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The role of the chromatin organization in DNA double strand break repair in mouse embryonic stem cells

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Table of Contents

Acknowledgements 3

List of figures 6

List of abbreviations 8

Thesis summary 13

Thesis summary in French 18

Introduction 24

- DNA damage and repair 24
  - DNA damage 24
  - DNA repair 26
- DSB recognition and DNA damage response 30
  - DSB sensing and DDR activation 30
  - γH2AX and MDC1 foci formation 31
  - Role of parylation in DDR 33
  - Signal transduction and amplification. DDR kinases 33
  - Role of ubiquitylation and SUMOylation in DSB repair 36
  - DDR activation consequences 39
- Double-strand break repair pathways 40
  - Non-homologous end joining 40
  - Homologous recombination 42
  - Microhomology-mediated end joining 45
  - Single-strand annealing 46
  - Double-strand break repair pathway choice 47
- Chromatin 49
  - Euchromatin and heterochromatin 52
  - DNA repair in the chromatin context 54
  - Bivalency 57
- Embryonic stem cells 58
  - Chromatin in embryonic stem cells 59
  - DNA repair in embryonic stem cells 60
- Genome editing 62
- Genome editing fidelity studies 64

Goals 72

Results 73
Establishing an experimental system 73
Target choice and guide RNA design 77
TIDE analysis. Indel pattern is sequence-specific and is not influenced by a cell type or a chromatin context 78
3T3 cells have a higher rate of error-prone repair in housekeeping genes but not in pluripotency regulators 83
HR-TIDE: improving methodological procedures in order to detect HR 87
Overall cutting efficiency is slightly higher in 3T3 than in ES cells 88
HR rates are higher in ES than in 3T3 cells 90
Bivalent and inactive genes 93
Exceptions 95
LADs 95
The cell cycle is partially responsible for the low HR rate in 3T3. Cell cycle block causes an increase in HR utilisation but cannot fully explain differences between cell types 96
Discussion 99
Sequence predominantly defines the DSB repair outcome. Chromatin and cell type might have no more than moderate influence on the DSB repair outcome 100
ES cells use HR more actively than differentiated cells 101
HR is enhanced in transcriptionally active genes. It is more prominent in ES than in differentiated cells 101
HR levels are low in bivalent domains and facultative heterochromatin 102
Influence of the cell cycle on the enhanced HR utilization in ES cells 102
Perspectives 104
Materials and methods 107
Cell culture 107
Cell cycle analysis 110
Western blotting 110
Chromatin Immunoprecipitation (ChIP) 111
TIDE 113
Bibliography 117

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List of figures

1. Types of DNA lesions, their sources and repair pathways
2. Schematic representation of early steps of DDR activation
3. Roles of PI3KKs in the maintenance of genome stability
4. Consequences of DNA damage-induced Chk1 and Chk2 mediated cell cycle checkpoint activation
5. Schematic representation of cNHEJ pathway
6. Schematic representation of HR pathway
7. Schematic representation of MMEJ pathway
8. Schematic representation of SSA pathway
9. DSB repair pathway choice
10. Chromatin environment influence over DNA repair pathway choice
11. Schematic representation of CRISPR-Cas0 system mechanism of action
12. A scheme of the construct used to create stable cell lines
13. FACS results for analysed clones
14. γH2AX levels are increased after transfecting with a guide RNA for major satellites
15. ChIP-qPCR results for selected targets
16. A scheme of TIDE experiment
17. Reproducibility of indel patterns
18. TIDE results: indel pattern
19. Indel size assessment
20. Indel size assessment. Larger insertions are more prominent in ES than in 3T3 cells
21. TIDE results comparison between cell types
22. Quantification of a cell cycle distribution with or without transfection
23. RT-qPCR in ES cells
24. Scheme of HR-TIDE approach
25. A representative graph returned by TIDE software after co-transfecting guide RNA for Oct4 and a corresponding HR template
26. Comparison of cutting efficiency
27. Comparison between HR efficiencies in 3T3 and ES cells

28. Comparison of HR use between cell types on exampl of single guide RNAs
29. Comparison of arrays of guide RNAs targeting housekeeping genes or pluripotency regulators between cell lines
30. Comparison betwee different target groups in the same cell type
31. Comparison of bivalent and heterochromatic targets between two cell lines on single guide RNAs and on combined arrays
32. Comparison of targets located in LADs targets between two cell types on single guide RNAs
33. HR proportion grows considerably after cell cycle block with RO inhibitor
34. Cell cycle distribution difference between cells cultured in the presence of serum or in serum-free 2i medium
35. Comparison of HR proportion in two culture mediums shows that it is not consistent between different targets and no conclusion can be drawn

List of abbreviations

2i	cocktail of two inhibitors: for MEK1 and GSK3β
53BP1	TP53-binding protein 1
ABRA1	BRCA1-A complex subunit Abraxas 1
Alt-EJ	alternative end-joining
AP	apurinic-apirimidinic
APE1	Apurinic-apyrimidic endonuclease 1
APTX	Aprataxin
ATR	Ataxia telangiectasia and Rad3-related protein
ATRIP	ATR-interacting protein
BARD1	BRCA1-associated RING domain protein 1
BER	base excision repair
BIR	break-induced replication
BLM	Bloom syndrome helicase
Bmi1	Polycomb complex protein BMI-1 (B cell-specific Moloney murine leukemia virus integration site 1)
BRCA1, 2	Breast cancer type 1, 2 susceptibility protein
BRCC36	BRCA1/BRCA2-containing complex subunit 36
Cas9	CRISPR-associated protein 9
Cdc25A	M-phase inducer phosphatase 1
Cdks	cyclin-dependent kinases
CENPA	Histone H3-like centromeric protein A
CETN2	Centrin 2
CHFR	checkpoint protein with FHA and RING domain
Chk1,2	Checkpoint kinase 1,2
cNHEJ	classical non-homologous end-joining
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA

CSA-B	Cockayne Syndrome protein A-B
CTCF	CCCTC-binding factor
CtIP	CtBP-interacting protein
DD	destabilisation domain
DDR	DNA damage response
DNA	deoxyribonucleic acid
DNA2	DNA replication ATP-dependent helicase/nuclease DNA2
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	double strand break
dsDNA	double-stranded DNA
DYNLL1	Dynein light chain 1
ERCC1	DNA excision repair protein ERCC1
ERK	Extracellular signal-regulated kinase
ES cells	embryonic stem cells
ETAA1	Ewing's tumor-associated antigen 1
EXO1	Exonuclease 1
EZH2	Enhancer of zeste homolog 2
FA	Fancony anemia
FAAP24	FA associated protein of 24 kDA
FANCA-M	Fanconi anemia group A-M protein
FEN1	Flap endonuclease 1
FGF	Fibroblast growth factor
GG-NER	global genome NER
GSK3β	Glycogen synthase kinase-3 beta
HERC2	HECT domain and RCC1-like domain-containing protein 2
HMGB1	High mobility group box 1 protein
HMT	Histone methyltransferases
HP1	Heterochromatin protein 1

HR	homologous recombination
ICL	interstrand crosslink
Indels	insertions and deletions
IR	ionizing radiation
Kap1	KRAB-associated protein 1
Ku 70/80	Ku autoantigen protein p70/80 homolog
LADs	lamina-associated domains
LEDGF	Lens epithelium-derived growth factor
LIF	Leukemia inhibitory factor 1
Lig1-4	DNA ligases 1-4
LTGC	long-tract gene conversion
MDC1	Mediator of DNA damage checkpoint protein 1
Mec1	Mitosis entry checkpoint 1
MEK1	MAPK/ERK kinase 1
MFH	Forkhead box protein C2
MLL2	Myeloid/lymphoid or mixed-lineage leukemia protein 2
MMEJ	microhomology-mediated end joining
MMR	mismatch repair
Mre11	Meiotic recombination 11 homolog 1
MRN	Mre11-Rad50-Nbs1 complex
MSH	MutS homolog
Mst 1,2	Mammalian STE20-like protein kinase 1,2
Myc	Myc proto-oncogene protein
Nbs1	Nijmegen breakage syndrome protein 1
NER	nucleotide excision repair
NHEJ	Non-homologous end joining
Oct4	Octamer-binding protein 4
PALB2	Partner and localizer of BRCA2
PARP	Poly (ADP-ribose) polymerase

PCNA	Proliferating cell nuclear antigen
PIKK	phosphoinositide 3-kinase (PI3K)-related kinase
PNKP	polynucleotide kinase 3'-phospate
PolII	RNA polymerase II
Polα-σ	DNA polymerase α-σ
Pou5f1	POU domain, class 5, transcription factor 1
PRC1, 2	Polycomb repressive complex 1, 2
PTIP	PAX transactivation activation domain-interacting protein
Rad51, 52, 54	DNA repair protein RAD51, 52, 54 homolog
RAP80	Receptor-associated protein 80
REV1, 7	Rev1, 7-like terminal deoxycytidyl transferase
RFC	Replication factor C
RIF1	Rap1-interacting factor 1 homolog
RNAPII	RNA polymerase II
Rnf2, 8 , 168	RING finger protein 2, 8, 168
ROS	reactive oxigene species
RPA	Replication protein A
Sall4	Sal-like protein 4
SHLD1-3	Shieldin complex subunit 1-3
SMC1	Structural maintenance of chromosomes protein 1
SSA	single-strand annealing
SSB	single-strand break
SSBR	single strand break repair
SDSA	Synthesis-dependent strand annealing pathway
Sox2	SRY-box 2
ssDNA	single-strand DNA
STAT3	Signal transducer and activator of transcription 3
SUMO	Small Ubiquitin-like Modifier

TAD	topologically associated domain
TALENs	Transcription activator-like effector nucleases
TC-NER	transcription-coupled NER
TDP1	tyrosyl-DNA phosphodiestherase 1
TFIIH	Trancription factor II H
TIDE	tracking indels by decomposition
Tip60	60 kDa Tat-interactive protein
TLS	translesion synthesis
TOP1	DNA topoisomerase 1
TopBP1	DNA topoisomerase 2-binding protein 1
tracrRNA	trans-activating RNA
Ubc13	ubiquitin-conjugating enzyme 13
UBQLN4	Ubiquilin-4
USP3, 11, 16	ubiquitin-specific proteases 3, 11, 16
UV	ultraviolet
XLF	XRCC4-like factor
XPA-G	Xeroderma Pigmentosum, complementation group A-G
XPF	Excision repair cross-complementing rodent repair deficiency, complementation group 4
XRCC1-4	X-ray repair cross-complementing protein 1-4
ZNFs	zinc-finger nucleases

Thesis summary

Thousands of acts of DNA damage happen in multicellular organisms every day. This makes the process of DNA repair, particularly of double-strand breaks, extremely important to study. Evidence grows to support the hypothesis that chromatin organization plays a notable role in repair pathway choice. It has been shown by multiple groups that the local chromatin organization around the site of a double-strand break (DSB) can influence the repair outcome. For example, transcriptionally active chromatin marks such as trimethylation of the lysine 36 of histone H3 (H3K36me3) and acetylation of a lysine 16 of the histone H4 (H4K16ac) has been observed to promote homologous recombination (HR). Moreover, it has recently been demonstrated that in case of DSBs induced in transcrtiptionally active regions in G1, where HR is not possible due to the absence of a sister chromatid that should serve as a template, repair can even be postponed till G2 phase where HR is enabled. On the other hand, it has been shown that the presence of repressive chromatin marks, such as mono- and dimethylation of the lysine 20 of histone H4 (H4K20me1 and H4K20me2) favours the choice of non-homologous end joining (NHEJ) as the main repair pathway. Results obtained in our laboratory a few years ago showed that in lamina-associated domains (LADs) that represent highly repressed chromatin type HR is absolutely prohibited and cells even use potentially deleterious microhomology-mediated end-joining (MMEJ) instead. It has also been observed in the lab that in pericentric heterochromatin, another case when HR can potentially be deleterious, there are certain precautions cells take to prevent undesirable consequences of its use by translocating breaks to the periphery of a chromocenter for repair. DSB repair in heterochromatin, especially constitutive, is much better studied than in euchromatin or facultative heterochromatin. It has been shown that euchromatin is more susceptible to DNA damage and generally promotes HR but overall it requires more detailed studies.

One of the chromatin types, relatively abundant in stem cells, that has yet never been studied in the field of DNA repair is so-called bivalent chromatin. Bivalent domains is a name for chromatin stretches marked with both permissive and repressive histone modifications. At first, the combination of H3K4me3 and H3K27me3 was observed, which is now also named ‘classic bivalent domains’; later on H3K4me3 and H3K9me3 or H3K36me2/3 and H3K9me3 containing bivalent domains were found in human mesenchymal stem cells and preadipocytes and HEK293 cells respectively. For some years after their discovery, they

were considered a cell culture artifact by many scientists. However, within several last years, they were observed *in vivo* by several groups, both during normal development and in cancer. All in all, we consider it an interesting phenomenon, worth thorough investigation.

In our laboratory we are working on various aspects of DSB repair regulation by the chromatin context, using various chromatin types, experimental approaches, and model organisms. In my project, I used mouse ESCs as a model to study stem cell-specific features of chromatin influence over the DNA repair process and study the specific features of DNA repair in bivalent chromatin.

The main questions I wanted to answer are the following:

Do bivalently marked regions represent a distinct chromatin type in terms of DNA repair?

Does chromatin structure affect repair of the same loci in the same cell line during differentiation?

Are there ES-specific features of DNA repair in the chromatin context?

The goal of my project was to study the kinetics and mechanisms of DSB repair and its relation to the chromatin context that the breaks are induced. To this end, I was using CRISPR-Cas9 to induce double-strand breaks (DSBs) in various chromatin contexts in ESCs or differentiated cells. I have generated stable mES cell lines expressing wtCas9 fused to GFP and to a destabilisation domain (DD) that leads to constant degradation of the wtCas9. In the presence of the chemical molecule called Shield1, Cas9 is stabilized. I have observed that 8-10h after shield addition, Cas9GFP reaches its max levels and 10h after shield withdrawal it drops almost to the levels observed in the absence of Shield1.

To induce DSBs at the different chromatin contexts, I use plasmids or in vitro transcribed guide RNAs that target genomic locations decorated by different chromatin modifications. As one of the chromatin types of our interest is bivalent domains that have not been studied before in terms of DNA repair and are often observed at developmental regulators and thought to enable fast differentiation, we chose to induce breaks at several bivalently marked genes. These genes (Pax6, Zic1, and Ngn2) are reported to be expressed at very low levels in ESCs and in high levels in some differentiated cell types and regulate neuronal development. These loci were compared with genes that are considered to be markers of

pluripotency (Nanog, Pou5f1, Tfcp2l1, Zfp42) that are expressed highly in mESCs and are shut down during differentiation and with housekeeping genes (Actb, Gapdh) that are expressed all the time, and genes that are reported to be totally repressed by H3K27me3 in ES cells (Hoxb1, Tdrd1, Mc4r). We also sought to include some genes that belong to LADs in ES cells (Sox6, Ptn, Nrp1) into the comparison.

To this end, we designed two guide RNAs per gene to introduce DSBs into their promoters. We used the Cas9-expressing NIH 3T3 cell line previously established in the lab to get a comparison with a differentiated cell type.

To measure NHEJ efficiently as well as repair fidelity of the breaks induced in the above-described chromatin contexts in mESCs and NIH3T3 cells we have employed a method named TIDE (Tracking of Indels by Decomposition). TIDE is a sequencing-based method allowing us to quantify a percentage of incorrectly repaired sequences in the population, as well as a percentage of particular insertions or deletions. Our results from TIDE showed that ESCs are using non-homologous end joining (NHEJ) or other error-prone pathways more than expected from the literature. Interestingly, we also observed that the relative frequency of erroneous repair varies depending on a type of gene where the break was introduced. ESCs are using error-prone repair less than 3T3 cells for housekeeping genes and developmental regulators but, surprisingly, not for pluripotency markers, suggesting that both the chromatin structure as well as the levels of transcription influence TIDE efficiency. The possibility that disrupting a promoter of a pluripotency regulator we interfere with the cell cycle and increase the proportion of cell in G1 leading to increase of NHEJ use was discharged as we could show that there are no changes at the ESCs cell cycle profiles in all conditions.

We also noticed although the overall TIDE efficiency depends on the cell type, the pattern of insertions and deletions (indels) is quite similar between ESCs and 3T3 cells and depends on guide RNAs *per se*. As deletions less than 10nt are considered products of NHEJ and more than 10nt alternative end-joining (AltEJ), our results suggest that the NHEJ/AltEJ balance does not largely depend on chromatin structure or cell type.

Unfortunately, the TIDE method did not allow us to distinguish between the DNA that was not cut or repaired using HR. To measure HR efficiency, we needed to modify the method, taking advantage of the TIDE sensitivity of measuring small insertion efficiency after a break induction. To this end, for each guide

RNA, we designed a specific HR template that consists of 1000bp homology arms (500bp at each side of the break) and a 9 bp unique DNA that is inserted by HR at the break site. ESCs and 3T3 cells that stably express Cas9 were co-transfected with a guide RNA together with a template, and the locus was amplified by PCR and subjected to TIDE. The frequency of 9 bp insertion at each genomic location indicated the HR frequency. Using this modified TIDE method (which we called HR-TIDE) we were able to get a comparison of HR efficiency between different chromatin types, as well as between 3T3 and ES cells. We could see that HR frequency in 3T3 cells was generally low, and never exceeded 20%. ES cells demonstrated greater variability, from as low as 3% in some inactive up to 40% in active genes. In general, using HR-TIDE we could not confirm previous reports that 80% of DSBs are repaired using HR, although it was giving a greater contribution than in differentiated cells, which goes in line with current ideas in the field.

Comparing HR efficiency between different chromatin contexts we could confirm the observation that transcriptionally active chromatin is rather promotive for it, whereas facultative heterochromatin represents a repressive environment. We also observed that bivalent domains show intermediate levels of HR, supporting the idea that they represent an intermediate state between active and repressed chromatin. As for genes located in LADs, we saw some variability, with some genes totally repressed and some demonstrating higher HR proficiency, which might be explained by their location inside or at a border of domains.

Seeing differences in HR frequency we bared in mind that the cell cycle differs between ES and 3T3 cells, the former having a much shorter G1 and thus a larger percentage of cells capable of this way of repair. To address this question, we first decided to block 3T3 cells in the G2 phase of the cell cycle using RO inhibitor. In accordance with previous reports, we could see a considerable increase in the percentage of HR. However, we required complementary proof that extending the G1 phase of ES cells would lead to an HR efficiency drop. In order to model such a situation without driving cells into a commitment for differentiation, we decided to use 2i medium. 2i medium for culturing ES cells is serum-free and includes a cocktail of two inhibitors (MEK inhibitor and GSK-3 inhibitor, that block MEK/ERK and Wnt/β-Catenin signalling pathways respectively). However, we could not observe a significant drop in HR efficiency in active genes, which indicates that cell cycle differences between cell types cannot be on their own accountable for differences in a repair pathway choice. At the same time, we have noticed a decrease in HR usage in inactive genes, which goes in line with the fact that bivalency is lost in 2i conditions.

All in all, we have established an inducible and degradable system to assess DNA double-strand break repair and we have put forwards several assays to study DNA repair pathway choice. Our data shed light on the role of bivalent chromatin and facultative heterochromatin in the process of DNA repair pathway choice. At the same time, we have proposed and optimized an easy and quick method of accessing HR proficiency of a particular locus in a cellular context, which can be of practical use in designing knock-ins using the CRISPR-Cas9 system.

As future perspectives, several experiments can be planned. Taking advantage of the shield inducible and degradable Cas9 system I described at the beginning it is possible to perform ChIP experiments monitoring the kinetics of appearance and disappearance of several DDR factors (γH2AX and 53BP1) at different times after shield addition and withdrawal. As above, these experiments should be performed in ES cells and 3T3 cells. To correlate actual break induction and repair with DDR mounting and switch off at these breaks, LM-PCR should be performed at the same time points after shield addition and withdrawal in all breaks and cell types.

However, to get a broader picture of the chromatin influence over DNA repair pathway choice, it would be advantageous to perform a larger-scale experiment. Having optimized template design and cloning process on the one hand, and with the availability of data on genome-editing efficiencies of different guides genome-wide on the other hand, it can be possible to combine these two approaches. For this, a library containing guides and corresponding templates could be cloned into non-integrating viral vectors and used for infection of ES or differentiated cells and subsequent analysis of a repair profile using NGS.

Thesis summary in French

Des milliers de dommages à l'ADN se produisent chaque jour au sein des organismes multicellulaires. C'est pourquoi il est important d'étudier le processus de réparation de l'ADN, en particulier les cassures des doubles brins. De plus en plus de preuves appuient l'hypothèse selon laquelle l'organisation de la chromatine joue un rôle notable dans le choix de la voie de réparation. Il a été démontré que l'organisation locale de la chromatine autour du site d'une cassure double brin de l'ADN (Double Strand Break soit DSB en anglais) peut influencer le résultat de la réparation. Par exemple, la présence des marques d'histones telles que la triméthylation de la lysine 36 de l'histone H3 (H3K36me3) et l'acétylation d'une lysine 16 de l'histone H4 (H4K16ac), signe d'une chromatine transcriptionnellement active, ont été observées pour promouvoir une réparatoin par le mécanisme de recombinaison homologue (Homologous Recombination soit HR en anglais). De plus, il a récemment été démontré que dans le cas de DSBs induites dans des régions transcrtiptionnellement actives en G1, où la HR n'est pas possible en raison de l'absence d'une chromatide soeur, la réparation peut être différée à la phase G2 où la HR est activée. D'autre part, il a été démontré que la présence de marques d'histones corrélées avec de la chromatine répressives, telles que la mono- et diméthylation de la lysine 20 de l'histone H4 (H4K20me1 et H4K20me2), favorise le choix de l'assemblage non homologue des extrémités (Non Homologous End Joining soit NHEJ en anglais) comme voie de réparation principale. Au sein de notre laboratoires, il a été montrer que dans les domaines associés aux lamines (Lamins Associated Domains soit LADs en anglais) ou la chromatine fortement réprimées, la réparation par HR est inhibée favorisant l'utilisation du mécanisme alternatif de jonction des extrémités (alternativ End Joining soit alt-EJ an anglais) potentiellement délétère. Il a également été observé en laboratoire que dans l'hétérochromatine péricentrique, le mécanisme de HR est inhibé car il est potentiellement délétère, lors de translocations de séquences pouvant conduire à des ruptures de chromosomes. La réparation de la DSB dans l'hétérochromatine, surtout constitutive, est beaucoup mieux étudiée que dans l'euchromatine ou l'hétérochromatine facultative. Il a été démontré que l'euchromatine est plus sensible aux dommages causés par l'ADN et qu'elle favorise généralement les RH, mais dans l'ensemble, elle nécessite des études plus détaillées.

L'un des types de chromatine, relativement abondant dans les cellules souches, qui n'a encore jamais été étudié dans le domaine de la réparation de l'ADN est la chromatine dite bivalente. Domaines bivalents est

un nom pour les tronçons de chromatine marqués à la fois par des modifications d'histones permissives et répressives. Les premières observations de domaines bivalents concernent la combinaison des marques d'histone H3K4me3 et H3K27me3. Par la suite, les marques H3K4me3 et H3K9me3 ou H3K36me2/3 et H3K9me3 contenant des domaines bivalents furent trouvés respectivement dans des cellules souches mésenchymateuses humaines et des cellules préadipocytes et HEK293. Pendant quelques années après leur découverte, ces marques ont été considérées comme un artefact de culture cellulaire par de nombreux scientifiques. Cependant, au cours des dernières années, ils ont été observés in vivo par plusieurs groupes, à la fois pendant le développement normal et dans lors de cancer.

Dans notre laboratoire, nous étudions comment le contexte chromatinien affecte la régulation des mécanismes de réparation des DSB. Dans mon projet, j'ai étudiée l'influence des caractéristiques spécifiques de la chromatine bivalente au sein de cellules souches de souris (Embryonic Stem Cell soit ESC en anglais) sur le processus de réparation de l'ADN.

Au cours de ma thèse, j'ai essayé de répondre aux questions telles que : les régions bivalentes représentent elles un type de chromatine distinct en termes de réparation de l'ADN ? Le rôle de la structure de la chromatine lors de la réparation des mêmes loci est-il important au cours de la différenciation cellulaire ? Il y a-t-il des caractéristiques spécifiques aux ESC lors de la réparation de l'ADN dans le contexte de la chromatine ?

Le but de mon projet était d'étudier la cinétique et les mécanismes de réparation du DSB et sa relation avec le contexte chromatinien dans lequel des DSB sont induites. À cette fin, j'ai utilisé le systeme CRISPR-Cas9 pour induire des DSB dans divers contextes chromatiniens dans des ESC ou des cellules différenciées. J'ai généré des lignées cellulaires mES stables exprimant une protéine Cas9 fusionnées à une GFP et à un domaine dégron (DD). En présence de la drogue appelée Shield1, le domaine dégron est masqué, stabilisant l'expression de la protéine Cas9 dans les cellules. J'ai observé que entre 8 et 10h après de la drogue shield, la protéine Cas9GFP atteint son niveau maximum d'expression. De plus, 10h après le retrait de la drogue shield1, le niveau d'expression de la protéine Cas9GFP retombe à un niveau basal.

Pour induire des DSB dans les différents contextes chromatiniens, j'utilise des plasmides ou des ARN guides transcrits in vitro qui ciblent des sites génomiques comportant différentes modifications chromatiniennes. Nous avons choisi d'étudier des domaines bivalents qui n'ont jamais été étudiés

auparavant en termes de réparation de l'ADN. Ces derniers sont souvent observés au niveau des régulateurs du développement et sont impliqués dans la différenciation rapide des cellules ESC. Ainsi, nous avons choisi d'induire des DSB sur plusieurs gènes bivalemment marqués. Ces gènes (Pax6, Zic1 et Ngn2) sont exprimés à des niveaux très faibles dans les ESCs à des niveaux élevés dans certains types de cellules différenciées et régulent le développement neuronal. Ces loci ont été comparés à des gènes considérés comme des marqueurs de pluripotence (Nanog, Pou5f1, Tfcp2l1, Zfp42) qui s'expriment fortement dans les mESCs et sont éteint pendant la différenciation. Nous avons également choisi des gènes domestiques (Actb, Gapdh) qui sont exprimés en permanence, et des gènes que l'on rapporte comme totalement réprimés en présence des marques d'histones H3K27me3 dans les cellules ES (Hoxb1, Tdrd1, Mc4r). Nous avons également cherché à inclure dans la comparaison certains gènes appartenant aux LADs dans les cellules ES (Sox6, Ptn, Nrp1).

À cette fin, nous avons conçu deux guides d'ARN par gène pour introduire les DSB dans leurs promoteurs. Nous avons utilisé la lignée cellulaire « Cas9-expressing NIH 3T3 » précédemment établie en laboratoire pour obtenir une comparaison avec un type cellulaire différencié.

Pour mesurer efficacement la NHEJ ainsi que la fidélité de réparation des cassures induites dans les contextes chromatiniens décrits ci-dessus dans les cellules mESCs et NIH3T3, nous avons utilisé une méthode appelée TIDE (Tracking of Indels by Decomposition). TIDE est une méthode basée sur le séquençage qui nous permet de quantifier un pourcentage de séquences mal réparées dans la population, ainsi qu'un pourcentage d'insertion ou de suppression de séquences particulières. Les résultats de l'étude TIDE ont montré que les ESC utilisent la NHEJ ou d'autres voies sujettes aux erreurs plus que prévu dans la littérature. Fait intéressant, nous avons également observé que la fréquence relative des réparations erronées varie selon le type de gènes où la cassure a été introduite. Les ESC utilisent moins les voies de réparation sujettes aux erreurs que les cellules 3T3 pour les gènes de ménage et les régulateurs du développement. Cependant, nous avons pu observer l'inverse pour les gènes marqueurs de pluripotence, suggèrant que la structure chromatinienne ainsi que les niveaux de transcription influencent tous les deux l'efficacité de TIDE. Ce résultat s'explique par la possibilité qu'en perturbant un promoteur d'un régulateur de

pluripotence, on interfère avec le cycle cellulaire, augmentant la proportion de cellules dans la phase G1. Les conséquences de cette perturbation pourrait être une augmentation de l'utilisation de la NHEJ, Cependant nous avons pu écarter cette hypothèse en démontrant qu'il n'y a aucun changement dans les profils de cycle cellulaire des ESCs dans toutes les conditions.

Nous avons également remarqué que bien que l'efficacité globale de TIDE dépend du type de cellule, le schéma des insertions et des suppressions (indels) est assez similaire entre les cellules ESC et 3T3 et dépend des ARN guides utilisé pour cibler la protéine Cas9. Comme les délétions inférieures à 10nt sont considérées comme des produits de NHEJ et de plus de 10nt de AltEJ, nos résultats suggèrent que l'équilibre NHEJ/AltEJ ne dépend pas directement de la structure chromatiniennes ou du type cellulaire.

Malheureusement, la méthode TIDE ne nous a pas permis de distinguer l'ADN non coupé par Cas9 ou réparé à l'aide de HR. Pour mesurer l'efficacité des RH, nous avons dû modifier la méthode en tirant parti de la sensibilité de TIDE pour mesurer l'efficacité des petites insertions après une induction de DSB. À cette fin, pour chaque ARN guide utilisés, nous avons conçu une séquence donneuse pour la HR spécifique au locus ciblé contenant une homologie de 1000bp entourant une séquence ADN unique de 9bp. Les ESC et les cellules 3T3 qui expriment de façon stable Cas9 ont été co-transfectées avec un guide ARN et la séquence homologue donneuse. Ensuite, le locus a été amplifié par PCR et soumis à une analyse TIDE. La fréquence d'insertion de 9 pb à chaque emplacement génomique indiquait la fréquence des HR. En utilisant la méthode TIDE ainsi modifiée (que nous avons appelée HR-TIDE), nous avons pu obtenir une comparaison de l'efficacité HR entre différents types de chromatine, ainsi qu'entre les cellules 3T3 et ES. Nous avons pu constater que la fréquence HR dans les cellules 3T3 était généralement faible et ne dépassait jamais 20 %. Nous avons pu observer une plus grande variabilité dans les cellules ES, allant d'aussi peu que 3 % chez certains inactifs jusqu'à 40 % chez les gènes actifs. En général, en utilisant HR-TIDE, nous n'avons pas pu confirmer les rapports précédents selon lesquels 80 % des ORD sont réparés à l'aide de HR, bien qu'ils apportent une plus grande contribution que dans les cellules différenciées, ce qui correspond aux idées actuelles sur le terrain.

En comparant l'efficacité de la HR entre différents contextes chromatiniens, nous avons pu confirmer l'observation que les régions de chromatine activent transcriptionnellement sont plutôt promotrice du m, alors que l'hétérochromatine facultative représente un environnement répressif. Nous avons également

observé que les domaines bivalents présentent des niveaux intermédiaires de HR, soutenant l'idée qu'ils représentent un état intermédiaire entre la chromatine active et la chromatine refoulée. En ce qui concerne les gènes situés dans les LAD, nous avons observé une certaine variabilité, certains gènes étant totalement réprimés et d'autres présentant une compétence plus élevée en matière de ressources humaines, ce qui pourrait s'expliquer par leur emplacement à l'intérieur ou à la limite de domaines.

Voyant des différences dans la fréquence des HR, nous avons réalisé que le cycle cellulaire diffère entre les cellules ES et 3T3, la première ayant un G1 beaucoup plus court et donc un plus grand pourcentage de cellules capables de ce mode de réparation. Pour répondre à cette question, nous avons d'abord décidé de bloquer les cellules 3T3 en phase G2 du cycle cellulaire à l'aide d'un inhibiteur RO. Conformément aux rapports précédents, nous avons pu constater une augmentation considérable du pourcentage des ressources humaines. Cependant, nous avions besoin d'une preuve complémentaire que l'extension de la phase G1 des cellules ES entraînerait une baisse de l'efficacité RH. Afin de modéliser une telle situation sans engager les cellules dans une démarche de différenciation, nous avons décidé d'utiliser un milieu de culture cellulaire appelé « 2i medium ». Le 2i pour la culture des cellules ES est exempt de sérum et comprend un cocktail de deux inhibiteurs (inhibiteur MEK et inhibiteur GSK-3, qui bloquent respectivement les voies de signalisation MEK/ERK et Wnt/-Catenin). Cependant, nous n'avons pas pu observer une baisse significative de l'efficacité des ressources humaines dans les gènes actifs, ce qui indique que les différences de cycle cellulaire entre les types cellulaires ne peuvent être à elles seules responsables des différences dans le choix d'une voie de réparation. Dans le même temps, nous avons remarqué une diminution de l'utilisation du mécanisme de HR dans les gènes inactifs, ce qui correspond au fait que la bivalence est perdue dans les conditions 2i.

Dans l'ensemble, nous avons établi un système inductible et dégradable pour évaluer la réparation des DSB et nous avons proposé plusieurs essais pour étudier le choix de la voie de réparation de l'ADN. Nos données ont mis en lumière le rôle de la chromatine bivalente et de l'hétérochromatine facultative dans le processus de choix de la voie de réparation de l'ADN. En même temps, nous avons proposé et optimisé une méthode simple et rapide d'accès à la compétence HR d'un lieu particulier dans un contexte cellulaire, qui peut être d'une utilité pratique dans la conception de mutants knock-in utilisant le système CRISPR-Cas9.

Comme perspectives d'avenir, plusieurs expériences peuvent être planifiées. En tirant parti du système Cas9 inductible et dégradable (DD/shield1), il est possible d'effectuer des expériences ChIP pour surveiller la cinétique d'apparition et de disparition de plusieurs facteurs de réparation de l'ADN et des voies de signalisation appelées DDR (Damage Response Repair) (H2AX et 53BP1) à différents moments après ajout et retrait de shield1. Comme ci-dessus, ces expériences doivent être effectuées dans des cellules ES et des cellules 3T3. Pour corréler l'induction et la réparation des DSB réelles avec l'augmentation de la DDR et l'arrêt de l'induction de ces DSB, la technique de LM-PCR, permettant de quantifier précisément le nombre de cassures ADN, doit être effectuée en même temps après l'ajout et le retrait du shield1 dans toutes cassures et types de cellules.

Cependant, pour obtenir une image plus large de l'influence de la chromatine sur le choix de la voie de réparation de l'ADN, il serait avantageux d'effectuer une expérience à plus grande échelle. L'optimisation de la conception de séquences homologue pour le HR-TIDE, d'une part, et la disponibilité de données sur l'efficacité de l'édition du génome de différents guides à l'échelle du génome, d'autre part, permettent de combiner ces deux approches. Pour ce faire, une bibliothèque contenant des ARN guides et des séquences homologues correspondantes pourrait être clonée dans des vecteurs viraux non intégratifs et utilisée pour l'infection de cellules ES ou cellules différenciées, suivit d'une analyse des profils de réparation à l'aide de techniques NGS.

Introduction

DNA damage and repair

DNA is constantly assaulted by various endogenous and exogenous damaging agents. Timely and faultless correction of acquired damage is necessary for the health and normal survival of an organism. Failure to properly repair mutilated DNA can lead to severe consequences such as mutagenesis, ageing, and cancer. A big variety of DNA damaging factors leads to different kinds of damage.

DNA damage

As I previously mentioned, DNA damage can be classified by its origin as endogenous and exogenous. Endogenous DNA damage is the one that our organism faces the most frequently, and it cannot be avoided. One of the major sources of endogenous damage is reactive oxygen species (ROS). They form as by-products of normal cellular processes such as cellular respiration and at low levels are involved in cellular homeostasis as messengers in redox signalling reactions (Friedberg et al., 2006). However, at higher concentrations, they can react with DNA bases causing damage by reacting with double bonds, methyl groups, or sugar residues (Chatterjee & Walker, 2017; Winterbourn, 2008). Influence of ROS species, such as electrophilic –OH radicals, lead to residues chemical modification, such as thymine glycol residue generation of formamidopyrimidine formation (Chatterjee & Walker, 2017; Friedberg et al., 2006) or 8-oxo-guanine formation (Chatterjee & Walker, 2017; Kasai & Mishimura, 1983). ROS can also break the DNA backbone and induce single-strand break (SSB) formation (Chatterjee & Walker, 2017; Henner et al., 1983).

Another common endogenous cause of DNA damage is DNA replication. It can lead to base mismatches due to replicative polymerase errors (which happens at rates between 10^{-6} and 10^{-8} per cell per generation) (Chatterjee & Walker, 2017; T.A. Kunkel, 2009; Thomas A. Kunkel, 2004), or replication fork stalling or collapse (Chatterjee & Walker, 2017; Viguera et al., 2001), which can lead to double-strand break (DSB) formation. DNA can also be mutilated by various topoisomerase enzymes that act to remove superhelical tension or other inappropriate DNA structures by introducing nicks or DSBs (Chatterjee & Walker, 2017; Pommier et al., 2006; J. C. Wang, 2002).

Finally, spontaneous base deamination and DNA methylation (or rather, removal of methylated DNA bases) can threaten genome integrity and need mechanisms for correction (Chatterjee & Walker, 2017; T. Lindahl & Barnes, 2000; Tomas Lindahl, 1993; Yonekura et al., 2009).

Exogenous DNA damaging agents can be more or less commonly encountered, and it is not possible to completely avoid their influence during the lifespan. Perhaps the most abundant exogenous cause of DNA damage is ultraviolet (UV) radiation. UV radiation is capable of affecting biological molecules in two ways: by direct absorption and by energy transfer. In the case of absorption, energy received by a molecule can cause photochemical alterations. Otherwise, UV energy is absorbed by molecules called photosensitizers and then transferred to nearby molecules. Both ways could lead to DNA damage (Chatterjee & Walker, 2017). One of the main outcomes is a covalent link formation between two adjacent pyrimidines (so-called bulky dimers), primarily cyclobutane pyrimidine dimers and pyrimidine-pyrimidone (6-4) photoproducts (Chatterjee & Walker, 2017; Davies, 1995). Another possible outcome of UV exposure is DNA-protein crosslinks and SSBs (Chatterjee & Walker, 2017; Friedberg et al., 2006).

Ionizing radiation of various kinds, alpha, beta, gamma, neutrons or X-rays, is also abundant in the environment, and can both direct (SSB occurrence) and indirect (by ROS production such as water radiolysis) DNA damage (Chatterjee & Walker, 2017; Desouky et al., 2015; Friedberg et al., 2006). SSBs caused by ionizing radiation (IR) have are unique as they tend to have 3' phosphate or 3'phosphoglycolate and not 3'-OH ends. Also, fragmented sugar derivatives can accumulate around break sites, additionally complicating the repair process. Such modified ends mutt be processed by endonucleases such as Apurinic-apirimidinic (AP) endonucleases, Polynucleotide kinase 3'-phospate (PNKP) or Tyrosyl-DNA phosphodiestherase 1 (TDP1) prior to repair (Chatterjee & Walker, 2017; El-Khamisy et al., 2007; Friedberg et al., 2006; Jilani et al., 1999; T. Zhou et al., 2005). IR can also cause DSBs by inducing multiple damage events close to each other at a short interval (Chatterjee & Walker, 2017; Hutchinson, 1985).

Food, tobacco smoke, industrial pollution, and byproducts from burning fuel can contain exogenous agents that are harmful to DNA while microorganisms and fungi can produce natural toxins. Together, these factors can lead to various DNA mutilations (Chatterjee & Walker, 2017). Exemplarily, alkylating agents present as by-products of tobacco smoke or organic material burning, as well as in food or medication, could result in adducted DNA base formation (Chatterjee & Walker, 2017; Friedberg et al., 2006; Singer & Kusmierek, 1982) and aromatic amines present in tobacco, colourants, fuels, etc. lead to base substitution and frameshift mutations (Chatterjee & Walker, 2017; Skipper et al., 2010). Additionally, environmental stress factors such as oxidative stress, hypoxia, heat or cold could result in DNA damage (Chatterjee & Walker, 2017; Gafter-Gvili et al., 2013; Kantidze et al., 2016; Luoto et al., 2013; Neutelings et al., 2013).

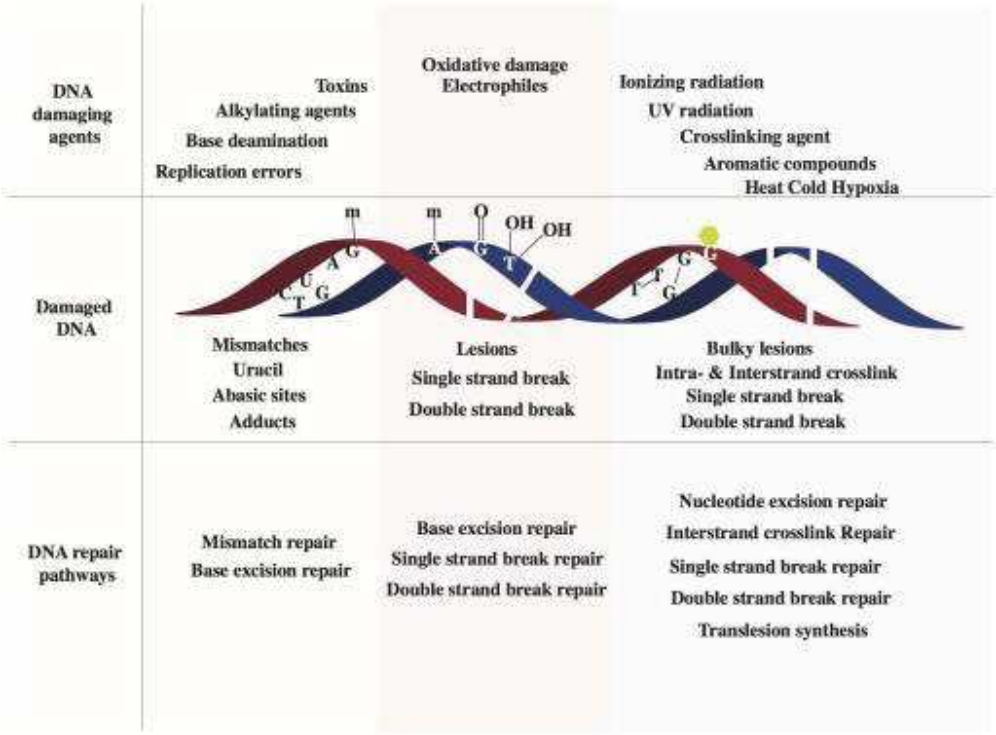

Fig. 1 Types of DNA lesions, their sources and repair pathways. Adapted from (Chatterjee & Walker, 2017)

DNA repair

Subsequently, multiple repair mechanisms are required to combat the variety and frequency of genomic lesions.

Reversal of DNA damage in an error-free way is possible with UV photolesions by non-enzymatic light-induced photoreversal and with alkylated bases by specialized enzymes. These enzymes belong to two families: O^6-alkylguanine-DNA alkyltransferase that repairs O-alkylated and AlkB-related α-ketoglutarate-dependent dioxygenases that repair N-alkylated DNA lesions (Chatterjee & Walker, 2017; Friedberg et al., 2006).

Base excision repair (BER) pathway is used to correct lesions that do not cause large distortion of DNA helix such as abasic sites and some base modifications. It begins with removing a modified base by DNA glycosylases to create an abasic site by cleaving N-glycosylic bond linking the base to a sugar phosphate backbone and proceeds with the cleavage of this apurinic-apyrimidinic (AP) site by an AP nuclease that generates an SSB by cleaving the phosphodiester bond 5’ to it. The created SSB is then repaired with either

the short-patch or long-patch repair pathway. An abasic site gets removed and the gap is filled by DNA polymerase beta (Polβ) and is followed by DNA ligase 1 (LIG1) -mediated ligation. BER is predominantly used in the G1 phase of the cell cycle (Chatterjee & Walker, 2017; Dianov & Hübscher, 2013).

Nucleotide excision repair (NER) acts to repair bulky lesions and is divided into two mechanisms: global genome NER (GG-NER) and transcription-coupled NER (TC-NER). They differ in a recognition step: while in GG-NER a complex of XPC (Xeroderma Pigmentosum, complementation group C), RAD23b and CETN2 (Centrin2) scans genome for the presence of transient ssDNA caused by DNA helix unwinding at the place of damage (Chatterjee & Walker, 2017; Nishi et al., 2005), TC-NER is initiated by PolII and involves CSA and CSB proteins (Cockayne Syndrome proteins A and B) (Chatterjee & Walker, 2017; Friedberg et al., 2006; Marteijn et al., 2014). Following repair process is the same for both pathways and involves pre-incision complex (consisting of TFIIH (Transcription factor II H), XPA (Xeroderma Pigmentosum, complementation group A), RPA (Replication protein A), and XPG (Xeroderma Pigmentosum, complementation group G)) formation, XPF (Excision repair cross-complementing rodent repair deficiency, complementation group 4)-ERCC1 and XPC-induced cleavage, gap filling by Polδ,ε or κ, and ligation by Lig1 of XRCC1-Lig3 (Chatterjee & Walker, 2017; Friedberg et al., 2006).

Mismatch repair (MMR) is a post-replicative repair mechanism active in S and G2 phases of the cell cycle. It is used to correct replication errors and thus plays a role in replication fidelity and genome maintenance through generations (Chatterjee & Walker, 2017; T.A. Kunkel, 2009). It acts to correct mismatches that occur due to replication errors as well as insertion-deletion loops at repetitive regions(Chatterjee & Walker, 2017; Friedberg et al., 2006). Various MSH (MutS homolog) proteins act to recognise lesions and initiate Exonuclease 1 (EXO1)-mediated excision. Resulting gaps are processed by Polδ, RFC (Replication factor C), HMGB1 (high mobility group box 1 protein), and Lig1 (Chatterjee & Walker, 2017).

Interstrand crosslink (ICL) repair is required when a covalent bond is formed between bases of two complementary strands. It is mediated by FA (Fanconi anemia) proteins. FA family contains 21 functional complementation groups, which are involved in ICL resistance (Chatterjee & Walker, 2017; Clauson et al., 2013). Damage sites are recognised by FANCM protein together with FAAP24 (FA associated protein of 24 kDA) and Forkhead box protein C2 (MFH) (Chatterjee & Walker, 2017; Ciccia et al., 2007; Clauson et al., 2013). MFH stimulates fork remodelling and FANCM is responsible for Holliday junction migration and

creation of ssDNA gaps (Chatterjee & Walker, 2017; Clauson et al., 2013; Gari et al., 2008; Huang et al., 2010). Presence of ssDNA results in RPA recruitment and ATR (Ataxia telangiectasia and Rad3-related protein) signalling activation. In the context of the FA pathway, it leads to FANCE, FANCD2, and FANCI activation by ATR target checkpoint kinase 1 (CHK1) as well as Mre11-Rad50-Nbs1 (MRN) complex assembly (discussed in details later) (Chatterjee & Walker, 2017; Clauson et al., 2013; Duquette et al., 2012; Smogorzewska et al., 2007; X. Wang et al., 2007). Other FA pathway core components get recruited to the lesion and stimulate the excision of the DNA strand of the lesion by structure-specific endonucleases (Chatterjee & Walker, 2017; Clauson et al., 2013). In replicating cells following repair is carried out by translesion synthesis polymerases Pol ι,κ,ν, and Rev1-like terminal deoxycytidyl transferase (REV1). These polymerases are capable of carrying synthesis through aberrant DNA fragments, although with lower fidelity (Chatterjee & Walker, 2017; Clauson et al., 2013; Minko et al., 2008; Räschle et al., 2008; Yamanaka et al., 2010). In non-replicating cells, it depends on both GG-NER and TC-NER pathways and TLS polymerases (Chatterjee & Walker, 2017; Clauson et al., 2013).

Translesion synthesis (TLS) is performed by a highly-conserved Y-family of DNA polymerases (consisting of Pol ι,κ,ν, and REV1) or some polymerases belonging to other families (B, X or A), such as Pol θ,μ,λ, or ζ. These polymerases are capable of carrying replication through DNA lesions but have considerably lower fidelity as damaged bases often provide a misleading template (Chatterjee & Walker, 2017; Sale, 2013). It has been shown that despite in some cases it is possible for a single polymerase to bypass a lesion (Johnson et al., 1999; Sale, 2013) the bypass might also involve cooperation of different polymerases (Sale, 2013; Shachar et al., 2009). Two models have been proposed for this phenomenon. The first one, the polymerase switch model, suggests that TLS polymerases come sequentially in a two-step process, where an inserter enzyme (usually Pol η, ι, or κ) incorporates a nucleotide at the place of the DNA lesion and then is replaced by an extender enzyme (Pol ζ) (Chatterjee & Walker, 2017; Korzhnev & Hadden, 2016; Washington et al., 2002). The second one, the gap-filling model, implies that ssDNA stretches are left by replicative polymerases and are subsequently filled by TLS polymerases (Chatterjee & Walker, 2017; Quinet et al., 2016; Sale et al., 2009). As translesion synthesis is a highly mutagenic process it must be tightly regulated. In mammalian cells it is achieved by concentrating them in replication factories (Sabbioneda et al., 2008; Sale, 2013). As previously mentioned, TLS polymerases are also known to play a role in other repair

pathways including NER, BER, and FA pathway, which further emphasises the importance of this phenomenon (Chatterjee & Walker, 2017).

SSBs are repaired via three different pathways. First is the long patch single strand break repair (SSBR) pathway where SSBs are detected by poly (ADP-ribose) polymerase 1 (PARP1) which is poly(ADP)-ribosylated and quickly dissociates (Chatterjee & Walker, 2017; D'Amours et al., 1999), and the ends are further processed by APE1 (apurinic-apyrimidinic endonuclease 1), PNKP (polynucleotide kinase 3'-phosphate) and APTX (aprataxin). Subsequently, Flap endonuclease 1 (FEN1) removes the mutilated 5' end and the resulting ssDNA gap is filled by Polβ and Polδ/ε and ligated by Lig1. In the short patch SSBR pathways, breaks are recognised by APE1, and following steps converge with the long patch SSB pathway. Another particularity of this pathway is the fact that gap-filling is performed exclusively by Polβ and ligation by Lig3 (Chatterjee & Walker, 2017; McKinnon & Caldecott, 2007). And the third one, the DNA topoisomerase 1 (TOP1)-SSB pathway is a modification of the long-patch SSB repair where end processing is performed by the TDP1, which acts to remove TOP1 (Keith W. Caldecott, 2008; Chatterjee & Walker, 2017).

Double-strand breaks are considered to be among the most deleterious and toxic kinds of DNA lesions as in that case the second strand is not available as a repair template. Failure to repair them might lead to severe consequences such as cancer or ageing, and it is therefore absolutely essential for cells to mend them efficiently (Chatterjee & Walker, 2017; Mladenov et al., 2016; Thompson, 2012). Despite they pose a serious threat to a cell in particular and to an organism in general, DSBs are quite abundant. They could result from exposure to IR, UV, or small molecules (for example chemotherapy drugs), from environmental stresses such as hyperosmotic stress, hypoxia or heat shock, and from replication stress, or be created on purpose during lymphocyte maturation (V(D)J recombination of class-switch recombination) (Fillingham et al., 2006; Lamarche et al., 2010; Ohnishi et al., 2009). As my work was focusing on DSB repair, I will discuss mechanisms of their recognition and repair in more details in the next section.

DSB recognition and DNA damage response

The process of DSB repair can be conceptually divided into three sequential steps: DSB recognition and DNA damage response (DDR) signalling activation, repair pathway choice, and repair itself. We shall consider all of them one by one.

DSB sensing and DDR activation

Unlike other kinds of DNA lesions, DSB recognition is thought to be based on the altered chromatin structure rather than on the recognition of mutilated DNA by sensor proteins. Chromatin relaxation, an essential step for DSB repair, is promoted by certain covalent histone modifications, as well as by ATP-dependent chromatin remodelling allowing repair factors to assess the damage site (Murr et al., 2006; Thompson, 2012; van Attikum & Gasser, 2009). ATM (Ataxia telangiectasia mutated) activation seems to be uniformly seen as the initial step of DDR. However, the exact mechanism of this event remains controversial (Blackford & Jackson, 2017; Thompson, 2012). It has been proposed that it may result from changes in chromatin structure due to relieving topological constraints caused by supercoiling (Bakkenist & Kastan, 2003; Thompson, 2012).

It has been observed that in eukaryotes DDR proteins often accumulate in conglomerates called ionizing radiation-induced foci (IRIF) as they were first observed in cells treated with IR. IRIFs are considered to be an indication of ongoing repair of one or more DSB (Bekker-Jensen et al., 2006; Carney et al., 1998; Fernandez-Capetillo et al., 2003; van Attikum & Gasser, 2009; Vignard et al., 2013). Among others, components of MRN (MRE11-RAD50-NBS1) complex appear to accumulate at IRIFs (Fernandez-Capetillo et al., 2003; van Attikum & Gasser, 2009).

It is widely accepted that in higher eukaryotic cells DSBs are sensed by MRN complex (Ji Hoon Lee & Paull, 2005; Ohnishi et al., 2009; Thompson, 2012; van Attikum & Gasser, 2009). Despite influencing the repair pathway choice at later stages by favouring one of the pathways, homologous recombination (HR), it has been shown that MRN complex is involved at the earliest steps of DSB recognition and is essential for DDR activation in all repair pathways. It binds to free DNA ends at the break site and promotes ATM activation (Blackford & Jackson, 2017; Carney et al., 1998; Dupré et al., 2006; Ji Hoon Lee & Paull, 2004; Lou et al., 2006; Stucki et al., 2005; van Attikum & Gasser, 2009). Along with two other phosphoinositide 3-kinase (PI3K)-related kinases (PIKKs), ATR (ATM and Rad3-related kinase), and DNA-PKcs (DNA-dependent protein kinase catalytic subunit), ATM plays a major role in DSB repair (Blackford & Jackson, 2017).

In an inactive form, ATM exists as a catalytically inactive homodimer (Bakkenist & Kastan, 2003). Its activity is modulated by post-translational modifications. Autophosphorylation at S1981 and acetylation at K3016 acetylation by Tip60 (60 kDa Tat-interactive protein) have been shown to lead to its dissociation into active monomers (Bakkenist & Kastan, 2003; Dupré et al., 2006; Sun et al., 2005, 2007; Thompson, 2012). However, the role of ATM autophosphorylation is controversial, which will be discussed later. Ribosylation of ATM is required for its further activation and is performed by PARP1 in response to DNA damage. Impairing this process may lead to delays in phosphorylation of ATM targets and abolished ATM foci formation (Aguilar-Quesada et al., 2007; Haince et al., 2007; Núñez et al., 1998; Thompson, 2012). Thus, the whole range of reactions from DDR signal spreading and DSB repair to an induction of a cell cycle block is triggered by a pioneering sensory complex binding and ATM activation (fig. 2).

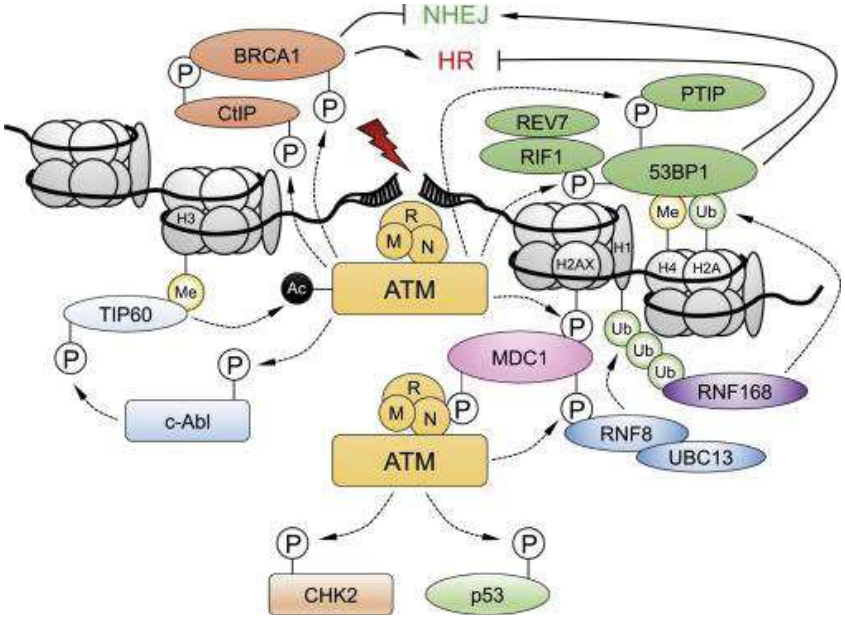

Fig. 2 Schematic representation of early steps of DDR activation. DSBs are sensed by MRN complex, which binds to free DNA ends and promotes ATM activation. This leads to multiple DNA repair proteins activation, including Mdc1, 53BP, CtIP and BRCA1 and promotes DNA repair. Intra-S and G2-M checkpoints and a cell cycle arrest are activated by phosphorylation of checkpoint kinases Chk1 and Chk2, and p53. Adapted from (Blackford & Jackson, 2017)

γH2AX and MDC1 foci formation

ATM, ATR, and DNA-PKcs phosphorylate and thus activate a number of repair and checkpoint proteins but one of its most important targets is the serine residue S139 of histone variant H2AX (Burma et al., 2001; Fillingham et al., 2006; Hanasoge & Ljungman, 2007; Hongyan Wang et al., 2005; Ward & Chen, 2001). The

roles of ATM and DNA-PKcs in phosphorylating H2AX are largely overlapping. However, there some aspects specific for each kinase (to be discussed later) (Stiff et al., 2004; Thompson, 2012). This phosphorylated form is called γH2AX and it is one of the main signalling hallmarks of a DSB, independently of the way of its induction and on whether it was caused by a hostile environment or induced in a controlled manner as a part of cell homeostasis (Fillingham et al., 2006; Hua Tang Chen et al., 2000; Nazarov et al., 2003; Petersen et al., 2001; Rogakou et al., 1998, 1999; Tomilin et al., 2001). In mammalian cells, it forms large domains, up to megabases size (Rogakou et al., 1998; van Attikum & Gasser, 2009). γH2AX foci arise quickly, within minutes after DNA damaging event has taken place, but continue to expand further for one to several hours according to different studies (Y. Lee et al., 2019; Löbrich et al., 2010; Sharma et al., 2012; Staszewski et al., 2008). Although H2AX-deficient mice are viable, they show increased genome instability and phenotypical abnormalities, underlining its importance for DNA repair (Celeste et al., 2002, 2003; Thompson, 2012; Weyemi et al., 2018). Another histone that is phosphorylated in the course of DDR signalling activation is H2B. H2B phosphorylation on S14 is induced by IR and progresses to form foci. They colocalise with those of γH2AX, although accumulating slower (Fernandez-Capetillo et al., 2004; Thompson, 2012). It has recently been shown that kinases responsible for this process are Mst1 and 2 (Bitra et al., 2017; Pefani et al., 2018).

As DDR progresses, γH2AX is bound by the Mediator of DNA damage checkpoint protein 1 (MDC1), which serves as a platform for a repair machinery assembly (Thompson, 2012). It directly interacts with multiple proteins involved in DSB repair, such as ATM, MRN complex, 53BP1, and Structural maintenance of chromosomes protein 1 (Smc1) (Stewart et al., 2003; Thompson, 2012). H2AX phosphorylation, as well as MDC1 recruitment, also acts as a signal for activation of intra-S and G2-M checkpoints and a cell cycle arrest, notably for phosphorylation of checkpoint kinases Chk1 and Chk2, KRAB-associated protein 1 (Kap1) and Smc1 (Celeste et al., 2003; Fernandez-Capetillo et al., 2002; Stewart et al., 2003; Thompson, 2012). It has been shown that phosphorylation and foci formation of H2AX and MDC1 is interdependent (Stewart et al., 2003; Thompson, 2012). Direct interaction of MDC1 and γH2AX has been proven by co-crystallization, and the relevance of this interaction has been demonstrated by analysing MDC1 mutant or null cells, which have impaired accumulation of other DDR factors including 53BP1, NBS1 (Nijmegen breakage syndrome protein 1), and phosphorylated ATM (Lou et al., 2006; Stucki et al., 2005; van Attikum & Gasser, 2009). Therefore, MDC1 can be considered an early DDR factor, important for both recruitment and retention of other players (Thompson, 2012; van Attikum & Gasser, 2009).

Role of parylation in DDR

Another important process involved in DDR is poly-ADP-ribosylation, or parylation, a post-translational modification crucial for DSB recognition. This modification is catalysed by PARP (Poly-ADP-Ribosyl Polymerase) enzymes that covalently link poly-ADP-ribosyl chains to protein substrates. PARP1 and PARP2 are involved in both SSB and DSB recognition, whereas PARP3 specifically responds to DSBs (Beck, Robert, et al., 2014; Boehler et al., 2011; Boulton et al., 1999; K. W. Caldecott, 2014; Rulten et al., 2011). PARP enzymes mediate DDR activation in several ways. First, some DDR factors have a PAR motif and are therefore recruited to damage sites in a PAR-dependent manner. Second, parylation of DDR and downstream repair factors might influence their catalytic activity or capability to bind to DNA or their interaction factors. In particular, parylation facilitates the recruitment of MRN complex and ATM kinase to a break site (Beck, Robert, et al., 2014; Bryant et al., 2009). Further on repair process, parylation plays a role in a pathway choice by promoting end resection, and therefore favouring homologous recombination and microhomology-mediated end joining (Beck, Boehler, et al., 2014; Langelier et al., 2014; Luijsterburg et al., 2016). However, PARP3 is involved in non-homologous end-joining by limiting end-resection as well as by promoting X-ray repair cross-complementing protein 1 (XRCC4)-Lig4 mediated ligation (Beck, Boehler, et al., 2014; K. W. Caldecott, 2014; Fenton et al., 2013; Rulten et al., 2011). Therefore, PARP enzymes play an important and complex role in DSB repair from the first to the last steps of this process.

Signal transduction and amplification. DDR kinases

As I mentioned before, break recognition leads to an activation of ATM, ATR, and DNA-PKcs kinases that all belong to the phosphatidylinositol-3-kinase family. These kinases mediate signal transduction and amplification via γH2AX foci formation and play a role in downstream DNA repair proteins activation.

ATM is the central kinase involved in DDR. It was identified in 1995 a single mutated gene responsible for a rare genetic disorder, ataxia telangiectasia occurrence (Blackford & Jackson, 2017; K. Savitsky et al., 1995). Ataxia telangiectasia is characterised by dilated blood vessels, progressive neurodegeneration, and immunodeficiency (Blackford & Jackson, 2017). The activation of ATM proceeds as dissociation of catalytically inactive homodimers into active monomers (Bakkenist & Kastan, 2003; Blackford & Jackson, 2017; Thompson, 2012). Among post-translational modifications that lead to its activation autophosphorylation at S1981 was the first to be discovered (Bakkenist & Kastan, 2003). However, its role remains controversial as on the one hand it has been reported to be the process initiated as the very first

step of DDR stimulated by chromatin changes upon the DSB occurrence, and on the other hand, it has been shown dispensable for ATM functioning (Bakkenist & Kastan, 2003; Blackford & Jackson, 2017; Kozlov et al., 2006; Pellegrini et al., 2006; Thompson, 2012). Another possible mechanism for early ATM activation is acetylation by Tip60, which is attracted to the break site by exposed H3K9me3 nucleosomes, on K3016 (Blackford & Jackson, 2017; Sun et al., 2005, 2007). It is certain, however, that whatever causes its initial activation, it is later enhanced by ATM direct interaction with the C-terminal domain of Nbs1, a component of MRN complex. This interaction is stabilised in the presence of free DNA ends (Blackford & Jackson, 2017; Falck et al., 2005). Nevertheless, MRN-independent mechanisms of ATM activation have also been shown in multiple studies (Blackford & Jackson, 2017; Guo et al., 2010; Olcina et al., 2013). Activated ATM phosphorylates a vast variety of targets, influencing the process of DSB repair at all levels: break recognition and DDR activation, DDR enhancement, repair pathway choice and stimulation, cell cycle checkpoint activation, and apoptosis. Its role in break recognition and DDR activation has already been discussed. Further on ATM promotes DDR by phosphorylating H2AX into γH2AX and MDC1, the major γH2AX reader, promoting its dimerization and stabilisation (Blackford & Jackson, 2017; Burma et al., 2001; Jungmichel et al., 2012; Jinping Liu et al., 2012; Thompson, 2012). MDC1 further recruits the MRN complex, thus promoting additional ATM accumulation and H2AX phosphorylation, thus providing a positive feedback loop for repair foci formation (Blackford & Jackson, 2017). Moreover, ATM has also been shown to be involved in chromatin relaxation and remodelling around the break (Goodarzi et al., 2008; Moyal et al., 2011; Ziv et al., 2006). ATM also promotes recruitment downstream repair proteins to the break site. It was shown to predominantly stimulate HR by phosphorylating CtBP-interacting protein (CtIP), a major MRN interactor that promotes end-resection (Blackford & Jackson, 2017; Sartori et al., 2007; Shibata et al., 2011). However, ATM is also able to stimulate DSB repair in G1 in a way that requires ATM, DNA-PK, MRN complex and Artemis activity and by promoting NHEJ due to its redundant functions with XRCC4-like factor (XLF) (Blackford & Jackson, 2017; Riballo et al., 2004; Zha et al., 2011). Additionally, it phosphorylates Chk2 and p53 causing a cell cycle block at G2/M transition (Banin et al., 1998; Blackford & Jackson, 2017; Matsumura et al., 2015; Siliciano et al., 1997).

The existence of DNA-PK was presupposed in 1985 when it was observed that the addition of double-stranded DNA (dsDNA) into *Xenopus* and *Arbacia* egg extracts promoted phosphorylation of several proteins and named DNA-dependent protein kinase (Blackford & Jackson, 2017; Walker et al., 1985). This

kinase was purified five years later (Blackford & Jackson, 2017; Carter et al., 1990; Jackson et al., 1990; Lees-Miller et al., 1990). DNA-PKcs is recruited to DNA damage sites by Ku70/Ku80 (Ku autoantigen protein p70/80 homolog) heterodimers and form DNA-PK complex that enhances the catalytic activity of the kinase (Blackford & Jackson, 2017; Gell & Jackson, 1999; Singleton et al., 1999). DNA-PKcs acts cooperatively with ATM to promote γH2AX signal spreading and DDR activation (Firsanov et al., 2011; Stiff et al., 2004; Hongyan Wang et al., 2005). It also plays a crucial role in NHEJ, and multiple target proteins have been suggested (Blackford & Jackson, 2017; Enriquez-Rios et al., 2017). Some prominent NHEJ players as Artemis, are among the confirmed targets (Blackford & Jackson, 2017; W. Jiang et al., 2015; Malu et al., 2012). While DNA-PKcs phosphorylation by ATM leads to end-processing by promoting Artemis recruitment, additional autophosphorylation is necessary for it to act on other targets. The exact target proteins are unknown but the importance of DNA-PKcs is affirmed by the fact that its knock-out is lethal in mice during embryonic development by E16.5 and on the cellular level leads to an inability to multiple NHEJ defects similar to XRCC4 and XLF deficient cells (Blackford & Jackson, 2017; W. Jiang et al., 2015). Particularly, it was shown to play an important role in regulating end-ligation by promoting the transition between two synaptic complexes (Blackford & Jackson, 2017; Graham et al., 2016; W. Jiang et al., 2015). At the same time, DNA-PKcs phosphorylates RPA and activates the S-phase cell cycle checkpoint (Ashley et al., 2014). Activated RPA mediates the recruitment of PALB2 (Partner and localizer of BRCA2), thus promoting DSB repair progression (Murphy et al., 2014). On the other hand, it has also been shown that Chk1 promotes DNA-PK activity in order to stimulate fast repair by NHEJ and prevent cell cycle block (Goudelock et al., 2003; Shimura et al., 2007).

ATR is an analogue of the yeast protein Mec1 (Mitosis entry checkpoint 1) that was first discovered as Rad3 in a radiosensitivity screen and later as being essential for cell cycle checkpoints (Blackford & Jackson, 2017; Nasim & Smith, 1975; Weinert et al., 1994). A human ATR protein was cloned independently by two different groups (Bentley et al., 1996; Blackford & Jackson, 2017; Cimprich et al., 1996). ATR kinase plays a crucial role in replication stress response. It gets recruited ssDNA in response to various kinds of genotoxic stress. Therefore, it accumulates at DSB sites after resection but also at stalled replication forks. Interaction of ATR with RPA allows an ATRIP (ATR-interacting protein) cofactor recruitment and binding to ATR (Blackford & Jackson, 2017; Zou & Elledge, 2003). ATR-ATRIP heterodimer formation is essential for ATR activation. ATR recruitment alone is not sufficient for the optimal response and requires cofactors such as

TopBP1 (DNA topoisomerase 2-binding protein 1) or ETAA1 (Ewing's tumor-associated antigen 1) (Bass et al., 2016; Blackford & Jackson, 2017; S. Feng et al., 2016; Haahr et al., 2016; Kumagai et al., 2006; Mordes et al., 2008). Activated ATR promotes γH2AX signal spreading and leads to Chk1 phosphorylation and activation, which also causes cell cycle block in the G2/M phase (Blackford & Jackson, 2017; Hui & Helen, 2001; Q. Liu et al., 2000). ATR has also some unique targets related to replication stress management, including FANCI protein, which promotes dormant origin firing and FANCD2 mono-ubiquitylation and therefore FA pathway progression (Andreassen et al., 2004; Blackford & Jackson, 2017; Y. H. Chen et al., 2015; Ishiai et al., 2008).

In G1/S checkpoint is thought to be mainly regulated by ATM, whereas intra-S and G2/M are controlled by both ATM and ATR cooperatively (Adams et al., 2006; Blackford & Jackson, 2017; Cuadrado et al., 2006; Jazayeri et al., 2006; Myers & Cortez, 2006). All PIKKs are strictly regulated by cofactors required for DNA binding: Nbs1, ATRIP, and Ku80 for ATM, ATR, and DNA-PKcs respectively. Therefore their appropriate involvement is achieved throughout the cell cycle and three DNA repair PIKKs are acting cooperatively in the process of DSB repair (fig. 3).

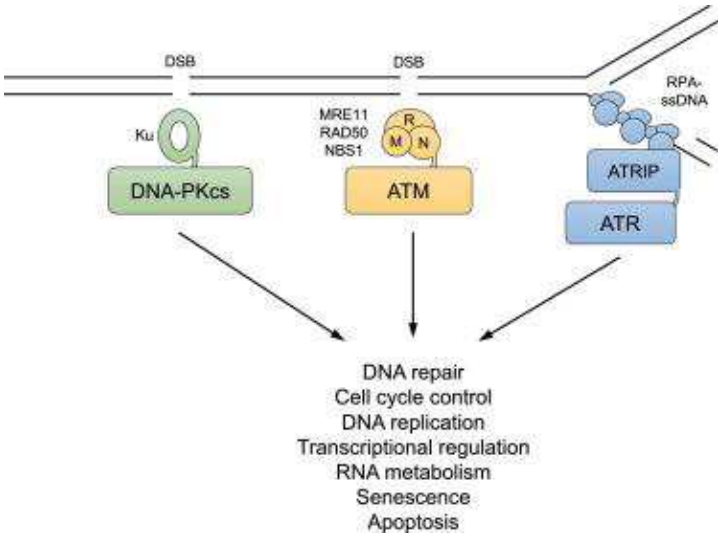

Fig. 3 Roles of PI3KKs in the maintenance of genome stability. Adapted from (Blackford & Jackson, 2017)

Role of ubiquitylation and SUMOylation in DSB repair

Histone ubiquitylation and SUMOylation (Small ubiquitin-like modifier) play a prominent role in DRR and repair progression. As it was mentioned before, γH2AX recruits MDC1, which plays a role in both signal spreading by creating a positive feedback loop with ATM and DNA repair progression by serving as a

platform for downstream repair complexes assembly. Multiple ubiquitylation steps are performed to ensure this process. As a first step, H2AX acetylation enabled by Tip60 facilitated H2AX monoubiquitylation at K118/119. Not all players involved in this process are known to date but Ubc13 (ubiquitin-conjugating enzyme 13) E2 ligase have been shown to play a role in it (Ikura et al., 2007; X. Jiang et al., 2010; Thompson, 2012; G. Y. Zhao et al., 2007).

Also, PRC1 (Polycomp repressive complex 1) gets recruited to DSBs and its subunits, Rnf2 (RING finger protein 2) and Bmi1 (B cell-specific Moloney murine leukemia virus integration site 1), have been shown to mediate the monoubiquitylation of H2A and H2AX on K119 (Ismail et al., 2010; Thompson, 2012). This modification plays a role in the recruitment of DDR proteins and IRIFs formation. RNF2/BMI1 complex controls DDR in an ATM-dependent manner and their knockdown leads to increased sensitivity to IR and compromised DSB repair (Facchino et al., 2010; Pan et al., 2011; Thompson, 2012).

The best-established E3 ligases involved in DSB repair are RNF8 (RING finger protein 8), CHFR (checkpoint protein with FHA and RING domain), and RNF168 (RING finger protein 168). All three of them use Ubc13 an E2 ligase (Panier & Durocher, 2009; Thompson, 2012). RNF8 recruitment is facilitated by MDC1 (Huen et al., 2007; Mailand et al., 2007; Thompson, 2012). RNF8 ubiquitylates histones H1 and H2A, H2AX, and H2B to promote binding of repair factors such as 53BP1 (TP53-binding protein1), RAP80 (Receptor-associated protein 80) and BRCA1 (Breast cancer type 1 susceptibility protein) (Huen et al., 2007; Mandemaker et al., 2017; Sobhian et al., 2007; Thompson, 2012).

Another E3-ligase, RNF168, is recruited in an RNF8-dependent manner to enhance the signal by ubiquitylation of H1 and H2A histones (Mandemaker et al., 2017; Thompson, 2012). Upon irradiation, RNF168 gets stabilized by HERC2 (HECT domain and RCC1-like domain-containing protein 2) (Bekker-Jensen & Mailand, 2010; Thompson, 2012). It colocalizes with γH2AX at damage sites and promotes amplification of the ubiquitylation established by RNF8 and PRC1 complex (Doil et al., 2009; Thompson, 2012).

Finally, CHFR (checkpoint protein with FHA and RING domain) E3 ligase, which has considerable structural similarity to RNF8, has been shown to act synergistically with it in promoting ATM activation and cell cycle checkpoints activation (Bothos et al., 2003; Thompson, 2012; J. Wu et al., 2011).

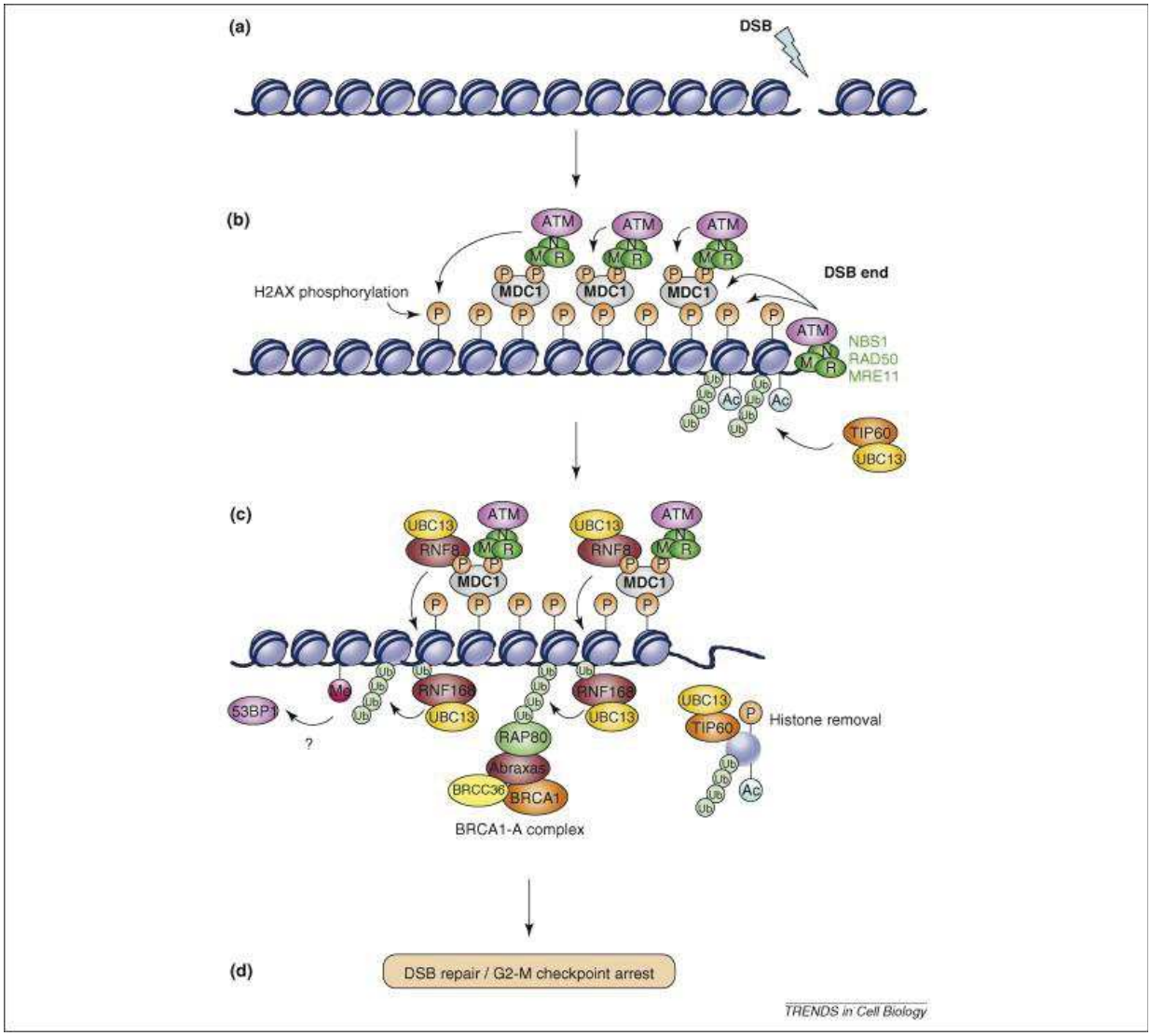

Fig. 3 Polyubiquitylation in the process of DSB repair. Adapted from (van Attikum & Gasser, 2009).

Ubiquitination-dependent DDR promotion is limited by deubiquitylation enzymes. One of the enzymes involved in this process is BRCC36 (BRCA-containing complex 36), a member of the RAP80-ABRA1-BRCA1-BARD1-BRCC36 complex (Ng et al., 2016; Thompson, 2012; B. Wang & Elledge, 2007), Other prominent players such as USP3, USP11 and USP16 (ubiquitin-specific proteases 3, 11 and 16) help to regulate ubiquitylation-mediated signalling and fine-tune the extent of DDR activation (Nicassio et al., 2007; Thompson, 2012; Wiltshire et al., 2010; Z. Zhang et al., 2014).

DDR activation consequences

The presence of mutilated DNA regions is unfavourable in the course of a cell cycle progression. Therefore, cell cycle arrest mechanisms, known as cell cycle checkpoints have developed in cells. Such precautions ensure that existing DNA damage is repaired before the transition to the next stage. However, if DNA damage is too profound or impossible to fix, cells undergo senescence or apoptosis.

Cell cycle arrest is one of the main and immediate DDR activation consequences. In the course of a cell cycle that constitutes from post-mitotic gap (G1) DNA replication (S), post-growth (G2), and mitotic (M) phases, all transitions are tightly orchestrated by cyclins and cyclin-dependent kinases (Cdks) (Schäfer, 1998). Cell cycle checkpoints are signaling pathways that are intended to sense DNA damage or other mistakes such as erroneous chromosome segregation and delay cell cycle progression to facilitate the repair or eliminate cells that are beyond it (Bartek & Lukas, 2003; B. S. Zhou & Elledge, 2000). There are three checkpoints. G1/S checkpoint is activated after ATM-dependent Chk2 phosphorylation (Bartek & Lukas, 2003; Falck et al., 2001; Matsuoka et al., 1998). ATM and Chk2 in turn mediate Cdc25A (M-phase inducer phosphatase 1) phosphorylation and sequential degradation which promotes p53 stabilisation followed by p21 expression and leads to Cdk inhibition and cell cycle progression abrogation (Bartek & Lukas, 2003; D'Adda Di Fagagna, 2008; Falck et al., 2001). Intra-S checkpoint is activated by ATR-dependent Chk1 phosphorylation (Bartek & Lukas, 2003; Hui & Helen, 2001). It results in inhibition of origin firing and therefore slowing down the replication process in unperturbed conditions to avoid replication stress (Ge & Blow, 2010; Moiseeva et al., 2019; Sørensen et al., 2003). Activation of G2/M checkpoint relies on ATM- and ATR-dependent phosphorylation for Chk2 and Chk1 (Ahn et al., 2000; Bahassi et al., 2006; Bartek & Lukas, 2003; Gatei et al., 2003; Z. Xiao et al., 2003). Activated Chk1 blocks Cdc2/CyclinB activation through targeting Cdc25C to prevent mitosis (Stanford & Ruderman, 2005).

It has been shown that too prolonged checkpoint activation is perceived as unrepairable damage by a cell. One of the proteins activated by ATM is p53, which has a dual role in balancing between repair and apoptosis depending on the degree and continuance of activation (D'Adda Di Fagagna, 2008).

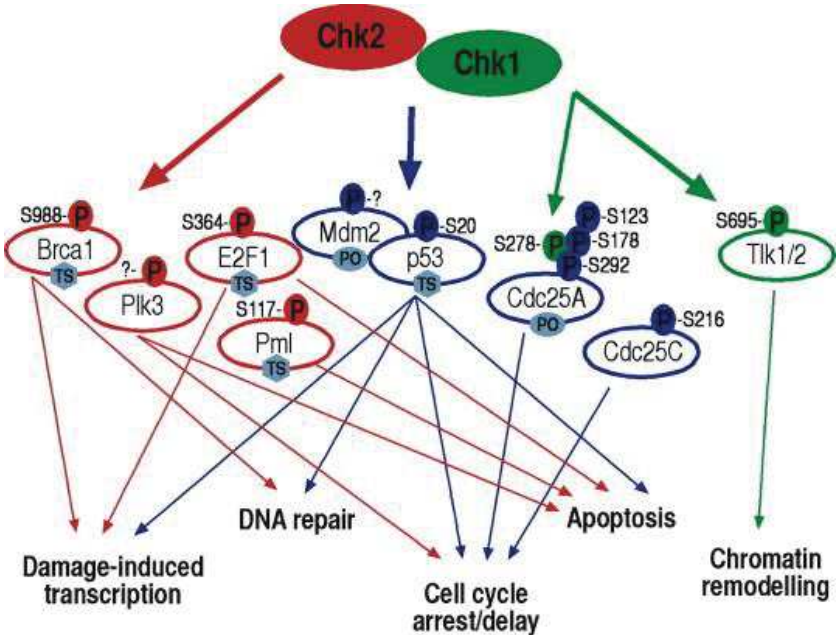

Fig. 4 Consequences of DNA damage-induced Chk1 and Chk2 mediated cell cycle checkpoint activation. Together, they phosphorilate multiple targets, including Cdc25Aand Cdc25C, resulting in DNA repair activation, and finally cell cycle arrest or apoptosis. Adapted from (Bartek & Lukas, 2003).

Double-strand break repair pathways

As discussed above, DNA damage and particularly DSBs can represent a considerable danger for a cell, and therefore repair should be guaranteed, so several pathways have been developed in mammalian cells to ensure it. After a break recognition and DDR activation one of either two major pathways, non-homologous end joining (NHEJ), homologous recombination (HR), or two minor pathways, microhomology-mediated end joining (MMEJ), also sometimes referred to as alternative end joining (Alt-EJ), or single-strand annealing (SSA) can be employed (Sallmyr & Tomkinson, 2018; Scully et al., 2019).

Non-homologous end joining

Non-homologous end joining, which is also called classical non-homologous end-joining (cNHEJ) in contrast to alternative end-joining (Alt-EJ), is considered to be a predominantly used pathway in higher eukaryotes (Ferguson et al., 2000; Karanjawala et al., 1999; Scully et al., 2019). It has been reported that approximately 80% of DSBs are repaired using this mechanism (Tichy et al., 2010). NHEJ is initiated by Ku70/Ku80 heterodimer binding to free DNA ends (Britton et al., 2013; Scully et al., 2019). Ku proteins are abundant in

the nucleus and have a high affinity to free DNA ends which allows them to bind to a break site within 5 seconds after its occurrence (Fell & Schild-Poulter, 2015; Mari et al., 2006). It serves a dual role of, on the one hand, protecting DNA ends from degradation and keeping them together for further repair, and on the other hand, preventing resection (Fell & Schild-Poulter, 2015; Mimitou & Keeney, 2018). It also serves as a platform for DNA repair machinery assembly (Costantini et al., 2007; Fell & Schild-Poulter, 2015; Hsu et al., 2002; Rivera-Calzada et al., 2007; Yano et al., 2008). Another important function of Ku70/80 heterodimer is in DDR regulation by modulating ATM activity and in preventing apoptosis by binding and inhibiting a pro-apoptotic factor Bax (Amsel et al., 2008; Fell & Schild-Poulter, 2015; Tomimatsu et al., 2007; X. Y. Zhou et al., 2002). DNA-PKcs binds to Ku70/80 heterodimer to form an active DNA-PK complex. DNA-PKcs mediates tethering of broken ends (Fell & Schild-Poulter, 2015; Hammel et al., 2010). DNA-PK autophosphorylates its own subunits and phosphorylates several other target molecules including Artemis, PNKP, XRCC4, XLF, and DNA ligase IV (Fell & Schild-Poulter, 2015; Goodarzi et al., 2006; Hammel et al., 2010; Y. G. Wang et al., 2004; Y. Yu et al., 2003, 2008). In case end-processing is required before ligation it can be performed by various enzymes including PNKP (polynucleotide kinase/phosphatase), Artemis, Exo1, Tdp1, and others in order to remove 3' phosphate ends, 3'-phosphoglycolates, or 5'-hydroxyl groups and generate 5' phosphate ends required for further ligation (Fell & Schild-Poulter, 2015; Mahaney et al., 2009). The next step is ligation, which is performed by a ligase complex of DNA Ligase IV with XRCC4 and XLF, co-factors required for its stabilisation and stimulation (Ahnesorg et al., 2006; Fell & Schild-Poulter, 2015; Grawunder et al., 1997; Nick McElhinny et al., 2000). However, it has recently been shown that a small subset of breaks undergoes resection-dependent NHEJ repair enabled by Artemis and CtIP cooperation and resection performed by MRE11 (Meiotic recombination 11 homolog 1) and EXO1 (Biehs et al., 2017; Shibata et al., 2018). Finally, Ku dimers have to be removed from repaired DNA. The exact mechanism of how that happens remains unclear. However, two mechanisms have been proposed: ubiquitylation-driven degradation or DNA nicking that would allow Ku to escape (Fell & Schild-Poulter, 2015; Langerak et al., 2011; Postow et al., 2008).

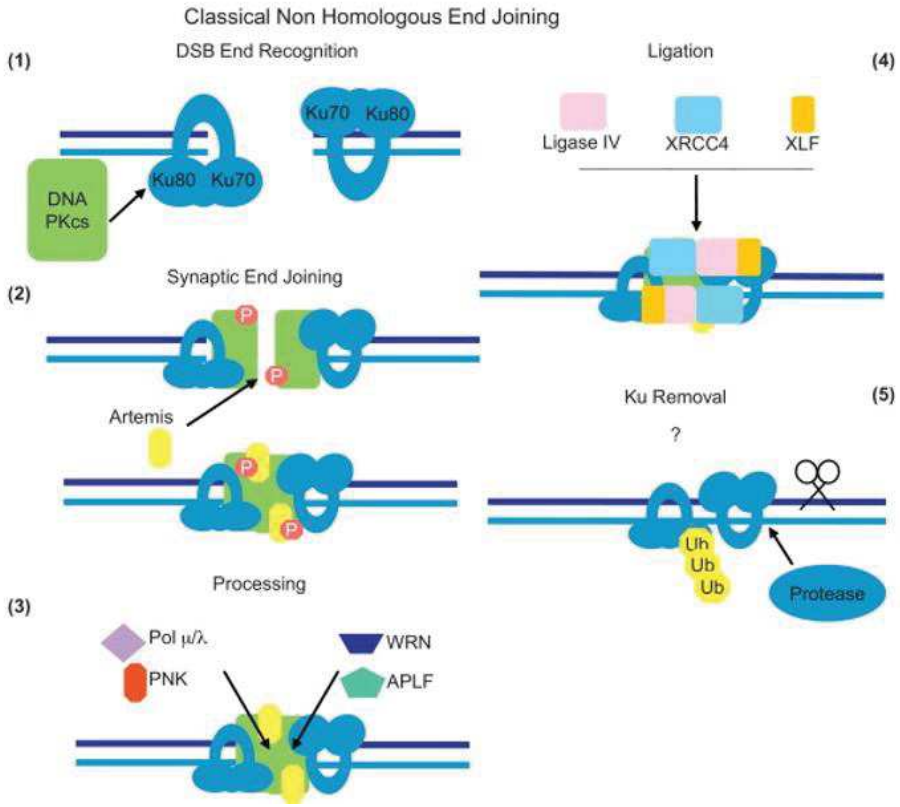

Fig. 5 Schematic representation of cNHEJ pathway. It is initiated by Ku70/Ku80 binding to free DNA ends and a subsequent DNAPKcs recruitment and an active DNA-PK complex formation. DNA-PK mediates tethering of broken ends, autophosphorylates its own subunits and phosphorylates other targets including Artemis, PNKP, XRCC4, XLF, and DNA ligase IV. If necessary, end-processing is performed by PNKP, Artemis, WRN, APLF, Pol μ/λ, and others. DNA Ligase IV, XRCC4, and XLF perform ligation. Finally, Ku heterodimers are removed, presumably by proteasomal degradation. Adapted from (Fell & Schild-Poulter, 2015).

Homologous recombination

The homologous recombination repair pathway uses a sister chromatid as a template for restoring mutilated DNA. Therefore, this pathway is error-free but restricted to S and G2 phases of the cell cycle (Scully et al., 2019; Takata et al., 1998). HR is promoted by MRN complex and resection is initiated by its subunit MRE11(T. Liu & Huang, 2016; Shibata et al., 2014; Stracker & Petrini, 2011). MRN complex also recruits CtIP (CTBP interacting protein) that further promotes end-resection (Escribano-Díaz et al., 2013; Sartori et al., 2007; Stracker & Petrini, 2011). CtIP recruits BRCA1 to prevent binding of RIF1 (Rap1-interacting factor 1 homolog), a protein involved in NHEJ initiation (Escribano-Díaz et al., 2013; T. Liu &

Huang, 2016). End-resection is then performed by EXO1 (Exonuclease1) by 5' to 3' digestion of DNA ends, which leads to the formation of single-strand DNA (ssDNA) overhangs (Cannavo et al., 2013; Eid et al., 2010; T. Liu & Huang, 2016). ssDNA is then bound by RPA (replication protein A) heterotrimer that protects it from degradation (San Filippo et al., 2008; Van Komen et al., 2002). MRN complex promotes Bloom syndrome helicase (BLM) recruitment, which together with DNA2 (DNA replication ATP-dependent helicase/nuclease DNA2) promotes extended resection (Daley et al., 2017; Nimonkar et al., 2011; Scully et al., 2019). Rad51 recombinase must be recruited to a break site by interacting with RPA, and later replace it as to HR could proceed (San Filippo et al., 2008; Scully et al., 2019). To that end, BRCA2 competes with RPA for binding ssDNA and interacts with RAD51 monomers and BRCA1 via its partner PALB2 to promote RAD51 filament formation (Jensen et al., 2010, 2013; Prakash et al., 2015; Scully et al., 2019; Stark et al., 2004; Wright et al., 2018; Xia et al., 2006; Yang et al., 2005; F. Zhang et al., 2009). RAD51 nucleoprotein filaments stabilized by RAD54 are involved in the homology search process that is a key step of HR facilitated by BRCA1-BARD1 (BRCA1-associated RING domain protein 1) (Sanchez et al., 2013; Scully et al., 2019; Wright et al., 2018; W. Zhao et al., 2017). RAD51 nucleofilaments invade a sister chromatid in a RAD54-dependent manner and form a three-strand helix intermediate which is further resolved into a heteroduplex formed by an invading strand and a complementary strand (Z. Chen et al., 2008; Mazina & Mazin, 2004; van der Heijden et al., 2008; Wright et al., 2018). The dissociated DNA strand is displaced into a displacement-loop (D-loop) and protected by RPA (Daley et al., 2014; Wright et al., 2018). At this step RAD51 filament is disassembled in an ATP-dependent manner (Scully et al., 2019; van der Heijden et al., 2008). Invading strand extension is performed by DNA polymerase δ, although translesion synthesis DNA polymerases could also be involved in this process (McVey et al., 2016; Scully et al., 2019). PCNA (Proliferating cell nuclear antigen) and its loader RFC1-5 has been shown to be required for the initiation of DNA synthesis by Polδ during HR (X. Li et al., 2009; Wright et al., 2018). The final steps of HR can be conducted in three different ways. Synthesis-dependent strand annealing pathway (SDSA), where only one strand undergoes invasion. This results in the annealing of the non-invading strand with the displaced strand once D-loop is unwound and promotes HR termination. This is a non-crossover and therefore preferable pathway as it does not involve a Holliday junction formation (Scully et al., 2019; Westmoreland & Resnick, 2013; Wright et al., 2018). Another pathway is double Holliday junction formation which happens due to the second strand invasion and can

be resolved with or without crossover (Osman et al., 2003; Scully et al., 2019; Wright et al., 2018; Wyatt et al., 2017).

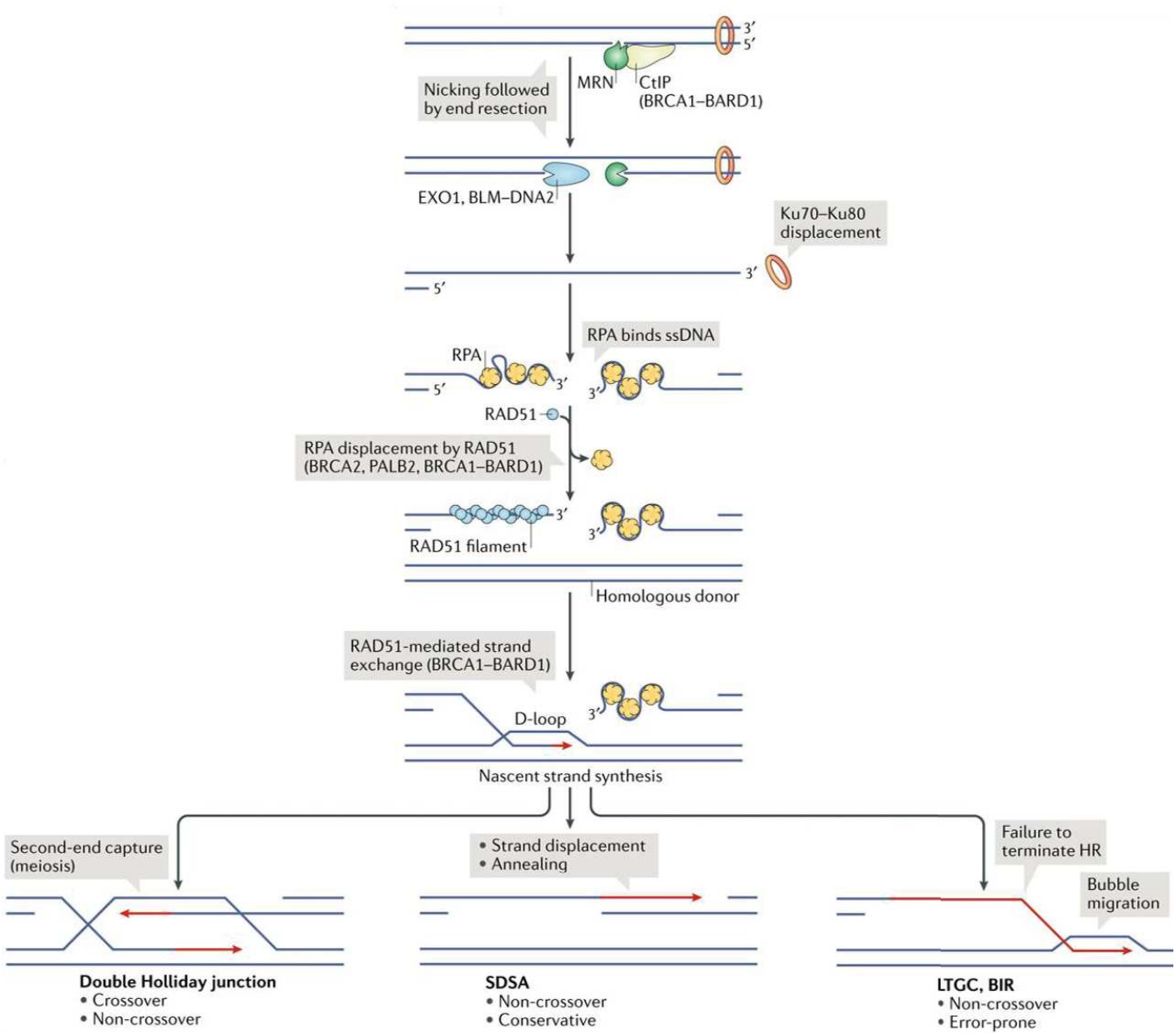

Fig. 6 Schematic representation of HR pathway. DSB is sensed by MRN complex, which recruits CtIP. Mre11 initiates resection, which is further conducted by EXO1, BLM, and DNA2, and Ku heterodimers are displaced from ssDNA. Resected DNA is protected by RPA, which is further displaced by Rad51, resulting in RAD51 nucleofilament formation. After RAD51-mediated strand invasion, facilitated by BRCA1 and BARD1, nascent strand is synthesised by either SDSA, Holliday junction formation, or LTGC. Adapted from (Scully et al., 2019).

Finally, during replication long-tract gene conversion (LTGC) and break-induced replication (BIR) might be deployed, in which DNA synthesis induced by HR proceeds to a large distance from the break sites. Those pathways are highly mutagenic and are therefore suppressed in mammals and could mostly be observed if one of the key HR players is dysfunctional (Chandramouly et al., 2013; Scully et al., 2019; Willis et al., 2017).

Microhomology-mediated end joining

MMEJ (or Alt-EJ) pathway was for a long time considered as a backup for the major ones. It is initiated by end resection and requires short (2-20 bp) 3' ssDNA overhangs (Seol et al., 2018; Welcker et al., 2000; Wright et al., 2018). It is Ku-independent and require proteins known to be a part of HR end resection machinery and is more prominent if NHEJ or HR is impaired (e.g. in KU or BRCA1-deficient cells) and utilizes microhomologies in close proximity to a break site (Boboila et al., 2010; Ceccaldi et al., 2015; Scully et al., 2019; Wright et al., 2018). This pathway leads to small deletions so it was also assumed that other mechanisms, which are error-free or cause minimal error would be preferable. Break recognition is thought to be mediated by PARP1 that competes with Ku dimer (Badie et al., 2015; Mansour et al., 2010, 2013). After break recognition, the initial steps of MMEJ are common with HR. It is also dependent on Mre11 and CtIP resection activity (Badie et al., 2015; Truong et al., 2013; Wright et al., 2018; Xie et al., 2009). However, BLM/Exo1 complex that promotes long-range resection is inhibiting MMEJ (Daley et al., 2015; Wright et al., 2018; Y. Wu et al., 2015). Mechanisms of microhomology search are not yet clear, but once it is found ssDNA gaps are filled by Polθ (Kent et al., 2015; Mateos-Gomez et al., 2017; Scully et al., 2019; Wright et al., 2018). Polθ contains a Rad51 domain that allows it to inhibit Rad51-mediated recombination as well as misplaces RPA that is known to promote HR and negatively regulate MMEJ (Ahrabi et al., 2016; Ceccaldi et al., 2015; S. K. Deng et al., 2014; Mateos-Gomez et al., 2015, 2017; Scully et al., 2019; Wright et al., 2018). The final ligation step is performed by the XRCC1-Lig3 complex (Audebert et al., 2004; Okano et al., 2003, 2005; Wright et al., 2018).

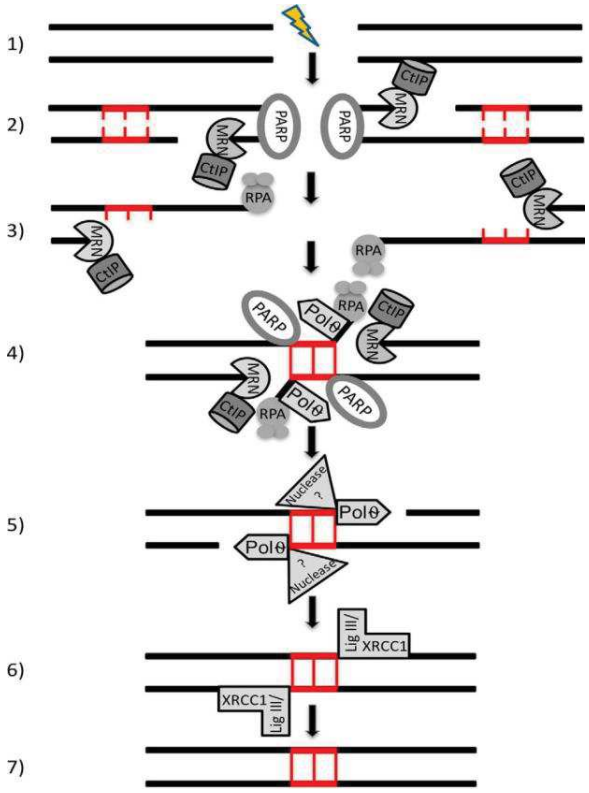

Fig. 7 Schematic representation of MMEJ pathway. After PARP1-mediated break recognition, resection is performed by Mre11, aided by CtIP. ssDNA is protected by RPA until gaps are filled by Polθ. Ligation is carried out by Lig III and XRCC1. Adapted from (Wright et al., 2018).

Single-strand annealing

Finally, another pathway that could perform a repair of resected ends is single-strand annealing (SSA). This pathway is carried out through annealing of homologous regions of 3' ssDNA on the same chromosome (i.e. within repetitive regions). Therefore SSA is considered to be a highly deleterious pathway (Iliakis et al., 2019; Scully et al., 2019). In the course of SSA resection could be carried to a considerable distance, sometimes longer than during HR (Iliakis et al., 2019; Ochs et al., 2016). Although the mechanisms are poorly understood in mammals, it has been shown that SSA initiation requires PARP1 and resection that precedes it relies on CtIP and RPA (Grimme et al., 2010; Iliakis et al., 2019; Sullivan-Reed et al., 2018; Truong et al., 2013; Wright et al., 2018; Xie et al., 2009). Annealing of ssDNA after resection is mediated by Rad52 (Grimme et al., 2010; Iliakis et al., 2019; Iyama & Wilson, 2013; Rothenberg et al., 2008; Wright et al., 2018). It has been shown that non-homologous 3' end is removed by a NER-associated nuclease complex

ERCC1/XPF (Ahmad et al., 2008; Al-minawi et al., 2008; Iyama & Wilson, 2013). However, downstream polymerases and ligases remain unknown (Iyama & Wilson, 2013).

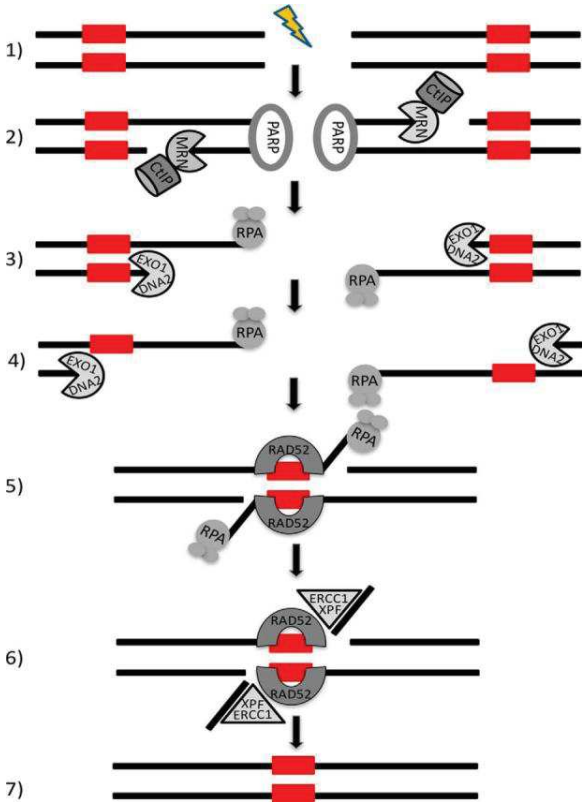

Fig. 8 Schematic representation of SSA pathway. After PARP1-mediated break recognition, initial resection is performed by Mre11, aided by CtIP. It is further extended by EXO1 and DNA2. ssDNA is protected by RPA, and Rad52 mediates annealing. ERCC1/XPF complex removes 3' overhangs. Adapted from (Wright et al., 2018)

Double-strand break repair pathway choice

The availability of multiple pathways for DSB repair leads to the necessity to choose between them, preferably selecting the most appropriate one. This choice is very complex and it is regulated at multiple levels. In most of the cases, NHEJ and HR are considered to be predominant pathways (exceptions to be discussed later), and the backup pathways step in occasionally when two preferential pathways failed to complete repair (Jachimowicz, Goergens, et al., 2019; Scully et al., 2019).

The crucial decision point is the decision for resection as Ku70/80 binding to ssDNA is weak, therefore predisposing such breaks to be repaired by HR or backup pathways, and its binding to dsDNA is strong, so they would be likely repaired by NHEJ (Mimori & Hardin, 1986; Scully et al., 2019). As previously mentioned,

one of the key factors in the repair pathway choice is the cell cycle state. This arises from HR requiring a sister chromatid and therefore is limited to S and G2 phases of the cell cycle. Therefore many of the resection-initiating factors that are essential for commitment to HR are regulated by CDKs (Aylon et al., 2004; Ira et al., 2004; Scully et al., 2019; Tomimatsu et al., 2014). The competition between Ku70/80 binding and repair machinery is ensured by multiple proteins, the 53BP1 and CtIP being the major players. CtIP phosphorylation is essential for MRE11 endonuclease activity initiation and BRCA1 binding, whereas 53BP1 suppresses resection and promotes NHEJ (Anand et al., 2016; Bunting et al., 2010; Jachimowicz, Goergens, et al., 2019; Scully et al., 2019; Xie et al., 2007; X. Yu & Chen, 2004). UBQLN4 (Ubiquilin-4) promotes MRE11 proteasomal degradation and DYNLL1 (Dynein light chain 1) blocks its function via direct interaction, therefore inhibiting HR activation (He et al., 2018; Jachimowicz, Beleggia, et al., 2019; Jachimowicz, Goergens, et al., 2019). 53BP1 acts in cooperation with its interactor PTIP (PAX transactivation activation domain-interacting protein) and effectors RIF1 and REV7 (Rev7-like terminal deoxycytidyl transferase) (Bunting et al., 2010; Escribano-Díaz et al., 2013; L. Feng et al., 2013; Jachimowicz, Goergens, et al., 2019; Munoz et al., 2007; J. Wang et al., 2014; G. Xu et al., 2015; Zimmermann et al., 2013). PTIP directly interacts with Artemis and ensures its retention at the break site (Jachimowicz, Goergens, et al., 2019; J. Wang et al., 2014). The mechanism by which the 53BP1-RIF1-REV7 pathway promotes NHEJ was recently identified to be via the Shieldin complex, which consists of REV7, SHLD1, SHLD2, and SHLD3 (Shieldin complex subunit 1-3). This complex is recruited to break sites in 53BP1 and RIF1 dependent way and protects them from extensive resection. The loss of its components leads to PARP inhibition resistance in cancers due to HR reinstatement (Findlay et al., 2018; Ghezraoui et al., 2018; Gupta et al., 2018; Jachimowicz, Goergens, et al., 2019; Noordermeer et al., 2018; Tomida et al., 2018). It has also been proposed that it fills in ssDNA ends to enable them for NHEJ (Mirman et al., 2018; Scully et al., 2019). REV7 and SHLD3 have been shown to inhibit CtIP-dependent resection and therefore represses HR (Gupta et al., 2018; Jachimowicz, Goergens, et al., 2019; G. Xu et al., 2015).

Misregulation of the repair pathway choice leads to severe consequences, including various syndromes and cancer (Jachimowicz, Goergens, et al., 2019; Stewart et al., 2009; Terabayashi & Hanada, 2018).

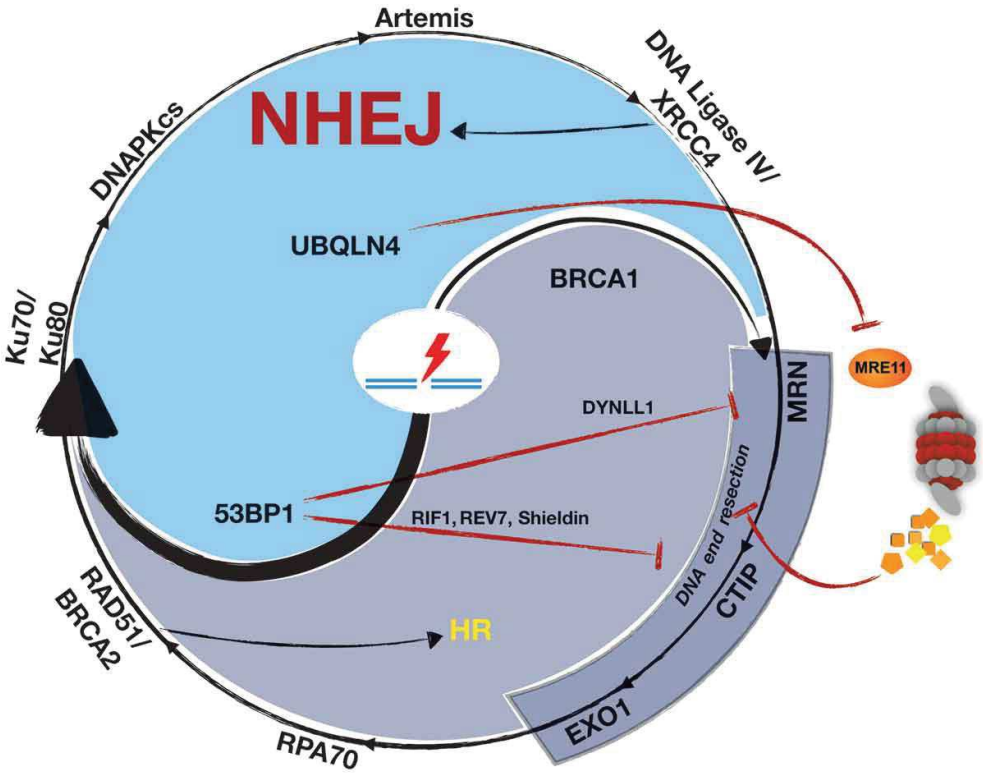

Fig. 9 DSB repair pathway choice. Adapted from (Jachimowicz, Goergens, et al., 2019)

Chromatin

DNA organization in eukaryotic cells is organized in chromatin, which is an intricate nucleoprotein structure. Studies on chromatin began as early as the 1880s when Flemming in his experiments on cellular division discovered an easily-stainable structure that he named 'chromatin' and Miescher, Kossel, and Hoppe-Seylercontinued found that this substance consists of nucleic acid and proteins that were termed 'histones' and continued in increasing intensity and details ever since (D. E. Olins & Olins, 2003; Paweletz, 2001). After many years of elaborate exploration and major discoveries that included identification of DNA as a carrier of inherited information and deciphering of its structure, chromatin research came to a new level in 1960s when the chromosome structure, as well as the role of histone modifications, were defined (Allfrey et al., 1964; Avery et al., 1944; Gall, 1963; D. E. Olins & Olins, 2003; Watson & Crick, 1953). Another key advance was achieved in the 1970s by identifying and characterizing a nucleosome as a basic chromatin subunit (A. L. Olins & Olins, 1974; D. E. Olins & Olins, 2003; Oudet et al., 1975). Since that time our understanding of both the principles and the importance of chromatin organization has been steadily increasing.

Chromatin structure is extremely complex and includes several levels of organization. Each of these levels represents an additional mean for modulating the use of inherited information as long as its structure is in order. However, they also pose additional obstacles for maintaining it or modifying it in the desired way. First, DNA is wrapped around histone octamers to form “beads on a string” structure, with H1 histone bound to nucleosome-free parts (Allan et al., 1980; Baldi et al., 2020; Felsenfeld & Groudine, 2003; Fyodorov et al., 2018; Kornberg, 1974; Woodcock et al., 1976). Each nucleosome represents a globular structure with two copies of each histone, H2A, H2B, H3, and H4, and 174 base pair-long DNA is wrapped around it (Arents & Moudrianakis, 1993; Fyodorov et al., 2018; Luger et al., 1997). However, first three of the abovementioned histones, as well as a linker histone H1, have several known structural variances, and each of the core histones can be chemically modified, so this structure represents a broad layer of regulation (Biterge & Schneider, 2014; Marzluff et al., 2002). The abovementioned H2AX variant is of the utmost interest in relation to DNA repair, but other histone variants play an equally important role in other aspects of cellular homeostasis. For instance, H3 variant CENPA is a hallmark of a centromere and is essential for a kinetochore complex assembly, and H2A.Z and H3.3 are predominantly found in transcriptionally active chromatin whereas macro H2A is linked to a repressed chromatin state (Biterge & Schneider, 2014; Chakravarthy & Luger, 2006; Rogakou et al., 1998; Thakar et al., 2009; Yoda et al., 2000). Posttranslational modification of histone tails has long been known to play an important role in chromatin state regulation (Allfrey et al., 1964; Allis & Jenuwein, 2016; Hebbes et al., 1994; Jeppesen & Turner, 1993; Kayne et al., 1988). It has been shown that interaction of histone octamer has an ionic nature and is influenced by the charge, therefore those modifications that affect protein charge either by being charged such as acetylation of lysine or by affecting a residue they bind to. This allows DNA accessibility modulation simply at a level of physical interactions (Davey et al., 2002; Korolev et al., 2012; North et al., 2012; Shimko et al., 2011; M. Simon et al., 2011; K. Zhou et al., 2019). To add more complexity, each modification can promote or inhibit interaction with multiple proteins, which underlies multiple processes in the nucleus such as the abovementioned H2AX phosphorylation that targets all downstream repair machinery or heterochromatic domain formation with H3K9me3-mediated recruitment of HP1 (Heterochromatin protein 1) proteins. The principles of chromatin modifications influence on the biological outcome were formulated as “histone code hypothesis” which was further expanded into the “epigenetic code” theory. It postulates that epigenetic landscape features, which are created by specialized enzymes (“writers” that introduce

modifications and “erasers” that remove them), are recognized by an array of dedicated proteins (“readers”) in order to control biological functions (Allis & Jenuwein, 2016; Jenuwein & Allis, 2001; Strahl & Allis, 2000).

For many years the consensus was that “beads on a string“ structures further fold into 30 nm fibers (Fyodorov et al., 2018; Ghirlando & Felsenfeld, 2008). However, recent studies using super-resolution microscopy have shown that *in vivo* nucleosomes do not form a regular fiber but are arranged into heterogeneous clusters of various sizes (Fyodorov et al., 2018; Ou et al., 2017; Ricci et al., 2015). This is in accordance with another recent study where relaxed nucleosome zigzag chains rather than 30 nm fibers were observed in vivo using electron microscopy (Fyodorov et al., 2018; Grigoryev et al., 2016).

Organized or not, chromatin fibers are further looped into topologically associated domains (TADs). TADs represent chromatin regions with a high frequency of interactions as demonstrated by Hi-C experiments (Bonev & Cavalli, 2016; Dixon et al., 2012; Nora et al., 2012; Sexton et al., 2012). It has been shown that TADs are correlating with many genomic features such as transcription or chromatin marks, and that promoter-enhancer interactions are mostly occurring within a TAD (Bonev & Cavalli, 2016; Dixon et al., 2012; Shen et al., 2012). They are separated by boundaries established by architectural proteins, the most prominent of which are CTCF (CCCTC-binding factor) and Cohesin (Bonev & Cavalli, 2016; Dixon et al., 2012; Rao et al., 2014). In mammalian cells, TADs have a complex structure containing smaller domains called subTADs (Bonev & Cavalli, 2016; Phillips-Cremins et al., 2013; Wijchers et al., 2016). Two chromatin compartments have been specified based on inter-TAD interactions, active A compartment and inactive B compartment (Bonev & Cavalli, 2016; Lieberman-Aiden et al., 2009). However, it has later been shown that these compartments can be further divided into subtypes (Bonev & Cavalli, 2016; S. Wang et al., 2016; Wijchers et al., 2016). TADs were shown to be predominantly conserved between cell types (Bonev & Cavalli, 2016; Dixon et al., 2012; Rao et al., 2014). However, this is not the case regarding compartments, which tend to switch in a cell type-specific manner (Bonev & Cavalli, 2016; Dixon et al., 2015; Lieberman-Aiden et al., 2009). Other well-defined domains, such as lamina-associated domains (LADs), nucleolus-associated domains, pericentromere-associated domains as well as the nucleolus itself and chromocenters can be defined within the nucleus (Guelen et al., 2008; Németh et al., 2010; Solovei et al., 2016; Wijchers et al., 2015). Finally, at larger scale chromatin is organized into chromosome territories (Bonev & Cavalli, 2016; Lieberman-Aiden et al., 2009; P et al., 1988; Pinkel et al., 1988).

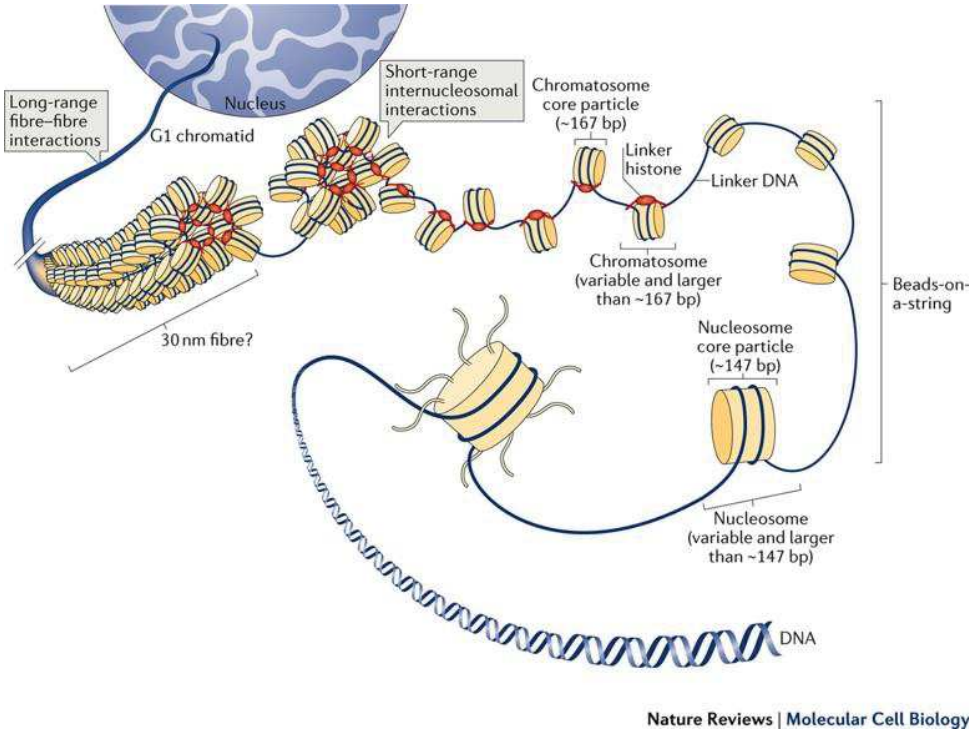

Fig. 9 Levels of chromatin organization. Adapted from (Fyodorov et al., 2018)

Such a complex structure obviously leads to a complicated hierarchy of interactions and interconnections but in the simplest and the most intuitive classification would be discerning active and inactive parts, accessible and not accessible. And this, with a huge simplification, is a division to euchromatin and heterochromatin.

Euchromatin and heterochromatin

Euchromatin is gene-rich, transcriptionally active, and belongs to early replicating parts of a genome. As a contrast, heterochromatin is transcriptionally inactive, gene-poor, and late-replicating (Dileep & Gilbert, 2018; Hildebrand & Dekker, 2020; Jing Liu et al., 2020; Passarge, 1979; Schultz & Dobzhansky, 1934; Solovei et al., 2016; Wakimoto & Hearn, 1990). Nucleosomes within euchromatin carry post-translational histone modifications (histone marks) characteristic for transcription (Hildebrand & Dekker, 2020; Lawrence et al., 2016). Prominent among these marks are H4K16ac that enhances transcription, H3K4me3 that marks active TSS (transcription start sites), H3K36me3 that is present throughout the hole actively transcribed region, H3K27ac that is a part of active enhancers and directly opposes establishing a repressive H3K27me3 modification, H3K9ac that marks active promoters and many others (Akhtar et al., 2000; Bannister et al., 2005; Bannister & Kouzarides, 2011; Creyghton et al., 2010; Karmodiya et al., 2012; Lawrence et al., 2016;

Noma et al., 2001; Schneider et al., 2004; Stepanik & Harte, 2012; Tie et al., 2009). It is not compacted and easily accessible, which is largely promoted by histone acetylation (Bassett et al., 2009; Görisch et al., 2005; Lawrence et al., 2016; Otterstrom et al., 2019; Shogren-Knaak et al., 2006). As long as euchromatin is in focus, the matter seems to be relatively straightforward. But heterochromatin comes to the spotlight, no simplicity is left. Heterochromatin is a very complex and complicated concept, as there is no such thing as just heterochromatin. Heterochromatin is often claimed to be compacted but not all data support this theory, and it is possible that heterochromatic regions are inaccessible, not due to physical compaction but rather an environment created by associated proteins (Boettiger et al., 2016; Hildebrand & Dekker, 2020; Jing Liu et al., 2020; Otterstrom et al., 2019; Ou et al., 2017; J. Xu et al., 2018). There are two distinct heterochromatin types, constitutive and facultative, LADs are considered separately as they are distinct from both of them (Brown, 1966; Fu et al., 2020; Hildebrand & Dekker, 2020; Nagano et al., 2013).

Constitutive heterochromatin, marked with H3K9me3, is predominantly formed around genomic regions that have to be strictly inhibited, such as repetitive elements such as satellite DNA, ribosomal DNA, and transposable elements, elements of viral origin or inactivated X chromosome (Litt et al., 2001; Jing Liu et al., 2020; Nakayama et al., 2001; Noma et al., 2001; M. Savitsky et al., 2002; Whitehead & Moran, 1949). H3K9me3 promotes recruitment of HP1, which silences transcription and ensures formation and maintenance of heterochromatic domains (Bannister et al., 2001; Lachner et al., 2001; Lehnertz et al., 2003; Peters et al., 2003; Schotta et al., 2004; Zeng et al., 2009). Constitutive heterochromatin plays an important role in regulating genome stability by protecting telomeres and pericentromeres from inappropriate treatment by DNA repair machinery, recombination, and chromosomal rearrangements and by preventing transposable elements activation (Eberhart et al., 2013; Fu et al., 2020; Peng & Karpen, 2007, 2009; M. Savitsky et al., 2002; Sentmanat & Elgin, 2012).

On the contrary, facultative heterochromatin is formed in those parts of a genome that might be transcribed, but have to be inactivated in a particular cell type (Hübner et al., 2015; Lewis, 1978; Jing Liu et al., 2020; Ou et al., 2017; Solovei et al., 2016; Jiang Zhu et al., 2013). A hallmark of facultative heterochromatin is H3K27me3 histone modification, which is established and maintained by Polycomb repressive complexes, PRC1 and PRC2 complexes (Boyer et al., 2006; Bracken et al., 2006; Czermin et al., 2002; Kuzmichev et al., 2002; Jing Liu et al., 2020; Müller et al., 2002; J. A. Simon & Kingston, 2013; Wiles & Selker, 2017). The only methyltransferase that is capable of creating this mark is a PRC2 catalytic subunit

EZH2 (Enhancer of zeste homolog 2) (Kuzmichev et al., 2002; J. A. Simon & Kingston, 2013). Once marked, facultative heterochromatic regions are recognised and bound by PRC1 or PRC2 complex to maintain transcriptional repression (Boyer et al., 2006; Bracken et al., 2006; Cao et al., 2002; T. I. Lee et al., 2006; Hengbin Wang et al., 2004; Wiles & Selker, 2017). Polycomb complexes play a crucial role in establishing and maintaining expression and silencing patterns in the course of development, and either their loss or malfunction leads to developmental abnormalities or cancer (Akasaka et al., 1996; Ben-Porath et al., 2008; Bracken et al., 2006; Conway et al., 2015; Nikoloski et al., 2010; Ohm & Baylin, 2007; Sneeringer et al., 2010).

And finally, LADs are the most repressed part of a genome. They are known to be strictly repressive for transcription, and were proven to be physically compacted (Dixon et al., 2012; Ou et al., 2017; Reddy et al., 2008; Solovei et al., 2016). LADs are enriched for repressive chromatin marks like H3K9me2/3 and H3K27me3 and directly interact with nuclear lamins (Guelen et al., 2008; Harr et al., 2015; Kind et al., 2013; Lochs et al., 2019; Meuleman et al., 2013; Solovei et al., 2016; F. Wu & Yao, 2017).

All these levels of the organization are interconnected and influence all processes that take place in the nucleus, including DNA repair.

DNA repair in the chromatin context

It has been shown multiple times by many researchers that chromatin context surrounding a break site has a strong influence over the flow of repair.

Chromatin sensitively responds to DNA damage. DDR activation leads to immediate chromatin relaxation that results from PARP1 activity and chromatin remodelers (Burgess et al., 2014; Clouaire & Legube, 2019; Luijsterburg et al., 2016; Sellou et al., 2016; Smith et al., 2018). However, this relaxation is only transient, it is followed by an ATM-dependent re-condensation, and this re-condensation is crucial for normal DDR propagation (Burgess et al., 2014; Clouaire & Legube, 2019). Even the most prominent DNA damage marker, γH2AX, is itself a chromatin modification, and therefore chromatin is an effector for DDR. Genome-wide studies of chromatin response to double-strand break introduction revealed a plethora of induced chromatin changes, some local and some ranging megabases (Clouaire et al., 2018; Clouaire & Legube, 2019). Large-scale modifications are common for all mechanisms of repair include γH2AX foci formation (discussed above) linked with *de novo* H2AX deposition and the loss of H1 (Clouaire & Legube, 2019; Piquet

et al., 2018; Sellou et al., 2016; Strickfaden et al., 2016). Conversely, local chromatin changes are repair pathway specific (Clouaire & Legube, 2019). H3.3 histone variant deposition, were reported to contribute to Ku and XRCC4 recruitment and thus promote NHEJ (Clouaire & Legube, 2019; Luijsterburg et al., 2016). H2A.Z loading also plays a role in the Ku70 deposition albeit it has to be removed later in order for repair the process to proceed (Clouaire & Legube, 2019; Gursoy-Yuzugullu et al., 2015; Y. Xu et al., 2012). Histone modifications that promote NHEJ include H4Y51 phosphorylation and H2BK120 acetyl to monoubiquitin transition and H3K36 dimethylation (Clouaire et al., 2018; Clouaire & Legube, 2019; Fnu et al., 2011; Hossain et al., 2016). H2AK15 monoubiquitylation by RNF168 and H4K20me1/2 facilitate recruitment of resection restraining factors including 53BP1 (Clouaire & Legube, 2019; Fradet-Turcotte et al., 2013; Wilson et al., 2016). Histone variant loaded in order to promote HR is macroH2AX, and H2A.Z is depleted from the break vicinity (Clouaire et al., 2018; Clouaire & Legube, 2019; Khurana et al., 2014; C. Xu et al., 2012). Changes in histone modification include H2BK120ac and H2AK127/129ub establishment and H2BK120ub, H3K4me3, H3K79me2, and H4K12ac decrease (Clouaire et al., 2018; Clouaire & Legube, 2019; Densham et al., 2016; Uckelmann & Sixma, 2017). H2AK15 and H4K16 acetylation performed by TIP60 has been shown to promote resection (Clouaire & Legube, 2019; Jacquet et al., 2016; Tang et al., 2013).

However, not only is the chromatin environment affected DNA damage, but it can also influence the way this damage would be repaired. It has been proposed that euchromatic regions are more prone to HR (Aymard et al., 2014; Mitrentsi et al., 2020). It is achieved by the affinity of HR promoting proteins, namely BRCA1 and CtIP interactor LEDGF (Lens epithelium-derived growth factor), to active chromatin marks (Daugaard et al., 2012; Mitrentsi et al., 2020; Tamburini & Tyler, 2005; Wilson & Durocher, 2017). It has also been shown that proteins involved in R-loops resolution can recruit RAD51 and BRCA2 (Cohen et al., 2018; D'Alessandro et al., 2018; Mitrentsi et al., 2020). It has even been suggested that if DSBs are acquired at transcriptionally active regions during G1 when HR cannot be engaged they are held unrepaired until S or G2 phase (Aymard et al., 2017; Mitrentsi et al., 2020). Another hypothesis implies the use of RNA as a template for DSB repair (Meers et al., 2016; Mitrentsi et al., 2020). At the same time, heterochromatin has been shown to be not a very HR-friendly environment and rather promotes NHEJ (Kalousi & Soutoglou, 2016; Pfister et al., 2014). Despite its compaction might represent a barrier to repair factor recruitment, it is unclear whether heterochromatin proteins such as HP1 are dismissed or the repair process can proceed without it (Kalousi et al., 2015; Mitrentsi et al., 2020; Noon et al., 2010; Tsouroula et al., 2016). Some studies

even show an increase of H3K9me3 in close proximity to DSBs (Ayrapetov et al., 2014; Mitrentsi et al., 2020). It was shown that LADs repress HR and a double-strand break that occurs there could be repaired by other pathways, even though more mutagenic, because in this case MMEJ became an alternative for NHEJ, but HR is inhibited in this nuclear compartment. At the same time, breaks around nuclear pores that also reside at the nuclear periphery but are surrounded by transcriptionally active chromatin, are capable of utilizing HR normally (Kalousi & Soutoglou, 2016; Lemaître et al., 2014). It has also been shown that when a break occurs in heterochromatinised chromocenters it can only be repaired by HR at the periphery of a chromocenter, thus escaping a heterochromatic environment (Kalousi & Soutoglou, 2016; Tsouroula et al., 2016).

However, facultative heterochromatin is relatively poorly investigated in the context of DNA repair.

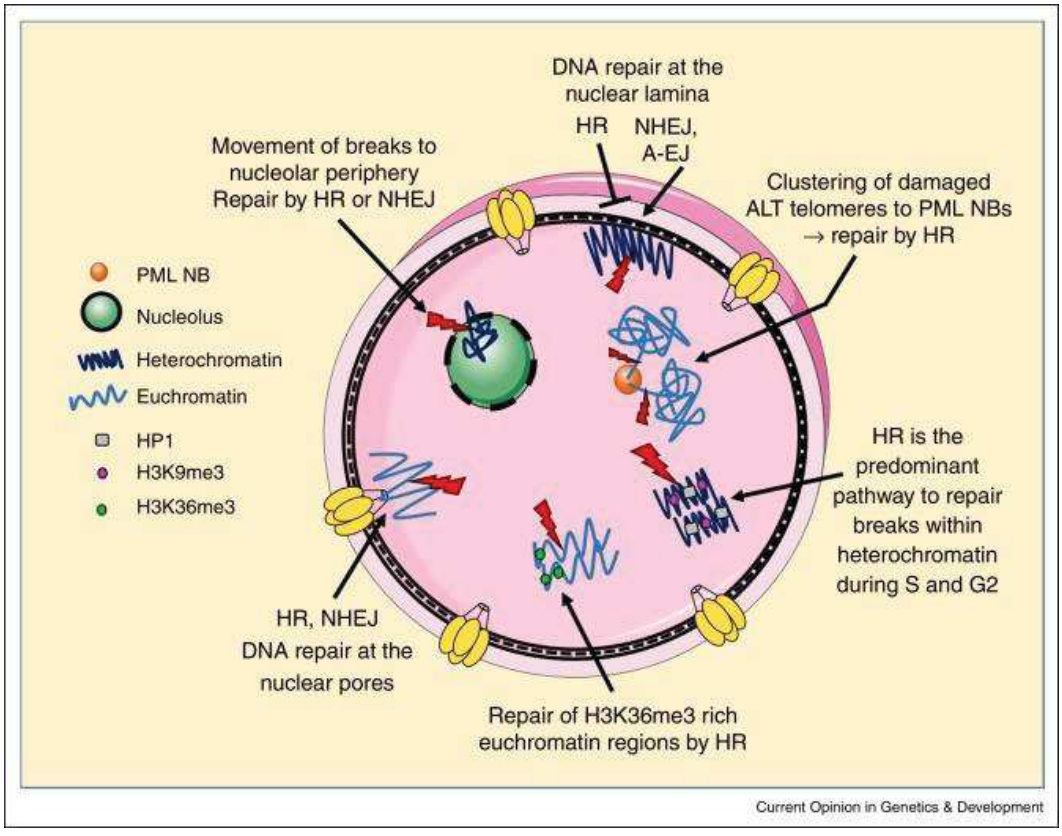

Fig. 10 Chromatin environment influence over DNA repair pathway choice. Adapted from (Kalousi & Soutoglou, 2016)

Bivalency

Another type of chromatin that has never to my knowledge been investigated in the context of DNA repair is bivalent chromatin. Generally, any kind of state that combines active and repressive chromatin marks is bivalent chromatin. To date, these include a simultaneous presence of H3K4me3 and H3K27me3, H3K4me3 and H3K9me3, H3K36me2/3 and H3K9me3, or a combination of H3K9acK14ac, H3K4me3 and H3K27me3 (Azuara et al., 2006; Bernstein et al., 2006; Harikumar & Meshorer, 2015; Matsumura et al., 2015; Mauser et al., 2017; Roh et al., 2006; Vakoc et al., 2005). But the first kind of such chromatin state that has been observed (currently termed 'classic' bivalent domains) is a combination of H3K4me3 and H3K27 me3. They were initially found in mouse embryonic stem (ES) cells in culture and for the first several years it was rather common to consider them a culture artifact (Azuara et al., 2006; Bernstein et al., 2006; Harikumar & Meshorer, 2015). However, later bivalent domains has been observed in other cell types, and even *in vivo* during development and in cancer (Béguelin et al., 2016; Kampilafkos et al., 2015; Matsumura et al., 2015; Minoux et al., 2017; Sachs et al., 2013; Sin et al., 2015). In the course of differentiation, one of the marks is normally lost from bivalent promoters (Bernstein et al., 2006). Another question was addressed, whether real bivalency exists, it is only resulted from a heterogeneity of cells in the population or from the close proximity of two differentially marked genomic region. However, the existence of bivalency has later been proven by genome-wide assessing individual bivalent nucleosomes. Sequential immunoprecipitation of single nucleosomes confirmed that both marks can coexist on the same nucleosome, although on different histone H3 tails (Sen et al., 2016).

A currently accepted explanation of this phenomenon is based on the observation that many of the bivalently marked regions found in ES cells are promoters of developmental genes. The hypothesis is that such a chromatin state is needed to enable rapid activation or complete inactivation of a respective gene (Harikumar & Meshorer, 2015). However, RNA polymerase II (RNAPII) pausing was not observed on these promoters in accordance with RNAPII association with actively transcribed and not bivalent genes (Mantsoki et al., 2018). Additionally, it has also been shown that bivalency is linked to the cell cycle. MLL2 (Myeloid/lymphoid or mixed-lineage leukemia protein 2), as a part HMT (Histone methyltransferases) complex, establishes H3K4me3 at developmental genes and thus real bivalency, and during the rest of the cell cycle genes are robustly repressed (Singh et al., 2015). According to other studies, exit from

pluripotency accrues in a cell cycle-dependent manner, so these two studies go in line (Boward et al., 2016; Pauklin & Vallier, 2013).

This chromatin type has not been studied extensively yet and the best model for it are embryonic stem cells where this type of chromatin is rather abundant.

Embryonic stem cells

Stem cells are defined by the ability of both self-renewal and differentiation. There are three classes of stem cells (although the last one is sometimes divided into two). The first one, totipotent stem cells, are capable of differentiation into any type of adult, embryonic, or extraembryonic tissue. The second one, pluripotent stem cells, keeps the potential and can give rise to any adult or embryonic cell type but not some of the extraembryonic tissues. Finally, multipotent stem cells are tissue-specific and usually reside in mouse embryonic stem cells. Unipotent stem cells, that are sometimes considered separately from multipotent ones, can only differentiate on one cell type (Alison & Islam, 2009). Embryonic stem (ES) cells represent pluripotent stem cells derived from a preimplantation blastocyst (Martello & Smith, 2014). Mouse ES cells were first obtained by Evans and Kaufman in 1981 and until now stay a very attractive research model and potentially powerful therapeutic tool as well as a fascinating research subject (Evans & Kaufman, 1981).

The pluripotent state is maintained by a regulatory network of transcription factors. The core factors are Oct4 (Octamer-binding protein 4), Sox2 (SRY-box 2), and Nanog, and their key role has been proven many times both by demonstrating their importance for stemness and viability and by their capacity to initiate somatic cell reprogramming (Kehler et al., 2004; M. Li & Izpisua Belmonte, 2018; Mitsui et al., 2003; Nichols et al., 1998; Takahashi & Yamanaka, 2006). Oct4 and Sox2 form heterodimers and induce numerous targets including their own genes (Chew et al., 2005; M. Li & Izpisua Belmonte, 2018; Rodda et al., 2005). However, change of expression levels or interaction partners of Oct4 and Sox2 drive mesendodermal or ectodermal differentiation respectively (Avilion et al., 2003; Ferri et al., 2004; M. Li & Izpisua Belmonte, 2018; Niwa et al., 2000; M. Thomson et al., 2011). Nanog is required for maintenance of pluripotency and appear to be the key LIF/STAT3 (Leukemia inhibitory factor 1, Signal transducer and activator of transcription 3) pathway as its overexpression makes LIF redundant (Chambers et al., 2003; M. Li & Izpisua Belmonte, 2018; Mitsui et al., 2003). Together Oct4, Sox2, and Nanog binding cover the majority of pluripotency regulatory network

transcription factors (X. Chen et al., 2008; M. Li & Izpisua Belmonte, 2018; Loh et al., 2006). Their synergistic action plays a role in both propagating pluripotency by activating transcription of pluripotency-related genes and preventing differentiation by counter-balancing each other and blocking transcription of developmental regulators (Boyer et al., 2005; Loh et al., 2006; Young, 2011).

Another curious feature of ES cells should be mentioned. They have a distinct cell cycle profile with a very short G1 phase, which is tightly controlled by the pluripotency regulatory network and, in turn, has been shown to suppress differentiation (Coronado et al., 2013; Kareta et al., 2015; V. C. Li & Kirschner, 2014; Savatier et al., 2002). One of the core network targets, Myc (Myc proto-oncogene protein), enhances proliferation by activating transcription of cell-cycle related genes as well as directly represses differentiation by inhibiting FGF/ERK (Fibroblast growth factor, Extracellular signal-regulated kinase) signaling pathway (Cartwright et al., 2005; Chappell et al., 2013; Kareta et al., 2015; Kim et al., 2008; Loh et al., 2006). A collective action of Myc and core pluripotency factors leads to a compromised G1/S cell cycle checkpoint that contributes to G1 shortening (Kanai et al., 2015; Kareta et al., 2015; Kim et al., 2010; Suvorova et al., 2016; F. Zhang et al., 2009).

Among multiple regulatory measures than ensure stem cell proficiency for pluripotency and differentiation of them unique chromatin organization is a prominent one.

Chromatin in embryonic stem cells

As previously mentioned, one of the prominent features of stem cells is the enrichment for bivalent domains. However, it is not the only unique feature of ES cell chromatin.

Pluripotent stem cells have less compacted chromatin and higher dynamics of interaction with chromatin-associated proteins and increased turnover of a linker histone H1, which most likely results from overexpression of chromatin remodelers. Even the distribution of chromatin in the nucleus is more even in pluripotent than in somatic cells. These features have been observed both *in vitro* and *in vivo* (Ahmed et al., 2010; Azuara et al., 2006; Bouwman & De Laat, 2015; Efroni et al., 2008; Fussner et al., 2011; Gaspar-Maia et al., 2011; Meshorer et al., 2006; Meshorer & Misteli, 2006; Otterstrom et al., 2019; Ricci et al., 2015; Schlesinger & Meshorer, 2019). As a result, basal transcription is also elevated in pluripotent cells (Efroni et al., 2008; Kobayashi & Kikyo, 2015; Ramalho-Santos et al., 2002). This includes exonic, intronic, and non-genic regions, causing a considerable profusion of nascent RNA as compared to differentiated cells

(Bouwman & De Laat, 2015; Efroni et al., 2008). Reduced levels of heterochromatic histone marks and increased levels of those associated with active transcription characteristic for ES cells fall in line with these observations (Efroni et al., 2008; Kobayashi & Kikyo, 2015; Jeong Heon Lee et al., 2004; Meshorer et al., 2006; Schlesinger & Meshorer, 2019; Wen et al., 2009). PRC2 complex plays an important role in maintaining pluripotency as in ES cells it is predominantly bound to promoters of genes related to differentiation (Boyer et al., 2006; Bracken et al., 2006; Denholtz et al., 2013; Kobayashi & Kikyo, 2015). Lower levels of DNA methylation, which is largely associated with repressed chromatin state, were observed in ES cells (Leitch et al., 2013; Marks et al., 2012; Schlesinger & Meshorer, 2019). Finally, one of the prominent features observed both in early embryos and in ES cells is a reactivation of an imprinted X chromosome (Mak et al., 2004; Okamoto et al., 2004; Surani et al., 2007; Wutz & Jaenisch, 2000).

Unraveling a great number of pluripotent stem cell-specific long-range interactions resulted from genome architecture studies (Apostolou et al., 2013; De Wit et al., 2013; Dixon et al., 2012; Kobayashi & Kikyo, 2015; Wei et al., 2013). Moreover, whereas the genome of differentiated cells is predominantly shaped in 3D around repressed heterochromatic regions, in ES cells the main structure-forming entities are hubs of active transcription dependent on core pluripotency factors (de Wit et al., 2013; Dixon et al., 2012; Kobayashi & Kikyo, 2015). These changes are notable even on a whole-nucleus level. For example, chromocenters, prominent in differentiated cells, show higher numbers and are less distinct in embryonic stem cells (Aoto et al., 2006; Bouwman & De Laat, 2015; Mayer et al., 2005; Schlesinger & Meshorer, 2019; Wiblin et al., 2005). Heterochromatin protein HP1β is almost diffused in ES cells but becomes accumulated at heterochromatic foci in the course of differentiation (Mattout et al., 2015; Schlesinger & Meshorer, 2019). Even interactions with nuclear lamina are massively reorganized between ES and differentiated cells (Kobayashi & Kikyo, 2015; Peric-Hupkes et al., 2010). Nuclear lamina itself lacks Lamin A and is less organized in pluripotent cells (Melcer et al., 2012; Pagliara et al., 2014; Schlesinger & Meshorer, 2019).

All in all, the evidence point that chromatin structure in pluripotent stem cells is distinct and is tightly interconnected with their undifferentiated state.

DNA repair in embryonic stem cells

ES cells represent a fascinating model for us not only for having unique chromatin features but also for their special mechanisms for maintaining genome integrity. Similar to other cell types, they are permanently

exposed to DNA damaging agents of both exogenous and endogenous origin. However, due to their rapid proliferation (as discussed above) they also undergo constant replication stress. These factors combined lead to constant activation of DDR (Ahuja et al., 2016; Juan et al., 2016; Nagaria et al., 2013; Savatier et al., 2002). However, ES cells are highly tolerant of this stress and show a lower level of spontaneously induced mutations and karyotypic aberrations than somatic cells (Brimble et al., 2004; Y. Hong et al., 2007; Nagaria et al., 2013; J. A. Thomson, 1998). Enhanced maintenance of genome integrity appears to be a characteristic of pluripotent cells (Y. Hong et al., 2007; Nagaria et al., 2013). In order to protect the genome of the whole pool stem cells readily sacrifice themselves by initiating apoptosis or differentiation (Aladjem et al., 1998; Yiling Hong & Stambrook, 2004; Lin et al., 2005; Jinping Liu et al., 2012; Nagaria et al., 2013). Of note, p53 plays a less role in ES cells, particularly it does not activate G1/S or intra-S checkpoint, and processes related DNA damage response and repair are regulated by a stem-cell specific protein Filia (Aladjem et al., 1998; Chuykin et al., 2008; Nagaria et al., 2013; Suvorova et al., 2016; vanderLaan et al., 2013; Vitale et al., 2017; B. Zhao et al., 2015). It has also been shown that some known pluripotency markers such as Sall4 (Sal-like protein 4) are directly involved in the process of DNA repair. It gets recruited to DSBs and helps to stabilize MRN complex and enhance ATM activation (Xiong et al., 2015). ES cells have enriched levels of DNA repair proteins, including HR and NHEJ factors, and enhanced levels of pro-apoptotic factors that are lost upon differentiation (Cooper et al., 2014). Together with constant activation of DDR, it is capable of sufficiently safeguarding their genome (Ahuja et al., 2016). Mouse ES cells were shown to have a strong preference towards HR and NHEJ pathway in DSB repair (Bañuelos et al., 2008; Nagaria et al., 2013; Serrano et al., 2011; Tichy et al., 2010). They have been shown to repair 80% of DSBs by HR and only 20% by NHEJ, whereas in somatic cells the ratio is inverted. Alt-EJ was equally both cases, which means a shifted balance towards the precise repair pathway choice (Nagaria et al., 2013; Serrano et al., 2011; Tichy et al., 2010). Therefore, it is of no surprise that knockouts of key HR players usually result in embryonic lethality (Lim & Hasty, 1996; Nagaria et al., 2013; Tsuzuki et al., 1996; Y. Xiao & Weaver, 1997; Jie Zhu et al., 2001).

Taken together, the presence of unique mechanisms of replications stress and DNA damage tolerance makes ES cells a fascinating model for investigation.

Genome editing

Artificial genome editing in a variety of species has long been an important task for both medical and research purposes. Medical applications include perspectives of gene editing for correction of disease-related mutations, disruption of disease-promoting genes or even introducing novel genes (e.g. for sensitising the immune system to tumour cells). The research application range is even broader: it includes creating knock-outs, knock-ins, and introducing mutations to study the role of particular proteins, processes, create disease models, and more.

Given tempting perspectives of practical use, it is of no surprise that the development of methods that would allow gene editing has been going on for a considerable time. At first, the strategy implied the use of random recombination, either to change the sequence or to allow further use of nucleases (such as Cre) (Carroll, 2017; ROTHSTEIN, 1989; Scherer & Davis, 1979; Smithies et al., 1985; Thomas et al., 1986). The discovery of zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) allowed a considerable advance if the field. However, the largest step has been done with the discovery of CRISPR-Cas9.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) are parts of bacterial mechanisms of antiviral defence (Carroll, 2014; Jansen et al., 2002; Sorek et al., 2013). Shortly after their discovery, the system was adopted as a tool of genome editing in other organisms, such as plants, fish, human, mice, and many more (Carroll, 2014; Cho et al., 2013; Hall et al., 2018; Hwang et al., 2013; Jinek et al., 2012; Ma et al., 2015).

The editing system consists of Cas9 protein and a short non-coding RNA that contains two parts, a target-specific CRISPR RNA (crRNA) and a helper trans-activating RNA (tracrRNA) (Karvelis et al., 2013; Memi et al., 2018). In the guide RNA, tracrRNA component is responsible for binding Cas9 and crRNA acts in sequence recognition by pairing to the target sequence (also called protospacer) (Memi et al., 2018; Pattanayak et al., 2013). However, Cas9 protein only performs catalytic reaction if a protospacer-adjacent motif (PAM) is present at the 5’ side of the protospacer (Memi et al., 2018; O’Connell et al., 2014). Later on single guide RNAs containing both crRNA and tracRNA sequences were designed to simplify the practical use (Mali et al., 2013; Memi et al., 2018).

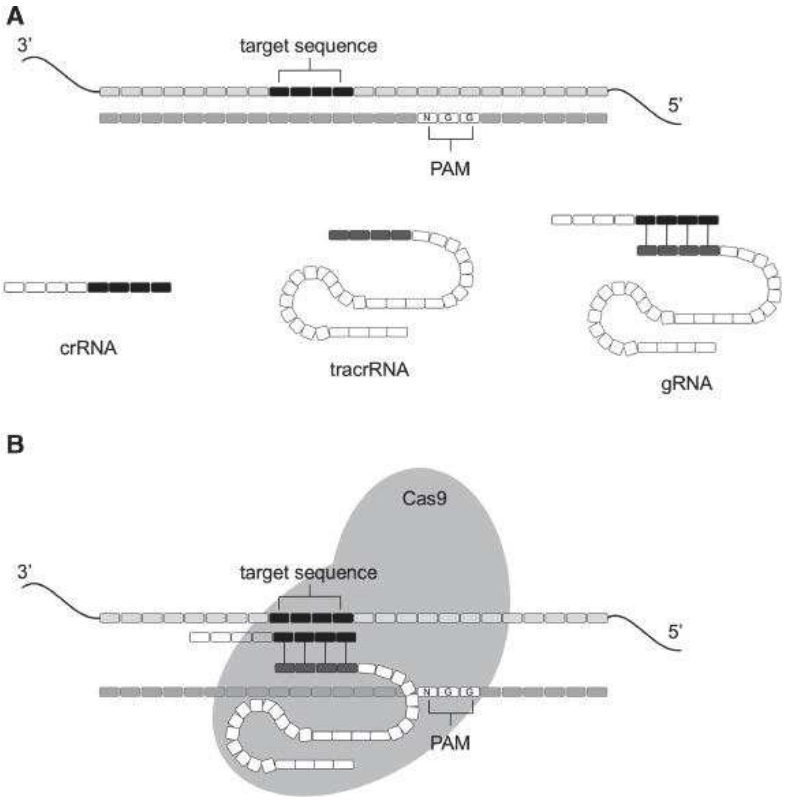

Fig. 11 Schematic representation of CRISPR-Cas0 system mechanism of action. Adapted from (Memi et al., 2018)

Promising as it is, this system still requires a thorough investigation, for the mechanism of action to be fully understood. Cas9 applications lie beyond genome editing and also include modulation of transcription, epigenetic modifications, genomic imaging, and lineage tracing (W. Deng et al., 2015; Gilbert et al., 2013; Hilton et al., 2015; Spanjaard et al., 2018; Haifeng Wang et al., 2016).

It is important for the procedures both in research and in clinical applications is that Cas9-mediated genome editing is achieved by introducing a break in a selected location. All the following events leading to repair of this break, either in a desired or in an undesired way, are carried out by the cellular mechanisms of DNA repair. In order to predict a repair outcome, we need an excellent understanding how these mechanisms work.

Genome editing fidelity studies

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REVIEW
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Genome Editing Fidelity in the Context of DNA Sequence and Chromatin Structure

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Genome editing by Clustered Regularly Inter Spaced Palindromic Repeat (CRISPR) associated (Cas) systems has revolutionized medical research and holds enormous promise for correcting genetic diseases. Understanding how these Cas nucleases work and induce mutations, as well as identifying factors that affect their efficiency and fidelity is key to developing this technology for therapeutic uses. Here, we discuss recent studies that reveal how DNA sequence and chromatin structure influences the different steps of genome editing. These studies also demonstrate that a deep understanding of the balance between error prone and error free DNA repair pathways is crucial for making genome editing a safe clinical tool, which does not induce further mutations to the genome.

Keywords: chromatin, dna editing crispr, knock in, DNA repair, nucleus

INTRODUCTION

Genome editing is very valuable for both medical and research purposes. Future medical applications include the correction of disease-related mutations, disruption of disease-promoting genes or even introducing novel genes (e.g., for sensitising immune system to tumour cells). Research applications range from creating knock-out/knock in cell line or organisms, and/or introducing mutations, to study the role of a particular protein, pathway or processes to creating humanized disease models. Given the tempting scope of practical use, it is of no surprise that there has been considerable effort in developing genome editing methods. The traditional way for introducing changes to the genome was by the use of spontaneous recombination, either to introduce DNA mutations or to insert sequences that would allow further use of recombinases (such as Cre) to excise genes [reviewed in Sauer (2002)]. Subsequent discoveries of zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) allowed a considerable advance in the field by allowing the introduction of DNA breaks at desired, rather than random, genomic locations [reviewed in Gaj et al. (2013)]. Nevertheless, the biggest advance in genome editing has been the more recent discovery of clustered regularly interspaced palindromic repeat (CRISPR) associated (Cas) systems (Ishino et al., 1987; Jansen et al., 2002; Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013).

Shortly after its discovery, the CRISPR-Cas9 system, a bacterial defense mechanism, was repurposed as a powerful tool for genome editing in plant, animal and human cells due to its specificity and its easier implementation. Current and future potential uses cover a wide range of application in research and clinical areas, by allowing substitution, insertion or deletion to the DNA sequence in a targeted genomic location [reviewed in Hsu et al. (2014) and Wang and Qi (2016)]. The CRISPR-Cas9 system operates through the recruitment of the RNA-guided Cas9 nuclease at a specific genomic position. The targeting relies on the complementarity between the guide RNA and the targeted sequences and the presence of an adjacent DNA protospacer motif (PAM). The Cas9 nuclease generates a DNA double strand break (DSB) at the targeted sequence adjacent to the PAM sequence (Jiang and Doudna, 2017), which then leads to recruitment of DNA repair machinery to fix the break.

Typically, DNA DSBs are repaired by (i) the error free homologous recombination (HR) pathway, which occurs in S/G2 phases of the cell cycle as it uses the homologous sequences of the sister chromatids as a repair template, and (ii) the error prone non-homologous end joining (NHEJ) pathway, which occurs throughout the cell cycle and religates DNA ends without the presence of an undamaged template (Ciccia and Elledge, 2010). In addition, other alternative end joining pathways, which rely on the presence of microhomologies (MH mediated end joining, MMEJ), have been described, these DSB repair pathways are error prone and are often associated with long deletions (Decottignies, 2013; Chang et al., 2017).

DNA end resection is a major determinant influencing DNA repair pathway choice. Unresected DNA ends, processed by the NHEJ pathway, are bound to the Ku complex (Ku70-Ku80 heterodimer) which recruits NHEJ factors including DNA-PKcs (DNA dependent protein kinase catalytic subunit), XRCC4 (X ray repair cross complementing 4) and LIG IV (DNA ligase IV) to catalyze DNA ends ligation. In contrast, the MMEJ pathway requires minimal DNA ends resection (through the CtIP-MRN complex) that reveals homologies on opposite strands that will be further involved in annealing. DNA portion between homologies is removed, leading to deletion scars. Other MMEJ factors are further recruited to resolve the break, including DNA polymerase θ (POL Q), and the DNA ligases I and III (Decottignies, 2013; Chang et al., 2017).

DNA repair pathway choice is regulated at different levels: cell cycle stage, availability and post translational modifications of DNA repair factors, chromatin status and the position within the nucleus of the break [reviewed in Kalousi and Soutoglou (2016)]. The choice of pathway can have critical consequences for the cell, since the use of error prone pathways can lead to unwanted deleterious mutations. Despite the many efforts put into characterizing repair pathways, Cas9-induced DSB repair outcomes have not been yet extensively investigated. It is crucial, for both research and clinical purposes, to precisely understand how mutation profiles observed following Cas9-induced DSB are generated, to be able to predict repair outcomes. In this review, we will focus on recent work highlighting the outcome of CRISPR-Cas9-induced DSBs in mammalian cells. Interestingly, the CRISPR-Cas9 mutational pattern appears to be non-random, highly reproducible and mainly dependent on the targeted DNA sequence.

CAS 9-MUTATIONAL PROFILES ARE LARGELY DEPENDENT ON THE TARGET DNA SEQUENCE

Several studies have revealed the prominent role of the target DNA sequence in Cas9-dependent DNA repair outcomes. In these studies, repair outcomes were profiled by classifying the mutations generated at Cas9 target sites by the type of insertion or deletion (indel) that occurred (e.g., size, position, microhomology), and monitoring the frequency of each class of indel. van Overbeek et al. (2016) were the first to conduct a systematic study of DNA repair profiles following Cas9 cleavage in human cell lines. They followed the repair outcomes after guide RNAs delivery targeting 69 different genomic sites and demonstrated that indel patterns differed from one targeted site to another and were very reproducible among replicates and between cell types. Nevertheless, the mutation frequencies of a given indel class varied with cell type. Taken together, this suggests that the characteristic DNA repair profile associated with a genomic location is influenced by the DNA sequence around the targeted area (van Overbeek et al., 2016). To further confirm this conclusion, guide RNAs matching multiple locations in human genome ("multiple target single spacers," MTSS) were designed and the associated indel profiles were assessed. In line with their previous observations, similarities between repair profiles for each site targeted by the same guide RNA are observed across replicates and cell type (van Overbeek et al., 2016).

Allen et al. (2018) confirmed such observations by specifically interrogating the influence of the DSB-flanking DNA sequence on repair outcomes. The authors designed and delivered synthetic constructs containing both a guide RNA and its target sequence flanked by variable DNA sequences, in human K562 cells. Indel profile analysis revealed that indels were highly reproducible and sequence-specific. Moreover, shorter deletions were more prominent compared to longer deletions, with nucleotide insertions (+1) and deletions (-1) being the most common. 58% of all Cas9-generated deletions, however, were at least 3 bp long and about a half of them occurred between at least two nucleotide repeats, referred to as microhomology (MH). The deletion frequency resulting from MH presence was inversely correlated with the distance between MH sequences. Introducing point mutation(s) in MH regions led to a remarkable drop in the associated repair outcome frequency (Allen et al., 2018). Intriguingly, although the indel patterns were similar across most cell types, stem cells had more large deletions and MH mediated products, whereas single nucleotide insertions (+1 insertions) were more frequent in differentiated cells. It was proposed that such observations correlate with different activities for the DNA repair pathways in different cell types.

Furthermore, indel profiling revealed that for almost half (49%) of the guide RNAs with a T (thymidine) before the cut site, a + 1 insertion involving another T dominates the repair outcome. A bias was also observed regarding small deletions: 77% of -1 deletions are associated with the removal of a repeated nucleotide at the break site. For half of the dinucleotide deletions, the removal of a two- base repeat was also quite common (Allen et al., 2018). These results are in agreement with Lemos et al. (2018), who demonstrated that single base insertions were shown to preferably repeat a PAM-distal nucleotide at the break site in yeast.

A recent large-scale study shed further light on the influence of genetic and epigenetic factors in CRISPR-Cas9 repair outcomes (Chakrabarti et al., 2019). Analysis of indel patterns at approximately 1,500 targeted locations in human cells (HepG2), revealed again that DNA editing precision differs across sites in a non-random and reproducible manner. The majority of examined targeted sites showed a preference for small indels (44% for 1 bp insertion and 26% for 1 bp deletion). However, a preference for large deletions (up to 41 nucleotides) was also observed for some sites. As a consequence of single nucleotide modifications, a considerable bias toward frameshifting mutations was observed (average of 80.1% compared to 66% of a random outcome).

Editing precision (recurrence of a specific indels) varied considerably between different targets with some targets associated with a large number (up to 79) of distinct, infrequent, deletions. In contrast, other targets showed one dominant mutation (representing up to 94% of all repair events). Overall, one fifth of all analyzed targets had at least a 50% chance of leading to a specific indel. Based on the distribution of commonest indel frequencies, the targeted sites were categorized into three groups: imprecise (commonest indel frequency below 25%), middle (commonest indel frequency below 50%), and precise (commonest indel frequency above 50%) sites. The vast majority of recurrent indels in precise targets (68.4%) are associated with a strong preference for insertions with a bias toward single nucleotide indels. In agreement with Allen et al. insertion, of a single nucleotide homologous to a PAM distal nucleotide (at position -4) at the break site was very common, especially when this nucleotide is T. These observations are consistent with Taheri-Ghahfarokhi et al. (2018), who also highlighted the importance of the 4th nucleotide before the PAM in the single nucleotide indel frequencies.

Strikingly, not only the indel pattern but also the editing precision could be predicted from the target site DNA sequence. Using a neural network Chakrabarti et al. found a significant correlation between the computational (estimated) and the observed indel frequencies. Despite a moderate predictive power of the model, it allowed the identification of key sequencing features. This computational quest also led to the conclusion that the nucleotide at position -4 from the PAM strongly influences the repair outcome in accordance with all previous experimental observations.

All in all, both by experimental studies and computer simulations, the Cas9-associated indel pattern and a presence of a dominant pattern appear to be mostly dependent on the DNA sequence around a break site, with the presence of MH in the target DNA sequence one of the main cues for predictability.

CAS 9-MUTATIONAL PROFILES RELY ON MMEJ

The types of indel observed upon CRISPR-Cas9 cleavage suggest that Cas9-induced breaks are mainly repaired by NHEJ and MMEJ. It is generally assumed that small indels (<3 bp) occur via NHEJ and longer deletions occur via MMEJ. When analyzing the indel distribution following CRISPR-Cas9 activity over for a 48 h period, van Overbeek et al. showed that larger deletions are more prevalent at later points. They also observed that upon inhibition of NHEJ, +1 insertions and small indel (<3 bp) frequencies were decreased and, in contrast, large deletions (>3 bp) frequencies were increased (van Overbeek et al., 2016). The fact that alteration of NHEJ leads to increased MMEJ usage points to a tight balance between NHEJ and MMEJ pathways in repairing these breaks. Similar studies were performed later by Brinkman et al. for a single locus in human K562 cells. Targeting the *LBR* locus, the indel pattern analysis revealed a +1 insertion in balance with a -7 bp deletion. Addition of the NHEJ inhibitor NU7441 led to an increase of -7 deletions concomitant to a decrease in +1 insertions. Addressing the kinetics of the two processes revealed that MMEJ is delayed and initiated after NHEJ, and the delay is not observed when NHEJ is inhibited arguing for MMEJ predominantly being used as a back-up to repair breaks that, for unknown reason, failed to engage NHEJ (Brinkman et al., 2018).

Aiming to characterize in detail the contribution of the MMEJ pathway in the repair outcomes following Cas9 activity, Taheri et al. developed a computational platform called RIMA (Rational Indel Meta Analysis). Two datasets from the literature were reanalyzed using RIMA to validate their approach. They confirmed MMEJ pathway involvement in DNA repair after Cas9 cleavage and MMEJ-associated indels enrichment upon NU7441 (Bae et al., 2014; van Overbeek et al., 2016; Taheri-Ghahfarokhi et al., 2018). They also confirmed that larger indels and other MMEJ events relied on the activity of the known MMEJ factor POLQ (Taheri-Ghahfarokhi et al., 2018).

Experiments to determine the contribution of MH to the CRISPR-cas9 dependent DNA repair outcome by Chakrabarti et al. revealed that microhomologies of different sizes were responsible for a majority of deletions (73.3%). Strikingly, deletions associated with short microhomologies (1–4 bp), typically not considered as a substrate for MMEJ, were also enriched indicating a role for homology regions of any length MH, not restricted only to long regions of MH as had previously been believed (Chakrabarti et al., 2019). In line with these observations, Bae et al. found that a large subset of all observed deletions upon Cas9 activity were associated with 2–8 bp MH sequences. Based on this observation, the authors developed a computer program to predict MH-dependent deletions at a given site in order to increase the frequency of gene disruption (Bae et al., 2014).

Despite how incomplete our understanding of the exact role of MH involvement in the repair process is, it has already been flagged for its potential practical applications. In their recent work, Kim et al. demonstrated the possibility of using this genomic feature for obtaining a desired genome editing effect. They suggested an elegant two-step scheme for introducing point mutations in human iPS cells, associated with scar-less selection marker excision. Initially the desired mutation is introduced into the locus of interest as engineered MH sequences flanking a selection marker used as a donor. Although positive selection based on the presence of the selective marker represents an easy way to obtain clonal population, some applications require the removal of the selective marker. Therefore after positive selection, the selection marker can be excised using CRISPR-Cas9 induced DSBs targeting the region adjacent to the MH sequences, promoting the use of MMEJ for the selection marker excision while preserving the point mutation (Kim et al., 2018).

Overall, based on both computational and experimental studies, MH arises as a major factor influencing the DNA repair outcome at CRISP-Cas9 lesions. However, whether it is indeed an underestimated role of the MMEJ pathway or a lack of a deep understanding of NHEJ pathway functioning remains to be seen.

CAS 9-MEDIATED LARGE DELETIONS AND COMPLEX REPAIR OUTCOMES

Most of the studies addressing repair of Cas9-induced breaks were focused on deletions of a relatively small size, based on the belief that NHEJ and MMEJ are the main pathways involved. However, large-scale indel pattern analysis highlights the complexity of Cas9-dependent repair outcomes. Such complexity is well depicted in the Shin et al. study where they analyzed the consequence of CRISPR-Cas9-mediated genome editing in founder mice (Shin et al., 2017). They showed that the majority of detected deletions were asymmetric (1.5-fold or more difference between deletion up-and downstream of the cutting site). Prevalence of asymmetric indels was observed for almost all targeted sites. Symmetric deletions were infrequent and tended to be small (less than 10 bp). Moreover, the deletions mostly occurred at repetitive regions, which is consistent with the conclusions of the above-mentioned studies relating to the role of MH in DSB repair.

Induction of DSBs with single guide RNAs in murine zygotes also revealed a 9 bp median deletion size, but larger deletions (up to 600 bp) were also present (Kim et al., 2018).

Testing whether sequential or simultaneous guide RNAs delivery would have any effect on an indel pattern and on a balance between small and large deletions, revealed that sequential guide RNAs delivery is more reliable than simultaneous in precisely deleting juxtaposed sites. Moreover, while no difference was observed for smaller deletions (less than 400 bp) between the two delivery strategies, deletions larger than 400 bp (up to 24 kb) were only present after simultaneous delivery. These large deletions didn't appear to rely on the presence of MH (Kim et al., 2018).

In light of the potential therapeutic use of Cas9, the findings of Kosicki et al., 2018 are especially striking. The authors explored large genetic alterations observed after CRISPR-Cas9 activity, focusing primarily on large deletions, which often are missing from repair outcome analysis due to a strong focus on a region proximal to the break (Kosicki et al., 2018). They performed knock-out experiments in mESC with single guide RNAs and observed that more than 20% of resulting alleles carried large (>250 bp and up to 6 kb) deletion. Even more surprisingly, in more than 15% of cases they observed additional DNA alterations (point mutations, large or small indels), distal to the cut site. Large inversions and duplications were also observed. Using mESCs obtained from a cross between two murine strains, Kosicki et al. also observed cases of loss of heterozygosity, presumably caused by using a homologous chromosome as a template. Despite differences in indel profile frequencies observed between stem cells and differentiated cells (Allen et al., 2018), larger deletions are not a unique feature associated with stem cells since they were observed in mouse hematopoietic progenitors cells and human RPE-1 cells (Kosicki et al., 2018).

Together, these data suggest, Cas9-mediated genome editing appears to be more complex and involves larger genome regions than was thought before. Thus, it is extremely important to understand the reasons for such an effect, and to take this into account while assessing using Cas9 for any medical purpose.

CHROMATIN STRUCTURE INFLUENCES CAS 9 BINDING

The chromatin structure around DNA breaks influences DNA repair pathway choice (Kalousi and Soutoglou, 2016). However, regarding the repair of Cas9-mediated breaks, the question arises; which step of Cas9 editing (binding, cutting and/or repair) is most influenced by chromatin state? To dissect this, some *in vitro* and *in vivo* studies have been performed. First, Isaac et al. developed a biochemical assay to determine how nucleosomes and chromatin remodellers influence Cas9 activity. Using nucleosome assembly associated with poor breathing (a term that defines the dynamic binding of histones to DNA), they observed that Cas9 binding activity and cutting is inhibited. In contrast, Cas9-induced cleavage is achieved near to the entry/exit of a nucleosome assembly associated with higher breathing. Furthermore, the authors demonstrated that different classes of chromatin remodellers enhanced Cas9 activity, with an increase of Cas9-mediated cleavage in the presence of remodellers from the ISWI family promoting nucleosome sliding (SNF2h) or histone octamer eviction (RSC) (Isaac et al., 2016).

At the same time, a study conducted by Horlbeck et al. led to the same observations *in vivo* and *in vitro*. The authors first overlaid data obtained from a CRISPR screen (Gilbert et al., 2015) with MNase-seq experiments publicly available at ENCODE (performed in K562 human cells) and observed that high nucleosome occupancy is associated with low CRISPR interference activity (for CRISPR interference, catalytically inactive Cas9 is fused to a transcriptional repressor and guided to the targeted site in order to interfere with

gene transcription) (Horlbeck et al., 2016). Along similar lines, *in vitro* experiments argued for a block of Cas9 activity in the presence of DNA assembled into nucleosomes (Hinz et al., 2015; Horlbeck et al., 2016). Using an inducible system to control chromatin state (open or close) in human cells at a specific locus, Daer et al. observed reduced editing efficiency associated with heterochromatin (closed state) due to a reduction in Cas9 binding, for six over a total of nine guide RNAs used. This observation suggests that the effect of closed chromatin on Cas9 editing is guide RNA dependent or that in such inducible system the closed chromatin spreading is not covering equally all targeted sequences. Nevertheless, the mutation signature was not affected by the chromatin state. Interestingly, editing efficiency could be restored by artificial transcription activation (Daer et al., 2017).

Cas9 binding has also been studied in ChIP experiments in mouse ESC in which catalytically inactive Cas9 (dead Cas9) has been expressed. These studies also revealed that chromatin accessibility (assessed by DNAse I hypersensitivity experiments) is an important determinant of Cas9 binding *in vivo* and the vast majority of Cas9 off target sites are associated with active genes (Wu et al., 2014). Such findings were later confirmed by Kuscu et al. (2014) and O'Geen et al. (2015) that demonstrated a correlation between open chromatin and Cas9 off target binding in human and mouse cell lines, respectively.

Thus there is a general agreement that Cas9 activity is influenced by chromatin structure both *in vivo* and *in vitro*, with closed chromatin associated with less Cas9 binding and editing.

THE ROLE OF CHROMATIN IN CAS 9-MEDIATED GENOME EDITING

The degree of influence of chromatin state over Cas9-induced mutagenesis has been the subject of studies by several research teams over the last few years. Chen et al. interrogated how chromatin status influences TALEN and CRISPR-Cas9 genome editing activity. For this purpose, a cellular system carrying a reporter in which chromatin status can be switched from compacted (H3K9me3 marked) to relaxed was used. Lower editing efficiency was observed when targeted sites were associated with heterochromatin for both TALENs and Cas9 nucleases, but the impact of chromatin state on editing was higher for TALENs. Interestingly, the efficiency of DSB formation was quite comparable (Chen et al., 2016). Subsequently, Chen et al. assessed the influence of chromatin structure on Cas9 editing in whole organisms. Zebrafish embryos were co-injected with guide RNAs and Cas9 mRNA. Editing efficiency positively correlated with chromatin accessibility (determined by ATAC-seq), and mutation rates were higher in an open chromatin. However, there was no correlation between nucleosome-occupancy and editing efficiency (Chen et al., 2017). The latter can be explained by high nucleosome dynamics in early zebrafish embryos, which is in line with the observations of Isaac et al., 2016 that pointed out that Cas9 activity is influenced by nucleosome breathing (Isaac et al., 2016). A study conducted by Kallimasioti-Pazi et al. induced Cas9 breaks at three different imprinted genes in mESC and demonstrated a delayed accumulation of mutations in heterochromatin compared to euchromatin. The allele-specific editing bias toward the active allele was particularly apparent in the case of low Cas9 expression or short Cas9 expression periods. In cells in which imprinting at the targeted locus had been lost, due to prolonged culture, there was a restoration of Cas9 editing efficiency, which again implies an heterochromatic environment impairs editing (Kallimasioti-Pazi et al., 2018). It does not appear to be the DNA methylation status of heterochromatin that is responsible for affecting cas9-mediated break editing, since Hsu et al. demonstrated that Cas9 mediated cleavage is not affected by CpG DNA methylation as supported by indel detection (around 8%) at the silent highly methylated SERPINB5 targeted locus (Hsu et al., 2013).

Kallimasioti-Pazi et al. (2018) could detect by allele-specific ChIP, that Cas9 binding was lower in heterochromatin, which correlated with the slowed rate of mutagenesis, thus confirming conclusions of Isaac et al. (2016) and Daer et al. (2017). Interestingly, despite distinct epigenetic statuses, the same mutation pattern was observed on maternal or paternal alleles arguing for an influence of heterochromatin on the kinetics but not on the outcome of Cas9 editing (Kallimasioti-Pazi et al., 2018). In line with such observations, using live cell single-molecule tracking in mouse cells, Knight et al. (2015) have demonstrated that even if Cas9 search efficiency is reduced in heterochromatic regions, Cas9 is still able to access successfully such regions (Knight et al., 2015).

Chakrabarti et al. have also come to similar conclusions. They observed that upon treatment with the histone deacetylase inhibitor TSA, indel formation is increased suggesting that chromatin decompaction augments Cas9 binding and editing efficiency (Chakrabarti et al., 2019). These results are in line with previous observations arguing for a lower editing efficiency associated with heterochromatin status (Chen et al., 2016; Daer et al., 2017; Kallimasioti-Pazi et al., 2018). In contrast, inhibition of the H3K27me3 methyltransferase EZH2, reduced indel formation, but with a less pronounced impact than TSA treatment. The fact that HDAC inhibition leads to the loss of constitutive heterochromatin and EZH2 inhibition, of facultative heterochromatin, suggests that different types of heterochromatin affect Cas9 editing in distinct ways (Chakrabarti et al., 2019). Nevertheless, these differences might not reflect only direct chromatin changes but indirect alterations on gene expression of DNA repair or other relevant genes. In agreement with this notion, even though both TSA and Ezh2i had an effect on indel formation, the authors were able to observe changes only in chromatin acetylation and not in H3K27me3 methylation. The same study demonstrated differences in ratios of different indels depending on a chromatin context. However, this did not affect dominant indels, suggesting that these changes are minor (Chakrabarti et al., 2019). Such results support the notion that in addition to the sequence around the break, certain chromatin context can modulate editing effectiveness.

Therefore, based on multiple studies with different experimental approaches and systems, we can conclude

that chromatin state influences Cas9-mediated genome editing efficiency with heterochromatin being an obstacle for this process. However, indel patterns are mostly unaffected.

CAS 9 FOR KNOCK INS (KIS)

Utilization of the CRISPR-cas9 system for genetic replacement is particularly exciting as it can be implemented in the clinical setting for the cure of genetic diseases. Genetic replacement or KI is mediated by homology-directed repair (HDR).

Several recent studies have investigated the best ways to increase KI potential using Cas9. The most efficient way described so far is incorporation of a single stranded oligonucleotide DNA (ssODN), via single-strand template repair (SSTR). Farboud et al. performed a study in *C. elegans* to determine an efficient strategy to increase knock in efficiency. Their initial goal was to introduce point mutations as it is often required for therapeutic reasons. They used short single-stranded oligonucleotides as a template for recombination matching with the protospacer or with the spacer strand. Interestingly, they found that single nucleotide polymorphism (SNP) insertion was strongly biased toward $5'$ or $3'$ of the PAM according to the use of the protospacer or the spacer strand (respectively) as a repair template (Farboud et al., 2019). Such polarity can be mainly explained by synthesis-dependent strand annealing (SDSA) mechanism, an HDR pathway in which resected end is annealed to the repair template and extended. After template dissociation the extended end anneals to the other DSB end followed by DNA synthesis to fill the gap (Farboud et al., 2019).

Richardson et al. (2016) also discovered that the binding kinetics of Cas9 with the target DNA is asymmetric. Although Cas9 has a slow release from the template, it releases first the 3' end of the cleaved DNA strand that is not complementary to the sgRNA (or non-target strand). They observed that the use of an asymmetric donor DNA, complementary to the non-target strand, with 90 nt and 30 nt overlapping the PAM proximal and distal sites respectively, is associated with a higher HDR rate (Richardson et al., 2016). Such findings highlighted the importance for an optimal donor DNA design to ensure high HDR. The same strategy was used to increase HDR efficiency when using ssODN as a donor to correct the β-globin gene (HBB) carrying a mutation responsible for the sickle cell disease (SCD) in human hematopoietic stem/progenitor cells (DeWitt et al., 2016). Another recent study by Okamoto et al. demonstrated the influence of the Cas9 re-cutting capacity of the template DNA on the knock in efficiency using ssODNs. The authors found that either by introducing mutations at the donor sequences that resulted in blocking the re-cutting or either by expressing Cas9/sgRNA transiently using Cas9 protein/sgRNA ribonucleoprotein complexes had a substantial increase on the knock in efficiency (Okamoto et al., 2019).

The use of short single-strand templates was more efficient than a double-strand templates for knock in Farboud et al. (2019). It has recently been demonstrated that in human cells, repair based on a short single-stranded template is Rad51-independent and managed by the Fanconi anemia pathway (Richardson et al., 2018). Thus, differences in efficiencies could be explained by the use of different pathways, and potentially by differential requirements for the length of a template. In the case of a large DNA fragment insertion, the use of a double-stranded template becomes a requirement. For large fragments insertions, Farboud et al. were able to introduce a 9.3 kb fragment by adding a second DSB 340 bp from the initial DSB site. Interestingly, HR efficiency is influenced by the orientation of PAMs. Efficiency was much higher when recognition sites were selected on different strands rather than a single strand. These results suggest that the sequence around the break is important for Cas9-mediated knock in efficiency using larger DNA sequences as donors (Farboud et al., 2019). Insertion efficiency mediated by HR, for DNA fragment as long as 800 bp is also increased after NHEJ inhibition (using Scr7 ligase IV inhibitor treatment) in a bone marrow derived dendritic cell line (DC2.4) (Maruyama et al., 2015). Similarly, SSTR was increased in several genes and cell types when cells were baring a mutation into the human PRKDC gene (encoding for the DNA-PKcs protein) that suppress DNA-PKcs kinase activity (Riesenberg et al., 2019). Promoting homology directed repair (HDR) was also achieved through 53BP1 (a pro-NHEJ factor) inhibition in both human and mouse cells (Canny et al., 2018). This observation might be useful for knock in experimental design.

Since HR takes place during replicative and post replicative stages of the cell cycle, Gutschner et al. developed a system to restrict Cas9 expression to S/G2/M cell cycle phases. By fusing the Cas9 nuclease to geminin they were able to convert Cas9 into a substrate for the APC/Cdh1 complex, which promotes proteins ubiquitination and therefore degradation during late M and G1 phases. In a reporter assay, they monitored HDR-mediated EGFP expression restoration and showed an increase in HDR rate (up to 1.87-fold compare to wt Cas9). They also observed an increase of HDR at a target endogenous locus in HEK293T cells (Gutschner et al., 2016). Along the same lines, delivery of the Cas9/sgRNA ribonucleoprotein complex in cells arrested with nocodazole and aphidicolin and then released, increased SSTR (Lin et al., 2014).

Other groups developed strategies to increase HDR efficiency, allowing spatial proximity between the DSB site and the repair template. By fusing Cas9 to the PCV protein (porcine circovirus 2 rep), forming robust covalent link to a donor DNA, Aird et al. were able to increase HDR efficiency in human cell lines. Using different assays, they showed that covalent tethering of donor DNA template enhances (i) HDR mediated peptide-tag insertion (up to 30-fold) and (ii) HDR mediated mCherry fluorescence restoration (in reporter cells expressing a mutant mCherry) (Aird et al., 2018). Savic et al. came to the same conclusion using snap-tag technology to link donor DNA template to Cas9 and showed that repair template linkage enhances HDR efficiency in a fluorescent reporter cell line and, importantly, also at targeted endogenous loci in K562 and mES cells (Savic et al., 2018).

Another approach to increase HDR efficiency using the Cas9 nuclease fused to CtIP protein (an essential factor promoting

DNA end resection) has been described by Charpentier et al. They revealed that tethering CtIP next to the DSB site enhances GFP transgene integration in human fibroblasts. HDR stimulation was also observed in human iPS cells and rat oocytes but depends on the guide RNA (Charpentier et al., 2018).

Chromatin structure has a big influence on homologous recombination (Clouaire et al., 2018; Mitrentsi et al., 2020) but weather it has any influence on Cas9-mediated KI still remains elusive. The expectation is that it will be largely affected by the pre-existing structure of the chromatin surrounding the break. Kallimasioti-Pazi et al. however, found no consistent influence of pre-existing chromatin state on HDR efficiency across several imprinted genes. Systematic analysis on different genomic sites corresponding to different chromatin states will shed more light into the issue.

CONCLUSION

In conclusion, genome editing using targeted nucleases, including Cas9, is a complex process, and its success depends on our understanding of specific mechanisms of DSB repair. It has become clear that repair outcome is predominantly sequence-specific and can minimally be altered by other factors. On the other hand, editing efficiency can be influenced by local chromatin structure and therefore can be improved by a change in the chromatin environment.

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LC and OM collected the literature and wrote the manuscript. ES wrote the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Goals

In my project, I intended to investigate two different questions.

First, I wanted to elucidate the influence of the pluripotent state on the fidelity of DSB repair and a pathway choice. It has been previously reported that HR is enhanced in ES cells, which provides the necessary level of genome stability. However, current knowledge is mostly based on either a study of indirect factors, such as proteins involved in certain DNA repair pathways, or studying single loci, which cannot always be extrapolated to the whole genome. Therefore, we found it relevant to perform a study tackling several targets and relying on repair outcomes rather than factors recruiting.

Second, I aimed to investigate the role of a local chromatin structure on a repair outcome and a balance between different repair pathways. Bivalent chromatin was one of the types of particular interest for me as its influence on the process of DNA repair has never been studied yet. Other chromatin types, like euchromatin and facultative heterochromatin, appeared important to study as well. On the one hand, they could serve as a reference to compare behaviour of bivalent chromatin to. On the other hand, existing studies provided incomplete information, as they were based on indirect detection of a pathway use rather than assessing a direct repair outcome.

Therefore, the goals of my work were as following:

1. Compare fidelity of double-strand break repair between different chromatin contexts, and particularly in bivalent chromatin
2. Compare fidelity of double-strand break repair between pluripotent and differentiated cells.

Results

Establishing an experimental system

In my project, I set a goal to follow the process of a single break repair, contrary to many previous studies that were inducing DNA damage globally or at repetitive elements. Those experiments were leading to a large number of simultaneous breaks, which models certain extreme situations cells might face during the lifespan but not physiological conditions. The discovery of the CRISPR-Cas9 system provided an excellent tool for creating DSBs at any selected single locus. Being particularly interested in DSB repair in mouse ES cells I, therefore, needed to establish a mouse ES cell line stably expressing Cas9. As an original plan for the project included an option of time-course experiments, we decided to use a degradable system. Therefore, I cloned a construct that consisted of Cas9 fused to GFP and a mutated FKBP12-derived destabilization domain (DD), and NeoR gene as a selective marker (fig. 12). Fusion to DD is often used for controlled protein function perturbation, as it allows rapid proteasomal degradation in mammalian cells, unless bound by a synthetic ligand Shield 1 (Banaszynski et al., 2006).

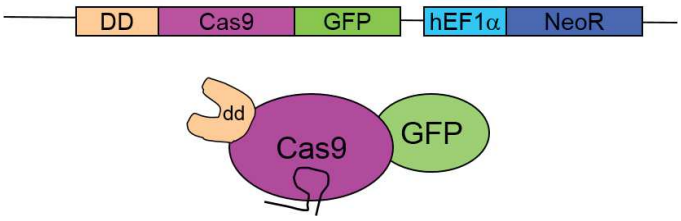


Fig. 12 A scheme of the construct used to create stable cell lines

Clones obtained after antibiotics selection and single-cell FACS sorting were tested by FACS in the presence and absence of Shield1, and two clones with robust induction and minimal leakiness were selected (fig. 13).

Two selected clones were additionally transfected with the suicidal cassette (kindly provided by Dr. Alexey Tomilin). This cassette consists of PuroR and thymidine kinase (TK) genes under the control of the minimal Oct4 promoter. Such cassette allows selective elimination of either pluripotent of differentiated cells by applying ganciclovir or puromycine respectively (Liskovykh et al., 2011).

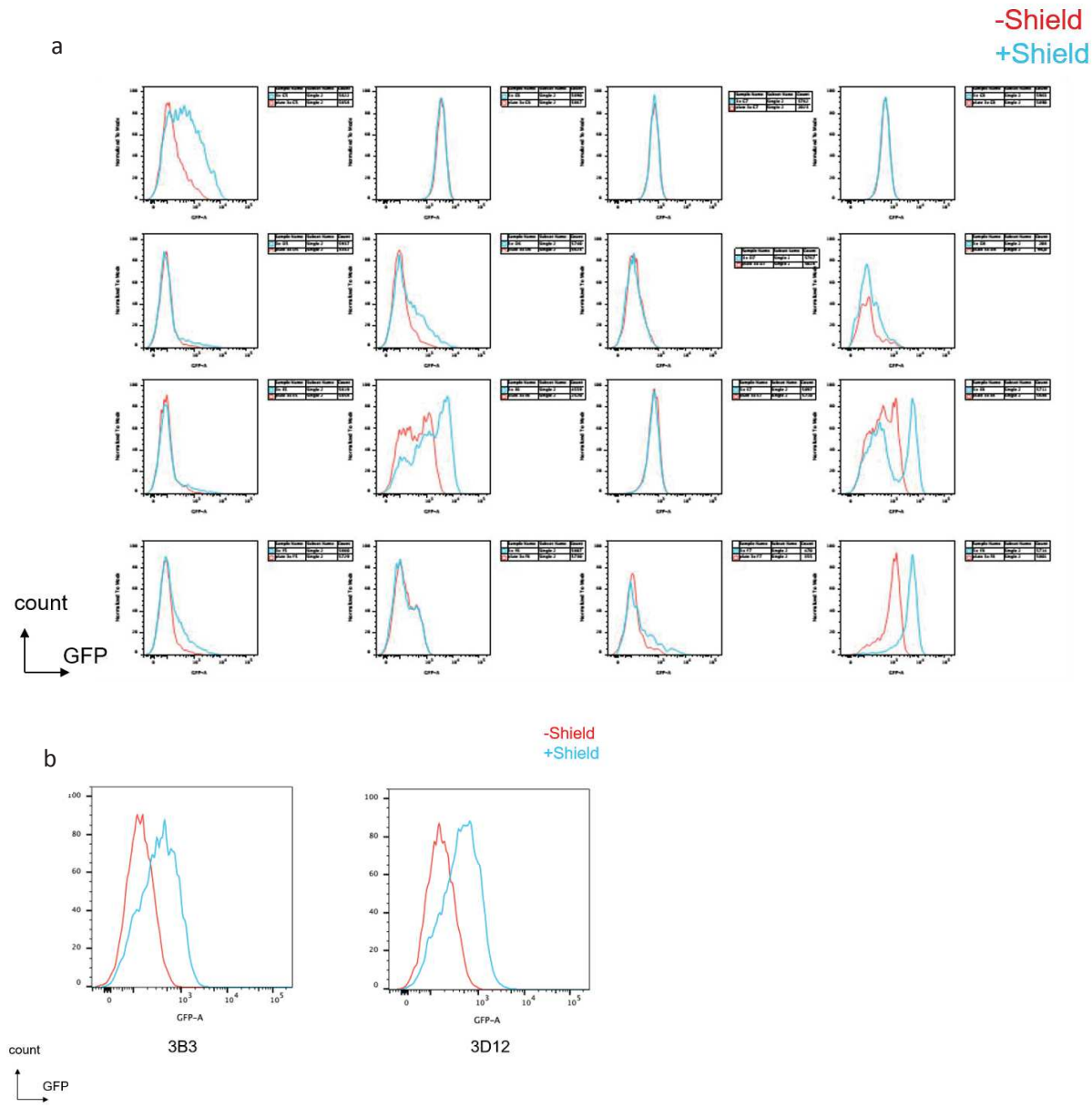


Fig. 13 a) Typical FACS results for several analysed clones; b) FACS results for clones selected for analysis

The functionality of the system was proven by transfecting selected clones with the guide RNA for major satellite repeats previously designed in the lab (Tsouroula et al., 2016). Bound to this guide RNA, Cas9 is capable of causing hundreds of DSBs per cell, and the amount of DNA damage is enough to allow γH2AX increase detection by Western Blotting (fig. 14).

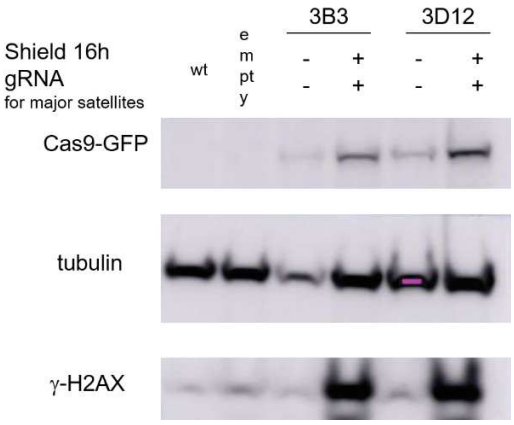


Fig. 14 Confirming Cas9 functionality by WB: γH2AX levels are increased after transfecting with a guide RNA for major satellites.

After confirming that the system is functional I tested the kinetics of Cas9 activation and inactivation. The test was performed by FACS analysis of GFP fluorescence, and it showed that both activation and inactivation reach a plateau after 10 hours after Shield1 addition or withdrawal respectively (fig. 14 a, b) Titration of Shield1 allowed me to find the working concentration necessary and sufficient for the full activation (fig. 14 c).

Having the system set up and tested I was able to switch to experiments with single-cutting guide RNAs.

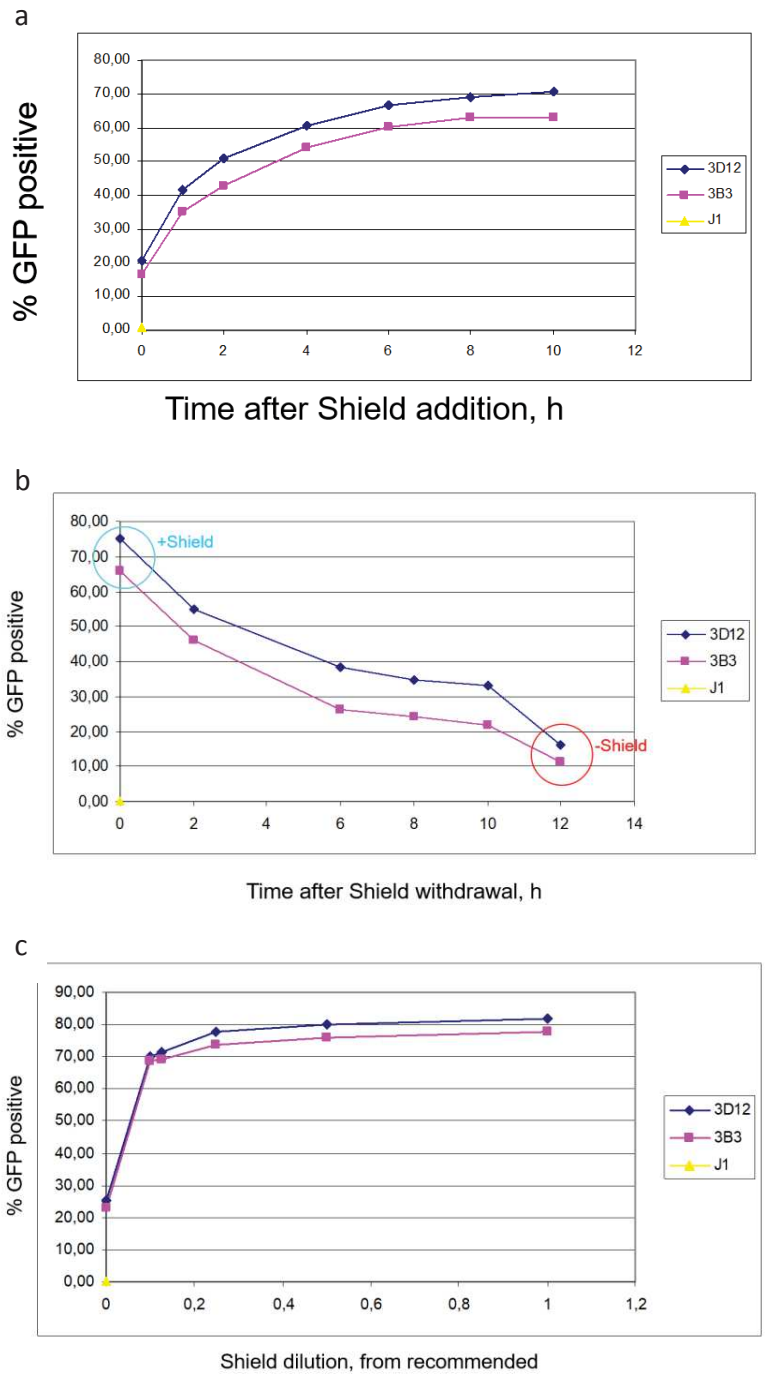


Fig. 14 a) Cas9-GFP stabilisation after Shield1 addition for both selected clones; b) Cas9-GFP degradation after washing out Shield1; c) Titration curve for Shield1 for both selected clones. All results are obtained by measuring GFP fluorescence by FACS analysis

Target choice and guide RNA design

The goal of my project was to compare DSB repair in different chromatin contexts. Therefore, targets were selected so that they would represent different chromatin types. I chose several groups of genes depending on their functionality and epigenetic status:

- housekeeping genes (Gapdh and Actin), further colourcoded as pink
- pluripotency regulators (Pou5f1, Nanog, and Tfcp2l1), further colourcoded as green
- developmental regulators that are bivalent in ES cells (Pax6, Ngn2, Zic1, Dlx2, Zfpm2, Foxa), further colourcoded as yellow
- facultative heterochromatic genes (Hoxb1, Tdrd1, and Mc4r), further colourcoded as red
- genes belonging to LADs (Sox6, Ptn, and Nrp1), further colourcoded as purple

Given that one of the chromatin types of interest was bivalent, and these domains are typically found at promoter regions, most of the guide RNAs were designed within a proximal promoter of a corresponding gene. The only exception was the group of genes located in LADs, as this part was connected to another project running at the lab, and introducing breaks at the introns was required. Proximal promoters were defined as 1 kb up- and downstream of the TSS according to the BindDB web tool (Livyatan et al., 2015). Guide RNAs were designed using the Benchling website (https://www.benchling.com/). guide RNA sequences are provided in Table 1 in the Materials and methods section.

Chromatin status at targeted sites was confirmed by ChIP. I used H3K4me3 as a mark of active chromatin, H3K27me3 as a mark of facultative heterochromatin, and H3K9me2 as a mark for LADs. I also tested H3K9me3 as a marker of constitutive heterochromatin. I performed ChIP in ES cells. 3T3 cells were used as a differentiated control. I could observe an expected chromatin pattern in ES cells. In 3T3 cells only housekeeping genes of all tested were active, and the rest represented facultative heterochromatin (fig. 15).

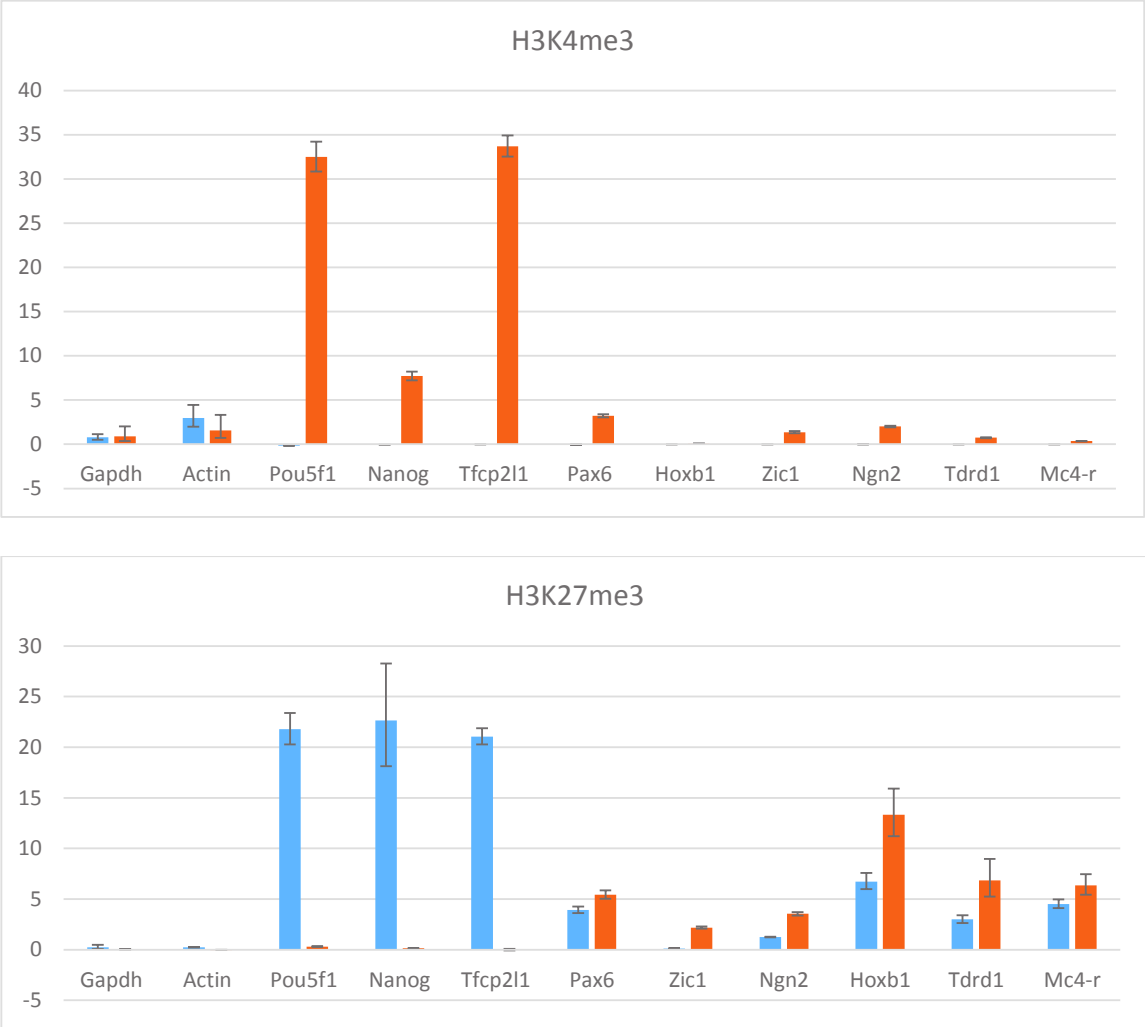


Fig. 15 ChIP-qPCR results for selected targets. Results for 3T3 cells are shown in blue and for ES cells in orange. Error bars represent s.d. from 3 technical replicates.

TIDE analysis. Indel pattern is sequence-specific and is not influenced by a cell type or a chromatin context

To analyse repair fidelity after introducing a break I employed the Tracking indels by decomposition (TIDE) method (Brinkman et al., 2014). This method is based on PCR and Sanger sequencing and allows calculating the percentage of insertions or deletions (indels) of a particular length out of the total pool of DNA present in the sample. In this method, a fragment around the expected cut is PCRed from gDNA extracted from cells transfected with the guide RNA and from non-transfected (control) cells. PCR products are then sent for Sanger sequencing. If a break was introduced by Cas9 and repaired with indels, a corresponding shift of the

following sequence occurs. As a result of a merge of sequences with different repair outcomes, superposed nucleotide picks appear on a chromatogram. The original sequence and height of individual peaks available from the control sequence allow TIDE software to decompose the signal and return ratios of different indels (fig. 16). This technique gave us an opportunity to estimate both total employment of error-prone repair and an indel pattern for each condition.

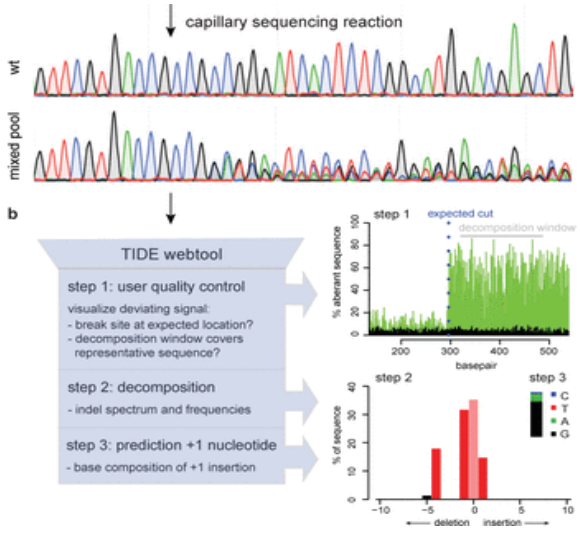
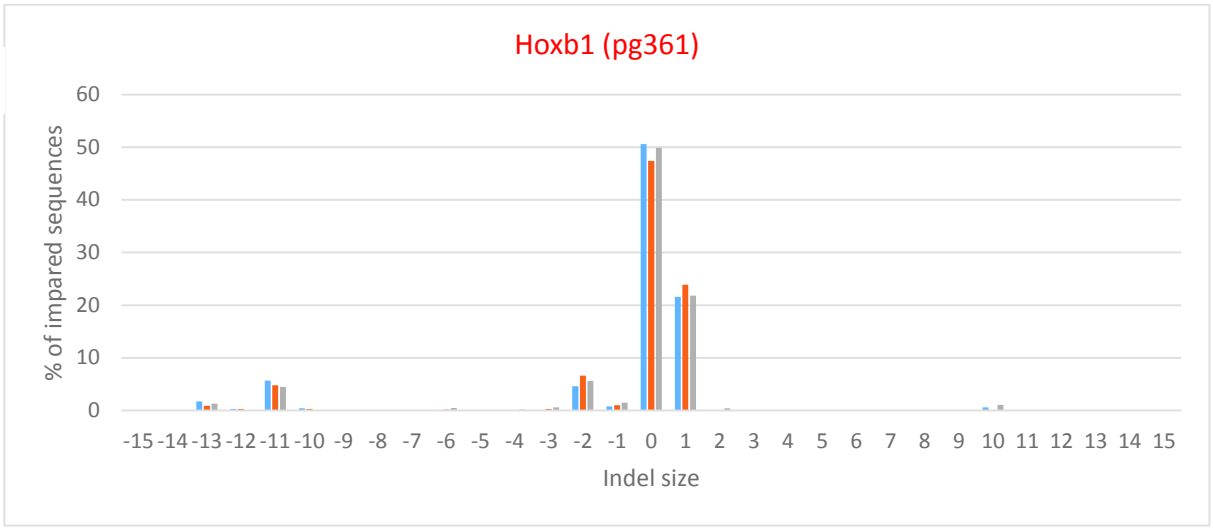


Fig. 16 A scheme of TIDE experiment. Adapted from (Brinkman et al., 2014)

The first observation I could make was the high reproducibility of the indel pattern for each guide RNA (fig. 17 a). Using a Hoxb1 guide RNA as a representative example, we can see that significant indels do not vary between replicates. Variations between non-significant indels (and possibly even their presence) can be explained by the sample processing or sequencing. This goes in line with observations of other teams discussed in the introduction. It also became apparent that the indel pattern had only slight variations between 3T3 and ES cells (fig 17 b). Using Actin and Hoxb1 guide RNAs as a representative example, the comparison between 3T3 and ES cells clearly shows that although ratios might vary, the overall array of significant indels remains largely unchanged.

a



b

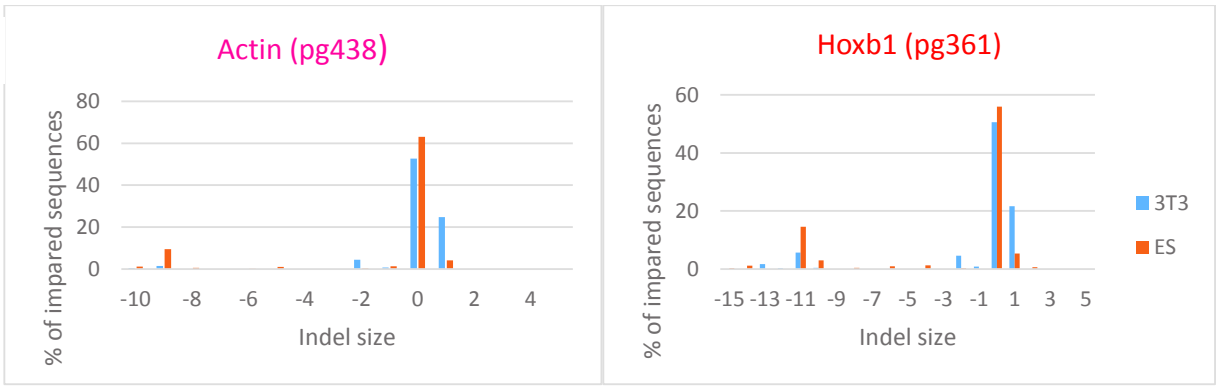
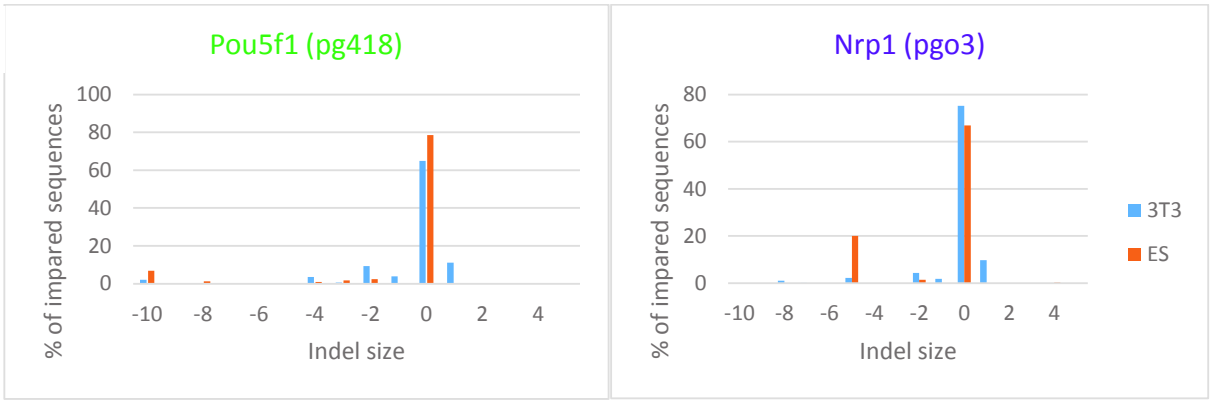


Fig. 17 Reproducibility of indel patterns. a) 3 biological replicates of transfecting the same guide RNA on the example of a guide RNA targeting Hoxb1 in 3T3 cells; b) Indel pattern comparison between 3T3 (blue) and ES (orange) cells. Actin and Hoxb1 guide RNAs are taken as representative example.

However, in some examples, notably pg418 guide targeting Pou5f1, pg450 guide targeting Hoxb1, and pgo3 guide targeting Nrp1, a considerable contribution of +1 insertion was observed in 3T3 but not ES cells (fig. 18 a). However, the number of guide RNAs manifesting such changes was low, so I think we can consider them as an exception, which also fits the general idea in the field. At the same time, no link could be made between indel patterns of two guide RNAs for the same gene even in the same cell type (fig. 18 b), despite in some cases distance between their cutting sites was rather short. This observation also fits the hypothesis of the indel pattern being mostly dictated by the surrounding sequence.

a



b

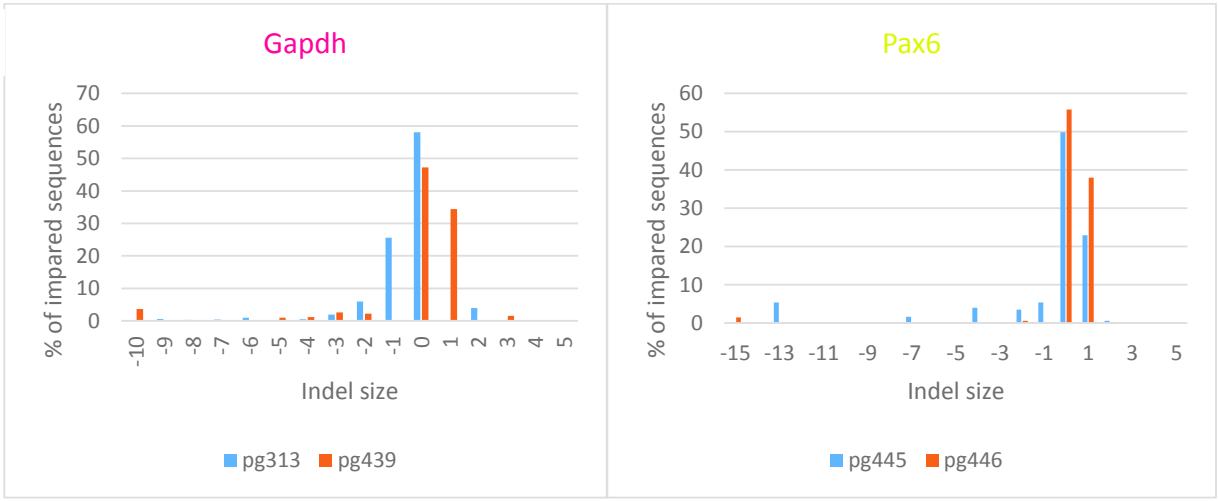
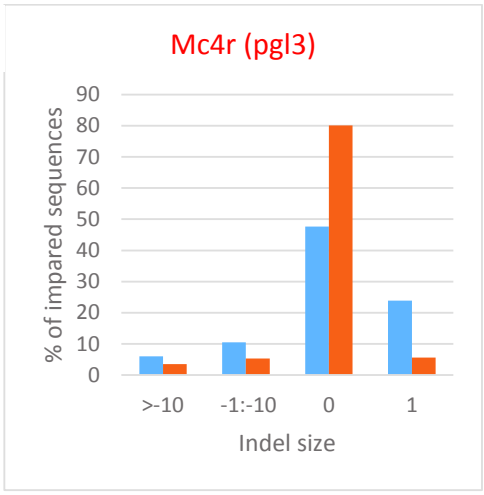
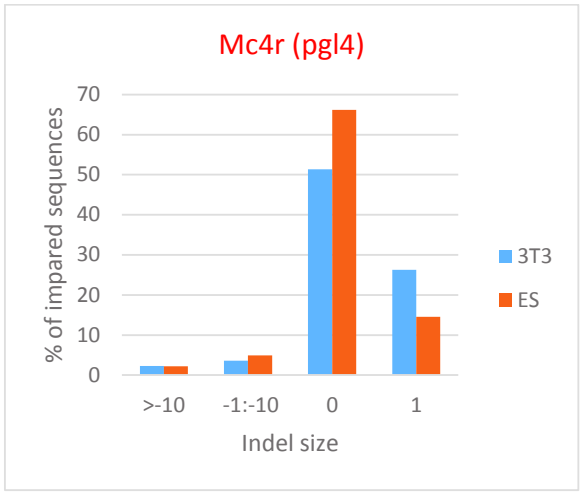


Fig. 18 TIDE results: indel pattern. a) guide RNAs targeting Pou5f1 and Nrp1. +1 insertion can be seen in 3T3 but not in ES cells; b) Comparison of indel patterns of two guide RNAs targeting the same locus in the same cell type.

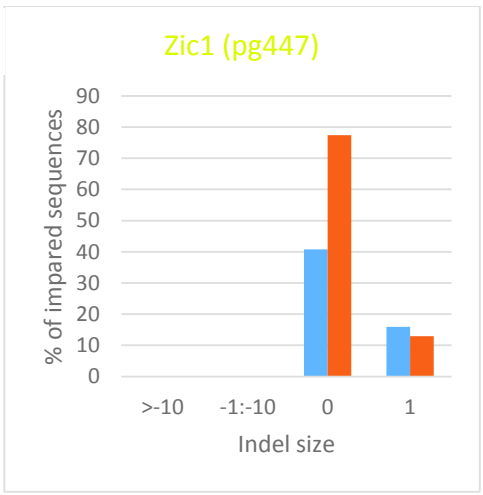
The size of deletions has been largely debated in the context of the balance between error-prone repair pathways, NHEJ and MMEJ. While it remains under debate which of these pathways could be the source of relatively small indels, there is no doubt that deletions larger than 10 b.p. are generated by MMEJ. Therefore, I tried to estimate MMEJ recruitment in different cell types by analysing deletions larger than 10 b.p (fig. 19 a). It can be seen from comparison between 3T3 and ES cells for the same guide RNA that most of the time large deletions either occur in both cell types or are absent in either. Moreover, sometimes even two guide RNAs for the same gene (fig. 19 b) can demonstrate these two different patterns.

a





b



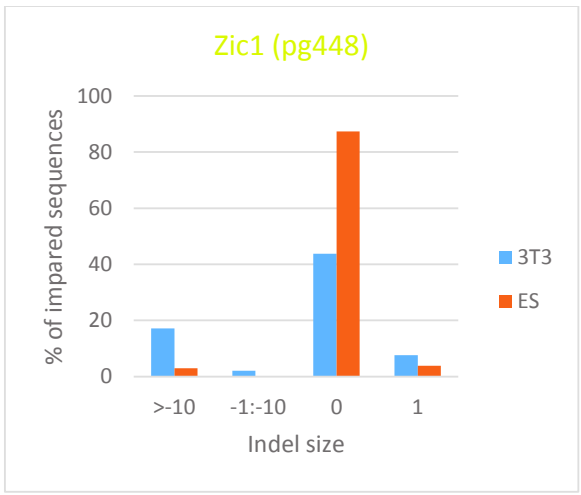


Fig. 19. Indel size assessment. a) Large deletions are notable in both cell types on an example of Mc4r; b) large insertions can be seen for one but not another guide RNA for the same locus on an example of Zic1.

Nevertheless, it was a general trend among most of the guide RNAs that the +1 insertion level was slightly reduced in ES cells and a proportion of deletions was increased as compared to 3T3 cells (fig. 20). This may result from a generally more open chromatin state allowing to shift a balance towards MMEJ rather than NHEJ or from ES cells highly relying on resection-dependent methods. This hypothesis would require additional experiments to prove this. However, these changes related to exact proportions of indels rather than overall pattern, which stayed constant between cell types.

All in all, experiments I have performed using multiple guide RNAs to introduce breaks in a variety of loci in 3T3 and ES cells lead to the conclusion that indel pattern is predominantly defined by the sequence surrounding the break site and the influence of a cell type and a chromatin context is minor. This fits the concept currently accepted in the field and can be considered another evidence supporting it.

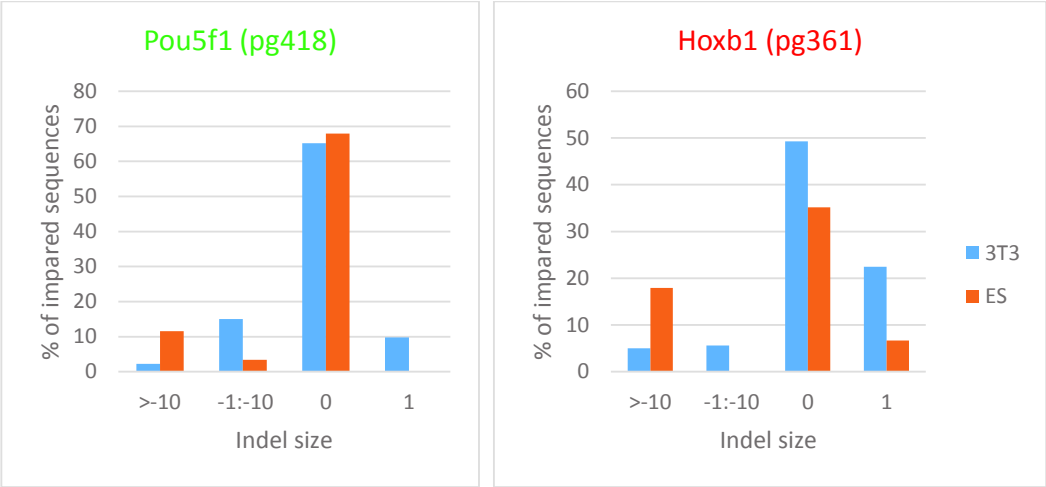


Fig. 20 Indel size assessment. Larger insertions are more prominent in ES than in 3T3 cells

3T3 cells have a higher rate of error-prone repair in housekeeping genes but not in pluripotency regulators

Assuming that the total percentage of mutated sequences gives us the means to estimate the overall employment of error-prone repair, I compared this parameter in 3T3 and ES cells for some of the target genes (fig. 21). Interestingly, we observed higher rates of error-prone repair than it was reported in the literature. The proportion of mutated sequences was going over 20% in some cases, which would mean at least 30% of breaks repaired by NHEJ of MMEJ, considering that neither induction nor transfection was absolute, and efficiency of both processes can be considered close to 80% judging from previous tests.

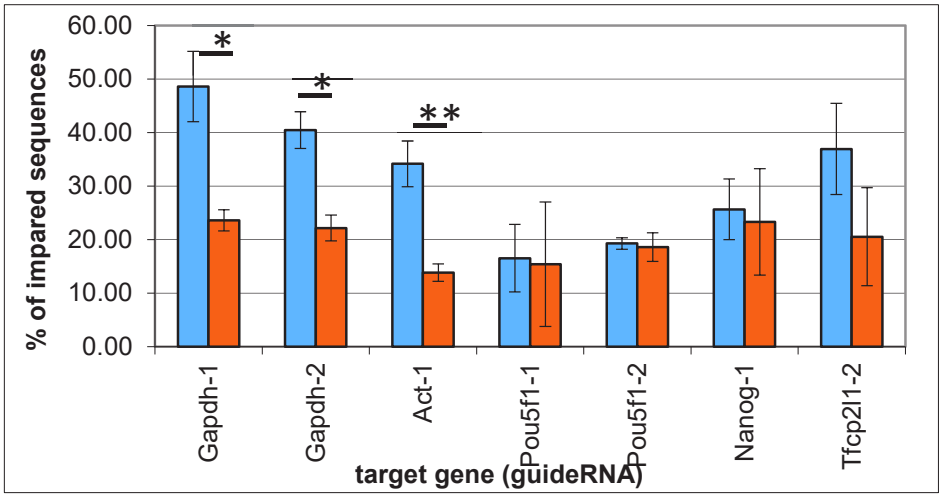


Fig. 21 TIDE results comparison between cell types. A rate of impaired sequences is significantly higher in analysed housekeeping genes (Gapdh and Actin). No significant difference can be seen for analysed pluripotency regulators (Pou5f1, Nanog, Tfcp2l1). Results for 3T3 ells are shown in blue, and for ES cells in orange. * p<-0.5, **p<0.01. n>3

Housekeeping genes demonstrated a result that could be expected from literature: levels of error-prone repair were significantly higher in differentiated cells than in ES cells. Surprisingly, when I looked at pluripotency regulators the picture changed dramatically, and no difference between cell types could be observed. This observation was rather surprising, considering that pluripotency regulators are transcriptionally inactivate and heterochromatinised in 3T3 cells, which should lead to reduced use of HR and therefore increased mutagenesis according to literature.

One possible explanation for that discrepancy was the influence of transcriptional regulators on the cell cycle. It is known that the cell cycle of mouse ES cells is dramatically different from the cell cycle of differentiated cells, including 3T3 (fig. 22 a). ES cells are characterised by a short G1 phase and an absence of G1-S checkpoint. As a consequence, a larger proportion of cells in a population is in S and G2 phases of the cell cycle, hence HR-proficient. It was therefore important to rule out the possibility that mutations in pluripotency regulators lead to changes in the cycle that would prevent ES cells from employing error-free repair. I performed a cell cycle analysis after transfecting cells with guide RNAs targeting Gapdh as a housekeeping gene or Nanog as a pluripotency regulator (fig. 22 b, c). The cell cycle was not affected after targeting Nanog as compared to targeting Gapdh at the usual collection time (48h), as well as at an earlier time point (24h). Moreover, cell cycle distribution in both cases matched those of non-treated cells. Therefore, we could conclude that an observed effect was not an artifact caused by a cell cycle perturbation.

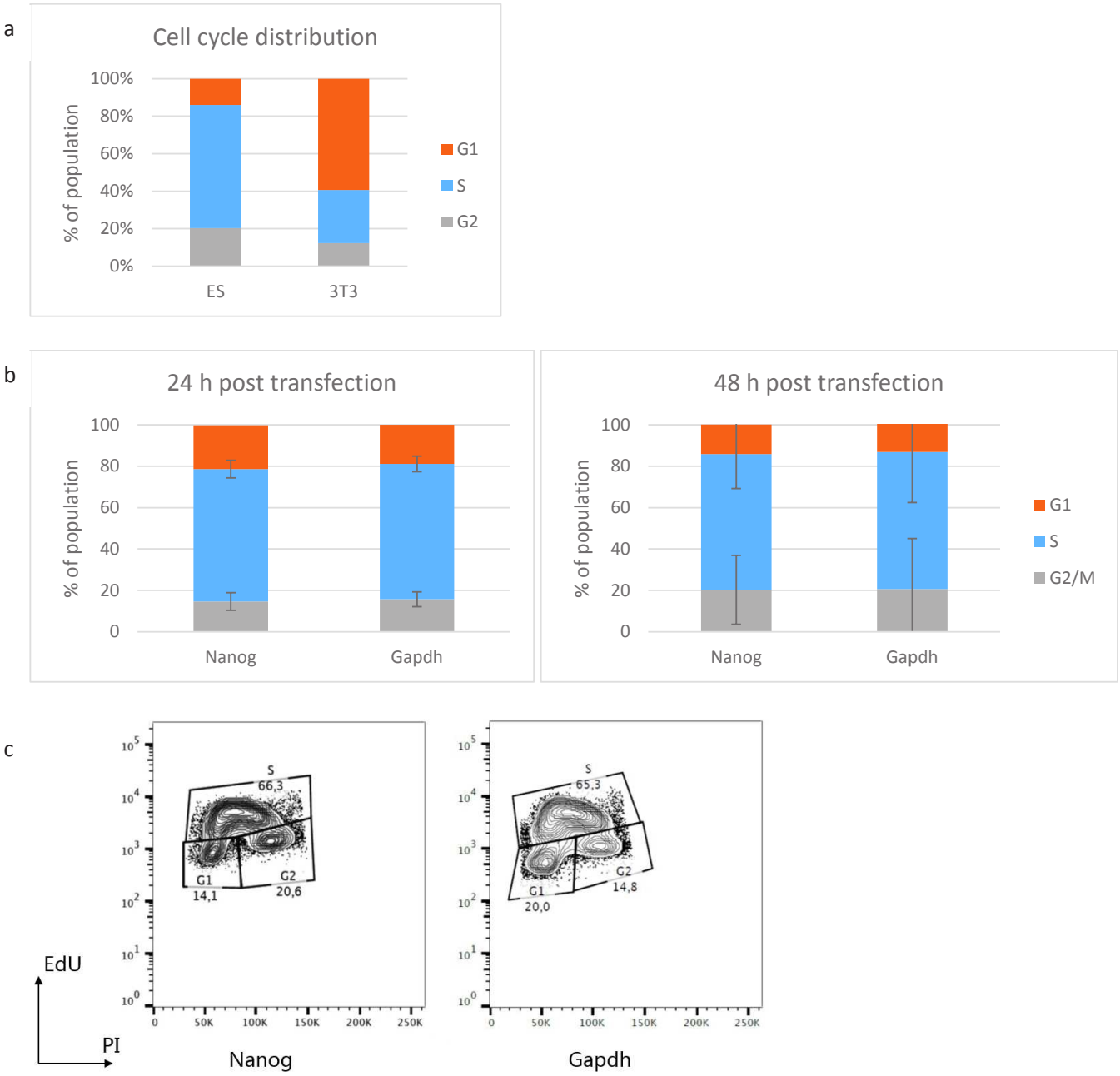
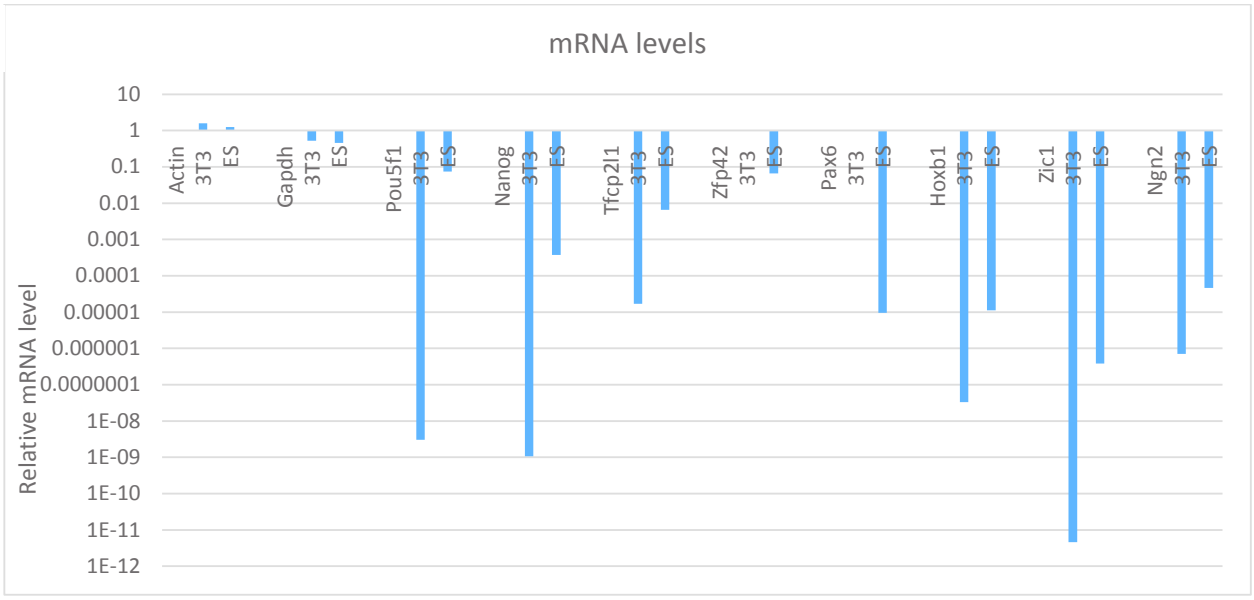


Fig. 22 Quantification of a cell cycle distribution of a) non-transfected ES and 3T3 cells; b) ES cells transfected withguide RNAs for Nanog or Gapdh 24 h or 48 h post transfection (error bars represent s.d., n=3) c) representative FACS plots for ES cells transfected with Nanog and Gapdh 24 hours post transfection.

Another potential explanation could have been a collapse between transcriptional and repair machinery. Pluripotency regulators are actively transcribed in ES cells, and it could have caused problems with the repair, and a shift towards faster repair pathways such as NHEJ. To check whether this was the case I

performed RT-qPCR in 3T3 and ES cells (fig. 23 a). I found that mRNA levels of housekeeping genes are considerably higher than those of pluripotency regulators.

a



b

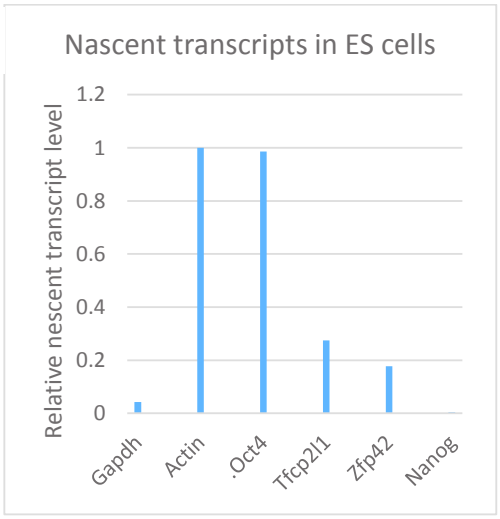


Fig. 23 a) RT-qPCR (enrichment shown in logarithmic scale) and b) RT-qPCR for nascent transcripts in ES cells.

As transcription levels can be different from mRNA rates, which depend not only on transcription but also on mRNA degradation, I performed nascent transcript qPCR. However, transcription levels were not necessarily higher for pluripotency regulators than for housekeeping genes as well (fig. 23 b)

A technical problem we were facing was our inability to detect HR repair outcomes using TIDE, as it is indistinguishable from uncut alleles for methods that involve analysis of the sequence. Therefore we were

not able to say whether differences we observed resulted from differential Cas9 binding, HR involvement, or even transfection efficiency variability between different replicates. Therefore, we needed to modify our experimental approach.

HR-TIDE: improving methodological procedures in order to detect HR

We decided to modify the TIDE method to allow the detection of HR outcomes (fig. 24). This modified TIDE (HR-TIDE) implied an addition of templates for HR, that contended a 9 b.p. insert (to avoid a frameshift when the cut was introduced in an exon) located at the place of a break and 500 b.p.-long homology arms at both sides of it.

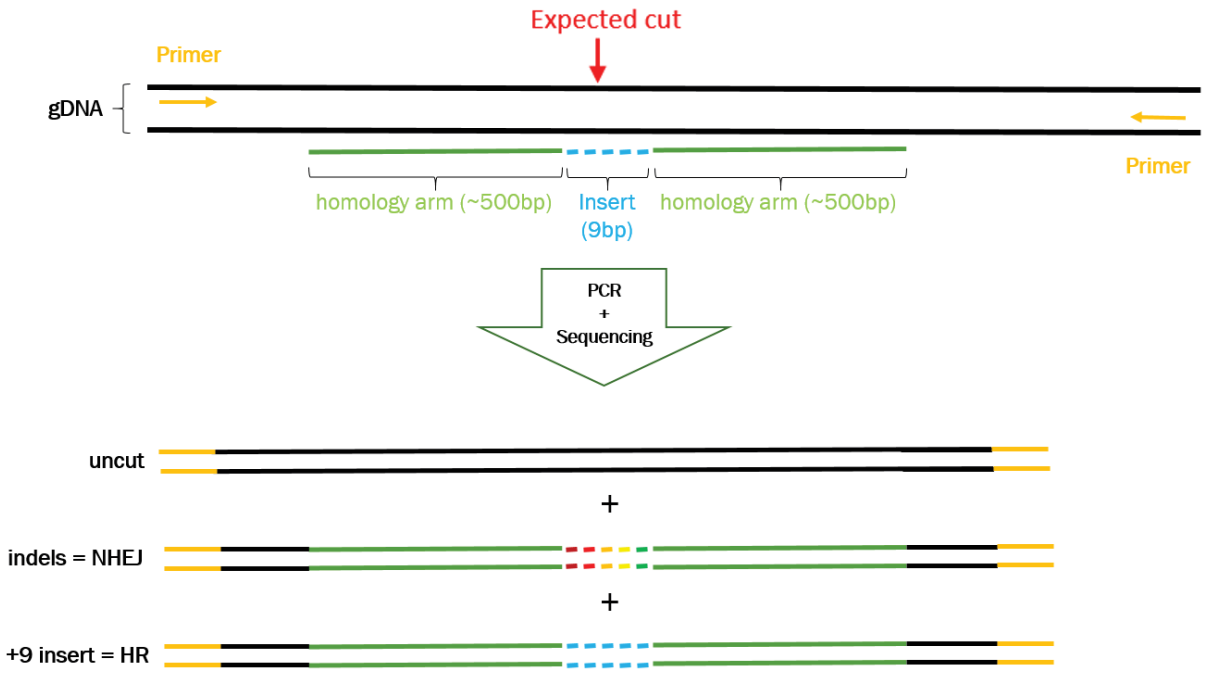


Fig. 24 Scheme of HR-TIDE approach

Therefore, as an outcome of an experiment, we would now receive a mix of wild type sequences, which are non-cut, different indels indicating error-prone repair pathways, and +9 insertion, which results from the use of HR (fig. 25). After cloning templates for all guide RNAs, both 3T3 and ES cells were transfected with both pairs of guide RNAs and templates.

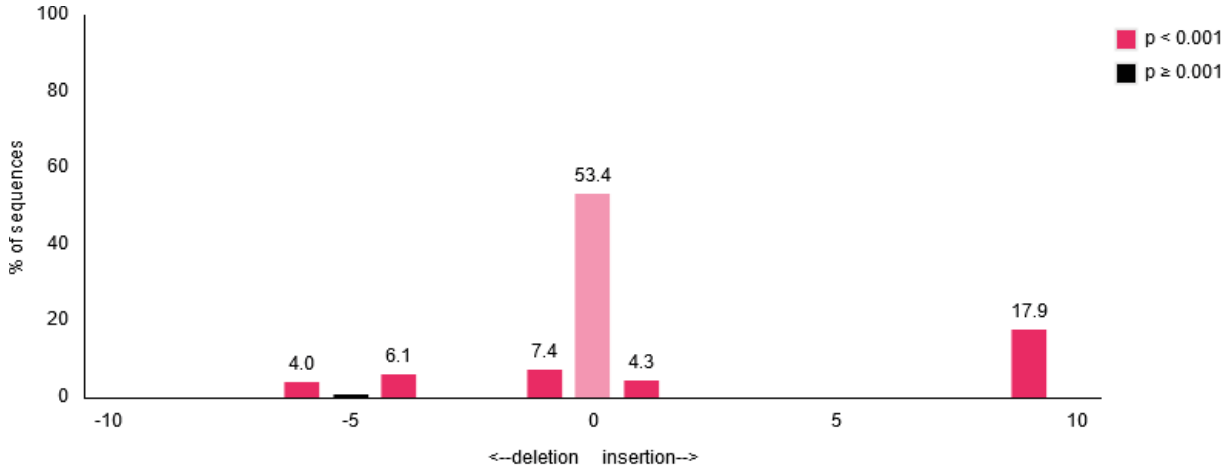


Fig. 25 A representative graph returned by TIDE software after co-transfecting guide RNA for Oct4 and a corresponding HR template.

So as by using HR templates we were taking into account all repair pathways, we assumed that the percentage of perturbed sequences represented total cutting efficiency. We could see that it was sometimes varying between cell types (which could be explained both by differential Cas9 binding caused by different chromatin context and by transfection efficiency fluctuations) and even between different replicates (definitely resulting from transfection variations).

Overall cutting efficiency is slightly higher in 3T3 than in ES cells

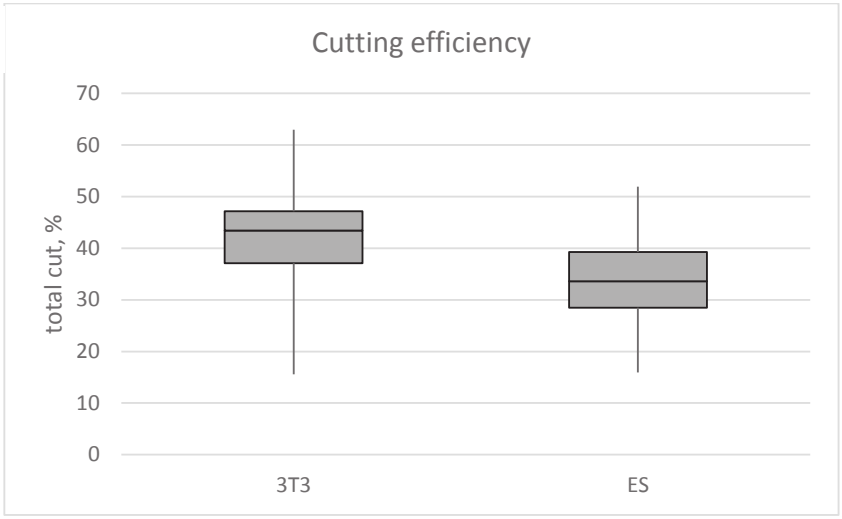
However, it was interesting to evaluate whether there is an actual difference in Cas9-mediated cleavage between cell types. To this end, I looked at the whole array of results to find out whether any trend could be observed from a comparison between two cell types. In this analysis, I considered cutting efficiency for each guide RNA, calculated as an average from all biological replicates, as a single measurement. This allowed me to assess the break introduction as a parameter of interest, where cutting efficiencies for genome locations recognised by different guide RNAs became single measurements of this parameter.

I observed that the median value in ES cells was close to 34%, whereas in 3T3 cells it was around 43% (fig. 26). The increase of the break introduction efficiency in 3T3 cells was statistically significant ($p=0.0065$, two-tailed Mann-Whitney U-criteria).

In order to check whether this held true when we compare results in two cell types for the same guide RNA, I found a ratio between cutting efficiency in 3T3 and ES cells and considered this as an experimental array.

The median of this array was 1.24, which shows an increase at the level of individual guide RNAs. Therefore Cas9-mediated DSB introduction is slightly efficient in 3T3 cells as compared to ES cells. Most likely, this difference resulted from differential transfection efficiency.

a



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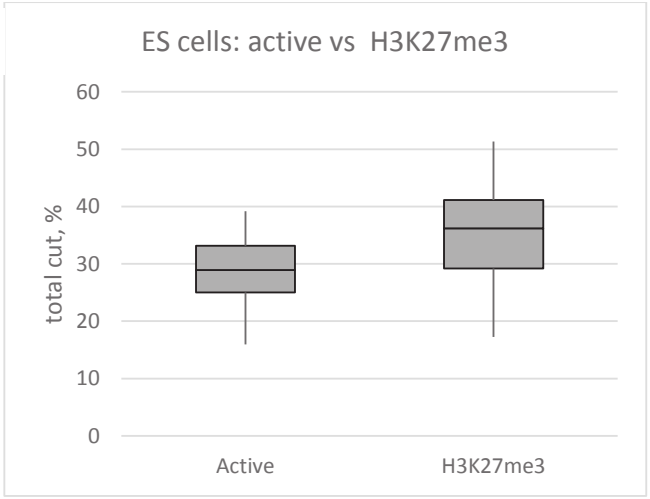

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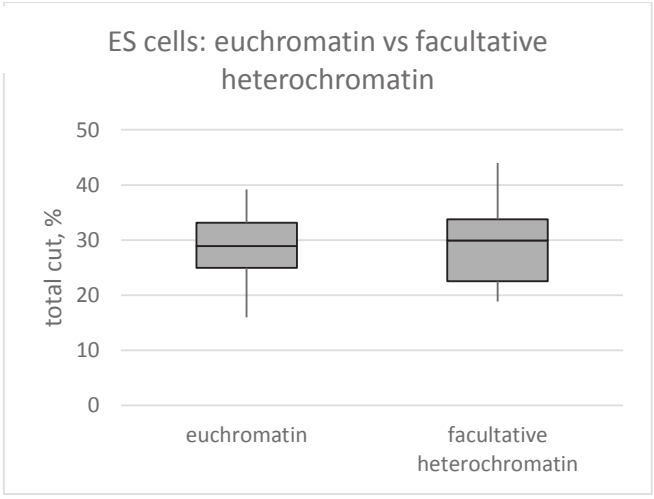

Fig. 26 Comparison of cutting efficiency in a) 3T3 and ES cells, b) euchromatin and H3K27me3-marked regions in ES cells and c) euchromatin and facultative heterochromatin in ES cells.

The efficiency of Cas9-mediated break introduction in heterochromatin as compared to euchromatin is broadly discussed. It has been previously shown that constitutive heterochromatin considerably reduces break introduction by Cas9 (Kallimasioti-Pazi et al., 2018). There is also evidence that facultative heterochromatin, and particularly the presence of H3K27me3 has a similar effect (Schep et al., 2020). Due to the number of analysed guide RNAs, I could perform such a comparison only in ES but not 3T3 cells. I

could not observe any reduction while considering either in cutting efficiency, and the difference was not significant while comparing either all H3K27me3-marked targets (bivalent and facultative heterochromatin combined, fig. 26 b) or facultative heterochromatin only (fig. 26 c)

HR rates are higher in ES than in 3T3 cells

In order to eliminate the abovementioned sources of variation, I chose to use the cutting efficiency as a normalisation factor for HR and error-prone pathways (NHEJ and MMEJ) outcome values and a further look at their proportions.

By analysing HR efficiency in the same way as the cutting efficiency, I observed that the median value in ES cells was close to 11%, whereas in 3T3 cells it was around 2% (fig. 27). This increase was statistically significant (p=0.0024, two-tailed Mann-Whitney U-criteria). Interestingly, taking advantage of an opportunity to directly measure a proportion of HR in several different locations we could see again that despite its higher levels in ES as compared to differentiated cells, it still does not match previous evaluations, and the vast majority of breaks is repaired by error-prone methods.

Fig. 27 Comparison between HR efficiencies in 3T3 and ES cells.

After assessing the ratio between HR level in ES and 3T3 cells I found that the median ratio was 5.3, which shows an increase at the level of individual guide RNAs. Therefore ES cells demonstrate a five-fold increase of HR employment as compared to cycling differentiated cells.

Pairwise comparisons of HR employment after break induction with the same guide RNA between different cell types showed a significant increase in ES cells compared to 3T3 cells for housekeeping genes and pluripotency regulators (fig. 28) with one exception. Also, one guide for each target group showed abnormally low HR in ES cells.

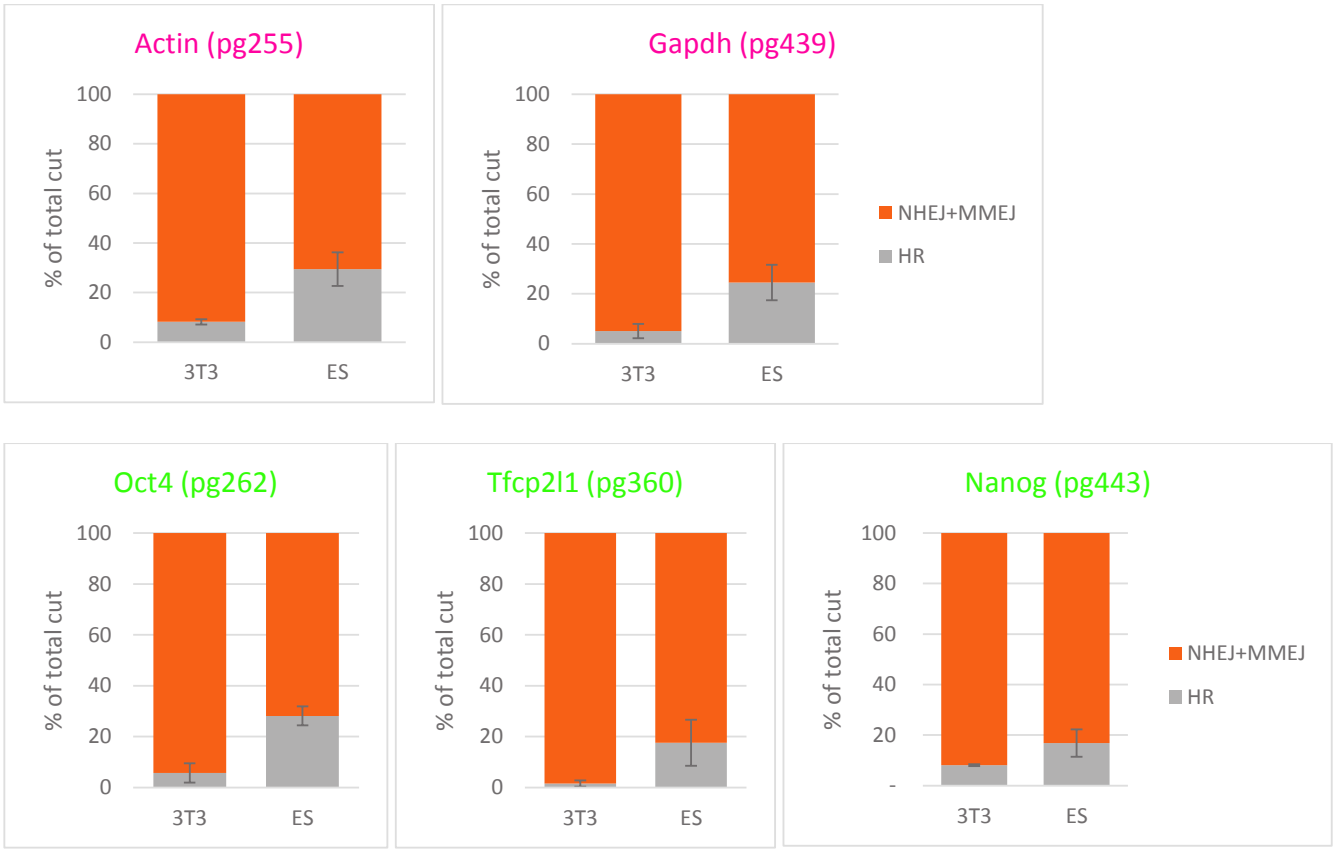

Fig. 28 Comparison of HR use between cell types on exampl of single guide RNAs. HR utilisation is higher in ES than in 3T3 cells for both housekeeping genes and pluripotency regulators.

This may have resulted from surrounding sequence or chromatin features. Using the same strategy as before, I compared arrays of HR proportions from ES and 3T3 cells for each of these target groups (fig. 29). In both groups the median of HR utilisation was higher in ES than in 3T3 cells (27% versus 9% for housekeeping genes and 18% versus 1% for pluripotency). However, statistical analysis using Mann-Whitney criteria showed the significance of the increase for pluripotency regulators (p=0.0214) but not housekeeping genes. This could have been caused either by a lower number of guide RNAs analysed for

housekeeping genes or by higher variability in this group. In any case, analysing more targets for this group might solve this question.

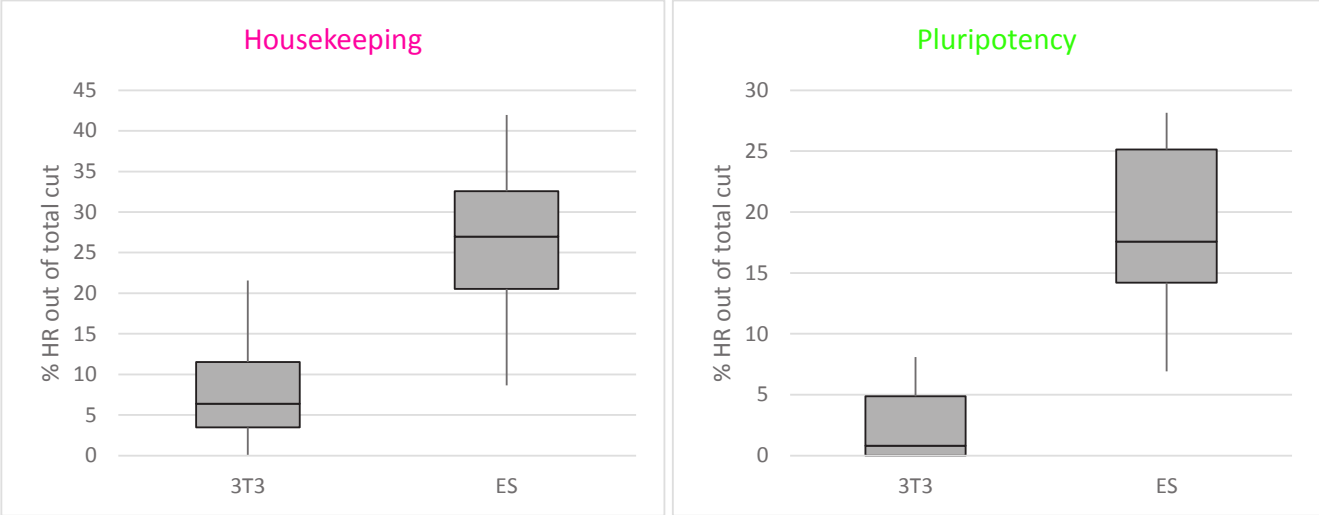

Fig. 29 Comparison of arrays of guide RNAs targeting housekeeping genes or pluripotency regulators between cell lines.

Thus, we can claim with confidence that active genes in ES cells show considerably higher HR rates than they do in differentiated cells, independently of their activation status in the latter case.

I also plotted all investigated groups on the same graph for each of analysed cell types. Despite the inability to do statistical analysis due to the lower number of targets per group, I could notice as a trend that for each cell type those groups of targets, which are active in this cell type, had elevated HR levels as compared to groups of inactive genes (fig. 30). This goes in line with other published data demonstrating that transcriptionally active chromatin is an HR-permissive environment. However, adding more targets to each group to enable proper statistical analysis is needed to secure this conclusion.

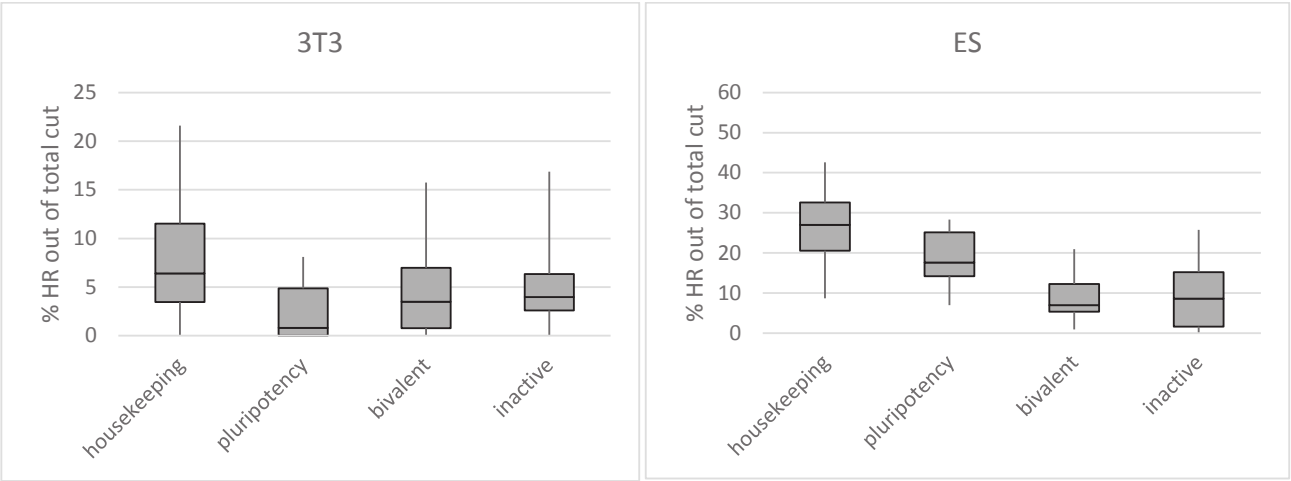

Fig. 30 Comparison betwee different target groups in the same cell type.

Bivalent and inactive genes

Looking at other chromatin types of interest (bivalent domains, facultative heterochromatin, and LADs) we observed a completely different picture.

Pairwise comparison for each guide RNA would show a significant increase for half of target sites analysed in both bivalent domains and facultative heterochromatin (fig. 31 a and b respectively) so no clear conclusion could be drawn on whether HR is more actively employed by ES cells to repair these loci. Moreover, one gene in each of these two arrays can potentially be exceptional. Hoxb1 demonstrated surprisingly high HR rates in both target sites and both cell types analysed. This could potentially result from differences of Hox locus organisation compared to other genomic regions. However, this assumption would require to assess more Hox genes to confirm it.

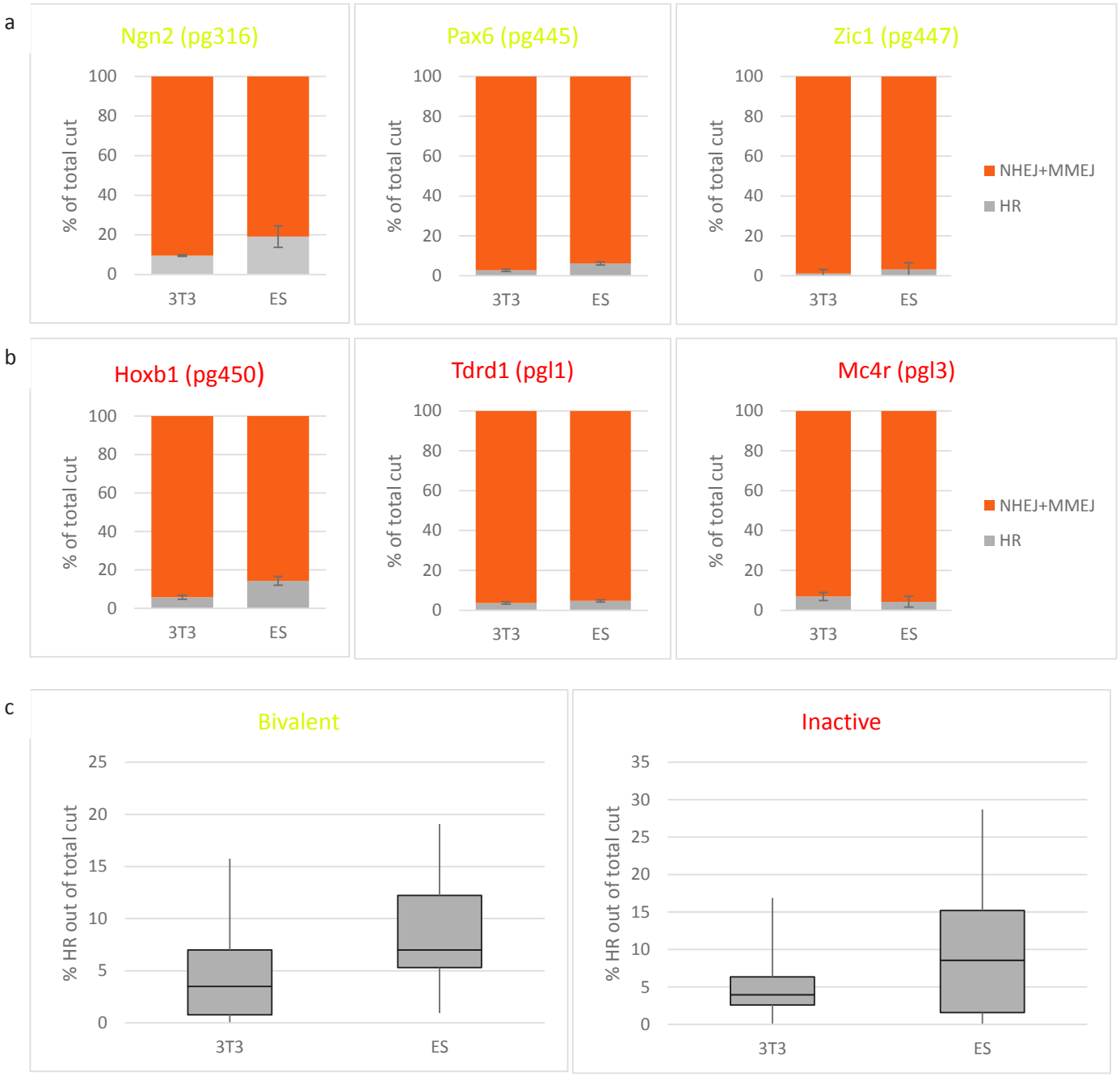

Fig. 31 Comparison of a) bivalent and b) heterochromatic targets between two cell lines on single guide RNAs and c) on combined arrays. There is no significant difference in HR efficiency in different cell types.

Considering bivalent regions and facultative heterochromatic targets as independent arrays (fig. 31 c) we found no significant difference between analysed cell types. Moreover, medians of an array of one cell type lied within a confident interval of another cell type for both chromatin environment, and the increase of HR utilisation in ES cells was approximately two-fold (close to 2 and 2.16 respectively). This led us to a

conclusion that in terms of DNA repair bivalent chromatin predominantly behaves similarly to facultative heterochromatin and that repressive chromatin marks tend to play a decisive role in a repair pathway choice.

Exceptions

It can be noticed that in ES cells HR in bivalent targets seems to have high variability (fig 31 c). This may result from one of the selected targets, Pax6, demonstrating very low HR levels. It is possible that this locus is an exception due to its DNA sequence or another factor, but assessing more bivalent targets would be necessary to check it.

The same problem can be observed for the group of facultative heterochromatic targets. HR level in this group goes surprisingly high in ES cells (fig. 31 c) and demonstrate a rather prominent variability. It is caused by results from two guide RNAs for the same gene, Hoxb1. However, the Hox cluster is known to have a rather distinct chromatin structure and is sometimes even thought to represent a separate chromatin type (Aaronson et al., 2016). Therefore, it would help to increase the number of heterochromatinised targets as well as to add some more Hox genes into analysis to check whether it stands the same in the context of DNA repair.

LADs

We could observe no significant increase in HR employment for any of analysed loci within LADs (fig. 32).

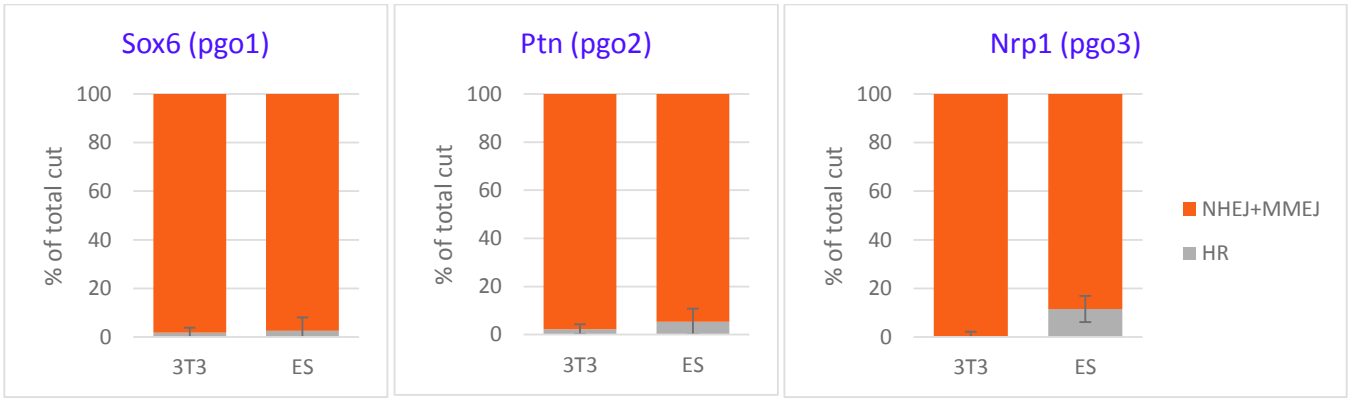

Fig. 32 Comparison of targets located in LADs targets between two cell types on single guide RNAs. Different targets behave in a different way, making it impossible to draw a conclusion based on such a small number of targets.

However, it was not completely blocked in any of the targets. This observation does not fit results previously obtained in the lab. However, such a discrepancy could be explained if genes in question are located at the

periphery of LADs. This assumption requires further experimental investigation. However, the fact that two of the genes of interest were reported as bivalent in literature can be considered indirect proof for it.

The cell cycle is partially responsible for the low HR rate in 3T3. Cell cycle block causes an increase in HR utilisation but cannot fully explain differences between cell types

As it was noted before, mouse ES cells are characterised by a specific cell cycle distribution, with a very short G1 phase. As a consequence, only 20% of cells in a population are in this phase at any time point, and the rest of the cells is in S/G2 phases, therefore 80% of cells are HR-proficient. In differentiated cells, these ratios are different, as G1 is longer. 60% of cells in a population are in this state and therefore cannot perform HR. This could potentially be the cause of different ratios of HR in 3T3 and ES cells. To test this hypothesis we first decided to enrich a population of 3T3 cells for G2 cells. To this end, we blocked cells in the G2 phase with RO inhibitor and analysed changes in HR utilisation. We could indeed detect an increase of HR ratios comparable or even larger than those observed I ES cells (fig. 33). However, from previous observations we knew that the degree of HR enhancement varies between different chromatin contexts, and would the cell cycle be the only explanation, it would cause a similar shift in all cases.

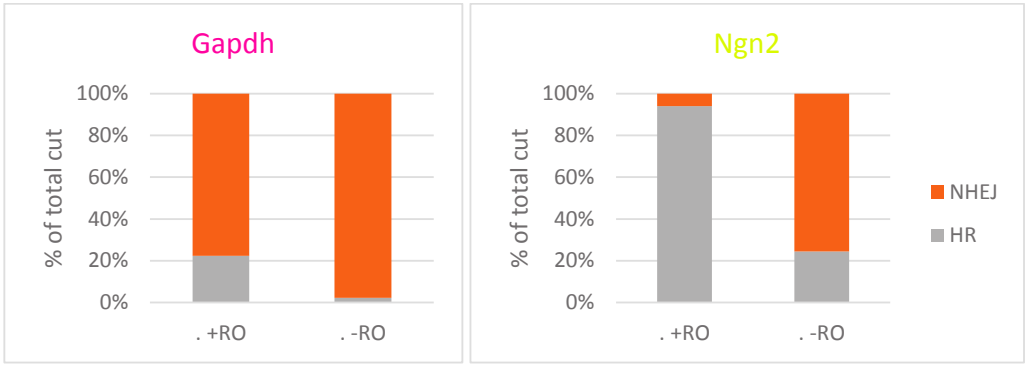

Fig. 33 HR proportion grows considerably after cell cycle block with RO inhibitor.

To further test the degree to which perturbed cell cycle is responsible for higher HR rates in ES cells, we needed to bring it to the state close to those of differentiated cells. This can be achieved by culturing ES cells in a serum-free medium with an addition of MEK and GSK3β inhibitors (so-called 2i medium) (Ying et al., 2008). It has been demonstrated that in this medium a G1-S checkpoint is restored in ES cells and the length of the G1 phase is increased, and 40% of a cell population is in this phase (ter Huurne et al., 2017). We could also observe these changes (fig. 34).

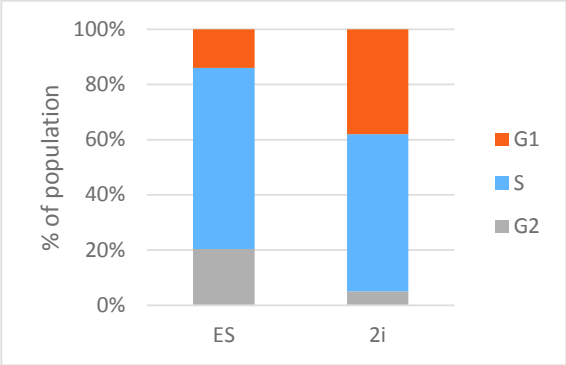

Fig. 34 Cell cycle distribution difference between cells cultured in the presence of serum or in serum-free 2i medium

We performed transfections of some selected guide RNAs in these culture conditions and analysed HR rates. For most of the analysed target sites, we could detect no difference in HR rates between two culture mediums (fig. 35), although in a few cases we could see a slight decrease or increase. When results from all analysed target sites were combined for statistical analysis, no significant difference was observed. Also, when a ratio between HR levels in serum-containing medium and 2i medium was calculated for each guide RNA, the median ratio amounted to a 1.16-fold increase, which is lower than differences observed between 3T3 and ES cells.

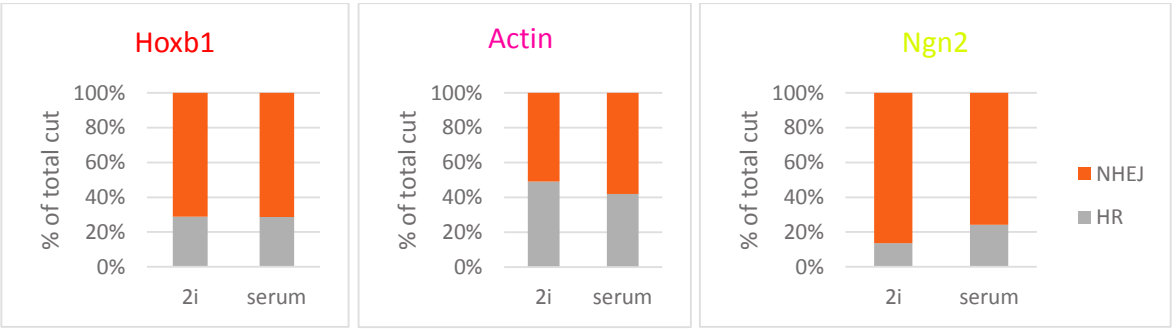

Fig. 35 Comparison of HR proportion in two culture mediums shows that it is not consistent between different targets and no conclusion can be drawn.

All in all, our results point that altered cell cycle might be one of the mechanisms that contribute to the ability of ES cells to maintain their genome stability. However, it does not entirely explain a present difference and allows us to assume that other factors might be involved. One such mechanism could be a decreased level of repressive chromatin marks that is observed in ES cells. Another potential mechanism is

an increased level of proteins involved in DNA repair and particularly in HR that was previously reported. However, a precise answer to this question would require further investigation.

Discussion

In my project, I investigated the role of sequence and chromatin context in the process of DNA double-strand break repair and the influence of the cell type on it. Cell types in the focus were mouse ES cells and 3T3 cells. ES cells were selected as a model because they were reported to have unique pluripotency-specific features of DNA repair, and because of the abundance of bivalent chromatin. 3T3 cells were used as a model of differentiated yet cycling cells as a line stably expressing Cas9 was previously acquired in the team. Targets for a break induction were selected at euchromatic, bivalent regions, facultative heterochromatin, and LADs. The first three target types were dictated by our wish to compare bivalent regions to chromatin types a mix of which they represent. I performed TIDE and HR-TIDE experiments for all selected targets in both cell types, in normal conditions or with cell cycle perturbations. I could confirm existing hypothesis of error-prone repair outcome being greatly dependent on the sequence around the break site, and having variations depending on a cell type.

The tendency seems to be in accordance with previous observations that euchromatin seems to shift the balance between repair pathway towards HR, as compared to transcriptionally inactive regions such as facultative heterochromatin and bivalent domains, although due to the small number of investigated targets I cannot claim statistical significance. However, these observations come into contradiction with the results of a study by Schep et al. that was recently published on BioRxiv (Schep et al., 2020). They addressed the question of the role of the chromatin environment in repair pathway choice by inserting a short barcoded reporter cassette with a well-characterised Cas9 target site into multiple genomic locations in a human immortalised cancer cell line. Analysing thousands of target sites, they observed a negative correlation between the presence of H3K27me3 chromatin mark and NHEJ employment and could also see that the euchromatic environment promotes the use of NHEJ. In our investigation we could see prominent recruitment of NHEJ in euchromatic targets in 3T3 but not in ES cells. However, somatic 3T3 cells seem to be a more proper line to compare to another differentiated cell line, such as K562 utilised by Schep et al. At the same time, we did not see the anticorrelation between H3K27 trimethylation and the use of NHEJ in either of the model cell types. However, a small number of targets does not allow us to draw such broad conclusions.

In the same study, a template-dependent repair has been tackled. It was observed that the chromatin environment influences it the same way as it does for NHEJ. However, the pathway chosen for that

investigation was single-strand templated repair, so we could not directly compare it to our results as we were interested in HR. We could see that HR utilization was influenced both by a chromatin type and a cell type. Heterochromatic marks appeared to dominate over euchromatic in control of a repair pathway choice and to be repressive for HR. I found that cell cycle characteristics are one of the factors leading to the abovementioned differences between pluripotent and differentiated cells but they are unlikely to be the only factor involved. Therefore, further experiments to dissect this issue might be necessary.

Altogether, despite certain similarities in the experimental setup, we mostly addressed different questions from those of Schep et al. Our studies could therefore be considered as complementary for clarification of the role of chromatin features in defining DSB repair strategy.

Sequence predominantly defines the DSB repair outcome. Chromatin and cell type might have no more than moderate influence on the DSB repair outcome

After analysing indel patterns for all target sites (26 guide RNAs) designed to induce DSBs in different chromatin contexts in two different cell types I could see no correlation with any of these factors. All types of repair outcomes, including small insertions and deletions, thought to be resulting from NHEJ employment, and larger deletions normally connected to MMEJ could be observed in all chromatin types. Indel patterns between different cell types bore strong similarity for every guide RNA, whereas no resemblance could be detected for two different guide RNAs even in a case of a short distance between break sites. This goes in line with previously published data indicating that DNA sequence around the break site, particularly a few nucleotides surrounding it, and a presence of microhomologies define specific indels found after a break repair.

However, an overall proportion of larger deletions, presumably associated with MMEJ, appeared to be higher in ES cells than in 3T3 cells. This is likely to indicate a shift in a balance between NHEJ and MMEJ. One possible explanation for this effect could be a more open chromatin organisation in ES cells, considering that such a state is generally reported to be prone to HR and at the same time MMEJ being also resection-dependent. This shift of balance could also be a consequence of an increased level of some proteins simultaneously involved in both HR and MMEJ repair pathways, such as CtIP. Nevertheless, this would require additional experiments to prove.

ES cells use HR more actively than differentiated cells

Focusing more on the balance between error-free and error-prone repair pathways I performed HR-TIDE experiments and analyzed a proportion of sequences that incorporated a provided template for HR. Despite numbers varying for individual guide RNAs, analysis on the whole array revealed that HR is significantly higher used across the genome in ES cells as compared to 3T3 cells. This corresponds to the current paradigm in the field. However, unlike some other cases, in my setup, I could still see a vast contribution of error-prone pathways in the repair process. Therefore, my results support the hypothesis of HR being more actively employed in ES cells but do not confirm the degree of its mobilization previously claimed. This is likely to result from the difference between experimental setups. Studies leading to conclusions that HR is utilised to repair up to 80% of breaks in ES cells were performed on one single break on a transcriptionally active gene, and I checked several target sites across the genome, in both active and inactive states. Another possible explanation could be the use of Cas9 to introduce breaks in my experiments, as it has previously been suggested that the presence of Cas9 at the DNA after a break was introduced might potentially influence the pathway choice.

HR is enhanced in transcriptionally active genes. It is more prominent in ES than in differentiated cells

A comparison between transcriptionally active regions and other types of chromatin led me to the conclusion that the euchromatic environment is promoting HR. Despite I cannot prove this observation statistically due to a low number of loci explored for each group, it is supported by several other studies, which makes it likely to be correct. Nevertheless, it would be preferable to increase the number of targets to enable a solid conclusion. Therefore, finding that pluripotency regulators demonstrate significantly higher levels of HR in ES than 3T3 cells is not surprising. However, I could also see a threefold increase for housekeeping genes, which supports the previous conclusion that HR levels are generally elevated in pluripotent cells.

Despite both conclusions fit within currently existing views, I think they add considerably to previously developed theories as to my knowledge it is the first time when HR contribution is assessed directly at the level of sequence and not based on the recruitment of different factors, which could also be involved into some other processes. Moreover, it disconfirms a recent study by Janssen et al. that claimed no influence

of the chromatin state on the process of HR and therefore sheds light on a still controversial topic (Janssen et al., 2019).

HR levels are low in bivalent domains and facultative heterochromatin

Of different types of heterochromatin constitutive heterochromatin is the most studied and it has been shown that DSBs in such an environment are less likely to be repaired by HR. However, facultative heterochromatin has been less studied. We assessed several loci marked with H3K27me3 and found that HR levels, in this case, were low, and more interestingly, they were not significantly increased in ES cells as compared to 3T3 cells. Therefore, we can conclude that facultative heterochromatin represents an environment predominantly repressive for HR.

As euchromatin represents an HR-promoting environment and heterochromatin is repressive to it, it was especially interesting for us to see what results we might observe in the case of bivalent domains, where marks of both are combined. Interestingly, we could see that HR levels stay low and there is no significant difference between cell types. Therefore, the same outcome is observed for bivalent domains behave as for facultative heterochromatin. In other words, in regulating the process of DSB repair repressive chromatin marks appear to be a stronger driver than permissive ones. It is an interesting observation as to our knowledge no one has yet studied the influence of this type of chromatin on the process of DNA repair.

Influence of the cell cycle on the enhanced HR utilization in ES cells

As a cell cycle is known to strongly influence the process and it is also changed in ES cells as compared to differentiated cells, we decided to check to what extent it contributes to the difference in repair pathway choice we see between cell types. For this we blocked 3T3 cells in the G2 phase to check if increasing a proportion of HR-proficient cells in the population would change the balance between repair pathways. It is hardly surprising that we found it to lead to an increase in the proportion of HR products. Therefore, in consonance with existing knowledge, we could see that blocking cells in a cell cycle stage permissive for HR would lead to increase utilisation of this pathway up to levels similar or even exceeding observed in ES cells.

Another apparent question was whether modifying the cell cycle of ES cells to resemble one of the differentiated cells would cause the opposite effect. Fortunately, we had a way to do it, as it has been

reported that in 2i medium ES cells acquire a cell cycle that resembles one of the differentiated cells, and what was of particular interest for us, in such culture conditions larger proportion of cells in a population stays in G1 phase. After introducing DSBs using several selected guide RNAs we only found a moderate drop in HR efficiency, which was not statistically significant. However, it has to be taken into account that the share of cells in the G1 phase is smaller in ES cells even in 2i than in 3T3 cells.

All in all, we came to the conclusion that it is very likely that a distinct cell cycle that is known to be characteristic for ES cells is likely to be one of the mechanisms to ensure higher genome stability. Nevertheless, it is highly doubtful it is the only underlying mechanism, and further experiments would be required to uncover other ways utilised by ES cells to ensure their genome is preserved through generations.

Perspectives

It has to be noted that I performed all experiments in my project on a relatively small number of targets, which limits our ability to generalise conclusions. Therefore, my perspectives could be divided into short-term, aiming to prove established results without the addition of a large number of other target sites or a major change in the experimental setup, and long-term, aiming to broaden our observations to a genome-wide level.

I would like to start discussing the suggested experiments with short-term perspectives. First, considering the heterogeneity of results for bivalent domains and facultative heterochromatin target groups I think adding several more targets for each group to ensure that none of the results for selected targets was impaired by any factor other than chromatin structure (in other words, that we do not deal with any target representing an exception from the general trend) and reduce the influence of outliers. Adding 3 or 4 more targets should be sufficient to complement already performed HR-TIDE-based experiments. Considering the fact that most of the time using two guide RNAs designed for the same region leads to very similar results, it might be profitable to design one guide RNA per each selected target. Choosing some additional targets within the Hox cluster would also allow evaluating the hypothesis of exceptional DSB repair pathway preference (and therefore indirectly confirm an exceptional chromatin organisation in this part of a genome). Considering the differences observed between publications it would be more tenable to perform ChIP-qPCR for all newly selected targets to confirm their chromatin status.

Second, adding several more guide RNAs for housekeeping genes would enable us to perform a proper statistical analysis to compare different target groups within the same cell type. It ought to be remarked that the target group of LADs was excluded from all comparisons with other chromatin types due to a small size of the group. Therefore, it also requires enlargement. However, considering highly heterogenic results observed for this target group it is also necessary to perform FISH to prove that all of the targeted regions indeed belong to LADs in the utilised cell line.

In my analysis, I have set the NHEJ/MMEJ border voluntary. To stratify this question as well as to prove that +9 incorporation is indeed facilitated by HR, it would be necessary to perform TIDE or HR-TIDE experiments while knocking down or chemically inhibiting key players of investigated pathways, such as

DNA-PKcs, DNA Ligase IV, or XRCC4 for NHEJ, Polθ, DNA Ligase III, or XRCC1 for MMEJ, and Rad51 or BRCA1 for HR.

According to the obtained results, we could formulate a hypothesis that in case of bivalency, i.e. coexistence between euchromatic and heterochromatic marks DSB pathway balance is kept similar to those of facultative heterochromatin. However, this assumption requires direct confirmation. In order to achieve it, we plan to remove H3K27me3 from bivalent genes by recruitment of histone demethylases such as JMJD3 or UTX to target sites by fusing them to dCas9 (a catalytically inactive form of Cas9) prior to performing HR-TIDE experiments. Demethylases recruitment can alternatively be substituted by chemical inhibition or knock-down of PRC2 complex subunits. However, it would cause genome-wide perturbations, therefore, targeted demethylation is preferable. On the other hand, targeting PRC2 complex to transcriptionally active targets to form bivalent domains *de novo* would complement proposed experiments in order to prove a proposed theory. However, if this strategy is chosen, it would be important to confirm that bivalent domain formation and not replacement of euchromatic marks by heterochromatic follows such a recruitment. Therefore, a confirmation by ChIP-qPCR would be required.

We could conclude from our results that HR rates are higher in actively transcribed regions marked by H3K4me3. However, we cannot draw a line between roles transcriptional activity and chromatin status. Performing HR-TIDE experiments after chemical transcription inhibition would allow us to dissect between these two factors. Triptolide use would be preferable compared to other commonly used compounds, such as α-amanitin, actinomycin D, DRB, or flavopiridol, due to the fact that it inhibits transcription initiation, while other inhibitors block elongation. Considering that selected targets lie within proximal promoter regions, an attitude that blocks transcription inhibition should preferentially be used. Potential downsides of such an approach are massive perturbation caused in a cell by a prolonged transcription block. One cannot exclude that effects caused by such a block might mask analyzed influence on DNA repair.

Regarding long-term perspectives, I would like to mention a potential practical application of the developed approach. In my opinion, it could be used for predicting knock-in efficiency. Analysing obtained results I have noticed that in some cases HR efficiency differs from what can be expected from the chromatin environment surrounding the introduced break. Knocking-in large fragments, such as

fluorescent reporters, is often necessary for research. However, the introduction of large fragments often results in reduced efficiency compared to smaller ones. Therefore, I think that prior testing of designed homology arms with smaller insert might be a good strategy to predict their efficiency before engaging in a large experiment to enable the optimal setup. To allow this, homology arms should be cloned with a small insert containing digestion sites that would enable further replacement by a larger insert of interest. This way, knock-in efficiency could be tested by HR-TIDE prior to the real experiment. To verify that HR-TIDE results are a good predictor for a larger fragment, it would be good to replace an insert in several of previously used templates with mCherry and evaluate knock-in efficiency by FACS to test whether it correlates with HR proportion observed in HR-TIDE experiments.

Finally, all experiments performed in the course of my doctoral project can be used as a basis to design a genome-wide test of HR utilization. Selecting one or two hundreds of well-cutting guide RNAs targeting different genome regions based on available data and cloning them together with corresponding HR templates into a viral library based on an integration-deficient lentiviral vector could be a solution for such a goal. This library should be used for infecting cells of interest and further submitted to NGS analysis. Such an experiment would provide a map of HR utilisation efficiency and allow assessment of its dependence on a chromatin state as well as other factors. It would also help to select a group of targets for further dissection of mechanisms underlying observed correlations.

Materials and methods

Cell culture

Mouse ES cells were cultured on a feeder layer of mitotically inactive MEFs, growing conditions were set to 37°C, 5% CO_2. Cells were passaged every 2-3 days and kept in culture for no more than 10 passages. Passaging involved trypsinisation for 5 minutes at 37°C, trypsin inactivation with at least equal volume of medium, achieving a state of single-cell suspension, separation from feeders by gravity (after 10 minutes incubation in a 15 ml Falcon tube, top fraction was used for plating) and plating on a fresh dish at 1:10 to 1:20 rate depending on the desired final confluency.

Standard ES cell medium composition:

High glucose DMEM

15% ES-grade FCS

1mM NEAA

1 mM Sodium Pyruvate

40μg/ml Gentamycin

100 μM β-mercaptoethanol

2x recombinant LIF

For creating stable cell lines, antibiotics selection begun 24 h post transfection and continued for 2 days with Puromycin (at a final concentration of 2μM) or for 10 days with G418 (at a final concentration of 400 μM).

Suicidal cassette: for all following experiments cells were treated with puromycine for 2 days starting 24h after thawing.

For experiments in serum-free conditions 2i medium was used.

2i medium composition:

DMEM/F12 + Neurobasal mixed 1:1

1x N2

1x B27

25 μg/ml BSA

100 μM β-mercaptoethanol

2x recombinant LIF

1 μM PD 98059

3 μM SB 216763

For a plasmid transfection, reverse transfection protocol using Lipofectamine 2000 was applied. Cells were pre-treated with Shield1 (at a final concentration of 1:2000) overnight prior transfection. For the transfection, cells were trypsinised and separated from feeders by gravity in 15 ml Falcon tubes for 15 minutes. ES cell containing supernatant was separated from a pellet of feeders and transferred into fresh Falcon tubes. $0.5*10^6$ cells per sample were taken if transfecting cells cultured in the standard ES cell medium. In the time of incubation, transfection mixes were prepared. Transfection mix 1 contained total of 5 μg of DNA (when transfecting for HR-TIDE experiments, HR template and a guide RNA were co-transfected at a 3:2 ratio, for template only or guide samples a corresponding plasmid was replaced by the same amount of the empty vector), 10 μl of Lipofectamine 2000, and 250 μl of OptiMEM medium. Transfection mix 2 contained 12,5 μl of Lipofectamine 2000, and 250 μl of OptiMEM medium. Transfection mixes 1 and 2 were incubated for 5 min at room temperature, then mixed and incubated for 10 more min at room temperature. For transfection, cells were centrifuged for 3 min at 1200 rpm, and the supernatant removed. Resulting pellets were carefully disloged, and then gently resuspended in corresponding transfection mixes. Transfection was performed with 15 minutes of incubation in suspension at 37°C. In order to prevent a pellet formation, tubes were gently shaken every 5 min. Cells

were plated on 6-well plates pre-coated with gelatine with addition of 0,5 ml of OptiMEM per well for 4 hours, than medium was replaced by standard ES cell culture medium supplemented with Shield1.

For transfections of cells cultured in 2i medium 1*10^6 cells was taken per sample and transfected with total of 2 μg of DNA. Reverse transfection was performed with reducing incubation time to 7 minutes. Cells were plated in 2i medium supplemented with 2% serum.

Growing conditions for 3T3 cells were set to 37°C, 5% CO_2. Cells were passaged every 3-4 days and kept in culture for no more than 8 passages.

3T3 cell medium composition:

High glucose DMEM

10% NCS

40μg/ml Gentamycin

Transfection of 3T3 cells was performed using Lipofectamine 2000. Cells were plated at a confluency of $1*10^5$ cells per sample on a well of a 6-well plate 18 h prior transfection. On the day of transfection, medium in each well was replaced by 1 ml of a fresh 3T3 cell medium and transfection mixes were prepared. Transfection mix 1 contained total of 2 μg of DNA (when transfecting for HR-TIDE experiments, HR template and a guide RNA were co-transfected at a 3:2 ratio, for template only or guide samples a corresponding plasmid was replaced by the same amount of the empty vector) and 150 μl of OptiMEM medium. Transfection mix 2 contained 10 μl of Lipofectamine 2000 and 150 μl of OptiMEM medium. Transfection mixes 1 and 2 were incubated for 5 min at room temperature, then mixed and incubated for 10 more min. Next, each transfection mix was added to a corresponding well.

Cell cycle analysis

Cell cycle analysis was performed using Click-iT EdU incorporation kit (Invitrogen) according to a slightly modified manufacturer's protocol. Cell culture medium was changed to fresh medium pre-warmed to 37°C and supplemented with EdU at a final concentration of 50 μM. After 30 min, cells were harvested by trypsinisation, achieving a state of single-cell suspension, washed with 1% BSA in PBS, and fixed with a 100 μl of a provided fixative solution (Component D) for 15 min at room temperature. Then cells were washed with 1% BSA in PBS and incubated with a 100 μl of a provided permeabilization and wash reagent (prepared according to manufacturer's protocol) for 15 min at room temperature. Next, cells were washed with 1% BSA in PBS and a total 200 μl a reaction cocktail was added per sample, followed by a 30 min incubation. Then samples were washed with 1,2 ml per sample of a permeabilization and wash reagent and pelleted, and supernatant was removed. Resulting pellets were resuspended in 200 μl of a permeabilization and wash reagent, supplemented by RNaseA at a final concentration of 4 μg/ml, and PI at a final concentration of 4 μg/ml. Samples were further analysed by FACS.

Reaction cocktail composition (per sample):

175,2 μl of PBS

4 μl of $CuSO_4$ (Component F)

2 μl of Fluorescent dye azide (prepared according to manufacturer's protocol)

20 μl of Reaction Buffer Additive (prepared according to manufacturer's protocol)

Western blotting

Cells were harvested by trypsinisation and lysed in RIPA buffer containing HEPES pH7.6 50mM, Sodium dexicholate 0.5%, NP40 1%, EDTA 1mM, LiCl 0.5M. Quantification was performed with Bradford assay according to manufacturer's protocol. Samples were mixed with NuPAGE lysing buffer and pre-heated for 20 minutes. Samples were loaded on pre-cast 4-12% gradient gels for migration. Wet transfer was performed on nitrocellulose membrane at 400 mA for 90 minute. Membrane was blocked in 5% non-fat dry milk in PBS for 1 hour at room temperature. Primary antibodies were diluted in 3% non-fat dry milk in PBS-0.1% Tween20 and incubated at 4°C overnight. Primary antibodies used were γH2AX (Abcam

ab2893) diluted 1:1000, tubulin (Sigma T9026) diluted 1:10000 and GFP (Santa Cruz sc-9,996 (clone B-2)) diluted 1:500. Membranes were washed with PBS-0.1% Tween20 and incubated with secondary antibodies for 1 hour at room temperature. After incubations were washed with PBS-0.1% Tween20 and developed using ECL reagent.

RIPA buffer composition:

50mM HEPES pH7.6

0,5% Sodium dexicholate

1% NP40

1mM EDTA

0.5M LiCl

Chromatin Immunoprecipitation (ChIP)

Cells were rinsed with PNS and fixed with 1% PFA in PBS for 15 minutes at 37°C. Cells were washed twice with ice-cold PBS and collected by scraping on ice in PBS with PIC and PhoSTOP. Scraped cells were pulled down at 4°C for 5 minutes at 1200 rpm. Starting from scraping, all samples and buffers were kept on ice, except for SDS-containing sonication buffer. After removing supernatant cells were incubated for 10 minutes of ice in Lysis buffer 1 and centrifuged at 4°C for 2 minutes at 6000 rpm. After removing supernatant cells were incubated for 10 minutes in Lysis buffer 2 and centrifuged at 4°C for 2 minutes at 6000 rpm. Supernatant was removed and after addition of Sonication buffer cells were transferred into Covaris sonication tubes and sonicated for 15 minutes. 50 µl of Protein G Dynabeads was used for each sample. Beads were washed 3 times with 5 mg/ml BSA in PBS, blocked in 5 mg/ml BSA in PBS for 30 minutes at 4°C on a rotating wheel and coated with antibodies for 4 hours. Immunoprecipitation was performed with 20 µg of sonication materiel. Samples were diluted with ChIP dilution buffer and immunoprecipitated overnight at 4°C. Samples were washed twice with SDS wash buffer, once in high salt buffer and once in TE buffer. Samples were eluted with Elution buffer and decrosslinked overnight at 65°C with vigorous shaking.

Lysis buffer 1 composition:

10 mM Tris-HCl pH 8.0

0.25% TritonX100

100 mM EDTA

Lysis buffer 2 composition:

10 mM Tris-HCl pH 8.0

200 mM NaCl

20 mM EDTA

Sonication buffer composition:

10 mM Tris-HCl pH 8.0

100 mM NaCl

1 mM EDTA

1% SDS

SDS wash buffer composition:

2% SDS in water

High salt buffer composition:

50 mM HEPES pH 7.5

500 mM NaCl

1 mM EDTA

1% TritonX100

0.1% Sodium deoxycholate

TE buffer composition:

10 mM Tris-HCl pH 7.5

1 mM EDTA

Elution buffer:

50 mM Tris-HCl pH 8.0

10 mM EDTA

1% SDS

TIDE

Cells for analysis were collected 48h post transfection.

gDNA extraction was performed by Machry-Nagel NucleoSpin tissue kit according to manufacturer's protocol.

PCR amplification was performed using Invitrogen Taq Polymerase.

PCR cycle:

Stage 1 (repeated once): 95°C, 5 min

Stage 2 (repeated 35 cycles): 95°C, 30 sec; 60°C, 30 sec; 72°C, 1 min 30 sec

Stage 3 (repeated once): 72°C, 10 min; 10°C, indefinitely

PCR cleanup was performed using Beckman AMPure XP kit according to manufacturer's protocol.

Table1. Guide RNAs used

Gene	Guide RNA number	Guide RNA sequence
Actin	Pg255	GAACGGCGGGCGCTGAT
Pou5f1	Pg261	ACGTCCCCAACCTCCGTC
Gapdh	Pg313	TCACCCACTGTAGCCCCA
Ngn2	Pg316	ACAATCAGATCTGCCCCG
Tfcp2l1	Pg360	CTTAGCTACTGACCCTG
Hoxb1	Pg361	ATCCATCTGAGAGCGACA
Pou5f1	Pg418	AACCTCCGTCTGGAAGACAC
Actin	Pg438	TTTTATAGGACGCCACAG
Gapdh	Pg439	CACTACCGAAGAACAACG
Nanog	Pg442	AGAACTAGGCAAACTGTG
Nanog	Pg443	CTGAGATGCTCTGCACAG
Pax6	Pg445	GCTGGAGGATGATGACAG
Pax6	pG446	GCGCGAGCCACAACAGCG
Zic1	pG447	CGGCGTCCAGGAGCATCG
Zic1	pG448	CGTGGCCGAGAGAGACGT
Ngn2	pG449	CATGCACACTTACCTACG
Hoxb1	pG450	GAAAGAAACATGGAATGG
Tdrd1	pgl1	AACCTCAGTGACTCTCAGCG
Tdrd1	pgl2	TCTCTAGAAAGGTGTCCCGG
Mc4r	pgl3	CAGAGTCACAAACACCTCGG
Mc4r	pgl4	GAGGTTGGATCAGTTCAAGG
Sox6	pgo1	TCAAACCCATGTGTGCAGAG
Ptn	pgo2	AGTATGGAAATCTCACACCG
Nrp1	Pgo3	catatgtactctcagtacag

Table 2. Primers used for HR-TIDE

Gene	Guide RNA number	Forward primer	Reverse primer
Actin	Pg255	CGTAGCGTCTGGTTCCCAAT	AGGTGCGTGTCCTTCTAAGC
Pou5f1	Pg266	CCCTCCTCCTAATCCCGTCT	TTCTAGTCCACACTGCGTCG
Gapdh	Pg313	GAGGAGTCCTTGGAGTGTGC	TGCTGAGTCACTTGGAGCAG
Ngn2	Pg316	CTCTCTCACAACGTGCCTCC	GGTGAGCGCCCAGATGTAAT
Tfcp2l1	Pg360	ATGTCACACGAGCCCAGTTT	GCTAGCGAAATCCCCACAGA
Hoxb1	Pg361	AGACAGTGTCACACGTAGGC	GATGCAAAGGTTGCGGTCTG
Pou5f1	Pg418	CCCTCCTCCTAATCCCGTCT	TTCTAGTCCACACTGCGTCG
Actin	Pg438	CGTAGCGTCTGGTTCCCAAT	AGGTGCGTGTCCTTCTAAGC
Gapdh	Pg439	TGGAACTCACCCGTTCACAC	GCAGGGCATCCTGACCTATG
Nanog	Pg442	TTGCGTTAAAAAGCCGCACT	GAGCTTCAGACCTTGGCTCC
Nanog	Pg443	TTGCGTTAAAAAGCCGCACT	GAGCTTCAGACCTTGGCTCC
Pax6	Pg445	AAGCAGCCGCACTTAGTCAA	TAGTGGCTTCTTTCACCGCC
Pax6	pG446	AGGGAGAGGGAGCATCCAAT	GGGAACACACCAACTTTCGC
Zic1	pG447	GCACGACTTTTTGGGGTTGG	TGGCAGCCCTGTTAGTCAAA
Zic1	pG448	GCACGACTTTTTGGGGTTGG	TGGCAGCCCTGTTAGTCAAA
Ngn2	pG449	CTCTCTCACAACGTGCCTCC	GGTGAGCGCCCAGATGTAAT
Hoxb1	pG450	CTCTTGCCCTCCTGGACTTG	TCCATGTAGAGGCCGAAGGA
Tdrd1	pgl1	GAGGTGGGGCATAACGACTT	CAGGAGGGGTTGCACGTTTA
Tdrd1	pgl2	GAGGTGGGGCATAACGACTT	CAGGAGGGGTTGCACGTTTA
Mc4r	pgl3	TCGATGACGGCGTTACACAT	TGAGCCTTCCGTCATTCAGG
Mc4r	pgl4	TCGATGACGGCGTTACACAT	TGAGCCTTCCGTCATTCAGG
Sox6	pgo1	CGTACTGCACCTCAGTGTGA	CTATAGTGTGCGTGAGGCGA
Ptn	pgo2	CACCACACCACTTAGCCCAT	CAGGGAGGTGACAGAAACCC
Nrp1	Pgo3	GCCTGCTGGGCAAATTGAAA	AGGGAGGAATGGGGCATACT

Table3. Primers used for sequencing

Gene	Guide RNA number	Sequencing primer
Actin	Pg255	CGTAGCGTCTGGTTCCCAAT
Pou5f1	Pg266	GGGGACATATCTGGTTGGGG
Gapdn	Pg313	GATCTCACCCTGTGTCCACG
Ngn2	Pg316	CCCGAGTCTCGTGTGTTGTC
Tfcp2l1	Pg360	GTCAGTGTTCAGAGCGAGGA
Hoxb1	Pg361	TGCCATCGTTTTCCCTCCTC
Pou5f1	Pg418	GGGGACATATCTGGTTGGGG
Actin	Pg438	CGTAGCGTCTGGTTCCCAAT
Gapdh	Pg439	AGCTACGTGCACCCGTAAAG
Nanog	Pg442	AGCCGTTGGCCTTCAGATAG
Nanog	Pg443	AGCCGTTGGCCTTCAGATAG
Pax6	Pg445	GGCAGAGCCGAAAACAAGTG
Pax6	pG446	GGAGCCTTGACAACGACGA
Zic1	pG447	CGGGTAGAATTGAAAGCAGCG
Zic1	pG448	CGGGTAGAATTGAAAGCAGCG
Ngn2	pG449	GCACGAGAACGACAACACAC
Hoxb1	pG450	GCCATCGTTTTCCCTCCTCT
Tdrd1	pgl1	TCTCTGAGTTCACGGCCAAC
Tdrd1	pgl2	TCTCTGAGTTCACGGCCAAC
Mc4r	pgl3	TGGTACTGGAGCGCGTAAAA
Mc4r	pgl4	ATGACGATGGTTTCCGACCC

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The role of the chromatin organization in DNA double strand break repair in mouse embryonic stem cells

Résumé

De plusier facteurs influencent la réparation de l'ADN, y compris l'organisation locale de la chromatine, l'état de différenciation et du cycle cellulaire. En développant un système CRISPR/Cas9 dans des cellules souches de souris pour induire des cassures des doubles brins dans divers contextes chromatiniens, et HR-TIDE, une méthode pour détecter le résultat de la réparation, nous avons pu montrer que la fréquence de l'utilisation du recombinaison homologue est plus élevée chez les cellules souches que dans les cellules différenciées. Nous avons pu voir qu'il est partiellement causé par des différences de cycle cellulaire. Nous pourrions également confirmer que la chromatine active favorise plutôt la recombinaison homologue, alors que l'hétérochromatine facultative et les domaines bivalents la répriment. Dans l'ensemble, nos données ont mis en lumière le rôle de la chromatine bivalente et de l'hétérochromatine facultative dans le processus de choix de la voie de réparation de l'ADN.

Mots clés : cellules souches de souris, l'hétérochromatine facultative, domaines bivalents, HR, NHEJ, MMEJ, fidélité de réparation, CRISPR/Cas9

Résumé en anglais

Multiple parameters can influence DNA repair, including local chromatin organization around the damage site, cell differentiation status, and a cell cycle state. Developing a CRISPR/Cas9 system in mouse embryonic stem cells for specific targeting chromatin types of interest, and HR-TIDE, a method to detect repair outcome, we were able to show that homologuos recombination frequency, despite often being low, is higher in embryonic stem cells than in differentiated cells. However, we could see that it is at least partially caused by cell cycle differences. We could also confirm that transcriptionally active chromatin is rather promoting homologous recombination, whereas facultative heterochromatin and bivalent domains represent a repressive environment. All in all, our data shed light on the role of bivalent chromatin and facultative heterochromatin in the process of DNA repair pathway choice.

Keywords : mouse ES cells, facultative heterochromatin, bivalent domains, HR, NHEJ, MMEJ, repair fidelity, CRISPR/Cas9