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NOUVEAUX ACTEURS A' L'INTERFACE DE LA TRANSCRIPTION ET DE LA REPARATION

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THESE

Présentée par

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Pour obtenir le grade de

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CONTENTS

ABBREVIATIONS

DDR -DNA damage response
NER -nucleotide excision repair
siRNA -short interfering/silencing RNA
GGR -global genome repair
TCR -transcription-coupled repair
6-4PPs -pyrimidine-(*6*-4)-pyrimidone photoproducts
CPDs -cyclobutane pyrimidine dimers
XP -xeroderma pigmentosum
TTD -trichothiodystrophy
CS -Cockayne syndrome
UV(A/B/C) -ultraviolet light (400 – 315 /315 – 280 /280 – 100 nm)
FRAP -fluorescence recovery after photobleaching
MEF -mouse embryonic fibroblasts
TFIIH -transcription factor II H, multisubunit complex
TFIIH core subunits:

- p62 -General transcription factor IIH subunit 1
- p52 -General transcription factor IIH subunit 4
- p44 -General transcription factor IIH subunit 2
- p34 -General transcription factor IIH subunit 3
- p8 -General transcription factor IIH subunit 5
- XPB -ERCC3, ATP dependent human DNA helicase
- XPD -ERCC2, ATP dependent human DNA helicase

CAK -cyclin activating kinase-subcomplex of TFIIH

- CDK7 -Cell division protein kinase 7
- MAT1 -CDK-activating kinase assembly factor
- Cyclin H

POLII -RNA polymerase II
CTD -carboxy-terminal domain
DOT1L -DOT1-like, histone H3 lysine 79 methyltransferase

RESUME DE LA THESE DOCTORAT

NOUVEAUX ACTEURS A' L'INTERFACE DE LA TRANSCRIPTION ET DE LA REPARATION

INTRODUCTION

Les dommages à l'ADN proviennent de sources environnementales (irradiations UV ou X, polluants de l'atmosphère) ou endogènes (radicaux libres oxygénés, instabilité intrinsèque de la molécule d'ADN). Les lésions interférent avec la progression des ADN ou ARN polymérases, et compromettent ainsi la fidélité de la réplication et de la transcription[1,6]. Ainsi, la prévention et la réparation des dommages de l'ADN sont essentielles pour la survie des cellules. La plupart des voies de réparation de l'ADN englobe un même ensemble de processus étroitement coordonnés: la détection des lésions de l'ADN, l'accumulation des facteurs de réparation sur le site de dommages et enfin l'élimination de la lésion[2,4]. Parmi les voies de réparation, la réparation par excision de nucléotides (NER) est la voie la plus polyvalente en réparant des lésions aussi variées que les lésions UV ou les lésions chimiques encombrantes. Alors que les bactéries n'ont besoin que de trois protéines pour compléter l'étape d'incision de la NER, les eucaryotes utilisent environ 30 protéines pour mener à bien cette étape. La réparation de l'ADN dans les cellules eucaryotes est compliquée par le fait que l'ADN génomique est intégré dans une structure chromatinienne le rendant inaccessibles. Les cellules utilisent des modifications post-traductionnelles des histones et le remodelage de la chromatine dépendant de l'ATP afin de moduler la structure de la chromatine et d'augmenter l'accessibilité de la machinerie de réparation aux lésions[3,5].

L'avènement de la technique d'interférence à l'ARN (ARNi) a permis de développer des outils de choix, les banques de siARN, afin d'identifier de nouveaux régulateurs de voies cellulaires fondamentales. Bien que les facteurs de base de la NER ont été identifiés et que le système ait été reconstitué sur de l'ADN nu "in vitro", il reste encore beaucoup à comprendre sur la régulation de cette voie de réparation fondamentale "in vivo", sur de la chromatine. Dans ce contexte, nous avons initié un criblage siARN basé sur le suivi de l'efficacité de réparation des lésions UV-induites 6-4 photoproduits (6-4PPs). Le but de

ce screening était de mettre en évidence de nouvelles enzymes impliquées dans la réponse aux dommages d'ADN et le maintien de l'intégrité du génome par la voie NER.

D'autre part, nous nous sommes également concentrés sur DOT1L, une histone-méthyltransférase impliquée dans la régulation du silencing télomérique, dans le contrôle du cycle cellulaire et dans l'étape d'élongation de la transcription. Nous avons montré que l'absence de DOT1L dans des fibroblastes embryonnaires de souris (MEF^{DOT1L}) conduit à une sensibilité de ces cellules aux irradiations UV.
Enfin, lors d'un travail en collaboration avec un thésard du laboratoire, nous avons étudié le rôle des sous-unités XPB et XPD de TFIIH, gardiennes essentielles du génome, dans la réparation par excision de nucléotides.

RESULTAS

Les résultats du criblage siRNA destiné à identifiés de nouveaux acteurs de la NER, sont en court d'exploitation mais nous mettons déjà en évidence le rôle de certains gènes impliqués dans la biochimie des ARNm comme ceux empêchant la formation des hybrides ARN/ADN dans l'efficacité de réparation des lésions UV.

En étudiant le rôle de la methyltransférase DOT1L, nous avons montré que son absence dans des fibroblastes embryonnaires de souris (MEF ^{DOT1L}) conduit à une sensibilité de ces cellules aux irradiations UV alors que la réparation des lésions produites par cette irradiation est intacte. L'absence de DOT1L conduit en réalité à une inhibition de l'initiation de la transcription des gènes après irradiation. Au niveau mécanistique, des expériences de STRIP-FRAP ont établit que DOT1L assurait l'association de l'ARN polymérase II à la chromatine après irradiation UV. Dans une analyse plus détaillée, nous avons montré que DOT1L favorisait la formation du complexe de pré-initiation au niveau du promoteur des gènes de ménage ainsi que l'apparition de marques d'euchromatine transcriptionnellement actives. Bien que l'expression des gène de ménage soit inhibée, une analyse transcriptomique montre que les gènes pro-apoptotiques sont fortement transactivés chez les MEF ^{DOT1L} après irradiation. Le traitement à la trichostatine A, qui relaxe la chromatine, diminue la transactivation des gènes apoptotiques et restore l'initiation de la transcription et la survie aux UV.

Pour comprendre la fonction de XPB et XPD, nous avons étudié des lignées cellulaires exprimant des formes mutées de ces protéines dans l'activité ATPasique. Nous avons montré l'implication de XPB, mais pas d'XPD, dans le recrutement de TFIIH sur des sites d'ADN endommagé. Le recrutement de TFIIH se produit indépendamment de l'activité hélicase d'XPB, mais nécessite deux nouveaux motifs récemment identifiés, une boucle « R-E-D » et un motif « thumb ». Par ailleurs, nous montrons que ces motifs sont spécifiquement impliqués dans la stimulation de l'activité ATPase de XPB qui a lieu en présence de l'ADN.

CONCLUSION

Le criblage d'une banque de siRNA s'est révélé un outil précieux dans l'identification de nouveaux acteurs de la NER. Faute de temps nous n'avons pas encore pu aller en profondeur dans l'étude du rôle précis de ces gènes mais nous reviendrons dans le manuscrit sur les premières conclusions de notre étude.

L'Histone methyltransférase DOT1L permet la restauration de l'initiation de la transcription après une attaque génotoxique. Nous proposons que DOT1L permette la présence d'une structure ouverte de la chromatine afin de réactiver l'initiation de la transcription des gènes de ménage suite à un stress génotoxique.

Nous avons étudié les détails moléculaires du recrutement de TFIIH aux sites d'ADN endommagé. L'ensemble de nos données montre que le recrutement de TFIIH aux sites de dommages est un processus actif, sous le contrôle de l'activité ATPasique de XPB. Nos résultats suggèrent également que XPB fonctionne comme un crochet ATP dépendant servant à stabiliser la liaison de TFIIH avec l'ADN endommagé.

LISTE DES PUBLICATIONS ET POSTERS

1. Nicolas Le May, Izarn Iltis, Jean-Christophe Ame, **Alexander Zhovmer**, Denis Biard, Jean-Marc Egly, Valerie Schreiber and Frederic Coin (2012) Poly (ADP-ribose) glycohydrolase regulates retinoic acid receptor-mediated gene expression. **Molecular Cell.**
2. **Alexander Zhovmer**, Valentyn Oksenych, Frederic Coin. Chromatin remodelers, kinases and phosphatases mediate DNA damage response and nucleotide excision repair(*in preparation*).
3. Valentyn Oksenych*, **Alexander Zhovmer***, Jitka Eberova, Tiziana Nardo, Miria Stefanini, Jean-Marc Egly and Frédéric Coin (2012) Histone methyltransferase DOT1L drives recovery of transcription initiation after a genotoxic attack(*reviewing in JCB*) ***These authors contributed equally to this work.**
4. **Alexander Zhovmer**, Valentyn Oksenych, Frédéric Coin (2010) Two sides of the same coin: TFIIH complexes in transcription and DNA repair. **ScientificWorldJournal**. Review.
5. Valentyn Oksenych, Bruno Bernardes de Jesus, **Alexander Zhovmer**, Jean-Marc Egly Frédéric Coin (2009) Molecular insights into the recruitment of TFIIH to sites of DNA damage. **The EMBO journal**.
6. Poster: Genome wide survey of protein kinases required for nucleotide excision repair. **Alexander Zhovmer**, Valentyn Oksenych, Frederic Coin**.** Best poster, selected for oral presentation (2009) The “3rd EU - IP DNA Repair Workshop for Young Scientists".

REFERENCES

1. Hanawalt, P. C., & Spivak, G. (2008). Transcription-coupled DNA repair: two decades of progress and surprises. Nature reviews. Molecular cell biology, 9(12), 958-70.
2. Jones, R. M., & Petermann, E. (2012). Replication fork dynamics and the DNA damage response. The Biochemical journal, 443(1), 13-26.
3. Li, J., Bhat, A., & Xiao, W. (2011). Regulation of nucleotide excision repair through ubiquitination. Acta biochimica et biophysica Sinica, 43(12), 919-29.
4. Lord, C. J., & Ashworth, A. (2012). The DNA damage response and cancer therapy. Nature, 481(7381), 287-94.
5. Luijsterburg, M. S., & van Attikum, H. (2011). Chromatin and the DNA damage response: the cancer connection. Molecular oncology, 5(4), 349-67.
6. Lukas, J., Lukas, C., & Bartek, J. (2011). More than just a focus: The chromatin response to DNA damage and its role in genome integrity maintenance. Nature Cell Biology, 13(10), 1161-1169. Nature Publishing Group.

FIGURES AND TABLES

INTRODUCTION

PROJECT I

FOREWORD

The problem, often not discovered until late in life, is that when you look for things like love, meaning, motivation, it implies they are sitting behind a tree or under a rock. The most successful people recognize, that in life they create their own love, they manufacture their own meaning, they generate their own motivation.

Comment on "I am Neil deGrasse Tyson - AMA" (March 01, 2012).

INTRODUCTION

PART I - A NEW LOOK AT A VERY OLD COMPLEX

TFIIH COMPLEXES IN TRANSCRIPTION AND DNA REPAIR

Our genome is vulnerable to an array of DNA-damaging agents that affect fundamental cellular processes, such as DNA replication and transcription. To counteract the deleterious effects of these agents, cells are armed with several DNA repair pathways that protect us from cancer and accelerated aging[1]. Each of these DNA repair pathways removes structure-specific DNA lesions.

The nucleotide excision repair (NER) pathway removes bulky adducts, including cisplatin lesions and 6-4 photoproducts generated by UV light, through two related subpathways[2].

The general global genome repair (GGR) removes DNA damage from the entire genome, while the transcription-coupled repair (TCR) corrects lesions located on actively transcribed genes[3]. The importance of DNA repair mechanisms in genome stability is emphasized by the existence of several repair-deficient disorders. Deficiency in NER results in three rare genetic diseases: xeroderma pigmentosum (XP), trichothiodystrophy (TTD), and Cockayne syndrome (CS)[4]. XP patients are highly photosensitive and display a 1000-fold increased risk of developing skin cancers[4]. TTD patients are mildly photosensitive, but present neurological disorders and sulfur-deficient brittle hair and nails caused by the reduced level of cysteine-rich matrix proteins[5]. CS patients are also mildly photosensitive, but harbor neurological problems, growth failure, and premature aging[6]. XP and TTD patients are deficient both in GGR and TCR (with the exception of XP-C patients who are only deficient in GGR, (see below). Pure CS patients are deficient only in TCR. GGR is then fully functional in persons with this syndrome, but they die prematurely from progeria. This leads to the hypothesis that the low level of residual lesions that block transcription in CS cells promotes apoptosis. This premature cell death would protect CS patients from cancer at the expense of aging.

In GGR, XPC-HR23B detects the damage-induced DNA distortion in the genome,

followed by the opening of the DNA by the XPB and XPD ATPases/helicases of the transcription/repair factor TFIIH[7]. XPA and RPA are then recruited to the repair complex and assist in the expansion of the DNA bubble around the damage[8,9]. Next, the endonucleases XPG and XPF generate cuts in the 3′ and 5′ sides of the lesion, respectively[10,11], thereby causing the removal of a 27-nts(±2)-long damaged oligonucleotide[12,13]. Finally, the resynthesis machinery fills the DNA gap[14]. In TCR, blockage of transcribing RNA polymerase II (RNPII) on the damaged DNA template is thought to initiate the repair reaction in a process that requires, in addition to TFIIH, XPA, XPG and XPF, the TCR-specific proteins CSB and CSA[2].

The transcription-coupled NER pathway is independent from the XPC-HR23B complex, thereby explaining the strict GGR defect harbored by the XP-C patients. Among the NER factors, TFIIH has attracted the most interest, owing to its additional roles in several fundamental cellular processes.

TFIIH: A MULTISUBUNIT FACTOR WITH SEVERAL CELLULAR TASKS

TFIIH is a ten-subunit complex[15,16], essential for the transcription of RNAI- and II-dependent genes and for the NER pathway[17,18]. The TFIIH complex can be divided into two subcomplexes: the core and the CAK. The core TFIIH includes XPB, p62, p52, p44, p34, and the repair-specific TTDA subunit. XPD links the core to the Cdk-activating kinase (CAK) module composed of Cdk7 (cyclin-dependent kinase), cyclin H, and MAT1 (ménage à trois) (Figure 1).

Mutations in three TFIIH subunits give rise to XP, TTD, or CS; mutations in XPB are associated with a combined XP-CS phenotype or with TTD; mutations in XPD are associated with XP, XP-CS, or TTD; and mutations in TTDA are associated only with TTD[4]. In RNPII transcription, TFIIH joins the preinitiation complex composed of RNPII and the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, and TFIIF (Figure 1). There, TFIIH is involved in several processes ranging from initiation, promoter escape, and early elongation stages[19], to transcription reinitiation[20] and formation of gene loops[21]. Besides its roles in RNPII transcription, TFIIH is also involved in the transcription of ribosomal genes by RNPI. The reconstituted "in vitro" RNPI transcription

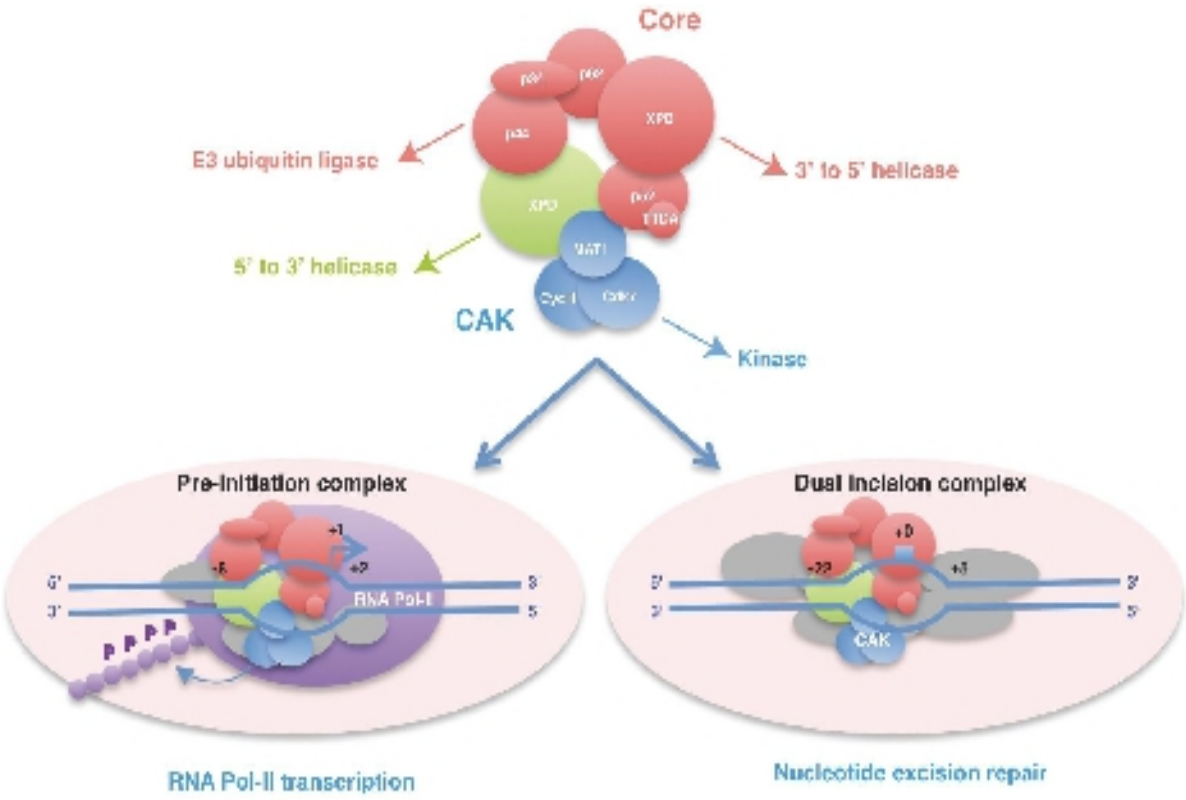

Figure 1. A multisubunit and tasks complex,TFIIH is a ten-subunit complex composed of a core (in red; XPB, p62, p52, p44, p34, and TTDA) associated to the CAK (in blue; Cdk7, CycH, and MAT1) through MAT1 and the XPD subunit (in green). Four enzymatic activities are found in TFIIH: XPB and XPD are 3' to 5' and 5' to 3' helicases, Cdk7 is a kinase, and p44 has been described as an E3 ubiquitin ligase in yeast. The complex is involved both in RNPI- and II-dependant transcription and in nucleotide excision repair. The role of TFIIH in RNPI transcription in unknown. In RNPII-dependant transcription, TFIIH opens DNA around the promoter in the preinitiation complex (–8 to +2 relatively to the transcription start site +1) and phosphorylates the carboxyl terminal domain of the RNPII (purple) to license transcription. In NER, TFIIH opens DNA around the lesion (–22 to +5 relatively to the lesion in blue square) and assists ERCC1-XPF for the 5' incision[77].

system requires TFIIH[18] and the complex localizes in the nucleolus at sites of active ribosomal gene transcription[22]. TFIIH was also reported to be part of a complex containing RNPI and the NER factors CSB and XPG[23], but the precise role of TFIIH during RNPI transcription yet remains unknown. In NER, TFIIH belongs to the dual incision complex composed of XPC-HR23B, XPA, RPA, XPG, and ERCC1-XPF, and is involved in the opening of the DNA around the damage (Figure 1). Four TFIIH subunits harbor enzymatic activities required for transcription and DNA repair (Figure 1). Cdk7 phosphorylates RNPII and certain nuclear receptors, and thereby regulates basal and

activated transcription[24,25]. XPB and XPD are ATPases/helicases involved in DNA opening[7]. The ATPase activity of XPB is required for anchoring TFIIH to the damaged DNA[26,27], while the helicase activity of XPD opens DNA around the lesion[28]. The enzymatic activity of XPD is not required for RNPII transcription and this subunit is therefore believed merely to act as a structural component of the TFIIH complex and to regulate the kinase activity of Cdk7[29,30]. Finally, p44 was described as an E3 ubiquitin ligase in yeast[31]. This activity is suggested to be important for the survival of cells after exposure to UV light or methyl methanesulfonate (MMS).

The enzymatic activities of TFIIH are highly regulated either by members of the complex or by DNA repair factors (Table 1). The p52 subunit of TFIIH stimulates the ATPase activity of XPB[28]. TTDA is a NER-specific factor that is recruited to the TFIIH complex after a genotoxic attack[32,33].

Subunit	Activity	Function	Partners	Regulators
XPB	DNA-dependent ATPase	Opening of the DNA around the promoter; anchoring of TFIIH to damaged DNA	p52, p62*	p52 (stimulates the ATPase)[28], TTDA (stimulates the ATPase in a TFIIH context)[33], XPC (stimulates the ATPase on DNA)[36]
XPD	3' to 5' helicase	Opening of DNA around the damage	p44, p52, MAT1	p44 (stimulates the helicase)[37,39], MAT1 (inhibits the helicase)[38]
p44	E3 ubiquitin ligase**	Transcription answer after damage	XPD, p34	p34 (stimulates the E3 ubiquitin ligase)[31]**
Cdk7	Kinase	Basal transcription; activated transcription	Cyclin H, MAT1	Cyclin H (stimulates the kinase activity)[52], TFIIE(stimulates the kinase)[52], MAT1 (stabilizes the association between Cdk7 and cyclin H)[52]

* Unpublished data

** In yeast.

Table 1. TFIIH enzymatic activities, their functions, and their regulators.

During NER, TTDA stimulates the ATPase activity of XPB through a direct interaction with p52[34]. The TTDA-interacting domain of p52 also binds DNA, and the addition of TTDA triggers dissociation of p52 from DNA. We hypothesize that the recruitment of TTDA to TFIIH provokes a conformational change of the complex that leads to a "bona fide" interaction between p52 and XPB, and to the stimulation of its ATPase activity[35]. The repair factor XPC also regulates the ATPase activity of XPB[36]. On the other hand, p44 stimulates XPD helicase activity, required to unwind DNA around the damage[37]. The MAT1 protein of the CAK module has been shown to inhibit XPD helicase and this

negative effect might be compensated by p44 stimulation[38]. The importance of these regulations is pointed out by the existence of mutations in XP patients that impair the XPB-p52[28] or XPD-p44[37,39] interactions, leading to cancers and premature aging.

THE CAK COMPLEX: THE DOUBLE LIFE OF A KINASE

The CAK complex is composed of Cdk7, cyclin H, and MAT1[40] associated with U1 snRNA[41]. Cdk7 can be found in three different complexes: in CAK alone (50% of Cdk7), in CAK together with core TFIIH (40% of Cdk7), or in CAK with XPD alone (10% of Cdk7) (Figure 2). Several factors, including cyclin H, MAT1, TFIIE, the Mediator[42], XPD[43], and U1 snRNA[44], regulate the activity of Cdk7. Phosphorylation of Cdk7 at threonine 170 is required for the CAK activity[45], while phosphorylation of serine 164 by Cdk1 and Cdk2 inhibits Cdk7 activity in cell cycle progression[46]. MAT1 interacts with both Cdk7 and cyclin H, and thereby stabilizes the assembly of the CAK complex[47,48]. Surprisingly, free CAK and CAK interacting with core TFIIH have significantly different substrate specificity. Free CAK acts as a Cdk-activating kinase and phosphorylates Cdk1, Cdk2, Cdk4, and Cdk6 involved in cell cycle progression[49,50,51,52]. When it interacts with the core, CAK preferentially phosphorylates the carboxy-terminal domain (CTD) of the RNPII large subunit rpb1[53,54]. The CTD consists of multiple repeats of the conserved sequence YSPTSPS, which attract both mRNA- and histone-modifying enzymes, depending on their phosphorylation state[55]. Whereas the serine 5 of the CTD is phosphorylated by TFIIH during transcription initiation, Rtr1 phosphatase removes it during elongation[56]. Recently, another Cdk7-dependent phosphorylation was demonstrated on serine 7 of the CTD in both yeast and mammalian cells[57,58,59]. As a component of TFIIH, CAK also phosphorylates nuclear receptors, including the retinoic acid receptors α and ɣ, the estrogen receptor α[25,60,61], the peroxysome proliferator-activated receptor[62], and the thyroid receptor[63], thereby supporting the transcription of the nuclear receptor–dependent genes. Cdk7 also activates the vitamin D receptor indirectly by phosphorylating the Ets1 coactivator[64]. The role of the CAK-XPD complex is more elusive, but it seems to represent an inactive version of the CAK complex. Indeed, evidence points to a negative regulation of CAK activity by XPD. By this means, XPD

would regulate the progression of the cell cycle at the mitotic phase[65]. While the function of CAK in transcription is well documented, its role in NER is contradictory.

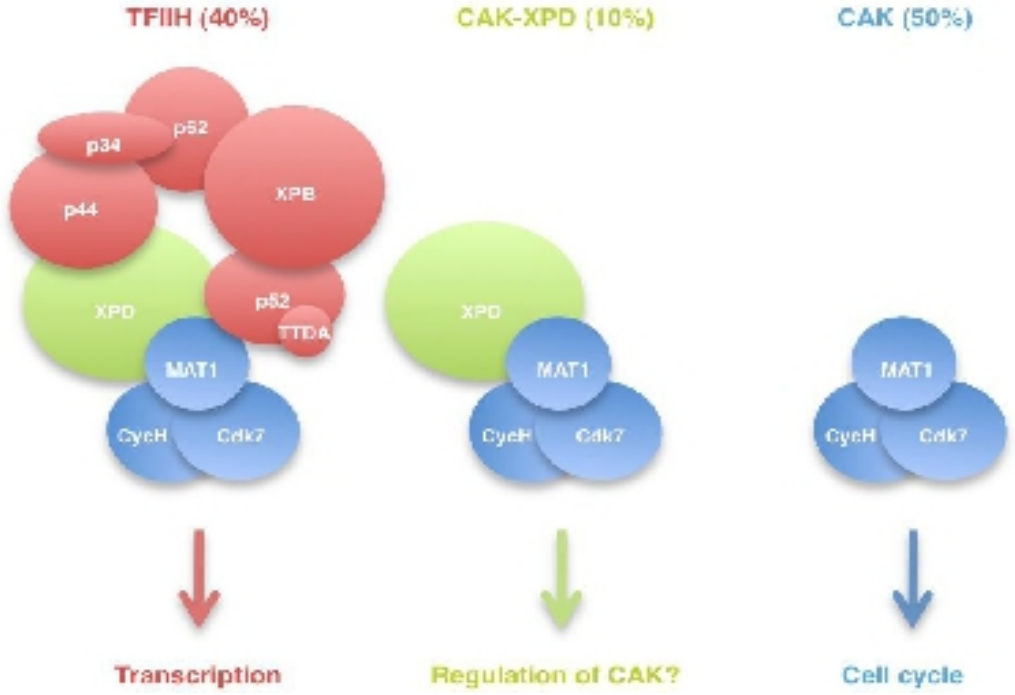

Figure 2. Three Cdk7-containing complexes. Cdk7 kinase can be found in three different complexes, TFIIH, CAK-XPD, and CAK, that represent 40, 10, and 50% of the Cdk7-containing complexes (personal data), respectively. When Cdk7 is in TFIIH, it functions in transcription, while it functions in cell cycle progression when it is in CAK alone or associated with XPD.

Microinjection of anti-Cdk7 immunoglobulins into human fibroblasts affects both transcription and NER[66], but dual incision assay can be reconstituted without the CAK complex. These data suggest that the TFIIH core is sufficient to perform excision of the damaged DNA "in vitro"[67]. Indeed, when Cdk7 expression is knocked down using siRNA, fully functional repair of UV lesions is maintained, whereas transcription activation of UV-inducible genes is significantly inhibited[68]. Also, the kinase activity of CAK is detrimental to the dual incision assay efficiency[67], but UV irradiation affects this activity[48]. In yeast, two TFIIH complexes can be purified in undamaged cells: a core TFIIH associated with repair factors that is active in NER and a CAK-associated TFIIH complex that is active in transcription[69]. Do these two TFIIH complexes coexist in mammalian cells? Is the CAK module physically engaged in NER? How does a single TFIIH complex face the task of participating in both transcription and NER?

To understand how the ten subunits that constitute the TFIIH complex participate in repair and/or transcription processes, we utilized an original approach combining chromatin immunoprecipitation and western blotting (ChIP-Western)[70]. With this technique, we were able to analyze the composition of TFIIH on the chromatin before or after UV irradiation. Surprisingly, we observed a dynamic dissociation/reassociation of the CAK complex onto the core TFIIH that correlates with the recruitment/release of the repair factors during the DNA repair reaction[68] (Figure 3). In the absence of formaldehyde cross-linking, the dissociation of CAK is still observed in the soluble cell fractions, but the UV dose required to dissociate CAK from the core under this condition is much higher than the dose needed to detect the dissociation by ChIP-Western. Also, while repair factors accumulate on the core TFIIH by ChIP, no accumulation was observed on TFIIH in the soluble fraction after UV. These observations advantageously reconcile the yeast and human models by showing that in humans, the free core TFIIH is transient and exists only in the chromatin, during the short period of DNA repair, while in yeast, it exists in the soluble fraction and in absence of DNA damage. In both cases, it is the core TFIIH that participates in DNA repair, in the absence of CAK. Fifteen minutes after 20J/m2 UV-C irradiation, CAK bound to the TFIIH core reaches a minimum level, impaired in XP-C cells, in which TFIIH is not recruited to the damaged DNA[71], suggests that the change in TFIIH subunit composition is due to its participation in the removal of the 6-4PP lesions that are eliminated in the first hours after UV irradiation[72]. After the removal of the 6-4PP lesions (6–8 h post irradiation), we observed the dissociation of the repair factors from the core and the return of the CAK. We noticed that mutations in NER proteins, as found in XP patients, cause an accumulation of intermediate repair complexes that can persist for several hours on the chromatin (Figure 3) whereas the recruitment of repair factors to the TFIIH core reaches its maximum level. At that specific time point, about 70% of TFIIH is involved in DNA repair and does not contain CAK. This early event, together with the fact that the release of CAK is the persistence of these intermediate poised complexes may potentially cause prolonged transcription and replication arrests, and their impact on cell survival should be investigated.

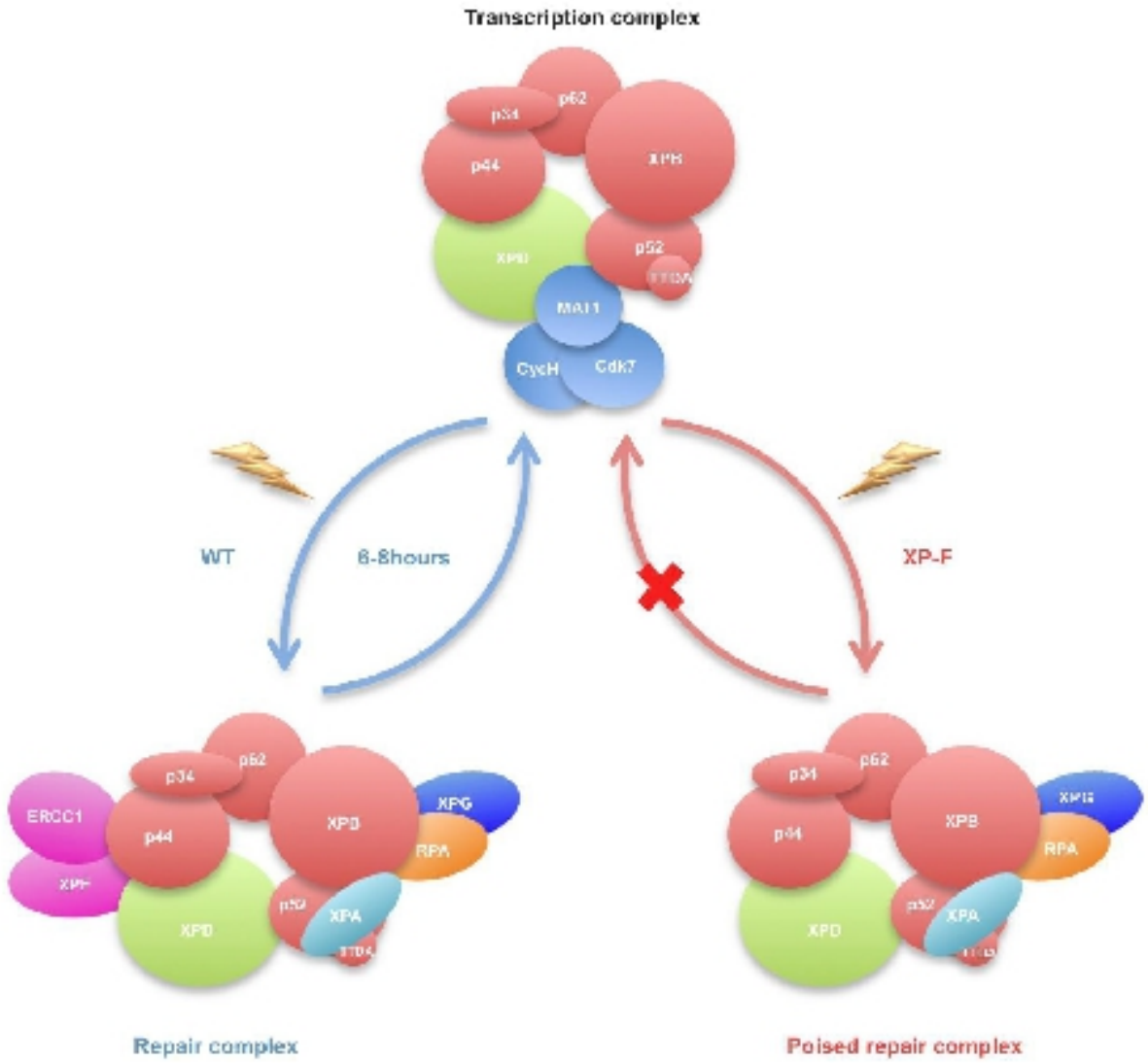

Figure 3. Dynamic composition of the TFIIH complex. When TFIIH functions in transcription, it contains ten subunits, including the CAK module. When it functions in repair, it does not contain the CAK module, but associates with various DNA repair factors, including XPA, RPA, XPG, and ERCC1-XPF. In a wild-type cell, the transcription complexes are present without DNA damage. The repair complexes appear following the generation of damage on the DNA and persist until the lesions are removed (6–8 h). Mutations in NER proteins, as found in XP patients (here XP-F), cause an accumulation of intermediate poised repair complexes that can persist in the chromatin.

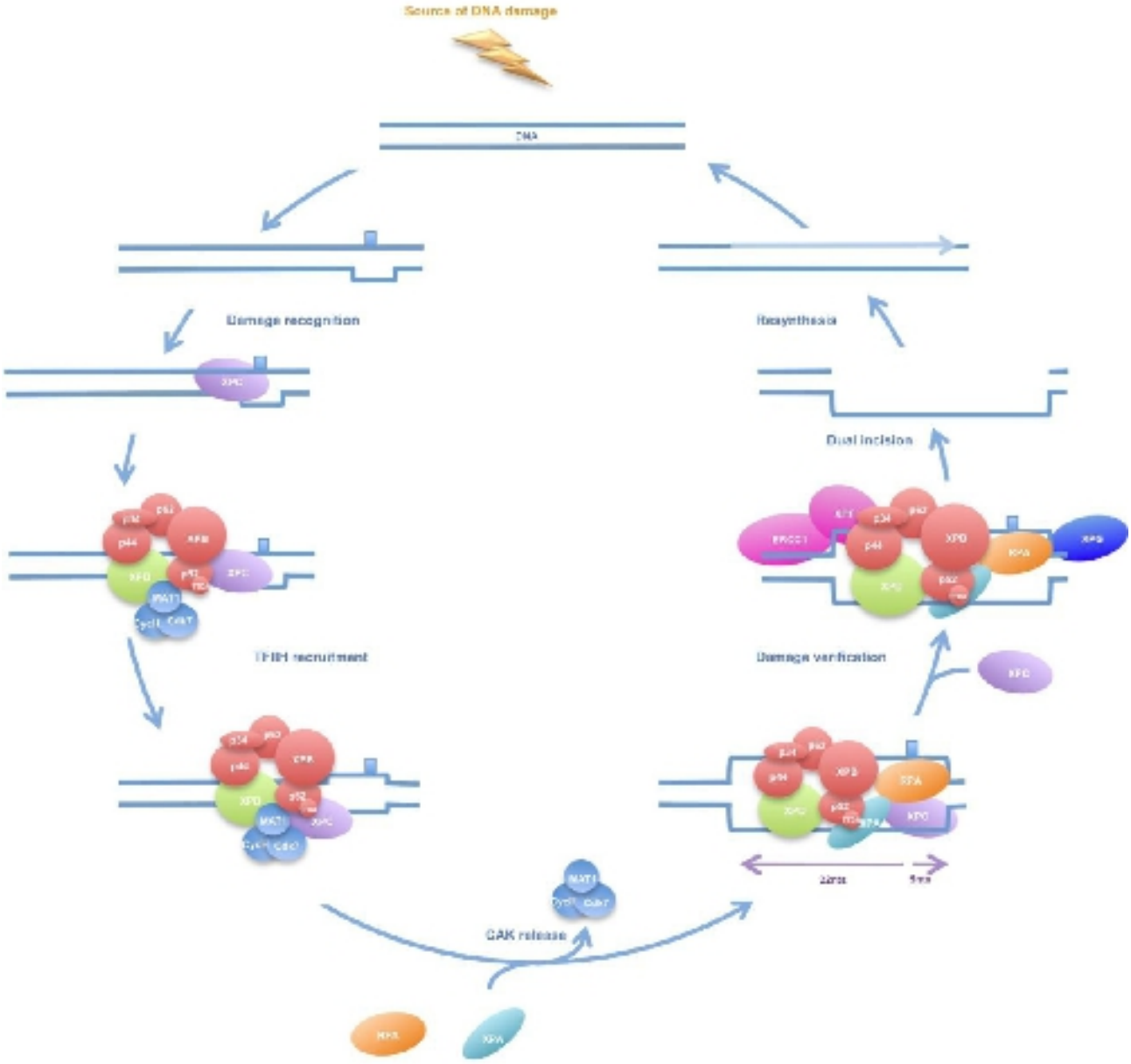

Figure 4. Nucleotide excision repair pathway. Following exposure to genotoxic agents (e.g., sunlight), a lesion (blue square) is created on the DNA. Then, the damage recognition factor XPC-HR23B interacts with the damaged DNA structure on the opposite strand of the lesion. TFIIH joins XPC-HR23B on the damaged DNA. In the presence of ATP, XPB, and XPD, helicases in TFIIH are involved in the opening of the DNA, allowing the stable association of XPA and RPA, which help to enlarge the opened structure and drive the dissociation of the CAK complex from TFIIH. This dissociation is a prerequisite for the enlargement of the DNA opening that favors the arrival of XPG, mediating the release of XPC-HR23B. 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.

The question remains on how and when the CAK is released from the core TFIIH. We

reconstituted the dissociation of the CAK from the core TFIIH *in vitro* and provided evidence that the transformation is catalyzed by the presence of XPA, in an ATP-dependent manner. XPA is known as a scaffold protein without enzymatic activity that nevertheless shows preferential association to damaged DNA and is indispensable for DNA incision[73,74]. Our "in vitro" system demonstrated that the release of CAK by XPA is required to trigger the dual incision of the damaged DNA. The recruitment of XPA and the dissociation of CAK may then constitute a major checkpoint in NER that will elicit the removal of lesions (Figure 4). Consistent with this model, CAK inhibits the helicase activity of XPD[38]. In addition, the recruitment of XPA to the XPC/TFIIH intermediate preincision complex promotes the opening of the damaged DNA[75]. In light of our latest discoveries about CAK release during DNA repair, we propose that detachment of CAK from the core by the damage verification factor XPA will stimulate the helicase/ATPase activities of TFIIH that will lead to opened DNA structures, thereby facilitating DNA repair.

CONCLUSION

Altogether, our results dispel a broadly accepted idea that large nuclear complexes are stable and do not experience large-scale alterations in composition when switching between different cellular processes or different cellular conditions[22]. The flexibility of the TFIIH complex makes it able to participate in various distinct cellular processes. Based on our discovery, we propose that the core TFIIH is involved in the various functions of the complex. The association of the core with different modules enables its engagement in different functions, such as transcription or repair. The two modules, CAK for transcription and TTDA for repair, confer to the core TFIIH and to its helicases the capacity to open unrelated DNA structures, such as promoters or damaged DNA, respectively (Figure 5). The CAK and the TTDA modules seem to coexist in the same TFIIH complex, but how one negatively influences the activity of the other is not known (Figure 5). Whether or not the flexibility in the composition of TFIIH is a more general aspect of its biology that may explain some aspects of the spatial and selective deregulation of nuclear receptor target genes in specific organs[63], or the lesion-specific DNA repair defect observed in XP, TTD, or CS patients[76], merits further

investigations.

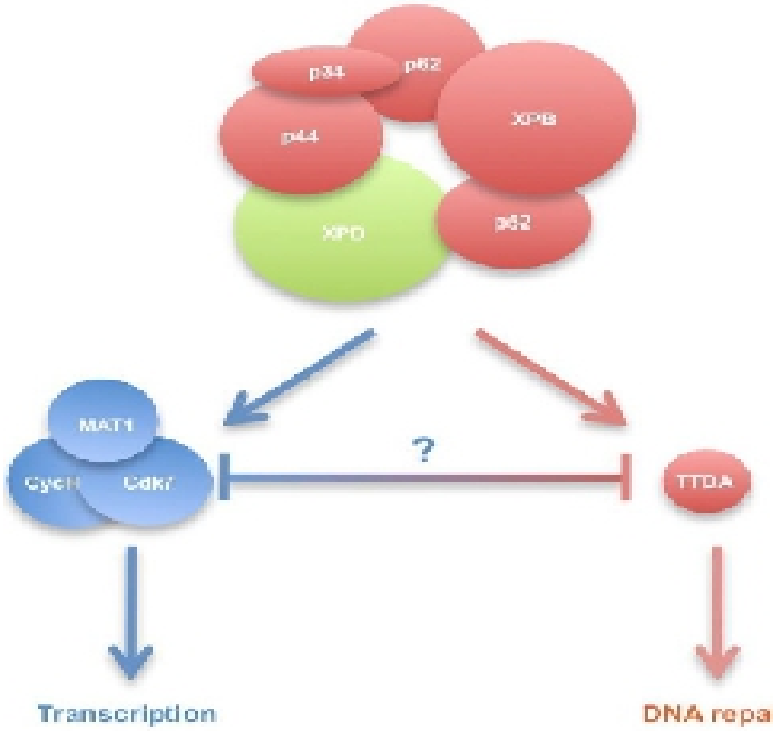

Figure 5. CAK and TTDA modules engage the core TFIIH in different cellular pathways. TFIIH is composed of a core that associates with different modules to enable its engagement in different cellular processes. The presence of CAK in TFIIH engages the core in transcription[29], while the presence of TTDA engages the core in DNA repair[33]. The CAK and the TTDA modules coexist in the same TFIIH complex[33], but the negative influence of one module on the other is not known.

REFERENCES

1. Hoeijmakers, J.H. (2009) DNA damage, aging, and cancer. N. Engl. J. Med. 361, 1475–1485.
2. Lindahl, T. and Wood, R.D. (1999) Quality control by DNA repair. Science 286, 1897–1905.
3. Hanawalt, P. (2002) Subpathways of nucleotide excision repair and their regulation. Oncogene 21, 8949–8956.
4. Lehmann, A.R. (2003) DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. Biochimie 85, 1101–1111.
5. Itin, P.H., Sarasin, A., and Pittelkow, M.R. (2001) Trichothiodystrophy: update on the sulfur-deficient brittle hair syndromes. J. Am. Acad. Dermatol. 44, 891–920; quiz 921–894.
6. Andressoo, J.O., Hoeijmakers, J.H., and Mitchell, J.R. (2006) Nucleotide excision repair disorders and the balance between cancer and aging. Cell Cycle 5, 2886–2888.
7. Zurita, M. and Merino, C. (2003) The transcriptional complexity of the TFIIH complex. Trends Genet. 19, 578–584.
8. 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.
9. 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.
10. 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.
11. 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., Rademakers, S., de Rooij, J., Jaspers, N.G., Hoeijmakers, J.H., and Wood, R.D. (1996) Xeroderma pigmentosum group F caused by a defect in a structure-specific DNA repair endonuclease. Cell 86, 811–822.
12. 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 22 nd phosphodiester bond 5' and the 6th phosphodiester

bond 3' to the photodimer. Proc. Natl. Acad. Sci. U. S. A. 89, 3664–3668.
13. 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.
14. 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.
15. 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., Botta, E., Stefanini, M., Egly, J.M., Aebersold, R., Hoeijmakers, J.H., and Vermeulen, W. (2004) A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A. Nat. Genet. 36, 714–719.
16. Ranish, J.A., Hahn, S., Lu, Y., Yi, E.C., Li, X.J., 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.
17. 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.
18. 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.
19. Dvir, A., Conaway, J.W., and Conaway, R.C. (2001) Mechanism of transcription initiation and promoter escape by RNA polymerase II. Curr. Opin. Genet. Dev. 11, 209–214.
20. Yudkovsky, N., Ranish, J.A., and Hahn, S. (2000) A transcription reinitiation intermediate that is stabilized by activator. Nature 408, 225–229.
21. O'Sullivan, J.M., Tan-Wong, S.M., Morillon, A., Lee, B., Coles, J., Mellor, J., and Proudfoot, N.J. (2004) Gene loops juxtapose promoters and terminators in yeast. Nat. Genet. 36, 1014–1018.
22. 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.
23. Bradsher, J., Auriol, J., Proietti de Santis, L., Iben, S., Vonesch, J.L., Grummt, I., and Egly, J.M. (2002) CSB is a component of RNA pol I transcription. Mol. Cell 10, 819–829.
24. Lu, H., Zawel, L., Fisher, L., Egly, J.M., and Reinberg, D. (1992) Human general transcription factor IIH phosphorylates the C-terminal domain of RNA polymerase II. Nature 358, 641–645.
25. Rochette-Egly, C., Adam, S., Rossignol, M., Egly, J.M., 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.
26. 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.
27. Oksenych, V., de Jesus, B.B., Zhovmer, A., Egly, J.M., and Coin, F. (2009) Molecular insights into the recruitment of TFIIH to sites of DNA damage. EMBO J. 28(19), 2971–2980.
28. Coin, F., Oksenych, V., and Egly, J.M. (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.
29. 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.
30. Seroz, T., Perez, C., Bergmann, E., Bradsher, J., and Egly, J.M. (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, 33260–33266.
31. Takagi, Y., Masuda, C.A., Chang, W.H., Komori, H., Wang, D., Hunter, T., Joazeiro, C.A., and Kornberg, R.D. (2005) Ubiquitin ligase activity of TFIIH and the transcriptional response to DNA damage. Mol. Cell 18, 237–243.
32. 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.
33. Coin, F., Proietti De Santis, L., 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.
34. Kainov, D.E., Vitorino, M., Cavarelli, J., Poterszman, A., and Egly, J.M. (2008) Structural basis for group A trichothiodystrophy. Nat. Struct. Mol. Biol. 15(9), 980–984.
35. Kainov, D.E., Selth, L.A., Svejstrup, J.Q., Egly, J.M., and Poterzsman, A. (2009) Interacting partners of the Tfb2 subunit from yeast TFIIH. DNA Repair (Amst.) 9(1), 33–39.
36. Bernardes de Jesus, B.M., Bjoras, M., Coin, F., and Egly, J.M. (2008) Dissection of the molecular defects caused by pathogenic mutations in the DNA repair factor XPC. Mol. Cell. Biol. 28, 7225–7235.
37. 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.
38. 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.
39. 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.
40. Roy, R., Schaeffer, L., Humbert, S., Vermeulen, W., Weeda, G., and Egly, J.M. (1994) The DNA-dependent ATPase activity associated with the class II transcription factor BTF2/TFIIH. J. Biol. Chem. 269, 9826–9832.
41. Kwek, K.Y., Murphy, S., Furger, A., Thomas, B., O'Gorman, W., Kimura, H., Proudfoot, N.J., and Akoulitchev, A. (2002) U1 snRNA associates with TFIIH and regulates transcriptional initiation. Nat. Struct. Biol. 9, 800–805.
42. Svejstrup, J.Q., Li, Y., Fellows, J., Gnatt, A., Bjorklund, S., and Kornberg, R.D. (1997) Evidence for a mediator cycle at the initiation of transcription. Proc. Natl. Acad. Sci. U. S. A. 94, 6075–6078.
43. 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.
44. O'Gorman, W., Thomas, B., Kwek, K.Y., Furger, A., and Akoulitchev, A. (2005) Analysis of U1 small nuclear RNA interaction with cyclin H. J. Biol. Chem. 280, 36920–36925.
45. Larochelle, S., Chen, J., Knights, R., Pandur, J., Morcillo, P., Erdjument-Bromage, H., Tempst, P., Suter, B., and Fisher, R.P. (2001) T-loop phosphorylation stabilizes the CDK7-cyclin H-MAT1 complex in vivo and regulates its CTD kinase activity. EMBO J. 20, 3749–3759.
46. Akoulitchev, S. and Reinberg, D. (1998) The molecular mechanism of mitotic inhibition of TFIIH is mediated by phosphorylation of CDK7. Genes Dev. 12, 3541–3550.
47. Devault, A., Martinez, A.M., Fesquet, D., Labbé, J.C., Morin, N., Tassan, J.P., Nigg, E.A., Cavadore, J.C., and Dorée, M. (1995) MAT1 ('ménage à trois') a new RING finger protein subunit stabilizing cuclin H-cdk7 complexes in starfish and Xenopus CAK. EMBO J. 14, 5027–5036.
48. Adamczewski, J.P., Rossignol, M., Tassan, J.P., Nigg, E.A., Moncollin, V., and Egly, J.M. (1996) MAT1, cdk7 and cyclin H form a kinase complex which is UV light-sensitive upon association with TFIIH. EMBO J. 15, 1877–1884.
49. Nigg, E.A. (1996) Cyclin-dependent kinase 7: at the cross-roads of transcription, DNA repair and cell cycle control? Curr. Opin. Cell Biol. 8, 312–317.
50. Kaldis, P. (1999) The cdk-activating kinase (CAK): from yeast to mammals. Cell. Mol. Life Sci. 55, 284–296.
51. Harper, J.W. and Elledge, S.J. (1998) The role of Cdk7 in CAK function, a retro-retrospective. Genes Dev. 12, 285–289.
52. Fisher, R.P. (2005) Secrets of a double agent: CDK7 in cell-cycle control and transcription. J. Cell Sci. 118, 5171–5180.
53. 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.
54. Yankulov, K.Y. and Bentley, D.L. (1997) Regulation of CDK7 substrate specificity by MAT1 and TFIIH. EMBO J. 16, 1638–1646.
55. Komarnitsky, P., Cho, E.J., and Buratowski, S. (2000) Different phosphorylated forms of RNA polymerase II and associated mRNA

processing factors during transcription. Genes Dev. 14, 2452–2460.
56. Qiu, H., Hu, C., and Hinnebusch, A.G. (2009) Phosphorylation of the Pol II CTD by KIN28 enhances BUR1/BUR2 recruitment and Ser2 CTD phosphorylation near promoters. Mol. Cell 33, 752–762.
57. Akhtar, M.S., Heidemann, M., Tietjen, J.R., Zhang, D.W., Chapman, R.D., Eick, D., and Ansari, A.Z. (2009) TFIIH kinase places bivalent marks on the carboxy-terminal domain of RNA polymerase II. Mol. Cell 34, 387–393.
58. Glover-Cutter, K., Larochelle, S., Erickson, B., Zhang, C., Shokat, K., Fisher, R.P., and Bentley, D.L. (2009) TFIIH-associated Cdk7 kinase functions in phosphorylation of C-terminal domain Ser7 residues, promoter-proximal pausing, and termination by RNA polymerase II. Mol. Cell. Biol. 29, 5455–5464.
59. Kim, M., Suh, H., Cho, E.J., and Buratowski, S. (2009) Phosphorylation of the yeast Rpb1 C-terminal domain at serines 2, 5, and 7. J. Biol. Chem. 284, 26421–26426.
60. Bastien, J., Adam-Stitah, S., Riedl, T., Egly, J.M., Chambon, P., and Rochette-Egly, C. (2000) TFIIH interacts with the retinoic acid receptor gamma and phosphorylates its AF-1-activating domain through cdk7. J. Biol. Chem. 275, 21896–21904.
61. Chen, D., Riedl, T., Washbrook, E., Pace, P.E., Coombes, R.C., Egly, J.M., 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, 127–137.
62. Compe, E., Drane, P., Laurent, C., Diderich, K., Braun, C., Hoeijmakers, J.H., and Egly, J.M. (2005) Dysregulation of the peroxisome proliferator-activated receptor target genes by XPD mutations. Mol. Cell. Biol. 25, 6065–6076.
63. Compe, E., Malerba, M., Soler, L., Marescaux, J., Borrelli, E., and Egly, J.M. (2007) Neurological defects in trichothiodystrophy reveal a coactivator function of TFIIH. Nat. Neurosci. 10, 1414–1422.
64. Drane, P., Compe, E., Catez, P., Chymkowitch, P., and Egly, J.M. (2004) Selective regulation of vitamin D receptor-responsive genes by TFIIH. Mol. Cell 16, 187–197.
65. Chen, J., Larochelle, S., Li, X., and Suter, B. (2003) Xpd/Ercc2 regulates

CAK activity and mitotic progression. Nature 424, 228–232.
66. 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.
67. 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.
68. Coin, F., Oksenych, V., Mocquet, V., Groh, S., Blattner, C., and Egly, J.M. (2008) Nucleotide excision repair driven by the dissociation of CAK from TFIIH. Mol. Cell 31, 9–20.
69. 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.
70. 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.
71. 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.
72. 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.
73. de Vries, A., van Oostrom, C.T., Hofhuis, F.M., Dortant, P.M., Berg, R.J., de Gruijl, F.R., Wester, P.W., van Kreijl, C.F., Capel, P.J., van Steeg, H., et al. (1995) Increased susceptibility to ultraviolet-B and carcinogens of mice lacking the DNA excision repair gene XPA. Nature 377, 169–173.
74. Missura, M., Buterin, T., Hindges, R., Hubscher, U., Kasparkova, J., Brabec,

V., and Naegeli, H. (2001) Double-check probing of DNA bending and unwinding by XPA-RPA: an architectural function in DNA repair. EMBO J. 20, 3554–3564.

75. 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.

76. 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.

77. Coin, F. et al. (2004) Phosphorylation of XPB helicase regulates TFIIH nucleotide excision repair activity. EMBO J. 23(24), 4835–4846.

PART II - DNA REPAIR IN VIVO

CHROMATIN REMODELLING

Chromatin is an ensemble of genomic DNA and DNA binding proteins, primary histones, that package it into a smaller volume. Upon DNA damage the highly condensed state of chromatin is an obstacle for DNA repair machinery that have to promptly detect and remove lesion hidden among billions base pairs. Thus it is not surprisingly that eukaryotic cells possess specialized proteins that rearrange nucleosomes and modify histones tails alleviating access to DNA for DNA repair proteins[1].

Nucleosome rearrangement and subsequent chromatin reorganization induced by DNA damage involve recycling of parental histones and incorporation of new histones. H3.1 histones, the good illustration of this process, get incorporated "in vivo" at repair sites and the deposition depends on nucleotide excision repair occurring at the post repair stage. Histone chaperone chromatin assembly factor 1 (CAF-1) is tightly involved in that histone deposition process and shows distinct localization to the damage sites there CAF-1 associates with several NER factors: TFIIH, PCNA and RPA in chromatin[2,3]. The RPA, particularly N-terminal domain of the RPA 70-kDa subunit (RPA70N), physically interacts with another chromatin remodeler - human DNA helicase B. Similarly to CAF-1, exposure to UV radiation induces accumulation of the HELB on chromatin in a dose - and time - dependent manner[4].

CAF-1 and HELB are not the only proteins that change chromatin structure and interact with NER proteins upon DNA damage. A large group of SWI/SNF chromatin remodelers, ISWI, INO80, CHD proteins are shown to have role in DDR and DNA repair. Thus in nematode, C. elegans, four SWI/SNF and four ISWI/Cohesin family chromatin remodeling factors are known to be implicated in the UV damage response[8].

In yeast, S. cerevisiae, NER damage-recognition heterodimer RAD4-RAD23 copurifies with the SNF5 and SNF6, subunits of the SWI/SNF chromatin-remodeling complex. And the interaction between SWI/SNF and RAD4-RAD23 is stimulated by the UV radiation. When SWI/SNF is inactivated, NER and UV - induced nucleosome rearrangement at the silent HML locus, region of condensed chromatin, is markedly attenuated[5]. Human SWI/SNF0, particularly its SNF5 component, also interacts with

UV damage recognition factor XPC (RAD4 in yeast) and colocalizes with it at the damage sites where SNF5 facilitates the access of ATM, which in turn promotes H2AX and BRCA1 phosphorylation[6]. Moreover, in mammalian cells, knockdown of BRG1, the ATPase subunit of SWI/SNF, negatively affects the elimination of CPD following UV irradiation[7].

In yeast, RAD4-RAD23 interacts with chromatin-remodeling complex INO80. And INO80 is recruited to the chromatin by RAD4 in a UV damage-dependent manner. Remarkably, INO80 mutant cells have a defect in restoration of nucleosome structure after the finishing of DNA repair[10]. Also mammalian INO80 alongside with ARP5 are enriched to UV-damaged DNA. Deletion of these two core components of INO80 complex, INO80 and ARP5, significantly hampers cellular removal of UV-induced photo lesions[11].

Some experiments show ISWI and CHD proteins to play a role in DNA repair. Human ISWI proteins, SNF2H and SNF2L ISWI proteins as well as ACF1 accumulate at UV-induced DNA damage sites within tens of seconds[9]. Uncertainty exists for NER role of CHD family chromatin remodelers - based on the analogy to DSB repair, it is expected that these complexes are involved in NER[13]. Thus chromodomain helicase DNA-binding protein CHD2 mutant cells are defective in their ability to repair DNA damage induced by ionizing and ultraviolet radiation[12].

Summarizing the foregoing data biologists start to compile molecular model of action for SWI/SNF and INO80 remodeling complexes(Figure 6). However the role of other complexes, such as ISWI and CHD, needs more attention. Although possible vagueness, it is certainly known that chromatin remodeling is an essential part of proper DDR(Table2). Chromatin remodelers are indispensable for repair machinery to access and correct DNA lesions at damage sites. Once the repair processes are finished chromatin remodelers reestablish chromatin structure at these sites restarting normal cellular transcription and replication.

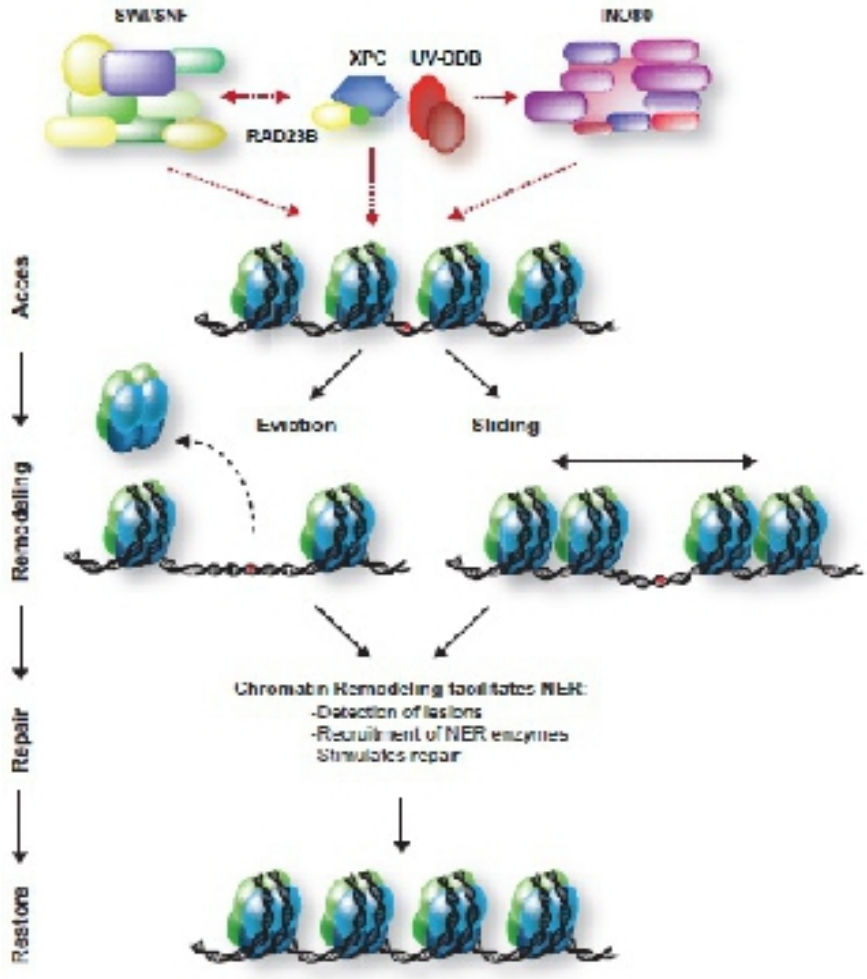

Figure 6. Mammalian nucleotide excision repair (NER)-associated chromatin remodeling. Both the SWI/SNF and the INO80 ATP-dependent chromatin-remodeling complexes are recruited to sites of UV-induced DNA damage, and are implicated in mammalian global genome NER (GG-NER). SWI/SNF may interact with the damage-detection complexes XPC/RAD23 and UV-DDB, and stimulate recruitment of XPC to the damage. Recruitment of SWI/SNF is also stimulated by XPC. In addition, mammalian INO80 interacts with UV-DDB, and stimulates recruitment of XPC. Together, SWI/SNF and INO80 are thought to regulate accessibility of DNA by sliding or eviction of nucleosomes at the damaged site. Red dotted arrows depict chromatin recruitment and protein-protein interactions. Adapted from [13].

CHROMATIN MODIFICATIONS

Probably, the very first histone modification induced by DNA damage, phosphorylation of the histone H2A variant, H2AX, at Serine 139 to generate γ-H2AX, was discovered in 1998[15,16]. Since that time much of our present understanding of

nucleotide excision repair mechanism has been obtained from biochemical investigations “in vitro”. Biochemical reaction, as it occurs on naked DNA substrate, is significantly distinctive from that on DNA heavily loaded with proteins as it is presented “in vivo” in the nucleus of living cell. Therefore “in vitro” studies generally miss histone modifications and modifications of other chromatin-associated proteins. And notwithstanding great advancement in comprehension of NER in recent years we are still lacking the information about DNA repair in context of chromatin[17,18].

Remodelling complex	Snf2-protein subunit	DDR-associated activities
INO80	Ino80 (yeast)	Recruited via phospho-H2AX (γ-H2AX). Evicts nucleosome at damage sites. Participates in the DNA-damage cell cycle checkpoint pathway.
SWR1	Swr1 (yeast)	Recruited via γ-H2AX. Important for error-free non-homologous end-joining (NHEJ) pathway.
SWI/SNF	Snf2 (yeast) Brg1 or hBrm (human)	Associated with the homologous recombination (HR) repair pathway – possibly to clear nucleosomes and expose donor sequences to repair machinery. Human SWI/SNF facilitates γ-H2AX accumulation at DSBs.
RSC	Sth1 (yeast)	Linked with both HR and NHEJ repair pathways. May remodel DSB sites to facilitate loading of other repair factors.
Alc1	Alc1 (human)	Recruitment to damage sites by PARylation. Remodelling activity (*in vitro*) stimulated by PARylation of PARP1 and/or nucleosomes. Alc1-depletion leads to hypersensitivity to damaging agents.
NuRD	CHD4 (human)	Recruitment to damage sites by PARylation. CHD4 is phosphorylated by ATM kinase – a major DSB checkpoint kinase. CHD4-depletion leads to hypersensitivity to damaging agents and deficient DSB repair.
ACF	Snf2H (human)	ACF accumulates at sites of DNA damage. ATPase activity of Snf2H required for efficient DSB repair.

Table2. SNF2-protein complexes involved in DDR. Adapted from [14].

DNA damage response is associated with such modifications of histones and histone - binding proteins as poly(ADP-rybosyl)ation, phosphorylation, acetylation, methylation, ubiquitination and SUMOylation. They all are typical chromatin modifications that could be found at sites of DNA damage(Figure 7, Figure 8).

In yeast, importance of histone modifications for NER is demonstrated by histone H3K4R and H3K79R modification sites mutants. These mutants show decreased UV survival and impaired NER at the transcriptionally silent HML locus, maintaining normal NER in the constitutively expressed RPB2 gene. They have normal repair of UV lesions by photolyase and nucleotide excision repair in minichromosomes and slightly enhanced repair in the subtelomeric region. H3K79 methylation is shown to participate in GGR in both nucleosomal core regions and internucleosomal linker DNA playing no role in TCR. More precisely, H3K79 trimethylation contributes to but is not absolutely

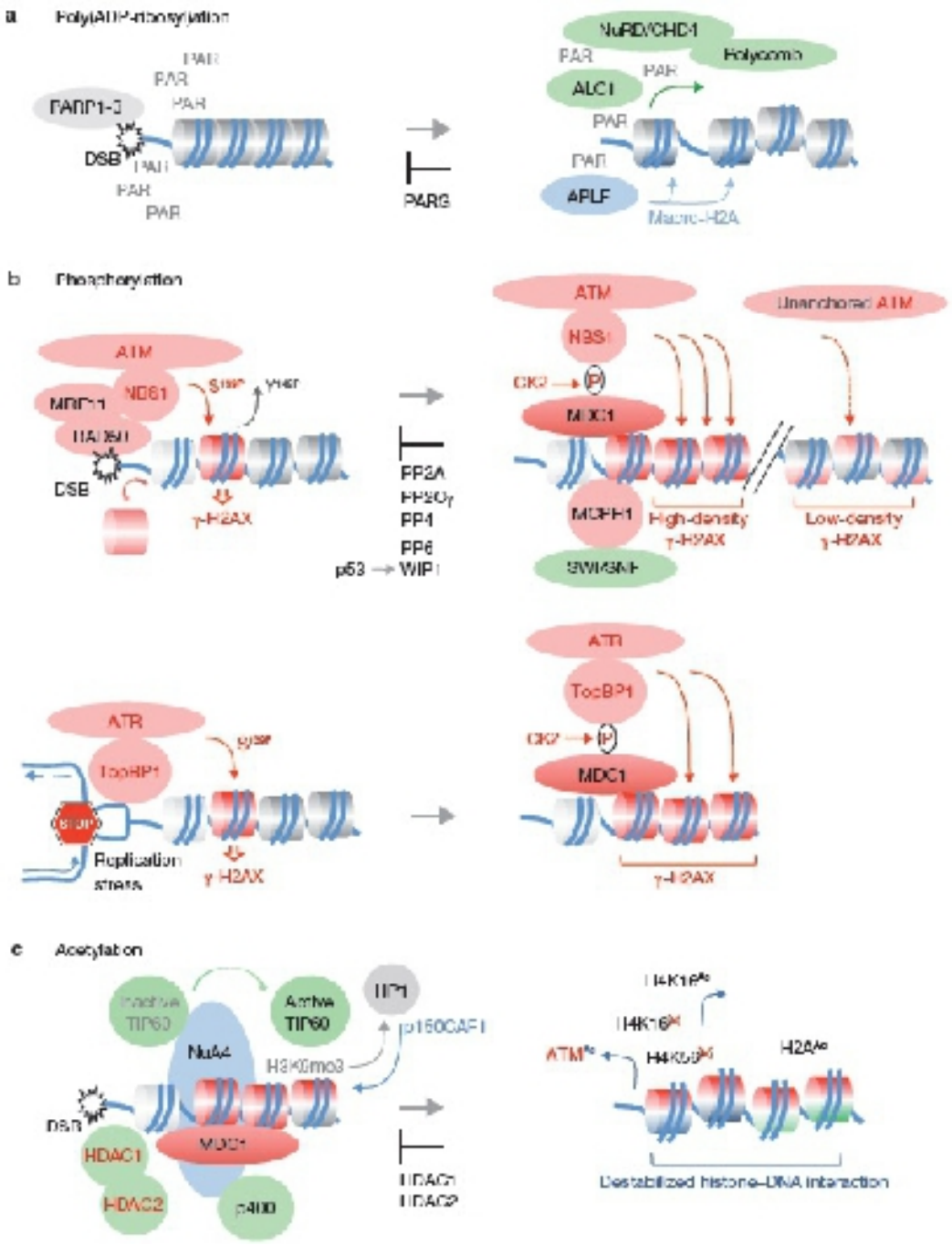

Figure 7. Chromatin responses to DNA damage are orchestrated by a series of post-translational modifications. These include (a) poly(ADP-ribosyl)ation, (b) phosphorylation and (c) acetylation. Left panels depict the key protein complexes involved in a given modification together with the mechanisms regulating their recruitment to the sites of DNA damage. Right panels indicate the impact of a given modification on chromatin restructuring and/ or recruitment of proteins to this compartment. Grey; poly(ADP-ribosyl)ation. Red; phosphorylation (MDC1 is depicted in dark red to highlight its central coordinating role in most steps of DNA-damage-induced chromatin development). Green; activities that alter chromatin compaction or topology. The activities that reverse a given modification are highlighted in black at the transition between left and right panels. Adapted from [19].

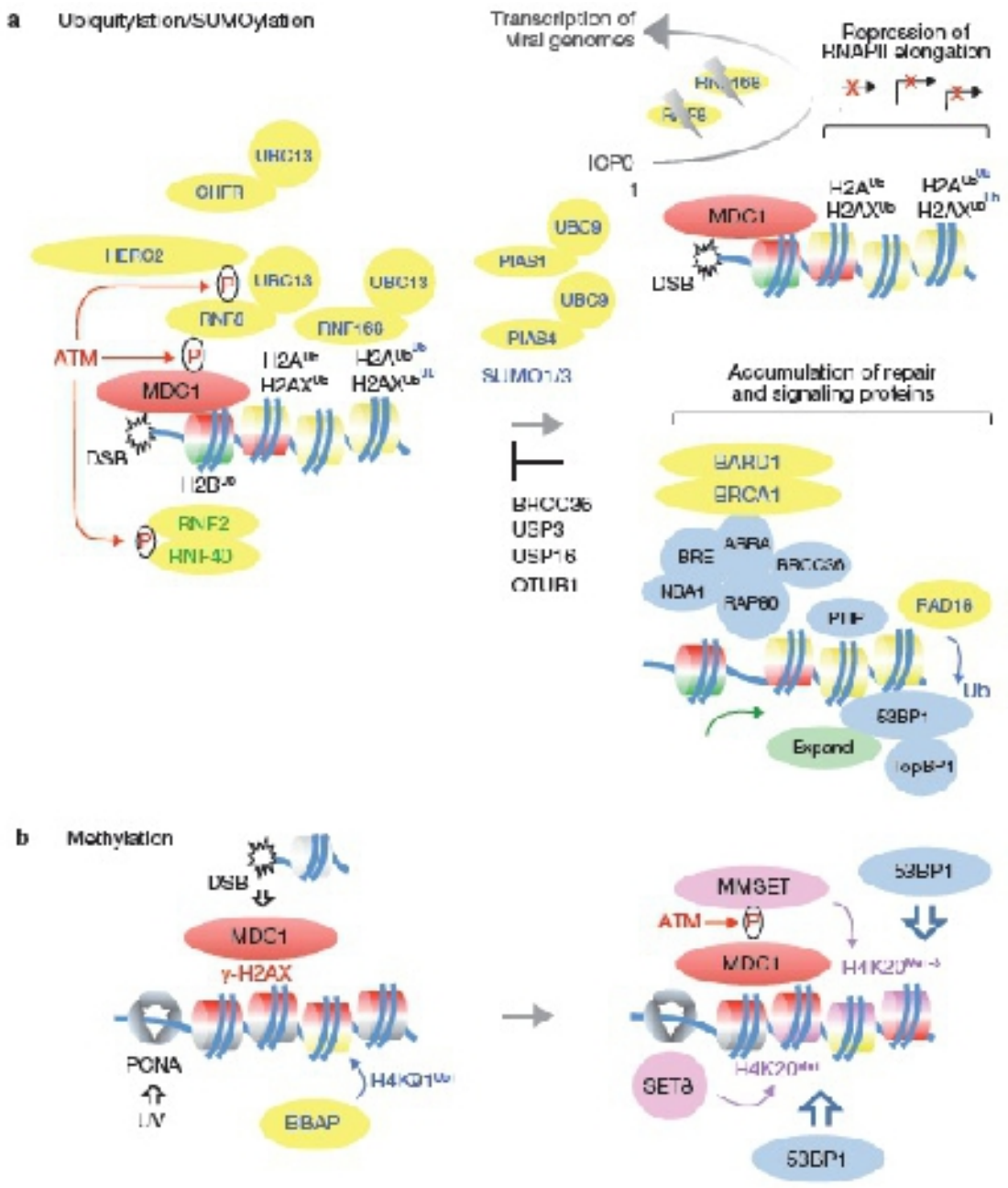

Figure 8. Post-translational modifications that affect chromatin organization and recruit additional genome caretakers to promote repair and suppress transcription in the vicinity of DNA lesions. (a) Ubiquitylation with SUMOylation. (b) Methylation. Yellow; enzymes involved in ubiquitylation and SUMOylation. Pink; enzymes involved in methylation. Adapted from [19].

required for GGR and may serve as an additional docking site for the GGR machinery on the chromatin[20-22].

Acetylation of histone H3 and histone H4 is also important for NER. It is significantly more efficient in the non-repressed subtelomere regions where UV

radiation stimulates both histones H3 and H4 acetylation. Particularly, in response to UV radiation-induced DNA damage, increased histone H3 acetylation at lysine 9 and 14 correlates with changes in chromatin structure and efficiency of GGR in yeast, and depends on the presence of the RAD16 and GCN5 proteins[23-24]. Beside the GCN5, molecular mechanism of histone acetylation involves E2F1 protein that accumulates at sites of both DNA double-strand breaks and UV radiation-induced damage. Moreover, it is E2F1 that associates with the GCN5 acetyltransferase in response to UV radiation and recruits GCN5 to sites of damage[25]. Acetylation is equivalently important for non - histone proteins. Cell-cycle arrest, apoptosis, senescence- they all are widely accepted as the major mechanisms by which p53 inhibits tumor formation. Study in mice bearing lysine to arginine mutations at one p53 ^{K117R} or three p53 ^{3KR}(K117R+K161R+K162R) of p53 acetylation sites shows that although p53 $^{K117R/K117R}$ cells are competent for p53-mediated cell-cycle arrest and senescence, but not apoptosis, all three of these processes are ablated in p53 $^{3KR/3KR}$ cells. Surprisingly, unlike p53 null mice, which rapidly succumb to spontaneous thymic lymphomas, early-onset tumor formation does not occur in either p53 $^{K117R/K117R}$ or p53 $^{3KR/3KR}$ animals. Notably, p53 ^{3KR} retains the ability to regulate energy metabolism and reactive oxygen species production[26]. Thus acetylation modulates activities of p53 protein as well as other non - histone and histone proteins in response to DNA damage.

Chromatin phosphorylation involves action of different DNA-dependent protein kinase complexes(DNA-PKcs) and their subunits during DDR. Depletion of DNA-PKcs mediated by siRNA significantly decreases the TCR capacity of repairing the UV-induced CPDs damage in DHFR gene in HeLa cells, indicating that DNA-PKcs may also be involved in the TCR pathway of DNA damage repair. Probable mechanisms are not clear but the presence of DSB at a human RNAPII - transcribed gene leads to inhibition of transcription elongation and reinitiation. Upon inhibition of DNAPK, RNAPII bypasses the break and continues transcription elongation, suggesting that role of DNAPK is inhibition of RNAPII processivity[28-29]. Phosphorylation by p38 MAPK is also required for the prompt repair of UV-induced DNA damage CPD. Moreover p38 MAPK mediates UV-induced histone H3 acetylation, chromatin relaxation, UV-induced DDB2 ubiquitylation and degradation via phosphorylation of the target protein. p38 MAPK is

required for the recruitment of NER factors XPC and TFIIH to UV-induced DNA damage sites showing great importance of phosphorylation for proper coordination of DDR[27].

Another type of DDR linked modifications mentioned above is poly(ADP-ribosyl)ation. In mammalian cells, it rapidly appears in response to ultraviolet radiation. UV activates a nuclear enzyme poly(ADP-ribose) polymerase-1. And human fibroblasts depleted of PARP show a decreased UV survival. That depletion of PARP in NER-proficient human fibroblasts also decreases the host cell reactivation of UVB - or UVC-damaged reporter gene to a level closer to that in the XP-C and CS-B cell lines, which are deficient in the lesion recognition steps of the global genome repair and transcription-coupled repair sub-pathways of NER, respectively[30].

Ubiquitin and ubiquitin - like modifiers such as SUMO are as important for DDR and DNA repair as other modifications. They mediate signal transduction through post - translational modification of substrate proteins in pathways that control differentiation, apoptosis, cell cycle and responses to stress. In yeast, two SUMO E3 ligases SIZ1 and SIZ2 are responsible for the vast majority of SUMOylation and siz1Δ siz2Δ mutants are sensitive to ultraviolet light and are deficient in repair of both the transcribed and non-transcribed strands of the DNA. Mechanism of this deficiency is unclear but several factors that participate in NER are SUMOylated, including RAD4, RAD16, RAD7, RAD1, RAD10, SSL2, RAD3, and RPB4. And SUMO attachment to most of these NER factors is significantly increased by DNA damage[33]. Additionally, In yeast, the proliferating cell nuclear antigen PCNA is modified by ubiquitin and by SUMO. Ub–PCNA signal for recruitment of translesion DNA polymerases and SUMO–PCNA signals for recruitment of the anti-recombinogenic DNA helicase SRS2[31]. Ub-PCNA is also specifically recognized by Spartan that is recruited to sites of UV damage in a manner dependent upon the PCNA ubiquitylation. Spartan colocalizes and interacts with RAD18, the E3 ubiquitin ligase that modifies PCNA. Knockdown of Spartan compromises chromatin association of RAD18, monoubiquitylation of PCNA, and localization of Pol η to UV damage. Thus, a “reader” of ubiquitylated PCNA, Spartan creates a feed-forward loop to enhance PCNA ubiquitylation and translesion DNA synthesis[32].

UV induces modifications of histones and histone - associated proteins. Methylation, acetylation, phosphorylation, poly(ADP-rybosyl)ation, ubiquitination and

SUMOylation are just on top of iceberg of many unstudied and even undiscovered types of protein modifications that mediate DDR. They helps DNA repair machinery to access DNA preserved in high-order chromatin structures and remove lesions. Modifications serve as dynamic instructions that insure proper coordination of different signaling pathways acting in DDR.

RNA IN DAMAGE RESPONSE

DDR involves a complex network of processes that detect and repair DNA damage, in which miRNAs, siRNAs and other classes of regulatory RNAs, could play intriguing roles. There is very little known about whether and how miRNA expression is regulated in the DNA damage response(Figure 9)[34]. Thus Piwil2-deficient mouse embryonic fibroblasts are defective in cyclobutane pyrimidine dimers repair after UV treatment[35]. Also some striking results were shown for~21-nucleotide small RNAs that are produced from the sequences in the vicinity of DSB sites in Arabidopsis and in human cells. Biogenesis of these RNAs, in Arabidopsis, requires the PI3 kinase ATR, RNA polymerase IV (Pol IV), and Dicer-like proteins. Mutations in these proteins as well as in Pol V cause significant reduction in DSB repair efficiency. In Arabidopsis, these RNAs are recruited by Argonaute 2 (AGO2) to mediate DSB repair. Knock down of Dicer or Ago2 in human cells also reduces DSB repair. Therefore small RNAs may function as guide molecules directing chromatin modifications or the recruitment of protein complexes to DSB sites to facilitate repair[36]. In human, mouse and zebrafish, DICER and DROSHA, but not downstream elements of the RNAi pathway, are necessary to activate the DDR upon exogenous DNA damage and oncogene-induced genotoxic stress, as studied by DDR foci formation and by checkpoint assays. DDR foci are sensitive to RNase A treatment, and DICER- and DROSHA - dependent RNA products are required to restore DDR foci in RNase A - treated cells. RNA deep sequencing and the study of DDR activation at a single inducible DNA double-strand break, demonstrate that DDR foci formation requires site-specific DICER- and DROSHA-dependent small RNAs, named DDRNAs, which act in a MRE11-RAD50-NBS1-complex-dependent manner (MRE11 also known as MRE11A; NBS1 also known as NBN). DDRNAs, either chemically synthesized or in vitro generated by DICER cleavage, are sufficient to restore the DDR in RNase A-

treated cells, also in the absence of other cellular RNAs. These results describe an unanticipated direct role of a novel class of ncRNAs in the control of DDR activation at sites of DNA damage[55]. And probably one day, based on the analogy to DSB repair, RNAs will be found to play a role in NER as well.

NEW ELEMENTS FIT NER JIGSAW PUZZLE

Eukaryotic cells harbor a large complex of molecules to identify lesions and to launch a cascade of reactions in order to perform adaptive response to the DNA damage (Table 3). Among these enzymes recently discovered UV - DDB proteins, CENTRIN2, and well-known XPC - HHR23B have a very specific role in lesion detection.

Damage-specific DNA binding protein 1 (DDB1) was first isolated as a subunit of a heterodimeric complex that recognizes the UV-induced DNA lesions in the nucleotide excision repair pathway. DDB1 and CULLIN 4A (DDB2) form a complex that promotes the global genome repair, whereas DDB1 and Cockayne syndrome group A protein form a complex that contributes to the transcription-coupled repair pathway. DDB1, as a component of an ubiquitin-E3 ligase complex, functions as substrate or adapter protein between DDB2 and CUL4 - associated factors to target substrates for ubiquitination. This CUL4 – DDB1 - E3 - ligase complex regulates the selective proteolysis of key proteins in DNA repair, replication and transcription, thus DDB1 plays a role in transcriptional regulation of UV-induced genes[39]. Mechanistically, CUL4B-DDB1 E3 ligase specifically binds to mononucleosomes assembled with human recombinant histone octamers and nucleosome - positioning DNA containing CPD or 6-4PP photolesions. Subsequent ubiquitination of H2A Lys119/Lys120 is a necessary step for destabilization of nucleosomes and concomitant release of CUL4B-DDB1 from photolesion - containing DNA[37]. Additional regulation of this step is offered by CSN protein, a negative regulator of cullin-based ubiquitin ligases, that dissociates from the DDB2 complex when the complex binds to damaged DNA as well as XPC and Ku that oppositely regulate the ubiquitin ligase activity, especially around damaged sites[38]. The structures of the fully assembled DDB1-DDB2-CUL4A/B-RBX1 ligases also reveal that the mobility of the ligase arm creates a defined ubiquitination zone around the damage, which precludes direct ligase activation by DNA lesions.

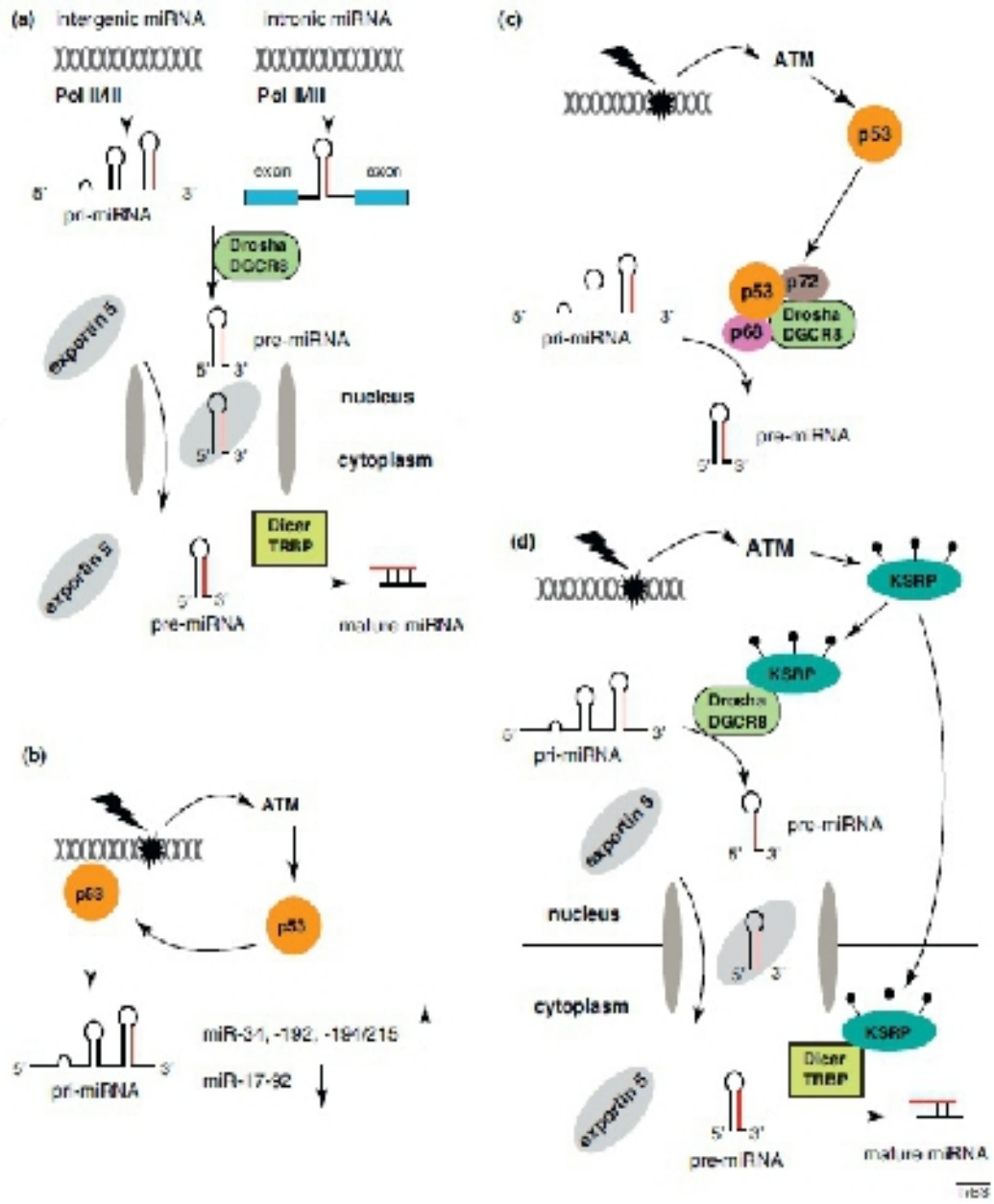

Figure 9. miRNA biogenesis in the DNA damage response. (a) miRNA biogenesis without DNA damage stress. Intergenic miRNA genes are transcribed by RNA polymerase II/III (Pol II/III) into pri-miRNAs, whereas intronic miRNAs are transcribed together with their host genes. The Drosha–DGCR8 complex recognizes and cleaves pri-miRNAs into pre-miRNAs in the nucleus. Pre-miRNA is exported to the cytoplasm by exportin-5 in a RanGTP-dependent manner, and then is further processed by the Dicer–TRBP complex into the mature miRNA duplex. The mature miRNA incorporates into the Ago2 complex to repress gene expression. (b) p53 transcriptionally activates miRNA genes after DNA damage. (c) ATM-induced p53 promotes the post-transcriptional processing of pri-miRNAs via p68 and p72 after DNA damage. (d) The ATM kinase upregulates miRNA maturation by phosphorylating and activating KSRP. ATM-mediated phosphorylation of KSRP enhances the activity of KSRP in the processing of miRNAs by the Drosha and Dicer complexes. Adapted from [34].

Human gene*	Role	Comments	Rodent‡	Saccharomyces cerevisiae‡	Escherichia coli‡
GGR genes					
XPE (also known as DDB2)	Lesion recognition	Recruits XPC and is p53 inducible	Ddb2 (also known as Xpe)	Unknown	Unknown
DDB1	Lesion recognition	Forms a complex with DDB2	Ddb1	Unknown	Unknown
XPC	Lesion recognition	Opens DNA and is p53 inducible	Xpc	Rad4	UvrA
RAD23B	Lesion recognition	Forms a complex with XPC	Rad23b	Rad23	Unknown
Centrin-2	Lesion recognition	Forms a complex with XPC	Unknown	Unknown	Unknown
CGR and TCR genes					
XPB	Helicase and ATPase	TFIIH subunit	Ercc3 (also known as Xpb)	Rad25	UvrB
XPD	Helicase and ATPase	TFIIH subunit	Ercc2 (also known as Xpd)	Rad3	UvrB
XPA	Lesion verification	Stabilizes pre-incision complex in GGR and TCR	Xpa	Rad14	Unknown
RPA p70, p32 and p14	ssDNA binding	Binds to XPA	Rpa	RFA1, RFA2 and RFA3	Ssb?
XPG	Structure-specific endonuclease	3′ incision	Ercc5 (also known as Xpg)	Rad2	UvrC
ERCC1	Forms a complex with XPF	5′ incision	Ercc1	Rad10	Unknown
XPG	Structure-specific endonuclease	5′ incision and stabilization of TFIIH	Ercc5 (also known as Xpg)	Rad2	UvrC
PCNA	DNA replication sliding clamp	Three subunits; contains docking sites for DNA pol	Pcna	PCNA	β-clamp
RFC1	Loads PCNA on DNA	RFC large subunit	Rfc1	CDC44	Unknown
Unknown	Removal of incised oligo	None	Unknown	Unknown	UvrD‖
DNA pol δ or ε	DNA replication and repair	None	DNA pol δ or ε	DNA pol δ or ε	DNA pol I
DNA pol ε	Bypass polymerase	None	DNA pol ε	DNA pol ε	Dpo1
DNA ligase-I	Ligase	None	Unknown	CDC9	Ligase
DNA ligase-III	Ligase complex	Might be associated with dividing or non-dividing cells	Unknown	Unknown	Ligase
XRCC1	Ligase complex	Might be associated with dividing or non-dividing cells	Xrcc1	Unknown	Unknown
TCR genes					
CSA	Ubiquitin-ligase complex	WD repeat	Ercc8 (also known as Csa)	Rad28	Unknown
CSB	TCR coupling factor and chromatin remodelling	Transcription elongation factor	Ercc6 (also known as CSA)	Rad26	Mfd
XAB2	Transcription factor	Link between XPA and RNAPII	Unknown	Unknown	Unknown
TTDS	RNAPII elongation factor	Stimulation of transcript cleavage by RNAPII	Unknown	Unknown	GreA and GreB
HMGN1	Chromatin relaxation	Nucleosome removal?	Unknown	Unknown	Unknown
p300	Chromatin remodelling	Nucleosome removal?	Unknown	Unknown	Unknown

Table 3. Complexes are indicated by the same adjacent background colors. Other gene products, such as FEN1, MMS19L and p53, might participate in NER or induce expression of NER genes. ‡Genetic and functional homologues. Although only XPB and XPD (and TTDA, which is not listed) have NER functions, all ten TFIIH subunits are essential for NER. ||There are no mammalian homologues of UvrD; its role in removing the damaged oligonucleotide might be carried out by DNA polymerases. ERCC, excision repair cross-complementing; CDC, cell-division cycle; GGR, global genomic repair; NER, nucleotide excision repair; PCNA, proliferating cell nuclear antigen; Pol, polymerase; Ssb, single-stranded DNA-binding protein; ssDNA, single-stranded DNA; RF, replication factor; RNAP, RNA polymerase; RPA, replication protein A; TCR, transcription-coupled repair; TF, transcription factor; XAB2, XPA-binding protein-2; XP, xeroderma pigmentosum. Adapted from [39].

The COP9 signalosome (CSN) mediates the DDB2 inhibition in a CSN5 independent, non-enzymatic, fashion. In turn, CSN inhibition is relieved upon DNA damage binding to the DDB2 module within CSN-DDB2. The Cockayne syndrome A DCAF complex crystal structure shows that DCAF(WD40) ligases share common architectural features. Indeed ligase activation is induced by CSN displacement from DCAF on substrate binding to the DCAF[40]. Also, in plants, DET1, component of the E3 ubiquitin ligase DCX DET1-COP1 complex, acts with CULLIN4-based ubiquitin E3 ligase, and appropriate dosage of DET1 protein is necessary for efficient removal of UV photoproducts through the NER pathway. DET1 is required for CULLIN4-dependent targeted degradation of the UV-lesion recognition factor DDB2. Finally, DET1 protein is degraded concomitantly with DDB2 upon UV irradiation in a CUL4-dependent mechanism. Thus DET1, as well as other regulators of E3 ligase activity and DDB2 cooperate during the excision repair process[41]. Proteins that recognize DNA lesions continuously probe DNA strands. Photobleaching experiments show that XPC constantly associates with and dissociates from chromatin in the absence of DNA damage. To avoid excessive DNA probing by the low specificity of the protein, that could interfere with transcription and replication, the steady-state level in the nucleus is controlled by nucleus-cytoplasm shuttling, allowing temporally higher concentrations of XPC in the nucleus under genotoxic stress conditions[47]. And probably it is UV-DDB that adopts a ubiquitin - independent function as it is evidenced by domain mapping and “in situ” protein dynamics studies, revealing direct but transient interactions that promote a thermodynamically unfavorable β-hairpin insertion of XPC into substrate DNA[48]. The β-hairpin is so far one of the most commonly used feature in damage detection and verification in NER. UvrB, XPC, XPA and DDB2 all contain at least one β -hairpin structure that could be or has been shown to be involved in critical interactions with the site of damage (Figure 10) [49]. Subsequently, CENTRIN2 is recruited onto nuclear damaged areas quickly after irradiation. And XPC plays an important role during its internalization into the nucleus of human cells[50]. UV - induced modification of RAD4, yeast form of XPC, is strongly increased in cells deleted for RAD33. The predicted structure of RAD33 shows resemblance to the CENTRIN homologue CDC31. In human cells, CENTRIN2 binds to XPC and is involved in NER. In yeast, RAD4 binds RAD33 directly and via the same conserved amino acids required for the interaction of XPC with CENTRIN2. Disruption of the RAD4-

RAD33 interaction is sufficient to enhance the modification of RAD4 and results in a repair defect similar to that of a RAD33 mutant. Thus role of RAD33 in the RAD4-RAD23 complex has parallels with the role of CENTRIN2 in the XPC-HHR23B complex[52]. Going deeper inside yeast-mammalian parallels, another yeast protein ELC1, the homologue of the mammalian elongation factor Elongin C, has been shown to be a component of a ubiquitin ligase complex that contains RAD7 and RAD16, two factors that are specifically required for GGR. ELC1 has also been suggested to be present in another ubiquitin ligase complex that lacks RAD7 and RAD16 and is involved in UV-induced ubiquitylation and subsequent degradation of RNA polymerase II. Cells deleted for ELC1 show normal NER in the transcribed strand of an active gene but have no detectable NER in the non-transcribed strand[52]. Therefore ELC1 and autonomously replicating sequence-binding factor 1, another protein that forms a stable complex with RAD7 and RAD16 proteins, promote efficient GGR in yeast[53].

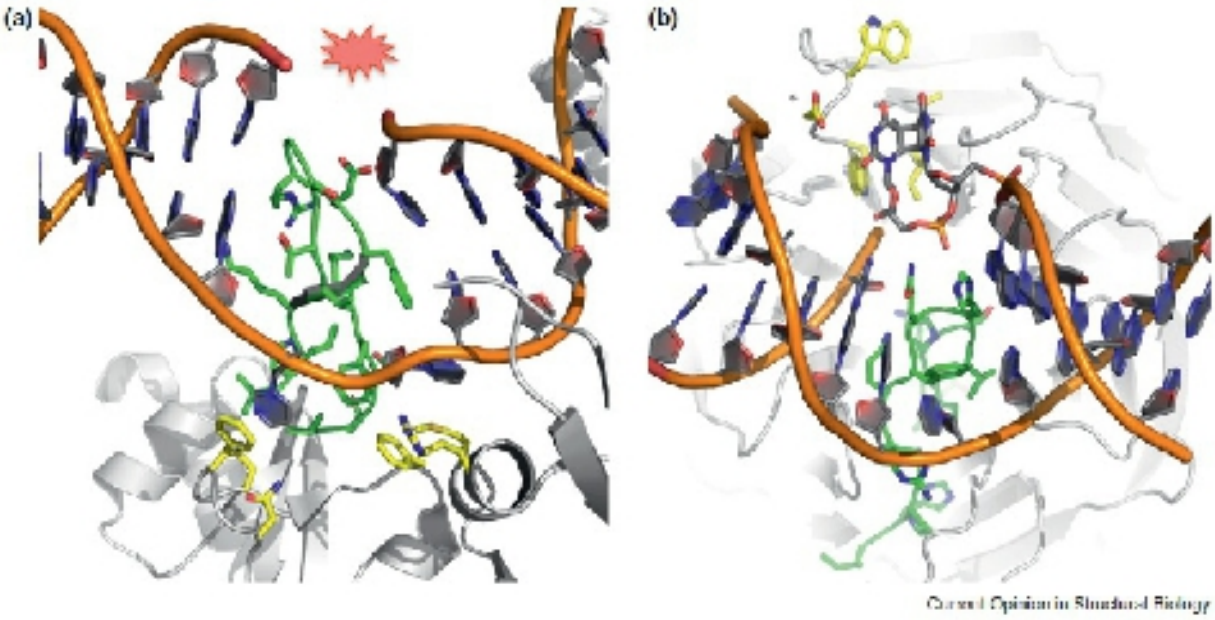

Figure 10. β-hairpin (in green) structures of XPC (a) and DDB2 (b) bound to DNA. Adapted from[49].

Thereafter we should also mention UVSSA, a newly discovered part of a UV-induced ubiquitinated protein complex. Knockdown of UVSSA results in TC-NER deficiency. UVSSA protein interacts with elongating RNA polymerase II, localizes specifically to UV - induced lesions, resides in chromatin-associated TC-NER complexes and is implicated in stabilizing the TC-NER master organizing protein CSB by delivering

the deubiquitinating enzyme USP7 to TC-NER complexes[43]. TC-NER regulation also involves indirect mechanism of up-regulation in gene expression of CSA and HR23A in a stress - induced transcription manner that occurs through a p53 independent mechanism and is coordinated by USF-1 which loss was shown to compromise DNA repair[44]. On other hand, TCR-NER p53-dependent mechanisms acts through CSB regulation. It includes interaction of p53 and CSB there both proteins are implicated in overlapping biological processes, such as DNA repair and aging. Interaction of CSB with the C-terminal region of p53 facilitates the sequence-independent association of p53 with chromatin when p53 concentrations are low. p53 protein prevents CSB from binding to nucleosomes when p53 concentrations are elevated excluding CSB from nucleosomes by occluding a nucleosome interaction surface on CSB[42].

Efficient DDR and DNA repair critically depends on numerous links between various signaling pathways. Recently Cep164, a chromatin binding mediator protein that functions in ATR - mediated checkpoint activation upon UV damage, was shown to localizes to cyclobutane pyrimidine dimers. Recruitment of Cep164 to CPD sites or photoproduct formation region requires a XPA, showing an important juncture between checkpoint pathways and repair systems on chromatin[45]. RPA protein also acts on crossroad of checkpoint and DNA repair. As a regulator of the transition from dual incision to repair-synthesis in UV-irradiated non-cycling cells, RPA averts the generation of unprocessed repair intermediates. These intermediates could lead to recombinogenic events and trigger a persistent ATR - dependent checkpoint signaling due to the formation of endonuclease APE1-mediated DNA strand breaks in addition to the strong DNA damage signaling leading to cell cycle arrest, apoptosis and increased mutagenesis from stalled RNA polymerase II[46]. An finally, exonuclease 1 (EXO1), a highly conserved from yeast to human protein, that is implicated in numerous DNA metabolic pathways, including repair, recombination, replication, telomere maintenance, cellular response to UV irradiation in human cells. Thus after local UV irradiation, fluorescent-tagged hEXO1 localizes, together with NER factors, at the sites of damage. hEXO1 accumulation requires XPF - dependent processing of UV-induced lesions and is enhanced by inhibition of DNA repair synthesis. Depletion of hEXO1 impairs activation of the checkpoint signal transduction cascade in response to UV damage[54].

CONCLUSION

Molecular details of NER and DDR obtained from investigations “in vivo” show that in the chromatin context plausible mechanisms are significantly different from the models based exclusively on experimental results obtained for naked DNA substrates. Upon DNA damage chromatin is dynamically modified favoring DNA repair and serving as platform for the cooperation of various signaling pathways induced by UV radiation. Chromatin remodelers orchestrate numerous NER - associated enzymes to properly organize damage detection and repair in space and time. After all DNA lesions were repaired they restore perturbed epigenetic landscape subsequently changing marks for switch from DDR and DNA repair to normal programs of cellular growth and division.

REFERENCES

1. Guo R, Chen J, Mitchell DL, Johnson DG (2011) GCN5 and E2F1 stimulate nucleotide excision repair by promoting H3K9 acetylation at sites of damage. Nucleic acids research 39: 1390–1397.
2. Polo SE, Roche D, Almouzni G (2006) New histone incorporation marks sites of UV repair in human cells. Cell 127: 481–493.
3. Zhu Q, Wani G, Arab HH, El-Mahdy MA, Ray A, et al. (2009) Chromatin restoration following nucleotide excision repair involves the incorporation of ubiquitinated H2A at damaged genomic sites. DNA repair 8: 262–273.
4. Guler GD, Liu H, Vaithiyalingam S, Arnett DR, Kremmer E, et al. (2011) Human DNA Helicase B (HDHB) binds to replication protein A and facilitates cellular recovery from replication stress. The Journal of biological chemistry.
5. Gong F, Fahy D, Smerdon MJ (2006) Rad4-Rad23 interaction with SWI/SNF links ATP-dependent chromatin remodeling with nucleotide excision repair. Nature structural & molecular biology 13: 902–907.
6. Ray A, Mir SN, Wani G, Zhao Q, Battu A, et al. (2009) Human SNF5/INI1, a component of the human SWI/SNF chromatin remodeling complex, promotes nucleotide excision repair by influencing ATM recruitment and downstream H2AX phosphorylation. Molecular and cellular biology 29: 6206–6219.
7. Zhao Q, Wang Q-E, Ray A, Wani G, Han C, et al. (2009) Modulation of nucleotide excision repair by mammalian SWI/SNF chromatin-remodeling complex. The Journal of biological chemistry 284: 30424–30432.
8. Lans H, Marteijn JA, Schumacher B, Hoeijmakers JHJ, Jansen G, et al. (2010) Involvement of global genome repair, transcription coupled repair, and

chromatin remodeling in UV DNA damage response changes during development. PLoS genetics 6: e1000941.

9. Erdel F, Rippe K (2011) Binding kinetics of human ISWI chromatin-remodelers to DNA repair sites elucidate their target location mechanism. Nucleus (Austin, Tex) 2: 105–112.

10. Sarkar S, Kiely R, McHugh PJ (2010) The Ino80 chromatin-remodeling complex restores chromatin structure during UV DNA damage repair. The Journal of cell biology 191: 1061–1068.

11. Jiang Y, Wang X, Bao S, Guo R, Johnson DG, et al. (2010) INO80 chromatin remodeling complex promotes the removal of UV lesions by the nucleotide excision repair pathway. Proceedings of the National Academy of Sciences of the United States of America 107: 17274–17279.

12. Rajagopalan S, Nepa J, Venkatachalam S (2012) Chromodomain helicase DNA-binding protein 2 affects the repair of X-ray and UV-induced DNA damage. Environmental and molecular mutagenesis 53: 44–50.

13. Lans H, Marteijn J a, Vermeulen W (2012) ATP-dependent chromatin remodeling in the DNA-damage response. Epigenetics & chromatin 5: 4.

14. Ryan DP, Owen-Hughes T (2011) Snf2-family proteins: chromatin remodellers for any occasion. Current opinion in chemical biology 15: 649–656.

15. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM (1998) DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. The Journal of biological chemistry 273: 5858–5868.

16. Kinner A, Wu W, Staudt C, Iliakis G (2008) Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. Nucleic acids

research 36: 5678–5694.

17. Reed SH (2005) Nucleotide excision repair in chromatin: the shape of things to come. DNA repair 4: 909–918.
18. Reed SH (2011) Nucleotide excision repair in chromatin: damage removal at the drop of a HAT. DNA repair 10: 734–742.
19. Lukas J, Lukas C, Bartek J (2011) More than just a focus: The chromatin response to DNA damage and its role in genome integrity maintenance. Nature Cell Biology 13: 1161–1169.
20. Chaudhuri S, Wyrick JJ, Smerdon MJ (2009) Histone H3 Lys79 methylation is required for efficient nucleotide excision repair in a silenced locus of Saccharomyces cerevisiae. Nucleic acids research 37: 1690–1700.
21. Tatum D, Li S (2011) Evidence that the histone methyltransferase Dot1 mediates global genomic repair by methylating histone H3 on lysine 79. The Journal of biological chemistry 286: 17530–17535.
22. Fink M, Thompson JS, Thoma F (2011) Contributions of histone H3 nucleosome core surface mutations to chromatin structures, silencing and DNA repair. PloS one 6: e26210.
23. Irizar A, Yu Y, Reed SH, Louis EJ, Waters R (2010) Silenced yeast chromatin is maintained by Sir2 in preference to permitting histone acetylations for efficient NER. Nucleic acids research 38: 4675–4686.
24. Yu S, Teng Y, Waters R, Reed SH (2011) How chromatin is remodelled during DNA repair of UV-induced DNA damage in Saccharomyces cerevisiae. PLoS genetics 7: e1002124.
25. Biswas AK, Johnson DG (2011) Transcriptional and Nontranscriptional

Functions of E2F1 in Response to DNA Damage. Cancer research 72: 13–17.

26. Li T, Kon N, Jiang L, Tan M, Ludwig T, et al. (2012) Tumor Suppression in the Absence of p53-Mediated Cell-Cycle Arrest, Apoptosis, and Senescence. Cell 149: 1269–1283.

27. Zhao Q, Barakat BM, Qin S, Ray A, El-Mahdy MA, et al. (2008) The p38 mitogen-activated protein kinase augments nucleotide excision repair by mediating DDB2 degradation and chromatin relaxation. The Journal of biological chemistry 283: 32553–32561.

28. An J, Yang T, Huang Y, Liu F, Sun J, et al. (2011) Strand-specific PCR of UV radiation-damaged genomic DNA revealed an essential role of DNA-PKcs in the transcription-coupled repair. BMC biochemistry 12: 2.

29. Pankotai T, Bonhomme C, Chen D, Soutoglou E (2012) DNAPKcs-dependent arrest of RNA polymerase II transcription in the presence of DNA breaks. Nature structural & molecular biology.

30. Ghodgaonkar MM, Zacal N, Kassam S, Rainbow AJ, Shah GM (2008) Depletion of poly(ADP-ribose) polymerase-1 reduces host cell reactivation of a UV-damaged adenovirus-encoded reporter gene in human dermal fibroblasts. DNA repair 7: 617–632.

31. Armstrong AA, Mohideen F, Lima CD (2012) Recognition of SUMO-modified PCNA requires tandem receptor motifs in Srs2. Nature 483: 59–63.

32. Centore RC, Yazinski SA, Tse A, Zou L (2012) Spartan/C1orf124, a Reader of PCNA Ubiquitylation and a Regulator of UV-Induced DNA Damage Response. Molecular Cell 46: 625–635.

33. Silver HR, Nissley JA, Reed SH, Hou Y-M, Johnson ES (2011) A role for SUMO in

nucleotide excision repair. DNA repair 10: 1243–1251.

34. Wan G, Mathur R, Hu X, Zhang X, Lu X (2011) miRNA response to DNA damage. Trends in biochemical sciences 36: 478–484.

35. Yin D-T, Wang Q, Chen L, Liu M-Y, Han C, et al. (2011) Germline stem cell gene PIWIL2 mediates DNA repair through relaxation of chromatin. PloS one 6: e27154.

36. Wei W, Ba Z, Gao M, Wu Y, Ma Y, et al. (2012) A Role for Small RNAs in DNA Double-Strand Break Repair. Cell 149: 101–112.

37. Lan L, Nakajima S, Kapetanaki MG, Hsieh CL, Fagerburg M, et al. (2012) Monoubiquitinated H2A destabilizes photolesion-containing nucleosomes with the concomitant release of the UV-damaged DNA-binding protein E3 ligase. The Journal of biological chemistry.

38. Takedachi A, Saijo M, Tanaka K (2010) DDB2 complex-mediated ubiquitylation around DNA damage is oppositely regulated by XPC and Ku and contributes to the recruitment of XPA. Molecular and cellular biology 30: 2708–2723.

39. Hanawalt PC, Spivak G (2008) Transcription-coupled DNA repair: two decades of progress and surprises. Nature reviews Molecular cell biology 9: 958–970.

40. Fischer ES, Scrima A, Böhm K, Matsumoto S, Lingaraju GM, et al. (2011) The Molecular Basis of CRL4(DDB2/CSA) Ubiquitin Ligase Architecture, Targeting, and Activation. Cell 147: 1024–1039.

41. Castells E, Molinier J, Benvenuto G, Bourbousse C, Zabulon G, et al. (2011) The conserved factor DE-ETIOLATED 1 cooperates with CUL4-DDB1DDB2 to maintain genome integrity upon UV stress. The EMBO journal 30: 1162–1172.

42. Lake RJ, Basheer A, Fan H-Y (2011) Reciprocally Regulated Chromatin

Association of Cockayne Syndrome Protein B and p53 Protein. The Journal of biological chemistry 286: 34951–34958.

43. Schwertman P, Lagarou A, Dekkers DHW, Raams A, van der Hoek AC, et al. (2012) UV-sensitive syndrome protein UVSSA recruits USP7 to regulate transcription-coupled repair. Nature Genetics advance on.

44. Baron Y, Corre S, Mouchet N, Vaulont S, Prince S, et al. (2012) USF-1 Is Critical for Maintaining Genome Integrity in Response to UV-Induced DNA Photolesions. PLoS genetics 8: e1002470.

45. Pan Y-R, Lee EY-HP (2009) UV-dependent interaction between Cep164 and XPA mediates localization of Cep164 at sites of DNA damage and UV sensitivity. Cell cycle (Georgetown, Tex) 8: 655–664.

46. Lagerwerf S, Vrouwe MG, Overmeer RM, Fousteri MI, Mullenders LHF (2011) DNA damage response and transcription. DNA repair 10: 743–750.

47. Hoogstraten D, Bergink S, Ng JMY, Verbiest VHM, Luijsterburg MS, et al. (2008) Versatile DNA damage detection by the global genome nucleotide excision repair protein XPC. Journal of cell science 121: 2850–2859.

48. Fei J, Kaczmarek N, Luch A, Glas A, Carell T, et al. (2011) Regulation of Nucleotide Excision Repair by UV-DDB: Prioritization of Damage Recognition to Internucleosomal DNA. PLoS Biology 9: e1001183.

49. Kuper J, Kisker C (2012) Damage recognition in nucleotide excision DNA repair. Current opinion in structural biology 22: 88–93.

50. Renaud E, Miccoli L, Zacal N, Biard DS, Craescu CT, et al. (2011) Differential contribution of XPC, RAD23A, RAD23B and CENTRIN 2 to the UV-response in human cells. DNA repair 10: 835–847.

51. den Dulk B, van Eijk P, de Ruijter M, Brandsma JA, Brouwer J (2008) The NER protein Rad33 shows functional homology to human Centrin2 and is involved in modification of Rad4. DNA repair 7: 858–868.

52. Lejeune D, Chen X, Ruggiero C, Berryhill S, Ding B, et al. (2009) Yeast Elc1 plays an important role in global genomic repair but not in transcription coupled repair. DNA repair 8: 40–50.

53. Yu S, Smirnova JB, Friedberg EC, Stillman B, Akiyama M, et al. (2009) ABF1-binding sites promote efficient global genome nucleotide excision repair. The Journal of biological chemistry 284: 966–973.

54. Sertic S, Pizzi S, Cloney R, Lehmann AR, Marini F, et al. (2011) Human exonuclease 1 connects nucleotide excision repair (NER) processing with checkpoint activation in response to UV irradiation. Proceedings of the National Academy of Sciences of the United States of America 108: 13647–13652.

55. Francia S, Michelini F, Saxena A, Tang D, de Hoon M, et al. (2012) Site-specific DICER and DROSHA RNA products control the DNA-damage response. Nature. 10.1038

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Molecularinsightsintotherecruitmentof TFIIHtositesofDNAdamage

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XPBandXPDsubunitsofTFIIHarecentralgenomecare-takers involved innucleotide excisionrepair (NER), althoughtheir respectiverolewithinthis DNArepair pathwayremainsdifficulttodelineate. Toobtaininsight intothefunctionof XPBandXPD, westudiedcell lines expressingXPBorXPDATPase-deficient complexes.We showtheinvolvementofXPB, butnotXPD, intheaccu-mulationofTFIIHtositesofDNAdamage.Recruitmentof TFIIHoccursindependentlyofthehelicaseactivityofXPB, butrequirestworecentlyidentifiedmotifs,aR-E-Dresidue loopandaThumb-likedomain. Furthermore,weshow that thesemotifsarespecificallyinvolvedintheDNA-induced stimulation of the ATPase activity of XPB. Together,ourdatademonstratethat therecruitment of TFIIHtositesof damageisanactiveprocess, underthe controloftheATPasemotifsofXPBandsuggestthatthis subunitfunctionsasanATP-drivenhooktostabilizethe bindingoftheTFIIHtodamagedDNA.
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Introduction

DNAandRNAhelicasesareaubiquitous,yetdiverse,group of enzymespresentinviruses, prokaryotesandeukaryotes (DelagoutteandvonHippel, 2003).Theyconvertchemical energyofnucleosidetriphosphatehydrolysistothemechan-ical energynecessarytotransientlyseparatethestrandsof duplexnucleicacids(TutejaandTuteja,2004).Bythismean, theyprovidethesingle-strandedDNAorRNAintermediates necessaryfor replication,transcription,recombinationor repair.Furthermore,it hasbeenshownthat helicasescan alsoeffectivelydisplaceboundproteinsfromDNAorRNA (vonHippel,2004).Thereareseveralknownhumandiseases causedbydefectivehelicases(Ellis, 1997). Amongthese disorders,thecancer-proneXerodermapigmentosum(XP), aloneorincombinationwiththeCockaynesyndrome(CS), andtheTrichothiodystrophy(TTD)arenoteworthyasthey entailmutationsintheXPBandXPDsuperfamily2helicases. BothofthesehelicasesarepartofthesameTFIIHcomplex. TFIIHiscomposedofaseven-subunitcore(XPB,XPD,p62, p52, p44, p34andp8/TTD-A) associatedwiththeCAK subcomplex(Cdk7,cyclinH, andMAT1)(Giglia-Mari etal, 2004;Ranishetal,2004).TFIIHfunctionsinbothtranscrip-tioninitiationsofmRNAandrRNA(Ibenetal,2002),aswell asinnucleotideexcisionrepair(NER)(Schaefferetal,1993).

XPBandXPDpatientsarephotosensitiveanddisplaya 1000-foldincreaseinmelanomariskbecauseofdefectsinthe NERfunctionof TFIIH(Lehmann, 2003).NERremovesa broadspectrumof DNAlesionsincludingUV-inducedpyr-imidinedimersandbulky,helix-distortingadductscausedby toxicchemicalssuchastheanticancerdrugcisplatin(Sancar, 1996).Inmammaliancells, theproteinsnecessaryforthe incisionreactionincludeXPC-HR23b,TFIIH, XPA, RPAand thenucleasesXPGandERCC1-XPF(Araujoetal,2000).The removalof lesionsrequirestheirrecognitionbytherepair factorXPC-HR23bandthesubsequentopeningoftheDNA duplexbyTFIIH.Thesingle-strandedstructureisthenstabi-lizedbyXPAandRPA,andthemarginsoftheresultingDNA bubble are recognizedbyXPGandERCC1-XPF, thereby generating3′and5′incisionsrelativetothedamage,respec-tively(O'Donnovanetal,1994;Sijbersetal,1996).

AsXPBandXPDhelicasesarebothintegralpartsofTFIIH, theirindividual molecularrolesinNERremaindifficult to delineate.As XPBandXPDare helicaseswithopposite polarities,itwasoriginallysuggestedthattheycouldcoop-eratetoopenDNAonthe5′ and3′ sides of alesion, respectively(Schaefferetal,1994).Indeed,mutationofthe ATPaseactivityofeitherXPBorXPDresultsintheinabilityto removeDNAlesions(Sungetal,1988;Guzderetal,1994). Refiningtheseproposals,recentdatabringintoquestionthe direct role of the helicaseactivityof XPBinNERand transcription,andsuggestthat onlytheATPaseactivityis required(Linet al, 2005;Coinet al, 2007;Richardsetal, 2008).Supportingtheprimeroleof theATPaseactivityof XPBinTFIIHfunctions,werecentlyshowedthatthisactivity wasregulatedbythep52subunitofTFIIH(Coinetal,2007) andbythedamagerecognitionfactor XPC(Bernardesde Jesusetal, 2008).ContrarytoXPB, thehelicaseactivityof XPD, whichisregulatedbythep44subunitofTFIIH(Coin et al, 1998),isrequiredfor efficientopeningof theDNA aroundthe damage,but is dispensablefor transcription (Tirodeetal,1999;Coinetal,2007).

Tofurtherourunderstandingofthemechanisticdetailsof XPBandXPDfunction,weanalysedthebehaviourofATPase-deficientTFIIHcomplexesinvivo. Wefoundthat aTFIIH complexdeficientintheATPaseofXPBwasnotrecruitedto sitesof DNAdamage,whereasacomplexdeficientinthe ATPaseofXPDdid.Moresurprisingly,wediscoveredthatthe recruitmentof TFIIHtothesesites does not requirethe helicaseactivityofXPBbutdependsontwomotifs,aR-E-D residueloopandapositivelychargedflexibleThumb(ThM)

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motifthatwereidentifiedinahomologueofXPBfromthe thermophilicorganismArchaeoglobusfulgidus(Fanet al, 2006).WeanalysedthemoleculardetailsofR-E-DandThM impactonXPBactivitiesandfoundthattheywererequiredto stimulateATPhydrolysisinthepresenceofDNA.Wepropose amechanisminwhichXPBfunctionsasanATP-dependent hookthatusestheATPase,R-E-DandThMmotifstoanchor TFIIHtothesitesofDNAdamageduringDNArepair.

Results

TheATPaseactivityofXPBanchorsTFIIHtothesites ofDNAdamageinvivo

TofunctionallydiscriminatebetweentheATPaseactivitiesof XPBandXPD,weproducedrecombinantTFIIH/XPD(K48R) andTFIIH/XPB(K346R)inbaculovirus-infectedcells(Tirode etal,1999)andtestedtheminDNArepairandtranscription assays.Thesecomplexesaremutatedin theATPaseWalkerA motifofXPDandXPB,respectively.Whenincubatedinthe presenceofrecombinantTBP,TFIIA,TFIIB,TFIIEandTFIIF transcriptionfactorsinadditiontopurifiedRNApolymerase IIandalinearizedDNAtemplatecontainingtheadenovirus majorlatepromoter(Tirodeetal,1999), TFIIH/XPD(K48R) supported transcription,contrary to TFIIH/XPB(K346R), whichwastotallyinactive(Figure1A,upperpanel).Totest therepaircapacityofthedifferentTFIIHcomplexes,weused areconstituteddualincisionassaycomposedoftherecombi- nantXPC-HR23b,XPA,RPA,XPG,ERCC1-XPFfactorsanda closed-circularplasmid(Pt-DNA) containingasingle1,3- intra-strandd(GpTpG)cisplatin-DNAcrosslinkasatemplate (Araujoetal,2000).Noneofthemutatedcomplexeswasable toexcisethedamagedoligonucleotide(Figure1A, lower panel).Inapermanganatefootprintingassaythatmeasures theopeningoftheDNAaroundthelesion(Tapiasetal,2004), additionof TFIIH(WT)inducedanincreasedsensitivityof nucleotidesatpositionsTþ 5, Tþ 6, T–4, T–5, and, toa lesserextent,T–7andT–10(Figure1B,lane2),indicativeof DNAopening. Incontrast, neitherTFIIH/XPB(K346R)nor TFIIH/XPD(K48R)wereabletoopendamagedDNA(com- parelanes3–4withlane2).

ToanalysethebehaviourofATP-deficientTFIIHcomplexes invivo,weusedastablytransfectedChinesehamsterovary (CHO)-UV5celllineexpressinganHA-taggedversionofthe humanXPD(K48R)protein(Winkleretal, 2000).Usingthe CHO-27-1cellsmutatedinthehamsterhomologueof XPB (Maetal,1994),wealsogeneratedastablytransfectedcell lineexpressingaC-terminallyGFP-taggedversionof the humanXPBWTorK346Rprotein(seealsoFigure2B).The functionalityofanXPB–GFPfusionconstructwasestablished earlier(Hoogstratenetal,2002)(seealsoFigure2).Weused immunofluorescentlabellingafter local UVirradiationof stablytransfectedcells(Volker et al, 2001)toassess the nuclear distribution pattern of XPB and XPD. Immunostainingwithantibodiesagainstcyclobutanepyrimi- dinedimers(CPDs)showedthatUVdamageswerelocatedin discretelocalspotsinthenucleus(Figure1C,panelsa,e,i, m). Both, humanwild-typeXPBandXPDproteinscoloca- lizedwithCPDspots, indicatingthatTFIIHwasefficiently recruitedtothedamagedsites inthesecells (Figure1C, panels a–d and i–l). Surprisingly,although signals of XPD(K48R)colocalizedwithCPDspotsinCHO-UV5cells (panelsm–p), signalsof XPB(K346R)showedanhomoge- nousdistributionpatternthroughthenucleus(panelse–h), indicatingthatTFIIH/XPB(K346R)complexwasnotrecruited tothedamagedsites(Figure1D).Thesedatasuggestthatthe accumulationofTFIIHtositesofDNAdamagetakesplacein theabsenceofanactiveXPDproteinbutrequiresfunctional XPB.

NewmotifsinXPBrequiredfortheactivityofTFIIH inNER

Byintroducingmutationsinsomeof theseven canonical helicasemotifsof XPB, wedemonstratedrecentlythatits helicaseactivitywasnotrequiredforTFIIHrepairfunction (Coinet al, 2007).Recently,threeadditionalmotifswere identifiedinahomologueof XPBfromthethermophilic organismArchaeoglobusfulgidus(Fanetal,2006).Todeter- minewhetherthesenewlyidentifiedmotifshaveafunction intheactivitiesofthehumanTFIIHcomplexintranscription andrepair,wedesignedfourmutants(E253A,E253A/R283A, E473AandD516–526)depictedinFigure3.WeintroducedAn E253Asubstitutionlocatedattheendofthefirstb strandthat wascombined, whenindicated, withanR283Amutation locatedatthebeginningofthea-helixofaputativedamage recognitiondomain(DRD). We alsodesignedanE473A substitutionin the R-E-Dresidue loop to change the local negativechargeof the motif, andwe deletedthe positivelychargedThMdomainfromaminoacid516to526 (D516–526).

ToinvestigatetheimportanceoftheDRD,R-E-DandThM motifs of XPBintherepair functionof TFIIH, wefirst performeda host-cell reactivationassay(Carreauet al, 1995).AUVdamagedreporterconstruct,carryingaluciferase gene(pLuc)wastransientlytransfectedintoCHO-27-1cells, togetherwithvectors codingfor b-galactosidaseandfor humanXPBproteins.TransfectionofeitherXPB(E253A)or XPB(E253A/R283A) restored luciferase expression that reachedthelevelobservedwithXPB(WT)(Figure2A,lanes 1–5). Inmarkedcontrast,XPB(E473A)andXPB(D516–526) werenotabletorestoreluciferaseexpression(lanes7–8),a defectalsoobservedwithXPB(K346R)(lane6).Thevarious XPBwereexpressedatasimilarlevelwiththeexceptionof XPB(D516–526)whoseexpressionwaslightlyreducedcom- paredwiththewildtype(Figure2A,WB).

Next, wecarriedout aUV-survival assayandfor that purposeweestablishedCHO-27-1cells stablyexpressing thenewXPB–GFPmutant proteins.Immunoprecipitations usingarabbitpolyclonal antibodyrecognizingthehamster homologueofthecoreTFIIHsubunitp62,demonstratedthat thevariousXPBwereefficientlyincorporatedintotheham- ster TFIIHcomplex (Figure 2B). The stably transfected CHO-27-1cellswereUVirradiatedat differentdoses(3, 6 and9J/m ^{2})andtheirsurvivalwasmeasured.Expressionof XPB(WT), XPB(E253A)andXPB(E253A/R283A)induceda substantialrescueoftheUVsurvival oftheCHO-27-1cells comparedwithnontransfectedcontrol(Figure2C). Onthe other hand, the UV-survival curve of XPB(E473A) and XPB(D516–526)transfectedcellsfellintotherangeof both thenontransfectedparentalCHO-27-1cellsandthosetrans- fectedwiththeNER-deficient XPB(K346R)control.These dataindicatethattheR-E-DandThMdomainsof XPBare crucialfortherepairactivityofTFIIH,whiletheputativeDRD isdispensable.

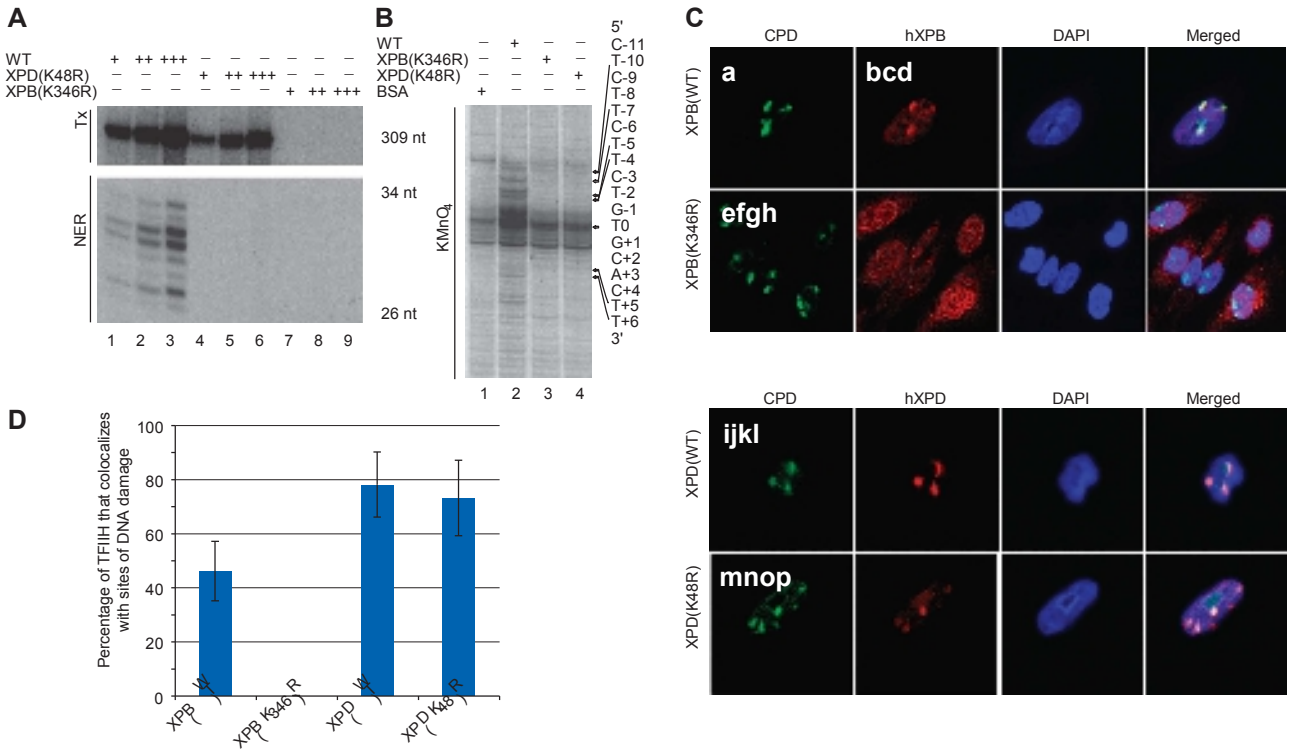

Figure1TheATPaseactivityofXPBisrequiredtoanchorTFIIHtodamagedchromatin.(A)Ameasureof25,50and100ngofTFIIH(WT), TFIIH/XPD(K48R)orTFIIH/XPB(K346R)weretestedeitherinareconstitutedtranscriptionassay(Tx,upperpanel)orinadualincisionassay (NER,lowerpanel)asdescribed(Coinetal,2004).Thesizesoftheincisionandtranscriptionproductsareindicated.(B)TFIIH(WT),TFIIH/ XPD(K48R)orTFIIH/XPB(K346R)(100ng)wereincubatedwitharadio-labelledlinearDNAfragmentfromthePt-DNAplasmidand40ngof XPC-HR23b,25ngof XPA, 50ngof RPAand150ngof XPGinaKMnO $_4$ footprintingassay(Lane1; Pt-DNAwithBSAonly. Residuesare numberedwiththecentralthymineofthecrosslinkedGTGsequencedesignatedT0.ArrowsindicateKMnO$_4$sensitivesites.Adductedstrand residuestothe3^{0}and5^{0}ofT0aredenotedbypositiveandnegativeintegers(3^{0}N,–N),respectively.(C)StablytransfectedCHO-27-1expressing aGFP-taggedversionofthehumanWTorK346RXPBproteins(upperpanel)andstablytransfectedCHO-UV5cellsexpressinganHA-tagged versionofthehumanWTorK48RXPDproteins(bottompanel)(Winkleretal,2000)wereUVirradiatedat100J/m ^{2} throughthe3mmpore filterandfixed30minlater.Immunofluorescentlabellingwasperformedusingarabbitpolyclonalanti-GFP(panelsbandf),aratmonoclonal anti-HA(panelsjandn)oramousemonoclonalanti-CPD(panelsa,e,i,m).Nucleiwerecounterstainedwith DAPI(panelsc,g,k,o),and slideswereemerged(panelsd,h,l,p).(D)Quantitativeanalysisoftherecruitmentof TFIIHtositesofDNAdamageintransfectedcells.Values representaverages±s.d.(n ¼100sitesofDNAdamage)fromthreeindependentexperiments.

R-E-DandThMmotifsareneededforanoptimalATPase activityofXPB

Todecipherthemoleculardetailsoftherepairdefectgener-atedbytheE473Aand D516–526mutations,weproduced recombinant TFIIH complexes in baculovirus-infected cells containingthecorrespondingmutatedXPBsubunits (Tirodeetal,1999).Westernblotanalysisoftherecombinant TFIIH/XPB(E253A), XPB(E253A/R283A),XPB(E473A) and XPB(D516–526)complexesrevealedasimilarsubunitcom-positioncomparedwiththeTFIIH(WT)complex(datanot shown). Whentestedineither adual incisionor ina transcriptionassays,TFIIH/XPB(E253A) andXPB(E253A/ R283A)wereasactiveasTFIIH(WT)inexcisingdamaged DNA(Figure4A,upperpanel,comparelanes5–8withlanes 3–4)orsynthesizingRNA(lowerpanel).Incontrast,TFIIH/ XPB(E473A)andXPB(D516–526)wereinactiveinrepairing damagedDNAandinsynthesizingRNA(Figure4A,compare lanes11–12and13–14withlanes3–4), similarlytoTFIIH/ XPB(K346R)(lanes9–10).

Inapermanganatefootprintassay,TFIIH/XPB(E473A)and XPB(D516–526)wereunabletoopenthedamagedDNA (Figure 4B, lanes 5–6), comparedwith TFIIH(WT) or XPB(E253A/R283A)(lanes2–3).Altogether,theabovedata drewourattentiontothecriticalroleofboththeR-E-Dand ThMmotifsofXPBindamagedDNAopening.

R-E-DandThMmotifsareneededfortheanchoring ofTFIIHtothesitesofDNAdamage

Wenext measuredtherecruitment of theTFIIHcomplexes carryingmutationsinthenewlyidentifiedmotifstositesof DNAdamageinvivo. XPB(E253A/R283A)colocalizedwith CPDspots(Figure5, panelse–h), indicatingthatthecorre-spondingTFIIHcomplexestranslocatedtothesitesofDNA photolesions.XPB(E473A) andXPB(D516–526),however, displayedahomogeneousdistributionpatternthroughthe nucleus(panelsi–l andm–p), whichparallelsthepattern observedinFigure1CwithXPB(K346R). Thishomogenous distributioncontrastedwiththelocal accumulationtothe damagedsitesofaTFIIH/XPB(T469A)complex(panelsq–t) containingamutationthathasbeenshowntoimpedethe helicaseactivityofXPBbut not theNERfunctionof TFIIH (Coinetal,2007).WeconcludefromthesedatathattheR-E-D andThMmotifsarerequired, togetherwiththeWalkerA

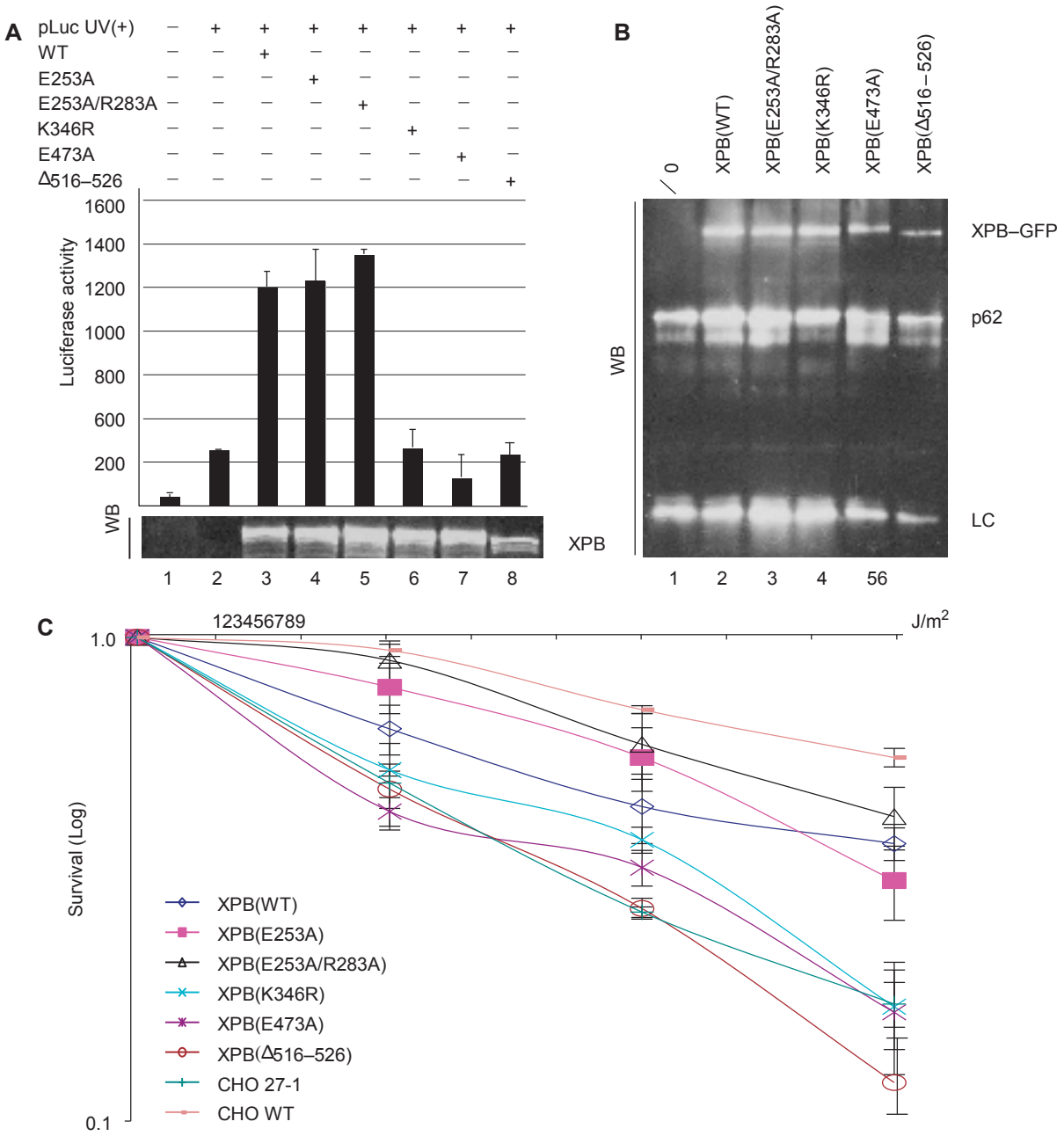

Figure2Analysisofthefunctionofthenewmotifsin NERinvivo.(A)CHO-27-1cellsweretransfectedwithUV-irradiatedpLucplasmid expressingtheluciferasegene(lanes2–8)incombinationwithvectorexpressingeitherXPB(WT)(lane3),XPB(E253A)(lane4),XPB(E253A/ R283A)(lane5), XPB(K346R)(lane6), XPB(E473A)(lane7) or XPB(D516–526)(lane8). Theluciferaseactivityincell lysates(48h posttranfection),normalizedwiththeinternalb-galactosidasestandard,assessesrepaircomplementation.Resultsareexpressedasrelative luciferaseactivity.Valuesrepresentaverages±s.d.fromthreeindependentexperiments.Ameasureof50%oftotalextractwereresolvedby SDS–PAGEandwesternblotted(WB)withamouseanti-humanXPBantibody(Coinetal,2004).NotethatXPB(D516–526)migratesslightly lowerthantheothersbecauseofthedeletion.(B)TFIIHfrom100mgofextractspreparedfromuntransfectedCHO-27-1(lane1)orCHO-27-1 stablyexpressingXPB(lanes2–6)wasimmunoprecipitatedwithapolyclonalantibodyagainstthep62subunitofTFIIHandresolvedbySDS– PAGE, followedbyWesternblottingwithamouseanti-humanXPBandamouseanti-p62antibody. LC; light chainof theantibody. (C)QuantitativeUV-survivalanalysisoftransfectedCHO-27-1celllines.Resultsareexpressedasaratiobetweenthecellsthatsurvivedafter irradiationandthecellsthatsurvivedwithouttreatment.Valuesrepresentaverages±s.d.fromthreeindependentexperiments.

motif,fortherecruitmentof theTFIIHcomplextositesof DNAdamage.

R-E-DandThMmotifsstimulatetheDNA-dependent TFIIHATPaseactivity

Dataaboveshowcommonbiochemicalandbiologicaldefects forTFIIHcomplexesmutatedeitherintheATPase,R-E-Dor ThMmotifsof XPBandsuggest alinkbetweenthenewly identifiedmotifsofXPBanditsATPaseactivity.Toassessthe contributionsofthesemotifsonthehydrolysisof ATPby XPB, weperformedATPaseassay.Ourdatashowthat the TFIIH/XPB(E473A) and TFIIH/XPB(D516–526) displayed about 40%of residual ATPase activity comparedwith TFIIH(WT)(Figure6A, comparelanes1–3with10–12and 13–15).TFIIH/XPB(E253A)orTFIIH/XPB(E253A/R283A)ex- hibitedthesameactivityasTFIIH(WT)(lanes1–6anddata

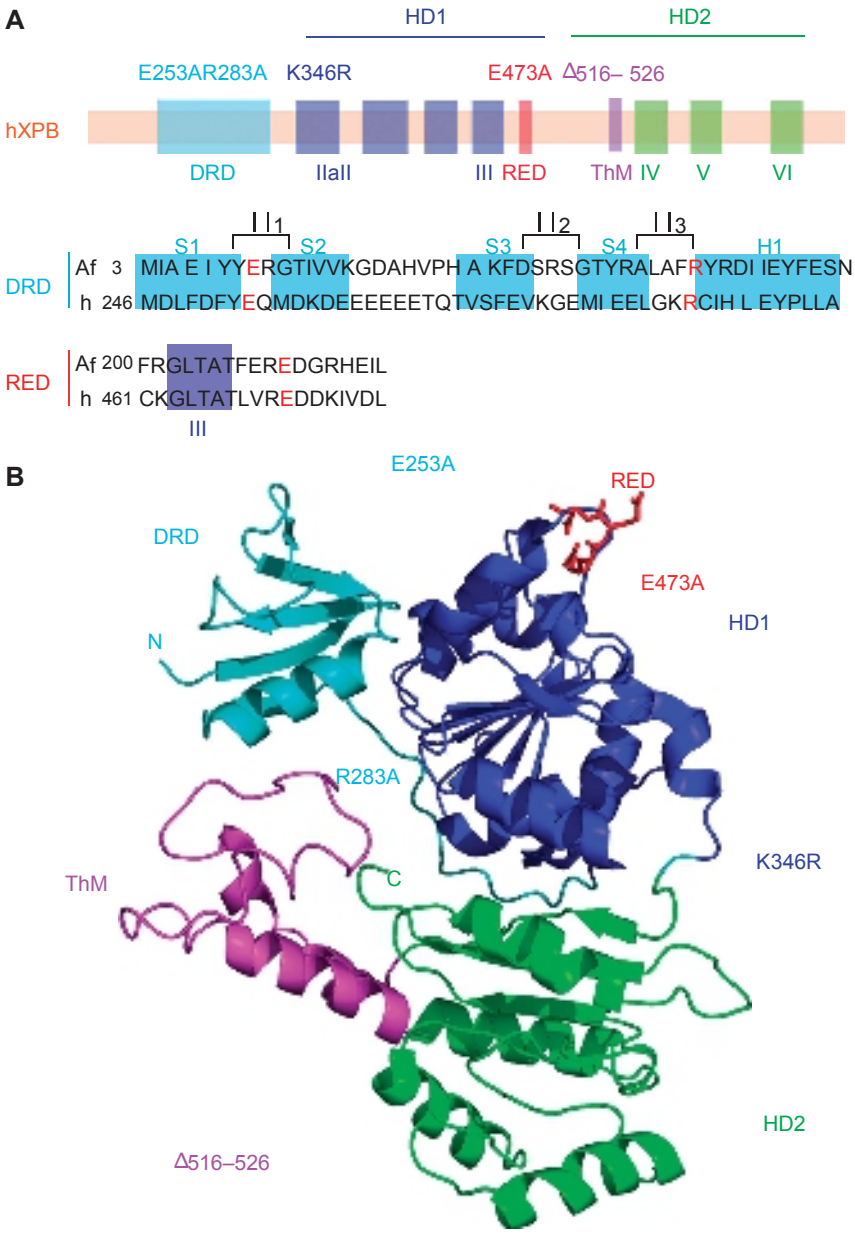

Figure3XPBconservedsequences,motifsandstructuralarchitecture(A)Upperpanel showsthelocationof thehumanXPBstructural domains:thefourclassicalhelicasemotifs(I,Ia,IIandIII)ofthefirsthelicasemodule(HD1)areindicatedinblue.Thethreehelicasemotifs (IV,VandVI)ofthesecondhelicasemodule(HD2)areindicatedingreen.Theputativedamagerecognition(DRD),R-E-DandThumb(ThM) domainsidentifiedinanhomologueof XPBfromthethermophilicorganismArchaeoglobus fulgidus(Fanet al, 2006)areindicated, respectively,inlightblue,redandpurple.ThemutationsE253A,R283A, K346R,E473Aand D516–526areannotated.Lowerpanelshows thesequenceconservationoftheDRDandR-E-Dmotifsbetweenhuman(h)andArchaeoglobusfulgidus(Af)XPBproteins.Theb-strands (S1–4)andthea-helix(H1)areindicated.Identicalresiduesaremarkedinboldletters.Theconservedhelicasemotif IIIindicatedbyablue squareislocatedclosetotheR-E-Dmotifindicatedbyaredopenedsquare.Thethreehairpinloopspotentiallyinvolvedin DNAbindingare indicated(1, 2, 3).Residuesmutatedinthisstudyaremarkedinred.(B)Viewoftheribbonrepresentation ofAfXPB.TheHD1is indicatedinblue,HD2ingreen.TheputativeDRD, R-E-DandThMdomainsareindicatedinlightblue,redandpurple,respectively. The positionsofthenewmutationsareindicated.

not shown).TFIIH/XPB(K346R)showedonly20%residual ATPaseactivity(correspondingtothatof XPD(Coinet al, 2006)), comparedwithTFIIH(WT) (comparelanes 1–3 with7–9).

XPBandXPDareDNA-dependent ATPases(Royet al, 1994).TheATPaseactivityofTFIIH(WT),lowintheabsence of DNA, isstimulatedbydouble-strandedDNA(Figure6B, comparelanes1–2with7–8).Incontrast,TFIIH/XPB(E473A) showed almost no DNA-induced ATPase stimulation (Figure6B, comparelanes5–6with11–12). Moreimportantly,intheabsenceofDNA, TFIIH/XPB(WT)andTFIIH/ XPB(E473A) displayed similar specific ATPase activities (Figure6B, comparelanes1–2and5–6)that wereslightly higherthantheTFIIH/XPB(K346R)activity(comparelanes 1–2,5–6and3–4).Similarobservationswereobtainedwith TFIIH/XPB(D516–526)(Supplementarydata1).Altogether,

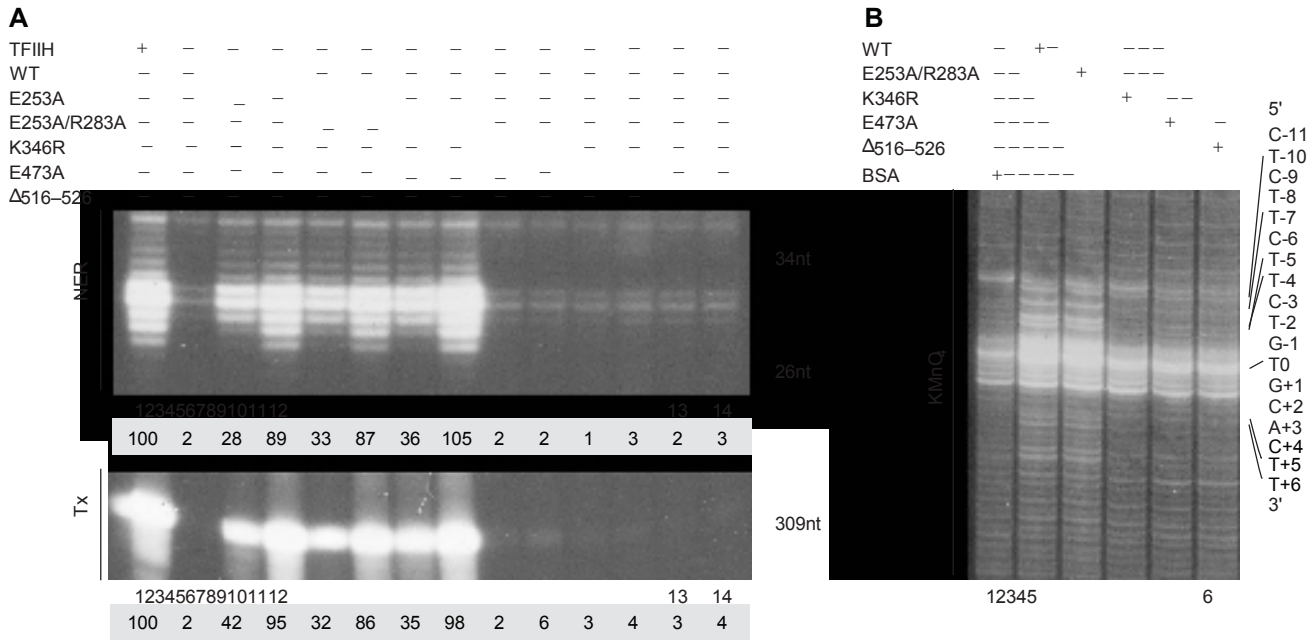

Figure4MutationsinR-E-DandThMmotifsimpairtheATPaseactivityof XPB. (A)Ameasureof 25and75ngof TFIIH(WT), TFIIH/XPB(E253A),/XPB(E253A/R283A),/XPB(K346R),/XPB(E473A)orXPB(Δ516–526)weretestedinadualincisionassay(NER,upperpanel)or inareconstitutedtranscriptionassay(Tx,lowerpanel)asdescribed(Coinetal,2004).Lane1containshighlypurifiedHelaTFIIH(Giglia-Mari etal,2004).Lane2containsallthefactorsexceptTFIIH.Thesizesoftheincisionortranscriptionproductsareindicated.Thetranscriptionand repairsignalswerequantifiedusingGenetool(Syngene).(B)Ameasureof100ngofthevariousTFIIHcomplexesweretestedinaKMnO$_4$ footprintassay(seeFigure1B). Lane1; Pt-DNAwithBSAonly. Residuesarenumberedwiththecentralthymineof thecrosslinkedGTG sequencedesignatedT0.ArrowsindicateKMnO$_4$sensitivesites.Adductedstrandresiduestothe3^{0}and5^{0}ofT0aredenotedbypositiveand negativeintegers(+N,–N).

thesedataindicatethattheR-E-DandThMmotifsdonot affectthebasal intrinsicATPaseactivityof XPBbut are requiredforthestimulationofthisactivitybyDNA.

Discussion

Toefficientlyprotectthegenome,cellsneedtodetectalltypes ofDNAstructuralalterationsembeddedinbillionsofnormal basepairs. Theidentificationof thevariousproteinsthat executeNERwasdonethroughextensivestudiesofhuman cellsdeficientinthisrepairpathway(Maillardetal, 2007). Bothinvivoandinvitroexperimentsidentified XPCasthe first factorthat bindsthedamagedDNA(Sugasawaet al, 1998;Volkeretal,2001;Riedletal,2003).TFIIHisrecruited tothelesionimmediatelyafterXPC(Yokoietal,2000;Riedl etal,2003),presumablythroughdirectprotein–proteininter-
Q2 action(BernardesdeJesusetal,2008).TheroleofTFIIHis devotedtotheopeningoftheDNAaroundthedamagedsite, buttheindividualfunctionofitshelicasesubunitsinthisstep remainsdifficulttodelineate.

Earlierstudiesfromourlaboratoryhaveshownthatmuta-tionsinthehelicasemotifsIII(T469A)orVI(Q638A),which impairedthehelicaseactivityof theXPBsubunit, didnot inhibit theNERactivityof TFIIH(Coinet al, 2007),thus raisingthequestionof theroleof XPBinNER. Here,we showedthatTFIIHcontainingmutationinthemotifIIIofXPB isrecruitedtotheDNArepair sites after UVirradiation. However,amutationinthehelicasemotifIa,whichabolishes theATPaseactivityof XPB, thwartstheaccumulationof TFIIHtothesesites. Thisimpliesthat therecruitmentof TFIIHtositesof damageisanactiveprocessthat requires ATPhydrolysis.Incontrast,theATPaseactivityofXPD,the secondhelicaseofTFIIH,isnotrequiredtorecruitTFIIHto thedamagesites,althoughitisneededforDNArepair.

Inadditiontotheaforementioned ATPasemotif,wefound thattwoadditional motifs,theR-E-DandThMmotifs, are implicatedintherecruitment of TFIIHtosites of DNA damage.Thesetwodomains, highlyconservedinhuman XPB, wereidentifiedinanhomologueof XPBfromthe thermophilicorganismArchaeoglobusfulgidusandweresug-gestedtobeinvolvedinTFIIHfunctions(Fanet al, 2006). MutationsintheR-E-DandThMmotifsmimickedthebio-chemicalandbiologicaldefectsobtainedwithamutationin theATPasemotif.ThissuggeststhattheATPase,R-E-Dand ThMmotifsworktogethertoensureacorrectrecruitmentof TFIIHtothedamagedsitesbeforetheopeninganddual incisionstepstakeplaceduringNER. HowtheR-E-Dand ThMmotifsparticipatetotheanchoringofTFIIH?TheThM domainhasnotbeenfoundinotherhelicases,includingXPD (Bienstocketal,2002;Fanetal,2008;Liuetal,2008;Wolski et al, 2008),but asimilarhelical protrusionhasbeenob-servedinDNApolymerases(Doublieet al, 1998) andin SulfolobussolfataricusSWI2/SNF2ATPaseRad54(Durretal, 2005),inwhichitisexpectedtogripdouble-strandedDNA fromtheminorgroove.ThestructureofXPBsuggeststhatthe energyfurnishedbytheATPhydrolysisisusedtoinducea flipof170 $\AA$ oftheHD2domainafterthebindingofXPBto DNA(Fanetal,2006)(Figure7).TheR-E-D(presentinHD1) andtheThM(presentinHD2)aretheninclosevicinityand areusedtostabilizeTFIIHontheDNAbyintroducinga wedge(the E473residue) inthe double-strandedDNA, grippedbytheThMmotif.Toobtainexperimentalevidence

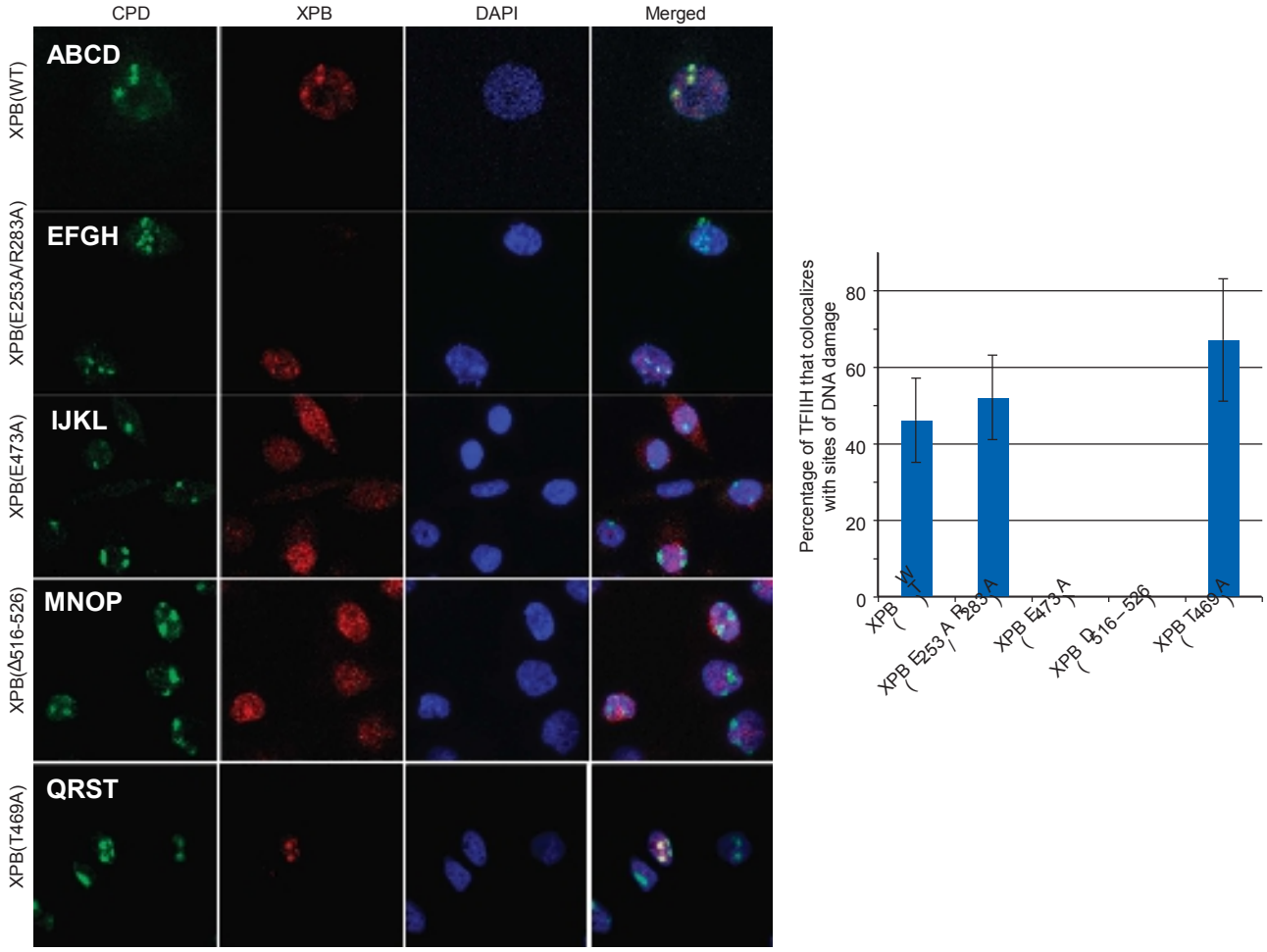

Figure5Recruitment of TFIIHtolocal sitesof DNAdamage.(Left panel)CHO-27-1cellswerestablytransfectedwithpEGFPplasmids expressingvariousformsofGFP-taggedXPBproteins.Thesecellswereirradiatedwith UVlight(100J/m2)throughthe3-mmporefilterand fixed30minlater. Immunofluorescentlabellingwasperformedusingeitheramousemonoclonal anti-CPD(panelsA, E, I, M, Q)ora rabbitpolyclonalanti-GFP(panelsB,F,J,N,R).Nucleiwerecounterstainedwith DAPI(panelsC,G,K,O,S),andslidesweremerged(panels D,H,L,P,T).(Rightpanel)Quantitativeanalysisoftherecruitmentof TFIIHtositesofDNAdamageintransfectedcells.Valuesrepresent averages±s.d.(n ¼100sitesofDNAdamage)fromthreeindependentexperiments.

forthismodel,wecomparedtheATPaseactivitiesoftheWT andmutatedcomplexeswithorwithout DNA. Indeed,like most SF1andSF2members, DNAstimulatestheATPase activityofTFIIH(Royetal,1994).InthepresenceofDNA, mutationsintheR-E-DandThMmotifsinduces60%inhibitionoftheATPaseactivitycomparewithTFIIH(WT).Inthe absenceof DNA, thethreeATPaseactivities arestrictly identicalandareslightlyhigherthantheATPaseactivityof theTFIIHcomplexmutatedintheATP-bindingsiteofXPB. Thesedatafurthersupportthemodel of theconformation changeproposedabove,asit demonstratesthatR-E-Dand ThMareusedtostabilizethebindingof XPBtoDNA. Furthermore,the fact that these mutationsinhibit both, TFIIHtranscriptionandrepairactivities,suggestsacommon modeof recruitmentof TFIIHtothepromotersandtothe damagesites.

TherecruitmentofTFIIHthroughtheactionoftheATPase activityof XPBmayalsoinduceareorganizationof the protein–DNAcomplexesintranscriptionandrepairthatwill allownewprotein–proteinorprotein–DNAcontacts.Indeed, usingphotocrosslinkexperiments,wehaveshownthatadditionofATPinNERinducedare-positioningofXPConthe damagedDNA, whichdependentonTFIIH(Tapiaset al, 2004).Aftertherecruitmentof TFIIHtothedamagedDNA throughtheenergyfurnishedbytheATPaseactivityofXPB, theDNAwouldbeopenedbyXPD, whichhasaprocessive androbusthelicaseactivitystimulatedbythep44subunitof thecoreTFIIH(Coinet al, 1998).Here,amutationinthe ATPase activityof XPDstill allowedTFIIHtobindthe damagedsitesinvivobut wasunabletoopentheDNA aroundthelesion. Altogether,ourdatabringsanewconceptualviewoftherolesof XPBandXPDinNERbyrevealing their different molecular functions within this genome caretakingevent.

Materialsandmethods

Celllines

CHO-27-1isaCHOmutantcell linebelongingtothethirdrodent complementationgroup(thehamsterERCC3geneisthehomologue ofthehumanXPBgene)(Halletal,2005).CHO-UV5belongstothe secondrodentcomplementationgroup(thehamsterERCC2isthe homologueofthehumanXPDgene)(Winkleretal,2000).

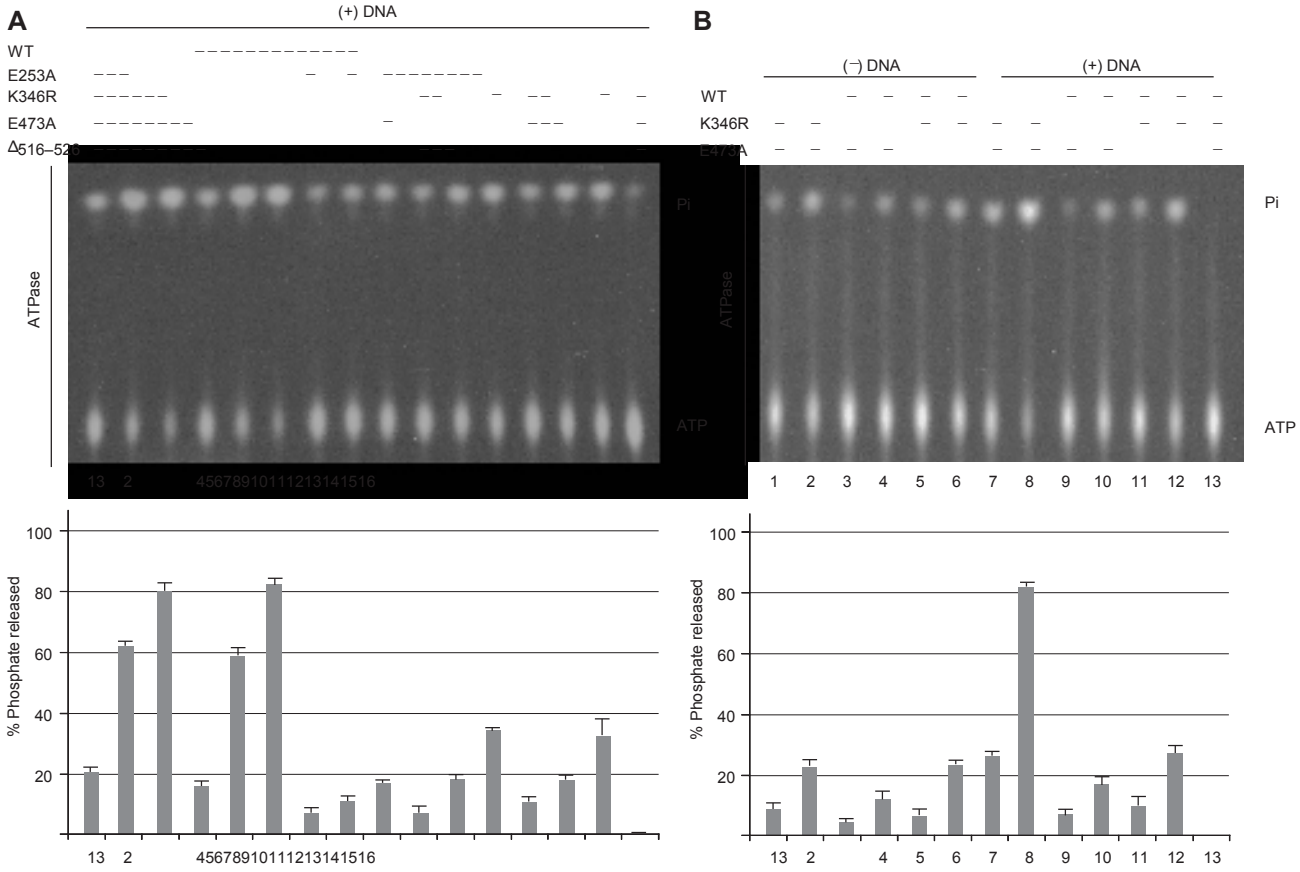

Figure6MutationsintheR-E-Dmotif impair DNA-dependantTFIIHATPaseactivity. (A) 50, 100, and150ngof TFIIH(WT), TFIIH/XPB(E253A),XPB(K346R),XPB(E473A)orXPB(Δ516–526)weretestedinanATPaseassayinthepresenceof200ngofdouble-strandcircularDNA(Coinetal,2007).Theaveragepercentage±s.d.ofphosphatereleased(Pi/(ATPþ Pi))fromthreeindependentexperimentsisrepresentedinthegraph.(B)50and150ngofTFIIH(WT),TFIIH/XPB(K346R)orTFIIH/XPB(E473A)weretestedinanATPaseassaywithout(lanes1–6)or with(lanes7–12)200ngof double-strandcircularDNA.Theaveragepercentage±s.d. of phosphatereleased(Pi/(ATPþ Pi))fromthree independentexperimentsisrepresentedinthegraph.

Constructionoftheplasmids

Baculovirus allowing the expression of mutated XPB were constructedintheFLAGtagpSK278vector(BDBiosciences).XPB wasinsertedattheBamHI/EcoRIsite,infusionwiththeFLAGtagat its5′ side.Themutantswereobtainedbysite-directedmutagenesis (Quickchange,Stratagene).Theresultingvectorswererecombined withbaculovirusDNA(BaculoGoldDNA,PharMingen)inSpodoptera frugiperda9(Sf9)cells. Invivoexperimentswerecarriedout withthepEGFP-N1plasmid(Clontech)containingtheXPBcDNA insertedinframewiththegreenfluorescentproteintag(Hoogstratenetal,2002).

Stablecelllines

CHO-27-1cells(10^5)weretransfectedwith2 mgofpEGFP-N1/XPB plasmidin10cmPetri dishes usinglipofectamine(Invitrogen). Fortyhoursaftertransfection,thefluorescentcellsweresortedon the FACS DIVa (BD; Becton, Dickinsonand Company). The cellswiththehighestleveloffluorescence(about5%oftotalcells) weremaintainedintheselectivemediumwithG418(Geniticin, 800 mg/ml),expandedandanalysedforXPBexpression.

DamagedDNAsubstrates

Covalentlyclosedcircular Pt-DNAcontainingasingle1,3-intrastrandd(GpTpG) cisplatin–DNAcrosslinkwas preparedas described(Fritetal,2002).

Dualincisionassay

Dualincisionassaywascarriedoutin25 mlofRepairbuffer(45mM Hepes-KOH(pH7.8),5mMMgCl2,1mMDTT,0.3mMEDTA,10% glycerol,2.5 mgBSA, 50mMKCl)supplementedwith2mMATP. Eachreactioncontained5ngofXPG,15ngofXPF/ERCC1,10ngof XPC-HR23b,50ngof RPAand25ngof XPA. Afterpre-incubation 10minat 30°C, 30ngof Pt-DNAwas addedandreactionwas continuedfor90minat 30°C.Theexcisedfragmentwasdetectedon a14%urea-acrylamideafterannealingwith9ngof thecomplementaryoligonucleotideandadditionoffourradiolabelleddCMP P^{32} (3000 mCi/mmol)residuesbySequenaseV2.1(USB).

$KMnO_4$ footprintassay

Thedamagedstrandprobewasobtainedon Age1/Ase1digestionof thePt-DNAandradiolabellingatthe 3′ endinaKlenowreaction,the Ptadductislocatedat156bpfromthelabelledend.Theresulting fragmentwas purifiedbythe 'crushandsoak' methodafter migrationina5%nondenaturatingPAGE. Reactions(75 ml) were carriedoutin20 ml ofRepairbuffer(þ 2mMATP)containingthe labelledcisplatinatedprobe(40fmol) and40ngof XPC-HR23b, 25ngofXPA,50ngofRPAand150ngofXPG.Afterincubationat 30°C for15min,3 ml of120mM $KMnO_4$ wasadded,andoxidation was allowedtoproceedfor 3minat roomtemperaturebefore reductionbyadding6 ml of14.6M b-mercaptoethanolfor5minon ice.Afterorganicextractionandethanolprecipitation,driedpellets wereresuspendedin100 ml ofasolutioncontaining1Mpiperidine, 1mMEDTAand1mMEGTAandincubatedat 90°C for25min. Sampleswerenext ethanolprecipitated,andfinal pelletswere recoveredin10 ml ofloadingbufferandanalysedin8%ureaPAGE.

ATPaseassay

Proteinfractionswereincubatedfor2hat 30°C inthepresenceof 1 mCi [g-32P]ATP(7000Ci/mmol,ICNPharmaceuticals)ina20 ml reactionvolumein20mMTris–HCl pH7.9, 4mM $MgCl_2$,1mM DTT, 50 mg/ml BSAandwhenindicated200ngof double-strand DNA(pcDNA3 þ). Reactionswerestoppedbyadditionof EDTA

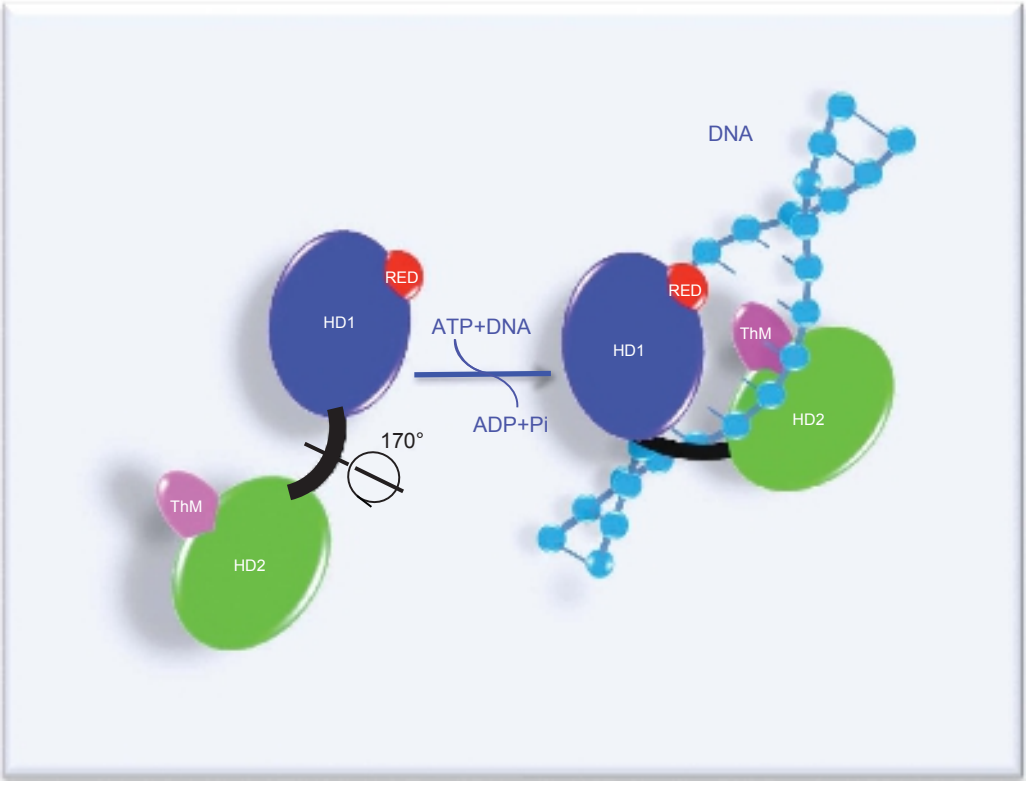

Figure7Proposedstructure-basedmechanismforbindingofXPBtoDNA.Inthismodel,adaptedfromFanetal(2006),XPBisinanopened conformationintheabsenceofDNA.WhenXPBbindstoDNA,therotation(170)ofthesecondhelicasedomain(HD2)togetherwiththeThM domain,facilitatedbyHD1-mediatedATPhydrolysis,formstheclosedandstableXPB–DNAcomplex.

(50mM)andSDS(1%(w/w)). Thereactionswerethendiluted five-fold,spottedontopolyethylenimine(PEI)TLCplates(Merck), runin0.5MLiCl/1Mformicacidandautoradiographed.

LocalUVirradiationandimmunofluorescence

Thecells wererinsedwithPBSandcoveredwithanisopore polycarbonatefilter with pores of 3mmdiameter (Millipore, Badford,MA). Cellswerethenexposedto UVirradiationwitha PhilipsTUVlamp(predominantly254nm)at adoseof 100J/m^2 (Volker et al, 2001).Subsequently,thefilter wasremoved, the mediumwasaddedbacktothecells, andtheywerereturnedto culture conditions for 30min. Then, cells were fixedin2% formaldehydefor15minat roomtemperatureandpermeabilized withPBS/0.5%TritonX-100for5min. AfterwashingwithPBS-Tween(0.05%),theslideswereincubatedfor1hwiththeindicated antibodies.After extensivewashingwithPBS-Tween,theywere incubatedfor1hwithCy3-conjugateddonkeyanti-rabbitIgG,goat anti-mouseAlexa488IgGorgoatanti-ratAlexa488IgG(Jackson Laboratories)diluted1:400inPBS-Tween/0.5%FoetalCalfSerum. Theslideswerecounterstainedfor DNAwithDAPI preparedin Vectashieldmountingmedium(Vector lab). All images were collectedusingaLeicaConfocalTCS4Dmicroscopeequippedwith bothUVlaserandanArgon/Kriptonlaser, andstandardfilterstoo allowcollectionofthedataat488and568nm.ThesoftwareTCSTK was used for three-colourreconstructions,and figures were generatedusingthePLCHTKsoftware.

Host-cellreactivationassay

ThepGL3vectorexpressingPhotinuspyralis(firefly)luciferasewas purchasedfromPromegaandthepCH110vectorexpressingthe galactosidasefromInvitrogen.ThepGL3vectorwasUVirradiated (254nm,1000J/m^2)ataconcentrationof1mg/mlin10mMTris–HCl(pH8.0)and1mMEDTA.CHO-27-1cellsweretransfectedina six-well plateat aconfluenceof 95%usingLipofectaminePlus (Invitrogen).Eachtransfectionmixturecontained500ngofpGL3 (UVþ/Aþ), 100ngof pCH110(nonirradiated)and10ngof the variouspcDNAXPBplasmids.After4hofincubation,thetransfection reagentswerereplacedbymedium.Cellswerelysedafter24h tomeasureluciferaseactivityonamicrotiterplateluminometer (Dynex).All results(meanvaluesof at leastfivemeasurements) werenormalizedbycalculatingtheratiosbetweenluciferaseand galactosidaseactivities.

UV-survivalassay

Cells(10^3)wereplatedper6cmpetridishes,culturedovernightand UVirradiatedat 254nmat variousdoses(0.5J/m^2/s). After 14 days,thecellsarestainedbytrypanblueandcounted.

Antibodies

MousemonoclonalantibodiestowardsTFIIHsubunitswereusedas described(Coinetal,2007).Primaryantibodies(thefinaldilutions areindicatedinparentheses)usedinfluorescent labellingwere purifiedrabbit anti-GFP(TorreyPinesBiolabs, Inc)(1:1000), rat monoclonal anti-HA3F10 (Roche) (1:1000) and mouse IgG monoclonalanti-ThymineDimer(H3)(1:2000)(Abcam).

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Conflictofinterest

Theauthorsdeclarethattheyhavenoconflictofinterest.

References

AraujoSJ, TirodeF, CoinF, PospiechH, SyvaojaJE, StuckiM, HubscherU, EglyJM, WoodRD (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 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, 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, 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 and I 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, Cubedduc L, Roberts J, Liu H, White MF (2008) The archaeal XPB protein is a ss DNA-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, Schaefferl, 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

Schaefferl, 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

Schaefferl, 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 to damaged DNA of transcription factor IIH. J Biol Chem 275: 9870–9875

PUBLICATION 2:

« DOT1L/KMT4 drives recovery of transcription after UV irradiation »

Histone methyltransferase DOT1L drives recovery of gene expression after a genotoxic attack

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Summary

UV-induced DNA damage invokes repression of RNA synthesis. Following DNA damage removal, transcription recovery operates through a process that is not understood yet. Here we show that knocking-out of the histone methyltransferase DOT1L in mouse embryonic fibroblasts (MEF^{DOT1L}) leads to a high UV sensitivity coupled to a deficient recovery of transcription initiation after UV irradiation. However DOT1L is not implicated in the removal of the UV-induced DNA damage by the nucleotide excision repair pathway. Vice, using strip-FRAP and ChIP experiments we established that DOT1L promotes the formation of the pre-initiation complex to the promoter of a housekeeping gene and the appearance of transcriptionally active chromatin marks there. Treatment with Trichostatin A, which merely relaxes chromatin, recovers transcription initiation and restores normal UV sensitivity in MEF^{DOT1L}cells. Our data suggest that DOT1L secures an open chromatin structure in order to reactivate RNA Pol II transcription initiation after the genotoxic stress.

Introduction

Short-wave UV light may serve as a significant source of mutagenic and cytotoxic DNA damage. UV irradiation induces two major types of DNA lesions; the cis-syn cyclobutane-pyrimidine dimers (CPD) and the pyrimidine (6-4) pyrimidone photoproducts (6-4PP) {Friedberg, 2006 #969} . Through the deformation of the DNA structure, these lesions implement repressive effect on various nuclear processes including replication and transcription. As a matter of fact, the removal of these lesions is a priority for the cell and takes place at the expense of fundamental cellular processes that are paused to circumvent the risks of mutations or aberrant gene products. The molecular mechanism underlying transcription inhibition and recovery is not understood yet but it includes proteins such as CSB, a member of the SWI2/SNF2 family of chromatin remodeling proteins, which promote transcription re-initiation at the promoters of housekeeping genes {Citterio, 2000 #980 ; Proietti-De-Santis, 2006 #1}.

UV lesions are removed from DNA by the nucleotide excision repair (NER) mechanism through two sub-pathways. A general global genome repair (GG-NER) removes DNA damage from the entire genome, while a transcription-coupled repair (TC-NER) corrects lesions located on the actively transcribed genes {Hanawalt, 2002 #95}. In the TC-NER, an elongating RNA polymerase II (RNA Pol II) stalled by a lesion triggers efficient repair of the cytotoxic damage that block transcription while lesion anywhere else in the genome are detected by the XPC/hHR23B complex for the GG-NER {Fousteri, 2008 #52} . Then, both sub-pathways funnel into a common process involving XPA, RPA, TFIIH, XPG and XPF-ERCC1 to excise damaged oligonucleotides from DNA.

Post-translational histone modifications modulate promoter activity. Histone acetylation, phosphorylation, ubiquitination, and methylation dictate the transcriptional fate of any given locus {Shilatifard, 2006 #11}. Inactive chromatin is associated with high levels of methylation at H3K9, H3K27, and H4K20 lysine residues and low levels of histone acetylation, while actively transcribed chromatin shows a high level of acetylation of H4K16 and H4K20 and methylation of H3K4, H3K36, and H3K79 residues {Kouzarides, 2007 #13;Campos, 2009 #12}.

The *dot1* gene (disruptor of telomeric silencing-1), also called *kmt4* (lysine methyltransferase-4), encodes a protein that exclusively methylates lysine 79 of histone H3 (H3K79) {Feng, 2002 #55;Lacoste, 2002 #237;van Leeuwen, 2002 #31}. Unlike most modified histone residues that are located within the N-terminal tail, H3K79 is found within the globular core of the histone octamer {Luger, 1997 #39}. The DOT1 protein is the only histone lysine methyltransferase that does not contain the conserved SET domain but exhibits a methyltransferase fold that is responsible for its activity {Min, 2003 #40;Sawada, 2004 #41}.

Several lines of evidence suggest that DOT1 plays an important role in genomic stability. DOT1L has been reported to favor the recruitment of double strand breaks sensor 53BP1 to DNA lesions {Huyen, 2004 #989}. DOT1 is also required for the activation of the *RAD9/RAD53* checkpoint function by UV and ■radiation {Giannattasio, 2005 #42;Wysocki, 2005 #43;Toh, 2006 #44}. DOT1 plays also a role in the yeast cellular response to UV damage but its specific function in this mechanism is unclear {Bostelman, 2007 #45}.

Here, we provide evidence that the mammalian DOT1L (DOT1-like) protein is required to re-initiate transcription after UV irradiation. Knocking-out of DOT1L results in high sensitivity to UV irradiation in mouse embryonic fibroblasts (MEF ^{DOT1L}), however preserves an accurate repair of the two main types of UV-induced DNA damage. Instead, MEF ^{DOT1L} cells are unable to recover transcription of constitutively expressed genes after UV irradiation. Using fluorescence recovery after photobleaching (FRAP), we have shown that DOT1L regulates the recruitment of RNA Pol II to chromatin after UV irradiation. Applying chromatin immunoprecipitation assay, we additionally revealed that DOT1L triggers the formation of the pre-initiation complex to the promoters of housekeeping genes after UV irradiation, as well as the appearance of active transcriptional marks on histones. Altogether, our results highlight a new role for DOT1L in the protection of an open chromatin structure in order to reactivate the formation of the pre-initiation complex (PIC) on the promoter of constitutive genes after a genotoxic stress.

Results

DOT1L deficient mammalian cells are UV-sensitive.

To investigate the role of DOT1L in the repair of UV-induced DNA damage, we used knocked-out MEF^{DOT1L} cells carrying a homozygous gene trap insertion in *Dot1l* {Steger, 2008 #46}. Together with a decrease in *Dot1l* expression to the background level, the mono- and tri-methylation of H3K79 was abolished in MEF ^{DOT1L} cells(Figure 1A). In a UV survival assay, MEF^{DOT1L} cells were more sensitive to UV irradiation as compared to MEF ^{WT} cells but less than MEF^{XPG}cells , that were knocked-out for the NER factor XPG and deficient both for GG- and TC-NER {Shiomi, 2005 #148} (Figure 1B, in black&white two squares looks odd, maybe circle will do better). However, MEF ^{DOT1L} cells showed UV sensitivity similar to that achieved in cells with knock-out of the CSB protein (MEF^{CSB}), which were deficient in TC-NER.

We further investigated whether DOT1L affected UV survival by sustaining the repair of UV-induced DNA damage. We performed unscheduled DNA synthesis assay (UDS), which was mainly a measure of the GG-NER efficiency {Stefanini, 1992 #97}. The UDS of MEF ^{DOT1L} cells was identical to that of the MEF ^{WT} cells(Figure 2A what is a unit in y-axis ?). We also used an assay based on immunofluorescence coupled to quantification of DNA lesions directly in the cell nucleus to measure repair of the main type of UV-induced DNA damage (see experimental procedures). MEF^{XPG} cells showed higher level of (6-4)PPs in the genomic DNA along the time course of repair, compared to MEF ^{WT} or MEF ^{CSB} cells in which lesions were gradually removed (Figures 2B). When measured in MEF ^{DOT1L} cells, the removal rate of (6-4)PP lesions was identical to that of MEF ^{WT} cells(Figures 2B), which implied that the absence of DOT1L did not impair the GG-NER.

To determine whether MEF^{DOT1L} cells were able to perform the TC-NER, we measured cell survival following incubation with Ecteinascidin 743 (et743), an anti-tumor drug that was shown to be highly cytotoxic only for TC-NER proficient cells {Takebayashi, 2001 #104}. Indeed, in our experimental conditions, cells with knock-out of the TC-NER factor CSB demonstrated higher et743 tolerance(Figure 2C). In contrast, MEF ^{DOT1L} cells showed sensitivity to et743 which

wasequivalent to that of MEF ^{WT} cells (Figure 2C). Overall, these results indicated that the absence of DOT1L induced sensitivity to UV irradiation that was not the consequence of the defects in GG- and TC-NER.

DOT1L promotes transcription recovery after UV irradiation.

To further investigate the role of DOT1L in cell survival, we analyzed whether DOT1L was involved in the recovery of RNA synthesis (RRS) after UV irradiation. Cells were UV-irradiated and their ability to synthesize RNA was monitored by incubation with radioactive [^{3}H]uridine during a 30 minutes pulse performed 1, 8 and 24 hours after exposure to UV-C light (20J/m ^{2}) {Stefanini, 1992 #47}. RNA synthesis was depressed in MEF ^{WT} cells immediately after UV irradiation to a level 80% of that in mock-treated cells (Figure 3A). Over the next 8 hours, RNA synthesis recovered completely. In contrast, the RNA synthesis in irradiated MEF ^{DOT1L} cells progressively decreased to a level 50% of that in mock-treated cells and did not recover significantly with time (Figure 3A). To demonstrate visual data, we measured RNA synthesis by autoradiography following incubation with radioactive uridine {Stefanini, 1992 #47}. Mock-treated MEF ^{WT} and MEF ^{DOT1L} cells showed similar levels of RNA synthesis, visualized by equivalent number of black dots in their nuclei (～100 dots/nucleus, Figure 3B, graph and autoradiography, compare panels a and c). In contrast, we observed a marked deficiency of RNA synthesis in MEF ^{DOT1L}cells , 24 hours after UV treatment, as compared with MEF ^{WT} cells(Figure 3B, graph and autoradiography, compare panels b and d). We estimated the residual transcription activity in the MEF^{DOT1L} cells to a level of 30% of that in mock-treated cells, 24 hours after irradiation with 20J/m^2.

DOT1L ensures RNA Pol II binding to chromatin after UV irradiation.

To determine the molecular mechanisms that led to the inhibition of transcription in MEF^{DOT1L} cells after UV irradiation, we examined live-cell protein mobility of RNA Pol II by

fluorescence recovery after photobleaching (strip-FRAP). In brief, a small region in the middle of the nucleus was bleached and the subsequent fluorescence recovery was measured in time (Figure 4A). For that purpose, the largest RNA Pol II subunit RPB1 was fused with GFP and expressed either in MEF ^{WT} or MEF ^{DOT1L}cells . In these conditions, we found an equivalent mobility of RNA Pol II in mock-treated and UV-irradiated (16J/m ^{2}) MEF ^{WT} cells(Figure 4B). In marked contrast, strip-FRAP analysis of UV-damaged MEF ^{DOT1L} cells revealed a significant increase in the amount of fluorescence recovery when compared to mock-treated cells (Figure 4C), indicating that a fraction of RNA Pol II became mobile in the absence of DOT1L, after UV irradiation.

Defect in transcription initiation after UV irradiation in cells depleted of DOT1L.

We next wondered whether DOT1L was required to mobilize RNA pol II either during the initiation or elongation steps of transcription. For this purpose, we examined the step of transition from initiation to elongation by the RNA Pol II *in vivo* on an endogenous mouse gene. Briefly, we reversibly blocked gene transcription in cells by incubation with the P-TEFb inhibitor DRB (5,6-dichloro-1- ✔-D-ribobenzimidazole), which inhibited the transition from initiation to elongation but did not block elongation of ongoing mRNA transcripts allowing RNA Pol II and splicing machinery to finish initiated RNA synthesis and excise introns from all remaining unmature transcripts{Singh, 2009 #151} (Figure 5A). Following the removal of DRB, RNA Pol II was released from promoter-proximal regions and the level of newly synthesized pre-mRNA had been measured owing to the presence of introns. As we measured the transition from initiation to elongation on the *Utrophin* gene that possessed a very short Exon1 (170bp) andsince ~1 transcription blocking (6-4)PP lesion was created per 30kb of DNA at 20J/m^{2} {Coin, 2008 #153}, less than 5% of cells had the UV-lesion in this exon following a used dose of 15J/m ^{2} of UV-C. Therefore, any significant inhibition of transcription initiation couldn't be explained by the blockage of RNA Pol II in front of a lesion in the Exon 1.

We treated MEF cells for 3 hours with DRB and extracted RNA at 10 minutes intervals after removal of the drug (Figure 5B). Next, we performed RT-PCR using primers spanning Exon1-Intron1 junctions to detect newly synthesized pre-mRNA of *Utrophin gene*. In the absence of genotoxic stress, MEF^{WT} and MEF^{DOT1L} cells were able to recover transcription of the Exon1 region within 10 to 20 minutes after the DRB removal (Figure 5C), indicating that the transcriptional initiation rate in MEF ^{WT} and MEF ^{DOT1L} cells was identical, in the absence of the genotoxic attack. Then, we irradiated MEF cells with UV-C (15J/m ^{2}) after the DRB treatment (Figure 5B). In these experimental conditions, MEF ^{WT} cells were able to recover transcription of the Exon1 within 60 minutes after the removal of DRB and UV-treatment, while MEF ^{DOT1L} cells showed no recovery of the Exon1 transcription throughout the entire time course (Figure 5D).

Since DOT1L was shown to be involved in the chromatin remodeling, we tested whether a chromatin relaxation could overcome the absence of DOT1L. For that purpose, we treated MEF^{DOT1L} cells with Trichostatin A (TSA, 20nM), a class I HDAC inhibitor that relaxed chromatin nonspecifically (Figure 5B). Following the TSA treatment, we observed a recovery of the Exon1 pre-mRNA expression in MEF ^{DOT1L}cells, which peaked between 30 to 40 minutes after UV irradiation (Figure 5E) and paralleled the transcription of the Exon1 in MEF ^{WT} cells. Together with the recovery of rhe Exon 1 expression, pre-treatment of MEF ^{DOT1L} cells with TSA (10nM) also induced a potent recovery of their UV survival (Figure 5F) suggesting that transcription inhibition in MEF^{DOT1L} cells was indeed responsible for the UV-sensitivity which was observed in these cells. Altogether, our data suggested that DOT1L relaxed chromatin and allowed RNA Pol II transcription re-initiation after UV irradiation.

Repressive chromatin marks in cells depleted of DOT1L after UV irradiation.

The above data prompted us to perform a detailed analysis of PIC formation on the promoter of housekeeping genes throughout the time, after UV irradiation. We studied the promoter of *DHFR* gene that was commonly used in many laboratories as well as in our group as a model system for studying the assembly/disassembly of the PIC after UV-irradiation

{Proietti-De-Santis, 2006 #1}. Using chromatin immunoprecipitation (ChIP), we observed a slight decrease in RNA Pol II and TFIIB occupancy at the *DHFR* promoter in UV-irradiated MEF^{WT}cells , 2 hours post-UV irradiation (Figures 6B and 6C). The transcription machinery started to re-assemble on the promoter between 6 and 10 hours after UV irradiation (Figures 6B and 6C) and the steady state level of DHFR mRNA did not varied significantly with time in UV-irradiated MEF^{WT}cells ,(Figure 6A). In contrast, RNA Pol II and TFIIB were not back to the housekeeping gene promoter throughout the entire time course in MEF ^{DOT1L} cells (Figures 6B and 6C), while the steady state level of *DHFR* mRNA decreased with time after UV irradiation to a level 40% of that of the mock-treated MEF^{DOT1L} cells (Figure 6A), in agreement with results obtained in Figure 3 where we measured global transcription.

We then analyzed chromatin modifications associated with active or inactive chromatin in the promoter region of the *DHFR* gene. We observed that accumulation of acetylated histone H4, an active mark of transcription, followed the dynamic of the RNA Pol II, with the first phase of decrease 2 hours post-UV, and the phase of increase from 6 to 24 hours (Figure 6D). In contrast, no phase of recovery was observed in MEF ^{DOT1L} cells (Figure 6D). Di-methylation of H3K9 residue, a mark of inactive transcription, was stable in MEF ^{WT} cells throughout the time course after UV-treatment, while it increased just after UV irradiation in MEF ^{DOT1L} cells (Figure 6E). Finally, di-methylation of H3K79, performed by DOT1L, was observed transiently in the promoter region of *DHFR* and peaked at 6 hours after UV irradiation(Figure 6F), when RNA Pol II and TFIIB started to come back to the DHFR promoter in MEF ^{WT} cells. Altogether these data suggested that DOT1L was required to drive the recovery of PIC formation at the promoters of housekeeping genes after UV irradiation.

DiscussionTranscription inhibition and its subsequent recovery that operate after the genotoxic attack are thought to limit the risks that lesions represent for the cell and its genome. Molecular mechanisms which are responsible for the turn-off/turn-on switch of transcription after the DNA damage are not understood well. Here we show that the methyltransferase DOT1L is required for the re-initiation of transcription after UV-irradiation supporting the re-formation of the PIC. Our data supports the hypothesis that transcription recovery after a genotoxic attack is an active cellular process which involves specific chromatin remodelers, the actors which insure not only the repair of the DNA but also the recovery of fundamental cellular processes such as transcription.

DOT1L participates to UV-survival but not to DNA repair.

Our study demonstrates that the knock-out of DOT1L causes hypersensitivity to UV irradiation in mammalian cells. There are several plausiible mechanisms that could explain the increased sensitivity to UV irradiation conferred by DOT1L depletion. If DOT1L is directly involved in the DNA repair, its absence may results in the increased level of DNA damage leading to cellular apoptosis. A function for yeast Dot1 in GG-NER has been described recently {Tatum, 2011 #987}. However, we don't find any DNA repair defect associated with the absence of DOT1l in mammalian cells. Indeed, MEF cells depleted of DOT1L exhibit normal UDS efficiency, in assay that mainly measure GG-NER. Furthermore, these cells repair (6-4)PPs, the main UV-induced NER substrate, at the same rate that wild-type cells do. MEF ^{DOT1L} Indeed are also proficient in TC-NER, we show it via sensitivity of the cells towards the drug et743, a natural marine product isolated from the Caribbean see squirt. Antiproliferative activity of et743 is shown to be dependent on active TC-NER {Takebayashi, 2001 #104}. In our experimental conditions, we observe that MEF cells with knock-out of CSB, one of the two TC-NER specific factors, exhibit a higher resistance to et743 treatment than MEF ^{WT}cells do , confirming previous observation obtained with patient cells {Takebayashi, 2001 #104}. Using et743 on MEF ^{DOT1L}, we show that these cells are as sensitive to treatment as MEF ^{WT} cells and we conclude that it is unlikely that high sensitivity to UV irradiation in DOT1L-deficient mammalian cells is due to a

GG-NER or TC-NER defects.

DOT1L controls transcription recovery after UV irradiation.

Alternatively, DOT1L may serve in reactivation ofmRNA synthesis after UV irradiation. Transcriptional arrest has been shown to lead to a highly cytotoxic cellular response to stress {Fousteri, 2008 #52}. This response has multiple causes and is likely not only the result of DNA lesions that block RNA Pol II in elongation. Previous studies have challenged the relationship between efficient repair of a lesion in the transcribed strand of active genes and the recovery of transcription inhibited by DNA damage . For instance, cells carrying mutations in the Cockayne syndrome B protein (CSB) were unable to recover lesion-inhibited transcription while they efficiently repaired acetylaminofluorene lesions in transcriptionally active genes {van Oosterwijk, 1996 #53}. In addition, CSB was shown to accumulate to the promoters of housekeeping genes after UV irradiation, where it stimulated the recovery of inhibited transcription independently of the presence of lesions {Proietti-De-Santis, 2006 #1}. This finding led to the hypothesis that removal of transcription blocking lesions was insufficient to restore transcription after DNA damage and that in addition, chromatin changes in the promoters of housekeeping genes could be required. In our study we, using RRS assay, show the inhibition of global gene expression in cells which are depleted of DOT1L. We detect only30% of residual transcription there 24 hours post UV irradiation (20J/m ^{2}). This inhibition is confirmed at the single gene level since *DHFR* show the similar level of inhibition at the same time point. Furthermore, transcription inhibition does not depend on the cell cycle since no difference in the distribution of the phases is observed between MEF^{WT} and MEF^{DOT1L} cells before or after UV irradiation (Unpublished Data).

DOT1L allow PIC formation on housekeeping gene promoters after UV irradiation.

Early steps of mRNA expression include formation of PIC, transcription initiation and escape of RNA Pol II form the promoter at the elongation step. Using the DRB assay that measure the expression of newly synthetized first short exon of *Utrophin* gene *in vivo*, we

demonstrate that the transition from initiation to elongation iq deficient in the absence of DOT1L after UV. We further analyse both the transcription machinery occupancy and the chromatin modifications on the promoter of the housekeeping gene, DHFR, with time after UV irradiation and we show that this promoter is temporally depleted of basal transcription factors in the first hours after irradiation in wild-type cells. Aand we demonstate that recovery of occupancy after DNA repair take place, 6-10 hours after UV irradiation. However in the absence of DOT1L, RNA Pol II and TFIIB, basal transcription factor, do not come back to the promoter of DHFR gene , Moreover increase in heterochromatin mark such as methylation of H3K9 residue appears on this promoter. Using GFP-tagged RPB1 subunit of RNA Pol II in strip-FRAP experiment we show that a fraction of RNA Pol II becomes mobile after UV-irradiation in the absence of DOT1L, indicating that dissociation of RNA Pol II from promoters of housekeeping gene after UV irradiation in the absence of RNA Pol II is a broad phenomenon.

Altogether, these data suggest that DOT1L favors an open chromatin structure around the promoter in housekeeping genes allowing efficient transcription re-initiation. In line with this hypothesis, H3K79me2, the mark of DOT1L activity, peaks transiently on the promoters of housekeeping genes in wild-type cells after UV irradiation, keeping the open chromatin. In addition, the absence of DOT1L can be bypassed by the class I HDAC inhibitor TSA which barely relaxes chromatin. TSA restores both transcription initiation and normal UVsensitivity in MEF^{DOT1L} cells, creating thus a link between these two events.

Among the sites of histone H3 methylation, H3K79 is unique as it is not located within the H3 N-terminal tail domain but is found in the core region. Specifically, this methylation occurs on the surface of the nucleosome and may serve as a platform to recruit additional chromatin modifiers and DNA damage response factors {Luger, 1997 #39}. On the other hand, regions of chromatin where transcription is repressed are depleted of H3K79 methylation, indicating that silencing of chromatin probably requires hypomethylation of H3K79 {van Leeuwen, 2002 #31;Ng, 2003 #54}. The mechanism that links euchromatin to H3K79 methylation is not fully understood but it is believed that in addition to recruiting chromatin

modifiers, this histone mark plays an important role in confining the Sir proteins to heterochromatic regions {Ng, 2002 #56;van Leeuwen, 2002 #31}. In yeast, Sir3 binds to nucleosomes containing deacetylated histone H4K16 and promotes spreading of heterochromatin along the chromatin {Johnson, 2009 #57}. Based on these observations and our data, we propose that RNA Pol II re-accumulation at promoters of housekeeping genes after UV irradiation depends on the chromatin changes orchestrated by DOT1L, including the emergence of active chromatin transcription marks around the promoters of these genes. In the absence of DOT1L, facultative heterochromatin marks such as H3K9me2 appear and RNA Pol II does not get to the promoters. Through the recruitment of chromatin modifiers and subsequent histone modifications, DOT1L serves to limit the spreading of heterochromatin to housekeeping genes immediately after UV irradiation and allowing re-association of the basal transcription machinery to the promoters of these genes and re-activation of their transcription.

We also believe that our observation will lead to the identification of additional factors relevant for the regulation and timing of transcription recovery of housekeeping genes after DNA damage that appears more complicated than anticipated.

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Experimental Procedures

Cell lines. MEF cells were cultured at 37°C in the presence of 5% CO2 in Dulbecco's modified medium supplemented with 10% FCS.

Quantitative UV survival analysis. Ten thousand cells were plated on a 3.5 cm Petri dish, cultured overnight and UV-irradiated with UV-C light (254nm) at various doses (0.5J/m^2/s). After 4 days, cells were dried and stained by crystal violet, then lysed and quantified by spectrometry at 570nm wavelength. When indicated, MEF cells were incubated with TSA (10nM final concentration) for 3 hours before UV irradiation and during the 4 days of the post-irradiation period.

Immunofluorescent-based DNA lesion quantification. Five thousand MEF cells were plated in 96 well plates (OptiPlates-96, Perkin Elmer). Twenty-four hours later, cells were UV-irradiated with UV-C lamp (10J/m^2) and recovered in fresh medium for the indicated period of time at 37°C, 5% CO2. Immuno-labeling of (6-4)PP was performed using mouse 64M-2 antibodies respectively. For this, cells were treated as described below but prior to labeling, DNA was denatured with 2M HCl for 30 minutes at room temperature (RT) and blocked in 10% BSA in PBS for 15 minutes. (6-4)PP lesions were quantified using an INCell Analyser 1000 imaging system (GE Healthcare) and the percentage of (6-4)PP removal was determined (100% represents the amount of lesions determined just after UV irradiation).

Unscheduled DNA synthesis (UDS) and Transcription recovery after UV irradiation (RRS). UDS was determined by counting the number of grains on at least 50 non-S-phase cells in autoradiographic preparations of cultures incubated for 3 h after UV-irradiation in medium containing ^{3}H-thymidine (^{3}H-TdR, Amersham, specific activity 25 Ci/mmol) {Stefanini, 1992 #97}. In RRS, cells were incubated in the presence of 20µCi/ml [^{14}C]thymidine (PerkinElmer Life and Analytical Sciences, Boston MA 02118 USA) for 2 days to uniformly label the DNA. The UV-

irradiated cells (10J/m ^{2}) were pulse labeled with 5µCi/ml of [^{3}H]uridine (PerkinElmer Life and Analytical Sciences, Boston MA 02118 USA) for 30 minutes at different time points. 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 hours 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.

Fluorescence recovery after photobleaching (FRAP). Cell lines stably expressing GFP-RPB1$^{\text{-amaR}}$ were generated by transfection of MEF^{WT} or MEF^{DOT1L} cells with 1 ug of pAT7h1$^{\text{-amaR}}$ {Nguyen, 1996 #208} using FuGEN6 (Roche Diagnostics, Mannheim, Germany). One day after transfection, cells expressing GFP-RPB1$^{\text{-amaR}}$ were selected by overnight incubation with 20 ug/ml of alpha-amanitin. Three days prior to microscopy experiments, cells were seeded onto 24 mm diameter coverslips. Imaging and FRAP were performed on a Zeiss LSM 710 meta confocal laser scanning microscope (Zeiss, Oberkochen, FRG).

FRAP analysis was performed at high time resolution as previously described {Giglia-Mari, 2006 #207}. Briefly, a strip spanning the nucleus was photo-bleached for 20ms at 100% laser intensity (laser current set at 6.1Å). We monitored the recovery of fluorescence in the strip every 20 msec for 20 sec at 0.5% of laser intensity. Twenty independent measurements were performed and the average values were used for every mobility curve. Mobility curves are plotted as relative fluorescence (fluorescence post-bleach divided by fluorescence pre-bleach) measured against time. Error bars included in all the plotted FRAP data represent the SEM. Whenever two distinct FRAP curves were not easily dissociable, the statistical significance of their difference was checked by using Student's t-test (two-sample, two-tailed) within an appropriate time window: right after the photobleaching when evaluating mobility differences or after complete recovery when immobile fractions were being evaluated.

Initiation-Elongation transition assay *in vivo(DRB assay)*. We grew cells overnight on 60mm plates to 70-80% confluency and treated them with 100 μM of 5,6-Dichlorobenzimidazole 1- β-D-ribofuranoside (DRB) (Sigma) in culture medium for 3 hours. The cells were washed twice with PBS and incubated in fresh medium for various periods of time and RNA was extracted. Trichostatin A (TSA) (Sigma) was used at a concentration of 20nM and was added 12 hours before treatment with DRB and maintained during the time of the experiment. When indicated, cells were UV-irradiated (15J/m^2) after the DRB treatment.

Reverse Transcription and Real-Time Quantitative PCR. cDNA synthesis was performed using hexamere 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.

Chromatin Immunoprecipitation (ChIP). Cells were crosslinked with a 1% formaldehyde solution for 10 minutes at RT. Crosslinking was stopped by addition of glycine at 125mM final concentration for 5 minutes at RT. Samples were sonicated to generate 500bp DNA fragments. For immunoprecipitations, 100 μg of protein extract was pre-cleared for 2 hours with 50 μl of protein G-sepharose before addition of the indicated antibodies. Then, 2 μg of antibody was added to the reactions and incubated over night at 4°C in the presence of 50 μl of protein A/G beads. After serial washings, the immunocomplexes were eluted twice for 10 minutes at 65°C and crosslinking was reversed by adjusting to 200mM NaCl and incubating 5 hours at 65°C. Further proteinase-K digestion was performed for 2 hours at 42°C. DNA was purified using Quiagen columns (QIAquick PCR Purification Kit). Immunoprecipitated DNA was quantified by real-time PCR. Primer sequences are available upon request.

Antibodies. Primary antibodies (the final dilutions are indicated in parentheses) used were

mouse monoclonal anti-6–4PP (Cosmobio; 64M-2, dilution 1/500), ,anti-TFIIB C-18 (Santa Cruz, sc-225), anti-H3K79me1 (Cell Signaling, 9757S) (1/1.000), anti-H3K79me3 (Abcam, ab2621) (1/1.000) and anti Histone H3 (Abcam, ab1791). Anti-Acetylated H4 is a mouse monoclonal antibodies produced at the IGBMC.

References

Ardehali MB, Lis JT (2009) Tracking rates of transcription and splicing in vivo. *Nat Struct Mol*

Biol **16**(11)**:** 1123-1124

Bostelman LJ, Keller AM, Albrecht AM, Arat A, Thompson 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**(3)**:** 383-395

Campos EI, Reinberg D (2009) Histones: annotating chromatin. *Annu Rev Genet* **43:** 559-599

Coin F, Oksenych V, Mocquet V, Groh S, Blattner C, Egly JM (2008) Nucleotide excision repair driven by the dissociation of CAK from TFIIH. *Mol Cell* **31**(1)**:** 9-20

Feng Q, Wang H, Ng HH, Erdjument-Bromage H, Tempst P, Struhl K, Zhang Y (2002) Methylation of H3-lysine 79 is mediated by a new family of HMTases without a SET domain. *Curr Biol* **12**(12)**:** 1052-1058

Fousteri M, Mullenders LH (2008) Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects. *Cell Res* **18**(1)**:** 73-84

Giannattasio M, Lazzaro F, Plevani P, Muzi-Falconi M (2005) The DNA damage checkpoint response requires histone H2B ubiquitination by Rad6-Bre1 and H3 methylation by Dot1. *J Biol Chem* **280**(11)**:** 9879-9886

Giglia-Mari G, Miquel 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**(6)**:** e156

Hanawalt PC (2002) Subpathways of nucleotide excision repair and their regulation. *Oncogene* **21**(58)**:** 8949-8956

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**(5)**:** 1163-1174

Johnson A, Li G, Sikorski TW, Buratowski S, Woodcock CL, Moazed D (2009) Reconstitution of heterochromatin-dependent transcriptional gene silencing. *Mol Cell* **35**(6)**:** 769-781

Kouzarides T (2007) Chromatin modifications and their function. *Cell* **128**(4)**:** 693-705

Lacoste N, Utley RT, Hunter JM, Poirier GG, Cote J (2002) Disruptor of telomeric silencing-1 is a chromatin-specific histone H3 methyltransferase. *J Biol Chem* **277**(34)**:** 30421-30424

Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389**(6648)**:** 251-260

Min J, Feng Q, Li Z, Zhang Y, Xu RM (2003) Structure of the catalytic domain of human DOT1L, a non-SET domain nucleosomal histone methyltransferase. *Cell* **112**(5)**:** 711-723

Mone MJ, Volker M, Nikaido O, Mullenders LH, van Zeeland AA, Verschure PJ, Manders EM, van Driel R (2001) Local UV-induced DNA damage in cell nuclei results in local transcription

inhibition. *EMBO Rep* **2**(11)**:** 1013-1017

Muse GW, Gilchrist DA, Nechaev S, Shah R, Parker JS, Grissom SF, Zeitlinger J, Adelman K (2007) RNA polymerase is poised for activation across the genome. *Nat Genet* **39**(12)**:** 1507-1511

Ng HH, Ciccone DN, Morshead KB, Oettinger MA, Struhl K (2003) Lysine-79 of histone H3 is hypomethylated at silenced loci in yeast and mammalian cells: a potential mechanism for position-effect variegation. *Proc Natl Acad Sci U S A* **100**(4)**:** 1820-1825

Ng HH, Feng Q, Wang H, Erdjument-Bromage H, Tempst P, Zhang Y, 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**(12)**:** 1518-1527

Nguyen AT, Zhang Y (2011) The diverse functions of Dot1 and H3K79 methylation. *Genes Dev* **25**(13)**:** 1345-1358

Nguyen VT, Giannoni F, Dubois MF, Seo SJ, Vigneron M, Kedinger C, Bensaude O (1996) In vivo degradation of RNA polymerase II largest subunit triggered by alpha-amanitin. *Nucleic Acids Res* **24**(15)**:** 2924-2929

Proietti-De-Santis L, Drane P, Egly JM (2006) Cockayne syndrome B protein regulates the transcriptional program after UV irradiation. *Embo J* **25**(9)**:** 1915-1923

Sawada K, Yang Z, Horton JR, Collins RE, Zhang X, Cheng X (2004) Structure of the conserved core of the yeast Dot1p, a nucleosomal histone H3 lysine 79 methyltransferase. *J Biol Chem* **279**(41)**:** 43296-43306

Shilatifard A (2006) Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. *Annu Rev Biochem* **75:** 243-269

Shiomi N, Mori M, Kito S, Harada YN, Tanaka K, Shiomi T (2005) Severe growth retardation and short life span of double-mutant mice lacking Xpa and exon 15 of Xpg. *DNA Repair (Amst)* **4**(3)**:** 351-357

Singh J, Padgett RA (2009) Rates of in situ transcription and splicing in large human genes. *Nat Struct Mol Biol* **16**(11)**:** 1128-1133

Stefanini M, Giliani S, Nardo T, Marinoni S, Nazzaro V, Rizzo R, Trevisan G (1992) DNA repair investigations in nine Italian patients affected by trichothiodystrophy. *Mutat Res* **273**(2)**:** 119-125

Steger DJ, Lefterova MI, Ying L, Stonestrom AJ, Schupp M, Zhuo D, Vakoc AL, Kim JE, Chen J, Lazar MA, Blobel GA, Vakoc CR (2008) DOT1L/KMT4 recruitment and H3K79 methylation are ubiquitously coupled with gene transcription in mammalian cells. *Mol Cell Biol* **28**(8)**:** 2825-2839

Takebayashi Y, Pourquier P, Zimonjic DB, Nakayama K, Emmert S, Ueda T, Urasaki Y, Kanzaki A, Akiyama SI, Popescu N, Kraemer KH, Pommier Y (2001) Antiproliferative activity of ecteinascidin 743 is dependent upon transcription-coupled nucleotide-excision repair. *Nat Med* **7**(8)**:** 961-966

Toh GW, O'Shaughnessy AM, Jimeno S, Dobbie IM, Grenon M, Maffini S, O'Rorke A, 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**(6)**:** 693-703

Tomicic MT, Christmann M, Kaina B (2005) Apoptosis in UV-C light irradiated p53 wild-type, apaf-1 and p53 knockout mouse embryonic fibroblasts: interplay of receptor and mitochondrial pathway. *Apoptosis* **10**(6)**:** 1295-1304

van der Wees C, Jansen J, Vrieling H, van der Laarse A, Van Zeeland A, Mullenders L (2007) Nucleotide excision repair in differentiated cells. *Mutat Res* **614**(1-2)**:** 16-23

van Hoffen A, Balajee AS, van Zeeland AA, Mullenders LH (2003) Nucleotide excision repair and its interplay with transcription. *Toxicology* **193**(1-2)**:** 79-90

van Leeuwen F, Gafken PR, Gottschling DE (2002) Dot1p modulates silencing in yeast by methylation of the nucleosome core. *Cell* **109**(6)**:** 745-756

van Oosterwijk MF, Versteeg A, Filon R, van Zeeland AA, Mullenders LH (1996) The sensitivity of Cockayne's syndrome cells to DNA-damaging agents is not due to defective transcription-coupled repair of active genes. *Mol Cell Biol* **16**(8)**:** 4436-4444

Wansink DG, Schul W, van der Kraan I, van Steensel B, van Driel R, de Jong L (1993) Fluorescent labeling of nascent RNA reveals transcription by RNA polymerase II in domains scattered throughout the nucleus. *J Cell Biol* **122**(2)**:** 283-293

Wysocki R, Javaheri A, Allard S, Sha F, Cote J, 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**(19)**:** 8430-8443

Figure legends

Figure 1. DOT1L is involved in UV-survival in mammalian cells.

(A) Left panel: Relative quantification of *DOT1L* mRNA in MEF ^{WT} and MEF ^{DOT1L}. Right Panel: Histone acid-extraction was performed on MEF ^{WT} or MEF ^{DOT1L} cells followed by SDS-PAGE and Western-blotting of H3, H3K79me1 or H3K79me3.

(B) MEF ^{WT}, MEF ^{DOT1L}, MEF ^{XPG} and MEF ^{CSB} cells were assayed in a UV-survival assay. Cells were treated with increasing dose of UV-C and cell survival was determined 96 hours later, as detailed in the Experimental Procedures. Data were normalized to the mock treatment controls (as value of 1). The values are the means of three independent experiments (± SD).

Figure 2. DNA repair takes place in the absence of DOT1L.

(A) Unscheduled DNA synthesis (UDS) assay. Left panel; UDS expressed as mean number of autoradiographic grains/nucleus. UDS was measured by incubating MEF ^{WT} or MEF ^{DOT1L} cells with radioactive [^{3}H]thymidine before treatment with increasing doses of UV-C light and autoradiography {Stefanini, 1992 #97}. The values are the means of two independent experiments +/-SEM. Right Panel; Auto-radiography of an UDS experiment (20J/m^2).

(B) (6-4)PP removal was carried out in MEF ^{WT}, MEF ^{DOT1L}, MEF ^{XPG} and MEF ^{CSB}cells, harvested at different time points after UV irradiation at 20J/m ^{2}. Cells were labeled with an anti-(6-4)PP antibody and signals were measured using IN Cell 1000 analyzer (GE Healthcare). Graph represents the % of lesions that remains in the genome at different time points. The values are the means of three independent experiments (± SD). For each time point, a mean of 4000 cells has been analysed.

(**C**) MEF ^{WT}, MEF ^{DOT1L}, MEF ^{XPG} and MEF ^{CSB} cells were treated with increasing concentration of et743 and cell survival was determined 96 hours later, as detailed in the Experimental Procedures. Data were normalized to the mock treatment controls (as value of 1). The values are the means of three independent experiments (± SD).

Figure 3. Deficient transcription recovery after UV irradiation in the absence of DOT1L.

(A) Recovery of RNA synthesis assay (RRS). After pre-labeling with [^{14}C]thymidine for 2 days, mock treated or UV-C (10 J/m^2) irradiated cells were pulse labeled for 30 minutes with [^{3}H]uridine at different incubation times after irradiation, and the acid-insoluble radioactivity was determined. The ^{3}H/^{14}C ratio was taken as a measure of RNA synthesis. The values are the means of three independent experiments (± SD).

(**B**) Top, Graph of the RRS experiment. The mean numbers of auto-radiographic grains per nucleus of mock treated or UV-irradiated cells (10 or 20J/m^2) from two independent experiments are expressed (± SEM, measured on at least 150 cells). Below the graph, the results are expressed as percentage of grains per nucleus relative to mock treated cells. Bottom, Auto-radiography of an RRS experiment. Twenty four hours after UV irradiation (20J/m ^{2}), MEF^{WT} or MEF^{DOT1L} cells were pulse labeled 30 minutes with [^{3}H]uridine followed by autoradiography.

Figure 4. DOT1L ensures binding of RNA Pol II to chromatin after UV irradiation

(**A**) Schematic diagram showing the strip-FRAP assay. Cells were transfected with a GFP-RNA Pol II construct (on RPB1). A small region in the middle of the nucleus was bleached and the subsequent fluorescence recovery was followed in time. When indicated, cells were UV-irradiated, 1 hour before the photobleaching.

(**B-C**) Strip-FRAP curves of RNA Pol II-GFP protein stably expressed in either MEF ^{WT} (**B**) or MEF^{DOT1L} (**C**) untreated (green) or treated (red) with UV (16J/m^2), 1 hour before photobleaching. Cells were photobleached with a 488 nm laser at maximum power 4 sec after the beginning of the acquisition. One image per 20 msec was taken during 20 sec in the photobleached area. Error bars represent the SEM obtained from more than 10 cells.

Figure 5. Inhibition of the initiation-to-elongation transition phase in the absence of DOT1L, after UV irradiation.

(A) Measure of the rate of initiation-to-elongation transition phase by RNA Pol II transcription *in*

vivo on endogenous genes. We reversibly blocked gene transcription by incubating cells with DRB. Cells are depleted of their pre-mRNA pool within few hours of incubation with the drug {Singh, 2009 #151}. Following the chase of DRB, RNA Pol II is released from promoter-proximal regions and newly synthesized pre-mRNA appear; the level of pre-mRNA is measured using oligonucleotides targeting respectively the exon/intron junctions of the gene.

(**B**) Schematic diagram showing the experimental approach used to measured the rate of initiation-to-elongation transition phase by RNA Pol II after UV irradiation. MEF cells were treated with DRB for 3 hours before chase and addition of fresh medium at t=0 hour. When indicated, cells were UV irradiated at t=0 hour, before the addition of fresh medium or treated with TSA for 12 hours before the addition of DRB.

(**C**) Expression levels of the newly synthesized Exon1 of the *Utrophin* gene in MEF ^{WT} and MEF^{DOT1L} cells treated with 100 µM of DRB for 3 hours before the addition of fresh medium. The cells were harvest at intervals of 10 minutes for RNA isolation and qRT-PCR was performed using oligonucleotides targeting respectively the Exon1 and Intron1 of the gene. The expression values are plotted relatively to the expression level of mock treated cells that is set to 1 in all experiments (±SD).

(**D**) Expression levels of the newly synthesized Exon1 of the *Utrophin* gene in either MEF ^{WT} or MEF^{DOT1L} cells irradiated with UV-C (15J/m^2) after treatment as in (**C**).

(**E**) Expression levels of the newly synthesized Exon1 of the *Utrophin* gene in either MEF ^{WT} or MEF^{DOT1L} cells treated with TSA (20nM) for 12 hours before addition of DRB for 3 hours and UV-C irradiation (15J/m ^{2}). TSA was maintained in the medium during DRB treatment and time course.

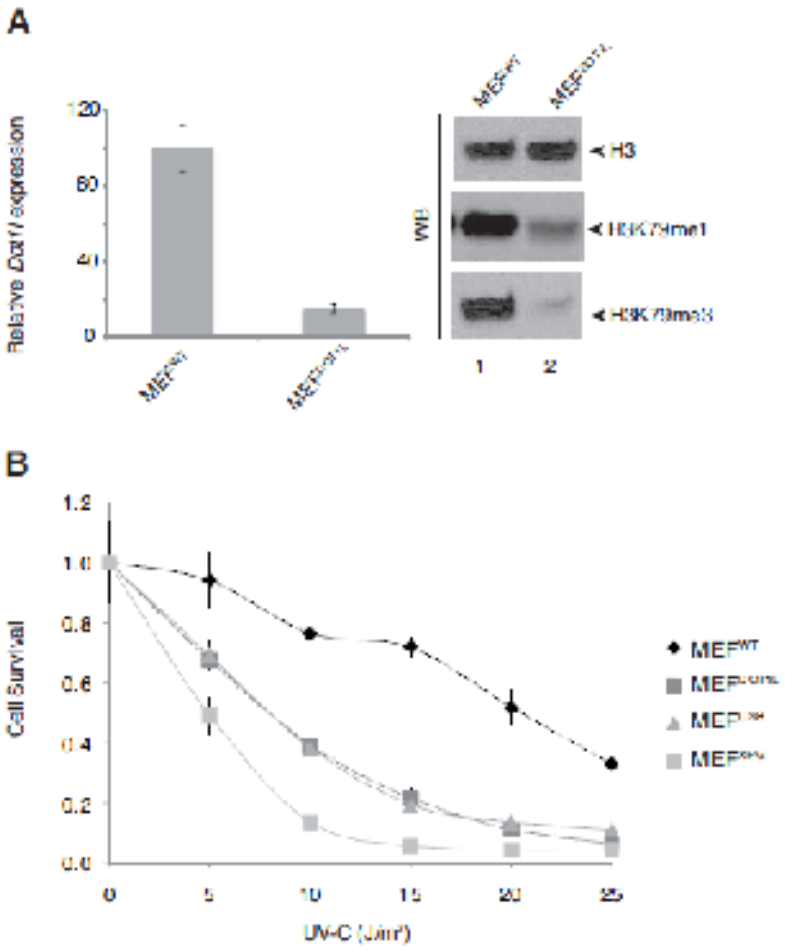
(**F**) MEF^{WT} and MEF ^{DOT1L} cells were irradiated with increasing doses of UV-C light. Cell survival was determined 96 hours later, as detailed in the Experimental Procedures. Data were normalized to the mock treatment controls (as value of 1). The values are the means of three independent experiments (± SD). When indicated, cells were treated with TSA (10nM), 12 hours before UV irradiation, and TSA was maintained for the time of the experiment.

Figure 6. Pre-initiation complex assembly and chromatin modification after UV irradiation.

(**A**) Relative mRNA expression of *DHFR* gene in MEF ^{WT} and MEF ^{DOT1L} cells after UV irradiation (10J/m^2). The values are the means of three independent experiments (± SD).

(**B-F**) Time-dependent occupancy of RNA Pol II (**B**), TFIIB (**C**), H4Ac (**D**), H3K9me2 (**E**) and H3K79me2 (**F**) at the promoter of the *DHFR* gene following UV irradiation (10J/m ^{2}). Soluble chromatin was prepared from MEF ^{WT} and MEF ^{DOT1L} cells at indicated time points after UV-C treatment and subjected to ChIP assay using the indicated antibodies. Real-time PCR using specific primers was performed to test the relative enrichment at the proximal promoter of the DHFR gene. The results are expressed as folds of enrichment relative to the untreated cells. The values are the means of a triplicate experiment (± SD).

Figure 1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2
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Cell Survival
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0.2
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10
15
20
25
UV-C (J/m²)

Figure 2

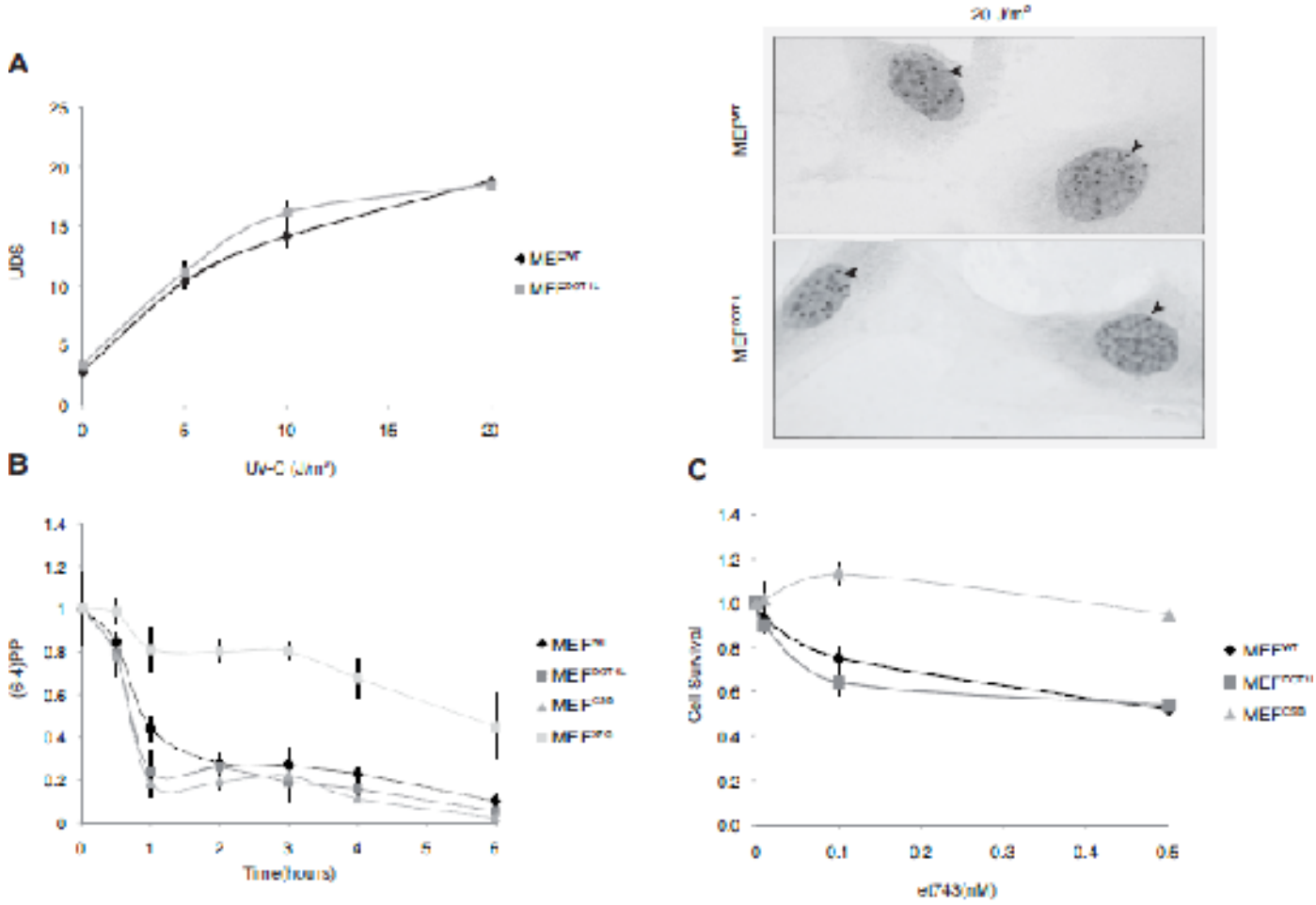
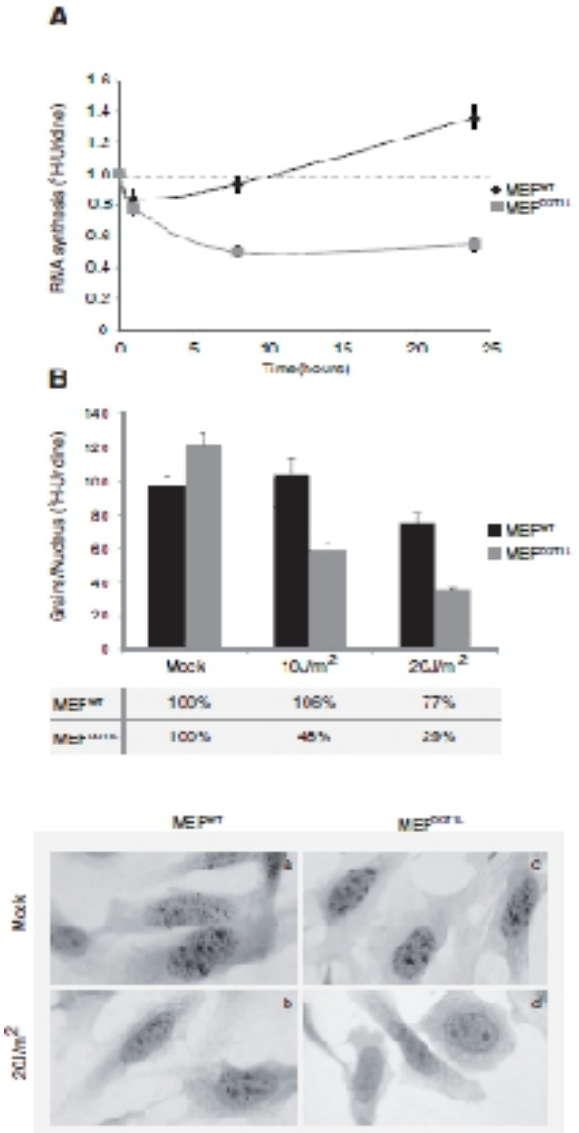


Figure 3


A
RNA synthesis (³H-Uridine)
Time (hours)
B
Grain/Nucleus (³H-Uridine)
Mock
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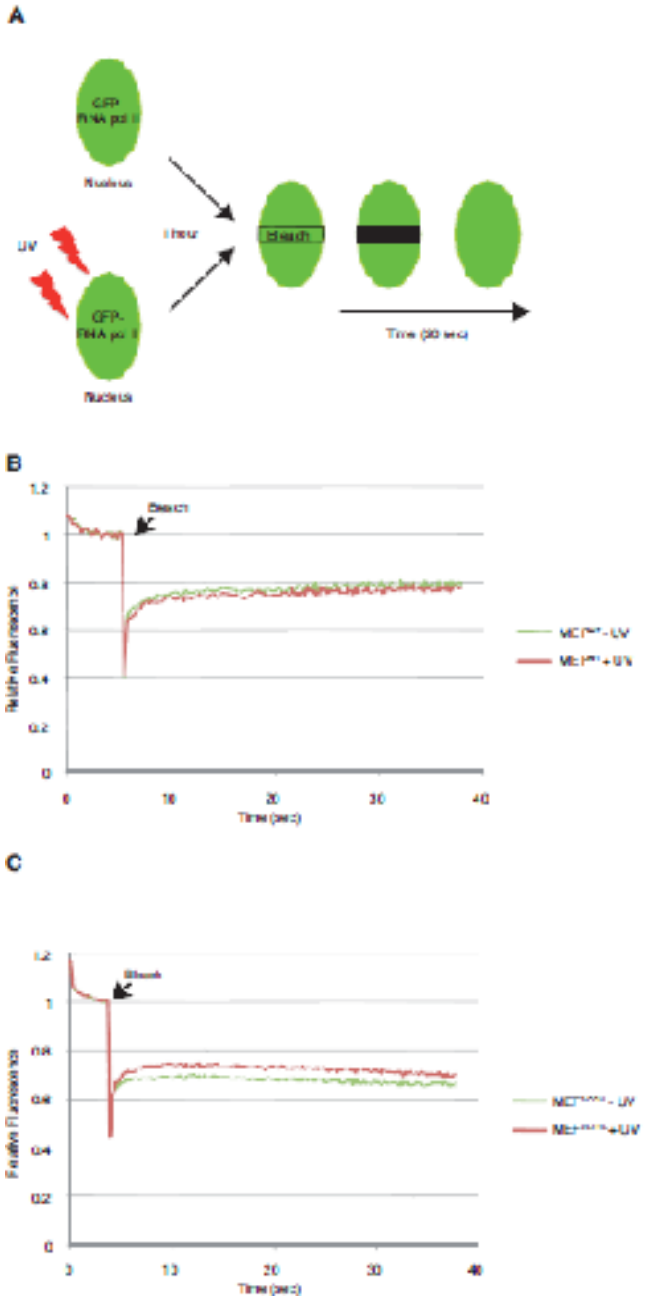
20J/m²
100%
166%
77%
100%
48%
29%
Mock
20J/m²

Figure 4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GFP-
Nucleus
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1 hour
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1.2
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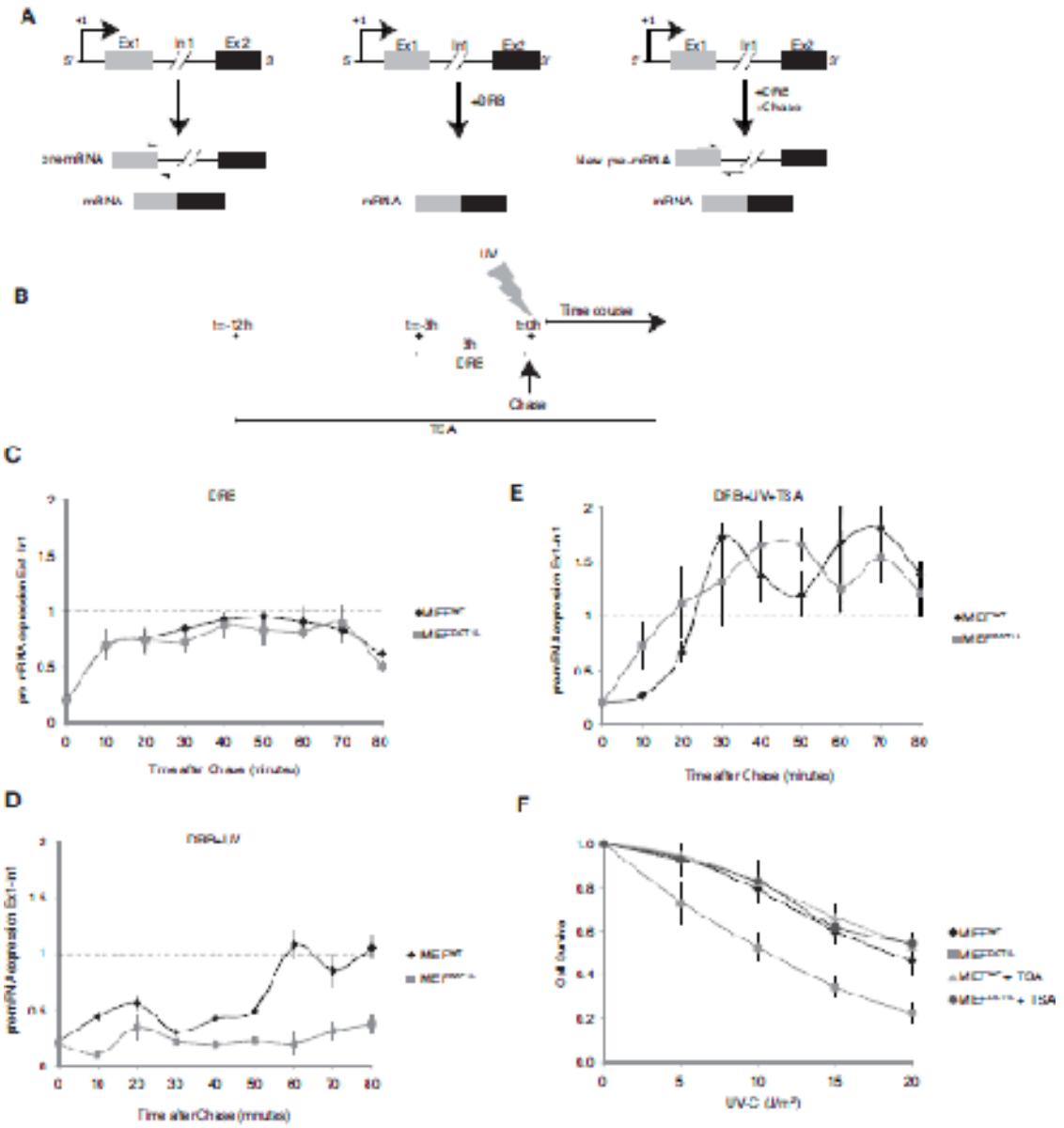
Figure 5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Ex1
In1
Ex2
mRNA
B
UV
Time course
Chase
TSA
C
DRE
Time after Chase (minutes)
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Time after Chase (minutes)
E
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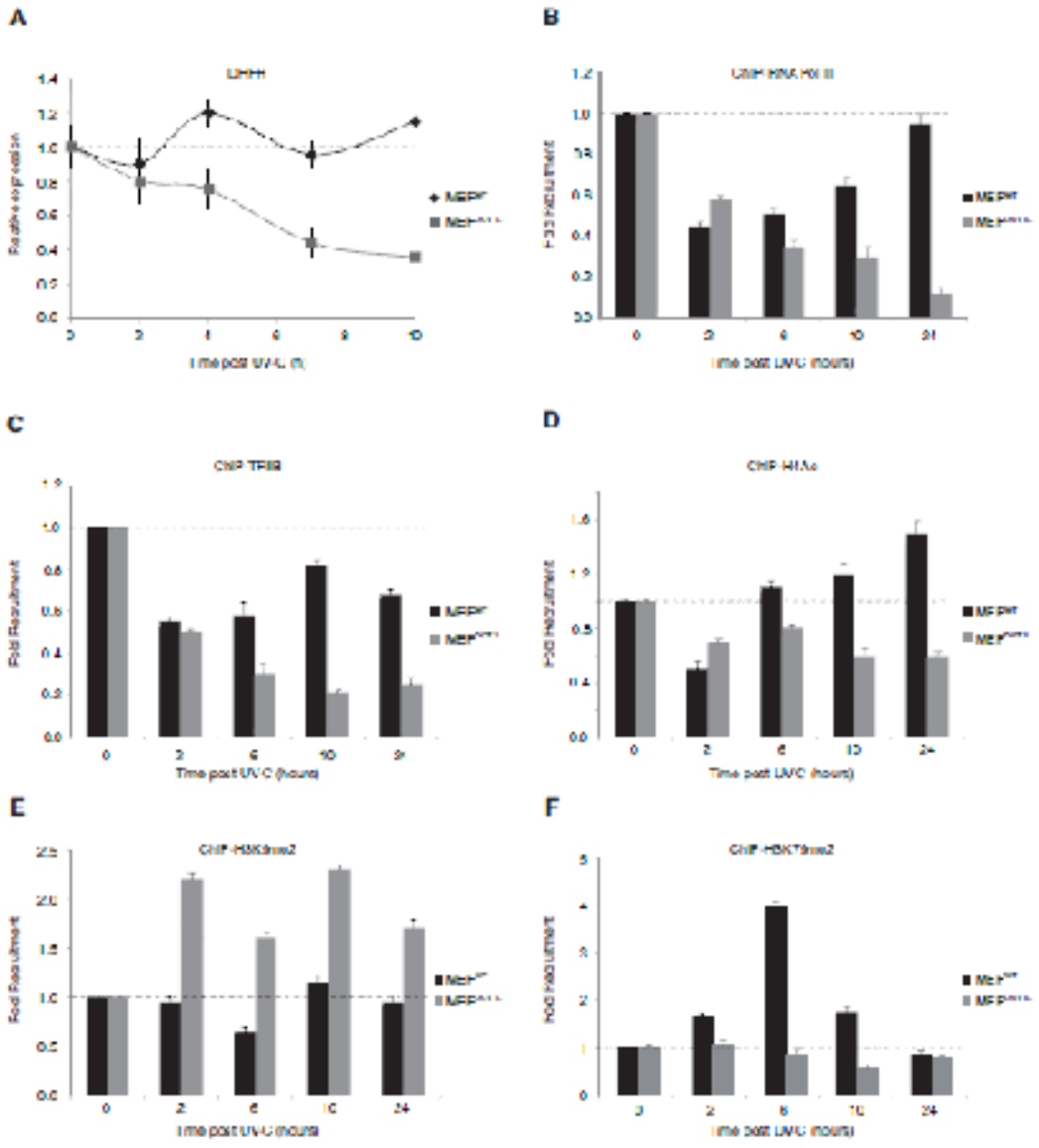
Time after Chase (minutes)
F
UV-C (J/m²)

Figure 6



PROJECT I:

« Chromatin remodelers, kinases and phosphatases mediate DNA damage response and nucleotide excision repair »

Chromatin remodelers, kinases and phosphatases mediate DNA damage response and nucleotide excision repair

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SUMMARY

DNA replication and transcription are under constant pressure of numerous environmental factors that damage DNA and could lead to cell death or cancer transformation. And we address our siRNA screening, based on monitoring of efficient nucleotide excision repair (NER) of UV light - induced 6-4 photoproducts (6-4PPs), to get insight into complicated universe of enzymes involved in DNA damage response (DDR) and maintenance of genome integrity by NER pathway. Groups of genes, whose downregulation led to defect in repair of 6-4PPs after UV irradiation, demonstrate plausible links between numerous biochemical cellular pathways and DDR. We demonstrate that many previously unrelated to TFIIH or NER genes modulate both directly and indirectly DDR and efficient photodamage removal. We highlight the role of gene networks presenting the STAT3, histone acetylation and mRNA – processing associated genes in preventing excessive DNA damage, which in some cases could be due to preventing of abnormal RNA:DNA hybrids formation, ribonucleotides incorporation or damage signaling regulation. Our results clearly show that DDR and NER are more complicated due to chromatin context than it is appreciated from “in vitro” experiments and they are intimately linked to basics of cellular metabolism.

INTRODUCTION

Damage to DNA arises from environmental and endogenous sources, including

genotoxic chemicals, radiation, reactive oxygen species and the intrinsic instability of the DNA molecule. DNA lesions can trigger histone alterations, nucleosome repositioning and changes in higher-order folding of the chromatin fiber. These processes interfere with the progression of DNA and RNA polymerases, and compromise the fidelity of replication and transcription[1,2]. Prevention and repair of DNA damage are essential for cell survival and DDR pathways insure this encompassing a similar set of tightly coordinated processes: namely the detection of DNA damage, the accumulation of DNA repair factors at the site of damage and finally the physical repair of the lesion[3,4]. Among them NER is the most versatile DNA repair pathway in all organisms. While bacteria require only three proteins to complete the incision step of NER, eukaryotes employ about 30 proteins to complete the same step. Efficient DNA repair in eukaryotic cells is complicated by the packaging of genomic DNA into a condensed, often inaccessible structure of chromatin. Cells widely utilize post-translational histone modifications and ATP-dependent chromatin remodeling to modulate chromatin structure and increase the accessibility of the repair machinery to lesions embedded in chromatin[5,6].

Hereafter, trying to reveal the intrinsic mechanism of these modifications, we report the results of the screening on chromatin remodelers, kinases and phosphatases in HeLa cells using 6-4PPs as a sensor of efficient DDR and NER. Loss-function study for these genes shows that set of triggers that compromise DDR and NER is not restricted to canonical NER or other DNA damage sensing proteins. But notably, most of well-known enzymes engaged in NER/DDR, that contemporaneously served as quality control, are present in genes selected with screening. Beside these hits, cells are no longer able to deal with UV - induced lesions in a proper way due to malfunctioning of genes that previously were unlinked to DDR or NER. Thus our results show a plausible way for prediction and explanation of pathological conditions that cause cellular death, senescence and cancer transformation as well as we could employ these findings to promote developing of new therapy and drugs against these mortal diseases.

RESULTS AND DISCUSSION

We performed siRNA screening using the QIAGEN siLibrary (Figure 1a). The library

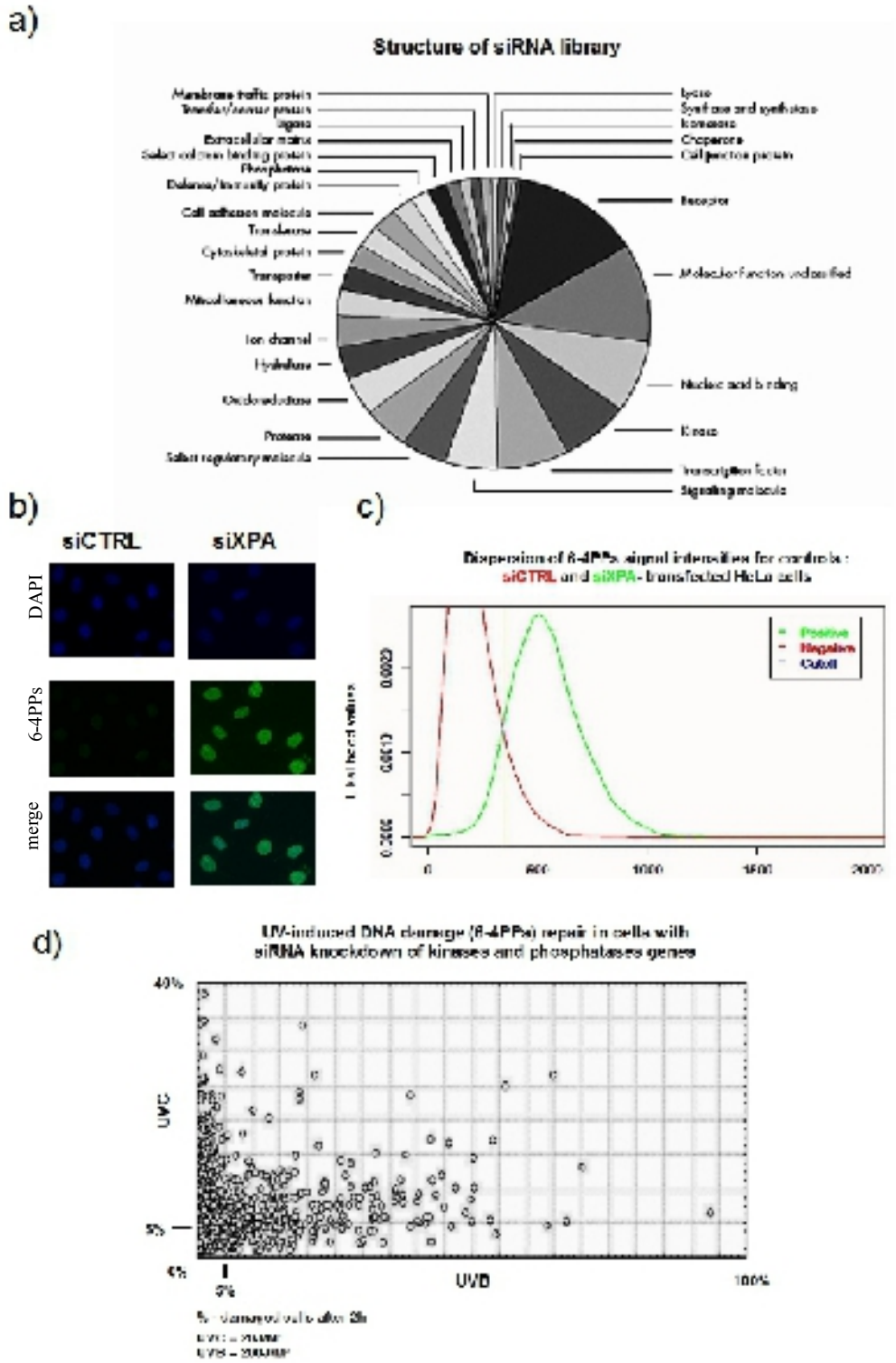


Figure 1. a) siRNA library structure. b) Negative control, nonsense siRNA transsfected HeLa celss (siCTRL) and positive control, HeLa cells transfected with siRNA against NER factor XPA (siXPA) were transfected for 24 hours prior to irradiation with 20J/M^2 of UVC, and subsequently fixed 2 hours after UV treatment, stained with DAPI(blue) and antibodies against 6-4PPs(green). c) Distributions of 6-4PPs signals in siCTRL(red) and siXPA(green) transfected cells, cutoff line(blue) that divide positive(more likely to be damaged) cells from negative(more likely to be undamaged) cells. d) UVB and UVC data plot for double positive kinase and phosphatase genes

targets about 1800 genes by pools of four individual siRNA duplexes per gene. Cells were stained with antibodies against 6-4PPs to measure DNA damage induced by UV irradiation, 20 J/M^2 - 254nm wavelength. Experiment was triplicated on independent plates for each gene with a non - targeting siRNA pool as the negative control and siRNA pool targeting the NER factor XPA as a positive control (Figure 1b). Data concerning the efficiency of DNA repair were obtained using a INCELL1000, laser-scanning microscopy platform, allowing analysis of cell number and quantification of 6-4PPs intensity with a single cell resolution. A Mann-Whitney test was performed to check how different positive and negative controls are (Figure 1c). Cutoff line was determined in each plate for subsequent estimation of the positive cell portion for every siRNA. Pools were used at 20nM total siRNA concentration to minimize off-target effects. Additionally part of the library representing the kinases and phosphatases was tested with 200J/M^2 dose and 312nm wavelength to verify the hypothesis of non-equivalent activation of DDR pathways depending on the energy of UV light(Figure 1d).

UVC and UVB induce DDR via kinases and phosphatases in ways different from direct 6-4PPs or CPD sensing

We found that transient perturbations in cellular homeostasis raised by siRNA knockdown lead to compromised repair of UV induced lesions making cells more sensitive to UV radiation. Kinases and phosphatases are involved in amino acid, lipid, carbohydrate metabolism and processing of nucleobases, and they are indispensable for efficient DDR. Impaired production of nitric oxide, reactive oxygen species alongside with inhibition of ERK/MAPK signaling, that is involved in cell cycle transition, apoptosis, gene expression, respond to proinflammatory cytokines, osmotic and heat shock, are the molecular keys that result in repair deficiency both upon UVC and UVB. We found that UV induces DDR via NFkB pathway and oxidative stress response(Table 1a) regardless the wavelength. And genes like GK2 or OXSR1 are equivalently important for proper DDR both in UVC or UVB experiments. Meanwhile knockdown of certain genes: DGUOK, RIPK3 or PNCK, ACP5 gives repair deficiency in test with a particular source of UV damage, UVB or UVC respectively(Table 1b, Figure 1d).

Discussing these findings we have to remember that for a long time DNA has been

regarded as the only molecular target for UV. However, ultraviolet light can also affect cytoplasmic and membrane structures. And particularly UV radiation can directly affect cytoplasmically located transcription factors, kinases closely located to the cellular membrane and membrane receptors interfering with cytokine signaling and inducing apoptosis via direct activation of apoptosis - related surface receptors[7]. We generally assume this idea that we tested using UVB radiation and kinome - phosphatome part of the library. Our findings support data published by other researches. And speaking about cytoplasmic and membrane effects of UVB we can mention that exposure to UVB radiation is shown to cause inflammatory tissue damage and skin cancer. A plausible molecular link between inflammation and cancer is the NF - κB (Figure 2).

a)

Diseases and Disorders

	p-value
Cancer	2,23E-04 - 4,40E-02
Reproductive System Disease	2,23E-04 - 4,40E-02
Dermatological Diseases and Conditions	1,00E-03 - 1,40E-02
Developmental Disorder	1,00E-03 - 1,70E-02
Hematological Disease	1,80E-03 - 4,40E-02

Molecular and Cellular Functions

	p-value
Amino Acid Metabolism	1,00E-03 - 4,40E-02
Post-Translational Modification	1,00E-03 - 4,40E-02
Small Molecule Biochemistry	5,00E-03 - 2,20E-02
Cell Cycle	7,00E-03 - 4,20E-02
Cell Signaling	1,53E-03 - 1,61E-02

Physiological System Development and Function

	p-value
Digestive System Development and Function	1,00E-03 - 1,00E-03
Embryonic Development	1,00E-03 - 1,10E-02
Hepatic System Development and Function	1,00E-03 - 1,25E-02
Lymphoid Tissue Structure and Development	1,40E-03 - 1,80E-03
Nervous System Development and Function	1,00E-03 - 4,40E-02

Molecular pathways

	p-value
Mitotic Roles of Polo-Like Kinase	1,2E-04
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1,42E-04
Dopamine Receptor Signaling	2,94E-04
CDK5 Signaling	5,27E-04
Ribonucleoside Metabolism	5,70E-04
FPAR/RXR Activation	5,75E-03
G2/M Transition of the Cell Cycle	5,94E-03
Molecular Mechanisms of Cancer Regulation by Transcription Factors of PPARs	1,58E-01
NF-κB Signaling Pathway	1,83E-01
Hepatic Cholestasis	2,10E-01

b)

Top 20 UVB :

DCLK1
GK2
TLK2
OXGR1
DUSP10
PPP2R3A
ILK
PRPF4B
PMVK
CRK7
GNE
ENPP1
CALM2
PPP3CA
LRRK2
MTMR4
TNK2
ROCK1
NRK
HCHD1

Top 20 UVC :

IKBKG
PNCK
ACTR5
GK
SIK2
PPP1R10
MOS
CK2
PDK1
GRK7
EPHA3
CDK7
GRSF1
WEE1
KIDINS220
G6PC3
PACSIN1
ALPK2
CDC25A
HOK1

Table 1. a) Biological functions and pathways for UVB-UVC double-positive shortlisted genes (Ingenuity). b) Top 20 shortlisted molecules selected in 6-4 repair efficiency – based screening with UVB and UVC irradiation.

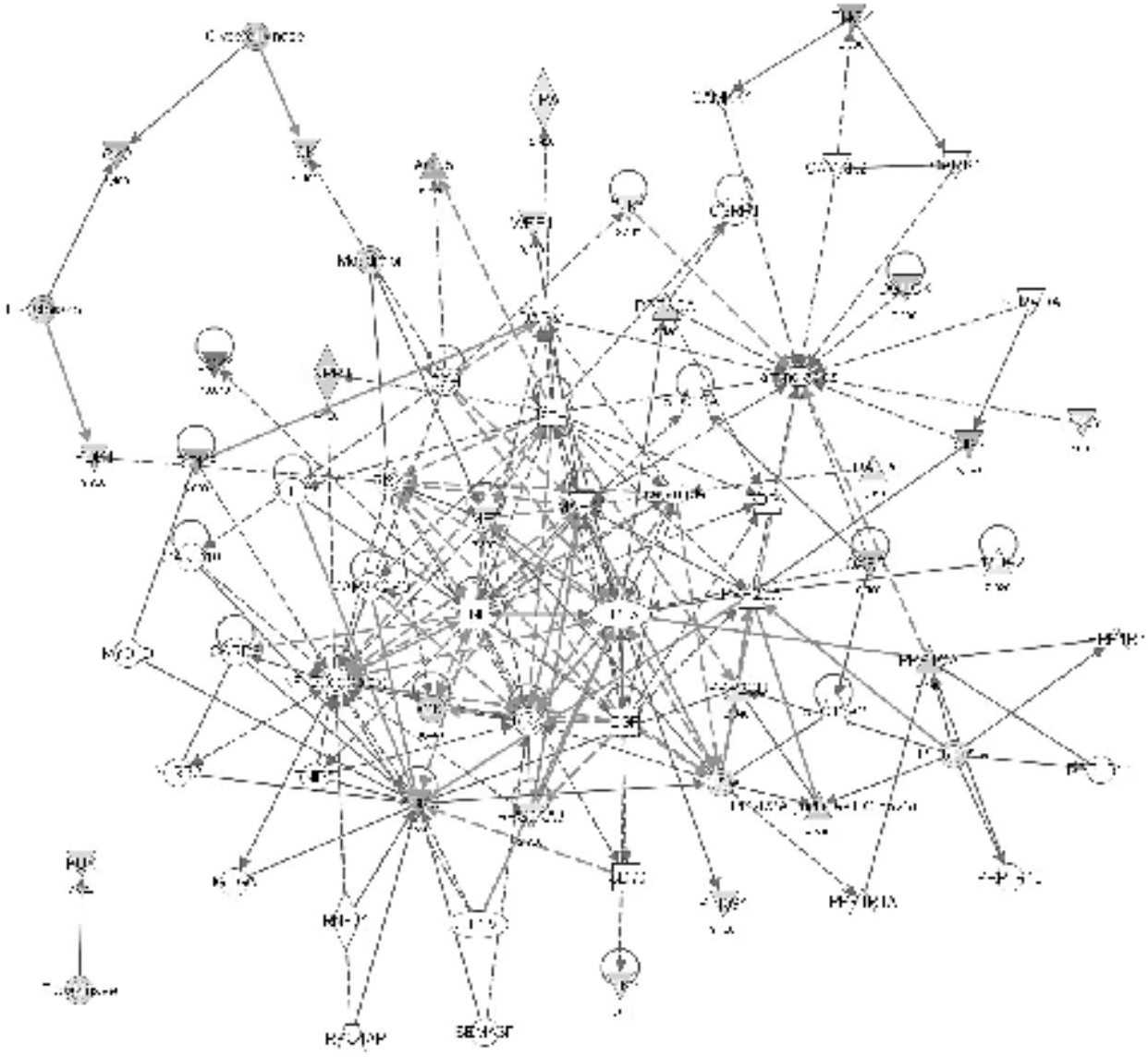


Figure 2. Ingenuity kinome&phosphatome gene network analysis for UVB and UVC screening.

UV radiation results in increased degradation of IκBα, nuclear translocation of p65 and p50, and the DNA binding by NF - κB which regulates expression of pro-inflammatory genes including inducible nitric oxide synthase (iNOS).[9]. Three types of UV: UVA, UVB and UVC all induce a dose- and time-dependent phosphorylation of eIF2α-Ser. But they act through distinct signaling mechanisms. Thus UVA - induced eIF2α phosphorylation occurs through MAPKs, including ERKs, JNKs and p38 kinase, and phosphatidylinositol (PI)-3 kinase. UVB - induced eIF2α phosphorylation is through JNKs and p38 kinase, but not ERKs or PI-3 kinase. And UVC stimulates eIF2α phosphorylation via JNKs alone. ATM is involved in induction of the intracellular responses to UVA and UVB, rather than UVC.

Wavelength-specific UV irradiation activates differential response signaling pathways converged on the eIF2α phosphorylation. Direct eIF2α kinase PKR is activated though phosphorylation by either RSK1 or MSK1, two downstream kinases of MAPKs/PI-3 kinase - mediated signaling pathways[11]. Additional complexity arises with idea that carcinogenic and mutagenic sunlight effects depend not only on cyclobutane pyrimidine dimers and 6-4PPs but also on another types of UV-induced lesions. All three kinds of UV: UVA, UVB and UVC are known to increase the mutation frequency of purified single-stranded phage DNA and cellular DNA in human cells showing that even UVA exposure, lowest energy wavelength, may induce mutation probably via non - dipyrimidine lesion in DNA[8,37]. Apoptosis could be logical a consequence of both inflammation and unrepaired DNA damage and its induction in keratinocytes by ultraviolet irradiation is considered to be a protective function against skin cancer. UV-induced apoptosis is a crucial event both for UVB and UVC. However there are some specific features that rise again for each UV wavelength. Thus caspase-8 activation occurs only in UVB - irradiated cells. The activation of caspase-8 cannot be inhibited by caspases-9 and -3 specific tetrapeptide inhibitors, indicating that the caspase-8 cleavage is not due to feedback from activation of caspases-9 and -3. Thus, despite the lower production of photoproducts in DNA by UVB irradiation, UVB irradiation could induce apoptosis through both mitochondrial (intrinsic) and caspase-8 activation (extrinsic) pathways, while UVC induces apoptosis only via the intrinsic pathway[10]. When apoptosis is compromised UV radiation could strongly induce cancer, as UV is also linked to the activation of putative cancer genes like PPP2R2A, MTAP and MAP2K4[12].

DDR is also mediated through cellular metabolism. Mammalian transcription factors of the AP-1 family are activated by either stress signals such as UV radiation, or mitogenic signals such as growth factors. A similar situation exists in the yeast Saccharomyces cerevisiae, there AP-1 transcriptional activator GCN4, known to be activated by stress signals such as UV radiation and amino acids starvation and is also induced by growth stimulation such as glucose. Glucose-dependent GCN4 activation is mediated through the Ras/cAMP pathway. This pathway is also responsible for UV-dependent GCN4 activation but is not involved in GCN4 activation by amino acid starvation. The finding that GCN4 is activated in response to glucose via the Ras/cAMP pathway suggests that this cascade coordinates glucose metabolism with amino acids

and purine biosynthesis and thereby ensures availability of both energy and essential building blocks for continuation of the cell cycle[13]. There is a link to metabolism for a most studied UV-DDR player, p53 protein. LPIN1 is a p53-responsive gene that is induced in response to DNA damage and glucose deprivation. It is essential for adipocyte development and fat metabolism. p53 and LPIN1 regulate fatty acid oxidation in mouse. LPIN1 expression in response to nutritional stress is controlled through the ROS-ATM-p53 pathway and is conserved in human cells. LPIN1 could provide a link between p53 and metabolism that may be an important component in mediating the tumor suppressor function of p53 in DDR(Figure2)[14,15].

In conclusion, we demonstrated plausible links of UV radiation to inflammation and cancer via certain kinases and phosphatases. Moreover, we proposed how kinases and phosphatases could act in DDR and NER through regulation of cellular metabolism in a wavelength specific ways. Therefore,we could assume that all previous and future experimental data concerning the role of cytoplasmic proteins in DDR and NER should be discussed with respect to specific features of DDR activation by UVB and UVC.

DDR acts via chromatin remodelers

Ingenuity and STRING-DB analysis tools reveal that our findings are well - correlated with results of other teams that studied DDR using models different from UV and 6-4PPs detection. We detected most of well - known DDR and NER proteins presented in siRNA library, and regardless any possible flows of our experimental design or procedures, we rested in perfect accordance with broadly accepted UV - DDR and DNA repair models validating that the screening found the genes it was aimed to find. Therefore we show new potentially important proteins in TFIIH gene network and highlight genes in new promising networks like regulation of histone acetylation that could act in genotoxic stress response(Figure 3, Figure 4). We would like to specially concentrate on groups that correspond to STAT3 and RNA - processing associated gene networks(Figure 4a). Role of these nets as well as role of RNA:DNA hybrids in NER and R-loops in transcription will be discussed hereafter. Role of well-known groups like TFIIH network, histone modifications network (Figure 3) which were also detected in screening needs further investigation but not an additional discussion as long as they

have been noted to directly participate in NER and discussed for a long time as well as perfectly reviewed in number of specialized publications[16–18].

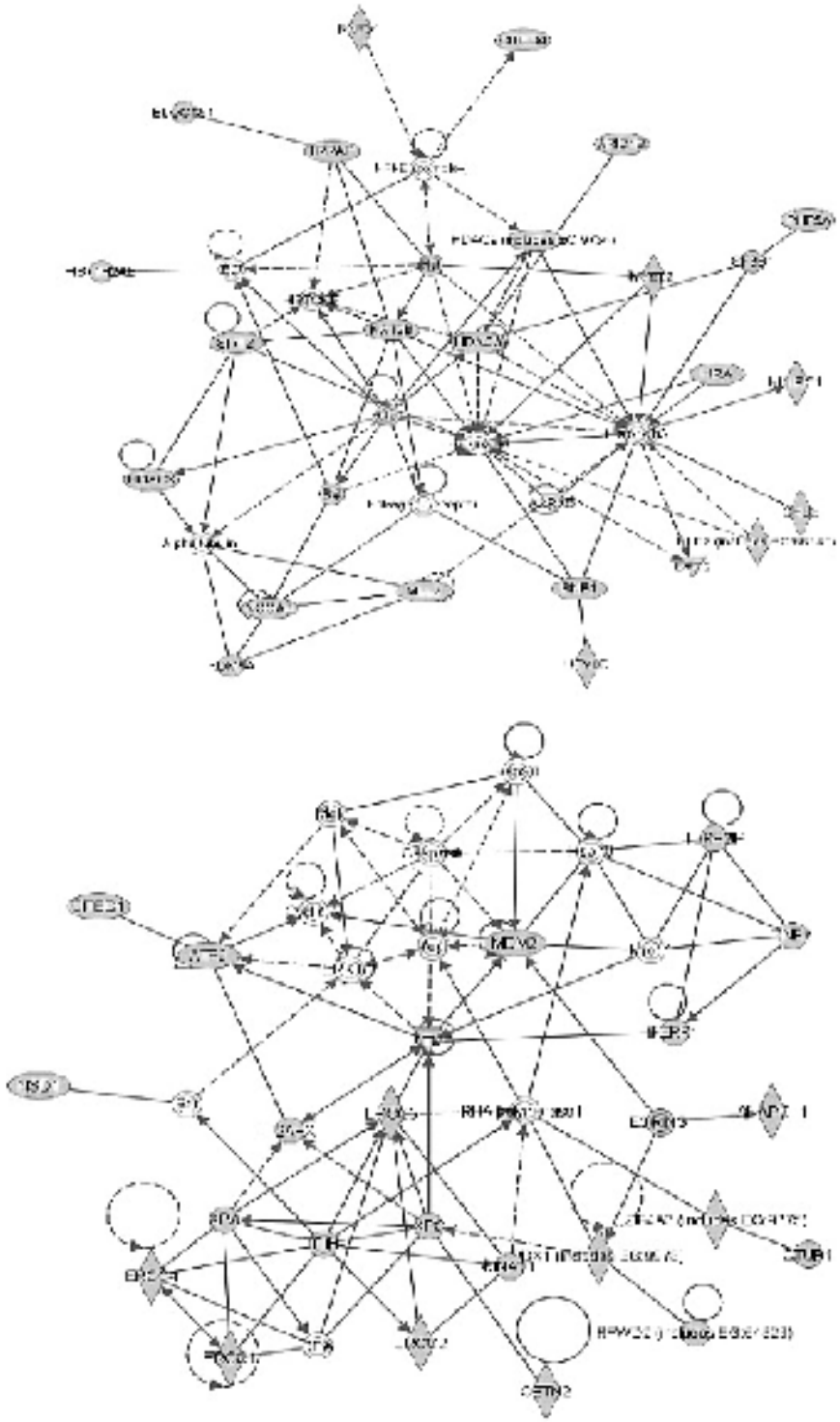
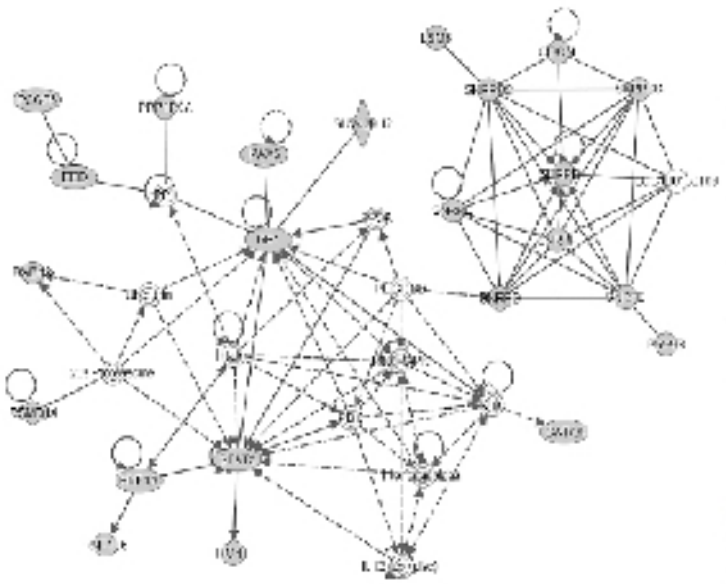


Figure 3. Ingenuity gene network analysis for chromatin remodelers. Histone modifications (upper net) and TFIIH - related proteins (bottom net).

a)



b)

Diseases and Disorders

	p-value
Dermatological Diseases and Conditions	2.18E-06 - 2.45E-02
Genetic Disorder	2.18E-06 - 2.45E-02
Cancer	9.30E-06 - 2.45E-02
Renal and Urological Disease	1.50E-04 - 8.35E-03
Gastrointestinal Disease	1.54E-04 - 8.35E-03

Molecular and Cellular Functions

	p-value
Gene Expression	6.43E-11 - 2.45E-02
DNA Replication, Recombination, and Repair	7.01E-11 - 2.45E-02
RNA Post-Transcriptional Modification	1.31E-06 - 1.66E-02
Nucleic Acid Metabolism	2.70E-06 - 1.66E-02
Small Molecule Biochemistry	2.70E-06 - 2.45E-02

Physiological System Development and Function

	p-value
Hair and Skin Development and Function	3.29E-06 - 1.66E-02
Connective Tissue Development and Function	2.90E-04 - 2.45E-02
Tumor Morphology	2.54E-04 - 2.45E-02
Organ Morphology	5.00E-04 - 1.66E-02
Reproductive System Development and Function	5.00E-04 - 1.66E-02

Molecular pathways

	p-value
Mismatch Repair Pathway	1.32E-03
Hereditary Breast Cancer Signaling	5.54E-03
Cell Cycle: G1/S Checkpoint Regulation	1.02E-04
Estrogen Receptor Signaling	3.17E-04
Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency	6.36E-04
p53 Signaling	3.81E-04
G2/M Transition of the Cell Cycle	3.98E-03
RAR Activation	5.75E-03
G1/S Transition of the Cell Cycle	7.92E-03
TR/RXR Activation	9.33E-03

c)

Name	Score	Name	Score	Name	Score	Name	Score	Name	Score
XPA	0.90-0.95	PUM1	0.76	PPIL2	0.62	MLF2	0.58	EIF4G3	0.54
XPC	0.72-0.74	SYNM	0.74	HAL2	0.62	UBE2L3	0.57	YTHDC2	0.53
ERCC1	0.69	UPF1	0.72	SNRPF	0.61	RBM39	0.57	TRIM5	0.52
ERCC2/XPD	0.64	SNRNP1	0.70	SNRPB	0.61	TPR	0.56	UBR5	0.52
ERCC3/XPB	0.66	SF3B1	0.70	PUM3	0.61	TTF2	0.56	DKFZP434A129	0.52
ERCC4/XPF	0.69	CSTF2	0.68	SNRNP	0.59	STAT3	0.55	LCP2	0.52
MNAT1/MAT1	0.61	SNRPG	0.67	NAP1L4	0.59	SFRS1A	0.55	TUBB	0.52
		SNRPD2	0.66	TZFP	0.58	PAX5	0.55	RNF148	0.51
Negative CTRL	0.00-0.06	ANAPC11	0.65	TRIM22	0.58	TIRA	0.55	FLJ21128	0.51
		PPIH	0.63	SMCA1	0.58	PPP1R8A	0.54	TBT	0.50

Figure 4. a)Ingenuity gene network analysis (continiue). b) Ingenuity gene functions and pathways analysis for chromatin remodelers. c) Control genes and top 40 chromatin remodelers associated with 6-4 PPs repair. Score, 0 to 1, shows portion of "positive" XPA-like cells.

In the following discussion we will have a closer look on less popular but very intriguing STAT3 protein and RNA - splicing associated events and their possible contribution to NER and DDR.

STAT3 is a prosurvival protein, that responds to cytokines and growth factors. Constitutive activation of NF-kB and STAT3 depends on upstream signaling through PI3K, and is important for cell survival and proliferation, as well as for maintaining the

level of Myc[19]. Disruption of the STAT3 pathway abolishes the apoptosis resistance against UVC and MMC[20]. Cells lacking STAT3 are also more sensitive to oxidative stress and are less efficient in repairing damaged DNA. Moreover, STAT3 deficient cells show reduced activity of the ATM-Chk2 and ATR-Chk1 pathways, both important pathways in sensing DNA damage. MDC1, a regulator of the ATM-Chk2 pathway and facilitator of the DNA damage response, is modulated by STAT3 at the transcriptional level. Thus importance of STAT3 for efficient repair of damaged DNA could be partly explained by modulating the ATM-Chk2 and ATR-Chk1 pathways. On other hand there are some facts that STAT3 could acts in different ways. Generally, UV-induced STAT3 activation is mediated by both reactive oxygen species and DNA damage. In human epidermal keratinocytes and dermal fibroblasts dominancy of ROS and DNA damage to activate STAT3 depended on the wavelength of UV. And UVB activates STAT3 via both ROS and DNA damage, while UVC does so mainly via DNA damage[21]. Thus STAT3 is a promising target for further studies of DDR in its UV - wavelength dependent modes.

Plausible roles of RNA, RNA splicing and spliceosome proteins in DDR and NER are extremely intriguing. Notably, we observe that loss function for almost every RNA processing gene leads to deficient 6-4PPs repair. And analysis with STRING-DB tools shows the splicing proteins are as important for NER as TFIIH and histone modifications(Figure 4a, Figure 5). And it could be even a direct role of RNAs in DNA repair and maintenance of genome stability. Probably one of the most exciting opportunities to exploit is a role of RNA in DNA repair. Thus repair of double-strand breaks (DSBs) in Arabidopsis thaliana is shown to involve small RNAs of ~21 nucleotides in length, derived from both sense and antisense sequences flanking DSBs, that are upregulated following DNA damage. Their production depends on the kinase ATR, which promotes DNA repair in response to single-stranded DNA, and RNA polymerase IV, which transcribes siRNAs in plants. RNA-dependent RNA polymerases that produce double-stranded RNA (dsRNA) from single-stranded RNA are also necessary for diRNA production, as are Dicer - like proteins, which cleave dsRNAs into shorter fragments. Argonaute 2 (AGO2), a component of the RNA-induced silencing complex, binds mature diRNAs, and loss of AGO2 significantly reduces diRNA levels and impedes DSB repair. However, diRNA expression does not affect RNA-directed DNA methylation, histone H2AX phosphorylation, or the levels of key DNA repair proteins, so the mechanism

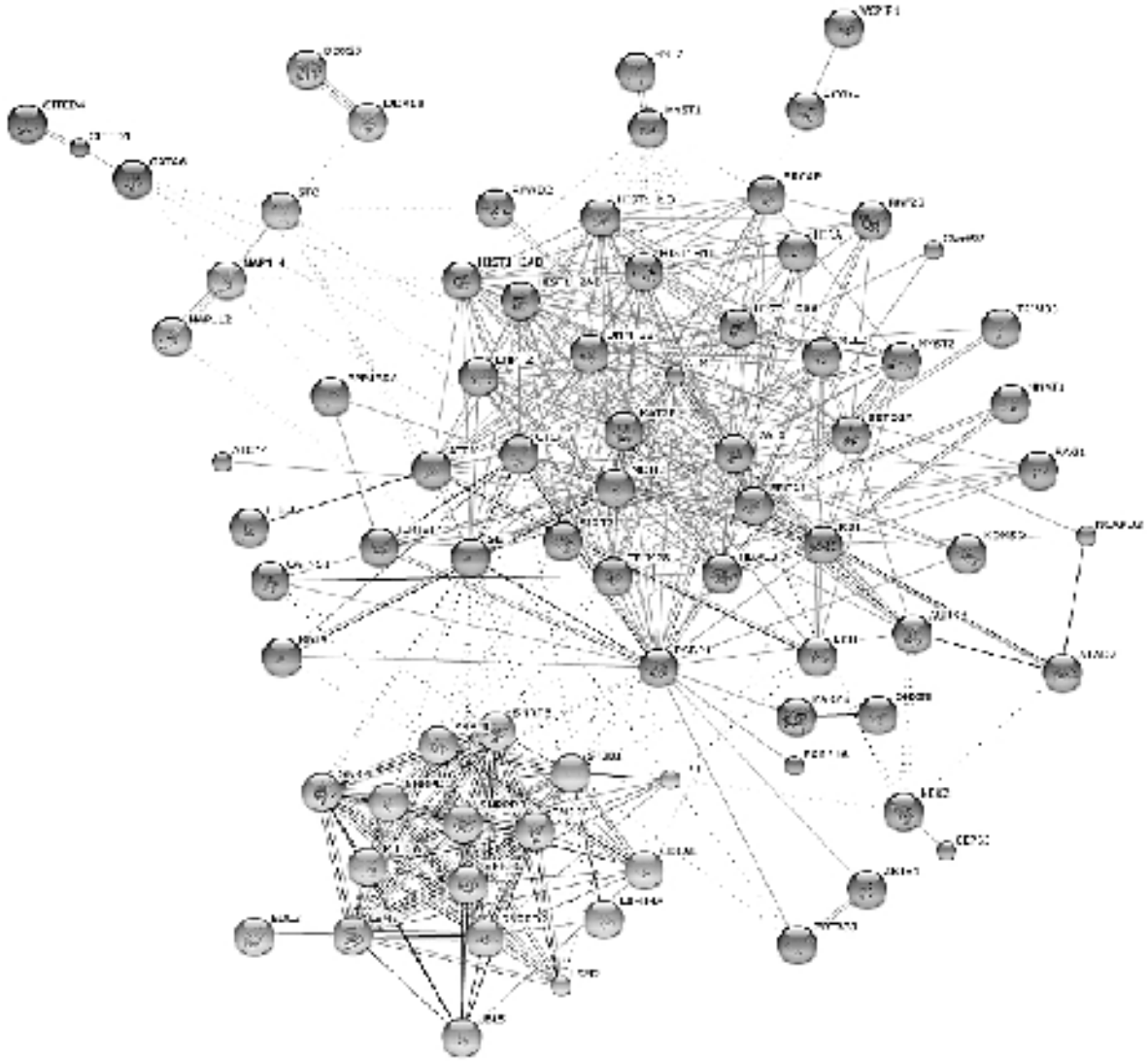


Figure 5. STRING-DB analysis for chromatin remodelers.

for their effects on DNA repair is still unclear. But importantly, diRNAs are also detected in human cells, where DSB repair depends on DICER and AGO2. This suggests that diRNAs have an evolutionarily conserved role in DNA repair, and may recruit specific repair factors [22]. In human, mouse and zebrafish, DICER and DROSHA, but not downstream elements of the RNAi pathway, are necessary to activate the DDR upon exogenous DNA damage and oncogene-induced genotoxic stress, as studied by DDR foci formation and by checkpoint assays. DDR foci are sensitive to RNase A treatment, and DICER- and DROSHA-dependent RNA products are required to restore DDR foci in RNase-A-treated cells. RNA deep sequencing and the study of DDR activation at a single inducible DNA double-strand break, demonstrate that DDR foci formation requires site-

specific DICER - and DROSHA - dependent small RNAs, named DDRNAs, which act in a MRE11-RAD50-NBS1-complex-dependent manner (MRE11 also known as MRE11A; NBS1 also known as NBN). DDRNAs, either chemically synthesized or in vitro generated by DICER cleavage, are sufficient to restore the DDR in RNase-A-treated cells, also in the absence of other cellular RNAs. These results describe an unanticipated direct role of a novel class of ncRNAs in the control of DDR activation at sites of DNA damage[36]. And based on analogy for proteins that are involved both in UV damage and ionizing damage response we could expect to find small RNAs acting in NER pathway in the near future.

Genome instability, a hallmark of cancer progression and another action point for RNA processing genes, often arises through DSBs. A mechanism proposed by studies in yeast and mammalian cells shows that DSBs and instability can occur through RNA:DNA hybrids generated by defects in RNA elongation and splicing. Yeast RNA:DNA hybrids naturally form at many loci in wild-type cells, likely due to transcriptional errors, but are removed by two evolutionarily conserved RNase H enzymes. Mutants defective in transcriptional repression, RNA export and RNA degradation show increased hybrid formation and associated genome instability[23]. Studies in mutants with chromosome instability identify seven essential subunits of the mRNA cleavage and polyadenylation machinery. RNA:DNA hybrid formation is also directly detected in polyadenylation machinery mutants. And chromosome instability is suppressed by expression of the R-loop-degrading enzyme RnaseH[24]. On other side, it is generally assumed that, the direct presence of ribonucleotides in genomic DNA is undesirable given their increased susceptibility to hydrolysis. Therefore there is no surprise that RNase H enzymes that recognize and process such embedded ribonucleotides are present in all domains of life. Indeed, in humans, RNase H2 hypomorphic mutations cause the neuroinflammatory disorder Aicardi-Goutières syndrome. In mice, RNase H2 is an essential enzyme required for embryonic growth from gastrulation onward. RNase H2 null embryos accumulate large numbers of single (or di-) ribonucleotides embedded in their genomic DNA (>1,000,000 per cell), resulting in genome instability and a p53-dependent DNA-damage response. Thus these results indicate that RNase H2 is a key mammalian genome surveillance enzyme required for ribonucleotide removal and also that ribonucleotides are the most commonly occurring endogenous nucleotide base lesion in replicating cells[25].

Besides the genome instability and direct involvement in DDR or DNA repair RNA:DNA hybrids participate in pausing of transcription, an important regulation step of gene expression in bacteria and eukaryotes. Mechanism of transcription pausing is determined by the ability of the elongating RNA polymerase to recognize the sequence of the RNA-DNA hybrid. RNA polymerase directly 'senses' the shape and/or identity of base pairs of the RNA-DNA hybrid. Recognition of the RNA-DNA hybrid sequence delays translocation by RNA polymerase, and thus slows down the nucleotide addition cycle through 'in pathway' mechanism. This phenomenon is conserved among bacterial and eukaryotic RNA polymerases, and is involved in regulatory pauses, such as a pause regulating the production of virulence factors in some bacteria and a pause regulating transcription/replication of HIV-1[26]. Additional point for RNA:DNA acting in transcription is methylation regulation of CpG islands (CGIs) that function as promoters for approximately 60% of human genes. Most of these elements remain protected from CpG methylation, a prevalent epigenetic modification associated with transcriptional silencing. Methylation - resistant CGI promoters are characterized by significant strand asymmetry in the distribution of guanines and cytosines (GC skew) immediately downstream from their transcription start sites. Transcription through regions of GC skew leads to the formation of long RNA:DNA loop structures. And loop formation protects from DNMT3B1, the primary de novo DNA methyltransferase in early development[27]. On contrary, certain ncRNAs are implicated in the regulation of RNAi-directed heterochromatin in fission yeast. ncRNAs transcribed from heterochromatin are thought to recruit the RNAi machinery to chromatin for the formation of heterochromatin. Heterochromatic ncRNA associates with chromatin via the formation of a RNA:DNA hybrid and bound to the RNA-induced transcriptional silencing (RITS) complex. Over-expression and depletion of RNase H in vivo decreased and increased the amount of DNA-RNA hybrid formed, respectively, and both disturbed heterochromatin. [28]. Transcription of NER factors is also under control of RNA:DNA loops and RNAi mechanisms we discussed above but additionally it is regulated via proper splicing. Thus XPG gene contains multiple splice sites with low information content which are components of the minor (U12) spliceosome in association with multiple alternatively spliced isoforms of XPG mRNA. Missense mutations in XPG, human endonuclease that cuts 3' to DNA lesions during nucleotide excision repair, can lead to xeroderma

pigmentosum, whereas truncated or unstable XPG proteins cause Cockayne syndrome, normally yielding life spans of <7 years[30,31]. Mutations in intron 3 of the XPC DNA repair gene affect pre-mRNA splicing in association with xeroderma pigmentosum with many skin cancers (XP101TMA) or no skin cancer (XP72TMA), respectively. These mutations disrupt U2 snRNP-BPS interaction and lead to abnormal pre-mRNA splicing and reduced XPC protein. At the cellular level these changes were associated with features of reduced DNA repair including diminished NER protein recruitment, reduced post-UV survival and impaired photoproduct removal[32]. Some xeroderma pigmentosum patients exhibit a new G→C homozygous substitution at 3'-end of XPC intron 12 leading to the abolition of an acceptor splicing site and the absence of the XPC protein. [33]. Loss of this 42-bp splicing variant of ERCC1 sequence also changes its expression and is associated with increased ERCC1 mRNA expression, in an assessment of ovarian cancer specimens[34]. A regulation mechanism through direct interference of NER and splicing proteins probably could exist. Thus conditionally-lethal pso4-1 mutant targets allele of the spliceosomal - associated PRP19 gene. RAD2 encodes an endonuclease indispensable for nucleotide excision repair, RLF2 encodes the major subunit of the chromatin assembly factor I, whose deletion results in sensitivity to UVC radiation, while DBR1 encodes the lariat RNA splicing debranching enzyme, which degrades intron lariat structures during splicing. And characterization of mutagen-sensitive phenotypes of rad2Delta, rlf2Delta and pso4-1 single and double mutant strains showed enhanced sensitivity for the rad2Delta pso4-1 and rlf2Delta pso4-1 double mutants, suggesting a functional interference of these proteins in DNA repair processes in S. cerevisiae[35]. Additionally, XPA binding protein – XAB2, that interacts with Cockayne syndrome group A and B proteins and RNA polymerase II, as well as XPA, and is involved in TCR and transcription, purifies as a multimeric protein complex consisting of hAquarius, XAB2, hPRP19, CCDC16, hISY1, and PPIE, which are involved in pre - mRNA splicing. Enhanced interaction of XAB2 with RNA polymerase IIo or XPA was observed in cells treated with DNA-damaging agents. And knockdown of XAB2 with small interfering RNA in HeLa cells results in a hypersensitivity to killing by UV light and a decreases recovery of RNA synthesis after UV irradiation and regular RNA synthesis. [29]. Therefore we see that RNA itself, RNA:DNA hybrids and splicing proteins could act in DDR mechanism in a various ways modulating DNA damage signaling and repair,

transcription and splicing both directly and indirectly.

In conclusion, we demonstrated that our loss-function study highlighted regulation networks that corresponded to THIIH, histone modification, RB1, STAT3 and spliceosome related genes. We linked our results with available research data concerning the DDR and NER and assumed that all studies of cytoplasmic part of DDR should be concerned with respect to UV wavelength. Further peer investigation of these gene clews will provide us new important details of intrinsic mechanism cells engage to withstand mutagenic and carcinogenic effects of UV radiation. These studies will fructify with new approaches and therapeutic targets concerning the treatment of aging - related diseases and cancer as these pathologies so often arise from compromised DNA repair.

PERSPECTIVE

Altogether, results of our study indicate that pathways and processes affecting genome stability have more regulation nodes, involve more proteins and different cellular pathways are connected much broader to favor proficient DNA repair and adaptive DDR than it was previously anticipated from “in vitro” experiments. Our data provide links between the maintenance of genome stability and metabolic homeostasis as well as highlight intriguing role in XPC regulation for RB1, UV specific DDR for STAT3 pathway, plausible direct involvement of small RNAs in DDR and NER, role of RNA:DNA hybrids and spliceosome complex proteins in maintenance of genome integrity and transcription under the UV radiation. We enthusiastically expect that soon we will achieve a great advance in understanding regulation of UV induced DNA damage response and nucleotide excision repair in chromatin context.

EXPERIMENTAL PROCEDURES

siRNA screening

The siRNA screening was performed using the QIAGEN siLibrary. HeLa cells were transfected using Lipofectamin2000 reagent. After 24 hours, cells were irradiated and 2 hours after fixed and stained with 6-4PPs antibody and DAPI. Cells were imaged on

INCELL 1000 laser scanning platform. The total fluorescence intensity of each cell was calculated for both channels by integrating the pixel values associated with the cell and subtracting the average background intensity of the well.

Data analysis and Statistical Analysis

Genome data were analyzed and normalized to account for two sources of variability in the data: cell-to-cell and plate-to-plate variations. A statistical analysis was carried out to estimate the significance of a given result. A Mann-Whitney test was performed to check how different positive and negative controls are. We expect to see a shift to the right when compare positive and negative controls distribution. The p-Value should be at most 0.01 depending on the sample size. Cutoff line was determined for each plate for estimation of siRNA - induced gene silencing effect on efficiency of 6-4PPs repair. Cell which 6-4PPs signal was located after the cutoff line were concerned as positive(damaged) and a portion of these cells gave the score to the certain gene. siRNAs that induced cell death prior to UV-irradiation were not used for the subsequent analysis.

Bioinformatics

Functional classification was determined using Ingenuity pathway analysis on those genes whose 6-4PPs signal scored with highest significance. Enriched groups were then put into String database analyzer for protein-protein interaction network identification. Gene functions were assigned using resources from above programs.

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REFERENCES

1. Lukas J, Lukas C, Bartek J (2011) More than just a focus: The chromatin response to DNA damage and its role in genome integrity maintenance. Nature Cell Biology 13: 1161–1169.
2. Hanawalt PC, Spivak G (2008) Transcription-coupled DNA repair: two decades of progress and surprises. Nature reviews Molecular cell biology 9: 958–970.
3. Jones RM, Petermann E (2012) Replication fork dynamics and the DNA damage response. The Biochemical journal 443: 13–26.
4. Lord CJ, Ashworth A (2012) The DNA damage response and cancer therapy. Nature 481: 287–294.
5. Li J, Bhat A, Xiao W (2011) Regulation of nucleotide excision repair through ubiquitination. Acta biochimica et biophysica Sinica 43: 919–929.
6. Luijsterburg MS, van Attikum H (2011) Chromatin and the DNA damage response: the cancer connection. Molecular oncology 5: 349–367.
7. Schwarz T (1998) UV light affects cell membrane and cytoplasmic targets. Journal of photochemistry and photobiology B, Biology 44: 91–96.
8. Izumizawa Y, Yang SJ, Negishi T, Negishi K (2000) DNA lesion and mutagenesis induced in phageM13mp2 by UVA, UVB and UVC irradiation. Nucleic acids symposium series: 73–74.
9. Chang E-J, Kundu JK, Liu L, Shin J-W, Surh Y-J (2011) Ultraviolet B radiation activates NF-κB and induces iNOS expression in HR-1 hairless mouse skin: role of IκB kinase-β. Molecular carcinogenesis 50: 310–317.
10. Takasawa R, Nakamura H, Mori T, Tanuma S (2005) Differential apoptotic pathways in human keratinocyte HaCaT cells exposed to UVB and UVC.

Apoptosis : an international journal on programmed cell death 10: 1121–1130.

11. Zhong JL, Yang L, Lü F, Xiao H, Xu R, et al. (n.d.) UVA, UVB and UVC induce differential response signaling pathways converged on the eIF2α phosphorylation. Photochemistry and photobiology 87: 1092–1104.
12. Curtis C, Shah SP, Chin S-F, Turashvili G, Rueda OM, et al. (2012) The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature advance on.
13. Marbach I, Licht R, Frohnmeyer H, Engelberg D (2001) Gcn2 mediates Gcn4 activation in response to glucose stimulation or UV radiation not via GCN4 translation. The Journal of biological chemistry 276: 16944–16951.
14. Assaily W, Rubinger DA, Wheaton K, Lin Y, Ma W, et al. (2011) ROS-mediated p53 induction of Lpin1 regulates fatty acid oxidation in response to nutritional stress. Molecular cell 44: 491–501.
15. Qian Cheng JC (2010) Mechanism of p53 stabilization by ATM after DNA damage. Cell cycle (Georgetown, Tex) 9: 472.
16. Egly J-M, Coin F (2011) A history of TFIIH: two decades of molecular biology on a pivotal transcription/repair factor. DNA repair 10: 714–721.
17. Lagerwerf S, Vrouwe MG, Overmeer RM, Fousteri MI, Mullenders LHF (2011) DNA damage response and transcription. DNA repair 10: 743–750.
18. Greenberg RA (2011) Histone tails: Directing the chromatin response to DNA damage. FEBS letters 585: 2883–2890.
19. Han S-S, Yun H, Son D-J, Tompkins VS, Peng L, et al. (2010) NF-κB/STAT3/PI3K signaling crosstalk in iMycEμ B lymphoma. Molecular Cancer 9: 97.

20. Zushi S, Shinomura Y, Kiyohara T, Miyazaki Y, Kondo S, et al. (1998) STAT3 mediates the survival signal in oncogenicras-transfected intestinal epithelial cells. International Journal of Cancer 78: 326–330.
21. Barry SP, Townsend PA, Knight RA, Scarabelli TM, Latchman DS, et al. (2010) STAT3 modulates the DNA damage response pathway. International Journal of Experimental Pathology 91: 506–514.
22. Mason S (2012) Small RNA-mediated DNA repair. Nature Structural & Molecular Biology 19: 470–470.
23. Wahba L, Amon JD, Koshland D, Vuica-Ross M (2011) RNase H and multiple RNA biogenesis factors cooperate to prevent RNA:DNA hybrids from generating genome instability. Molecular cell 44: 978–988.
24. Stirling PC, Chan YA, Minaker SW, Aristizabal MJ, Barrett I, et al. (2012) R-loop-mediated genome instability in mRNA cleavage and polyadenylation mutants. Genes & development 26: 163–175.
25. Reijns MAM, Rabe B, Rigby RE, Mill P, Astell KR, et al. (2012) Enzymatic Removal of Ribonucleotides from DNA Is Essential for Mammalian Genome Integrity and Development. Cell.
26. Bochkareva A, Yuzenkova Y, Tadigotla VR, Zenkin N (2012) Factor-independent transcription pausing caused by recognition of the RNA-DNA hybrid sequence. The EMBO journal 31: 630–639.
27. Ginno PA, Lott PL, Christensen HC, Korf I, Chédin F (2012) R-Loop Formation Is a Distinctive Characteristic of Unmethylated Human CpG Island Promoters. Molecular Cell 45: 814–825.

28. Nakama M, Kawakami K, Kajitani T, Urano T, Murakami Y (2012) DNA-RNA hybrid formation mediates RNAi-directed heterochromatin formation. Genes to cells : devoted to molecular & cellular mechanisms 17: 218–233.
29. Kuraoka I, Ito S, Wada T, Hayashida M, Lee L, et al. (2008) Isolation of XAB2 complex involved in pre-mRNA splicing, transcription, and transcription-coupled repair. The Journal of biological chemistry 283: 940–950.
30. Steffen Emmert TDS (2001) The human XPG gene: gene architecture, alternative splicing and single nucleotide polymorphisms. Nucleic Acids Research 29: 1443.
31. Thorel F, Constantinou A, Dunand-Sauthier I, Nouspikel T, Lalle P, et al. (2004) Definition of a Short Region of XPG Necessary for TFIIH Interaction and Stable Recruitment to Sites of UV Damage. Molecular and Cellular Biology 24: 10670–10680.
32. Khan SG, Yamanegi K, Zheng Z-M, Boyle J, Imoto K, et al. (2010) XPC branch-point sequence mutations disrupt U2 snRNP binding, resulting in abnormal pre-mRNA splicing in xeroderma pigmentosum patients. Human Mutation 31: 167–175.
33. Cartault F, Nava C, Malbrunot A-C, Munier P, Hebert J-C, et al. (2011) A new XPC gene splicing mutation has lead to the highest worldwide prevalence of xeroderma pigmentosum in black Mahori patients. DNA Repair 10: 577–585.
34. Yu JJ, Thornton K, Guo Y, Kotz H, Reed E (2001) An ERCC1 splicing variant involving the 5′-UTR of the mRNA may have a transcriptional modulatory function. Oncogene 20: 7694–7698.
35. L. F. Revers JMC (2002) Thermoconditional modulation of the pleiotropic sensitivity phenotype by the Saccharomyces cerevisiae PRP19 mutant allele

pso4-1. Nucleic Acids Research 30: 4993.

36. Francia S, Michelini F, Saxena A, Tang D, de Hoon M, et al. (2012) Site-specific DICER and DROSHA RNA products control the DNA-damage response. Nature. 10.1038

37. Robert C, Muel B, Benoit A, Dubertret L, Sarasin A and Anne Stary A (1996) Cell Survival and Shuttle Vector Mutagenesis Induced by Ultraviolet A and Ultraviolet B Radiation in a Human Cell Line. Journal of Investigative Dermatology 106: 721–728.

DISCUSSION AND CONCLUSION

To protect their genomes efficiently, 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[1]. We performed a loss-function screening on chromatin remodelers, kinases and phosphatases discovering new genes involved in DDR and NER. First, we found that UVC and UVB induced DDR via kinases and phosphatases in different ways as cytoplasmic proteins were activated independently from sensing of 6-4PPs or CPD in the nucleus. We showed that genes involved in amino acids, lipids, carbohydrates metabolism and processing of nucleobases, were indispensable for efficient DDR. We discovered that impaired production of nitric oxide or reactive oxygen species resulted in repair deficiency both upon UVB and UVC. Particularly, while studying knockdown of genes like DGUOK, DUSP15 or PNCK, ACP5 we demonstrated that repair deficiency appeared in test with a particular source of UV damage, UVB or UVC respectively. Meanwhile other genes in our study, e.g. GK2 and OXSR1, were equivalently important for proper 6-4PPs repair regardless UV wavelength.

Discussing our findings, we should mention that UV directly affects cytoplasmatically located transcription factors, kinases closely located to the cellular membrane and membrane receptors interfering with cytokine signaling and inducing apoptosis via direct activation of apoptosis - related surface receptors[2]. Thus UVB radiation is shown to cause inflammatory tissue damage and skin cancer and NF-κB could be a plausible molecular link, which regulates expression of pro-inflammatory genes including inducible nitric oxide synthase (iNOS)[3]. Also even lowest energy UV radiation - UVA is able to induce mutations via non- CPD or 6-4PPs dipyrimidine lesions in DNA and that could give additional signals for DDR[8]. Moreover, DDR signaling is linked to cellular metabolism. Thus AP-1 transcriptional activator Gcn4 that is shown to be activated by stress signals such as UV radiation is also induced by amino acids starvation and growth stimulation such as glucose[4]. Similar findings exists for well-known in DDR pathway p53 protein. Lpin1, a p53-responsive gene, is known to be induced in response to DNA damage and glucose deprivation. Lpin1 expression in

response to nutritional stress is controlled through the ROS-ATM-p53 pathway and is conserved in human cells. Lpin1 provides a bridge between p53 and metabolism that could be an important component in mediating the tumor suppressor function of p53[5,6].

We showed that DDR acted via certain chromatin modifying enzymes. We found new DDR related genes that corresponded to RB1, STAT3 gene interaction networks and RNA splicing - associated enzymes as well as we also found new nodes in networks that corresponded to TFIIH complex and histones modifications related genes.

We anticipate to verify in further experiments role of RB1 protein in XPC regulation and reparation of both cyclobutane pyrimidine dimers and 6-4 photoproducts[7-8]. We expect to test findings on STAT3 dependent apoptosis resistance against UVC, MMC and oxidative stress and also findings that indicate impaired activity of the ATM-Chk2 and ATR-Chk1 pathways in STAT3 deficient cells. We would like to investigate in details mechanisms of UVB and UVC activation of STAT3[9-11]. Our results concerning the plausible link of RNA-processing genes to DDR and NER are supported by recently published studies that showed role of RNAs in DNA repair, genome instability and transcription[12-14] as well as importance of RNA processing proteins in splicing and transcription of NER genes[15-19].

Next, we investigated the recruitment of TFIIH to the lesion, that occurred immediately after XPC and supposedly via direct protein-protein interaction[20,21]. The role of this factor was devoted to the opening of the DNA around the damaged site but the individual function of its helicases subunits in this step remained difficult to delineate. Previous studies from our lab showed that mutations in the helicase motifs III (T469A) or VI (Q638A) that impaired the helicase activity of the XPB subunit, did not inhibit the NER activity of TFIIH[22], raising the question of the role of XPB in NER. We showed that TFIIH containing mutation in the motif III was recruited to the DNA repair sites after UV irradiation and mutation in helicase motif Ia that abolished ATPase activity of XPB, thwarted the accumulation of TFIIH to these sites. We implied that the recruitment of TFIIH to sites of damage was an active process that required ATP hydrolysis. We showed that ATPase activity of XPD, the second helicase of TFIIH, was not required to recruit TFIIH to the damage sites, although it was needed for DNA repair. In addition to the aforementioned ATPase motif, we found that two additional motifs were

implicated in the recruitment of TFIIH to sites of DNA damage. Mutations in the R-E-D and ThM motifs fully mimicked the biochemical and biological defects obtained with a mutation in the ATPase motif. We suggested that the ATPase, R-E-D and ThM motifs worked together to ensure a correct recruitment of TFIIH to the damaged sites before the opening and dual incision steps take place. ThM domain was not found in other helicases, including XPD[23-26], but a similar helical protrusion was observed in DNA polymerases[27] and in Sulfolobus solfataricus SWI2/SNF2 ATPase Rad54[28] where it was expected to grip double-stranded DNA from the minor groove. The structure of XPB suggested that the energy furnished by the ATP hydrolysis could be used to induce a flip of 170° of the HD2 domain following the binding of XPB to DNA[29]. The R-E-D (present in HD1) and the ThM (present in HD2) were then in close vicinity and were 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 evidences 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, the ATPase activity of TFIIH was stimulated by DNA[30]. In the presence of DNA, mutations in the R-E-D and ThM motifs induced 50% inhibition of the ATPase activity compare to TFIIH(WT). In the absence of DNA, the three ATPase activities were strictly identical and were still slightly higher than the ATPase activity of the TFIIH complex mutated in the ATP binding site. These data accredited the model of the conformation change proposed above since they demonstrated that R-E-D and ThM were mainly used to stabilize the binding of XPB to DNA. Furthermore, that these mutations inhibited both TFIIH transcription and repair activities suggested a common mode of recruitment of TFIIH to the promoters and to the damage. The recruitment of TFIIH through the action of the ATPase activity of XPB could also induce a reorganization of the protein-DNA complexes in transcription and repair that could allow new protein-protein or protein-DNA contacts. Indeed, using photo - crosslink experiments, we showed that addition of ATP in NER induced a re-positioning of XPC on the damaged DNA that was dependent on TFIIH[31]. Following 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 had a processive and robust helicase activity stimulated by the p44 subunit of the core TFIIH[32]. 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 gave a new view of the roles of XPB and XPD in NER by revealing their different molecular functions within this event.

Finally, we studied DOT1L, a chromatin modifying enzyme with histone methyltransferase activity that was only one known protein responsible for methylation of histone 3 at lysine 79. We showed that disruption of DOT1L caused hypersensitivity to UV-irradiation in mammalian cells. Applying assays either for unscheduled DNA synthesis or direct measurement of lesion removal, we demonstrated that cells depleted of DOT1L repaired (6-4)PP or CPD at the same rate that wild-type cells, arguing that GG-NER was not deficient in these cells. To show that MEF ^{DOT1L} were also proficient in TC-NER we used the sensitivity of cells towards the drug et743. Indeed, it was shown that cells with defect in TC-NER were resistant to treatment with the drug et743[33]. However, MEF^{DOT1L} were as sensitive to treatment with et743 as MEF ^{WT}. It was then unlikely that UV irradiation sensitivity in DOT1L-deficient mammalian cells was due to a GG-NER or TC-NER defect. DOT1L could serve to reactivate mRNA synthesis after UV-irradiation. Transcriptional arrest was shown to lead to a highly cytotoxic cellular response to stress[34]. This response had multiple causes and was likely not only the result of DNA lesions that block RNA Pol II in elongation. Previous studies challenged the relationship between efficient repair of a lesion in the transcribed strand of active genes and the restoration of DNA damage inhibited transcription. For instance, cells carrying mutations in the Cockayne syndrome B protein (CSB) were unable to recover lesion-inhibited transcription while they efficiently repaired acetylaminofluorene lesions in transcriptionally active genes. In addition, CSB was shown to accumulate on the promoters of housekeeping genes after UV-irradiation, where it stimulated the recovery of inhibited transcription[35, 36]. This finding led to the hypothesis that removal of transcription blocking lesions was insufficient to restore transcription after DNA damage and that in addition, chromatin changes in the promoters of housekeeping genes could be required. Our studies showed that expression of housekeeping genes was deficient in cells depleted of DOT1L. This inhibition did not depend on the cell cycle since no difference in the distribution of the phases was observed between MEF ^{WT} and MEF ^{DOT1L} when compared before or after UV-irradiation. Using a transcription initiation “in vivo” assay, we demonstrated that transcription re-initiation of constitutively expressed genes

was deficient in the absence of DOT1L. Further we analyzed both the occupancy and the chromatin modifications at the corresponding promoters with time after UV-irradiation and showed that these promoters were temporally depleted of basal transcription factors in the first hours after irradiation in wild-type cells and showed recovery of occupancy after DNA repair took place, 6-10 hours after UV-irradiation. In the absence of DOT1L, we observed a lost of basal transcription factor occupancy and increased heterochromatin marks such as methylation of H3K9 residue at the promoters of constitutively expressed genes, even 24 hours after UV-irradiation. These data suggested that DOT1L favored an opened chromatin structure around the promoter of housekeeping genes to allow transcription re-initiation. In line with this hypothesis, we observed that H3K79me2, the mark of DOT1L activity, was found transiently on the promoters of housekeeping genes in wild-type cells after UV-irradiation, to keep the chromatin opened. In addition, the absence of DOT1L could be circumvented by the class I HDAC inhibitor TSA that relaxed chromatin. TSA restored both the transcription initiation of housekeeping genes and the UV-survival of MEF ^{DOT1L} cells, creating thus a link between these two events. Among the sites of histone methylation, H3K79 was unique as it was not located within the H3 N-terminal tail domain but in the core region. Specifically, this methylation occurred on the surface of the nucleosome and could serve as a platform to recruit additional chromatin modifiers and DNA damage response factors[37]. On the other hand, regions of chromatin where transcription was repressed were depleted of H3K79 methylation, indicating that silencing of chromatin probably required hypomethylation of H3K79. The mechanism that links euchromatin to H3K79 methylation was not fully understood but it was believed that in addition to recruiting chromatin modifiers, this histone mark played an important role in confining the Sir proteins to heterochromatic regions[38-40]. In yeast, Sir3 bound to nucleosomes containing deacetylated histone H4K16 and promoted spreading of heterochromatin along the chromatin[41]. Based on these observations and our data, we proposed that RNA Pol II re-accumulation at promoters of housekeeping genes after UV-irradiation depended on the chromatin changes orchestrated by DOT1L, including the emergence of active chromatin transcription marks around the promoters of these genes. In the absence of DOT1L, facultative heterochromatin marks such as H3K9me2 appeared and RNA Pol II did not get to the promoters. Through the recruitment of chromatin modifiers

and subsequent histone modifications, DOT1L served to limit the spreading of heterochromatin to housekeeping genes immediately after UV-irradiation and to allow re-association of the basal transcription machinery to the promoters of these genes to re-activate their transcription. This model was supported by the results obtained with the STRIP - FRAP experiments. It demonstrated that a fraction of RNA Pol II became mobile in the nucleus in the absence of DOT1L after UV-irradiation. This indicated that the binding of RNA Pol II to the chromatin and as thus, its re - engagement in transcription after UV-irradiation depended on DOT1L. It should be mentioned that the behavior of RNA Pol II in the absence of DOT1L after UV-irradiation mimicked the behavior of basal transcription factors such as TFIIH when cells were treated with the transcription inhibitor DRB that disengaged TFIIH from the PIC[42]. While DOT1L was needed for the re-activation of housekeeping gene transcription after irradiation, our results also demonstrated that DOT1L was dispensable for the transcription of most of the UV-inducible genes. RNA sequencing data indicated that 44% of the genes that were activated in MEF ^{WT}, 2 hours after UV-irradiation were also activated in MEF ^{DOT1L}. Previously published results suggested that the transcription of DNA damage-induced genes was regulated at a post-initiation stage when RNA Pol II was already engaged and poised after transcription start site[43]. Biologically, this regulatory mechanism would result in rapid transcription of DNA-damage induced genes in response to a genotoxic attack. Based on this, we anticipated that transcription initiation of UV-inducible genes takes place without large scale chromatin remodeling and is thus independent of DOT1L activity. Furthermore, a large set of genes was specifically activated in MEF^{DOT1L} following UV-irradiation. Using the String database we showed that these genes were connected to the fos and fas gene families, involved in apoptosis. It was then tempting to speculate that these inductions were part of the specific cellular response of MEF ^{DOT1L} cells to the transcription inhibition induced by UV-irradiation. Induction of these apoptotic genes could be responsible to the cell death observed in MEF ^{DOT1L}, after UV-irradiation. In line with this hypothesis, treatment of MEF ^{DOT1L} with TSA, which restored transcription initiation and UV-survival, also strongly reduced the induction of fosB to a level similar to that of MEF ^{WT} cells. Using our assay to measure the rate of RNA Pol II transcription initiation and elongation “in vivo” on the long ubiquitously expressed Utrophin gene, we also clearly demonstrated that transcription of such constitutively expressed long genes

could take place without DOT1L in the absence of a genotoxic stress. In both MEF ^{WT} and MEF^{DOT1L}, we found that RNA Pol II transcribes Utrophin at a rate of 2.2kb min $^{-1}$, which was consistent with the transcriptional rates measured in other mammalian wild-type cells[44]. A role of DOT1L in transcription elongation was proposed based on the facts that, first, H3K79me2 positively correlated with active gene transcription and second, that mammalian DOT1L-containing complexes were composed of elongation factors[45]. Even if a role of DOT1L in transcription elongation was plausible, our results suggested that DOT1L was probably not required for transcription of all genes. To our knowledge, these were the first reported "in vivo" experiments that allowed direct evaluation of the participation of DOT1L in transcription elongation. Finally, our results made DOT1L an attractive target in chemotherapeutic strategies that rely on the inability of the cells to eliminate drug-induced DNA lesions and lead to apoptosis. The inhibition of DOT1L, in addition to the presence of drugs that saturate DNA repair, could increase cellular toxicity and thus reduce the overall side effects of high doses of chemotherapeutic agents.

In conclusion, we performed a screening of chromatin remodelers, kinases and phosphatases and found new factors that could modulate DDR and DNA repair. Furthermore we demonstrated molecular details of TFIIH recruitment to the damaged DNA in process of NER. We showed importance of H3K79 histone methyltransferase DOT1L for recovery of the transcription after the genotoxic stress. And now we look forward to study in details molecular mechanisms of NER and DDR regulation using perspective genes from networks we detected in our siRNA screening trying to pinpoint potential targets for future therapy and drug design.

REFERENCES

1. Maillard, O., Camenisch, U., Clement, F. C., Blagoev, K. B., and Naegeli, H. (2007). DNA repair triggered by sensors of helical dynamics. Trends Biochem Sci *32*, 494-499.
2. Schwarz T (1998) UV light affects cell membrane and cytoplasmic targets. Journal of photochemistry and photobiology B, Biology 44: 91–96.
3. Izumizawa Y, Yang SJ, Negishi T, Negishi K (2000) DNA lesion and mutagenesis induced in phageM13mp2 by UVA, UVB and UVC irradiation. Nucleic acids symposium series: 73–74.
4. Marbach I, Licht R, Frohnmeyer H, Engelberg D (2001) Gcn2 mediates Gcn4 activation in response to glucose stimulation or UV radiation not via GCN4 translation. The Journal of biological chemistry 276: 16944–16951.
5. Assaily W, Rubinger DA, Wheaton K, Lin Y, Ma W, et al. (2011) ROS-mediated p53 induction of Lpin1 regulates fatty acid oxidation in response to nutritional stress. Molecular cell 44: 491–501.
6. Qian Cheng JC (2010) Mechanism of p53 stabilization by ATM after DNA damage. Cell cycle (Georgetown, Tex) 9: 472.
7. Lin PS, McPherson LA, Chen AY, Sage J, Ford JM (2009) The role of the retinoblastoma/E2F1 tumor suppressor pathway in the lesion recognition step of nucleotide excision repair. DNA repair 8: 795–802.
8. Hardy TM, Kumar MS, Smith ML (2010) RB stabilizes XPC and promotes cellular NER. Anticancer research 30: 2483–2488.
9. Han S-S, Yun H, Son D-J, Tompkins VS, Peng L, et al. (2010) NF-κB/STAT3/PI3K signaling crosstalk in iMycEμ B lymphoma. Molecular Cancer 9: 97.
10. Zushi S, Shinomura Y, Kiyohara T, Miyazaki Y, Kondo S, et al. (1998) STAT3 mediates the survival signal in oncogenicras-transfected intestinal epithelial cells. International Journal of Cancer 78: 326–330.
11. Barry SP, Townsend PA, Knight RA, Scarabelli TM, Latchman DS, et al. (2010) STAT3 modulates the DNA damage response pathway. International Journal of Experimental Pathology 91: 506–514.
12. Mason S (2012) Small RNA-mediated DNA repair. Nature Structural &

Molecular Biology 19: 470–470.

13. Wahba L, Amon JD, Koshland D, Vuica-Ross M (2011) RNase H and multiple RNA biogenesis factors cooperate to prevent RNA:DNA hybrids from generating genome instability. Molecular cell 44: 978–988.
14. Nakama M, Kawakami K, Kajitani T, Urano T, Murakami Y (2012) DNA-RNA hybrid formation mediates RNAi-directed heterochromatin formation. Genes to cells : devoted to molecular & cellular mechanisms 17: 218–233.
15. Steffen Emmert TDS (2001) The human XPG gene: gene architecture, alternative splicing and single nucleotide polymorphisms. Nucleic Acids Research 29: 1443.
16. Thorel F, Constantinou A, Dunand-Sauthier I, Nouspikel T, Lalle P, et al. (2004) Definition of a Short Region of XPG Necessary for TFIIH Interaction and Stable Recruitment to Sites of UV Damage. Molecular and Cellular Biology 24: 10670–10680.
17. Khan SG, Yamanegi K, Zheng Z-M, Boyle J, Imoto K, et al. (2010) XPC branch-point sequence mutations disrupt U2 snRNP binding, resulting in abnormal pre-mRNA splicing in xeroderma pigmentosum patients. Human Mutation 31: 167–175.
18. Cartault F, Nava C, Malbrunot A-C, Munier P, Hebert J-C, et al. (2011) A new XPC gene splicing mutation has lead to the highest worldwide prevalence of xeroderma pigmentosum in black Mahori patients. DNA Repair 10: 577–585.
19. Yu JJ, Thornton K, Guo Y, Kotz H, Reed E (2001) An ERCC1 splicing variant involving the 5′-UTR of the mRNA may have a transcriptional modulatory function. Oncogene 20: 7694–7698.
20. 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.
21. Bernardes de Jesus, B. M., Bjoras, M., Coin, F., and Egly, J. M. (2008). Dissection of the Molecular Defects Caused by Pathogenic Mutations in the DNA Repair Factor XPC. Mol Cell Biol *28*, 7225-7235.
22. Coin, F., Oksenych, V., and Egly, J. M. (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.

23. Bienstock, R. J., Skorvaga, M., Mandavilli, B. S., and 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.
24. Fan, L., Fuss, J. O., Cheng, Q. J., Arvai, A. S., Hammel, M., Roberts, V. A., Cooper, P. K., and Tainer, J. A. (2008). XPD helicase structures and activities: insights into the cancer and aging phenotypes from XPD mutations. Cell *133*, 789-800.
25. Liu, H., Rudolf, J., Johnson, K. A., McMahon, S. A., Oke, M., Carter, L., McRobbie, A. M., Brown, S. E., Naismith, J. H., and White, M. F. (2008). Structure of the DNA repair helicase XPD. Cell *133*, 801-812.
26. Wolski, S. C., Kuper, J., Hanzelmann, P., Truglio, J. J., Croteau, D. L., Van Houten, B., and Kisker, C. (2008). Crystal structure of the FeS cluster-containing nucleotide excision repair helicase XPD. PLoS Biol *6*, e149.
27. Doublie, S., Tabor, S., Long, A. M., Richardson, C. C., and Ellenberger, T. (1998). Crystal structure of a bacteriophage T7 DNA replication complex at 2.2 A resolution. Nature *391*, 251-258.
28. Durr, H., Korner, C., Muller, M., Hickmann, V., and Hopfner, K. P. (2005). X-ray structures of the Sulfolobus solfataricus SWI2/SNF2 ATPase core and its complex with DNA. Cell *121*, 363-373.
29. 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.
30. Roy, R., Schaeffer, L., Humbert, S., Vermeulen, W., Weeda, G., and Egly, J. M. (1994). The DNA-dependent ATPase activity associated with the class II basic transcription factor BTF2/TFIIH. J Biol Chem *269*, 9826-9832.
31. Tapias, A., Auriol, J., Forget, D., Enzlin, J., Scharer, O., Coin, F., Coulombe, B., and Egly, J. (2004). Ordered conformational changes in damaged DNA induced by nucleotide excision repair factors. J Biol Chem, in press.
32. 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.

33. Takebayashi, Y., Pourquier, P., Zimonjic, D.B., Nakayama, K., Emmert, S., Ueda, T., Urasaki, Y., Kanzaki, A., Akiyama, S.I., Popescu, N.*, et al.* (2001). Antiproliferative activity of ecteinascidin 743 is dependent upon transcription-coupled nucleotide-excision repair. Nat Med *7*, 961-966.
34. Fousteri, M., and Mullenders, L.H. (2008). Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects. Cell Res *18*, 73-84.
35. van Oosterwijk, M.F., Versteeg, A., Filon, R., van Zeeland, A.A., and Mullenders, L.H. (1996). The sensitivity of Cockayne's syndrome cells to DNA-damaging agents is not due to defective transcription-coupled repair of active genes. Mol Cell Biol *16*, 4436-4444.
36. 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.
37. Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature *389*, 251-260.
38. van Leeuwen, F., Gafken, P.R., and Gottschling, D.E. (2002). Dot1p modulates silencing in yeast by methylation of the nucleosome core. Cell *109*, 745-756.
39. Ng, H.H., Ciccone, D.N., Morshead, K.B., Oettinger, M.A., and Struhl, K. (2003). Lysine-79 of histone H3 is hypomethylated at silenced loci in yeast and mammalian cells: a potential mechanism for position-effect variegation. Proc Natl Acad Sci U S A *100*, 1820-1825.
40. Ng, H.H., 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*, 1518-1527.
41. Johnson, A., Li, G., Sikorski, T.W., Buratowski, S., Woodcock, C.L., and Moazed, D. (2009). Reconstitution of heterochromatin-dependent transcriptional gene silencing. Mol Cell *35*, 769-781.
42. 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.

43. Muse, G.W., Gilchrist, D.A., Nechaev, S., Shah, R., Parker, J.S., Grissom, S.F., Zeitlinger, J., and Adelman, K. (2007). RNA polymerase is poised for activation across the genome. Nat Genet *39*, 1507-1511.
44. Singh, J., and Padgett, R.A. (2009). Rates of in situ transcription and splicing in large human genes. Nat Struct Mol Biol *16*, 1128-1133.
45. Nguyen, A.T., and Zhang, Y. (2011). The diverse functions of Dot1 and H3K79 methylation. Genes Dev *25*, 1345-1358.

AFTERWORD

The worthwhile problems are the ones you can really solve or help solve, the ones you can really contribute something to. … No problem is too small or too trivial if we can really do something about it.

Letter from Feynman to Koichi Mano (3 February 1966); published in *Perfectly Reasonable Deviations from the Beaten Track : The Letters of Richard P. Feynman* (2005)

Alexander ZHOVMER

NOUVEAUX ACTEURS A' L'INTERFACE DE LA TRANSCRIPTION ET DE LA REPARATION

Résumé

Les résultats du criblage siRNA destiné à identifiés de nouveaux acteurs de la NER, sont en court d'exploitation mais nous mettons déjà en évidence le rôle de certains gènes impliqués dans la biochimie des ARNm comme ceux empêchant la formation des hybrides ARN/ADN dans l'efficacité de réparation des lésions UV. En étudiant le rôle de la methyltransférase DOT1L, nous avons montré que son absence conduit en réalité à une inhibition de l'initiation de la transcription des gènes après irradiation. Dans une analyse plus détaillée, nous avons montré que DOT1L favorisait la formation du complexe de pré-initiation au niveau du promoteur des gènes de ménage ainsi que l'apparition de marques d'euchromatine transcriptionnellement actives. Le traitement à la trichostatine A, qui relaxe la chromatine, diminue la transactivation des gènes apoptotiques et restore l'initiation de la transcription et la survie aux UV. Nous proposons que DOT1L garde structure de la chromatine ouverte après UV.

Mots-clés : methyltransférase, DOT1L, DDR, NER, chromatine

Résumé en anglais

As a result of siRNA screening we identified new players at the interface between NER machinery and chromatin. Despite it is ongoing study we already highlighted that certain genes which are involved in the biochemistry of mRNA such as splicing and preventing the formation of RNA:DNA hybrids are important for efficient repair of UV damage. Studying the role of histone H3 lysine 79 methyltransferase DOT1L, we have shown that its absence leads to an inhibition of the initiation of gene transcription after UV irradiation. In a more detailed analysis, we show that DOT1L favors the formation of pre-initiation complex at the promoter of housekeeping genes as well as the appearance of marks of the transcriptionally active euchromatin. Treatment with trichostatin A, which relaxes the chromatin, lowers the transactivation of proapoptotic genes and restores the transcription initiation as well as cell survival after UV. We propose that DOT1L keeps the opened chromatin structure after UV irradiation.

Keywords : methyltransferase, DOT1L, DDR, NER, chromatin