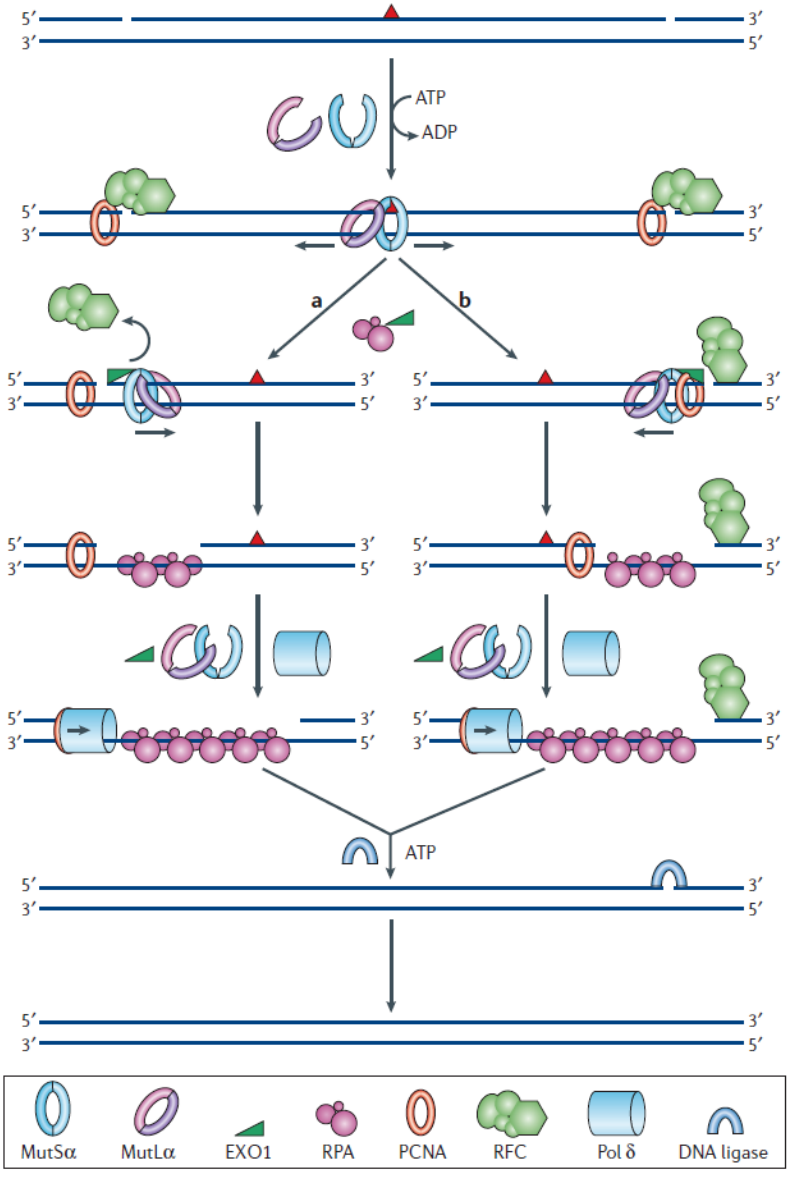

Figure 4. **The reconstituted human MMR system**.

(**a**) Clamps that diffuse upstream encounter RFC that is bound at the 5' terminus of the strand break, and will displace it and load EXO1. The activated exonuclease starts the degradation of the strand in a 5′–>3′ direction. The single-stranded gap is stabilized by RPA. When themismatch is removed, EXO1 activity is no longer stimulated by MutSα, and is actively inhibited by MutLα. Pol δ loads at the 3′ terminus of the original discontinuity, which carries a bound PCNA molecule. This complex fills the gap and DNA ligase I seals the remaining nick to complete the repair process.

(**b**) Clamps that migrate downstream encounter a PCNA molecule that is bound at the 3′ terminus of the strand break. The recruitment and the activation of EXO1 results in the degradation of the region between the original discontinuity and the mismatch, possibly through several iterative EXO1-loading events. RFC that is bound at the 5′ terminus of the discontinuity prevents degradation in the 5′–>3′ direction (away from the mismatch). Once the mismatch is removed and the EXO1 activity is inhibited by bound RPA and MutLα, the gap is filled by Pol δ. DNA ligase I seals the remaining nick to complete the repair process.

(Adapted from Jiricny, 2006)

leads to less severe phenotype, compare with *Msh2*$^{-/-}$ animals because MutSβ can deal with most IDL. *Msh3*$^{-/-}$ mice are not tumor-prone, presumably because MutSα can initiate repair of most replication errors.

MutL homologues

The MutL proteins are ATPases of the GHKL (gyrase/Hsp90/histidine-kinase/MutL) family, with the ATPase situated in the N-terminal domain and the dimerization domain at the C terminus. Human cells express four MutL homologous: MLH1, MLH3, PMS1 (post-meiotic segregation protein 1) and PMS2, which functions as three distinct heterodimers. The complex composed of MLH1 and PMS2-MutLα has the most important role in the MMR, as cells that lack either protein exhibit mutator phenotypes and micro-satellite instability (MSI) that is comparable to cells with mutated MSH2 (Marra and Jiricny, 2005).

$Mlh1^{-/-}$ and $Pms2^{-/-}$ mice do not have identical phenotypes. It was postulated that there must be a backup for PMS2 in DNA metabolism. MutLβ, composed of MLH1 and PMS1, could conceivably fulfill this function. Although this heterodimer could not be shown to participate to MMR *in vitro*, *Pms1*-knockdown mice exhibit MSI in mononucleotide runs (Raschle et al., 1999), so MutLβ might be involved in MMR to some extent. Similarly, MutLγ, which is composed of MLH1 and MLH3, might also have a backup role in mammalian MMR. *Mlh3*-knockout mice exhibit weak instability in mononucleotide microsatellites and are tumor prone. Recombinant MutLγ participates in the repair of base-base mismatches and single nucleotide IDL. The role of these factors differs from that of the yeast MutLγ, which was implicated in the repair of a subset of IDL (Flores-Rozas and Kolodner, 1998).

How do MutL homologues function in MMR? In *E. coli*, the ATP-activated mismatch-bound MutS sliding clamp was shown to first interact with a homodimer of MutL. This interaction is believed to modulate the ATP-hydrolysis-dependent turnover of the complex and/or its interaction with the MMR factors bound at the excision-initiation site, such as MutH and UvrD (reviewed in Kunkel and Erie, 2005). How this interaction is mediated – this is a subject of discussion. The first model indicates that the MutS-MutL complex forms at the mismatch and then translocates in either direction and helps to initiate the excision process on encountering MutH and UvrD. The second model indicates that MutS interacts with MutL at the site of repair initiation. The third one indicates that the MutS-MutL complex remains bound at the mismatch and interacts with

the MutH and UvrD proteins by looping out the intervening DNA. Evidence in support of these models is indirect. The MutS-MutL complex was shown to migrate during *E. coli* MMR *in vivo*, which argues against the third scenario (Smith et al., 2001).

Similar to the bacterial MutS and MutL proteins, human MutSα and MutLα can form relatively stable ATP-dependent ternary complexes on oligonucleotide substrates that have free ends. This evidence, coupled with the *in vitro* finding that mismatch-provoked degradation of the nicked strand can be initiated on substrates that contain physical barriers between the mismatch and the excision-initiation site, has led to the proposal that MutSα-MutLα complex remains bound at the mismatch and initiates the repair reaction by looping out the intervening DNA. However, surface-plasmon-resonance studies that have been carried out with the human and yeast MutSα-MutLα complexes provided evidence that this proteins travel along the DNA contour similarly to the MutSα sliding clamp.

None of the experimental systems deployed so far show where the ternary complex assembles. The propensity of the proteins to associate also with DNA ends complicates the interpretation of the results. The formation of the ternary complexes was mismatch independent and was very susceptible to changes in experimental conditions. The fact that the ternary complex possesses four ATP-binding sites on the MutL and MutS homologous further complicates data interpretation.

Exonucleases in MMR

Mismatch-dependent degradation of heteroduplex substrates can initiate at a strand break or gap that is either 5'–>3' from the mispair. It was anticipated that the MMR process would involve exonucleases of both 3'–>5' and 5'–>3' polarity. In agreement with this expectation, yeast genetic studies implicated the 3'–>5' proofreading activities of DNA polymerase δ and/or DNA polymerase ε in MMR. Recent findings indicated that the 3'–>5' exonuclease activity of MRE11 might also be required *in vivo*. As far as 5'–>3' exonucleases are concerned, the only enzyme with this directionality that has been implicated in MMR *in vivo* and *in vitro* is exonuclease-1 (EXO1, Tran et al., 2004). Unlike mice that lack MSH2 or MLH1, *Exo1*$^{-/-}$ mice are prone to lymphomas but not to gastrointestinal tumors. They are sterile, like *Mlh1*$^{-/-}$ animals, but the meiotic

defect seems to take place later. The extracts of embryonic stem cells are deficient in the repair of base-base mismatches and one-nucleotide IDL, but not of larger IDL. The mutator phenotype of the cells is weaker than that seen in MSH2- and MSH1-deficient cells and MSI is limited to mononucleotide repeats. Taken together, the above evidence indicates that EXO1 is involved in some, but not all, MMR-dependent events *in vivo*. It was therefore surprising to learn that a reconstituted MMR system requires only EXO1 for both 5'–>3' and 3'–>5' mismatch correction (Genschel et al., 2002).

The MRR system has received a considerable amount of attention during the past decade, primarily thanks to its link with hereditary non-polyposis colon cancer (HNPCC), one of the most common inherited cancer-predisposition syndromes. The initial investigations into MMR focused on its role in mutation avoidance. Although the MMR system could be reconstituted from eight recombinant proteins, there are undoubtedly other redundant or non-essential factors that participate in the process *in vivo*, and these remain to be identified.

It is now clear that the MMR system is multifaceted and that it participates in several different pathways of DNA metabolism, namely those that involve recombination. Given the importance of these processes in the maintenance of genomic stability, it would be of substantial interest to learn how MMR proteins affect the outcome of meiotic and mitotic recombination events. Most of our insights come from yeast studies, and it is desirable to understand these processes in mammalian cells.

The study of MutL homologous deserves particular attention, as the biological roles of these proteins remain enigmatic and as the malfunction of different heterodimeric combinations of MLH proteins gives rise to very diverse phenotypes, which range from genomic instability to sterility.

The involvement of MMR proteins in DNA-damage signaling also requires further study, as this process could have an important role in spontaneous cell transformation and cancer. Moreover, the MMR status affects, in some cases by several orders of magnitude, the response of cells to certain classes of therapeutics. One of the greatest challenges in modern molecular cancer research is to understand how one and the same system guards genomic stability on the one hand, while contributing to cell death on the other (reviewed in Jiricny, 2006).

Double-strand breaks repair

DNA double-strand breaks (DSB) are critical DNA modifications. They are obstacles for essential biological processes such as replication, transcription and chromosome segregation. A variety of different sources could lead to the formation of DSB (O'Driscoll and Jeggo, 2006; Franco et al., 2006). The different nature of these modifications influences both which processing factors are needed before DNA rejoining and which mechanism is used for the repair (O'Driscoll and Jeggo, 2006). The failure to repair DSB can lead to cell death or chromosome changes, including deletions, translocations, and chromosome fusions. Subsequent genome instability is a mark of cancer cells (Shrivastav et al., 2008; Su, 2006).

Mammalian cells are using two different DSB repair pathways, homologous recombination (HR) and non-homologous end-joining (NHEJ), as presented in Figure 5. They interact with a DNA damage response signaling pathway, initiated by the lesions. The relative contribution of HR and NHEJ pathways is still unclear; it depends on the cell cycle phase and the DNA damage acquirement. HR acts predominantly during the late S-phase and G2, while NHEJ acts during the others cell cycle phases (Shrivastav et al., 2008; Rothstein et al., 2000).

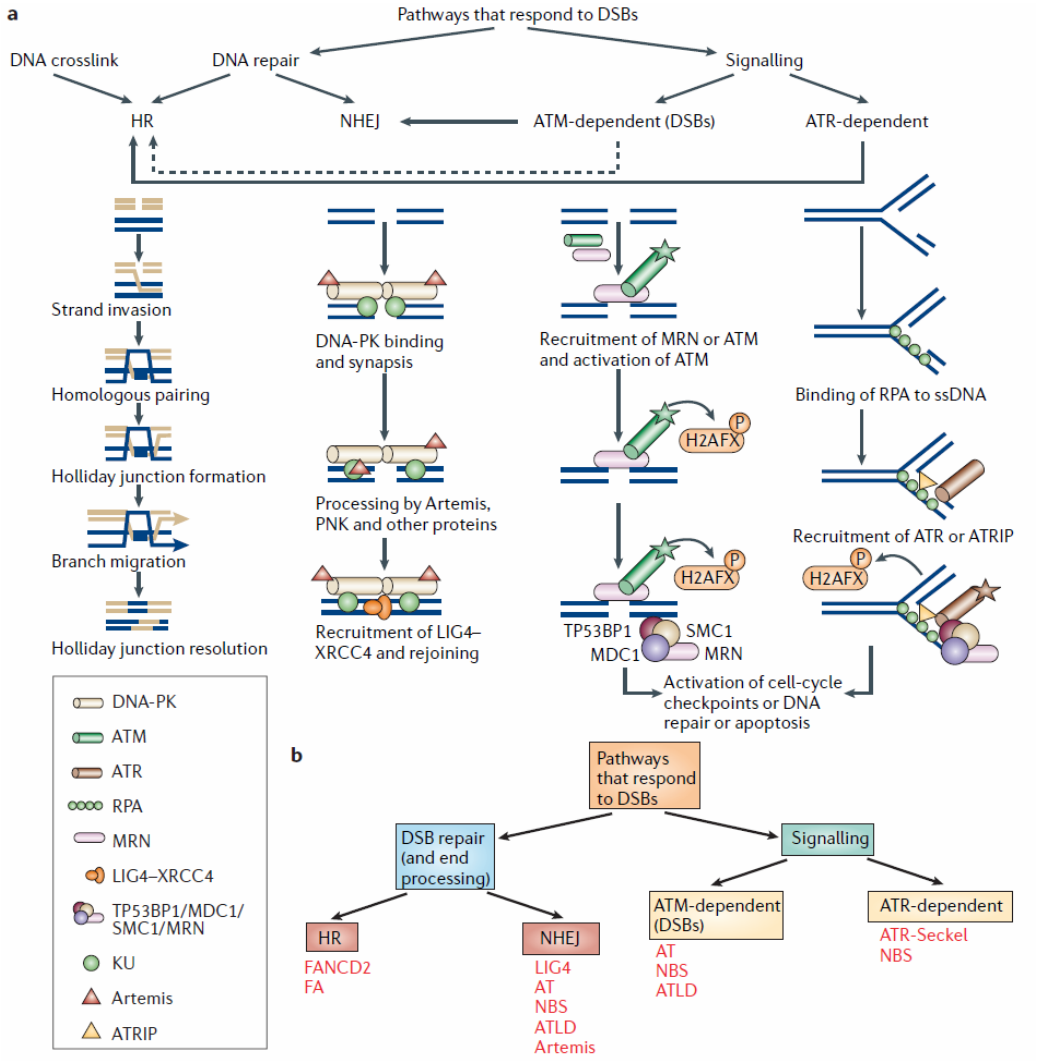

Figure 5. Double-strand breaks repair pathways.

(**a**) ATM signaling is the main signal-transduction process that responds to a DSB. ATR signaling is activated later after irradiation, when radiation-induced lesions block replication. Both pathways lead to cell-cycle checkpoint activation, which allows more time for repair or permanently prevents the proliferation of damaged cells. An important step in ATM- and ATR-dependent signaling is phosphorylation of H2A histone family member X (H2AX, or **H2AFX**) and recruitment of the mediator proteins. (**b**) DDR syndromes with affected DSB response pathway. AT, ataxia telangiectasia; ATLD, ataxia-telangiectasia-like disorder; FA, Fanconi anemia; FANCD2, Fanconi anemia complementation group D2; NBS, Nijmegen breakage syndrome; PNK, polynucleotide kinase.

Adapted from O'Driscoll and Jeggo, 2006.

The cells with defected HR are highly sensitive to DNA crosslinking agents. It fits with an idea that HR is more devoted to lesions at replication forks, and NHEJ pathway deals with the whole genome.

Homologous recombination

Homologous recombination (Figure 5) consists of three stages, which are common for prokaryotes and eukaryotes. First, presynapsis, where double-strand breaks or gap is formed, and the resulting DNA end is being prepared for recombination. Second, synapsis, where physical connection between the recombinogenic substrate and an intact homologous duplex DNA template is generated leading to the formation of heteroduplex (hybrid) molecules. Third, postsynapsis, where DNA synthesis from the invading 3' end takes place followed by the resolution of junction intermediates (reviewed in Nowosielska, 2007).

Homologous recombination (HR) rejoins the DNA double strand breaks in an error-free manner. The HR takes place during the late S and G2 phases, and a sister chromatid is used as repair template, allowing a high-fidelity process (Dronkert et al., 2000; Kadyk and Hartwell, 1992). HR involves the recognition of the double-strand breaks and generation of a single-strand region, which is followed by creation of a Holliday junction and subsequent branch migration and resolution (O'Driscoll and Jeggo, 2006). The protein from Rad51 family mediates this process. In addition, RPA, Rad54, BRCA1 and BRCA2 participate to this process. Rad51 is loaded onto the single-stranded DNA and promotes strand invasion. BRCA2 can have a role in facilitating the binding of Rad51 to the DNA (Pellegrini et al., 2002), and BRCA1 can have a regulatory function. Most of the proteins involved in the HR are essential for the cell viability, perhaps because of their role during the cell cycle and replication (Cha and Kleckner, 2002; Casper et al., 2002). HR deficient cells are highly sensitive to DNA crosslinking reagents and possess only mild radio-sensitivity (Thompson and Schild, 2001).

Non-homologous end joining

Another pathway of DSB repair, the non-homologous end-joining (NHEJ), does not require a template and is a source of mutations (error-prone DNA repair). NHEJ works throughout the cell cycle and is the main DSB repair pathway in the G1 phase (Shrivastav et al., 2008). It also plays a role during V(D)J recombination, which assures genetic variability of immunoglobins and T and B lymphocytes (Franco et al., 2006, Chaudhuri et al., 2007; Chaudhuri and Alt, 2004). NHEJ-deficient mice (if viable)

present severe combined immunodeficiency, SCID (Biedermann et al., 1991; Blunt et al., 1996).

NHEJ starts by the recruitment of the Ku heterodimer complex (Ku70/Ku80) to the free DNA ends (Walker et al., 2001). This association leads to the involvement of other factors important for NHEJ, such as DNA-PK (DNA-dependent protein-kinase), XRCC4, XLF and DNA Ligase IV (Ding et al., 2003; Reddy et al., 2004; Mari et al., 2006). The binding of DNA-PK is favoured by the conformational changes occurred after the Ku heterodimer association with the DNA breaks. DNA-PK is autophosphorylated leading to physical approach between both DNA ends (Chan et al., 2002). DNA-PK phosphorylates the XRCC4, and this leads to the Ligase IV–XRCC4 complex formation, which assures the DNA ligation step (Moreno-Herrero et al., 2005; Hopfner et al., 2002; de Jager et al., 2001).

The DNA ends from DSB generated by mutagenic agents are damaged and cannot be joined directly (about 10% of all DSB). In this situation, the DSB are processed by nucleases and polymerases before NHEJ. This process involves new factors, including Artemis (5'–>3' exonuclease), FEN-1 and the complex MRN, MRE11/Rad50/NBS1 (Chappell et al., 2002; Koch et al., 2004). The error-prone features of the NHEJ arise from this DNA "trimming" process, which leads to DNA errors, deletions or insertions. BRCA1 protein is also implicated in HR and decreases the errors amount in NHEJ (Bau et al., 2006). The recruitment of BRCA1 inhibits the nucleolitic activity of the DSB associated complex, limiting the DNA-trimming process (Zhuang et al., 2006).

DNA double-strand breaks damage response (DDR)

DNA damage response (DDR) is more than just a DNA repair (HR and NHEJ). It includes also the signal transducing mechanisms, alerting the cell for the presence of the lesions (Jackson, 2002). The activation by DSB is mediated and depends on the phosphatidylinositol-3-kinase related kinases (PIKK) members: ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related). DNA repair and signal transducing pathways interplay and recent studies indicate that HR and NHEJ are regulated in an ATM and ATR dependent manner (Riballo et al., 2004; Bolderson et al., 2004; Wang et al., 2004).

Damage sensing pathways enclose proteins required for damage sensing, signal transducers and effectors. The effectors carry an adequate cellular response to DNA damage, which includes cell cycle arrest and apoptosis. DNA damage leads to changes in gene-expression, protein synthesis, degradation and trafficking (Amundson et al., 2001).

ATM activation

The main response to DSB is mediated by ATM. This protein corresponds to the product of a gene, mutated in the genetic disorder ataxia-telangiectasia (Savitsky et al., 1995). ATM responds to DSB starting a cascade of phosphorylations (Kim et al., 1999), enhancing and repressing the activity of target proteins. ATM activation involves fast autophosphorylation (Kozlov et al., 2003). It was suggested, that in the absence of DNA damages ATM forms homodimers or higher order multimers. These comlexes are kinase inactive (Bakkenist et al., 2003). After the DSB formation and phosphorylation, each ATM separates from the dimers or multimers. During a few minutes, DSB can "switch on" almost all the ATM molecules.

The list of ATM substrates includes already more that a dozen factors. For instance, ATM phosphorylates p53 protein to activate and stabilize it (Kozlov et al., 2003; Banin et al., 1998). The p53 protein activates p21, an inhibitor of Cdk2, which is involved in the cell cycle regulation (Shiloh, 2003). Another substrate of ATM is BRCA1, which is phosphorylated at the different residues (Cortez et al., 1999; Gatei et al., 2000). Depending on the phosphorylation status, BRCA1 is involved in the regulation of the intra-S-phase checkpoint, G2-M checkpoint or activate the expression of some damage responsive genes (Jasin, 2002; Yarden et al., 2002).

Some of the responses involving ATM engage more than one controlled pathway. Divergent pathways can converge to a single process, increasing an efficiency of the response. For instance, the intra-S checkpoint involves, at least, five ATM-mediated pathways: in addition to BRCA1, the phosphorylation of NBS1 (component of the MRN complex), SMC1 or FANCD2 have a role in the checkpoint (Lim et al., 2000; D'Amours and Jackson, 2002; Taniguchi et al., 2002).

At the same time, one substrate could be involved in different responsive pathways; Chk1 and Chk2 both phosphorylate CDC25A which leads to it degradation

(McGowan, 2002; Bartek et al., 2001). CDC25A is involved in the maintenance of Cdk1 and Cdk2 activity, two proteins involved in the G1-S transition, and G2 to mitosis mobilization, respectively. The degradation of CDC25A leads directly to the G1-S, intra-S and G2-M checkpoints, without further activation of gene expression or protein synthesis.

A few things are known about these pathways. They were characterized at the systems defective activation of specific checkpoints leading to the abrogation of specific phosphorylation substrates.

ATR

ATM-mediated pathways are responsible for the cellular answer after DNA damages. ATM acts during one or two hours after DNA damage (Shiloh, 2003), but longer responses have been reported *in vivo*. This observation leads to the identification of other factors, involved later in DNA damage response (DDR). They continue phosphorylations started by ATM. One of these late factors in DDR is ATR. It can phosphorylate some of the ATM substrates (Abraham, 2001; Tibbetts et al., 1999; Zou et al., 2002).

ATR is not restricted to the late DDR response. It is also involved in the answer to stalled replication forks or UV irradiation (Abraham, 2001; Hammond et al., 2002; Shiloh, 2001), phosphorylating different substrates such as p53 and BRCA1 (Tibbetts et al., 1999, Tibbetts et al., 2000). An important ATR characteristic is requirements of a cofactor, ATRIP (ATR interacting protein). Immediately after the damage, ATRIP is one of the first substrates of ATR phosphorylation (Cortez et al., 2001), and the interaction between these proteins is important for ATR stability. In budding yeast, the ATR orthologue Mec1p, needs the ATRIP equivalent for its recruitment to the damaged sites and for its subsequent activation (Rouse and Jackson, 2000; Kondo et al., 2001). This pathway is well conserved through evolution (Edwards et al., 1999).

The effectors respond differently to ATM or ATR, and this has an important role in the general downstream response. For instance, whereas ATM prefers Chk2, the ATR protein chooses Chk1 as a target (Zhao and Piwnica-Worms, 2001; Lopez-Girona et al.,

2001). The activation of Chk2 by ATM leads to a stabilization of p53. Indeed, ATR does not have a significant influence on p53 stabilization and G1-S checkpoint activation.

Another important ATR substrate is Rad17 (Zou et al., 2002), a protein related to the replication factor C (RF-C) subunits. Rad17 replaces the large subunit of RFC, p140, in an alternative form of the clamp-loading complex that interacts with the PCNA-like heterotrimeric Rad9-Rad1-Hus1 (9-1-1) complex. This complex is linked with the checkpoint activation and acts as a sliding clapm being probably a damage sensor (Bermudez et al., 2003). The mediated phosphorylation of Rad17 and Chk1 is dependent on the Hus1 (Zou et al., 2002; Weiss et al., 2002). The recruitment of the 9-1-1 complex to the chromatin does not involve ATR or ATR-dependent Rad17 phosphorylation. It helps ATR to recognize its substrates at the chromatin level.

Nucleotide excision repair (NER)

Nucleotide excision repair (NER) is one of the major DNA repair mechanisms. This is a versatile multi-step DNA repair pathway that serves to remove a broad range of bulky, helix-distorting lesions (reviewed in Hoeijmakers, 2001; Lindahl and Wood, 1999; Maddukuri et al., 2007). It is ubiquitous from the simplest organisms such as mycoplasma to well developed ones like mammals. The majority of environmental carcinogens, including UV irradiation, antitumor drugs (cis-platin) and food-borne genotoxins, form bulky DNA adducts which may block replication and transcription. Among other helix-distorting lesions, NER is responsible for the repair of UV light-induced photoproducts (cyclobutane pyrimidine dimers), lipid peroxidation-induced DNA adducts, cigarette smoke-induced benzo[α]pyrene DNA adducts, chemical carcinogen-induced 4-nitroquinoline and other DNA adducts (Kraemer et al., 2007a). NER is also involved in the repair of oxidative DNA lesions (Brooks, 2007; Johnson et al., 2004) and in *Escherichia coli*, single strand breaks by UvrABC system (Truglio et al., 2006).

NER process in mammals is carried out by a multi-protein comlex reffered to as nucleotide excision repairosome, consisting of over 30 proteins which function in a stepwise manner. The main steps of NER are DNA damage recognition, assembly of the protein complex that carries out excision of damaged DNA, and synthesis and ligation of

a stratch of DNA strand for gap filling (Park and Choi, 2006). The key event in eukaryotic NER is an excision of approximately 30-nucleotide DNA fragment containing the damaged site (Wood, 1997; Leibeling et al., 2006). In mammalian cells, NER consists of two distinct sub-pathways, namely global-genome repair (GGR) and transcription-coupled repair (TCR). The difference between these two processes is DNA damage recognition. In TCR, RNA polymerase II stalled at the damaged site constitutes the signal for the recruitment of DNA repair proteins, while in GGR, the DNA damage-induced helical distortion is recognized by a specific protein complex. TCR specifically repairs transcription-blocking lesions in actively transcribed DNA regions. In contrast, GGR eliminates DNA lesions from the entire genome (Hanawalt, 2002).

Global genome repair (GGR)

In global genome repair (GGR), the major DNA damage recognition factor is the XPC-hHR23 complex. The transcription factor IIH (TFIIH), XPA and replication protein A (RPA) sequentially bind to the site of the damage to form a pre-incision complex. There are two TFIIH subunits, XPB and XPD, which possess ATPase and helicase activities. These proteins unwind the DNA double helix at the damaged site. The dual incision is performed by endonuclease activities of XPG and heterodimer XPF-ERCC1. They hydrolyze phosphodiester bonds 2-8 nucleotides downstream and 15-24 nucleotides upstream of the damaged site. The resulting gap is filled in by DNA polymerases delta/epsilon which require proliferating cell nuclear antigen (PCNA), RPA and replication factor C (RFC). Finally, the DNA fragments are ligated by DNA ligase I (LIG1) (Friedberg, 2001; Sancar and Reardon, 2004; Mocquet et al., 2008).

Transcription-coupled repair

For the first time transcription-coupled repair (TCR) was described in mammalian cells as removal of the lesions from the transcribed strands of active genes, which is more efficient than from the rest of genome (Bohr et al., 1985; Mellon and Hanawalt, 1989). The TCR specifically repairs transcription-blocking lesions from the actively transcribed DNA regions. RNA polymerase II, stalled at the damaged site, is a signal for the recruitment of TCR proteins. The lesion is removed only in the presence of CSB in an

ATP-dependent manner (Sarker et al., 2005; Laine and Egly, 2006a). Recent models of the TCR propose that the CSB protein interacts with lesion-stalled RNA polymerase II and recruits other TCR factors to the damaged site (Sarasin and Stary, 2007; Hanawalt and Spivak, 2008). XPG also interacts with CSB (Sarker et al., 2005). Subsequently, TFIIH, RPA and XPA arrive at the site of the damage. It appears that TFIIH and XPA stabilize each other's interaction with RNA polymerase II. A recent study suggests that an ATP-dependent activity of TFIIH is required to release the stalled RNA polymerase II (Laine and Egly, 2006a, 2006b). After, XPF-ERCC1 heterodimer is recruited to the damaged site and the CSB-dependent incision takes place (performed by XPG and XPF-ERCC1). Functional CSB is also required for the assembly of CSA-DDB1 E3 ubiquitin-ligase CSN complex (Fousteri et al., 2006).

DNA damage recognition in GGR

Several proteins are involved in the DNA damage recognition step of GGR, including the XPC-HR23B-centrin 2 heterotrimeric complex, which plays the major role (Figures 6 and 7). The human XPC is a 125 kDa protein. Its function is restricted to GGR (van Hoffen et al., 1995). It is known to form a stable complex with a 58 kDa HR23B protein (human homologue of yeast Rad23) at the damaged site. In this complex, XPC alone is responsible for the binding to the site of the damage, while HR23 stimulates XPC function and is required for the displacement of the complex in the next steps of GGR. HR23B can be substituted by HR23A, suggesting a functional redundancy of these two proteins (Sugasawa et al., 1998; You et al., 2003). The main role of the XPC-HR23B complex is a direct recognition and binding to the damaged DNA which causes a change of the DNA conformation and enables the recruitment of the entire repairosome to the damaged site (Batty and Wood, 2000; Yokoi et al., 2000; Min and Pavletich, 2007). Earlier studies implied that the XPC-HR23B complex was also involved in the open complex formation and stabilization (Aboussekhra et al., 1995; Mu et al., 1997).

Recently, Camenisch et al. (2009) described a two-stage process, when XPC recognizes damaged DNA. The authors exploited fluorescence-based imaging techniques to visualize the mobility of XPC protein at work at the chromatin context of living cells.

The rapid DNA quality check driven by a dynamic sensor of non-hydrogen bonded bases precedes the final engagement of the entire XPC with lesion site (see Figure 6).

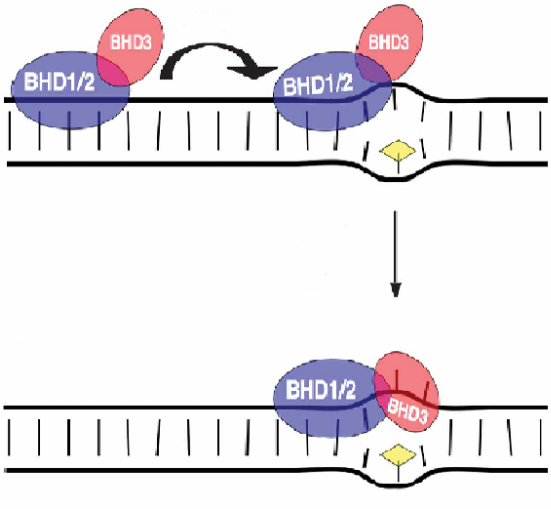

Figure 6. **Two-stage detection of DNA lesion by XPC protein.**
Model depicting switch from a dynamic damage sensor intermediate (top) to the ultimate recognition complex (bottom). BHD1,2, and 3 – beta hairpin domains 1,2, and 3 of XPC protein. Yellow diamond corresponds to a DNA lesion. Adapted from (Camenisch et al., 2009).

Besides the XPC–HR23B complex, other proteins, including XPA and XPE, may also be involved in the damage recognition.

DNA damage binding (DDB) factor is also a sensor of GGR. DDB consists of DDB1, 127 kDa subunit, as well as 48 kDa DDB2 (or XPE). The XPE is required only for *in vivo* but not *in vitro* repair process and is characteristic for human and absent in hamster cells that fail to repair CPD in GGR (Tang et al., 2000; Sancar and Reardon, 2004; Reardon and Sancar, 2005).

DNA damage recognition in TCR

In TCR (Figure 7), the XPC–HR23B complex is not required for the damage recognition step. The transcription arrest caused by stalled RNA polymerase II itself is a strong signal for TCR and recruits the repair machinery to the damaged site (Laine and Egly, 2006a; Sarker et al., 2005). After transcription blockage, the CSB–RNA polymerase II interaction is established or a pre-existing complex of the two proteins is further stabilized (van den Boom et al., 2004). The CSB is a 168 kDa DNA-dependent ATPase belonging to the SNF2 protein family. One of its roles is remodeling of the DNA–RNA polymerase II interface by interacting with chromatin factors such as XPA-binding protein 2 (XAB2) and histone acetyltransferase (HAT) p300. However, previous

reports indicating its participation in the release of RNA polymerase II was not confirmed (Fousteri et al., 2006). For intrinsic paused sites, and perhaps small base damage such as 8-oxoguanine, the influence of CSB may allow RNA polymerase II to bypass the site of pausing. If the blocking lesion is bulky, such as the UV-induced lesions or cisplatin crosslink, and RNA polymerase II bypass is impossible, the polymerase becomes more permanently arrested. At this juncture, the presence of CSB induces the recruitment of other TCR factors to the damaged site (Kamiuchi et al., 2002).

In vivo studies of Fousteri et al. (2006) showed that following UV irradiation CSB recruits the CSA–DDB1 E3 ubiquitin ligase–CSN complex to the damaged site. CSA is a 48 kDa protein with seven WD-40 repeats which are known to function as a protein-protein interaction interface (Henning et al., 1995). Together with DDB1, COP9 signalosome (CSN) and other subunits, CSA form an E3 ubiquitin ligase complex (Groisman et al., 2003). In CS cells, ubiquitilation of RNA polymerase II after UV irradiation is not observed, which implies that in normal cells this modification may depend on CSA and CSB (Bregman et al., 1996). This post-translation modification might be a mechanism of RNA polymerase II function regulation, possibly leading to protein degradation. However, it has not been shown whether this E3-ligase activity can cause polyubiquitination of RNA polymerase II and what is the molecular function of the ubiquitylated stalled RNA polymerase II is (Groisman et al., 2003). Moreover, CSA can also bind to the p44 subunit of TFIIH, suggesting a possible role in the assembly of the TCR repair machinery (Henning et al., 1995).

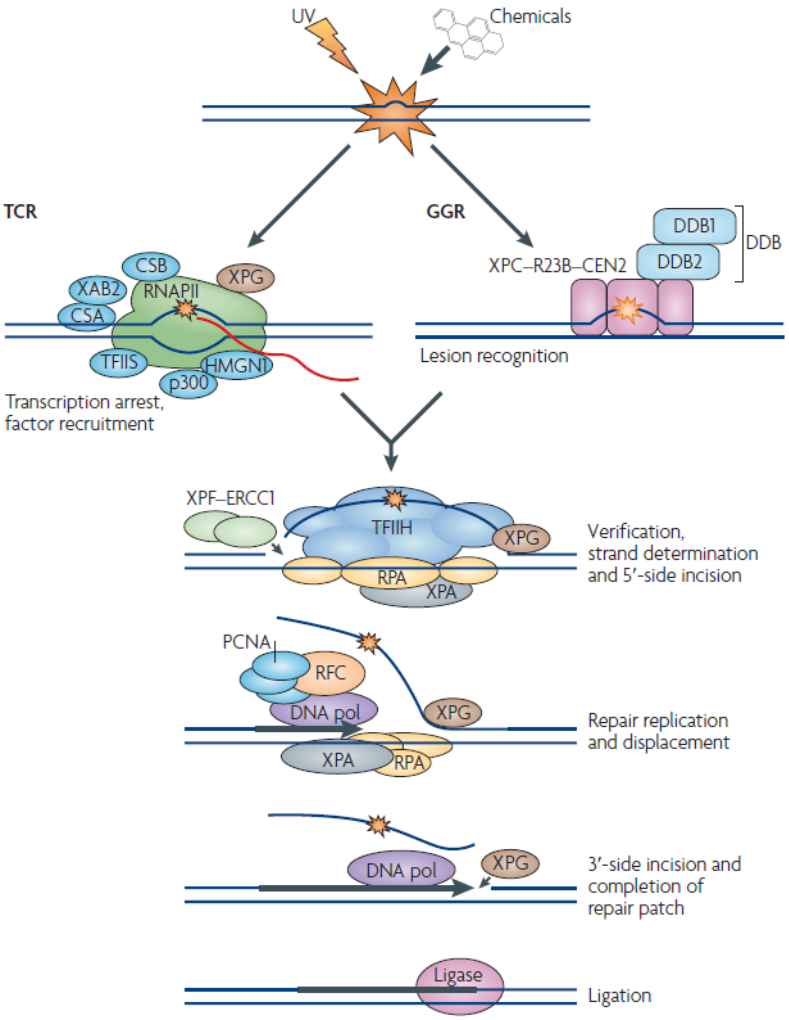

Figure 7. **Sequence of events in mammalian TCR and GGR** (more detailed GGR is at the discussion part). Lesions are initially recognized, either by a translocating RNA polymerase (RNAP) (for transcriptioncoupled repair (TCR); left) or through the binding of the lesion sensor DNA damage-binding-2 (DDB2; the product of xeroderma pigmentosum complementation group E (*XPE*)) — which forms a heterodimer with DDB1 to constitute the DDB complex — and XPC in complex with RAD23B and centrin-2 (CEN2) (for global genomic repair (GGR); right). The subpathways converge to the following steps for NER: TFIIH is recruited (with XPG, which stabilizes TFIIH); and the helicase and ATPase activities of its subunits XPD and XPB, respectively, are stimulated for further opening of the damaged DNA. RPA and XPA might be present before and/or after the appearance of TFIIH. The XPF–ERCC1 endonuclease complex is recruited and incises the damaged DNA strand at the 5′ side of the bubble, whereas XPG incises on the 3′ side. Once lesion verification has occurred, the 5′-side incision is made, and then repair replication begins before the 3′-side incision is produced by XPG. RFC loads the PCNA to accommodate DNA polymerases δ, ε or κ. Excision of damaged oligonucleotide in mammals may occur by the translocating DNA polymerase. Ligation step is done by ligase-I and flap endonuclease-1 or by the ligase-III–XRCC1 complex. CS, Cockayne syndrome; HMGN1, high-mobility group nucleosome-binding domain-containing protein-1; XAB2, XPA-binding protein-2. Adapted from Hanawalt (2008).

DNA unwinding in NER

The further steps of the NER pathway are similar in both GGR and TCR (Figures 7 and 12). The XPC–HR23B complex in GGR, and CSB factor in TCR, recruits other NER factors such as TFIIH, XPA, RPA, XPG, and XPF-ERCC1 in a sequential manner to the damaged site (Laine and Egly, 2006a; Mocquet et al., 2008; Publication 2). In GGR, the damage recognition XPC–HR23B complex interacts with the p62 subunit of TFIIH to bring it to the site of the damage (Yokoi et al., 2000). In addition, XPC interacts with XPB subunit of TFIIH modulating its ATPase activity (Bernardes de Jesus, 2008; Coin et al, 2006).

The XPB and XPD subunits of TFIIH are helicases with DNA-dependent ATPase activity. The XPB, 89 kDa subunit, has 3'–>5' DNA unwinding activity, and the XPD, 80 kDa subunit, functions in the opposite direction, 5'–>3' (Schaeffer et al., 1994). XPB appears to have a weaker helicase activity than XPD (Schaeffer et al., 1993) and is not involved directly in the unwinding and repair of damaged DNA (Publication 1 and Publication 3). The XPD helicase unwinds approximately 30-nt fragment of the DNA double helix around a lesion with the requirement of the XPB ATPase activity (Evans et al., 1997; Publication 1).

The damage-recognition XPC–HR23B complex can also interact with XPA (You, 2003). It is a 36 kDa protein which is required for both GGR and TCR (Hanawalt, 1994) and is known to interact with RPA, ERCC1 and TFIIH, as well as with the damaged DNA. An *in vitro* study shows that XPA protein preferentially binds to the damaged DNA through its central loop-rich domain (Ikegami et al., 1998). The zinc finger central region of XPA is also responsible for the binding of the RPA70 subunit of RPA protein, while the N-terminal domain mediates a strong interaction with RPA32 (Lee and Hurwitz, 1990; Stigger et al., 1998). The ERCC1-binding region of XPA forms a transient intra-molecular interaction with its DNA-binding region (Buchko et al., 2001).

RPA, also known as replication factor A (RFA), or human single-strand DNA binding protein (HSSB), consists of three subunits, RPA14, RPA32, and RPA70. RPA has an ssDNA-binding activity, which is responsible for the stabilization of the ssDNA produced by the TFIIH (de Laat et al., 1998). RPA70 has an affinity to the undamaged DNA strand opposite the lesion. Therefore, RPA may protect the intact DNA from the nuclease attack (Lee et al., 2003).

Due to its affinity to the ssDNA and the strong interaction with XPA, RPA plays an important role in the open complex formation and stabilization, but also participates to the next steps of NER. It interacts with XPG and XPF–ERCC1 complex, coordinating the function of these nucleases during incision step.

Binding of the XPA factor to the repair complex (including damaged DNA, TFIIH, XPC, and RPA) leads to the CAK TFIIH sub-complex dissociation. The CAK leaving, in turn, stimulates the DNA opening around the lesion and prepares incision step (Publication 2).

Dual incision in NER

Following DNA unwinding, sequential recruitment of the nucleases XPG and XPF–ERCC1 complex leads to a dual incision in close proximity to a damaged site. XPG hydrolyses a phosphodiester bond 2–8 nucleotides 3' to the damaged site (Habraken et al., 1993) while XPF–ERCC1 cuts 15–24 nucleotides 5' to the lesion (Mu et al., 1995).

XPG and XPF–ERCC1 are structure-specific nucleases that hydrolyse duplex substrates preferentially near the junction between the ssDNA and dsDNA (Sijbers et al., 1996). The XPF–ERCC1 and XPG binding to XPA (through ERCC1) and RPA (through XPF) (Bessho et al., 1997) not only facilitates the correct positioning of these proteins at the damaged sites but also stimulates the junction-cutting endonuclease activity of XPG and XPF–ERCC1 (Matsunaga et al., 1996). In TCR, XPG seems to be recruited at the earlier step and binds together with CSB to a stalled RNA polymerase II (Sarker et al., 2005).

XPG is a 133 kDa protein, a member of the FEN1 family of structure-specific endonucleases which incise a varity of DNA substrates, including bubbles, flap, splayed arms and stem-loops (Scherly et al., 1993). XPG possesses two highly conserved nuclease motifs separated by a spacer region, which serves as a protein-protein interaction interface and determinates the substrate specificity (Dunand-Sauthier et al., 2005). Binding of XPG to the pre-incision complex induces a conformational change that is required for the recruitment of the XPF–ERCC1 complex, the last factor joining the incision complex.

XPF is a 115 kDa protein that forms a tight complex with the 38 kDa ERCC1 protein (Park et al., 1995). The strong interaction between the two proteins is mediated through the C-terminal helix-hairpin-helix domains of both proteins. XPF has a nuclease activity while a central region of ERCC1 is similar to the XPF nuclease domain but does not contain residues crucial for the enzymatic activity (Choi et al., 2005). The XPF–ERCC1 heterodimer incises a variety of DNA substrates such as bubbles, stem-loops and flaps. The XPF and XPG nucleases require Mg^{2+} or Mn^{2+} but not ATP for the specific cleavage activity (Evans et al., 1997).

DNA resynthesis during the NER

After incision of the damaged DNA, the resulting gap of about 30 nucleotides is filled in by DNA polymerase δ or ε, and subsequently the DNA fragments are ligated by DNA ligase I (Wood et al., 2000).

After the dual incision, an RPA remains bound to the single strand DNA intermediates and is involved in the recruitment of PCNA and RPA to the repair synthesis site (Yuzhakov et al., 1999; Riedl et al., 2003). The resynthesis step by these polymerases occurs in a PCNA-dependent manner and with a recruitment of RFC. The last factor preferentially binds to the 3'-hydroxyl end of DNA primer and assembles PCNA onto the DNA template in an ATP-dependent manner. In addition, XPG has also been shown to interact with PCNA and facilitate its loading to the repair synthesis site. PCNA is 37 kDa protein belonging to the DNA sliding clamp protein family (Wyman and Botchan, 1995), which forms a ring-shaped homo-trimeric sliding clamp that encircles DNA. The RFC-PCNA complex serves as a docking platform that links polymerase to the DNA template and initiates chain elongation. PCNA also stabilizes the interaction of polymerases with the DNA template. The interaction of polymerase with PCNA and RFC allows an accurate and efficient DNA synthesis (Waga and Stillman, 1998). DNA polymerases δ and ε exhibit low processivity in the absence of PCNA (Burgers, 1991). Recently, a low fidelity Y family DNA polymerase kappa (κ) was found to participate in the resynthesis step of NER (Ogi and Lehmann, 2006).

Polymerases δ and ε belonging to the B family polymerases show an intrinsic proof-reading exonuclease activity (3'–>5'). Mammalian DNA polymerase δ is a complex of four subunits: 125, 68, 50 and 12 kDa. DNA polymerase ε consists of a large catalytic subunit of 261 kDa and three associated subunits of 59, 17 and 12 kDa. Both replicative polymerases δ and ε are highly processive, can polymerase long DNA stretches without dissociating from the template, and are stimulated by RPA and PCNA (reviewed in Maddukuri et al., 2007).

RFC is a heteromeric protein complex composed of one large subunit, RFC145, and four smaller subunits (RFC40, RFC38, RFC37 and RFC36; Uhlmann et al., 1996). The genes encoding these subunits share homology among themselves, although each of them is necessary for the proper function of RFC.

After a synthesis of new DNA strand by DNA polymerase, the remaining nick is sealed by ligase 1 (Lig1) to complete the repair. The enzyme binds to PCNA, encircles and partially unwinds nicked DNA and catalyzes the esterification of the 3'-hydroxyl and 5'-phosphoryl termini of the nick in DNA. The reaction also requires ATP and a diavalent cation. Human Lig1 is a 102 kDa monomer, composed of a highly conserved C-terminal domain with the active site and an N-terminal domain which contains the nuclear localization signal and directs the enzyme to the sites of DNA replication (Ranalli et al., 2002).

TFIIH and its role in the nucleotide excision repair

Transcription factor II H (BTF2 – basal transcription factor 2, Gerard et al., 1991) is a dynamic complex of ten polypeptides with different functions (see Figures 10-12). There are core subunits XPB, XPD, p62, p52, p44, p34, and TTD-A/p8, and CAK sub-complex consisting of Cdk7, cyclin H and MAT1. TFIIH was initially purified from the rat liver (Conaway and Conaway, 1989), HeLa cells (Gerard et al., 1991) and yeast (Feaver et al., 1991) by conventional column chromatography, and later by a single step immunoaffinity purification method (Kershnar et al., 1998). It has three enzymatic activities required for transcription: DNA-dependent ATPase (XPB and XPD), ATP-dependent helicase (XPD and XPB), and CTD kinase (Cdk7). In addition to the enzymatic activities essential for transcription, some components of TFIIH are involved in nucleotide excision repair (XPB and XPD) DNA damage response. CAK sub-complex of TFIIH is responsible for the phosphorylation of C-terminal domain (CTD) of RNA polymerase II (Rpb1 subunit). CAK and core TFIIH are linked by the XPD helicase, which is essential for DNA repair activity and has rather a structural role in transcription (Rossignol et al., 1997; Coin et al., 1999).

XPB

XPB (ERCC3; Ssl2; Rad25) is the largest subunit of the eukaryotic transcription factor TFIIH. It is essential for both initiation of transcription by RNA polymerase II and nucleotide excision repair. XPB belongs to SF2 superfamily of monomeric helicases. It is thought to have evolved in eukaryotes, however, a gene highly homologous to human XPB can be found in a number of bacteria, for instance, *Mycobacterium tuberculosis* and *Kineococcus radiotolerans* (Biswas et al., 2009). Similarly to eukaryotic XPB, bacterial homologous are DNA-dependant ATPases with 3'–>5' DNA helicase activity.

The ERCC3 gene (excision repair cross-complementing group 3) was initially characterized by Weeda et al. (Weeda et al., 1990a; Weeda et al., 1990b). It was observed, that product of this gene is involved in the early steps of nucleotide excision repair. The gene was cloned after transfection of human chromosomal DNA to the UV-sensitive, incision-defective Chinese hamster ovary (CHO) mutant 27-1. This mutant

belongs to the complementation group 3 of repair-deficient rodent mutants. CHO 27-1 cells have an *A1075G* transition (K359E) located at the very beginning of the Ia helicase domain which causes deficiency in open complex formation and in dual incision during NER (Hall et al., 2006). After selection of UV-resistant CHO transformants, human sequences associated with the induced UV resistance were analyzed, and the gene size between 35 kb and 45 kb was proposed. The ERCC3 cDNA induced specific and almost complete correction of the UV sensitivity and unscheduled DNA synthesis of complementation group 3 mutants (Weeda et al., 1990b).

Later, the ERCC3 factor was characterized as 782 amino acids protein with putative nucleotide, chromatin, and helix-turn-helix DNA binding domains and seven consecutive motifs conserved between two superfamilies of DNA and RNA helicases (Wood et al., 1990), strongly suggesting that this is DNA repair helicase. ERCC3-deficient rodent mutants phenotypically resemble the human repair syndrome xeroderma pigmentosum (XP). ERCC3 specifically corrects the excision defect in one of the eight xeroderma pigmentosum groups, XP-B. Patients of the group known at that time presented an exceptional conjugation of two rare genetic disorders, XP and Cockayne syndrome (CS). As XP is leading to cancer, ERCC3 was considered as a tumor-preventing gene.

The XPB protein is known to be important for both DNA repair and transcription (Winkler et al., 2000). In particular, it was proposed that the XPB enzymatic activity plays an important role in the promoter escape during transcription initiation by RNA polymerase II (Bradsher et al., 2000). Mutations targeted to the ATPase and helicase activities of *Schizosaccharomyces pombe* XPB have demonstrated that helicase activity is not required for promoter opening, but ATPase activity is essential (Lin et al., 2005). It fits with the crosslinking studies, which suggest that XPB may not be close enough to the melted DNA to act as conventional helicase (Kim et al., 2000). *In vitro* studies of XPB function used mutants that act indirectly by targeting the ATPase activity (motifs I and II), needed to drive helicases (Ma et al., 1994). The helicase motifs (III–VI) directly were targeted later, and results confirmed the XPB ATPase involvement to the NER and transcription function (Lin et al., 2005; Publication 1; Publication 3).

Sulfolobus solfataricus and related archaea have two homologues of the XPB protein, XPB1 (Sso0959) and XPB2 (Sso0473) (Richards JD. et al., 2007). They reported that these enzymes bind single-strand DNA and have DNA-dependent ATPase activity, but neither has helicase activity against a range of damaged and undamaged DNA substrates *in vitro*.

Recently, Fan et al. (2006) have solved the structure of XPB homolog from the termophilic organism *Archaeoglobus fulgidus* (AfXPB). Structures of the amino (N)-terminal and carboxy (C)-terminal parts of AfXPB revealed two RecA-like helicase domains (HD1 and HD2), as well as associated motifs and extensions. The structure of the full-length protein characterized AfXPB architecture and domain flexibility, including the flexible hinge connection joining HD1 and HD2. The structures unveil a putative DNA damage recognition domain (DRD) with conservative residues E253 and R283. However, the following study (Publication 3) showed no nucleotide excision repair or transcription defect, when mutated E253A and R283A. In addition, the structure unveiled a positively charged Thumb-like domain, and an R-E-D residue loop implicated in XPB-specific activities (Publication 3).

The XPB activity inside of TFIIH is highly regulated by others TFIIH subunits, such as directly by p52 (Publication 1), or indirectly by p8/TTD-A through p52 (Coin et al., 2006). The p52–XPB interaction is weakening in the XP-B patients with F99S mutation, and the defected regulation of TFIIH function could explain the XP/CS phenotype at the molecular level (Publication 1). In addition, the NER damage recognition factor XPC-HR23B stimulates XPB ATPase activity and DNA opening around the lesion (Coin et al., 2006).

Mutations in XPB gene lead to the rare genetic disorders in human. There are six families known with the XP-B defects (Oh et al., 2006). However, these patients have different clinical phenotypes. Three patients from two families have the xeroderma pigmentosum combined with Cockayne syndrome (XP/CS), with skin features of XP including increased frequencies of skin cancer and neurological abnormalities of CS (Rapin et al., 2000). Two siblings in the third family had trichothiodystrophy (TTD) with sulfur deficient, brittle hair, and neurological abnormalities without increased frequency of skin cancer (Riou et al., 1999). Later, Oh et al. (2006) described two sisters (XP33BR

and XP1SA) with relatively mild XP without CS (Vivian et al., 1993) and two severe XP/CS complex families (XP131MA (Bartenjev et al., 2000), XP183MA) with defects in XPB. The cells from the milder XP sisters had the same mutation (296T>C, F99S) that was previously reported in two mild XP/CS complex brothers (XP1BA and XP2BA). The severely affected XP/CS complex families all have the same splice acceptor site mutation (2218-6C>A, Q739insX42) in one allele, leading to a frame-shift and alteration of last 41 amino acids. Oh et al. (2006) described five different alleles in the XP-B patients: c.1273C>T, R425X; c.471+1G>A, K157insTSDSX; c.807–808delTT, F270X;

c.1421–1422insA, D474EfsX475; and c.1633C>T, Q545X. The heterogeneity of XPB is associated with partially active missense mutations in milder patients. At the same time, severe XP/CS complex patients have nonsense mutations in both alleles with low levels of altered XPB proteins.

XPD

The XPD (xeroderma pigmentosum group D) helicase family comprises a number of superfamily 2 DNA helicases with members found in eukaryotes, prokaryotes, and archaea (reviewed in White, 2009). The founding member, the XPD helicase (ERCC2, Rad3), is conserved in archaea and eukaryotes, whereas the closest homologue in bacteria is the DinG (damage-inducible G) helicase. Three XPD paralogues, FancJ (Fanconi's anemia complementation group J), RTEL (regulator of telomere length) and Chl1, have evolved in eukaryotes and function in a variety of DNA recombination and repair pathways. All family members are believed to be 5'–>3' DNA helicases with a structure that includes an essential iron-sulfur-cluster-binding domain. Recent structural, mutational and biophysical studies have provided a molecular framework for the mechanism of the XPD helicase and help to explain the phenotypes of a considerable number of mutations in the XPD gene that can cause different genetic disorders.

XPD is an essential structural component of TFIIH and acts as a bridging subunit between the core TFIIH and the CAK complex. The XPD helicase is found as part of ten-subunits TFIIH, as well as another complex, XPD–CAK (Reardon et al., 1996). The helicase activity of XPD is important for NER, and is not required for the transcription initiation (Tirode et al., 1999).

There are at least three paralogues of XPD in eukaryotes. All of them share the four conserved cystein residues and therefore likely to be iron-sulfur proteins. The best characterize is helicase FancJ which is important for the DNA cross-link repair pathway that is mutated in Fanconi's anemia patients (reviewed in Wu et al., 2009). FancJ interacts with the breast cancer susceptibility protein BRCA1 and may have a role in double-strand break repair. Probably, A349P mutation of FancJ in humans causes Fanconi's anemia by disrupting the iron-sulfur cluster-binding domain and thus inactivating the FancJ helicase (Rudolf et al., 2006). RTEL paralogue of XPD is important for the telomere and genome stability (Ding et al., 2004). Chl1, found in yeast and metazoan, also contains four conserved cysteine residues and is therefore probably an iron-sulfur-dependant helicase. Chl1 is reported to be important for the chromosome segregation (a step in cell reproduction or division, where chromosomes pair off with their similar homologous chromosome) (Skibbens, 2004).

Crystal structures of archaeal XPD have been solved by three different research groups (Fan et al., 2008; Liu et al., 2008; Wolski et al., 2008). All the structures revealed a four-domain organization, with the iron-sulfur-cluster-binding domain and an Arch domain arising from the first of two canonical helicase motor domains. Together, the Arch and iron-sulfur domains form a channel through which single-strand DNA is dragged by the action of the motor domains in a cyclical ATP-dependant reaction. This is consistent with a role for the iron-sulfur domain in breaking the DNA duplex. However, it is not yet clear, which specific part of the protein is utilized to separate the DNA strands, and it remains a formal possibility that the DNA helix is broken at the other side of the protein on the motor domain 2 (Wolski et al., 2008).

The discovery of an iron-sulfur cluster domain in XPD was unexpected, and the role of the cluster is the subject of discussion. Iron-sulfur clusters are comparatively rare in DNA-repair proteins. The only other example is the cluster found in the family 4 of the uracil-DNA glycosilases (Hinks et al., 2002). The cluster has been postulated to have a structural role and potentially a role in DNA damage detection. Since XPD could be one of the factors responsible for the DNA damage detection or verification during NER, it was proposed that the iron-sulfur cluster might play an active role in this process (photoproducts detection). However, for the XPD paralogues with similar iron-sulfur

clusters detection of similar DNA damages is not expected. The clusters could also confer a redox-sensing function of the protein. In this case, the XPD enzymatic activity is controlled by the oxidation state of the iron-sulfur clusters (inactivating the protein under oxidative stress by decomposing the clusters). However, it would be strange to deactivate a DNA repair enzyme at the time, when it is the most required. Finally, the role of the cluster could be in the stabilization of XPD domains structure, to provide the DNA duplex separation during helicase function (reviewed in White, 2009). Iron-sulfur clusters have been discovered in several other proteins, including DNA primase (an enzyme involved in DNA replication), RNA polymerase, as well as bacterial AddAB (helicase/exonuclease) and eukaryotic Dna2 (multifunctional protein with endonuclease activity) enzymes (Klinge et al., 2007; Hirata et al., 2008; Yeeles et al., 2009).

The XPD protein is regulated by TFIIH subunit p44 (Coin et al., 1998). XPD helicase activity is stimulated by p44 association with its carboxyl-terminal part. Mutations in the XPD (most of them are at the C-terminal region) prevent p44 binding, and provoke XP or TTD disorders.

Several rare genetic diseases are caused by changes in the XPD sequence. Cleaver discussed three of them (Cleaver, 1994), xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). Later, XPD/ERCC2 was reported to be involved in the UV-sensitive Cerebro-oculo-facio-skeletal syndrome (COFS) (Graham et al., 2001).

p62

The Tfb1/p62 is a structural component of TFIIH core. In yeast, mutations in the *tfb1* gene result in intermediate UV light sensitivity and defects in both global genome repair (GGR) and transcription coupled repair (TCR) (Sweder et al., 1996). Later, Castro et al. (2002) described *Drosophila melanogaster* model with mutated p62 homologue Dmp62. The heterozygous flies had higher UV sensitivity if compare to wild type flies. In addition, severe cuticular damage was produced by irradiation of the *Dmp62* mutant.

The p62 gene product is highly conserved in the evolution as are the others components of TFIIH (Castro et al., 2002). There is a clear correlation of conservative motifs position in plants and animals. p62 has multiple contacts with another TFIIH

subunits and different transcription factors (Tong et al., 1995; Tremeau-Bravard et al., 2001). The yeast p62 homolog (Tfb1) requires 112 C-terminal amino acids to contact Ssl1 (p44) (Matsui et al., 1995).

In human, the p62 plays a central role in the architecture and function of TFIIH (Fischer et al., 1992; Tirode et al., 1999). In transcription, TFIIH is recruited at a late stage of the preinitiation complex formation by the general transcription factor TFIIE (reviewed in Svejstrup et al., 1996). The interaction between TFIIE and TFIIH, which has been characterized in yeast and mammalian cells, involves the direct binding of the p62 subunit of TFIIH to the alpha subunit of TFIIE (Bushnell et al., 1996). This interaction markedly stimulates TFIIH-dependent phosphorylation of the C-terminal domain of RNA polymerase II. Moreover, the p62 subunit of TFIIH contacts transcription activators, viral transactivators and the tumor suppressor p53 (reviewed in Zurita and Merino, 2003). These interactions modulate transactivation in several cases. Point mutations in the acidic transcriptional activation domain of VP16 that reduce its transactivation activity also weaken its binding to TFIIH. In the case of E2F-1, a loss of TFIIH binding via p62 results in 60-65% reduction in transactivation. Nuclear receptors, like other transcriptional regulators (p53 and E2F-1), are phosphorylated by Cdk7 subunit of TFIIH, and this reaction requires association with p62 (estrogen receptor ER-alpha) (Xiao et al., 1994; Vandel and Kouzarides, 1999; Chen et al., 2000). In addition, p62 directly interacts with thyroid hormone receptor, enhancing T3-mediated transcription (Liu et al., 2005). In DNA repair, Tfb1 interacts with damage recognition complex XPC-HR23B, as well as a structure-specific endonuclease that cleaves DNA at the 3' end of lesion, XPG (Yokoi et al., 2000; Iyer et al., 1996).

Gervais et al. (2004) reported a structure of TFIIH p62 subunit (solved by nuclear magnetic resonance, NMR). They described a pleckstrin homology and phosphotyrosine binding N-terminal (PHD/PTB) domain (amino acids 1-108), required for the NER activity of TFIIH because of interaction with XPG. However, this domain is not important for the architecture of TFIIH and transcription initiation. Further NMR structural analysis of p62 showed p62-p53 interaction mechanism, which provides p53 transcription activation ability. This interaction occurs through N-terminal region of p53, residues 47-55, and PH domain of p62. In addition, phosphorylation of p53 Serine 46 and

Threonine 55 leads to a significant enhancement of p53-p62 association (Di Lello et al., 2006). The same PH-domain of p62 interacts with TFIIE-alpha C-terminal residues 336-439 (Di Lello et al., 2008), and VP16 C-terminal sub-domain residues 456-490 (Langlois et al., 2008).

There are no described mutations in human p62 that leads to disorders. However, the correlation between p62 single nucleotide polymorphisms (SNP) and the lung cancer risk have been reported (Wu et al., 2009).

p52

Human p52 protein (homologue of yeast Tfb2) was characterized as one of the TFIIH subunits (the ninth out of ten known for the moment, Marinoni et al., 1997). It was co-purified and co-immunoprecipitated with others TFIIH subunits. In a cDNA library correspondent gene was described as an open-reading frame (ORF) of 1386 base pairs that encodes a protein of 462 amino acids (52 186 Da). In yeast, p52 homologue is Tfb2 protein, 513 amino acids and about 59 kDa.

Addition of antibodies towards p52 to the *in vitro* transcription or NER systems inhibited these TFIIH dependent reactions (Marinoni et al., 1997).

To further understanding of the TFIIH function, Jawhari et al. (2002) examined the role of p52 subunit. Using completely reconstituted *in vitro* transcription or DNA repair (NER) systems, it was shown that deletion of the C-terminal region of p52 results in a dramatic reduction of TFIIH activities (Feaver et al., 1997; Jawhari et al., 2002). This mutation prevents promoter opening. In addition, it was shown that intact p52 is needed to anchor the XPB subunit within TFIIH complex. p52/Tfb2 protein physically interacts with XPB and stimulates its activity during transcription and DNA repair (Feaver et al., 2000; Jawhari et al., 2002). The complex p34/p44/p62 (Tfb4/Ssl1/Tfb1) does not interact with XPB in the absence of p52 (Tfb2). XPB binding involves two independent regions of p52: the first is located within residues 1-135 and contains a few residues strictly conserved among p52 orthologs. The second binding region is formed by residues 303-381, as p52(381-362) interacts with XPB, and p52(381-462) does not (Jawhari et al., 2002). This second binding region is highly conserved from human to yeast.

The fact that p52(381-462) does not interact with XPB correlates with the observation that the C terminus of p52 is rapidly digested in a mild proteolysis assay. It seems that this domain is crucial for the architecture and/or the stability of TFIIH, as a deletion of residues 358-462 weakens the binding of XPB within the core TFIIH. This instability can explain the NER defect and transcriptional impairment as well as the phenotype observed in yeast cells containing a similar deletion. It has been proposed that the C terminal domain of p52 might possess a three-dimensional structure that locks up XPB inside TFIIH. The consequence of the p52 deletion would be a weaker and/or inappropriate positioning of the XPB within TFIIH, and transcription or DNA repair complex. This leads to the DNA opening defect (Jawhari et al., 2002).

Yeast Tfb2 (transcription factor b 2nd subunit) have been reported by Feaver et al. (1997). This subunit was a product of mRNA with open-reading frame (ORF) 1539 and includes 513 amino acids (58,6 kDa). Tfb2 deficient yeast did not survived, as p52 protein is essential for the transcription. However, yeast strain with deletion of 60 amino acid residues from the C-terminal part was viable with no apparent phenotype (Feaver et al., 1997). When exposed to UV irradiation, this strain was about ten folds more sensitive than the wild type one. This kind of sensitivity is similar to one observed with mutations in Tfb1 (p62) and Ssl2 (XPB). So, the Tfb2 (p52) is required for NER in yeast.

No human genetic disorder has been associated with the gene encoding p52. A change of the last 40 amino acids in the XP-B/CS patient results in a similar defect in promoter opening and RNA synthesis (Coin et al., 1999). Positioning of this mutated XPB onto promoter DNA is impaired. XPB contains three modules, an N-terminal domain (DNA binding domain), a catalytic core domain with conserved helicase motifs, and a C-terminal domain (Poterszman et al., 1997). Jawhari et al. (2002) have shown that p52 interacts with putative DNA binding region of XPB Deletion of C-terminal part of p52 abolishes NER and transcription *in vitro*.

Later, Aguilar-Fuentes et al. (2008) described *Drosophila melanogaster* carrying mutated p52 protein (Dmp52). These animals had neurological defects, brittle bristles phenotype, UV-irradiation hypersensitivity, and cuticle defects, as well as reduced amount of TFIIH, which made them similar to trichothiodystrophy (TTD) patients at the

molecular level. Overexpression of p8/TTD-A proteins in that animals corrected lethal *Dmp52*-deficient phenotypes, suggesting the importance of p8–p52 interaction *in vivo*.

Studying Tfb5 (p8/TTD-A yeast homologue), Zhou et al. (2007) observed a p8–p52 interaction inside of TFIIH and proved the role of that association for the efficient NER. They proposed a model when p8 stabilizes core-TFIIH in yeast to support its function.

Tfb5/p8 protects a hydrophobic surface of p52 (C-terminal domain) from solvent, and may explain why lack of p8/TTD-A leads to TFIIH instability and trichothiodystrophy (Kainov et al., 2008).

p44

The p44 subunit of human TFIIH (or Ssl1 in yeast) have been initially cloned and characterized by Humbert et al. (1994). The protein has a zinc finger domain, which is important for the protein-DNA interaction (Jacobs, 1992). Inside of TFIIH p44 interacts with others subunits (p34, described and summarized in Feaver et al., 2000), p62 (Feaver et al., 1993) and XPD (Bardwell et al., 1994). The binding of XPD to the zinc-finger containing p44 led to the suggestion that, after binding to the DNA, p44 could modify the XPD ATPase/helicase activity and processivity (Bardwell et al., 1994). Later, Coin et al. (1998) demonstrated XPD stimulation by p44 *in vitro* using human proteins. Mutations in C-terminal region of XPD weaken that protein-protein interaction leading to the xeroderma pigmentosum and trichothiodystrophy.

The ubiquitin ligase activity of p44/Ssl1 has been uncovered due to the presence of a RING finger domain at the C-terminal part, residues 403 to 454 (Takagi et al., 2005). The NMR structure of p44 RING finger domain (residues 321 to 395) shows two zinc-binding sites coordinated by eight cysteine residues (Kellenberger et al., 2005). These conserved cysteine residues are also found in the RING finger domain of Ssl1 and are critical for its E3 ubiquitin ligase activity, since mutations in the first two cysteine residues (C403A and C406A) abolished Ssl1 enzymatic activity in an *in vitro* polyubiquitination assay performed in the presence of E1 and E2 (Ubc4) enzymes and further reduced the yeast survival rate following UV irradiation or methyl

methanesulfonate (MMS) treatment. The Ssl1 ubiquitin ligase activity can be enhanced by the inclusion of another RING finger-containing protein p34 (Takagi et al., 2005).

The C-terminal region of p44 was shown to be important for the TFIIH integrity (Fribourg et al., 2000) and transcription activity dependent on the intact N-terminal part, which interacts with XPD (Seroz et al., 2000). Mutations in the region between amino acid residues 66 and 200 of p44 weaken the p44–XPD interaction and lead to the defect of the first step of transcription reaction, the phosphodiester bound formation and promoter clearance. The p44–XPD interaction stimulates transcription, increasing an activity of CAK TFIIH sub-complex (Seroz et al., 2000).

The p44 genes were described in the relation to spinal muscular atrophies (SMA), autosomal recessive disorders characterized by degradation of spinal cord anterior horn cells and proximal muscle wasting (Carter et al., 1997). Werdnig-Hoffmann disease is SMA type I, the most severe if compare with two others SMA types (II and III). The gene encoding p44 was mapped to the SMA region of chromosome 5 (Buckler et al., 1991). Two highly homologous copies of p44 are described. They differ by two non-conservative amino acids changes, Ile–>Met and Leu–>Val. Both forms of p44 appeared to be ubiquitously expressed and are present in fetal and adult tissues. The copies of p44 genes were named after their location at the chromosomes, T-p44 (telomeric gene) and C-p44 (centromeric gene). T-p44 is missing in the 15% of SMA patients with no correlation to the severity of the disorder (Carter et al., 1997). At the same time, Burglen et al. (1997) made a similar conclusion, that T-p44 deletion is related to the large-scale deletions in the SMA Type I disorder. However, deletion of T-p44 gene did not affect neither TFIIH complex stability, no activity (transcription and DNA repair).

p34

After the TFIIH (BTF2) was isolated (Gerard et al., 1991), the p34 polypeptide has been identified. Later, it was characterized by trypsin digestion followed by reversed phase chromatography. Amino acid sequences obtained from the digestion of p34 were used to create oligonucleotides and to screen HeLa cDNA library. The p34 cDNA possesses 909 base pairs encoding 303 amino acids with molecular weight 33,920 Da (Humbert et al., 1994). The p34 was described as a hydrophobic protein with high

leucines and isoleucines (about 20%). It possesses zinc-finger domain, which is usually important for the interaction with DNA. No TFIIH-related enzymatic activity has been described for this protein. However, antibody to recombinant p34 recognized the 34 kDa polypeptide of purified TFIIH (Humbert et al., 1994). At the same study, the antibody to p34 was used to deplete HeLa extract, and the NER activity of this extract was significantly reduced. In order to determine if additional NER factors were also removed, the treated extract was mixed with rodent repair deficient extracts (ERCC2, ERCC3 and ERCC4). The depleted HeLa extracts had lost the ability to restore repair activity of ERCC2 (XPD) and ERCC3 (XPB), but restored ERCC4 (XPF). These finding indicated that anti-p34 removed at least p34, XPB and XPD TFIIH components, and that the p34 is a *bona fide* TFIIH subunit.

Later, yeast homologue of p34, Tfb4, has been characterized (Feaver et al., 1997). To test the requirement of this protein in yeast, a diploid strain was made in which the Tfb4 gene was replaced with LEU2. After the sporulation, only Tfb4 proficient (and leu2-deleted) cells survived, demonstrating that p34 is important for the cell growth.

Feaver et al. (1999) generated yeast strain carrying a temperature sensitive p34 allele. That yeast revealed UV sensitivity, impaired NER *in vitro*, as well as defected RNA polymerase II dependent transcription. This way, p34 was shown to be important both for the DNA repair and transcription. However, the exact function of p34 in transcription and NER remains unknown. Perhaps, the role of p34 there is not direct, and is important, as this protein is a structural part of TFIIH complex.

Tfb4 (p34) interacts with another TFIIH subunit, Ssl1 (p44) (Feaver et al., 2000). When yeast TFIIH expression was firstly established in the insect cells (Sf21) as a complex (Takagi et al., 2003), it became clear that Tfb4 (p34) is an important core component stabilizing the factor. If p34 was replaced by MAT1 (Tfb3), initially reported as TFIIH core protein in yeast, oligopeptides expressed in the insect cells rapidly degraded. Immunoprecipitation with antibody to p34 confirmed the presence in the TFIIH core complex Rad3 (XPD), Ssll1 (p44), Tfb1 (p62) and Tfb2 (p52), together with Tfb4 (p34) (Takagi et al., 2003).

p8/TTD-A

Applying the method of quantitative proteomics, Ranish et al. (2003) analyzed a yeast RNA polymerase II preinitiation complex. A small protein with 72 amino acids and 8 kDa (p8), has been identified (Ranish et al., 2004) as a product of uncharacterized open-reading frame YDR079c-a. Later, the polypeptide was confirmed as *bona fide* component of RNA polymerase II preinitiation complex, required for efficient transcription both *in vitro* and *in vivo* (Ranish et al., 2004). In addition, YDRO79c-a was shown to be a part of transcription factor II H (TFIIH) and required for the TFIIH recruitment to the promoter region. Yeast lacking YDR079c-a grow slowly and are sensitive to UV light, as others TFIIH mutated strains. This 8 kDa polypeptide is evolutionarily conserved. Mutations in the human orthologue of this gene lead to DNA repair deficient form of the trichothiodystrophy disorder called TTD-A (Giglia-Mari et al., 2004).

In the p8-deficient human cells the level of TFIIH complex is significantly reduced if compare with the wild type cells (Vermeulen et al., 2000). Microinjections of human cDNA encoding TFB5 (*GTF2H5*, also called TTD-A) corrected the DNA repair defect of TTD-A cells. Also, three functional inactivating mutations in this gene in three unrelated families with TTD syndrome group A have been described (Giglia-Mari et al., 2004). Correspondent cell lines are TTD99RO (R56stop), TTD1BR (R56stop + L21P), and TTD13/14PV (M1T – no translation start). These findings identified the p8 as an important factor in the DNA repair, transcription and human disorders formation. Also, it became clear, that the mutations in *GTF2H5* gene causes the NER defect in the TTD-A and provide an opportunity to begin to dissect the molecular details underlying the disease.

Later, Coin et al. (2006) described human p8 as a DNA repair specific factor. It stimulates XPB ATPase activity together with a damage recognition factor XPC–HR23B. Contrary, p8 is not important for the RNA synthesis and does not interfere with the transcription TFIIH sub-complex CAK, although both interact with XPD subunit. Overexpression of p8 in the XPD deficient cells increased cellular TFIIH concentration (Coin et al. 2006), and TFIIH-stabilizing function of TTD-A/p8 subunit has been proposed.

For further understanding the p8 function in the cells, Giglia-Mari et al. (2006) labeled this protein with green fluorescent protein (GFP) tag. Under non-challenging conditions, the p8–GFP protein is present in two distinct kinetic pools: one bound to TFIIH and another, free fraction that shuttles between cytoplasm and nucleus. After induction of NER specific DNA lesions, the equilibrium between these two pools dramatically shifts towards a more stable association of p8 to TFIIH. Modulating transcriptional activity in cells (Giglia-Mari et al., 2006) did not induce a similar shift in this equilibrium. Surprisingly, DNA conformations that only provoke an abortive-type of NER reaction do not result into a more stable incorporation of p8 into TFIIH. These findings also identify p8 as NER specific TFIIH subunit *ex vivo*, and indicate that its interaction with TFIIH reflects productive NER.

Finally, the structure of p8 alone or in the complex with p52 TFIIH subunit has been solved (Vitorino et al., 2007; Kainov et al., 2008). In the first work, using a nuclear magnetic resonance (NMR), p8/TTD-A was described as a small alpha/beta protein built around an antiparallel beta-sheet that forms a homodimer with an extended interface. Mutation at position F44A destabilized the dimeric form of the protein, and had no effect on the NER activity *in vitr*o. However, it affects the capacity of p8 to restore TFIIH concentration in TTD-A fibroblasts (Vitorino et al., 2007). Later, the crystal structure of minimal p8-p52 yeast complex was solved (Kainov et al., 2008). The structure revealed that these two polypeptides adopt the same fold, forming a compact pseudosymmetric heterodimer via a beta-strand addition and coiled coils interactions between terminal alpha-helices. Furthermore, p8 protects a hydrophobic surface in p52 from solvent. This provides a rationale for the influence of p8 in the stabilization of p52 and explains why mutations that weaken p8-p52 interactions lead to a reduced intracellular TFIIH concentration and a NER defect.

In addition, this p8–p52 structure explains well the observation made in p52-deficient *Drosophila melanogaster* (Aguilar-Fuentes et al., 2008). Drosophila mutants in the Dmp52 TFIIH subunit exhibit phenotypic effects similar to those observed in TTD patients with defected p8 or XPD, including reduced levels of TFIIH. Several p52 phenotypes, including lethality, developmental defects and sterility, can be suppressed by overexpression of p8. TFIIH level is also recovered in rescued flies. In addition, p8

overexpression suppresses a lethal allele of the Drosophila XPB homologue. Furthermore, transgenic flies expressing p8 are more resistant to UV irradiation than wild type flies are. Probably, this can be explained by the more efficient CPD and 6-4PP photolesions repair. Thus, p8 can complement mutated XPD, XPB, and p52 subunits, stabilize TFIIH and correct or enhance NER activity of this complex.

CAK

CAK is Cyclin-dependent kinase – activating kinase. Cdk7 is a kinase phosphorylating the serine 5 and 7 residues of the RNA polymerase II C-terminal domain (CTD). Activity of Cdk7 is regulated by cyclin H, MAT1, TFIIE, Mediator (Svejstrup et al., 1996), XPD (Keriel et al., 2002), and U1 small nuclear RNA (snRNA, O'Gorman et al., 2005). The Cdk7-cyclin H-MAT1 complex in the context of TFIIH has higher activity in phosphorylating the CTD compared with the free form of CAK (Yankulov and Bentley, 1997). Phosphorylation of serine 5 leads to the recruitment of 5' capping enzyme (Cho et al., 1997) and is implicated in promoter clearance. That CTD phosphorylation regulates the transition from transcription initiation to elongation. RNA polymerase II enters pre-initiation complex (PIC) assembly as the hypophosphorylated IIA form and escapes the promoter as the hyperphosphorylated IIO form (Hampsey, 1998). Besides phosphorylating CTD, TFIIH CAK has been shown to phosphorylate transcripton activators, such as p53 (Lu et al., 1997), retinoic acid receptor alpha (Rochette-Egly et al., 1997), retinoic acid receptor gamma, Ets-1, estrogen receptor alpha, and general cofactor PC-4 (reviewed in Thomas and Chiang, 2006).

Through the MAT1–XPD interaction CAK is linked to the core-TFIIH (Coin et al., 1999). After the genotoxic attack the kinase activity of the complex is reduced (Adamczewski et al., 1996). When core TFIIH participates to DNA repair, the CAK is leaving chromatin (Figure 11). Following the DNA repair (NER), CAK is again associated with the chromatin fraction of core-TFIIH (Publication 2).

Cdk7

Cdk7 (MO15; Kin28 in yeast), as others cyclin-dependent kinases (Cdk), is a member of Serine/threonine protein kinase family. Cdk first appeared as the controllers of two major transitions of the cell cycle: initiation of the DNA synthesis (S) phase and

entry into mitosis (M). Subsequently, Cdk-cyclin complexes were also identified as conserved components of RNA polymerase II transcription machinery. Two sets of Cdk were described in metazoans. First, Cdk1, Cdk2, Cdk4 and Cdk6, which are dedicated to cell division control. Second, Cdk8 and Cdk9, which are primarily implicated in the transcription. Cdk7 is both a Cdk-activating kinase, which phosphorylates cell cycle cyclin-dependent kinases within the activation segment (T-loop), and a component of a general transcription factor TFIIH, phosphorylating the C-terminal domain of RNA polymerase II (reviewed by Harper and Elledge, 1998).

Early studies of Cdk regulation revealed phosphorylation within the T-loops of Cdk (Krek and Nigg, 1991), and a genetic requirement for such phosphorylation in *S. pombe* cell cycle progression (Gould et al., 1991). Studies the Cdk activation *in vitro* indicated that both T-loop phosphorylation and cyclin binding are required to generate high levels of kinase activity and that metazoan cell extracts contain CAK activity. Biochemical purification of CAK from animal cell extracts led to the first identification of Cdk7 complex, and its reconstitution *in vitro* from recombinant Cdk7 and its partner cyclin H (Fesquet et al., 1993). Cdk7, cyclin H and the RING finger protein MAT1 were found to be associated with TFIIH (Roy et al., 1994) as was the yeast ortholog of Cdk7, Kin28 (Feaver et al., 1994). Kin28 has no CAK activity *in vitro* and it mutants have no cell cycle progression defects (Cismowski et al., 1995; Valay et al., 1995). The CAK of *S. cerevisiae* even was described as Cak1, a single-subunit kinase related very distantly to the CDK. Because of conservation of transcription function between CAK and Cak1 (Cdk7 and Kin28), it was suggested that Cdk7 might be a CAK only *in vitro*, and Cak1-like kinase have to be identified in higher eukaryotes (Kaldis et al., 1996). However, no Cak1-like protein appeared upon sequencing of human, worm and fly genomes (Murray and Marks, 2001). Genetics also provide data that Cdk7 is a CAK *in vivo*. In *Drosophila*, inactivation of temperature-sensitive Cdk7 mutant (P140S) specifically prevents activation of Cdk1 by T-loop phosphorylation and causes embryonic and larval lethality and a block to mitosis in the germline. Adult flies bearing the temperature-sensitive allele are viable, and apart from a cessation of egg laying, are phenotypically nearly normal (Larochelle et al., 1998). Both in flies and worms Cdk7 mutations lead to the phenotype

similar to the Cdk1 mutations, linking these two kinases in one process (reviewed in Fisher, 2006).

The two enzymatic functions of Cdk7 are quite different. In the cell cycle role, Cdk7 is an upstream regulator, causing a conformational change. It boosts Cdk activity about 300 folds in the case of the human Cdk2-cyclin A complex (Russo et al., 1996). In transcription, Cdk7 apparently behaves more like one of its targets, as a downstream effector kinase. The heptad repeat unit of CTD consists of the sequence $Y\mathbf{S_2}PT\mathbf{S_5}P\mathbf{S_7}$, within which Cdk7 is selective to residues serine 5 (Roy et al., 1994) and serine 7 (Akhtar et al., 2009). Cdk7 probably adopts different mechanism to target its Cdk substrates (Cdk1, Cdk2, Cdk4 and Cdk6).

Early reports suggested that Cdk7 changes its substrate specificity upon binding to MAT1 or integrating to the TFIIH complex (Adamczewski et al., 1996; Rossignol et al., 1997). Subsequent studies revealed that CTD kinase activity is selectively stimulated, with no loss of CAK activity. This requires both the presence of MAT1 and phosphorylation of Cdk7 itself at its activating residue, Thr170 (Larochelle et al., 2001). It is unlikely that the activity of Cdk7 is influenced by cycles of MAT1 association/dissociation, but its regulation by T-loop phosphorylation is quite probable. Mutations of *Drosophila* Cdk7 (T170A) lead to anomalies in gene expression during embryogenesis and morphogenetic defects of the wing when the mutant protein is overexpressed (Leclerc et al., 2000). The Serine 164 of Cdk7 is a proven site for the phosphorylation by Cdk1 and Cdk2 (reviewed in Fisher, 2006), and this phosphorylation may inhibit the Cdk7 activity during the cell cycle (Akoulitchev and Reinberg, 1998). Another proposed mode of CAK inhibition is the phosphorylation of cyclin H by Cdk7 (Lolli et al., 2004).

Cyclin H

The activity of Cyclin-dependent kinases is regulated by transiently associated cyclines, binding of regulatory protein or phosphorylation/dephosphorylation reactions (Morgan, 1995). Such association leads to phosphorylation of specific protein substrates, involved in DNA replication or transcription. Different cyclin-kinase pairs are described. Among them are cyclin A–Cdk2, cyclin B–Cdk1, cyclin D–Cdk6, cyclin D–Cdk4, cyclin

E–Cdk2, cyclin H–Cdk7. This association is followed by phosphorylation of the kinase at the threonine located between amino acids 160 and 170, by a distinct kinase activity (Cdk-activating kinase, CAK).

Human cyclin H (Ccl1 yeast homologue) is a polypeptide which consists of 323 amino acids, with molecular weight 38 kDa. When cyclin H is associated with Cdk7, the kinase is able to phosphorylate substrates (for instance, Cdk2 or RNA polymerase II CTD). Andersen et al. (1997) presented a crystal structure of cyclin H, shedding light to the composition of TFIIH complex.

Cyclin H and MAT1 were firstly characterized as TFIIH components by Adamczewski et al. (1996). The ternary complex, including also the Cdk7 kinase, has been identified.

Studying yeast TFIIK, a complex with kinase activity, similar to mammalian CAK, the Ccl1 (cyclin H) gene product was described (Svejstrup et al., 1996). It was reported two proteins, 45 kDa and 48 kDa, originated from one gene, probably, because of alternative translation start sites. So, the Kin28 and Ccl1 complex was suggested to be different from the mammalian CAK with three polypeptides. However, the yeast MAT1 protein, Tfb3, is also described (Feaver et al., 1997).

TFIIH interacts with U1 small nuclear RNA (Kwek et al., 2002). Later, O'Gorman et al. (2005) confirmed and further described U1–cyclin H association. snRNA U1 is supposed to regulate TFIIH kinase activity through interaction with CAK.

MAT1

The MAT1 (yeast Tfb3) protein, "ménage à trois" is a third component of CAK complex, known to stabilize Cdk7–cyclin H complex (Devault et al., 1995) and to link CAK to XPD subunit of TFIIH (Sandrock and Egly, 2001). This third component of CAK was a RING finger protein with a characteristic domain at the N-terminal part. Stabilization of CAK by MAT1 does not involve phosphorylation of threonine Cdk7 T176, as mutation T176A still provided three-component complex formation. Analysis of the starfish and *Xenopus* MAT1 (Devault et al., 1995) showed that the RING finger domain has 68% of similarity, while the rest molecule keeps only 45%.

Both the RING finger N-terminal domain and central coiled-coil domain are potentially involved in protein-protein interactions. The MAT1 interacts with Cdk7 through the hydrophobic C-terminal domain, and this region is sufficient to activate Cdk7 towards synthetic substrates phosphorylation. The RING finger motif is not important for the TFIIH complex formation, but is required for the CTD phosphorylation and basal transcription activity (Busso et al., 2000).

MAT1 is able to interact separately with either Cdk7 or cyclin H. In a structural model of Andersen et al. (1997), there is a vacant cleft between Cdk7 and cyclin H. In this model, MAT1 could bridge and stabilize the complex. This binding mainly involves C-terminal part of MAT1 (Busso et al., 2000). In addition, it was proposed that coiled coil central region of MAT1 may interact with XPB and XPD subunits of TFIIH (Busso et al., 2000). Later, it was proved for XPD subunit (Sandrock and Egly, 2001). Having demonstrated, that MAT1 is crucial for the formation of CAK–XPD interaction, the question remained concerning the domain of MAT1 involved in the interaction. There are three parts of MAT1, the N-terminal RING finger domain (1–66), the central coiled-coil domain (67–189) and the C-terminal hydrophobic domain (191–309). Sandrock and Egly, (2001) proposed that coiled-coil domain interacts with XPD, while hydrophobic one – with Cdk7 and cyclin H. Is there any biological meaning of the CAK–XPD interaction? The known role for XPD in transcription and NER is 5'–>3' unwinding of DNA around either start site or lesion, respectively. This activity is stimulated by p44 TFIIH-core subunit. Surprisingly, the addition of CAK to XPD substantially diminished the XPD helicase activity. The inhibition occurs upon interaction either with MAT1 alone, carried by cyclin H or carried by both Cdk7 and cyclin H. However, when p44 is also associated with XPD, there is no CAK-induced inhibition of helicase activity (Sandrock and Egly, 2001).

DOT1

Histone methyltransferase from the DOT1 family has several names. They vary depending on organism of origin or the physiological role in correspondent process (Table 1):

Table 1. Examples of proteins from the DOT1 histonemethyltransferase family

Name	Organism or function
DOT1	Disruptor of telomeric silencing 1, *Saccharomyces cerevisiae*
DOT1p	DOT1 homolog, *Schizosaccharomyces pombe*
DOT1L	DOT1-like protein, *Homo sapiens*
Grappa	*Drosophila melanogaster*
HMT	Histone methyltransferase
KMT4	Lysine methyltransfrase 4
mDOT1	Mice DOT1, *Mus musculus*
Pch1	Pachytene 1

Chromatin structure and histone modifications

Chromatin structure is critical to the organization of DNA in eukaryotic cells, and essential for many nuclear processes (Luger, 2006). Chromatin is comprised of the highly conserved histone proteins, which are assembled along the DNA strand (Figure 8). The chromatin fiber can adopt a variety of structural forms, ranging from highly condensed heterochromatin to weakly condensed euchromatin, which can influence the functionality of the DNA. A wide variety of nuclear activities, including transcription, replication and DNA repair are influenced and regulated by the manner of chromatin organisation. An understanding of these processes will be more complete in the context of chromatin.

There are four core histones, H2A, H2B, H3 and H4, which assemble as heterodimers to form an octameric complex (Davey et al., 2002). Each octamer is wrapped with a DNA sequence about 146 basepairs long. DNA makes 1.65 superhelical

turns around the octamer to form nucleosome. Later it is further secured by non-core histone H1. Histones are highly targeted for posttranslation modifications, including acetylation, methylation, phosphorylation, ubiquitilation, and sumoylation (Kouzarides 2007). The majority of these modifications are made to amino acids residues in the histone N and C termini, although modification sites have also been identified on the surface of the nucleosome core. These modifications are believed to serve as regulators to influence chromatin structure and function. It was proposed that specific combinations of modifications serve as "histone code" to influence specific nuclear processes (Jenuwein and Allis, 2001). Histone acetylation serves as a key regulator of transcription and correlates with transcriptionally active domains, although distinct acetylation patterns are believed to underlie specific transcriptional functions (Li et al., 2007). Histone phosphorylation may be important for the chromatin condensation prior mitosis (McManus and Hendzel, 2006).

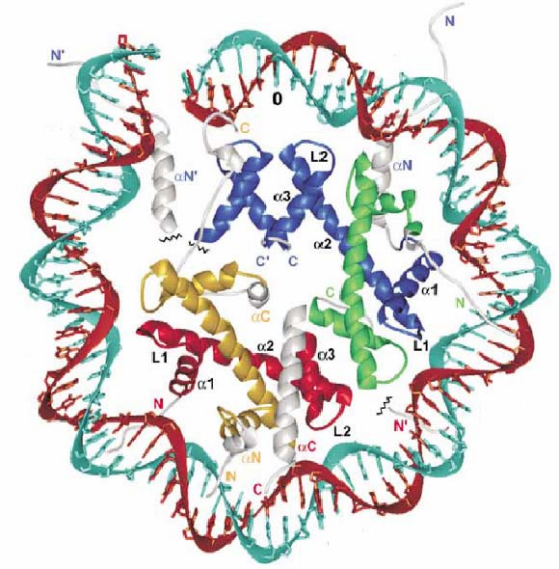

Figure 8. **Structure of the 147 bp nucleosome core particle**.
View down the DNA superhelix axis. The DNA strands are cyan and brown. The histone-fold domains of the histone proteins are **blue for H3**, **green for H4**, **yellow for H2A** and **red for H2B**. The histone-fold extensions and N-terminal tail regions shown are white.
Adapted from Davey et al., (2002).

Histone modifications are also important for the DNA repair pathways (Figure 9). Some of these modifications are involved in chromatin remodeling to enable repair proteins to recognize and gain access to damaged DNA (Thoma, 2005). Others are likely serve to regulate the binding of damage response proteins to chromatin (Downs, 2007). One of the modifications is phosphorylation of histone variant H2AX that is found in higher eukaryotes (Fillingham et al., 2006). It is observed shortly after induction of

double-stranded DNA breaks and serves as a signal to trigger various checkpoint and repair processes (Rogakou et al., 1998; Cowell et al., 2007). Histone acetylation has also been proposed as an important damage response modification. Histone acetyltransferases Esa1/Tip60, Hat1, and Gcn5 are involved in DNA repair (Ikura et al., 2000; Bird et al., 2002; Qin and Parthun, 2002; Teng et al., 2002; Qin and Parthun, 2006). Hyperacetylation of histones in response to DNA damage has been observed, and studies in yeast indicate that these modifications play direct roles in repair rather than influence indirectly through effects on transcription (Yu et al., 2005).

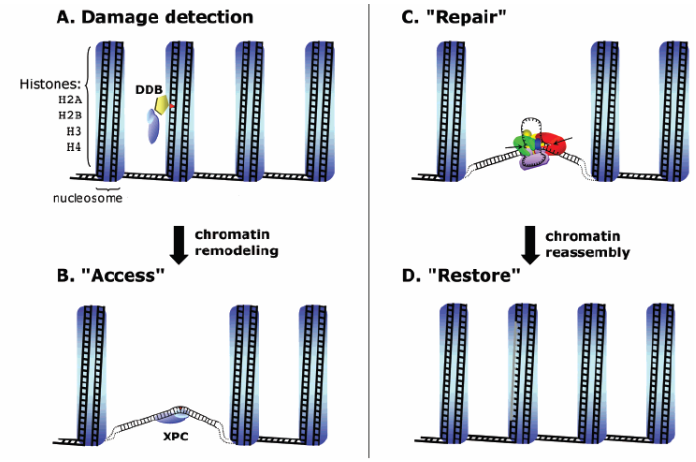

Figure 9. **Model for DNA repair (NER) in the chromatin context**.
A. The damage is detected (for instance by DDB, XPC-HR23B). **B.** Nucleosome unfolding. DNA is accessible for repair machinery. **C**. DNA repair. **D**. Re-assembly of nucleosome and chromatin (Adapted from Gillet and Scharer, 2006).

Histone methylation has emerged as an important player in regulating gene expression and chromatin function. Histone methylation occurs on arginine and lysine residues at the N-terminal tails of histones H3 and H4 and is catalyzed by three distinct families of proteins. They include PRMT1 (protein arginine methyltransferase 1), SET-domain containing family of lysine methyltransferases, and DOT1 family. DOT1 is also lysine methyltransferase, but has no SET domain.

Methylation of histones is important for the transcription regulation and DNA repair. For instance, transcription activaton correlates with methylation of histone 3 lysines K4, K36 and K79. Contrary, methylation of lysines H3 K9 and K27 is associated with low transcription level. Methylation of H3 K79 is also important for the checkpoints in DNA repair and meiosis, as well as survival after X-ray and UV light irradiation in yeast. The only described enzyme responsible for the H3K79 methylation is DOT1.

DOT1

DOT1 was originally identified in a genetic screen for genes which overexpression disrupts telomeric silencing (Singer et al., 1998). The sole yeast DOT1 cDNA isolated in this study was 1882 bp and found to contain ORF encoding the entire 582-amino-acids predicted protein. Disruption or overexpression of DOT1 both impaired telomeric silencing and reduced silencing at mating type and rDNA loci (Singer et al., 1998). DOT1 also plays an important role in meiotic checkpoint control (San-Segundo and Roeder, 2000). DOT1-like proteins are identified in different eukaryotic organisms, including yeast, trypanosome, fly, mouse and human (Feng et al, 2002; Zhang et al., 2004; Shanower et al., 2005).

Human DOT1-like (DOT1L) protein

Feng et al. (2002) identified human DOT1-like protein as a polypeptide including 1537 amino acids residues. Mass of this protein is 165 kDa. The polypeptide is encoded by a gene with chromosome location 19p13.3. Analysis of the hDOT1L protein sequence revealed no known functional motif other than a putative SAM (S-Adenosyle methionine) binding domain. N-terminal part of human DOT1L protein, 1-472 amino acids, exhibits significant HMT (histone methyltransferase) activity *in vitro* in the presence of nucleosomes. N-terminal hDOT1L peptide lost its activity when highly conserved amino acids GSG(163-165) were changed to RCR (Feng et al., 2002).

DOT1 protein in mice

BLAST search with the conserved Dot1 methyltransferase motif against the mouse database produced three significant alignments (Zhang et al., 2004). After extensive search and further alignment procedures, the open reading frame to encode 1540 amino acids was predicted. This protein was named mDOT1a. Also, as a combination of different exones and as a result of alternative splicing several isoforms were proposed. There are mDOT1b, mDOT1c, mDOT1d, and mDOT1e. Comparison of mDOT1a cDNA with human hDOT1L and yeast yDOT1 demonstrates that they encode protein consisting of 1540, 1537 and 582 amino acids respectively. Both proteins mDOT1a and hDOT1L share 84% identity, but are similar to yeast DOT1 only around the methyltransferase motif. The N-terminal region (amino acids 1-353) is extreamly

conserved with only one amino acid change between mDOT1a and hDOT1L. In addition, a putative leucine zipper motif is present in mDOT1a and hDOT1L (amino acids 574-595 and 576-597, respectively), but is absent from yDOT1. Mammalian DOT proteins may have additional functions ascribed to leucine zippers, such as DNA-binding activity. The mDOT1a and hDOT1L are most divergent in two small regions, residues 789-840 and 1439-1469. They have only 57% identity there. The physiological role of these regions is not clear.

The mouse mDOT1 mRNA was identified in different tissues and organs (lymph node, diaphragm, retina, testis, mammary gland, placenta and Wolffian duct). Also, mRNA was detected during the different steps of embryonic development, starting from *in vitro* fertilized eggs and two-cell stage to 19.5 days organism.

Overexpression of plasmid encoding mDOT1a fused with EGFP increased histone H3K79 methylation. Similar results were obtained using only N-terminal part of mDOT1a (2-478 amino acids). At the same time, mutations in the putative SAM-binding site (GSG to RCR) abolished the methyltransferase activity of both full-size and truncated mDOT1a, as was shown for human DOT1L by Ng et al. (2002).

Grappa

The Drosophila protein GRAPPA (gpp) was identified as methyltransferase required for histone H3 lysine 79 methylation (Shanower et al., 2005).

The homeotic genes of the *Antennapedia* (ANT-C) and *Bithorax* (BX-C) complexes are responsible for specifying parasegment identity in the fly. Early in development *gap* and *pair-rule* genes initiate parasegment-specific patterns of ANT-C and BX-C gene activity. The expression pattern established during the initiation phase is then sustained during development by a maintenance system consisting of the *tritorax-group* (trx-G) and the *Polycomb-group* (Pc-G) genes, which have antagonistic functions (Kennison, 1995; Simon and Tamkun, 2002). Proteins from trx-G are required to maintain gene activity. Conversely, Pc-G proteins function as silencers. The antagonistic activities and phenotypes associated with trx-G and Pc-G genes are a function of their distinct effect on chromatin structure. The trx-G protein ASH1 is a histone methyltransferase that modifies H3K4 and H4K20 (Beisel et al., 2002). It is believed that

this epigenetic mark functions to recruit another trx-G protein, Brahma, the fly homolog of SNF2/SWI2 protein, which facilitates transcription via chromatin remodeling. Like ASH1, the Pc-G protein E(Z) is also a histone methyltransferase methylating H3K9 and H3K27 (Czermin et al., 2002). Nucleosomes possessing histone H3 methylated at these sites function to recruit Polycomb and other components of Pc-G silencing complex.

In genetic screens designed to identify novel factors required for Pc-G-mediated silencing, Shanower et al. (2005) discovered several alleles of a Drosophila gene grappa (gpp). It has an unusual property of exhibiting phenotypes and genetic interactions that are characteristic of both Pc-G and trx-G genes and is a memberof Enhancer of *trithorax* and *polycomb* class of genes (ETP). GRAPPA mutants (*gpp*) dominantly suppress silencing by telomeric, but not centromeric heterochromatin.

Potentially, there could be 12 different mRNAs coding for GRAPPA, but practically only 6 of have been identified (Shanower et al., 2005). These mRNAs code for proteins with a predicted mass ranging from 171 to 232 kDa (1848 amino acids protein was described by List et al., 2009). Common N-terminal domain of GPP shares similarity (about 42%) to the *Saccharomyces cerevisiae* DOT1 protein. GRAPPA has additional protein domains that are not present in yeast DOT1. These include a putative coiled coil domain and ATP/GTP binding domain. Potentially GPP may dimerize or interact with other proteins. In addition, there is a proline-rich region. The coiled-coil motif and similar region with prolines are found in human, *Caenorhabditis elegans*, *Drosophila pseudoobscura*, and *Anopheles gambia* DOT1-like proteins (Shanower et al., 2005).

At the early Drosophila development little if any H3K79 methylation is detected, until germband extended embryos stage (stage 11). In polytene chromosomes, methylated H3K79 is enriched in puffs and interbands, which correspond to active chromatin. At the same time, this methylation is underrepresented at the telomeres (Shanower et al., 2005). Only mono- and di-methylation of H3K79 were reported in fly tissue (Mckittrick et al., 2004). Probably, in the past Drosophila possesses a system similar to Trypanosoma, when second variant of DOT1 protein (DOT1B) is required for the H3K79 trimethylation.

List et al. (2009) have shown that *gpp* mutant flies were short-lived and hypersensitive to oxidative stress (1% H_2O_2). At the same time, overexpression of GRAPPA enhances stress resistance and behavioural activity without affecting the expression level of oxidative stress resistnace genes. When kept in the nutrient-poor conditions, the young wild type flies survived much longer (15% to 38%) if compare to *gpp* mutants. However, in two weeks old animals this difference was not significant.

DOT1A and DOT1B in Trypanosoma

Little is known about chromatin structure, epigenetic regulation and cell cycle control in *Trypanosoma brucei*, the unicellular parasitic protozoan that is responsible for African Sleeping Sickness. Studies in the Cross laboratory suggest that epigenetic mechanisms regulate expression of variant surface glycoproteins (VSG), the major surface proteins of African trypanosomes (Cross et al., 1998). By regularly switching the VSG, trypanosome population escapes the host's immune system. The active VSG gene is transcribed from 1 of about 20 essentially identical telomere-proximal expression sites (Barry and McCulloch, 2001). Trypanosome telomeres repress the reporter gene expression, and this position effect might contribute to the regulation of telomeric VSG genes (Horn and Cross, 1995). DOT1-like histone methyltransferases are involved in the telomeric silencing. In *Trypanosoma brucei*, there are two DOT1 homologues identified, DOT1A and DOT1B (the 33 and 31 kDa polypeptides, respectively, Janzen et al., 2006). They have significant similarity to the catalytic domain of human hDOT1L. *Trypanosoma cruzi* and *Leishmania major* also contain both homologs. DOT1A and DOT1B methylate histone H3 lysine 76 (H3K76, correspondent to H3K79 in yeast and mammals). There are several conserved domains, which are found in DOT1A, DOT1B and others proteins from DOT1 family. One of the most concerved sequences is DxGxGxG motif I, which is found in S-adenosyl-L-methionine (SAM) binding proteins. Dimethylation of H3K76 is a marker for mitosis (Janzen et al., 2006). The methylation of H3K76 is provided only by DOT1B, as there is no such a mark in the DOT1B mutants. Trimethylation was only found in active genes, whereas dimethylation occurs in both active and inactive chromatin. In mice, dimethylation of H3K9 is dependent on the methyltransferase G9a, whereas trimethylation is directed by Suv39h1 and Suv39h2

(Peters et al., 2003; Rice et al., 2003). The different methylation states of H3K9 are associated with different domains of silent chromatin, but the function is the same – gene silencing. In contrast, dimethylation and trimethylation of H3K76 in *T. brucei* appear to affect fundamentally different processes. H3K76me2 by DOT1A is essential for viability. DOT1B is not as important, although its absence causes defects in mitosis (Janzen et al., 2006). DOT1B-dependent trimethylation of H3K76 is required for complete differentiation into the insect stage of the life cycle in culture.

Figuieredo et al. (2008) reported that DOT1B is important for VSG transcriptional regulation. DOT1B is required for complete BES silencing (bloodstream expression sites, located in the telomere regions), but not for others gene expression regulation. In DOT1B deficient cells, two VSG were expressed at the same time. Only with continuous selection the switching was completed. Therefore, the DOT1B is necessary for monoallelic VSG expression and rapid switching kinetics (one telomere re-silencing, and other telomere-located gene activation).

DOT1 and H3K79 methylation

Many damage related histone modifications are found at the N termini, comparable modifications have also been identified within the core portion of these proteins (Altaf et al., 2007). One such modification is methylation of lysine 79 (K79) in histone H3 (H3K76 in trypanosomes), a highly conserved modification that is catalyzed exclusively by the histone methyltransferase DOT1 (Feng et al., 2002; van Leeuwen et al., 2002; Ng et al., 2003). H3K79 methylation exists in distinct states throughout the yeast euchromatin, ranging from monomethylated to trimethylated (van Leeuwen et al., 2002). DOT1 is responsible for adding all three methyl groups to histone H3, while its ability to add the second and the third methyl group to monomethylated K79 is dependent on ubiquitylation of yeast lysine K123 (mammalian K120) in histone H2B (Ng et al., 2002; Sun and Allis, 2002; Shahbazian et al., 2005). H2B ubiquitylation is catalyzed by Rad6 (Robzyk et al., 2000), a ubiquitin conjugating enzyme and one of the regulators of post-replication repair (Broomfield et al., 2001).

Many studies indicate that Rad6/Bre1 mediated H2B ubiquitination is required for histone H3 lysines K4 and K79 methylation both in yeast and higher eukaryotes (Osley,

2006). This histone cross-talk appears to function unidirectional: mutations affecting H2B ubiquitination, including htbK123R, reduce the level of these methylated H3 modifications. At the same time, deletion of either of the relevant methyltransferases, or mutation of the H3 methylation sites, has no reciprocal effect on H2B ubiquitination (Briggs et al., 2002; Sun and Allis, 2002).

In yeast, H3K79 methylation is catalyzed by Dot1 (Feng et al., 2002; Ng et al., 2002; van Leeuwen et al., 2002), and H3K4 methylation by Set1, which functions as part of the COMPASS complex (MLL complex in humans). Set1-COMPASS associates with elongating form of RNA polymerase II in a manner, dependent on the Cdk7 (Kin28) kinase and the PAF complex (Shilatifard, 2006; and Kim and Roeder, 2009). PAF is required for the Rad6 association with the COMPASS and subsequent H2B monoubiquitination (Wood et al., 2003).

H2B ubiquitination specifically affects di- and trimethylation of H3K4 and H3K79 but does not eliminate monomethylation, provided by Set1 and DOT1 methyltransferases, respectively (Shahbazian et al., 2005).

53BP1 and H3K79

In the context of DNA damage, methylated H3K79 serves as a binding site for Rad9/53BP1 (Huyen et al., 2004, Grenon et al., 2007), a central signaling factor in several damage checkpoints (Toh and Lowndes, 2003). H3K79 methylation is important for Rad9 checkpoint function (Giannattasio et al., 2005; Toh et al., 2006). Loss of this methylation causes defects in G1 and intra-S checkpoints in response to double-stranded breaks (Wysocki et al., 2005). H3K79 methylation is not altered in response to double-stranded breaks. It has been proposed that changes in chromatin structure caused by breaks might expose constitutive H3K79 methylation, enabling Rad9/53BP1 to bind chromatin in order to initiate damage response (Huyen et al., 2004). Genetic analysis of DOT1 indicates that H3K79 methylation additionally participates in recombination repair in response to double-stranded breaks (Game et al., 2006), although specific function of this modification in the repair pathway has not been determined.

53BP1/Rad9 is a conserved checkpoint protein with properties of a DNA double-strand breaks (DSB) sensor (Schultz et al., 2000). The structure of the 53BP1 domain that

recruits it to sites of DSB has been recently solved (Huyen et al., 2004). This domain consists of two tandem tudor folds with a deep pocket at their interface formed by residues conserved in the budding yeast Rad9, fission yeast Rhp9/Crb2, and *Caenorhabditis elegans* Hsr-9/TO5F1 orthologues. *In vitro*, the 53BP1 tandem tudor domain binds histone H3 methylated on K79 using residues that form the walls of the pocket. These residues were also required for recruitment of 53BP1 to DSB. Suppression of DOT1L decreases histone H3K79 methylation, and this also inhibits recruitment of 53BP1 to DSB.

DOT1 and UV-irradiation

Methylation of H3K79 is important for the response to damage caused by ultraviolet (UV) radiation in the yeast *Saccharomyces cerevisiae*, with properties that extend beyond its role in Rad9 checkpoint function (Bostelman et al., 2007). UV causes the formation of cyclopyrimidine and 6-4 photoproduct dimers (CPD and 6-4PP, correspondently). These lesions can inhibit transcription and replication. UV damages are repaired by nucleotide excision repair. Recombination repair and post-replication repair (PRR) can additionally serve as bypass pathways to enable the completion of replication of damaged DNA. Deletion of DOT1 causes sensitivity to UV in yeast. Methylation of H3K79 plays overlapping roles in NER and recombination repair (Bostelman et al., 2007), and Rad9 checkpoint.

Mutations of four amino acids close to K79 of histone H3 are shown to provoke sensitivity to UV irradiation (Evans et al., 2008; Chaudhuri et al., 2009). There are histone H3 L70S, E73D, Q76R, and T80A *Saccharomyces cerevisiae* mutants that are slightly more UV sensitive at high (lethal) UV doses (100 – 200 J/m²) than wild type cells. However, after irradiation with 2 – 10 J/m² these mutations shown no effect on the survival, contrary to *rad1* and *rad18* deficient yeast, which did not survive even after these respectively low doses of UV.

Recently, Chaudhuri et al (2009) analyzed NER impact of two lysine residues important for transcription. They produced H3K4R and H3K79R mutations to avoid Set1-dependent and DOT1-dependent histone methylation. H3K4R does not show any additional UV-sensitivity if compare with wild type cells. The double mutant, H3K4R-

K79R is more sensitive to UV irradiation than either of the single mutants. It is possible that H3K79 methylation will compensate for the effect of caused by the loss of H3K4 methylation, or *vice versa*. The combined methylation states of H3K4 and H3K79 may have a cumulatively greater effect on UV sensitivity than either residue individually. It has also been shown previously that H3K79 mehtylation plays a role in UV-induced Rad9-mediated checkpoint function and affects nucleotide excision repair and Rad5 repair pathway (Bostelman et al., 2007). The DNA repair is affected at the silenced loci (in the H3K4R-K79R cells), and it is recovered in the absence of Sir comlex (Sir2 deacetylase, Chaudhuri et al., 2009). It leads to the suggestion, that after UV-irradiation, to open chromatin and to repair DNA at the silen loci, the Set1-COMPASS and DOT1 are required. However, in the absence of Sir complex the chromatin is already accessible for the repair machinery, and additional switch of chromatin state is not required.

Sirt1/Sir2 and DOT1

Sirt1 (sirtuin [silent mating type information regulation 2 homolog S. cerevisiae]-1), as several other Sirt forms, is involved in the protein deacetylation, including histone deacetylation. The yeast homolog is Sir2. This protein was identified in the budding yeast as a crucial gene for regulation of the replicative lifespan. Indeed deletion of Sir2 causes premature senescence, whereas the expression of extra copies of Sir2 extends the yeast replicative lifespan (Sinclair and Guarente, 1997; Kaeberlein et al., 1999). Subsequent studies revealed important roles for Sir2 in lifespan determination in nematodes and fruit flies. In mammalian cells, Sir2 homologue Sirt1 appears to control the cellular response to stress through regulation of the FOXO family of Forkhead transcription factors (Brunet et al., 2004). FOXO family proteins are also involved in insulin/insulin-like growth factors signalling, a major pathway that controls lifespan and stress resistance in worms and flies (Longo and Finch, 2003; Giannakou and Partridge, 2007). However, the mechanism by which the chromatin silencers extend lifespan is not simple. Lifespan extension occurs when Sir2 is overexpressed, and at the same time *Sir2* deletion increase chronological lifespan and inactivation of this enzyme causes the upregulation of many stress resistance genes (Fabrizio et al., 2005).

Silent information regulatory proteins 1-4 mediate transcription silencing in yeast. Deletion of Sir2, Sir3, or Sir4 abolishes both silent loci repression and telomeric position effect (Aparicio et al., 1991). The Sir factors are structurally unrelated to each other. Their recruitment to silenced loci requires the origin recognition complex and sequence specific transcription factors Abf1 and Rap1 that bind flanking silencer elements. Sir1 bridges between origin recognition complex and Sir4. The latter recruits Sir2 and Sir3 (Hoppe et al., 2002). At telomeres, Sir4 is recruited by Rap1 and Ku70/Ku80 complex, a heterodimer that binds the single-strand double-strand DNA junction (Cockell et al., 1995; Fisher and Zakian, 2005). Once nucleated, Sir2, Sir3 and Sir4 appear to spread along nucleosomes to silence promoters up to 3 kb from the telomeric repeat (Strahl-Bolsinger et al., 1997). Whereas Sir3 and Sir4 are assumed to be structural components of silent chromatin, Sir2's histone deacetylase activity is essential for repression (Tanny et al., 1999).

The tails of histones H3 and H4 are primary targets for deacetylation by Sir2 (Imai et al., 2000), which presumably generates high-affinity binding sites for Sir3 and Sir4. Deletion analysis of the N-terminal tail of H4 identified a basic stretch from lysine K16 to K20 that is critical for repression (Kayne et al., 1988). Any basic-to-neutral amino acid change in this region or any substitution at K16 disrupted telomeric repression. However, Sir3 and Sir4 still bind recombinant H4 tails bearing the derepressing H4K16Q mutation (Hecht et al., 1995). This raised the possibility that H4K16 does more than attract Sir proteins. In higher eukaryotes, unmodified H4K16 appears to mediate interactions between neighboring nucleosomes (Dorigo et al., 2003).

Not only histone tail modifications, but also changes near and around histone H3K79, disrupt Sir-mediated repression *in vivo* (Ng et al., 2002). The Sir3 interacts with unmethylated H3K79-containing peptides (Altaf et al., 2007a). Sir2-3-4 complex binding to the unmodified chromatin is only weakly sensitive to the removal of histone tails, probably due to Sir4 high affinity to DNA (Martino et al., 2009). At the same study, Sir3 has been shown to have lower affinity than the holocomplex for the nucleosomal template. By methylating of H3K79 with DOT1 methyltransferase, Martino et al. (2009) found, that the presence of one methylated H3K79 per nucleosome is sufficient to reduce the binding of either Sir3 or Sir2-3-4. The product of Sir2-mediated NAD hydrolysis, O-

acetyl-ADP-ribose (O-AADPR), increases the affinity of Sir3 or SIR holocomplex to chromatin in yeast.

Methylation of histone H3 lysines, H3K4me and H3K79me, is a mark of active chromatin. The absence of this methylation marks will disregulate SIR complex binding to the silent loci, probably because of "dilution" effect. The loss of H3K4 and H3K79 methylation is an intermediate event during the formation of silent chromatin (Yang et al., 2008). The global loss of histone modifications leads to silencing defects by redistributing Sir proteins to inappropriate chromosomal loci. Mutations, causing defects in histone acetylation (H3K14R, H4K8,16R) facilitated Sir protein recruitment, spreading, and silencing at silenced loci (HML, HMR, telomeres), whereas defects in histone methylation did not (Yang et al., 2008). In the absence of H3K79 methylation SIR complex binds across of genome. However, H3K79R with a positive charge at the position 79, supported usual silencing, leading to the suggestion that other chromatin modifications are also important for the silent chromatin formation (Yang et al., 2008).

Yeast Sir3 binds to the nucleosome by interacting with a basic patch around lysine 16 on the H4 tail (H4K16). Acetylation of H4K16 reverses or prevents this binding (Carmen et al., 2002). DOT1 interacts with the same H4 basic region, R17, H18 and R19 (Altaf et al., 2007a; Fingerman et al., 2007). Binding of DOT1 to this region is not blocked by H4K16 acetylation. This H4 interaction is necessary for DOT1-catalyzed methylation of H3K79 and protection of chromatin from SIR-mediated silencing.

Upon establishment of silencing, lysine methylation at H3K4 and H3K79 decreases following drops in H4K16 acetylation (Katan-Khaykovich and Struhl, 2005). However, it is unclear, whether removal of H3K4 and H3K79 methylation promotes silencing or the loss of this marks is a consequence of silencing. To resolve this issue, Osborne et al., (2009) assayed the kinetics and pattern of silencing establishment in single in single cells lacking DOT1, Set1, Sas2 or the JmjC-domain containing histone demethylase Jhd2. Loss of DOT1 or Set1 methyltransferases increased the spead of silencing establishment. At the same time, loss of Sas2 lysine acetylase (forms H4K16-acetylation, recognized by Sir2) and Jhd2 (H3K4me1,2,3 demethylase) significantly delayed silenced chromatin formation in yeast. As conclusion to the work of Osborne et al. (2009) we may suggest that the demethylation is a limiting step in the silencing

formation. Why absence of acetylase Sas2 decreased the spead of heterochromatin formation? There are two possible answers. First, loss of H4K16 acetylation creates additional chromatin sites for SIR complex binding. This leads to dilution of silenced proteins. Second explanation is that the active deacetylation of H4K16 by Sir2 may guide the SIR complex into an optimal conformation to promote silencing. Studying the establishment of silencing in individual cells, Osborne et al. (2009) supported the model, where the silencing in yeast is completed within two cell cycles.

Relation of histone modifications to leukemia

Leukemia is a cancer of the blood or bone marrow and is characterized by an abnormal proliferation (production by multiplication) of blood cells, usually white blood cells (leukocytes). Leukemia is a broad term covering a spectrum of disease.

Table 2. Human leukemia and genes involved in leukemogenesis

Short name	Explanation
ABL	Acute biphenotypic leukemia
AF4	ALL1 fused gene from chromosome 4
AF9	ALL1 fused gene from chromosome 9
AF10	ALL1 fused gene from chromosome 10
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
CALM	Clathrin assembly lymphoid myeloid leukemia
ENL	Eleven-nineteen leukemia
MLL	Mixed-lineage leukemia
PML	Promyelocytic leukemia
RARA	Retinoic acid receptor alpha (RARα)

The mixed-lineage leukemia gene (MLL, here and later for this part see Table 2) encodes a histone methyltransferase. It can fuse with more than 50 different partners in multiple-lineage leukemias, for instance acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and acute biphenotypic leukemia (ABL). MLL fusions such as MLL-ENL can target either committed myeloid progenitors or hematopoietic stem cells to induce AML but fail to give rise to ALL or ABL (reviewed in Zeisig et al., 2008). MLL-AF4, the most common MLL fusion, is capable to induce lymphoid malignancy (Chen et al., 2006). However, the disease is predominantly limited to mature B cell lymphoma, which is very different from pro-B ALL/ABL.

MLL leukemias, in particular patients with MLL-AF4, respond poorly to current cancer terapeutics. While directly targeting an oncogenic transcription factor has proven to be difficult, emerging evidence indicates that MLL fusions activate downstream target genes via specific recruitment of histone modification enzymes, which can be potential therapeutic targets. DOT1L and PRMT1 (protein arginine methyltransferase 1), two distinctive histone modification enzymes, mediating H3K79 and H4R3 methylation, are independently linked to MLL-mediated pathogenesis (Cheung et al., 2007; Okada et al., 2005). Direct fusion of PRMT1 or part of DOT1L to truncated MLL is sufficient to transform primary hematopoietic cells, and the transformations are dependent on the catalytic activity of these enzymes. Downregulation of PRMT1 expression can specifically suppress transformation mediated by certain MLL fusions, revealing the potential of targeting specific epigenetic modifying enzymes for cancer terapeutics (Cheung et al., 2007). Krivtsov et al. (2008) demonstrated the significance of DOT1L and the associated histone mark for disease classification and activation of critical downstream targets. It is notable that DOT1L recruitment is ubiquitously associated with gene transcription and that a critical level of DOT1L is required for general cell survival (Okada et al., 2005). Hovewer, Krivtsov et al (2008) just related the H3K79 methylation at specific sites with cancerogenesis. As H3K79me correlates with gene expression, still there is a question, whether incorrect DOT1 recruitment leads to the MLL-AF4 related leukemia, or this methylation just reflects activated HOXA gene expression by MLL.

Different situation is in MLL-AF10 leukemias, when AF10 is a binding partner of DOT1L, and attracts it to the chromatin, activated by MLL (Okada et al., 2005). CALM-

AF10 fusion leads to histones hypomethylation, affecting DOT1L association to chromatin (Lin et al., 2009).

An octapeptide motif and a leucine zipper region of AF10 mediate interaction with DOT1. In general, the AF10 gene is involved in two leukemia-associated fusion genes. The t(10;11)(p12;q23) translocation results in the MLL-AF10 fusion and the t(10;11)(p12;q14) translocation results in CALM-AF10 fusion. While MLL-AF10 fusion is only observed in acute leukemia (AML), CALM-AF10 fusions have been reported in both acute lymphoblastic and acute myeloid leukemia (ALL and AML) as well as in lymphoma. The protein encoded by CALM (the Clathrin Assembly Lymphoid Myeloid leukemia gene) binds to clathrin and is involved in clathrin-mediated endocytosis and the trafficking of vesicles between the trans-Golgi network and endosomes. Mutations in the CALM gene are responsible for defects in the hematopoietic system and iron metabolism. The gene product of AF10 (ALL-1 Fused gene from chromosome 10, or MLLT10) is a putative transcription factor with plant homeo domain (PHD) and an OM-LZ (Octapeptide Motif-Leucine Zipper) region close to its C terminus.

Fusion of hDOT1L to MLL is capable of leukemic transformation in a DOT1L histone methyltransferase activity-dependent manner. Moreover, enzymatically defective DOT1L mutant is capable of suppressing growth of MLL-AF10-transformed cells. Transformation of progenitor bone marrow cells by MLL-AF10 or MLL-hDOT1L results in upregulation of a set of leukemia-relevant HOX genes, including HOXA9 (Okada et al., 2005).

Leukemic fusion CALM-AF10 causes global H3K79 hypomethylation by disturbing the association of hDOT1L with chromatin in human and murine cells (Lin et al., 2009). CALM-AF10-induced H3K79 hypomethylation leads to hypersensitivity of cells to radiation and increases chromosomal instability. In addition, HOXA genes are up-regulated in the CALM-AF10 fusion (HOXA5). These leukemia patients have a higher frequency of additional chromosomal aberrations, than patients with an MLL-AF9 or a PML-RARA fusion gene. The findings of Lin et al. (2009) suggest a novel pathway for CALM-AF10 to exert its leukemogenic effect partially by increasing genome instability through alterations in epigenetic marks and indicate that leukemias with

H3K79 hypomethylation might have increased sensitivity to treatment employing DNA damaging agents.

DOT1L in the embryonic stem cells

The chromatin of embryonic stem cells (ESC) has been studied extensively since large set of genes must be coordinately regulated during cellular differentiation and embryos formation. For instance, bivalent domains comprised of overlapping regions of H3K27me and H3K4me mark key developmentally-regulated genes that are poised for later expression (Bernstein et al., 2006). Chromatin is also crucial for other cellular functions such as chromosome dynamics, silencing of heterochromatic regions, and DNA damage responses. These other features of chromatin have been less extensively investigated in ESC.

Mouse embryos lacking DOT1L exhibit prenatal lethality (Botuyan et al., 2006). In addition, embryos homozygous for a targeted mutation in DOT1L exhibit a mid-gestation developmental arrest at 10.5 day (E10.5) with several developmental phenotypes including abnormal angiogenesis, yolk sac abnormalities, and cardiac malformation (Jones et al., 2008). The importance of chromatin for chromosome stability and cell cycle progression has been relatively under-studied in ESC. Since DOT1L is supposed to be involved in transcription as well as chromosomal and cell cycle function across several species and cell-types, Barry et al. (2009) performed a comprehensive investigation of the importance of DOT1L for murine ESC and their differentiated progenitors.

ESC deficient for DOT1L tolerate loss of H3K79 methylation without a substantial impact on proliferation or morphology. However, shortly after differentiation is induced, DOT1L-deficient cells cease proliferating and arrest in G2/M phase of the cell cycle, with increased levels of aneuplody. In addition, many aberrant mitotic spindles occur in DOT1L-deficient cells. Surprisingly, these mitotic and cell cycle defects fail to trigger apoptosis, indicating that mouse ESC lack stringent cell cycle checkpoint conrol during initial stages of differentiation. Transcriptom analysis indicates that DOT1L deficiency causes the misregulation of a select set of genes, including many with known roles in cell cycle control and cellular proliferation as well as markers of endoderm

differentiation. The data indicate requirement of DOT1L function for early stages of ESC differenciation where DOT1L is necessary for faithful execution of mitosis and proper transcription of many genes throughout the genome (Barry et al., 2009).

Publication 1

“Distinct roles for the XPB/p52 and XPD/p44 subcomplexes of TFIIH in damaged DNA opening during Nucleotide Excision Repair”

Publication 1

"Distinct roles for the XPB/p52 and XPD/p44 subcomplexes of TFIIH in damaged DNA opening during Nucleotide Excision Repair"

There are ten subunits in the transcription factor IIH (TFIIH). Two of them, XPB and XPD, possess ATPase and helicase motifs. DNA helicases are proteins that unwind double-strand DNA using nucleoside triphosphate hydrolysis as the energy source. These proteins have seven helicase motifs each. The motifs are characterized by conserved amino acid sequences.

Why there are two proteins with similar function inside the single TFIIH complex? It was hypothesized that XPB and XPD supply opposite direction of DNA unwinding, required for open DNA intermediates in NER. Mutations in the ATP binding site of these proteins inhibit NER *in vitro* and *in vivo* due to the defect in the opening of the damaged DNA structure.

The previous studies focused on the Walker A ATPase motif I, and did not expand to the others motifs (II to VI). Are both XPB and XPD helicases involved in the opening of short DNA duplex around the lesion?

Here, we analyzed the mutations found in XP patients or artificially introduced into the highly conserved domains of XPB and XPD. We found that the DNA opening does not require helicase activity of XPB and is driven by its ATPase, in combination with XPD helicase activity. Futhermore, we demonstrated, that the p52 subunit of TFIIH upregulates the ATPase activity of XPB *via* direct interaction. This p52/XPB interaction is impaired in some of the XP-B patients, and their TFIIH is unable to induce sufficient DNA opening around the lesion.

Our work started the major re-evaluation of the distinct roles of XPB and XPD proteins, including their role in the nucleotide excision repair. In addition, we expanded our knowledge about the function of certain domains of XPB and XPD.

Publication 2

“Nucleotide excision repair driven by the dissociation of CAK from TFIIH”

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“Nucleotide excision repair driven by the dissociation of CAK from TFIIH”

Transcription factor IIH consists of two sub-complexes. First, core, includes XPB, XPD, p62, p52, p44, p34, and p8/TTD-A. Three proteins, Cdk7, cyclin H and MAT1, form CAK, the second sub-complex. TFIIH plays crucial role in both transcription and DNA repair. How does TFIIH complex participate in these two different processes?

We studied the composition of TFIIH in human cells over time after UV irradiation. For this, a chromatin immunoprecipitation followed by western blot was used. We have shown that the CAK dissociates from the core after TFIIH recruitment to the DNA repair complex. At the same time, the core associates with NER-specific factors, including XPA. The latter catalyzes CAK detachment from the core. It triggers DNA opening around the lesion and incision/excision of the damaged oligonucleotide. When NER is completed, repair-specific factors dissociate from TFIIH core. After this, the CAK joins core again. Following UV-irradiation, transcription is inhibited and the CAK is not present on the chromatin. When DNA repair is completed, CAK re-appears on the chromatin, and transcription recovers. In some xeroderma pigmentosum cells, XP-C and XP-A, CAK does not leave the complex, as the repair process is defected at the initial steps. In the others, XP-G and XP-F, CAK do not re-join core-TFIIH, as NER is not completed.

Here, we demonstrated in details the dynamic of TFIIH composition during NER, association and dissociation of the core with other proteins.

Publication 3

“Molecular insights into the recruitment of TFIIH to sites of DNA damage”

Publication 3

“Molecular insights into the recruitment of TFIIH to sites of DNA damage”

Inside ten-subunit TFIIH complex, there are two proteins with helicase and ATPase domains, XPB and XPD. Previously, we have shown that the ATPase activity of XPB and the helicase activity of XPD drive the DNA opening around the damage during nucleotide excision repair. The exact points of action of both the XPB and XPD at NER remained unclear.

The structure of XPB homologue from archea *Archaeoglobus fulgidus* has been recently solved. The structural analysis revealed several new concerved XPB domains and motifs, including R-E-D motif, positively charged Thumb-like domain, and putative damage recognition domain (DRD).

Here, we deepen our knowledge about the mechanism of TFIIH recruitment to the damaged DNA, including the XPB and XPD involvement at each step. We propose a model, where XPB ATPase anchors TFIIH to the DNA, and subsequently, XPD helicase opens DNA around the lesion. In addition, we examine the role of newly described XPB domains and motifs. The mutations in R-E-D and Thumb-like motifs impair ATPase activity of XPB and TFIIH recruitment to the DNA. Contrary, the substitution of conserved amino acids in the DRD domain has no effect on the XPB and TFIIH function.

Publication 4 (in preparation)

Publication 4 (in preparation)

"The long unwinding road: XPB and XPD helicases in damaged DNA opening"

This is invited review to Cell Cycle Journal. It is currently in preparation.

TFIIH participates in the repair complex formation during NER. It binds to the XPC associated with damaged DNA. Then, the ATPase activity of XPB is used to anchor the ten-subunit complex to the DNA, and XPD ATP-dependent helicase opens DNA around the lesion to make it accessible for the XPG and ERCC1-XPF endonucleases, which provides subsequent incision/excision of damaged oligonucleotide (25 to 30 nucleotides long).

Here we discuss our recent data, concerning the regulation of TFIIH activities during the nucleotide excision repair. For instance, TTD-A/p8 subunit of TFIIH interacts with another subunit, p52. This binding leads to complex stabilization and stimulation of the XPB helicase activity. From another side, XPB ATPase is stimulated by XPC, associated with damaged DNA. The XPD helicase is stimulated by p44 subunit of TFIIH. Mutations in XP-B or XP-D patients impairs interaction with p52 and p44, correspondently.

Another question is recognition of damaged DNA during NER. The XPC-HR23B complex recognizes helix distortion. However, there are other candidates for the damage recognition and verification before the irreversible incision step. Among them, there are TFIIH (XPD, XPB or other subunits) and XPA.

Inhibition of TFIIH activities can be used during the cancer chemotherapy. Resistence to the treatment of late cancer stages is related to active nucleotide excision repair. Inhibition of XPB ATPase or XPD ATPase-dependent heicase may improve the medical treatment of many patients with cancer.

Results

“DOT1 histone methyltransferase in UV-light response”

Results (DOT1)

"DOT1 histone methyltransferase in UV-light response"

Methyltransferase DOT1 in yeast and DOT1-like protein in mammals is unique described enzyme that modifies histone H3 lysine K79. In yeast, deletion of DOT1 leads to significant X-ray sensitivity and mild UV-sensitivity, if compare to wild type cells. We initiated this study to clarify: does mammalian DOT1 is required for UV-response? And, if yes, what is the distinct role of DOT1 in this process?

At the first steps of research, we used mice cells with deleted DOT1L as a model. We observed hypersensitivity of these cells to the UV-C light in the survival assay. Moreover, DOT1L$^{-/-}$ cells were able to repair major UV-induced DNA lesions (CPD and 6,4-PP). However, DOT1L appeared to be important for the transcription recovery after genotoxic attack. Additional study is required to understand the role of DOT1L and methylation of H3K79 in the UV-response.

Discussion

TFIIH is a dynamic complex

Mammalian TFIIH is usually characterized as a complex of ten polypeptides (Figures 10 and 11). It includes core subunits XPB, p62, p52, p44, p34, and XPD, which links core to the CAK sub-complex (Cdk7, cyclin H and MAT1). Cyclin H interacts with snRNA U1, which is regulatory component of TFIIH kinase activity. However, at the different stages of cell life, the composition of TFIIH is not stable. It constantly associates with other transcription factors, RNA polymerase II and activators, including Gal4–VP16, E2F1, Rb, p53, ERα (estrogen receptor alpha), RARα (retinoic acid receptor alpha), RARγ, and AR (androgen receptor) (reviewed by Zurita and Merino, 2003; Thomas and Chiang, 2006), TFIIE (Okuda et al., 2008), and NER factors (Publication 2).

The XPG endonuclease forms a stable protein complex with TFIIH. This endonuclease maintains integrity of the complex in cooperation with XPD (Ito et al., 2007). The mutations of XPG found in the patients with severe xeroderma pigmentosum G (XP-G) and XP-G associated with Cockayne syndrome (XP-G/CS) resulted in an alteration of the architecture of TFIIH. It leads to the deregulation of gene expression, as illustrated in the case of transactivation by estrogene receptor alpha (ERα). XPG plays crucial role in transcription assisting TFIIH. The features of CS in XP-G/CS are at least partly because of transcriptional defect (Ito et al., 2007).

Following the genotoxic attack, CAK sub-complex disassociates from the chromatin fraction of core-TFIIH (Publication 2). Why this re-organization of TFIIH does occur? During the NER, XPD helicase opens the double-stranded DNA around the lesion. It was shown, that CAK (or MAT1 alone) inhibits XPD activity (Sandrock and Egly, 2001). Thus, the CAK leaving seems to be logical, when the DNA repair process is active. Contrary, the p44 TFIIH subunit stimulates XPD helicase activity (Coin et al., 1998), and alone with CAK dissociation, the p44–XPD interaction may have stronger impact on NER.

During the RNA polymerase II transcription, the presence of CAK stimulates this process, as it phosphorylates serines 5 (and serine 7) at the C-terminal domain of

polymerase subunit Rpb1. At this case, the XPD helicase activity might be lower than optimal. This helicase is important for NER, and is not required for the transcription initiation (Tirode et al., 1999). The DNA unwinding during transcription is performed by RNA polymerase II itself (Gnatt et al., 2001).

Transcription factor II D (TFIID) interacts with TFIIH, inhibiting the CAK kinase activity after RNA polymerase II phosphorylation (Gegonne et al., 2008). Why it would be important? In addition to TFIID role in pre-initiation complex, it inhibits transcription elongation and a switch between initiation and elongation, until transcription elongation complex is formed.

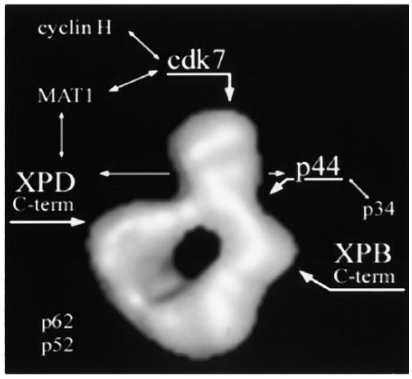

Figure 10. **Model of the TFIIH organization**.
The positions of subunits Cdk7, XPD, XPB, and p44 are indicated by arrows. The cyclin H, MAT1, and p34 subunits are positioned on the model according to intersubunit connection studies. p8 is associated with p52. Adapted from Schultz et al. (2000)

In addition to ten subunits TFIIH complex, the core-TFIIH (seven subunits) is detected during the NER at the chromatin fraction (Publication 2). In addition, free CAK and XPD–CAK are found as independent cellular polypeptide associations (Reardon et al., 1996). The CAK activity is different at different states: when it is free, upon association with XPD, and as a part of TFIIH complex (Adamczewski et al., 1996; Reardon et al., 1996; Rossignol et al., 1997).

The smallest TFIIH polypeptide p8/TTD-A, is also in the dynamic association with core complex. As was described by Giglia-Mari et al. (2006), p8 is quite mobile. In addition to nuclear fraction of p8/TTD-A, there is large fraction of the polypeptide detected in the cytoplasm under physiological conditions. During the NER, the equilibrium between nuclear and cytoplasmic p8 shifts dramatically, and polypeptide is associated with TFIIH. It marks p8 as NER-specific TFIIH subunit, which association with the complex is required during DNA repair.

TFIIH in the NER

During Nucleotide Excision Repair (Figures 6, 7 and 12) the XPC/HR23B/Centrine2 complex is recruited to the intact DNA strand (Min and

Pavletich, 2007; Camenisch et al., 2009) opposed the lesion. XPC attracts TFIIH-core, interacting with p62 and XPB subunits (Bernardes de Jesus et al., 2008). In addition, intact XPC is important for the fast and efficient XPA recruitment to the DNA repair complex, either because of direct XPC-XPA interaction or because of proper TFIIH recruitment, which precedes XPA binding (Bernardes de Jesus et al., 2008).

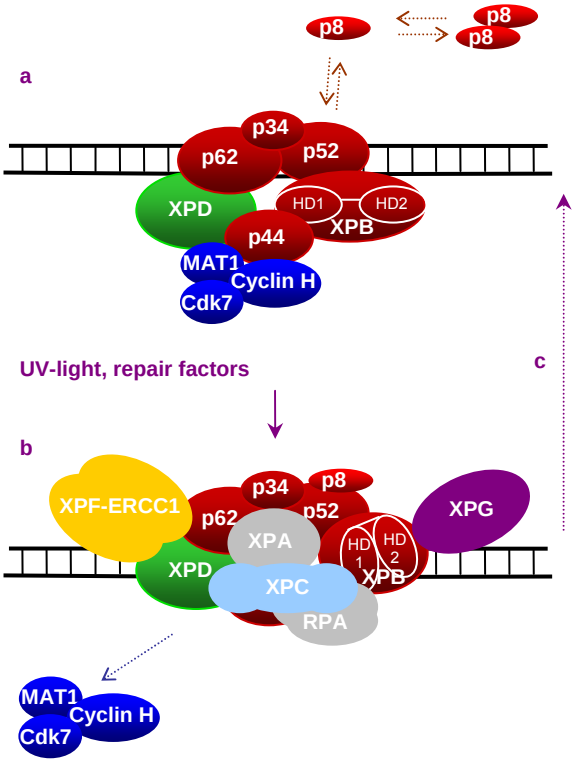

Figure 11. **TFIIH transformation after UV-irradiation**.
(**a**) Nine-subunit TFIIH is associated with chromatin fraction as a part of transcription machinery. CAK sub-coplex is bond to core via MAT1-XPD interaction. TTD-A/p8 is in dynamic association with core, it interacts with p52. Free TTD-A/p8 may be present as monomer or dimer. HD1 and HD2 domains of XPB subunits are marked.
(**b**) After UV-irradiation, TTD-A/p8 stably binds p52; p52 activates XPB ATPase activity, and HD1-HD2 domains turn (170°), anchoring TFIIH on the DNA. CAK leaves complex in an XPA-dependent manner. Other factors, including XPA, XPC, XPG, XPF/ERCC1 and RPA are stably associated with TFIIH-core during DNA repair.
When DNA repair is completed, TFIIH comes back to the initial state (**c**).
(Summarized from Publication 2, Publication 3, Giglia-Mari et al., 2006; Vitorino et al., 2007 and Kainov et al., 2008).

Attracted by XPC, TFIIH joins the NER complex. XPB subunit changes its conformation in ATP-dependent manner (Figures 11-12). There is a turn about 170°, when the angle is created between the helicase domains HD1 and HD2 (Fan et al., 2006). As result of this conformational modification, the conserved motif R-E-D of helicase domain HD1 moves closer to positively charged flexible domain Thumb, situated at the HD2. It is not clear, whether or not there is charge-based interaction between the R-E-D and Thumb regions. Hovewer, the Thumb motif enters between the DNA strands and fixes TFIIH factor inside the repair complex (Fan et al., 2006 and Publication 3). Recruitment of TFIIH to the damaged chromatin is abolished, when ATPase motif of XPB (K346R) is mutated. The same happens if R-E-D motif (R-A-D, E473A) or Thumb domain (Δ516-526) are mutated. If mutation in Walker A I motif (K346R) impairs

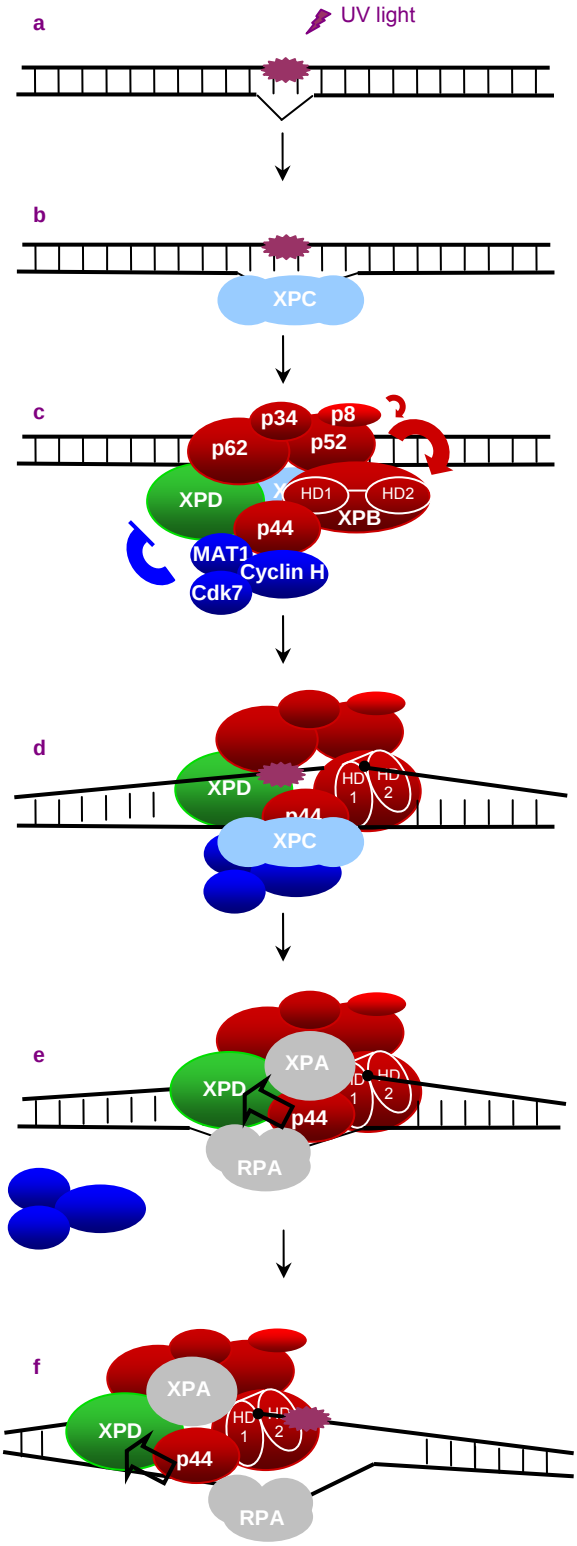

Figure 12. **TFIIH action during the NER.** (**a**). UV-induced DNA lesion (for instance, 6-4PP), which leads to small single-stranded DNA formation. (**b**) Heterotrimer including XPC, HR23B and Centrin 2, is recruited to the intact DNA strand infront of the lesion. (**c**) XPC attracts TFIIH through direct contacts with p62 and XPB. Inside of TFIIH, p8 stabilizes p52 subunit, which in turn stimulates XPB ATPase activity. CAK complex negatively regulates XPD helicase activity. (**d**) p52 stimulates XPB conformational change. Helicase domains HD1 and HD2 turn (170°), anchoring TFIIH to the DNA as an ATP-dependent hook. (**e**) XPA and RPA factors join the complex, attracted by TFIIH and single-stranded DNA, correspondently. Recruitment of XPA triggers CAK dissociation. In a CAK-free complex, p44 stimulates XPD helicase activity, and (**f**) DNA opening extends.

ATPase activity directly, the other two protein modifications could affect this activity indirectly. First, they may interaction with ATPase domain; second, the general conformation change of XPB may abolish proper ATPase function.

These observations suggest that XPB may work primarily as an ATP-dependent conformational switch or as a very limited helicase. Its role in NER may be to open the DNA sufficiently to allow the XPD helicase to gain access to and to extend the single-stranded region around the lesion, in turn allowing structure-specific nucleases to cleave the DNA. For the bacterial XPB-homologue activity, minimum four-nucleotides single-stranded DNA is required (Biswas et al., 2009). It means, XPB needs to be in the contact with any type of DNA to be active (and to provide work conditions for other components of NER machinery, including XPD).

One may ask several questions concerning the R-E-D and Thumb motifs effect on XPB ATPase activity. Why p89 protein has to be intact in those regions for the normal ATPase activity? Why the presence of DNA stimulates XPB ATPase activity? How many cycles of ATP hydrolyze occurs to anchor TFIIH to the DNA? Does the turn of XPB amino acids chain happen before or after the ATP hydrolysis? – If XPB conformation is modified after the interaction with DNA in ATP-independent manner, the subsequent ATP hydrolysis may be facilitated by intact R-E-D and Thumb motifs. It will stabilize the XPB-DNA complex, anchoring TFIIH on the DNA. Contrary, if XPB conformational change happens in the ATP-dependent manner, initial interaction of R-E-D or Thumb with DNA is required to provide efficient reaction only close to DNA.

The conformational change of XPB is similar to SWI/SNF ATPase (Fan et al., 2006). Additional function of XPB inside or outside of NER might be displacement of chromatin-associated proteins. For instance, removal of transcription and repair factors in the damaged regions. The Cockayne syndrome protein B (CSB) has domain composition similar to XPB. It possesses seven helicase motifs and a Thumb-like structure. It also might be an ATP-dependent anchor or participate to proteins displacement from chromatin during the transcription-coupled repair (TCR). However, neither for XPB, nor for CSB these functions are not demonstrated.

Is helicase activity of XPB required for nucleotide excision repair? To obtain an answer, several studies have been performed. There are seven conserved helicase motifs in XPB protein (I, Ia, II, III, IV, V, and VI). Mutation in the helicase motif III strongly impairs XPB DNA unwinding activity *in vitro* (Lin et al., 2005; Publication 1). The helicase motif VI is involved in the interaction with single stranded DNA (Tuteja and Tuteja, 2004).

The helicase domains of XPB are important for the transcription, but not required. We (Publication 1) and Lin et al. (2005) have shown that mutation of helicase motif III (T469A in human XPB and T478A in *Schizosaccharomyces pombe*) and helicase motif VI (Q638A for human and Q647A for *S. pombe*) affect RNA polymerase II transcription efficiency, showing 50% and 20% residual activity, respectively. The mutated helicases impair shift from transcription initiation to elongation (Lin et al., 2005). No negative effect has been observed at the promoter melting during transcription or DNA opening

during nucleotide excision repair. At the same time, mutation of ATPase (human K346R and yeast K354R) affects DNA opening, transcription and repair. When helicase motifs are mutated, ATPase activity is slightly affected (about 80 % of wild type cells activity). Why it happens? It is possible that ATPase activity is affected because of different structure of mutated XPB, when helicase motifs are destabilized. However, it will not explain significant transcription defect. Transcription is stronger affected when helicase motif VI is mutated. As the motif is required for the interaction with DNA, we may suggest that correct XPB–DNA interaction is required for efficient RNA synthesis by polymerase II.

We conclude that even if XPB helicase activity is required for RNA polymerase II transcription, it is not important for the NER. Here we can speak only about the global genome nucleotide excision repair. As XPB helicase activity correlates with transcription level, the efficiency of lesion recognition during transcription-coupled repair could be impaired.

When XPB anchors TFIIH on the DNA, the XPD helicase unwinds double-stranded helix around the lesion. DNA opening after the TFIIH recruitment to the repair complex also provokes questions. Does this opening occur in both directions? Does the process require at least two TFIIH molecules to prepare DNA for incision? Alternatively, one TFIIH is needed and the length of DNA from both sides of the lesion is not equal (asymmetric DNA opening)? Currently, we cannot discriminate between one or two (or more) TFIIH molecules participating to the damaged oligonucleotide excision. It is known that the DNA lesion *per se* modifies DNA structure. It provokes bending and DNA opening around the damaged area (+/- 3 nucleotides; Tapias et al., 2004). Recruitment of XPC/HR23B protein complex leads to further DNA opening. Subsequent accumulation of TFIIH and XPA at the damaged site makes single-stranded DNA region even longer. Simple recruitment of TFIIH to the damaged DNA according to the "XPB anchoring model" (Publication 3) would increase DNA loop around the lesion. However, this increase is not detected using $KMnO_4$ DNA opening assay (Publication 3, Figure 1). Later, XPD helicase expands ssDNA in an ATP-dependent manner. Finally, the lesion appears at the 3' side of the incision product (oligonucleotide), and ERCC1/XPF cuts the DNA first in the XPG-independent way (Staresincic et al., 2009). Does it mean that

TFIIH moves significantly to the 5' side from the lesion? And attracts XPF/ERCC1 complex? Or the DNA opening occurs mostly because of step-by-step proteins accumulation around the damage and depends mostly on the repair complex architecture? Further studies are required to answer these questions.

TFIIH is attracted to the repair complex as nine or ten subunit factor including core and CAK (Publication 2). The variation in subunit number is in agreement with model proposed by Giglia-Mari et al. (2006), when TFIIH is either alone or in the complex with TTD-A/p8, respectively. If the DNA repair process occurs, TTD-A/p8 subunit is stabilized at the p52 (Giglia-Mari et al., 2006; Kainov et al., 2008). At this time, XPA joins the repair complex. According to our unpublished data, XPA may interact with several TFIIH subunits (XPB, p62, p34 and p44; Bernardes de Jesus et al., In preparation). RPA complex stabilizes single-stranded DNA (Wang et al., 2000). The RPA recruitment occurs together with XPA. The XPA/RPA accumulation at the damaged chromatin leads to the CAK sub-complex dissociation from the repair machinery (Publication 2). The XPA-TFIIH interaction occurs through C-terminal region of XPA (amino acids 229-273; Park et al., (1995a)). Deletion of this XPA region leads to the situation, when CAK is not detached from the repair complex on DNA. One can propose that the CAK and the C-terminal part of XPA may compete for the TFIIH region. Nevertheless, it is not a case, as CAK joins TFIIH-core *via* MAT1–XPD interaction (Sandrock and Egly, 2001), and XPA interacts with XPB, p62, p34 and p44 subunits as mentioned above. However, we cannot exclude that XPA and CAK compete for the space inside of repair complex. The CAK leaving during NER is logical, as MAT1 inhibits XPD helicase activity (Sandrock and Egly, 2001). Thus, after CAK dissociation from the repair complex, p44 stimulation of XPD (Coin et al., 1998) can be more efficient.

During the preincision complex formation, nine or ten subunits TFIIH complex became seven-subunit complex without CAK and with TTD-A/p8. In addition, XPC, XPA and XPG proteins of the repair complex are shown to directly interact with TFIIH core. So, there is TFIIH transformation to another ten-subunit complex (XPB, XPD, p62, p52, p44, p34, TTD-A/p8; and XPA, XPC, XPG). However, this complex is stable only during the active repair of DNA lesion (in agreement with time required to remove 6-4

photoproducts, Publication 2). When rapid DNA repair is completed, TFIIH-core again binds CAK sub-complex on the chromatin.

Is there CAK dissociation or degradation during NER? To answer this question, we performed experiments *in vitro*, when degradation is not probable. In addition, we used inhibitor of proteasome MG132 to block protein degradation in cells, and the CAK left chromatin after UV-irradiation (Publication 2). We concluded that the CAK dissociation during DNA repair is an active process, dependent on XPA. Nevertheless, it is not clear, what happens with CAK after dissociation from core-TFIIH. First option, it is present as free CAK during the DNA repair and associates with core again when NER is completed. Another option, after the dissociation CAK degrades, and newly synthesized proteins join the core-TFIIH when DNA is repaired. This situation is not clarified yet.

The ChIP following by Western Blot assay ("ChIP–Western"), which we have used to study core-TFIIH–CAK interaction inside of TFIIH (Publication 2; see also Fousteri et al., 2006), is a perspective method to study other molecular complexes in cells. In frames of nucleotide excision repair, there are several stable protein associations. They include XPF-ERCC1 heterodimer, XPC-HR23B-Centrin heterotrimer and RPA heterotrimer (RPA70-RPA32-RPA14). However, during the reaction these complexes might dissociate or bind other partners. ChIP–Western blot method is also useful to study short-living complexes, such as core-TFIIH associated with XPC, XPA, XPG and, directly or indirectly, with XPF.

The fate of the CAK in cells of xeroderma pigmentosum patients is different. In the case of XP-A, there is no CAK leaving after the DNA is damaged. From one side, the full-size XPA recruitment to the repair complex is required for the CAK dissociation (Publication 2). Truncated XPA (1-228) can stimulate NER *in vitro* in the absence of CAK, but is not able to trigger the CAK leaving (Publication 2). The core–CAK TFIIH complex in the XP-A cells makes impossible further NER progression, including the DNA opening.

Different situation is in the XP-G and XP-F patients. Here, CAK leaving occurs normally after DNA damage. However, as incision reaction is not completed, the core TFIIH stays in the complex with XPA, XPC and XPG factors. TFIIH (as core–CAK

complex) is required for the transcription by RNA polymerase II. From another hand, damaged DNA stops RNA polymerase II. In the case of XP-G and XP-F patients, significant fraction of TFIIH-core might be blocked at the chromatin as part of repair machinery. As NER cannot be completed in these cells, transcription is also drops significantly and is not recovered after DNA damage (Publication 2).

Disorders associated with mutations in TFIIH and other NER factors

There are several rare genetic disorders associated with mutations in TFIIH factor subunits (XPB, XPD and TTD-A/p8, see Table 3 and Figure 13). They are characterized by deficient DNA repair and may have impaired transcription. For instance, XPB F99S mutation weakens p52–XPB interaction and affects XPB ATPase activity (Publication 1). In turn, low ATPase activity affects ability of XPB to anchor efficiently on the DNA (Publication 3).

Mutations at the C-terminal region of XPD affect XPD–p44 interaction and weaken this way helicase activity of complex. It leads to lower efficiency of DNA opening around the lesion and lower rate of damaged oligonucleotide excision (Coin et al., 1998; Publication 1).

TTD-A/p8 protein serves as a cap that covers and stabilizes p52 subunit of TFIIH (Kainov et al., 2008). Subsequently, p52 stimulates efficient XPB ATPase activity, leading to active NER (Publication 1; Coin et al., 2006). Mutations in TTD-A gene destabilize p8–p52 interaction and TFIIH complex in general (Giglia-Mari et al., 2004; Coin et al., 2006).

Mutated XPB protein leads to with xeroderma pigmentosum (XP), Cockayne Syndrome (CS) and trichothiodystrophy (TTD). Mutations in XPD provoke XP, CS, TTD and Cerebro-oculo-facio-skeletal syndrome (COFS). Amino acid changes in p8 are associated with TTD. Mutations in other proteins from the nucleotide excision repair sub-pathways (global genome repair and transcription-coupled repair) also lead to disorders from XP, CS and TTD groups (see Table 3).

There are many mutations reported for the XPD (ERCC2) gene, if compare with three described alleles in the XPB (ERCC3) gene. If mutation rate is statistically similar for both genes coding for p80 and p89 proteins, correspondently, the question is: why

there are more patients with wide spectrum of mutations in the XP-D group, and very few in the XP-B? Intact XPD protein is important for the TFIIH stability (DNA repair and transcription), core–CAK association (transcription) and NER efficiency. At the same time, XPB is important for the TFIIH recruitment to DNA (Publication 3), and destabilization of the protein immediately affects transcription and DNA repair. There are two possibilities. First, amino acid changes may less affect structure and function of XPB than XPD, and many such cases are considered rather as single nucleotide polymorphisms than as disease. Second, mutations in XPB different from F99S, T119P and a frame-shift FS740 are lethal in homozygotes, and it is hardly probable that the correspondent patients will be described.

Xeroderma pigmentosum (XP) is a multigenic multiallelic autosomal recessive disease that occurs at a frequency of about 1:250,000 (the USA) or higher (Japan and the Mediterranean region; Cleaver and Kraemer, 1995). Heterozygotes are unaffected, but homozygotes have severe sun sensitivity that leads to progressive degeneration of sun-exposed regions of the skin and eyes, usually leading to various forms of cutaneous malignancy (melanoma and nonmelanoma). Progressive neurological degeneration occurs in a significant number of patients. The disease begins in early life with the first exposures to sunlight, the median age of onset being 1-2 years of age, with skin rapidly exhibiting the signs associated with sun exposure. Pigmentation is patchy and skin shows atrophy and telangiectasia with development of basal and squamous cell carcinomas and melanomas. Cancer incidence for those individuals under 20 years of age is 2000 times that seen in general population (Lehmann, 2001). There may be a reduction of life span associated with a progression of cancer or neurological degeneration.

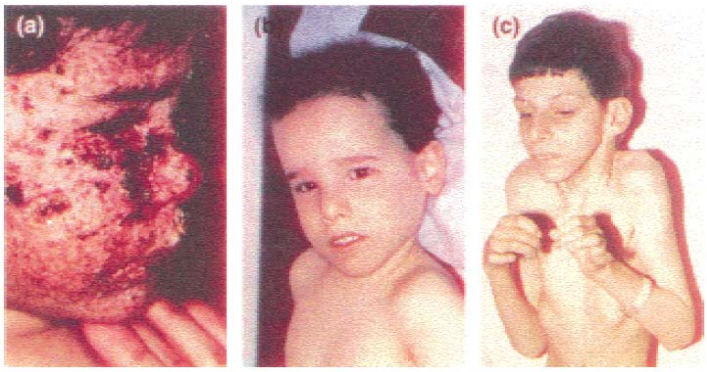

Figure 13. Patients with XP (a), TTD (b) and CS (c). Note the lack of skin pigmentation changes in CS and TTD patients compared with XP. Adapted from Lehmann, 2001.

Trichothiodystrophy is a term introduced by Price et al. (1980) for sulfur-deficient brittle hair. Patients with TTD have brittle hair and nails because of reduced content of cysteine-rich matrix proteins; ichthyotic skin, physical and mental retardation. Approximately half of the patients display photosensitivity correlated with a nucleotide excision repair defect. For the nonphotosensitive trichothiodystrophy there is a name TTDN (TTDN1, C7orf11). No increase of skin cancer is reported for TTD patients (Itin and Pittelkow, 1990; Stary and Sarasin, 1996).

Cockayne syndrome is characterized by abnormal and slow growth and development that becomes evident within the first few years after birth. “Cachectic dwarfism” describes the outward appearance of afflicted individuals. Other features include cutaneous photosensitivity, thin dry hair, a progeroid appearance, progressive pigmentary retinopathy, sensorineural hearing loss, dental caries, and a characteristic stance in the ambulatory patient. Patients often show disproportionally long limbs with large hands and feet, and flexion contractures of joints are usual skeletal features. Knee contractures result in a “horse-riding stance”. There is delayed neural development and severe progressive neurologic degeneration resulted in mental retardation. The mean age of death at reported cases is 12.5 years, although a few affected individuals have lived into their late teens or twenties. Patients with CS have no significant increase of skin cancer (Nance and Berry, 1992).

There are several examples of patients who exhibit combined symptoms of XP and other development and neurological disorders of CS or TTD. These have been found

to correspond to mutations in the XPB, XPD or XPG genes. For instance, among the XP-B patients there are XPB/CS (F99S and FS740) or XPB/TTD (T119P), and no pure XP form (Oh et al., 2006, Figure 14).

Table 3. Human hereditary diseases exhibiting TCR, GGR, or TCR and GGR deficiency (adapted from Hanawalt and Spivak, 2008; Nardo et al., 2009)

Disease	Implicated gene	Overlap with other disease	TCR	GGR	Cancer prone
XP	XPA	DSC	-	-	+
	XPB (ERCC3)	CS, TTD	-	-	+
	XPC		+	-	+
	XPD (ERCC2)	CS, TTD, COFS	-	-	+
	XPE (DDB2)		+	-	+
	XPF (ERCC4)		-	-	+
	XPG (ERCC5)	CS, COFS	-	-	+
CS	CSA (ERCC8)	UV^SS	-	+	-
	CSB (ERCC6)	UV^SS, COFS, DSC	-	+	-
	XPB (ERCC3)	XP, TTD	-	-	+
	XPD (ERCC2)	XP, TTD	-	-	+
	XPG (ERCC5)	XP	-	-	+
	ERCC1	COFS	-	-	Unknown
UV^SS	CSA (ERCC8)	CSA	-	+	-
	CSB (ERCC6)	CS, COFS, DSC	-	+	-
	Unknown		-	+	-
TTD	XPB (ERCC3)	XP, CS	-	-	-
	XPD (ERCC2)	XP, CS	-	-	-
	TTDA (GTF2H5)		-	-	-
	TTDN1 (C7orf11)		+	+	Unknown

Three complementation groups have been identified among UV-sensitive syndrome (UV^SS) patients. They are defined by mutations in an as-yet-identified gene in four cases, a CSB gene in two cases (reviewed in Spivak, 2005) and a CSA gene, as reported recently (Nardo et al., 2009). The UV^SS patients have acute sunburn, dryness

with freckling and pigmentation anomalies of sun-exposed skin, and telangiectasia without neurologic abnormalities or tumour. The patients have abnormal recovery of RNA synthesis (RRS) after UV irradiation. The cellular characteristics such as UV sensitivity, defected RRS after UV irradiation and normal unscheduled DNA synthesis are reminiscent of CS (Itoh et al., 1995). On this basis, the patients are included under the

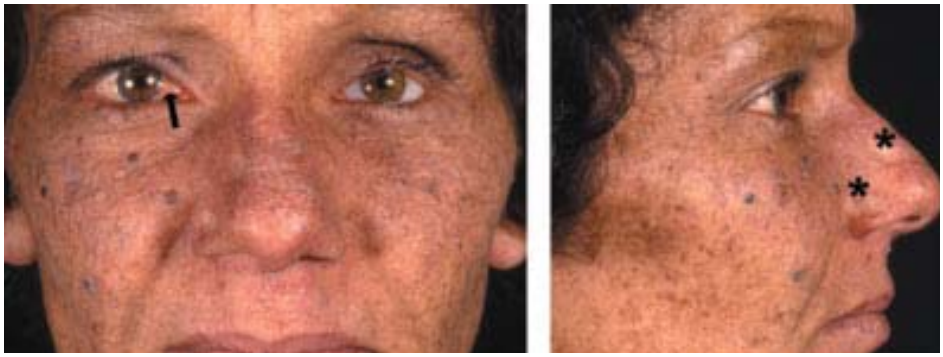

Figure 14. **Clinical feature of patient XP33BR, XPB-F99S (38 years old).**
Note the pterygiumin the inner canthus of her right eye (arrow). At the age of 22 years her left eye had a choroidalmelanoma and was enucleated and replaced with a prosthesis. The skin is dry with multiple pigmented seborrheic keratoses and scars (*) from previous excisions of basal cell carcinomas.
Adapted from Oh et al., 2006

general category designated UV-sensitive syndrome ($UV^{S}S$).

Cerebro-Oculo-Facio Skeletal Syndrome (COFS) is an autosomal, recessively inherited and rapidly progressive neurologic disorder. The disease leads to brain microcephaly and atrophy with calcifications, cataracts, microcornea, optic atrophy, progressive joint contractures, and growth failure. COFS appears to be particularly severe developmental and neurological expression of mutations in CSB, XPG and XPD (Graham et al., 2001).

Chromatin remodeling, transcription and DNA repair: DOT1L

Extremely long DNA molecules are packed into the compact chromatin together with histone proteins. The heterochromatin (packed form of DNA) is not a subject of transcription. The DNA repair in that regions occurs slower if compare with general rate of DNA repair (Chaudhuri et al., 2009). Methylation of histone H3 by MLL-COMPASS complex (histone H3 lysine 4, H3K4), Set2 (H3K36) and DOT1 (H3K79) correlates with active gene expression and opened chromatin state (euchromatin). The absence of DOT1

leads to defected DNA damage response and hypersensitivity to UV-light and X-ray irradiation (Game et al., 2005; Game et al., 2006; Chaudhuri et al., 2009). However, it is not clear yet, why the absence of DOT1 and H3K79 methylation leads to this defect.

It may occur because of higher heterochromatin level, which is more difficult to repair fast. If general repair of UV-light induced CPD and 6-4 photoproducts occurs normally in the DOT1 knocked-out cells (our non-published data), there is a delay of the DNA repair in silenced loci (Chaudhuri et al., 2009). This defect may lead to mutagenesis at the affected regions of DNA.

Histone methylation may be a mark to attract DNA repair proteins and to trigger DNA repair pathways. For instance, 53BP1 protein is efficiently recruited to the nucleosomes interacting with methylated H3K79. There might be other examples, and additional studies in this direction are required.

After UV-irradiation, transcription level in cells is low until the damages are removed. As we have shown, the recovery of transcription to the initial level is defected in the DOT1-deficient cells if compare to wild type cells (Oksenych et al., In preparation). Similar features are observed in the CS cells (CSA and CSB) and may suggest deficient TCR while GGR is not affected. In addition, transcription machinery (RNA polymerase II, TFIIB, mark of active transcription acetylated histone H4) is displaced from the promoter regions of genes following UV-irradiation. After DNA repair there is a recovery of the transcription factors level in the wild type cells. However, this is not a case for DOT1-deficient cells, where just a weak or no recovery is detected (Oksenych et al., In preparation). The absence of initial level of RNA polymerase II and acetylation of histone H4 in the DOT1-deficient cells correlates with accumulation of heterochromatin mark (methylated H3K9) at the promoter region of housekeeping genes as DHFR or GAPDH. We conclude that DOT1 is required for efficient recruitment of transcription machinery to the promoter regions after UV-irradiation, and for the subsequent recovery of transcription level at these cells. Deficient transcription by itself is not physiological condition, and may explain UV hypersensitivity of DOT1-deficient cells.

After UV-irradiation, there is a cell answer, and modulation of gene expression is a part of this response. When we checked the RNA level of p53-dependent genes,

including p21 and mdm2, we observed lower UV-inducible transcription activation at the DOT1-deficient cells if compare to wild type MEF (Oksenych et al., In preparation). At the same time, induction of c-Fos mRNA was higher in the absence of DOT1. What happens with transcription level of other UV-inducible genes? To answer this question, total mRNA sequencing will be performed, and the data will shed light to the deficient UV-response of the DOT1-/- cells.

Using antibodies directed to DOT1 protein in local UV-irradiation approach (Volker et al., 2001), we have observed accumulation of DOT1/KMT4 at the damaged spots of nuclei (Oksenych et al., In preparation). What is the role of DOT1 at the irradiated chromatin? This protein is not required for efficient global genome DNA repair. Instead, it can be required for transcription restart after DNA is repaired.

In general, we observed several aspects of cell life, when DOT1 protein is important: transcription recovery after UV-irradiation, including efficient position of transcription machinery to the promoter regions; activation of UV-inducible gene expression; preventing the heterochromatin spreading after DNA damage; specific accumulation of DOT1 at the UV-irradiated region. However, the role of DOT1 could either be related to its histone methyltransferase activity and methylation state of H3K79, or independent from it. As described (Feng et al., 2002), only 30% of N-terminal DOT1 protein is required for histone methylation. What is the role of other 70%? Is it regulatory region? Is it required for the interaction with other proteins, with nucleosomes or DNA?

As a general conclusion, my work contributed to the understanding of nucleotide excision repair mechanism as well as molecular defects in XP-B patients. We provided new data for the process of TFIIH recruitment to the repair complex, and for the dynamic composition and regulation of TFIIH subunits activity during the NER. In addition, we have expanded knowledge concerning the link between chromatin modifications and DNA repair.

References

A

Aboussekhra A., Biggerstaff M., Shivji MK., Vilpo JA., Moncollin V., Podust VN., Protic M., Hubscher U., Egly JM., and Wood RD. (1995). Mammalian DNA nucleotide excision repair reconstituted with purified protein components. Cell *80*, 859-68

Aboussekhra A., and Thoma F. (1999). TAT-binding protein promotes the selective formation of UV-induced (6-4)-photoproducts and modulates DNA repair in the TATA box. EMBO J. *18*, 433-43

Abraham RT. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev. *15*, 2177-96

Adamczewski JP., Rossignol M., Tassan JP., Nigg EA., Moncollin V., and Egly JM. (1996). MAT1, cdk7 and cyclin H form a kinase complex which is UV light-sensitive upon association with TFIIH. EMBO J. *15*, 1877-84

Aguilar-Fuentes J., Fregoso M., Herrera M., Reynaud E., Braun C., Egly JM., and Zurita M. (2008). p8/TTDA overexpression enhances UV-irradiation resistance and suppresses TFIIH mutations in a Drosophila trichothiodystrophy model. PLoS Genet. 4, e1000253

Akhtar MS., Heidemann M., Tietjen JR., Zhang DW., Chapman RD., Eick D., and Ansari AZ. (2009). TFIIH kinase places bivalent marks on the carboxy-terminal domain of RNA polymerase II. Mol Cell. *34*, 387-93

Akoulitchev S., and Reinberg D. (1998). The molecular mechanism of mitotic inhibition of TFIIH is mediated by phosphorylation of CDK7. Genes Dev. *12*, 3541-50

Altaf M., Saksouk N., and Cote J. (2007). Histone modifications in response to DNA damage. Mutat Res. *618*, 81-90

Altaf M., Utley RT., Lacoste N., Tan S., Briggs SD., and Cote J. (2007a). Interplay of chromatin modifiers on a short basic patch of histone H4 tail defines the boundary of telomeric heterochromatin. Mol Cell. *26*, 1002-14

Amundson SA., Bittner M., Meltzer P., Trent J., and Fornace AJ Jr. (2001). Induction of gene expression as a monitor of exposure to ionizing radiation. Radiat Res. *156*, 657-61

Andersen G., Poterszman A., Egly JM., Moras D., and Thierry JC. (1997). The crystal structure of human cyclin H. FEBS Lett. *397*, 65-9

Aparicio OM., Billington BL., and Gottschling DE. (1991). Modifiers of position effect are shared between telomeric and silent mating-type loci in S. cerevisiae. Cell *66*, 1279-87

Arczewska KD., and Kusmierek JT. (2007). Bacterial DNA repair genes and their eukaryotic homologous: 2. Role of bacterial mutator gene homologues in human disease. Overview of nucleotide pool sanitization and mismatch repair systems. Acta Biochim Pol. *54*, 435-57

B

Babcock GT., and Wikstrom M. (1992). Oxygen activation and the conservation of energy in cell respiration. Nature *356*, 301-9

Bakkenist CJ., and Kastan MB. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimmer dissociation. Nature *421*, 499-506

Banin S., Moyal L., Shieh S., Taya Y., Anderson CW., Chessa L., Smorodinsky NI., Prives C., Reiss Y., Shiloh Y., and Ziv Y. (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science *281*, 1674-7

Bardwell L., Bardwell AJ., Feaver WJ., Svejstrup JQ., Kornberg RD., and Friedberg EC. (1994). Yeast RAD3 protein binds directly to both SSL2 and SSL1 proteins: implications for the structure and function of transcription/repair factor b. Proc Natl Acad Sci USA. *91*, 3926-30

Barry JD., and McCulloch R. (2001). Antigenic variation in trypanosomes: enhanced phenotypic variation in a eukaryotic parasite. Adv Parasitol. *49*, 1-70

Barry ER., Krueger W., Jakuba CM., Veilleux E., Ambrosi DJ., Nelson CE., and Rasmussen TP. (2009). ES cell cycle progression and differentiation require the action of the histone methyltransferase Dot1L. Stem Cells. *27*, 1538-47

Bartek J., Falck J., and Lukas J. (2001). CHK2 kinase – a busy messenger. Nat Rev Mol Cell Biol. *2*, 877-86

Bartenjev I., Butina MR., and Potocnik M. (2000). Rare case of Cockayne syndrome with xeroderma pigmentosum. Acta Derm Venereol. *80*, 213-4

Batty DP., and Wood RD. (2000). Damage recognition in nucleotide excision repair of DNA. Gene *241*, 193-204

Bau DT., Mau YC., and Shen CY. (2006). The role of BRCA1 in non-homologous end-joining. Cancer Lett. *240*, 1-8

Beisel C., Imhof A., Greene J., Kremmer E., and Sauer F. (2002). Histone methylation by the Drosophila epigenetic transcriptional regulator Ash1. Nature *419*, 857-62

Bermudez VP., Lindsey-Boltz LA., Cesare AJ., Maniwa Y., Griffith JD., Hurwitz J., and Sancar A. (2003). Loading of the human 9-1-1 checkpoint complex onto DNA by the checkpoint clamp loader hRad17-replication factor C complex in vitro. Proc Natl Acad Sci USA. *100*, 1633-8

Bernardes de Jesus BM., Bjoras M., Coin F., and Egly JM. (2008). Dissection of the molecular defects caused by pathogenic mutations in the DNA repair factor XPC. Mol Cell Biol. *28*, 7225-35

Bernstein BE., Mikkelsen TS., Xie X., Kamal M., Huebert DJ., Cuff J., Fry B., Meissner A., Wernig M., Plath K., Jaenisch R., Wagschal A., Feil R., Schreiber SL., and Lander ES. (2006). Cell *125*, 315-26

Bessho T., Sancar A., Thompson LH., and Thelen MP. (1997). Reconstitution of human excision nuclease with recombinant XPF-ERCC1 complex. J Biol Chem. *272*, 3833-7

Biedermann KA., Sun JR., Giaccia AJ., Tosto LM., and Brown JM. (1991). scid mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair. Proc Natl Acad Sci USA. *88*, 1394-7

Bird AW., Yu DY., Pray-Grant MG., Qiu Q., Harmon KE., Megee PC., Grant PA., Smith MM., Christman MF. (2002). Acetylation of histone H4 by Esa1 is required for DNA double-strand break repair. Nature *419*, 411-5

Biswas T., Pero J., Joseph C., and Tsodikov O. (2009). DNA-dependent ATPase Activity of Bacterial XPB Helicases. Biochemistry *48*, 2839-48

Blunt T., Gell D., Fox M., Taccioli GE., Lehmann AR., Jackson SP., and Jeggo PA. (1996). Identification of a nonsense mutation in the carboxyl-terminal region of DNA-dependent protein kinase catalytic subunit in the scid mouse. Proc Natl Acad Sci USA. *93*, 10285-90

Bohr VA., Smith CA., Okumoto DS., and Hanawalt PC. (1985). DNA repair in an active gene: removal of pyrimidine dimersfrom the DHFR gene of CHO cells is much more efficient than in the genome overall. Cell *40*, 359-69

Bolderson E., Scorah J., Helleday T., Smythe C., and Meuth M. (2004). ATM is required for the cellular response to thymidine induced replication fork stress. Hum Mol Genet. *13*, 2937-45

Bostelman LJ., Keller AM., Albrecht AM., Arat A., and Thompon JS. (2007). Methylation of histone H3 lysine-79 by Dot1p plays multiple roles in the response to UV damage in *Saccharomyces cerevisiae*. DNA Repair (Amst). *6*, 383-95

Botuyan MV., Lee J., Ward IM., Kim JE., Thompson JR., Chen J., and Mer G. (2006). Structural basis for the methylation state-specific recognition of histone H4-K20 by 53BP1 and Crb2 in DNA repair. Cell *127*, 1361-73

Bradsher J., Coin F., and Egly JM. (2000). Distinct roles for the helicases of TFIIH in transcript initiation and promoter escape. J Biol Chem. *275*, 2532-8

Bregman DB., Halaban R., van Gool AJ., Henning KA., Friedberg EC., and Warren SL. (1996). UV-induced ubiquitination of RNA polymerase II: a novel modification deficient in Cockayne syndrome cells. Proc Natl Acad Sci USA. *93*, 11586-90

Briggs SD., Xiao T., Sun ZW., Caldwell JA., Shabanowitch J., Hunt DF., Allis CD., and Strahl BD. (2002). Gene silencing: trans-histone regulatory pathway in chromatin. Nature *418*, 498

Brooks PJ. (2007). The case for 8,5'-cyclopurine-2'-deoxynucleosides as endogenous DNA lesions that cause neurodegeneration in xeroderma pigmentosum. Neuroscience *145*, 1407-17.

Broomfield S., Hryciw T., and Xiao W. (2001). DNA postreplication repair and mutagenesis in *Saccharomyces cerevisiae*. Mutat Res. *486*, 167-84

Brunet A., Sweeney LB., Sturgill JF., Chua KF., Greer PL., Lin Y., Tran H., Ross SE., Mostoslavsky R., Cohen HY., Hu LS., Cheng HL., Jedrychowski MP., Gygi SP., Sinclair DA., Alt FW., and Greenberg ME. (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science *303*, 2011-5

Buchko GW., Isern NG., Spicer LD., and Kennedy MA. (2001). Human nucleotide excision repair protein XPA: NMR spectroscopic studies of an XPA fragment containing the ERCC1-binding region and the minimal DNA-binding domain (M59-F219). Mutat Res. *486*, 1-10

Buckler AJ., Pelletier J., Haber DA., Glaser T., and Housman DE. (1991). Isolation, characterization, and expression of the murine Wilms' tumor gene (WT1) during kidney development. Mol Cell Biol. *11*, 1707-12

Burcham PC. (1998). Genotoxic lipid peroxidation products: their DNA damaging properties and role in formation of endogenous DNA adducts. Mutagenesis *13*, 287-305

Burgers PM. (1991). Saccharomyces cerevisiae replication factor C. II. Formation and activity of complexes with the proliferating cell nuclear antigen and with DNA polymerases delta and epsilon. J Biol Chem. *266*, 22698-706

Burglen L., Seroz T., Miniou P., Lefebvre S., Burlet P., Munnich A., Pequiqnot EV., Egly JM., and Melki J. (1997). The gene encoding p44, a subunit of the transcription factor TFIIH, is involved in large-scale deletions associated with Werdnig-Hoffmann disease. Am J Hum Genet. *60*, 72-9

Bushnell DA., Bamdad C., and Kornberg RD. (1996). A minimal set of RNA polymerase II transcription protein interactions. J Biol Chem. *271*, 20170-4

Busso D., Keriel A., Sandrock B., Poterszman A., Gileadi O., and Egly JM. (2000). Distinct regions of MAT1 regulate cdk7 kinase and TFIIH transcription activities. J Biol Chem. *275*, 22815-23

C

Camenisch U., Trautlein D., Clement FC., Fei J., Leitenstorfer A., Ferrando-May E., and Naegeli H. (2009). Two-stage dynamic DNA quality check by xeroderma pigmentosum group C protein. EMBO J. *28*, 2387-99

Carmen AA., Milne L., and Grunstein M. (2002). Acetylation of the yeast histone H4 N terminus regulates its binding to heterochromatin protein SIR3. J Biol Chem. *277*, 4778-81

Carter TA., Bonnemann CG., Wang CH., Obici S., Parano E., De Fatima Bonaldo M., Ross BM., Penchaszadeh GK., Mackenzie A., Soares MB., Kunkel LM., and Gilliam TC. (1997). A multicopy transcription-repair gene, BTF2p44, maps to the SMA region and demonstrates SMA associated deletions. Hum Mol Genet. *6*, 229-36

Casper AM., Nghiem P., Arlt MF., and Glower TW. (2002). ATR regulates fragile site stability. Cell *111*, 779-89

Castro J., Merino C., and Zurita M. (2002). Molecular characterization and developmental expression of the TFIIH factor p62 gene from Drosophila melanogaster: effects on the UV light sensitivity of a p62 mutant fly. DNA Repair (Amst). *1*, 359-68

Czermin B., Melfi R., McCabe D., Seitz V., Imhof A., and Pirotta W. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell *111*, 185-96

Cha RS., and Kleckner N. (2002). ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones. Science *297*, 602-6

Chan DW., Chen BP., Prithivirajsingh S., Kurimasa A., Story MD., Qin J., and Chen DJ. (2002). Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA double-strand breaks. Genes Dev. *16*, 2333-8

Chappell C., Hanakahi LA., Karimi-Busheri F., Weinfeld M., and West SC. (2002). Involvement of human polynucleotide kinase in double-strand break repair by non-homologues end joining. EMBO J *21*, 2827-32

Chaudhuri J., and Alt FW. (2004). Class-switch recombination: interplay of transcription, DNA deamination and DNA repair. Nat Rev Immunol. *4*, 541-52

Chaudhuri J., Basu U., Zarrin A., Yan C., Franco S., Perlot T., Vuong B., Wang J., Phan RT., Datta A., Manis J., and Alt FW. (2007). Evolution of the immunoglobulin heavy chain class switch recombination mechanism. Adv Immunol. *94*, 157-214

Chaudhuri S., Wyrick JJ., and Smerdon MJ. (2009). Histone H3 Lys79 methylation is required for efficient nucleotide excision repair in a silenced locus of Saccharomyces cerevisiae. Nucleic Acids Res. *37*, 1690-700

Chen D., Riedl T., Washbrook E., Pace PE., Coombers RC., Egly JM., and Ali S. (2000). Activation of estrogen receptor alpha by S118 phosphorylation involves a ligand-dependent interaction with TFIIH and participation of CDK7. Mol Cell. *6*, 127-37

Chen W., Li Q., Hudson WA., Kumar A., Kirchhof N., and Kersey JH. (2006). A murine Mll-AF4 knock-in model results in lymphoid and myeloid deregulation and hematologic malignancy. Blood *108*, 669-77

Cheung N., Chan LC., Thompson A., Cleary ML., and So CW. (2007). Protein arginine-methyltransferase-dependent oncogenesis. Nat Cell Biol. *9*, 1208-15

Cho EJ., Takagi T., Moore CR., and Buratowski S. (1997). mRNA capping enzyme is recruited to the transcription complex by phosphorylation of the RNA polymerase II carboxy-terminal domain. Genes Dev. *11*, 3319-26

Choi YJ., Ryu KS., Ko YM., Chae YK., Pelton JG., Wemmer DE., and Choi BS. (2005). Biophysical characterization of the interaction domains and mapping of the contact residues in the XPF-ERCC1 complex. J Biol Chem. *280*, 28644-52

Cismowski MJ., Laff GM., Solomon MJ., and Reed SI. (1995). KIN28 encodes a C-terminal domain kinase that controls mRNA transcription in Saccharomyces cerevisiae but lacks cyclin-dependent kinase-activating kinase (CAK) activity. Mol Cell Biol. *15*, 2983-92

Cleaver JE. (1994). It was a very good year for DNA repair. Cell *76*, 1-4

Cleaver JE., and Kraemer KH. (1995). Xeroderma pigmentosum and Cockayne syndrome. In: Scriver CR., Beaudet AL., Sly WS., and Valle D. (Editors, 1995). *The Metabolic and Molecular Bases of Inherited Disease*, McGraw-Hill, New York, 4393–419

Cockell M., Palladino F., Laroche T., Kyrion G., Liu C., Lustig AJ., and Gasser SM. (1995). The carboxy termini of Sir4 and Rap1 affect Sir3 localization: evidence for a multicomponent complex required for yeast telomeric silencing. J Cell Biol. *129*, 909-24

Coin F., Marinoni JC., Rodolfo C., Fribourg S., Pedrini AM., and Egly JM. (1998). Mutations in the XPD helicase gene result in XP and TTD phenotypes, preventing interaction between XPD and thep44 subunit of TFIIH. Nat Genet. *20*, 184-8

Coin F., Bergmann E., Tremeau-Bravard A., and Egly JM. (1999). Mutations in XPB and XPD helicases found in xeroderma pigmentosum patients impair the transcription function of TFIIH. EMBO J. *18*, 1357-66

Coin F., Proietti de Santis L., Nardo T., Zlobinskaya O., Stefanini M., and Egly JM. (2006). p8/TTD-A as a repair-specific TFIIH subunit. Mol Cell. *21*, 215-26

Coin F., Oksenych V., and Egly JM. (2007). Distinct roles for the XPB/p52 and XPD/p44 subcomplexes of TFIIH in damaged DNA opening during nucleotide excision repair. Mol Cell. *26*, 245-56

Coin F., Oksenych V., Mocquet V., Groh S., Blattner C., and Egly JM. (2008). Nucleotide excision repair driven by the dissociation of CAK from TFIIH. Mol Cell. *31*, 9-20

Conaway JW., and and Conaway RC. (1989). A multisubunit transcription factor essential for accurate initiation by RNA polymerase II. J Biol Chem. *264*, 2357-62

Cortez D., Wang Y., Qin J., and Elledge SJ. (1999). Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. Science *286*, 1162-6

Cortes D., Guntuku S., Qin J., and Elledge SJ. (2001). ATR and ATRIP: partners in checkpoint signaling. Science *294*, 1713-6

Cowell IG., Sunter NJ., Singh PB., Austin CA., Durkacz BW., and Tilby MJ. (2007). GammaH2AX foci form preferentially in euchromatin after ionizing-radiation. PLoS ONE *2*, e1057

Cross GA., Wirtz LE., and Navarro M. (1998). Regulation of vsg expression site transcription and switching in Trypanosoma brucei. Mol Biochem Parasitol. *91*, 77-91

D

D'Amours D., and Jackson SP. (2002). The Mre11 complex: at the crossroads of dna repair and checkpoint signaling. Nat Rev Mol Cell Biol. *3*, 317-27

Davey CA., Sargent DF., Luger K., Maeder AW., and Richmond TJ. (2002). Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 a resolution. J Mol Biol. *319*, 1097-113

de Jager M., van Noort J., van Gent DC., Dekker C., Kanaar R., and Wyman C. (2001). Human Rad50/Mre11 is a flexible complex that can tether DNA ends. Mol Cell. *8*, 1129-35

de Laat WL., Appeldoorn E., Sugasawa K., Weterings E., Jaspers NG., and Hoeijmakers JH. (1998). DNA-binding polarity of human replication protein A positions nucleases in nucleotide excision repair. Genes Dev. *12*, 2598-2609

Dedon PC., and Goldberg IH. (1992). Free-radical mechanisms involved in the formation of sequence-dependent bistranded DNA lesions by the antitumor antibiotics bleomycin, neocarzinostatin, and calicheamicin. Chem Res Toxicol. *5*, 311-32

Demple B., and DeMott MS. (2002). Dynamics and diversions in base excision DNA repair of oxidized abasic lesions. Oncogene *21*, 8926-34

Devault A., Martinez AM., Fesquet D., Labbe JC., Morin N., Tassan JP., Nigg EA., Cavadore JC., and Doree M. (1995). MAT1 ('menage à trois') a new RING finger protein subunit stabilizing cyclin H-cdk7 complexes in starfish and Xenopus CAK. EMBO J. *14*, 5027-36

Dianov GL., and Parsons JL. (2007). Co-ordination of DNA single strand break repair. DNA Repair (Amst.) *6*, 454-460

Di Lello P., Jenkins LM., Jones TM., Nguyen BD., Hara T., Yamaguchi H., Dikeakos JD., Appella E., Legault P., and Omichinski JG. (2006). Structure of the Tfb1/p53 complex: Insights into the interaction between the p62/Tfb1 subunit of TFIIH and the activation domain of p53. Mol Cell. *22*, 731-40

Di Lello P., Miller Jenkins LM., Mas C., Langlois C., Malitskaya E., Fradet-Turcotte A., Archambault J., Legault P., and Omichinski JG. (2008). p53 and TFIIEalpha share a common binding site on the Tfb1/p62 subunit of TFIIH. Proc Natl Acad Sci USA. *105*, 106-11

Ding Q., Reddy YV., Wang W., Woods T., Douglas P., Ramsden DA., Lees-Miller SP., and Meek K. (2003). Autophosphorylation of the catalytic subunit of the DNA-dependent protein kinase is required for efficient end processing during DNA double-strand break repair. Mol Cell Biol. *23*, 5836-48

Ding H., Schertzer M., Wu X., Gertsenstein M., Selig S., Kammori M., Pourvali R., Poon S., Vulto I., Chavez E., Tam PP., Nagy A., Lansdorp PM. . (2004) Regulation of murine telomere length by Rtel: an essential gene encoding a helicase-like protein. Cell *117*, 873-86

Dizdaroglu M. (1992). Measurement of radical-induced damage to DNA at the molecular level. Int J Radiat Biol. *61*, 175-83

Doetsch PW. (2002). Translesion synthesis by RNA polymerases: occurrence and biological implications for transcriptional mutagenesis. Mutat Res. *510*, 131-40

Dorigo B., Schalch T., Bystricky K., and Richmond TJ. (2003). Chromatin fiber folding: requirement for the histone H4 N-terminal tail. J Mol Biol. *327*, 85-96

Downs JA., Nussenzweig MC., and Nussenzweig A. (2007). Chromatin dynamics and the preservation of genetic information. Nature *447*, 951-8

Drablos F., Feyzi E., Aas PA., Vaagbo CB., Kavli B., Bratlie MS., Pena-Diaz J., Otterlei M., Slupphaug G., and Krokan HE. (2004). Alkylation damage in DNA and RNA-repair mechanisms and medical significance. DNA Repair (Amst). *3*, 1389-1407

Dronkert ML., Beverloo HB., Johnson RD., Hoeijmakers JH., Jasin M., and Kanaar R. (2000). Mouse RAD54 affects DNA double-strand break repair and sister chromatid exchange. Mol Cell Biol. *20*, 3147-56

Dunand-Sauthier I., Hohl M., Thorel F., Jaquier-Gubler P., Clarkson SG., and Scharer OD. (2005). The spacer region of XPG mediates recruitment to nucleotide excision repair complexes and determines substrate specificity. J Biol Chem. *280*, 7030-7

E

Edwards RJ., Bentley NJ., and Carr AM. (1999). A Rad3-Rad26 complex reponds to DNA damage independently of other checkpoint proteins. Nat Cell Biol. *1*, 393-8

Engelward BP., Allan JM., Dreslin AJ., Kelly JD., Wu MM., Gold B., and Samson LD. (1998). A chemical and genetic approach together define the biological consequences of 3-methyladenine lesions in the mammalian genome. J Biol Chem. *273*, 5412-18

Evans E., Moggs JG., Hwang JR., Egly JM., and Wood RD. (1997). Mechanism of open complex and dual incision formation by human nucleotide excision repair factors. EMBO J *16*, 6559-73

Evans ML., Bostelman LJ., Albrecht AM., Keller AM., Strande NT., and Thompson JS. (2008). UV sensitive mutations in histone H3 in *Saccharomyces cerevisiae* that alter specific K79 methylation states genetically act through distinct DNA repair pathways. Curr Genet. *53*, 259-74

F

Fabrizio P., Gattazzo C., Battistella L., Wei M., Cheng C., McGrew K., and Longo VD. (2005). Sir2 blocks extreme life-span extension. Cell *123*, 655-67

Fan L., Arvai AS., Cooper PK., Iwai S., Hanaoka F., and Tainer JA. (2006). Conserved XPB core structure and motifs for DNA unwinding: implications for pathway selection of transcription or excision repair. Mol Cell. *22*, 27–37

Fan L., Fuss JO., Cheng QJ., Arvai AS., Hammel M., Roberts VA., Cooper PK., and Tainer JA. (2008). XPD helicase structures and activities: insights into the cancer and aging phenotypes from XPD mutations. Cell *133*, 789–800

Fang WH., and Modrich P. (1995). Human strand-specific mismatch repair occurs by a bidirectional mechanism similar to that of the bacterial reaction. J Biol Chem. *268*, 11838-44

Feaver WJ., Gileadi O., and Kornberg RD. (1991). Purification and characterization of yeast RNA polymerase II transcription factor b. J Biol Chem. *266*, 19000-5

Feaver WJ., Svejstrup JQ., Bardwell L., Bardwell AJ., Buratowski S., Gulyas KD., Donahue TF., Friedberg EC., and Kornberg RD. (1993). Dual roles of a multiprotein complex from S. cerevisiae in transcription and DNA repair. Cell *75*, 1379-87

Feaver WJ., Svejstrup JQ., Henry NL., and Kornberg RD. (1994). Relationship of CDK-activating kinase and RNA polymerase II CTD kinase TFIIH/TFIIK. Cell *79*, 1103-9

Feaver WJ., Henry NL., Wang Z., Wu X., Svejstrup JQ., Bushnell DA., Friedberg EC., and Kornberg RD. (1997). Genes for Tfb2, Tfb3, and Tfb4 subunits of yeast transcription/repair factor IIH. Homology to human cyclin-dependent kinase activating kinase and IIH subunits. J Biol Chem. *272*, 19319-27

Feaver WJ., Huang W., and Friedberg EC. (1999). The TFB4 subunit of yeast TFIIH is required for both nucleotide excision repair and RNA polymerase II transcription. J Biol Chem. *274*, 29564-7

Feaver WJ., Huang W., Gileadi O., Myers L., Gustafsson CM., Kornberg RD., and Friedberg EC. (2000). Subunit interactions in yeast transcription/repair factor TFIIH. Requirement for Tfb3 subunit in nucleotide excision repair. J Biol Chem. *275*, 5941-6

Fesquet D., Labbe JC., Derancourt J., Capony JP., Galas S., Girard F., Lorca T., Shuttleworth J., Doree M., and Cavadore JC. (1993). The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. EMBO J. *12*, 3111-21

Feng Q., Wang H., Ng H.H., Erdjument-Bromage H., Tempst P., Struhl K., and Zhang Y. (2002). Methylation of H3-lysine 79 is mediated by a new family of HMTases without a SET domain. Curr Biol. *12*, 1052-8

Figuieredo LM., Janzen CJ., and Cross GA. (2008). A histone methyltransferase modulates antigenic variation in African trypanosomes. PLoS Biol. *6*, e161

Fillingham J., Keogh MC., and Krogan NJ. (2006). GammaH2AX and its role in DNA double-strand break repair. Biochem Cell Biol. *84*, 568-77

Fingerman IM., Li HC., and Briggs SD. (2007). A charge-based interaction between histone H4 and Dot1 is required for H3K79 methylation and telomere silencing: identification of a new trans-histone pathway. Genes Dev. *21*, 2018-29

Fischer L., Gerard M., Chalut C., Lutz Y., Humbert S., Kanno M., Chambon P., and Egly JM. (1992). Cloning of the 62-kilodalton component of basic transcription factor BTF2. Science *257*, 1392-5

Fisher RP. (2006). Secrets of a double agent: CDK7 in cell-cycle control and transcription. J Cell Sci. *118*, 5171-80

Fisher TS., and Zakian VA. (2005). Ku: a multifunctional protein involved in telomere maintenance. DNA Repair (Amst). *4*, 1215-26

Flores-Rozas H., and Kolodner RD. (1998). The *Saccharomyces cerevisiae MLH3* gene functions in MSH3-dependent suppression of frameshift mutations. Proc Natl Acad Sci USA. *95*, 12404-9

Fousteri M., Vermeulen W., van Zeeland AA., and Mullenders LH. (2006). Cockayne syndrome A and B proteins differentially regulate recruitment of chromatin remodeling and repair factors to stalled RNA polymerase II *in vivo*. Mol Cell. *23*, 471-82

Franco S., Alt FW., and Manis JP. (2006). Pathways that suppress programmed DNA breaks from progressing to chromosomal breaks and translocations. DNA Repair (Amst.) *5*, 1030-41

Fribourg S., Kellenberger E., Rogniaux H., Poterszman A., Van Dorsselaer A., Thierry JC., Egly JM., Moras D., Kieffer B. (2000). Structural characterization of the cysteine-rich domain of TFIIH p44 subunit. J Biol Chem. *275*, 31963-71

Friedberg EC. (2001). How nucleotide excision repair protects against cancer. Nat Rev Cancer. *1*, 22-33

Fromme JC., BanerjeeA., and Verdine GL. (2004). DNA glycosylase recognition and catalysis. Curr Opin Struct Biol. *14*, 43-49

Funchain P., Yeung A., Stewart JL., Lin R., Slupska MM., and Miller JH. (2000). The consequence of growth of a mutator strain of Escherichia coli as measured by loss of function among multiple gene targets and loss of fitness. Genetics *154*, 959-70

G

Game JC., Williamson MS., Spicakova T., and Brown JM. (2006). The RAD6/BRE1 histone modification pathway in *Saccharomyces confers* radiation resistance through a Rad51-dependent process that is independent of RAD18. Genetics *173*, 1951-68

Gatei M., Scott SP., Filippovitch I., Soronika N., Lavin MF., Weber B., and Khanna KK. (2000). Role for ATM in DNA damage-induced phosphorylation of BRCA1. Cancer Res. *60*, 3299-304

Gegonne A., Weissman JD., Lu H., Zhou M., Dasgupta A., Ribble R., Brady JN., and Singer DS. (2008). TFIID component TAF7 functionally interacts with both TFIIH and P-TEFb. Proc Natl Acad Sci USA. *105*, 5367-72

Genschel J., Bazemore LR., and Modrich P. (2002). Human exonuclease I is required for 5' and 3' mismatch repair. J Biol Chem. *277*, 13302-11

Gerard M., Fischer L., Moncollin V., Chipoulet JM., Chambon P., and Egly JM. (1991). Purification and interaction properties of the human RNA polymerase B(II) general transcription factor BTF2. J Biol Chem. *266*, 20940-5

Gervais V., Lamour V., Jawhari A., Frindel F., Wasielewski E., Dubaele S., Egly JM., Thierry JC., Kieffer B., Poterszman A. (2004). TFIIH contains a PH domain involved in DNA nucleotide excision repair. Nat Struct Mol Biol. *11*, 616-22

Giannakou ME., and Partridge L. (2007). Role of insulin-like signalling in Drosophila lifespan. Trends Biochem Sci. *32*, 180-8

Giannattasio M., Lazzaro F., Plevani P., and Muzi-Falconi M. (2005). The DNA damage checkpoint response requires histone H2B ubiquitination by Rad6-Bre1and H3 methylation by Dot1. J Biol Chem. *280*, 9879-86

Giglia-Mari G., Coin F., Ranish JA., Hoogstraten D., Theil A., Wijgers N., Jaspers NG., Raams A., Argentini M., van der Spek PJ., Botta E., Stefanini M., Egly JM., Aebersold R., Hoeijmakers JH., and Vermeulen W. (2004). Nat Genet. *36*, 714-9

Giglia-Mari G., Miguel C., Theil AF., Mari PO., Hoogstraten D., Ng JM., Dinant C., Hoeijmakers JH., Vermeulen W. (2006). Dynamic interaction of TTDA with TFIIH is stabilized by nucleotide excision repair in living cells. PLoS Biol. *4*, e156

Gnatt AL., Cramer P., Fu J., Bushnell DA., and Kornberg RD. (2001). Structural basis of transcription: an RNA polymerase II elongation complex at 3.3 A resolution. Science *292*, 1876-82

Gould KL., Moreno S., Owen DJ., Sazer S., and Nurse P. (1991). Phosphorylation at Thr167 is required for Schizosaccharomyces pombe p34cdc2 function. EMBO J. *10*, 3297-309

Graham JM Jr., Anyane-Yeboa K., Raams A., Appeldoorn E., Kleijer WJ., Garritsen VH., Busch D., Edersheim TG., and Jaspers NG. (2001). Cerebro-oculo-facio-skeletal syndrome with a nucleotide excision-repair defect and a mutated XPD gene, with prenatal diagnosis in a triplet pregnancy. Am J Hum Genet. *69*, 291-300

Grenon M., Costelloe T., Jimeno S., O'Shaughnessy A., Fitzgerald J., Zgheib O., Degerth L., and Lowndes NF. (2007). Docking onto chromatin via the Saccharomyces cerevisiae Rad9 Tudor domain. Yeast *24*, 105-19

Groisman R., Polanowska J., Kuraoka I., Sawada J., Saijo M., Drapkin R., Kisselev AF., Tanaka K., and Nakatani Y. (2003). The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. Cell *113*, 357-67

Guillet M., and Boiteux S. (2003). Origin of endogenous DNA abasic sites in *Saccharomyces cerevisiae*. Mol Cell Biol. *23*, 8386-94

H

Habraken Y., Sung P., Prakash L., and Prakash S. (1993). Yeast excision repair gene RAD2 encodes a single-stranded DNAendonuclease. Nature *366*, 365-8

Hadi MZ., Ginalski K., Nguyen LH., and Wilson DM, 3rd. (2002). Determinants in nuclease specificity of Ape1 and Ape2, human homologous of *Escherichia coli* exonuclease III. J Mol Biol. *316*, 853-66

Hall H., Gursky J., Nicodemou A., Rybanska I., Kimlickova E., and Pirsel M. (2006). Characterization of ERCC3 mutations in the Chinese hamster ovary 27-1, UV24 and MMC-2 cell lines. Mutat Res. *593*, 177-86

Halliwell B. (1999). Oxygen and nitrogen are pro-carcinogenes. Damage to DNA by reactive oxygen, chlorine and nitrogen species: measurement, mechanism and the effects of nutrition. Mutat Res. *443*, 37-52

Halliwell B., and Aruoma OI. (1991). DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. FEBS Lett. *281*, 9-19

Hammond EM., Denko NC., Dorie MJ., Abraham RT., and Giaccia AJ. (2002). Hypoxia links ATR and p53 through replication arrest. Mol Cell Biol. *22*, 1834-43

Hampsey M. (1998). Molecular genetics of the RNA polymerase II general transcriptional machinery. Microbiol Mol Biol Rev. *62*, 465-503

Hanawalt PC. (1994). Transcription-coupled repair and human disease. Science *266*, 1957-8

Hanawalt PC. (2002). Subpathways of nucleotide excision repair and their regulation. Oncogene *21*, 8949-56

Hanawalt PC., and Spivak G. (2008).Transcription-coupled DNA repair: two decades of progress and surprises. Nat Rev Mol Cell Biol. *9*, 958-70

Harper JW., and Elledge SJ. (1998). The role of Cdk7 in CAK function, a retro-retrospective. Genes Dev. *12*, 285-9

Harrigan JA., Wilson DM. 3rd, Prasad R., Opresko PL., Beck G., May A., Wilson SH., and Bohr VA. (2006). The Werner syndrome protein operates in base excision repair and cooperates with DNA polymerase beta. Nucleic Acids Res. *34*, 745-54

Henning KA., Li L., Iyer N., McDaniel LD., Reagan MS., Legerski R., Schultz RA., Stefanini M., Lehmann AR., Mayne LV., and Friedberg EC. (1995). The Cockayne syndrome group A gene encodes a WD repeat protein that interacts with CSB protein and a subunit of RNA polymerase II TFIIH. Cell *82*, 555-64

Hill JW., Hazra TK., Izumi T., and Mitra S. (2001). Stimulation of human 8-oxoguanine-DNA glycosylase by AP-endonuclease: potential coordination of the initial steps in base excision repair. Nucleic Acids Res. *29*, 430-8

Hinks JA., Evans MC., De Miguel Y., Sartori AA., Jiricny J. and Pearl LH. (2002) An iron–sulfur cluster in the family 4 uracil–DNA glycosylases. J Biol Chem. *277*, 16936-40

Hirata A., Klein BJ., and Murakami KS. (2008). The X-ray crystal structure of RNA polymerase from Archaea. Nature *451*, 851-4

Hoeijmakers JH. (2001). DNA repair mechanisms. Maturitas *38*, 17-22; 22-3

Holmes JJ., Clark S., and Modrich P. (1990). Strand-specific mismatch correction in nuclear extracts of human and *Drosophila melanogaster* cell lines. Proc Natl Acad Sci USA. *87*, 5837-41.

Horn D., and Cross JA. (1995). A developmentally regulated position effect at a telomeric locus in Trypanosoma brucei. Cell *83*, 555-61

Hopfner KP., Craig L., Moncalian G., Zinkel RA., Usui T., Owen BA., Karcher A., Henderson B., Bodmer JL., McMurray CT., Carney JP., Petrini JH., and Tainer JA. (2002). The Rad50 zinc-hook is a structure joining Mre11 complexes in DNA recombination and repair. Nature *418*, 562-6

Hoppe .GJ, Tanny JC., Rudner AD., Gerber SA., Danaie S., Gygi SP., and Moazed D. (2002). Steps in assembly of silent chromatin in yeast: Sir3-independent binding of a Sir2/Sir4 complex to silencers and role for Sir2-dependent deacetylation. Mol Cell Biol. *22*, 4167-80

Humbert S., van Vuuren H., Lutz Y., Hoeijmakers JH., Egly JM., and Moncollin V. (1994). p44 and p34 subunits of the BTF2/TFIIH transcription factor have homologies with SSL1, a yeast protein involved in DNA repair. EMBO J. *13*, 2393-8

Huyen Y., Zgheib O., Ditullio RA Jr., Gorgoulis VG., Zacharatos P., Petty TJ., Sheston EA., Mellert HS., Stavridi ES., and Halazonetis TD. (2004). Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. Nature *432*, 406-11

I

Ikegami T., Kuraoka I., Saijo M., Kodo N., Kyogoku Y., Morikawa K., Tanaka K., and Shirakawa M. (1998). Solution structure of the DNA- and RPA-binding domain of the human repair factor XPA. Nat Struct Biol *5*, 701-6

Ikura T., Ogryzko VV., Grigoriev M., Groisman R., Wang J., Horikoshi M., Scully R., Qin J., and Nakatani Y. (2000). Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. Cell *102*, 463-73

Imai S., Armstrong CM., Kaeberlein M., and Guarente L. (2000). Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature *403*, 795-800

Itin PH., and Pittelkow MR. (1990). Trichothiodystrophy: review of sulfur-deficient brittle hair syndromes and association with the ectodermal dysplasias. J Am Acad Dermatol. *22*, 705-17

Ito S., Kuraoka I., Chymkowitch P., Compe E., Takedachi A., Ishigami C., Coin F., Egly JM., and Tanaka K. (2007). XPG stabilizes TFIIH, allowing transactivation of nuclear receptors: implications for Cockayne syndrome in XP-G/CS patients. Mol Cell. *26*, 231-43

Itoh T., Fujiwara Y., Ono T., and Yamaizumi M. (1995). UVs syndrome, a new general category of photosensitive disorder with defective DNA repair, is distinct from xeroderma pigmentosum variant and rodent complementation group I. Am J Hum Genet. *56*, 1267-76

Iyer N., Reagan MS., Wu KJ., Canagarajah B., and Friedberg EC. (1996). Interactions involving the human RNA polymerase II transcription/nucleotide excision repair complex TFIIH, the nucleotide excision repair protein XPG, and Cockayne syndrome group B (CSB) protein. Biochemistry *35*, 2157-67

J

Jackson AL., and Loeb LA. (2001). The contribution of endogenous sources of DNA damage to the multiple mutations in cancer. Mutat Res. *477*, 7-21

Jackson SP. (2002). Sensing and repairing DNA double-strand breaks. Carcinogenesis *23*, 687-96

Jacobs GH. (1992). Determination of the base recognition positions of zinc fingers from sequence analysis. EMBO J. *11*, 4507-17

Janzen CJ., Hake SB., Lowell JE., and Cross GA. (2006). Selective di- or trimethylation of histone H3 lysine 76 by two DOT1 homologs is important for cell cycle regulation in Trypanosoma brucei. Mol Cell. *23*, 497-507

Jasin M. (2002). Homologous repair of DNA damage and tumorigenesis: the BRCA connection. Oncogene *21*, 8981-93

Jawhari A., Laine JP., Dubaele S., Lamour V., Poterszman A., Coin F., Moras D., and Egly JM. (2002). p52 Mediates XPB function within the transcription/repair factor TFIIH. J Biol Chem. *277*, 31761-7

Jenuwein T., and Allis CD. (2001). Translating the histone code. Science *293*, 1074-80

Jiricny J. (2006). The multifaceted mismatch-repair system. Nat Rev Mol Cell Biol. *7*, 335-46

Johnson NA., McKenzie R., McLean L., Sowers LC., and Fletcher HM. (2004). 8-oxo-7,8-dihydroguanine is removed by a nucleotide excision repair-like mechanism in *Porphyromonas gingivalis* W83. J Bacteriol *186*, 7697-703

Jones B., Su H., Bhat A., Lei H., Bajko J., Hevi S., Baltus GA., Kadam S., Zhai H., Valdez R., Gonzalo S., Zhang Y., Li E., and Chen T. (2008). The histone H3K79 methyltransferase Dot1L is essential for mammalian development and heterochromatin structure. PLoS Genet. *4*, e1000190

K

Kadyk LC., and Hartwell LH. (1992). Sister chromatids are preferred over homologs as substrates for recombinational repair in *Saccharomyces cerevisiae*. Genetics *132*, 387-402

Kaeberlein M., McVey M., and Guarente L. (1999). The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. Genes Dev. *13*, 2570-80

Kainov DE., Vitorino M., Cavarelli J., Poterszman A., and Egly JM. (2008). Structural basis for group A trichothiodystrophy. Nat Struct Mol Biol. *15*, 980-4

Kaldis P., Sutton A., and Solomon MJ. (1996). The Cdk-activating kinase (CAK) from budding yeast. Cell *86*, 553-64

Kalvi B., Otterlei M., Slupphaug G., and Krokan HE. (2007). Uracil in DNA-general mutagen, but normal intermediate in acquired immunity. DNA Repair (Amst). *6*, 505-16

Kamiuchi S., Saijo M., Citterio E., de Jager M., Hoeijmakers JH., and Tanaka K. (2002). Translocation of Cockayne syndrome group A protein to the nuclear matrix: possible relevance to transcription-coupled DNA repair. Proc Natl Acad Sci USA. *99*, 201-6

Katan-Khaykovich Y., and Struhl K. (2005). Heterochromatin formation involves changes in histone modifications over multiple cell generations. EMBO J. *24*, 2138-49

Kayne PS., Kim UJ., Han M., Mullen JR., Yoshizaki F., and Grunstein M. (1988). Extremely conserved histone H4 N terminus is dispensable for growth but essential for repressing the silent mating loci in yeast. Cell *55*, 27-39

Kellenberger E., Dominguez C., Fribourg S., Wasielewski E., Moras D., Poterszman A., Boelens R., and Kieffer B. (2005). Solution structure of the C-terminal domain of TFIIH p44 subunit reveals a novel type of C4C4 ring domain involved in protein-protein interactions. J Biol Chem. *280*, 20785-92

Kennison JA. (1995). The Polycomb and trithorax group proteins of Drosophila: trans-regulators of homeotic gene function. Annu Rev Genet. *29*, 289-303

Keriel A., Stary A., Sarasin A., Rochette-Egly C., and Egly JM. (2002). XPD mutations prevent TFIIH-dependent transactivation by nuclear receptors and phosphorylation of RARalpha. Cell *109*, 125-35

Kershnar E., Wu SY., and Chiang CM. (1998). Immunoaffinity purification and functional characterization of human transcription factor IIH and RNA polymerase II from clonal cell lines that conditionally express epitope-tagged subunits of the multiprotein complexes. J Biol Chem. *273*, 34444-53

Kim TK., Ebright RH., and Reinberg D. (2000). Mechanism of ATP-dependent promoter melting by transcription factor IIH. Science *288*, 1418-22

Kim J., and Roeder RG. (2009). Direct Bre1-Paf1 complex interaction and RING finger independent Bre1-Rad6 interactions mediate H2B ubiquitylation in yeast. J Biol Chem. *284*, 20582-92

Kim JK., Patel D., and Choi BS. (1995). Contrasting structural impacts induced by *cis-syn* cyclobutane dimer and 6-4 adduct in DNA duplex decamers: implication in mutagenesis and repair activity. Photochem Photobiol. *62*, 44-50

Kim ST., Lim DS., Canman CE., and Kastan MB. (1999). Substrate specificities and identification of putative substrates of ATM kinase family members. J Biol Chem. *274*, 37538-43

Klinge S., Hirst J., Maman JD., Krude T., and Pellegrini L. (2007) An iron–sulfur domain of the eukaryotic primase is essential for RNA primer synthesis. Nat Struct Mol Biol. *14*, 875-7

Koch CA., Agyei R., Galicia S., Metalnikov P., O'Donnell P., Starostine A., Weinfeld M., and Durocher D. (2004). Xrcc4 physically links DNA end processing by polynucleotide kinase to DNA ligation by DNA ligase IV. EMBO J. *23*, 3874-85

Kondo T., Wakayama T., Naiki T., Matsumoto K., and Sugimoto K. (2001). Recruitment of Mec1 and Ddc1 checkpoint proteins to double-strand breaks through distinct mechanisms. Science *294*, 867-70

Kouzarides T. (2007). Chromatin modifications and their function. Cell *128*, 693-705

Kozlov S., Gueven N., Keating K., Ramsay J., and Lavin MF. (2003). ATP activates ataxia-telangiectasia mutated (ATM) in vitro. Importance of autophosphorylation. J Biol Chem. *278*, 9309-17

Kraemer KH., Patronas NJ., Schiffmann R., Brooks BP., Tamura D., and Digiovanna JJ. (2007). Xeroderma pigmentosum, trichothiodystrophy and Cockayne syndrome: A complex genotype-phenotype relationship. Neuroscience *145*, 1388-96

Krek W., and Nigg EA. (1991). Differential phosphorylation of vertebrate p34cdc2 kinase at the G1/S and G2/M transitions of the cell cycle: identification of major phosphorylation sites. EMBO J. *10*, 305-16

Krivtsov AV., Feng Z., Lemieux ME., Faber J., Vempati S., Sinha AU., Xia X., Jesneck J., Bracken AP., Silverman LB., Kutok JL., Kung AL., and Armstrong SA. (2008). H3K79 methylation profiles define murine and human MLL-AF4 leukemias. Cancer Cell *14*, 355-68

Krokan HE., Standal R., and Slupphaug G. (1997). DNA glycosylases in the base excision repair of DNA. Biochem J. *325*, 1-16

Kreutzer DA., and Essigmann JM. (1998). Oxidized, deaminated cytosines are a sourse of C-T transitions in vivo. Proc Natl Acad Sci USA. *95*, 3578-82

Kunkel TA. (1993). Nucleotide repeats. Slippery DNA and diseases. Nature *365*, 207-8

Kunkel TA., and Erie DA. (2005). DNA mismatch repair. Annu Rev Biochem.*74*, 681-710

Kwek KY., Murphy S., Furger A., Thomas B., O'Gorman W., Kimura H., Proudfoot NJ., and Akoulitchev A. (2002). U1 snRNA associates with TFIIH and regulates transcriptional initiation. Nat Struct Biol. *9*, 800-5

L

Lahue RS., Au KG., and Modrich P. (1989). DNA mismatch correction in a defined system. Science *245*, 160-4

Laine JP., and Egly JM. (2006a). Initiation of DNA repair mediated by a stalled RNA polymerase IIO. EMBO J. *25*, 387-97

Laine JP., and Egly JM. (2006b). When transcription and repair meet: a complex system. Trends Genet. *22*, 430-6

Langlois C., Mas C., Di Lello P., Jenkins LM., Legault P., and Omichinski JG. (2008). NMR structure of the complex between the Tfb1 subunit of TFIIH and the activation domain of VP16: structural similarities between VP16 and p53. *130*, 10596-604

Larochelle S., Pandur J., Fisher RP., Salz HK., and Suter B. (1998). Cdk7 is essential for mitosis and for in vivo Cdk-activating kinase activity. Genes Dev. *12*, 370-81

Larochelle S., Chen J., Knights R., Pandur J., Morcillo P., Erdjument-Bromage H., Tempst P., Suter B., and Fisher RP. (2001). T-loop phosphorylation stabilizes the CDK7-cyclin H-MAT1 complex in vivo and regulates its CTD kinase activity. EMBO J. *20*, 3749-59

Leclerc V., Raisin S., and Leopold P. (2000). Dominant-negative mutants reveal a role for the Cdk7 kinase at the mid-blastula transition in Drosophila embryos. EMBO J. *19*, 1567-75

Lee SH., and Hurwitz J. (1990). Mechanism of elongation of primed DNA by DNA polymerase delta, proliferating cell nuclear antigen, and activator 1. Proc Natl Acad Sci USA. *87*, 5672-6

Lee JH., Park CJ., Arunkumar AI., Chazin WJ., and Choi BS. (2003). NMR study on the interaction between RPA and DNA decamer containing cis-syn cyclobutane pyrimidine dimer in the presence of XPA: implication for damage verification and strand-specific dual incision in nucleotide excision repair. Nucleic Acids Res. *31*, 4747-54

Leibeling D., Laspe P., and Emmert S. (2006). Nucleotide excision repair and cancer. J Mol Histol. *37*, 225-8

Lehmann AR. (2001). The xeroderma pigmentosum group D (XPD) gene: one gene, two functions, three diseases. Genes Dev. *15*, 15-23

Leppard JB., and Champoux JJ. (2005). Human DNA topoisomerase I: relaxation, roles, and damage control. Chromosoma *114*, 75-85

Li B., Carey M., and Workman JL. (2007). The role of chromatin during transcription. Cell *128*, 707-19

Lim DS., Kim ST., Xu B., Maser RS., Lin J., Petrini JH., and Kastan MB. (2000). ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. Nature *404*, 613-7

Lin YC., Choi WS., and Gralla JD. (2005). TFIIH XPB mutants suggest a unified bacterial-like mechanism for promoter opening but not escape. Nat Struct Mol Biol. *12*, 603–7

Lin YH., Kakadia PM., Chen Y., Li YQ., Deshpande AJ., Buske C., Zhang KL., Zhang Y., Xu GL., and Bohlander SK. (2009). Global reduction of the epigenetic H3K79 methylation mark and increased chromosomal instability in CALM-AF10-positive leukemias. Blood *114*, 651-8

Lindahl T. (1993). Instability and decay of the primary structure of DNA. Nature 362, 709-15

Lindahl T., Karran P., and Wood RD. (1997). DNA excision repair pathways. Curr Opin Genet Dev. *7*, 158-69

Lindahl T., and Wood RD. (1999). Qulity control by DNA repair. Science *286*, 1897-905

List O., Togawa T., Tsuda M., Matsuo T., Elard L., and Aigaki T. (2009). Overexpression of grappa encoding a histone methyltransferase enhances stress resistance in Drosophila. Hereditas *146*, 19-28

Liu Y., Ando S., Xia X., Yao R., Kim M., Fondell J., and Yen PM. (2005). p62, A TFIIH subunit, directly interacts with thyroid hormone receptor and enhances T3-mediated transcription. Mol Endocrinol. *19*, 879-84

Liu H., Rudolf J., Johnson KA., McMahon SA., Oke M., Carter L., McRobbie AM., Brown SE., Naismith JH., and White MF. (2008) Structure of the DNA repair helicase XPD. Cell *133*, 801-12

Loeb LA., and Preston BD. (1986). Mutagenesis by apurinic/apyrimidinic sites. Annu Rev Genet. *20*, 201-30

Lolli G., Lowe ED., Brown NR., and Johnson LN. (2004). The crystal structure of human CDK7 and its protein recognition properties. Structure *12*, 2067-79

Longo VD., and Finch CE. (2003). Evolutionary medicine: from dwarf model systems to healthy centenarians? Science *299*, 1342-6

Lopes-Girona A., Tanaka K., Chen XB., Baber BA., McGowan CH., and Russel P. (2001). Serine-345 is required for Rad3-dependent phosphorylation and function of checkpoint kinase Chk1 in fission yeast. Proc Natl Acad Sci USA. *98*, 11289-94

Lu H., Fisher RP., Bailey P., and Levine AJ. (1997). The CDK7-cycH-p36 complex of transcription factor IIH phosphorylates p53, enhancing its sequence-specific DNA binding activity in vitro. Mol Cell Biol. *17*, 5923-34

Luger K. (2006). Dynamic nucleosomes. Chromosome Res. *14*, 5-16

M

Ma L., Westbroek A., Jochemsen AG., Weeda G., Bosch A., Bootsma D., Hoeijmakers JH., and van der Eb AJ. (1994). Mutational analysis of ERCC3, which is involved in DNA repair and transcription initiation: identification of domains essential for the DNA repair function. Mol Cell Biol. *14*, 4126–34

Maddukuri L., Dudzinska D., and Tudek B. (2007). Bacterial DNA repair genes and their eukaryotic homologues: 4. The role of nucleotide excision repair (NER) system in mammalian cells. Acta Biochimica Polonica *54*, 469-82

Marinoni JC., Roy R., Vermeulen W., Miniou P., Lutz Y., Weeda G., Seroz T., Gomez DM., Hoeijmakers JH., and Egly JM. (1997). Cloning and characterization of p52, the fifth subunit of the core of the transcription/DNA repair factor TFIIH. EMBO J. *16*, 1093-102

Marra G., and Jiricny J. (2005). DNA mismatch repair and colon cancer. Adv Exp Med Biol. *570*, 85-123

Mari PO., Flore BI., Persengiev SP., Verkaik NS., Bruggenwirth HT., Modesti M., Giglia-Mari G., Bezstarosti K., Demmers JA., Luider TM., Houtsmuller AB., and van Gent DC. (2006). Dynamic assembly of end-joining complexes requires interaction between Ku70/80 and XRCC4. Proc Natl Acad Sci USA. *103*, 18597-602

Martino F., Kueng S., Robinson P., Tsai-Pflugfelder M., van Leeuwen F., Ziegler M., Cubizolles F., Cockell MM., Rhodes D., and Gasser SM. (2009). Reconstitution of yeast silent chromatin: multiple contact sites and O-AADPR binding load SIR complexes onto nucleosomes in vitro. Mol Cell. *33*, 323-34

Matsui P., DePaulo J., and Buratowski S. (1995). An interaction between the Tfb1 and Ssl1 subunits of yeast TFIIH correlates with DNA repair activity. Nucleic Acids Res. *23*, 767-72

Matsunaga T., Hatakeyama Y., Ohta M., Mori T., and Nikaido O. (1993). Establishment and characterization of of a monoclonal antibody recognizing the Dewar isomers of (6-4) photoproducts. Photochem Photobiol. *57*, 934-40

Matsunaga T., Park CH., Bessho T., Mu D., and Sancar A. (1996). Replication protein A confers structure-specific endonuclease activities to the XPF-ERCC1 and XPG subunits of human DNA repair excision nuclease. J Biol Chem *271*, 11047-50

McGowan CH. (2002). Checking in on Cds1 (Chk2): checkpoint kinase and tumor suppressor. Bioessays *24*, 502-11

Mckittrick E., Gafken PR., Ahmad K., and Henikoff S. (2004). Histone H3.3 is enriched in covalent modifications associated with active chromatin. Proc Natl Acad Sci USA. *101*, 1525-30

Mellon I., and Hanawalt PC. (1989). Induction of the Escherichia coli lactose operon selectively increases repair of itstranscribed DNA strand. Nature *342*, 95-8

Michiels C., Raes M., Toussaint O., and Remacle J. (1994). Importance of Se-glutathione peroxidase, catalase, and Cu/Zn-SOD for cell survival against oxidative stress. Free Radic Biol Med. 17, 235-48

Min JH., and Pavletich NP. (2007). Recognition of DNA damage by the Rad4 nucleotide excision repair protein. Nature *449*, 570-5

Mocquet V., Laine JP., Riedl T., Yajin Z., Lee MY., and Egly JM. (2008). Sequential recruitment of the repair factors during NER: the role of XPG in initiating the resynthesis step. EMBO J. *27*, 155-67

Moreno-Herrero F., de Jager M., Dekker NH., Kanaar R., Wyman C., and Dekker C. (2005). Mesoscale conformational changes in the DNA-repair complex Rad50/Mre11/Nbs1 upon binding DNA. Nature *437*, 440-3

Morgan DO. (1995). Principles of CDK regulation. Nature *374*, 131-4

Mu D., Park CH., Matsunaga T., Hsu DS., Reardon JT., and Sancar A. (1995). Reconstitution of human DNA repair excision nuclease in a highly defined system. J Biol Chem. *270*, 2415-8

Mu D., Wakasugi M., Hsu DS., and Sancar A. (1997). Characterization of reaction intermediates of human excision repair nuclease. J Biol Chem. *272*, 28971-9

Murray AW., and Marks D. (2001). Can sequencing shed light on cell cycling? Nature *409*, 844-6

N

Nance MA., and Berry SA. (1992). Cockayne syndrome: review of 140 cases. Am J Med Genet. *42*, 68-84

Nardo T., Oneda R., Spivak G., Vaz R., Mortier L., Thomas P., Orioli D., Laugel V., Stary A., Hanawalt PC., Sarasin A., and Stefanini M. (2009). A UV-sensitive syndrome patient with a specific CSA mutation reveals separable roles for CSA in response to UV and oxidative DNA damage. Proc Natl Acad Sci USA. *106*, 6209-14

Ng HH., Feng Q., Wang H., Erdjument-Bromage H., Tempst P., Zhang Y., and Struhl K. (2002). Lysine methylation within the globular domain of histone H3 by Dot1 is important for telomeric silencing and Sir protein association. Genes Dev. *16*, 1518-27

Nowosielska A. (2007). Bacterial DNA repair genes and their eukaryotic homologues: 5. The role of recombination in DNA repair and genome stability. Acta Biochim Pol. *54*, 483-94

O

O'Driscoll M., and Jeggo PA. (2006). The role of double-strand break repair – insights from human genetics. Nat Rev Genet. *7*, 45-54

O'Gorman W., Thomas B., Kwek KY., Furger A., and Akoulitchev A. (2005). Analysis of U1 small nuclear RNA interaction with cyclin H. J Biol Chem. *280*, 36920-5

Ogi T., and Lehmann AR. (2006). The Y-family DNA polymerase kappa (pol kappa) functions in mammalian nucleotideexcision repair. Nat Cell Biol. *8*, 640-2

Oh KS., Khan SG., Jaspers NG., Raams A., Ueda T., Lehmann A., Friedmann PS., Emmert S., Gratchev A., Lachlan K., Lucassan A., Baker CC., and Kraemer KH. (2006). Phenotypic heterogeneity in the XPB DNA helicase gene (ERCC3): xeroderma pigmentosum without and with Cockayne syndrome. Hum. Mutat. *27*, 1092–1103.

Okada Y., Feng Q., Lin Y., Jiang Q., Li Y., Coffield VM., Su L., Xu G., and Zhang Y. (2005). hDOT1L links histone methylation to leukemogenesis. Cell *121*, 167-78

Okuda M., Tanaka A., Satoh M., Mizuta S., Takazawa M., Ohkuma Y., and Nishimura Y. (2008). Structural insight into the TFIIE-TFIIH interaction: TFIIE and p53 share the binding region on TFIIH. EMBO J. *27*, 1161-71

Osborne EA., Dudoit S., and Rine J. (2009). The establishment of gene silencing at single-cell resolution. Nat Genet. *41*, 800-6

Osley MA. (2006). Regulation of histone H2A and H2B ubiquitylation. Brief Funct Genomic Proteomic. *5*, 179-89

P

Park CH., Bessho T., Matsunaga T., and Sancar A. (1995). Purification and characterization of the XPF-ERCC1 complex of human DNA repair excision nuclease. J Biol Chem. *270*, 22657-60

Park CH., Mu D., Reardon JT., and Sancar A. (1995a). The general transcription-repair factor TFIIH is recruited to the excision repair complex by the XPA protein independent of the TFIIE transcription factor. J Biol Chem. *270*, 4896-902

Park CJ., and Choi BS. (2006). The protein shuffle. Sequential interactions among components of the human nucleotide excision repair pathway. FEBS J. *273*, 1600-8

Parsons JL., Dianova II., Allinson SL., and Dianov GL. (2005). Poly(ADP-ribose) polymerase-1 protects excessive DNA strand breaks from deterioration during repair in human cell extracts. FEBS J. *272*, 2012-21

Pearl LH. (2000). Structure and function in the uracil-DNA glycosylase superfamily. Mutat Res. *460*, 165-81

Pellegrini L., Yu DS., Lo T., Anand S., Lee M., Blundell TL., and Venkitaraman AR. (2002). Insights into DNA recombination from the structure of a RAD51-BRCA2 complex. Nature *420*, 287-93

Peters AH., Kubicek S., Mechtler K., O'Sullivan RJ., Derijck AA., Perez-Burgos L., Kohlmaier A., Opravil S., Tachibene M., Shinkai Y., Martens JH., and Jenuwein T. (2003). Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. Mol Cell. *12*, 1577-89

Poterszman A., Lamour V., Egly JM., Moras D., Thierry JC., and Poch O. (1997). A eukaryotic XPB/ERCC3-like helicase in Mycobacterium leprae? Trends Biochem Sci. *22*, 418-9

Price VH., Odom RB., Ward DH., and Jones FT. (1980). Trichothiodystrophy: sulfur-deficient brittle hair as a marker for a neuroectodermal symptom complex.Arch Dermatol. *116*, 1375-84

Purmal AA., Lampman GW., Bond JP., Hatahet Z., and Walace SS. (1998). Enzymatic processing of uracil glycol, a major oxidative product of DNA cytosine. J Biol Chem. *273*, 10026-35

Q

Qin S., and Parthun MR. (2002). Histone H3 and the histone acetyltransferase Hat1p contribute to DNA double-strand break repair. Mol Cell Biol. *22*, 8353-65

Qin S., and Parthun MR. (2006). Recruitment of the type B histone acetyltransferase Hat1p to chromatin is linked to DNA double-strand breaks. Mol Cell Biol. *26*, 3649-58

R

Ranalli TA., DeMott MS., and Bambara RA. (2002). Mechanism underlying replication protein A stimulation of DNA ligase I. J Biol Chem. *277*, 1719-27

Ranish JA., Yi EC., Leslie DM., Purvine SO., Goodlett DR., Eng J., and Aebersold R. (2003). The study of macromolecular complexes by quantitative proteomics. Nat Genet. *33*, 349-55

Ranish JA., Hahn S., Lu Y., Yi EC., Li XJ., Eng J., and Aebersold R. (2004). Identification of TFB5, a new component of general transcription and DNA repair factor IIH. Nat Genet. *36*, 707-13

Rapin I., Lindenbaum Y., Dickson DW., Kraemer KH., and Robbins JH. (2000). Cockayne syndrome and xeroderma pigmentosum. Neurology *55*, 1442-9

Raschle M., Marra G., Nystrom-Lahti M., Schar P., and Jiricny J. (1999). Identification of hMutLbeta, a heterodimer of hMLH1 and hPMS1. J Biol Chem. *274*, 32368-75

Reardon JT., Ge H., Gibbs E., Sancar A., Hurwitz J., and Pan ZQ. (1996). Isolation and characterization of two human transcription factor IIH (TFIIH)-related complexes: ERCC2/CAK and TFIIH. Proc Natl Acad Sci USA. *93*, 6482-7

Reardon JT., and Sancar A. (2005). Nucleotide excision repair. Prog Nucleic Acid Res Mol Biol. *79*, 183-235

Reddy YV., Ding Q., Lees-Miller SP., Meek K., and Ramsden DA. (2004). Non-homologous end joining requires that the DNA-PK complex undergo an autophosphorylation-dependent rearrangement at DNA ends. J Biol Chem. *279*, 39408-13

Riballo E., Kuhne M., Rief N., Doherty A., Smith GC., Recio MJ., Reis C., Dahm K., Fricke A., Krempler A., Parker AR., Jackson SP., Gennery A., Jeggo PA., and Lobrich M. (2004). Apathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci. Mol Cell. *16*, 715-24

Rice JC., Briggs SD., Ueberheide B., Barber CM., Shabanowitz J., Hunt DF., Shinkai Y., and Allis CD. (2003). Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. Mol Cell. *12*, 1591-8

Richards JD., Cubeddu L., Roberts J., Liu H., and White MF. (2007). The archaeal XPB protein is a ssDNA-dependent ATPase with a novel partner. J Mol Biol. *376*, 634-44

Riedl T., Hanaoka F., and Egly JM. (2003). The comings and goings of nucleotide excision repair factors on damaged DNA. EMBO J. *22*, 5293-303

Riou L., Zeng L., Chevallier-Lagente O., Stary A., Nikaido O., Taieb A., Weeda G., Mezzina M., and Sarasin A. (1999). The relative expression of mutated XPB genes results in xeroderma pigmentosum/ Cockayne's syndrome or trichothiodystrophy cellular phenotypes. Hum Mol Genet. *8*, 1125–33

Rochette-Egly C., Adam S., Rossignol M., Egly JM., and Chambon P. (1997). Stimulation of RAR alpha activation function AF-1 through binding to the general transcription factor TFIIH and phosphorylation by CDK7. Cell *90*, 97-107

Rogakou EP., Pilch DR., Orr AH., Ivanova VS., and Bonner WM. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem. *273*, 5858-68

Rossignol M., Colb-Cheynel I., and Egly JM. (1997). Substrate specificity of the cdk-activating kinase (CAK) is altered upon association with TFIIH. EMBO J. *16*, 1628-37

Rothstein R., Michel B., and Gangloff S. (2000). Replication fork pausing and recombination of "gimme a break". Genes Dev. *14*, 1-10

Rouse J., and Jackson SP. (2000). LCD1: an essential gene involved in checkpoint control and regulation of the MEC1 signalling pathway in Saccharomyces cerevisiae. EMBO J. *19*, 5801-12

Roy R., Adamczewski JP., Seroz T., Vermeulen W., Tassan JP., Schaeffer L., Nigg EA., Hoeijmakers JH., and Egly JM. (1994). The MO15 cell cycle kinase is associated with the TFIIH transcription-DNA repair factor. Cell *79*, 1093-101

Rudolf J., Makrantoni V., Ingledew WJ., Stark MJ. and White MF. (2006). The DNA repair helicases XPD and FancJ have essential iron–sulfur domains. Mol Cell. *23*, 801-8

Russo AA., Jeffrey PD., Patten AK., Massakue J., and Pavletich NP. (1996). Crystal structure of the p27Kip1 cyclin-dependent-kinase inhibitor bound to the cyclin A-Cdk2 complex. Nature *382*, 325-31

Rydberg B., and Lindahl T. (1982). Nonenzymatic methylation of DNA by the intracellular methyl group donor S-adenosyl-L-methionine is a potentially mutagenic reaction. EMBO J. *1*, 211-6

S

Sancar A., and Reardon JT. (2004). Nucleotide excision repair in *E. coli* and man. Adv Protein Chem. *69*, 43-71

San-Segundo P.A., and Roeder G.S. (2000). Role for the silencing protein DOT1 in meiotic checkpoint control. Mol Biol Cell. *11*, 3601-15

Sarasin A., and Stary A. (2007). New insights for understanding the transcription-coupled repair pathway. DNA Repair (Amst). *6*, 265-9

Sarker AH., Tsutakawa SE., Kostek S., Ng C., Shin DS., Peris M., Campeau E., Tainer JA., Nogales E., and Cooper PK. (2005). Recognition of RNA polymerase II and transcription bubbles by XPG, CSB, and TFIIH: insights for transcription-coupled repair and Cockayne syndrome. Mol Cell. *20*, 187-98

Sattler U., Frit P., Salls B., and Calsou P. (2003). Long-patch DNA repair synthesis during base excision repair in mammalian cells. EMBO Rep. *4*, 363-7

Savitsky K., Bar-Shira A., Gilad S., Rotman G., Ziv Y., Vanagaite L., Tagle DA., Smith S., Uziel T., Sfez S., *et al*. (1995). A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science *268*, 1749-53

Schaeffer L., Roy R., Humbert S., Moncollin V., Vermeulen W., Hoeijmakers JH., Chambon P., and Egly JM. (1993). DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor. Science *260*, 58-63

Schaeffer L., Moncollin V., Roy R., Staub A., Mezzina M., Sarasin A., Weeda G., Hoeijmakers JH., and Egly JM. (1994). The ERCC2/DNA repair protein is associated with the class II BTF2/TFIIH transcription factor. EMBO J. *13*, 2388-92

Scherly D., Nouspikel T., Corlet J., Ucla C., Bairoch A., and Clarkson SG. (1993).Complementation of the DNA repair defect in xeroderma pigmentosum group G cells by a human cDNA related to yeast RAD2. Nature *363*, 182-5

Schultz P., Fribourg S., Poterszman A., Mallouh V., Moras D., and Egly JM. (2000). Molecular structure of human TFIIH. Cell *102*, 599-607

Seroz T., Perez C., Bergmann E., Bradsher J., and Egly JM. (2000). p44/SSL1, the regulatory subunit of the XPD/RAD3 helicase, plays a crucial role in the transcriptional activity of TFIIH. J Biol Chem. *275*, 33260-6

Shahbazian MD., Zhang K., and Grunstein M. (2005). Histone H2B ubiquitilation controls processive methylation but not monomethylation by Dot1 and Set1. Mol Cell. *19*, 271-7

Shanower G.A., Muller M., Blanton J.L., Honti V., Gyurkovics H., and Schedl P. (2005). Characterization of the *grappa* gene, the Drosophila histone H3 lysine 79 methyltransferase. Genetics *169*, 173-84

Shilatifard A. (2006). Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. Annu Rev Biochem. *75*, 243-69

Shiloh Y. (2001). ATM and ATR: networking cellular responses to DNA damage. Curr Opin Genet Dev. *11*, 71-7

Shrivastav M., De Haro LP., and Nickoloff JA. (2008). Regulation of DNA double-strand break repair pathway choice. Cell Res. *18*, 134-47

Shultz LB., Chehab NH., Malikzay A., and Halazonetis TD. (2000). p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. J Cell Biol. *151*, 1381-90

Sijbers AM., de Laat WL., Ariza RR., Biggerstaff M., Wei YF., Moggs JG., Carter KC., Shell BK., Evans E., de Jong MC., Rademakers S., de Rooij J., Jaspers NG., Hoeijmakers JH., and Wood RD. (1996). Xeroderma pigmentosum group F caused by a defect in a structure-specific DNA repair endonuclease. Cell *86*, 811-22

Simon JA., and Tamkun JW. (2002). Programming off and on states in chromatin: mechanisms of Polycomb and trithorax group complexes. Curr Opin Genet Dev. *12*, 210-8

Simonelli V., Narciso L., Dogliotti E., and Fortini P. (2005). Base excision repair intermediates are mutagenic in mammalian cells. Nucleic Acids Res. *33*, 4404-11

Sinclair DA., and Guarente L. (1997). Extrachromosomal rDNA circles--a cause of aging in yeast. Cell *91*, 1033-42

Singer M.S., Kahana A., Wolf A.J., Meisinger L.L., Peterson S.E., Goggin C., Mahowald M., and Gottschling D.E. (1998). Identification of high-copy disruptor of telomeric silencing in Saccharomyces cerevisiae. Genetics *150*, 613-32.

Sinha RP, and Hader DP. (2002). UV-induced DNA damage and repair: a review. Photochem Photobiol Sci. *1*, 225-36

Skibbens RV. (2004) Chl1p, a DNA helicase-like protein in budding yeast, functions in sister-chromatid cohesion. Genetics *166*, 33–42

Smith BT., Grossman AD., and Walker GC. (2001). Visualization of mismatch repair in bacterial cells. Mol Cell. *8*, 1197-1206

Spivak G. (2005). UV-sensitive syndrome. Mutat Res. *577*, 162-9

Staresincic L., Fagbemi AF., Enzlin JH., Gourdin AM., Wijgers N., Dunand-Sauthier I., Giglia-Mari G., Clarkson SG., Vermeulen W., and Scharer OD. (2009). Coordination of dual incision and repair synthesis in human nucleotide excision repair. EMBO J. *28*, 1111-20

Stary A., and Sarasin A. (1996). The genetic basis of xeroderma pigmentosum and trichothiodystrophy syndromes. Cancer Surv. *26*, 155-71

Stigger E., Drissi R., and Lee SH. (1998). Functional analysis of human replication protein A in nucleotide excision repair. J Biol Chem. *273*, 9337-43

Strahl-Bolsinger S., Hecht A., Luo K., and Grunstein M. (1997). SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. Genes Dev. *11*, 83-93

Su TT. (2006). Cellular responses to DNA damage: one signal, multiple choices. Annu Rev Genet. *40*, 187-208

Sugasawa K., Ng JM., Masutani C., Iwai S., van der Spek PJ., Eker AP., Hanaoka F., Bootsma D., and Hoeijmakers JH. (1998). Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair. Mol Cell. *2*, 223-32

Sun ZW., and Allis CD. (2002). Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature, *418*, 104-8

Sung JS., and Demple B. (2006). Roles of base excision repair subpathways in correcting oxidized abasic sites in DNA. FEBS J. 273, 1620-29

Svejstrup JQ., Vichi P., and Egly JM. (1996). The multiple roles of transcription/repair factor TFIIH. Trends Biochem Sci. *21*, 346-50

Sweder KS., Chun R., Mori T., and Hanawalt PC. (1996). DNA repair deficiencies associated with mutations in genes encoding subunits of transcription initiation factor TFIIH in yeast. Nucleic Acids Res. *24*, 1540-6

T

Takagi Y., Komori H., Chang WH., Hudmon A., Erdjument-Bromage H., Tempst P., and Kornberg RD. (2003). Revised subunit structure of yeast transcription factor IIH (TFIIH) and reconciliation with human TFIIH. J Biol Chem. *278*, 43897-900

Takagi Y., Masuda CA., Chang WH., Komori H., Wang D., Hunter T., Joazeiro CA., and Kornberg RD. (2005). Ubiquitin ligase activity of TFIIH and the transcriptional response to DNA damage. Mol Cell. *18*, 237-43

Taniguchi T., Garcia-Higuera I., Xu B., Andreassen PR., Gregory RC., Kim ST., Lane WS., Kastan MB., and D'Andrea AD. (2002). Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. Cell *109*, 459-72

Tang JY., Hwang BJ., Ford JM., Hanawalt PC., and Chu G. (2000). Xeroderma pigmentosum p48 gene enhances global genomic repair and suppresses UV-induced mutagenesis. Mol Cell. *5*, 737-44.

Tanny JC., Dowd GJ., Huang J., Hilz H., and Moazed D. (1999). An enzymatic activity in the yeast Sir2 protein that is essential for gene silencing. Cell *99*, 735-45

Tapias A., Auriol J., Forget D., Enzlin JH., Scharer OD., Coin F., Coulombe B., and Egly JM. (2004). Ordered conformational changes in damaged DNA induced by nucleotide excision repair factors. J Biol Chem. *279*, 19074-83

Taylor J., Lu H., and Kotyk JJ. (1990). Quantitatiove conversion of the (6-4) photoproduct of TpdC to its Dewar valence isomer upon exposure to simulated sunlight. Photochem Photobiol. *51*, 161-7

Teng Y., Yu Y., and Water R. (2002). The *Saccharomyces cerevisiae* histone acetyltransferase Gcn5 has a role in the photoreactivation and nucleotide excision repair of UV-induced cyclobutane pyrimidine dimmers in the *MFA2* gene. J Mol Biol. *316*, 489-99

Thoma F. (2005). Repair of UV lesions in nucleosomes – intrinsic properties and remodeling. DNA Repair (Amst). *4*, 855-69

Thomas MC., and Chiang CM. (2006). The general transcription machinery and general cofactors. Crit Rev Biochem Mol Biol. *41*, 105-78

Thompson LH., and Schild D. (2001). Homologous recombination repair of DNA ensures mammalian chromosome stability. Mutat Res. *477*, 131-53

Tibbetts RS., Brumbaugh KM., Williams JM., Sarkaria JN., Cliby WA., Shieh SY., Taya Y., Prives C., and Abraham RT. (1999). A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev. 13, 152-7

Tibbetts RS., Cortez D., Brumbaugh KM., Scully R., Livingston D., Elledge SJ., and Abraham RT. (2000). Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress. Genes Dev. *14*, 2989-3002

Tirode F., Busso D., Coin F. and Egly JM. (1999). Reconstitution of the transcription factor TFIIH: assignment of functions for the three enzymatic subunits, XPB, XPD, and cdk7. Mol Cell. *3*, 87–95

Toh GW., O'Shaughnessy AM., Jimeno S., Dobbie IM., Grenon M., Maffini S., O'Rorke A., and Lowndes NF. (2006). Histone H2A phosphorylation and H3 methylation are required for a novel Rad9 DSB repair function following checkpoint activation. DNA Repair (Amst). *5*, 693-703

Tong X., Drapkin R., Reinberg D., and Kieff E. (1995). The 62- and 80-kDa subunits of transcription factor IIH mediate the interaction with Epstein-Barr virus nuclear protein 2. Proc Natl Acad Sci USA. *92*, 3259-63

Tran PT., Erdeniz N., Symington LS., and Liskay RM. EXO1-A multy-tasking eukaryotic nuclease. DNA Repair (Amst.) *3*, 1549-59

Tremeau-Bravard A., Perez C., and Egly JM. (2001). A role of the C-terminal part of p44 in the promoter escape activity of transcription factor IIH. J Biol Chem. *276*, 27693-7

Truglio JJ., Croteau DL., Van Houten B., and Kisker C. (2006). Prokaryotic nucleotide excision repair: the UvrABC system. Chem Rev. *106*, 233-252

Tudek B., Boiteux S., and Laval J. (1992). Biological properties of imidazole ring-opened N7-methylguanine in M13mp18 phage DNA. Nucleic Acids Res. *20*, 3079-84

Tudek B. (2007). Base excision repair modulation as a risk factor for human cancers. Mol Aspects Med. *28*, 258-75

Tuo J., Jaruga P., Rodriguez H., Dizdaroglu M., and Bohr VA. (2002). Tha cockayne syndrome group B gene product is involved in cellular repair of 8-hydroxyadenine in DNA. J Biol Chem. *277*, 30832-7

Tuteja N., and Tuteja R. (2004). Prokaryotic and eukaryotic DNA helicases. Essential molecular motor proteins for cellular machinery. Eur J Biochem. *271*, 1835-48

U

Uhlmann F., Cai J., Flores-Rozas H., Dean FB., Finkelstein J., O'Donnell M., and Hurwitz J. (1996). In vitro reconstitution of human replication factor C from its five subunits. Proc Natl Acad Sci USA. *93*, 6521-6

V

Valay JG., Simon M., Dubois MF., Bensaude O., Facca C., and Faye G. (1995). The KIN28 gene is required both for RNA polymerase II mediated transcription and phosphorylation of the Rpb1p CTD. J Mol Biol. *249*, 535-44

Valko M., Rhodes CJ., Moncol J., Izakovic M., and Mazur M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact. *160*, 1-40

van den Boom V., Citterio E., Hoogstraten D., Zotter A., Egly JM., van Cappellen WA., Hoeijmakers JH., Houtsmuller AB., and Vermeulen W. (2004). DNA damage stabilizes interaction of CSB with the transcription elongation machinery. J Cell Biol. *166*, 27-36

van Hoffen A., Venema J., Meschini R., van Zeeland AA., and Mullenders LH. (1995). Transcription-coupled repair removes both cyclobutane pyrimidine dimers and 6-4 photoproducts with equal efficiency and in a sequential way from transcribed DNA in xeroderma pigmentosum group C fibroblasts. EMBO J. *14*, 360-7

van Leeuwen F., Gafken PR., and Gottschling DE. (2002). Dot1p modulates silencing in yeast by methylation of the nucleosome core. Cell *109*, 745-56

Vandel L., and Kouzarides T. (1999). Residues phosphorylated by TFIIH are required for E2F-1 degradation during S-phase. EMBO J. *18*, 4280-91

Vermeulen W., Bergmann E., Auriol J., Rademakers S., Frit P., Appeldoorn E., Hoeijmakers JH., and Egly JM. (2000). Sublimiting concentration of TFIIH transcription/DNA repair factor causes TTD-A trichothiodystrophy disorder. Nat Genet. *26*, 307-13

Vitorino M., Coin F., Zlobinskaya O., Atkinson RA., Moras D., Egly JM., Poterszman A., and Kieffer B. (2007). Solution structure and self-association properties of the p8 TFIIH subunit responsible for trichothiodystrophy. J Mol Biol. *368*, 473-80

Vivian AJ., Ellison DW., and McGill JI. (1993). Ocular melanomas in xeroderma pigmentosum. Br J Ophthalmol. *77*, 597-8

W

Waga S., and Stillman B. (1998). The DNA replication fork in eukaryotic cells. Annu Rev Biochem. *67*, 721-51

Walker JR., Corpina RA., and Goldberg J. (2001). Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. Nature *412*, 607-14

Wang CI., and Taylor JS. (1991). Site-specific effect of thymine dimmer formation on dAn.dTn tract bending and its biological implications. Proc Natl Acad Sci USA. *88*, 9072-6

Wang M., Mahrenholz A., and Lee SH. (2000). RPA stabilizes the XPA-damaged DNA complex through protein-protein interaction. Biochemistry *39*, 6433-9

Wang H., Wang H., Powell SN., Iliakis G., and Wang Y. (2004). ATR affecting cell radiosensitivity is dependent on homologous recombination repair but independent of nonhomologous end joining. Cancer Res. *64*, 7139-43

Weeda G., van Ham RC., Vermeulen W., Bootsma D., van der Eb AJ., and Hoeijmakers JH. (1990a). A presumed DNA helicase encoded by ERCC-3 is involved in the human repair disorders xeroderma pigmentosum and Cockayne's syndrome. Cell *62*, 777-91

Weeda G., van Ham RC., Masurel R., Westerveld A., Odijk H., de Wit J., Bootsma D., van der Eb AJ., Hoeijmakers JH. (1990b). Molecular cloning and biological characterization of the human excision repair gene ERCC-3. Mol Cell Biol. *10*, 2570-81

Weiss RS., Matsuoka S., Elledge SJ., and Leder P. (2002). Hus1 acts upstream of chk1 in a mammalian DNA damage response pathway. Curr Biol. *12*, 73-7

White MF. (2009). Structure, function and evolution of the XPD family of iron-sulfur-containing 5'-->3' DNA helicases. Biochem Soc Trans. *37*, 547-51

Wiederhold L., Leppard JB., Kedar P., Karimi-Busheri F., Rasouli-Nia A., Weinfeld M., Tomkinson AE., Izumi T., Prasad R., Wilson SH., Mitra S., and Hazra TK. (2004). AP endonuclease-independent DNA base excision repair in human cells. Mol Cell. *15*, 209-20

Winkler GS., Araujo SJ., Fiedler U., Vermeulen W., Coin F., Egly JM., Hoeijmakers JH., Wood RD., Timmers HT., and Weeda G. (2000). TFIIH with inactive XPD helicase functions in transcription initiation but is defective in DNA repair. J Biol Chem. *275*, 4258–66

Wolski SC., Kuper J., Hanzelmann P., Truglio JJ., Croteau DL., Van Houten B., and Kisker C. (2008). Crystal structure of the FeS cluster-containing nucleotide excision repair helicase XPD. PLoS Biol. *6*, e149

Wood RD., and Lindahl T. (1990). Xeroderma pigmentosum. A gene or tumour prevention. Nature *348*, 13-4

Wood RD. (1997). Nucleotide excision repair in mammalian cells. J Biol Chem. *272*, 23465-8

Wood RD., Araujo SJ., Ariza RR., Batty DP., Biggerstaff M., Evans E., Gaillard PH., Gunz D., Koberle B., Kuraoka I., Moggs JG., Sandall JK., and Shivji MK. (2000). DNA damage recognition and nucleotide excision repair in mammalian cells. Cold Spring Harb Symp Quant Biol. *65*, 173-82

Wood A., Schneider J., Dover J., Johnston M., and Shilatifard A (2003). The Paf1 complex is essential for the histone monoubiquitination by the Rad6-Bre1 complex, which signal for histone methylation by COMPASS and Dot1p. J Biol Chem. *278*, 34739-42

Wu Y., Suhasini AN. and Brosh RM. (2009). Welcome the family of FANCJ-like helicases to the block of genome stability maintenance proteins. Cell Mol Life Sci. *66*, 1209–22

Wu W., Liu H., Lei R., Chen D., Zhang S., Lv J., Wang Y., Fan W., Qian J., Jin G., Ma H., Miao R., Hu Z., Wang H., Jin L., Wei Q., Shen H., Huang W., and Lu D. (2009). Genetic variants in GTF2H1 and risk of lung cancer: a case-control analysis in a Chinese population. Lung Cancer *63*, 180-6

Wyman C., and Botchan M. (1995). DNA replication. A familiar ring to DNA polymerase processivity. Curr Biol. *5*, 334-7

Wysocki R., Javaheri A., Allard S., Sha F., Cote J., and Kron SJ. (2005). Role of Dot1-dependent histone H3 methylation in G1 and S phase DNA damage checkpoint functions of Rad9. Mol Cell Biol. *25*, 8430-43

X

Xiao H., Pearson A., Coulombe B., Truant R., Zhang S., Regier JL., Triezenberg SJ., Reinberg D., Flores O., Ingles CJ., et al. (1994). Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. Mol Cell Biol. *14*, 7013-24

Y

Yankulov KY., and Bentley DL. (1997). Regulation of CDK7 substrat specificity by MAT1 and TFIIH. EMBO J. *16*, 1638-46

Yang B., Britton J., and Kirchmaier AL. (2008). Insights into the impact of histone acetylation and methylation on Sir protein recruitment, spreading, and silencing in Saccharomyces cerevisiae. J Mol Biol. *381*, 826-44

Yarden RI., Pardo-Reoyo S., Sgagias M., Cowan KH., and Brody LC. (2002). BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage. Nat Genet. *30*, 285-9

Yeeles JT., Cammack R., and Dillingham MS. (2009). An iron–sulfur cluster is essential for the binding of broken DNA by AddAB-type helicase–nucleases. J Biol Chem. *284*, 7746-55

Yokoi M., Masutani C., Maekawa T., Sugasawa K., Ohkuma Y., and Hanaoka F. (2000). The xeroderma pigmentosum group C protein complex XPC-HR23B plays an important role in the recruitment of transcription factor IIH to damaged DNA. J Biol Chem. *275*, 9870-5

You JS., Wang M., and Lee SH. (2003). Biochemical analysis of the damage recognition process in nucleotide excision repair. J Biol Chem. *278*, 7476-85

Yu Y., Teng Y., Liu H., Reed SH., and Waters R. (2005). UV irradiation stimulates histone acetylation and chromatin remodeling at a repressed yeast locus. Proc Natl Acad Sci USA. *102*, 8650-5

Yuzhakov A., Kelman Z., Hurwitz J., and O'Donnell M. (1999). Multiple competition reactions for RPA order the assembly of the DNA polymerase delta holoenzyme. EMBO J. *18*, 6189-99

Z

Zeisig DT., Bittner CB., Zeisig BB., Garcia-Cuellar MP., Hess JL., and Slany RK. (2008). The eleven-nineteen-leukemia protein ENL connects nuclear MLL fusion partners with chromatin. Oncogene *24*, 5525-32

Zhang W., Hayashizaki Y., and Kone B.C. (2004). Structure and regulation of the mDot1 gene, a mouse histone H3 methyltransferase. Biochem J. *377*, 641-51

Zhao H., and Piwnica-Worms H. (2001). ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Mol Cell Biol. *21*, 4129-39

Zhuang J., Zhang J., Willers H., Wang H., Chung JH., van Gent DC., Hallahan DE., Powell SN., and Xia F. (2006). Checkpoint kinase 2-mediated phosphorylation of BRCA1 regulates the fidelity of nonhomologous end-joining. Cancer Res. *66*, 1401-8

Zhou Y., Kou H., and Wang Z. (2007). Tfb5 interacts with Tfb2 and facilitates nucleotide excision repair in yeast. Nucleic Acids Res. *35*, 861-71

Zou L., Cortez D., and Elledge SJ. (2002). Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. Genes Dev. *16*, 198-208

Zurita M., and Merino C. (2003). The transcriptional complexity of the TFIIH complex. Trends Genet. *19*, 5–84

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Article

Distinct Roles for the XPB/p52 and XPD/p44 Subcomplexes of TFIIH in Damaged DNA Opening during Nucleotide Excision Repair

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SUMMARY

Mutations in XPB, an essential subunit of the transcription/repair factor TFIIH, lead to nucleotide excision repair (NER) defects and xeroderma pigmentosum (XP). The role of XPB in NER and the molecular mechanisms resulting in XP are poorly understood. Here, we show that the p52 subunit of TFIIH interacts with XPB and stimulates its ATPase activity. A mutation found among XP-B patients (F99S) weakens this interaction and the resulting ATPase stimulation, thereby explaining the defect in the damaged DNA opening. We next found that mutations in the helicase motifs III (T469A) and VI (Q638A) that inhibit XPB helicase activity preserve the NER function of TFIIH. Our results suggest a mechanism in which the helicase activity of XPB is not used for the opening and repair of damaged DNA, which is instead only driven by its ATPase activity, in combination with the helicase activity of XPD.

INTRODUCTION

The human transcription/repair factor IIH (TFIIH) consists of ten subunits. XPB, XPD, p62, p52, p44, p34, and p8/TTDA form the core complex, while cdk7, MAT1, and cyclin H form the cdk-activating kinase (CAK) subcomplex, linked to the core via XPD. Hereditary mutations in either XPB, XPD, or p8/TTDA yield the xeroderma pigmentosum (XP), XP combined with Cockayne syndrome (XP/CS), or trichothiodystrophy (TTD) syndromes (Lehmann, 2003; Giglia-Mari et al., 2004; Oh et al., 2006). These diseases exhibit a broad spectrum of clinical features including photosensitivity of the skin due to defects in nucleotide excision repair (NER) (Lehmann, 2003). NER is part of a cellular defense system that protects genome integrity by removing a wide diversity of helix-distorting DNA lesions induced by ultraviolet (UV) light and bulky chemical adducts. The removal of lesions requires their recognition by the repair factor XPC-HR23b and the subsequent unwinding of the DNA duplex by TFIIH. The single-stranded structure is then stabilized by XPA and RPA, and the margins of the resulting DNA bubble are recognized by XPG and ERCC1-XPF, thereby generating 3′ and 5′ incisions relative to the damage (O'Donnovan et al., 1994; Sijbers et al., 1996).

DNA helicases are motor proteins that can transiently catalyze the unwinding of the stable duplex DNA molecules using NTP hydrolysis as the source of energy. They are characterized by seven "helicase motifs," constituted of conserved amino acid sequences (Tuteja and Tuteja, 2004). It was always hypothesized that XPB and XPD helicase subunits of TFIIH supply opposite unwinding capacities required for local helix opening to form the open DNA intermediates in NER (Bootsma and Hoeijmakers, 1993). Indeed, mutations in the ATP binding site of these proteins inhibit NER in vivo and in vitro (Guzder et al., 1994; Sung et al., 1988), due to a defect in the opening of the damaged DNA structure (Coin et al., 2006). However, these studies used mutants that act by targeting the ATPase A Walker I motif, but not the other helicase motifs (from II to VI). Thus, questions remain as to whether DNA opening during repair requires both XPB and XPD helicases to open a short sequence of 24/32 nucleotides encompassing the lesion.

Thus far, investigations of the mechanistic defects leading to XP, CS, or TTD have been beneficial in understanding the function of XPB, XPD, and p8/TTDA in NER and in transcription (Evans et al., 1997; Keriel et al., 2002; Dubaele et al., 2003, Coin et al., 2006). In this study, we unveiled the role of both the XPB and XPD subunits of TFIIH in NER by analyzing several mutations found in XP patients or other engineered mutations introduced in highly conserved domains of the corresponding proteins. We found that the helicase activity of XPB was not used for damaged DNA opening, which is instead driven by its ATPase activity, in combination with the helicase activity of XPD. Furthermore, we demonstrated that the p52 subunit of TFIIH upregulates the ATPase activity of XPB through a direct XPB/p52 contact that is impaired in XP-B patients. The TFIIH from these patient is unable to induce the opening of the DNA around the lesion, due to the incorrect XPB/p52 interaction and ATPase stimulation.

RESULTS

The F99S Mutation in XPB Impairs Damaged DNA Opening

To provide insights into the role of XPB in NER, we investigated the DNA repair activity of two TFIIH complexes purified from cell extracts of XP-B patients carrying either the F99S (XP) or the T119P (TTD) mutations (Oh et al., 2006) (Figure 1A, left panel). Western blot analysis reveals a similar subunit composition of the immunopurified TFIIH/XPB(WT), TFIIH/XPB(F99S), and TFIIH/XPB(T119P) complexes (Figure 1A, right panel). Upon addition of TFIIH/XPB(F99S) to a reconstituted in vitro dual incision assay (Coin et al., 2004), a low level (10% activity) of excised damaged oligonucleotides was observed, compared with TFIIH/XPB(WT) (Figure 1B, NER, compare lanes 5 and 6 with lanes 3 and 4). TFIIH/XPB(F99S) was more efficient in a reconstituted transcription assay (Gerard et al., 1991) (75% activity) than in dual incision (Tx, compare lanes 5 and 6 with lanes 3 and 4). The T119P mutation did not affect either dual incision or transcription activities (compare lanes 7 and 8 with lanes 3 and 4).

Given the role of TFIIH in NER, we carried out a permanganate footprinting assay measuring the opening of the DNA around the damage (Evans et al., 1997). Addition of either TFIIH/XPB(WT) or TFIIH/XPB(T119P) to a reaction containing XPC-HR23b, in addition to the cisplatinated DNA fragment, resulted in an increased sensitivity of nucleotides at positions T-4, T-5, and, to a lesser extent, T-7 and T-10 indicative of DNA opening (Figure 1C, compare lane 2 with lanes 5 and 9). In contrast, addition of TFIIH/XPB(F99S) did not trigger a detectable opening of the damaged DNA (Figure 1C, lane 7). However, further addition of the NER factor XPA to TFIIH/XPB(F99S) promoted a weak but significant opening of the DNA, compared with the full opening obtained with either TFIIH/XPB(WT) or (T119P) (Figure 1C, compare lane 8 to lanes 6 and 10). This defect in DNA opening parallels and explains the low removal of damaged oligonucleotides seen in Figure 1B.

To dissect the molecular mechanism of the NER defect observed with the F99S mutation, we purified from baculovirus-infected insect cells a recombinant TFIIH complex (IIH6) containing the six subunits of the core TFIIH (XPB, XPD, p62, p52, p44, and p34). The following experiments were performed only with the core TFIIH, since the CAK complex did not play any role in our in vitro NER assay (Coin et al., 2006). Similarly to the endogenous TFIIH/XPB(F99S), the recombinant IIH6/XPB(F99S) showed a lower repair activity, compared with either IIH6/XPB(WT) or (T119P) (Figure 1D, compare lane 5 with lanes 1 and 7). Interestingly, the addition of the NER-specific TFIIH subunit p8/TTDA to IIH6/XPB(F99S) did not stimulate incision, compared with the increase in the removal of damaged oligonucleotides observed with either the IIH6/XPB(WT) or IIH6/XPB(T119P) complexes (Figure 1D, compare lane 6 with lanes 2 and 8). Next, we observed that addition of p8/TTDA and XPA to IIH6/XPB(F99S) did not trigger optimal opening of the damaged DNA in a permanganate footprinting assay, compared with either IIH6/XPB(WT) or IIH6/XPB(T119P) (Figure 1E, compare lanes 10–12 with lanes 4–6 and 13–15). As a control, mutation in the XPB ATPase A Walker I motif totally abolished the IIH6/XPB(K346R) repair activity (Figure 1D, lane 3), due to an inhibition of the damaged DNA opening (Figure 1E, lane 7) and regardless of the presence of p8/TTDA (Figure 1D, lane 4, and Figure 1E, lanes 8 and 9).

Finally, the recruitment of both TFIIH and XPA to the lesion was tested in vivo following local UV irradiation of wild-type MRC5 and XPCS2BA (bearing the F99S mutation) nuclei (Volker et al., 2001). Fluorescence signals of XPB colocalized with cyclobutane pyrimidine dimer (CPD) spots both in wild-type MRC5 and XPCS2BA cells (Figures 2A–2D), indicating that TFIIH/XPB(F99S) translocates to the sites of DNA photolesions. In contrast, XPA was not recruited to the lesions in XPCS2BA, compared to MRC5 cells (Figures 2E–2H). At this point, we concluded that the repair defect harbored by TFIIH/XPB(F99S) is at the opening step, following the binding of TFIIH to the damaged DNA.

p52 Stimulates the ATPase Activity of XPB

Having observed that the F99S mutation does not impair the helicase activity of the recombinant XPB protein (data not shown), we focused on the ATPase activity of XPB. We observed that the core IIH6/XPB(F99S) complex displayed a lower ATPase activity (30% activity) than those of IIH6/XPB(WT) and IIH6/XPB(T119P) (Figure 3A). Enigmatically, the free XPB(F99S) polypeptide exhibited a catalytic ATPase activity similar to those of XPB(WT) or XPB(T119P) (Figure 3B). These observations prompted us to examine if XPB-interacting subunits in TFIIH could modulate its ATPase activity. Addition of increasing amounts of p52, a partner of XPB in TFIIH (Jawhari et al., 2002), to a fixed amount of purified XPB significantly stimulated its ATPase activity (Figure 3C, lanes 2–4). To the contrary, addition of either p44 or p8/TTDA, two subunits of TFIIH that do not interact with XPB, had no effect on the ATPase (Figure 3C, lanes 5, 6, 8, and 9).

We next investigated if XPB(F99S) and XPB(T119P) were detrimental for the XPB/p52 interaction. Equal amounts of recombinant XPB(WT), XPB(F99S), and XPB(T119P), immobilized on agarose beads, were incubated with p52-expressing extracts. Following extensive washing, we observed in our experimental conditions that XPB(F99S) interacts much less with p52 than do XPB(WT) or XPB(T119P) (Figure 3D, compare lanes 8 and 9 and 5 and 6 with lanes 2 and 3). When tested in an ATPase assay, p52 weakly stimulated XPB(F99S), compared with XPB(WT) (Figure 3E, compare lanes 5–7 with lanes 2–4). Altogether, these results demonstrate first that p52 regulates XPB ATPase activity and second that a mutation found in XP-B/CS patients weakens the interaction between the regulatory subunit p52 and XPB, leading to a low stimulation of the ATPase activity and a reduced opening of DNA around the damage.

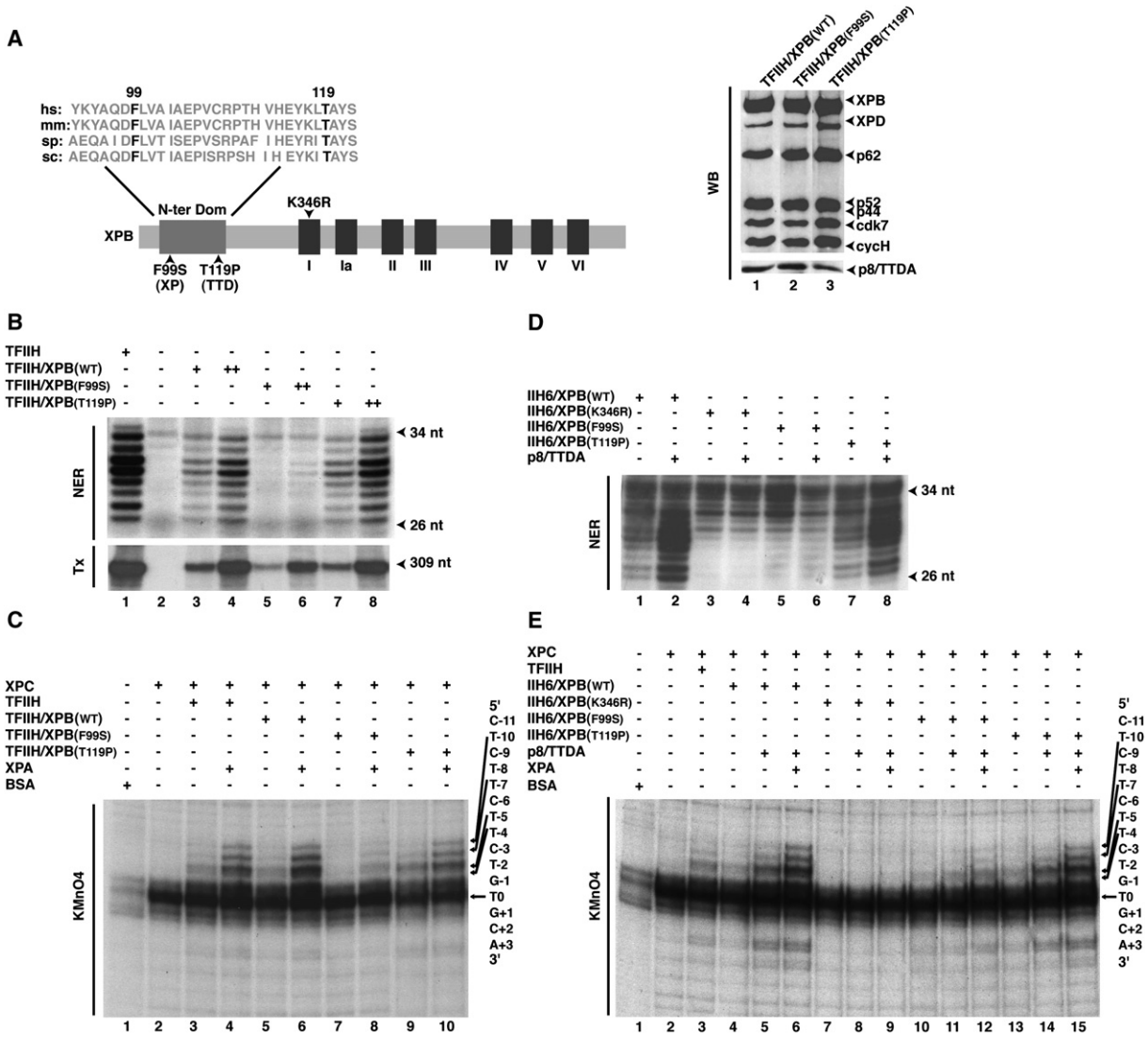


Figure 1. The F99S Mutation Impairs Damaged DNA Opening

(A) (Left) Schematic representation of XPB. The dark gray boxes indicate the helicase domains. The light gray box indicates the conserved N-terminal domain. Mutations found in XP-B patients (F99S and T119P) and mutation in the ATPase A Walker I motif (K346R) are depicted. (Right) Two established clones derived from the XPCS2BA cell line (mutation F99S) and expressing either the F99S (XP) or T119P (TTD) XPB (Riou et al., 1999) were used together with the MRC5 control cell line for TFIIH purification. TFIIH/XPB(WT), TFIIH/XPB(F99S), and TFIIH/XPB(T119P) were immunoprecipitated with antibody toward p44, a subunit of the core TFIIH, from whole-cell extracts and eluted with a competitor peptide (Coin et al., 1999). The samples were resolved by SDS-PAGE and western blotted (WB) with anti-TFIIH antibodies. The subunits of TFIIH are indicated.

(B) Fifty and one hundred nanograms of TFIIH/XPB(WT) (lanes 3 and 4), TFIIH/XPB(F99S) (lanes 5 and 6), or TFIIH/XPB(T119P) (lanes 7 and 8) were tested in a dual incision assay (NER) containing the recombinant XPC-HR23b, XPA, RPA, XPG, ERCC1-XPF factors and a closed-circular plasmid containing a single 1,3-intrastrand d(GpTpG) cisplatin-DNA crosslink (Pt-DNA) as a template (Frit et al., 2002) or in a reconstituted transcription assay (Tx) composed of recombinant TFIIB, TFIIF, TBP, TFIIE factors, the purified RNA polymerase II, and the adenovirus major late promoter template (Gerard et al., 1991). Sizes of the incision products or transcripts are indicated.

(C) TFIIH (100 ng) was incubated with a radiolabeled linear DNA fragment from the Pt-DNA plasmid and 40 ng of XPC-HR32b. XPA (25 ng) was added when indicated. Lane 1, Pt-DNA with BSA only. Residues are numbered with the central thymine of the crosslinked GTG sequence designated T0. Arrows indicate KMnO4-sensitive sites. Adducted strand residues to the 3′ and 5′ ends of T0 are denoted by positive and negative integers (+N, −N).

(D) The recombinant IIH6/XPB(WT), IIH6/XPB(K346R) (mutated in the ATPase A Walker I site), IIH6/XPB(F99S), and IIH6/XPB(T119P) lacking CAK and p8/TTDA were produced in baculovirus-infected insect cells (Tirode et al., 1999). TFIIH (100 ng) was tested in dual incision in the presence of 3 ng of recombinant p8/TTDA when indicated (lanes 2, 4, 6, and 8).

(E) A KMnO4 assay was performed as described in Figure 1C with 100 ng of recombinant IIH6 complex incubated with a radiolabeled linear DNA fragment from the Pt-DNA plasmid and XPC-HR32b. XPA (25 ng) and p8/TTDA (6 ng) were added when indicated. Lane 1, Pt-DNA with BSA only. Lane 2, positive control with TFIIH purified from HeLa.

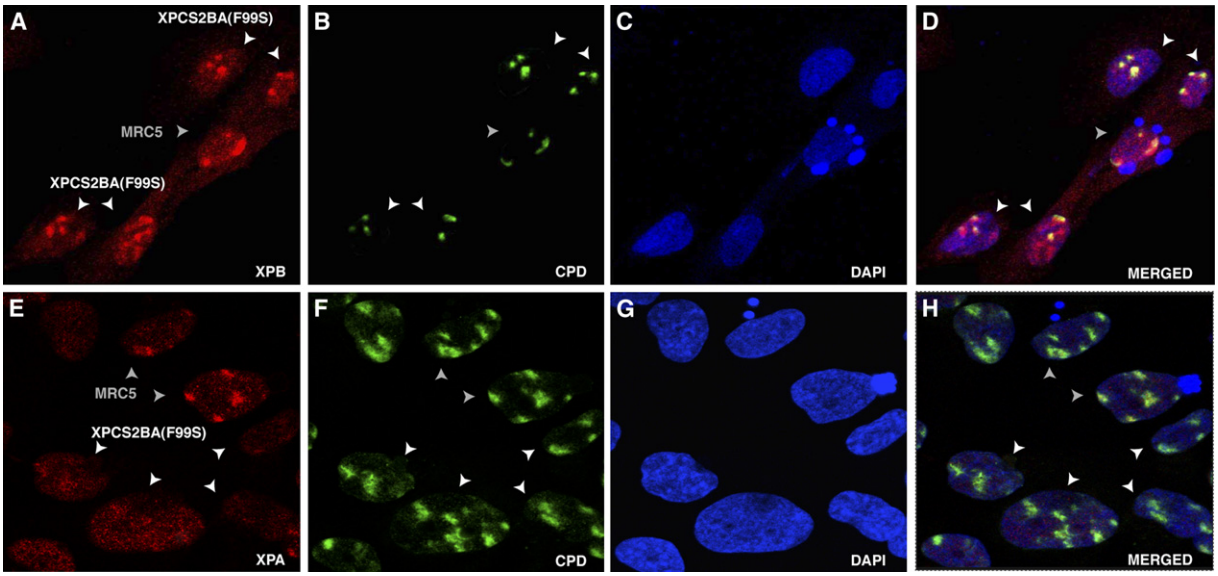


Figure 2. Recruitment of TFIIH and XPA at Sites of UV Damage

XPCS2BA(F99S) and wild-type MRC5 (labeled with blue beads) cells were plated on the same slide. Cells were UV irradiated with 70 J/m^2 through a 3 μm pore filter and fixed 30 min later. Immunofluorescent labeling was performed using a rabbit polyclonal anti-XPB (A), a mouse monoclonal anti-CPD (B and F) or a rabbit polyclonal anti-XPA (E). Nuclei were counterstained with DAPI (C and G), and slides were merged (D and H).

To map the region of p52 that is involved in the stimulation of XPB ATPase activity, we designed the p52(1–304) and the p52(305–462) truncated polypeptides (Figure 4A), knowing that p52 interacts with XPB through two distinct domains comprising the residues 1–135 and 304–381 (Jawhari et al., 2002). Equal amounts of purified recombinant p52(WT), p52(1–304), and p52(305–462) were incubated with fixed amount of purified recombinant XPB(WT) in an ATPase assay. Both p52(WT) and p52(305–462) stimulated XPB ATPase activity (Figure 4B, lanes 3–5 and 9–11, respectively), while addition of p52(1–305) did not show any significant effect (lanes 6–8). We also noticed that p52(1–358), a truncated p52 polypeptide mimicking a mutation found in yeast (Jawhari et al., 2002), efficiently stimulated the XPB ATPase (data not shown and Jawhari et al. [2002]). Altogether, our data indicate that the XPB ATPase stimulation depends on the second XPB-interacting domain in p52, delimited by residues 305 and 358.

Mutations in Helicase Domains of XPB Preserve TFIIH Repair Activity

We next explored the combined action, if any, of both the ATPase and helicase activities of XPB in NER. Since the helicase activity of XPB depends on the integrity of seven conserved motifs (Weeda et al., 1990), we designed two recombinant XPB proteins. The first T469A mutation is located in the helicase motif III, which is involved in the unwinding of the DNA. Such mutation in the domain III has been reported to impair the helicase activity of several SF2 helicase family members (Pause and Sonenberg, 1992; Papanikou et al., 2004), including XPB (Lin et al., 2005). The second Q638A mutation is located in the helicase motif VI, involved in the interaction with the single-stranded DNA (Tuteja and Tuteja, 2004) (Figure 5A), and was shown to be detrimental for XPB helicase activity (Lin et al., 2005). We found that both recombinant XPB(T469A) and (Q638A) displayed a very low 3′–5′ helicase activity, compared with XPB(WT) (Figure 5B, upper panel, compare lanes 5 and 6 and 8 and 9 with lanes 2 and 3), while neither T469A nor Q638A mutations interfered with XPB ATPase activity (Figure 5B, lower panel). Remarkably, IIH6/XPB(T469A) and IIH6/XPB(Q638A) removed damaged DNA as efficiently as did IIH6/XPB(WT) in a dual incision assay (Figure 5C, compare lanes 7–9 and 10–12 with lanes 1–3), while their ability to allow RNA synthesis was decreased when added to a reconstituted transcription system (50% and 20% activity, respectively) (Figure 5D, compare lanes 7–9 and 10–12 with lanes 1–3). In contrast, IIH6/XPB(K346R), deficient in the ATPase activity of XPB, was inactive both in DNA repair and transcription (Figures 5C and 5D, lanes 4–6). In a permanganate assay, addition of either TFIIH/XPB(WT) or TFIIH/XPB(T469A) to a reaction containing XPC-HR23b and p8/TTDA resulted in a DNA opening around the lesion, dependent on the addition of ATP (Figure 5E, lanes 3–5 and 9–11). By contrast, a mutation in the ATPase A Walker motif I (TFIIH/XPB[K346R]) inhibited the DNA-damaged opening (lanes 6–8) (Coin et al., 2006).

To assess the importance of the helicase activity of XPB during NER in vivo, a host cell reactivation assay was performed (Carreau et al., 1995). A reporter construct (pLuc), carrying a luciferase gene, was damaged by UV irradiation and transfected in the repair-deficient CHO27-1 cells, mutated in the XPB (Ma et al., 1994), together with an

undamaged control vector coding for β-galactosidase and an expression vector coding for the human XPB proteins of interest. Expression of the UV-irradiated reporter gene was suppressed in CHO27-1, due to their repair defect (Figure 5F, compare lane 2 with lane 3). Cotransfection of XPB(WT) cDNA partially restored luciferase gene expression (lanes 3 and 4), while cotransfection of XPB(fs740) cDNA containing a mutation that abolishes NER (Coin et al., 2004) did not (Figure 5F, lane 6). The recovery of luciferase activity is incomplete, probably due to species-specific differences between human and hamster XPB. Cotransfection of XPB(T469A) cDNA allowed an increase in the luciferase expression that reaches the level observed with XPB(WT) (lanes 4 and 5), demonstrating that the T469A mutation spares TFIIH repair activity in vivo. Altogether, we show that, while the helicase activity of XPB is dispensable for effective NER, its ATPase activity is required.

Mutations Impairing XPD Helicase Activity Thwart the Repair Activity of TFIIH

We next addressed if XPD, the other helicase of TFIIH, might be contributing to the opening of the damaged DNA. We designed recombinant IIH6 complexes with XPD containing either the R658H, R683W, or R722W mutations found within XP/TTD patients or the K48R mutation located in the ATPase A Walker I motif (Figure 6A). As they prevent the interaction of XPD with p44, the R683W and R722W mutations impair the helicase activity of XPD, while R658H conveys to its partial inhibition (Dubaele et al., 2003). The K48R mutation inhibits both ATPase and helicase activities of XPD (Tirode et al., 1999). Using the permanganate footprinting assay, we showed that damaged DNA opening was impeded in the absence of the XPD ATPase activity (Figure 6B, lanes 5 and 6). Similarly, R683W and R722W hindered damaged DNA opening, even in the presence of p8/TTDA (Figure 6B, lanes 7, 8, 11, and 12). In contrast, R658H is sensitive to the addition of p8/TTDA, and we observed a limited but significant opening of the DNA around the lesion with the corresponding mutated complex (Figure 6B, lane 10). Interestingly, the rate of dual incision activity obtained with the IIH6/XPD(R658H) complex (50% activity) (Figure 6C, compare lanes 6–8 with lanes 3–5) parallels the level of DNA opening. Finally, the presence of the XPB(T469A) subunit within IIH6/XPD(R658H) resulted in the IIH6/XPD(R658H)/XPB(T469A) complex exhibiting a dual incision activity similar to that of IIH6/XPD(R658H), regardless of the presence of p8/TTDA (Figure 6C, compare lanes 9–11 with lanes 6–8). In conclusion, our results reveal that DNA opening in NER depends on the ATPase, but not on the helicase, activity of XPB in combination with the helicase activity of XPD.

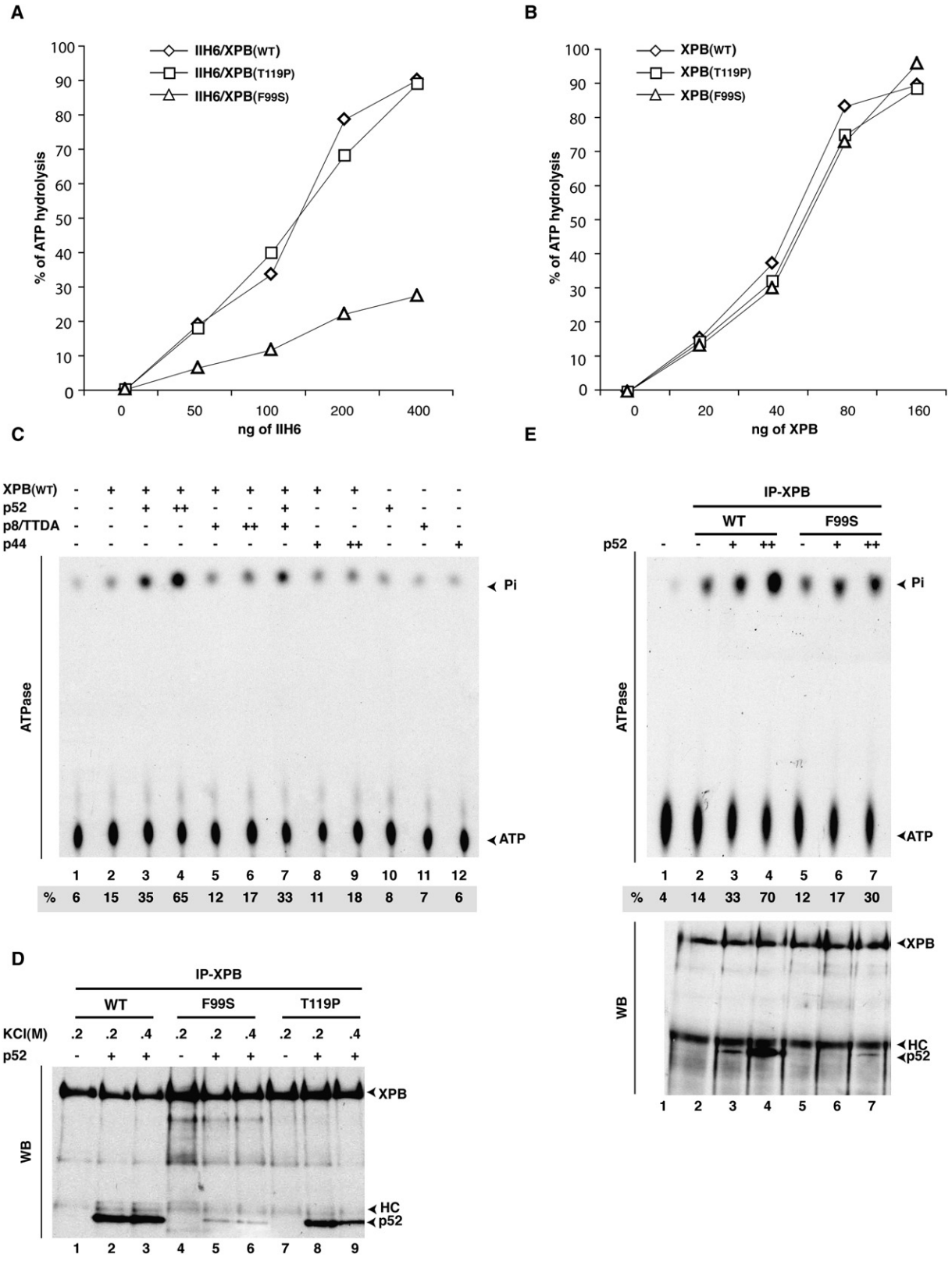
DISCUSSION

p52, a New Regulatory Subunit in TFIIH

By dissecting the repair defect induced by the F99S mutation found in XP-B patients, we have shed light on the importance of the partnership between XPB and p52 in NER. We demonstrated that the F99S mutation in XPB weakens the interaction with p52 and the resulting stimulation of its ATPase activity, thereby inhibiting the opening of the damaged DNA and the removal of the lesion. Given its role, p52 can be considered as a regulatory subunit of the ATPase activity of XPB within TFIIH. Recently, it was shown that p8/TTDA participates in the regulation of the ATPase activity of XPB within the TFIIH complex, even though these two subunits do not interact. However, p8/TTDA interacts with p52 (Coin et al., 2006), and it is likely that the free p8/TTDA, which was shown to shuttle between the cytoplasm and nucleus and to associate with TFIIH when NER-specific DNA lesions are produced (Giglia-Mari et al., 2006), would regulate or stabilize the XPB/p52 interaction within the TFIIH complex. Thus, the binding of p8/TTDA to TFIIH and the resulting stimulation of the XPB ATPase activity by p52 might constitute a crucial NER checkpoint, deciding whether or not a lesion will be removed. In the light of the 3D structure of an archea XPB homolog (Fan et al., 2006), it was proposed that ATP hydrolysis by XPB drives a large conformational change inducing a reorientation of a moiety of XPB and its wrapping around the DNA. Accordingly, it is likely that p52 together with p8/TTDA regulates this conformational change through the stimulation of the ATPase activity of XPB.

Is XPB a Conventional Helicase in NER?

The removal of lesions depends on the opening of the DNA around the damaged site. Natural mutations in either the XPB or the XPD proteins can disable DNA opening (Evans et al., 1997). A remaining question is this: do both DNA helicase activities function during the NER reaction? Mutations in the ATP binding site of XPB and XPD totally impede the formation of the open DNA structure in NER (Sung et al., 1988; Guzder et al., 1994; Coin et al., 2006). However, such observations indicate that the hydrolysis of ATP by XPB is essential for the function of TFIIH in repair but do not demonstrate that the helicase activity of XPB is required for NER. It raises the possibility that the ATPase activity is not only a provider of energy for the helicase action but also displays another independent and distinct function. This hypothesis is strengthened by the fact that the stimulation of the ATP hydrolysis by the XPB/p52 partnership does not increase XPB helicase activity (data not shown). Furthermore, TFIIH-bearing mutations in the helicase motifs III or VI of XPB are still functional in NER. This supports the idea that XPB doesn't act as a conventional helicase in NER, a role that is devoted to XPD, the other helicase of TFIIH. Indeed, we demonstrated that mutations weakening the contact of XPD with its p44 regulatory subunit (Coin et al., 1998; Dubaele et al., 2003) impair damaged DNA opening. In this context, we favor a model in which the wrapping of XPB around the DNA will allow for a local melting of the double-stranded DNA around the lesion that would favor the correct anchoring of the XPD helicase. XPB would therefore play the role of a wedge, using ATP to keep the two strands of the DNA around


A
IIH6/XPB(WT)
IIH6/XPB(T119P)
IIH6/XPB(F99S)
% of ATP hydrolysis
ng of IIH6
B
XPB(WT)
XPB(T119P)
XPB(F99S)
% of ATP hydrolysis
ng of XPB
C
XPB(WT)
p52
p8/TTDA
p44
ATPase
Pi
ATP
% 6 15 35 65 12 17 33 11 18 8 7 6
D
IP-XPB
WT
F99S
T119P
KCl(M)
p52
WB
XPB
HC
p52
E
IP-XPB
WT
F99S
p52
ATPase
Pi
ATP
% 4 14 33 70 12 17 30
WB
XPB
HC
p52

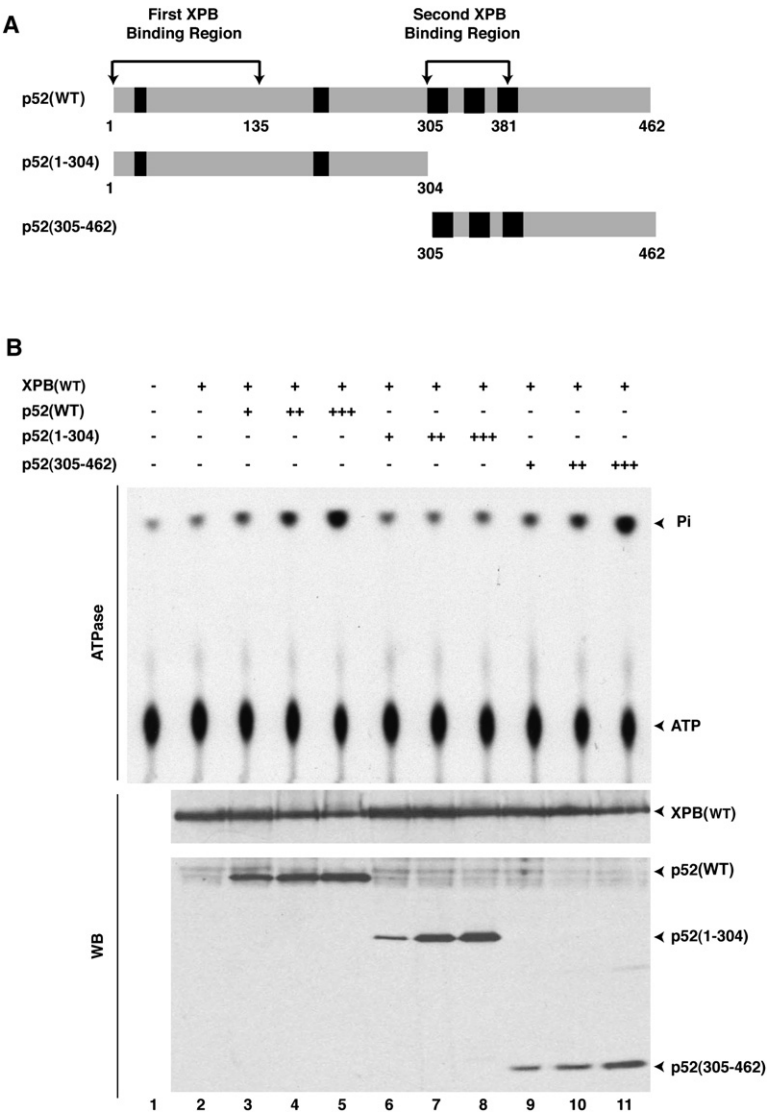


Figure 4. Mapping the Domain of p52 Involved in the Stimulation of the XPB ATPase

(A) Schematic representation of p52. The stretches of highly conserved residues in eukaryotes are indicated in black, and XPB binding regions are delimited.

(B) Purified FLAG-tagged p52(WT) (25, 50, and 100 ng) (lanes 3–5), p52(1–304) (lanes 6–8), and p52(305–462) (lanes 9–11) were incubated with 50 ng of recombinant XPB (lanes 2–11) and then resolved by SDS-PAGE and western blotted against XPB and the FLAG tag (WB) or incubated in an ATPase assay (ATPase).

the lesion apart, allowing XPD to unwind the DNA. This model also brings together the modes of action of XPB in the opening of the DNA around the promoter and around the lesion (Lin et al., 2005).

TFIIH Repair Disorders, a Matter of Interactions

So far in humans, viable TFIIH mutations have been found only in XPB, XPD, and p8/TTDA. Our work reveals that mutations found in XP-B and -D patients never affect the

Figure 3. The F99S Mutation Thwarts the Interaction between XPB and p52 and the Stimulation of XPB ATPase Activity

(A) Increasing amounts (50, 100, 200, and 400 ng) of either IIH6/XPB(WT), IIH6/XPB(F99S), or IIH6/XPB(T119P) were tested in an ATPase assay. The graph represents the percentage of phosphate released (Pi/[ATP+Pi]) from three independent experiments.

(B) Increasing amounts (20, 40, 80, and 160 ng) of either XPB(WT), XPB(F99S), or XPB(T119P) were tested in an ATPase assay. The graph represents the percentage of phosphate released (Pi/[ATP+Pi]) from three independent experiments.

(C) Purified XPB (50 ng) (lanes 2–9) was tested in an ATPase assay in the presence of 50 and 100 ng of purified p52 (lanes 3 and 4), 10 and 20 ng of purified p8/TTDA (lanes 5 and 6), or 50 and 100 ng (lanes 8 and 9) of purified p44 subunits of TFIIH.

(D) XPB(WT) (lanes 1–3), XPB(F99S) (lanes 4–6), or XPB(T119P) (lanes 7–9) from baculovirus-infected insect cell extracts were immunoprecipitated with anti-XPB antibody. Following washes, beads were incubated with baculovirus-infected insect cell extracts expressing p52, washed at 0.2 or 0.4 M KCl as indicated, and then resolved by SDS-PAGE and western blotted. HC, Ab heavy chain.

(E) XPB(WT) (lanes 2–4) or XPB(F99S) (lanes 5–7) from baculovirus-infected insect cell extracts were immunoprecipitated with anti-XPB antibody. Following washes, beads were incubated with increasing amounts of baculovirus-infected insect cell extracts expressing p52, washed at 0.4 M KCl, and then tested in an ATPase assay or resolved by SDS-PAGE and western blotted as indicated. HC, Ab heavy chain.

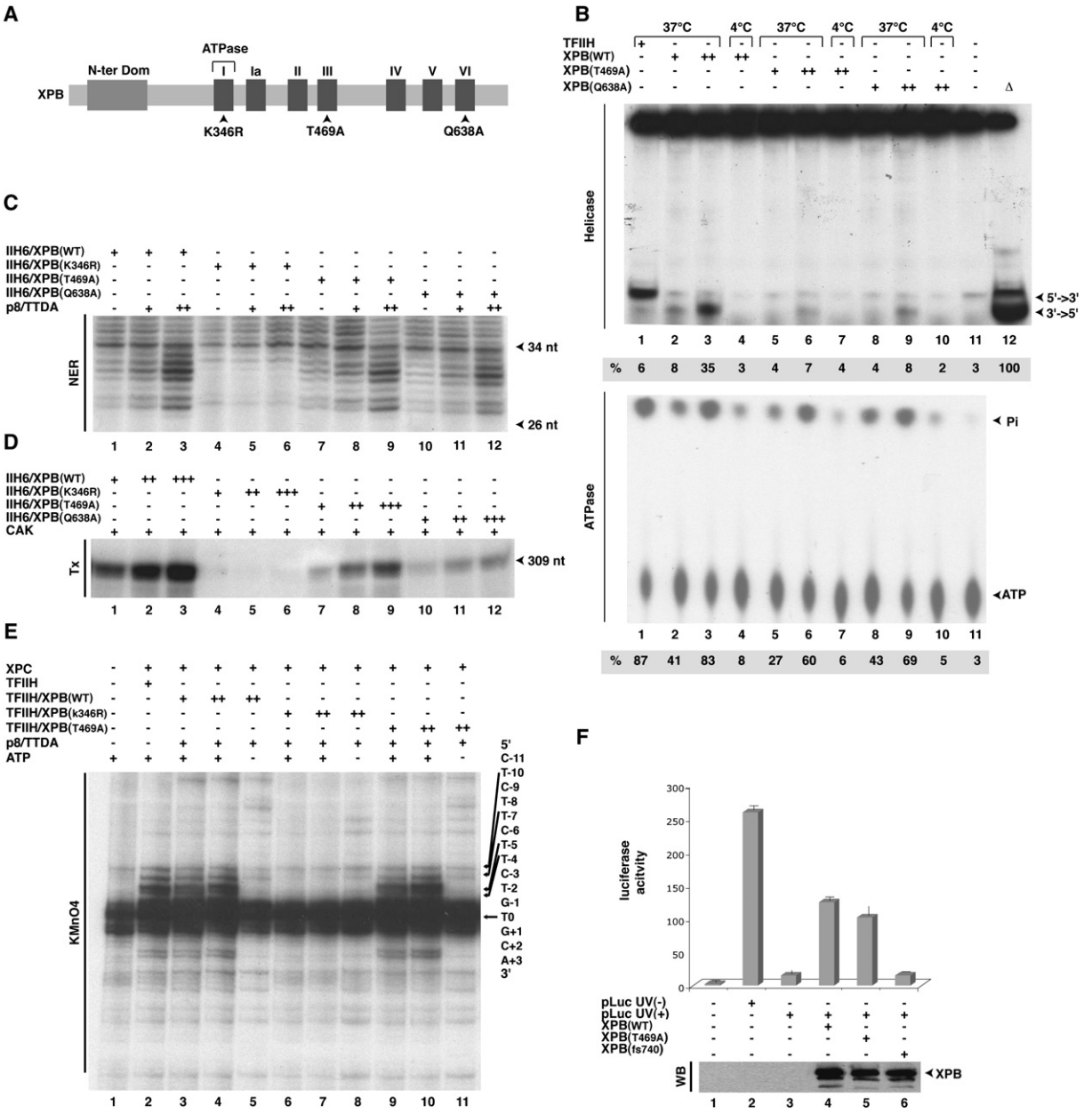


Figure 5. Mutations in Helicase Domains of XPB Spare TFIIH Repair Activity

(A) Schematic representation of XPB. Mutations introduced in conserved helicase domains are indicated.

(B) (Upper panel) Immunoprecipitated recombinant XPB (50 and 200 ng) (expressed in baculovirus-infected insect cells) were tested in helicase assay using a bidirectional probe (Coin et al., 1998) at either 37°C or 4°C as indicated. Lane 1 contains highly purified TFIIH from HeLa cells. Δ, probe has been heated 5 min at 100°C. Lane 11 is a control without XPB. Values under the autoradiograph represent the percentage of probe (3′→5′) released relative to control lane 12. (Lower panel) Immunoprecipitated XPB (50 and 200 ng) was tested in an ATPase assay at 30°C or 4°C when indicated. Values under the autoradiograph represent the percentage of phosphate released (Pi/[ATP+Pi]).

(C) One hundred nanograms of either IIH6/XPB(WT) (lanes 1–3), IIH6/XPB(K346R) (lanes 4–6), IIH6/XPB(T469A) (lanes 7–9), or IIH6/XPB(Q638A) (lanes 10–12) was tested in a dual incision assay in the presence of increasing amount of p8/TTDA (1.5 and 3 ng) as indicated.

(D) The TFIIH (50, 100, and 200 ng) tested in (C) was assessed in a reconstituted transcription assay as in Figure 1B, in the presence of 50 ng of recombinant CAK complex (Rossignol et al., 1997). The size of the transcript is indicated.

(E) Fifty and one hundred nanograms of IIH6/XPB(WT) (50 and 100 ng) (lanes 3–5), IIH6/XPB(K346R) (lanes 6–8), or IIH6/XPB(T469A) (lanes 9–11) were incubated with p8/TTDA and XPC-HR32b, with or without ATP, in a KMnO4 assay. Lane 1, Pt-DNA with BSA only. Lane 2, positive control with TFIIH purified from HeLa.

(F) CHO27-1 cells were transfected with pLuc plasmid expressing the luciferase gene previously irradiated (lanes 3–6) or not (lane 2) in combination with pcDNA expressing either XPB(WT) (lane 4), XPB(T469A) (lane 5), or XPB(fs740) (lane 6). Repair complementation was assessed by monitoring

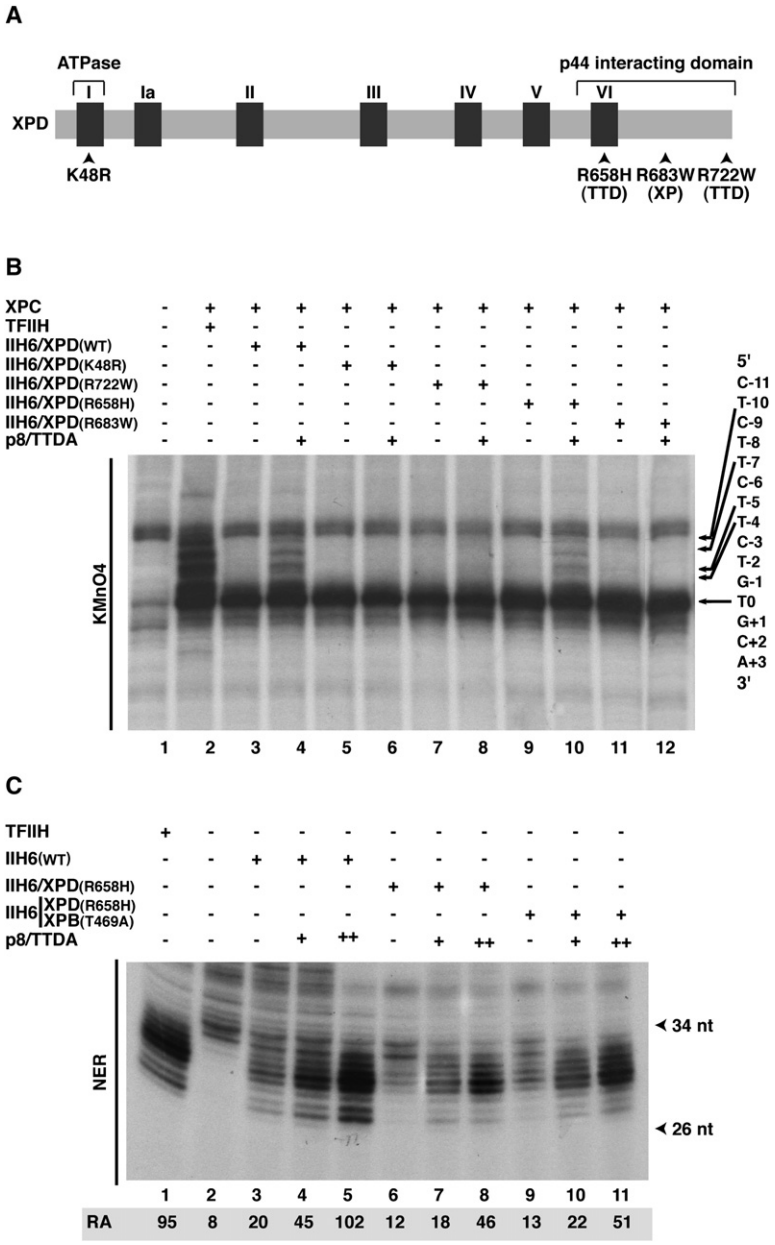


Figure 6. The XPD Helicase Activity Opens Damaged DNA in NER

(A) Schematic representation of XPD. The helicase domains are indicated by dark gray boxes. The region of interaction with the regulatory subunit p44 is highlighted (Coin et al., 1998; Dubaele et al., 2003).

(B) A KMnO4 assay, as described in Figure 1C, was performed with 100 ng of TFIIH incubated with the Pt-DNA, XPC-HR32b, and XPA. Eight nanograms of p8/TTDA was added when indicated. Lane 1, Pt-DNA with BSA only.

(C) One hundred nanograms of IIH6/XPD(WT) (lanes 3–5), IIH6/XPD(R658H) (lanes 6–8), or IIH6/XPD(R658H)/XPB(T469A) (lanes 9–11) was tested in a dual incision assay with increasing amounts of p8/TTDA (1.5 and 3 ng). Values under the autoradiograph represent the repair activity (RA) calculated from three independent experiments.

activity of the protein per se (helicase for XPD, ATPase for XPB) but rather disturb the interactions of these enzymes with their regulatory partners (p44 for XPD and p52 for XPB), explaining how patients with such mutations may exist. In this context, one could ask, why have no patients with mutations in p52 been described yet? Interestingly, point mutations in the *Drosophila* homolog of the human p52, destabilizing the interaction between Dmp52 and XPB and limiting the stimulation of the ATPase activity of the latter, give rise to UV sensitivity with melanotic tumors in larvae and pupae, and chromosome instability, all characteristics of a DNA repair defect (Fregoso et al., 2007). The study of such a fly model in association with a full characterization of the molecular defects associated to mutations found in humans will be useful to explain the clinical jumble associated with XP, CS, and TTD patients mutated in TFIIH genes.

Taken as a whole, our data show that some of the previously called ''structural'' subunits of TFIIH (i.e., p52, p44, p62, and p34) (Tirode et al., 1999) do indeed have crucial

luciferase activity in cell lysates (48 hr posttranfection) normalized with the internal β-galactosidase standard. Results are expressed as relative luciferase activity. The error bars were calculated on the basis of three independent experiments. Fifty micrograms of total extract was resolved by SDS-PAGE and western blotted (WB) with mouse anti-human XPB antibody (Coin et al., 2004).

functions inside the complex by regulating XPB and XPD enzymatic functions. Future studies will specially focus on posttranslational modifications of these regulatory subunits that may fine-tune TFIIH enzymatic activities, enabling this factor to participate in a remarkable variety of processes.

EXPERIMENTAL PROCEDURES

Construction of Plasmids

The cDNAs encoding XPB or p52, p52(1–304), and p52(305–462) were inserted at the BamHI/EcoRI sites of the FLAG-tagged pSK278 vector (BD Biosciences) in fusion with the FLAG peptide (MTKDDDDKH). The XPB mutants were obtained by site directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene). The resulting vectors were recombined with baculovirus DNA (BaculoGold DNA, Pharmingen) in Spodoptera frugiperda 9 (Sf9) cells. For hosT cell reactivation assay, XPB was inserted into the pcDNA3(+) expression vector (Invitrogen).

Protein Purification

Recombinant wild-type or mutated TFIIH, CAK, and p8/TTDA proteins were purified as described (Tirode et al., 1999). TFIIH from human cells was purified using mouse monoclonal anti-p44 antibody linked to protein A Sepharose. Following washes, the proteins were eluted from the resin with an excess of the corresponding competitor peptide (Coin et al., 1999). The FLAG-tagged p52 and XPB proteins were purified with the anti-FLAG M2 antibody agarose affinity gel (Sigma-Aldrich) followed by elution with an excess of competitor peptide.

Pull-Down Assay

Wild-type or mutant recombinant XPB from baculovirus-infected Sf9 cell lysates were immunoprecipitated O/N at 4°C in buffer A (50 mM Tris-HCl [pH 7.9], 20% glycerol, 0.1 mM EDTA, 0.5 mM DTT) containing KCl (0.2 M) with anti-XPB (1B3) antibody linked to protein A Sepharose beads. Beads were then extensively washed with buffer A (0.4 M KCl) and re-equilibrated in buffer A (0.2 M KCl). Beads were then incubated with baculovirus-infected Sf9 cell lysates expressing p52 for 2 hr at 4°C in buffer A (0.2 M KCl), washed extensively with buffer A (0.4 M KCl), and re-equilibrated in buffer A (0.05 M KCl) before being tested in western blot or ATPase assay.

DNA Substrates for NER Assays

DNA substrates and dual/single incision assays were performed as described (Riedl et al., 2003).

Host Cell Reactivation Assay

The pGL3 vector expressing *Photinus pyralis* (firefly) luciferase was purchased from Promega and the pCH110 vector expressing the β-galactosidase from Invitrogen. The pGL3 vector was UV irradiated (254 nm, 1000 J/m^2) at a concentration of 1 mg/ml in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. CHO27-1 cells were transfected in a 6-well plate at a confluence of 95% using Lipofectamine Plus (Invitrogen). Each transfection mixture contained 500 ng of pGL3 (UV+/−), 100 ng of pCH110 (nonirradiated), and 10 ng of pcDNAXPB(WT), (T469A) or (fs740). After 4 hr of incubation, the transfection reagents were replaced by medium. Cells were lysed after 24 hr to measure luciferase activity on a microtiter plate luminometer (Dynex). All results (mean values of at least five measurements) were normalized by calculating the ratios between luciferase and galactosidase activities.

KMnO4 Footprinting Assay

This assay has been described in Tapias et al. (2004). Briefly, the damaged strand probe was obtained upon AgeI digestion of the Pt-DNA and radiolabeling at the 3′ end in a Klenow reaction, the Pt adduct being located at 156 bp from the labeled end. The resulting fragment was purified by the "crush and soak" method after migration in a 5% non-denaturating PAGE. Reactions (75 μl) were carried out in 20 mM HEPES/KOH (pH 7.6), 60 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 0.3 mM EGTA, 1 mM ATP, 0.4% polyvinyl alcohol, and 0.4% polyethylene glycol 10,000 buffer containing the labeled cisplatinated probe (40 fmol) and, when indicated, the NER factors XPC/HR23b (40 ng) and XPA (25 ng). After incubation at 30°C for 15 min, 3 μl of 120 mM KMnO4 was added, and oxidation was allowed to proceed for 3 min at room temperature before reduction by adding 6 μl of 14.6 M β-mercaptoethanol for 5 min on ice. After organic extraction and ethanol precipitation, dried pellets were resuspended in 100 μl of a solution containing 1 M piperidine, 1 mM EDTA, and 1 mM EGTA and incubated at 90°C for 25 min. Next, samples were ethanol precipitated, and final pellets were recovered in 10 μl of loading buffer and analyzed in 8% urea-PAGE.

Helicase Assay

The helicase substrate was obtained by annealing 5 ng of an oligonucleotide corresponding to the fragment 6219–6255 of single-stranded M13mp18 (−) DNA to 1 μg of single-stranded M13mp18 (+). The resulting heteroduplex was digested for 1 hr at 37°C with EcoRI (New England Biolabs) and then extended to 21 and 20 bp, respectively, with the Klenow fragment (5 units) in the presence of 50 mM dTTP and 7 μCi [α-32P]dATP (3000 Ci/ mmol, Amersham). Helicase assay was then performed as described (Coin et al., 1998).

ATPase Assay

Protein fractions were incubated for 2 hr at 30°C in the presence of 1 μCi [γ-32P]ATP (7000 Ci/mmol, ICN Pharmaceuticals) in a 20 μl reaction volume in 20 mM Tris-HCl (pH 7.9), 4 mM $MgCl_2$, 1 mM DTT, 50 mg/ml BSA, and, when indicated, 120 ng of supercoiled double-strand DNA (pSK). Reactions were stopped by adding EDTA to 50 mM and SDS to 1% (w/w). The reactions were then diluted 5-fold, spotted onto polyethylenimine (PEI) TLC plates (Merck), run in 0.5 M LiCl/1 M formic acid, and autoradiographed.

Local UV Irradiation

The cells were rinsed with PBS and were covered with an isopore polycarbonate filter with pores of 3 μm diameter (Millipore, Bedford, MA). Cells were then exposed to UV irradiation with a Philips TUV lamp (predominantly 254 nm) at a dose of 70 J/m^2. Subsequently, the filter was removed, the medium was added back to the cells, and cells were returned to culture conditions for 30 min.

Fluorescence and Confocal Microscopy

Fibroblasts were grown for 2 days with fluorescent latex beads (Fluoresbrite Carboxylate Microspheres, Polysciences), fixed in 3% paraformaldehyde for 10 min at room temperature, and permeabilized with PBS/0.5% Triton for 5 min. After washing with PBS-Tween (0.05%), the slides were incubated for 1 hr with the indicated antibodies. After extensive washing with PBS-Tween, they were incubated for 1 hr with Cy3-conjugated goat anti-rabbit IgG (Jackson Laboratories) or with anti-mouse Alexa 488 IgG (Jackson Laboratories) diluted 1:400 in PBS-Tween (0.5%). The slides were counterstained for DNA with DAPI prepared in Vectashield mounting medium (Vector lab). All images were collected using a Leica Confocal TCS 4D microscope equipped with both UV laser and an Argon/Kripton laser and standard filters to allow collection of the data at 488 and 568 nm. The software TCSTK was used for three-color reconstructions, and figures were generated using the PLCHTK software.

Antibodies

Mouse monoclonal antibodies toward TFIIH subunits were used as described (Marinoni et al., 1997). Anti-FLAG M2 is from SIGMA. Primary antibodies (the final dilutions are indicated in parentheses) used in fluorescent labeling were rabbit IgG polyclonal anti-XPB (S-19, Santa Cruz Biotechnology) (1:200), rabbit IgG polyclonal anti-XPA (1:200) (FL-273, Santa Cruz Biotechnology), and mouse IgG monoclonal anti-CPD

(TDM2) (1:2000) (MBL International Corporation). Secondary antibodies used in this study were Alexa 488 anti-mouse IgG and Cy3-conjugated goat anti-rabbit IgG.

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REFERENCES

Bootsma, D., and Hoeijmakers, J.H.J. (1993). DNA repair. Engagement with transcription. Nature *363*, 114–115.

Carreau, M., Eveno, E., Quilliet, X., Chevalier-Lagente, O., Benoit, A., Tanganelli, B., Stefanini, M., Vermeulen, W., Hoeijmakers, J.H., Sarasin, A., et al. (1995). Development of a new easy complementation assay for DNA repair deficient human syndromes using cloned repair genes. Carcinogenesis *16*, 1003–1009.

Coin, F., Marinoni, J.C., Rodolfo, C., Fribourg, S., Pedrini, A.M., and Egly, J.M. (1998). Mutations in the XPD helicase gene result in XP and TTD phenotypes, preventing interaction between XPD and the p44 subunit of TFIIH. Nat. Genet. *20*, 184–188.

Coin, F., Bergmann, E., Tremeau-Bravard, A., and Egly, J.M. (1999). Mutations in XPB and XPD helicases found in xeroderma pigmentosum patients impair the transcription function of TFIIH. EMBO J. *18*, 1357–1366.

Coin, F., Auriol, J., Tapias, A., Clivio, P., Vermeulen, W., and Egly, J.M. (2004). Phosphorylation of XPB helicase regulates TFIIH nucleotide excision repair activity. EMBO J. *23*, 4835–4846.

Coin, F., De Santis, L.P., Nardo, T., Zlobinskaya, O., Stefanini, M., and Egly, J.M. (2006). p8/TTD-A as a repair-specific TFIIH subunit. Mol. Cell *21*, 215–226.

Dubaele, S., Proietti De Santis, L., Bienstock, R.J., Keriel, A., Stefanini, M., Van Houten, B., and Egly, J.M. (2003). Basal transcription defect discriminates between xeroderma pigmentosum and trichothiodystrophy in XPD patients. Mol. Cell *11*, 1635–1646.

Evans, E., Moggs, J.G., Hwang, J.R., Egly, J.-M., and Wood, R.D. (1997). Mechanism of open complex and dual incision formation by human nucleotide excision repair factors. EMBO J. *16*, 6559–6573.

Fan, L., Arvai, A.S., Cooper, P.K., Iwai, S., Hanaoka, F., and Tainer, J.A. (2006). Conserved XPB core structure and motifs for DNA unwinding: implications for pathway selection of transcription or excision repair. Mol. Cell *22*, 27–37.

Fregoso, M., Lainé, J.P., Aguilar-Fuentes, J., Mocquet, V., Reynaud, E., Coin, F., Egly, J.M., and Zurita, M. (2007). DNA repair and transcriptional deficiencies caused by mutations in the Drosophila p52 subunit of TFIIH generate developmental defects and chromosome fragility. Mol. Cell Biol. Published online March 5, 2007. 10.1128/MCB.00030-07.

Frit, P., Kwon, K., Coin, F., Auriol, J., Dubaele, S., Salles, B., and Egly, J.M. (2002). Transcriptional activators stimulate DNA repair. Mol. Cell *10*, 1391–1401.

Gerard, M., Fischer, L., Moncollin, V., Chipoulet, J.M., Chambon, P., and Egly, J.M. (1991). Purification and interaction properties of the human RNA polymerase B(II) general transcription factor BTF2. J. Biol. Chem. *266*, 20940–20945.

Giglia-Mari, G., Coin, F., Ranish, J., Hoogstraten, D., Theil, A., Wijgers, N., Jaspers, N., Raams, A., Argentini, M., van der Spek, P., et al. (2004). A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A. Nat. Genet. *36*, 714–719.

Giglia-Mari, G., Miquel, C., Theil, A.F., Mari, P.O., Hoogstraten, D., Ng, J.M., Dinant, C., Hoeijmakers, J.H., and Vermeulen, W. (2006). Dynamic interaction of TTDA with TFIIH is stabilized by nucleotide excision repair in living cells. PLoS Biol. *4*, e156. 10.1371/journal.pbio.0040156.

Guzder, S.N., Sung, P., Bailly, V., Prakash, L., and Prakash, S. (1994). RAD25 is a DNA helicase required for DNA repair and RNA polymerase II transcription. Nature *369*, 578–581.

Jawhari, A., Laine, J.P., Dubaele, S., Lamour, V., Poterszman, A., Coin, F., Moras, D., and Egly, J.M. (2002). p52 mediates XPB function within the transcription/repair factor TFIIH. J. Biol. Chem. *277*, 31761–31767.

Keriel, A., Stary, A., Sarasin, A., Rochette-Egly, C., and Egly, J.M. (2002). XPD mutations prevent TFIIH-dependent transactivation by nuclear receptors and phosphorylation of RARalpha. Cell *109*, 125–135.

Lehmann, A.R. (2003). DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. Biochimie *85*, 1101–1111.

Lin, Y.C., Choi, W.S., and Gralla, J.D. (2005). TFIIH XPB mutants suggest a unified bacterial-like mechanism for promoter opening but not escape. Nat. Struct. Mol. Biol. *12*, 603–607. Published online June 5, 2005. 10.1038/nsmb949.

Ma, L., Westbroek, A., Jochemsen, A.G., Weeda, G., Bosch, A., Bootsma, D., Hoeijmakers, J.H., and van der Eb, A.J. (1994). Mutational analysis of ERCC3, which is involved in DNA repair and transcription initiation: identification of domains essential for the DNA repair function. Mol. Cell. Biol. *14*, 4126–4134.

Marinoni, J.C., Rossignol, M., and Egly, J.M. (1997). Purification of the transcription/repair factor TFIIH and evaluation of its associated activities in vitro. Methods *12*, 235–253.

O'Donnovan, A., Davies, A.A., Moggs, J.G., West, S.C., and Wood, R.D. (1994). XPG endonuclease makes the 3′ incision in human DNA nucleotide excision repair. Nature *371*, 432–435.

Oh, K.S., Khan, S.G., Jaspers, N.G., Raams, A., Ueda, T., Lehmann, A., Friedmann, P.S., Emmert, S., Gratchev, A., Lachlan, K., et al. (2006). Phenotypic heterogeneity in the XPB DNA helicase gene (ERCC3): xeroderma pigmentosum without and with Cockayne syndrome. Hum. Mutat. *27*, 1092–1103.

Papanikou, E., Karamanou, S., Baud, C., Sianidis, G., Frank, M., and Economou, A. (2004). Helicase Motif III in SecA is essential for coupling preprotein binding to translocation ATPase. EMBO Rep. *5*, 807–811.

Pause, A., and Sonenberg, N. (1992). Mutational analysis of a DEAD box RNA helicase: the mammalian translation initiation factor eIF-4A. EMBO J. *11*, 2643–2654.

Riedl, T., Hanaoka, F., and Egly, J.M. (2003). The comings and goings of nucleotide excision repair factors on damaged DNA. EMBO J. *22*, 5293–5303.

Riou, L., Zeng, L., Chevallier-Lagente, O., Stary, A., Nikaido, O., Taieb, A., Weeda, G., Mezzina, M., and Sarasin, A. (1999). The relative expression of mutated XPB genes results in xeroderma pigmentosum/Cockayne's syndrome or trichothiodystrophy cellular phenotypes. Hum. Mol. Genet. *8*, 1125–1133.

Rossignol, M., Kolb-Cheynel, I., and Egly, J.M. (1997). Substrate specificity of the cdk-activating kinase (CAK) is altered upon association with TFIIH. EMBO J. *16*, 1628–1637.

Sijbers, A.M., de Laat, W.L., Ariza, R.R., Biggerstaff, M., Wei, Y.F., Moggs, J.G., Carter, K.C., Shell, B.K., Evans, E., de Jong, M.C., et al. (1996). Xeroderma pigmentosum group F caused by a defect in a structure-specific DNA repair endonuclease. Cell *86*, 811–822.

Sung, P., Higgins, D., Prakash, L., and Prakash, S. (1988). Mutation of lysine-48 to arginine in the yeast RAD3 protein abolishes its ATPase and DNA helicase activities but not the ability to bind ATP. EMBO J. *7*, 3263–3269.

Tapias, A., Auriol, J., Forget, D., Enzlin, J.H., Scharer, O.D., Coin, F., Coulombe, B., and Egly, J.M. (2004). Ordered conformational changes in damaged DNA induced by nucleotide excision repair factors. J. Biol. Chem. *279*, 19074–19083.

Tirode, F., Busso, D., Coin, F., and Egly, J.M. (1999). Reconstitution of the transcription factor TFIIH: assignment of functions for the three enzymatic subunits, XPB, XPD, and cdk7. Mol. Cell *3*, 87–95.

Tuteja, N., and Tuteja, R. (2004). Unraveling DNA helicases. Motif, structure, mechanism and function. Eur. J. Biochem. *271*, 1849–1863.

Volker, M., Mone, M.J., Karmakar, P., van Hoffen, A., Schul, W., Vermeulen, W., Hoeijmakers, J.H., van Driel, R., van Zeeland, A.A., and Mullenders, L.H. (2001). Sequential assembly of the nucleotide excision repair factors in vivo. Mol. Cell *8*, 213–224.

Weeda, G., van Ham, R.C.A., Vermeulen, W., Bootsma, D., van der Eb, A.J., and Hoeijmakers, J.H.J. (1990). A presumed DNA helicase encoded by ERCC-3 is involved in the human repair disorders Xeroderma Pigmentosum and Cockayne's syndrome. Cell *62*, 777–791.

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Nucleotide Excision Repair Driven by the Dissociation of CAK from TFIIH

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SUMMARY

The transcription/DNA repair factor TFIIH is organized into a core that associates with the CDK-activating kinase (CAK) complex. Using chromatin immunoprecipitation, we have followed the composition of TFIIH over time after UV irradiation of repair-proficient or -deficient human cells. We show that TFIIH changes subunit composition in response to DNA damage. The CAK is released from the core during nucleotide excision repair (NER). Using reconstituted in vitro NER assay, we show that XPA catalyzes the detachment of the CAK from the core, together with the arrival of the other NER-specific factors. The release of the CAK from the core TFIIH promotes the incision/excision of the damaged oligonucleotide and thereby the repair of the DNA. Following repair, the CAK reappears with the core TFIIH on the chromatin, together with the resumption of transcription. Our findings demonstrate that the composition of TFIIH is dynamic to adapt its engagement in distinct cellular processes.

INTRODUCTION

The genome of eukaryotes is vulnerable to an array of DNA-damaging agents. Cells possess several DNA repair pathways to avoid the harmful effects of DNA damage on replication and transcription. NER removes a broad variety of DNA lesions, including cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP) generated by UV light, through two related subpathways (Lindahl and Wood, 1999). The general global genome repair (GGR) removes DNA damages from the entire genome, and the transcription-coupled repair (TCR) corrects lesions located on actively transcribed genes (Hanawalt, 2002). Three repair-deficient disorders emphasize the importance of NER in genome stability: the xeroderma pigmentosum (XP), the trichothiodystrophy (TTD), and the Cockayne syndrome (CS) (Lehmann, 2003).

The widely accepted model of NER includes the detection of the damage-induced DNA distortion by XPC in GGR and by RNA polymerase II (RNA pol II) in TCR (Hanawalt, 2002). Both subpathways then funnel into a common process, and the DNA is unwound by the XPB and XPD ATPases/helicases of the transcription/repair factor TFIIH (Zurita and Merino, 2003). Such initiation of DNA opening favors the recruitment of XPA and RPA that assist in the expansion of the DNA bubble around the damage (Evans et al., 1997; Riedl et al., 2003). Next, the endonucleases XPG and XPF generate cuts in the 3′ and 5′ sides of the lesion, respectively (O'Donovan et al., 1994; Sijbers et al., 1996), thereby removing the damaged oligonucleotide (Huang et al., 1992; Moggs et al., 1996). Finally, the resynthesis machinery fills the DNA gap (Shivji et al., 1995). In TCR, two additional proteins, CSA and CSB, are required to assemble NER proteins and various TCR-specific factors onto the stalled RNA pol II (Fousteri et al., 2006).

Seminal studies on the TFIIH complex established its function in the transcription of protein-coding genes (Gerard et al., 1991) but provided only a glimpse of its cellular importance. Later, the unexpected finding that TFIIH was involved in NER and in the transcription of ribosomal genes made this factor a remarkable example of a multifunctional cellular complex (Feaver et al., 1993; Schaeffer et al., 1993; Drapkin et al., 1994; Guzder et al., 1994a, 1994b; Iben et al., 2002). Mammalian TFIIH includes a core, containing the seven subunits XPB, XPD, p62, p52, p44, p34, and p8/TTD-A (Giglia-Mari et al., 2004; Ranish et al., 2004; Coin et al., 2006) coupled to the cdk-activating kinase module (CAK) composed of the three subunits Cdk7, cyclin H, and MAT1 (Roy et al., 1994).

The major part of the work carried out on TFIIH relates to the pivotal role and the specific function of each of its subunits in either transcription or DNA repair (Dubaele et al., 2003; Giglia-Mari et al., 2006). Yet one of the most intriguing questions is how a single TFIIH complex faces the task of participating both in transcription and NER. In the present study, we have followed the makeup of TFIIH in vivo over time after a genotoxic attack. Using a chromatin immunoprecipitation (ChIP) approach, we showed that the CAK is released from the core TFIIH following the engagement of the complex in DNA repair. Meanwhile, the core TFIIH associates with NER-specific factors, including XPA, which catalyzes the detachment of the CAK from the core thereby triggering the incision/excision of the damaged oligonucleotide. Following damage removal, the NER factors are released from the complex and the CAK reappears with the core TFIIH on the chromatin, concomitantly with the recovery of the transcription formerly inhibited by UV irradiation.

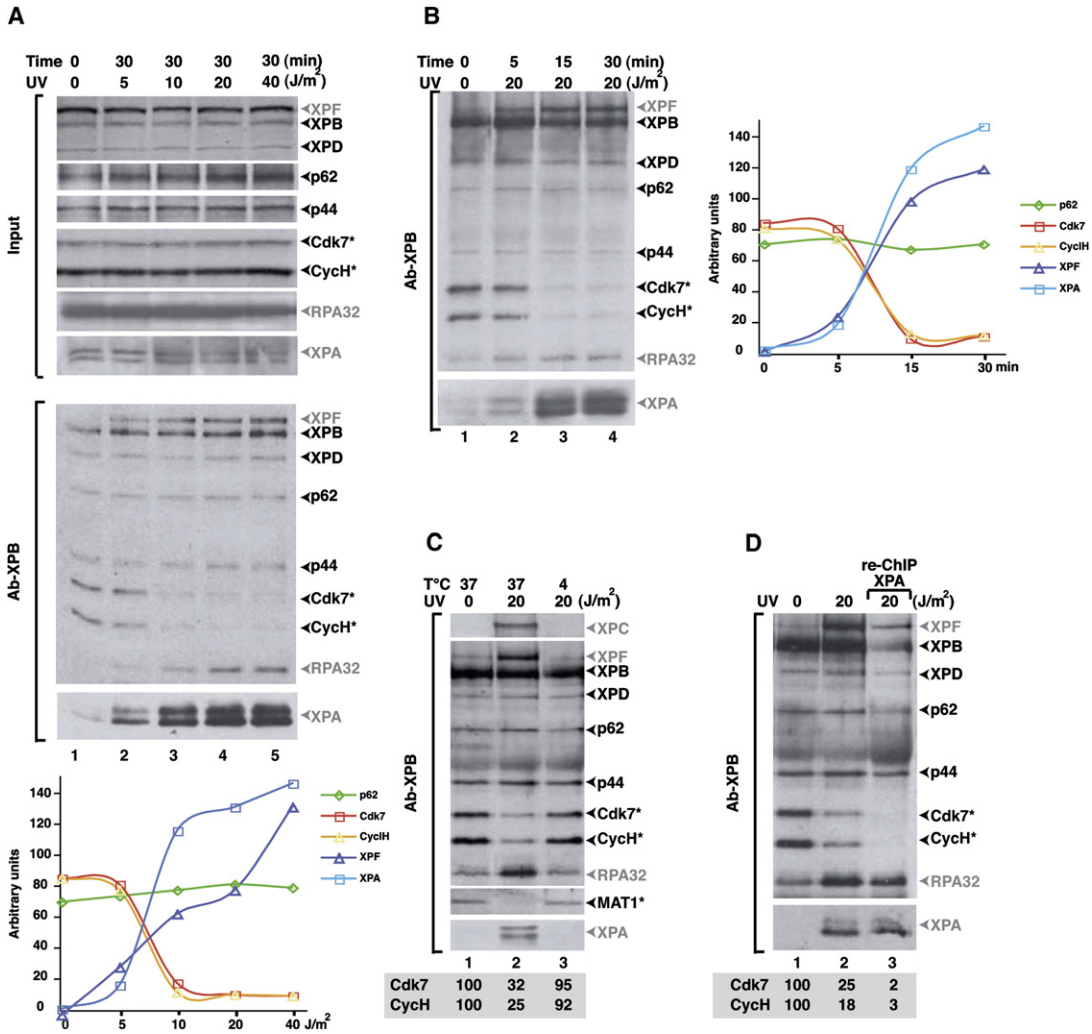

Figure 1. The Repair-Specific TFIIH Complex Is Depleted of the CAK Module

(A) Western-blotting analysis of Ab-XPB ChIP samples from chromatin extracts of untreated (lane 1) or UV-treated (lanes 2–5; 5, 10, 20, or 40 J/m^2) WT MRC5 fibroblasts, fixed 30 min later. The chromatin inputs are indicated. Subunits of TFIIH are in black; NER proteins in gray. Subunits of the CAK subcomplex are marked with an asterisk. Note that two bands are detected for XPA. The WB signals for XPF, XPB, p62, CyclH, Cdk7, and XPA were quantified using Genetool (Syngene) and plotted on the graphs. For each single lane, XPB was used as reference.

(B) ChIP was performed with an Ab-XPB on the chromatin of WT MRC5 fibroblasts, incubated over time (5, 15, and 30 min) after UV (20 J/m^2). A dose of 20 J/m^2 generates four photolesions per 10 KB of genomic DNA (van Hoffen et al., 1995). With DNA fragments of 200–400 bp (data not shown), we estimate that ~20% of the DNA fragments in our experiment contain a UV-induced lesion after sonication.

(C) ChIP was performed with an Ab-XPB on the chromatin of nontreated (lane 1) or treated WT MRC5 fibroblasts (20 J/m^2) incubated for 30 min after UV at 37°C (lane 2) or 4°C (lane 3).

(D) ChIP was performed as in (C), lanes 1 and 2, then pulled-down fractions from irradiated cells were eluted and re-ChIPed with an Ab-XPA (lane 3).

RESULTS

UV Irradiation Elicits a Change in the Composition of TFIIH

An antibody directed against XPB (Ab-XPB) was used to isolate TFIIH from the chromatin of wild-type (WT) fibroblasts 30 min after UV treatment. Western blotting (WB) revealed that though Cdk7 and Cyclin H of CAK were equally present in the inputs (Figure 1A, upper panel), their amount progressively decreased in the ChIP fractions on increasing UV dose (Figure 1A, lower panel). Meanwhile, the amount of XPD, p62, and p44 subunits of the core TFIIH remained constant. Together with the progressive diminution of CAK, the NER factors, including XPA, RPA, and XPF, assembled with the core TFIIH on the damaged chromatin (Figure 1A, lower panel; see also the graph). Similar findings were obtained with an antibody directed against p62 subunit of TFIIH (see Figure S1 available online). To investigate further the release of CAK, proteasome inhibitors were used and showed no effect on its disappearance from TFIIH, suggesting that it is detached from the core and not degraded (data not shown). The UV-induced change in TFIIH was also investigated at different time points and temperatures of incubation. When

cells were irradiated at 20 J/m^2, the changes were optimum within 15 min (Figure 1B, WB and graph) and did not occur at 4°C (Figure 1C, lanes 2 and 3), suggesting that the release of CAK was an active process. Next, the ChIP fractions isolated after irradiation were eluted and reimmunoprecipitated with an XPA antibody (re-ChIP). The ChIP re-ChIP assay demonstrated that the core TFIIH (XPB, XPD, p62, and p44) was present in a repair-specific complex containing XPA, XPF, and RPA, without CAK (Figure 1D, lanes 2 and 3). Eighty percent of TFIIH present on the chromatin did not contain CAK (Figure 1D) 15 min after irradiation. Together, these findings pinpoint that genotoxic attack induces a change in the composition of TFIIH with a release of the CAK from the core TFIIH, together with the arrival of the NER factors.

Next we checked TFIIH in the soluble fractions of noncrosslinked irradiated cells. The cells were irradiated and lysed in RIPA buffer, and immunoprecipitations were performed with Ab-p44 antibody followed by elution with an excess of competitor peptide (Coin et al., 1999). Western blotting, kinase assay (measuring Cdk7 activity; Tirode et al., 1999), and silver staining demonstrated that most of the CAK was released from the core TFIIH after irradiation (Figure S2). However, the dissociation was observed only after irradiation at 100 J/m^2, and no repair factors were found associated with the core TFIIH. These data indicate that soluble "repairosomes" are unstable in human and can be detected only on the chromatin, after fixation.

Release of the CAK Takes Place during NER

To assess whether the change in TFIIH was part of the NER reaction, we investigated the amount of CAK and repair factors associated to the core TFIIH in various repair-deficient cell lines by ChIP. First, we analyzed the composition of TFIIH in GGR defective XP-C versus TCR-defective CS-B cells. The Ab-XPB immunoprecipitated TFIIH pattern in XP-C cells was markedly different from the WT after UV: CAK was not released and none of the NER factors accumulated to TFIIH (Figure 2A, lanes 1–4). In CS-B cells, the TFIIH pattern after UV was similar to WT cells (cf. lanes 1 and 2 with 11 and 12). Then, ChIP was performed on XP-A cells. Under our experimental conditions, the release of the CAK (less than 20%, compared with 80% in WT cells) from the core and the recruitment of RPA were barely detectable after UV, and association of XPF with TFIIH was not observed (cf. lanes 1 and 2 with 5 and 6). In both UV-treated XP-G and XP-F cells, up to 80% of CAK was released together with the recruitment of XPA and RPA to the core TFIIH (cf. lanes 1 and 2 with lanes 7 and 8, and 9 and 10). Notably, XPF associated with TFIIH after UV in XP-G cells (lanes 7 and 8; Volker et al., 2001). To demonstrate the role of XPA in the CAK release, WT cells were treated with siRNA toward XPA (siXPA). The amount of XPA strongly decreased after siXPA transfection (Figure 2B, upper panel, lanes 1–3). ChIP using Ab-XPB showed that RPA was not recruited to the core TFIIH in the absence of XPA (Figure 2B, lower panel, lanes 2 and 3). Similar to what was observed in XP-A cells, the CAK (visualized by CyclinH) was barely released in siXPA-treated WT cells (cf. lane 1 with 3).

Our findings suggest that the recruitment of XPA to the preincision complex, which is composed of XPC and TFIIH, may promote the release of CAK. Therefore, we carried out ChIP analysis with an antibody against XPC in both the WT and XP-A cells. The association of the core TFIIH (XPB, XPD, and p62) with XPC took place both in WT and XP-A cells, after UV (Figure 2C, cf. lanes 1 and 2 with 3 and 4). However, the subunits of the CAK complex were found together with the core TFIIH and XPC in XP-A but not in WT cells (lanes 2 and 4).

In addition, we investigated whether mutations in the XPD helicase would modify the composition of TFIIH. In UV-treated XP-D cells, neither a release of the CAK from the core nor a recruitment of XPA and RPA to the TFIIH were observed (Figure 2D, lanes 1–4).

Overall, our findings suggest that the release of the CAK from the core TFIIH takes place during GGR, after the recruitment of XPA.

XPA and ATP Promote Release of CAK

To further dissect the change in TFIIH composition when engaged in the NER process, we set up an in vitro assay that allows us to analyze the composition of functionally active intermediate NER complexes. A linear DNA substrate containing a single 1,3-intrastrand d(GpTpG) cisplatin lesion was immobilized on magnetic beads and incubated with a repair-competent nuclear extract (NE) at 4°C for 15 min to allow the binding of XPC and TFIIH to the damaged DNA (Riedl et al., 2003). The samples were subsequently incubated at 30°C over time. Then, the proteins bound to the immobilized DNA were analyzed by WB. Without ATP, the composition of TFIIH was unchanged (Figure 3A, WB, lanes 1–4). ATP promoted a time-dependent release of CAK (85% of CAK is released after 10 min of incubation), together with the arrival of XPA, XPF, and RPA (Figure 3A,WB, lanes 5–8 and upper graph) and the removal of the damaged oligonucleotides (Figure 3A, NER, lanes 5–8). Note that the dissociation of the CAK was observed only in the presence of damaged DNA (data not shown).

We also investigated whether the core TFIIH engaged in DNA repair maintained its transcriptional activity. NER complex formation was allowed to proceed over the time in the presence of ATP. At the indicated times, the immobilized protein/DNA complexes were further incubated in a reconstituted transcription assay containing all the general transcription factors (except TFIIH) and the AdMLP template (Gerard et al., 1991). In this challenge experiment, we observed a progressive decrease of the TFIIH transcriptional activity over time, which correlates with the progressive increase of the elimination of the damaged oligonucleotide observed during NER (Figure 3A, Tx and NER panels; see also bottom graph). Addition of CAK restores the TFIIH transcription activity (Figure 3A, Tx; cf. lanes 5 and 6).

We further examined the role of XPA in the release of the CAK. An XP-A cell NE (that does not express the XPA protein; Koberle et al., 2006) was incubated with the immobilized damaged DNA at 30°C, in the presence of ATP. CAK (vizualized by Cyclin H) was not released from TFIIH over the time (Figure 3B, lanes 1–4). Addition of recombinant XPA WT catalyzed not only the release of CAK but also the recruitment of the repair factors on the DNA (lanes 5–8).

In another set of experiments, highly purified recombinant XPC and TFIIH were assembled on the damaged DNA. The immobilized DNA was subsequently washed to remove nonspecifically bound proteins, and the reactions were supplemented with a combination of repair factors, as indicated, in the presence of ATP. Addition of XPA-WT to the DNA/XPC/TFIIH complex was

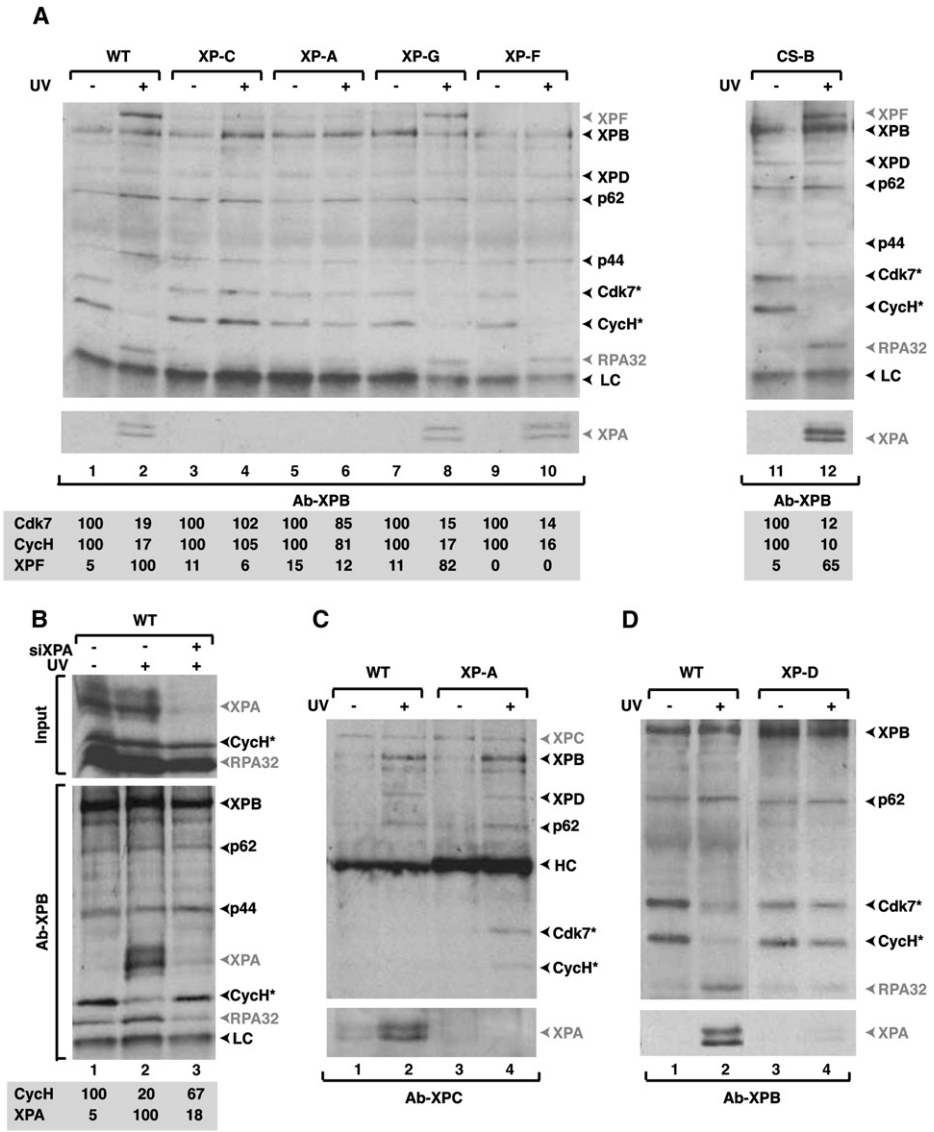

Figure 2. Analysis of the Release of the CAK Module in XP and CS Patient Cell Lines

(A) ChIP were performed with an Ab-XPB, 30 min after irradiation (20 J/m^2) of either WT (lanes 1 and 2), XP-C (GM14867; lanes 3 and 4), XP-A (XP12ROSV; lanes 5 and 6), XP-G (XPCS1RO; lanes 7 and 8), XP-F (GM08437; lanes 9 and 10), or CS-B (CS1ANSV; lanes 11 and 12) patient cell lines. LC indicates the light chain of the antibody. The percentage of CAK components that remain associated to TFIIH is shown; the amount of CAK in nonirradiated cells is considered 100%. For the quantification of XPF, the amount of XPF in wild-type irradiated cells was considered 100%.

(B) WT cells were transfected with siRNA toward XPA (siXPA). The chromatin inputs were analyzed by WB. ChIP were performed as in (A). For quantification, the amount of cyclinH in untreated cells was considered 100%, the amount of XPA in UV-treated cell was considered 100%. XPB was used as reference.

(C) ChIP was performed as in panel (A) with an Ab-XPC in the chromatin of either WT (lanes 1 and 2) or XP-A (lanes 3 and 4) cells.

(D) ChIP were performed as in (A) with chromatin of either WT (HeLa; lanes 1 and 2) or XP-D (HD2; lanes 3 and 4) cells.

sufficient to induce a time-dependent release of CAK (Figure 3C, cf. lanes 1 and 2 with 3 and 4) that was not observed following addition of RPA, XPG, and XPF (cf. lanes 1 and 2 with 5 and 6). Our in vitro data confirm that XPA catalyzes the dissociation of CAK from TFIIH during NER.

Release of CAK Drives NER

We then sought to identify which domain of XPA mediated the release of CAK from the core TFIIH. We designed constructs expressing XPA-WT and XPA(1-228) polypeptides (Figure 4A), knowing that TFIIH interacts with the carboxy-terminal part of XPA (Park et al., 1995; Nocentini et al., 1997). Equal amounts of purified XPA-WT and XPA(1-228) were incubated over time, with an XP-A NE and the damaged DNA. Contrary to XPA-WT, the truncated XPA(1-228) was unable either to catalyze the release of CAK (visualized by Cyclin H) from the core TFIIH or to join the preincision repair complex (Figure 4B).

XPA-WT and XPA(1-228) were next incubated in a dual incision assay containing either rIIH7 (the core TFIIH) or rIIH10 (the entire TFIIH, including CAK; Figure 4C, left panel). In the presence of XPA-WT, a similar dual incision activity was observed with either rIIH7 or rIIH10 (lanes 2 and 5). Dual incision was barely detectable with XPA(1-228) in the presence of rIIH10 but was clearly observed with rIIH7 (Figure 4C, right panel, lanes 3 and 6), although to a lower extent compared with XPA-WT.

Together, our data indicate that the detachment of the CAK from the core of TFIIH, mediated by the C-terminal part of XPA, drives dual incision.

Functional NER in CAK Knockdown Cells

The question remained as to whether CAK plays a role in NER before it is released from the preincision complex. Specific siRNA duplexes (siCdk7) were used to reduce endogenous Cdk7 expression in WT cells. Confocal microscopy revealed that siCdk7 transfected cells had a marked reduction in the amount of Cdk7 and Cyclin H as compared with either the untransfected or control siRNA (siCt)-treated cells (Figures 5Ab and 5Ae). In contrast, the amount of either XPB (Figures 5Aa and 5Ad) or p62 (data not shown) was not affected. Immunoprecipitation experiments performed with Ab-XPB showed the presence and the stability of the core TFIIH even in the absence of CAK (Figure 5B). To check for the efficiency of Cdk7 knockdown, we investigated the transcriptional response of UV-irradiated cells. Quantitative RT-PCR showed that the transactivation of the three UV-inducible genes *p21*, *Mdm2*, and *ATF3* (Fan et al., 2002) was significantly reduced in siCdk7-treated cells compared with siCt-treated cells (Table 1).

Next, the amount of 6-4PP lesions was measured in vivo using confocal microscopy (Mori et al., 1991), 4 hr after irradiation of either siCdk7- or siCt-treated cells. SiCdk7-treated cells (blue fluorescent latex beads) were fully capable of removing the 6-4PP lesions (Figures 5Cb and 5Ce), compared with either the repair-proficient siCt transfected (no beads) or repair-deficient XP-A (green fluorescent latex beads) cells (Figures 5Ca and 5Cd). Using an immuno-dot blot assay (Riou et al., 2004), we measured the amount of 6-4PP lesions remaining in the DNA at different time points after UV and observed identical repair activity in siCdk7- and siCt-treated cells (Figure 5D). Our data suggests that 6-4PP lesions are repaired regardless of the presence of CAK in vivo.

Return of CAK after DNA Repair

We also investigated the fate of TFIIH after DNA repair. ChIP assay using Ab-XPB established that the level of CAK in WT cells was shifted back to that of nonirradiated cells within 8 hr after UV (Figure 6A, upper panel). A progressive release of the NER factors was also observed, together with the recovery of a whole TFIIH. In parallel, we investigated RNA synthesis recovery by [^{3}H]uridine incorporation performed after UV. The kinetics of inhibition of transcription followed both the leaving of CAK from TFIIH and the coming of the NER factors. The resumption of transcription began at 4 hr and was complete at 8 hr, when both the CAK reassociated with TFIIH and the NER factors were released (Figure 6A, lower panel). The recovery of transcription started after the complete removal of 6-4PP lesions measured in Figure 5D.

Hereafter, we focused on CS-B, XP-G, and XP-F cell lines for which a release of CAK had been observed (Figure 2). In TCR-deficient CS-B cells, a whole TFIIH complex is present in the chromatin 8 hr after UV. Concomitantly, XPF, XPA, and RPA dissociated from TFIIH in a time course similar to that observed in WT cells (Figure 6B, WB and bottom graph). These data indicate that the restoration of TFIIH pattern is CSB independent. As previously observed (Mayne and Lehmann, 1982), RNA synthesis was not restored in CS-B cells because of the involvement of CSB in transcription (Proietti-De-Santis et al., 2006). In XP-G cells, the XPA, RPA, and XPF factors were dissociated from the core TFIIH in a time course similar to that of WT or CS-B cells (Figure 6C). However, we observed neither the return of a whole TFIIH complex on the chromatin nor the recovery of the transcription. A slightly different situation was obtained in XP-F cells, where XPA and RPA repair factors remained associated with the core TFIIH over time after treatment (Figure 6D). Collectively, these data suggest that the return of a whole TFIIH to the chromatin occurs once the removal of the DNA lesions is complete.

DISCUSSION

Dynamic State of the Human TFIIH Complex

Considerable efforts have been invested in the dissection of the molecular events that lead to the elimination of DNA lesions by the NER pathway. However, detailed information on the composition of the various complexes that participate in the repair process has been lacking. We were interested in understanding how the 10 subunits that constitute the TFIIH complex participate in the NER and/or transcription processes. In the present study, we have found that the composition of TFIIH changes according to various cellular responses. We have observed a dynamic dissociation/reassociation of the CAK complex, a crucial transcriptional module (Tirode et al., 1999), onto the core TFIIH that coincided with the recruitment/release of the NER factors during the DNA repair reaction. Thus, our findings dispel the broadly accepted idea that TFIIH is a stable complex without large-scale alterations in composition when switching between different cellular processes (Hoogstraten et al., 2002).

The release of the CAK from TFIIH was also observed when classical fractionations of the soluble fractions were performed in the absence of formaldehyde crosslinking. However, the dose of UV required to dissociate the CAK in this condition (100 J/m^2) was higher than the dose needed to detect such dissociation by ChIP (10 J/m^2). Also, though repair factors accumulate on the core TFIIH by ChIP, no accumulation was observed on the TFIIH in the soluble fraction after UV. These findings pinpoint the fragility of the repairosome complex in human cells and the difficulties in isolating this complex by traditional methods. They also explain why such dissociation was not observed in high eukaryotes until now.

The accumulation of NER factors with the core TFIIH depends on both the UV dose and the recovery time after UV exposure. It reached a maximum 15 min after UV treatment at 20 J/m^2 and occurred when cells were incubated at 37°C and not when they were kept at 4°C. In these conditions, we determined that

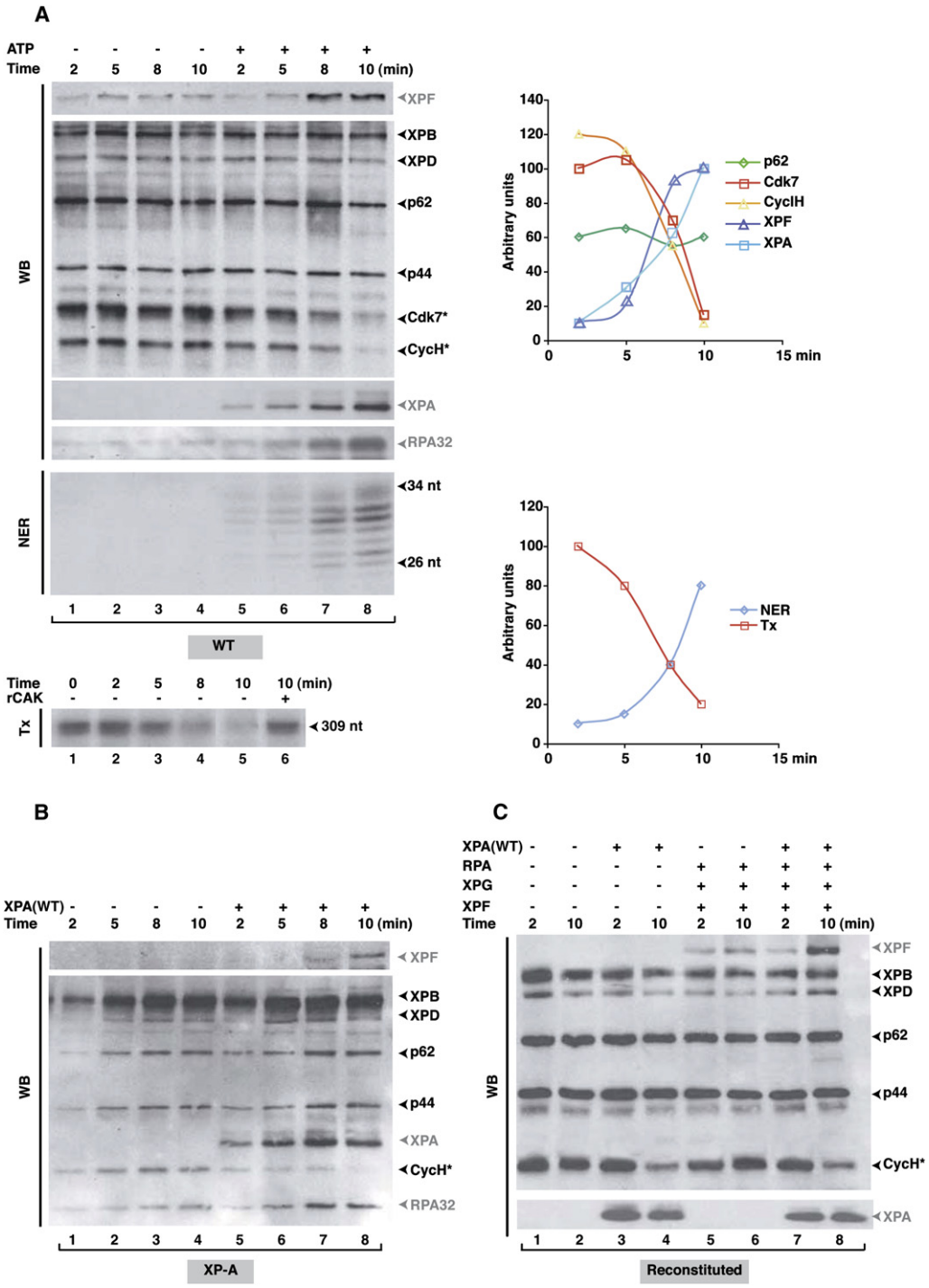

Figure 3. Progress of the CAK Release In Vitro

(A) HeLa cell extract (wild-type) was incubated 15 min at 4°C with damaged DNA coupled to magnetic beads. Reactions were then supplemented with ATP (lanes 6–9) and incubated at 30°C over the time indicated. After washes, aliquots were analyzed either by western blotting (WB) or in a dual-incision assay (NER). For transcription (Tx), reactions were allowed to proceed as described, and following washes at different times points as indicated, the magnetic beads were incubated in a reconstituted transcription assay containing an adenovirus major late promoter (AdMLP) template and the RNA pol II, TFIIA, TFIIB, TBP, TFIIE, TFIIF transcription factors (Gerard et al., 1991). mRNA products (309 nt) are visualized by the incorporation of radioactive nucleotides. In lane 6, 10 ng of recombinant CAK was added to the reaction. Signals for WB or NER and Tx were quantified and spotted on graphs. For WB, XPB was used as reference.

(B) XP-A cell extract (XP12ROSV) was used as in panel (A) with ATP and 50 ng of recombinant XPA-WT protein when indicated. After washes, aliquots were analyzed by western blotting.

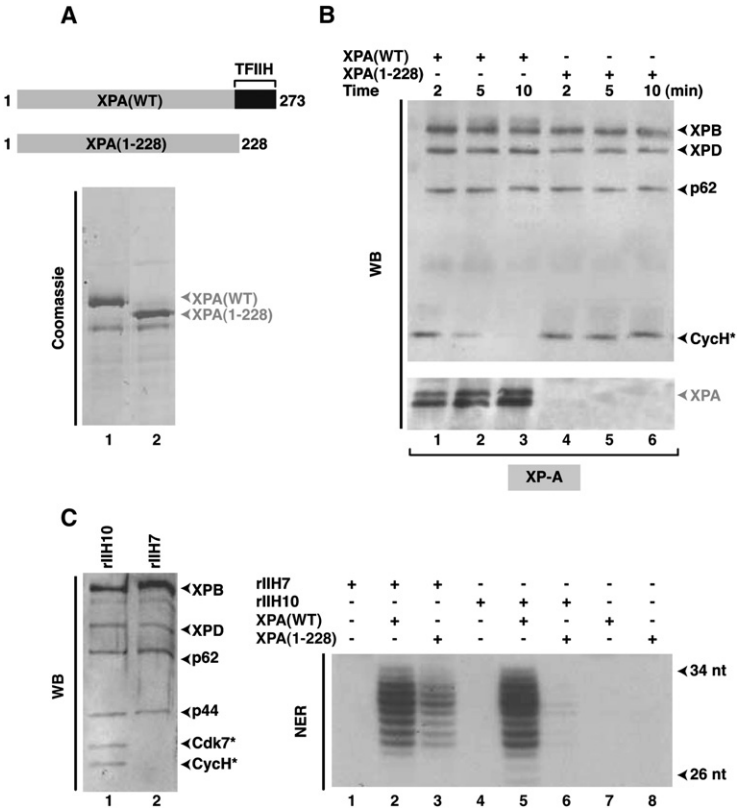

Figure 4. Release of the CAK Initiates DNA Repair

(A) Upper panel: Schematic representation of XPA-WT and XPA (1-228). The dark gray box indicates the TFIIH interacting domain. Lower panel: Coomassie staining of XPA-WT and XPA(1-228).

(B) XP-A cell extract was incubated as in Figure 3A, in the presence of ATP and 50 ng of recombinant XPA-WT or (1-228) polypeptides when indicated. After washes, aliquots were analyzed by western blotting (WB).

(C) Left panel: Purified recombinant TFIIH complexes were expressed in baculovirus infected cells with (rIIH10) or without (rIIH7) the CAK subcomplex and purified as described (Tirode et al., 1999). Complexes were resolved by SDS-PAGE and western blotted with TFIIH antibodies. rIIH7 (lanes 1–3) and rIIH10 (4–6) were added in a dual-incision assay containing XPC, RPA, XPG, and XPF, in the presence of either XPA-WT (lanes 2, 5, and 7) or XPA(1-228; lanes 3, 6, and 8).

~70% of the TFIIH complexes are missing the CAK and are involved in repair. That the remodeling of the complex is temperature dependent indicates that it is elicited by a cellular response to UV damage and not by a crosslinking effect of UV or by damaging the TFIIH complex itself. In addition, the synchronization between the release of the CAK from the core TFIIH and the arrival of the NER factors suggests that the change in TFIIH subunit composition is due to its participation in the removal of 6-4PP lesions that are repaired in the first hours after UV, and not in the removal of CPD lesions that takes place later (van Hoffen et al., 1995). This theory is strengthened by our finding that the release of CAK was impaired in XP-C cells in which TFIIH is not recruited to the damaged DNA (Volker et al., 2001). That the CAK is released and NER factors accumulate with the core TFIIH in CS-B but not in XP-C cells strongly suggests that the modifications we observed take place during GGR. In accordance with these findings, the release of the CAK was observed when cells were treated with actinomycin D or H8, two inhibitors of transcription (data not shown). It suggests that under our experimental condition, the TCR of UV-induced lesions attracts insufficient numbers of NER proteins to allow visualization of the incision complexes in XP-C cells, but it does not rule out the possibility that the CAK is detached from the core TFIIH during TCR. The reproducibility of our findings with the siRNA or XP cell lines validates the ChIP/western technology and its use in analyzing the composition of TFIIH after a cellular perturbation.

From a mechanistic standpoint, we assume that the CAK is detached from the core and not degraded because proteasome inhibitors did not alter the dynamic composition of TFIIH upon irradiation in vivo. Moreover, the disappearance of CAK from the core TFIIH engaged in NER was reconstituted in vitro, in a system that does not support active protein degradation. Unexpectedly, we observed that the NER factors are already present in the chromatin of untreated cells and are sequentially reallocated to the site of damage after irradiation to form intermediate repair complexes during the NER reaction (Figure 7). Notably, ChIP assays have shown several protein complexes containing TFIIH at different time points after UV. Before treatment, we observed a complex with TFIIH alone (or presumably present in heterogeneous transcription preinitiation complexes). Then, just after irradiation, a complex containing TFIIH and XPC was detected. Thirty minutes after irradiation, a complex containing only the core TFIIH in addition to XPA, RPA, XPF, and probably XPG was found on the chromatin. Finally, 8 hr after irradiation, when most of the 6-4PP lesions are removed, Ab-XPB captures a TFIIH complex that contains the CAK but that lacks all the NER factors. In a broader perspective, the findings presented here fully support the scenario of a sequential assembly of the repair factors to the damaged DNA, guided at each step by numerous protein-protein interactions (Riedl et al., 2003). However, contradictory findings remain about which factor initiates the subsequent recruitment of all NER proteins to the preincision complex, as both protein complexes of XPC-hHR23B and XPA-RPA have been proposed to play a key role in the first step of DNA damage recognition (Kesseler et al., 2007). Our ChIP experiments show that UV irradiation initiates the formation of a XPC/TFIIH complex in the chromatin in the absence of XPA, whereas a TFIIH/XPA complex is not detected without XPC. These findings suggest that XPC initiates the assembly of the preincision complex,

(C) Recombinant XPC and TFIIH were incubated 15 min at 4°C with damaged DNA coupled to magnetic beads. After washes, highly purified recombinant NER factors were added to the reaction mixture as indicated in the presence of ATP, and the reactions were allowed to proceed for 2 min or 10 min at 30°C. After a second wash, aliquots were analyzed by western blotting.

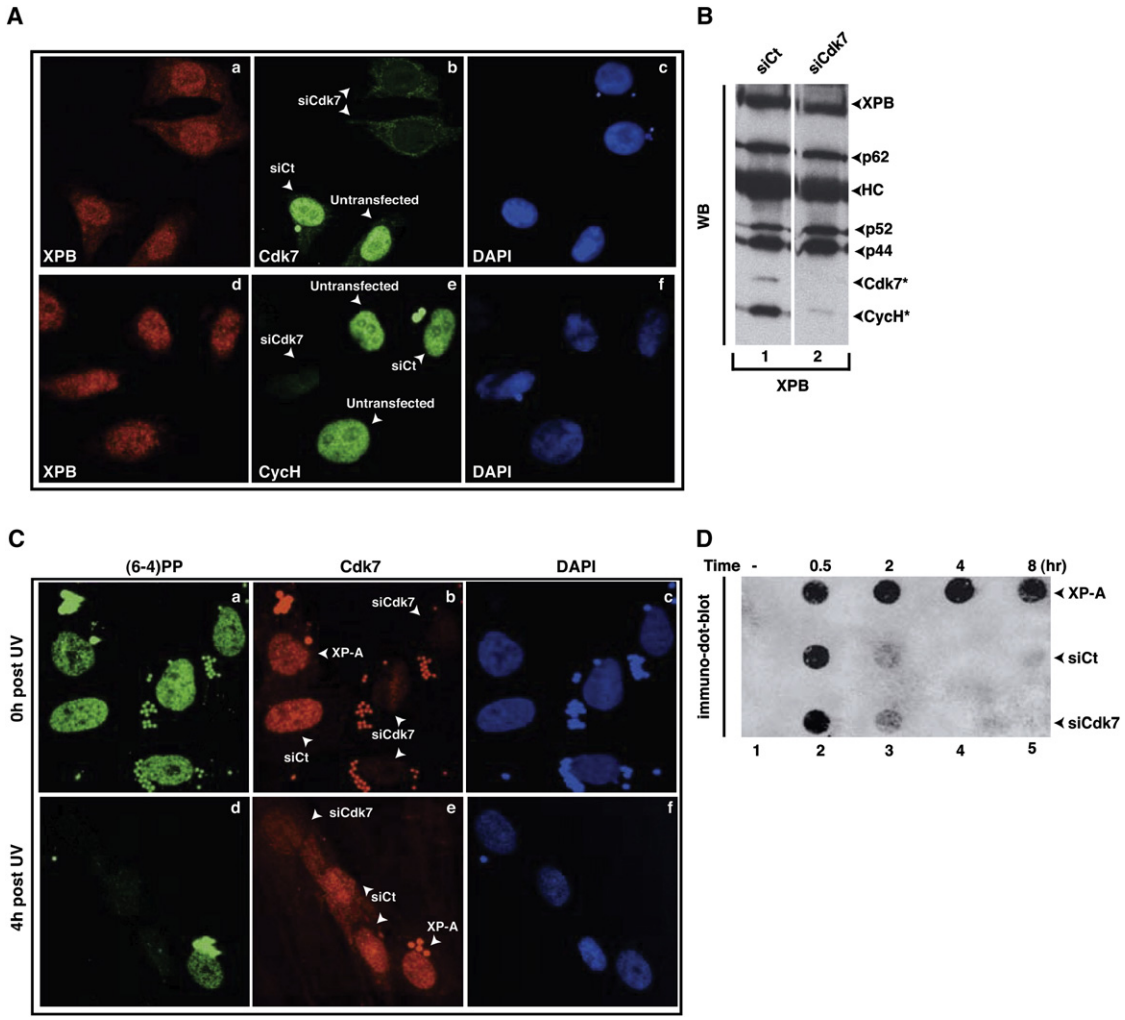

Figure 5. NER Takes Place without CAK Backup In Vivo

(A) siCdk7-treated WT cells prelabeled with small blue fluorescent latex beads, siCt-treated WT cells prelabeled with large green fluorescent latex beads, and untransfected WT cells (no beads) were plated on the same slide and analyzed by confocal microscopy using Ab-XPB (Aa and Ad), Ab-Cdk7 (Ab), or Ab-Cyclin H antibodies (Ae).

(B) WT cells were transfected either with a pool of siRNA oligonucleotides against Cdk7 (siCdk7) or with control siRNA (siCt). TFIIH from 200 μg of total extracts was immunoprecipitated with Ab-XPB and resolved by SDS-PAGE followed by western blotting. HC indicates heavy chain of the Ab.

(C) Blue fluorescent-labeled WT cells were transfected with siCdk7, and WT with no beads were transfected with siCt. Twenty-four hours later, these cells were plated on the same slide with green fluorescent-labeled XP-A cells. Forty-eight hours posttransfection, cells were irradiated with 10 J/m^2, and repair was measured after 4 hr of recovery by confocal microscopy using Ab-(6-4)PP (directed against the 6-4PP lesions; Ca and Cd) and Ab-Cdk7 (Cb and Ce).

(D) Immuno-dot blot experiments were carried out using Ab-(6-4)PP antibody with 500 ng of genomic DNA samples of siCdk7, siCt, or XP-A cells irradiated at 10 J/m^2. Post-UV recovery time is indicated. Lane 1 contains 500 ng of nonirradiated genomic DNA.

thereby supporting the conclusion that this protein is the earliest known NER factor to join the lesion.

Recruitment of XPA: A Checkpoint in NER

The question remains as to how and when the CAK is released from the core TFIIH. ChIP assays and the in vitro reconstituted incision system provide evidence that the detachment of the CAK from the core TFIIH is catalyzed by XPA, in the presence of ATP. This statement is based on the observations that mutations in either XPC or TFIIH, two repair factors that are recruited to the damaged DNA before XPA (Volker et al., 2001; Riedl et al., 2003), hinder the CAK release. The release of the CAK in an XP-A cell that does not express the XPA protein was barely observable. In contrast, the release of the CAK occurred in cell lines mutated in either XPG or XPF, two proteins that are recruited after XPA. Additionally, the detachment of the CAK from the core TFIIH was hardly detectable in a reconstituted in vitro system containing XPC, TFIIH, and a damaged DNA unless XPA and ATP were added to the reaction. In vitro studies have further revealed that the C-terminal region of XPA that interacts with TFIIH (Park et al., 1995) is required for the release of the CAK. The consequence of the detachment of the CAK

Table 1. Impaired UV-Dependent Transactivation of *p21*, *Mdm2*, and *ATF3* Genes in siCdk7-Treated Cells

Gene	siCt	siCdk7
p21	16.65 ± 1.0	5.61 ± 0.5
Mdm2	8.02 ± 0.5	2.30 ± 0.6
ATF3	22.29 ± 3.6	3.33 ± 1.0

RT-PCR analysis was performed 12 hr after UV irradiation (30 J/m^2) of siCt- or siCdk7-treated cells. The findings are expressed as fold induction (mRNA UV[+]/mRNA UV[−]) and are the mean of three independent experiments. *Mdm2*, murine double minute2; *ATF3*, activating transcription factor 3.

from the core TFIIH is the initiation of the incision/excision step of NER. The C-terminal truncated XPA, which was almost inactive in the presence of a whole TFIIH, catalyzed dual incision in the presence of a TFIIH lacking CAK. We thus propose that the accurate recruitment of XPA constitutes a major checkpoint in NER that will accelerate, in the appropriate situation, the removal of the damaged DNA by separating the CAK from the core TFIIH. This pinpoints one of the key roles of XPA, a scaffold protein that has no enzymatic activity on its own but is nevertheless indispensable for DNA incision.

How can the CAK be an obstacle to DNA repair? Strikingly, CAK was shown to negatively regulate the XPD DNA unwinding activity (Sandrock and Egly, 2001). It has also been established that the recruitment of XPA to the XPC/TFIIH intermediate preincision complex led to a full opening of the damaged DNA (Tapias et al., 2004; Andressoo et al., 2006). These observations suggest that the detachment of the CAK from the core may stimulate TFIIH helicase/ATPase activities, thereby accelerating the enlargement and the stabilization of the DNA-opened structure. Supporting this model, XPA has been shown to stimulate the ATPase activity of TFIIH specifically in the presence of damaged DNA (Winkler et al., 2001). Alternatively, it has been shown that CAK can negatively regulate NER by phosphorylation of one or more components of the reaction (Araujo et al., 2000). Even if the target(s) of Cdk7 in NER is

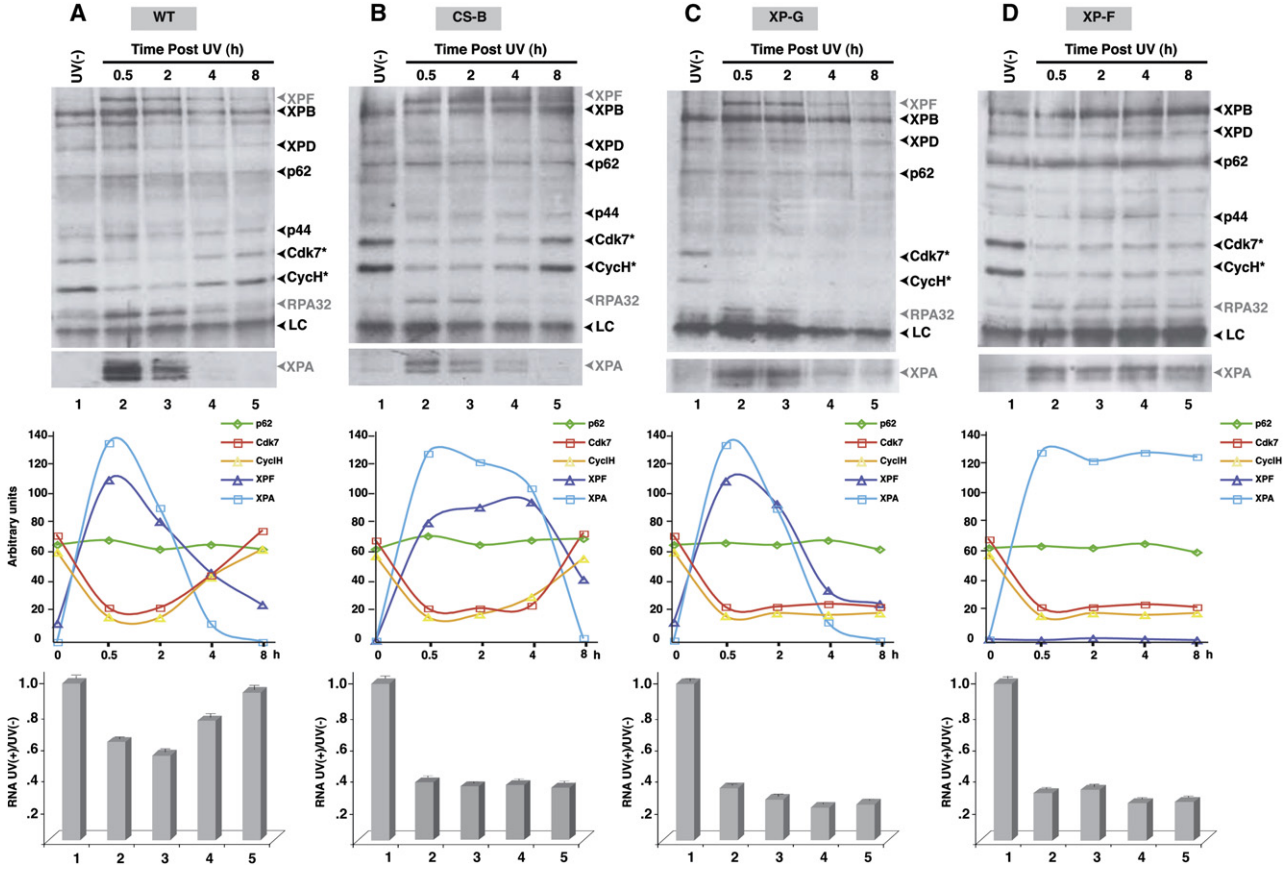

Figure 6. Return of the CAK in the TFIIH Complex

ChIP over the time of repair: ChIP with Ab-XPB was performed on (A) wild-type, (B) CS-B, (C) XP-G, and (D) XP-F cells incubated for different times after UV (20 J/m^2) as indicated. RNA synthesis recovery was also measured in each cell line. After prelabeling with [^{14}C]thymidine (0.02 μCi/ml) for 2 days, nonirradiated or UV (20 J/m^2)-irradiated cells were pulse labeled for 30 min with [3H] uridine at different time points after irradiation, and radioactivity was determined. The findings represent the relative RNA synthesis measured at different time points after UV compared with nonirradiated cells. The WB signals for XPF, XPB, p62, cyclH, Cdk7, and XPA were quantified using Genetool (Syngene) and plotted on the graphs. For each single lane, XPB was used as reference. Mean value and standard deviation of three independent experiments are shown.

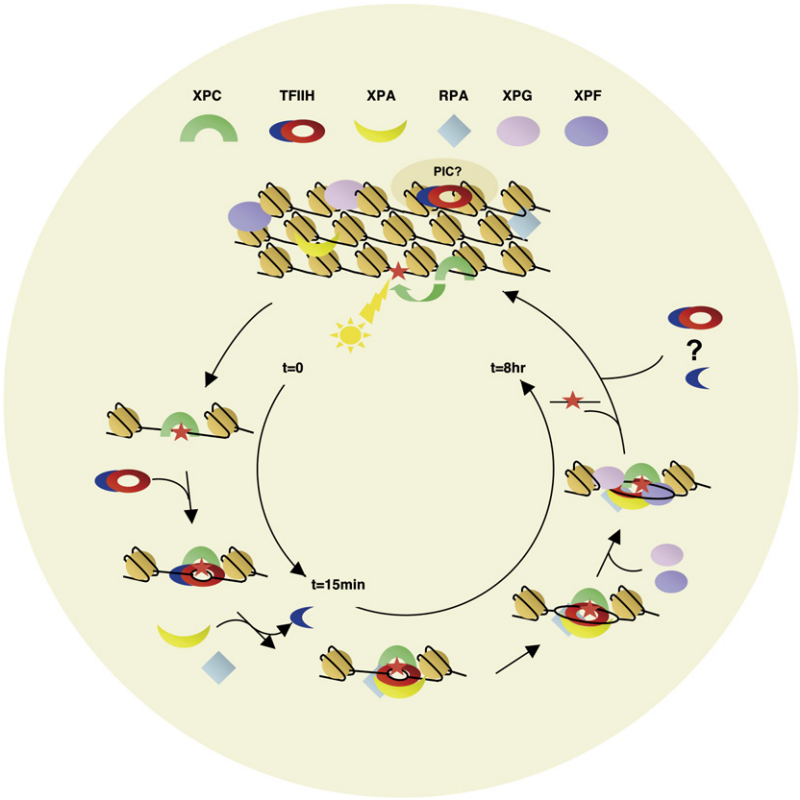

Figure 7. Model for the Assembly of the Human NER Incision Complex on the Chromatin

Schematic representation depicting the changes in the composition of TFIIH after a genotoxic attack. The transcription factor TFIIH (the core in red and the CAK in blue) is probably included in preinitiation complexes (PIC) containing either RNA pol I or II. Following irradiation and the formation of a lesion in the DNA (red star), the NER factors reallocate to the site of damage in sequential order. XPC and a whole TFIIH are recruited to the lesion, and a preopening of the damaged DNA is initiated. The arrival of XPA drives the release of the CAK within 15 min after the irradiation and promotes a full opening of the DNA around the damage to favor the binding of XPG and XPF. Following removal of the damage, a whole TFIIH is found in the chromatin, but whether it is a free CAK that reassociates with a core TFIIH formerly involved in NER, or a new whole TFIIH complex that is recruited to the chromatin, remains to be established. In our experimental conditions, the process of dissociation/reassociation of CAK to TFIIH takes 8 hr.

not known, the release of the CAK from the core TFIIH is a possible mechanism to eliminate the kinase from the preincision complex, thereby separating physically the enzyme and its substrate.

TFIIH: Two Sides of the Same Coin

Finally, our study reconciles several divergent observations obtained on the composition of TFIIH from studies in yeast and human models. In yeast extracts, two TFIIH-containing complexes with different principal tasks were characterized; the first form contains the core TFIIH and TFIIK (the homolog of CAK) and works mainly in transcription, whereas the second one contains only the core and is specifically involved in NER (Svejstrup et al., 1995). However, total extracts from human cells do not contain any free core TFIIH (unpublished data; Araujo et al., 2001). In light of our findings, an intriguing picture emerges that differentiates the yeast and human TFIIH complexes; in human, the free core TFIIH is transient and exists only during the short period of DNA repair. This repair-specific core TFIIH disappears when lesions are removed and transcription is ready to resume. We observed that the departure of the repair factors and the return of the CAK to the TFIIH complex are concomitant with the recovery of transcription in WT cells. It cannot be ruled out that the difference between yeast and human also indicates that yeast repair factors have a stronger intrinsic affinity for each other and for the core TFIIH than they do in man. In mammals, it remains to be established whether it is a free CAK that comes back to a core TFIIH formerly involved in NER, or if it is a new TFIIH complex that is recruited to the chromatin (Figure 7). Mutations in NER proteins cause an accumulation of intermediate repair complexes that can persist for several hours on the chromatin. Whether the persistence of these intermediate "poised" complexes are responsible for some of the phenotypes displayed by patients merits further investigation.

EXPERIMENTAL PROCEDURES

Chromatin Immunoprecipitation

Chromatin was prepared as described in Fousteri et al. (2006). The chromatin suspension was sonicated on ice (3 × 15 min) on buffer S (10 mM Tris-HCl [pH 8.0], 140 mM NaCl, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS) using a Diagenode (Liège, Belgium) Bioruptor (power setting 5) in 10 s pulse followed by 20 s of cooling. Samples were spun down (13,200 rpm, 15 min), and the supernatant that contained the crosslinked chromatin was stored at −80°C.

In each assay, 400 μg of protein from crosslinked chromatin was immunoprecipitated with 1 μg of antibody in buffer S, 2 hr at 4°C. The immunocomplexes were collected by adsorption to protein G Sepharose beads (Upstate; Billerica, MA) overnight at 4°C. The beads were next washed three times with 5 vol of buffer S and resuspended in 1× Laemli SDS buffer. Samples were incubated at 95°C for 90 min for decrosslinking prior electrophoresis.

In re-ChIP assay, 1.2 mg of protein from crosslinked chromatin extracted was immunoprecipitated with 6 μg of specific antibody as described, and fractions were eluted with 10 mM dithiothreitol at 37°C for 30 min. After centrifugation, the supernatant was diluted 10 times with buffer S and subjected to another round of immunoprecipitation.

RNA Interference

A pool of four RNA oligonucleotides (Dharmacon; Waltham, MA) forming a 19-base duplex core, specifically designed to target Cdk7 or XPA mRNA, was transfected in cells at the concentration of 50 nM. A pool of RNA oligonucleotides, without any target mRNA, was used as control. RNA transfection was performed by using Lipofectamine 2000 reagent (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. Specific target reduction was analyzed 48 hr posttransfection by western blotting.

Protein Binding Studies on Immobilized DNA

Dynabeads M-280 Streptavidin (Dynal; Carlsbad, CA) coupled to DNA were incubated in blocking buffer (10 mM HEPES, 100 mM glutamate, 10 mM MgOAc, 5 mM EGTA, 3.5% glycerol, 60 mg/ml casein, 5 mg/ml PVP, and 2.5 mM DTT), 15 min at room temperature to limit unspecific binding of proteins (Ranish et al., 2004). Immobilized DNA was then incubated in incision buffer (50 mM HEPES-OH [pH 7.6], 20 mM Tris-HCl [pH 7.6], 50 mM KCl, 2.5 mM MgCl2, 0.5 mM DTT, 0.5 mM EDTA, 10% glycerol) with recombinant XPC-hHR23B and HeLa purified or recombinant TFIIH at 4°C, 15 min. Upon incubation, beads were collected on a magnetic particle concentrator (Dynal MPC) and supernatants removed. Beads were then washed five times in 4 vol of cold incision buffer and resuspended in the same buffer with XPA, RPA, XPG, and ERCC1-XPF with 2 mM ATP as indicated. Following incubation at different times, beads were washed five times in 4 vol of cold incision buffer and resuspended in 1× Laemli SDS buffer for electrophoresis.

Determination of RNA Synthesis after UV Irradiation

Cells in exponential phase were grown in the presence of [^{14}C]thymidine (0.02mCi/ml) for 2 days to uniformly label the DNA. The UV-irradiated cells (10 J/m^2) were pulse labeled with 5 μCi/ml of [^{3}H]uridine for 30 min at different times. The cells were collected and washed once with ice-cold PBS and lysed in buffer containing 0.5% SDS and 100 μg/ml proteinase K for 2 hr at 37°C. After trichloroacetic acid precipitation (10% TCA), the samples were spotted onto glass fiber discs (Whatman; Maidstone, Kent, UK); the filters were next sequentially washed in 5% TCA, 70% ethanol/acetone, and counted for their radioactivity. The ^{3}H/^{14}C ratio was taken as a measure of RNA synthesis.

Dual Incision and Transcription Assays

Dual incision and transcription assays were carried out as described (Coin et al., 2006).

Protein Purification

Recombinant TFIIH complexes were purified as described (Tirode et al., 1999). XPA-WT and (1-228) were expressed in *E. coli* and purified using a N-terminal GST tag.

Reverse Transcription and Real-Time Quantitative PCR

cDNA synthesis was performed by using random hexanucleotides and AMV reverse transcriptase (Sigma; St. Louis, MO). Real-time quantitative PCR was done with the FastStart DNA Master SYBR Green kit and the Lightcycler apparatus (Roche Diagnostic; Basel, Switzerland). Primer sequences are available upon request.

Antibodies

Primary antibodies used in ChIP were rabbit IgG polyclonal anti-XPB (S-19), rabbit IgG polyclonal anti-XPA (FL-273, Santa Cruz Biotechnology; Santa Cruz, CA), and rabbit IgG polyclonal anti-XPC (Riedl et al., 2003). Primary antibodies (the final dilutions are indicated in parentheses) used in fluorescent labeling were mouse IgG monoclonal anti-6-4 (64M2; 1:2000) (MBL International Corp.) and mouse IgG monoclonal anti-Cdk7 (2F8) (Coin et al., 2006). Secondary antibodies used in this study were Alexa 488 anti-mouse IgG and Cy3-conjugated goat anti-rabbit IgG.

SUPPLEMENTAL DATA

Supplemental Data include two figures and are available at http://www.molecule.org/cgi/content/full/31/1/9/DC1/.

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REFERENCES

Andressoo, J.O., Jans, J., de Wit, J., Coin, F., Hoogstraten, D., van de Ven, M., Toussaint, W., Huijmans, J., Thio, H.B., van Leeuwen, W.J., et al. (2006). Rescue of progeria in trichothiodystrophy by homozygous lethal Xpd alleles. PLoS Biol. *4*, e322.

Araujo, S.J., Nigg, E.A., and Wood, R.D. (2001). Strong functional interactions of TFIIH with XPC and XPG in human DNA nucleotide excision repair, without a preassembled repairosome. Mol. Cell. Biol. *21*, 2281–2291.

Araujo, S.J., Tirode, F., Coin, F., Pospiech, H., Syvaoja, J.E., Stucki, M., Hubscher, U., Egly, J.M., and Wood, R.D. (2000). Nucleotide excision repair of DNA with recombinant human proteins: definition of the minimal set of factors, active forms of TFIIH, and modulation by CAK. Genes Dev. *14*, 349–359.

Coin, F., Bergmann, E., Tremeau-Bravard, A., and Egly, J.M. (1999). Mutations in XPB and XPD helicases found in xeroderma pigmentosum patients impair the transcription function of TFIIH. EMBO J. *18*, 1357–1366.

Coin, F., De Santis, L.P., Nardo, T., Zlobinskaya, O., Stefanini, M., and Egly, J.M. (2006). p8/TTD-A as a repair-specific TFIIH subunit. Mol. Cell *21*, 215–226.

Drapkin, R., Reardon, J.T., Ansari, A., Huang, J.C., Zawel, L., Ahn, K., Sancar, A., and Reinberg, D. (1994). Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II. Nature *368*, 769–772.

Dubaele, S., Proietti De Santis, L., Bienstock, R.J., Keriel, A., Stefanini, M., Van Houten, B., and Egly, J.M. (2003). Basal transcription defect discriminates between xeroderma pigmentosum and trichothiodystrophy in XPD patients. Mol. Cell *11*, 1635–1646.

Evans, E., Fellows, J., Coffer, A., and Wood, R.D. (1997). Open complex formation around lesion during nucleotide excision repair provides a structure for cleavage by human XPG protein. EMBO J. *16*, 625–638.

Fan, F., Jin, S., Amundson, S.A., Tong, T., Fan, W., Zhao, H., Zhu, X., Mazzacurati, L., Li, X., Petrik, K.L., et al. (2002). ATF3 induction following DNA damage is regulated by distinct signaling pathways and over-expression of ATF3 protein suppresses cells growth. Oncogene *21*, 7488–7496.

Feaver, W.J., Svejstrup, J.Q., Bardwell, L., Bardwell, A.J., Buratowski, S., Gulyas, K.D., Donahue, T.F., Friedberg, E.C., and Kornberg, R.D. (1993). Dual roles of a multiprotein complex from S. cerevisiae in transcription and DNA repair. Cell *75*, 1379–1387.

Fousteri, M., Vermeulen, W., van Zeeland, A.A., and Mullenders, L.H. (2006). Cockayne syndrome A and B proteins differentially regulate recruitment of chromatin remodeling and repair factors to stalled RNA polymerase II in vivo. Mol. Cell *23*, 471–482.

Gerard, M., Fischer, L., Moncollin, V., Chipoulet, J.M., Chambon, P., and Egly, J.M. (1991). Purification and interaction properties of the human RNA polymerase B(II) general transcription factor BTF2. J. Biol. Chem. *266*, 20940–20945.

Giglia-Mari, G., Coin, F., Ranish, J.A., Hoogstraten, D., Theil, A., Wijgers, N., Jaspers, N.G., Raams, A., Argentini, M., van der Spek, P.J., et al. (2004). A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A. Nat. Genet. *36*, 714–719.

Giglia-Mari, G., Miquel, C., Theil, A.F., Mari, P.O., Hoogstraten, D., Ng, J.M., Dinant, C., Hoeijmakers, J.H., and Vermeulen, W. (2006). Dynamic interaction of TTDA with TFIIH Is stabilized by nucleotide excision repair in living cells. PLoS Biol. *4*, e156.

Guzder, S.N., Qiu, H., Sommers, C.H., Sung, P., Prakash, L., and Prakash, S. (1994a). DNA repair gene RAD3 of S. cerevisiae is essential for transcription by RNA polymerase II. Nature *367*, 91–94.

Guzder, S.N., Sung, P., Bailly, V., Prakash, L., and Prakash, S. (1994b). RAD25 is a DNA helicase required for DNA repair and RNA polymerase II transcription. Nature *369*, 578–581.

Hanawalt, P.C. (2002). Subpathways of nucleotide excision repair and their regulation. Oncogene *21*, 8949–8956.

Hoogstraten, D., Nigg, A.L., Heath, H., Mullenders, L.H., van Driel, R., Hoeijmakers, J.H., Vermeulen, W., and Houtsmuller, A.B. (2002). Rapid switching of TFIIH between RNA polymerase I and II transcription and DNA repair in vivo. Mol. Cell *10*, 1163–1174.

Huang, J.C., Svoboda, D.L., Reardon, J.T., and Sancar, A. (1992). Human nucleotide excision nuclease removes thymine dimers from DNA by incising the 22nd phosphodiester bond 5′ and the 6th phosphodiester bond 3′ to the photodimer. Proc. Natl. Acad. Sci. USA *89*, 3664–3668.

Iben, S., Tschochner, H., Bier, M., Hoogstraten, D., Hozak, P., Egly, J.M., and Grummt, I. (2002). TFIIH plays an essential role in RNA polymerase I transcription. Cell *109*, 297–306.

Kesseler, K.J., Kaufmann, W.K., Reardon, J.T., Elston, T.C., and Sancar, A. (2007). A mathematical model for human nucleotide excision repair: Damage recognition by random order assembly and kinetic proofreading. J. Theor. Biol. *249*, 361–375.

Koberle, B., Roginskaya, V., and Wood, R.D. (2006). XPA protein as a limiting factor for nucleotide excision repair and UV sensitivity in human cells. DNA Repair (Amst.) *5*, 641–648.

Lehmann, A.R. (2003). DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. Biochimie *85*, 1101–1111.

Lindahl, T., and Wood, R.D. (1999). Quality control by DNA repair. Science *286*, 1897–1905.

Mayne, L.V., and Lehmann, A.R. (1982). Failure of RNA synthesis to recover after UV radiation: An early defect in cells from individuals with Cockayne's syndrome and xeroderma pigmentosum. Cancer Res. *42*, 1473–1478.

Moggs, J.G., Yarema, K.J., Essigmann, J.M., and Wood, R.D. (1996). Analysis of incision sites produced by human cell extracts and purified proteins during nucleotide excision repair of a 1,3-intrastrand d(GpTpG)-cisplatin adduct. J. Biol. Chem. *271*, 7177–7186.

Mori, T., Nakane, M., Hattori, T., Matsunaga, T., Ihara, M., and Nikaido, O. (1991). Simultaneous establishment of monoclonal antibodies specific for either cyclobutane pyrimidine dimer or (6–4)photoproduct from the same mouse immunized with ultraviolet-irradiated DNA. Photochem. Photobiol. *54*, 225–232.

Nocentini, S., Coin, F., Saijo, M., Tanaka, K., and Egly, J.-M. (1997). DNA damage recognition by XPA protein promotes efficient recruitment of transcription factor II H. J. Biol. Sci. *272*, 22991–22994.

O'Donovan, A., Davies, A.A., Moggs, J.G., West, S.C., and Wood, R.D. (1994). XPG endonuclease makes the 3′ incision in human DNA nucleotide excision repair. Nature *371*, 432–435.

Park, C.H., Mu, D., Reardon, J.T., and Sancar, A. (1995). The general transcription-repair factor TFIIH is recruited to the excision repair complex by the XPA protein independent of the TFIIE transcription factor. J. Biol. Chem. *270*, 4896–4902.

Proietti-De-Santis, L., Drane, P., and Egly, J.M. (2006). Cockayne syndrome B protein regulates the transcriptional program after UV irradiation. EMBO J. *25*, 1915–1923.

Ranish, J.A., Hahn, S., Lu, Y., Yi, E., Li, X., Eng, J., and Aebersold, R. (2004). Identification of TFB5, a new component of general transcription and DNA repair factor IIH. Nat. Genet. *36*, 707–713.

Riedl, T., Hanaoka, F., and Egly, J.M. (2003). The comings and goings of nucleotide excision repair factors on damaged DNA. EMBO J. *22*, 5293–5303.

Riou, L., Eveno, E., van Hoffen, A., van Zeeland, A.A., Sarasin, A., and Mullenders, L.H. (2004). Differential repair of the two major UV-induced photolesions in trichothiodystrophy fibroblasts. Cancer Res. *64*, 889–894.

Roy, R., Adamczewski, J.P., Seroz, T., Vermeulen, W., Tassan, J.P., Schaeffer, L., Nigg, E.A., Hoeijmakers, J.H., and Egly, J.M. (1994). The MO15 cell cycle kinase is associated with the TFIIH transcription-DNA repair factor. Cell *79*, 1093–1101.

Sandrock, B., and Egly, J.M. (2001). A yeast four-hybrid system identifies Cdk-activating kinase as a regulator of the XPD helicase, a subunit of transcription factor IIH. J. Biol. Chem. *276*, 35328–35333.

Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoeijmakers, J.H., Chambon, P., and Egly, J.M. (1993). DNA repair helicase: A component of BTF2 (TFIIH) basic transcription factor. Science *260*, 58–63.

Shivji, M.K.K., Podust, V.N., Hubsher, U., and Wood, R.D. (1995). Nucleotide excision repair DNA synthesis by DNA polymerase epsilon in the presence of PCNA, RFC, and RPA. Biochemistry *34*, 5011–5017.

Sijbers, A.M., de Laat, W.L., Ariza, R.R., Biggerstaff, M., Wei, Y.F., Moggs, J.G., Carter, K.C., Shell, B.K., Evans, E., de Jong, M.C., et al. (1996). Xeroderma pigmentosum group F caused by a defect in a structure-specific DNA repair endonuclease. Cell *86*, 811–822.

Svejstrup, J.Q., Wang, Z., Feaver, W.J., Wu, X., Bushnell, D.A., Donahue, T.F., Friedberg, E.C., and Kornberg, R.D. (1995). Different forms of TFIIH for transcription and DNA repair: Holo-TFIIH and a nucleotide excision repairosome. Cell *80*, 21–28.

Tapias, A., Auriol, J., Forget, D., Enzlin, J.H., Scharer, O.D., Coin, F., Coulombe, B., and Egly, J.M. (2004). Ordered conformational changes in damaged DNA induced by nucleotide excision repair factors. J. Biol. Chem. *279*, 19074–19083.

Tirode, F., Busso, D., Coin, F., and Egly, J.M. (1999). Reconstitution of the transcription factor TFIIH: assignment of functions for the three enzymatic subunits, XPB, XPD, and cdk7. Mol. Cell *3*, 87–95.

van Hoffen, A., Venema, J., Meschini, R., van Zeeland, A.A., and Mullenders, L.H. (1995). Transcription-coupled repair removes both cyclobutane pyrimidine dimers and 6–4 photoproducts with equal efficiency and in a sequential way from transcribed DNA in xeroderma pigmentosum group C fibroblasts. EMBO J. *14*, 360–367.

Volker, M., Mone, M.J., Karmakar, P., van Hoffen, A., Schul, W., Vermeulen, W., Hoeijmakers, J.H., van Driel, R., van Zeeland, A.A., and Mullenders, L.H. (2001). Sequential assembly of the nucleotide excision repair factors in vivo. Mol. Cell *8*, 213–224.

Winkler, G.S., Sugasawa, K., Eker, A.P., de Laat, W.L., and Hoeijmakers, J.H. (2001). Novel functional interactions between nucleotide excision DNA repair proteins influencing the enzymatic activities of TFIIH, XPG, and ERCC1-XPF. Biochemistry *40*, 160–165.

Zurita, M., and Merino, C. (2003). The transcriptional complexity of the TFIIH complex. Trends Genet. *19*, 578–584.

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Molecular insights into the recruitment of TFIIH to sites of DNA damage

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XPB and XPD subunits of TFIIH are central genome caretakers involved in nucleotide excision repair (NER), although their respective role within this DNA repair pathway remains difficult to delineate. To obtain insight into the function of XPB and XPD, we studied cell lines expressing XPB or XPD ATPase-deficient complexes. We show the involvement of XPB, but not XPD, in the accumulation of TFIIH to sites of DNA damage. Recruitment of TFIIH occurs independently of the helicase activity of XPB, but requires two recently identified motifs, a R-E-D residue loop and a Thumb-like domain. Furthermore, we show that these motifs are specifically involved in the DNA-induced stimulation of the ATPase activity of XPB. Together, our data demonstrate that the recruitment of TFIIH to sites of damage is an active process, under the control of the ATPase motifs of XPB and suggest that this subunit functions as an ATP-driven hook to stabilize the binding of the TFIIH to damaged DNA.

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Introduction

DNA and RNA helicases are a ubiquitous, yet diverse, group of enzymes present in viruses, prokaryotes and eukaryotes (Delagoutte and von Hippel, 2003). They convert chemical energy of nucleoside triphosphate hydrolysis to the mechanical energy necessary to transiently separate the strands of duplex nucleic acids (Tuteja and Tuteja, 2004). By this mean, they provide the single-stranded DNA or RNA intermediates necessary for replication, transcription, recombination or repair. Furthermore, it has been shown that helicases can also effectively displace bound proteins from DNA or RNA (von Hippel, 2004). There are several known human diseases caused by defective helicases (Ellis, 1997). Among these disorders, the cancer-prone *Xeroderma pigmentosum* (XP), alone or in combination with the Cockayne syndrome (CS), and the *Trichothiodystrophy* (TTD) are noteworthy as they entail mutations in the XPB and XPD superfamily 2 helicases. Both of these helicases are part of the same TFIIH complex. TFIIH is composed of a seven-subunit core (XPB, XPD, p62, p52, p44, p34 and p8/TTD-A) associated with the CAK subcomplex (Cdk7, cyclin H, and MAT1) (Giglia-Mari *et al*, 2004; Ranish *et al*, 2004). TFIIH functions in both transcription initiations of mRNA and rRNA (Iben *et al*, 2002), as well as in nucleotide excision repair (NER) (Schaeffer *et al*, 1993).

XPB and XPD patients are photosensitive and display a 1000-fold increase in melanoma risk because of defects in the NER function of TFIIH (Lehmann, 2003). NER removes a broad spectrum of DNA lesions including UV-induced pyrimidine dimers and bulky, helix-distorting adducts caused by toxic chemicals such as the anticancer drug cisplatin (Sancar, 1996). In mammalian cells, the proteins necessary for the incision reaction include XPC-HR23b, TFIIH, XPA, RPA and the nucleases XPG and ERCC1-XPF (Araujo *et al*, 2000). The removal of lesions requires their recognition by the repair factor XPC-HR23b and the subsequent opening of the DNA duplex by TFIIH. The single-stranded structure is then stabilized by XPA and RPA, and the margins of the resulting DNA bubble are recognized by XPG and ERCC1-XPF, thereby generating 3′ and 5′ incisions relative to the damage, respectively (O'Donnovan *et al*, 1994; Sijbers *et al*, 1996).

As XPB and XPD helicases are both integral parts of TFIIH, their individual molecular roles in NER remain difficult to delineate. As XPB and XPD are helicases with opposite polarities, it was originally suggested that they could cooperate to open DNA on the 5′ and 3′ sides of a lesion, respectively (Schaeffer *et al*, 1994). Indeed, mutation of the ATPase activity of either XPB or XPD results in the inability to remove DNA lesions (Sung *et al*, 1988; Guzder *et al*, 1994). Refining these proposals, recent data bring into question the direct role of the helicase activity of XPB in NER and transcription, and suggest that only the ATPase activity is required (Lin *et al*, 2005; Coin *et al*, 2007; Richards *et al*, 2008). Supporting the prime role of the ATPase activity of XPB in TFIIH functions, we recently showed that this activity was regulated by the p52 subunit of TFIIH (Coin *et al*, 2007) and by the damage recognition factor XPC (Bernardes de Jesus *et al*, 2008). Contrary to XPB, the helicase activity of XPD, which is regulated by the p44 subunit of TFIIH (Coin *et al*, 1998), is required for efficient opening of the DNA around the damage, but is dispensable for transcription (Tirode *et al*, 1999; Coin *et al*, 2007).

To further our understanding of the mechanistic details of XPB and XPD function, we analysed the behaviour of ATPase-deficient TFIIH complexes *in vivo*. We found that a TFIIH complex deficient in the ATPase of XPB was not recruited to sites of DNA damage, whereas a complex deficient in the ATPase of XPD did. More surprisingly, we discovered that the recruitment of TFIIH to these sites does not require the helicase activity of XPB but depends on two motifs, a R-E-D residue loop and a positively charged flexible Thumb (ThM)

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motif that were identified in a homologue of XPB from the thermophilic organism *Archaeoglobus fulgidus* (Fan *et al*, 2006). We analysed the molecular details of R-E-D and ThM impact on XPB activities and found that they were required to stimulate ATP hydrolysis in the presence of DNA. We propose a mechanism in which XPB functions as an ATP-dependent hook that uses the ATPase, R-E-D and ThM motifs to anchor TFIIH to the sites of DNA damage during DNA repair.

Results

The ATPase activity of XPB anchors TFIIH to the sites of DNA damage in vivo

To functionally discriminate between the ATPase activities of XPB and XPD, we produced recombinant TFIIH/XPD(K48R) and TFIIH/XPB(K346R) in baculovirus-infected cells (Tirode *et al*, 1999) and tested them in DNA repair and transcription assays. These complexes are mutated in the ATPase Walker A motif of XPD and XPB, respectively. When incubated in the presence of recombinant TBP, TFIIA, TFIIB, TFIIE and TFIIF transcription factors in addition to purified RNA polymerase II and a linearized DNA template containing the adenovirus major late promoter (Tirode *et al*, 1999), TFIIH/XPD(K48R) supported transcription, contrary to TFIIH/XPB(K346R), which was totally inactive (Figure 1A, upper panel). To test the repair capacity of the different TFIIH complexes, we used a reconstituted dual incision assay composed of the recombinant XPC-HR23b, XPA, RPA, XPG, ERCC1-XPF factors and a closed-circular plasmid (Pt-DNA) containing a single 1,3-intra-strand d(GpTpG) cisplatin-DNA crosslink as a template (Araujo *et al*, 2000). None of the mutated complexes was able to excise the damaged oligonucleotide (Figure 1A, lower panel). In a permanganate footprinting assay that measures the opening of the DNA around the lesion (Tapias *et al*, 2004), addition of TFIIH(WT) induced an increased sensitivity of nucleotides at positions T + 5, T + 6, T–4, T–5, and, to a lesser extent, T–7 and T–10 (Figure 1B, lane 2), indicative of DNA opening. In contrast, neither TFIIH/XPB(K346R) nor TFIIH/XPD(K48R) were able to open damaged DNA (compare lanes 3–4 with lane 2).

To analyse the behaviour of ATP-deficient TFIIH complexes *in vivo*, we used a stably transfected Chinese hamster ovary (CHO)-UV5 cell line expressing an HA-tagged version of the human XPD(K48R) protein (Winkler *et al*, 2000). Using the CHO-27-1 cells mutated in the hamster homologue of XPB (Ma *et al*, 1994), we also generated a stably transfected cell line expressing a C-terminally GFP-tagged version of the human XPB WT or K346R protein. The functionality of an XPB–GFP fusion construct was established earlier (Hoogstraten *et al*, 2002). We used immunofluorescent labelling after local UV irradiation of stably transfected cells (Volker *et al*, 2001) to assess the nuclear distribution pattern of XPB and XPD. Immunostaining with antibodies against cyclobutane pyrimidine dimers (CPDs) showed that UV damages were located in discrete local spots in the nucleus (Figure 1C, panels a, e, i, m). Both, human wild-type XPB and XPD proteins colocalized with CPD spots, indicating that TFIIH was efficiently recruited to the damaged sites in these cells (panels a–d and i–l). Surprisingly, although signals of XPD(K48R) colocalized with CPD spots in CHO-UV5 cells (panels m–p), signals of XPB(K346R) showed an homogenous distribution pattern through the nucleus (panels e–h), indicating that TFIIH/XPB(K346R) complex was not recruited to the damaged sites (Figure 1D). These data suggest that the accumulation of TFIIH to sites of DNA damage takes place in the absence of an active XPD protein but requires functional XPB.

New motifs in XPB required for the activity of TFIIH in NER

By introducing mutations in some of the seven canonical helicase motifs of XPB, we demonstrated recently that its helicase activity was not required for TFIIH repair function (Coin *et al*, 2007). Recently, three additional motifs were identified in a homologue of XPB from the thermophilic organism *Archaeoglobus fulgidus* (Fan *et al*, 2006). To determine whether these newly identified motifs have a function in the activities of the human TFIIH complex in transcription and repair, we designed four mutants (E253A, E253A/R283A, E473A and Δ516–526) depicted in Figure 2. We introduced an E253A substitution located at the end of the first β-strand that was combined, when indicated, with an R283A mutation located at the beginning of the α-helix of a putative damage recognition domain (DRD). We also designed an E473A substitution in the R-E-D residue loop to change the local negative charge of the motif, and we deleted the positively charged ThM domain from amino acid 516 to 526 (Δ516–526) (Figure 2).

To investigate the importance of the DRD, R-E-D and ThM motifs of XPB in the repair function of TFIIH, we first performed a host-cell reactivation assay (Carreau *et al*, 1995). A UV damaged reporter construct, carrying a luciferase gene (pLuc) was transiently transfected into CHO-27-1 cells, together with vectors coding for β-galactosidase and for human XPB proteins. Transfection of either XPB(E253A) or XPB(E253A/R283A) restored luciferase expression that reached the level observed with XPB(WT) (Figure 3A, lanes 1–5). In marked contrast, XPB(E473A) and XPB(Δ516–526) were not able to restore luciferase expression (lanes 7–8), a defect also observed with XPB(K346R) (lane 6). The various XPB were expressed at a similar level with the exception of XPB(Δ516–526) whose expression was slightly reduced compared with the wild type (Figure 3A).

Next, we carried out a UV-survival assay and for that purpose we established CHO-27-1 cells stably expressing the new XPB–GFP mutant proteins. Immunoprecipitations using a rabbit polyclonal antibody, recognizing the hamster homologue of the core TFIIH subunit p62, demonstrated that the various XPB were efficiently incorporated into the hamster TFIIH complex (Figure 3B). The stably transfected CHO-27-1 cells were UV irradiated at different doses (3, 6 and 9 J/m^2) and their survival was measured. Expression of XPB(WT), XPB(E253A) and XPB(E253A/R283A) induced a substantial rescue of the UV survival of the CHO-27-1 cells compared with nontransfected control (Figure 3C). On the other hand, the UV-survival curve of XPB(E473A) and XPB(Δ516–526) transfected cells fell into the range of both the nontransfected parental CHO-27-1 cells and those transfected with the NER-deficient XPB(K346R) control. These data indicate that the R-E-D and ThM domains of XPB are crucial for the repair activity of TFIIH, while the putative DRD is dispensable.

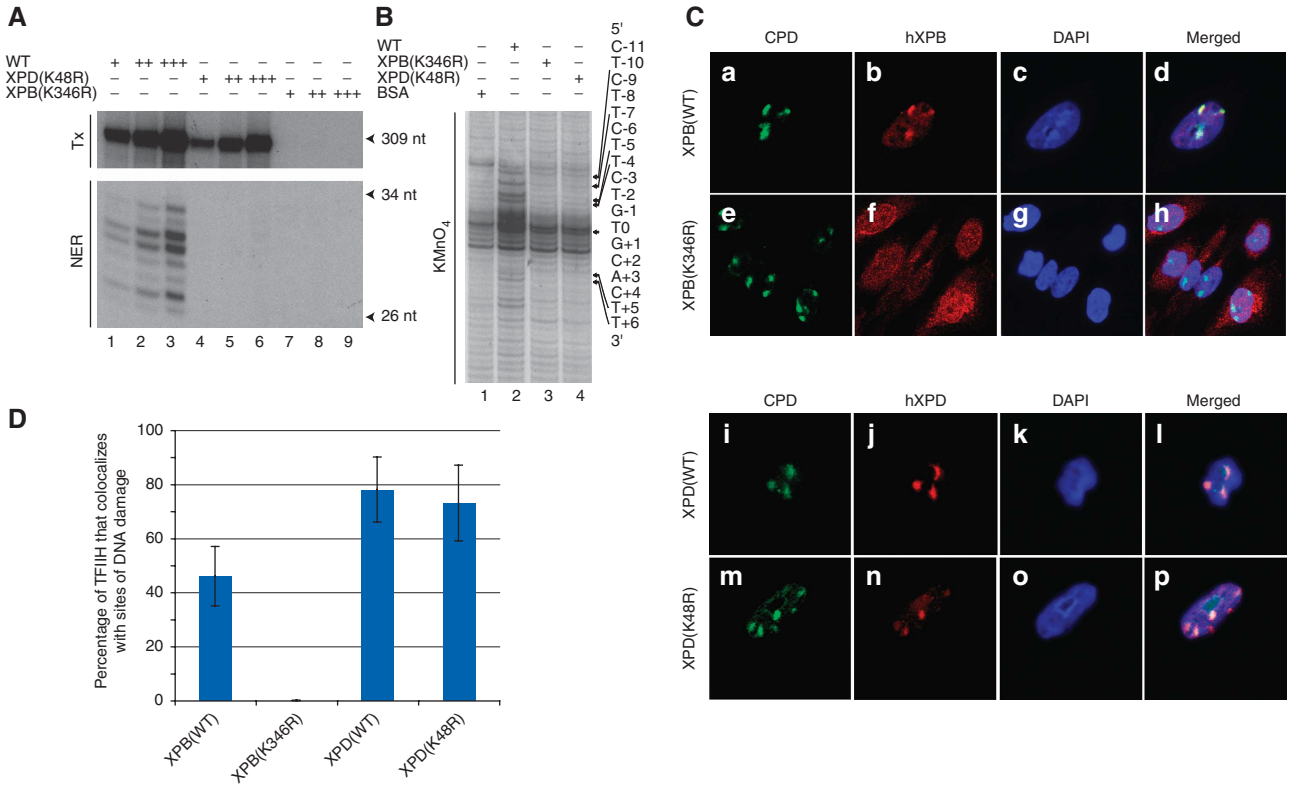

Figure 1 The ATPase activity of XPB is required to anchor TFIIH to damaged chromatin. (**A**) A measure of 25, 50 and 100 ng of TFIIH(WT), TFIIH/XPD(K48R) or TFIIH/XPB(K346R) was tested either in a reconstituted transcription assay (Tx, upper panel) or in a dual incision assay (NER, lower panel) as described (Coin *et al*, 2004). The sizes of the incision and transcription products are indicated. (**B**) TFIIH(WT), TFIIH/XPD(K48R) or TFIIH/XPB(K346R) (100 ng) were incubated with a radio-labelled linear DNA fragment from the Pt-DNA plasmid and 40 ng of XPC-HR23b, 25 ng of XPA, 50 ng of RPA and 150 ng of XPG in a $KMnO_4$ footprinting assay. Lane 1; Pt-DNA with BSA only. Residues are numbered with the central thymine of the crosslinked GTG sequence designated T0. Arrows indicate $KMnO_4$ sensitive sites. Adducted strand residues to the 3′ and 5′ of T0 are denoted by positive and negative integers (+N, –N), respectively. (**C**) Stably transfected CHO-27-1 expressing a GFP-tagged version of the human WT or K346R XPB proteins (upper panel) and stably transfected CHO-UV5 cells expressing an HA-tagged version of the human WT or K48R XPD proteins (bottom panel) (Winkler *et al*, 2000) were UV irradiated at 100 J/m^2 through the 3 μm pore filter and fixed 30 min later. Immunofluorescent labelling was performed using a rabbit polyclonal anti-GFP (panels b and f), a rat monoclonal anti-HA (panels j and n) or a mouse monoclonal anti-CPD (panels a, e, i, m). Nuclei were counterstained with DAPI (panels c, g, k, o), and slides were merged (panels d, h, l, p). (**D**) Quantitative analysis of the recruitment of TFIIH to sites of DNA damage in transfected cells. Values represent averages ± s.d. ($n = 100$ sites of DNA damage) from three independent experiments.

R-E-D and ThM motifs are needed for an optimal ATPase activity of XPB

To decipher the molecular details of the repair defect generated by the E473A and Δ516–526 mutations, we produced recombinant TFIIH complexes in baculovirus-infected cells containing the corresponding mutated XPB subunits (Tirode *et al*, 1999). Western blot analysis of the recombinant TFIIH/XPB(E253A), XPB(E253A/R283A), XPB(E473A) and XPB(Δ516–526) complexes revealed a similar subunit composition compared with the TFIIH(WT) complex (data not shown). When tested in either a dual incision or in a transcription assays, TFIIH/XPB(E253A) and XPB(E253A/R283A) were as active as TFIIH(WT) in excising damaged DNA (Figure 4A, upper panel, compare lanes 5–8 with lanes 3–4) or synthesizing RNA (lower panel). In contrast, TFIIH/XPB(E473A) and XPB(Δ516–526) were inactive in repairing damaged DNA and in synthesizing RNA (Figure 4A, compare lanes 11–12 and 13–14 with lanes 3–4), similarly to TFIIH/XPB(K346R) (lanes 9–10).

In a permanganate footprint assay, TFIIH/XPB(E473A) and XPB(Δ516–526) were unable to open the damaged DNA (Figure 4B, lanes 5–6), compared with TFIIH(WT) or XPB(E253A/R283A) (lanes 2–3). Altogether, the above data drew our attention to the critical role of both the R-E-D and ThM motifs of XPB in damaged DNA opening.

R-E-D and ThM motifs are needed for the anchoring of TFIIH to the sites of DNA damage

We next measured the recruitment of the TFIIH complexes carrying mutations in the newly identified motifs to sites of DNA damage *in vivo*. XPB(E253A/R283A) colocalized with CPD spots (Figure 5, panels e–h), indicating that the corresponding TFIIH complexes translocated to the sites of DNA photolesions. XPB(E473A) and XPB(Δ516–526), however, displayed a homogeneous distribution pattern through the nucleus (panels i–l and m–p), which parallels the pattern observed in Figure 1C with XPB(K346R). This homogenous distribution contrasted with the local accumulation to the damaged sites of a TFIIH/XPB(T469A) complex (panels q–t) containing a mutation that has been shown to impede the helicase activity of XPB but not the NER function of TFIIH (Coin *et al*, 2007). We conclude from these data that the R-E-D and ThM motifs are required, together with the Walker A

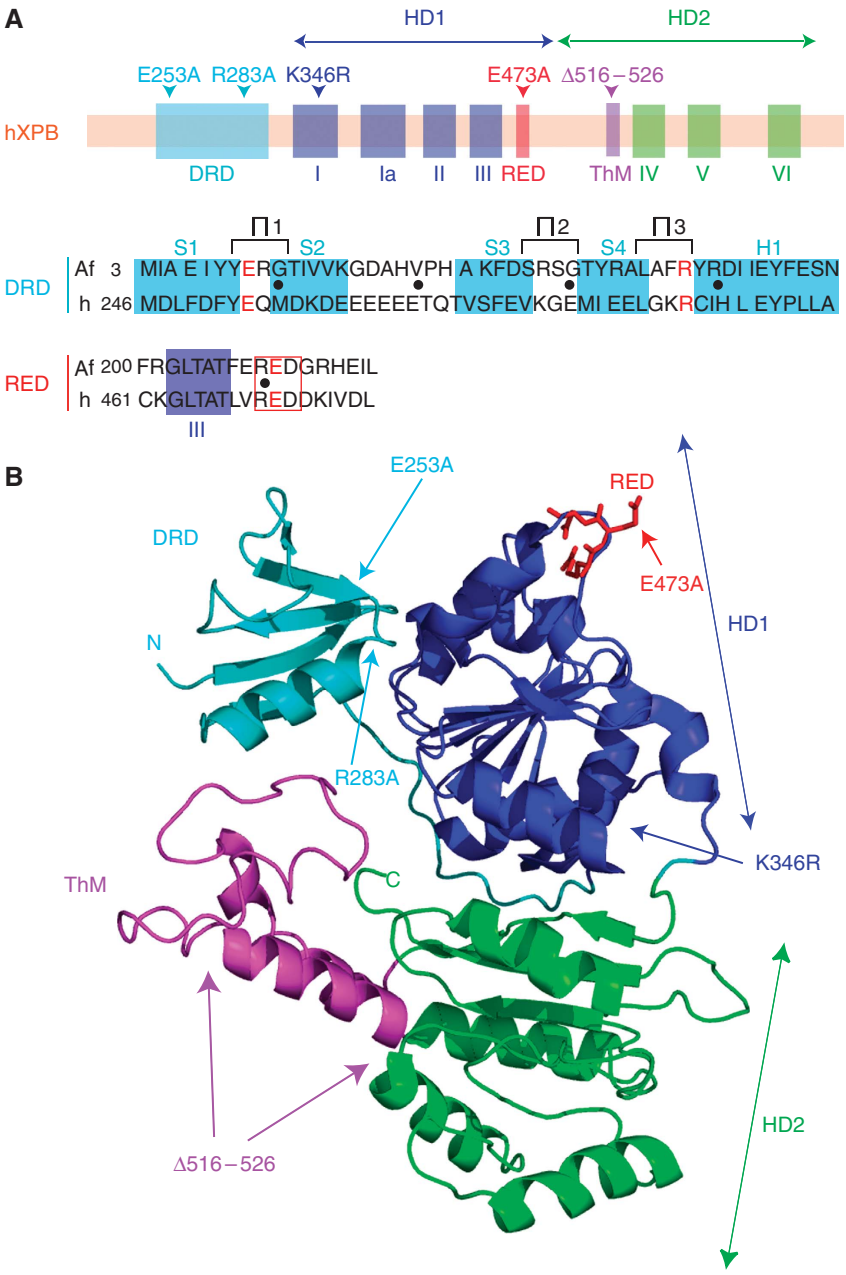

Figure 2 XPB conserved sequences, motifs and structural architecture. (**A**) Upper panel shows the location of the human XPB structural domains: the four classical helicase motifs (I, Ia, II and III) of the first helicase module (HD1) are indicated in blue. The three helicase motifs (IV, V and VI) of the second helicase module (HD2) are indicated in green. The putative damage recognition (DRD), R-E-D and Thumb (ThM) domains identified in an homologue of XPB from the thermophilic organism *Archaeoglobus fulgidus* (Fan *et al*, 2006) are indicated, respectively, in light blue, red and purple. The mutations E253A, R283A, K346R, E473A and Δ516–526 are annotated. Lower panel shows the sequence conservation of the DRD and R-E-D motifs between human (h) and *Archaeoglobus fulgidus* (Af) XPB proteins. The β-strands (S1–4) and the α-helix (H1) are indicated. The conserved helicase motif III indicated by a blue square is located close to the R-E-D motif indicated by a red opened square. The three hairpin loops potentially involved in DNA binding are indicated (∏1, ∏2, ∏3). Residues mutated in this study are marked in red. (**B**) View of the ribbon representation of AfXPB. The HD1 is indicated in blue, HD2 in green. The putative DRD, R-E-D and ThM domains are indicated in light blue, red and purple, respectively. The positions of the new mutations are indicated.

motif, for the recruitment of the TFIIH complex to sites of DNA damage.

R-E-D and ThM motifs stimulate the DNA-dependent TFIIH ATPase activity

Data above show common biochemical and biological defects for TFIIH complexes mutated either in the ATPase, R-E-D or ThM motifs of XPB and suggest a link between the newly identified motifs of XPB and its ATPase activity. To assess the contributions of these motifs on the hydrolysis of ATP by XPB, we performed ATPase assay. Our data show that the TFIIH/XPB(E473A) and TFIIH/XPB(Δ516–526) displayed about 40% of residual ATPase activity compared with TFIIH(WT) (Figure 6A, compare lanes 1–3 with 10–12 and 13–15). TFIIH/XPB(E253A) or TFIIH/XPB(E253A/R283A) exhibited the same activity as TFIIH(WT) (lanes 1–6 and data

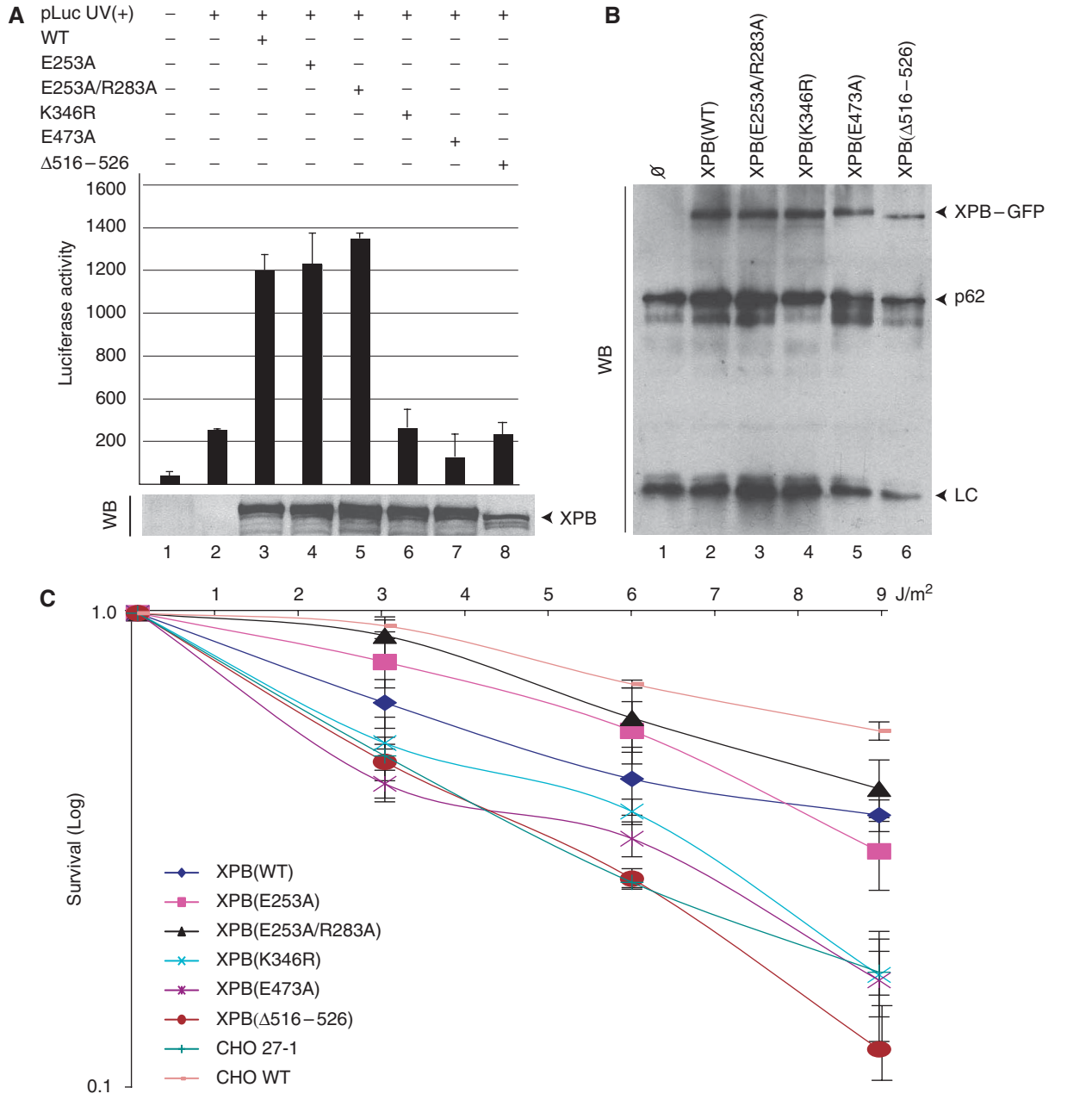

Figure 3 Analysis of the function of the new motifs in NER *in vivo*. (**A**) CHO-27-1 cells were transfected with UV-irradiated pLuc plasmid expressing the luciferase gene (lanes 2–8) in combination with vector expressing either XPB(WT) (lane 3), XPB(E253A) (lane 4), XPB(E253A/R283A) (lane 5), XPB(K346R) (lane 6), XPB(E473A) (lane 7) or XPB(Δ516–526) (lane 8). The luciferase activity in cell lysates (48 h post-transfection), normalized with the internal β-galactosidase standard, assesses repair complementation. Results are expressed as relative luciferase activity. Values represent averages ± s.d. from three independent experiments. A measure of 50 μg of total extract were resolved by SDS–PAGE and western blotted (WB) with a mouse anti-human XPB antibody (Coin *et al*, 2004). Note that XPB(Δ516–526) migrates slightly lower than the others because of the deletion. (**B**) TFIIH from 100 μg of extracts prepared from untransfected CHO-27-1 (lane 1) or CHO-27-1 stably expressing XPB (lanes 2–6) was immunoprecipitated with a polyclonal antibody against the p62 subunit of TFIIH and resolved by SDS–PAGE, followed by Western blotting with a mouse anti-human XPB and a mouse anti-p62 antibody. LC; light chain of the antibody. (**C**) Quantitative UV-survival analysis of transfected CHO-27-1 cell lines. Results are expressed as a ratio between the cells that survived after irradiation and the cells that survived without treatment. Values represent averages ± s.d. from three independent experiments.

not shown). TFIIH/XPB(K346R) showed only 20% residual ATPase activity (corresponding to that of XPD (Coin *et al*, 2006)), compared with TFIIH(WT) (compare lanes 1–3 with 7–9).

XPB and XPD are DNA-dependent ATPases (Roy *et al*, 1994). The ATPase activity of TFIIH(WT), low in the absence of DNA, is stimulated by double-stranded DNA (Figure 6B, compare lanes 1–2 with 7–8). In contrast, TFIIH/XPB(E473A) showed almost no DNA-induced ATPase stimulation (Figure 6B, compare lanes 5–6 with 11–12). More importantly, in the absence of DNA, TFIIH/XPB(WT) and XPB(E473A) displayed similar specific ATPase activities (Figure 6B, compare lanes 1–2 and 5–6) that were slightly higher than the TFIIH/XPB(K346R) activity (compare lanes 1–2, 5–6 and 3–4). Similar observations were obtained with TFIIH/XPB(Δ516–526) (Supplementary data 1). Altogether,

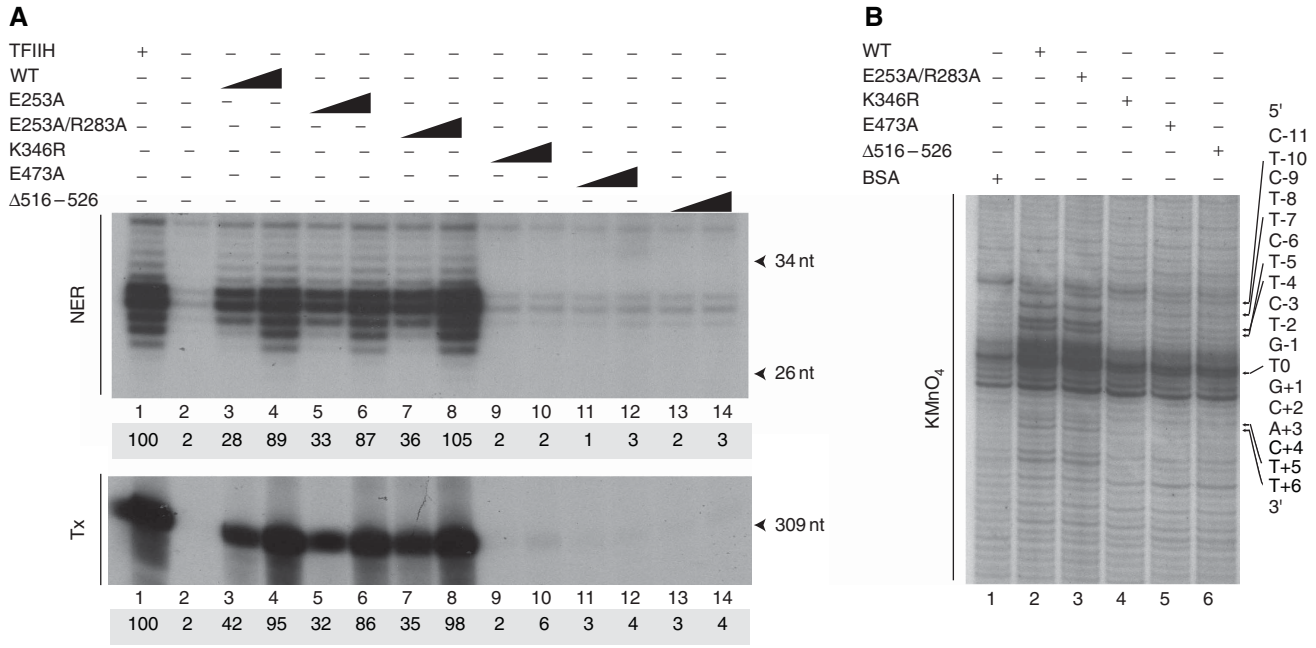

Figure 4 Mutations in R-E-D and ThM motifs impair the ATPase activity of XPB. (**A**) A measure of 25 and 75 ng of TFIIH(WT), TFIIH/XPB(E253A), XPB(E253A/R283A), XPB(K346R), XPB(E473A) or XPB(Δ516–526) was tested in a dual incision assay (NER, upper panel) or in a reconstituted transcription assay (Tx, lower panel) as described (Coin *et al*, 2004). Lane 1 contains highly purified Hela TFIIH (Giglia-Mari *et al*, 2004). Lane 2 contains all the factors except TFIIH. The sizes of the incision or transcription products are indicated. The transcription and repair signals were quantified using Genetool (Syngene). (**B**) A measure of 100 ng of the various TFIIH complexes were tested in a $KMnO_4$ footprint assay (see Figure 1B). Lane 1; Pt-DNA with BSA only. Residues are numbered with the central thymine of the crosslinked GTG sequence designated T0. Arrows indicate $KMnO_4$ sensitive sites. Adducted strand residues to the 3′ and 5′ of T0 are denoted by positive and negative integers (+N, –N).

these data indicate that the R-E-D and ThM motifs do not affect the basal intrinsic ATPase activity of XPB but are required for the stimulation of this activity by DNA.

Discussion

To efficiently protect the genome, cells need to detect all types of DNA structural alterations embedded in billions of normal base pairs. The identification of the various proteins that execute NER was done through extensive studies of human cells deficient in this repair pathway (Maillard *et al*, 2007). Both *in vivo* and *in vitro* experiments identified XPC as the first factor that binds the damaged DNA (Sugasawa *et al*, 1998; Volker *et al*, 2001; Riedl *et al*, 2003). TFIIH is recruited to the lesion immediately after XPC (Yokoi *et al*, 2000; Riedl *et al*, 2003), presumably through direct protein–protein interaction (Bernardes de Jesus *et al*, 2008). The role of TFIIH is devoted to the opening of the DNA around the damaged site, but the individual function of its helicase subunits in this step remains difficult to delineate.

Earlier studies from our laboratory have shown that mutations in the helicase motifs III (T469A) or VI (Q638A), which impaired the helicase activity of the XPB subunit, did not inhibit the NER activity of TFIIH (Coin *et al*, 2007), thus raising the question of the role of XPB in NER. Here, we showed that TFIIH containing mutation in the motif III of XPB is recruited to the DNA repair sites after UV irradiation. However, a mutation in the helicase motif Ia, which abolishes the ATPase activity of XPB, thwarts the accumulation of TFIIH to these sites. This implies that the recruitment of TFIIH to sites of damage is an active process that requires ATP hydrolysis. In contrast, the ATPase activity of XPD, the second helicase of TFIIH, is not required to recruit TFIIH to the damage sites, although it is needed for DNA repair.

In addition to the aforementioned ATPase motif, we found that two additional motifs, the R-E-D and ThM motifs, are implicated in the recruitment of TFIIH to sites of DNA damage. These two domains, highly conserved in human XPB, were identified in an homologue of XPB from the thermophilic organism *Archaeoglobus fulgidus* and were suggested to be involved in TFIIH functions (Fan *et al*, 2006). Mutations in the R-E-D and ThM motifs mimicked the biochemical and biological defects obtained with a mutation in the ATPase motif. This suggests that the ATPase, R-E-D and ThM motifs work together to ensure a correct recruitment of TFIIH to the damaged sites before the opening and dual incision steps take place during NER. How the R-E-D and ThM motifs participate to the anchoring of TFIIH? The ThM domain has not been found in other helicases, including XPD (Bienstock *et al*, 2002; Fan *et al*, 2008; Liu *et al*, 2008; Wolski *et al*, 2008), but a similar helical protrusion has been observed in DNA polymerases (Doublie *et al*, 1998) and in *Sulfolobus solfataricus* SWI2/SNF2 ATPase Rad54 (Durr *et al*, 2005), in which it is expected to grip double-stranded DNA from the minor groove. The structure of XPB suggests that the energy furnished by the ATP hydrolysis is used to induce a flip of 170° of the HD2 domain after the binding of XPB to DNA (Fan *et al*, 2006) (Figure 7). The R-E-D (present in HD1) and the ThM (present in HD2) are then in close vicinity and are used to stabilize TFIIH on the DNA by introducing a wedge (the E473 residue) in the double-stranded DNA, gripped by the ThM motif. To obtain experimental evidence

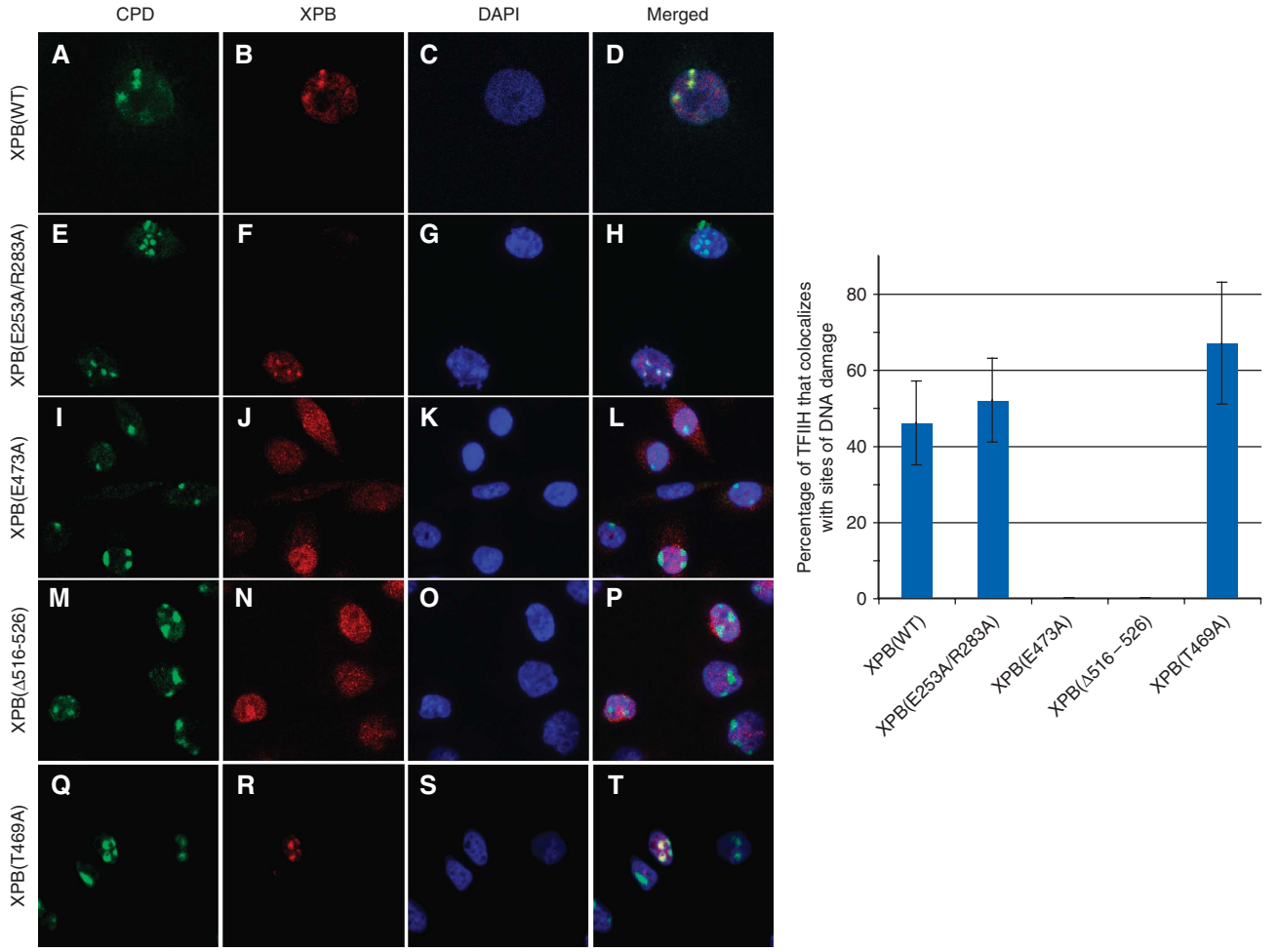

Figure 5 Recruitment of TFIIH to local sites of DNA damage. (Left panel) CHO-27-1 cells were stably transfected with pEGFP plasmids expressing various forms of GFP-tagged XPB proteins. These cells were irradiated with UV light (100 J/m^2) through the 3-μm pore filter and fixed 30 min later. Immunofluorescent labelling was performed using either a mouse monoclonal anti-CPD (panels **A, E, I, M, Q**) or a rabbit polyclonal anti-GFP (panels **B, F, J, N, R**). Nuclei were counterstained with DAPI (panels **C, G, K, O, S**), and slides were merged (panels **D, H, L, P, T**). (Right panel) Quantitative analysis of the recruitment of TFIIH to sites of DNA damage in transfected cells. Values represent averages ± s.d. ($n = 100$ sites of DNA damage) from three independent experiments.

for this model, we compared the ATPase activities of the WT and mutated complexes with or without DNA. Indeed, like most SF1 and SF2 members, DNA stimulates the ATPase activity of TFIIH (Roy *et al*, 1994). In the presence of DNA, mutations in the R-E-D and ThM motifs induces 60% inhibition of the ATPase activity compare with TFIIH(WT). In the absence of DNA, the three ATPase activities are strictly identical and are slightly higher than the ATPase activity of the TFIIH complex mutated in the ATP-binding site of XPB. These data further support the model of the conformation change proposed above, as it demonstrates that R-E-D and ThM are used to stabilize the binding of XPB to DNA. Furthermore, the fact that these mutations inhibit both, TFIIH transcription and repair activities, suggests a common mode of recruitment of TFIIH to the promoters and to the damage sites.

The recruitment of TFIIH through the action of the ATPase activity of XPB may also induce a reorganization of the protein–DNA complexes in transcription and repair that will allow new protein–protein or protein–DNA contacts. Indeed, using photocrosslink experiments, we have shown that addition of ATP in NER induced a re-positioning of XPC on the damaged DNA, which dependent on TFIIH (Tapias *et al*, 2004). After the recruitment of TFIIH to the damaged DNA through the energy furnished by the ATPase activity of XPB, the DNA would be opened by XPD, which has a processive and robust helicase activity stimulated by the p44 subunit of the core TFIIH (Coin *et al*, 1998). Here, a mutation in the ATPase activity of XPD still allowed TFIIH to bind the damaged sites *in vivo* but was unable to open the DNA around the lesion. Altogether, our data brings a new conceptual view of the roles of XPB and XPD in NER by revealing their different molecular functions within this genome caretaking event.

Materials and methods

Cell lines

CHO-27-1 is a CHO mutant cell line belonging to the third rodent complementation group (the hamster ERCC3 gene is the homologue of the human XPB gene) (Hall *et al*, 2005). CHO-UV5 belongs to the second rodent complementation group (the hamster ERCC2 is the homologue of the human XPD gene) (Winkler *et al*, 2000).

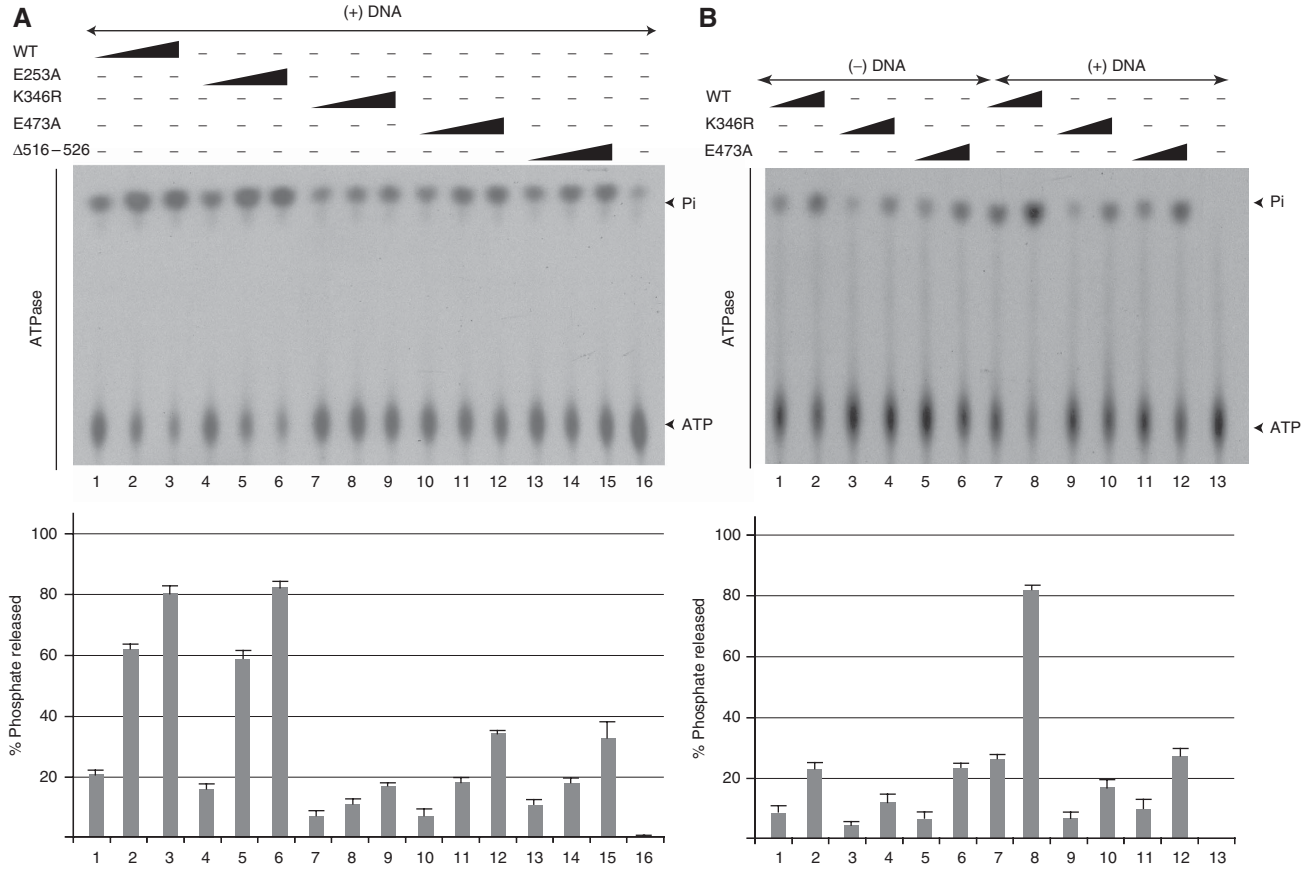

Figure 6 Mutations in the R-E-D motif impair DNA-dependant TFIIH ATPase activity. (**A**) 50, 100, and 150 ng of TFIIH(WT), TFIIH/XPB(E253A), XPB(K346R), XPB(E473A) or XPB(Δ516–526) were tested in an ATPase assay in the presence of 200 ng of double-strand circular DNA (Coin *et al*, 2007). The average percentage ± s.d. of phosphate released (Pi/(ATP + Pi)) from three independent experiments is represented in the graph. (**B**) 50 and 150 ng of TFIIH(WT), TFIIH/XPB(K346R) or TFIIH/XPB(E473A) were tested in an ATPase assay without (lanes 1–6) or with (lanes 7–12) 200 ng of double-strand circular DNA. The average percentage ± s.d. of phosphate released (Pi/(ATP + Pi)) from three independent experiments is represented in the graph.

Construction of the plasmids

Baculovirus allowing the expression of mutated XPB were constructed in the FLAG tag pSK278 vector (BD Biosciences). XPB was inserted at the BamHI/EcoRI site, in fusion with the FLAG tag at its 5′ side. The mutants were obtained by site-directed mutagenesis (Quickchange, Stratagene). The resulting vectors were recombined with baculovirus DNA (BaculoGold DNA, PharMingen) in *Spodoptera frugiperda* 9 (Sf9) cells. *In vivo* experiments were carried out with the pEGFP-N1 plasmid (Clontech) containing the XPB cDNA inserted in frame with the green fluorescent protein tag (Hoogstraten *et al*, 2002).

Stable cell lines

CHO-27-1 cells (10^6) were transfected with 2 μg of pEGFP-N1/XPB plasmid in 10 cm Petri dishes using lipofectamine (Invitrogen). Forty hours after transfection, the fluorescent cells were sorted on the FACS DIVa (BD; Becton, Dickinson and Company). The cells with the highest level of fluorescence (about 5% of total cells) were maintained in the selective medium with G418 (Geniticin, 800 μg/ml), expanded and analysed for XPB expression.

Damaged DNA substrates

Covalently closed circular Pt-DNA containing a single 1,3-intrastrand d(GpTpG) cisplatin–DNA crosslink was prepared as described (Frit *et al*, 2002).

Dual incision assay

Dual incision assay was carried out in 25 μl of Repair buffer (45 mM Hepes-KOH (pH 7.8), 5 mM MgCl2, 1 mM DTT, 0.3 mM EDTA, 10% glycerol, 2.5 μg BSA, 50 mM KCl) supplemented with 2 mM ATP. Each reaction contained 5 ng of XPG, 15 ng of XPF/ERCC1, 10 ng of XPC-HR23b, 50 ng of RPA and 25 ng of XPA. After pre-incubation 10 min at 30°C, 30 ng of Pt-DNA was added and reaction was continued for 90 min at 30°C. The excised fragment was detected on a 14% urea-acrylamide after annealing with 9 ng of the complementary oligonucleotide and addition of four radiolabelled dCMPα-P^{32} (3000 μCi/mmol) residues by Sequenase V2.1 (USB).

$KMnO_4$ footprint assay

The damaged strand probe was obtained on *Age*1/*Ase*1 digestion of the Pt-DNA and radiolabelling at the 3′end in a Klenow reaction, the Pt adduct is located at 156 bp from the labelled end. The resulting fragment was purified by the 'crush and soak' method after migration in a 5% nondenaturating PAGE. Reactions (75 μl) were carried out in 20 μl of Repair buffer (+2 mM ATP) containing the labelled cisplatinated probe (40 fmol) and 40 ng of XPC-HR23b, 25 ng of XPA, 50 ng of RPA and 150 ng of XPG. After incubation at 30 °C for 15 min, 3 μl of 120 mM $KMnO_4$ was added, and oxidation was allowed to proceed for 3 min at room temperature before reduction by adding 6 μl of 14.6 M β-mercaptoethanol for 5 min on ice. After organic extraction and ethanol precipitation, dried pellets were resuspended in 100 μl of a solution containing 1 M piperidine, 1 mM EDTA and 1 mM EGTA and incubated at 90°C for 25 min. Samples were next ethanol precipitated, and final pellets were recovered in 10 μl of loading buffer and analysed in 8% urea PAGE.

ATPase assay

Protein fractions were incubated for 2 h at 30°C in the presence of 1 μCi [γ-32P]ATP (7000 Ci/mmol, ICN Pharmaceuticals) in a 20 μl reaction volume in 20 mM Tris–HCl pH 7.9, 4 mM $MgCl_2$, 1 mM DTT, 50 μg/ml BSA and when indicated 200 ng of double-strand DNA (pcDNA3 +). Reactions were stopped by addition of EDTA

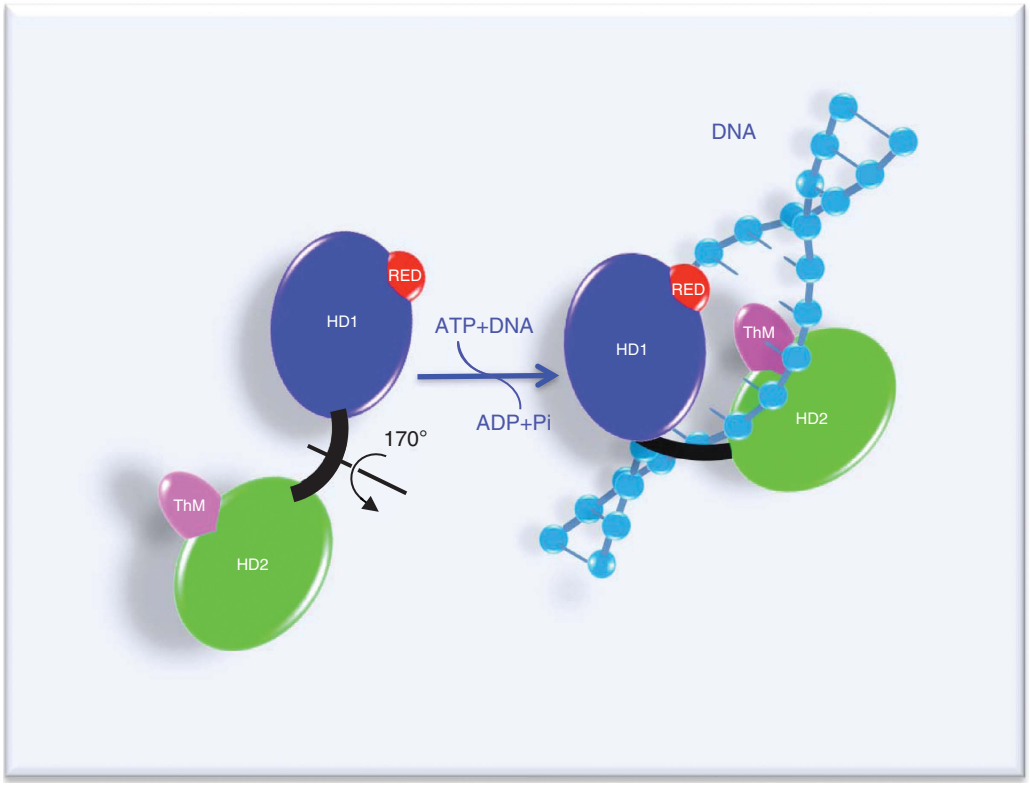

Figure 7 Proposed structure-based mechanism for binding of XPB to DNA. In this model, adapted from Fan *et al* (2006), XPB is in an opened conformation in the absence of DNA. When XPB binds to DNA, the rotation (170°) of the second helicase domain (HD2) together with the ThM domain, facilitated by HD1-mediated ATP hydrolysis, forms the closed and stable XPB–DNA complex.

(50 mM) and SDS (1% (w/w)). The reactions were then diluted five-fold, spotted onto polyethylenimine (PEI) TLC plates (Merck), run in 0.5 M LiCl/1 M formic acid and autoradiographed.

Local UV irradiation and immunofluorescence

The cells were rinsed with PBS and covered with an isopore polycarbonate filter with pores of 3 μm diameter (Millipore, Badford, MA). Cells were then exposed to UV irradiation with a Philips TUV lamp (predominantly 254 nm) at a dose of 100 J/m^2 (Volker *et al*, 2001). Subsequently, the filter was removed, the medium was added back to the cells, and they were returned to culture conditions for 30 min. Then, cells were fixed in 2% formaldehyde for 15 min at room temperature and permeabilized with PBS/0.5% Triton X-100 for 5 min. After washing with PBS-Tween (0.05%), the slides were incubated for 1 h with the indicated antibodies. After extensive washing with PBS-Tween, they were incubated for 1 h with Cy3-conjugated donkey anti-rabbit IgG, goat anti-mouse Alexa 488 IgG or goat anti-rat Alexa 488 IgG (Jackson Laboratories) diluted 1:400 in PBS-Tween/0.5% Foetal Calf Serum. The slides were counterstained for DNA with DAPI prepared in Vectashield mounting medium (Vector lab). All images were collected using a Leica Confocal TCS 4D microscope equipped with both UV laser and an Argon/Kripton laser, and standard filters to allow collection of the data at 488 and 568 nm. The software TCSTK was used for three-colour reconstructions, and figures were generated using the PLCHTK software.

Host-cell reactivation assay

The pGL3 vector expressing *Photinus pyralis* (firefly) luciferase was purchased from Promega and the pCH110 vector expressing the β-galactosidase from Invitrogen. The pGL3 vector was UV irradiated (254 nm, 1000 J/m^2) at a concentration of 1 mg/ml in 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA. CHO-27-1 cells were transfected in a six-well plate at a confluence of 95% using Lipofectamine Plus (Invitrogen). Each transfection mixture contained 500 ng of pGL3 (UV+/−), 100 ng of pCH110 (nonirradiated) and 10 ng of the various pcDNAXPB plasmids. After 4 h of incubation, the transfection reagents were replaced by medium. Cells were lysed after 24 h to measure luciferase activity on a microtiter plate luminometer (Dynex). All results (mean values of at least five measurements) were normalized by calculating the ratios between luciferase and galactosidase activities.

UV-survival assay

Cells (10^3) were plated per 6 cm petri dishes, cultured overnight and UV irradiated at 254 nm at various doses (0.5 J/m^2/s). After 14 days, the cells are stained by trypan blue and counted.

Antibodies

Mouse monoclonal antibodies towards TFIIH subunits were used as described (Coin *et al*, 2007). Primary antibodies (the final dilutions are indicated in parentheses) used in fluorescent labelling were purified rabbit anti-GFP (Torrey Pines Biolabs, Inc) (1:1000), rat monoclonal anti-HA 3F10 (Roche) (1:1000) and mouse IgG monoclonal anti-CPD (TDM2) (1:2000) (MBL international corp.).

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Araujo SJ, Tirode F, Coin F, Pospiech H, Syvaoja JE, Stucki M, Hubscher U, Egly JM, Wood RD (2000) Nucleotide excision repair of DNA with recombinant human proteins: definition of the minimal set of factors, active forms of TFIIH, and modulation by CAK. *Genes Dev* **14:** 349–359

Bernardes de Jesus BM, Bjoras M, Coin F, Egly JM (2008) Dissection of the molecular defects caused by pathogenic mutations in the DNA repair factor XPC. *Mol Cell Biol* **28:** 7225–7235

Bienstock RJ, Skorvaga M, Mandavilli BS, Van Houten B (2002) Structural and functional characterization of the human DNA repair helicase XPD by comparative molecular modeling and site-directed mutagenesis of the bacterial repair protein UvrB. *J Biol Chem* **27:** 27

Carreau M, Eveno E, Quilliet X, Chevalier-Lagente O, Benoit A, Tanganelli B, Stefanini M, Vermeulen W, Hoeijmakers JH, Sarasin A, Mezzina M (1995) Development of a new easy complementation assay for DNA repair deficient human syndromes using cloned repair genes. *Carcinogenesis* **16:** 1003–1009

Coin F, Auriol J, Tapias A, Clivio P, Vermeulen W, Egly JM (2004) Phosphorylation of XPB helicase regulates TFIIH nucleotide excision repair activity. *EMBO J* **23:** 4835–4846

Coin F, De Santis LP, Nardo T, Zlobinskaya O, Stefanini M, Egly JM (2006) p8/TTD-A as a Repair-Specific TFIIH Subunit. *Mol Cell* **21:** 215–226

Coin F, Marinoni JC, Rodolfo C, Fribourg S, Pedrini AM, Egly JM (1998) Mutations in the XPD helicase gene result in XP and TTD phenotypes, preventing interaction between XPD and the p44 subunit of TFIIH. *Nat Genet* **20:** 184–188

Coin F, Oksenych V, Egly JM (2007) Distinct roles for the XPB/p52 and XPD/p44 subcomplexes of TFIIH in damaged DNA opening during nucleotide excision repair. *Mol Cell* **26:** 245–256

Delagoutte E, von Hippel PH (2003) Helicase mechanisms and the coupling of helicases within macromolecular machines. Part II: Integration of helicases into cellular processes. *Q Rev Biophys* **36:** 1–69

Doublie S, Tabor S, Long AM, Richardson CC, Ellenberger T (1998) Crystal structure of a bacteriophage T7 DNA replication complex at 2.2 A resolution. *Nature* **391:** 251–258

Durr H, Korner C, Muller M, Hickmann V, Hopfner KP (2005) X-ray structures of the Sulfolobus solfataricus SWI2/SNF2 ATPase core and its complex with DNA. *Cell* **121:** 363–373

Ellis NA (1997) DNA helicases in inherited human disorders. *Curr Opin Genet Dev* **7:** 354–363

Fan L, Arvai AS, Cooper PK, Iwai S, Hanaoka F, Tainer JA (2006) Conserved XPB core structure and motifs for DNA unwinding: implications for pathway selection of transcription or excision repair. *Mol Cell* **22:** 27–37

Fan L, Fuss JO, Cheng QJ, Arvai AS, Hammel M, Roberts VA, Cooper PK, Tainer JA (2008) XPD helicase structures and activities: insights into the cancer and aging phenotypes from XPD mutations. *Cell* **133:** 789–800

Frit P, Kwon K, Coin F, Auriol J, Dubaele S, Salles B, Egly JM (2002) Transcriptional activators stimulate DNA repair. *Mol Cell* **10:** 1391–1401

Giglia-Mari G, Coin F, Ranish JA, Hoogstraten D, Theil A, Wijgers N, Jaspers NG, Raams A, Argentini M, van der Spek PJ, Botta E, Stefanini M, Egly JM, Aebersold R, Hoeijmakers JH, Vermeulen W (2004) A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A. *Nat Genet* **36:** 714–719

Guzder SN, Sung P, Bailly V, Prakash L, Prakash S (1994) RAD25 is a DNA helicase required for DNA repair and RNA polymerase II transcription. *Nature* **369:** 578–581

Hall H, Gursky J, Nicodemou A, Rybanska I, Kimlickova E, Pirsel M (2005) Characterization of ERCC3 mutations in the Chinese hamster ovary 27-1, UV24 and MMC-2 cell lines. *Mutat Res* **593:** 177–186

Hoogstraten D, Nigg AL, Heath H, Mullenders LH, van Driel R, Hoeijmakers JH, Vermeulen W, Houtsmuller AB (2002) Rapid switching of TFIIH between RNA polymerase I and II transcription and DNA repair *in vivo*. *Mol Cell* **10:** 1163–1174

Iben S, Tschochner H, Bier M, Hoogstraten D, Hozak P, Egly JM, Grummt I (2002) TFIIH plays an essential role in RNA polymerase I transcription. *Cell* **109:** 297–306

Lehmann AR (2003) DNA repair-deficient diseases, Xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. *Biochimie* **85:** 1101–1111

Lin YC, Choi WS, Gralla JD (2005) TFIIH XPB mutants suggest a unified bacterial-like mechanism for promoter opening but not escape. *Nat Struct Mol Biol* **12:** 603–607

Liu H, Rudolf J, Johnson KA, McMahon SA, Oke M, Carter L, McRobbie AM, Brown SE, Naismith JH, White MF (2008) Structure of the DNA repair helicase XPD. *Cell* **133:** 801–812

Ma L, Westbroek A, Jochemsen AG, Weeda G, Bosch A, Bootsma D, Hoeijmakers JH, van der Eb AJ (1994) Mutational analysis of ERCC3, which is involved in DNA repair and transcription initiation: identification of domains essential for the DNA repair function. *Mol Cell Biol* **14:** 4126–4134

Maillard O, Camenisch U, Clement FC, Blagoev KB, Naegeli H (2007) DNA repair triggered by sensors of helical dynamics. *Trends Biochem Sci* **32:** 494–499

O'Donnovan A, Davies AA, Moggs JG, West SC, Wood RD (1994) XPG endonuclease makes the 3′ incision in human DNA nucleotide excision repair. *Nature* **371:** 432–435

Ranish JA, Hahn S, Lu Y, Yi EC, Li XJ, Eng J, Aebersold R (2004) Identification of TFB5, a new component of general transcription and DNA repair factor IIH. *Nat Genet* **36:** 707–713

Richards JD, Cubeddu L, Roberts J, Liu H, White MF (2008) The archaeal XPB protein is a ssDNA-dependent ATPase with a novel partner. *J Mol Biol* **376:** 634–644

Riedl T, Hanaoka F, Egly JM (2003) The comings and goings of nucleotide excision repair factors on damaged DNA. *EMBO J* **22:** 5293–5303

Roy R, Schaeffer L, Humbert S, Vermeulen W, Weeda G, Egly JM (1994) The DNA-dependent ATPase activity associated with the class II basic transcription factor BTF2/TFIIH. *J Biol Chem* **269:** 9826–9832

Sancar A (1996) DNA excision repair. *Annu Rev Biochem* **65:** 43–81

Schaeffer L, Moncollin V, Roy R, Staub A, Mezzina M, Sarasin A, Weeda G, Hoeijmakers JHJ, Egly JM (1994) The ERCC2/DNA repair protein is associated with the class II BTF2/TFIIH transcription factor. *EMBO J* **13:** 2388–2392

Schaeffer L, Roy R, Humbert S, Moncollin V, Vermeulen W, Hoeijmakers JH, Chambon P, Egly JM (1993) DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor. *Science* **260:** 58–63

Sijbers AM, de Laat WL, Ariza RR, Biggerstaff M, Wei YF, Moggs JG, Carter KC, Shell BK, Evans E, de Jong MC, Rademakers S, de Rooij J, Jaspers NG, Hoeijmakers JH, Wood RD (1996) Xeroderma pigmentosum group F caused by a defect in a structure-specific DNA repair endonuclease. *Cell* **86:** 811–822

Sugasawa K, Ng J, Masutani C, Iwai S, van der Spek P, Eker A, Hanaoka F, Bootsma D, Hoeijmakers J (1998) Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair. *Mol Cell* **2:** 223–232

Sung P, Higgins D, Prakash L, Prakash S (1988) Mutation of lysine-48 to arginine in the yeast RAD3 protein abolishes its ATPase and DNA helicase activities but not the ability to bind ATP. *EMBO J* **7:** 3263–3269

Tapias A, Auriol J, Forget D, Enzlin J, Scharer O, Coin F, Coulombe B, Egly J (2004) Ordered conformational changes in damaged DNA induced by nucleotide excision repair factors. *J Biol Chem* **279:** 19074–19083

Tirode F, Busso D, Coin F, Egly JM (1999) Reconstitution of the transcription factor TFIIH: assignment of functions for the three enzymatic subunits, XPB, XPD, and cdk7. *Mol Cell* **3:** 87–95

Tuteja N, Tuteja R (2004) Unraveling DNA helicases. Motif, structure, mechanism and function. *Eur J Biochem* **271:** 1849–1863

Volker M, Mone MJ, Karmakar P, van Hoffen A, Schul W, Vermeulen W, Hoeijmakers JH, van Driel R, van Zeeland AA, Mullenders LH (2001) Sequential assembly of the nucleotide excision repair factors *in vivo*. *Mol Cell* **8:** 213–224

von Hippel P (2004) Helicases become mechanistically simpler and functionally more complex. *Nat Struct Mol Biol* **11:** 494–496

Winkler GS, Araujo SJ, Fiedler U, Vermeulen W, Coin F, Egly JM, Hoeijmakers JH, Wood RD, Timmers HT, Weeda G (2000) TFIIH with inactive XPD helicase functions in transcription initiation but is defective in DNA repair. *J Biol Chem* **275:** 4258–4266

Wolski SC, Kuper J, Hanzelmann P, Truglio JJ, Croteau DL, Van Houten B, Kisker C (2008) Crystal structure of the FeS cluster-containing nucleotide excision repair helicase XPD. *PLoS Biol* **6:** e149

Yokoi M, Masutani C, Maekawa T, Sugasawa K, Ohkuma Y, Hanaoka F (2000) The xeroderma pigmentosum group C protein complex XPC-HR23B plays an important role in the recruitment of transcription factor IIH to damaged DNA. *J Biol Chem* **275:** 9870–9875

The long unwinding road:

XPB and XPD helicases in damaged DNA opening

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Abstract

The mammalian nucleotide excision repair (NER) pathway removes dangerous bulky adducts from genomic DNA. Failure to repair these lesions can lead to oncogenesis, developmental abnormalities and accelerated ageing. TFIIH is a central NER factor that opens the damaged DNA prior to incision through the action of its two helicase subunits, XPB and XPD. Here we review our recently published data that suggest specific and distinct roles for these two helicases in NER. We also discuss the regulation of XPB and XPD enzymatic activities inside the TFIIH and repair complexes and show that mutations impeding enzyme-regulator interaction contribute to genetic disorders. Understanding the fundamental molecular mechanism regulating NER is a crucial aspect of cancer therapy since the resistance to chemotherapy treatment relies on the capacities of the cell to eliminate drug-induced DNA lesions.

Introduction: "Here comes the sun"

The genome of eukaryotes is vulnerable to an array of DNA-damaging agents. To avoid the harmful effects of DNA damage on fundamental cellular processes, cells are armed with several DNA repair pathways that represent the ultimate protection against damage-induced DNA mutations leading to cancer and ageing. Each of these DNA repair pathways removes structure-specific DNA lesions. For instance, the nucleotide excision repair pathway (NER) cleans off bulky adducts from DNA, including cisplatin lesions and photoproducts generated by UV-sunlight. Several repair-deficient disorders emphasize the importance of these survey mechanisms in genome stability. Deficiency in NER results in three rare genetic diseases; *Xeroderma pigmentosum* (XP), trichothiodystrophy (TTD) and Cockayne syndrome (CS).{Lehmann, 2003 #55} XP patients are highly photo-sensitive and display a 1000-fold increase in melanoma risk.{Lehmann, 2003 #55} TTD patients are mildly photo-sensitive and present neurological problems associated with sulphur-deficient brittle hair and nails caused by the reduced level of cysteine-rich matrix proteins.{Itin, 2001 #161} CS patients are also mildly photo-sensitive but harbor neurological problems, growth failure and premature ageing.{Andressoo, 2006 #5463}

The widely accepted model of NER includes the detection of the damage-induced DNA distortion by XPC-HR23B followed by the opening of the DNA by the XPB and XPD ATPases/helicases of the transcription/repair factor TFIIH.{Zurita, 2003 #65} Such initiation of DNA opening favors the recruitment of XPA and RPA that assist in the expansion of the DNA bubble around the damage.{Evans, 1997 #1016; Riedl, 2003 #68} Next, the endonucleases XPG and XPF generate cuts in the 3′ and 5′ sides of the lesion, respectively {O'Donnovan, 1994 #1784; Sijbers, 1996 #2059}, thereby removing a 25nts(+/-3) long damaged oligonucleotide.{Huang, 1992 #1265; Moggs, 1996 #458} Finally, the resynthesis machinery fills the DNA gap (Figure 1).{Shivji, 1995 #2053}

TFIIH is a multisubunit factor required for basal transcription initiation at RNA polymerase I and II promoters and in NER as part of the core incision machinery.{Zurita,

2003 #65}. TFIIH is composed of a core (XPB, p62, p52, p44, p34 and TTDA) associated to the Cdk-activating-kinase (CAK) by the XPD helicase. The importance of TFIIH during NER is highlighted by the fact that mutations in three of its subunits, XPB, XPD and TTDA, can give rise to XP, XP/CS or TTD disorders. The isolated XPB and XPD subunits are 3'–>5' and 5'–>3' DNA helicases, respectively. However, their individual roles in the NER reaction still remain unclear, since the presence of these proteins within the same complex renders their individual studies difficult. As XPB and XPD are helicases with opposite polarities, it was originally suggested that they could cooperate in damaged DNA opening on opposite sides of a lesion.{Schaeffer, 1994 #5354} In good agreement with this model, biochemical and genetic studies have shown the need of both XPB and XPD ATPase activities to open up DNA around a damaged site.{Guzder, 1994 #1166; Sung, 1988 #2128} However, recent data distinguished the function of the ATPase of XPB from that of its helicase.{Lin, 2005 #2614;Coin, 2007 #2751; Richards, 2008 #5357} They suggested that only the ATPase activity is required for the DNA opening in NER or in transcription, while the helicase would be devoted to promoter escape in transcription. {Dvir, 1997 #385} On the contrary, the XPD helicase activity has a minor role in transcription but is necessary for removing DNA damages.{Guzder, 1994 #1165; Tirode, 1999 #284} In addition to a role in damaged DNA opening, TFIIH may also have a role in damage recognition. Indeed, bulky DNA lesions inhibit the helicase activity of Rad3 (the XPD homolog in *Saccharomyces cerevisiae*) {Naegeli, 1993 #1735} and a putative damage recognition domain (DRD) has been found in the structure of a homolog of XPB in the thermophilic organism *Archaeoglobus fulgidus*. {Fan, 2006 #2682}

DNA damage recognition: “Please please me”

The broad substrate specificity of NER ranges from gross structural alterations in DNA to the minimal distortion caused by phosphorothiolate or methylphosphonate backbone modifications.{Branum, 2001 #748} Thus, the initial recognition of structurally unrelated

damaged sites is a crucial step in DNA repair. In mammalian NER, this key process is accomplished through the sequential actions of multiple proteins. The hypothesis that several factors could be involved in DNA damage recognition and verification stems from biochemical assays showing the preferential binding to damaged DNA of XPC, XPA and XPE, providing three actors for one role.{Jones, 1993 #1340; Sugasawa, 1998 #2120} This profusion of factors had boosted research on the recognition of DNA lesions in NER and had led to the identification of XPC as the first factor that binds the damaged DNA, joined subsequently by TFIIH and XPA.{Volker, 2001 #148; Riedl, 2003 #68} Further studies, including structural approaches, have shown that XPC-HR23B preferentially binds not only DNA lesions that distort the helix, but also mismatched base pairs that destabilize the DNA duplex.{Min, 2007 #5330; Sugasawa, 2007 #5331} It was then presumed that XPC-HR23B recognizes the destabilized Watson-Crick double helix induced by the damage rather than the lesion *per se*. Therefore, the XPC-HR23B protein can be defined as the initial damage sensor in human NER. The fact that the lesion does not interact directly with XPC-HR23B suggests that the lesion is accessible for binding by a second factor to authenticate its presence {Tapias, 2004 #2173; Min, 2007 #5330} and to further proceed with the reaction. Among the NER factors, the XPB and XPD polypeptides are the best candidates, since TFIIH is recruited to the lesion immediately after XPC-HR23B.{Riedl, 2003 #68}

The yeast XPD homologue Rad3 was shown to bind preferentially to UV-damaged over non-damaged DNA.{Sung, 1994 #2130} Furthermore, the Rad3 helicase activity is inhibited when it encounters damage in the DNA.{Naegeli, 1992 #1733} It was thus proposed that the stop of Rad3 in front of a lesion was a checkpoint ensuring that only fragments containing *bona fide* bulky adducts, and not mismatches, were excised by NER. However, and although structural studies identified a β-hairpin motif that participates in damage recognition by the XPD prokaryotic homologue UvrB {Skorvaga, 2002 #2065}, such motif is absent from the human protein.{Fan, 2008 #5350; Liu, 2008 #5349; Wolski, 2008 #5348; Bienstock, 2002 #708} Thus, while much of the evidence points to a key role for the XPD

subunit of TFIIH in damage verification, this remains highly speculative in the absence of the structure of an XPD-DNA complex containing a lesion.

XPB is the second candidate for the verification of DNA lesions. A putative DRD was found in a homolog of XPB from the thermophilic organism *Archaeoglobus fulgidus*. {Fan, 2006 #2682} To determine if this putative DRD had a role in the function of TFIIH in NER, we introduced two mutations to this domain at position E253 and R283 (Figure 2A and Table 1). These residues are positioned in two of the three hairpins that were suggested to be involved in DNA binding.{Fan, 2006 #2682} However, we detected no effect of these mutations on either NER or transcription both *in vivo* and *in vitro*.{Oksenych, 2009 #5384} Similarly, we did not detect preferential binding of an XPB(1-320) fragment, encompassing the DRD, to damaged *versus* non-damaged DNA (Unpublished data). Although we cannot exclude that XPB, or any other subunits of TFIIH, are involved in damage verification, it seems more plausible to assign this role to the XPA factor, which is recruited just before the arrival of the two endonucleases (XPG and XPF) that will generate irreversible cuts in the DNA.

Role of XPB and XPD in damaged DNA opening: "Come together"

DNA helicases are motor proteins that can transiently catalyze the unwinding of the stable duplex DNA molecules using NTP hydrolysis as the source of energy. They are characterized by seven "helicase motifs" (walker motif I, Ia, II, III, IV, V, and VI), constituted of conserved amino-acid sequences {Tuteja, 1996 #2244}. The helicases are then classified into three superfamilies (SF), SF1, SF2 and SF3, based on the extent of similarity and organization of these conserved motifs {Tanner, 2003 #5498}. One of the paradoxes of the NER pathway is the need of two helicases belonging to the SF2 (XPB and XPD) to open an asymmetrical (20nts in 5' and 5nts in 3') DNA bubble of 25 nucleotides around the damage. Indeed, most of the helicases are highly processive enzymes able to open hundreds of nucleotides in a short time. However, biochemical data clearly demonstrated that a mutation in the motif I (responsible for ATP binding) of either XPB or XPD totally abolished the

formation of the open DNA structure during NER.{Coin, 2006 #5355} Such observations indicate that the hydrolyse of ATP by XPB and XPD is essential for the function of TFIIH in NER, but do not demonstrate that the helicase activities of these proteins are required. It raises the possibility that the ATPase activity is not only a provider of energy for the helicase action but that it also displays another independent function. To understand if the helicase activities of XPB or XPD are important in NER, we introduced mutations directly in some of the helicase motifs of these enzymes. Surprisingly, our study demonstrated that the helicase activity of XPB was not crucial for NER since mutations in the motif III (T469) and VI (Q638A) that impair the helicase activity of XPB, do not inhibit NER *in vivo* (Table 1).{Coin, 2007 #2751} This supports the idea that XPB does not act as a conventional helicase in NER, but gives no explanation for its indispensable ATPase activity. We focused our attention on this question in our recent work and showed that a mutation in the motif I (K346R), which abolishes the ATPase activity of XPB, inhibits NER since it thwarts the accumulation of TFIIH to the damaged sites (Figure 2A and Table 1).{Oksenych, 2009 #5384} This implies that the recruitment of TFIIH to sites of damage is not due to a simple association of TFIIH to XPC.{Riedl, 2003 #68} Instead, it is an active process requiring ATP hydrolysis undertaken by XPB.

In addition to the aforementioned ATPase motif, we found that the highly conserved R-E-D and ThM motifs, which were identified in the structure of the homolog of XPB in *Archaeoglobus fulgidus,* were also required for the recruitment of TFIIH to the damaged sites. Indeed, mutations in the R-E-D (E473A) or in the ThM (Δ516-526) domains show similar biological defects than the K346R mutation in the motif I of XPB (Figure 2A and Table 1).{Oksenych, 2009 #5384} How the R-E-D and ThM motifs participate in the anchoring of TFIIH? The structure of XPB and our biochemical data suggest that the energy furnished by the ATP hydrolysis is used to induce a flip of 170° of the helicase domain 2 (HD2) following the binding of XPB to DNA.{Fan, 2006 #2682} The R-E-D and the ThM are then in close vicinity and are used to stabilize TFIIH on the DNA by introducing a wedge (the E473 residue) in the double stranded DNA, gripped by the ThM motif (Figure 2B). We

obtained experimental evidence for this model when we compared the ATPase activities of the WT and mutated complexes with or without DNA. In the presence of DNA, mutations in the R-E-D and ThM motifs induce a 50% inhibition of the ATPase activity compared to TFIIH(WT). In the absence of DNA, the ATPase activity of these three TFIIH complexes (WT, mutated in R-E-D or in ThM) are strictly identical and are still slightly higher than the ATPase activity of the TFIIH complex mutated in the motif I (K346R).{Oksenych, 2009 #5384} These data support the model of conformational change proposed above, since they demonstrate that the R-E-D and the ThM are functional only in the presence of DNA. Interestingly enough, the ThM domain is missing from other helicases, including XPD {Fan, 2008 #5350; Liu, 2008 #5349; Wolski, 2008 #5348; Bienstock, 2002 #708}, but a similar helical protrusion has been observed between the helicase domains III and IV of the *Sulfolobus solfataricus* SWI2/SNF2 ATPase Rad54 (ssRad54).{Durr, 2005 #5360} In ssRad54, the ThM like domain, called domain 2B, contacts DNA and is supposed to help to translate ATP-driven conformational changes between domains 1A and 2A (similar to HD1 and HD2 in the SF2) (Figure 3). These similarities, together with the fact that XPB displays a very low helicase activity *in vitro* {Coin, 1998 #306}, prompted us to propose that the mode of action of XPB is closer to the members of the SWI2/SNF2 family than to the SF2 helicases.

The anchoring of TFIIH through the conformational change in XPB may also induce a reorganization of the protein-DNA complexes in transcription and repair that will allow new protein-protein or protein-DNA contacts. Indeed, using photocrosslink experiments, we have shown that addition of ATP in NER induced a re-positioning of XPC on the damaged DNA that was dependant on TFIIH.{Tapias, 2004 #2173} In our model, following the anchoring of TFIIH by XPB, DNA opening around the lesion is performed by the helicase activity of XPD. As a matter of fact, we showed that a mutation in the ATPase or helicase activities of XPD still allowed TFIIH to bind to the damaged DNA *in vivo* but hampered the opening of the DNA around the lesion {Dubaele, 2003 #77} (Unpublished Data).

XPB and XPD regulation: “With a little help from my friends”

In agreement with a fundamental role of the ATPase activity of XPB and the helicase activity of XPD in the removal of DNA lesions, these activities are the targets of multiple regulations. TFIIH is recruited to the damaged DNA through direct interaction with the damage recognition factor XPC-HR23B.{Riedl, 2003 #68} Recently, we found that the recruitment of TFIIH to XPC-damaged DNA involves the XPB subunit of TFIIH. The binding of TFIIH to XPC-HR23B stimulates XPB ATPase activity that eventually leads to a conformational change fixing XPB to the DNA. A mutation found in XP-C patients abolishes the interaction between XPC-TFIIH and the stimulation of the ATPase.{Bernardes de Jesus, 2008 #5361}

We also showed that TTDA, a subunit mutated in some TTD patients, participates to the regulation of the ATPase activity of XPB within the TFIIH complex, even though these two subunits do not interact directly. However, TTDA interacts with the p52 subunit of TFIIH and may play a role in the stimulation of the ATPase activity of XPB through this direct interaction.{Coin, 2006 #5355} Indeed, we demonstrated that p52 was a regulatory subunit of the ATPase activity of XPB within TFIIH.{Coin, 2007 #2751} In addition, Giglia-Mari et al. previously demonstrated in an elegant paper that free TTDA exist in the cell and can shuttle between the cytoplasm and nucleus to associate with TFIIH when NER-specific DNA lesions are produced.{Giglia-Mari, 2004 #40} We then proposed that the recruitment of TTDA to the core TFIIH, bound to XPC-damaged DNA, regulates the XPB/p52 interaction within the complex and ensures a correct stimulation of the ATPase activity (Figure 4). In this model, the binding of TTDA to TFIIH serves as an important NER checkpoint regulating the conformational change of XPB described above and leading to the stable anchoring of TFIIH to the damaged DNA. Identically to XPC, a mutation (F99S) found in XP-B/CS patients impairs the interaction between XPB and p52 and the aforementioned ATPase stimulation, explaining the NER defect harbored by the patients (Table 1).{Coin, 2007 #2751}

On the other hand, several studies have shown that the helicase (and not the ATPase) activity of XPD was regulated through an interaction between the p44 subunit of TFIIH and the C-terminal end of XPD (Figure 4).{Coin, 1998 #306; Dubaele, 2003 #77} Most of the

mutations found in XP-D patients are located in the C-terminal domain and do not abolish the helicase activity of XPD *per se*, but prevent interaction with p44.{Coin, 1998 #306; Dubaele, 2003 #77} Altering this interaction in XP-D patients results in a decrease of the XPD helicase activity within TFIIH and consequently in NER defect.

Conclusion: "Things we said today"

Altogether, these results highlight the complex level of regulation of the enzymatic activities in TFIIH. They also reveal that mutations found in XP-B and XP-D patients never affect the activity of the protein *per se* (helicase for XPD, ATPase for XPB) but rather disturb the interactions of these enzymes with their regulatory partners (p44 for XPD and p52 for XPB), explaining how patients with such mutations may exist. Further studies will determine how these activities are regulated at the structural level. These data will establish if antagonists or agents targeting p52, p44 or TTDA can be developed to counteract the resistance of cancer cells to some chemotherapy. Cisplatin and related drugs are widely used in the treatment of non-small cell lung carcinoma and other late-stage tumors.{Martin, 2008 #5474} However, the high incidence of resistance to DNA-damaging chemotherapeutic drugs such as cisplatin are directly linked to an increased NER activity in these cells and have led to the search for molecules that would reduce their DNA repair activity. In the near future, chemotherapy could take into account not only the recently described circadian regulation of the NER process {Kang, 2009 #5420} but also the possibility to thwart some protein-protein interactions in order to increase the efficiency of the treatment.

Acknowledgments

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Figure 1. The nucleotide excision repair pathway.

Following exposure to a source of DNA lesions (i.e. sunlight), a lesion is created on the DNA. Then, the damage recognition factor XPC-HR23B interacts with the damaged DNA structure on the opposite strand of the lesion (black square). TFIIH joins XPC-HR23B on the damaged DNA. In the presence of ATP, XPB and XPD helicases in TFIIH unwind the damaged DNA, allowing the stable association of XPA and RPA that help to enlarge the opened DNA structure {Tapias, 2004 #2173} and drive the dissociation of the CDK activating kinase (CAK) complex from TFIIH. {Coin, 2008 #5346} This dissociation is a prerequisite for the enlargement of the DNA opening that favors the arrival of XPG, mediating the release of XPC-HR23B. {Riedl, 2003 #68} The recruitment of XPF-ERCC1 triggers dual incision and excision of the protein-free damaged oligonucleotide. The resynthesis machinery fills the gap and seals the DNA extremities.

Figure 2. Description of the structure and conformational change of XPB.

A. The four classical helicase motifs (I, Ia, II, III) of the first helicase domain (HD1) are indicated in blue. The three helicase motifs (IV, V and VI) of the second helicase domain (HD2) are indicated in green. The putative damage recognition (DRD), R-E-D and Thumb (ThM) domains identified in an homolog of XPB from the thermophilic organism *Archaeoglobus fulgidus* {Fan, 2006 #2682} are indicated respectively in light blue, red and purple. The engineered mutations E253A, R283A, K346R, E473A and Δ516-526 are marked. The mutations F99S (XP/CS), T119P (TTD) and FS740 (XP/CS) found in XP-B patients are highlighted in black.

B. In this model, the binding of XPB to DNA triggers the rotation of the second helicase domain (HD2) together with the ThM domain, facilitated by HD1-mediated ATP hydrolysis, to form the closed and stable XPB-DNA complex where the R-E-D motif intrudes between DNA strands.

Figure 3. Model of XPB and ssRad54 bound to a double-stranded DNA.

Model of XPB bound to a double-stranded DNA fragment (on the left), based on comparison of the full-length *Archaeoglobus fulgidus* XPB structure (PDB:2FWR, {Fan, 2006 #2682} with the *Sulfolobus solfataricus* SWI2/SNF2 ATPase core in complex with double-stranded DNA (on the right) (PDB:1Z63, {Durr, 2005 #5360}). Helicase domains 1 and 2 (HD1 and HD2) are in blue and green, respectively. The damage recognition domain (DRD) is cyan and the Thumb domain (ThM) in magenta. DNA is shown in orange. Af-XPB (PDB:2FWR) was superimposed onto the SWI2/SNF2 ATPase core of *Sulfolobus solfataricus* in complex with double-stranded DNA (chains A and BC of PDB:1Z63), using the first helicase domains of both proteins as a guide (Strands Sx-Sy of Af-XPB and Sc-Sv of SWI/SNF). To model the closed conformation of XPB, HD2 and the ThM domain were rotated as a rigid using the second helicase domains of Af-XPB and SsRad54 as templates (Strands Sx-Sy of Af-XPB and Sc-Sv of SsRad54). Superimpositions and graphic analysis were performed with moleman and o11, respectively. Molecular graphic figures were generated using PyMOL (http://www.pymol.org/).

Figure 4. Function of XPB and XPD in damaged DNA opening.

TFIIH is a ten subunit complex composed of a core (In red, XPB, p62, p52, p44, p34 and TTDA) associated to the Cdk-activating-kinase (CAK) (In Blue, cdk7, cyclinH and MAT1) and XPD (In green). When TFIIH binds to the damaged DNA, two sub-complexes participate in the stimulation of the XPB ATPase activity; XPC-HR23B and p52-TTDA. These stimulations help to stabilize TFIIH on the damaged DNA and position XPD. Whether TTDA is recruited to TFIIH following the association of the complex with the damaged DNA or comes together with it, is not known. Subsequently, the XPD helicase activity opens the DNA in the 5' to 3' direction. The p44 subunit interacts with XPD and stimulates its helicase activity.

Mutations	Domain	Phenotype	NER	Recruitment[2]	Opening	Helicase	ATPase
F99S[1]	-	XP/CS	-	+/-	+/-	+	+/-
T119P[1]	-	TTD	+/-	+	+/-	+	+
E253A	DRD	-	+	+	+	+	+
K346R	Ia	-	-	-	-	-	-
T469A	III	-	+	+	+	-	+
E473A	RED	-	-	-	-	+	+/-
Δ516-526	Thumb	-	-	-	-	+	+/-
Q638A	VI	-	+	+	+	-	+
FS740[1]	-	XP/CS	-	+[3]	+	+	+[3]

Table 1: Mutations in XPB and their consequences on TFIIH repair activity.

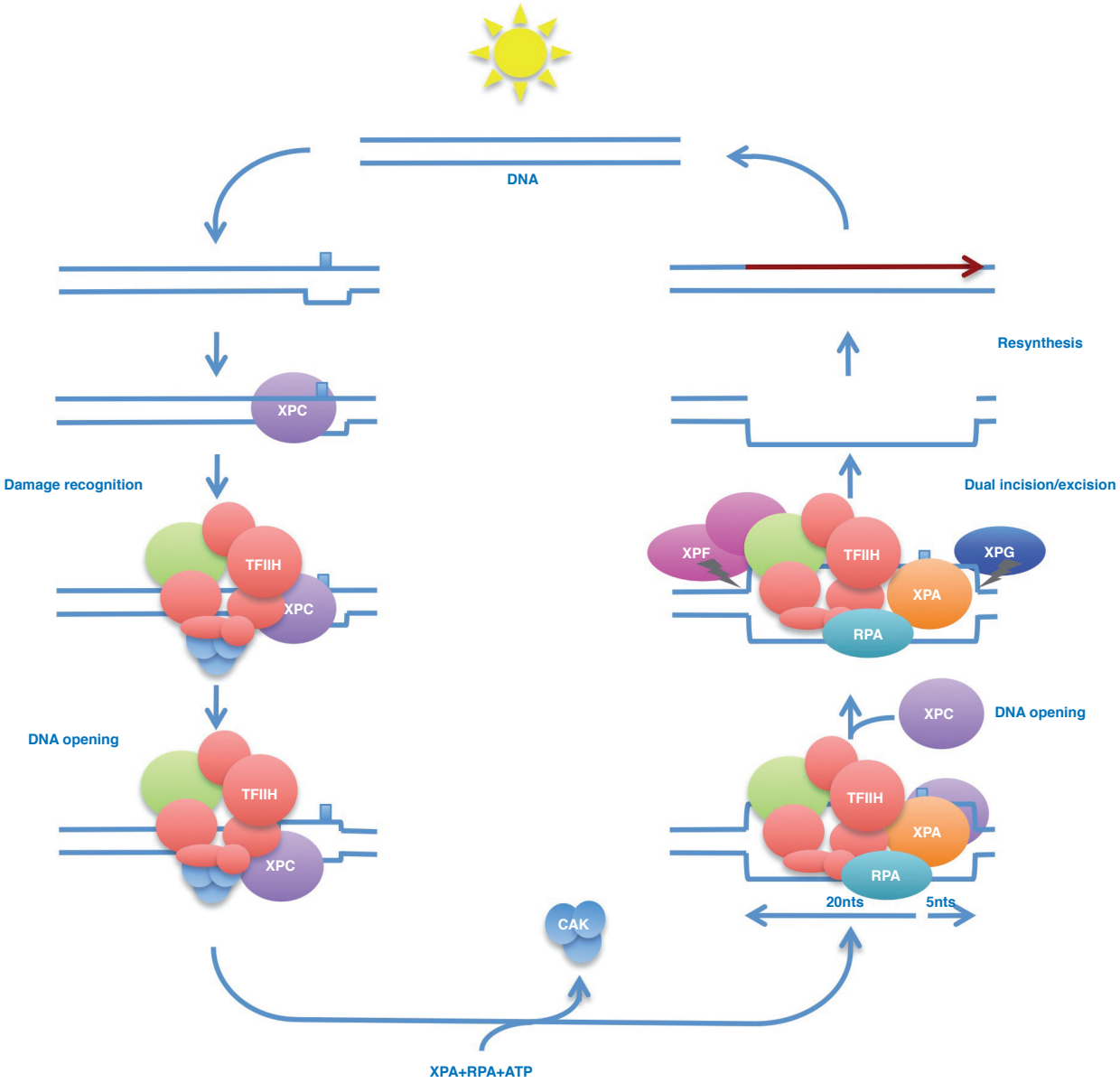
This table is a compilation of results obtained in our laboratory.

[1] Endogenous mutation found in XP-B patients.

[2] Recruitment of the TFIIH complex to the local damage sites *in vivo*.

[3] Unpublished data

Figure 1


DNA
XPC
Damage recognition
TFIIH
XPC
DNA opening
TFIIH
XPC
CAK
XPA+RPA+ATP
XPC
DNA opening
TFIIH
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5nts
Resynthesis
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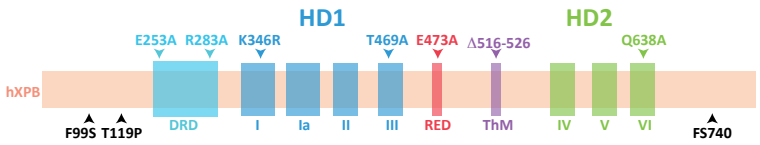
XPF
TFIIH
XPG
XPA
RPA

Figure 2

A


HD1
HD2
E253A
R283A
K346R
T469A
E473A
Δ516-526
Q638A
hXPB
F99S
T119P
DRD
I
Ia
II
III
RED
ThM
IV
V
VI
FS740

B

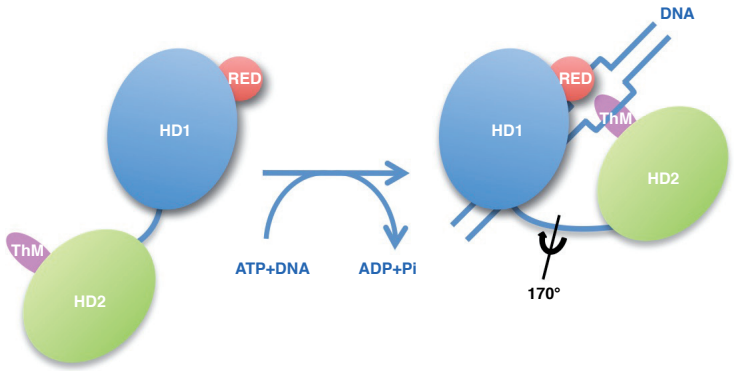

DNA
RED
HD1
ThM
HD2
ATP+DNA
ADP+Pi
RED
HD1
ThM
HD2
170°

Figure 3

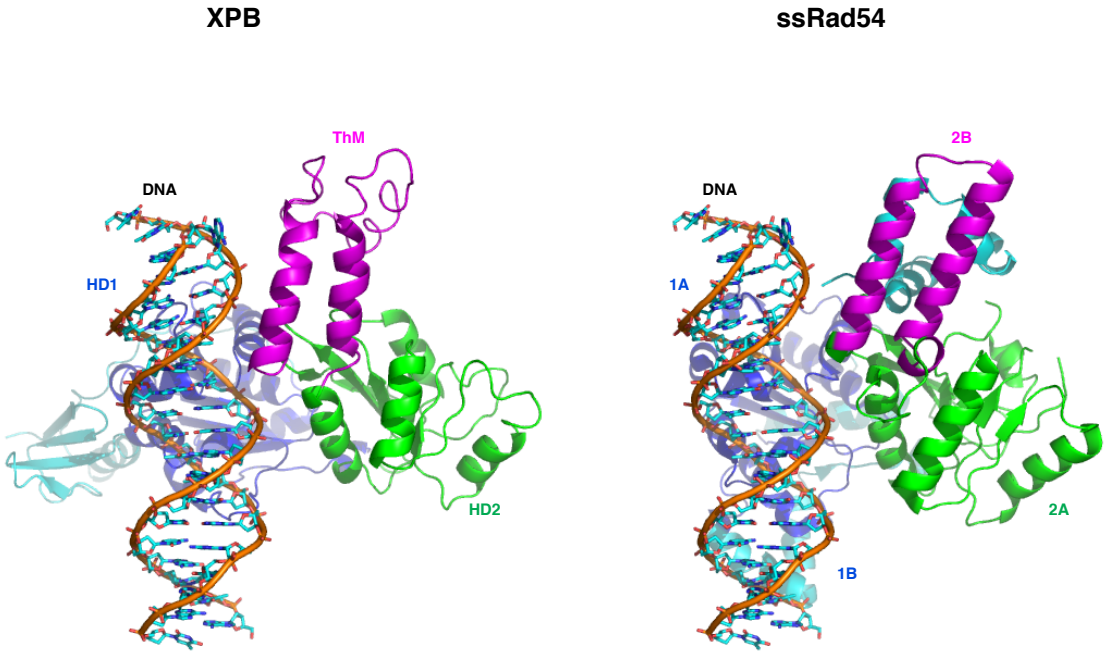
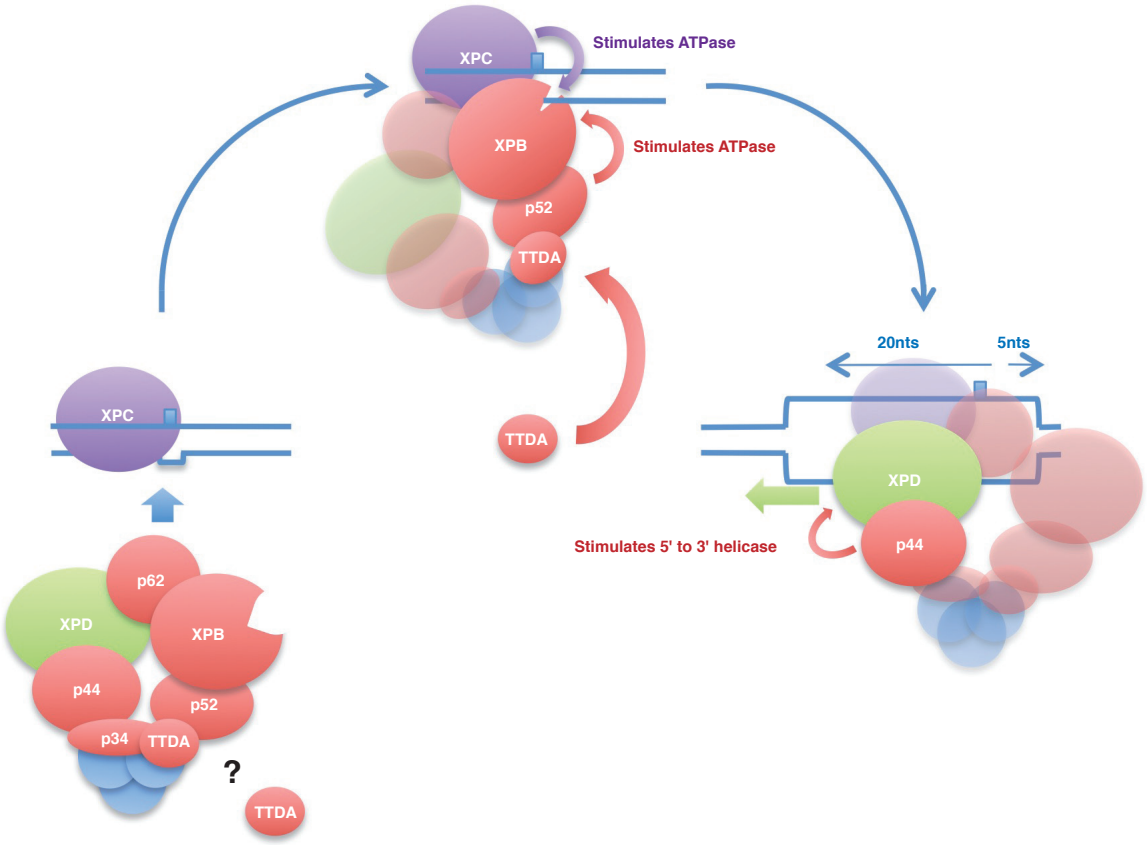


Figure 4


XPC
Stimulates ATPase
XPB
Stimulates ATPase
p52
TTDA
20nts
5nts
XPC
TTDA
XPD
p44
Stimulates 5' to 3' helicase
p62
XPD
XPB
p44
p52
p34
TTDA
?
TTDA

DOT1L supports transcription re-initiation after UV irradiation

Results and discussion

Mammalian DOT1L is required for UV light resistance in mammals

The DOT1 protein is involved into X-ray yeast resistance (Game et al., 2005, Game et al., 2006). However, almost nothing is known about the role of DOT1L in the UV-induced DNA damage response in mammals. We exposed mouse embryonic fibroblasts (MEF), either wild type (wt) or knocked-out ($DOT1L^{-/-}$) to different doses of UV-C light (254nm). The survival of $DOT1L^{-/-}$ cells was significantly affected if compare to wild type cells (Figure 1, A). Next, we treated human HeLa cells with short interference (si)RNA to DOT1L. Similarly, siDOT1L treated cells were more sensitive to UV light, as siXPA cells, if compare with controls (treated with random siCtrl or mock-treated) (Figure 1, B). As unscheduled DNA synthesis (UDS) had practically the same rate in wt and knocked-out MEF, the DOT1L is not required for efficient global-genome nucleotide excision repair (Figure 1, C). However, the recovery of RNA synthesis in the absence of DOT1L is significantly affected (Figure 1, D). This observation is similar to one obtained for CSB cells (Proietti-De-Santis et al., 2006). Moreover, after DNA damage, DHFR promoter occupancy by RNA polymerase II and transcription factor II B (TFIIB) is significantly reduced in the $DOT1L^{-/-}$ MEF (Figure 2, A, C). It correlates with decrease of histone H4 acetylation in this cell line (Figure 2, B). Thus, there is displacement of transcription machinery from the promoter of house-keeping genes, DHFR (Figure 2) and GAPDH (data not shown). Absence of polymerase II explains reduction of total RNA synthesis (Figure 1, D) and refers to defect in transcription-coupled repair (Proietti-De-Santis et al., 2006).

DOT1L is required for efficient transcription activation

As we observed UV-dependent displacement of transcription machinery from house-keeping gene promoters in the absence of DOT1L, next step was to check transcription activation after DNA damage. As expected, the activation of p53-dependent genes (p21 and mdm2) was lower in $DOT1L^{-/-}$ cells versus wt (Figure 3, D, E). Surprisingly, the activation of stress-related c-Fos gene was much stronger in the absence of DOT1L (Figure 3, F).

UV-independent transcription activation, induced by vitamin D (Cyp24 gene), also was different in wt and $DOT1L^{-/-}$ cells (Figure 3, A-C). The Cyp24 promoter occupancy by RNA polymerase II and

acetylation of histone H4 around transcription start region are significantly higher in wt cells. There was a delay in Cyp24 mRNA accumulation in DOT1L cells after vitamin D induction (Figure 3, A). Contrary, there was almost no change in the RNA polymerase II occupancy of the Cyp24 promoter in knocked-out cells (Figure 3, B). In addition, acetylation of histone H4 also was stable after induction of gene expression in the DOT1L$^{-/-}$ cells (Figure 3, C).

Accumulation of DOT1L on the damaged DNA

Next, we irradiated human cells (HeLa) with UV-C light (254nm) through filter with 3 μm pores (Volker et al., 2001). Already 10 minutes after DNA damage, we observed clear DOT1L co-localization with XPB, a subunit of transcription/repair factor TFIIH (Figure 4, A-D). Then, we tested DOT1L recruitment in cells expressing short-hairpin (sh)RNA to repair factors XPA and XPF, which models XP-A or XP-F repair-deficient phenotype. Ten minutes after UV-irradiation, we observed accumulation of DOT1L protein as efficient as in the HeLa cells (Figure 4, E-H and I-L). Thus, DOT1L recruitment to the damaged DNA does not depend on the DNA repair complex assembly, at least after XPC and TFIIH appearance on the chromatin. Still, it is not clear, does DOT1L require XPC, TFIIH, DDB, CSB or CSA to recruit efficiently on the area of damaged DNA. Further experiments will be performed to shed light on this question.

DOT1L methylates histone H3 lysine K79 (Feng et al., 2002; Ng et al., 2002). This histone, when methylated, attracts other repair and signaling factors, like 53BP1 (Huyen et al., 2004). The last protein is described in the context of double-strand DNA repair, but we detected it after UV-C irradiation (Figure 4, M-P) at the same condition, that DOT1L.

Additional questions

In this study we still have a number of questions to answer. We will identify the context of DOT1L involvement in the transcription recovery after UV-irradiation. For this, we will apply ChIP approach, using antibodies to transcription factors and marks of active and silent chromatin (histone modifications, including methylation of histones H3 K4, H3 K9, H3 K27 and H3 K36; correspondent histone methyltransferases and demethylases). Then, we will perform total RNA sequencing following UV-irradiation to compare gene induction in the wild type and DOT1L$^{-/-}$ cells. According to our preliminary data, the answer differs for different gene groups (for instance, p21 and c-Fos, see Figure 3, D-F).

Also, we will generate human model of DOT1L-deficient and rescued cells. For this, we will transfect and stabilize HeLa cells with vector expressing shRNA to silence DOT1L. To rescue $DOT1L^{-/-}$ MEF, we will stably transfect these cells with plasmid expressing DOT1L-GFP (Zhang et al., 2009).

One more question to solve will be quality of DNA damage after UV-C irradiation. DOT1L and 53BP1 are described mostly in the context of double-stranded (ds)DNA breaks repair, and we detect them on chromatin after UV-irradiation. So, either these two proteins play role in both kinds of DNA repair (nucleotide excision repair and repair of double-strand breaks), or except of CPD and 6,4-PP in our experiments we produce dsDNA breaks. To support or exclude first idea, we will verify the presence of dsDNA break repair-specific proteins in the locally irradiated foci. To exclude double-strand breaks, we will repeat experiments in the context of UV-B light (315nm), which will have less energy and less probability to form other DNA damages, but CPD and 6,4-PP.

Materials and methods

The DOT1L-/- cells were obtained from Vakoc (Steger et al., 2008). Briefly, murine embryonic stem cell line RRR032, obtained from BayGenomics, contains a gene trap integration within intron 12 of the Dot1l gene. RRR032 embryonic stem cells were injected into C57BL/6 blastocysts to generate chimeric mice, which were then bred to obtain heterozygous Dot1l mutant animals. Mouse embryonic fibroblast (MEF) cultures were derived from embryonic day 13.5 embryos obtained from matings between heterozygous mice. The genotype of homozygous Dot1l mutant MEFs was determined by PCR and reverse transcription (RT)-PCR, confirming integration of the gene trap between exons 12 and 13. Cells were immortalized by serial passaging.

The site of gene trap integration (intron 12) is expected to truncate Dot1L expression at amino acid 335, eliminating a critical nucleosome binding region at amino acids 390 to 407 required for full DOT1L methyltransferase activity *in vitro*.

Figures

Figure 1. DOT1L is required for the transcription recovery in the UV-irradiated cells.

Cell survival after UV irradiation (**A**, **B**). **A**. Mouse embryonic fibroblasts, MEF (wt) and DOT1L knocked-out MEF (DOT1L$^{-/-}$) were plated on 10cm Petri dishes and irradiated with various doses of 254nm UV light. After 96h the cells were stained with crystal violet and counted. Results are expressed as a ratio between the cells that survived after UV irradiation and the cells that survived without treatment.

B. HeLa cells mock treated or transfected with si RNA to DOT1L (siDOT1L), XPA (siXPA) or random si RNA (siCtrl) were irradiated with UV-C light and treated as described in (**1**, **A**).

C. DNA synthesis after UV irradiation. Wild type and DOT1L$^{-/-}$ MEF were irradiated with different doses of UV light. Unscheduled DNA repair synthesis (UDS) expressed as mean number of autoradiographic grains per nucleus as described in (Nardo et al., 2009).

D. RNA recovery analysis after UV-C irradiation. DNA in the cells (wt and DOT1L$^{-/-}$) was labeled with [^{14}C]-thymidine during 48h. Mock-irradiated and UV-irradiated (20J/m²) cells were pulse labeled for 1h with [^{3}H]-uridine at different time points after irradiation, and trichloroacetic acid (TCA)-insoluble radioactivity was determined. The curves represent relative RNA synthesis.

Figure 2. Transcription machinery at the DHFR promoter after UV irradiation.

MEF wt and DOT1L$^{-/-}$ cells were irradiated with 20 J/m² UV light. Soluble chromatin was prepared from wt and DOT1L$^{-/-}$ MEFs at indicated time points after UV irradiation. ChIP assays were performed using the antibodies to RNA polymerase II (**A**), acetylated histone H4 (**B**), and TFIIB (**C**). The results are expressed as fold enrichment related to the mock-treated cells.

Figure 3. Gene transactivation is DOT1L-dependent.

A. Cyp24 expression after vitamin D activation. MEF (wt) and DOT1L knocked-out cells (DOT1L$^{-/-}$) were treated with vitamin D (0.1 μM). Level of mRNA was quantified by RT-qPCR. The values represent the relative Cyp24 mRNA level measured at different time points after vitamin D treatment compared with mock-treated cells.

B. Recruitment of RNA polymerase II at the Cyp24 promoter in wt and DOT1L$^{-/-}$ MEFs after induction with vitamin D (0.1 μM). ChIP with antibody to RNA polymerase II.

C. Acetylation of histone H4 at the Cyp24 promoter after vitamin D induction. ChIP with anti-AcH4.

D. p21 and (**E**) mdm2 activation after UV irradiation. Wild type and DOT1L$^{-/-}$ MEF were irradiated with 20 J/m² UV light or mock-treated. Level of mRNA was quantified by RT-qPCR. The values represent the relative p21 and mdm2 mRNA level measured at different time points after UV irradiation compared with mock-treated cells.

F. Fos activation after UV irradiation. Wild type and DOT1L$^{-/-}$ MEF were irradiated with 20 J/m² UV light or mock-treated. Level of mRNA was quantified by RT-qPCR. The values represent the relative c-Fos mRNA level measured at different time points after UV irradiation compared with mock-treated cells.

Figure 4. Protein recruitment to the sites of damaged DNA.

Hela cells (A-B, and M-P) and HeLa cells expressing shRNA to XPA (E-H) or XPF (I-L) were irradiated with UV-C light (100 J/m²) through the 3µm pore filter and fixed 10 minutes later. Immunofluorescent labeling was performed using rabbit polyclonal anti-XPB (A, E, I, M), anti-DOT1L (B, F, J) or anti-53BP1 (N). The nuclei were counterstained with DAPI (C, G, K, O) and the images were merged (D, H, L, P).

References

Game JC., Williamson MS., and Baccari C. (2005). X-ray survival characteristics and genetic analysis for nine Saccharomyces deletion mutants that show altered radiation sensitivity. Genetics 169, 51-63

Game JC., Williamson MS., Spicakova T., and Brown JM. (2006). The RAD6/BRE1 histone modification pathway in Saccharomyces confers radiation resistance through a Rad51-dependent process that is independent of RAD18. Genetics *173*, 1951-68

Feng Q., Wang H., Ng H.H., Erdjument-Bromage H., Tempst P., Struhl K., and Zhang Y. (2002). Methylation of H3-lysine 79 is mediated by a new family of HMTases without a SET domain. Curr Biol. *12*, 1052-8

Huyen Y., Zgheib O., Ditullio RA Jr., Gorgoulis VG., Zacharatos P., Petty TJ., Sheston EA., Mellert HS., Stavridi ES., and Halazonetis TD. (2004). Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. Nature *432*, 406-11

Nardo T., Oneda R., Spivak G., Vaz R., Mortier L., Thomas P., Orioli D., Laugel V., Stary A., Hanawalt PC., Sarasin A., and Stefanini M. (2009). A UV-sensitive syndrome patient with a specific CSA mutation reveals separable roles for CSA in response to UV and oxidative DNA damage. Proc Natl Acad Sci USA. *106*, 6209-14

Ng HH., Feng Q., Wang H., Erdjument-Bromage H., Tempst P., Zhang Y., and Struhl K. (2002). Lysine methylation within the globular domain of histone H3 by Dot1 is important for telomeric silencing and Sir protein association. Genes Dev. *16*, 1518-27

Proietti-De-Santis L., Drane P., and Egly JM. (2006). Cockayne syndrome B protein regulates the transcriptional program after UV irradiation. EMBO J. *25*, 1915-23

Volker M., Mone MJ., Karmakar P., van Hoffen A., Schul W., Vermeulen W., Hoeijmakers JH., van Driel R., van Zeeland AA., and Mullenders LH. (2001). Sequential assembly of the nucleotide excision repair factors in vivo. Mol Cell. *8*, 213-24

Zhang D., Li S., Cruz P., and Kone BC. (2009). Sirtuin 1 Functionally and Physically Interacts with Disruptor of Telomeric Silencing-1 to Regulate {alpha}-ENaC Transcription in Collecting Duct. J Biol Chem. *284*, 20917-26

Figure 1

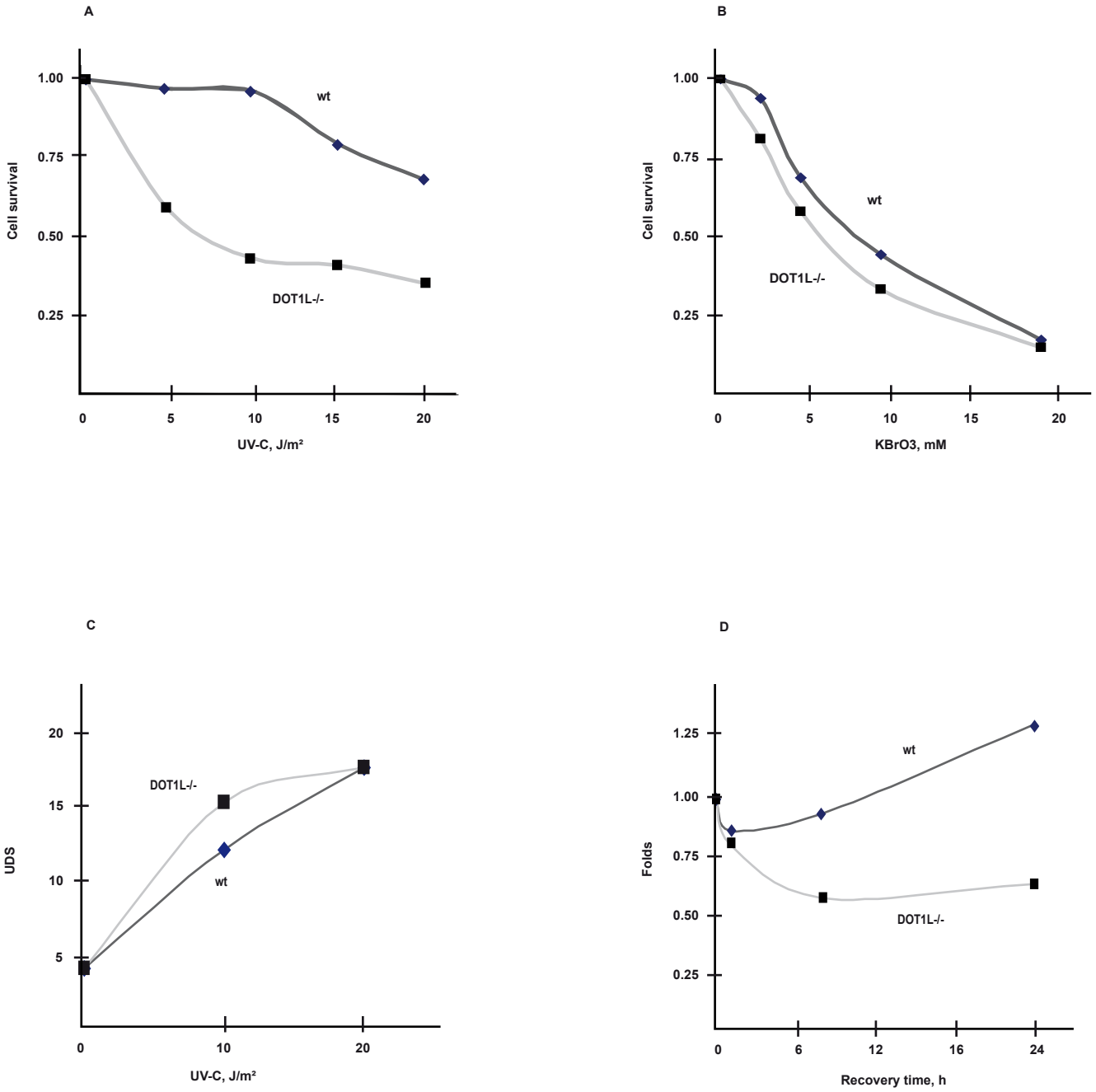

A
1.00
0.75
0.50
0.25
Cell survival
wt
DOT1L-/-
0
5
10
15
20
UV-C, J/m²
B
1.00
0.75
0.50
0.25
Cell survival
wt
DOT1L-/-
0
5
10
15
20
KBrO3, mM
C
20
15
10
5
UDS
DOT1L-/-
wt
0
10
20
UV-C, J/m²
D
1.25
1.00
0.75
0.50
0.25
Folds
wt
DOT1L-/-
0
6
12
16
24
Recovery time, h

Figure 2

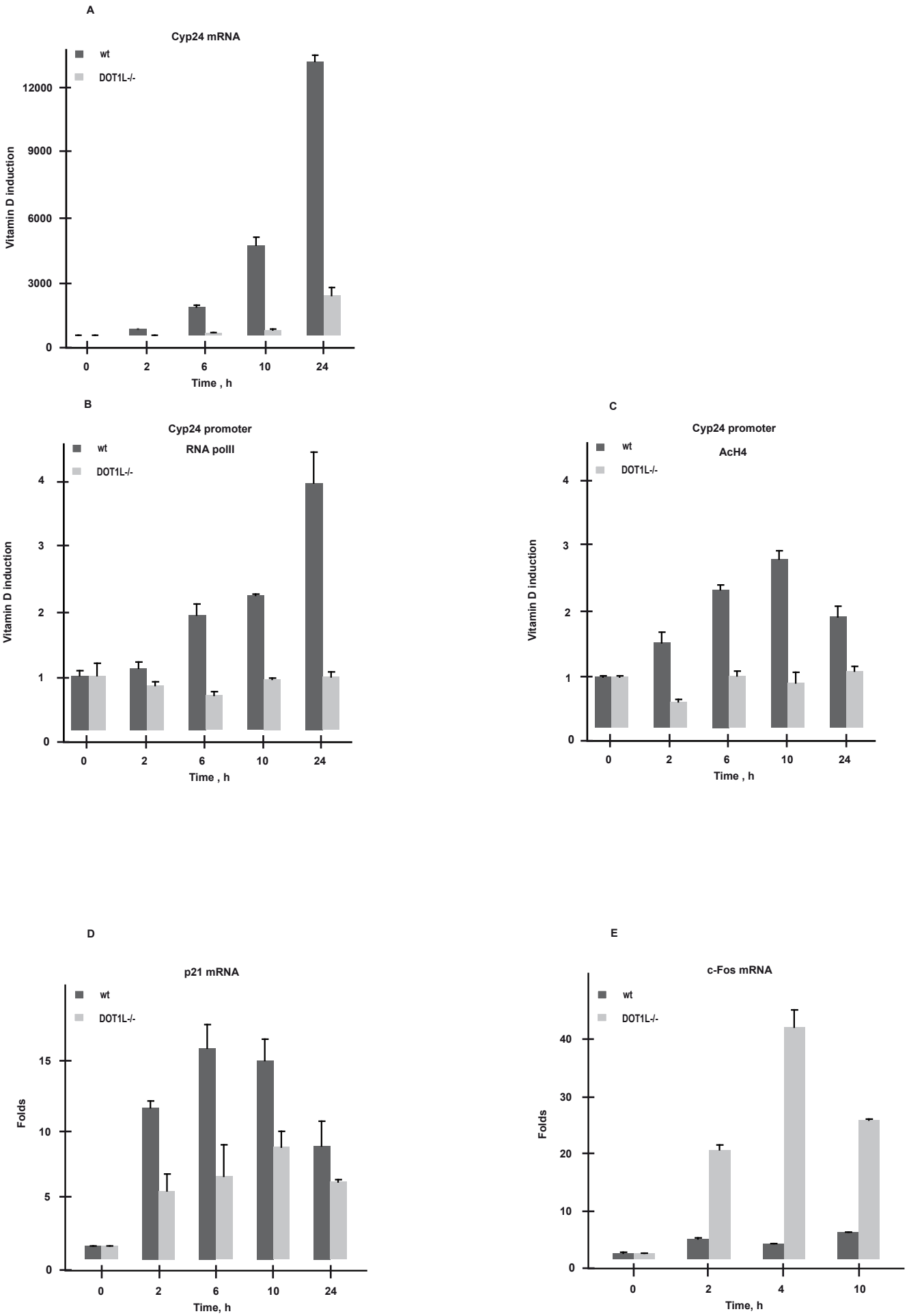

A
Cyp24 mRNA
wt
DOT1L-/-
Vitamin D induction
12000
9000
6000
3000
0
0
2
6
10
24
Time , h
B
Cyp24 promoter
RNA polII
wt
DOT1L-/-
Vitamin D induction
4
3
2
1
0
0
2
6
10
24
Time , h
C
Cyp24 promoter
AcH4
wt
DOT1L-/-
Vitamin D induction
4
3
2
1
0
0
2
6
10
24
Time , h
D
p21 mRNA
wt
DOT1L-/-
Folds
15
10
5
0
0
2
6
10
24
Time, h
E
c-Fos mRNA
wt
DOT1L-/-
Folds
40
30
20
10
0
0
2
4
10
Time, h

Figure 3

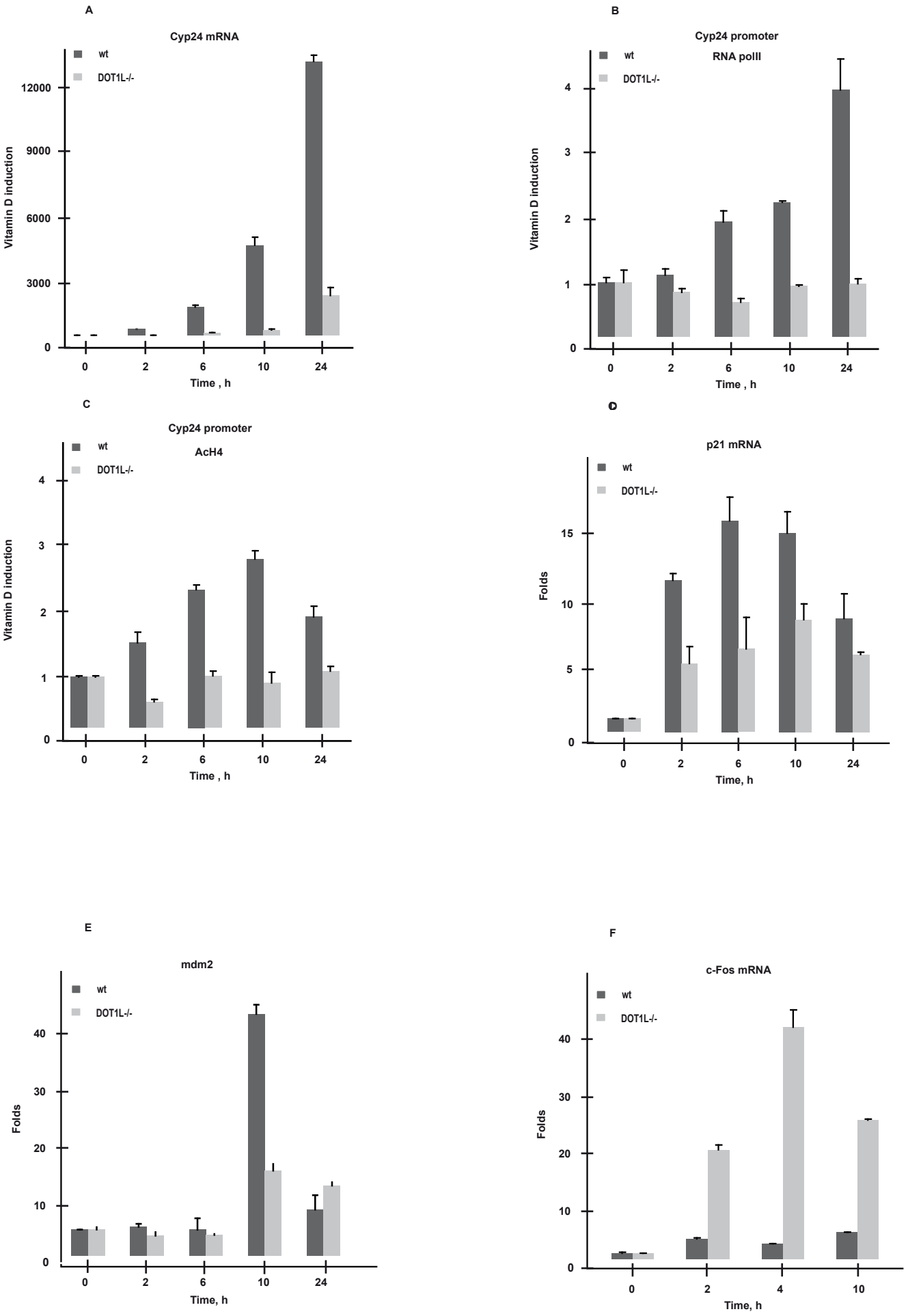

A
Cyp24 mRNA
wt
DOT1L-/-
Vitamin D induction
12000
9000
6000
3000
0
0
2
6
10
24
Time , h
B
Cyp24 promoter
RNA polII
wt
DOT1L-/-
Vitamin D induction
4
3
2
1
0
0
2
6
10
24
Time , h
C
Cyp24 promoter
AcH4
wt
DOT1L-/-
Vitamin D induction
4
3
2
1
0
0
2
6
10
24
Time , h
D
p21 mRNA
wt
DOT1L-/-
Folds
15
10
5
0
0
2
6
10
24
Time, h
E
mdm2
wt
DOT1L-/-
Folds
40
30
20
10
0
0
2
6
10
24
Time, h
F
c-Fos mRNA
wt
DOT1L-/-
Folds
40
30
20
10
0
0
2
4
10
Time, h

Figure 4

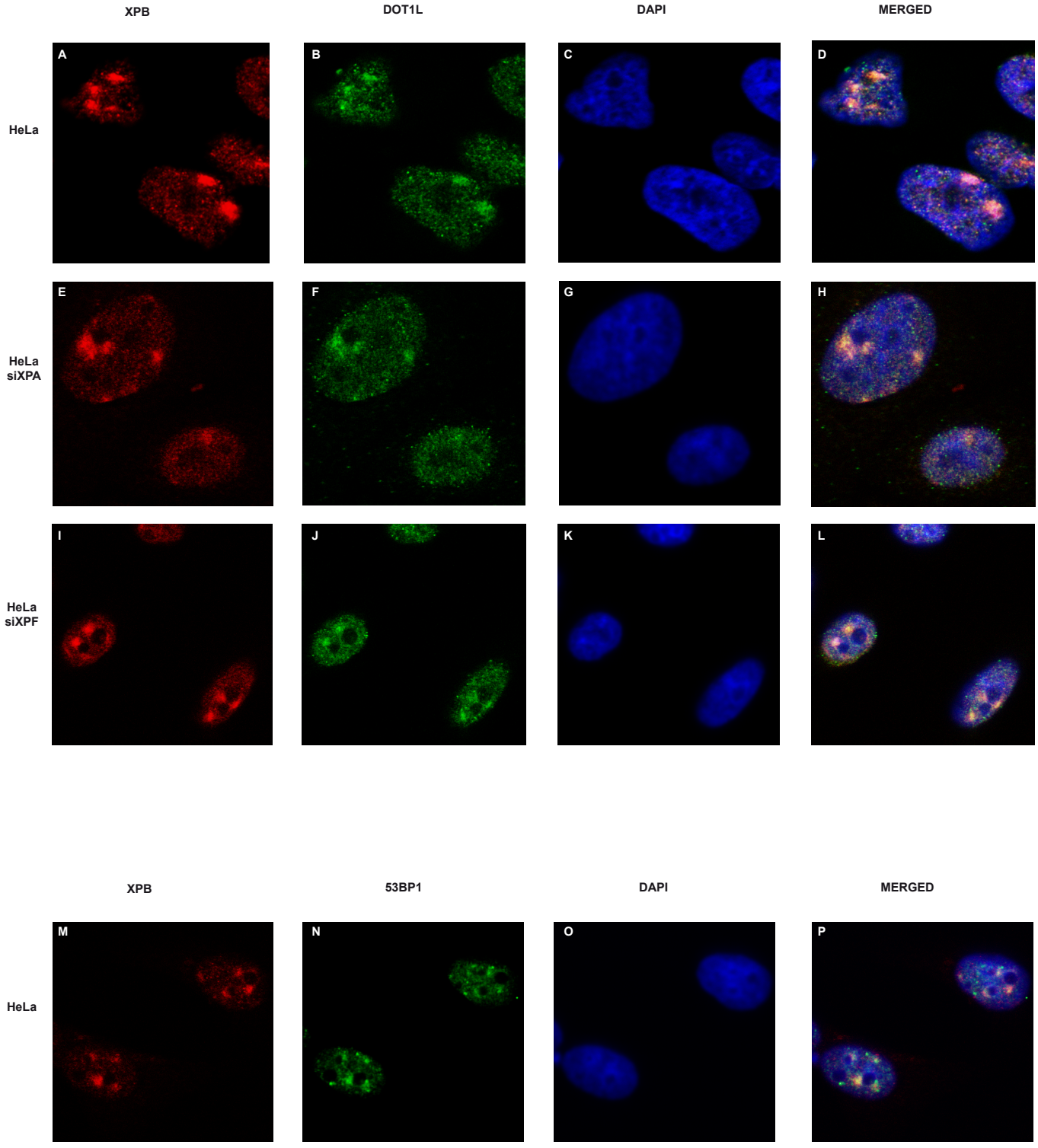
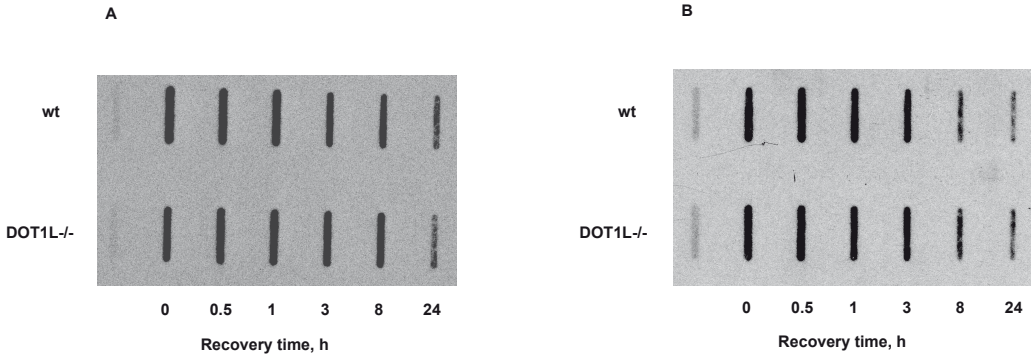
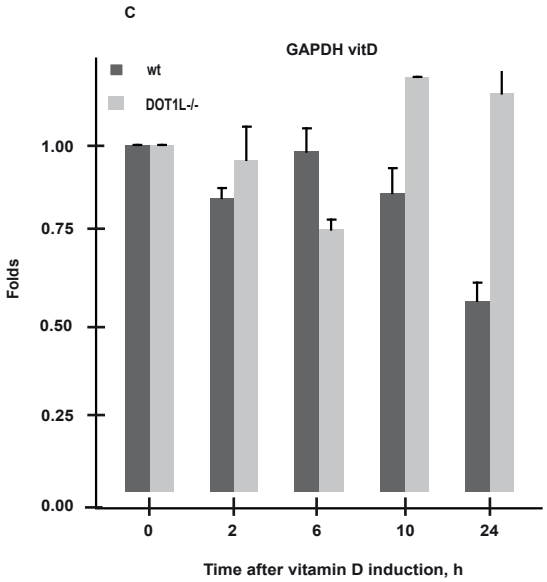
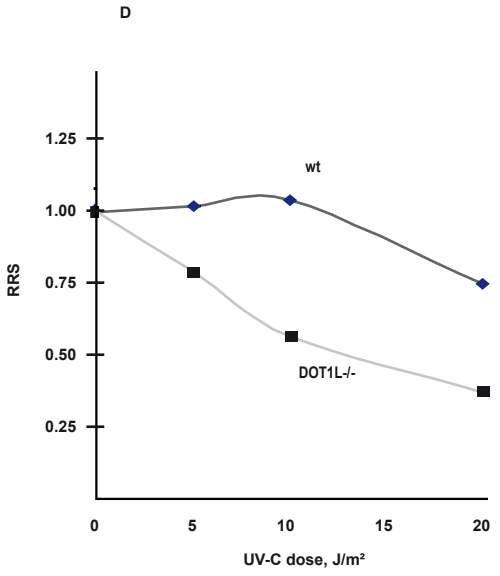
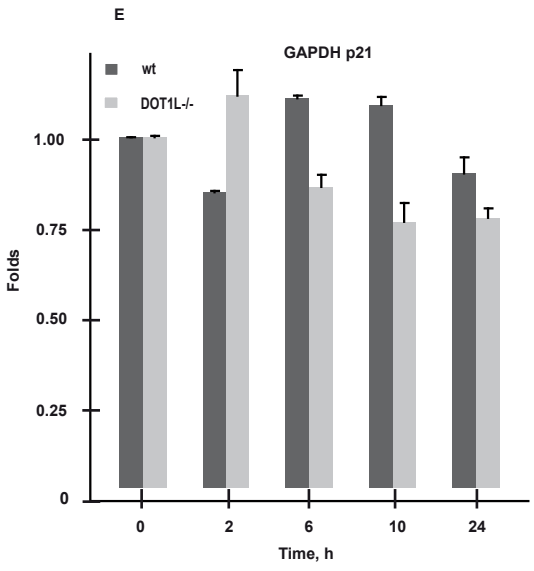

XPB
DOT1L
DAPI
MERGED
HeLa
HeLa siXPA
HeLa siXPF
A
B
C
D
E
F
G
H
I
J
K
L
XPB
53BP1
DAPI
MERGED
HeLa
M
N
O
P

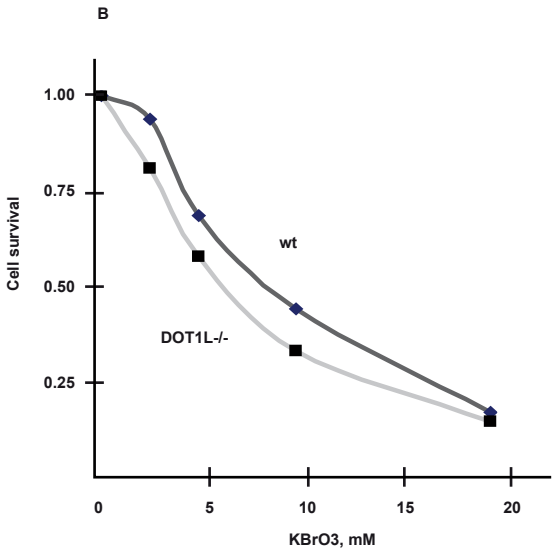
Figure S1


A
wt
DOT1L-/-
0 0.5 1 3 8 24
Recovery time, h
B
wt
DOT1L-/-
0 0.5 1 3 8 24
Recovery time, h


C
GAPDH vitD
wt
DOT1L-/-
Folds
1.00
0.75
0.50
0.25
0.00
0 2 6 10 24
Time after vitamin D induction, h


D
RRS
1.25
1.00
0.75
0.50
0.25
wt
DOT1L-/-
0 5 10 15 20
UV-C dose, J/m²


E
GAPDH p21
wt
DOT1L-/-
Folds
1.00
0.75
0.50
0.25
0
0 2 6 10 24
Time, h


B
Cell survival
1.00
0.75
0.50
0.25
wt
DOT1L-/-
0 5 10 15 20
KBrO3, mM