Functional analysis of XPE and XPD proteins in regard to the phenotypes of xeroderma pigmentosum patients

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Graduate School of Frontier Biosciences
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Videoconference for the DNA Repair Interest Group
NIH, September 21, 2010

Photo: 祇王寺、京都
Gi-ohji Temple, Kyoto

Topics:

The DDB2 complex-mediated ubiquitylation around DNA damage is oppositely regulated by XPC and Ku, and contributes to the recruitment of XPA

MMXD, a TFIIH-independent XPD-MMS19 protein complex involved in chromosome segregation

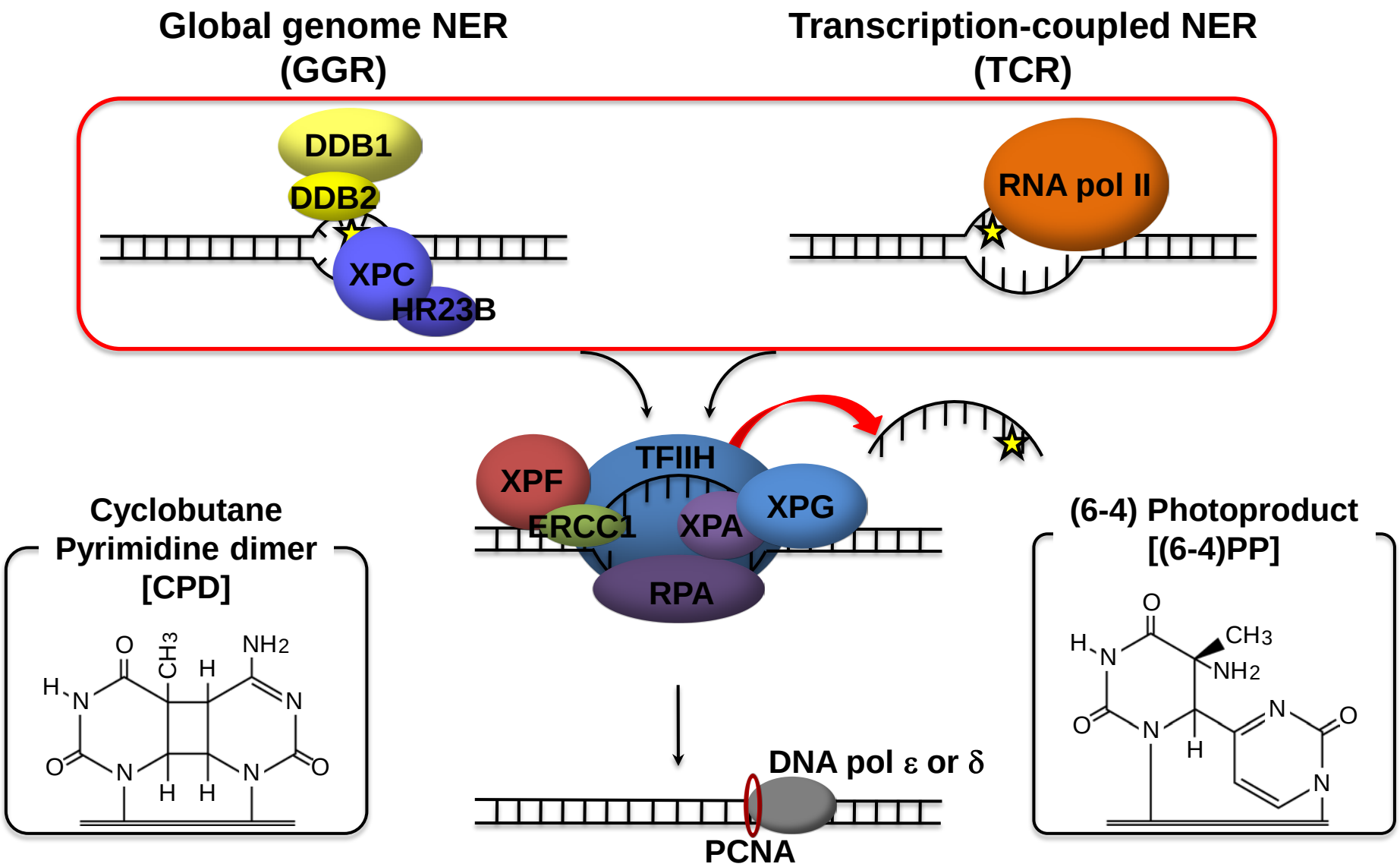
The DDB2 complex-mediated ubiquitylation around DNA damage is oppositely regulated by XPC and Ku, and contributes to the recruitment of XPA

Arato Takedachi

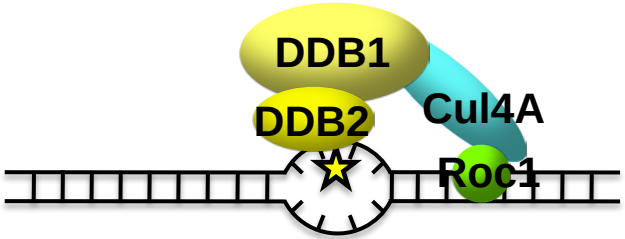
Masafumi Saijo

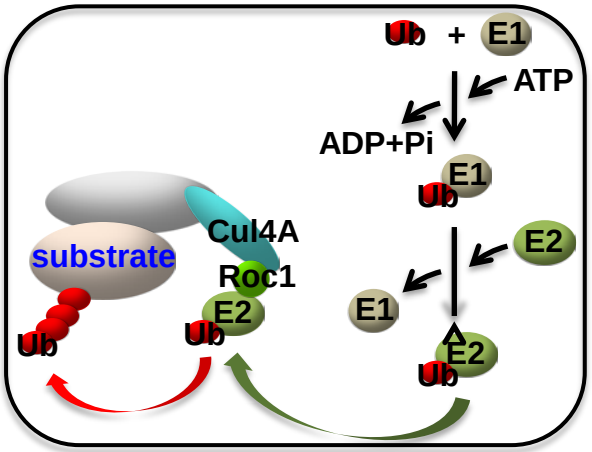
Takedachi et al., *Mol Cell Biol*, 2010

Nucleotide excision repair (NER)



UV-damaged DNA binding protein (UV-DDB)



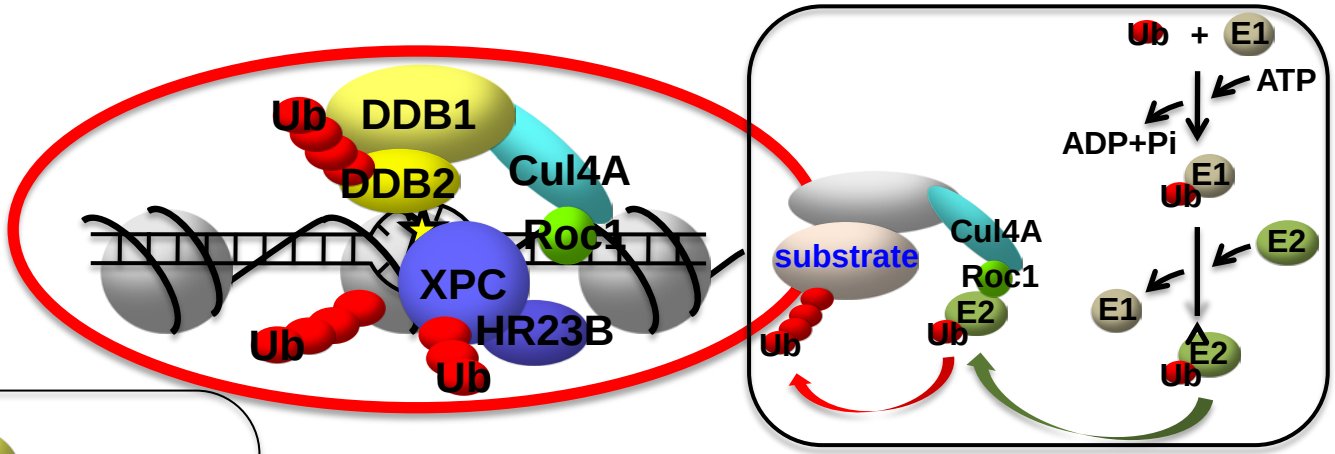


★ UV-damaged-DNA binding protein (UV-DDB) is a heterodimer composed of two subunits, DDB1 and DDB2, and plays a key role in DNA damage recognition at an early stage of global genome nucleotide excision repair (GGR) pathway.

★ Mutations in *DDB2* are responsible for xeroderma pigmentosum group E (XP-E).

★ UV-DDB is integrated in a complex including Cul4A and Roc1, and displays ubiquitin ligase (E3) activity.

UV-damaged DNA binding protein (UV-DDB)



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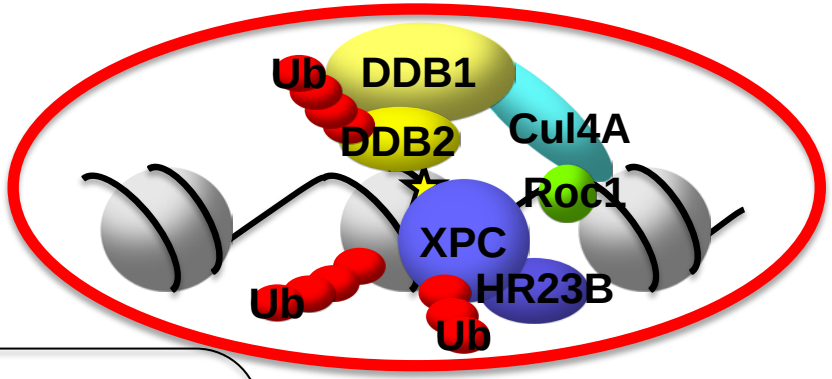
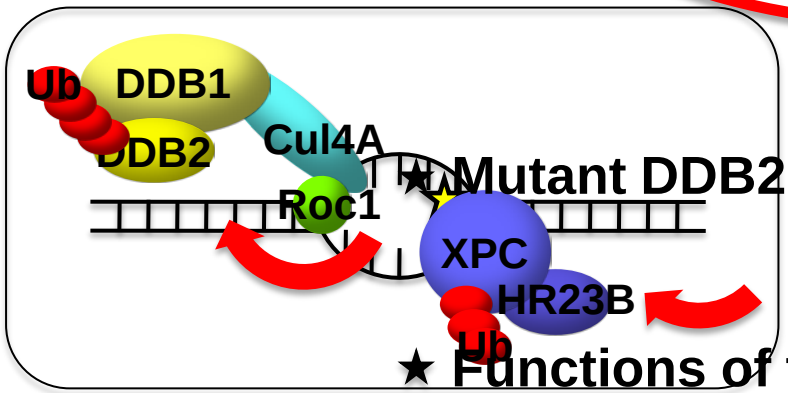
"Destabilization of chromatin structure"

UV-damaged DNA binding protein (UV-DDB)

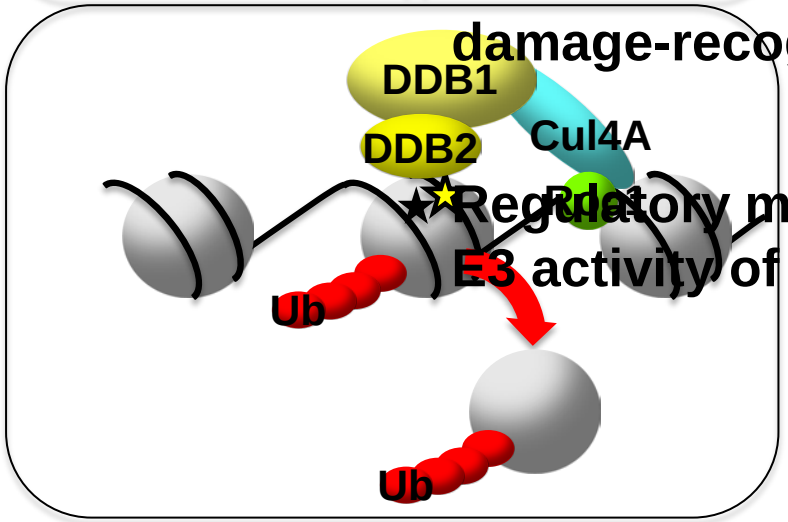




★ Mutant DDB2 derived from XP-E patients

"Hand-off from UV-DDB to the XPC/HR23B at the damaged site."

★ Functions of the DDB2 E3 complex in the damage-recognition step of GGR

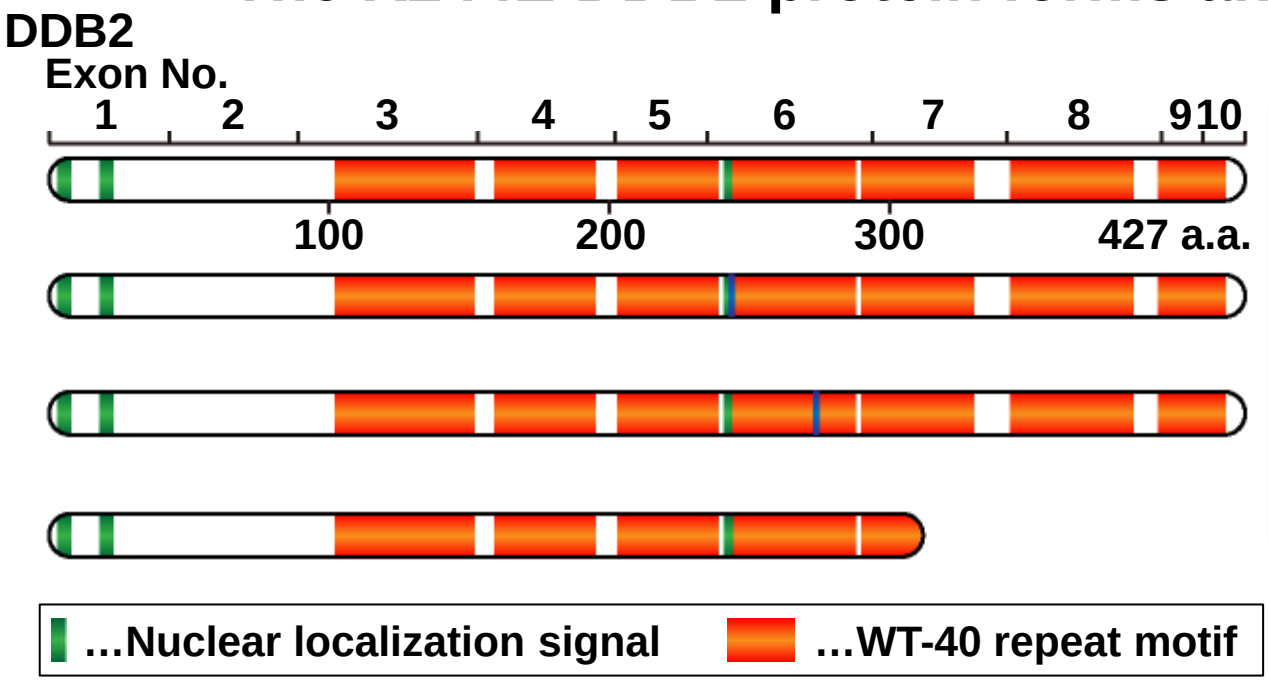


★ Regulatory mechanisms for the E3 activity of the DDB2 complex

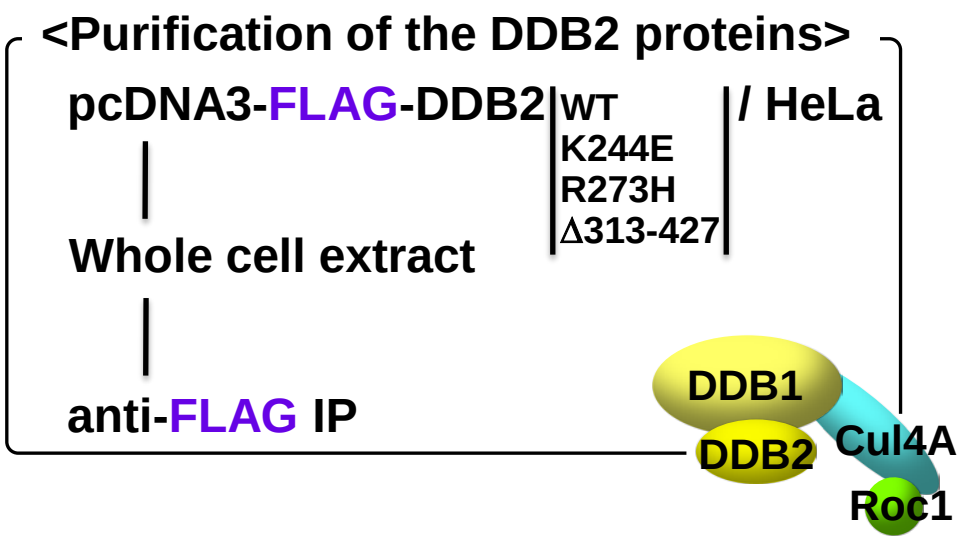
"Destabilization of chromatin structure"

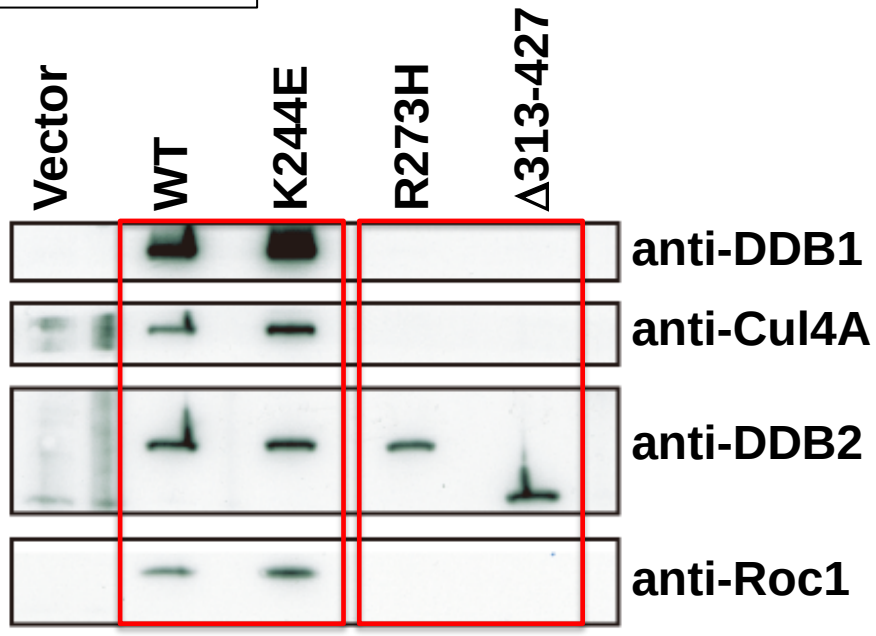
Mutant DDB2 derived from XP-E patients

The K244E DDB2 protein forms an E3 complex

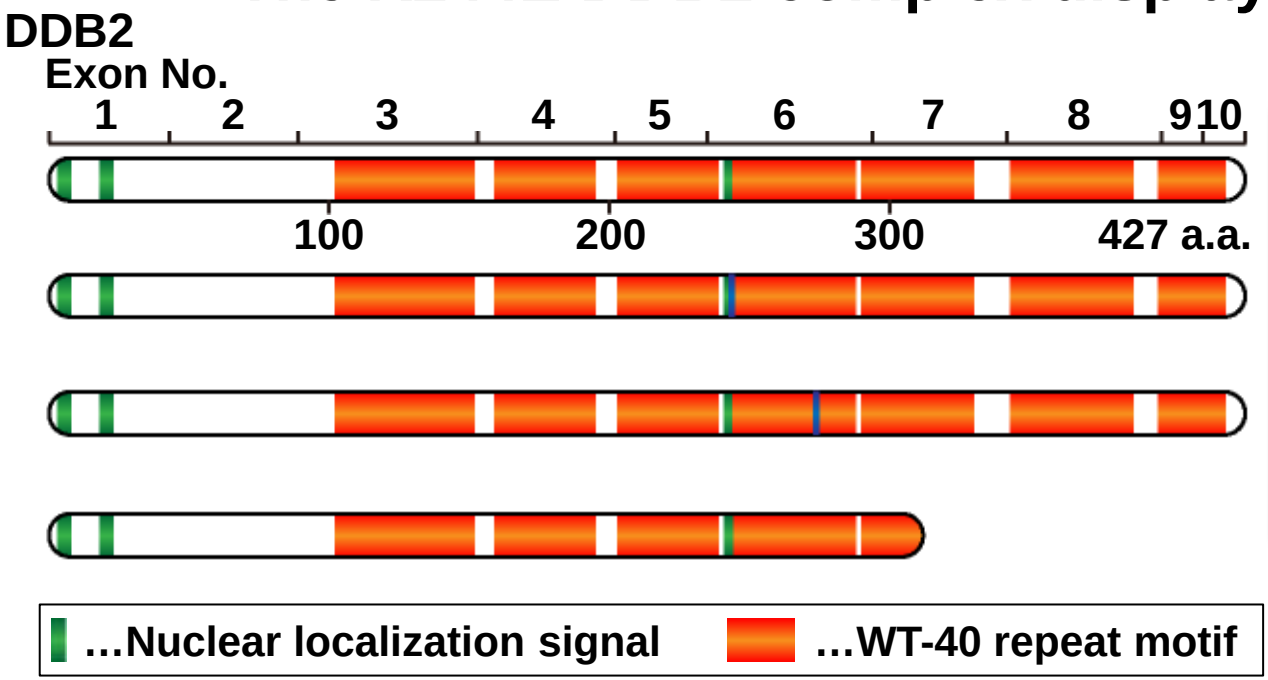


<mutation>	<patient>
WT	
K244E (A905G)	XP82TO
R273H (G933A)	XP2RO XP3RO
Δ313-427	Ops1





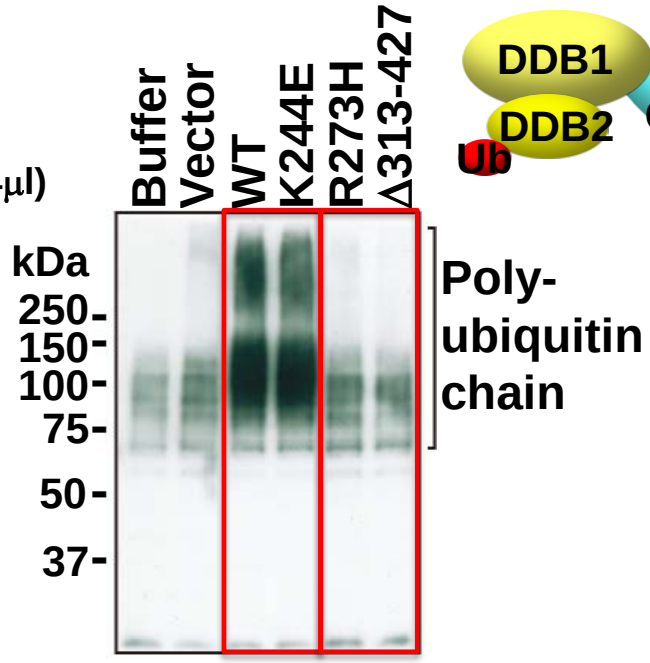
The K244E DDB2 complex displays E3 activity

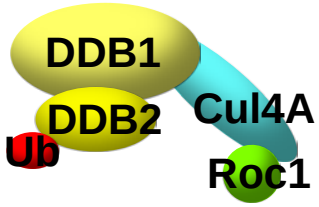


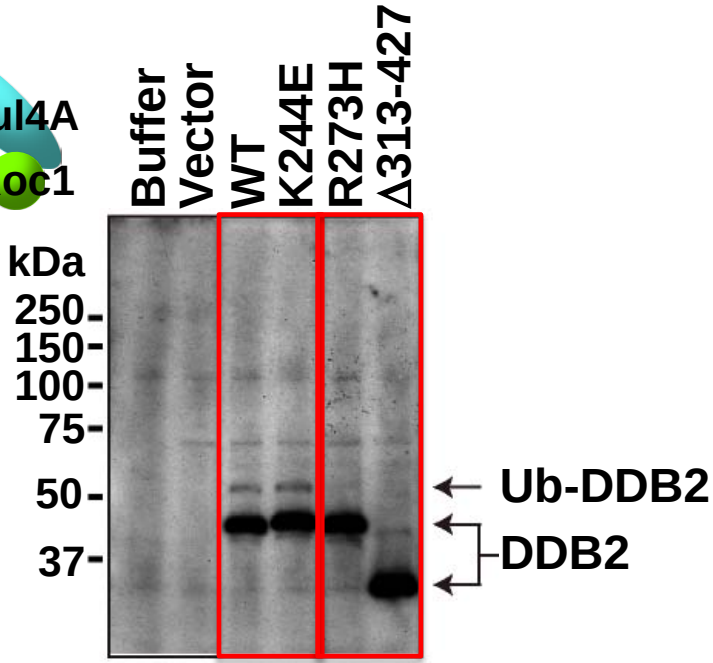
<mutation>	<patient>
WT	
K244E (A905G)	XP82TO
R273H (G933A)	XP2RO XP3RO
Δ313-427	Ops1

Ubiquitin ligase reaction (14μl)

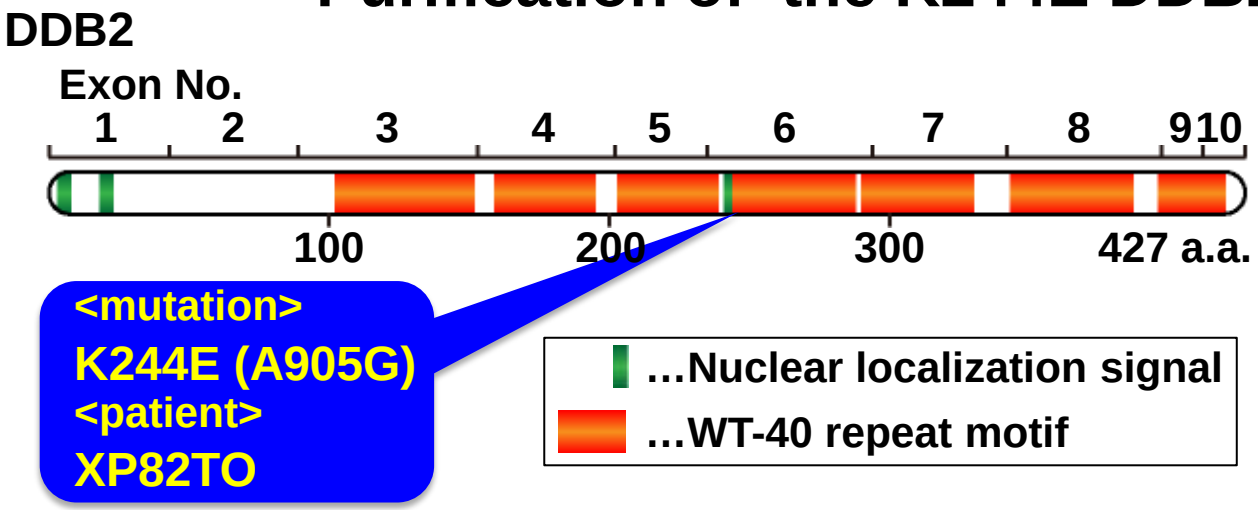
50 mM	Tris-HCl (pH 7.6)
10 mM	$MgCl_2$
1 mM	DTT
0.2 mM	$CaCl_2$
4 mM	ATP
5 μg	Ubiquitin
0.1 μg	E1
0.4 μg	UbcH5α; E2
	E3

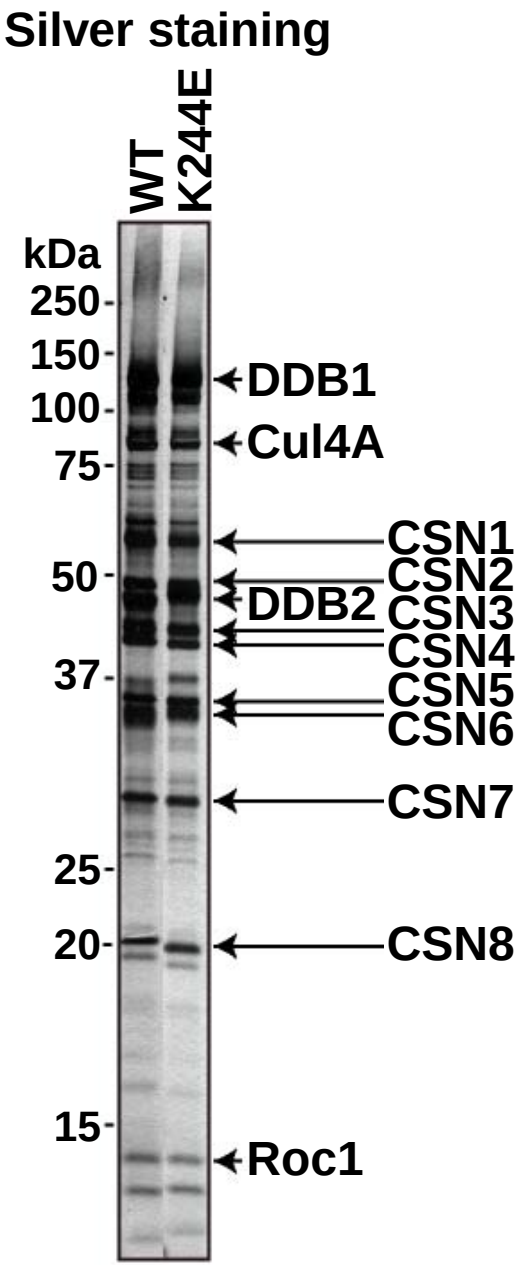






Purification of the K244E DDB2 complex





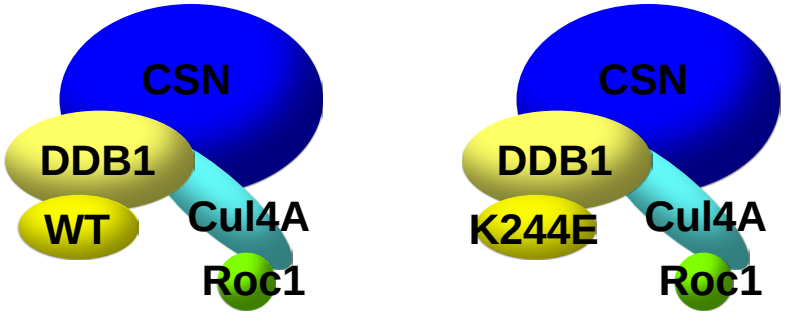
<Purification of the DDB2 complexes>

pCAGGS-FLAG-HA-DDB2 | WT / K244E | / HeLa

Whole cell extract

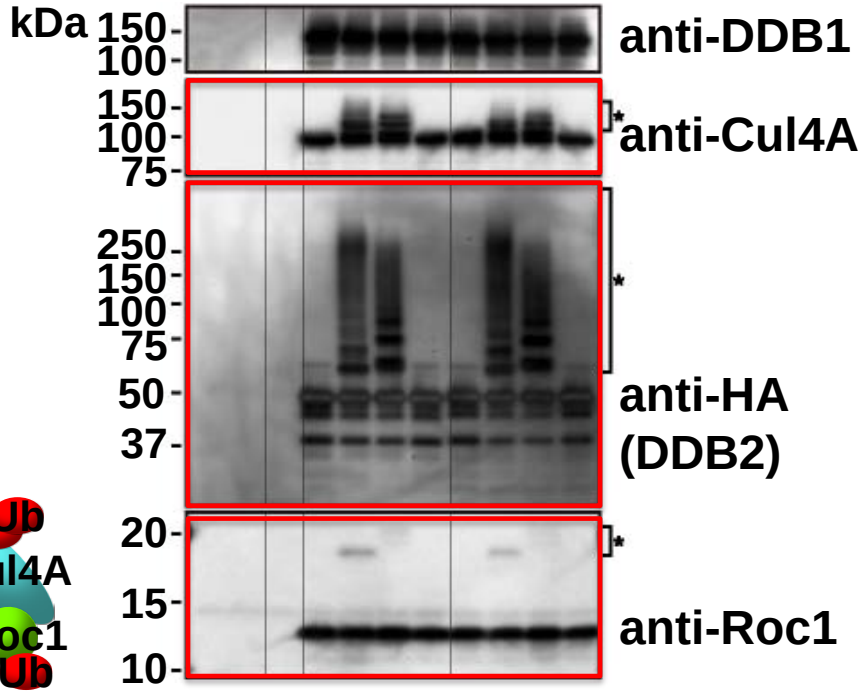
anti-FLAG IP

anti-HA IP

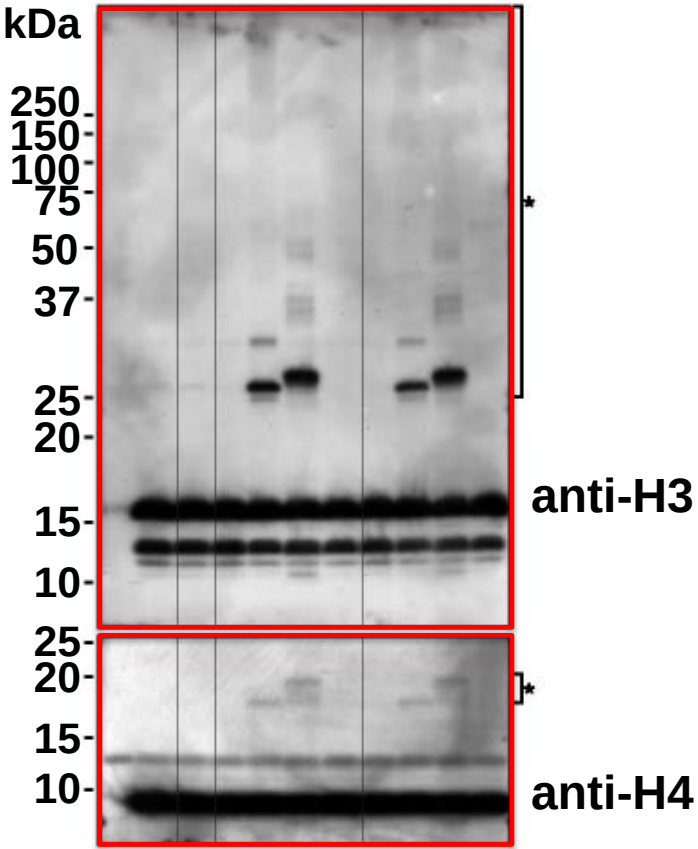


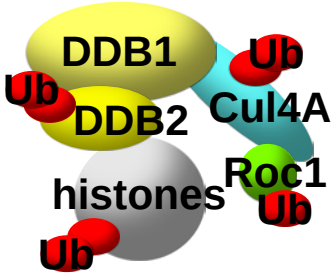
The K244E DDB2 complex mediates histone ubiqitylation *in vitro*

	Buffer		Mock	WT				K244E			
Ubiquitin	+	+	+	+	+	-	+	+	+	-	+
GST-Ubiquitin	-	-	-	-	-	+	-	-	-	+	-
E2 (UbcH5a)	+	+	+	+	+	+	-	+	+	+	-
E2 (UbcH5a d.n.)	-	-	-	-	-	-	+	-	-	-	+
Histone octamers	-	+	+	+	+	+	+	+	+	+	+
Reaction time (min)	60			0	60			0	60		



	Buffer		Mock	WT				K244E			
Ubiquitin	+	+	+	+	+	-	+	+	+	-	+
GST-Ubiquitin	-	-	-	-	-	+	-	-	-	+	-
E2 (UbcH5a)	+	+	+	+	+	+	-	+	+	+	-
E2 (UbcH5a d.n.)	-	-	-	-	-	-	+	-	-	-	+
Histone octamers	-	+	+	+	+	+	+	+	+	+	+
Reaction time (min)	60			0	60			0	60		





The K244E DDB2 complex does not bind to UV-damaged DNA

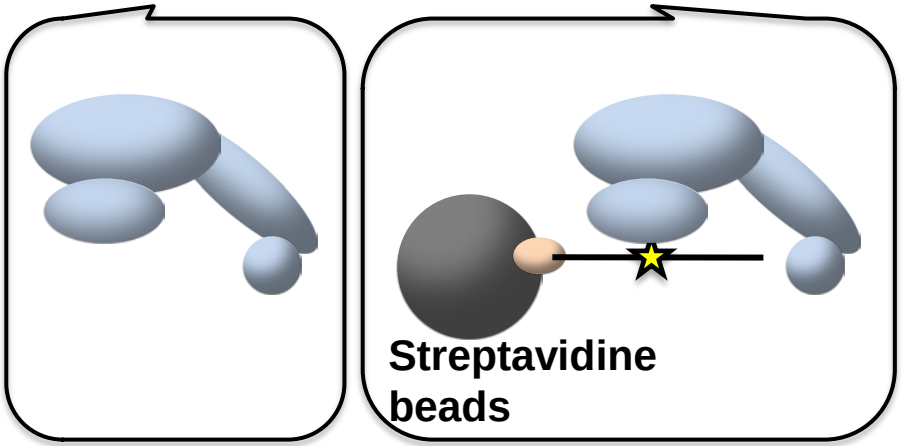
Amplification of the biotinylated DNA (165 bp)

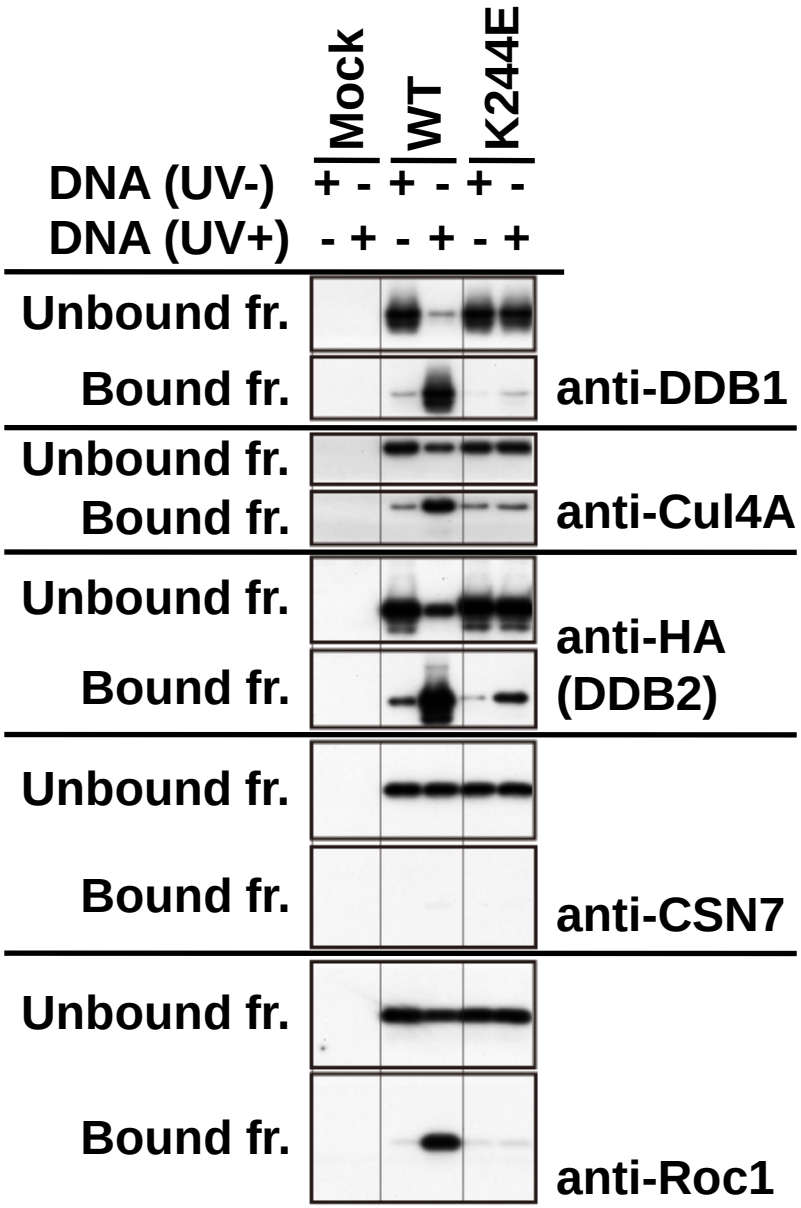
UV-irradiation (10 kJ/m^2)

Incubation with DDB2 (WT or K244E) complex

Immobilization on beads

Unbound fr. | Bound fr.





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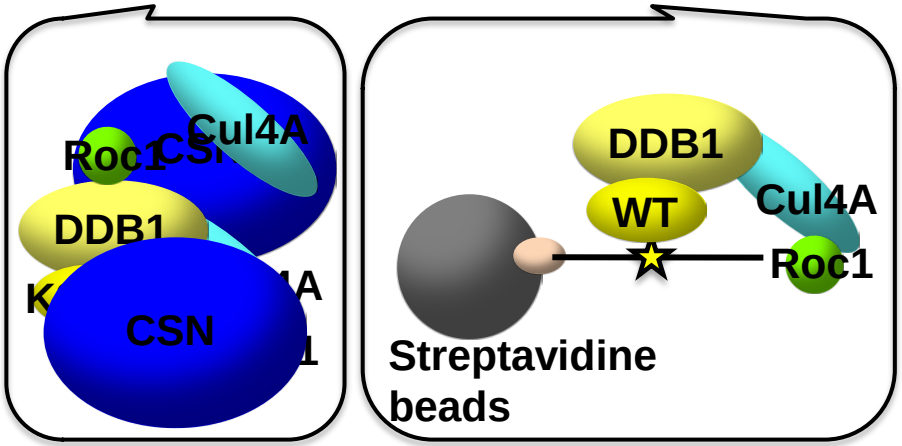
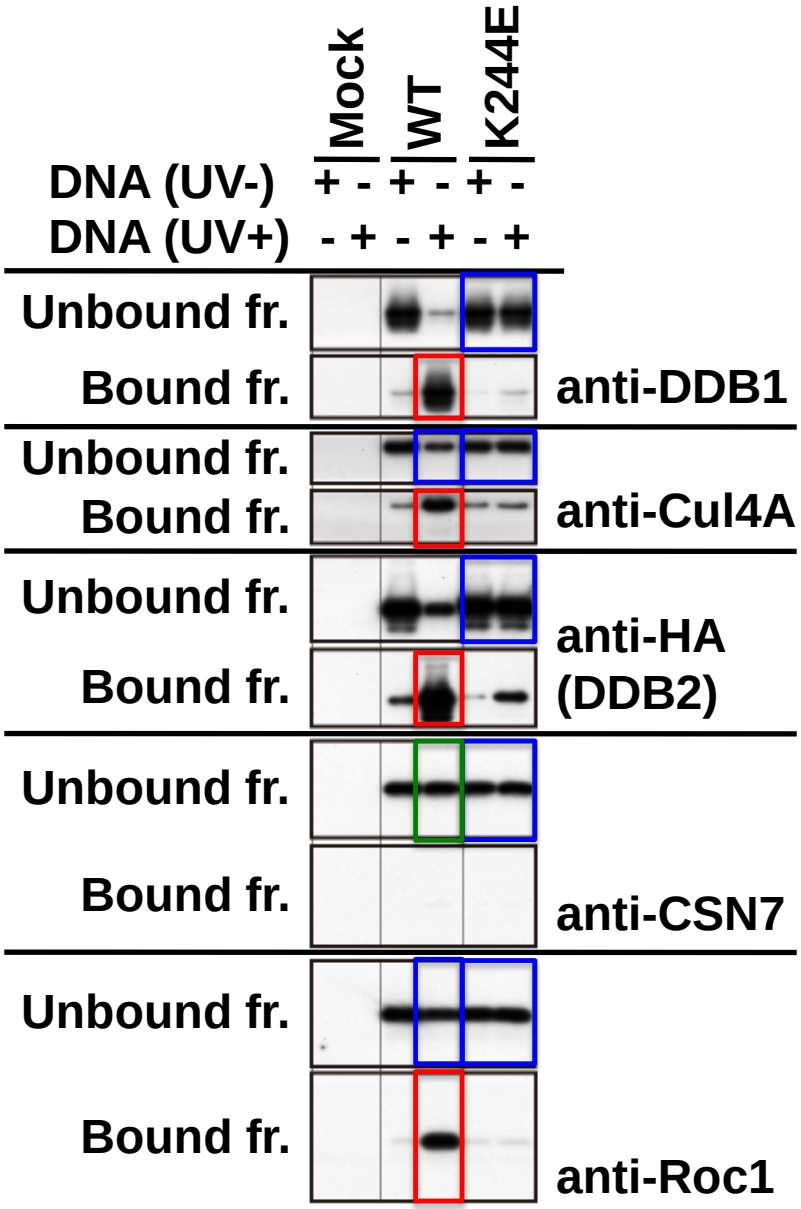




The wild-type, but not K244E DDB2 complex is associated with core histones in a UV-dependent manner

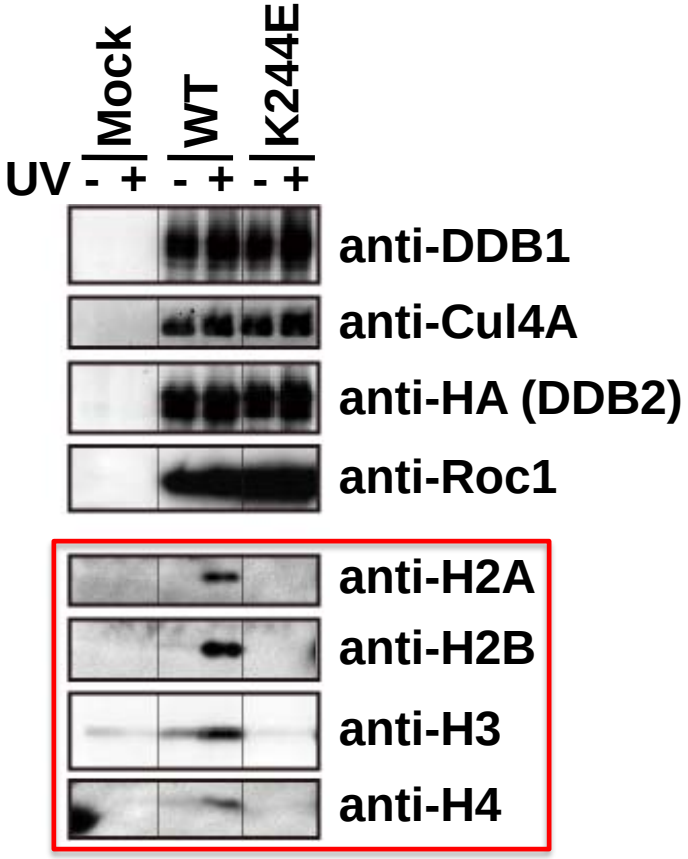
pCAGGS-FLAG-HA-DDB2 | WT / K244E | / HeLa

UV-irradiation (20 J/m^2, 0 min)

Solubilized chromatin fr.

anti-FLAG IP

anti-HA IP



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pCAGGS-FLAG-HA-DDB2 | WT / K244E | / HeLa

UV-irradiation (20 J/m^2)

0, 15, 30, 60, 120 min

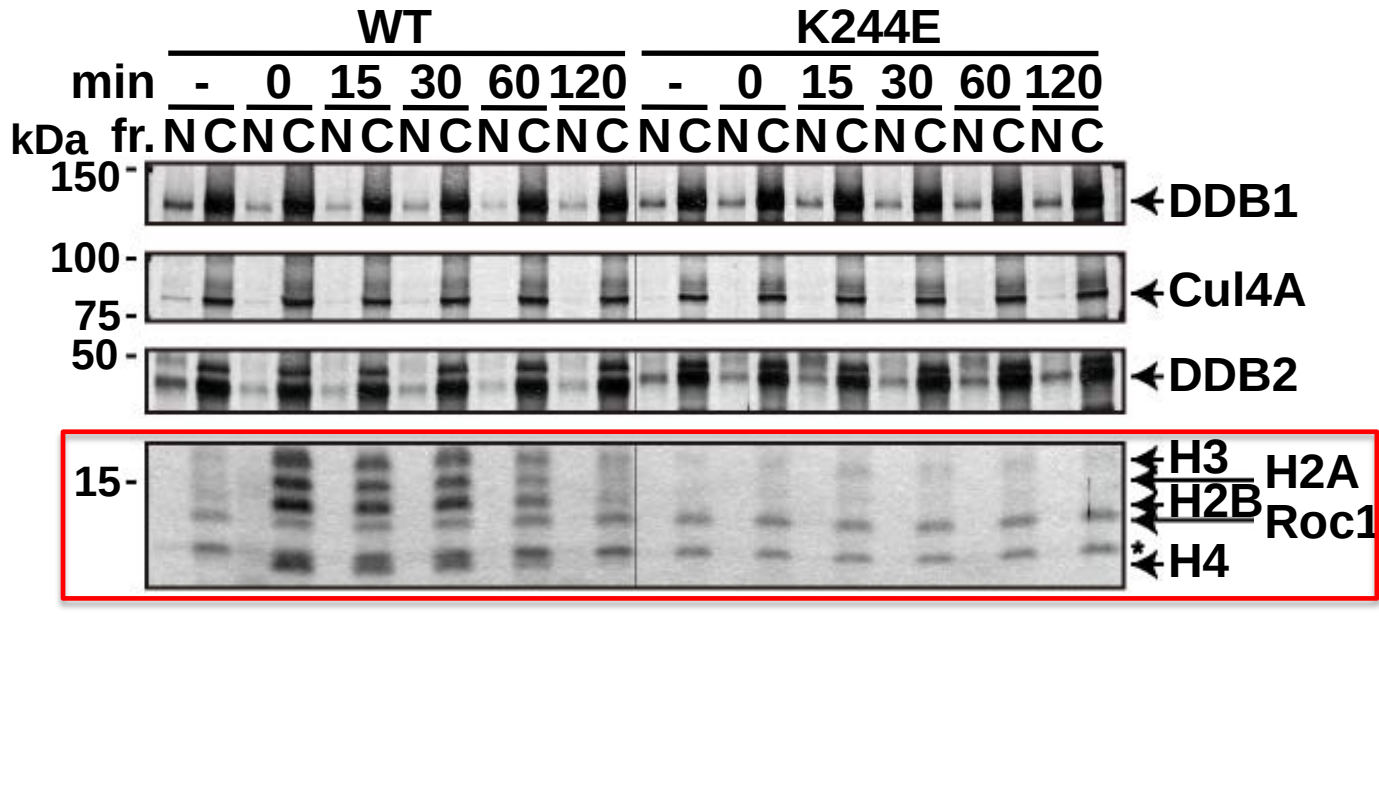
Nuclear extract (N)

Solubilized chromatin fr. (C)

anti-FLAG IP

anti-HA IP

Silver staining



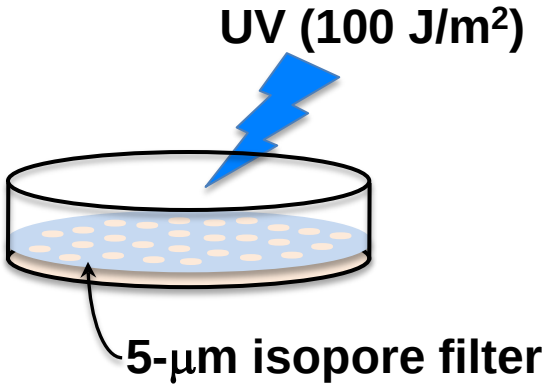
Wild-type, but not K244E DDB2 is distributed to UV-damaged sites in chromatin *in vivo*

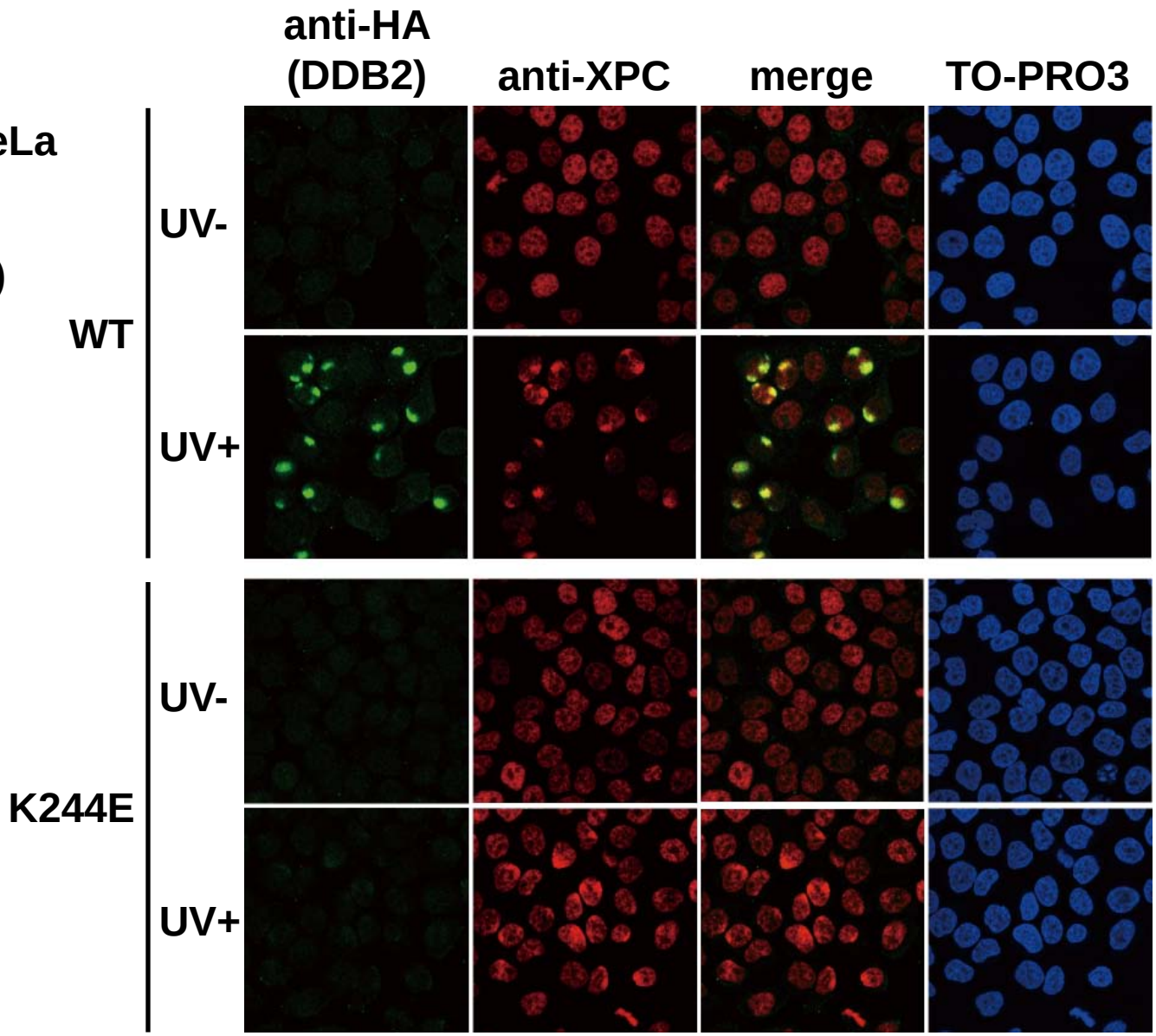
pCAGGS-FLAG-HA-DDB2 | WT / K244E | / HeLa

UV-irradiation (100 J/m^2)
[5-μm isopore filter]

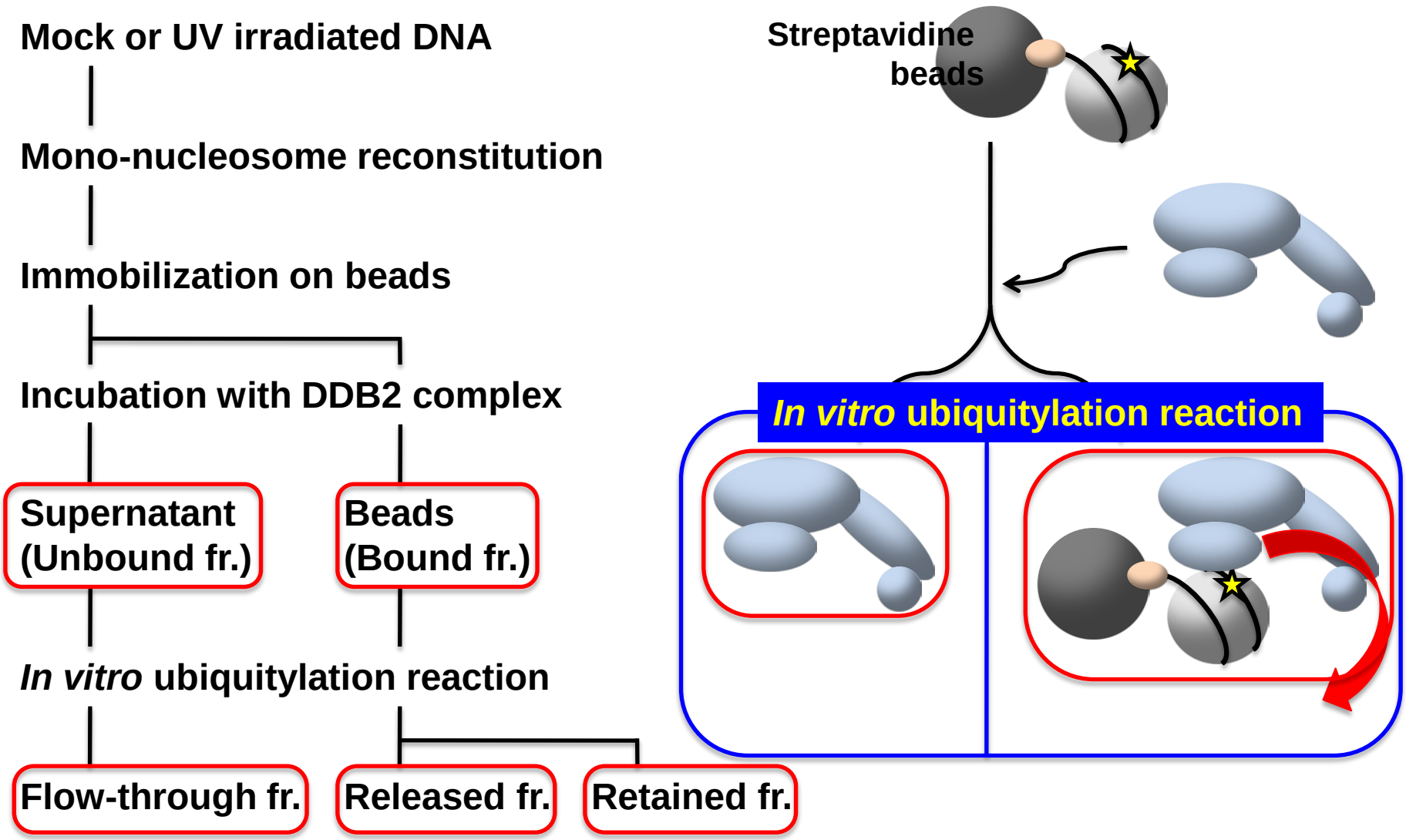
0 min

Immunostaining

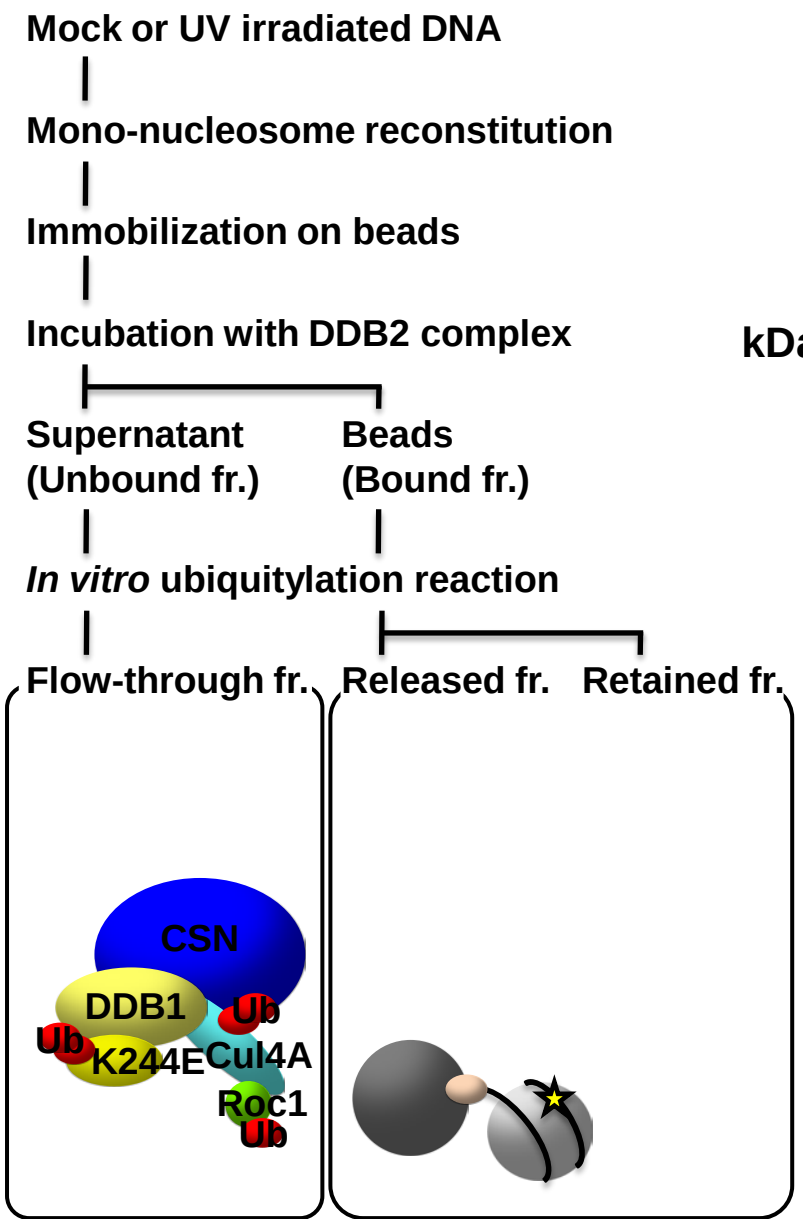


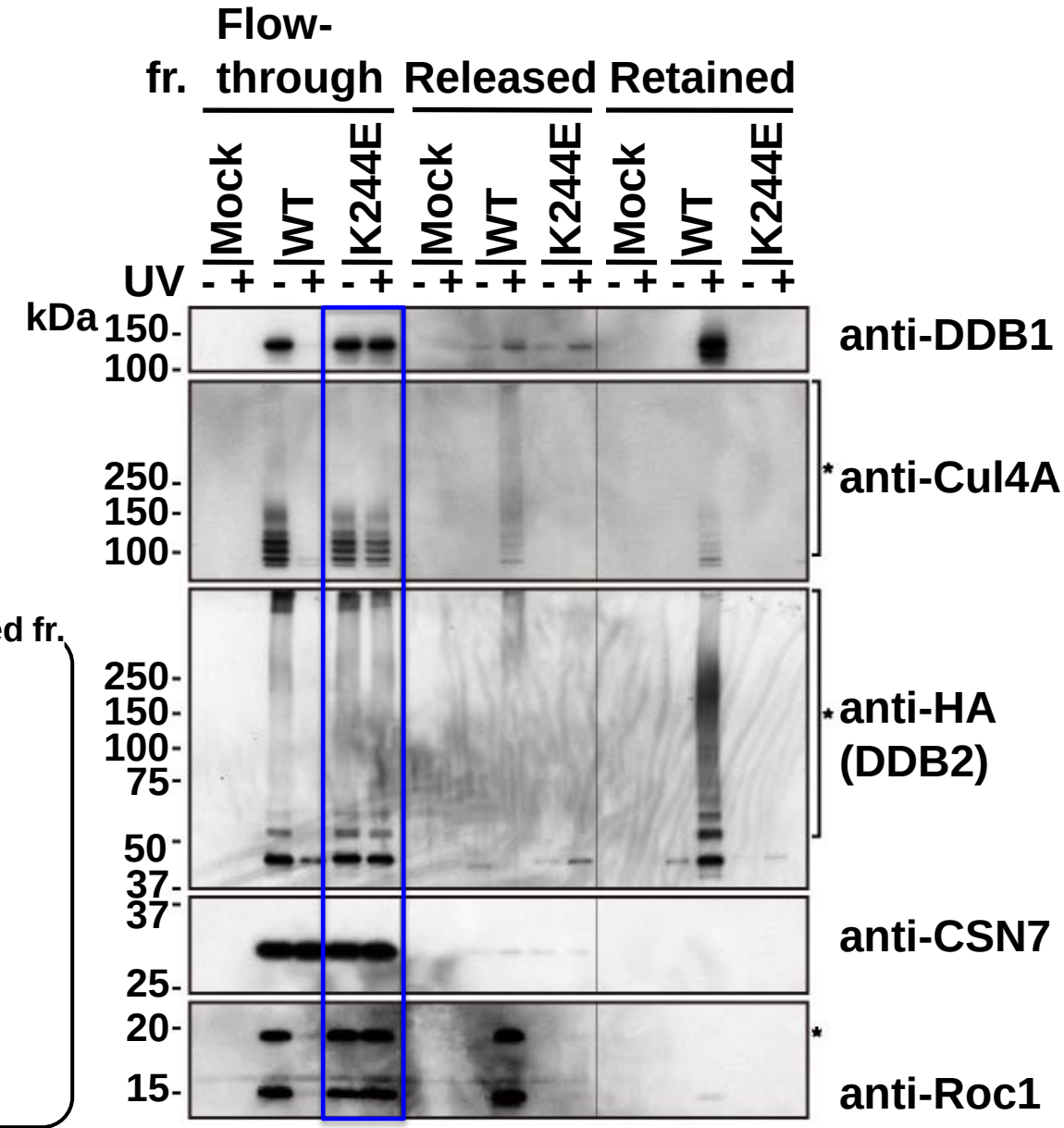


Schematic representation of the strategy to measure the *in vitro* binding of DDB2 complex to UV-damaged chromatin



The wild-type, but not K244E DDB2 complex can mediate histone ubiquitylation around damaged sites *in vitro*





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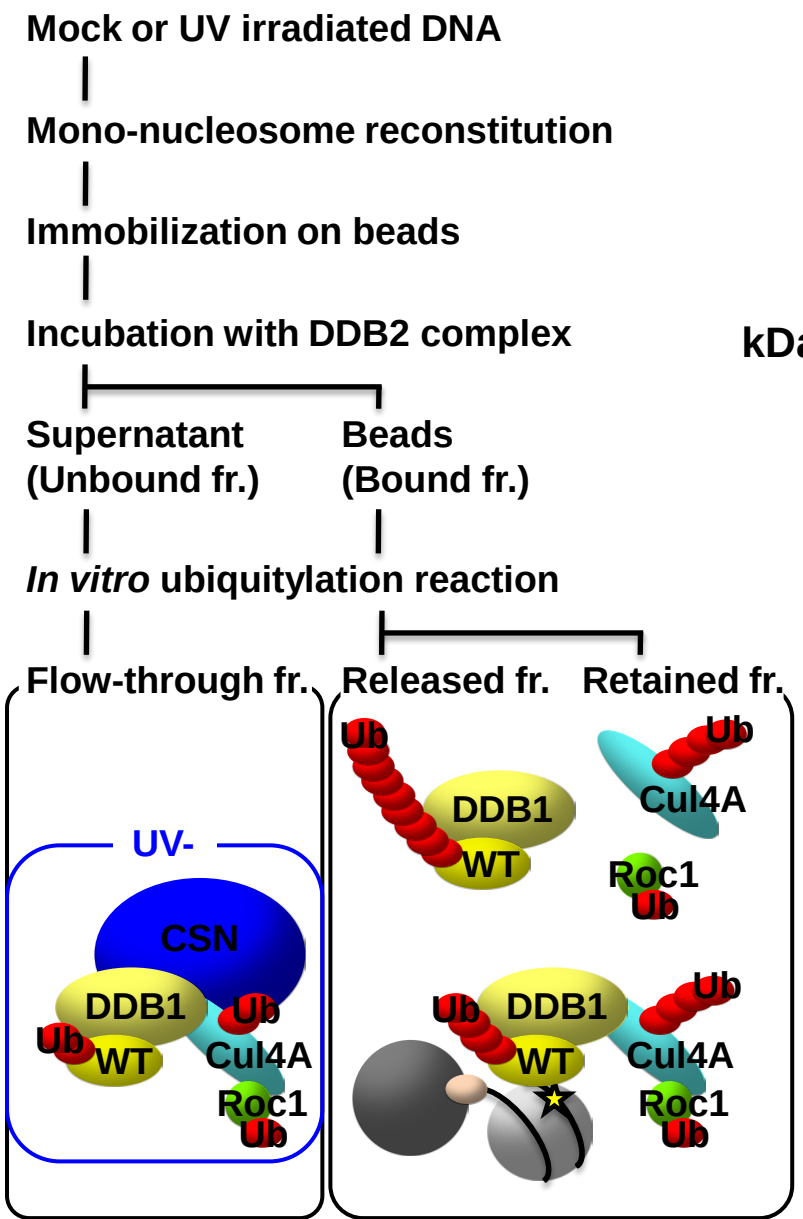

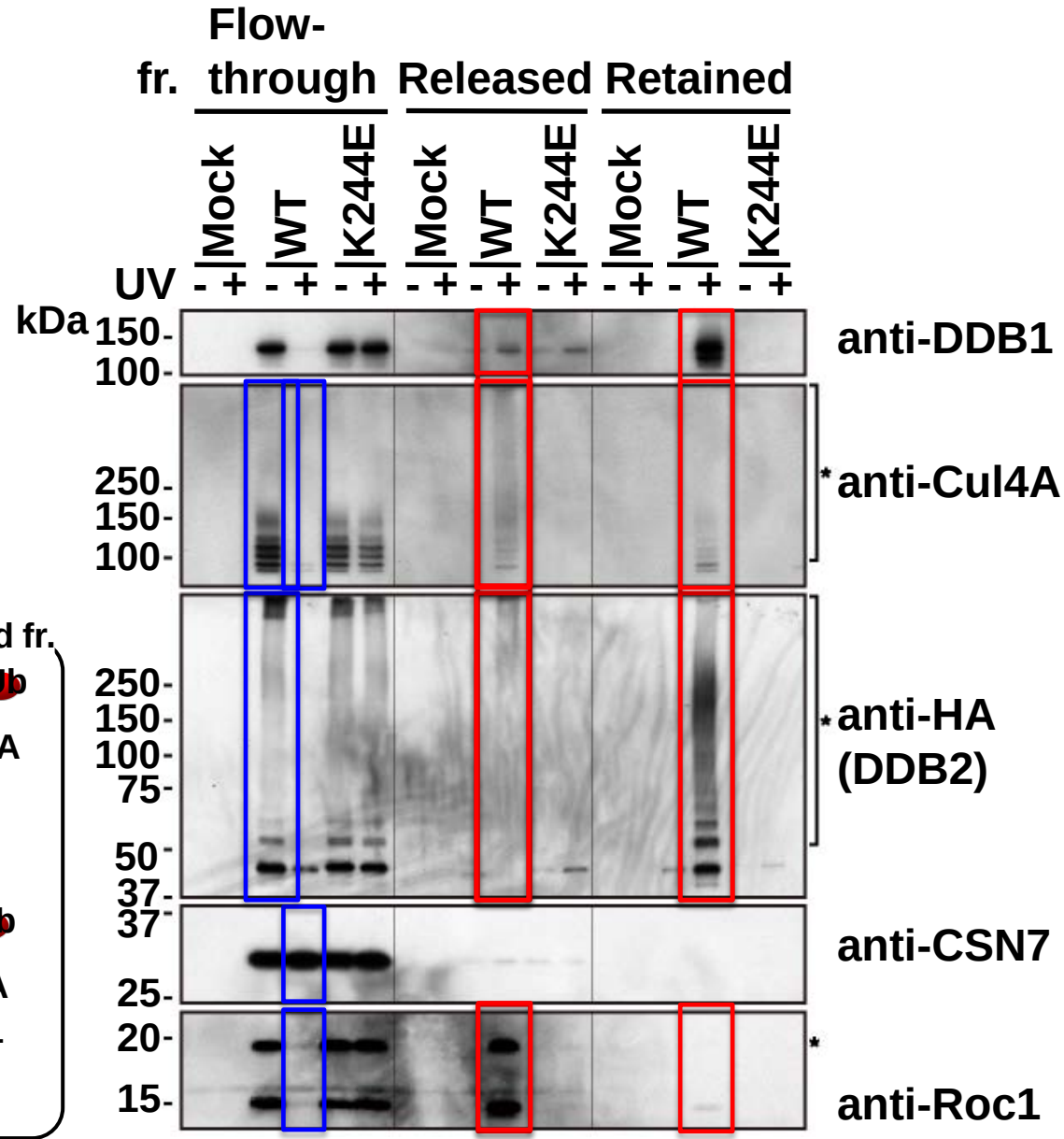

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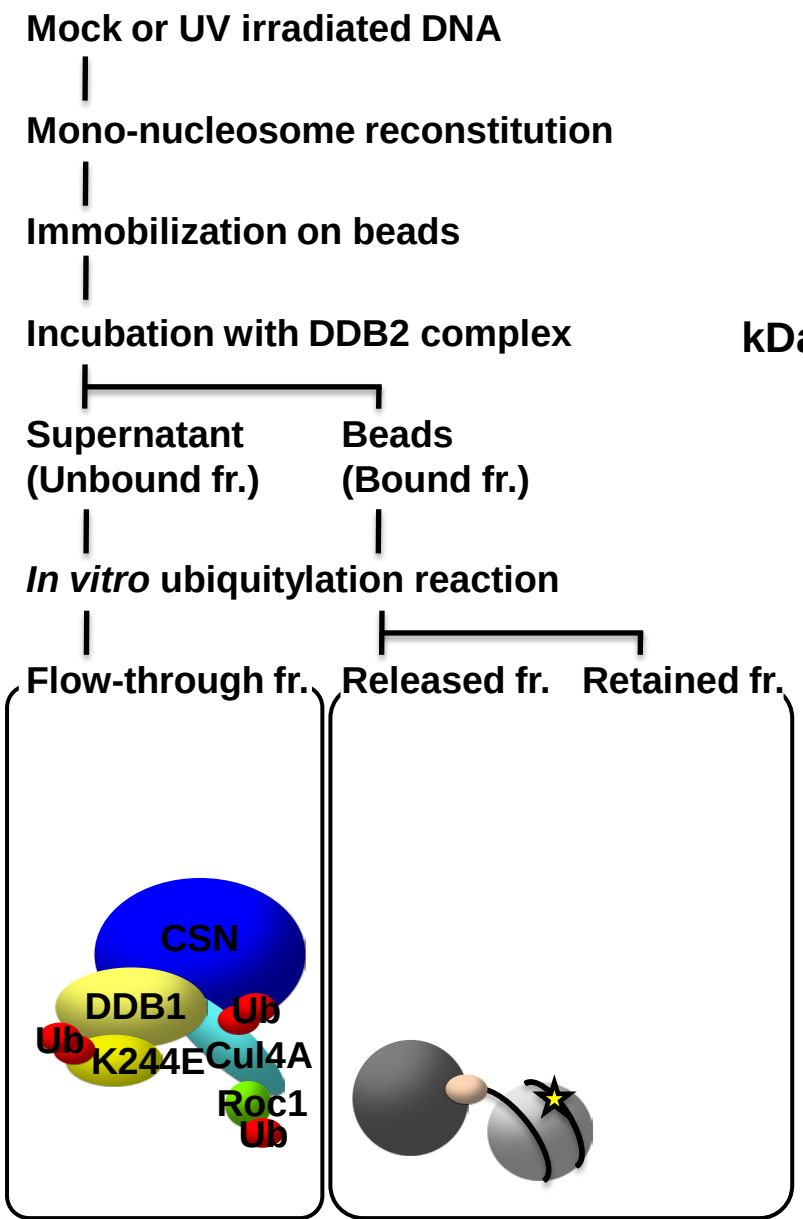

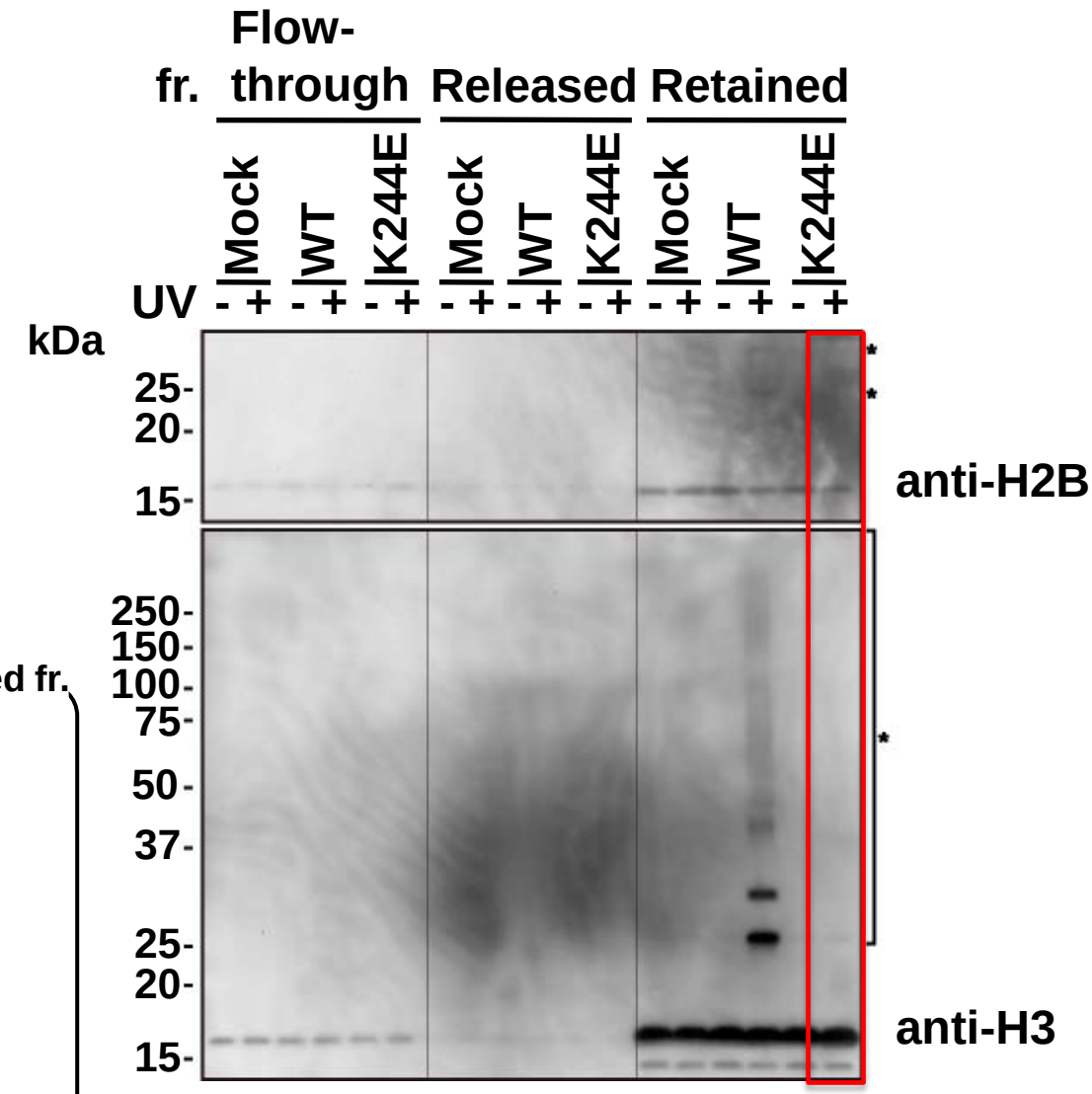

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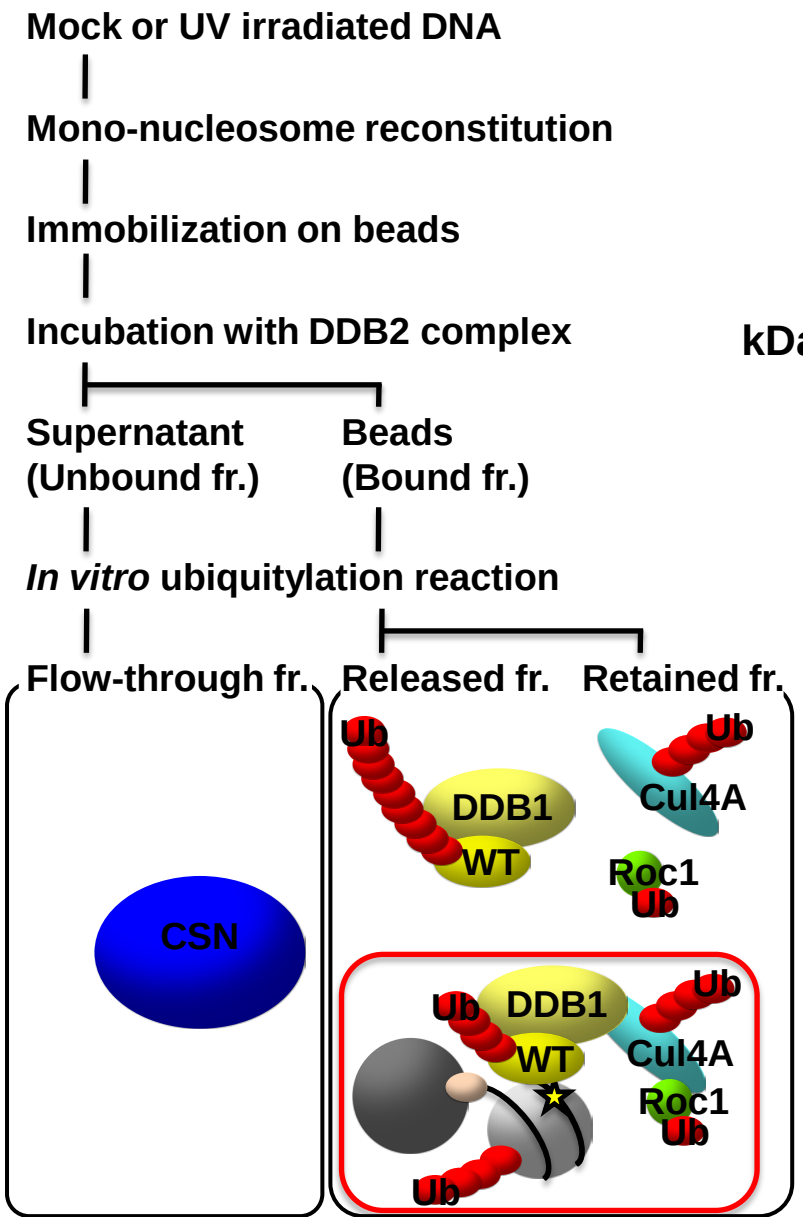
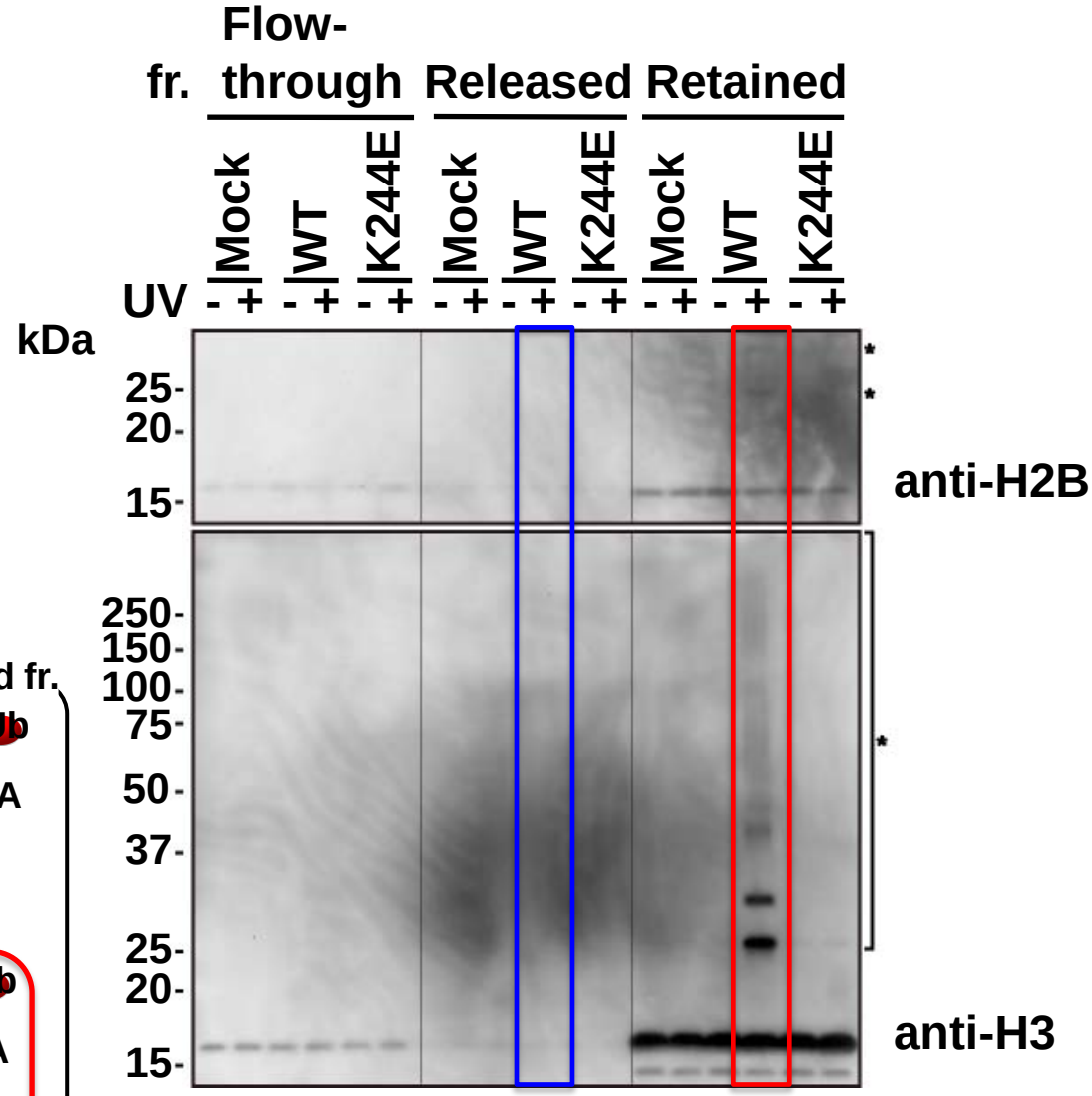




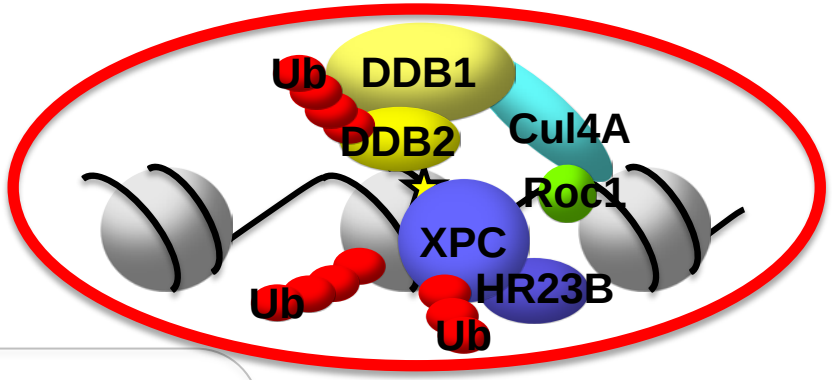
Conclusions

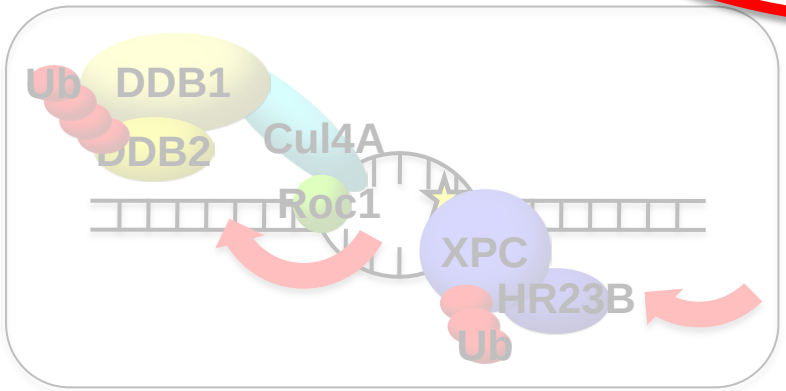
Both DNA-binding and E3 activities of the DDB2 complex are required to promote GGR in chromatin

- ★ **K244E DDB2 was able to form the cullin-based E3 complex. However, this complex did not bind to nucleosomes in the UV-irradiated cells.**
- ★ **The K244E DDB2 complex did not bind to the UV-damaged DNA or the nucleosomes bearing the UV-irradiated DNA, and consequently was not able to mediate the ubiquitylation of the nucleosomes assembled on UV-damaged DNA.**

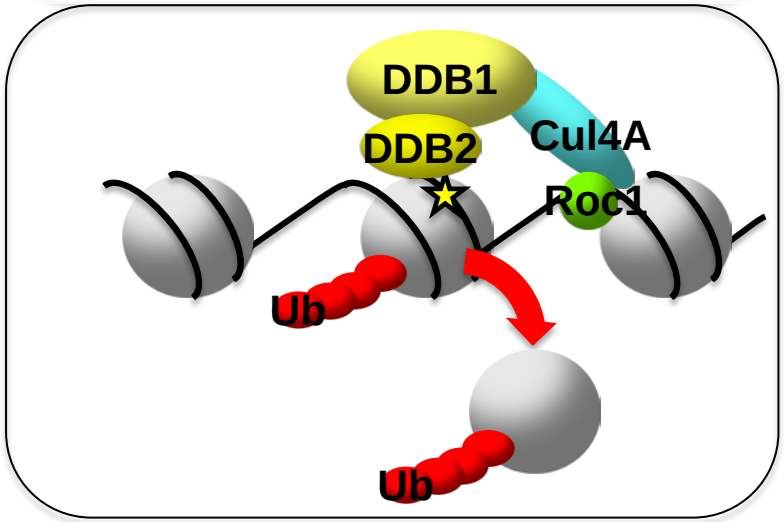
Functions of the DDB2 E3 complex in the DNA damage-recognition step of GGR

UV-damaged DNA binding protein (UV-DDB)





"Hand-off from UV-DDB to the XPC complex at the damaged site."



"Destabilization of chromatin structure"

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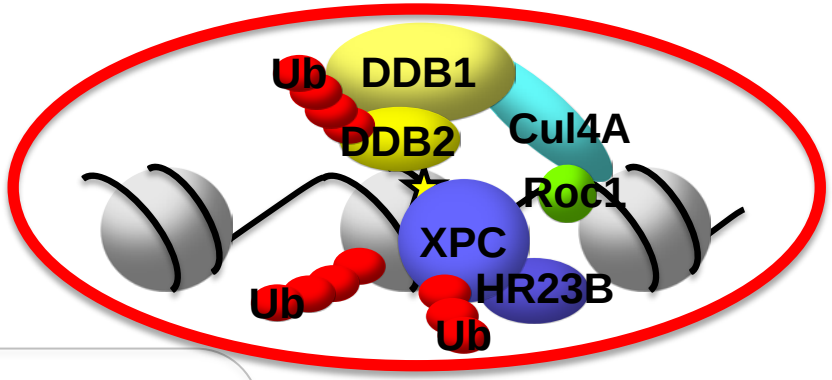
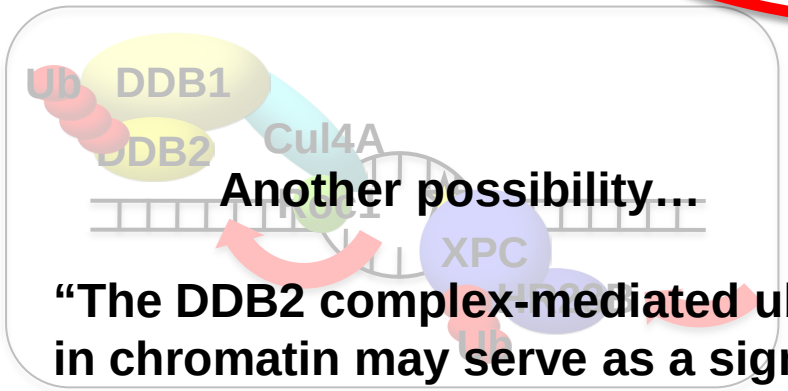
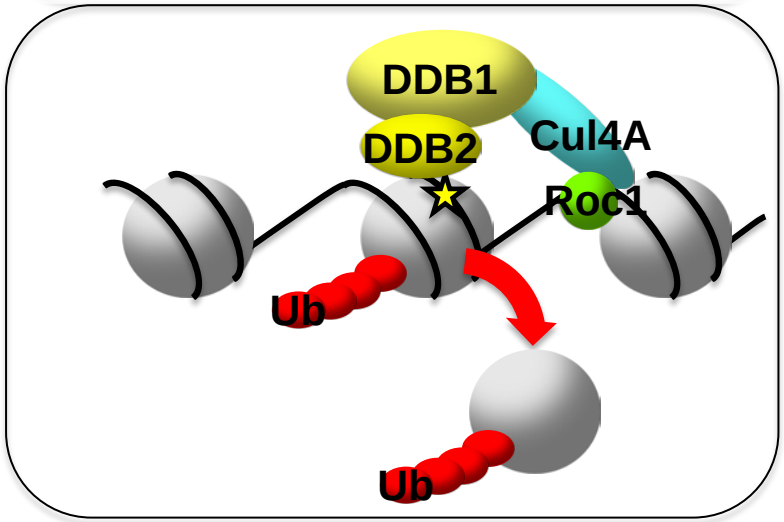




Another possibility…

"Hand-off from UV-DDB to the XPC complex at the damaged site."

"The DDB2 complex-mediated ubiquitylation around damaged sites in chromatin may serve as a signal for the recruitment of other NER factors."



"Destabilization of chromatin structure"

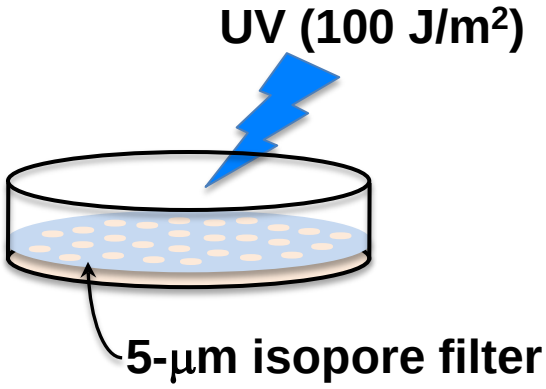
The ectopically expressed wild-type DDB2 is colocalized with endogenous XPA

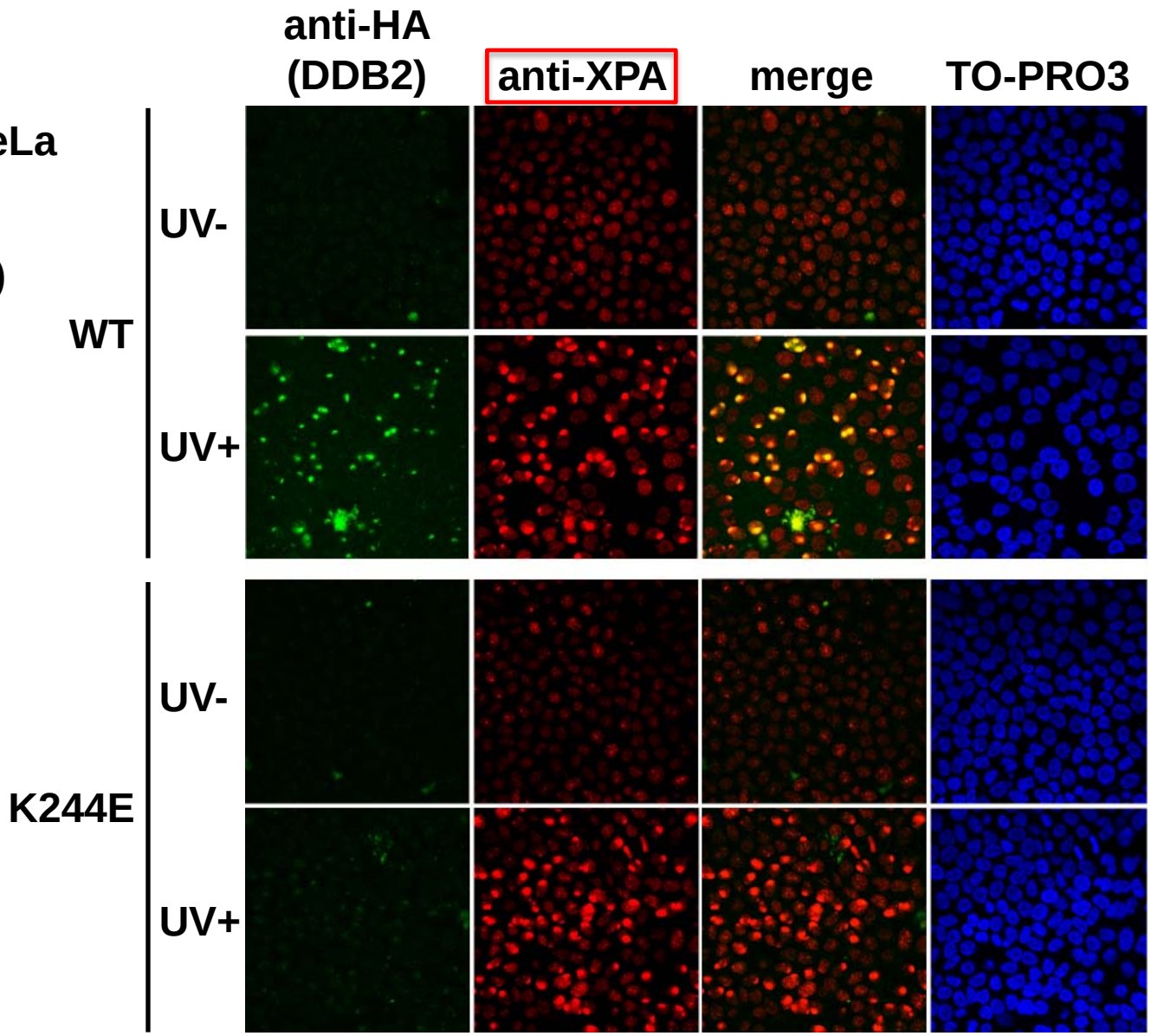
pCAGGS-FLAG-HA-DDB2 | WT / K244E | / HeLa

UV-irradiation (100 J/m^2)
[5-μm isopore filter]

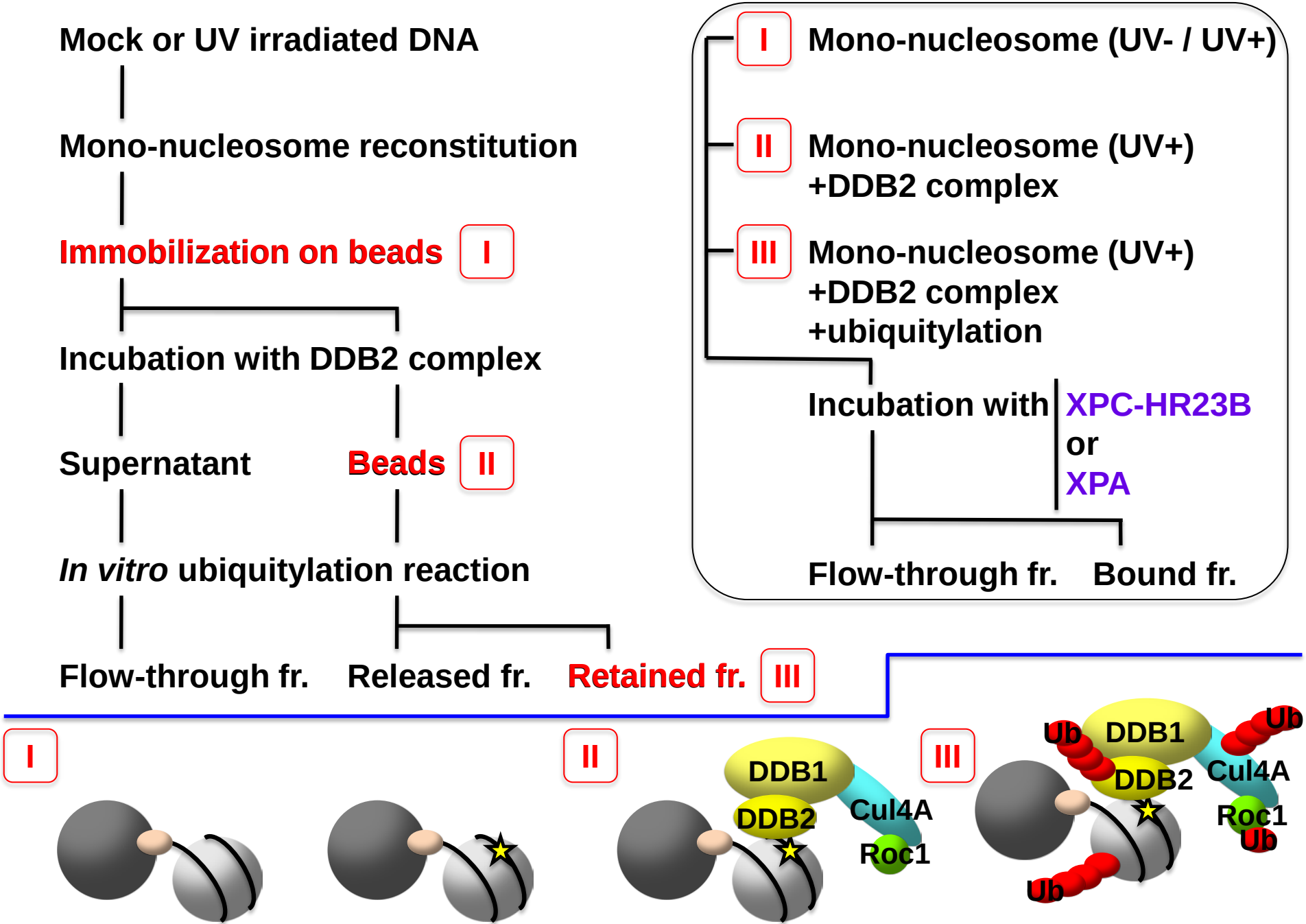
0 min

Immunostaining

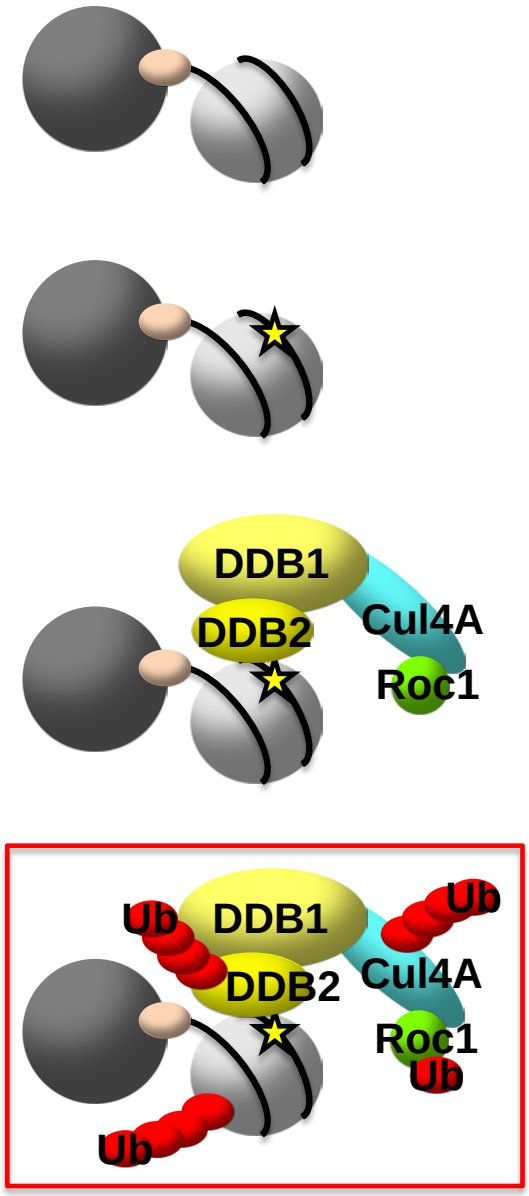


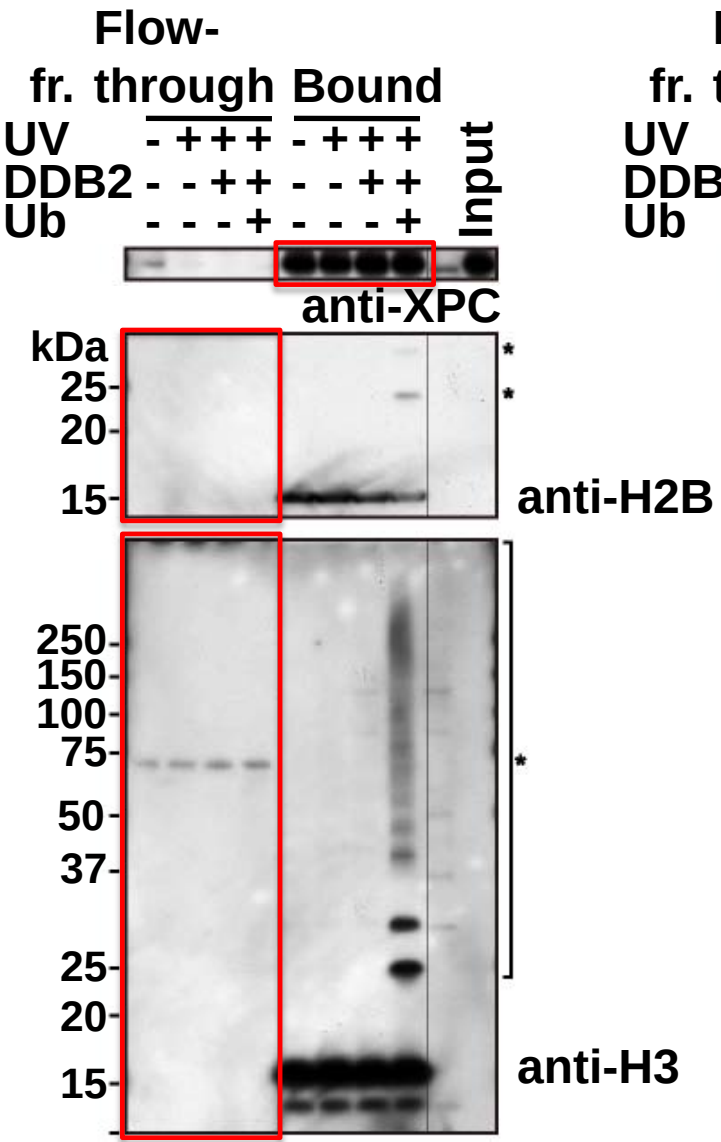


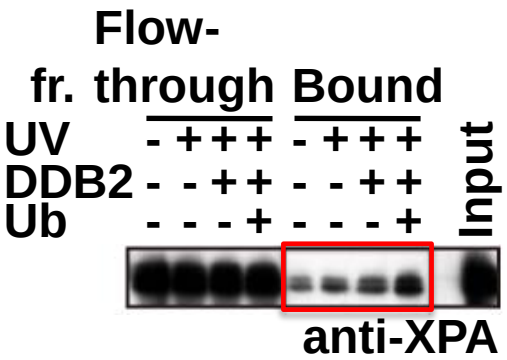
Schematic representation of the strategy



The DDB2 complex-mediated ubiquitylation around damaged sites facilitates the recruitment of XPA







Conclusions

Both DNA-binding and E3 activities of the DDB2 complex are required to promote GGR in chromatin

- ★ **K244E DDB2 was able to form the cullin-based E3 complex. However, this complex did not bind to nucleosomes in the UV-irradiated cells.**
- ★ **The K244E DDB2 complex did not bind to the UV-damaged DNA or the nucleosomes bearing the UV-irradiated DNA, and consequently was not able to mediate the ubiquitylation of the nucleosomes assembled on UV-damaged DNA.**

Implication for the DDB2 complex-mediated ubiquitylation in GGR

- ★ **The DDB2 complex-mediated ubiquitylation around damaged sites in nucleosomes enhanced the recruitment of XPA to lesions.**

  → **The DDB2 complex-mediated ubiquitylation around the damaged sites in chromatin functions in the signaling pathway during the recognition step in GGR.**

Regulatory mechanisms for the E3 activity of the DDB2 complex

The wild-type, but not K244E DDB2 complex interacts with the modified forms of XPC in a UV-dependent manner

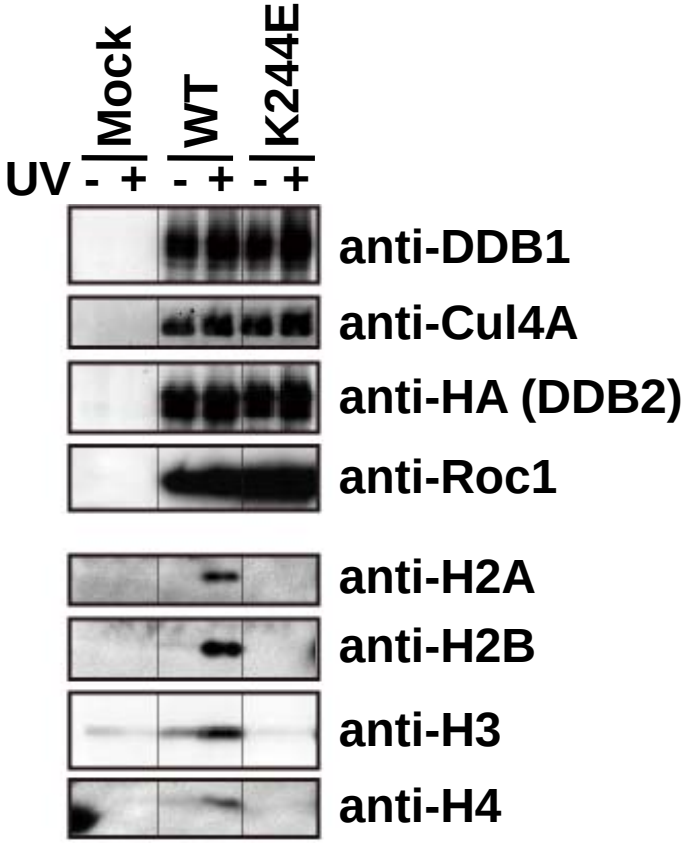
pCAGGS-FLAG-HA-DDB2 | WT / K244E | / HeLa

UV-irradiation (20 J/m^2, 0 min)

Solubilized chromatin fr.

anti-FLAG IP

anti-HA IP



The wild-type, but not K244E DDB2 complex interacts with Ku in a UV-dependent manner

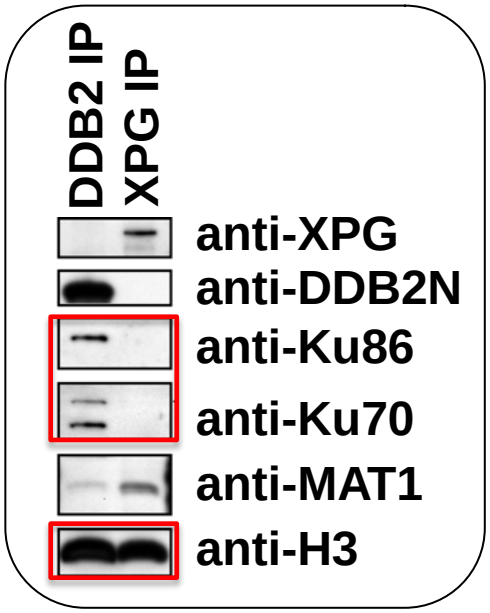
pCAGGS-FLAG-HA-DDB2 | WT / K244E | / HeLa

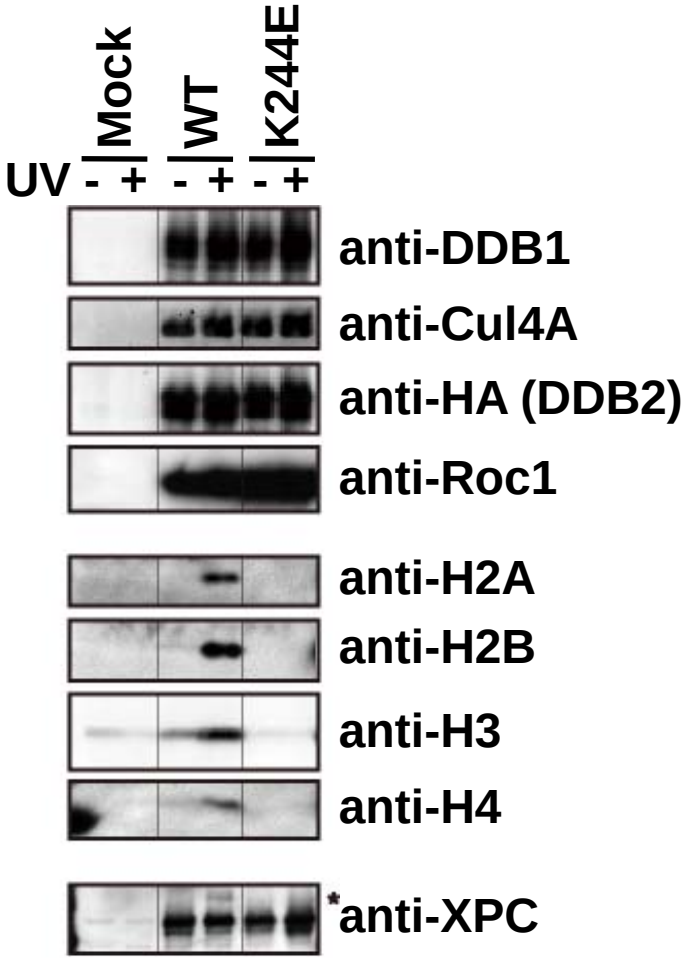
UV-irradiation (20 J/m^2, 0 min)

Solubilized chromatin fr.

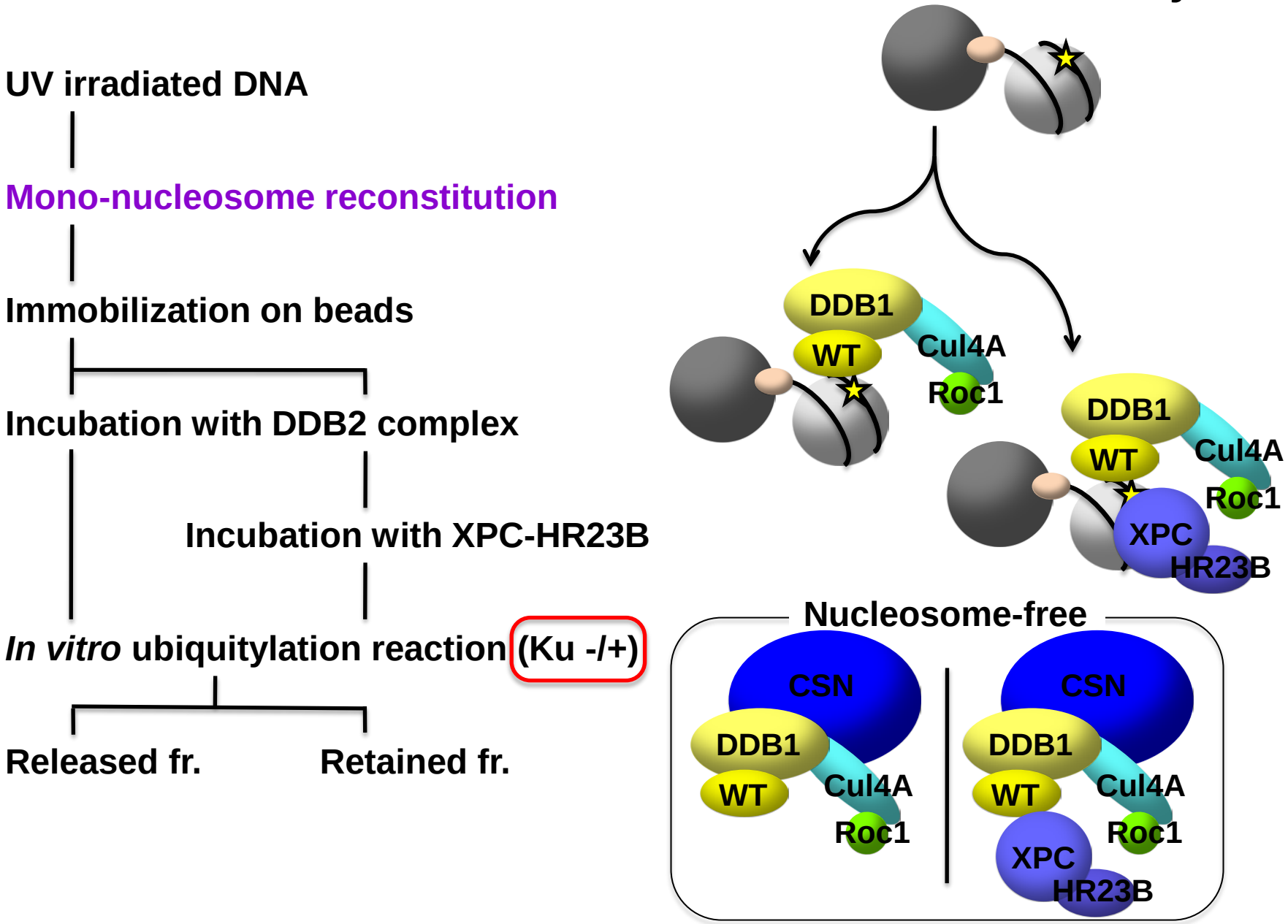
anti-FLAG IP

anti-HA IP

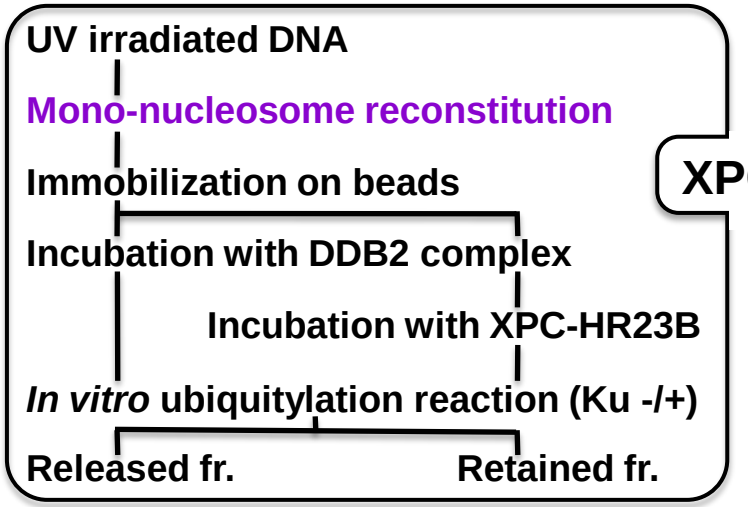


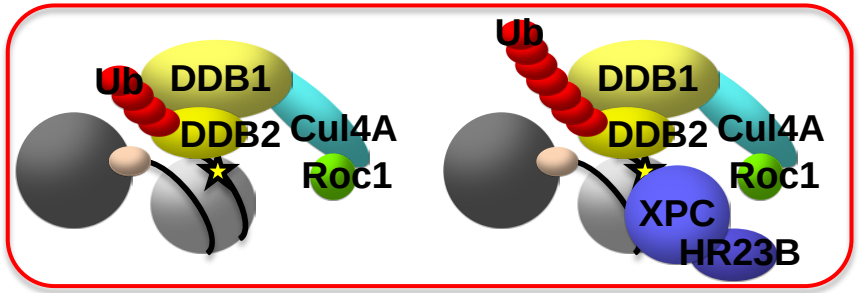


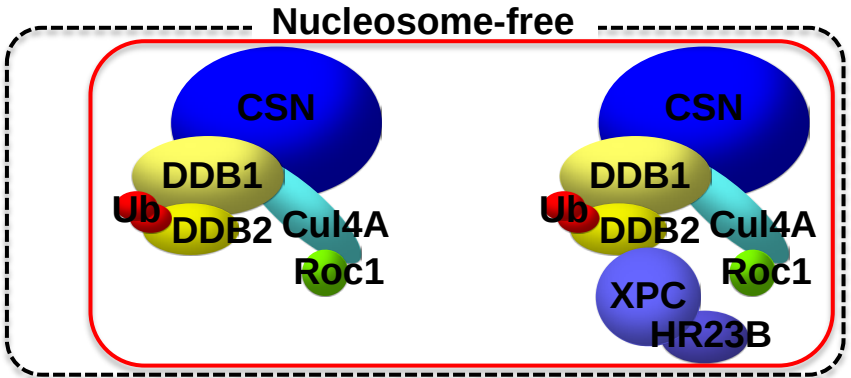
Schematic representation of the strategy to measure the effect of XPC/HR23B or Ku on the DDB2 E3 activity

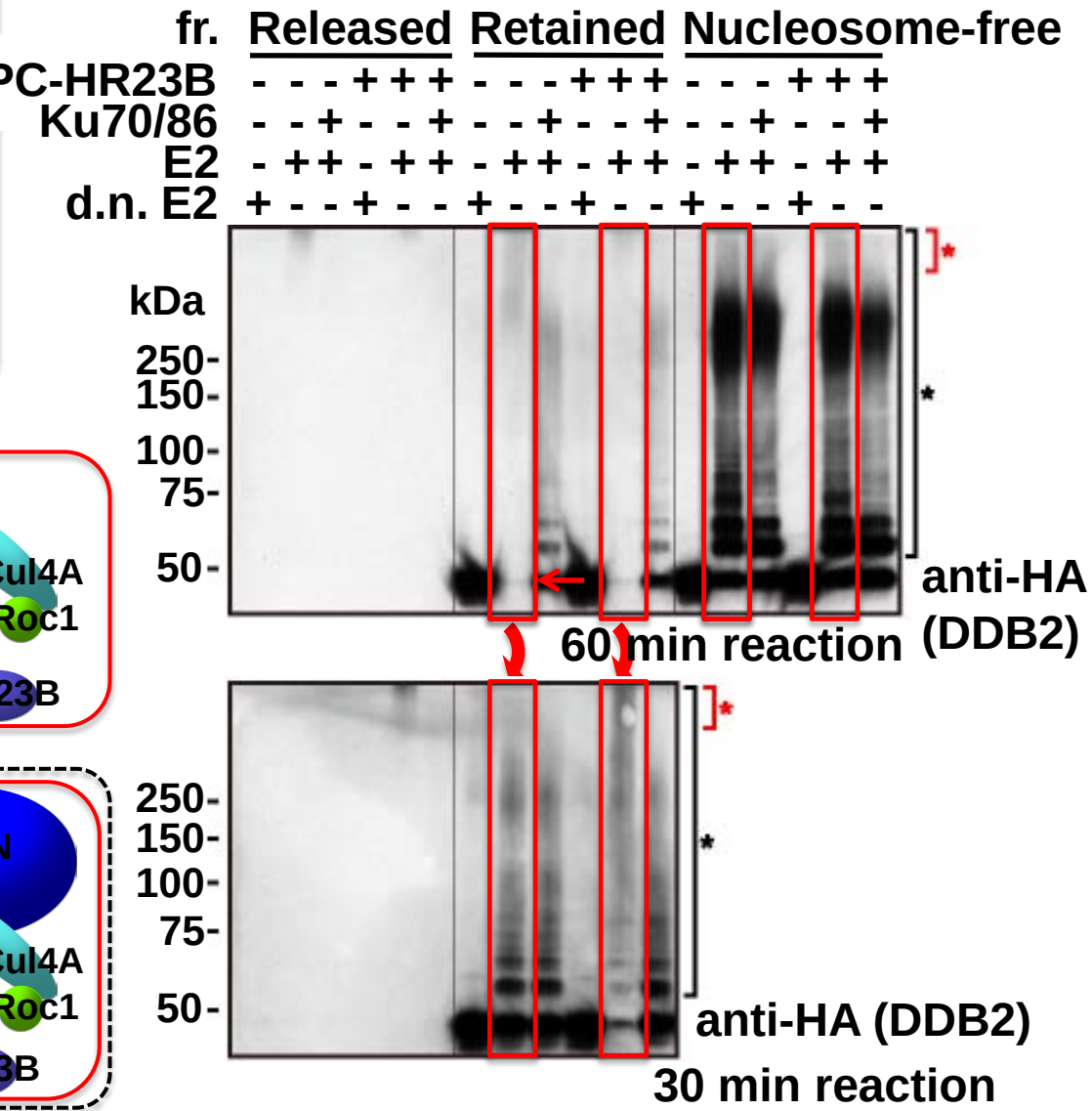


XPC stimulates the E3 activity of the DDB2 complex especially at damaged sites

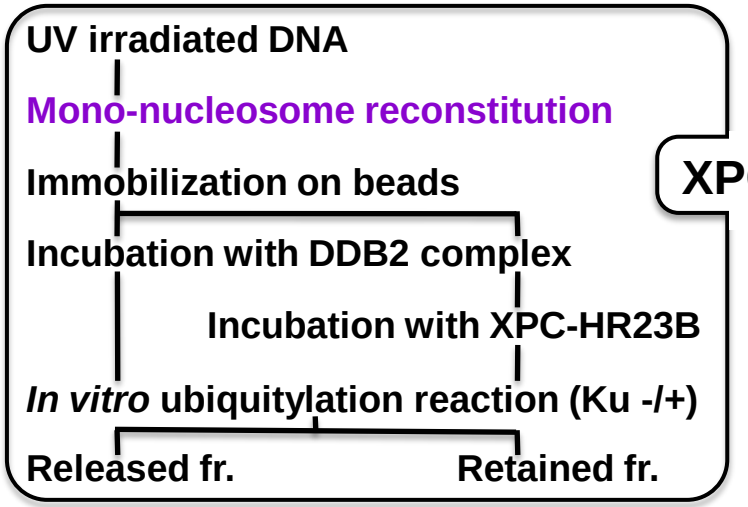


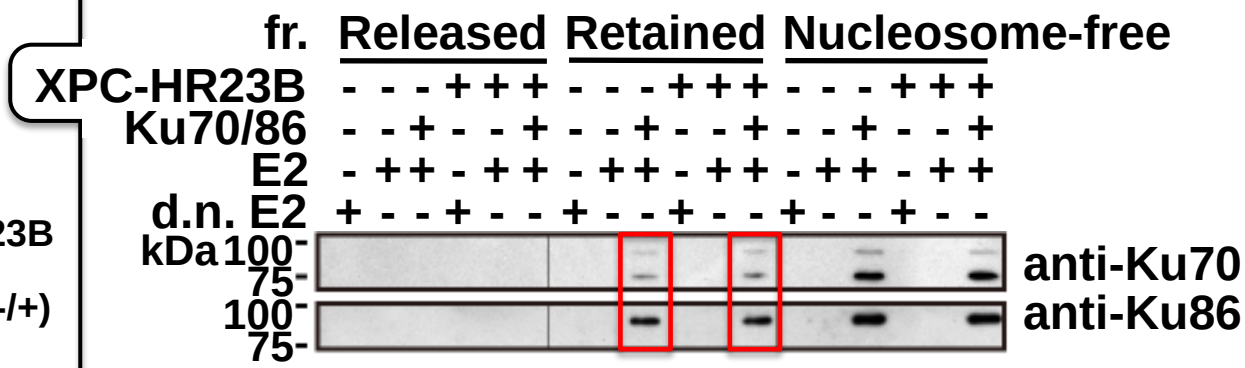


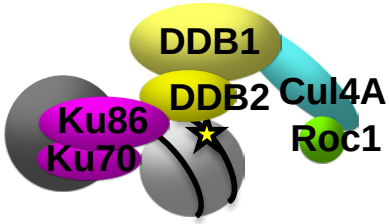


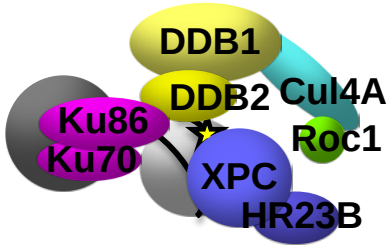


Ku inhibits the E3 activity of the DDB2 complex

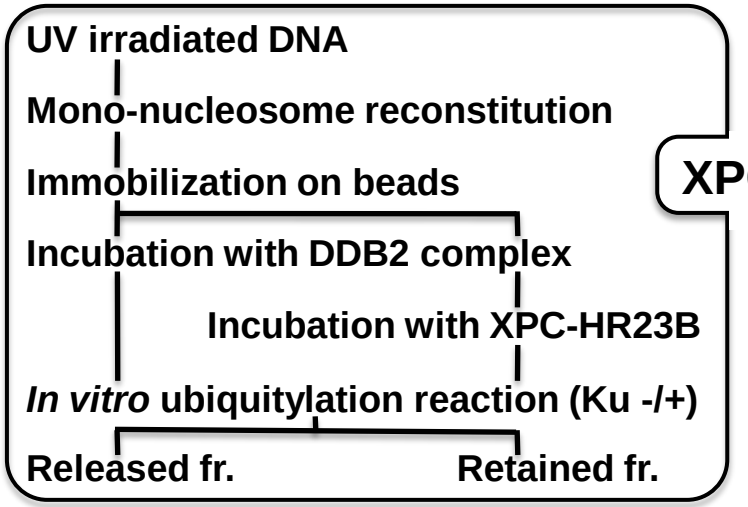


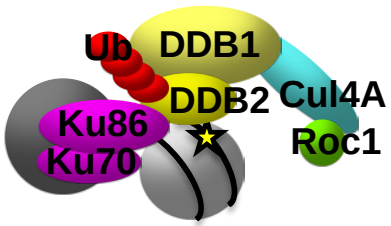


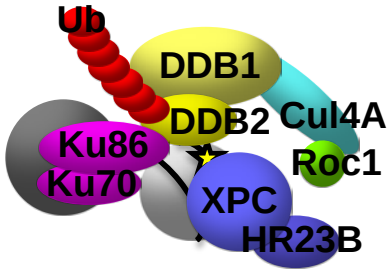


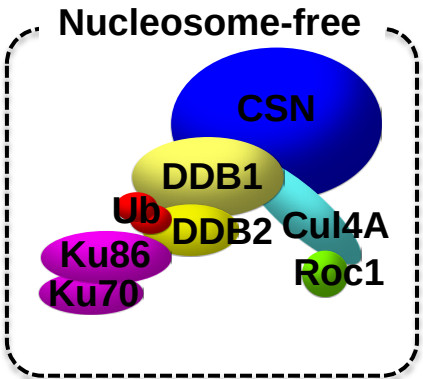


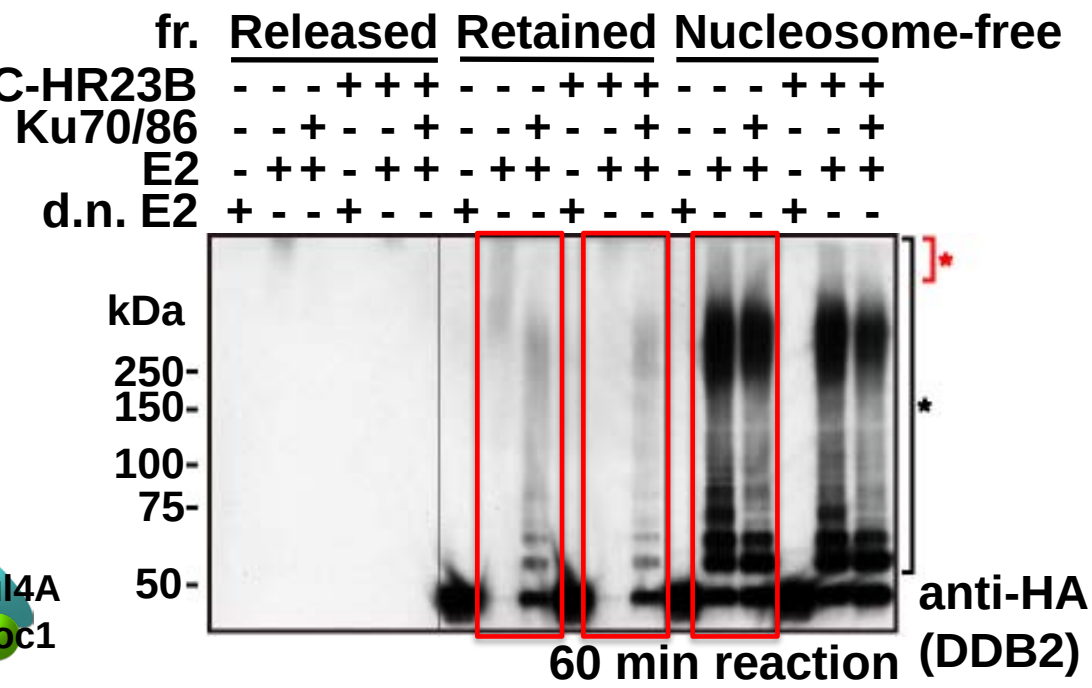
Ku inhibits the E3 activity of the DDB2 complex











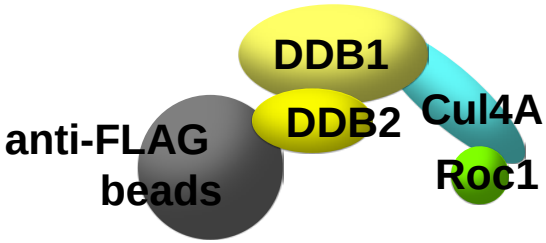
Ku physically interacts with the DDB2 complex and acts as a negative regulator for the E3 activity

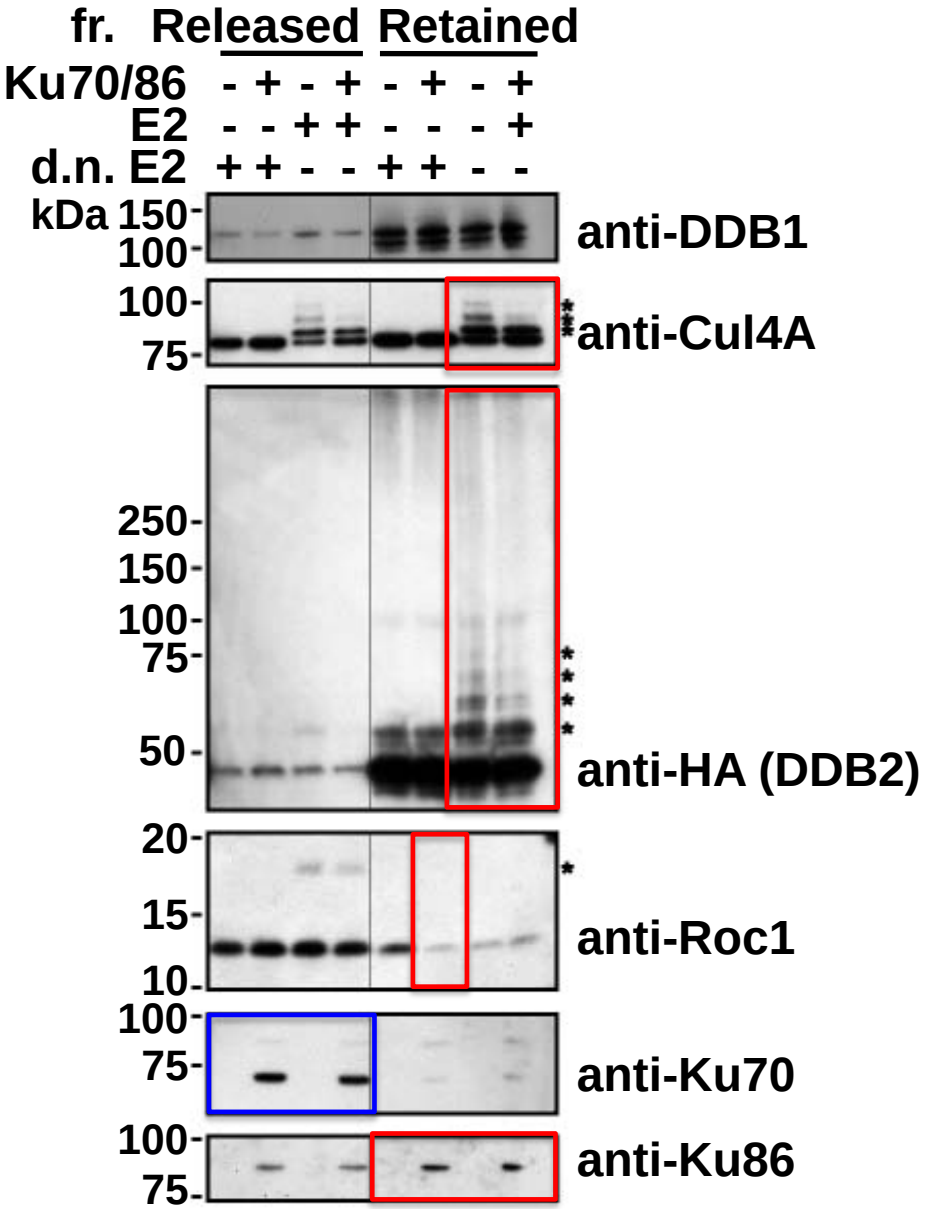
The DDB2 complex (FLAG-HA-DDB2 WT)

Immobilization on anti-FLAG beads

In vitro ubiquitylation reaction (Ku- / +)

Released fr. Retained fr.





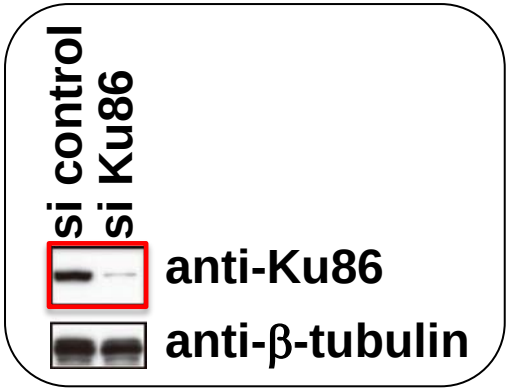
Ku affects the localization of DDB2 to the damaged sites *in vivo*

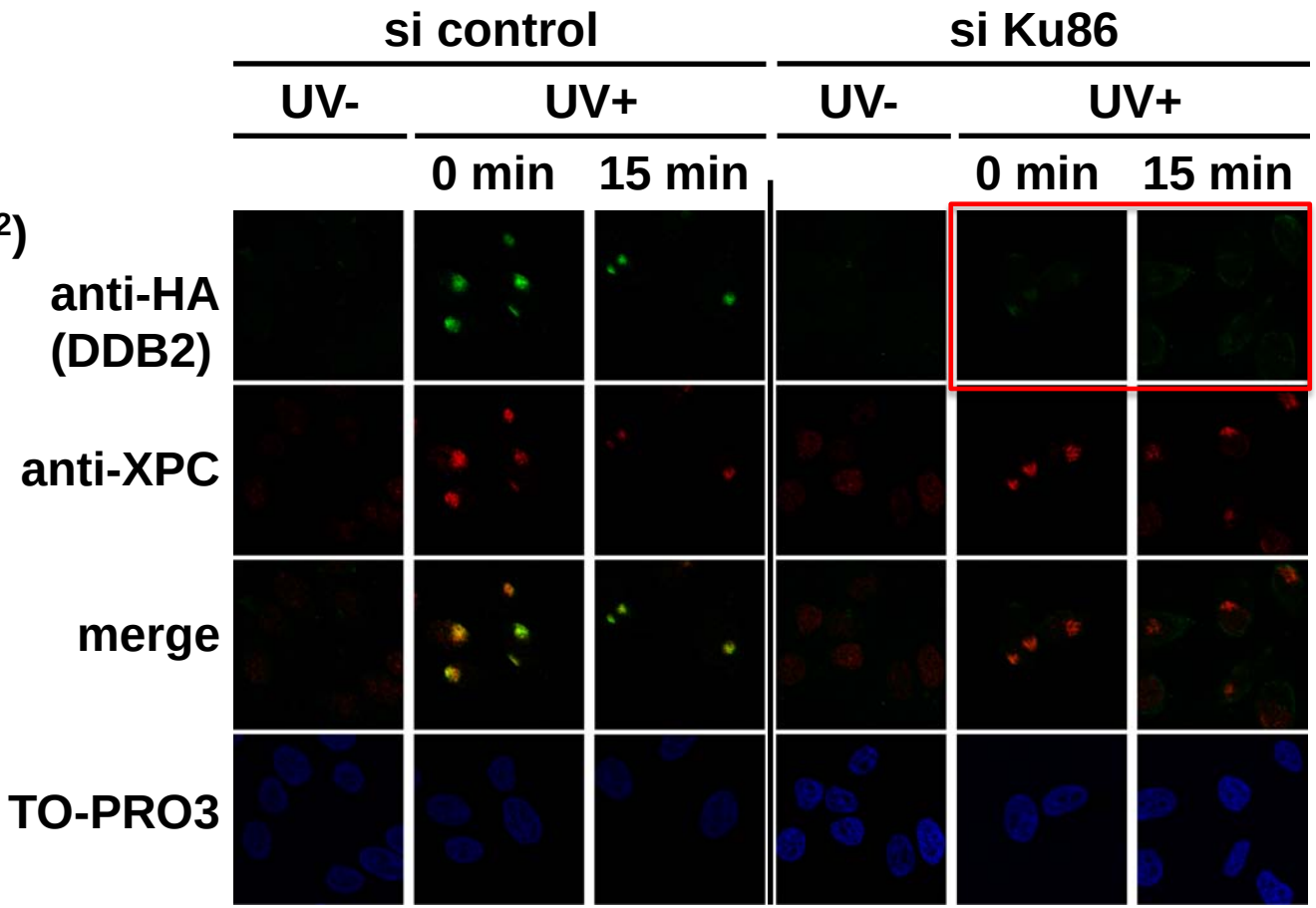
pCAGGS-FLAG-HA-DDB2 / HeLa

Transfection of siRNAs

UV-irradiation (100 J/m^2)
[5-μm isopore filter]

Immunostaining





Conclusions

Both DNA-binding and E3 activities of the DDB2 complex are required to promote GGR in chromatin

- ★ K244E DDB2 was able to form the cullin-based E3 complex. However, this complex did not bind to nucleosomes in the UV-irradiated cells.
- ★ The K244E DDB2 complex did not bind to the UV-damaged DNA or the nucleosomes bearing the UV-irradiated DNA, and consequently was not able to mediate the ubiquitylation of the nucleosomes assembled on UV-damaged DNA.

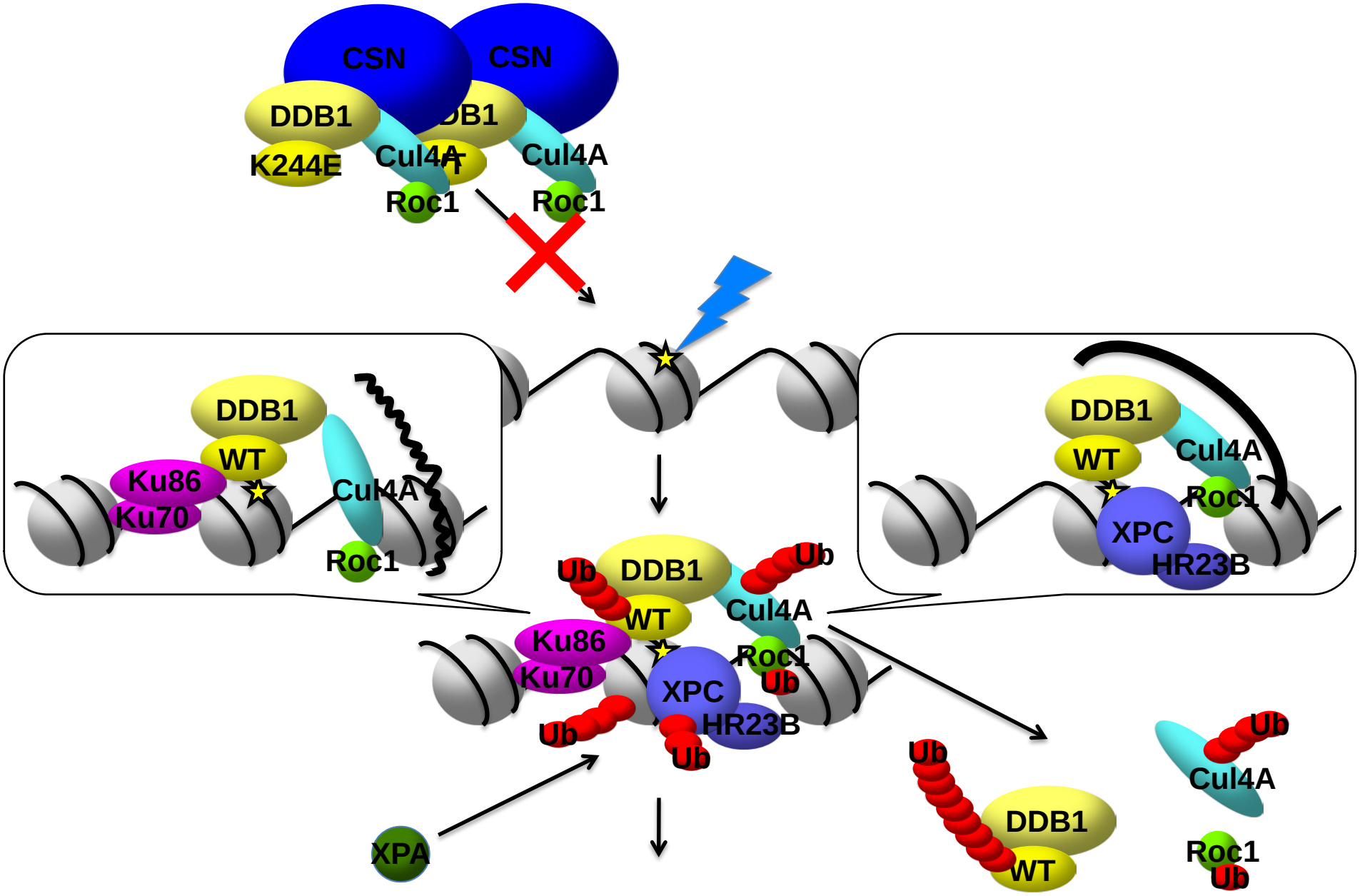
Implication for the DDB2 complex-mediated ubiquitylation in GG-NER

- ★ The DDB2 complex-mediated ubiquitylation around damaged sites in nucleosomes enhanced the recruitment of XPA to lesions.
  - ⟶ The DDB2 complex-mediated ubiquitylation around the damaged sites in chromatin functions in the signaling pathway during the recognition step in GGR.

Various regulatory mechanisms for the E3 activity of the DDB2 complex

- ★ CSN is released from the DDB2 complex when the complex binds to lesions. Correspondingly, the E3 activity of the DDB2 complex was stimulated.
- ★ XPC stimulated the E3 activity of the DDB2 complex especially at damaged sites by stabilizing it.
- ★ Ku negatively regulates the E3 activity of the DDB2 complex by destabilizing it.
  - ⟶ Ku particularly inhibits the extensive autoubiquitylation of DDB2, and consequently prevents the dissociation of the DDB2 complex from damaged sites.

Model for the mechanism of damage recognition in GGR



Topics:

The DDB2 complex-mediated ubiquitylation around DNA damage is oppositely regulated by XPC and Ku, and contributes to the recruitment of XPA

MMXD, a TFIIH-independent XPD-MMS19 protein complex involved in chromosome segregation

MMXD, a TFIIH-independent XPD-MMS19 protein complex involved in chromosome segregation

Shinsuke Ito
Li Jing Tan
Daisuke Andoh
Takashi Narita
Mineaki Seki

Ito S et al., *Mol Cell*, 2010

Genetic Complementation groups in XP, CS and TTD

Groups	Disorders	NER activity			Gene
		UV-sensitivity	GGR	TCR	
XP-A	XP	+ + +	—	—	XPA
XP-B	XP/CS, TTD	+ +	—	—	XPB(ERCC 3)
XP-C	XP	+	—	+	XPC
XP-D	XP, XP/CS, TTD	+ +	—	—	XPD(ERCC 2)
XP-E	XP	±	—	—	XPE(DDB 2
XP-F	XP	+	—	—	XPF
XP-G	XP, XP/CS	+ +	—	—	XPG(ERCC 5)
CS-A	CS	+	+	—	CSA(ERCC 8)
CS-B	CS	+	+	—	CSB(ERCC 6)
TTD-A	TTD	+	—	—	TTDA(p8)

Multi-functions of XPD protein

- is one of the components of TFIIH, which has roles in nucleotide excision repair, basal transcription, transactivation and possibly cell cycle control.

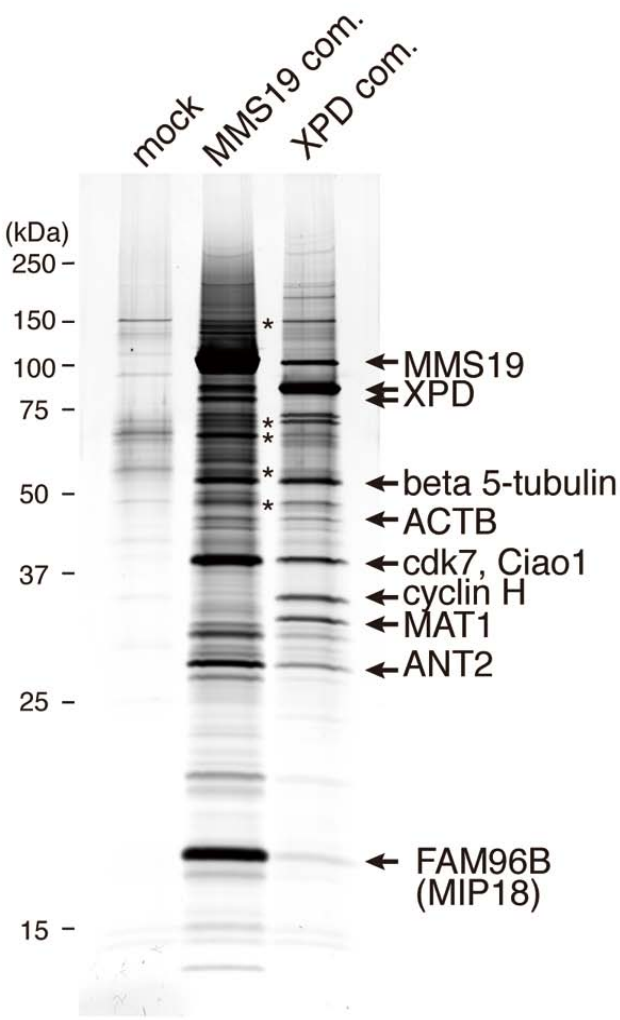
- has a 5' – 3' DNA helicase and ATPase activities essential for nucleotide excision repair but dispensable for transcription. (F Coin et al., *Mol Cell* 26: 245, 2007)

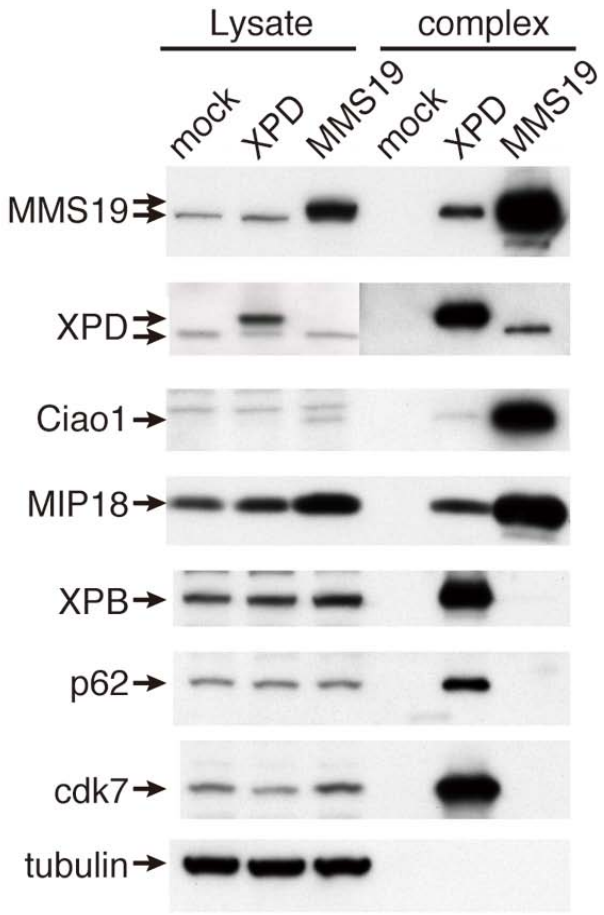
- directly interacts with p44 and MAT1, serves as molecular bridge of holo-TFIIH, and facilitate optimal transcription. (S Dubaele et al., *Nat Genet* 20: 184, 1998; P Schultz et al., *Cell* 102: 599, 2000; B Sandrock et al., *JBC* 276: 35328, 2001; F Coin et al, *Mol Cell* 11: 1635, 2003)

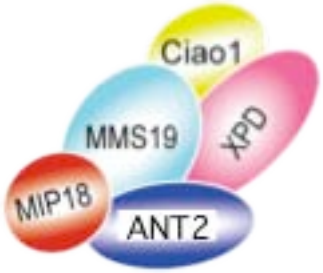
- a fraction of XPD is associated with CAK but not with the core TFIIH. (JT Reardon et al., *Proc Natl Acad Sci USA* 93: 6482, 1996)

-*Drosophila* Xpd was shown to negatively regulate CAK activity, resulting in decreased phosphorylation of the cdk1 T-loop and inhibition of mitotic progression, while Xpd expression was down-regulated at G2/M when cdk1 was most active (J Chen et al., *Nature* 424: 228, 2003)
Lack of *Drosophila* Xpd also cause defects in the dynamics of mitotic spindle and chromosomal instability, subcellular re-localizing of Cdk7 (X Li et al., *Plos Genetics* 6: e1000876, 2010)

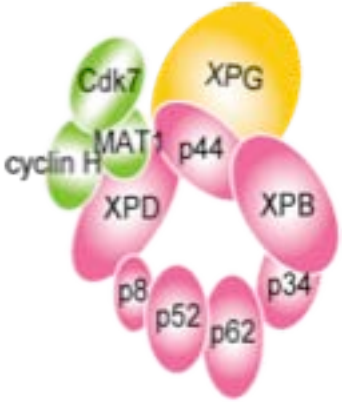
MMS19 and XPD complexes





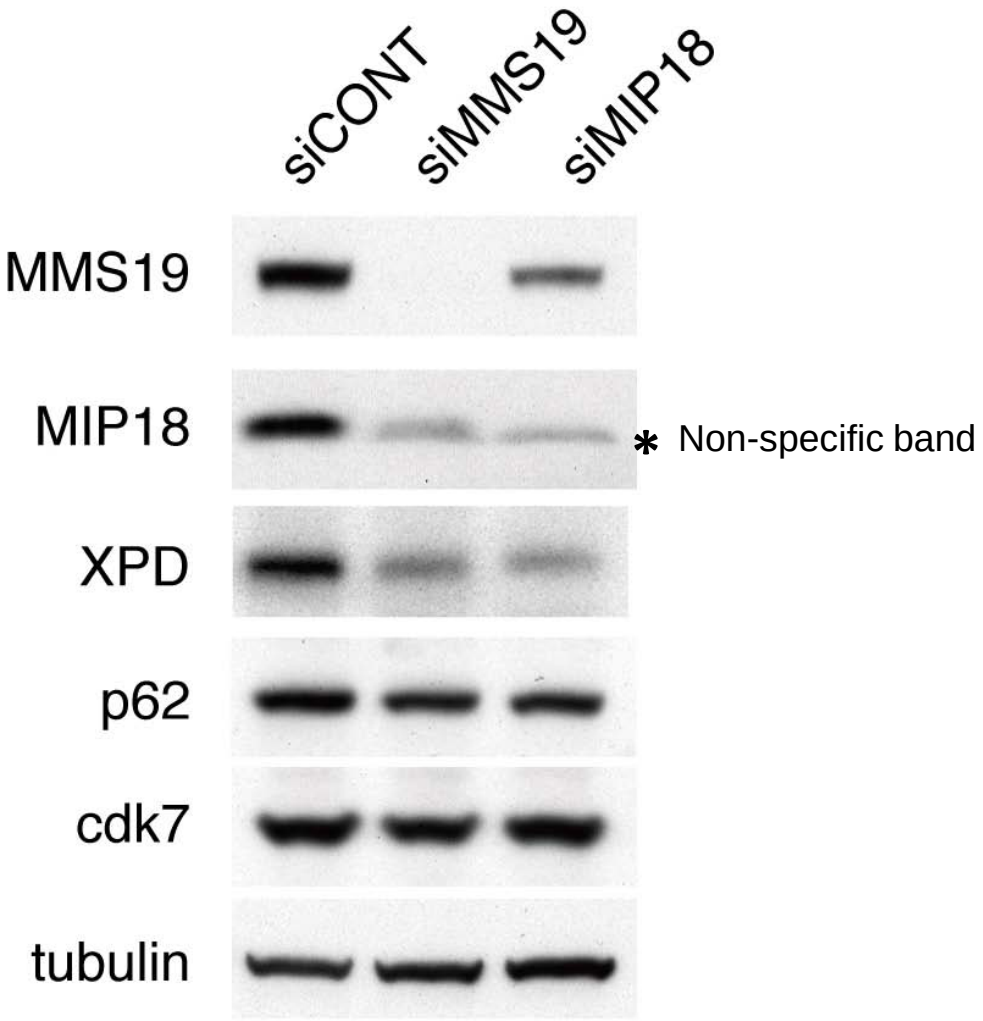


MMS19 complex (MMXD)

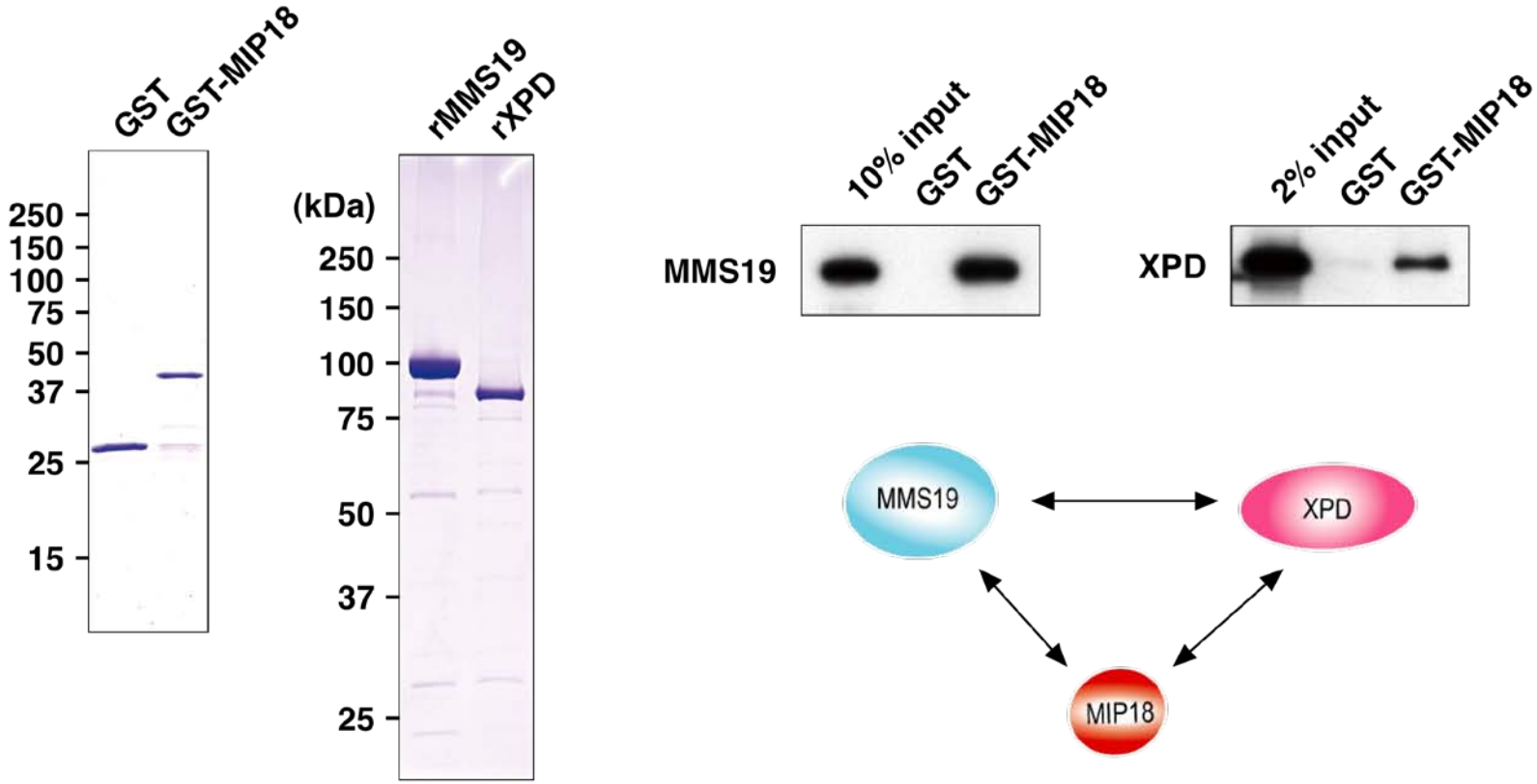


XPD complex (XPG-TFIIH)

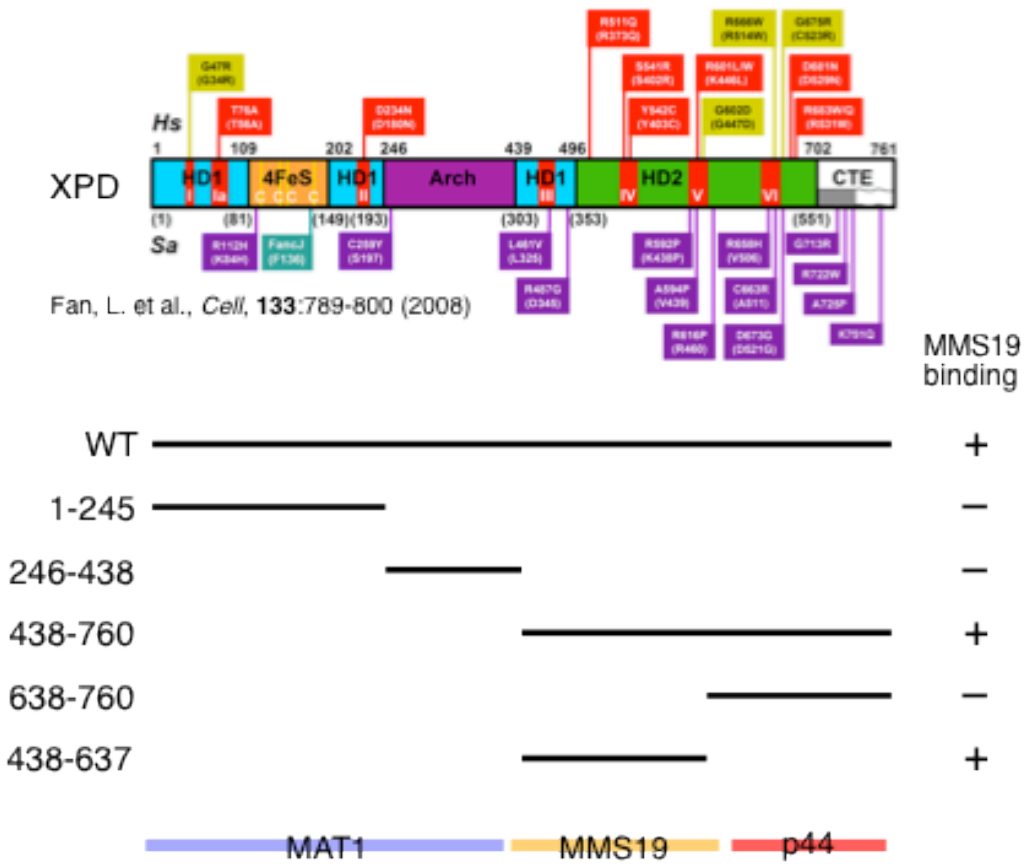
Knockdown of MMS19 and MIP18 in HeLa cells



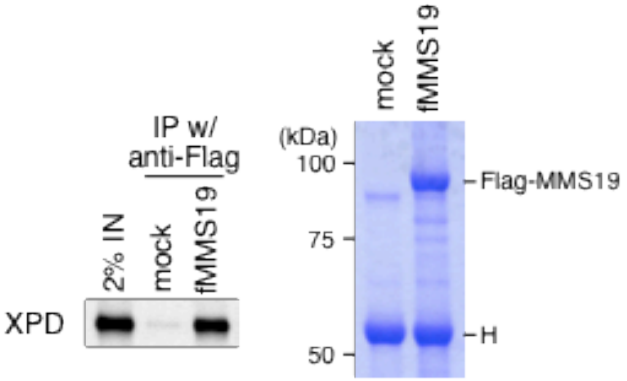
MIP18 can directly interact with MMS19 and XPD

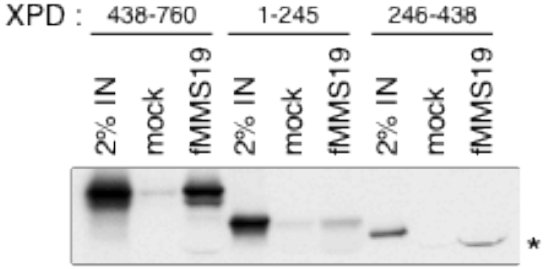


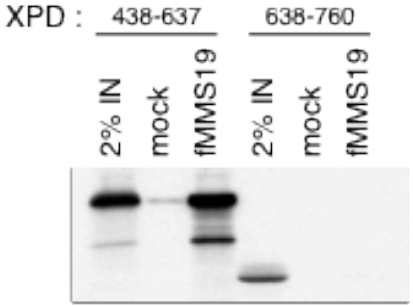
MMS19 interacts with XPD in the region between the MAT1- and p44-binding domains (aa 438–637)



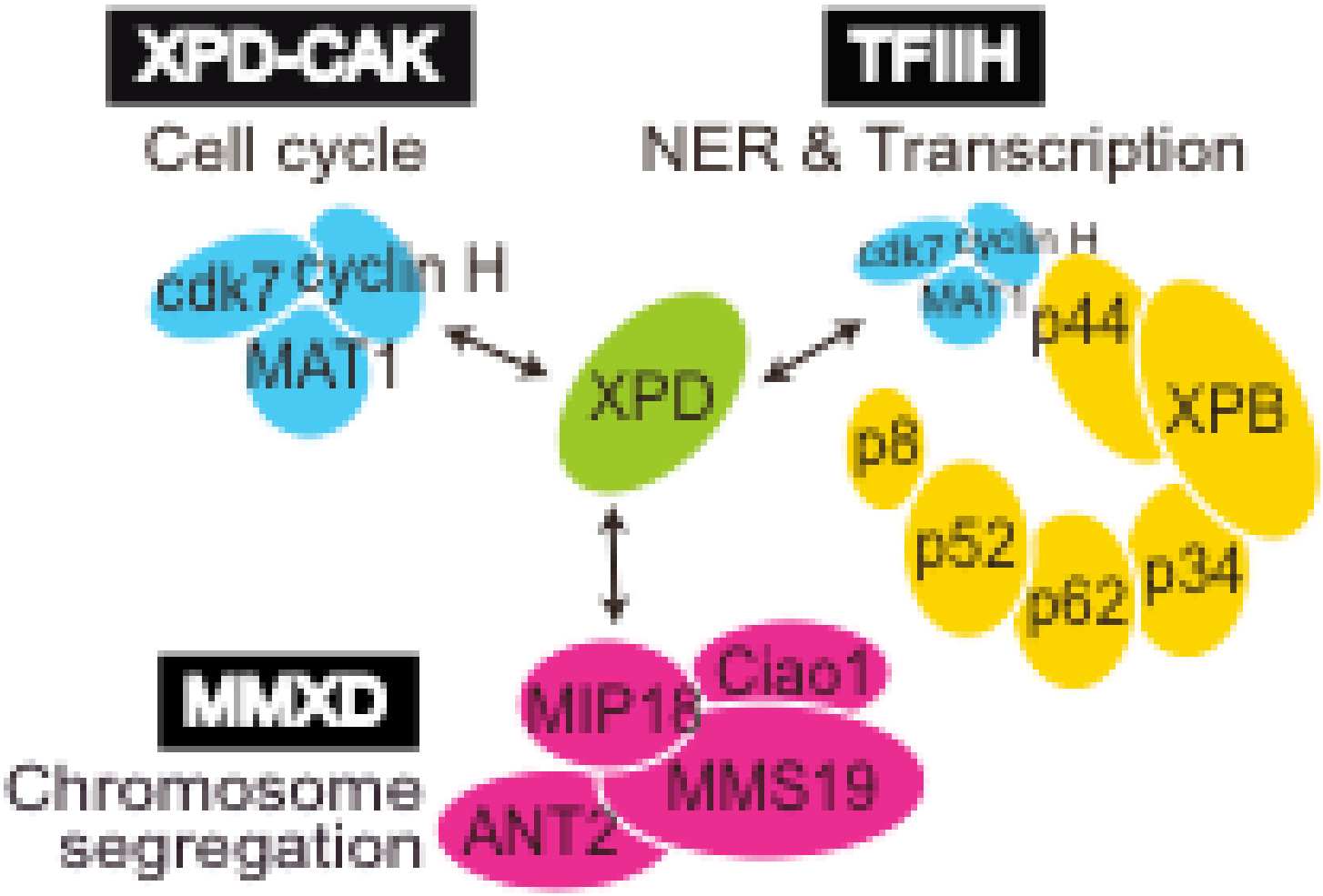
Fan, L. et al., *Cell*, **133**:789-800 (2008)



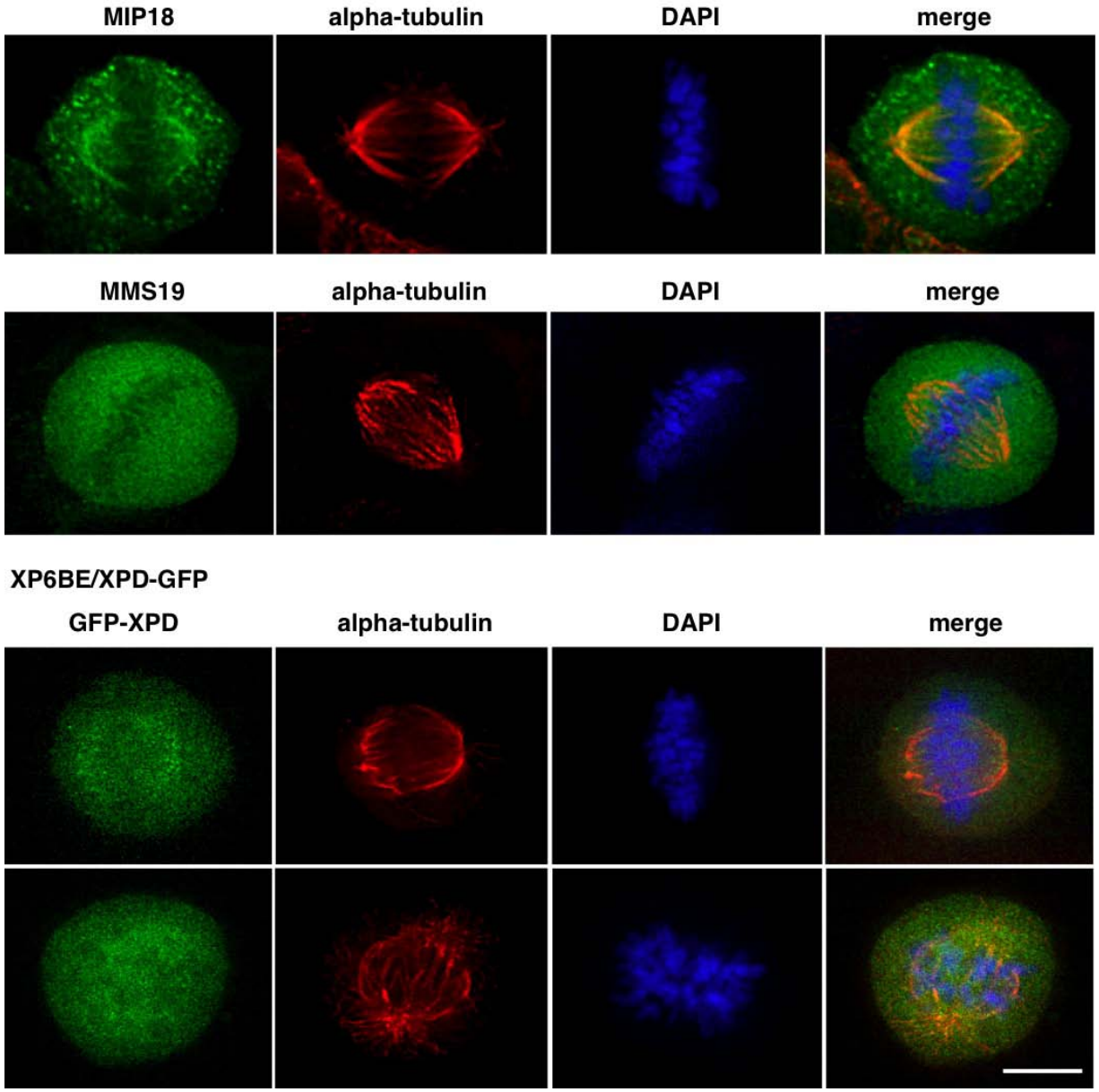




MMXD (*M*MS19-*M*IP18-*X*P*D*) complex

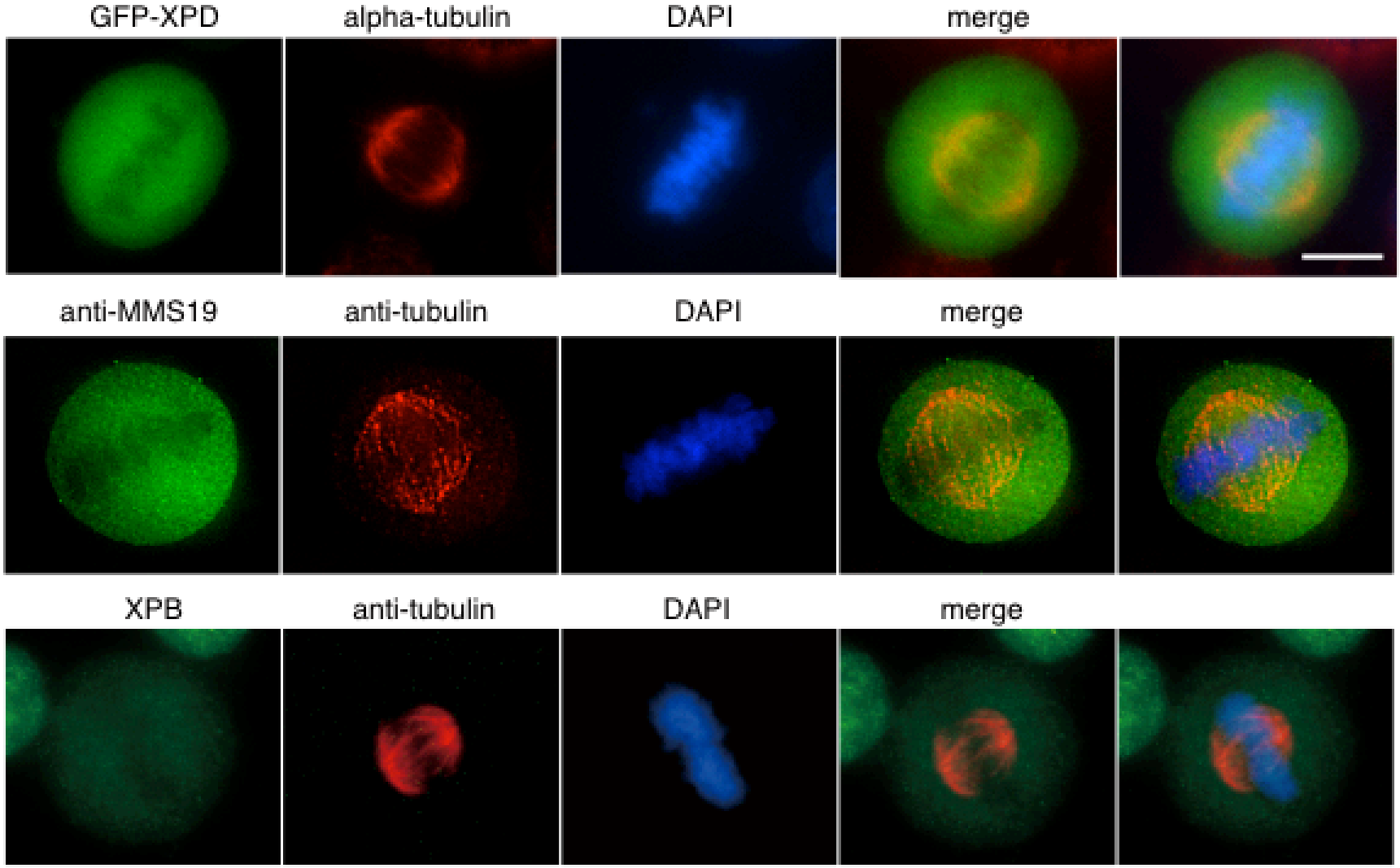


Co-localization of MIP18, MMS19 and GFP-XPD with alpha-tubulin in the mitotic phase of HCT116 cells



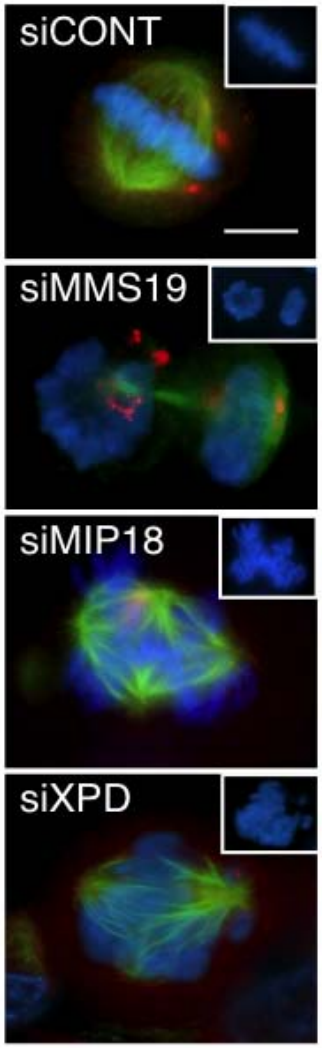
Scale bar: 10 μm

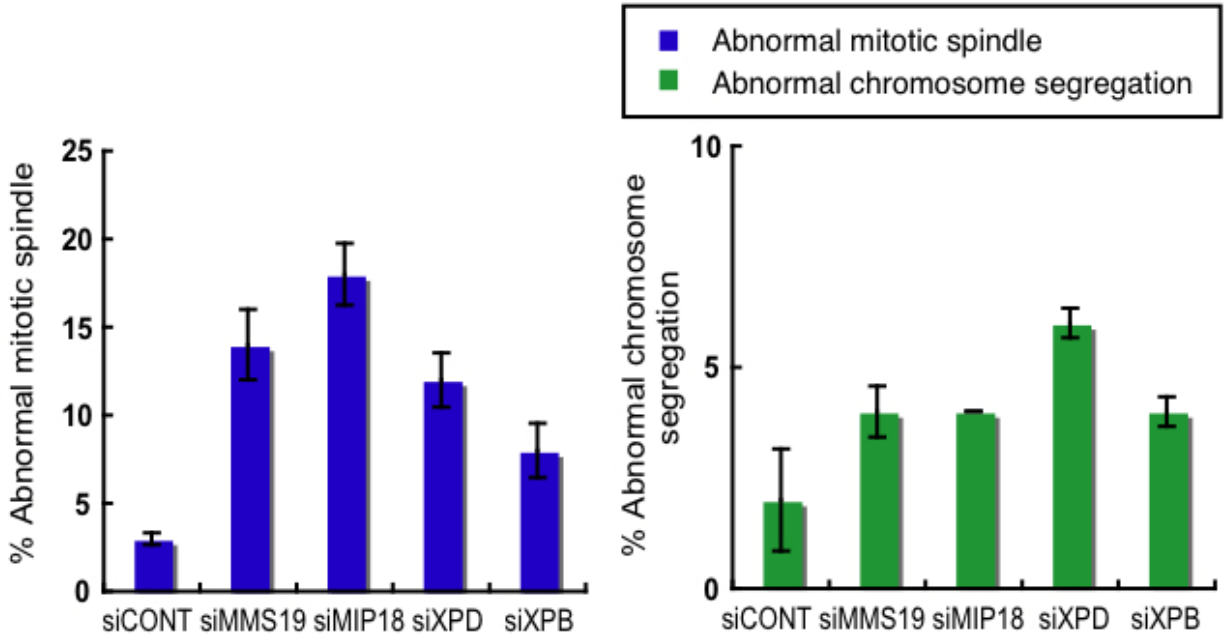
Co-localization of GFP-XPD and MMS19 with alpha-tubulin, but not XPB in the mitotic phase of HCT116 cells



scale bar: 10μm

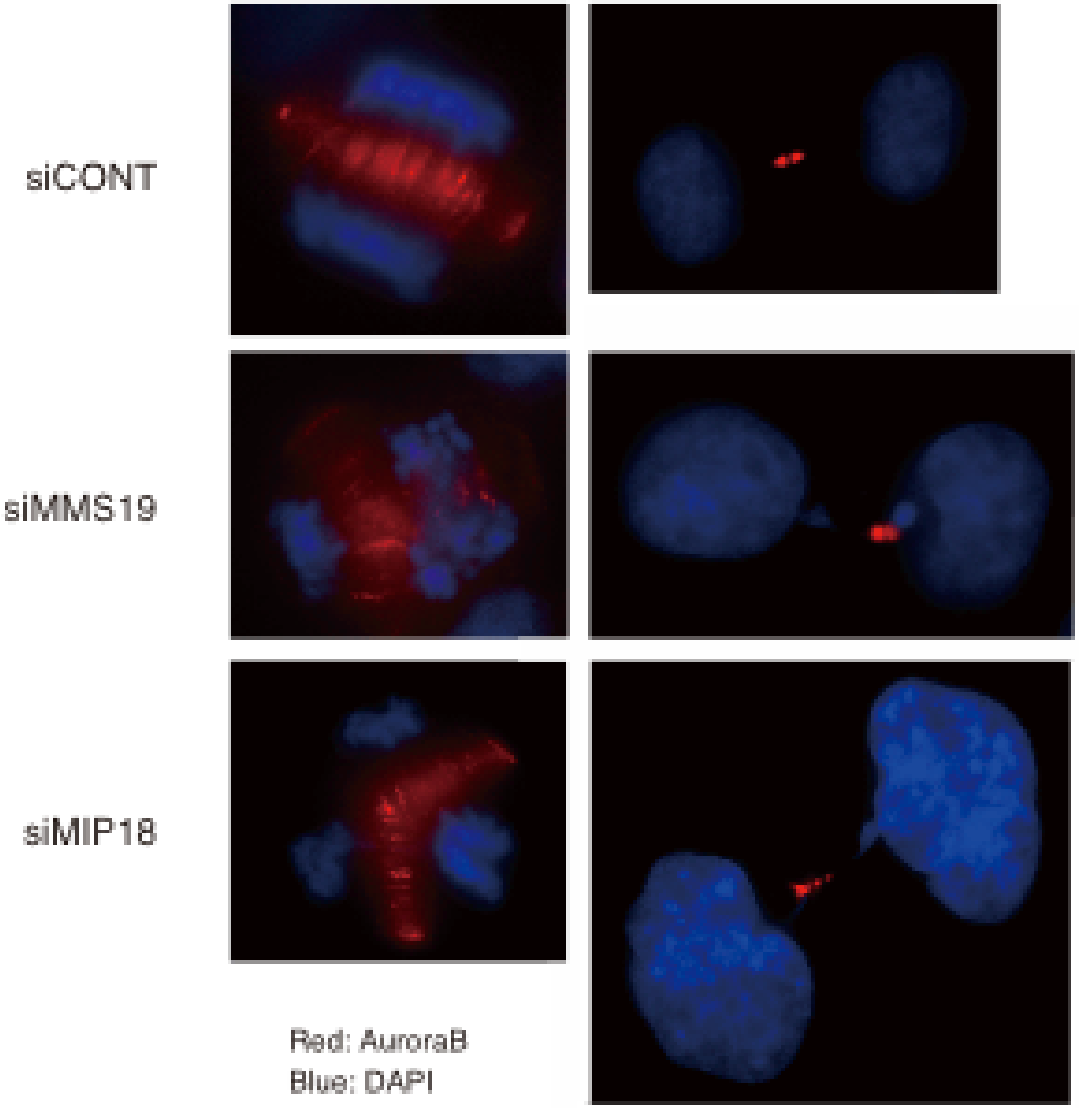
Enhanced frequency of abnormal mitotic spindle and chromosome segregation in MMS19-, MIP18-, and XPD-knockdown HCT116 cells



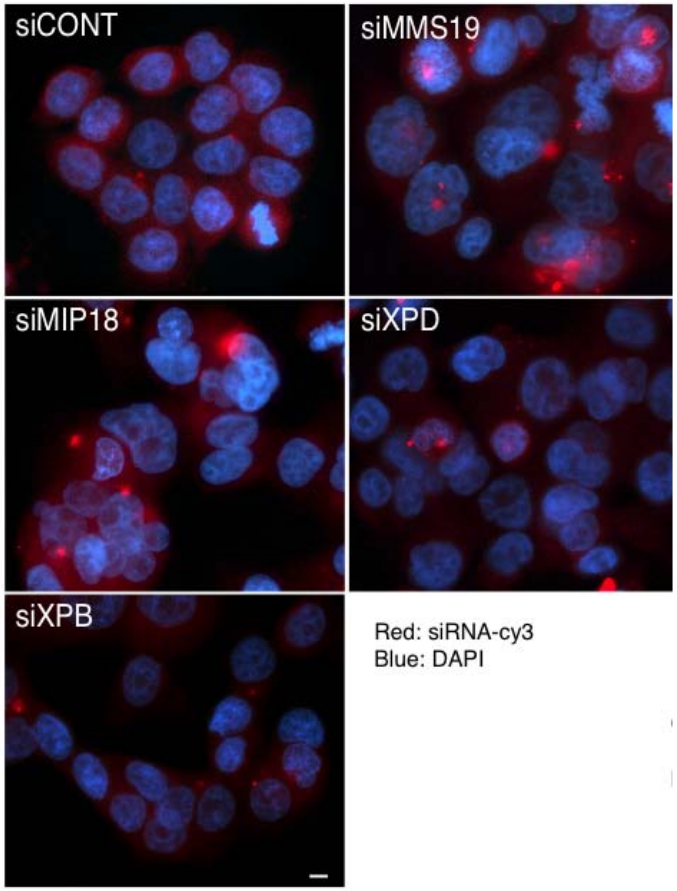


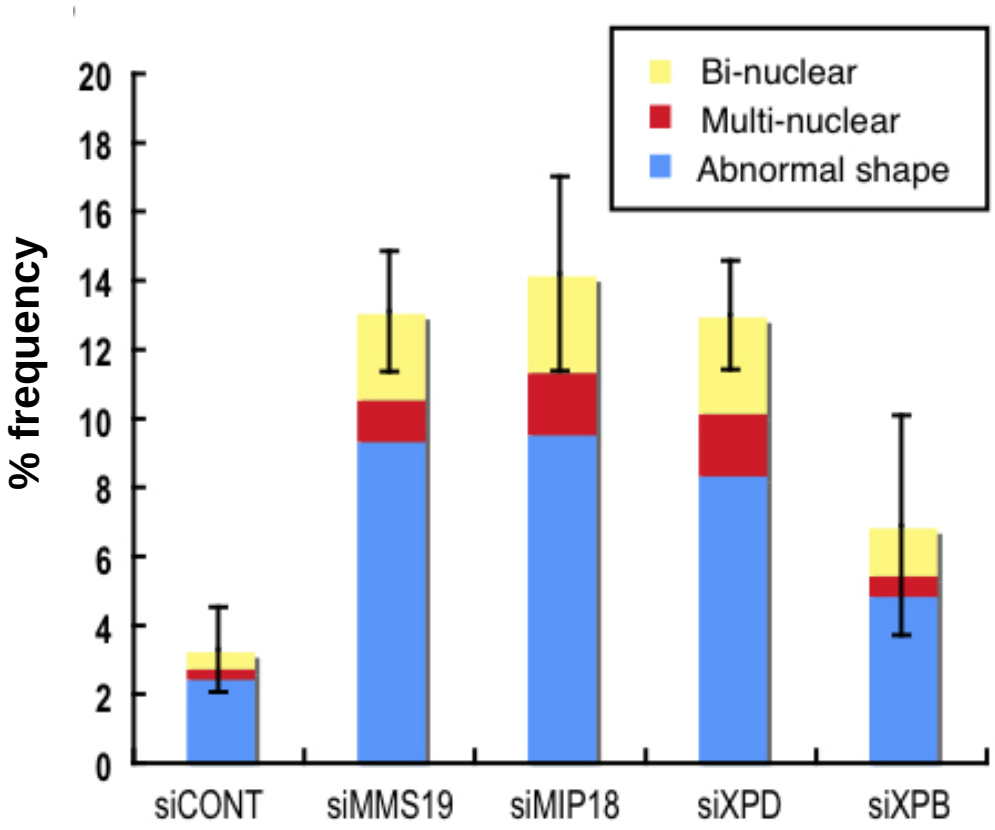
Blue: DAPI (chromosome)
Green: alpha-tubulin
Red:cy3-prelabeled siRNA

Abnormal localization of Aurora B in the MMS19- and MIP18-knockdown HeLa cells

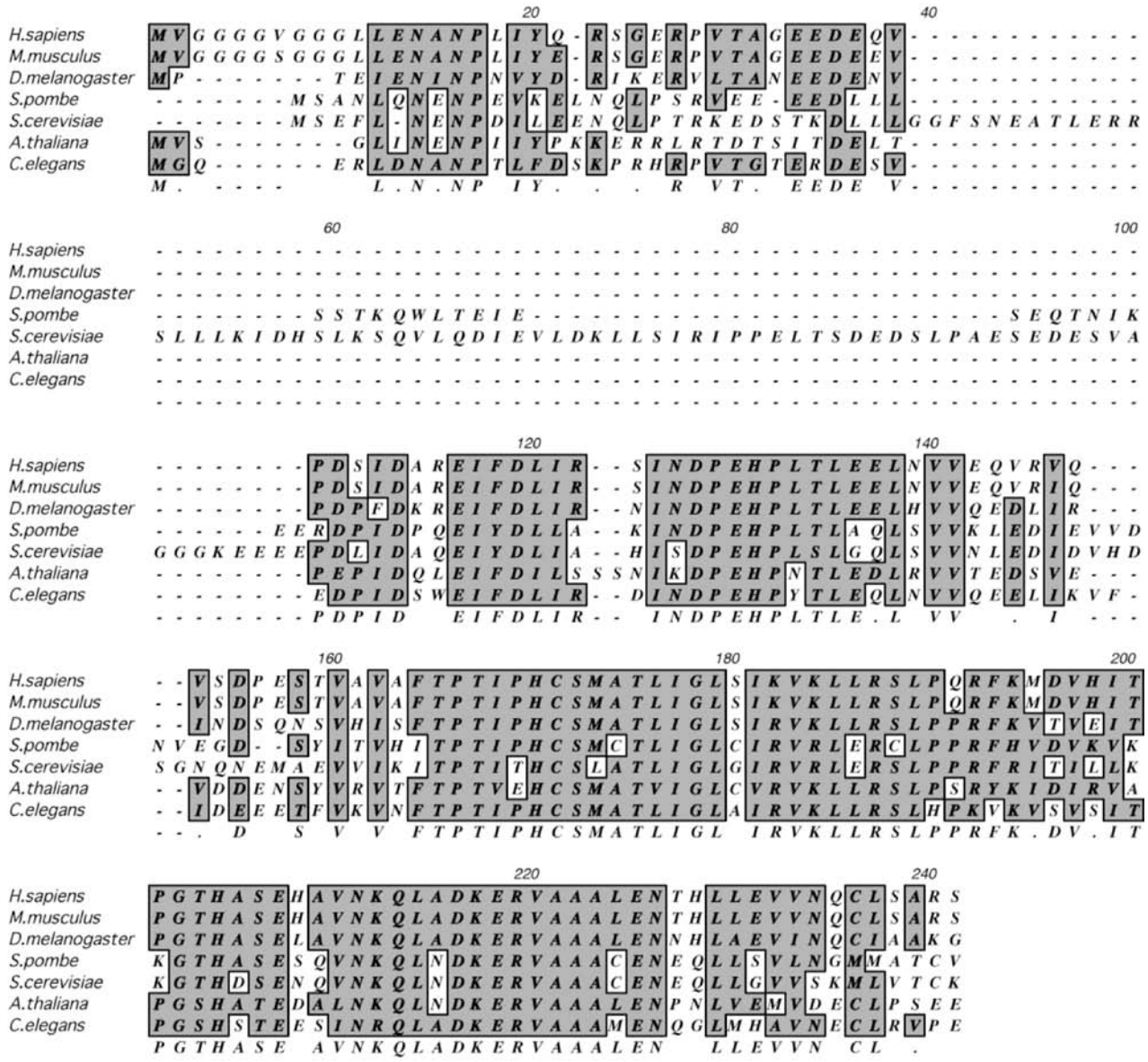


Enhanced frequency of abnormal nuclei in MMS19-, MIP18-, and XPD-knockdown HCT116 cells





FAM96B is designated MIP18 (MMS19-interacting protein 18 kDa)



Possible functions of the MMXD protein complex in chromosome segregation

- ❖ Cohesion between sister chromatids
- ❖ Formation of centrosome and/or mitotic spindles.
- ❖ Spindle checkpoint

Yeast *yhr122w* cells mutated in the putative yeast counterpart of MIP18 exhibited a chromosome instability phenotype, which is mostly associated with a defect in the establishment of sister chromatid cohesion.

Subunits of cohesins and condensins harbor HEAT repeats, which are frequently found in proteins required for chromosome dynamics including chromosome segregation, chromatin remodeling, and DNA repair. MMS19 contains HEAT repeat. It is plausible that the HEAT repeat in MMS19 functions with cohesins and condensins.

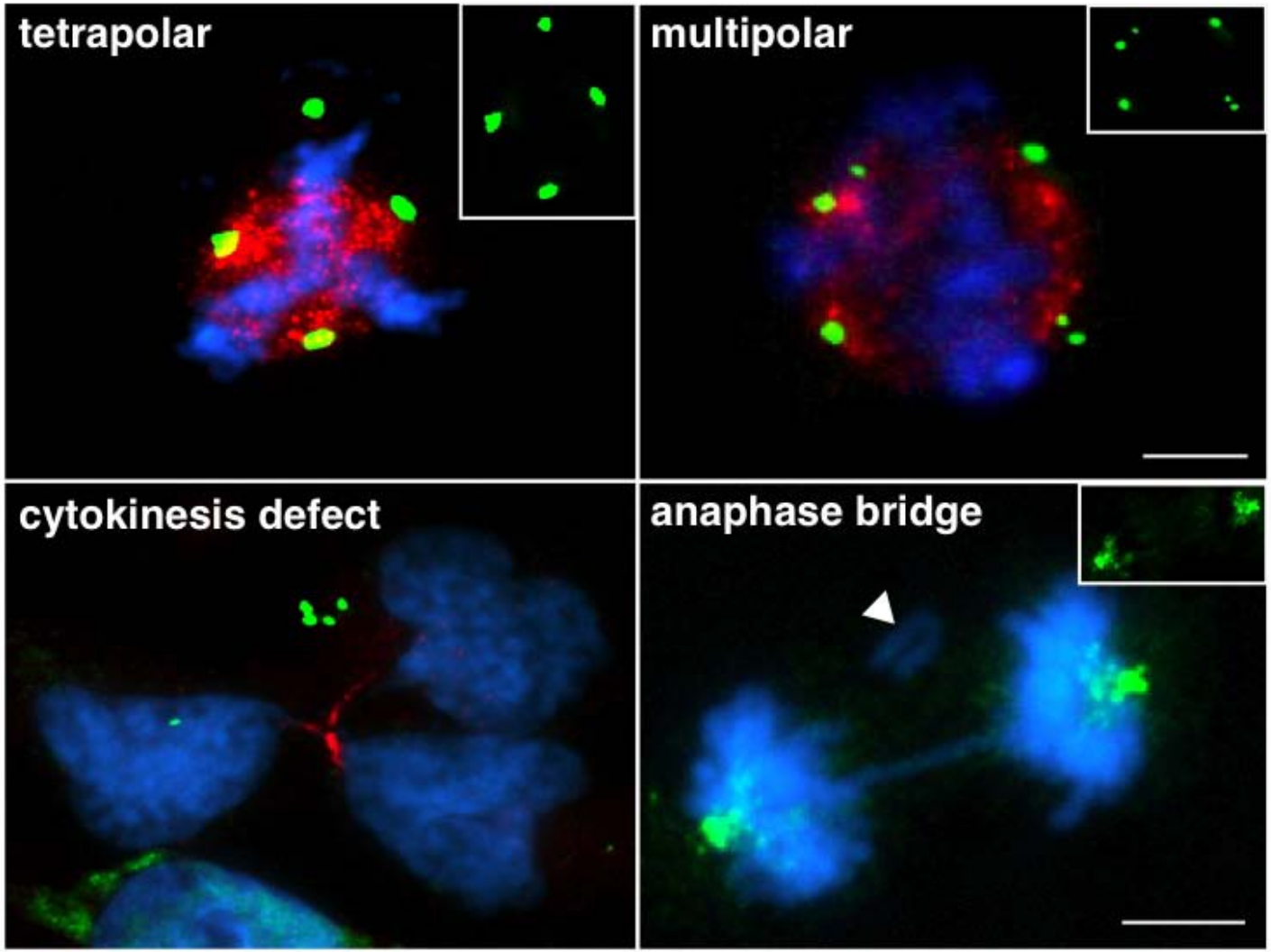
Relevance of MMXD-dysfunction to clinical features of XP-D, XP-D/CS and XP-D/TTD patients

Frequencies of abnormal nuclei at inter-phase, and aberrant mitotic spindle and chromosomal segregation were examined in the cells derived from patients with *XPD* mutations.

XPD-patients' cells:
XP6BE (XP-D)
XPCS-2 (XP-D/CS)
TTD1RO (XP-D/TTD)

Wild-type XPD cDNA-corrected isogenic cells:
XPD/XP6BE
XPD/XPCS-2
XPD/TTD1RO

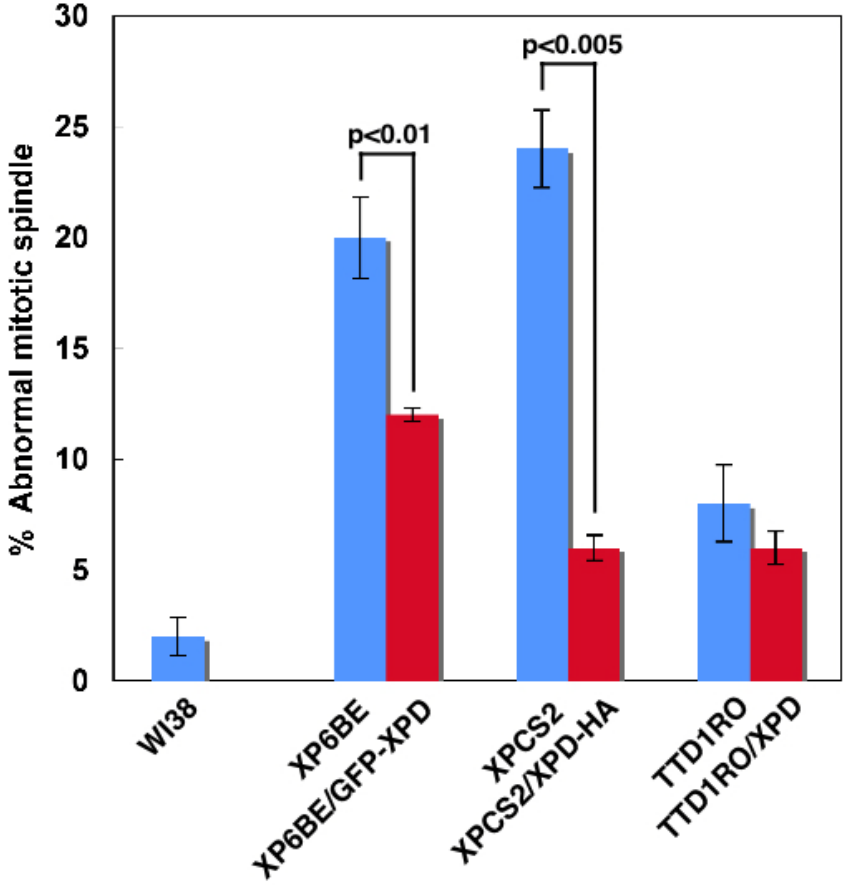
Abnormal mitotic spindle and chromosome segregation in the metaphase cells derived from XP-D and XP-D/CS patients



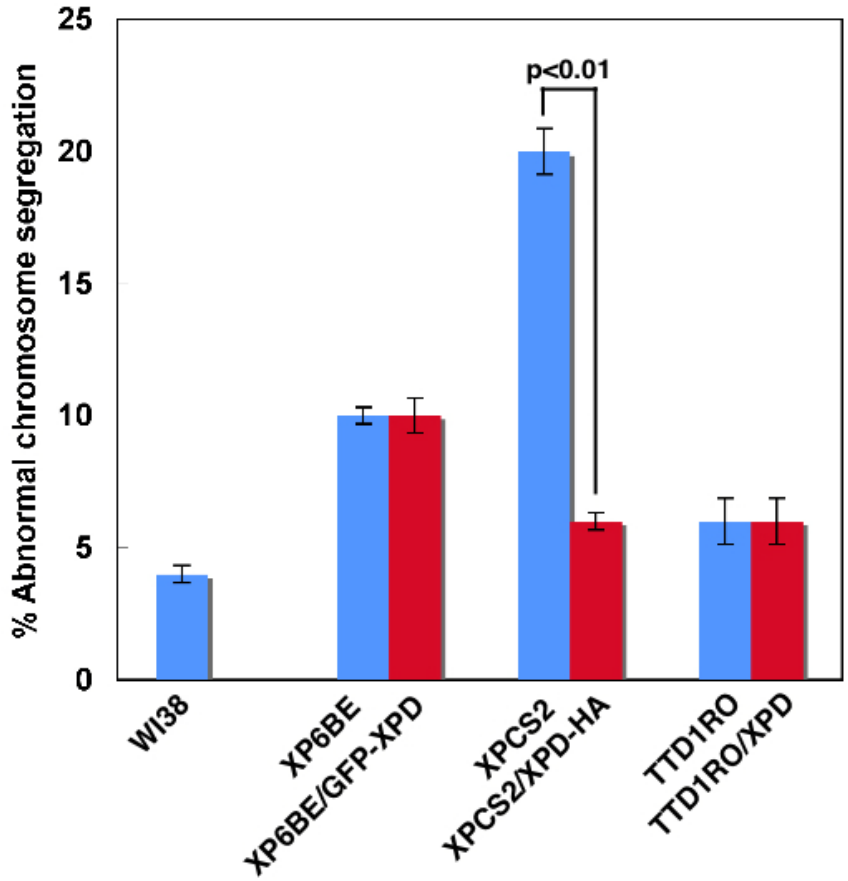
Green: gamma-tubulin Red: alpha-tubulin Blue: DAPI

Abnormal mitotic spindle and chromosome segregation in the metaphase cells derived from XP-D, XP-D/CS and XP-D/TTD patients

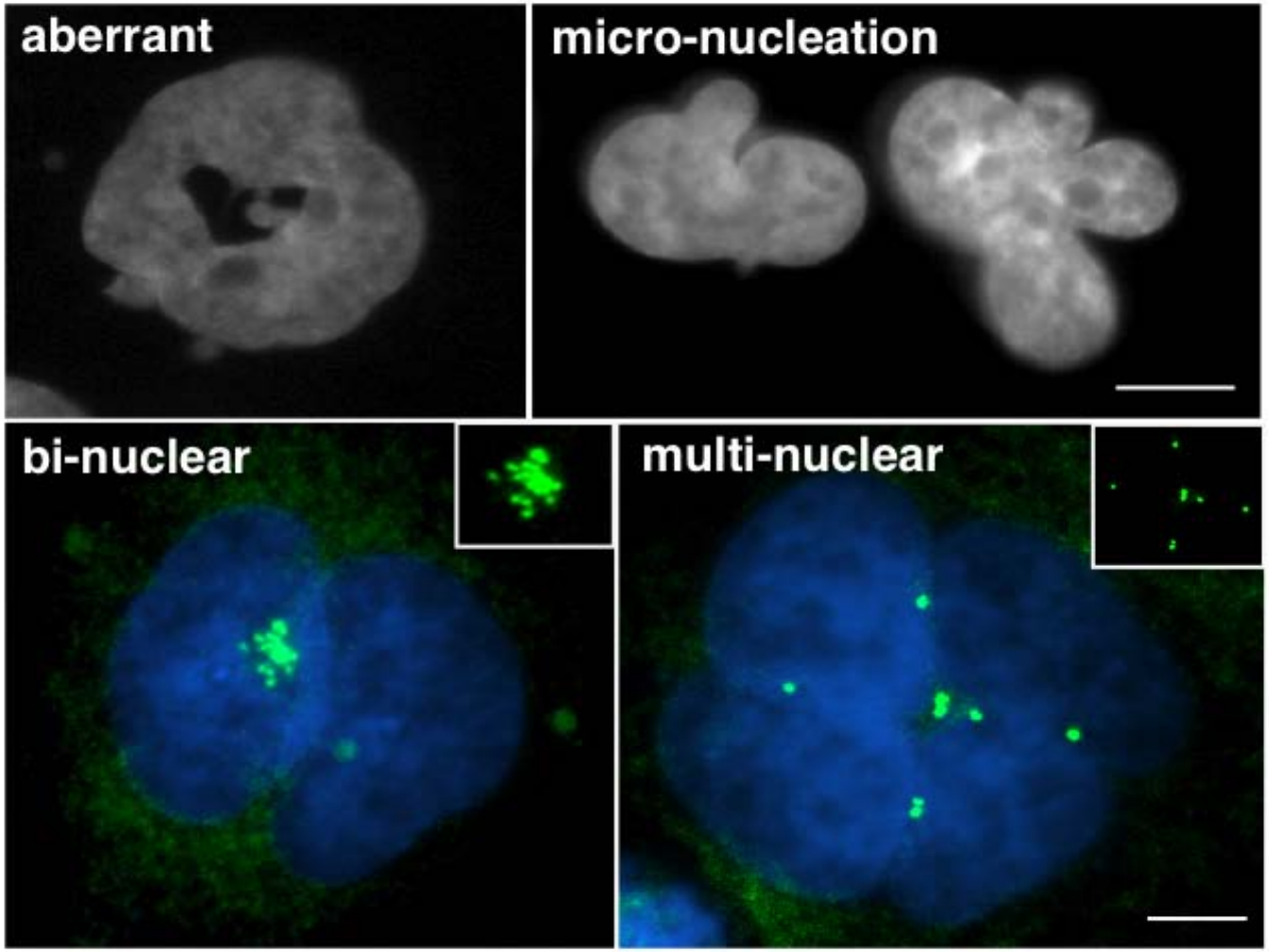
Abnormal mitotic spindle



Abnormal chromosome segregation

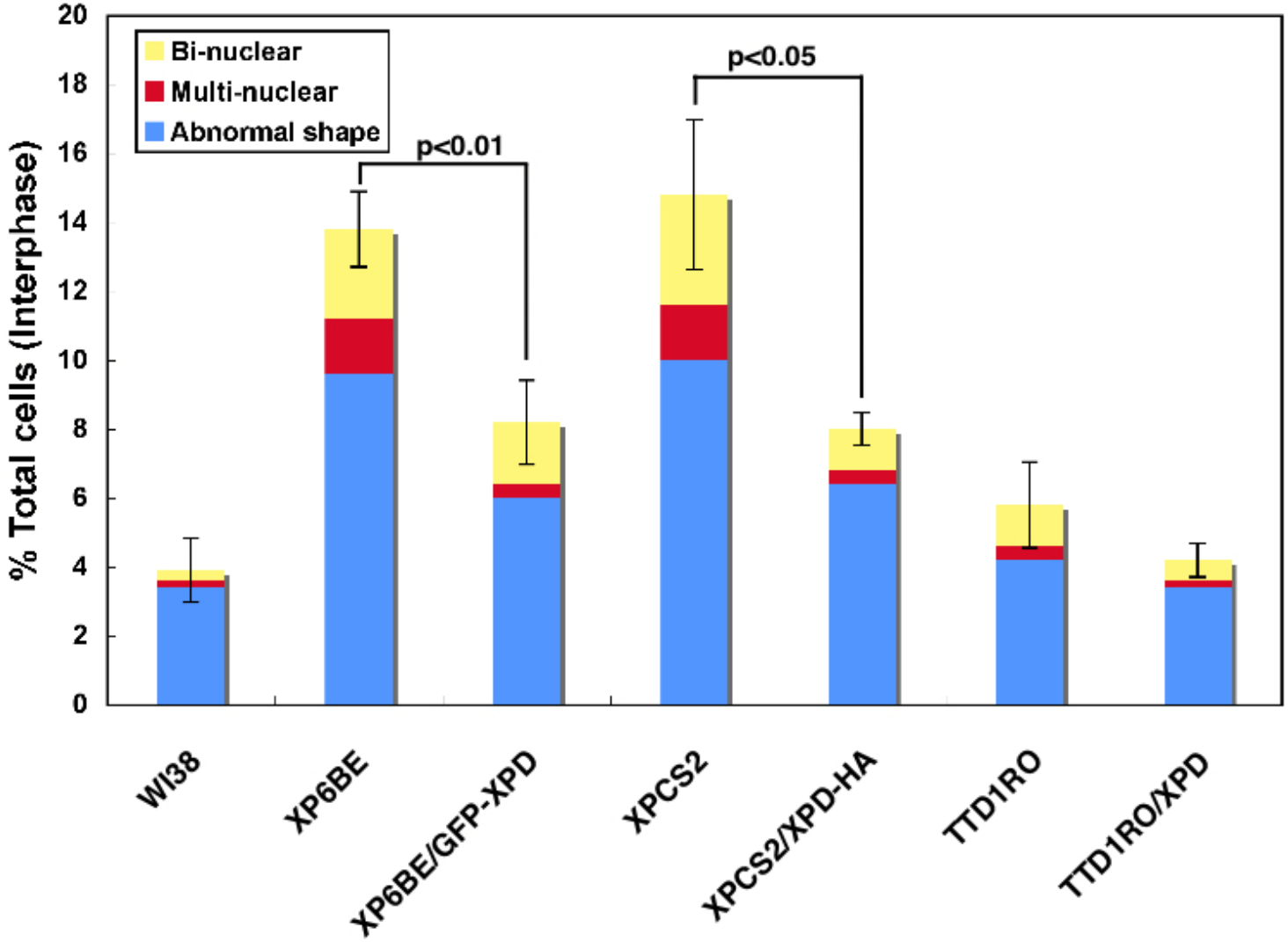


Abnormal nuclei in the interphase cells derived from XP-D(XP6BE) and XP-D/CS(XPCS-2) patients



Green: gamma-tubulin Blue: DAPI

Abnormal nuclei in the interphase cells derived from XP-D, XP-D/CS and XP-D/TTD patients



Clinical implication of MMXD-dysfunction:

The results indicate that the function of MMXD in chromosome segregation was affected in XP6BE (XP-D) and XPCS-2 (XP-D/CS) patients' cells, but not in TTD1RO (XP-D/TTD) patient cells.

It is worth noting that these three patients had defects in NER, while skin tumors were detected only in XP6BE and XPCS-2 patients, not in TTD1RO patient.

These findings suggest that the dysfunction of MMXD due to the *XPD* mutations lead to aneuploidy and/or cell death, contributing at least partly to the phenotypes in XP6BE and XPCS-2 patients such as a high incidence of skin tumors.

Collaboration

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