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# New players at the interface between transcription and DNA repair

Alexander Zhovmer

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**THÈSE** présentée par :

**Alexander ZHOVMER**

soutenue le : **28 Septembre 2012**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**  
Discipline/ Spécialité : **Aspects Moléculaires et Cellulaires de la Biologie**

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

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**THESE**



Présentée par  
**Alexander Zhovmer**

Pour obtenir le grade de  
**Docteur en Sciences de l'Université de Strasbourg**

Discipline : Sciences du Vivant  
Spécialité : Aspects Moléculaires et Cellulaires de la Biologie

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

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## CONTENTS

p.2	ACKNOWLEDGEMENTS
p.3	CONTENTS
p.5	ABBREVIATIONS
p.10	RESUME DE LA THESE DOCTORAT
	<ul style="list-style-type: none"><li>• INTRODUCTION</li><li>• RESULTAS</li><li>• CONCLUSION</li><li>• LISTE DES PUBLICATIONS ET POSTERS</li><li>• REFERENCES</li></ul>
p.11	FIGURES AND TABLES
p.12	FOREWORD
p.13	INTRODUCTION: PART I - A NEW LOOK AT A VERY OLD COMPLEX
	<ul style="list-style-type: none"><li>• TFIIH COMPLEXES IN TRANSCRIPTION AND DNA REPAIR</li><li>• TFIIH: A MULTISUBUNIT FACTOR WITH SEVERAL CELLULAR TASKS</li><li>• THE CAK COMPLEX: THE DOUBLE LIFE OF A KINASE</li><li>• TFIIH IN TRANSCRIPTION AND REPAIR: DR. JEKYLL AND MR. HYDE</li><li>• CONCLUSION</li><li>• REFERENCES</li></ul>
p.32	INTRODUCTION: PART II - DNA REPAIR IN VIVO
	<ul style="list-style-type: none"><li>• CHROMATIN REMODELING</li><li>• CHROMATIN MODIFICATIONS</li><li>• RNA IN DAMAGE RESPONSE</li><li>• NEW ELEMENTS FIT NER JIGSAW PUZZLE</li><li>• CONCLUSION</li><li>• REFERENCES</li></ul>
p.55	PUBLICATION I «Molecular insights into the recruitment of TFIIH to sites of DNA damage»
p.56	PUBLICATION II

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

p.57

PROJECT I

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

p.80

DISCUSSION AND CONCLUSION

REFERENCES

## ABBREVIATIONS

DDR	-DNA damage response
NER	-nucleotide excision repair
siRNA	-short interfering/silencing RNA
GGR	-global genome repair
TCR	-transcription-coupled repair
6-4PPs	-pyrimidine-(6-4)-pyrimidone photoproducts
CPDs	-cyclobutane pyrimidine dimers
XP	-xeroderma pigmentosum
TTD	-trichothiodystrophy
CS	-Cockayne syndrome
UV(A/B/C)	-ultraviolet light (400 – 315 /315 – 280 /280 – 100 nm)
FRAP	-fluorescence recovery after photobleaching
MEF	-mouse embryonic fibroblasts
TFIIH	-transcription factor II H, multisubunit complex TFIIH core subunits:
• p62	-General transcription factor IIH subunit 1
• p52	-General transcription factor IIH subunit 4
• p44	-General transcription factor IIH subunit 2
• p34	-General transcription factor IIH subunit 3
• p8	-General transcription factor IIH subunit 5
• XPB	-ERCC3, ATP dependent human DNA helicase
• XPD	-ERCC2, ATP dependent human DNA helicase
CAK	-cyclin activating kinase-subcomplex of TFIIH
• CDK7	-Cell division protein kinase 7
• MAT1	-CDK-activating kinase assembly factor
• Cyclin H	
POLII	-RNA polymerase II
CTD	-carboxy-terminal domain
DOT1L	-DOT1-like, histone H3 lysine 79 methyltransferase

# **RESUME DE LA THESE DOCTORAT**

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

### **INTRODUCTION**

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

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



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

D'autre part, nous nous sommes également concentrés sur DOT1L, une histone-méthyltransférase impliquée dans la régulation du silencing télomérique, dans le contrôle du cycle cellulaire et dans l'étape d'élongation de la transcription. Nous avons montré que l'absence de DOT1L dans des fibroblastes embryonnaires de souris (MEF<sup>DOT1L</sup>) conduit à une sensibilité de ces cellules aux irradiations UV.

Enfin, lors d'un travail en collaboration avec un thésard du laboratoire, nous avons étudié le rôle des sous-unités XPB et XPD de TFIIH, gardiennes essentielles du génome, dans la réparation par excision de nucléotides.

## RESULTAS

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

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

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

## CONCLUSION

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

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

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

## LISTE DES PUBLICATIONS ET POSTERS

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

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## **FIGURES AND TABLES**

### INTRODUCTION

- p.15 Figure 1. TFIIH, a multisubunit and multitasks complex
- p.16 Table 1. TFIIH enzymatic activities, their functions, and their regulators
- p.18 Figure 2. Three Cdk7-containing complexes
- p.20 Figure 3. Dynamic composition of the TFIIH complex
- p.21 Figure 4. Nucleotide excision repair pathway
- p.23 Figure 5. CAK and TTDA modules engage the core TFIIH in different cellular pathways
- p.34 Figure 6. Mammalian NER-associated chromatin remodeling
- p.35 Table 2. SNF2-protein complexes involved in DDR
- p.36 Figure 7. Post-translational modifications 1
- p.37 Figure 8. Post-translational modifications 2
- p.41 Figure 9. miRNA biogenesis in the DNA damage response
- p.42 Table 3. NER genes
- p.45 Figure 10.  $\beta$ -hairpin structures in XPC and DDB2

### PROJECT I

- p.60 Figure 1. Screening design
- p.62 Table 1. Ingenuity kinome&phosphatome gene gene network analysis: functions and pathways
- p.63 Figure 2. Ingenuity kinome&phosphatome gene network analysis
- p.66 Figure 3. Ingenuity gene network analysis for chromatin remodelers 1
- p.67 Figure 4. Ingenuity gene network analysis for chromatin remodelers 2
- p.69 Figure 5. STRING-DB analysis for chromatin remodelers

## **FOREWORD**

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

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

# **INTRODUCTION**

## **PART I - A NEW LOOK AT A VERY OLD COMPLEX**

### **TFIIH COMPLEXES IN TRANSCRIPTION AND DNA REPAIR**

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

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

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

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

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

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

#### TFIIH: A MULTISUBUNIT FACTOR WITH SEVERAL CELLULAR TASKS

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

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



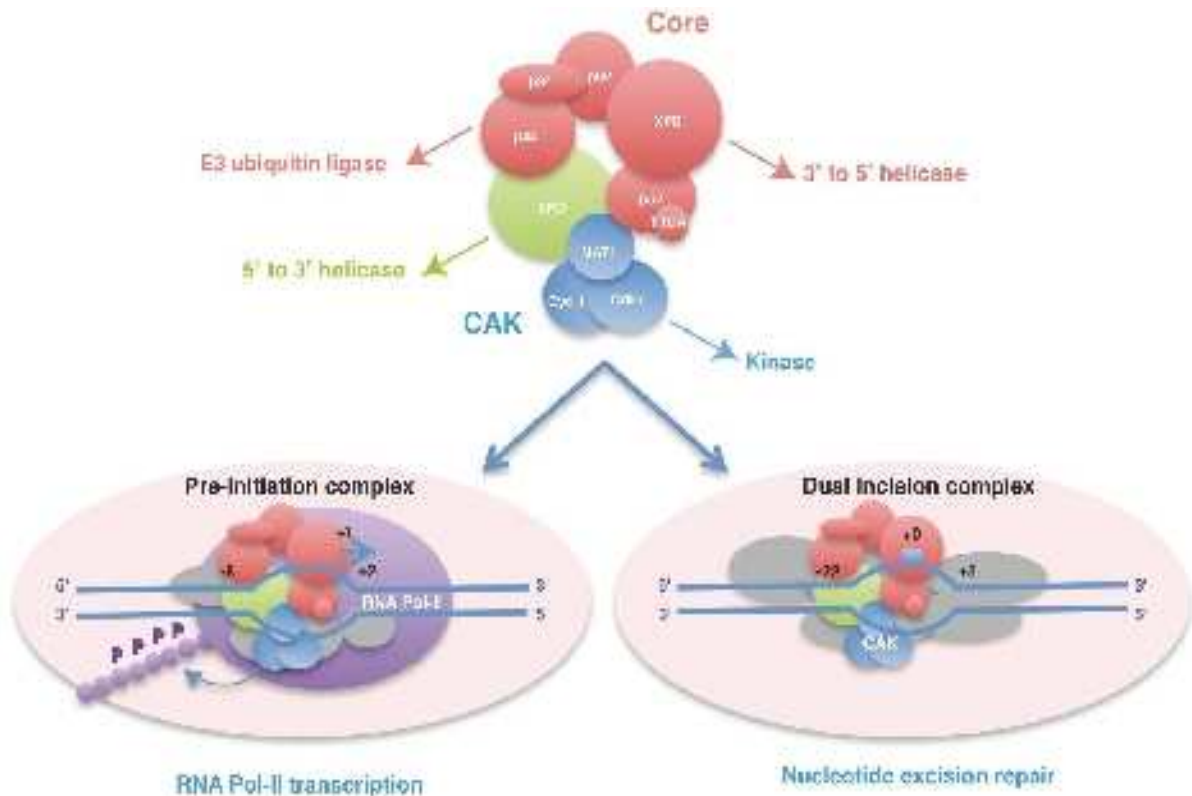


Figure 1. A multisubunit and tasks complex,TFIIH is a ten-subunit complex composed of a core (in red; XPB, p62, p52, p44, p34, and TTD4) associated to the CAK (in blue; Cdk7, CycH, and MAT1) through MAT1 and the XPD subunit (in green). Four enzymatic activities are found in TFIIF: XPB and XPD are 3' to 5' and 5' to 3' helicases, Cdk7 is a kinase, and p44 has been described as an E3 ubiquitin ligase in yeast. The complex is involved both in RNPI- and II-dependant transcription and in nucleotide excision repair. The role of TFIIF in RNPI transcription is unknown. In RNPII-dependant transcription, TFIIF opens DNA around the promoter in the preinitiation complex (-8 to +2 relatively to the transcription start site +1) and phosphorylates the carboxyl terminal domain of the RNPII (purple) to license transcription. In NER, TFIIF opens DNA around the lesion (-22 to +5 relatively to the lesion in blue square) and assists ERCC1-XPF for the 5' incision[77].

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

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

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

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

\* Unpublished data  
 \*\* In yeast.

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

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

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

#### THE CAK COMPLEX: THE DOUBLE LIFE OF A KINASE

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

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

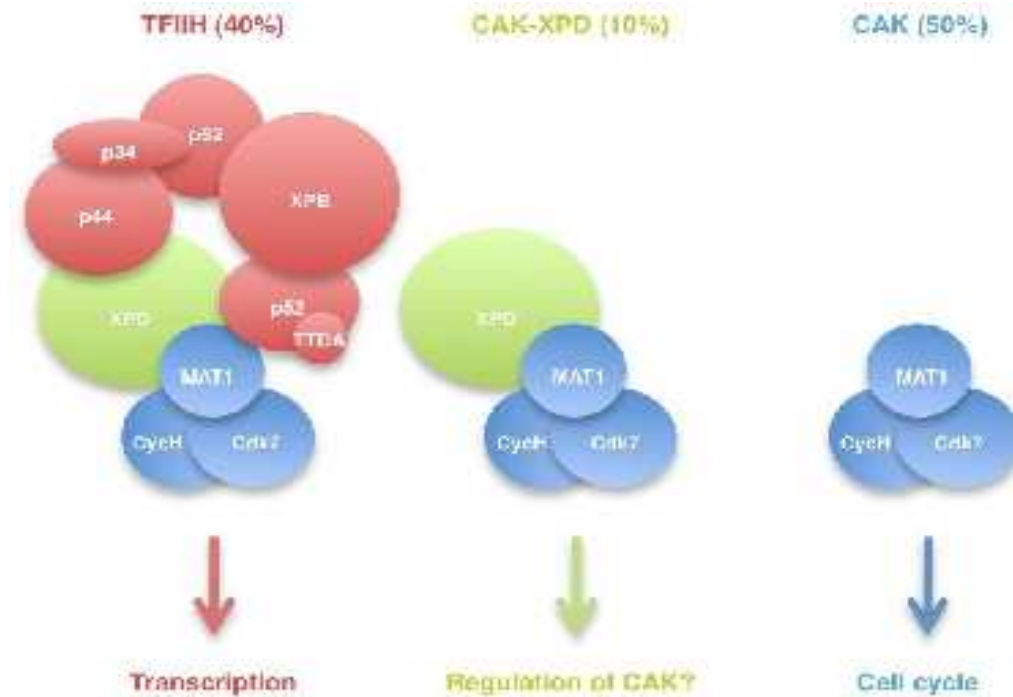


Figure 2. Three Cdk7-containing complexes. Cdk7 kinase can be found in three different complexes, TFIIH, CAK-XPD, and CAK, that represent 40, 10, and 50% of the Cdk7-containing complexes (personal data), respectively. When Cdk7 is in TFIIH, it functions in transcription, while it functions in cell cycle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

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

## TFIIH IN TRANSCRIPTION AND REPAIR: DR. JEKYLL AND MR. HYDE

To understand how the ten subunits that constitute the TFIIH complex participate in repair and/or transcription processes, we utilized an original approach combining chromatin immunoprecipitation and western blotting (ChIP-Western)[70]. With this technique, we were able to analyze the composition of TFIIH on the chromatin before or after UV irradiation. Surprisingly, we observed a dynamic dissociation/reassociation of the CAK complex onto the core TFIIH that correlates with the recruitment/release of the repair factors during the DNA repair reaction[68] (Figure 3). In the absence of formaldehyde cross-linking, the dissociation of CAK is still observed in the soluble cell fractions, but the UV dose required to dissociate CAK from the core under this condition is much higher than the dose needed to detect the dissociation by ChIP-Western. Also, while repair factors accumulate on the core TFIIH by ChIP, no accumulation was observed on TFIIH in the soluble fraction after UV. These observations advantageously reconcile the yeast and human models by showing that in humans, the free core TFIIH is transient and exists only in the chromatin, during the short period of DNA repair, while in yeast, it exists in the soluble fraction and in absence of DNA damage. In both cases, it is the core TFIIH that participates in DNA repair, in the absence of CAK. Fifteen minutes after 20J/m<sup>2</sup> UV-C irradiation, CAK bound to the TFIIH core reaches a minimum level, impaired in XP-C cells, in which TFIIH is not recruited to the damaged DNA[71], suggests that the change in TFIIH subunit composition is due to its participation in the removal of the 6-4PP lesions that are eliminated in the first hours after UV irradiation[72]. After the removal of the 6-4PP lesions (6–8 h post irradiation), we observed the dissociation of the repair factors from the core and the return of the CAK. We noticed that mutations in NER proteins, as found in XP patients, cause an accumulation of intermediate repair complexes that can persist for several hours on the chromatin (Figure 3) whereas the recruitment of repair factors to the TFIIH core reaches its maximum level. At that specific time point, about 70% of TFIIH is involved in DNA repair and does not contain CAK. This early event, together with the fact that the release of CAK is the persistence of these intermediate poised complexes may potentially cause prolonged transcription and replication arrests, and their impact on cell survival should be investigated.

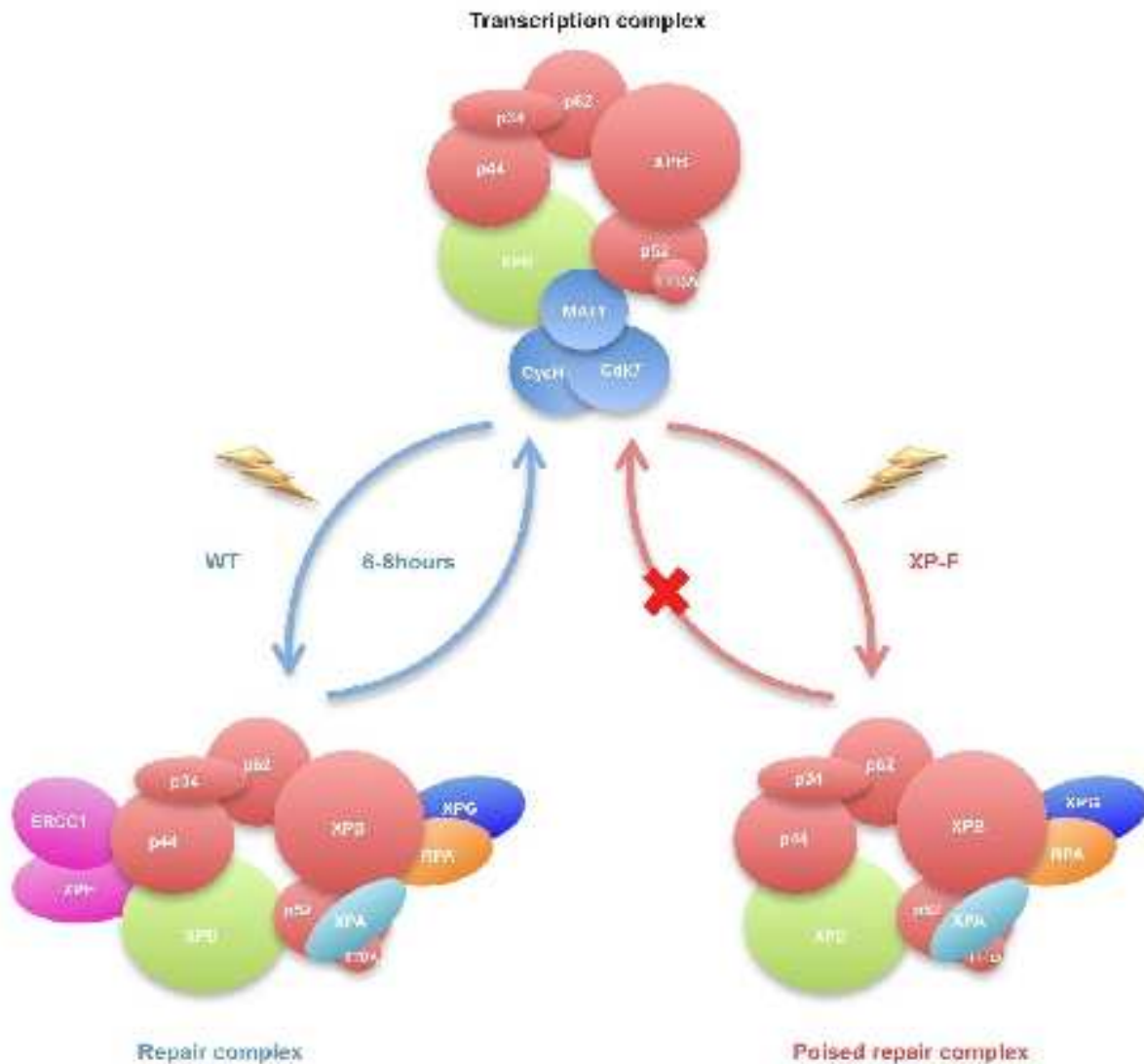


Figure 3. Dynamic composition of the TFIID complex. When TFIID functions in transcription, it contains ten subunits, including the CAK module. When it functions in repair, it does not contain the CAK module, but associates with various DNA repair factors, including XPA, RPA, XPG, and ERCC1-XPF. In a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

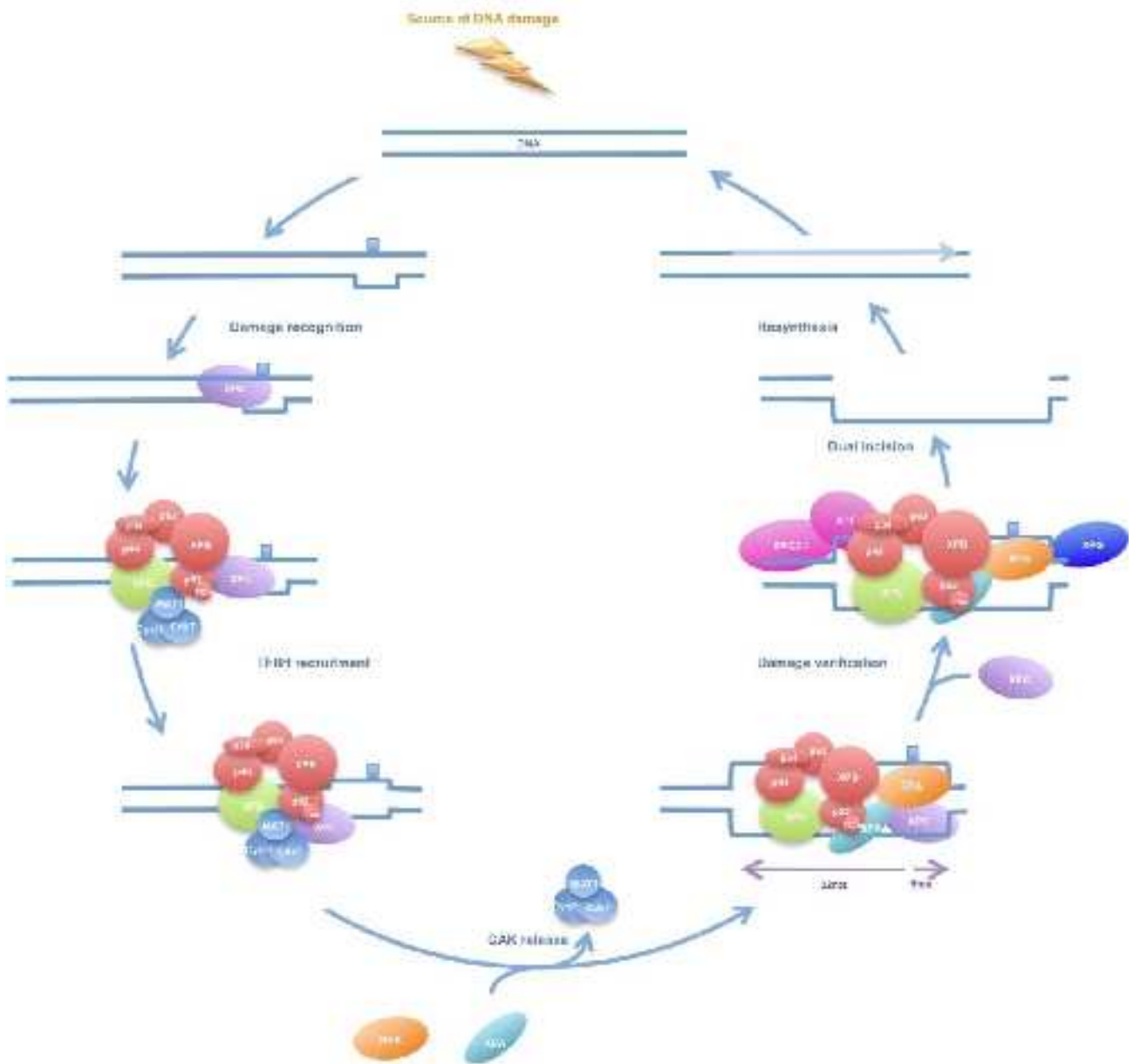


Figure 4. Nucleotide excision repair pathway. Following exposure to genotoxic agents (e.g., sunlight), a lesion (blue square) is created on the DNA. Then, the damage recognition factor XPC-HR23B interacts with the damaged DNA structure on the opposite strand of the lesion. TFIIH joins XPC-HR23B on the damaged DNA. In the presence of ATP, XPB, and XPD, helicases in TFIIH are involved in the opening of the DNA, allowing the stable association of XPA and RPA, which help to enlarge the opened structure and drive the dissociation of the CAK complex from TFIIH. This dissociation is a prerequisite for the enlargement of the DNA opening that favors the arrival of XPG, mediating the release of XPC-HR23B. The recruitment of XPF-ERCC1 triggers dual incision and excision of the protein-free damaged oligonucleotide. The resynthesis machinery fills the gap and seals the DNA extremities.

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

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

## CONCLUSION

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



investigations.

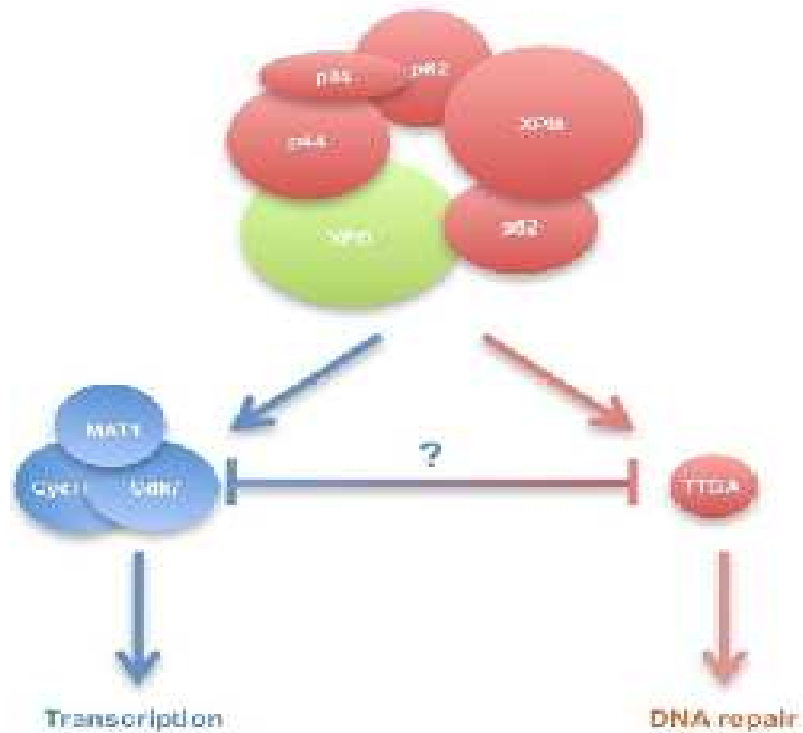


Figure 5. CAK and TTDA modules engage the core TFIID in different cellular pathways. TFIID is composed of a core that associates with different modules to enable its engagement in different cellular processes. The presence of CAK in TFIID engages the core in transcription[29], while the presence of TTDA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

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## PART II - DNA REPAIR IN VIVO

### CHROMATIN REMODELLING

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

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

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

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

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

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

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

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

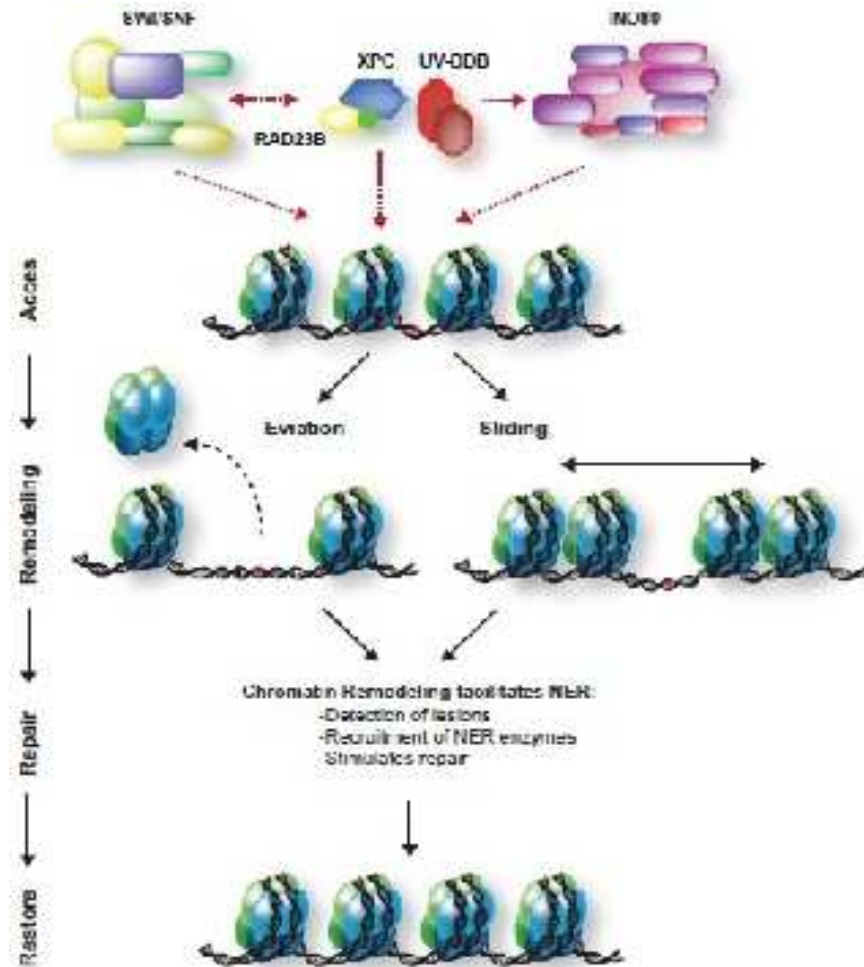


Figure 6. Mammalian nucleotide excision repair (NER)-associated chromatin remodeling. Both the SWI/SNF and the INO80 ATP-dependent chromatin-remodeling complexes are recruited to sites of UV-induced DNA damage, and are implicated in mammalian global genome NER (GG-NER). SWI/SNF may interact with the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

## CHROMATIN MODIFICATIONS

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

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

Remodeling complex	Snf2-protein subunit	DDR-associated activities
INO80	Ino80 (yeast)	Recruited via phospho-H2AX ( $\gamma$ -H2AX). Evicts nucleosome at damage sites. Participates in the DNA-damage cell cycle checkpoint pathway.
SWR1	Swr1 (yeast)	Recruited via $\gamma$ -H2AX. Important for error-free non-homologous end-joining (NHEJ) pathway.
SWI/SNF	Snf2 (yeast) Brg1 or BRG1 (human)	Associated with the homologous recombination (HR) repair pathway – possibly to clear nucleosomes and expose donor sequences to repair machinery. Human SWI/SNF facilitates $\gamma$ -H2AX accumulation at DSBs.
RSC	Sth1 (yeast)	Linked with both HR and NHEJ repair pathways. May remodel DSB sites to facilitate loading of other repair factors.
Aic1	Aic1 (human)	Recruitment to damage sites by PARylation. Remodelling activity (in vitro) stimulated by PARylation of PARP1 and/or nucleosomes. Aic1-depletion leads to hypersensitivity to damaging agents.
NJRD	CHD4 (human)	Recruitment to damage sites by PARylation. CHD4 is phosphorylated by ATM kinase – a major DSB checkpoint kinase. CHD4-depletion leads to hypersensitivity to damaging agents and deficient DSB repair.
ACF	Snf2H (human)	ACF accumulates at sites of DNA damage. ATPase activity of Snf2H required for efficient DSB repair.

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

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

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

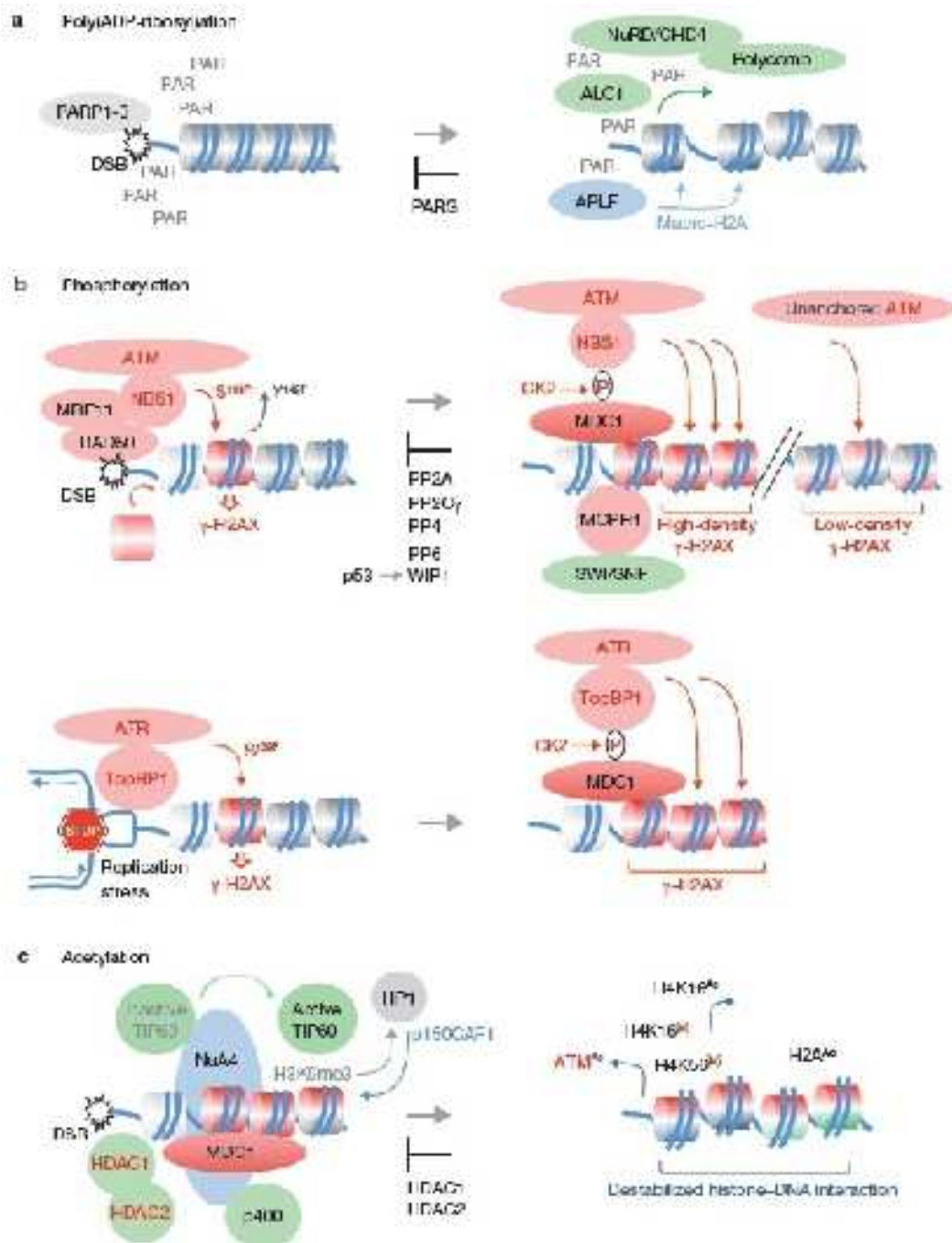


Figure 7. Chromatin responses to DNA damage are orchestrated by a series of post-translational modifications. These include (a) poly(ADP-ribosyl)ation, (b) phosphorylation and (c) acetylation. Left panels depict the key protein complexes involved in a given modification together with the mechanisms regulating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

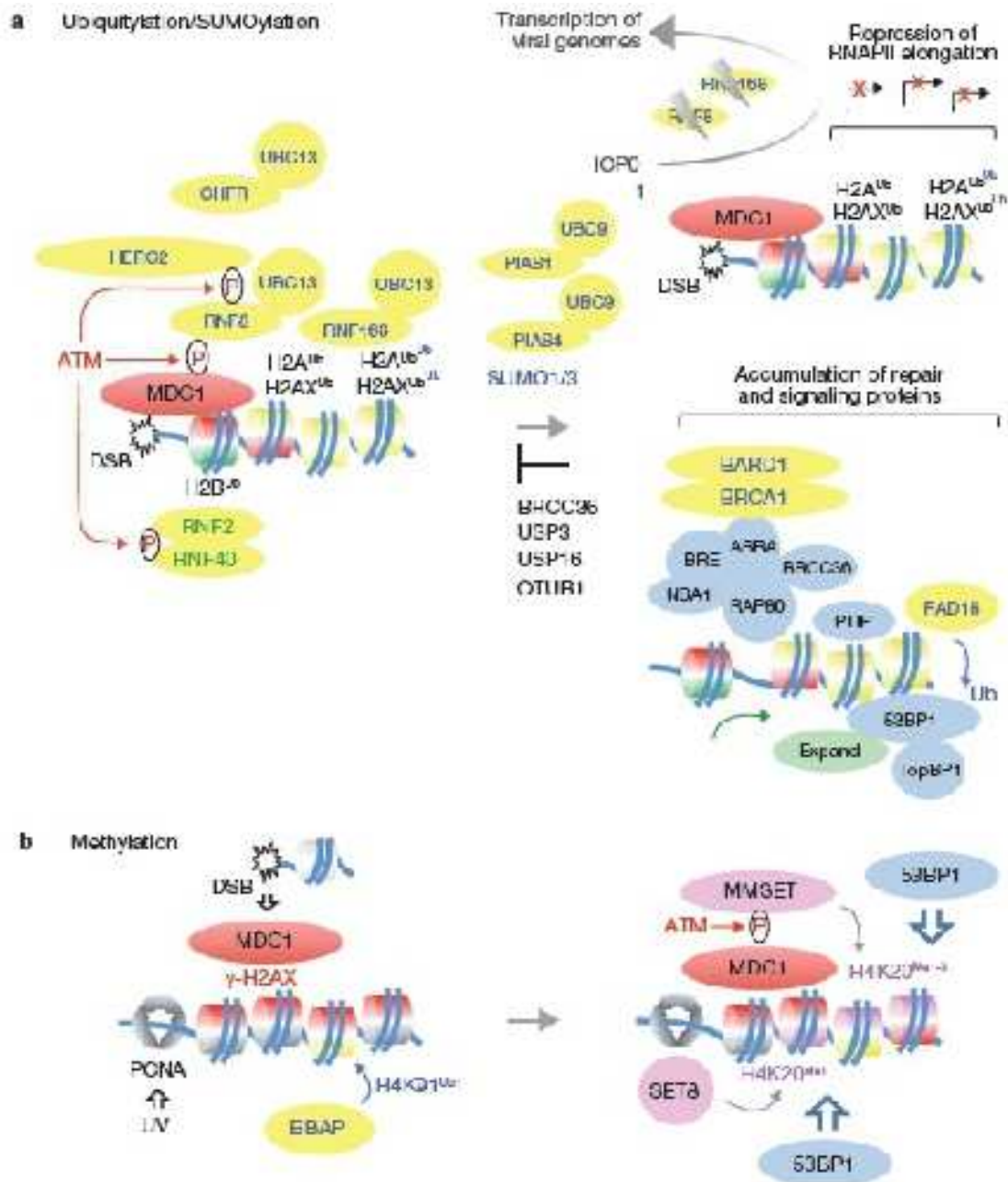


Figure 8. Post-translational modifications that affect chromatin organization and recruit additional genome caretakers to promote repair and suppress transcription in the vicinity of DNA lesions. (a) Ubiquitylation with SUMOylation. (b) Methylation. Yellow; enzymes involved in ubiquitylation and SUMOylation.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

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

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

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

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



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

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

Ubiquitin and ubiquitin - like modifiers such as SUMO are as important for DDR and DNA repair as other modifications. They mediate signal transduction through post-translational modification of substrate proteins in pathways that control differentiation, apoptosis, cell cycle and responses to stress. In yeast, two SUMO E3 ligases SIZ1 and SIZ2 are responsible for the vast majority of SUMOylation and *siz1*Δ *siz2*Δ mutants are sensitive to ultraviolet light and are deficient in repair of both the transcribed and non-transcribed strands of the DNA. Mechanism of this deficiency is unclear but several factors that participate in NER are SUMOylated, including RAD4, RAD16, RAD7, RAD1, RAD10, SSL2, RAD3, and RPB4. And SUMO attachment to most of these NER factors is significantly increased by DNA damage[33]. Additionally, In yeast, the proliferating cell nuclear antigen PCNA is modified by ubiquitin and by SUMO. Ub-PCNA signal for recruitment of translesion DNA polymerases and SUMO-PCNA signals for recruitment of the anti-recombinogenic DNA helicase SRS2[31]. Ub-PCNA is also specifically recognized by Spartan that is recruited to sites of UV damage in a manner dependent upon the PCNA ubiquitylation. Spartan colocalizes and interacts with RAD18, the E3 ubiquitin ligase that modifies PCNA. Knockdown of Spartan compromises chromatin association of RAD18, monoubiquitylation of PCNA, and localization of Pol η to UV damage. Thus, a “reader” of ubiquitylated PCNA, Spartan creates a feed-forward loop to enhance PCNA ubiquitylation and translesion DNA synthesis[32].

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

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

## RNA IN DAMAGE RESPONSE

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

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

#### NEW ELEMENTS FIT NER JIGSAW PUZZLE

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

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

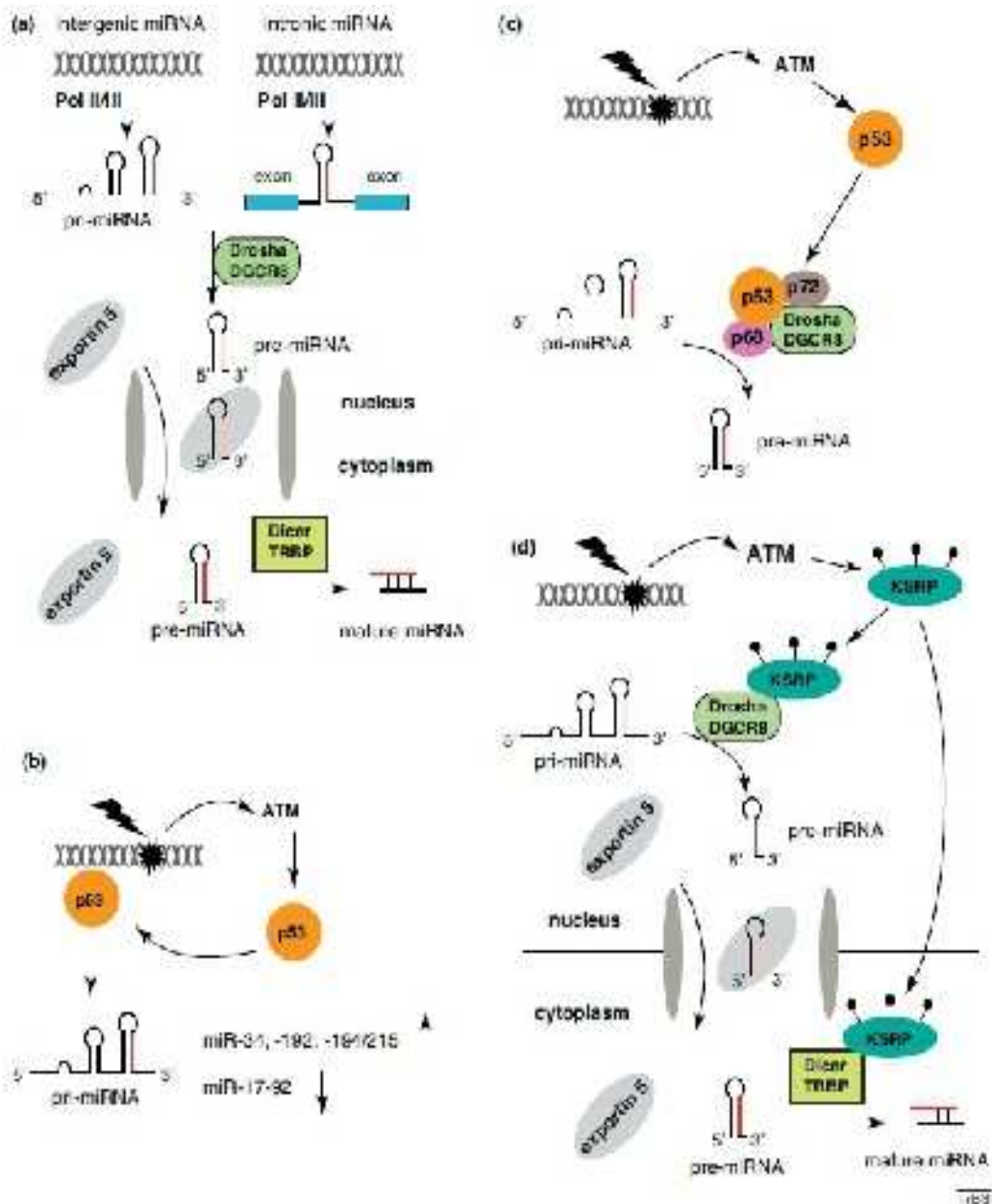


Figure 9. miRNA biogenesis in the DNA damage response. (a) miRNA biogenesis without DNA damage stress. Intergenic miRNA genes are transcribed by RNA polymerase II/III (Pol II/III) into pri-miRNAs, whereas intronic miRNAs are transcribed together with their host genes. The Drosha–DGCR8 complex recognizes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

Human gene*	Role	Comments	Rodent <sup>†</sup>	Saccharomyces cerevisiae <sup>‡</sup>	Escherichia coli <sup>§</sup>
<b>GGT genes</b>					
XPE (also known as ERCC1)	Lesion recognition	Recruits XPC and is p53 inducible	Dab2 (also known as Xpe)	Unknown	Unknown
DDIT1	Lesion recognition	Forms a complex with DDB2	Ddb1	Unknown	Unknown
XPC	Lesion recognition	Opens DNA and is p53 inducible	Xpa	RAD1	Him4
RAD23B	Lesion recognition	Forms a complex with XPC	Rad23b	RAD23	Unknown
Centin-2	Lesion recognition	Forms a complex with XPC	Unknown	Unknown	Unknown
<b>CCR and TCR genes</b>					
XPR	Helicase and TFIIH	TFIIH subunit <sup>¶</sup>	Frh3 (also known as Xpb)	RAD24	HimE
XPD	Helicase and TFIIH	TFIIH subunit <sup>¶</sup>	Ercc2 (also known as Xpd)	RAD3	UvrE
XPA	Lesion recognition	Stabilizes pre-replicative complexes in CCR and TCR	Xpa	RAD11	Unknown
RPA/p32, p32 and p24	ssDNA binding	Binds to XPA	Rpa	RP1, RP2 and RP3	Ssb2
XPG	Structure-specific endonuclease	3' incision	Lrrc1 (also known as Xpg)	RAD2	UvrC
FRCC1	Forms a complex with XPF	3' incision	Frh1	RAD10	Unknown
XPG	Structure-specific endonuclease	5' incision and stabilization of TFIIH	Ercc3 (also known as Xpg)	RAD2	UvrC
PCNA	DNA replication sliding clamp	Three subunits; contains clamping sites for DNA pol	Pcna	PCNA	$\beta$ -clamp
RFC1	Loads PCNA on DNA	RF; large subunit	Rfc1	CDC46	Unknown
Unknown	Removal of incised oligo	None	Unknown	Unknown	UvrD <sup>  </sup>
DNA pol $\alpha$ or $\epsilon$	DNA replication and repair	None	DNA pol $\alpha$ or $\epsilon$	DNA pol $\alpha$ or $\epsilon$	DNA pol I
DNA pol $\gamma$	Bypass polymerase	None	DNA pol $\gamma$	DNA pol $\gamma$	Pol II
DNA ligase-I	Ligase	None	Unknown	CDC10	Ligase
DNA ligase-III	Ligase complex	Might be associated with dividing or non-dividing cells	Unknown	Unknown	Ligase
XPC-H	TFIIH complex	Might be associated with dividing or non-dividing cells	Xpc-H	Unknown	Unknown
<b>TCR genes</b>					
CSA	Ubiquitin-ligase complex	WD repeat	Lrrc6 (also known as Csa)	RAD25	Unknown
CSB	TCR coupling factor and chromatin remodeling	Transcription elongation factor	Frh5 (also known as Csb)	RAD26	Mgi
XAB2	Transcription factor	Link between XPA and RNAP	Unknown	Unknown	Unknown
TTTS	RNAPII elongation factor	Stimulation of transcription cleavage by RNAPII	Unknown	Unknown	CraA and CraB
HMTN1	Chromatin relaxation	Nucleosome removal <sup>¶¶</sup>	Unknown	Unknown	Unknown
p100	Chromatin remodeling	Nucleosome removal <sup>¶¶</sup>	Unknown	Unknown	Unknown

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

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



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

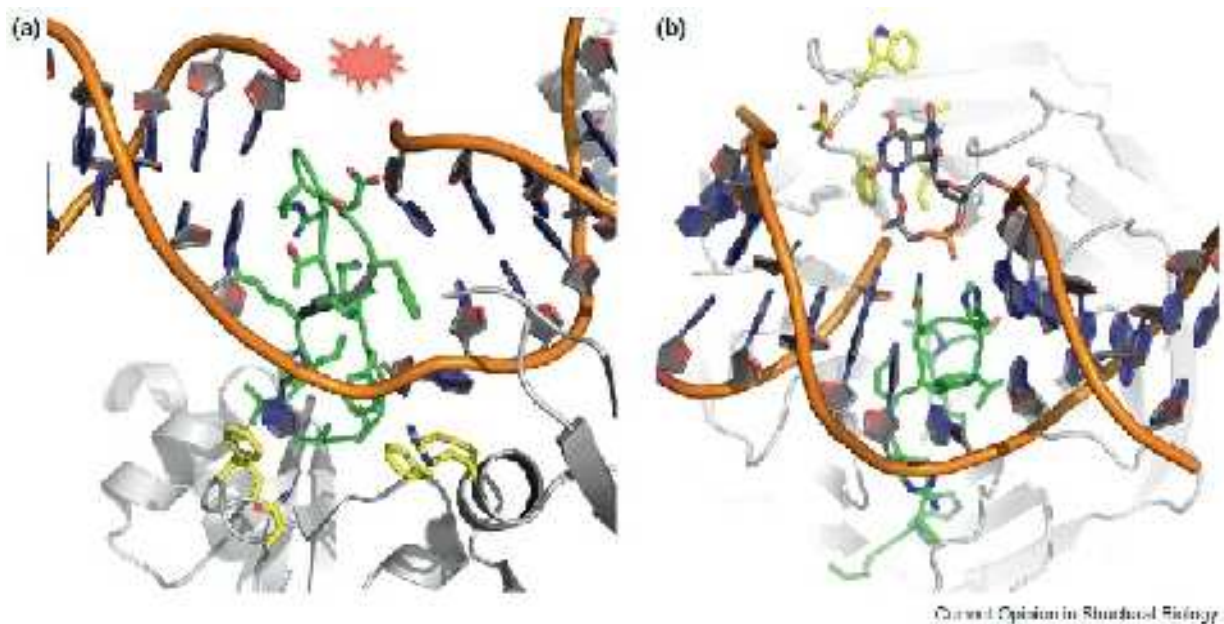


Figure 10.  $\beta$ -hairpin (in green) structures of XPC (a) and DDB2 (b) bound to DNA. Adapted from[49].

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

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

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



## CONCLUSION

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

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**PUBLICATION 1 :**

**«Molecular insights into the recruitment of TFIIH to sites of DNA damage»**

# Molecular insights into the recruitment of TFIIH to sites of DNA damage

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XPB and XPD subunits of TFIIH are central genome caretakers involved in nucleotide excision repair (NER), although their respective role within this DNA repair pathway remains difficult to delineate. To obtain insight into the function of XPB and XPD, we studied cell lines expressing XPB or XPD ATPase-deficient complexes. We show the involvement of XPB, but not XPD, in the accumulation of TFIIH to sites of DNA damage. Recruitment of TFIIH occurs independently of the helicase activity of XPB, but requires two recently identified motifs, a R-E-D residue loop and a Thumb-like domain. Furthermore, we show that these motifs are specifically involved in the DNA-induced stimulation of the ATPase activity of XPB. Together, our data demonstrate that the recruitment of TFIIH to sites of damage is an active process, under the control of the ATPase motifs of XPB and suggest that this subunit functions as an ATP-driven hook to stabilize the binding of the TFIIH to damaged DNA.

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## Introduction

DNA and RNA helicases are a ubiquitous, yet diverse, group of enzymes present in viruses, prokaryotes and eukaryotes (Delagoutte and von Hippel, 2003). They convert chemical energy of nucleoside triphosphate hydrolysis to the mechanical energy necessary to transiently separate the strands of duplex nucleic acids (Tuteja and Tuteja, 2004). By this means, they provide the single-stranded DNA or RNA intermediates necessary for replication, transcription, recombination or repair. Furthermore, it has been shown that helicases can also effectively displace bound proteins from DNA or RNA (von Hippel, 2004). There are several known human diseases caused by defective helicases (Ellis, 1997). Among these disorders, the cancer-prone Xeroderma pigmentosum (XP), alone or in combination with the Cockayne syndrome (CS),

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and the Trichothiodystrophy (TTD) are noteworthy as they entail mutations in the XPB and XPD superfamily 2 helicases. Both of these helicases are part of the same TFIIH complex. TFIIH is composed of a seven-subunit core (XPB, XPD, p62, p52, p44, p34 and p8/TTD-A) associated with the CAK subcomplex (Cdk7/cyclin H, and MAT1) (Giglia-Mari et al, 2004; Ranish et al, 2004). TFIIH functions in both transcription initiation of mRNA and rRNA (Iben et al, 2002), as well as in nucleotide excision repair (NER) (Schaeffer et al, 1993).

XPB and XPD patients are photosensitive and display a 1000-fold increase in melanoma risk because of defects in the NER function of TFIIH (Lehmann, 2003). NER removes a broad spectrum of DNA lesions including UV-induced pyrimidine dimers and bulky, helix-distorting adducts caused by toxic chemicals such as the anticancer drug cisplatin (Sancar, 1996). In mammalian cells, the proteins necessary for the incision reaction include XPC-HR23B/TFIIH, XPA, RPA and the nucleases XPG and ERCC1-XPF (Araujo et al, 2000). The removal of lesions requires their recognition by the repair factor XPC-HR23B and the subsequent opening of the DNA duplex by TFIIH. The single-stranded structure is then stabilized by XPA and RPA, and the margins of the resulting DNA bubble are recognized by XPG and ERCC1-XPF, thereby generating 3' and 5' incisions relative to the damage, respectively (O'Donovan et al, 1994; Sijbers et al, 1996).

As XPB and XPD helicases are both integral parts of TFIIH, their individual molecular roles in NER remain difficult to delineate. As XPB and XPD are helicases with opposite polarities, it was originally suggested that they could cooperate to open DNA on the 5' and 3' sides of a lesion, respectively (Schaeffer et al, 1994). Indeed, mutation of the ATPase activity of either XPB or XPD results in the inability to remove DNA lesions (Sun et al, 1988; Guzder et al, 1994). Refining these proposals, recent data bring into question the direct role of the helicase activity of XPB in NER and transcription and suggest that only the ATPase activity is required (Lin et al, 2005; Coin et al, 2007; Richard et al, 2008). Supporting the primer role of the ATPase activity of XPB in TFIIH functions, we recently showed that this activity was regulated by the p52 subunit of TFIIH (Coin et al, 2007) and by the damage recognition factor XPC (Bernardes de Jesus et al, 2008). Contrary to XPB, the helicase activity of XPD, which is regulated by the p44 subunit of TFIIH (Coin et al, 1998), is required for efficient opening of the DNA around the damage, but is dispensable for transcription (Tirode et al, 1999; Coin et al, 2007).

To further our understanding of the mechanistic details of XPB and XPD function, we analysed the behaviour of ATPase-deficient TFIIH complexes *in vivo*. We found that a TFIIH complex deficient in the ATPase of XPB was not recruited to sites of DNA damage, whereas a complex deficient in the ATPase of XPD did. More surprisingly, we discovered that the recruitment of TFIIH to these sites does not require the helicase activity of XPB but depends on two motifs, a R-E-D residue loop and a positively charged flexible Thumb (ThM)

motifs that were identified in a homologue of XPB from the thermophilic organism *Archaeoglobus fulgidus* (Fan et al., 2006). We analysed the molecular details of R-E-D and ThM impact on XPB activities and found that they were required to stimulate ATP hydrolysis in the presence of DNA. We propose a mechanism in which XPB functions as an ATP-dependent hook that uses the ATPase, R-E-D and ThM motifs to anchor TFIIH to the sites of DNA damaged during DNA repair.

## Results

### The ATPase activity of XPB anchors TFIIH to the sites of DNA damage in vivo

To functionally discriminate between the ATPase activities of XPB and XPD, we produced recombinant TFIIH/XPB (K48R) and TFIIH/XPB (K346R) in baculovirus-infected cells (Tirode et al., 1999) and tested them in DNA repair and transcription assays. These complexes are mutated in the ATPase Walker A motif of XPD and XPB, respectively. When incubated in the presence of recombinant TBP, TFIIA, TFIIIB, TFIIIE and TFIIIF transcription factors in addition to purified RNA polymerase II and a linearized DNA template containing the adenovirus major late promoter (Tirode et al., 1999), TFIIH/XPB (K48R) supported transcription, contrary to TFIIH/XPB (K346R), which was totally inactive (Figure 1A, upper panel). To test the repair capacity of the different TFIIH complexes, we used a reconstituted dual incision assay composed of the recombinant XPC-HR23b, XPA, RPA, XPG, ERCC1-XPF factors and a closed-circular plasmid (Pt-DNA) containing a single 1,3-intra-strand (GpTpG) cisplatin-DNA crosslink as a template (Araujo et al., 2000). None of the mutated complexes was able to excise the damaged oligonucleotide (Figure 1A, lower panel). In a permanganate footprinting assay that measures the opening of the DNA around the lesion (Tapias et al., 2004), addition of TFIIH (WT) induced an increased sensitivity of nucleotides at positions T<sub>p</sub> 5, T<sub>p</sub> 6, T-4, T-5, and to a lesser extent, T-7 and T-10 (Figure 1B, lane 2), indicative of DNA opening. In contrast, neither TFIIH/XPB (K346R) nor TFIIH/XPB (K48R) were able to open damaged DNA (compare lanes 3–4 with lane 2).

To analyse the behaviour of ATP-deficient TFIIH complexes in vivo, we used a stably transfected Chinese hamster ovary (CHO)-UV5 cell line expressing an HA-tagged version of the human XPD (K48R) protein (Winkler et al., 2000). Using the CHO-27-1 cells mutated in the hamster homologue of XPB (Ma et al., 1994), we also generated a stably transfected cell line expressing a C-terminally GFP-tagged version of the human XPB WT or K346R protein (see also Figure 2B). The functionality of an XPB-GFP fusion construct was established earlier (Hoogstraten et al., 2002) (see also Figure 2). We used immunofluorescent labelling after local UV irradiation of stably transfected cells (Volker et al., 2001) to assess the nuclear distribution pattern of XPB and XPD. Immunostaining with antibodies against cyclobutane pyrimidine dimers (CPDs) showed that UV damages were located in discrete local spots in the nucleus (Figure 1C, panels a, e, i, m). Both, human wild-type XPB and XPD proteins colocalized with CPD spots, indicating that TFIIH was efficiently recruited to the damaged sites in these cells (Figure 1C, panels a–d and i–l). Surprisingly, although signals of XPD (K48R) colocalized with CPD spots in CHO-UV5 cells (panels m–p), signals of XPB (K346R) showed an homoge-

nous distribution pattern through the nucleus (panels e–h), indicating that TFIIH/XPB (K346R) complex was not recruited to the damaged sites (Figure 1D). These data suggest that the accumulation of TFIIH to sites of DNA damage takes place in the absence of an active XPD protein but requires functional XPB.

### New motifs in XPB required for the activity of TFIIH in NER

By introducing mutations in some of these seven canonical helicase motifs of XPB, we demonstrated recently that its helicase activity was not required for TFIIH repair function (Coinet et al., 2007). Recently, three additional motifs were identified in a homologue of XPB from the thermophilic organism *Archaeoglobus fulgidus* (Fan et al., 2006). To determine whether these newly identified motifs have a function in the activities of the human TFIIH complex in transcription and repair, we designed four mutants (E253A, E253A/R283A, E473A and D516–526) depicted in Figure 3. We introduced an E253A substitution located at the end of the first strand that was combined, when indicated, with an R283A mutation located at the beginning of the  $\alpha$ -helix of a putative damage recognition domain (DRD). We also designed an E473A substitution in the R-E-D residue loop to change the local negative charge of the motif, and we deleted the positively charged ThM domain from amino acid 516 to 526 (D516–526).

To investigate the importance of the DRD, R-E-D and ThM motifs of XPB in the repair function of TFIIH, we first performed a host-cell reactivation assay (Carreau et al., 1995). A UV-damaged reporter construct, carrying a luciferase gene (pLuc) was transiently transfected into CHO-27-1 cells, together with vectors coding for  $\beta$ -galactosidase and for human XPB proteins. Transfection of either XPB (E253A) or XPB (E253A/R283A) restored luciferase expression that reached the level observed with XPB (WT) (Figure 2A, lanes 1–5). In marked contrast, XPB (E473A) and XPB (D516–526) were not able to restore luciferase expression (lanes 7–8), a defect also observed with XPB (K346R) (lane 6). The various XPB were expressed at a similar level with the exception of XPB (D516–526) whose expression was slightly reduced compared with the wild type (Figure 2A, WB).

Next, we carried out a UV-survival assay and for that purpose we established CHO-27-1 cells stably expressing the new XPB-GFP mutant proteins. Immunoprecipitations using a rabbit polyclonal antibody recognizing the hamster homologue of the core TFIIH subunit p62, demonstrated that the various XPB were efficiently incorporated into the hamster TFIIH complex (Figure 2B). The stably transfected CHO-27-1 cells were UV irradiated at different doses (3, 6 and 9 J/m<sup>2</sup>) and their survival was measured. Expression of XPB (WT), XPB (E253A) and XPB (E253A/R283A) induced a substantial rescue of the UV survival of the CHO-27-1 cells compared with nontransfected control (Figure 2C). On the other hand, the UV-survival curve of XPB (E473A) and XPB (D516–526) transfected cells fell into the range of both the nontransfected parent CHO-27-1 cells and those transfected with the NER-deficient XPB (K346R) control. These data indicate that the R-E-D and ThM domains of XPB are crucial for the repair activity of TFIIH, while the putative DRD is dispensable.

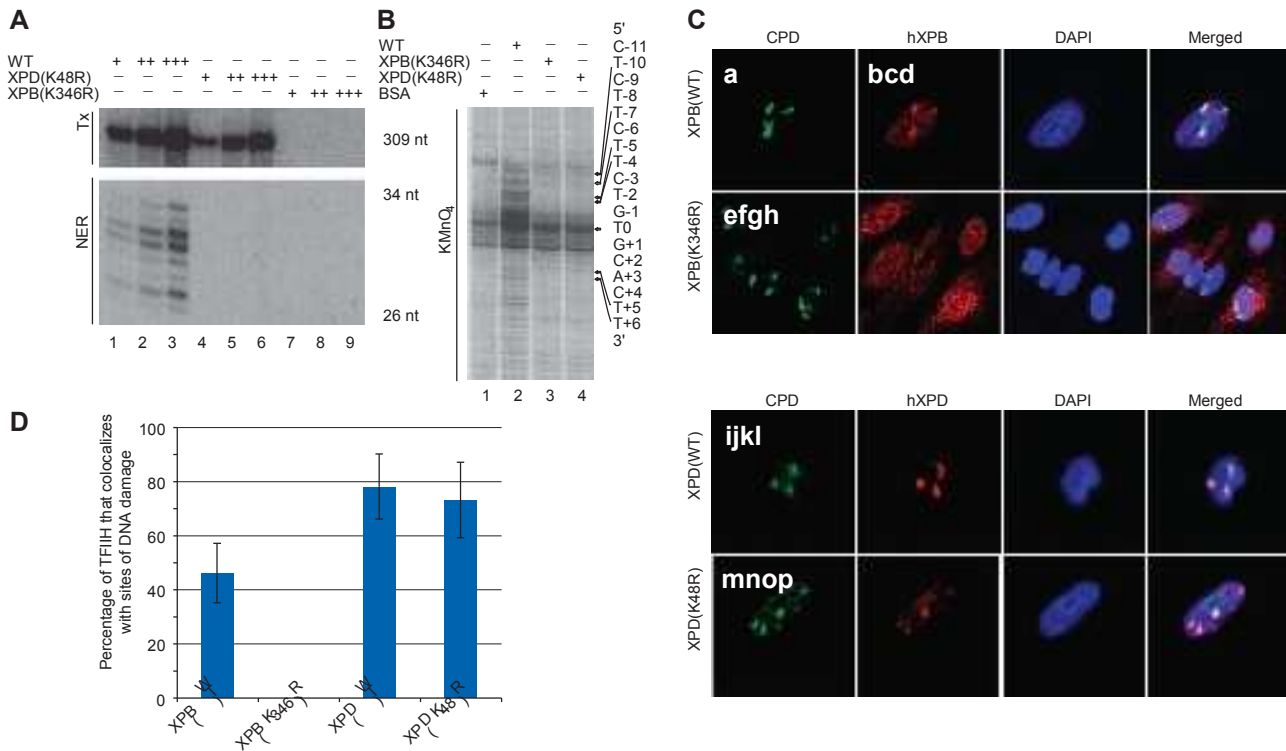


Figure 1. The ATPase activity of XPB is required to anchor TFIIH to damaged chromatin. (A) A measure of 25, 50 and 100 ng of TFIIH (WT), TFIIH/XPD(K48R) or TFIIH/XPB(K346R) were tested either in a reconstituted transcription assay (Tx, upper panel) or in a dual incision assay (NER, lower panel) as described (Coinetal, 2004). The sizes of the incision and transcription products are indicated. (B) TFIIH (WT), TFIIH/XPD(K48R) or TFIIH/XPB(K346R) (100 ng) were incubated with a radio-labelled linear DNA fragment from the Pt-DNA plasmid and 40 ng of XPC-HR23b, 25 ng of XPA, 50 ng of RPA and 150 ng of XPB in a  $KMnO_4$  footprinting assay. Lane 1; Pt-DNA with BSA only. Residues are numbered with the central thymine of the crosslinked GTG sequence designated T0. Arrows indicate  $KMnO_4$  sensitive sites. Adducted strand residues to the 3' and 5' of T0 are denoted by positive and negative integers (N, -N), respectively. (C) Stably transfected CHO-27-1 expressing a GFP-tagged version of the human WT or K346R XPB proteins (upper panel) and stably transfected CHO-UV5 cells expressing an HA-tagged version of the human WT or K48R XPD proteins (bottom panel) (Winkler et al., 2000) were UV irradiated at  $100 J/m^2$  through the 3 mm pore filter and fixed 30 min later. Immunofluorescent labelling was performed using a rabbit polyclonal anti-GFP (panels b and f), a rat monoclonal anti-HA (panels j and n) or a mouse monoclonal anti-CPD (panels a, e, i, m). Nuclei were counterstained with DAPI (panels c, g, k, o), and slides were merged (panels d, h, l, p). (D) Quantitative analysis of the recruitment of TFIIH to sites of DNA damage in transfected cells. Values represent averages  $\pm$  s.d. ( $n = 1/3$  of 100 sites of DNA damage) from three independent experiments.

### R-E-D and ThM motifs are needed for an optimal ATPase activity of XPB

To decipher the molecular details of the repair defect generated by the E473A and D516-526 mutations, we produced recombinant TFIIH complexes in baculovirus-infected cells containing the corresponding mutated XPB subunits (Tirode et al., 1999). Western blot analysis of the recombinant TFIIH/XPB(E253A), XPB(E253A/R283A), XPB(E473A) and XPB(D516-526) complexes revealed a similar subunit composition compared with the TFIIH(WT) complex (data not shown). When tested in either a dual incision or in a transcription assay, TFIIH/XPB(E253A) and XPB(E253A/R283A) were as active as TFIIH(WT) in excising damaged DNA (Figure 4A, upper panel, compare lanes 5-8 with lanes 3-4) or synthesizing RNA (lower panel). In contrast, TFIIH/XPB(E473A) and XPB(D516-526) were inactive in repairing damaged DNA and in synthesizing RNA (Figure 4A, compare lanes 11-12 and 13-14 with lanes 3-4), similarly to TFIIH/XPB(K346R) (lanes 9-10).

In a permanganate footprint assay, TFIIH/XPB(E473A) and XPB(D516-526) were unable to open the damaged DNA (Figure 4B, lanes 5-6), compared with TFIIH(WT) or

XPB(E253A/R283A) (lanes 2-3). Altogether, the above data drew our attention to the critical role of both the R-E-D and ThM motifs of XPB in damaged DNA opening.

### R-E-D and ThM motifs are needed for the anchoring of TFIIH to the sites of DNA damage

We next measured the recruitment of the TFIIH complexes carrying mutations in the newly identified motifs to sites of DNA damage in vivo. XPB(E253A/R283A) colocalized with CPD spots (Figure 5, panel e-h), indicating that the corresponding TFIIH complex translocated to the sites of DNA photolesions. XPB(E473A) and XPB(D516-526), however, displayed a homogeneous distribution pattern throughout the nucleus (panels i-l and m-p), which parallels the pattern observed in Figure 1C with XPB(K346R). This homogeneous distribution contrasted with the local accumulation to the damaged sites of a TFIIH/XPB(T469A) complex (panels q-t) containing a mutation that has been shown to impede the helicase activity of XPB but not the NER function of TFIIH (Coinetal, 2007). We conclude from these data that the R-E-D and ThM motifs are required, together with the Walker A

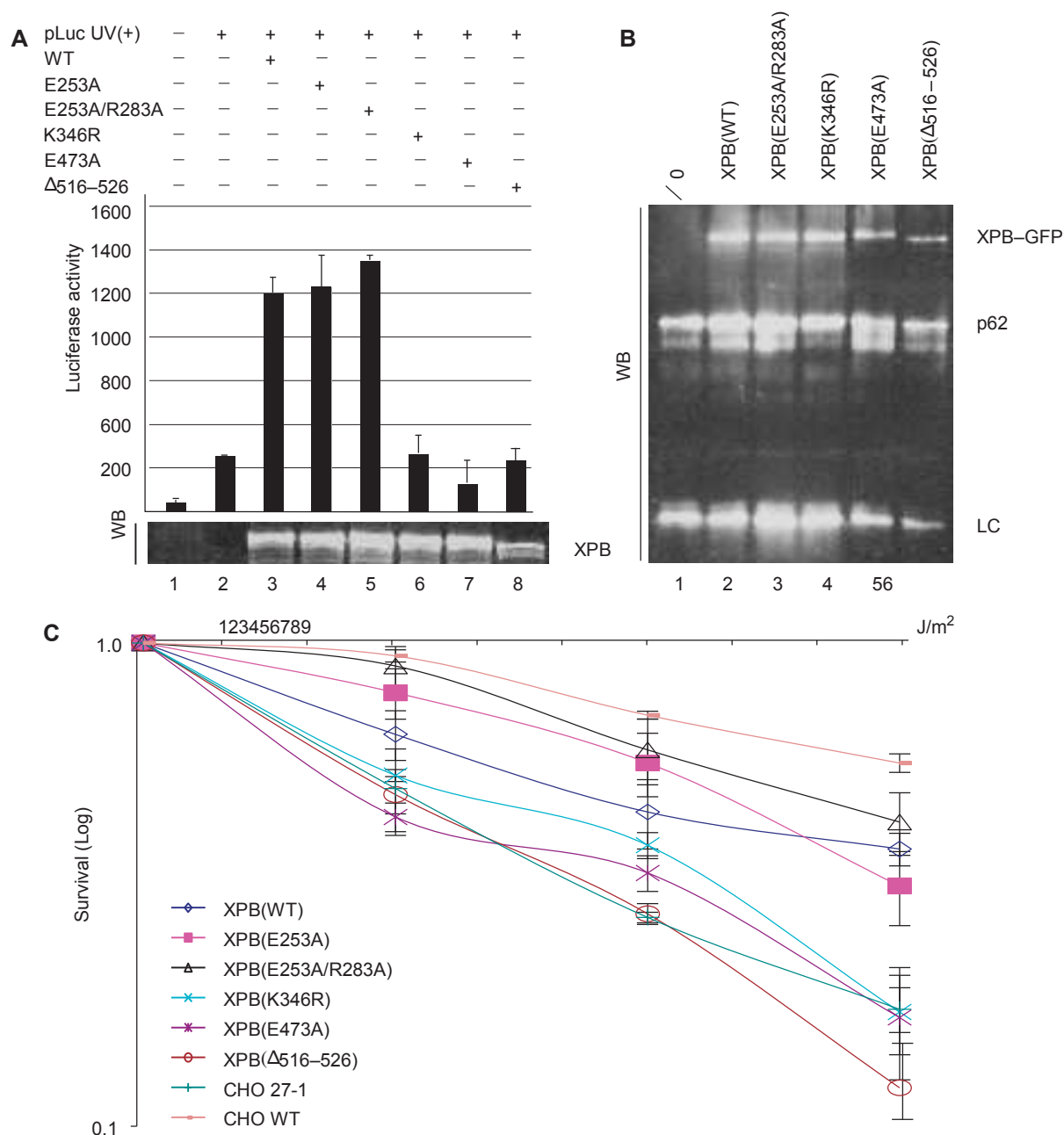


Figure 2 Analysis of the function of the new motifs in NER *in vivo*. (A) CHO-27-1 cells were transfected with UV-irradiated pLuc plasmid expressing the luciferase gene (lanes 2-8) in combination with vectors expressing either XPB(WT) (lane 3), XPB(E253A) (lane 4), XPB(E253A/R283A) (lane 5), XPB(K346R) (lane 6), XPB(E473A) (lane 7) or XPB(D516-526) (lane 8). The luciferase activity in cell lysates (48h posttransfection), normalized with the internal  $\beta$ -galactosidase standard, assesses repair complementation. Results are expressed as relative luciferase activity. Values represent average  $\pm$  s.d. from three independent experiments. A measure of 50% of total extract were resolved by SDS-PAGE and western blotted (WB) with mouse anti-human XPB antibody (Coinetal, 2004). Note that XPB(D516-526) migrates slightly lower than the others because of the deletion. (B) TFIIH from 100ng of extracts prepared from untransfected CHO-27-1 (lane 1) or CHO-27-1 stably expressing XPB (lanes 2-6) was immunoprecipitated with polyclonal antibody against the p62 subunit of TFIIH and resolved by SDS-PAGE, followed by Western blotting with mouse anti-human XPB and mouse anti-p62 antibody. LC; light chain of the antibody. (C) Quantitative UV-survival analysis of transfected CHO-27-1 cell lines. Results are expressed as a ratio between the cells that survived after irradiation and the cells that survived without treatment. Values represent average  $\pm$  s.d. from three independent experiments.

motif, for the recruitment of the TFIIH complex to sites of DNA damage.

### R-E-D and ThM motifs stimulate the DNA-dependent TFIIH ATPase activity

Data above show common biochemical and biological defects for TFIIH complexes mutated either in the ATPase, R-E-D or ThM motif of XPB and suggest a link between the newly

identified motifs of XPB and its ATPase activity. To assess the contributions of these motifs on the hydrolysis of ATP by XPB, we performed ATPase assay. Our data show that the TFIIH/XPB(E473A) and TFIIH/XPB(D516-526) displayed about 40% of residual ATPase activity compared with TFIIH(WT) (Figure 6A, compare lanes 1-3 with 10-12 and 13-15). TFIIH/XPB(E253A) or TFIIH/XPB(E253A/R283A) exhibited the same activity as TFIIH(WT) (lanes 1-6 and data



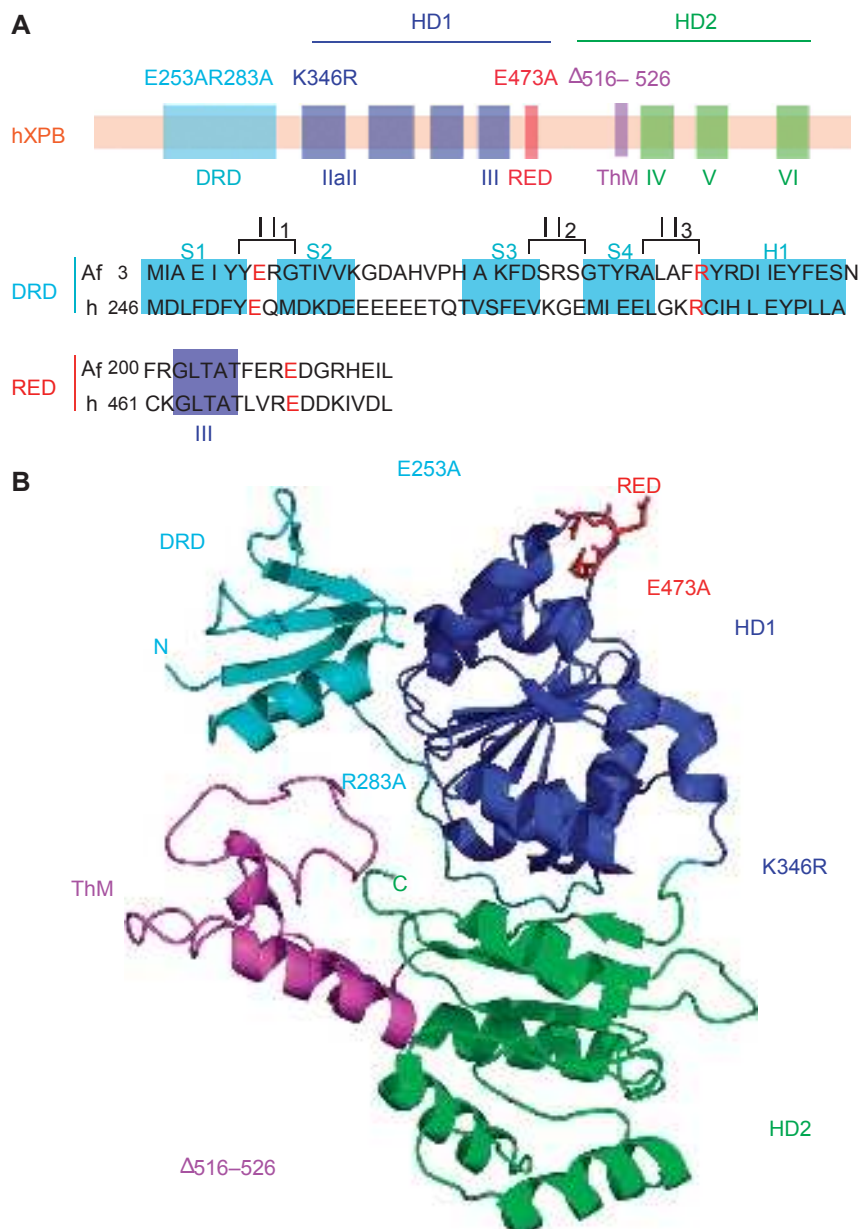
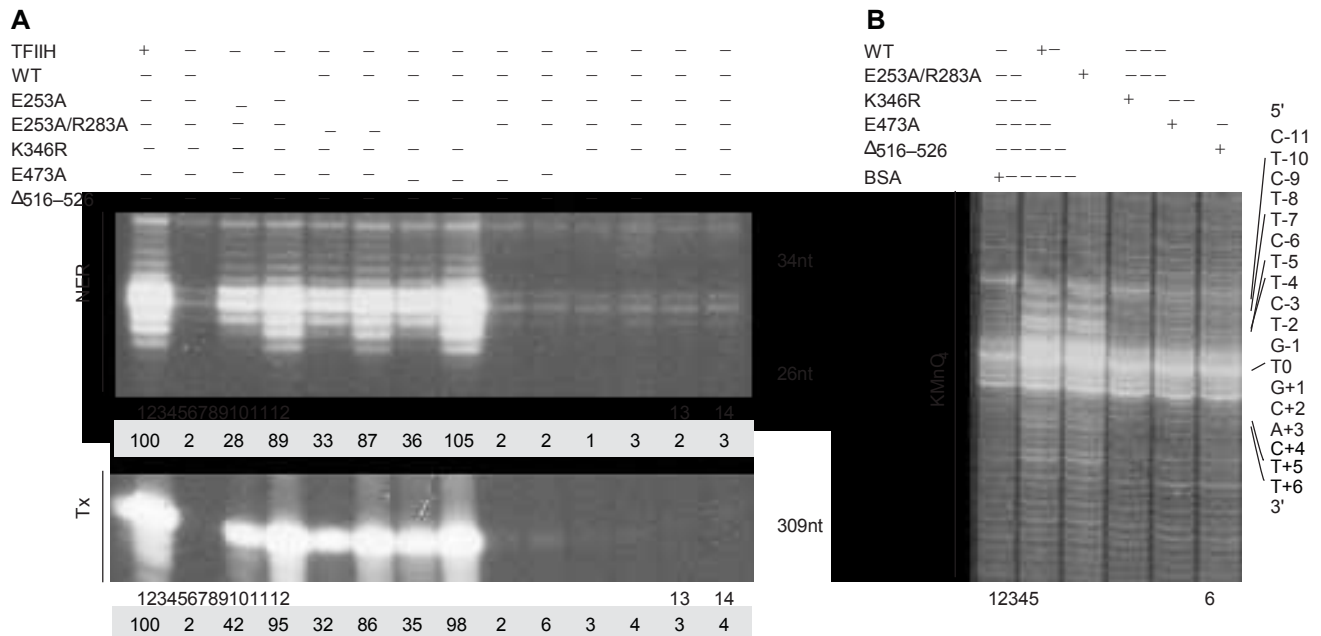


Figure 3 XPB conserved sequences, motifs and structural architecture (A) Upper panel shows the location of the human XPB structural domains: the four classical helicase motifs (I, II, III and IV) of the first helicase module (HD1) are indicated in blue. The three helicase motifs (IV, V and VI) of the second helicase module (HD2) are indicated in green. The putative damage recognition (DRD), R-E-D and Thumb (ThM) domains identified in a homologue of XPB from the thermophilic organism *Archaeoglobus fulgidus* (Fanet et al., 2006) are indicated, respectively in light blue, red and purple. The mutations E253A, R283A, K346R, E473A and Δ516–526 are annotated. Lower panel shows the sequence conservation of the DRD and R-E-D motifs between human (h) and *Archaeoglobus fulgidus* (Af) XPB proteins. The  $\alpha$ -strands (S1–4) and the  $\alpha$ -helix (H1) are indicated. Identical residues are marked in bold letters. The conserved helicase motif III indicated by a blue square is located close to the R-E-D motif indicated by a red open square. The three hairpin loops potentially involved in DNA binding are indicated (1, 2, 3). Residues mutated in this study are marked in red. (B) View of the ribbon representation of AfXPB. The HD1 is indicated in blue, HD2 in green. The putative DRD, R-E-D and ThM domains are indicated in light blue, red and purple, respectively. The positions of the new mutations are indicated.

not shown). TFIIH/XPB(K346R) showed only 20% residual ATPase activity (corresponding to that of XPD (Coinet et al., 2006)), compared with TFIIH(WT) (compare lanes 1–3 with 7–9).

XPB and XPD are DNA-dependent ATPases (Royet et al., 1994). The ATPase activity of TFIIH(WT), low in the absence of DNA, is stimulated by double-stranded DNA (Figure 6B, compare lanes 1–2 with 7–8). In contrast, TFIIH/XPB(E473A)

showed almost no DNA-induced ATPase stimulation (Figure 6B, compare lanes 5–6 with 11–12). More importantly, in the absence of DNA, TFIIH/XPB(WT) and TFIIH/XPB(E473A) displayed similar specific ATPase activities (Figure 6B, compare lanes 1–2 and 5–6) that were slightly higher than the TFIIH/XPB(K346R) activity (compare lanes 1–2, 5–6 and 3–4). Similar observations were obtained with TFIIH/XPB(Δ516–526) (Supplementary data 1). Altogether,



**Figure 4** Mutations in R-E-DandThM motifs impair the ATPase activity of XPB. (A) A measure of 25 and 75 ng of TFIIH (WT), TFIIH/XPB (E253A), XPB (E253A/R283A), XPB (K346R), XPB (E473A) or XPB (Δ516-526) were tested in dual incision assay (NER, upper panel) or in a reconstituted transcription assay (Tx, lower panel) as described (Coin et al., 2004). Lane 1 contains highly purified HeLa TFIIH (Giglia-Mari et al., 2004). Lane 2 contains all the factors except TFIIH. The sizes of the incision or transcription products are indicated. The transcription and repair signals were quantified using Genetool (Syngene). (B) A measure of 100 ng of the various TFIIH complexes were tested in a KMnO<sub>4</sub> footprint assay (see Figure 1B). Lane 1; Pt-DNA with BSA only. Residues are numbered with the central thymine of the crosslinked GTG sequence designated T0. Arrows indicate KMnO<sub>4</sub> sensitive sites. Adducted strand residues to the 3' and 5' of T0 are denoted by positive and negative integer (N, -N).

these data indicate that the R-E-D and ThM motifs do not affect the basal intrinsic ATPase activity of XPB but are required for the stimulation of this activity by DNA.

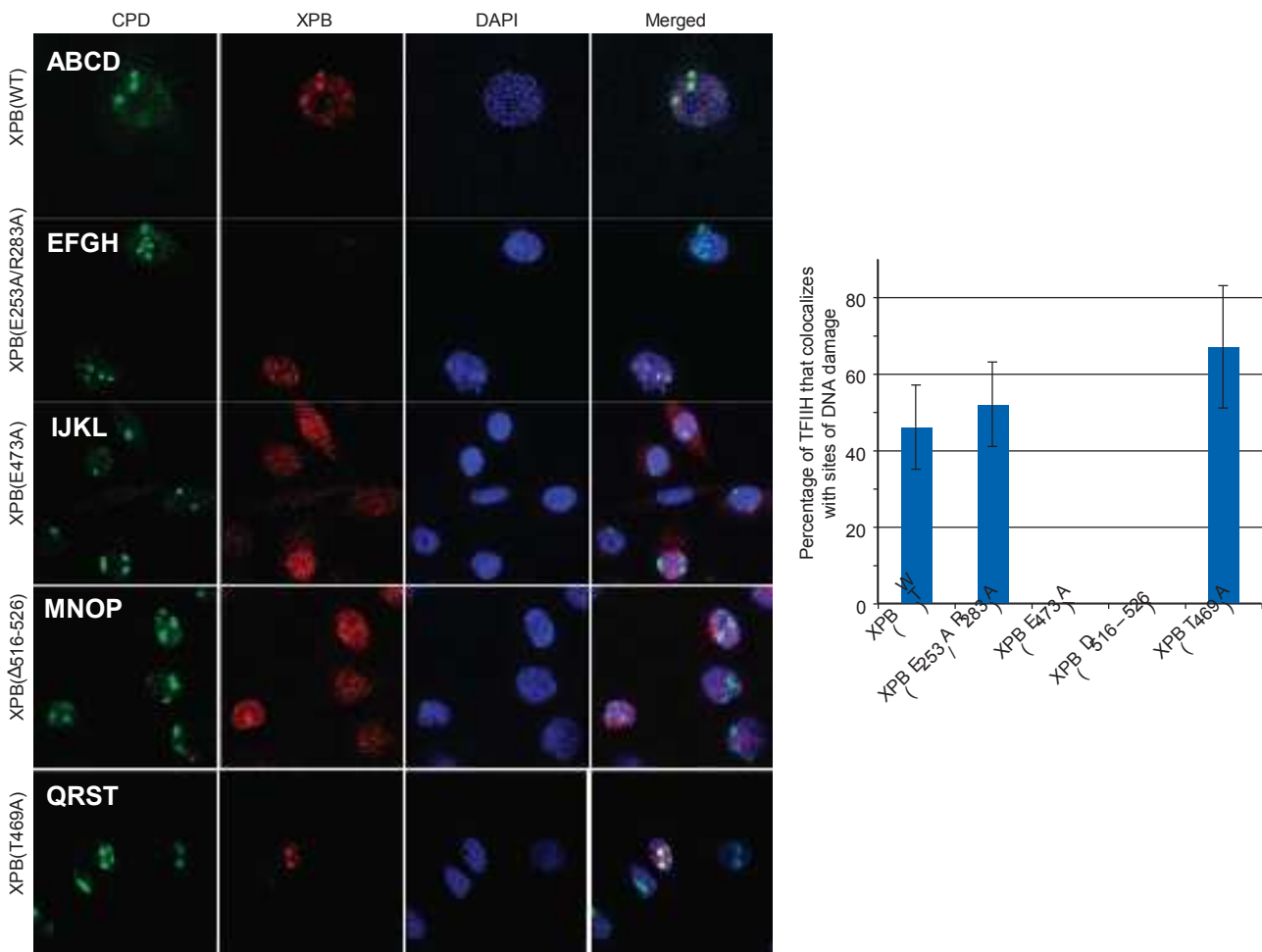
## Discussion

To efficiently protect the genome, cells need to detect all types of DNA structural alterations embedded in billions of normal basepairs. The identification of the various proteins that execute NER was done through extensive studies of human cells deficient in this repair pathway (Maillard et al., 2007). Both in vivo and in vitro experiments identified XPC as the first factor that binds the damaged DNA (Sugasawa et al., 1998; Volker et al., 2001; Riedl et al., 2003). TFIIH is recruited to the lesion immediately after XPC (Yokoi et al., 2000; Riedl et al., 2003), presumably through direct protein-protein interaction (Bernardes de Jesus et al., 2008). The role of TFIIH is devoted to the opening of the DNA around the damaged site, but the individual function of its helicase subunits in this step remains difficult to delineate.

Earlier studies from our laboratory have shown that mutations in the helicase motifs III (T469A) or VI (Q638A), which impaired the helicase activity of the XPB subunit, did not inhibit the NER activity of TFIIH (Coin et al., 2007), thus raising the question of the role of XPB in NER. Here, we showed that TFIIH containing mutation in the motif III of XPB is recruited to the DNA repair sites after UV irradiation. However, a mutation in the helicase motif I, which abolishes the ATPase activity of XPB, thwarts the accumulation of TFIIH to these sites. This implies that the recruitment of TFIIH to sites of damage is an active process that requires

ATP hydrolysis. In contrast, the ATPase activity of XPD, the second helicase of TFIIH, is not required to recruit TFIIH to the damage sites, although it is needed for DNA repair.

In addition to the aforementioned ATPase motif, we found that two additional motifs, the R-E-D and ThM motifs, are implicated in the recruitment of TFIIH to sites of DNA damage. These two domains, highly conserved in human XPB, were identified in an homologue of XPB from the thermophilic organism *Archaeoglobus fulgidus* and were suggested to be involved in TFIIH functions (Fan et al., 2006). Mutations in the R-E-D and ThM motifs mimicked the biochemical and biological defects obtained with a mutation in the ATPase motif. This suggests that the ATPase R-E-D and ThM motifs work together to ensure correct recruitment of TFIIH to the damaged sites before the opening and dual incision step take place during NER. How the R-E-D and ThM motifs participate to the anchoring of TFIIH? The ThM domain has not been found in other helicases, including XPD (Bienstock et al., 2002; Fan et al., 2008; Liu et al., 2008; Wolski et al., 2008), but a similar helical protrusion has been observed in DNA polymerases (Doubilet et al., 1998) and in *Sulfolobus solfataricus* SWI2/SNF2 ATPase Rad54 (Durr et al., 2005), in which it is expected to grip double-stranded DNA from the minor groove. The structure of XPB suggests that the energy furnished by the ATP hydrolysis is used to induce a flip of 170° of the HD2 domain after the binding of XPB to DNA (Fan et al., 2006) (Figure 7). The R-E-D (present in HD1) and the ThM (present in HD2) are then in close vicinity and are used to stabilize TFIIH on the DNA by introducing a wedge (the E473 residue) in the double-stranded DNA, gripped by the ThM motif. To obtain experimental evidence



**Figure 5** Recruitment of TFIIH to local sites of DNA damage. (Left panel) CHO-27-1 cells were stably transfected with EGFP plasmids expressing various forms of GFP-tagged XPB proteins. These cells were irradiated with UV light (100 J/m<sup>2</sup>) through the 3-mm pore filter and fixed 30 min later. Immunofluorescent labelling was performed using either a mouse monoclonal anti-CPD (panels A, E, I, M, Q) or a rabbit polyclonal anti-GFP (panels B, F, J, N, R). Nuclei were counterstained with DAPI (panels C, G, K, O, S), and slides were merged (panels D, H, L, P, T). (Right panel) Quantitative analysis of the recruitment of TFIIH to sites of DNA damage in transfected cells. Values represent average  $\pm$  s.d. (n = 100 sites of DNA damage) from three independent experiments.

for this model, we compared the ATPase activities of the WT and mutated complexes with or without DNA. Indeed, like most SF1 and SF2 members, DNA stimulates the ATPase activity of TFIIH (Roy et al, 1994). In the presence of DNA, mutations in the R-E-D and ThM motifs induces 60% inhibition of the ATPase activity compared with TFIIH (WT). In the absence of DNA, the three ATPase activities are strictly identical and are slightly higher than the ATPase activity of the TFIIH complex mutated in the ATP-binding site of XPB. These data further support the model of the conformational change proposed above as it demonstrates that R-E-D and ThM are used to stabilize the binding of XPB to DNA. Furthermore, the fact that these mutations inhibit both, TFIIH transcription and repair activities, suggests a common mode of recruitment of TFIIH to the promoters and to the damage sites.

The recruitment of TFIIH through the action of the ATPase activity of XPB may also induce reorganization of the protein-DNA complexes in transcription and repair that will allow new protein-protein or protein-DNA contacts. Indeed, using photocrosslink experiments, we have shown that addi-

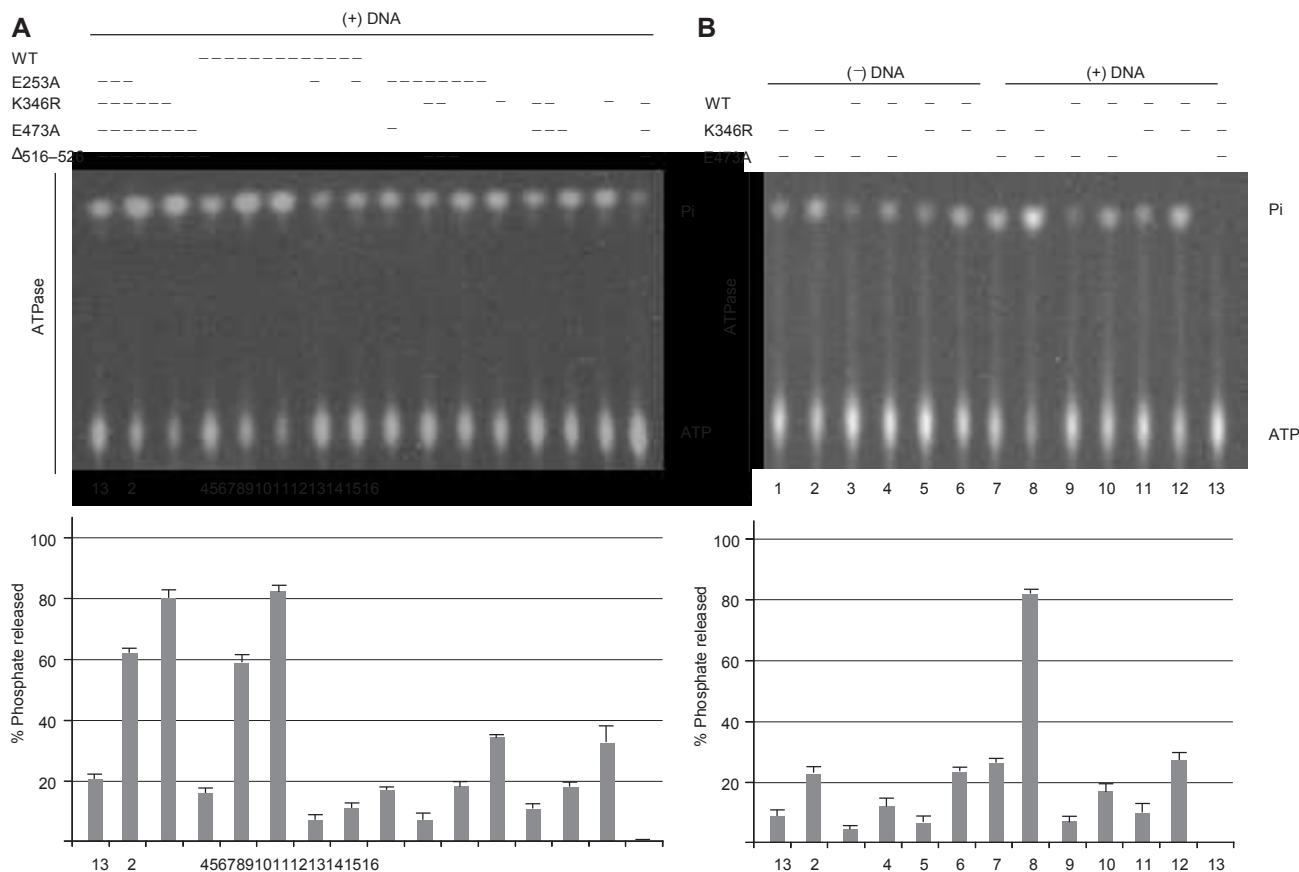
tion of ATP in NER induced re-positioning of XPC on the damaged DNA, which dependent on TFIIH (Tapias et al, 2004). After the recruitment of TFIIH to the damaged DNA through the energy furnished by the ATPase activity of XPB, the DNA would be opened by XPD, which has a processive and robust helicase activity stimulated by the p44 subunit of the core TFIIH (Coinet et al, 1998). Here, a mutation in the ATPase activity of XPD still allowed TFIIH to bind the damaged sites in vivo but was unable to open the DNA around the lesion. Altogether our data brings a new conceptual view of the roles of XPB and XPD in NER by revealing their different molecular functions within this genome caretaking event.

## Materials and methods

### Cell lines

CHO-27-1 is a CHO mutant cell line belonging to the third rodent complementation group (the hamster ERCC3 gene is the homologue of the human XPB gene) (Halle et al, 2005). CHO-UV5 belongs to the second rodent complementation group (the hamster ERCC2 is the homologue of the human XPD gene) (Winkler et al, 2000).





**Figure 6** Mutations in the R-E-D motif impair DNA-dependent TFIIH ATPase activity. (A) 50, 100, and 150 ng of TFIIH (WT), TFIIH/XPB (E253A), XPB (K346R), XPB (E473A) or XPB Δ516–526 were tested in an ATPase assay in the presence of 200 ng of double-strand circular DNA (Coinetal, 2007). The average percentages of phosphate released (Pi/(ATP + Pi)) from three independent experiments is represented in the graph. (B) 50 and 150 ng of TFIIH (WT), TFIIH/XPB (K346R) or TFIIH/XPB (E473A) were tested in an ATPase assay without (lanes 1–6) or with (lanes 7–12) 200 ng of double-strand circular DNA. The average percentages of phosphate released (Pi/(ATP + Pi)) from three independent experiments is represented in the graph.

### Construction of the plasmids

Baculovirus allowing the expression of mutated XPB were constructed in the FLAGtagpSK278 vector (BDBiosciences). XPB was inserted at the BamHI/EcoRI site, in fusion with the FLAGtag at its 5' side. The mutants were obtained by site-directed mutagenesis (Quickchange, Stratagene). The resulting vectors were recombined with baculovirus DNA (BaculoGold DNA, Pharmingen) in Spodoptera frugiperda 9 (Sf9) cells. In vivo experiments were carried out with the EGFP-N1 plasmid (Clontech) containing the XPB cDNA inserted in frame with the green fluorescent protein tag (Hoogstraeten et al., 2002).

### Stable cell lines

CHO-27-1 cells (10<sup>6</sup>) were transfected with 2 ng of pEGFP-N1/XPB plasmid in 10 cm Petri dishes using lipofectamine (Invitrogen). Forty hours after transfection, the fluorescent cells were sorted on the FACS DIVa (BD; Becton, Dickinson and Company). The cells with the highest level of fluorescence (about 5% of total cells) were maintained in the selective medium with G418 (Geneticin, 800 ng/ml), expanded and analysed for XPB expression.

### Damaged DNA substrates

Covalently closed circular Pt-DNA containing a single 1,3-intra-strand (GpTpG) cisplatin-DNA crosslink was prepared as described (Fritetal, 2002).

### Dual incision assay

Dual incision assay was carried out in 25 μl of Repair buffer (45 mM Hepes-KOH (pH 7.8), 5 mM MgCl<sub>2</sub>, 1 mM DTT, 0.3 mM EDTA, 10% glycerol, 2.5 mg BSA, 50 mM KCl) supplemented with 2 mM ATP. Each reaction contained 5 ng of XPG, 15 ng of XPF/ERCC1, 10 ng of

XPC-HR23b 50 ng of RPA and 25 ng of XPA. After pre-incubation 10 min at 30°C, 30 ng of Pt-DNA was added and reaction was continued for 90 min at 30°C. The excised fragment was detected on a 14% urea-acrylamide after annealing with 9 ng of the complementary oligonucleotide and addition of four radiolabelled dCMP P<sup>32</sup> (3000 Ci/mmol) residues by Sequenase V2.1 (USB).

### KMnO<sub>4</sub> footprint assay

The damaged strand probe was obtained on AgeI/AseI digestion of the Pt-DNA and radiolabelling at the 5' end in a Klenow reaction, the Pt adduct is located at 156 bp from the labelled end. The resulting fragment was purified by the 'crush and soak' method after migration in a 5% nondenaturing PAGE. Reactions (75 μl) were carried out in 20 μl of Repair buffer (pH 7.2) containing the labelled cisplatinated probe (40 fmol) and 40 ng of XPC-HR23b, 25 ng of XPA, 50 ng of RPA and 150 ng of XPG. After incubation at 30°C for 15 min, 3 μl of 120 mM KMnO<sub>4</sub> was added, and oxidation was allowed to proceed for 3 min at room temperature before reduction by adding 6 μl of 14.6 M β-mercaptoethanol for 5 min on ice. After organic extraction and ethanol precipitation, dried pellets were resuspended in 100 μl of a solution containing 1 M piperidine, 1 mM EDTA and 1 mM MEGTA and incubated at 90°C for 25 min. Samples were next ethanol precipitated and final pellets were recovered in 10 μl of loading buffer and analysed in 8% urea PAGE.

### ATPase assay

Protein fractions were incubated for 2 h at 30°C in the presence of 1 mCi [<sup>32</sup>P]ATP (7000 Ci/mmol, ICN Pharmaceuticals) in a 20 μl reaction volume in 20 mM Tris-HCl pH 7.9, 4 mM MgCl<sub>2</sub>, 1 mM DTT, 50 mg/ml BSA and when indicated 200 ng of double-strand DNA (pcDNA3 β). Reactions were stopped by addition of EDTA

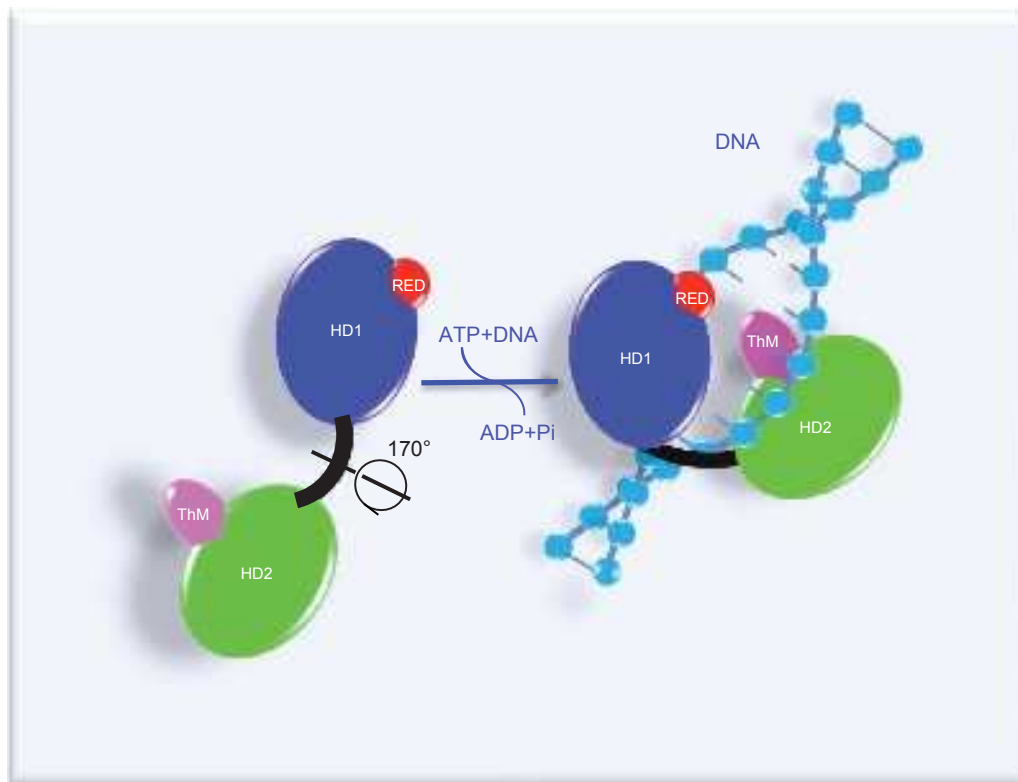


Figure 7 Proposed structure-based mechanism for binding of XPB to DNA. In this model, adapted from Fan et al (2006), XPB is in an opened conformation in the absence of DNA. When XPB binds to DNA, the rotation (170°) of the second helicase domain (HD2) together with the ThM domain, facilitated by HD1-mediated ATP hydrolysis, forms the closed and stable XPB-DNA complex.

(50 mM) and SDS (1% (w/w)). The reactions were then diluted five-fold, spotted onto polyethylenimine (PEI) TLC plates (Merck), run in 0.5 M LiCl/1 M formic acid and autoradiographed.

#### Local UV irradiation and immunofluorescence

The cells were rinsed with PBS and covered with an isopore polycarbonate filter with pores of 3 mm diameter (Millipore, Bedford, MA). Cells were then exposed to UV irradiation with a Philips TUV lamp (predominantly 254 nm) at a dose of 100 J/m<sup>2</sup> (Volker et al, 2001). Subsequently, the filter was removed, the medium was added back to the cells, and they were returned to culture conditions for 30 min. Then, cells were fixed in 2% formaldehyde for 15 min at room temperature and permeabilized with PBS/0.5% Triton X-100 for 5 min. After washing with PBS-Tween (0.05%), the slides were incubated for 1 h with the indicated antibodies. After extensive washing with PBS-Tween, they were incubated for 1 h with Cy3-conjugated donkey anti-rabbit IgG, goat anti-mouse Alexa488 IgG or goat anti-rat Alexa488 IgG (Jackson Laboratories) diluted 1:400 in PBS-Tween/0.5% Fetal Calf Serum. The slides were counterstained for DNA with DAPI prepared in Vectashield mounting medium (Vector lab). All images were collected using a Leica Confocal TCS4D microscope equipped with both UV laser and an Argon/Krypton laser, and standard filters to allow collection of the data at 488 and 568 nm. The software TCSTK was used for three-colour reconstructions and figures were generated using the PLCHTK software.

#### Host-cell reactivation assay

The pGL3 vector expressing Photinus pyralis (firefly) luciferase was purchased from Promega and the pCH110 vector expressing the galactosidase from Invitrogen. The pGL3 vector was UV irradiated (254 nm, 1000 J/m<sup>2</sup>) at a concentration of 1 mg/ml in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. CHO-27-1 cells were transfected in a six-well plate at a confluence of 95% using Lipofectamine Plus (Invitrogen). Each transfection mixture contained 500 ng of pGL3 (UVp/Å), 100 ng of pCH110 (nonirradiated) and 10 ng of the various pcDNA XPB plasmids. After 4 h of incubation, the transfection

reagents were replaced by medium. Cells were lysed after 24 h to measure luciferase activity on a microtiter plate luminometer (Dyner). All results (mean values of at least five measurements) were normalized by calculating the ratios between luciferase and galactosidase activities.

#### UV-survival assay

Cells (10<sup>6</sup>) were plated per 6 cm petri dishes, cultured overnight and UV irradiated at 254 nm at various doses (0.5 J/m<sup>2</sup>/s). After 14 days, the cells are stained by trypan blue and counted.

#### Antibodies

Mouse monoclonal antibodies towards TFIIH subunits were used as described (Coin et al, 2007). Primary antibodies (the final dilutions are indicated in parentheses) used in fluorescent labelling were purified rabbit anti-GFP (Torrey Pines Biolabs, Inc) (1:1000), rat monoclonal anti-HA3F10 (Roche) (1:1000) and mouse IgG monoclonal anti-Thymine Dimer (H3) (1:2000) (Abcam).

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#### Conflict of interest

The authors declare that they have no conflict of interest.

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**PUBLICATION 2:**

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

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

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## Summary

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

## Introduction

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

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

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

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

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

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



## Results

### DOT1L deficient mammalian cells are UV-sensitive.

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

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

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

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

### **DOT1L promotes transcription recovery after UV irradiation.**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

GG-NER or TC-NER defects.

### **DOT1L controls transcription recovery after UV irradiation.**

Alternatively, DOT1L may serve in reactivation of mRNA synthesis after UV irradiation. Transcriptional arrest has been shown to lead to a highly cytotoxic cellular response to stress {Fousteri, 2008 #52}. This response has multiple causes and is likely not only the result of DNA lesions that block RNA Pol II in elongation. Previous studies have challenged the relationship between efficient repair of a lesion in the transcribed strand of active genes and the recovery of transcription inhibited by DNA damage. For instance, cells carrying mutations in the Cockayne syndrome B protein (CSB) were unable to recover lesion-inhibited transcription while they efficiently repaired acetylaminofluorene lesions in transcriptionally active genes {van Oosterwijk, 1996 #53}. In addition, CSB was shown to accumulate to the promoters of housekeeping genes after UV irradiation, where it stimulated the recovery of inhibited transcription independently of the presence of lesions {Proietti-De-Santis, 2006 #1}. This finding led to the hypothesis that removal of transcription blocking lesions was insufficient to restore transcription after DNA damage and that in addition, chromatin changes in the promoters of housekeeping genes could be required. In our study we, using RRS assay, show the inhibition of global gene expression in cells which are depleted of DOT1L. We detect only 30% of residual transcription there 24 hours post UV irradiation (20J/m<sup>2</sup>). This inhibition is confirmed at the single gene level since *DHFR* show the similar level of inhibition at the same time point. Furthermore, transcription inhibition does not depend on the cell cycle since no difference in the distribution of the phases is observed between MEF<sup>WT</sup> and MEF<sup>DOT1L</sup> cells before or after UV irradiation (Unpublished Data).

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

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

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

Altogether, these data suggest that DOT1L favors an open chromatin structure around the promoter in housekeeping genes allowing efficient transcription re-initiation. In line with this hypothesis, H3K79me<sub>2</sub>, the mark of DOT1L activity, peaks transiently on the promoters of housekeeping genes in wild-type cells after UV irradiation, keeping the open chromatin. In addition, the absence of DOT1L can be bypassed by the class I HDAC inhibitor TSA which barely relaxes chromatin. TSA restores both transcription initiation and normal UV sensitivity in MEF<sup>DOT1L</sup> cells, creating thus a link between these two events.

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



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

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

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## **Experimental Procedures**

**Cell lines.** MEF cells were cultured at 37°C in the presence of 5% CO<sub>2</sub> in Dulbecco's modified medium supplemented with 10% FCS.

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

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

## **Unscheduled DNA synthesis (UDS) and Transcription recovery after UV irradiation (RRS).**

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

irradiated cells ( $10\text{J/m}^2$ ) were pulse labeled with  $5\mu\text{Ci/ml}$  of [ $^3\text{H}$ ]uridine (PerkinElmer Life and Analytical Sciences, Boston MA 02118 USA) for 30 minutes at different time points. The cells were collected and washed once with ice-cold PBS and lysed in buffer containing 0.5% SDS and  $100\text{ }\mu\text{g/ml}$  proteinase K for 2 hours at  $37^\circ\text{C}$ . After trichloroacetic acid precipitation (10% TCA), the samples were spotted onto glass fiber discs (Whatman; Maidstone, Kent, UK); the filters were next sequentially washed in 5% TCA, 70% ethanol/acetone, and counted for their radioactivity. The  $^3\text{H}/^{14}\text{C}$  ratio was taken as a measure of RNA synthesis.

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

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

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

**Reverse Transcription and Real-Time Quantitative PCR.** cDNA synthesis was performed using hexamere and AMV reverse transcriptase (Sigma; St. Louis, MO). Real-time quantitative PCR was done with the FastStart DNA Master SYBR Green kit and the Lightcycler apparatus (Roche Diagnostic; Basel, Switzerland). Primer sequences are available upon request.

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

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

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

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## Figure legends

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Figure 1

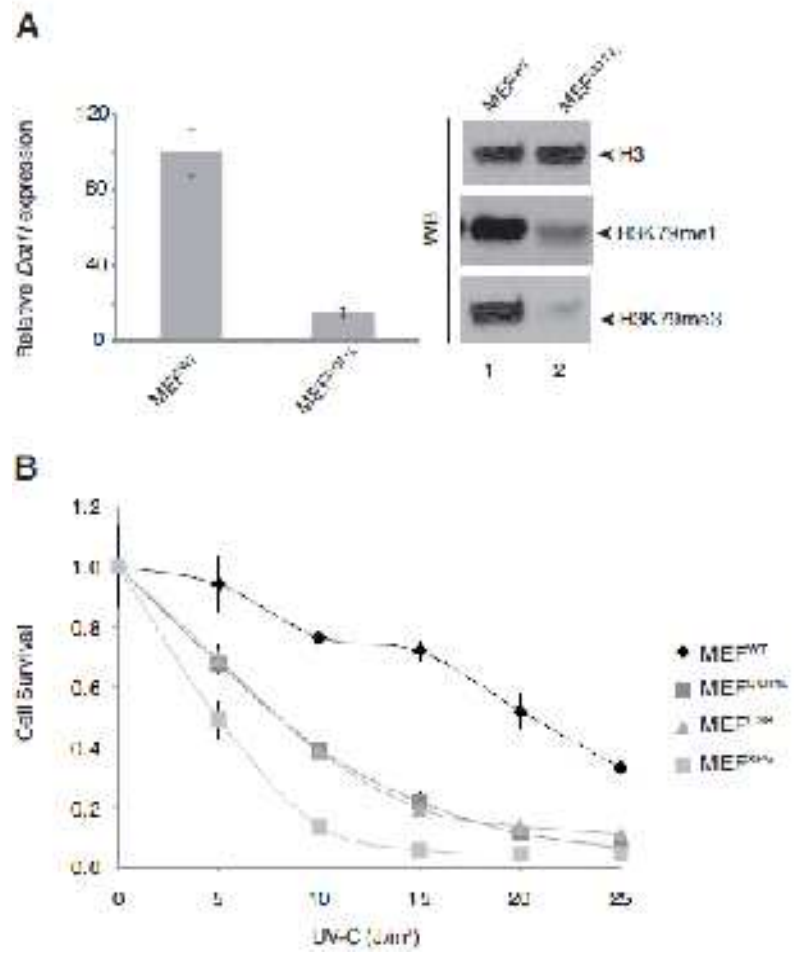


Figure 2

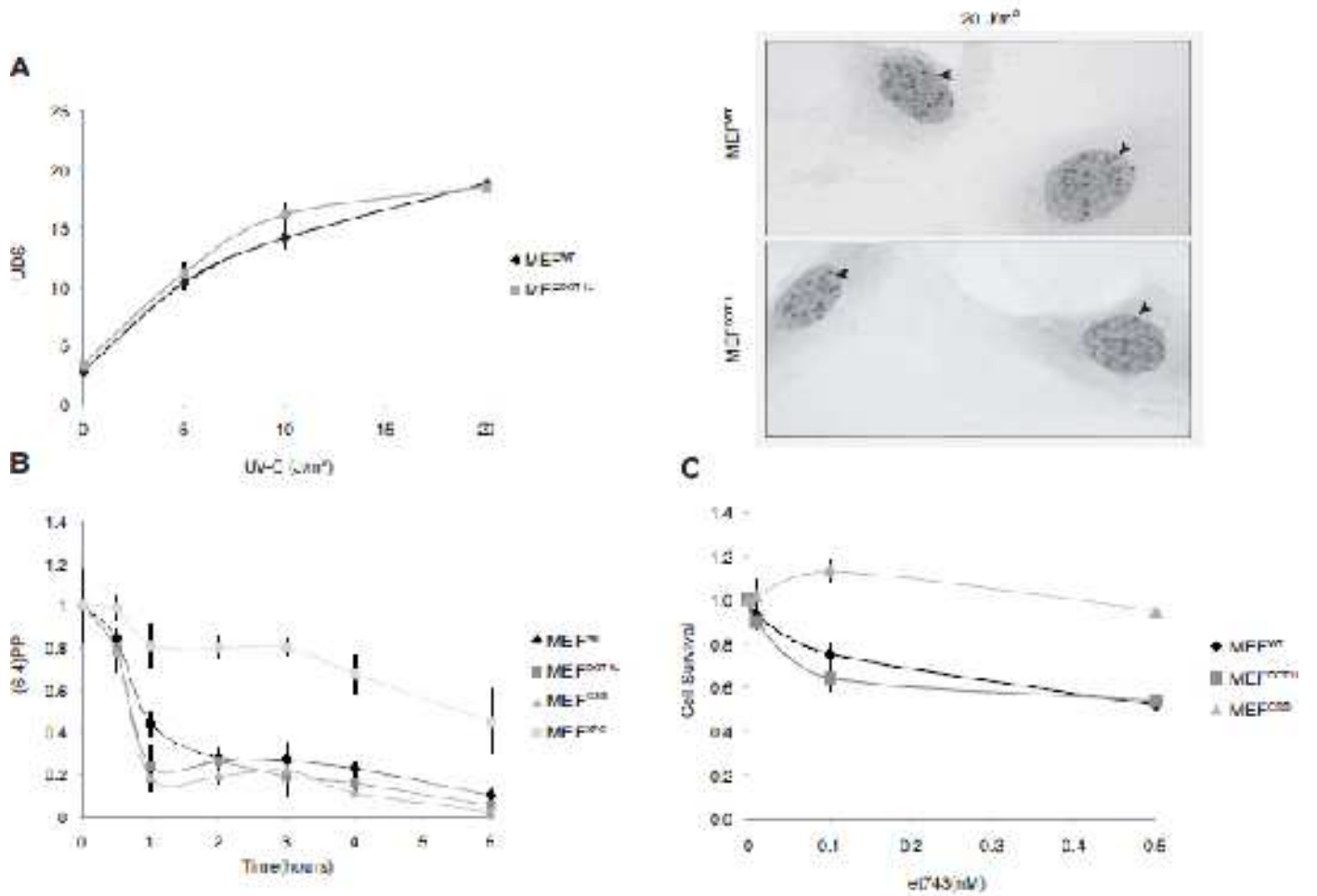


Figure 3

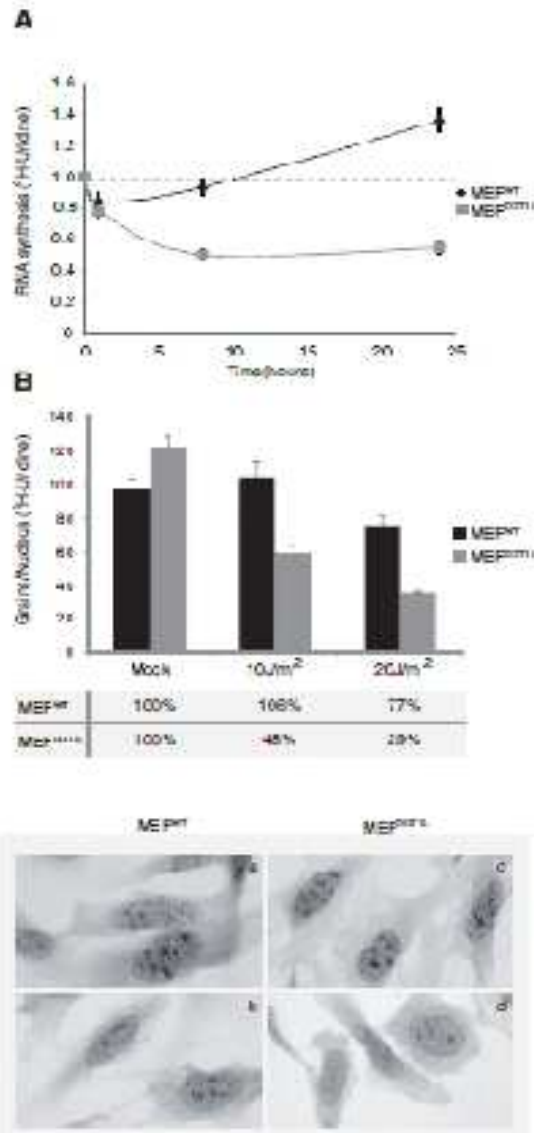


Figure 4

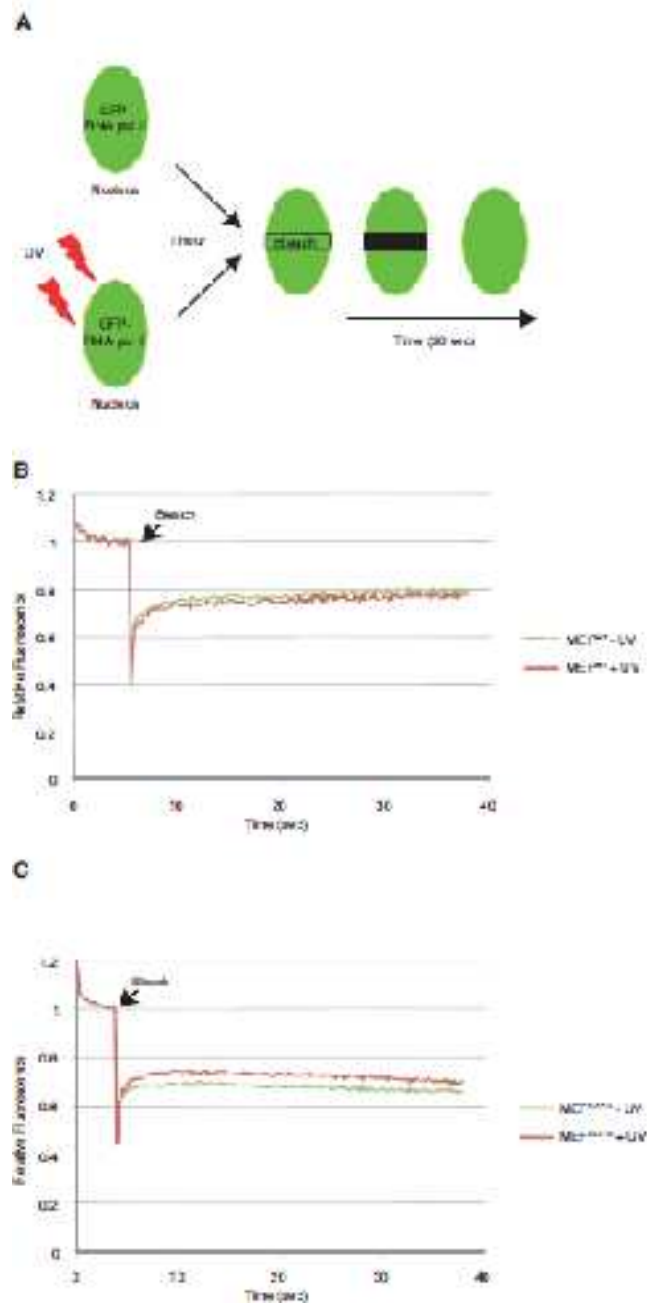




Figure 5

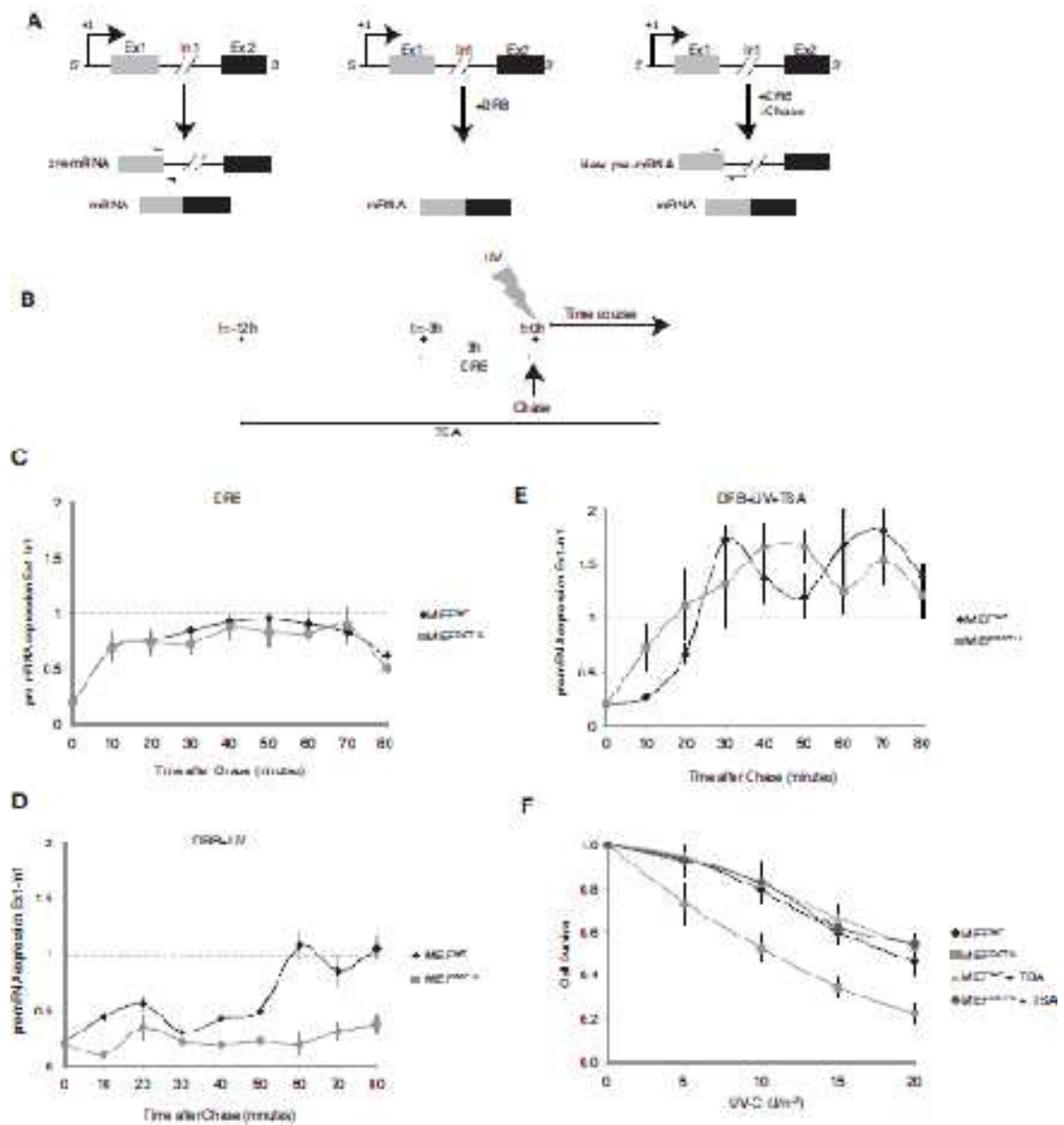
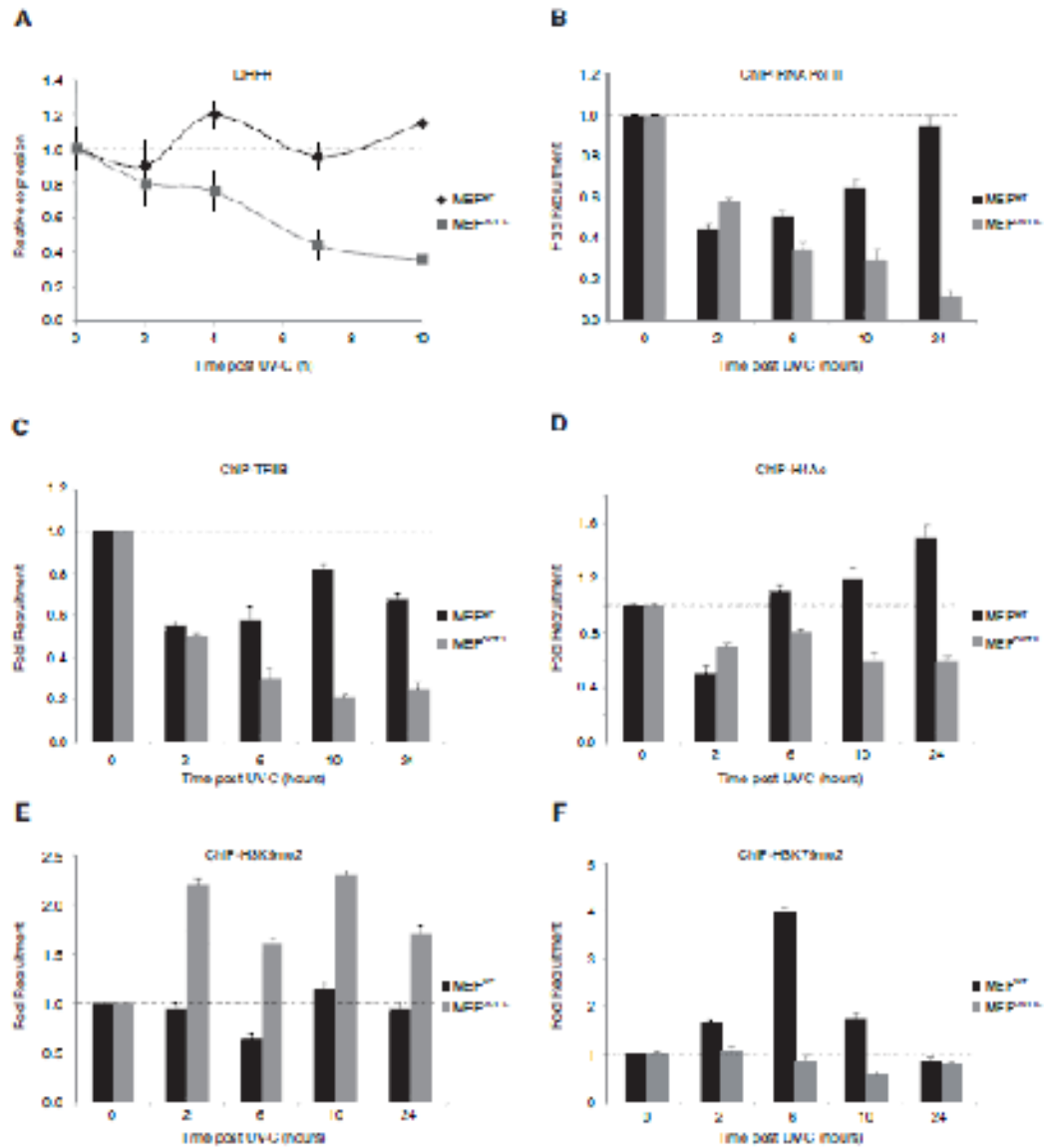


Figure 6



**PROJECT I:**  
**« Chromatin remodelers, kinases and phosphatases mediate DNA damage  
response and nucleotide excision repair »**

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

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## **SUMMARY**

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

## **INTRODUCTION**

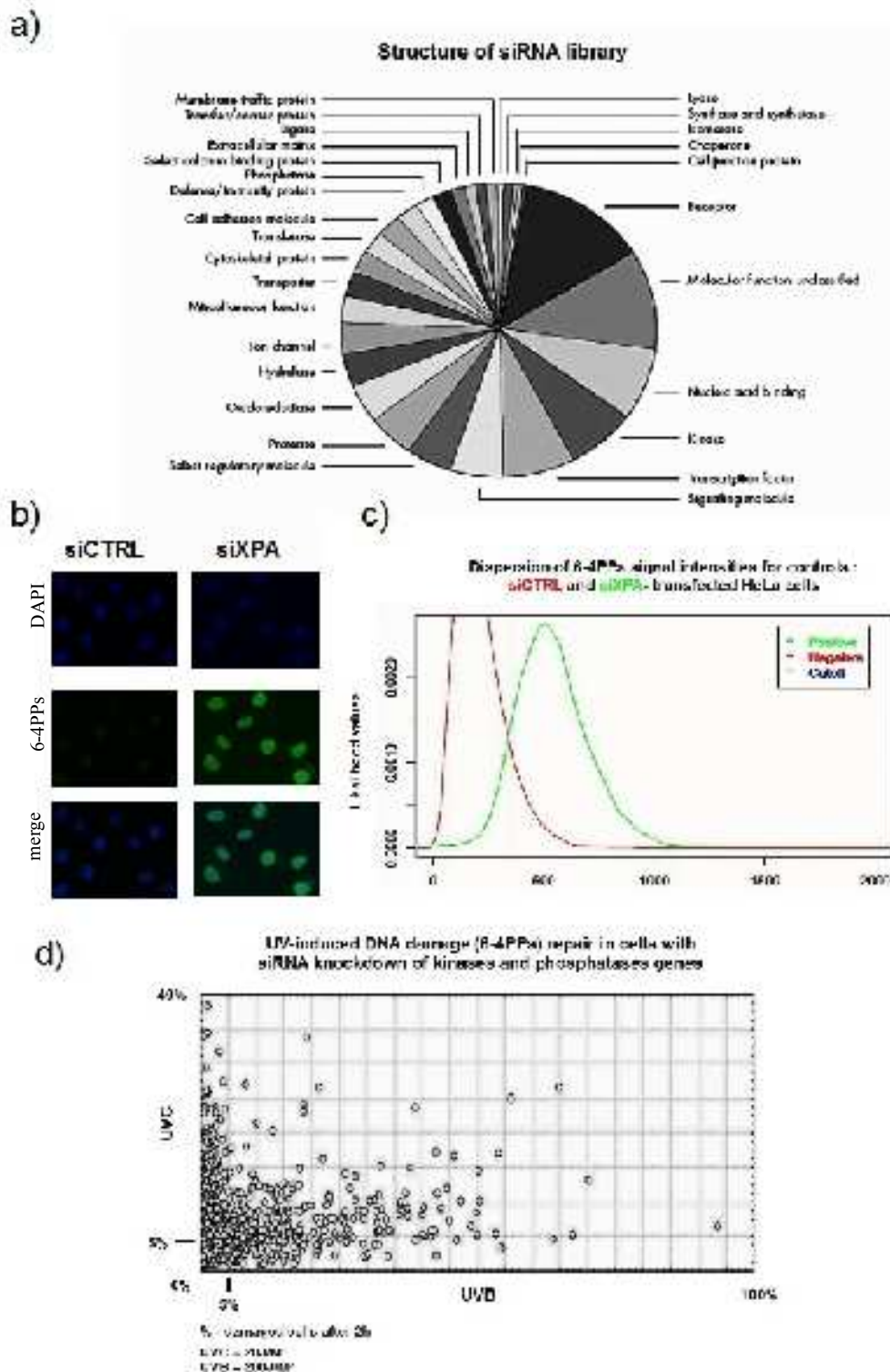
Damage to DNA arises from environmental and endogenous sources, including

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

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

## **RESULTS AND DISCUSSION**

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



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

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

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

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

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

a)		b)	
Diseases and Disorders		p-value	Top 20 UVB :
Cancer	2,23E-04	4,40E-02	DDJOK
Reproductive System Disease	2,21E-04	4,01E-02	SH2
Dermatological Diseases and Conditions	1,01E-02	1,41E-02	PIP3
Developmental Disorder	1,00E-02	1,70E-02	ORSR1
Histone acetylation	1,30E-03	4,40E-02	SHBP1
			PPP2R3A
			PIR
			SH3CA
			SHYK
			GRK7
			ONE
			CAFP1
			CALM2
			PPP3CA
			SH3D3
			MTMR4
			SH2
			SH3P2
			NRK
			HCHH1
Molecular and Cellular Functions		p-value	
Amino Acid Metabolism	5,01E-07	4,03E-01	
Post-Translational Modification	5,01E-07	4,03E-01	
Small Molecule Biochemistry	5,00E-07	3,92E-02	
Cell Cycle	7,05E-05	4,20E-02	
Cell Signaling	1,53E-04	1,87E-02	
Physiological System Development and Function		p-value	
Digestive System Development and Function	1,00E-02	1,00E-02	
Embryonic Development	1,00E-02	1,00E-02	
Health System Development and Function	1,41E-02	1,81E-02	
Lymphoid Tissue Structure and Development	1,01E-02	1,41E-02	
Nervous System Development and Function	1,01E-02	1,41E-02	
Molecular pathways		p-value	Top 20 UVC :
Mitotic Entry or Exit - K-Ras	1,2E-04		IKK $\alpha$
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1,4E-04		PNCK
Dopamine Receptor Signaling	2,94E-04		ACFS
CDK5 Signaling	5,27E-04		GK
Estrogen Receptors	5,70E-04		SH2
EPAF1/RXR Activation	0,74E-02		PPP2R10
G2M Transition of the Cell Cycle	0,94E-02		MOS
Mediation of Gene Regulation by Antisense Transcription via RNAi	1,58E-01		GR7
NF- $\kappa$ B Signaling Pathway	1,83E-01		DDK1
Epitaxial Differentiation	2,19E-01		GEF17
			EPHA3
			CEK7
			ORSR1
			WEE1
			KOING220
			GEPC3
			PACGIM1
			ALPK7
			CDC25A
			HOK1

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



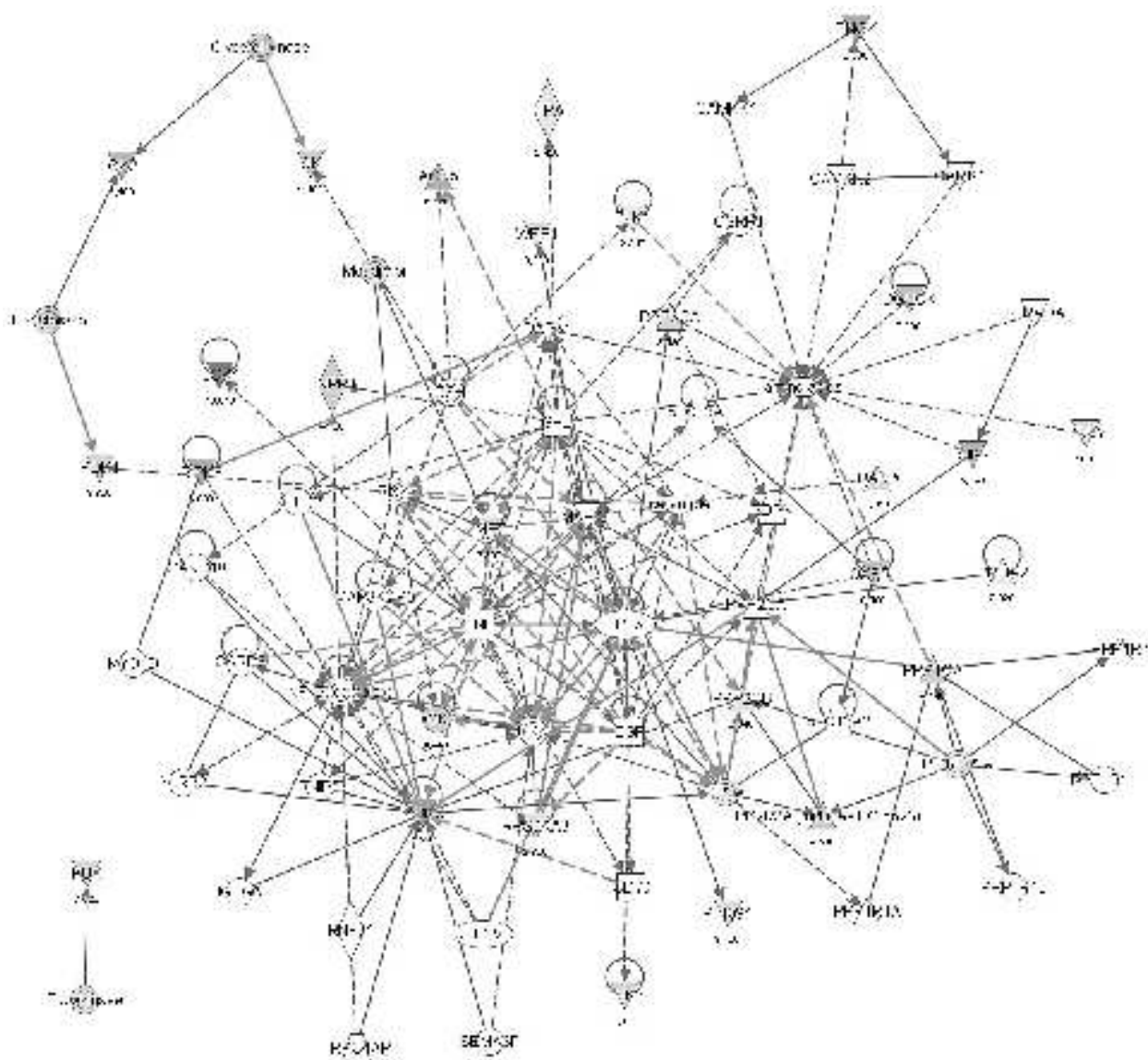


Figure 2. Ingenuity kinome&phosphatome gene network analysis for UVB and UVC screening.

UV radiation results in increased degradation of  $\text{I}\kappa\text{B}\alpha$ , nuclear translocation of p65 and p50, and the DNA binding by NF -  $\kappa\text{B}$  which regulates expression of pro-inflammatory genes including inducible nitric oxide synthase (iNOS).[9]. Three types of UV: UVA, UVB and UVC all induce a dose- and time-dependent phosphorylation of  $\text{eIF2}\alpha$ -Ser. But they act through distinct signaling mechanisms. Thus UVA - induced  $\text{eIF2}\alpha$  phosphorylation occurs through MAPKs, including ERKs, JNKs and p38 kinase, and phosphatidylinositol (PI)-3 kinase. UVB - induced  $\text{eIF2}\alpha$  phosphorylation is through JNKs and p38 kinase, but not ERKs or PI-3 kinase. And UVC stimulates  $\text{eIF2}\alpha$  phosphorylation via JNKs alone. ATM is involved in induction of the intracellular responses to UVA and UVB, rather than UVC.

Wavelength-specific UV irradiation activates differential response signaling pathways converged on the eIF2 $\alpha$  phosphorylation. Direct eIF2 $\alpha$  kinase PKR is activated through phosphorylation by either RSK1 or MSK1, two downstream kinases of MAPKs/PI-3 kinase - mediated signaling pathways[11]. Additional complexity arises with idea that carcinogenic and mutagenic sunlight effects depend not only on cyclobutane pyrimidine dimers and 6-4PPs but also on another types of UV-induced lesions. All three kinds of UV: UVA, UVB and UVC are known to increase the mutation frequency of purified single-stranded phage DNA and cellular DNA in human cells showing that even UVA exposure, lowest energy wavelength, may induce mutation probably via non - dipyrimidine lesion in DNA[8,37]. Apoptosis could be logical a consequence of both inflammation and unrepaired DNA damage and its induction in keratinocytes by ultraviolet irradiation is considered to be a protective function against skin cancer. UV-induced apoptosis is a crucial event both for UVB and UVC. However there are some specific features that rise again for each UV wavelength. Thus caspase-8 activation occurs only in UVB - irradiated cells. The activation of caspase-8 cannot be inhibited by caspases-9 and -3 specific tetrapeptide inhibitors, indicating that the caspase-8 cleavage is not due to feedback from activation of caspases-9 and -3. Thus, despite the lower production of photoproducts in DNA by UVB irradiation, UVB irradiation could induce apoptosis through both mitochondrial (intrinsic) and caspase-8 activation (extrinsic) pathways, while UVC induces apoptosis only via the intrinsic pathway[10]. When apoptosis is compromised UV radiation could strongly induce cancer, as UV is also linked to the activation of putative cancer genes like PPP2R2A, MTAP and MAP2K4[12].

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

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

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

### **DDR acts via chromatin remodelers**

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

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

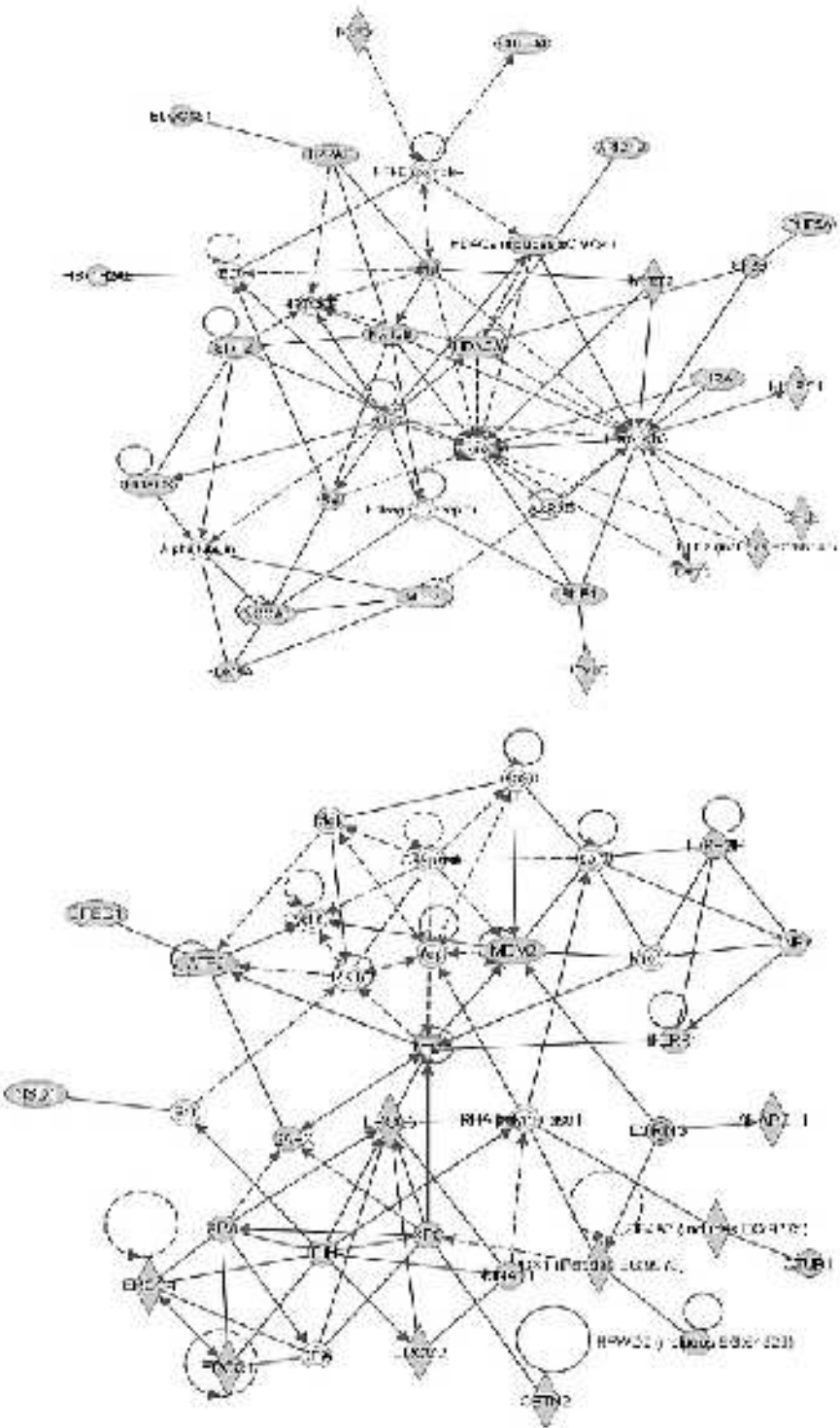


Figure 3. Ingenuity gene network analysis for chromatin remodelers. Histone modifications (upper net) and TFIID - related proteins (bottom net).

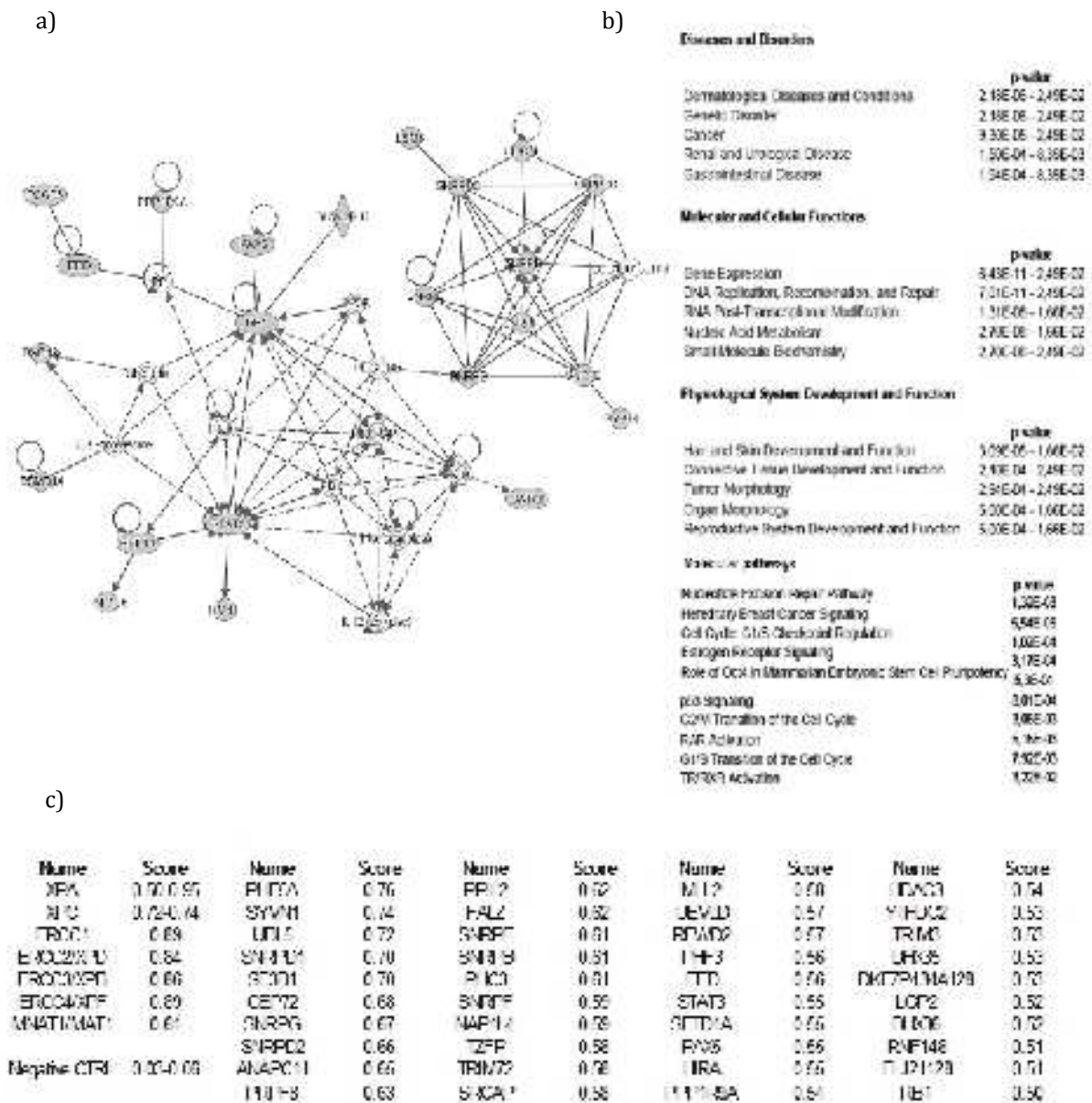


Figure 4. a) Ingenuity gene network analysis (continue). b) Ingenuity gene functions and pathways analysis for chromatin remodelers. c) Control genes and top 40 chromatin remodelers associated with 6-4 PPs repair. Score, 0 to 1, shows portion of "positive" XPA-like cells.

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

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

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

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

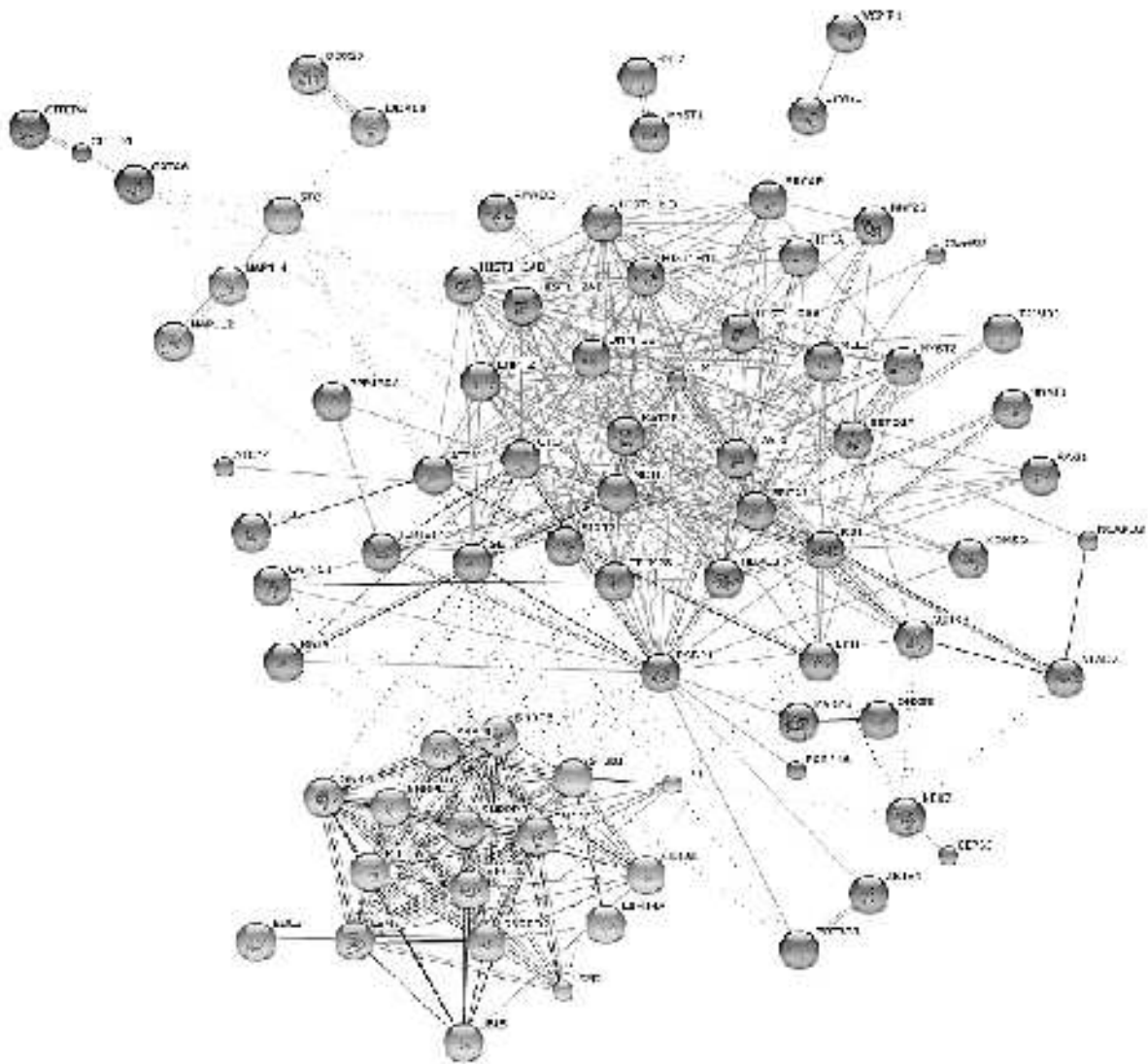


Figure 5. STRING-DB analysis for chromatin remodelers.

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

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

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



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

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

transcription and splicing both directly and indirectly.

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

## **PERSPECTIVE**

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

## **EXPERIMENTAL PROCEDURES**

### **siRNA screening**

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

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

### **Data analysis and Statistical Analysis**

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

### **Bioinformatics**

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

### **ACKNOWLEDGEMENTS**

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## **DISCUSSION AND CONCLUSION**

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

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

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

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

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

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

implicated in the recruitment of TFIIH to sites of DNA damage. Mutations in the R-E-D and ThM motifs fully mimicked the biochemical and biological defects obtained with a mutation in the ATPase motif. We suggested that the ATPase, R-E-D and ThM motifs worked together to ensure a correct recruitment of TFIIH to the damaged sites before the opening and dual incision steps take place. ThM domain was not found in other helicases, including XPD[23-26], but a similar helical protrusion was observed in DNA polymerases[27] and in *Sulfolobus solfataricus* SWI2/SNF2 ATPase Rad54[28] where it was expected to grip double-stranded DNA from the minor groove. The structure of XPB suggested that the energy furnished by the ATP hydrolysis could be used to induce a flip of 170° of the HD2 domain following the binding of XPB to DNA[29]. The R-E-D (present in HD1) and the ThM (present in HD2) were then in close vicinity and were used to stabilize TFIIH on the DNA by introducing a wedge (the E473 residue) in the double stranded DNA, gripped by the ThM motif. To obtain experimental evidences for this model, we compared the ATPase activities of the WT and mutated complexes with or without DNA. Indeed, like most SF1 and SF2 members, the ATPase activity of TFIIH was stimulated by DNA[30]. In the presence of DNA, mutations in the R-E-D and ThM motifs induced 50% inhibition of the ATPase activity compare to TFIIH(WT). In the absence of DNA, the three ATPase activities were strictly identical and were still slightly higher than the ATPase activity of the TFIIH complex mutated in the ATP binding site. These data accredited the model of the conformation change proposed above since they demonstrated that R-E-D and ThM were mainly used to stabilize the binding of XPB to DNA. Furthermore, that these mutations inhibited both TFIIH transcription and repair activities suggested a common mode of recruitment of TFIIH to the promoters and to the damage. The recruitment of TFIIH through the action of the ATPase activity of XPB could also induce a reorganization of the protein-DNA complexes in transcription and repair that could allow new protein-protein or protein-DNA contacts. Indeed, using photo-crosslink experiments, we showed that addition of ATP in NER induced a re-positioning of XPC on the damaged DNA that was dependent on TFIIH[31]. Following the recruitment of TFIIH to the damaged DNA through the energy furnished by the ATPase activity of XPB, the DNA would be opened by XPD, which had a processive and robust helicase activity stimulated by the p44 subunit of the core TFIIH[32]. Here, a mutation in the ATPase activity of XPD still allowed TFIIH to bind the damaged sites “in vivo” but was

unable to open the DNA around the lesion. Altogether, our data gave a new view of the roles of XPB and XPD in NER by revealing their different molecular functions within this event.

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

was deficient in the absence of DOT1L. Further we analyzed both the occupancy and the chromatin modifications at the corresponding promoters with time after UV-irradiation and showed that these promoters were temporally depleted of basal transcription factors in the first hours after irradiation in wild-type cells and showed recovery of occupancy after DNA repair took place, 6-10 hours after UV-irradiation. In the absence of DOT1L, we observed a loss of basal transcription factor occupancy and increased heterochromatin marks such as methylation of H3K9 residue at the promoters of constitutively expressed genes, even 24 hours after UV-irradiation. These data suggested that DOT1L favored an opened chromatin structure around the promoter of housekeeping genes to allow transcription re-initiation. In line with this hypothesis, we observed that H3K79me<sub>2</sub>, the mark of DOT1L activity, was found transiently on the promoters of housekeeping genes in wild-type cells after UV-irradiation, to keep the chromatin opened. In addition, the absence of DOT1L could be circumvented by the class I HDAC inhibitor TSA that relaxed chromatin. TSA restored both the transcription initiation of housekeeping genes and the UV-survival of MEF<sup>DOT1L</sup> cells, creating thus a link between these two events. Among the sites of histone methylation, H3K79 was unique as it was not located within the H3 N-terminal tail domain but in the core region. Specifically, this methylation occurred on the surface of the nucleosome and could serve as a platform to recruit additional chromatin modifiers and DNA damage response factors[37]. On the other hand, regions of chromatin where transcription was repressed were depleted of H3K79 methylation, indicating that silencing of chromatin probably required hypomethylation of H3K79. The mechanism that links euchromatin to H3K79 methylation was not fully understood but it was believed that in addition to recruiting chromatin modifiers, this histone mark played an important role in confining the Sir proteins to heterochromatic regions[38-40]. In yeast, Sir3 bound to nucleosomes containing deacetylated histone H4K16 and promoted spreading of heterochromatin along the chromatin[41]. Based on these observations and our data, we proposed that RNA Pol II re-accumulation at promoters of housekeeping genes after UV-irradiation depended on the chromatin changes orchestrated by DOT1L, including the emergence of active chromatin transcription marks around the promoters of these genes. In the absence of DOT1L, facultative heterochromatin marks such as H3K9me<sub>2</sub> appeared and RNA Pol II did not get to the promoters. Through the recruitment of chromatin modifiers

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

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

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



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## AFTERWORD

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

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



Alexander ZHOVMER



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

## Résumé

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

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

## Résumé en anglais

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

Keywords : methyltransferase, DOT1L, DDR, NER, chromatin