Evolutionarily Conserved Pathways Suppress Genomic Instability

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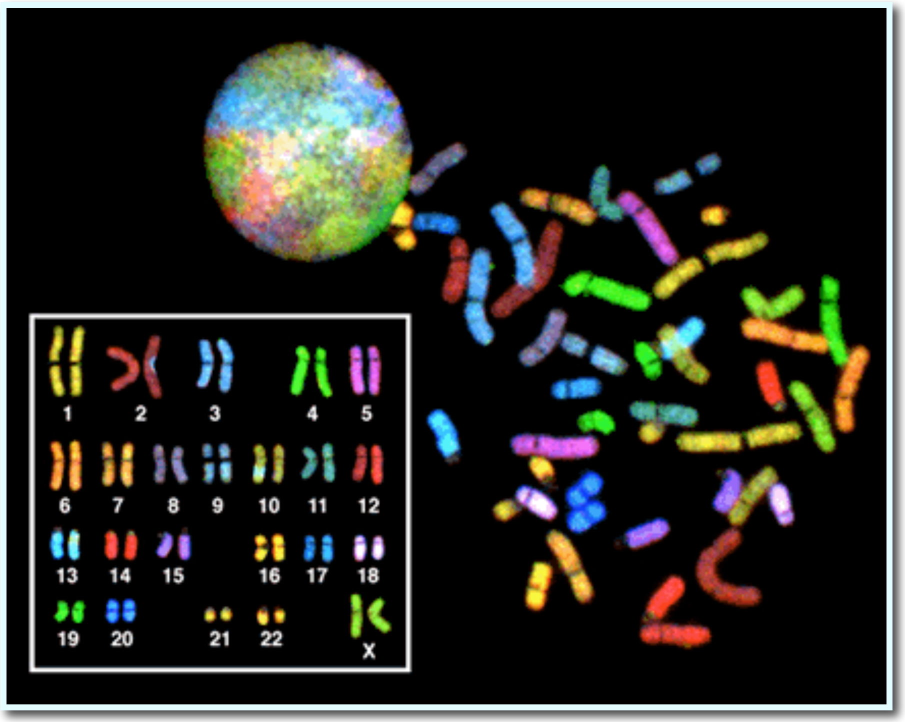
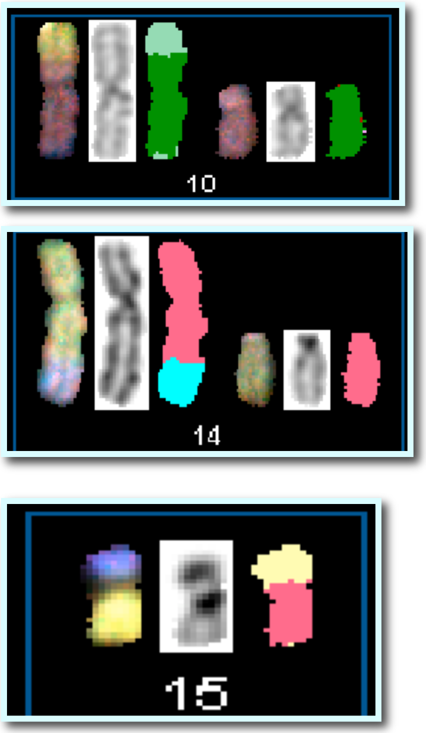
Genome Instability Section
NHGRI/NIH

GMBB

Gross Chromosomal Rearrangements (GCRs)



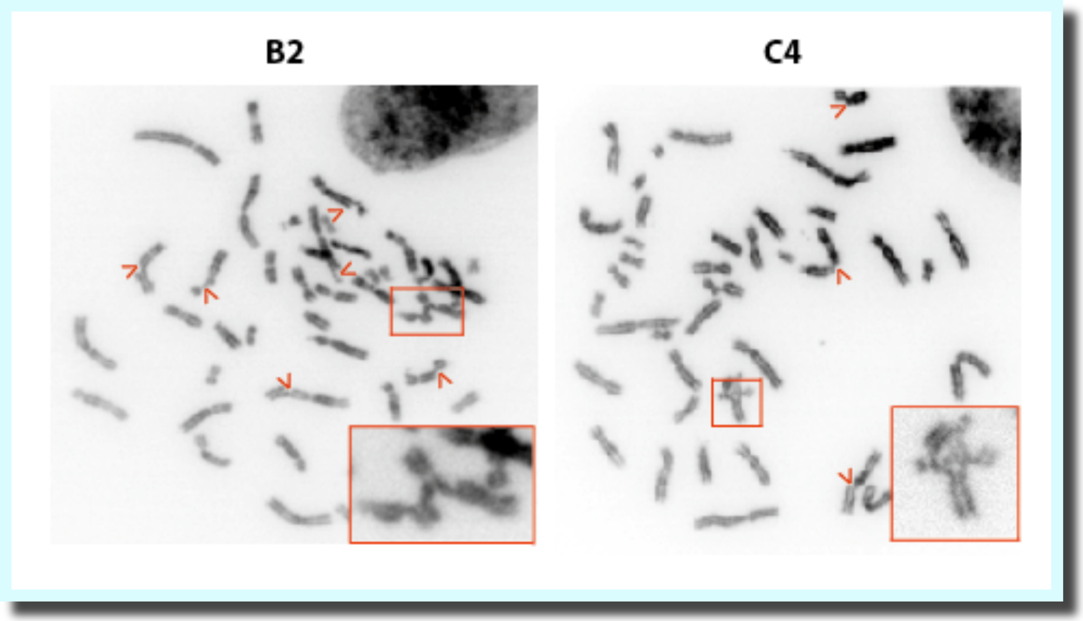
Wild type



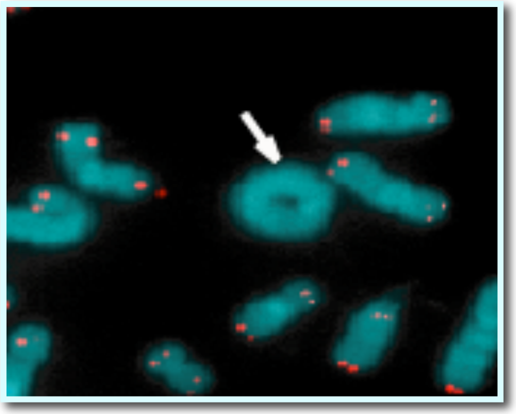
Translocations

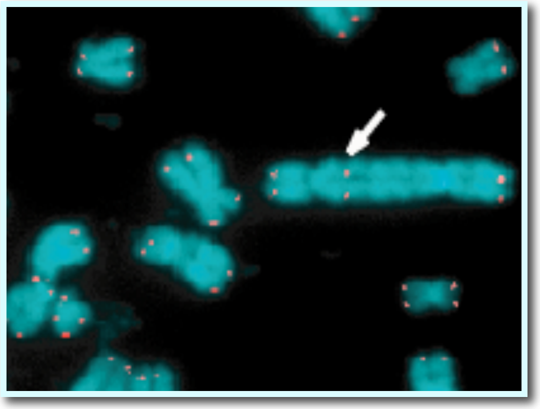
Myung, et al,, MCB (2004) 24:5050

Gross Chromosomal Rearrangements (GCRs)



Breaks

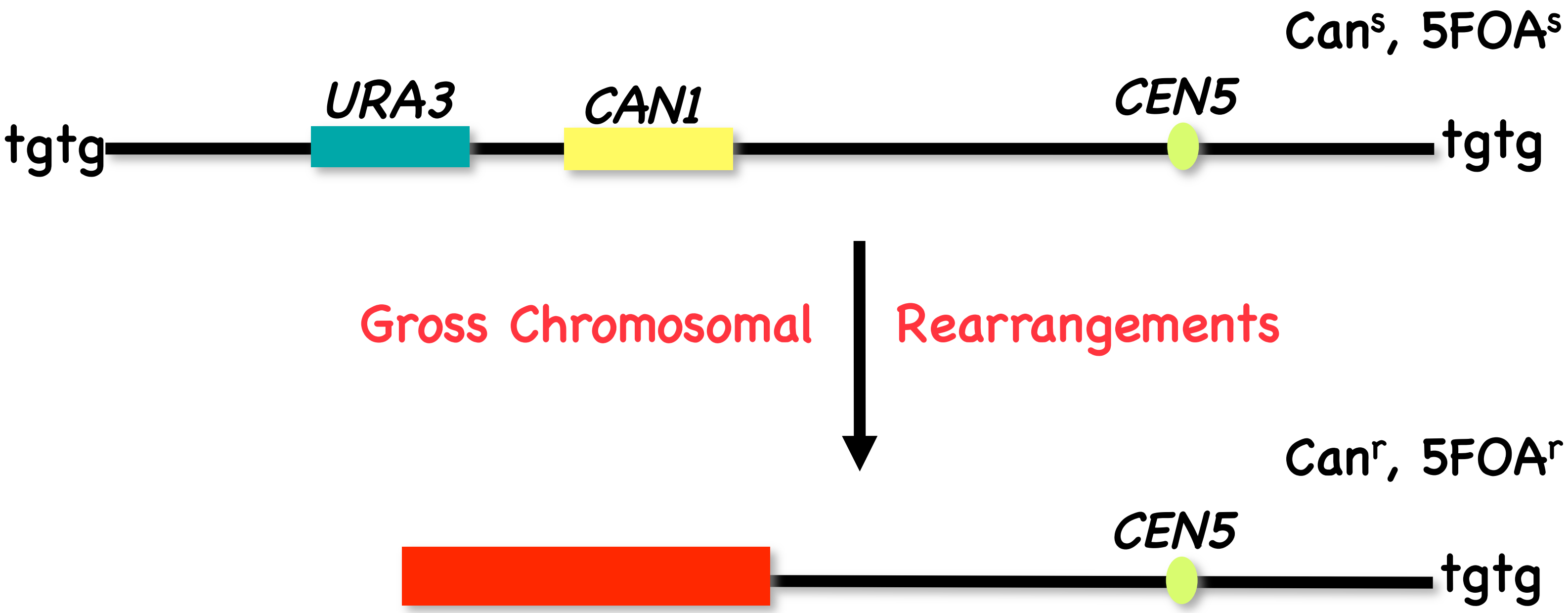




Fusions

Myung, et al,, MCB (2004) 24:5050 Motegi, et al., JCB (2006)175:703

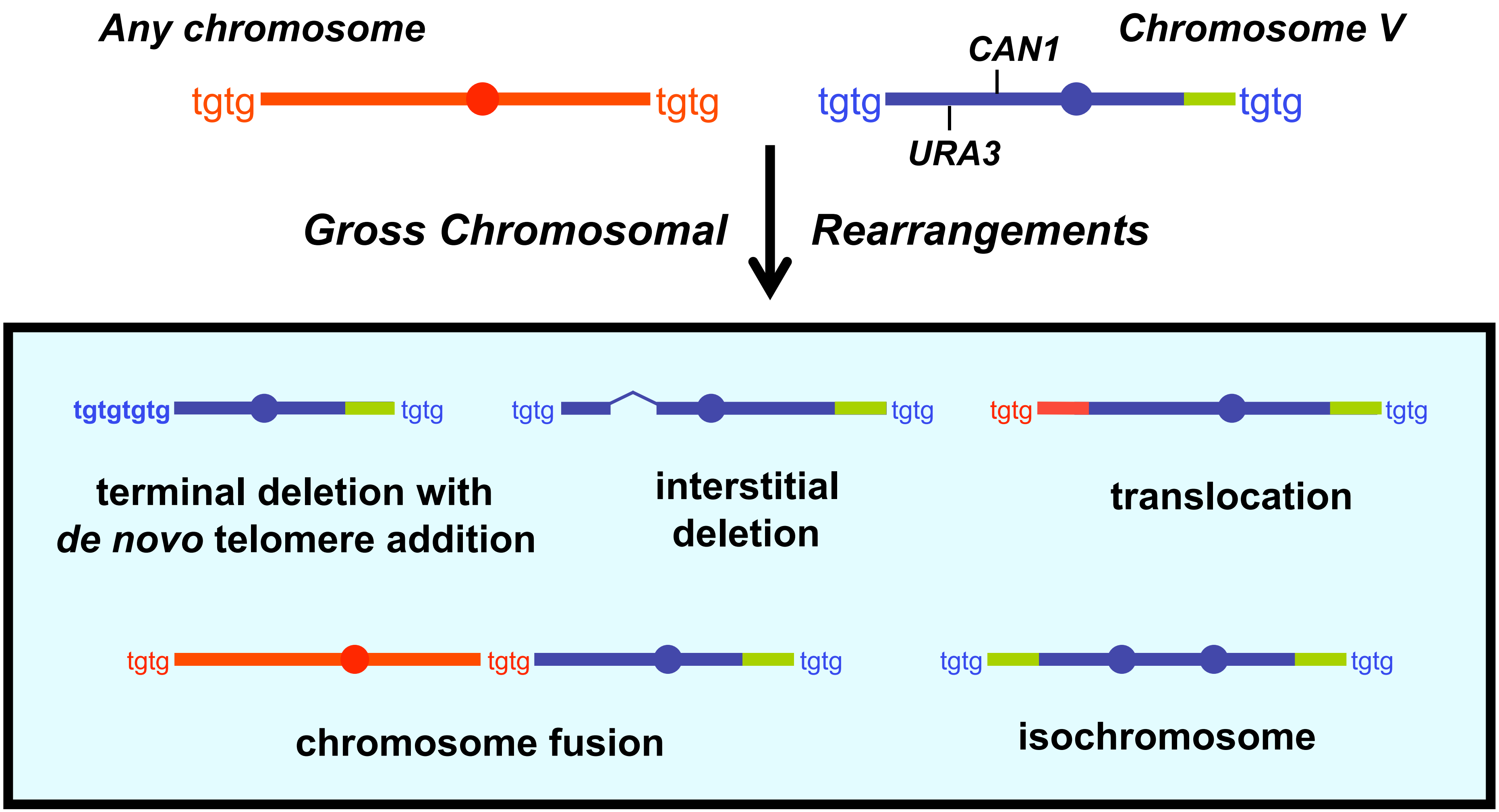
Assay for Measuring the Rate of Gross Chromosomal Rearrangements (GCRs)



Wild type rate of GCR = 3.5×10^{-10} per generation.

Chen & Kolodner., Nat. Genet. (1999) & Myung et al., Cell (2001)

GCRs Observed in Mutator Mutants



terminal deletion with
***de novo* telomere addition**

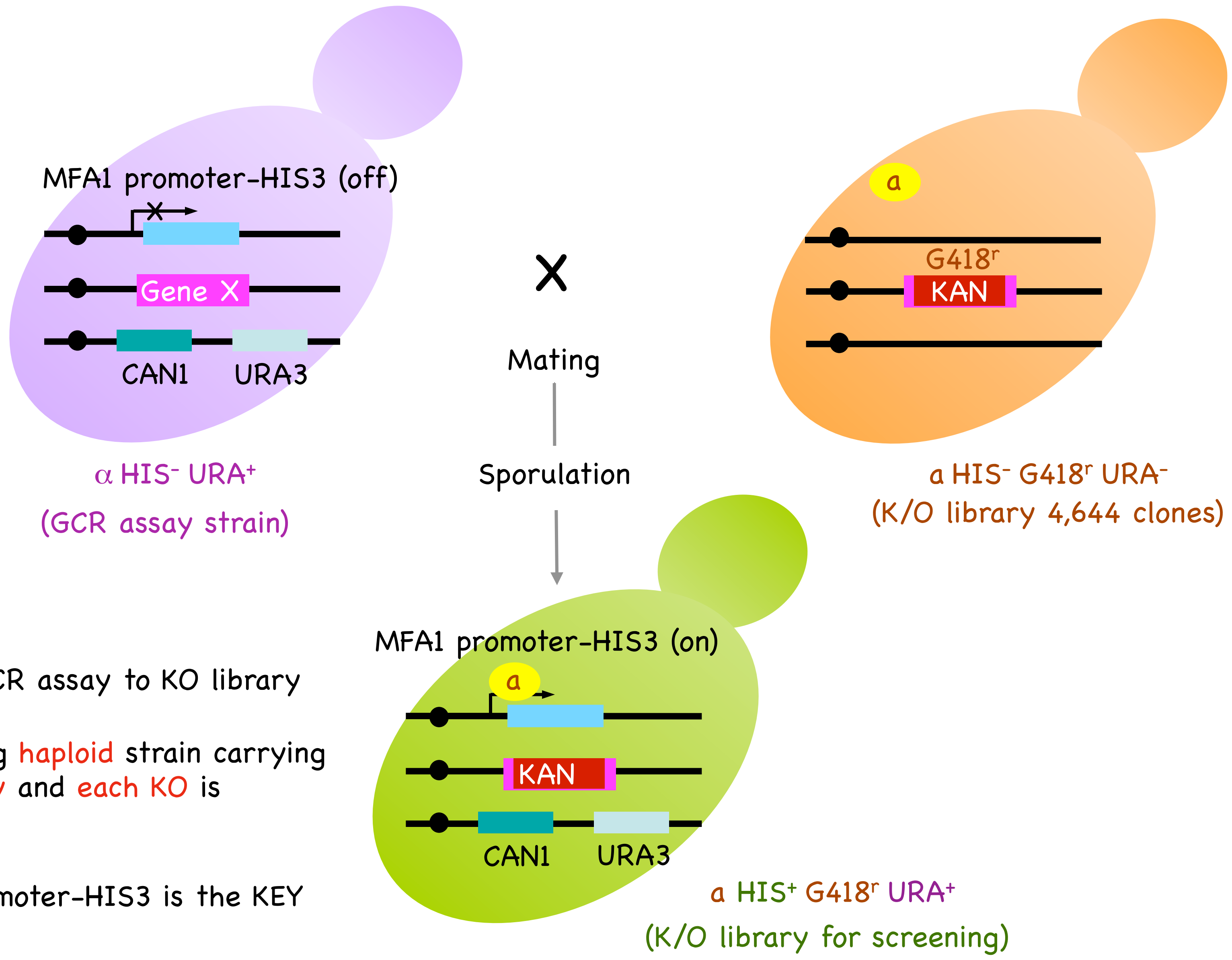
interstitial
deletion

translocation

chromosome fusion

isochromosome

A Scheme for Screening of All Non-Essential yeast ORFs for GCRs

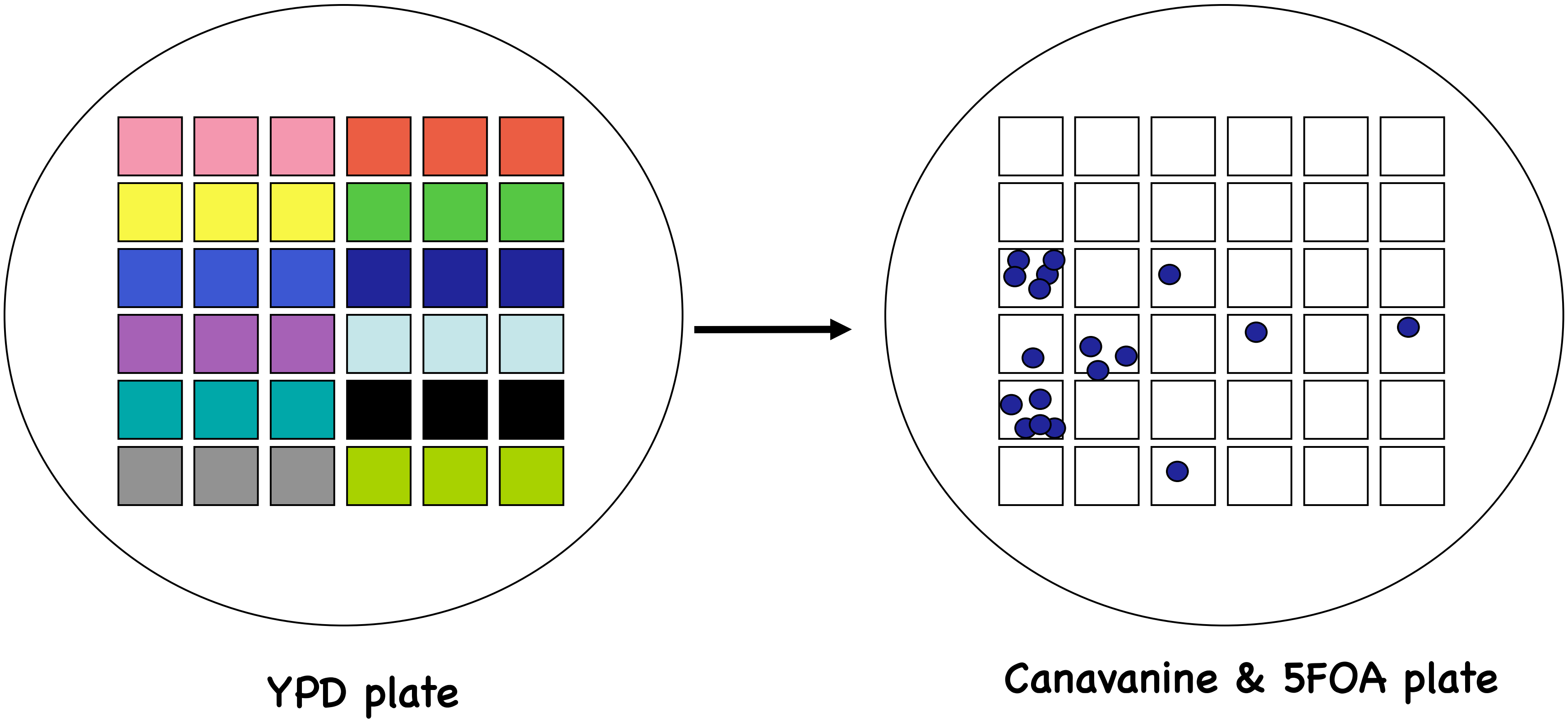


Moving GCR assay to KO library

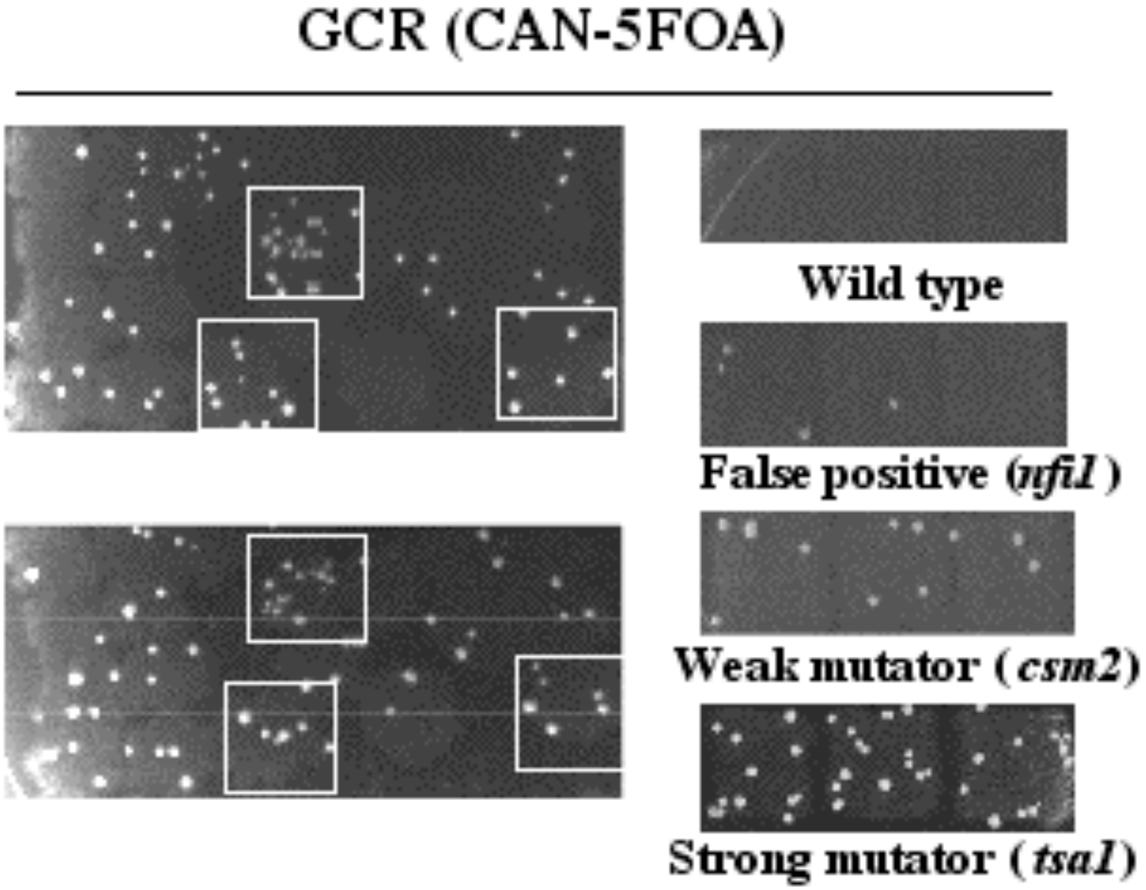
: Selecting haploid strain carrying GCR assay and each KO is Essential.

MFA1-Promoter-HIS3 is the KEY

Three Individual Patches for Each Primary Positives



Mutators Increased the Number of Resistant Colonies



GCR (CAN-5FOA)

Wild type

False positive (*nfi1*)

Weak mutator (*csm2*)

Strong mutator (*tsa1*)

Effect of New GCR Mutator Gene Defects on the GCR Rates

	WT	*pif1-m2*
Relevant Genotype	GCR rate (CAN^r-$5FOA^r$)	GCR rate (CAN^r-$5FOA^r$)
Wild type	3.5×10^{-10} (1)	6.3×10^{-8} (180)
alo1Δ	4.7×10^{-8} (134)	1.1×10^{-7} (314)
cdc50Δ	4.8×10^{-9} (14)	2.6×10^{-7} (743)
csm2Δ	2.7×10^{-9} (8)	1.6×10^{-7} (457)
elg1Δ	1.7×10^{-8} (49)	3.0×10^{-7} (857)
esc1Δ	2.3×10^{-9} (7)	1.1×10^{-7} (314)
mms4Δ	5.9×10^{-8} (169)	2.3×10^{-7} (657)
rad5Δ	2.4×10^{-8} (68)	2.2×10^{-7} (633)
rad18Δ	2.3×10^{-8} (65)	2.5×10^{-7} (714)
tsa1Δ	2.6×10^{-9} (7)	3.6×10^{-7} (1029)
ufo1Δ	2.6×10^{-8} (74)	1.4×10^{-7} (400)

Pif1-m2 mutation (Telomerase inhibitor) increased GCR rate synergistically with all currently known GCR mutator mutations

Smith *et al.*, PNAS (2004)

Effect of New GCR Mutator Gene Defects on the GCR Rates

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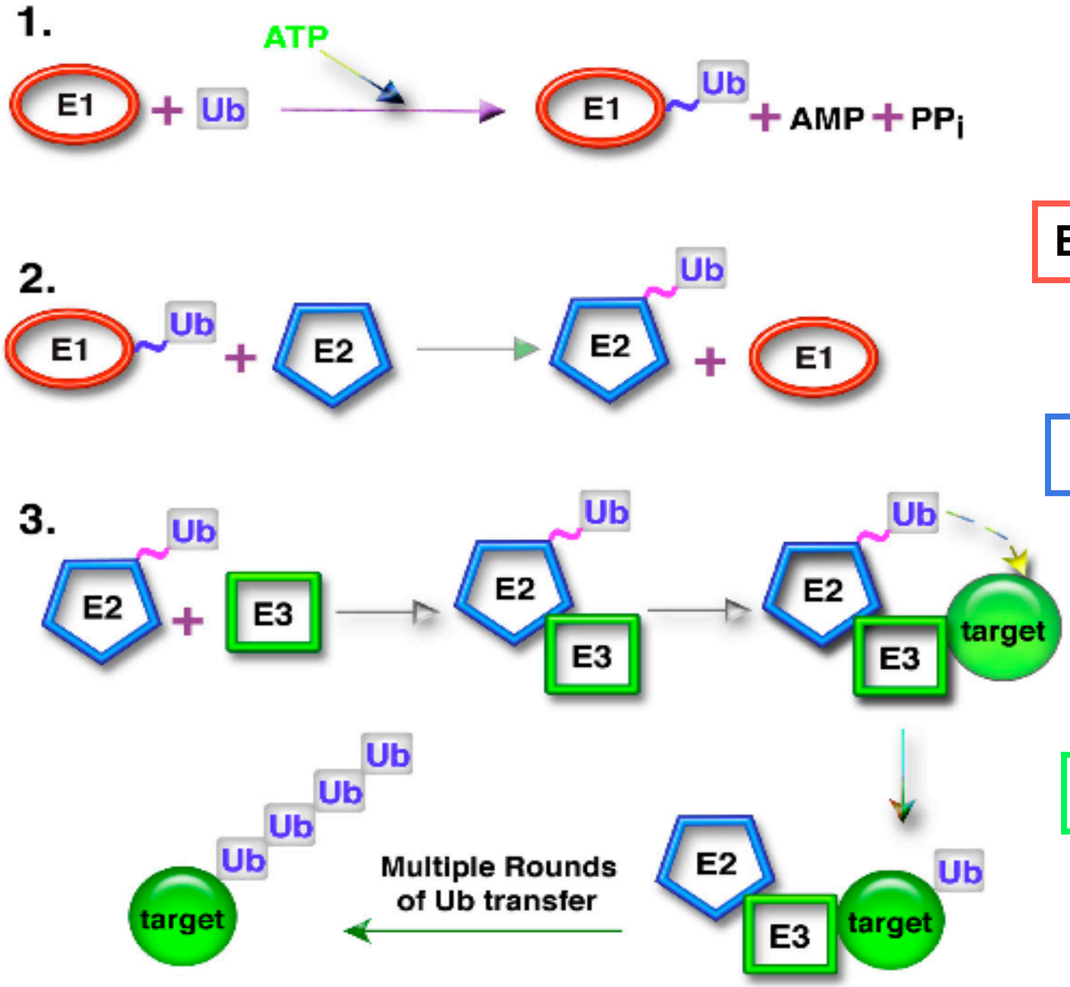
de novo **telomere addition (100%)**

Ubiquitinations

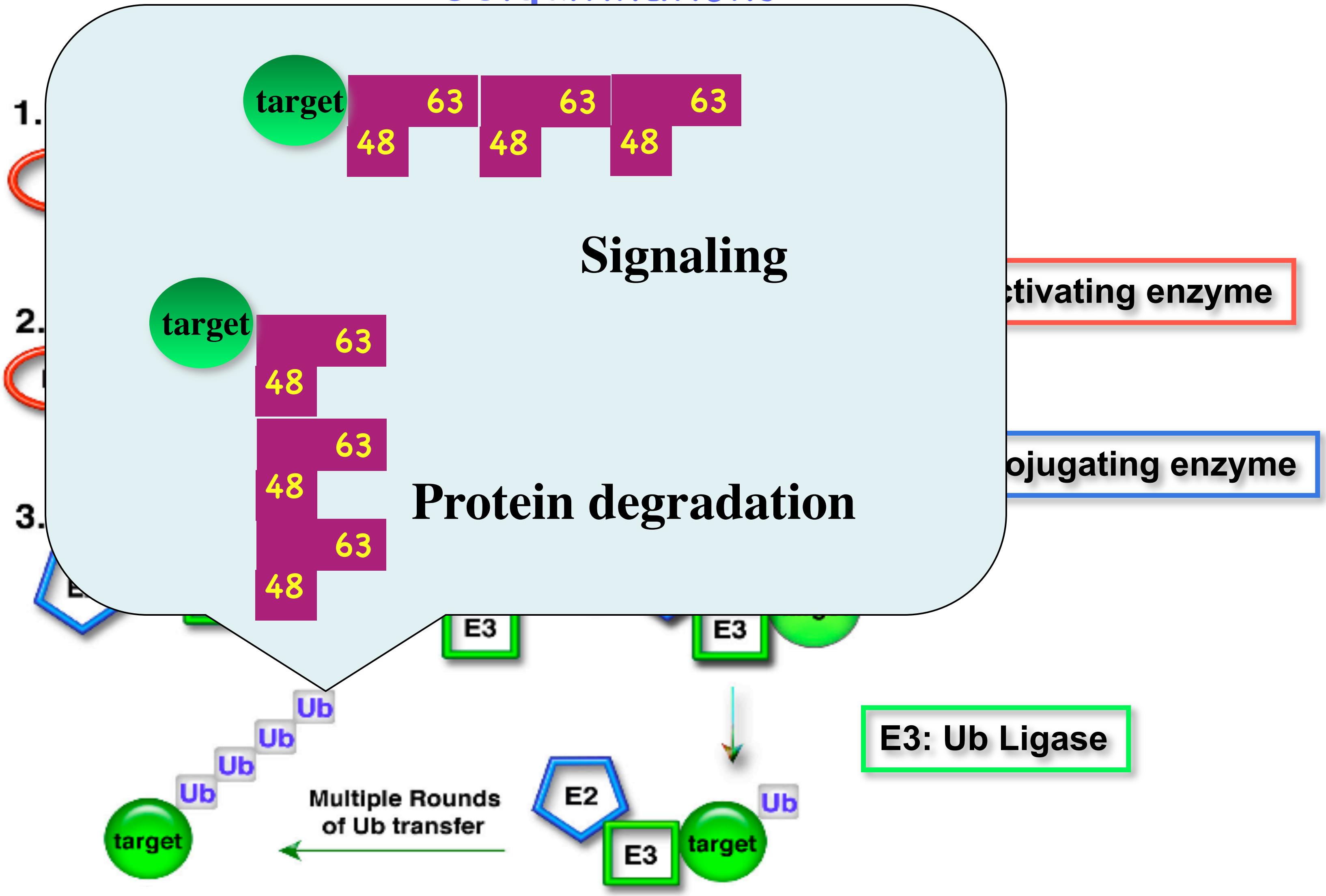


E1: Ub activating enzyme

E2: Ub cojugating enzyme

E3: Ub Ligase

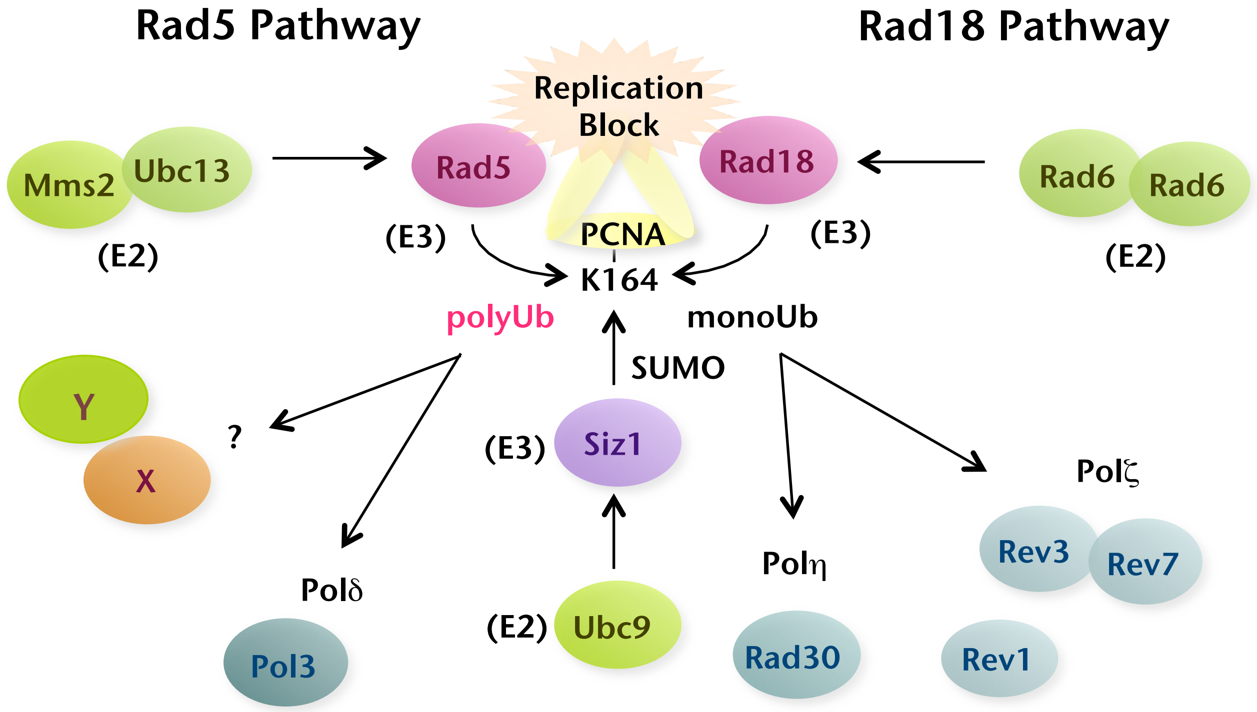
Ubiquitinations



Signaling

Protein degradation

Yeast *RAD5* Pathway Suppresses Gross Chromosomal Rearrangements



Rad5 Pathway

Rad18 Pathway

Replication Block

Mms2 Ubc13

(E2)

Rad5

(E3)

PCNA

K164

Rad18

(E3)

Rad6 Rad6

(E2)

polyUb

monoUb

SUMO

Y

X

?

Polδ

Pol3

(E3) Siz1

(E2) Ubc9

Polη

Rad30

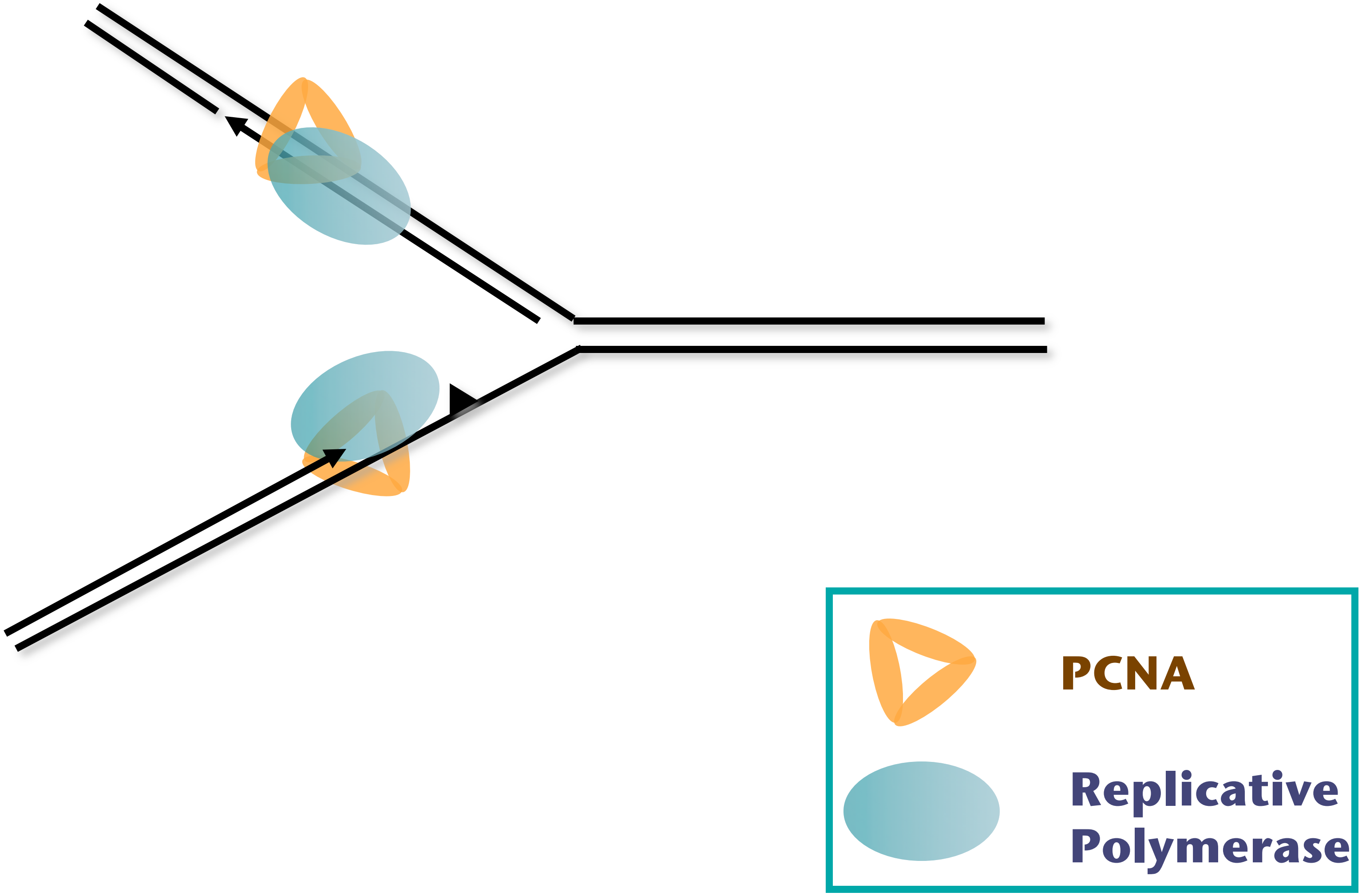
Polζ

Rev3 Rev7

Rev1

PCNA: Clamp for DNA replication polymerase and scaffold for DNA repair proteins

ScRAD18 Pathway: Translesion Synthesis (TLS)



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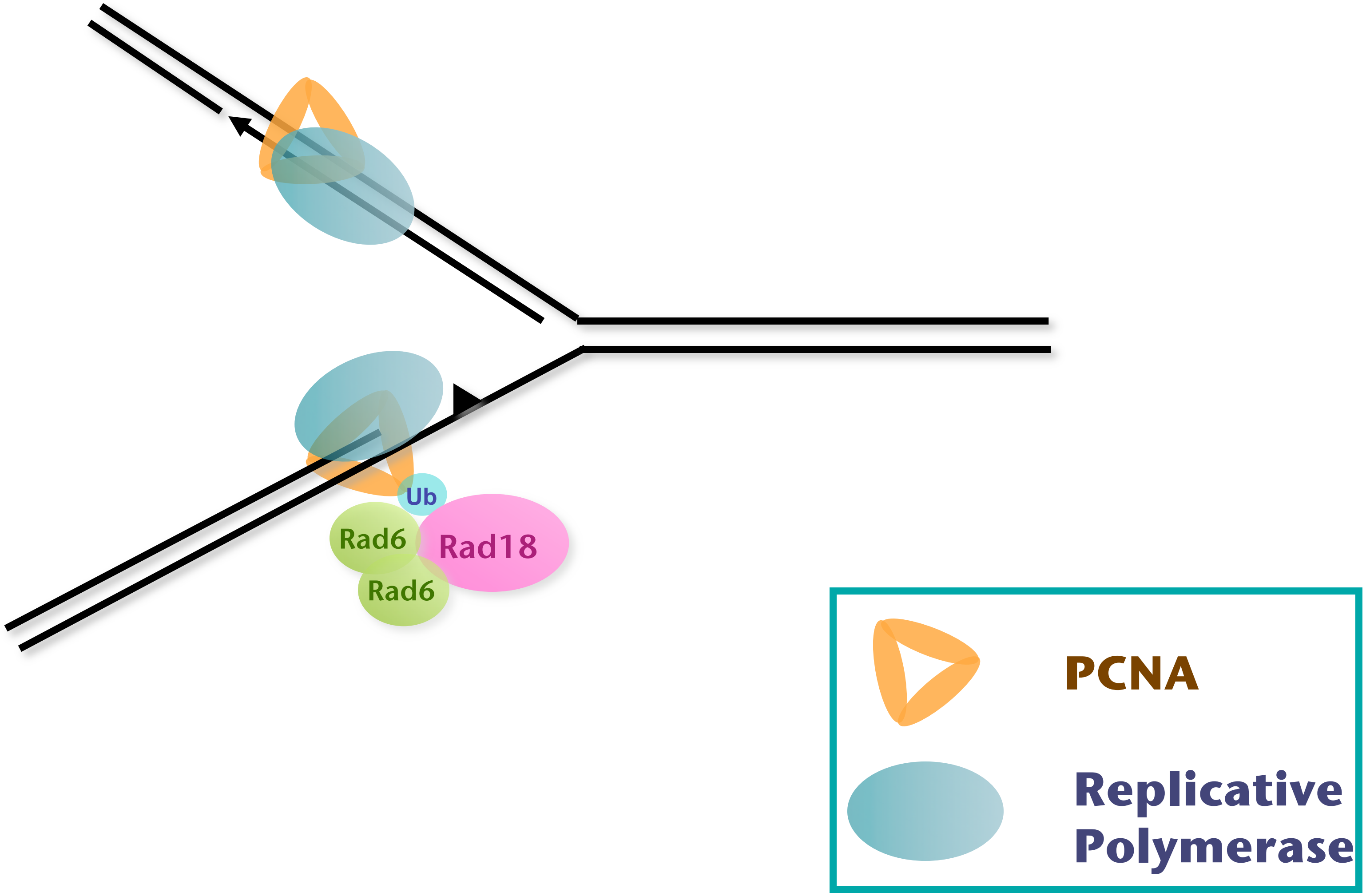

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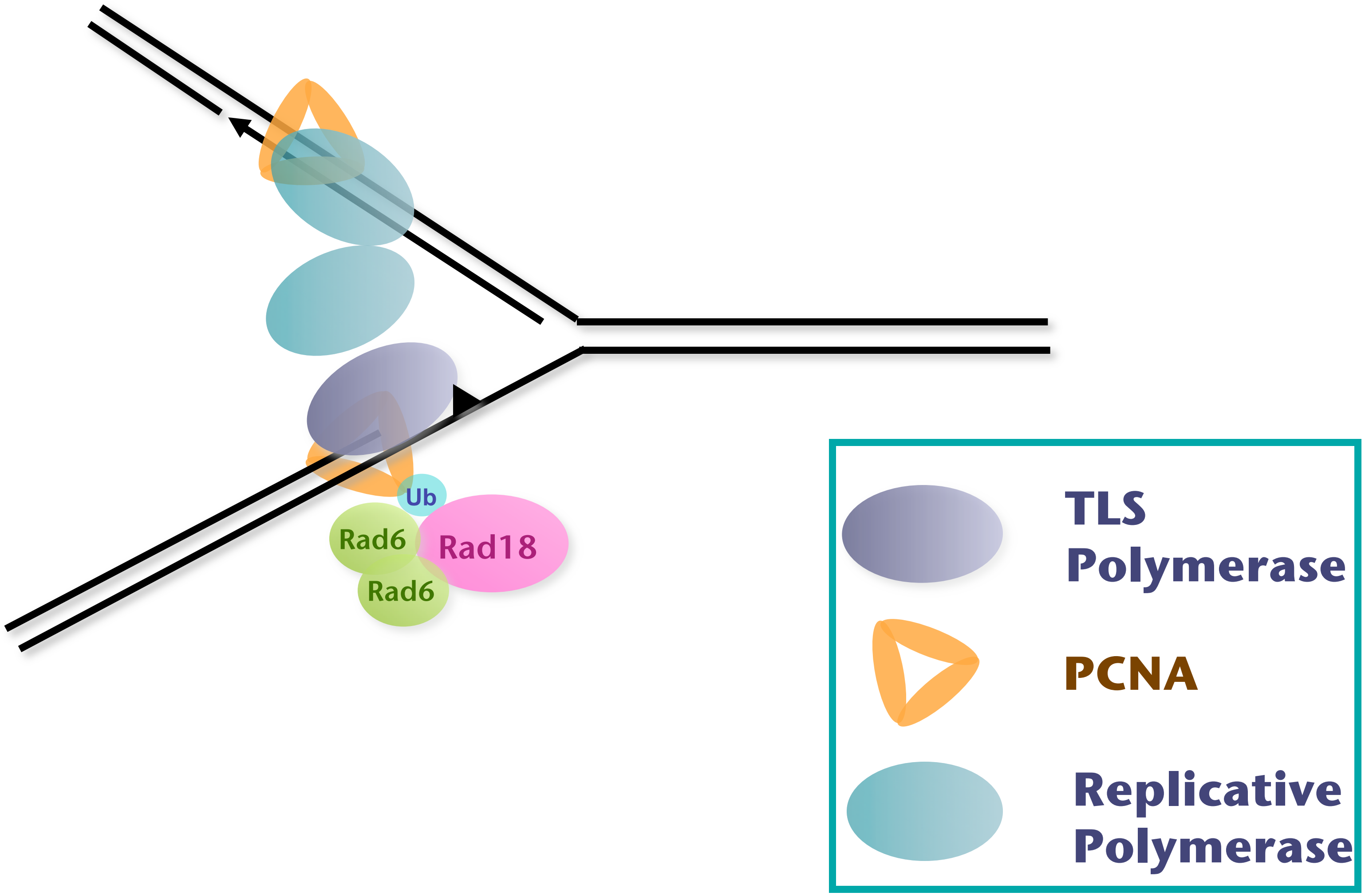

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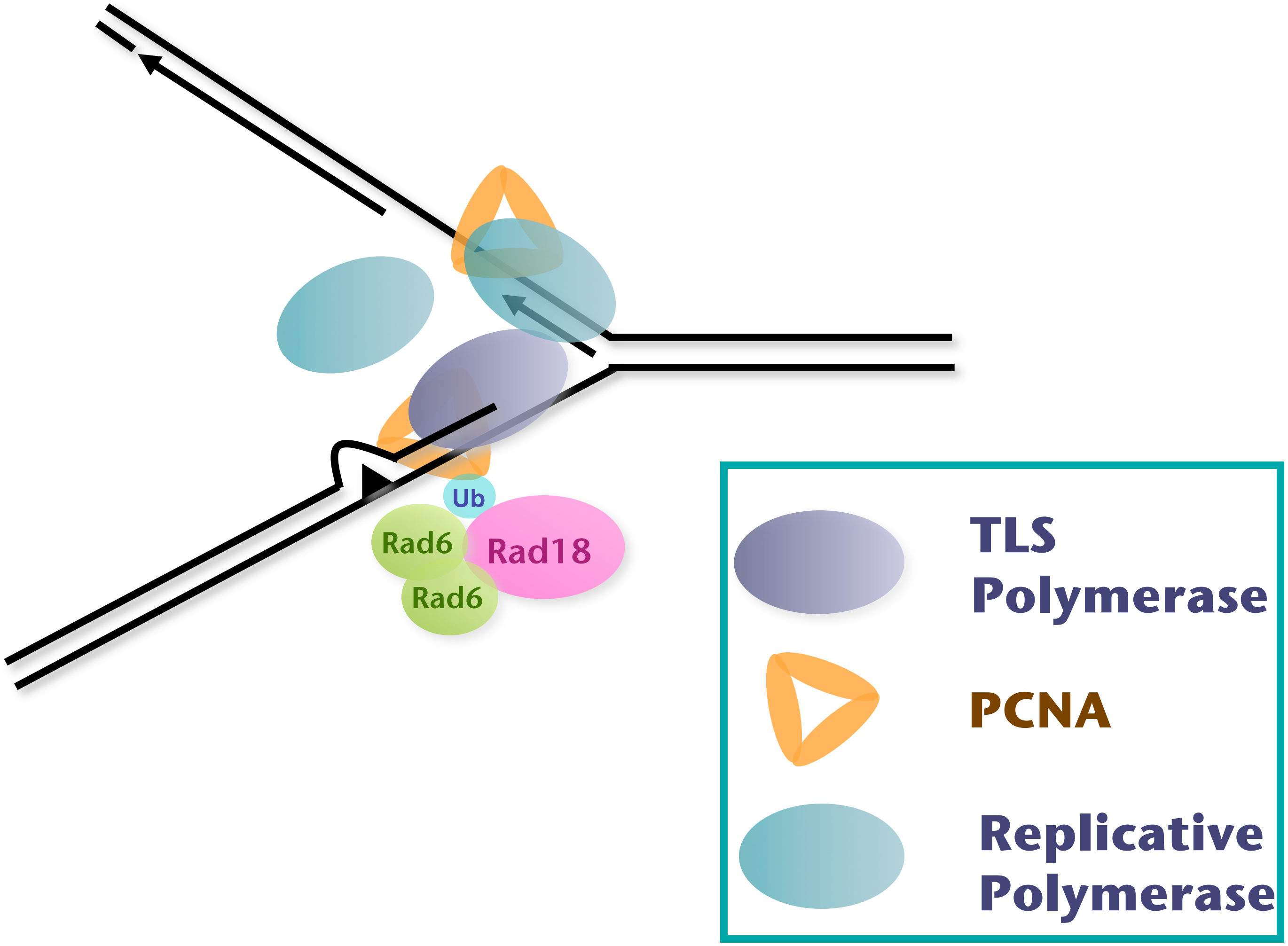

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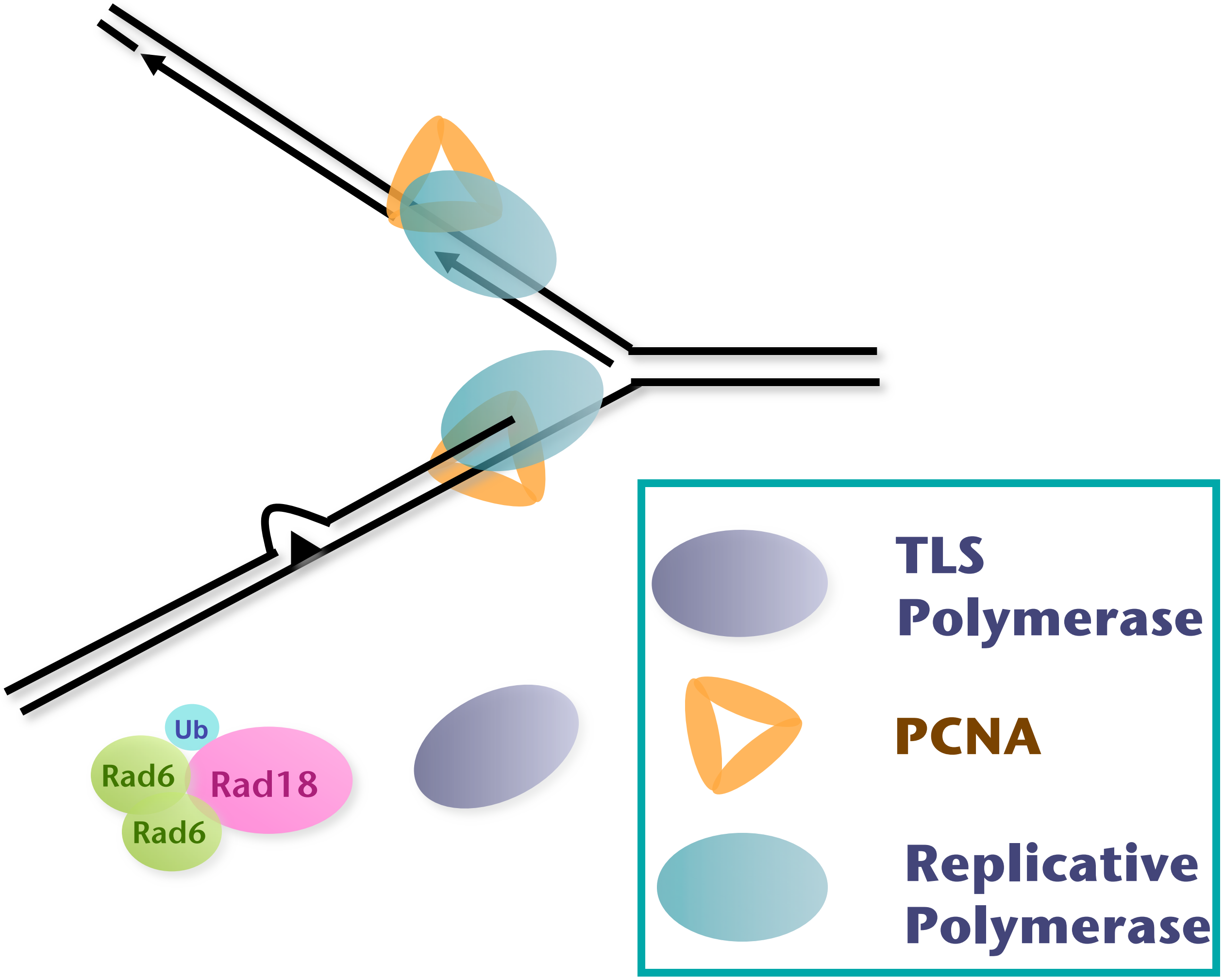
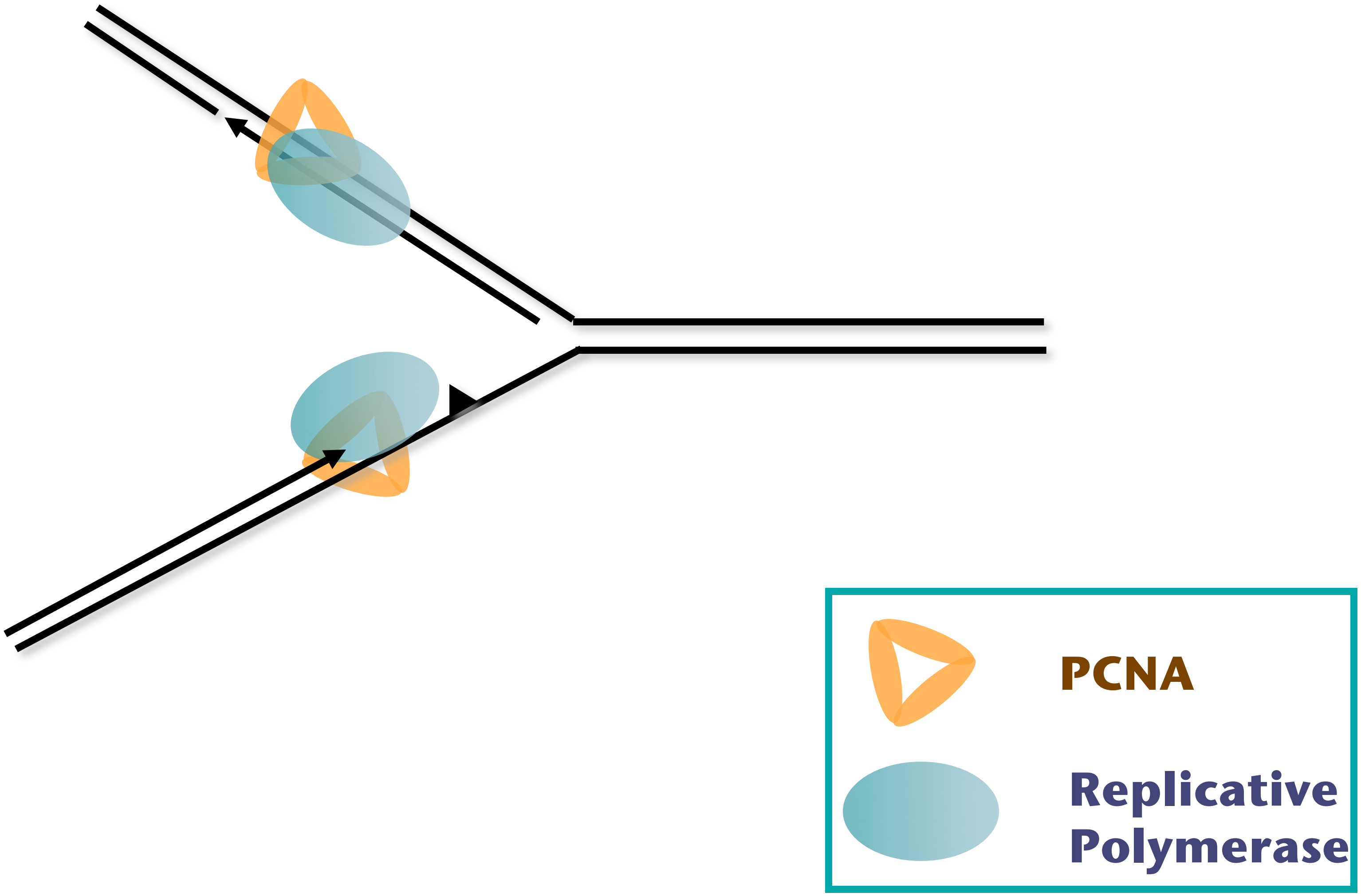


ScRAD5 Pathway: Fork Reversion (Damage in leading strand)?



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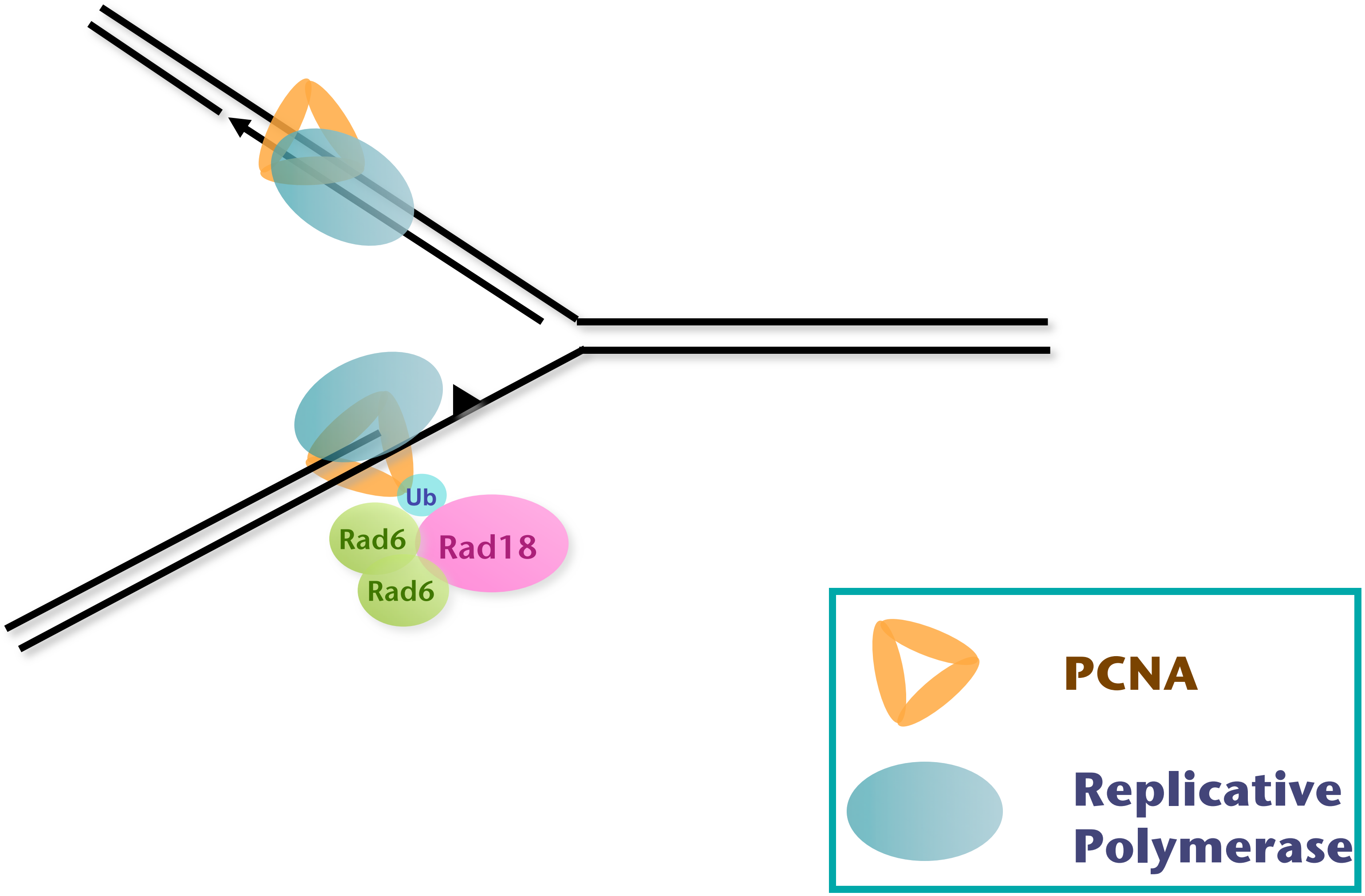

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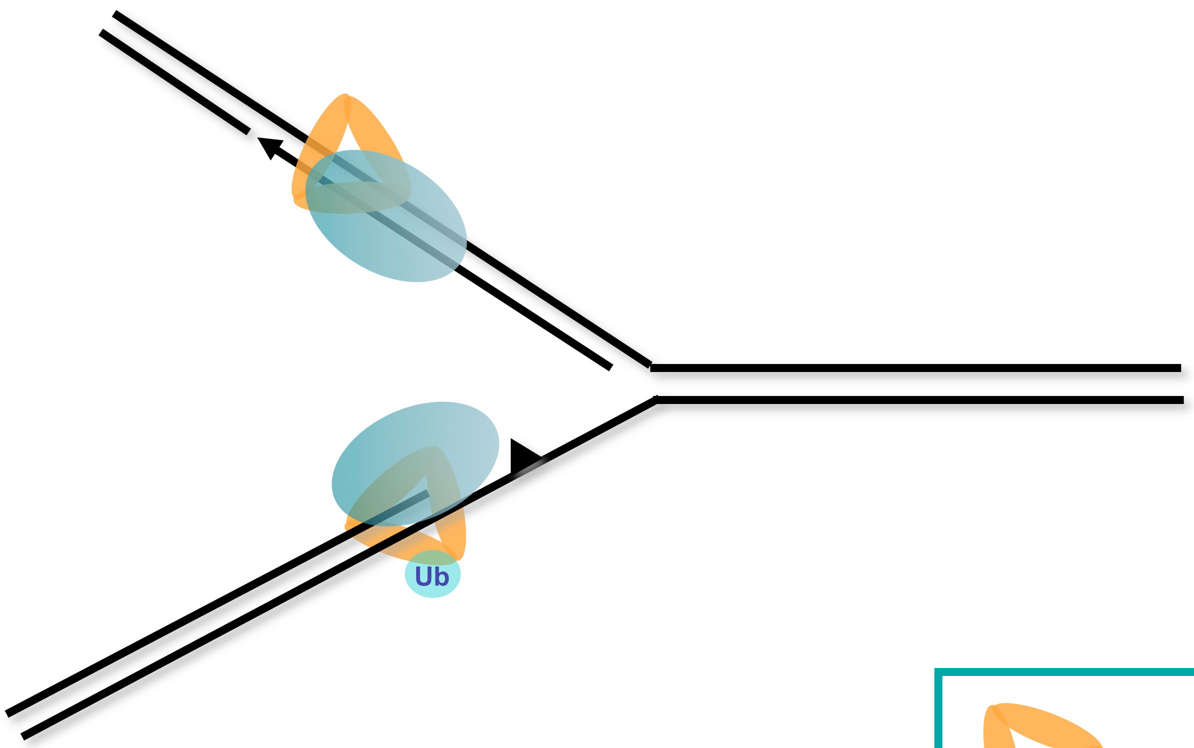
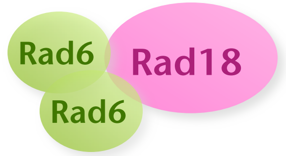
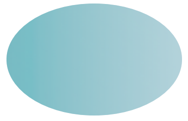




	**PCNA**
	**Replicative Polymerase**

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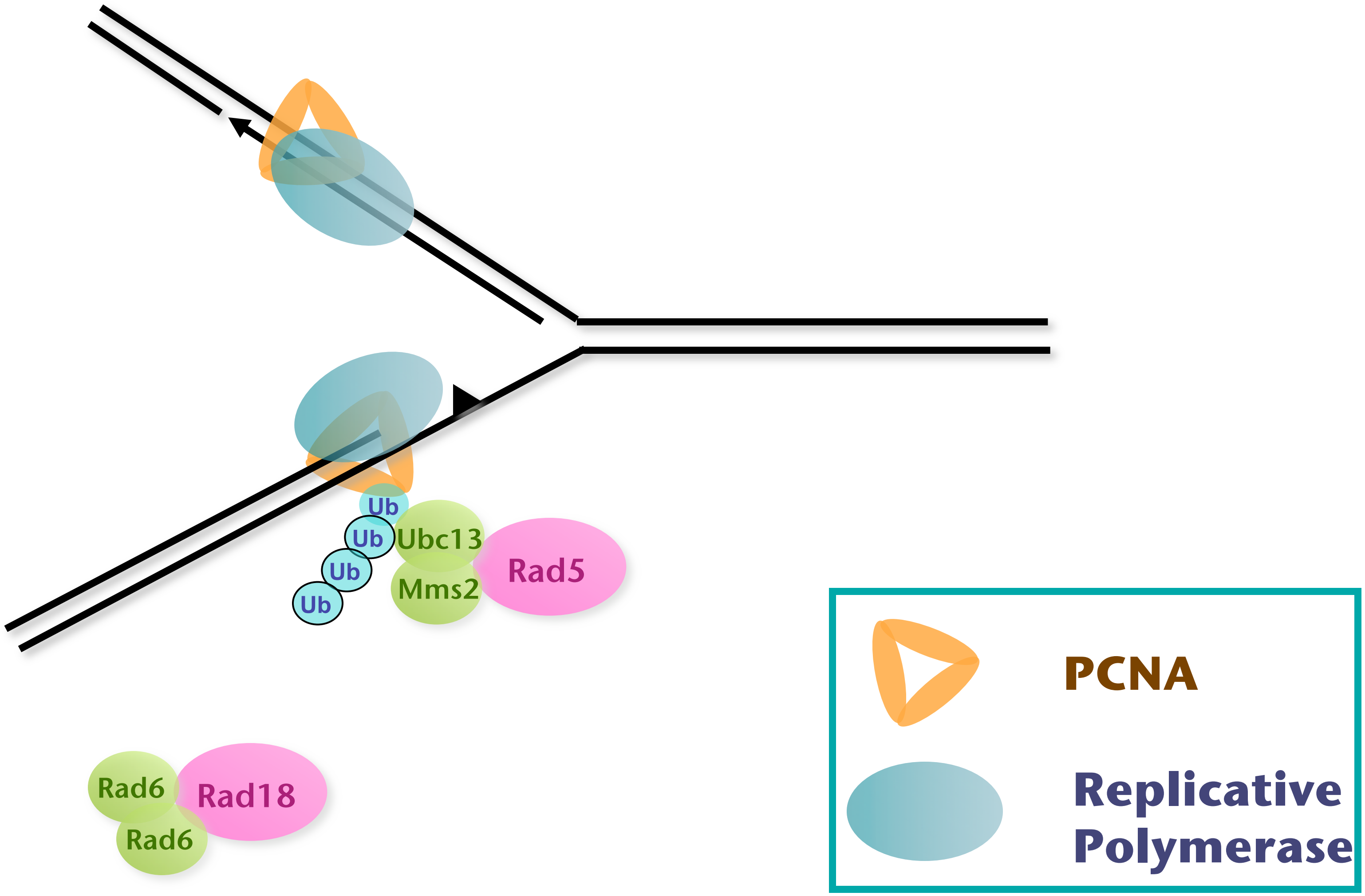

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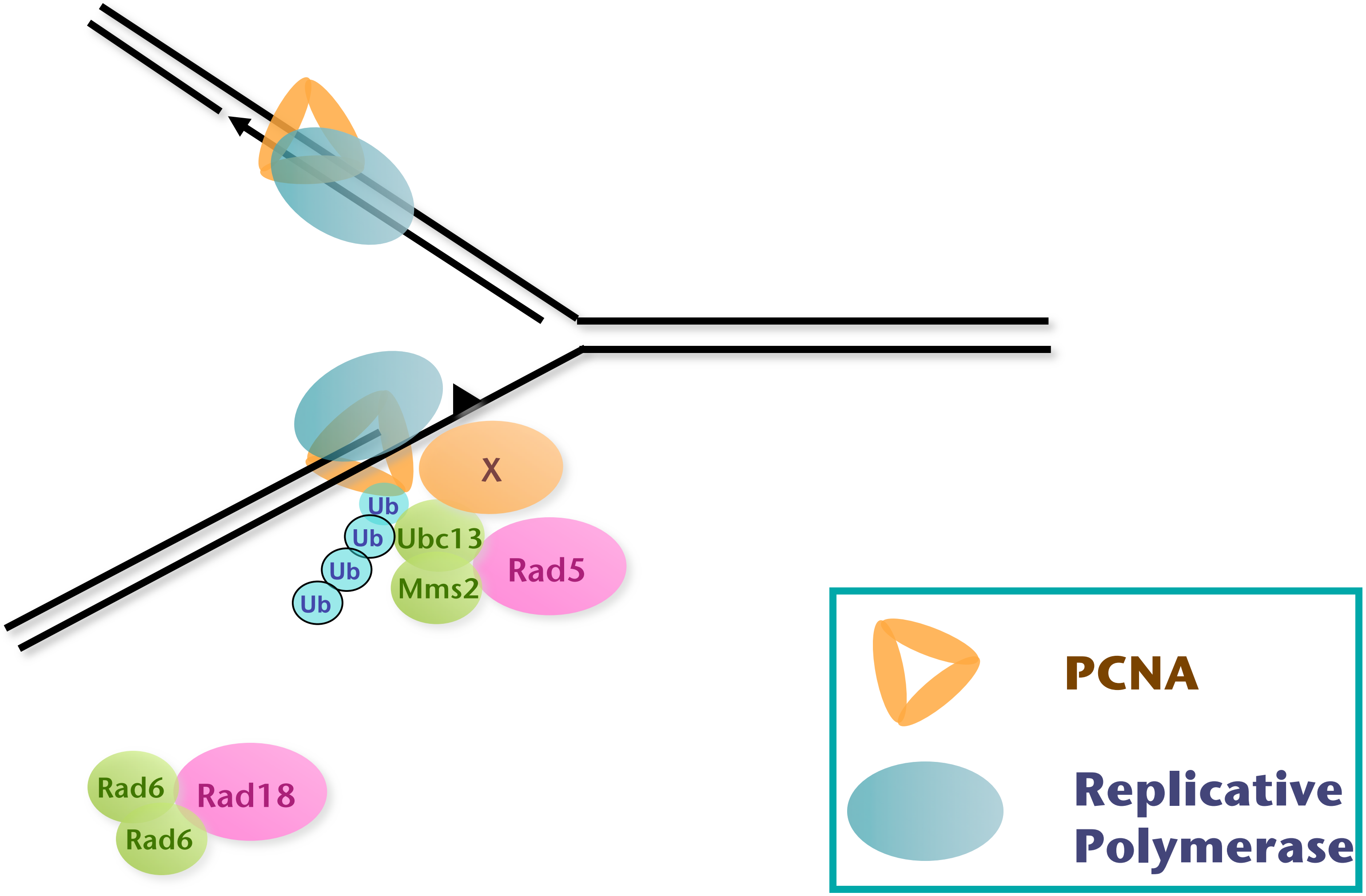

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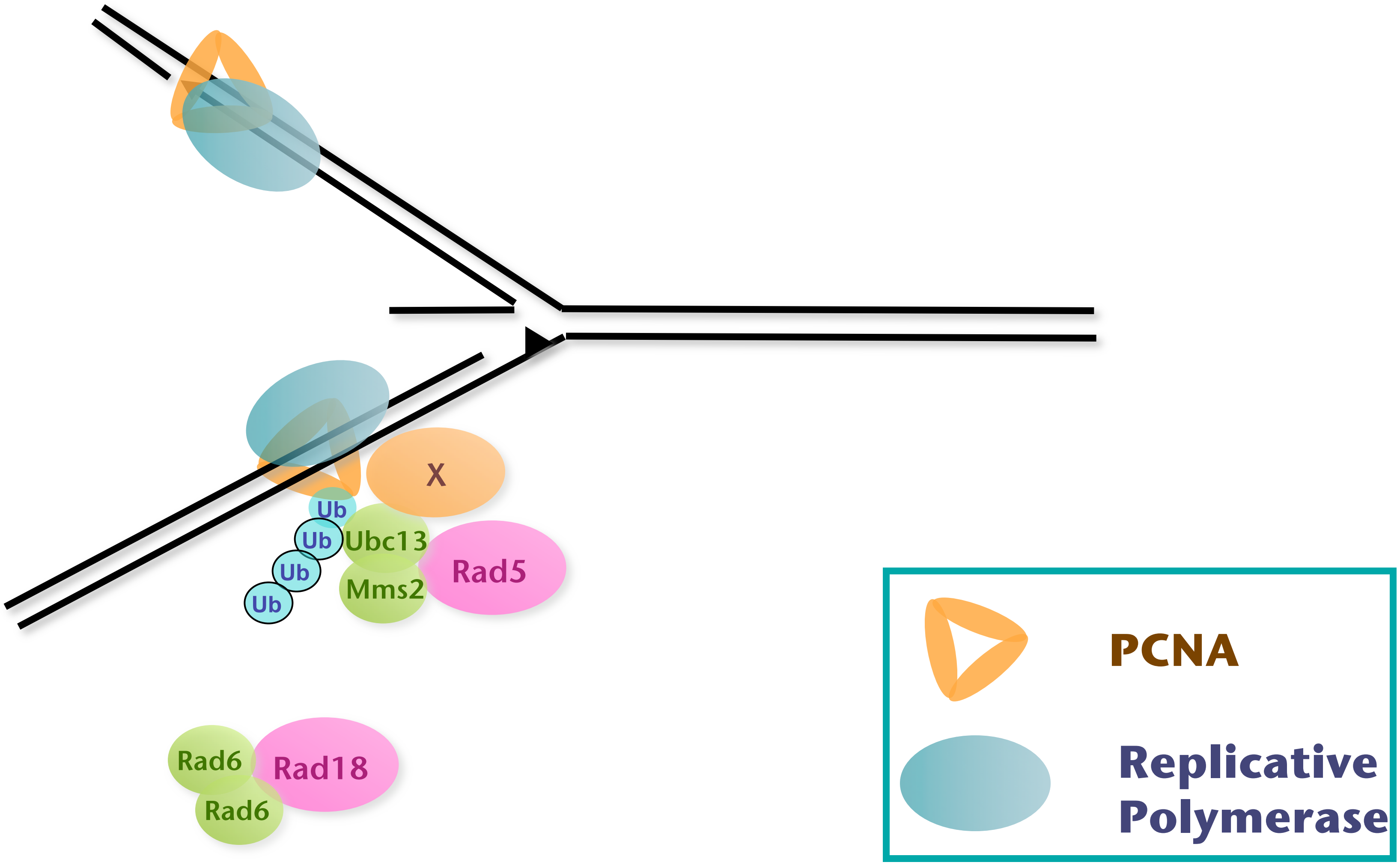

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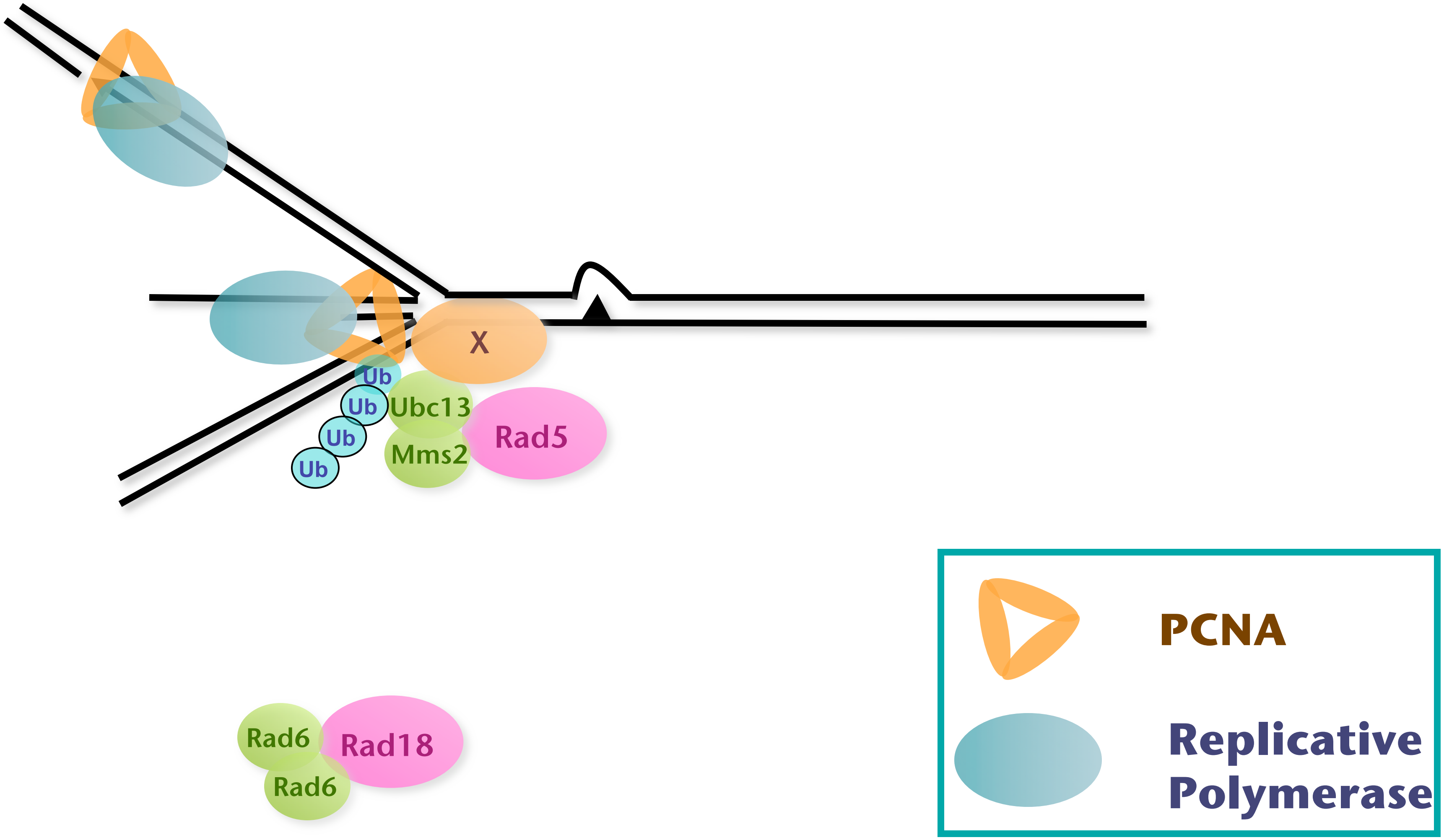

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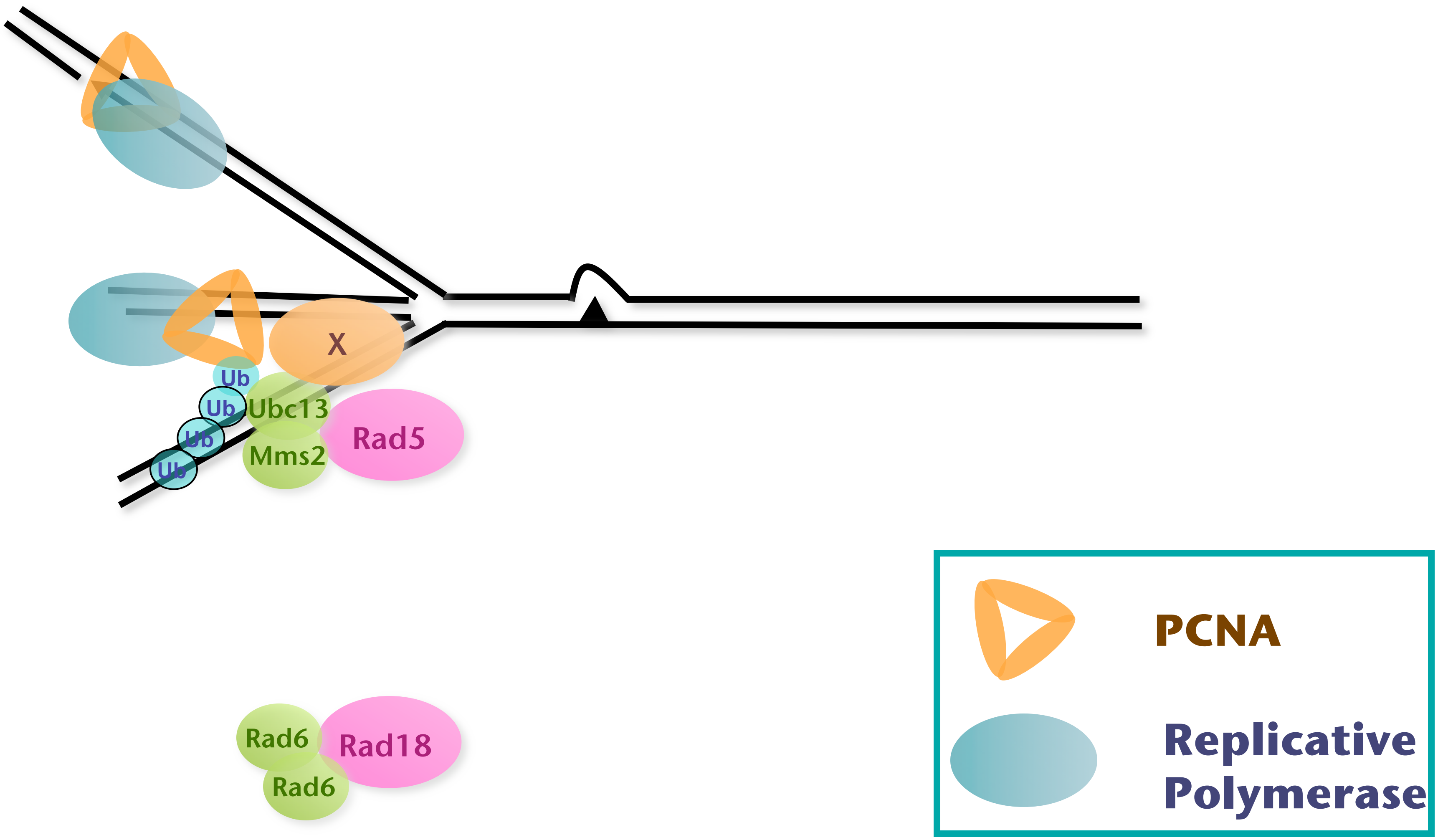

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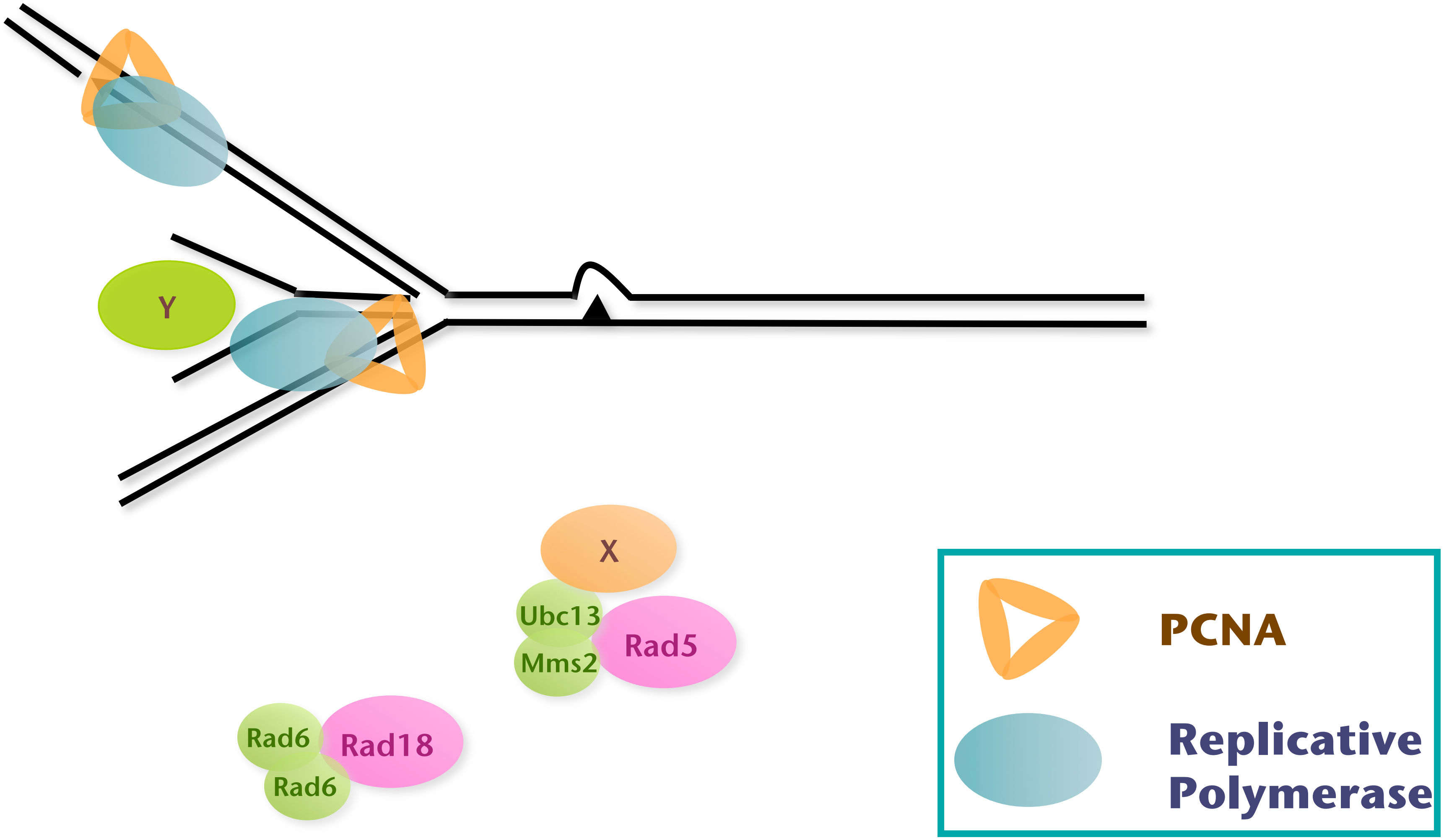

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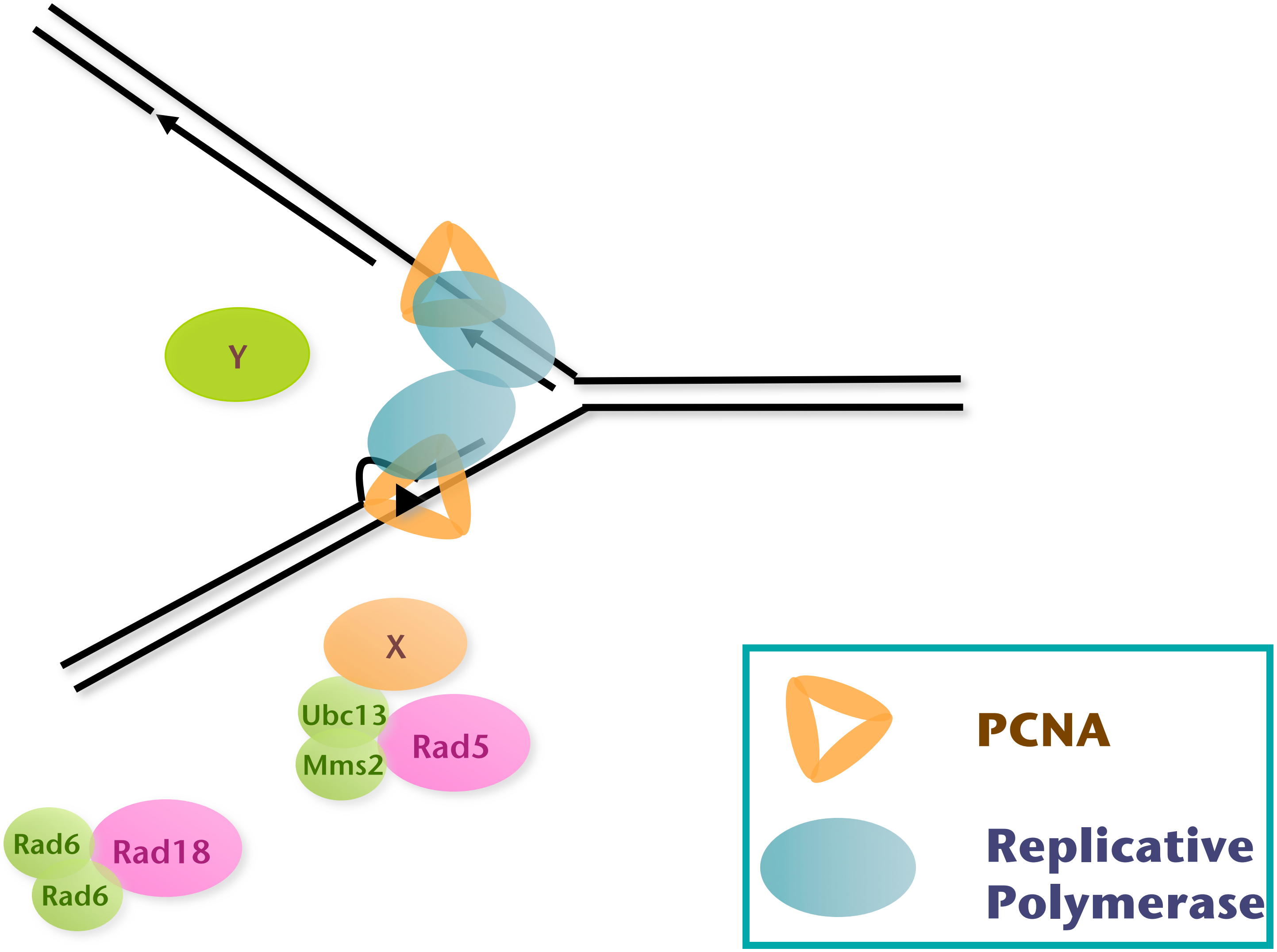
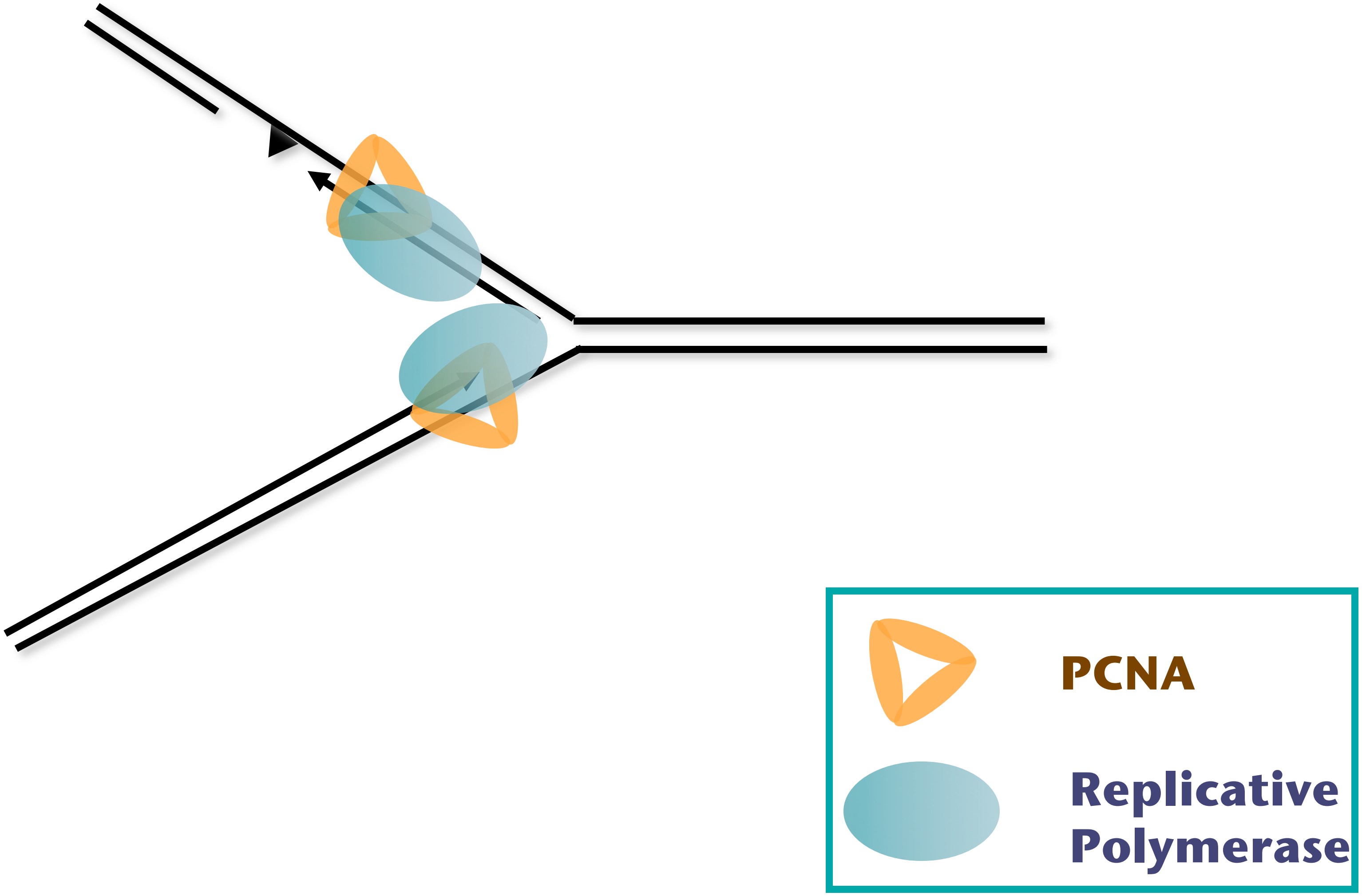


ScRAD5 Pathway: Template Switch (Damage in lagging strand)?



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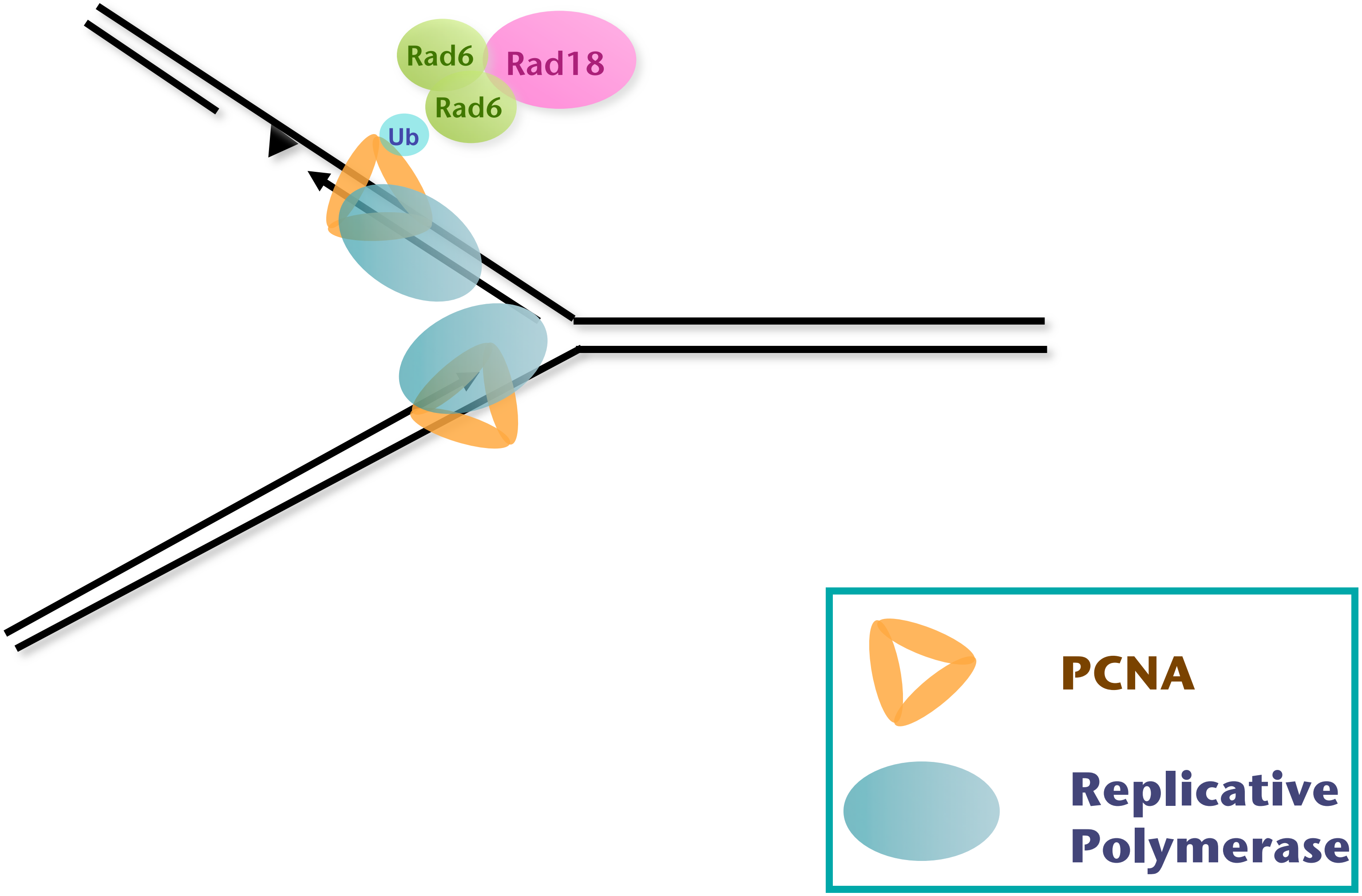

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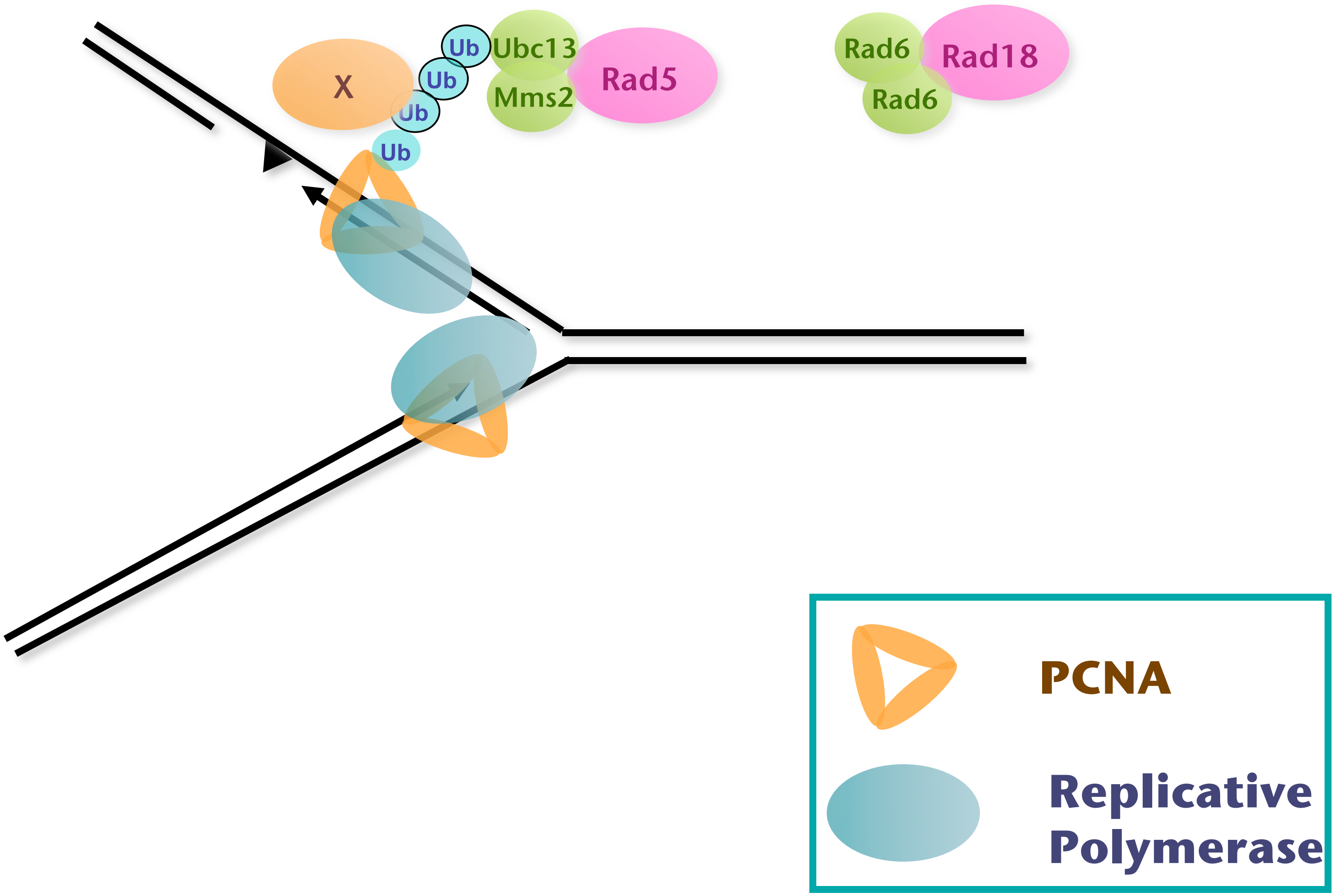

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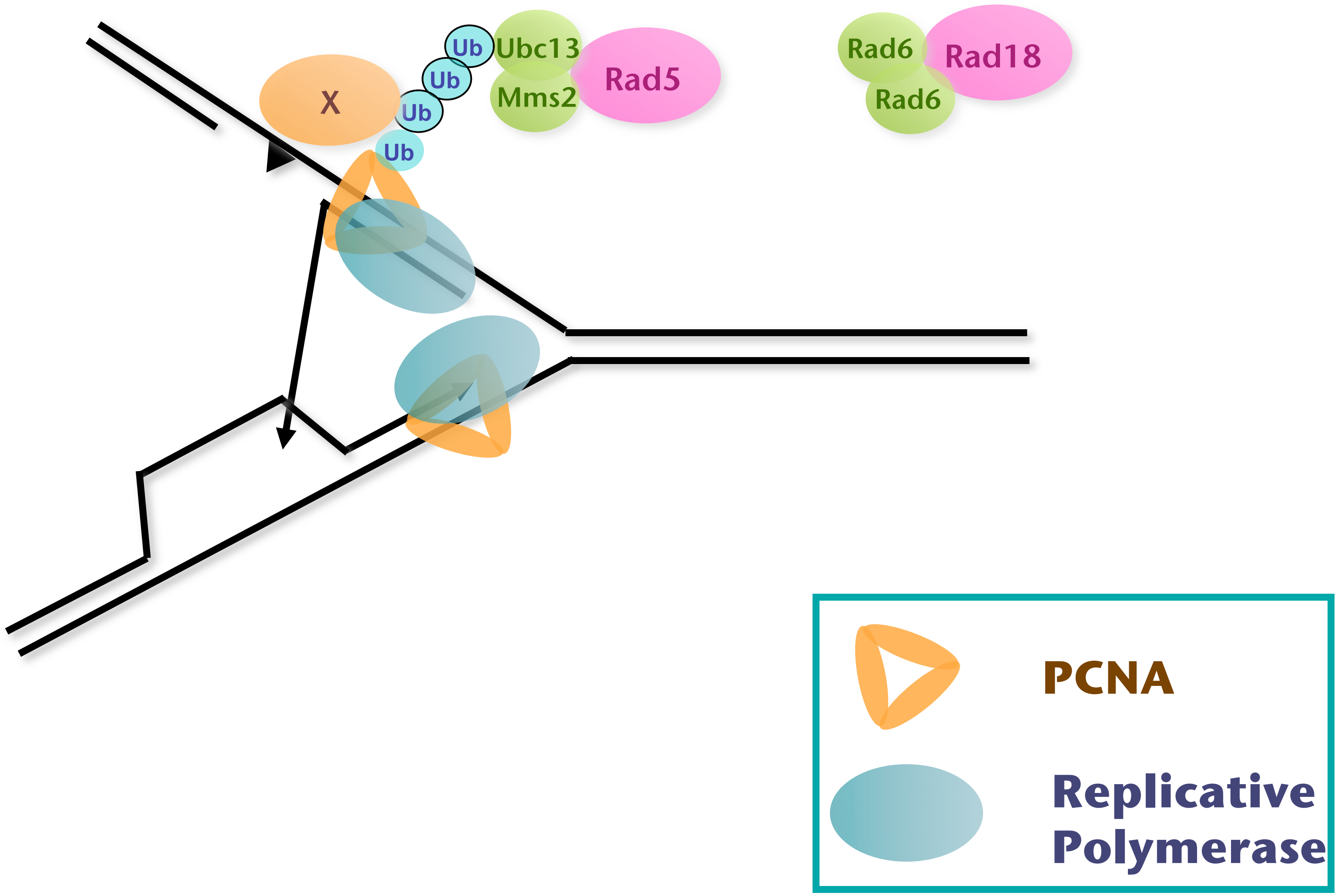

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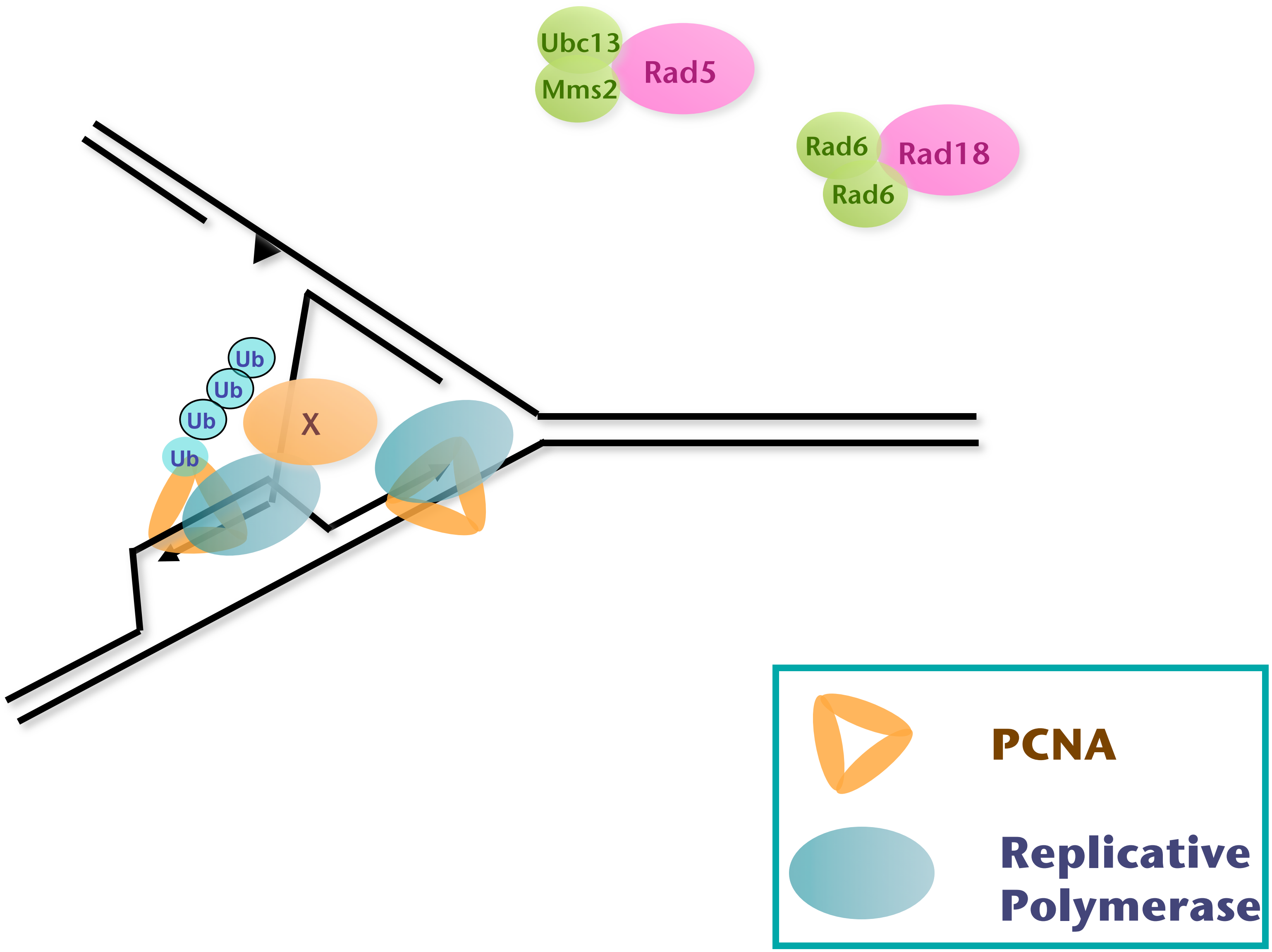

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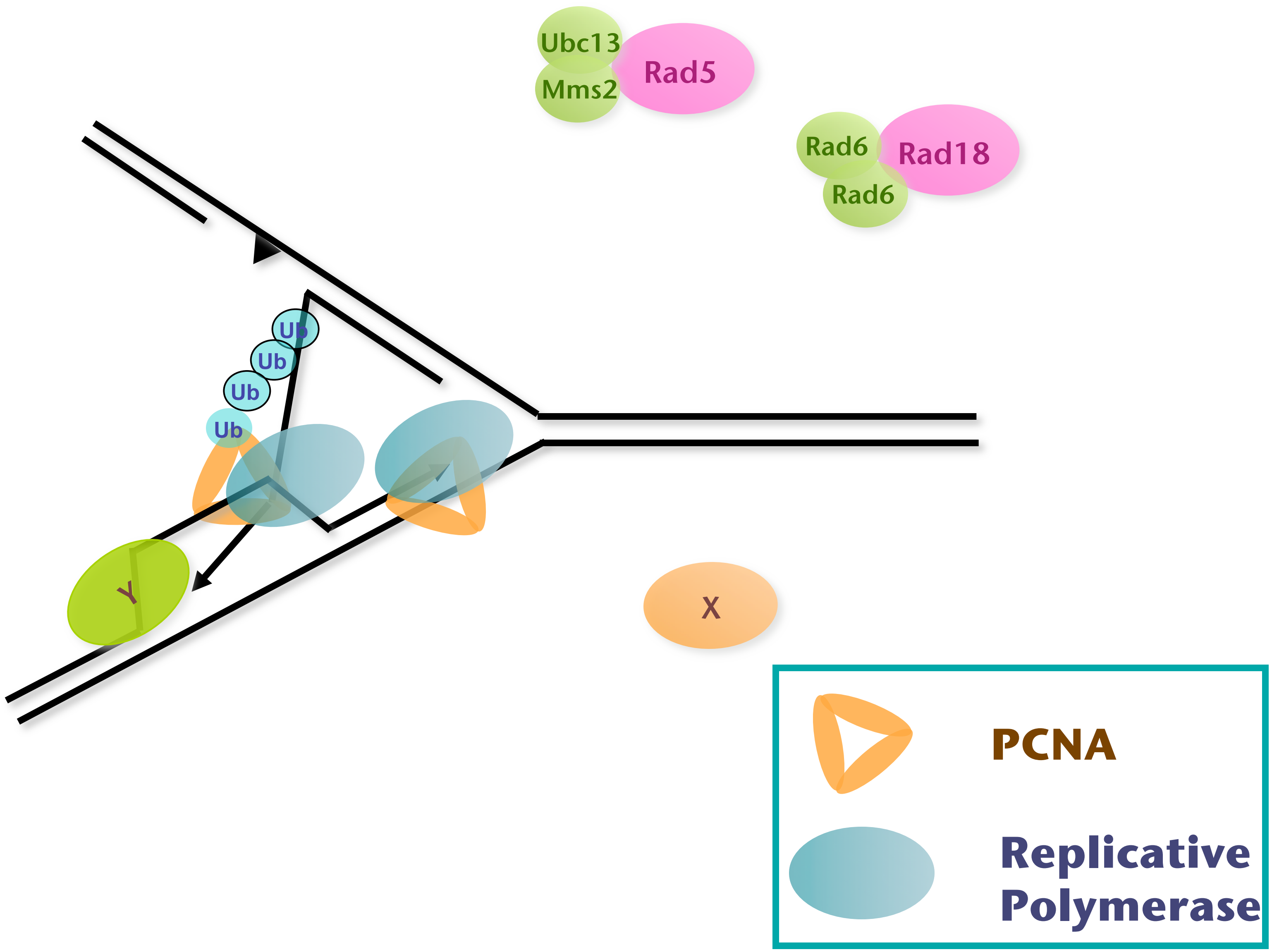

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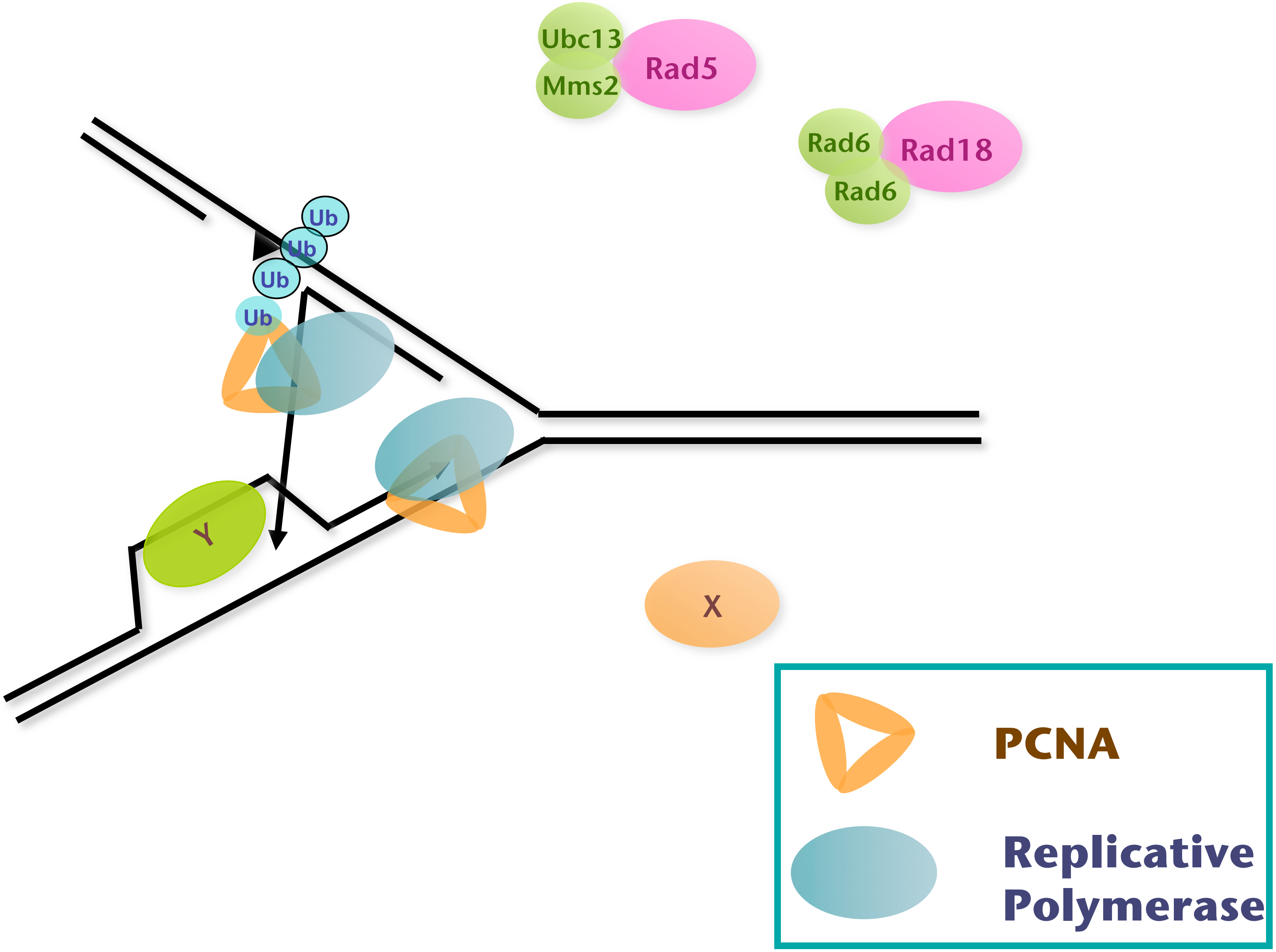

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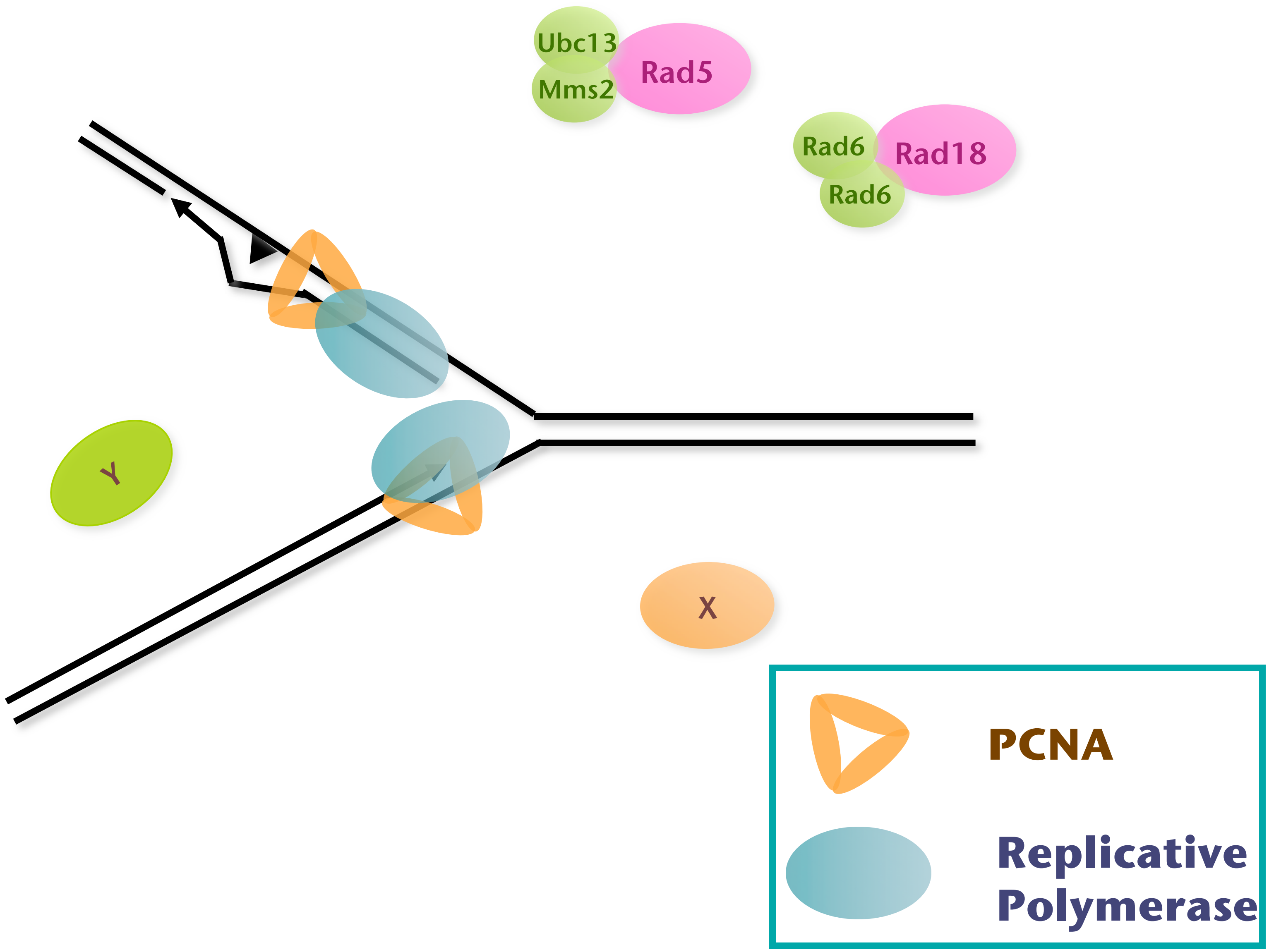
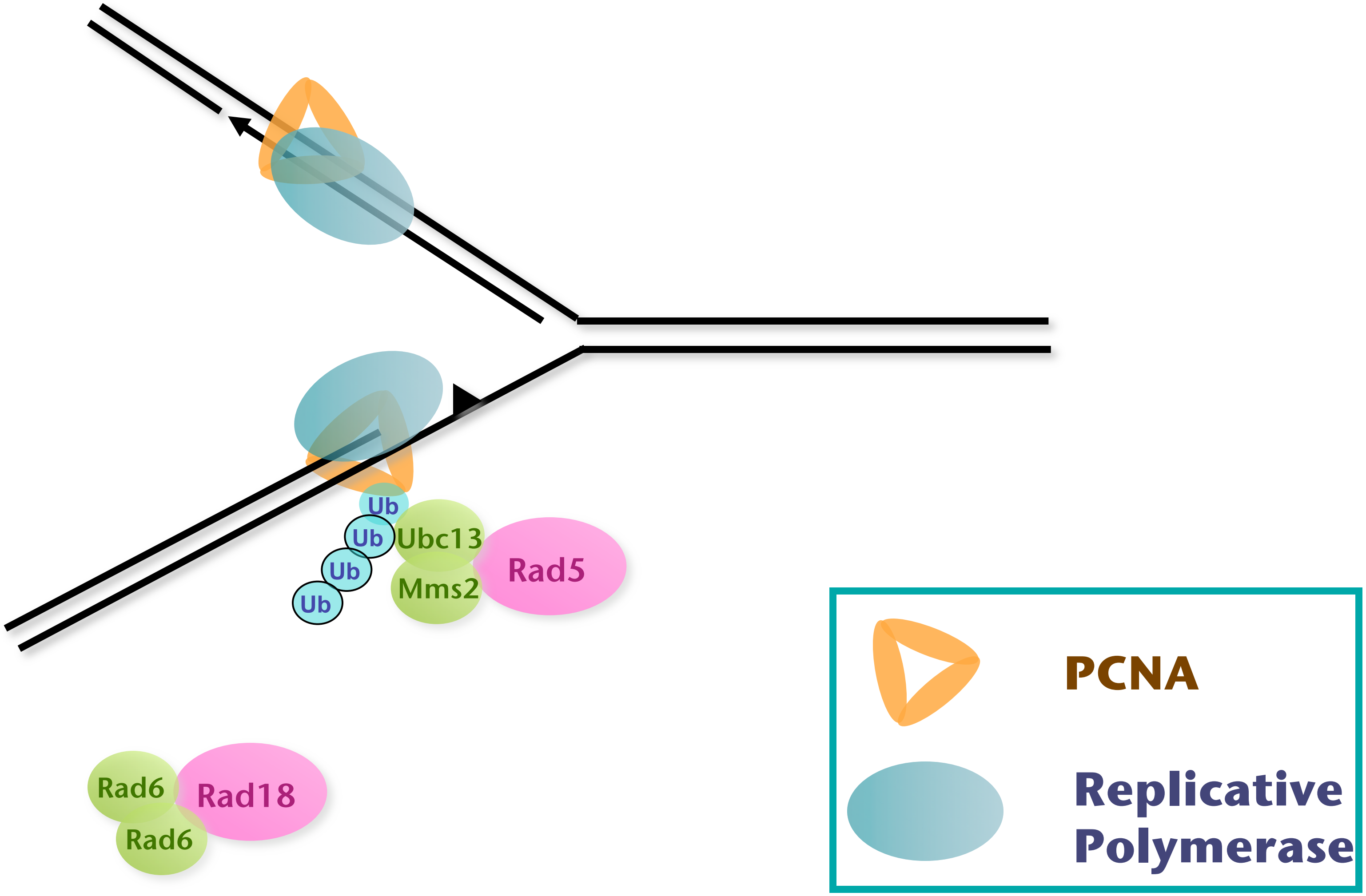


ScRAD5 Pathway: Firing New Origin?



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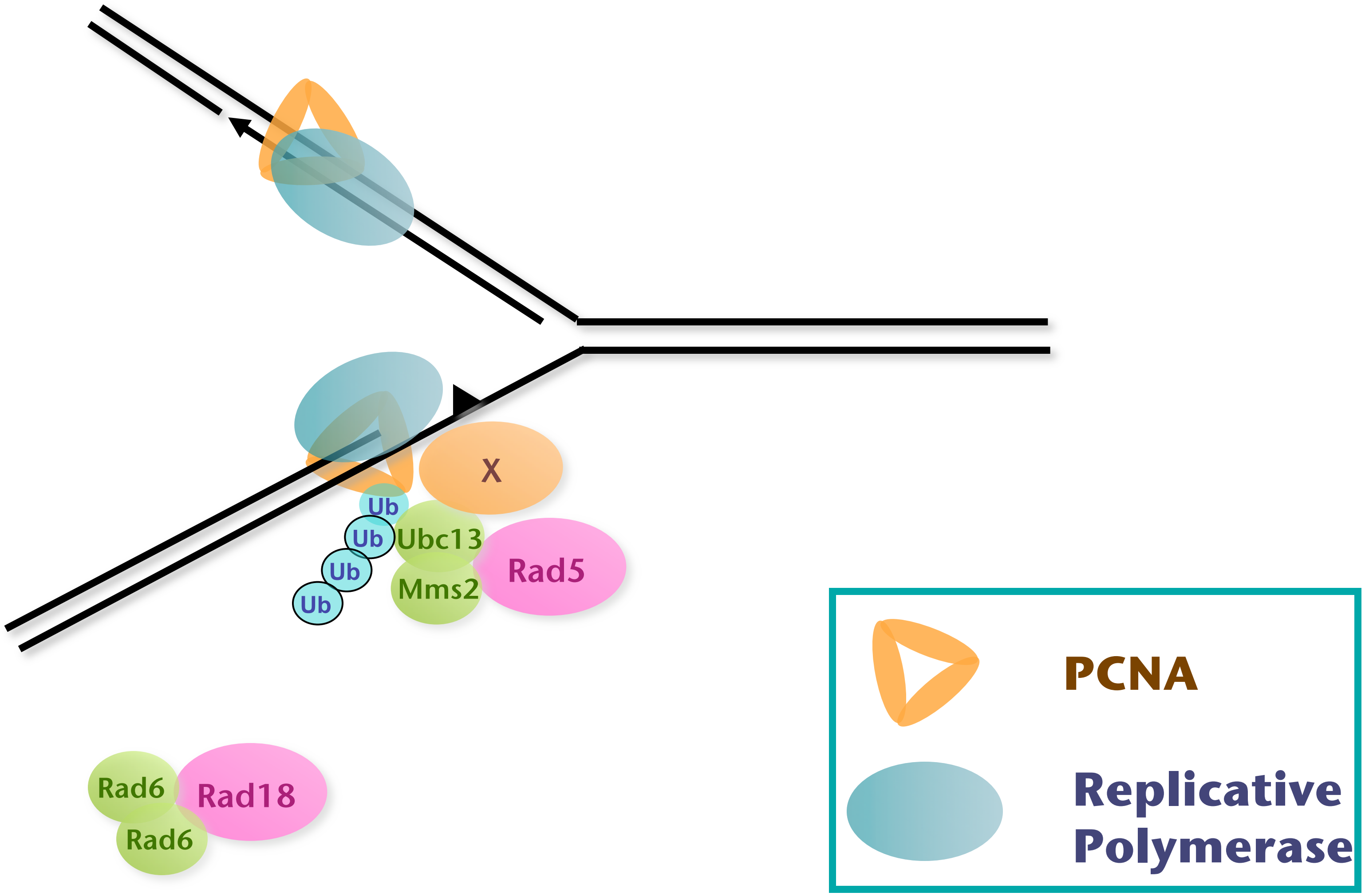

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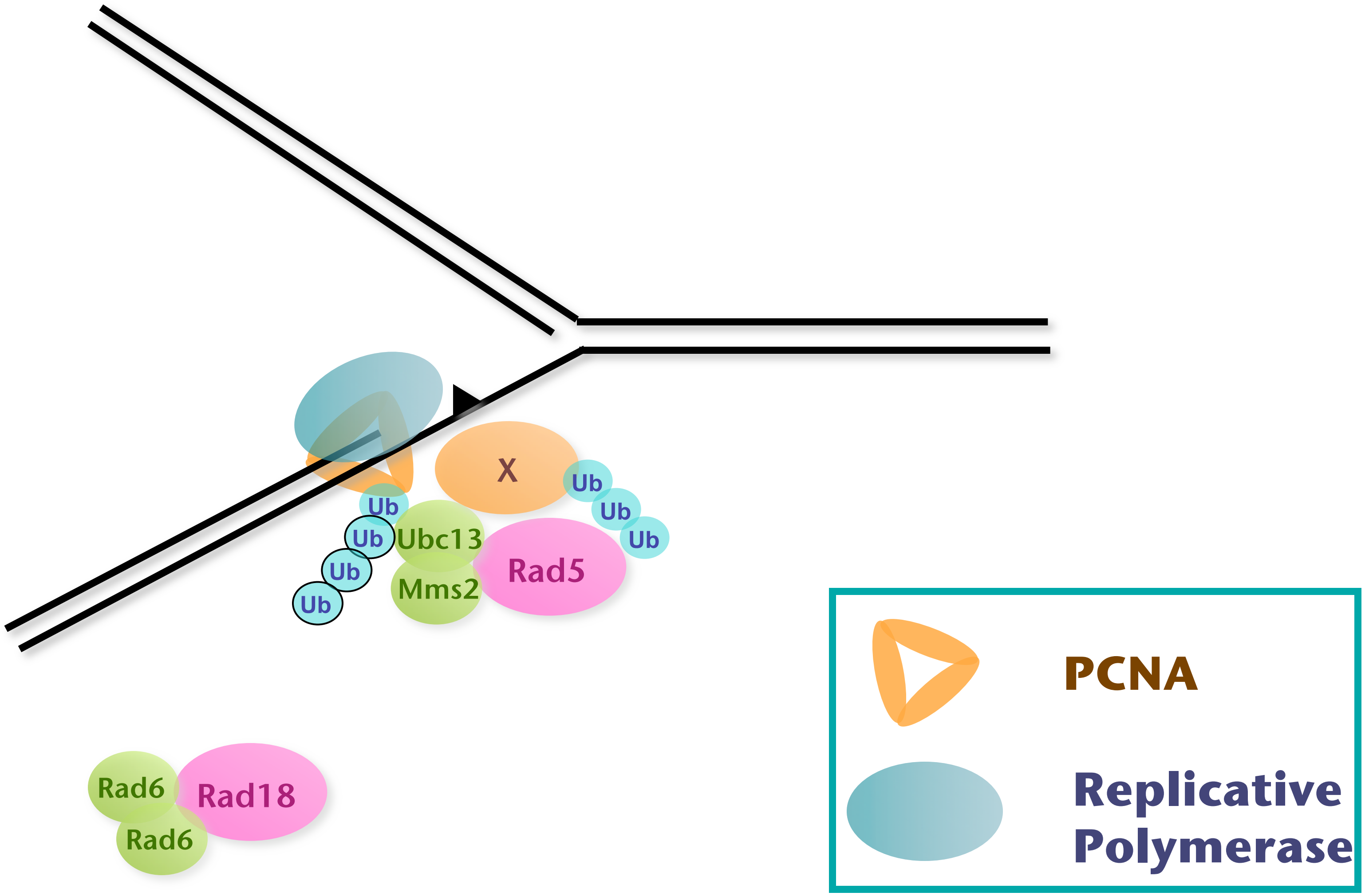

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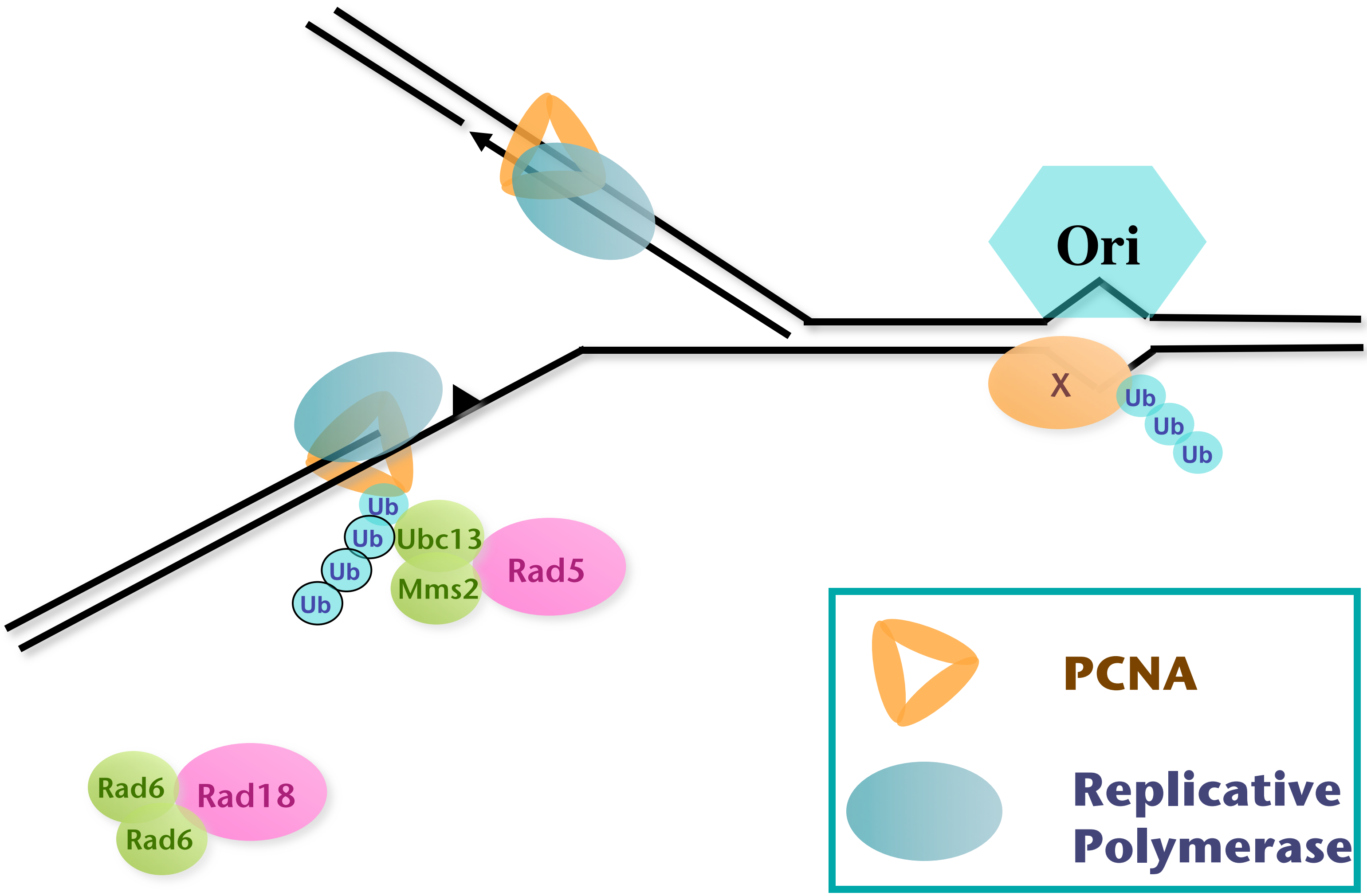

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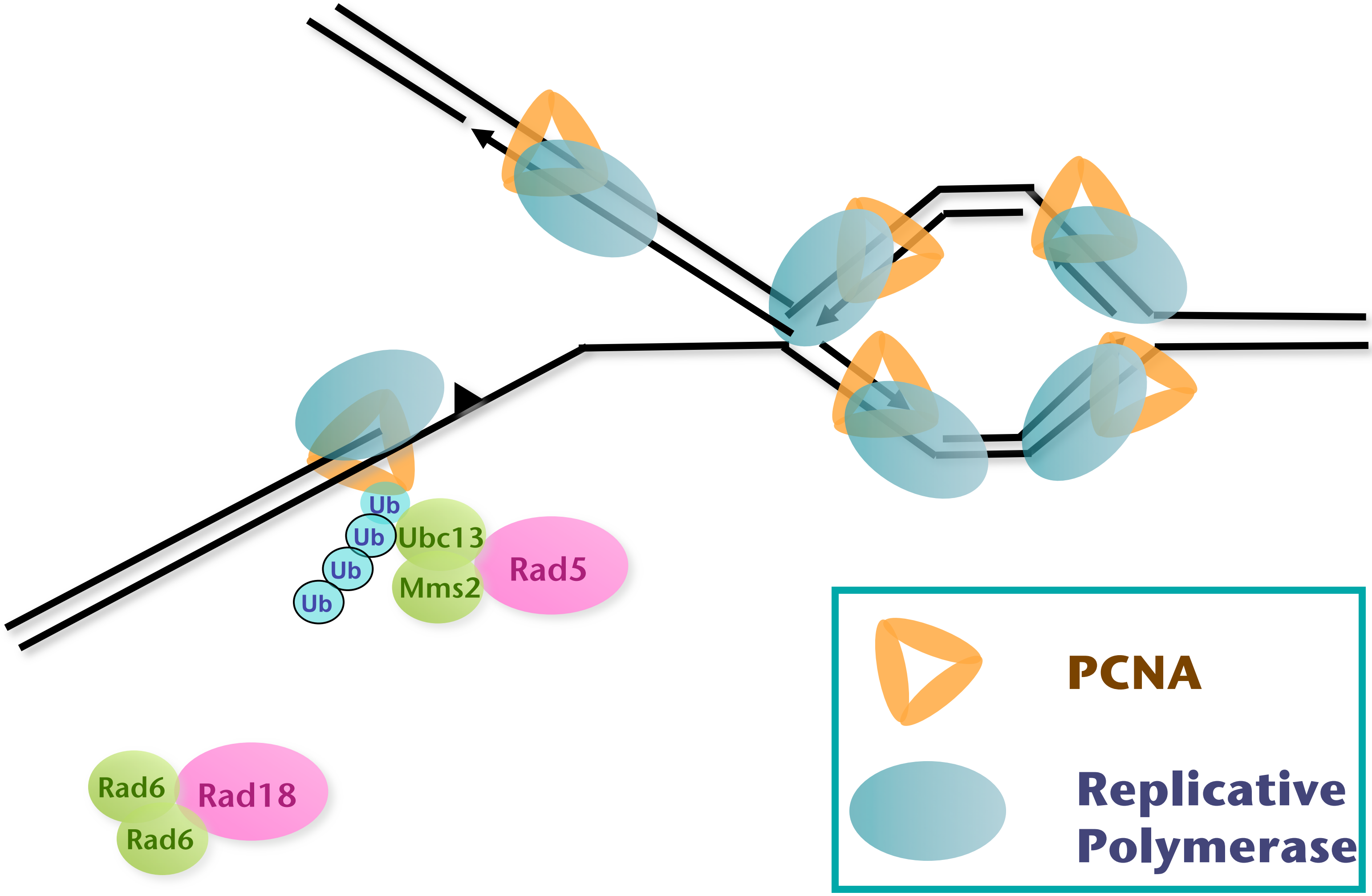

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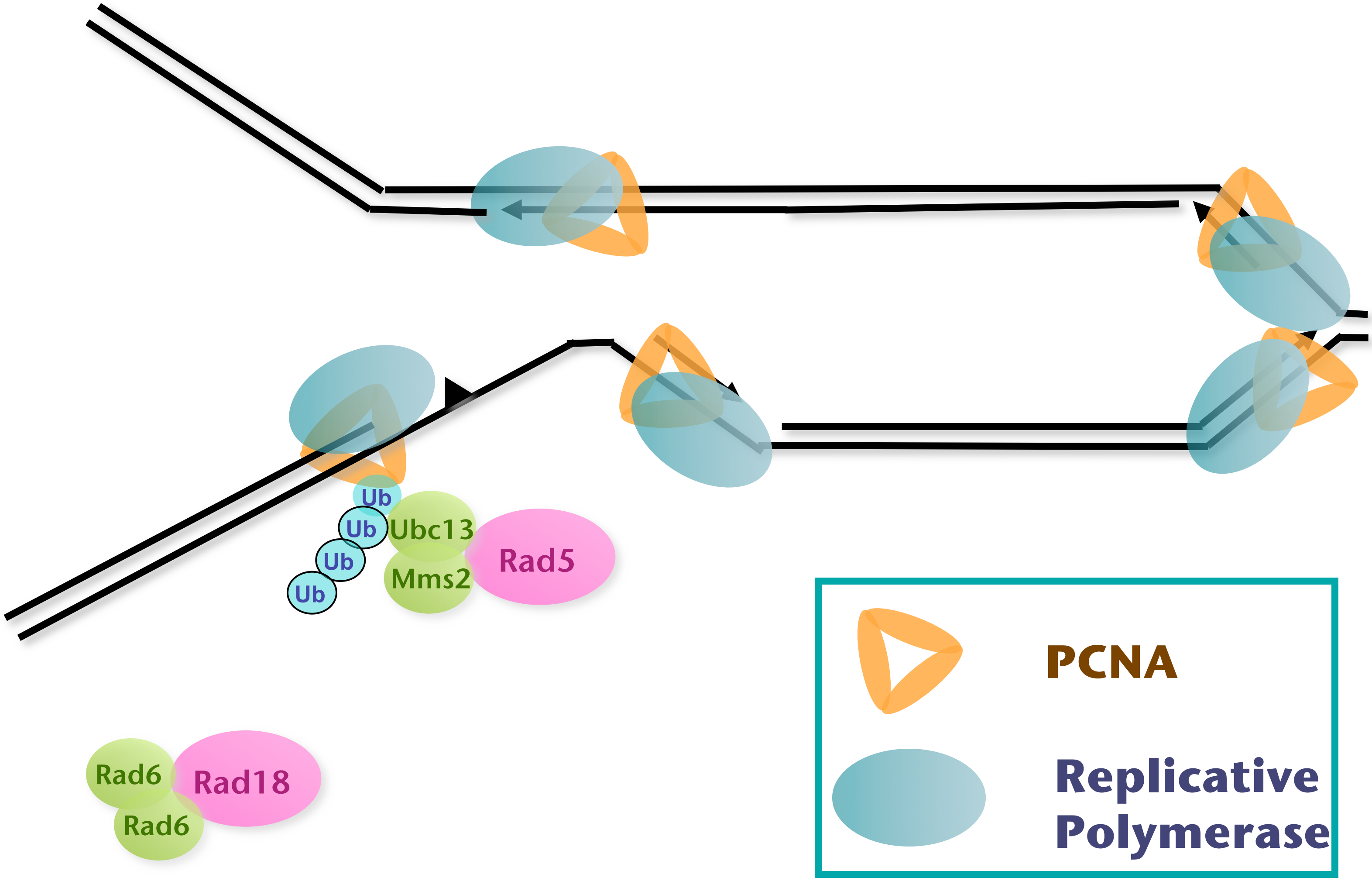

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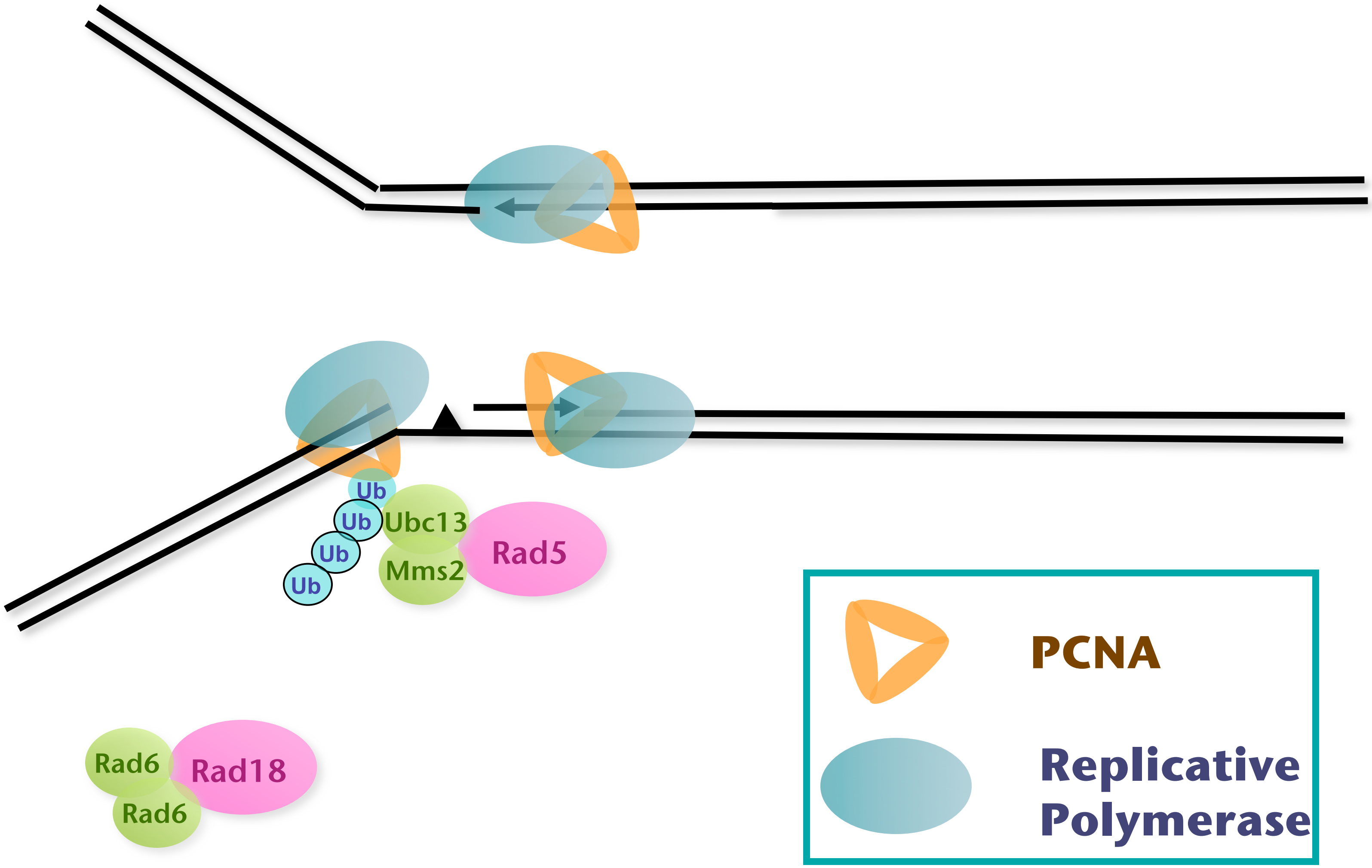

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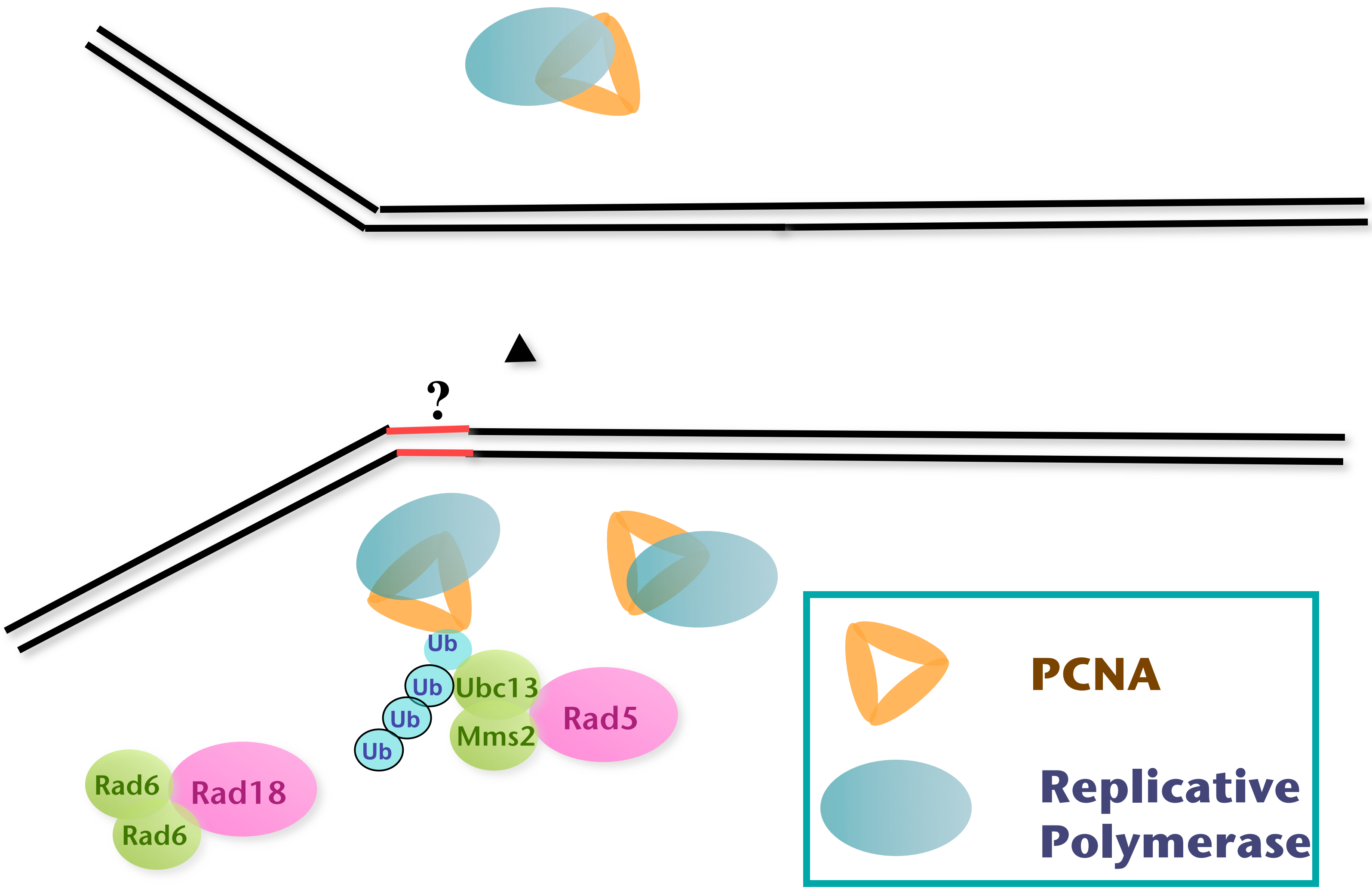
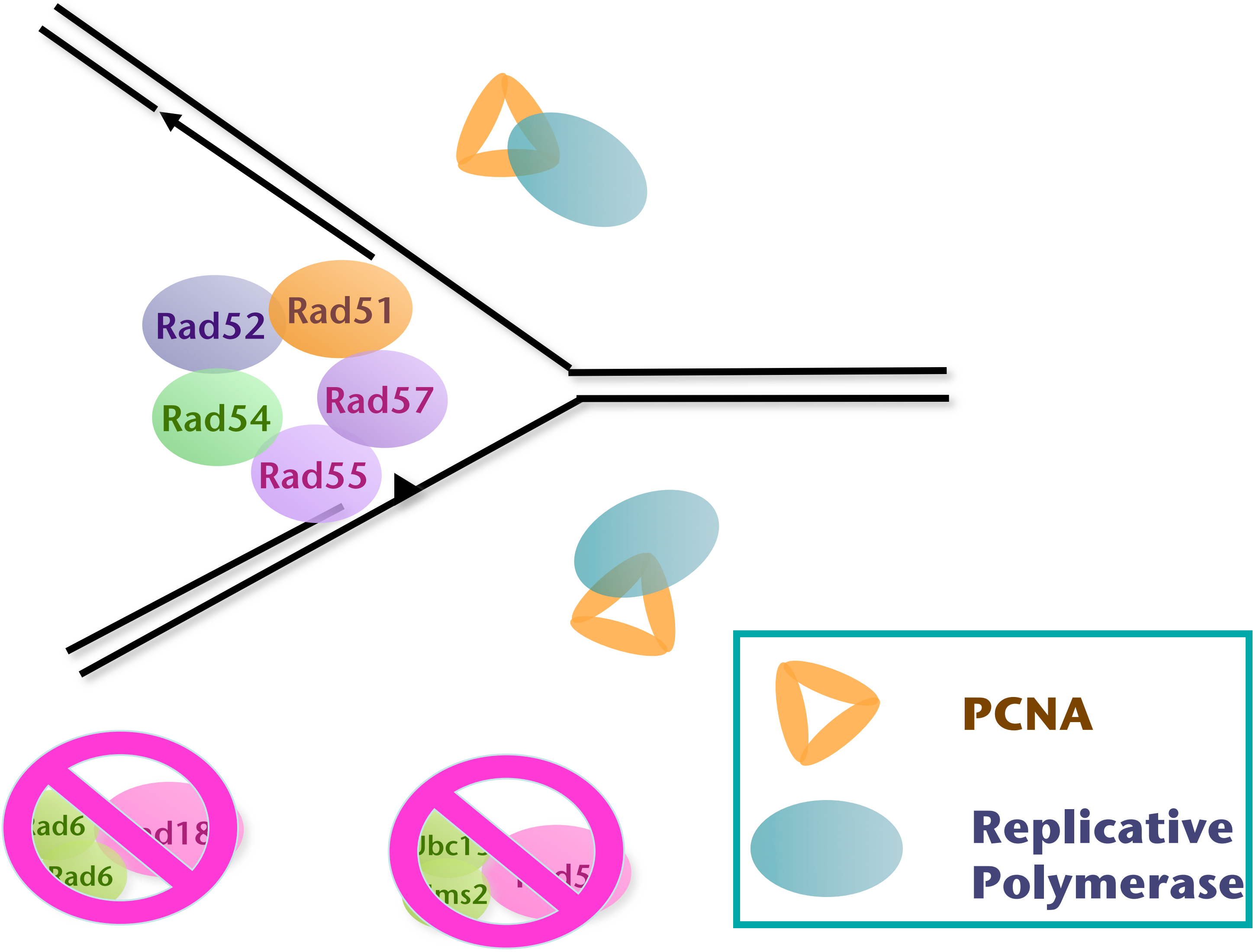


Homologous Recombination Is In Charge in the absence of both TLS and RAD5 Pathways



Defects in proteins functioning in the post-replication repair caused different effects in the rate of GCR formation

	WT	*pif1-m2*
Relevant Genotype	GCR rate (CAN^{r}-5FOA^{r})	GCR rate (CAN^{r}-5FOA^{r})
Wild type	3.5×10^{-10} (1)	4.8×10^{-8} (137)
rad5	2.4×10^{-8} (68)	2.2×10^{-7} (629)
rad18	2.3×10^{-8} (65)	2.5×10^{-7} (714)
rev1	8.3×10^{-10} (2)	6.5×10^{-8} (185)
rev3	6.4×10^{-10} (2)	5.0×10^{-8} (143)
rev7	$>1.2 \times 10^{-9}$ (3)	4.5×10^{-8} (130)
rad30	5.8×10^{-10} (2)	3.7×10^{-8} (106)
pol32	6.5×10^{-10} (2)	6.8×10^{-8} (194)

rad5 rad18: 2.9×10^{-8} (83)

rev1 rev3 rad30: $>3.9 \times 10^{-9}$ (4)

pol32 rev3 rad30: $>1.7 \times 10^{-9}$ (3)

rev3 rev7 rad30: 5.8×10^{-10} (2)

Motegi et al., MCB (2006) 26:1424

rad5 or *rad18* Mutations Interact Checkpoints and *de novo* Telomere Addition Pathways

		WT	*rad5*	*rad18*
Human homolog	Relevant Genotype	GCR rate (CAN^{r}-5FOA^{r})	GCR rate (CAN^{r}-5FOA^{r})	GCR rate (CAN^{r}-5FOA^{r})
	Wild type	3.5×10^{-10} (1)	2.4×10^{-8} (68)	2.3×10^{-8} (65)
ATR →	*mec1 sml1*	4.6×10^{-8} (131)	5.2×10^{-8} (149)	5.8×10^{-8} (166)
ATM →	*tel1*	2.0×10^{-10} (1)	1.4×10^{-9} (4)	3.0×10^{-9} (9)
Telomerase →	*est2*	1.2×10^{-10} (0.3)	5.9×10^{-10} (2)	$>3.4 \times 10^{-10}$ (1)
Telomerase inhibitor →	*pif1-m2*	4.8×10^{-8} (137)	2.2×10^{-7} (629)	2.5×10^{-7} (714)
	yku70	1.4×10^{-9} (4)	1.2×10^{-9} (3)	5.5×10^{-9} (16)

rad5 or *rad18* Mutations Interact Checkpoints and *de novo* Telomere Addition Pathways

		WT	*rad5*	*rad18*
	Relevant Genotype	GCR rate (CAN^r-$5FOA^r$)	GCR rate (CAN^r-$5FOA^r$)	GCR rate (CAN^r-$5FOA^r$)
Human homolog	Wild type	3.5×10^{-10} (1)	2.4×10^{-8} (68)	2.3×10^{-8} (65)
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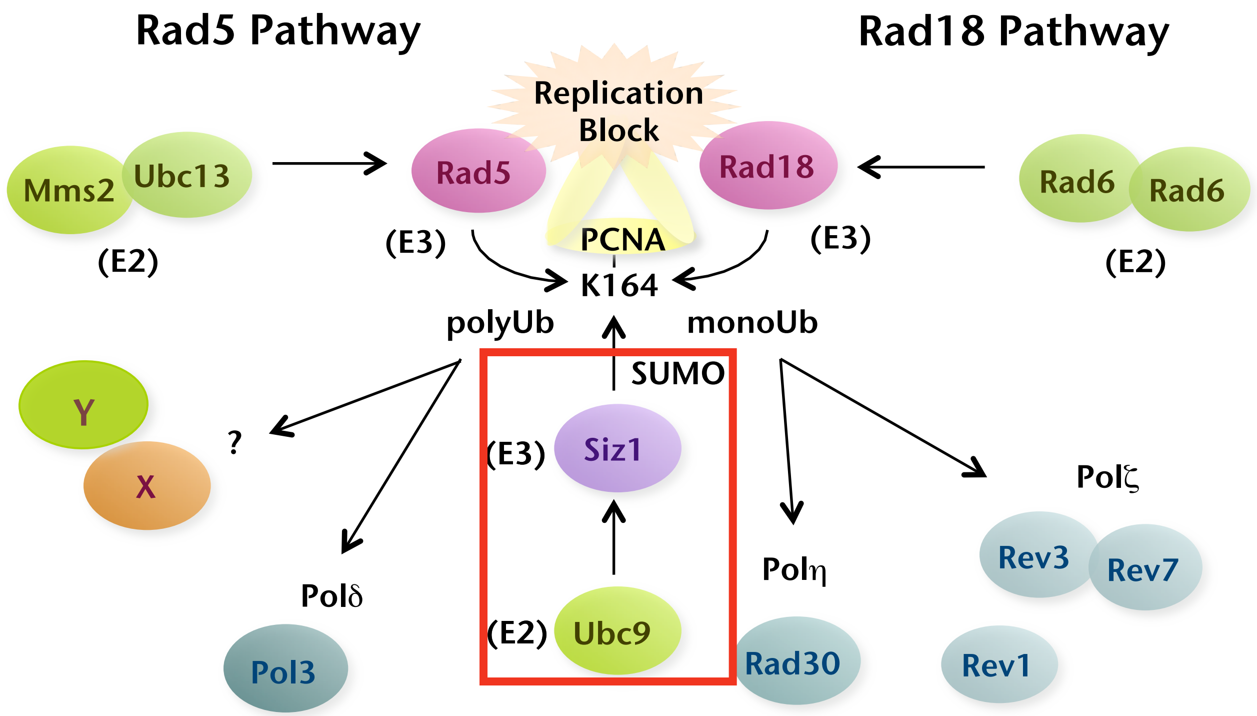
ATM →	*tel1*	2.0×10^{-10} (1)	1.4×10^{-9} (4)	3.0×10^{-9} (9)
Telomerase →	*est2*	1.2×10^{-10} (0.3)	5.9×10^{-10} (2)	$>3.4 \times 10^{-10}$ (1)
Telomerase inhibitor →	*pif1-m2*	4.8×10^{-8} (137)	2.2×10^{-7} (629)	2.5×10^{-7} (714)
	yku70	1.4×10^{-9} (4)	1.2×10^{-9} (3)	5.5×10^{-9} (16)

The *pol30-119* (K164R) PCNA did NOT Enhance The GCR Formation

<u>Genotype</u>	<u>GCR Rate</u>
Wild type	3.5×10^{-10} (1)
pol30-119 (K164R)	1.0×10^{-9} (3)
rad5	2.4×10^{-8} (68)
rad5 pol30-119 (K164R)	8.3×10^{-10} (2)
rad18	2.3×10^{-8} (65)
rad18 pol30-119 (K164R)	8.4×10^{-10} (2)

* *POL30* : Gene Name of PCNA in *Saccharomyces cerevisiae*

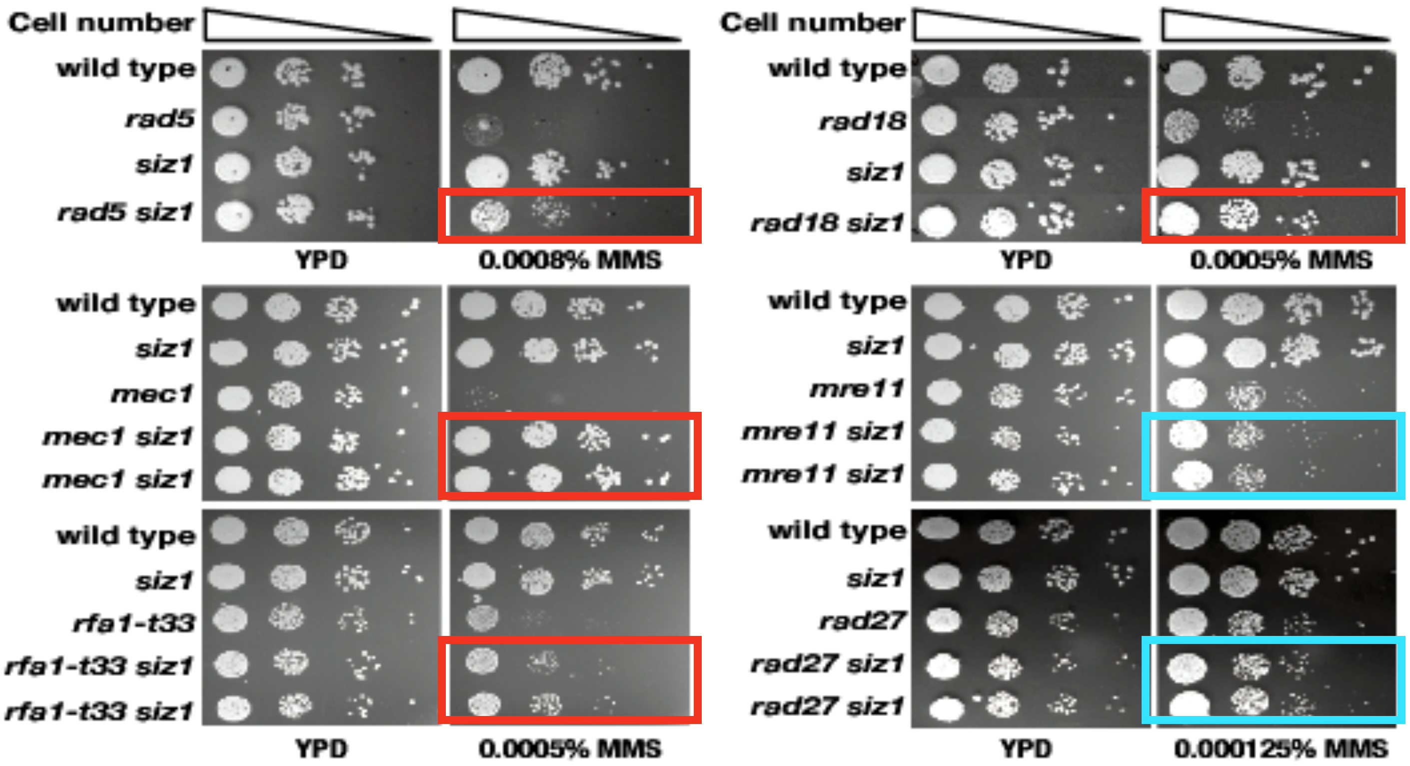
There is Another E3 Ligase Modifying PCNA K164



The *siz1* Mutation Reduced the GCR Rates Enhanced by Subset of GCR Mutator Gene Mutations

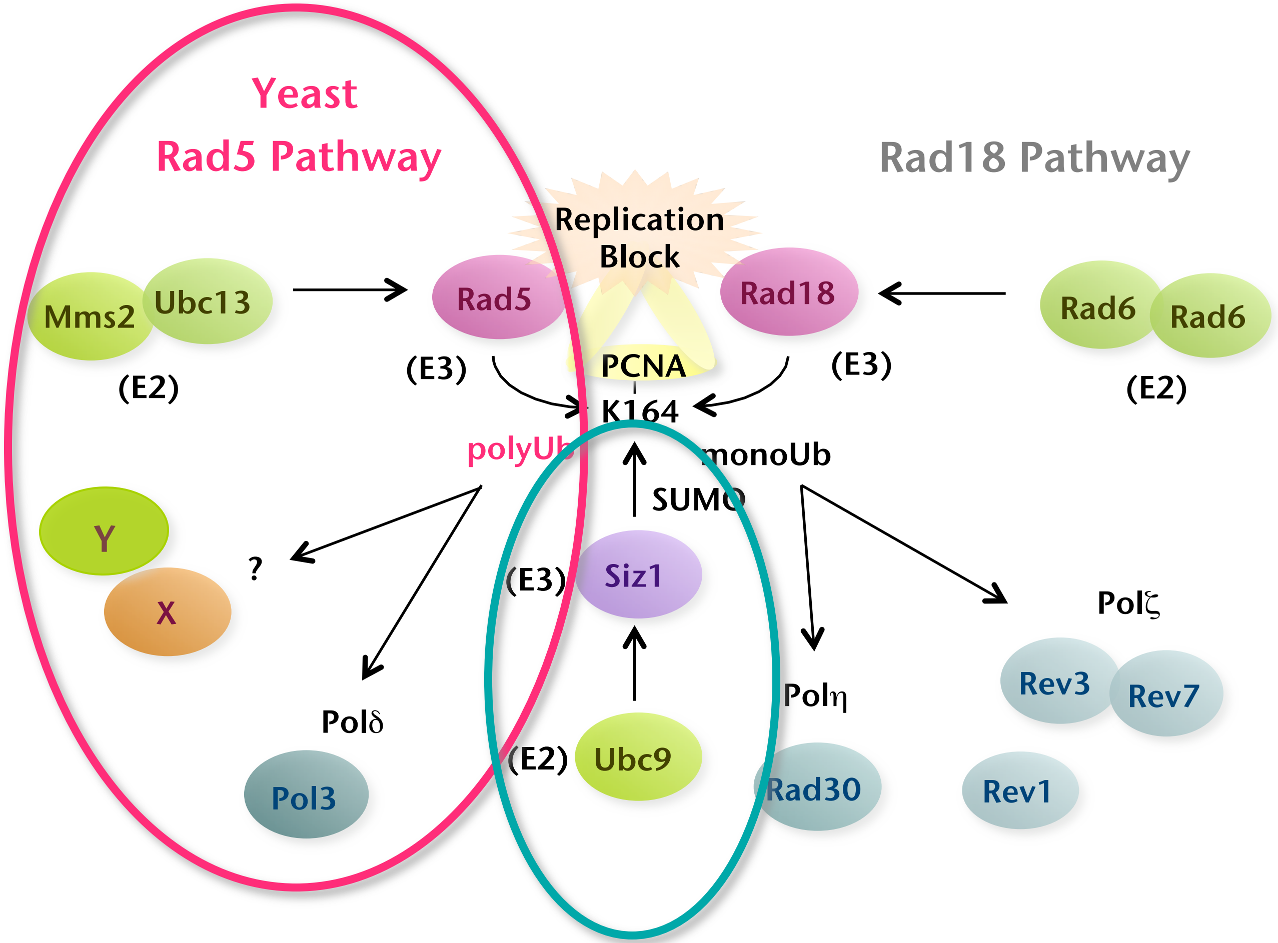
	WT	*siz1*
Relevant Genotype	GCR rate (CAN^r-$5FOA^r$)	GCR rate (CAN^r-$5FOA^r$)
Wild type	3.5×10^{-10} (1)	1.3×10^{-9} (4)
rad5	2.4×10^{-8} (68)	1.1×10^{-9} (3)
rad18	2.3×10^{-8} (65)	$>1.2 \times 10^{-9}$ (4)
mec1 sml1	4.6×10^{-8} (131)	1.1×10^{-9} (3)
rfa1-t33	2.7×10^{-7} (771)	1.3×10^{-7} (377)
rad27	3.4×10^{-7} (971)	4.3×10^{-7} (1229)
mre11	2.2×10^{-7} (629)	1.8×10^{-8} (514)
pif1-m2	4.8×10^{-8} (137)	3.7×10^{-8} (107)

Rescue of MMS Sensitivities in Different GCR Mutator Strains Showed Similar Genetic Interactions with GCR Suppression



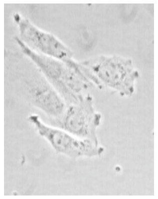
Yeast *RAD5* Pathway Suppresses Gross Chromosomal Rearrangements

GCR Suppression

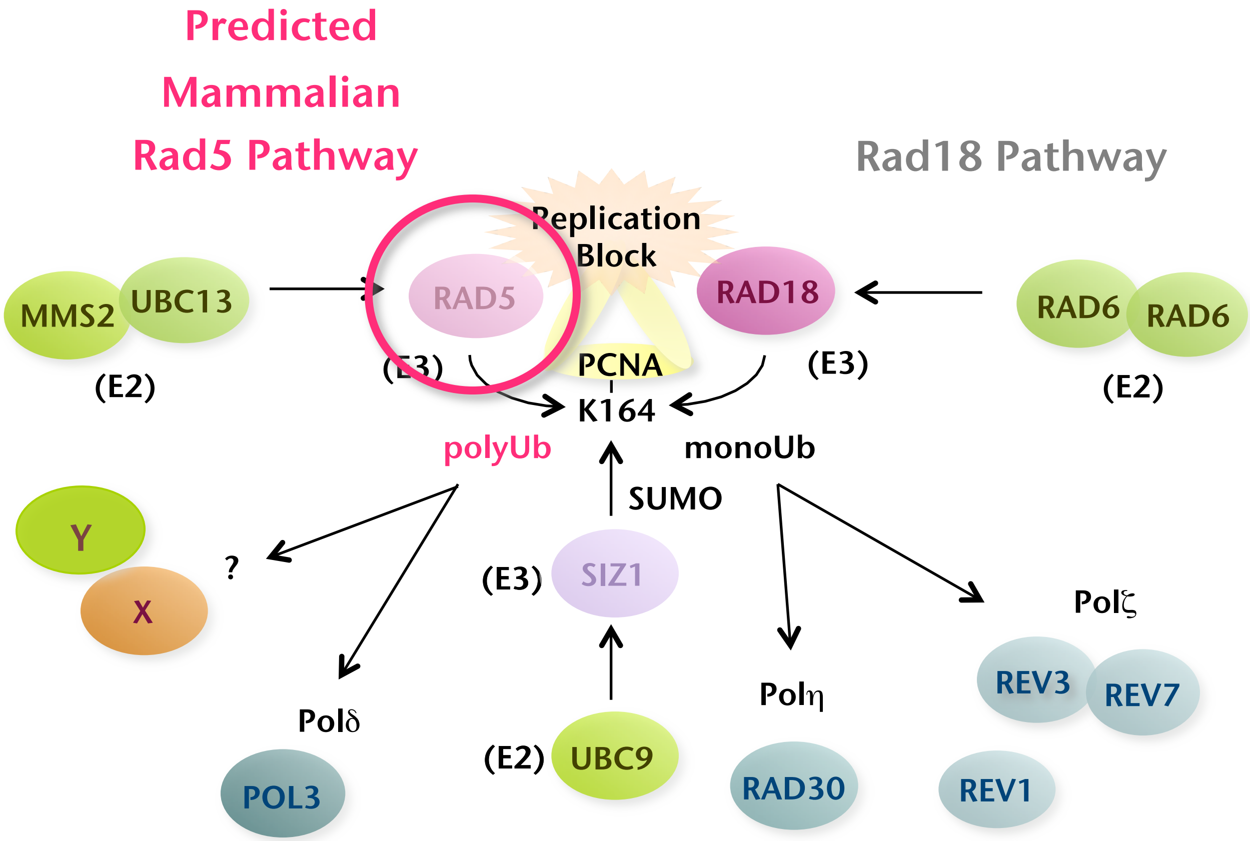


GCR Promotion

Motegi et al., MCB (2006) 26:1424



Rad5 Has been a Missing Piece in Mammalian System



Predicted
Mammalian
Rad5 Pathway

Rad18 Pathway

Replication
Block

MMS2
UBC13
(E2)

RAD5
(E3)

PCNA
K164

RAD18
(E3)

RAD6
RAD6
(E2)

polyUb

monoUb

SUMO

Y
X
?

Polδ
POL3

(E3) SIZ1

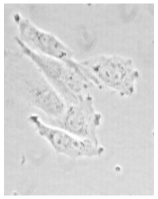
(E2) UBC9

Polη
RAD30

Polζ
REV3
REV7

REV1

* Candidate search in the databases did not identify a clear RAD5 homolog.

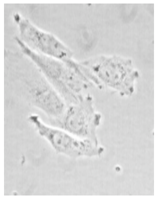


Search for Ortholg of Yeast RAD5

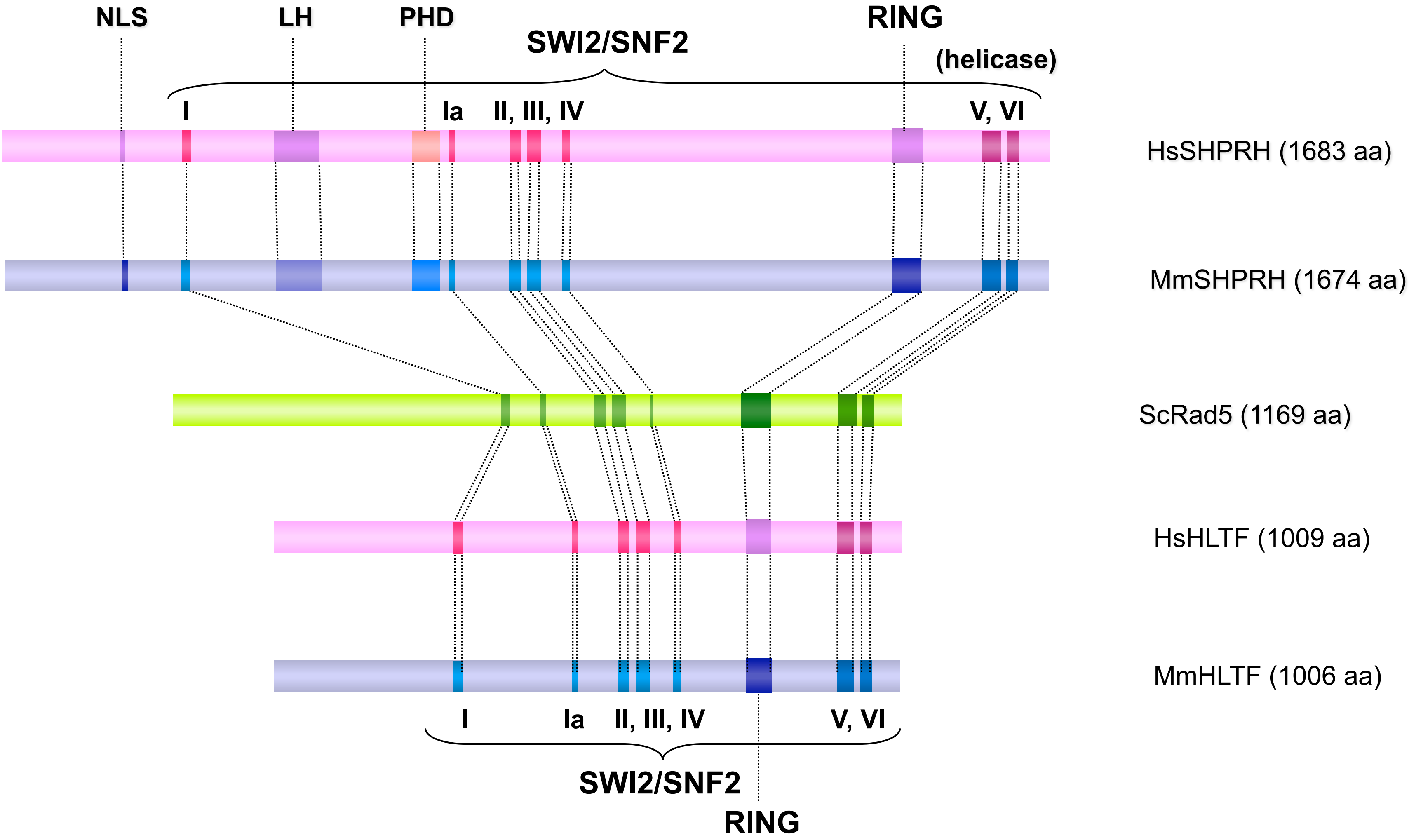
Regular Blast: None

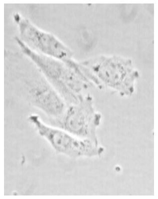
Domain Search
(SMART search: http://smart.embl-heidelberg.de/)
Query: RING and DEXDc (SWI2/SNF2 subdomains I, Ia, II, III, and IV)
& RING and HELICc (SWI2/SNF2 subdomains V and VI)

SHPRH : Four Mutations in Cancer Cells
HLTF : Silenced in many tumors by promoter
methylation



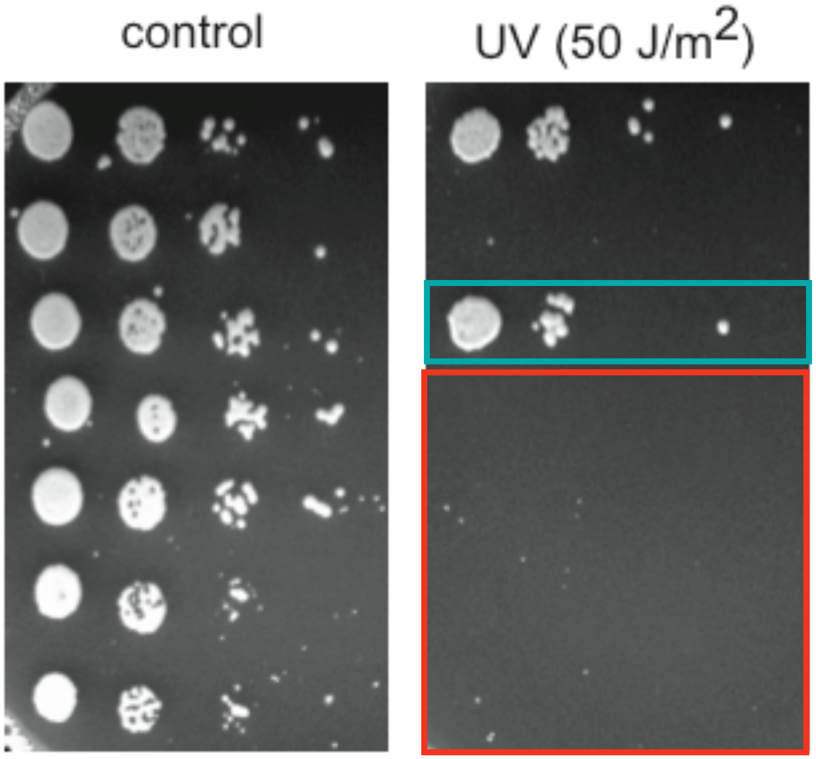
SHPRH and HLTF Share Domain Structure with ScRAD5

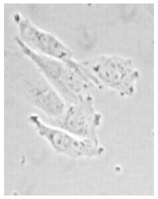




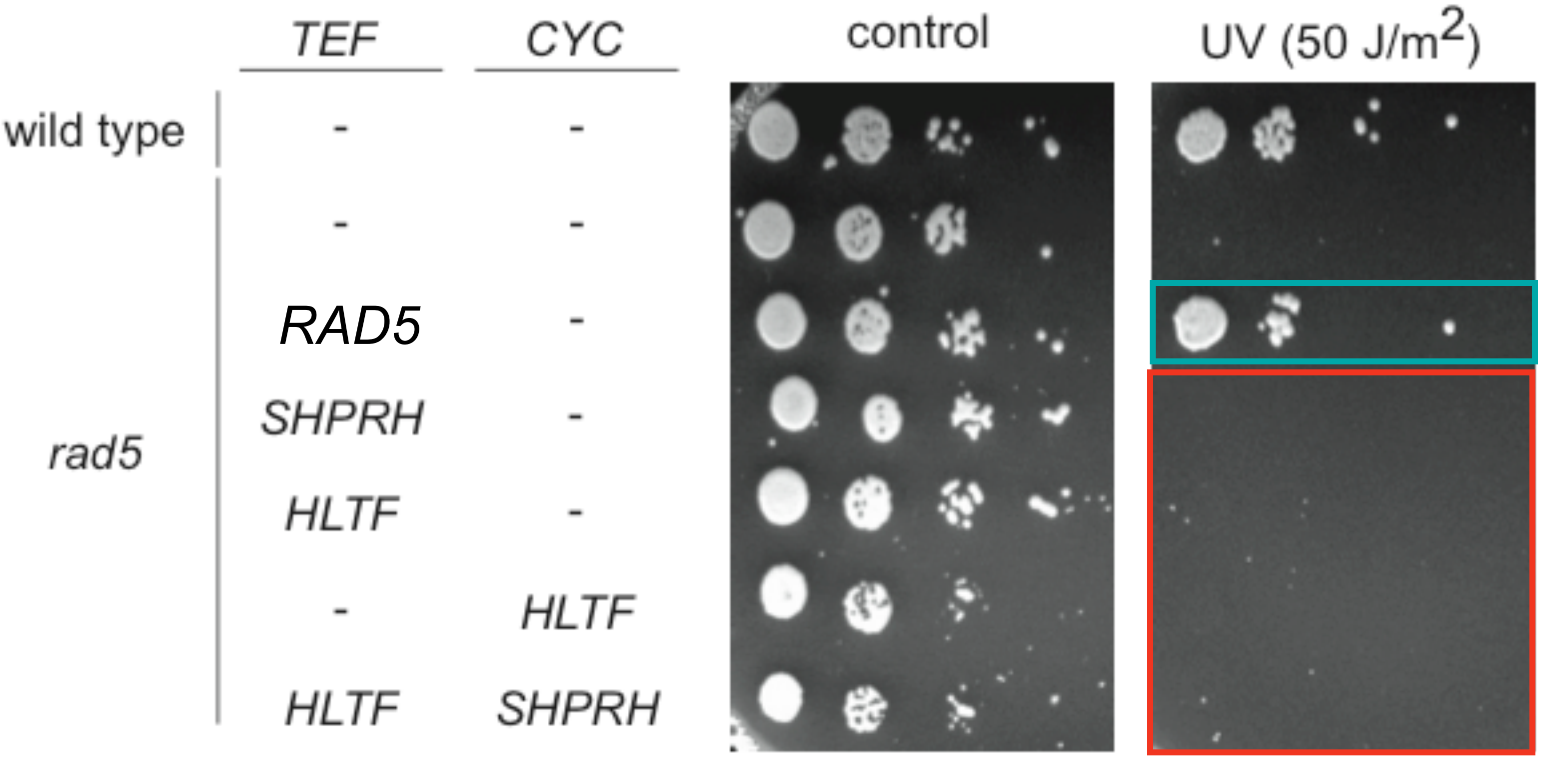
SHPRH and *HLTF* Do Not Complement *yrad5* Deficiency

	TEF	CYC
wild type	-	-
rad5	-	-
	RAD5	-
	SHPRH	-
	HLTF	-
	-	*HLTF*
	HLTF	*SHPRH*

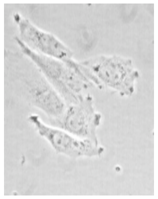




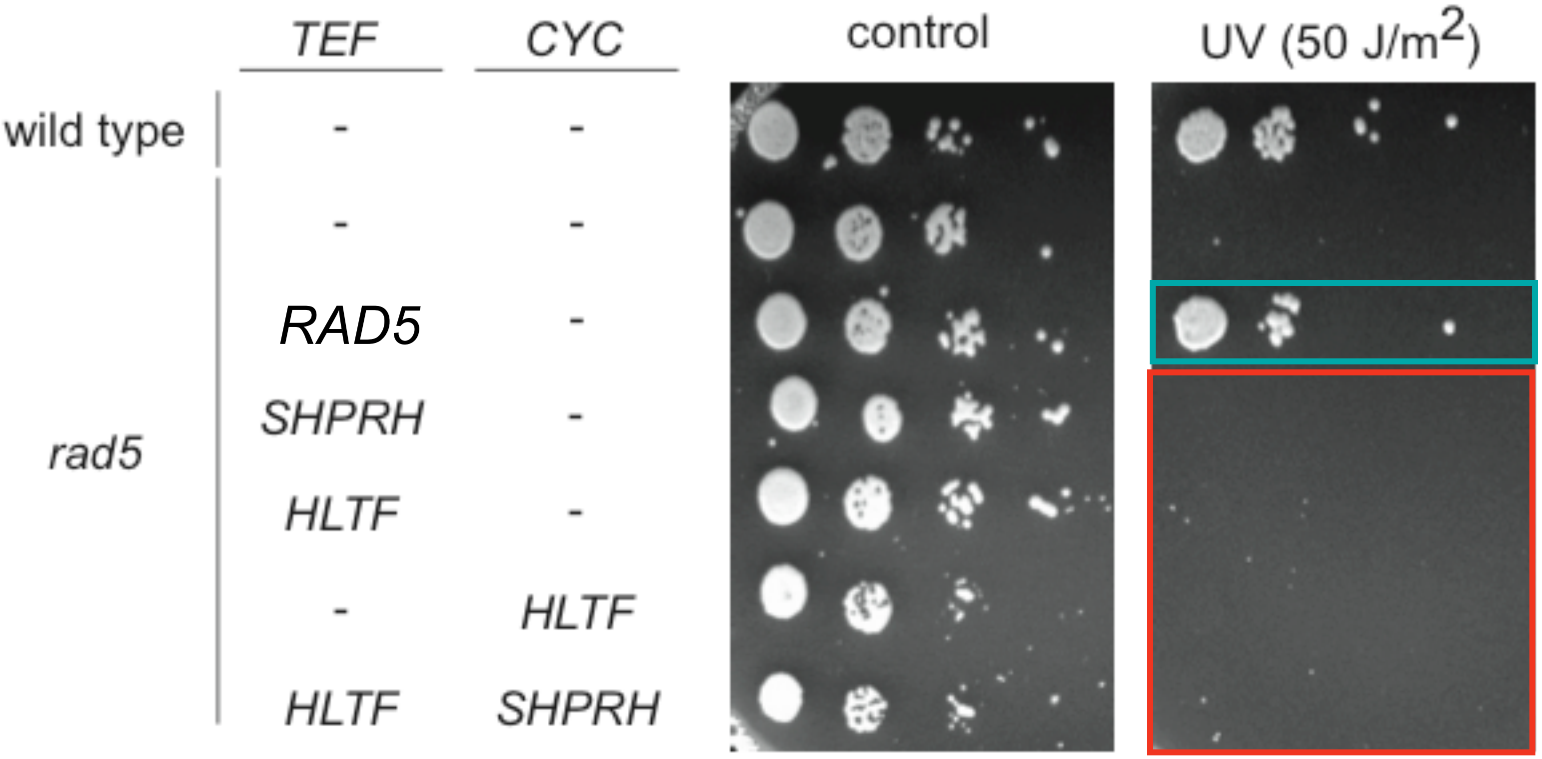
SHPRH and *HLTF* Do Not Complement *yrad5* Deficiency



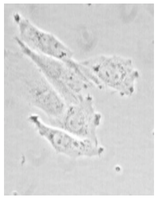
Human RAD18 also could not rescue the *rad18* deficiency in yeast.



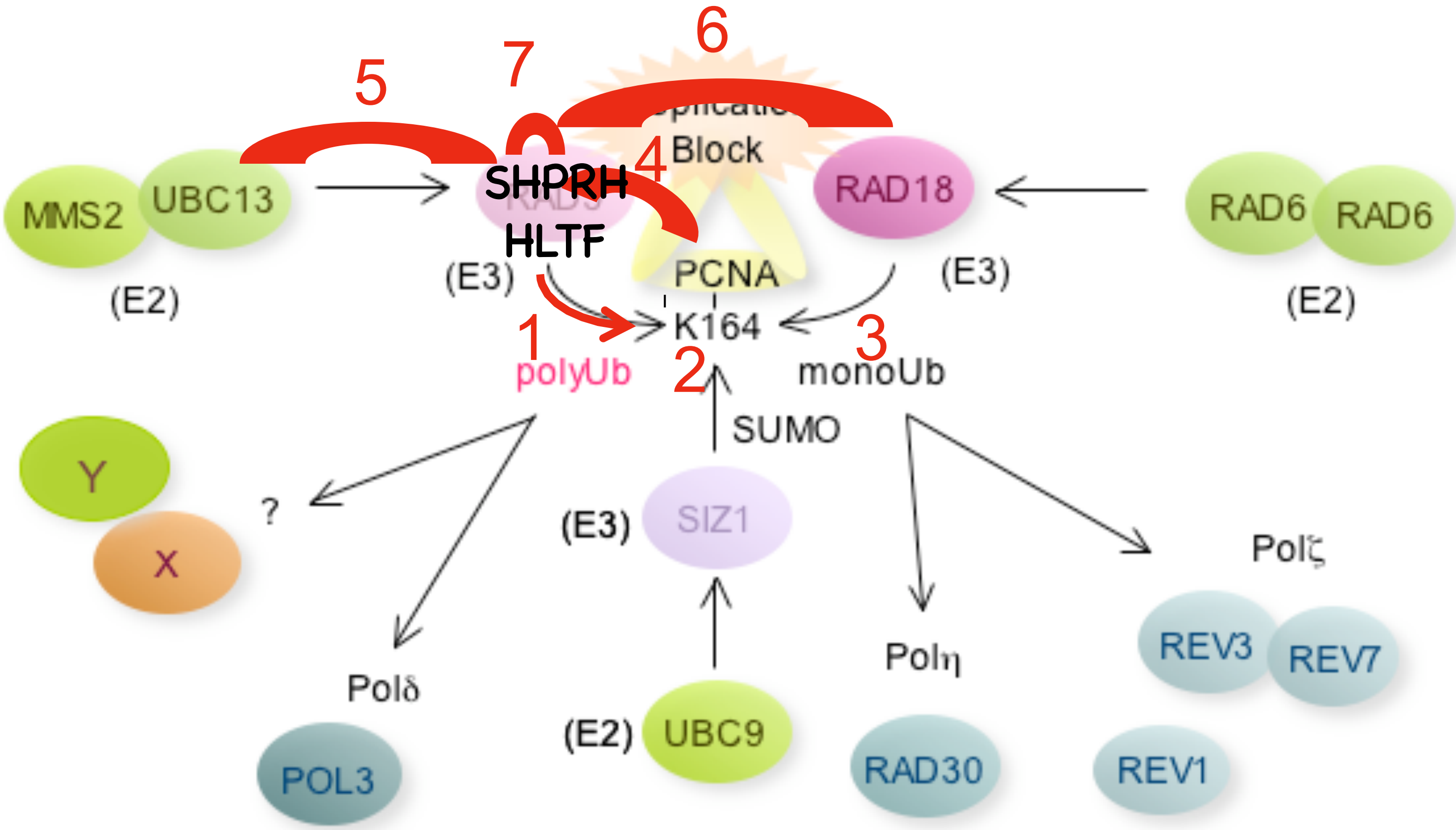
SHPRH and *HLTF* Do Not Complement *yrad5* Deficiency



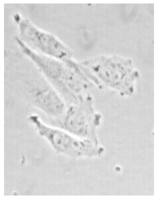
	Ub	E1	E2	E3
Number of yeast genes:	**4**	**8**	**15**	**68**
Number of human genes:	**4**	**16**	**53**	**527**
Complementation:	**Yes**	**Yes**	**Partial**	**No**



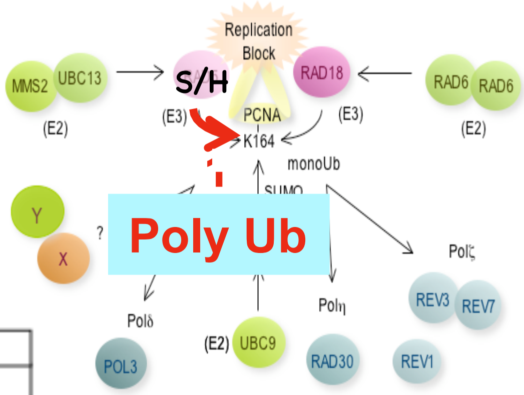
Seven Characteristics Found in Yeast Rad5



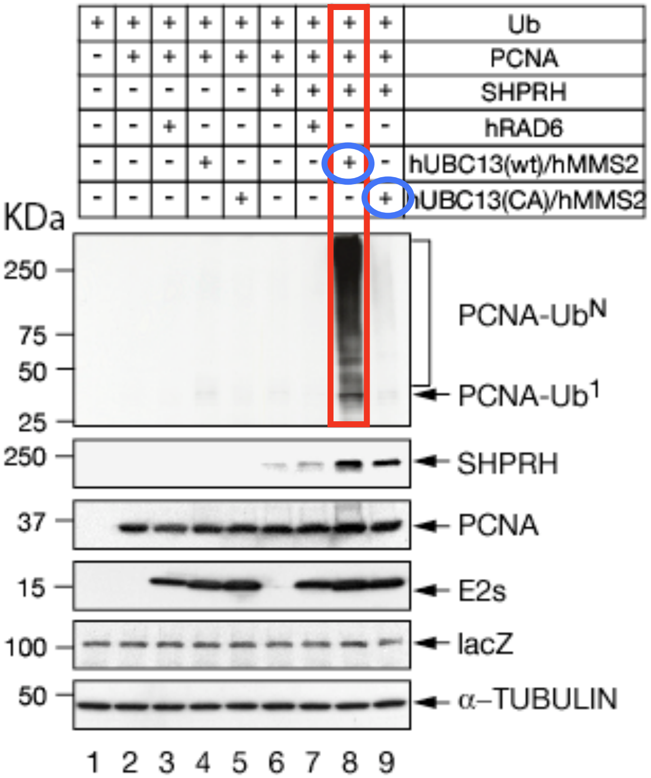
Mammalian RAD5 Should have similar characteristics.



SHPRH Poly-Ubiquitinates PCNA



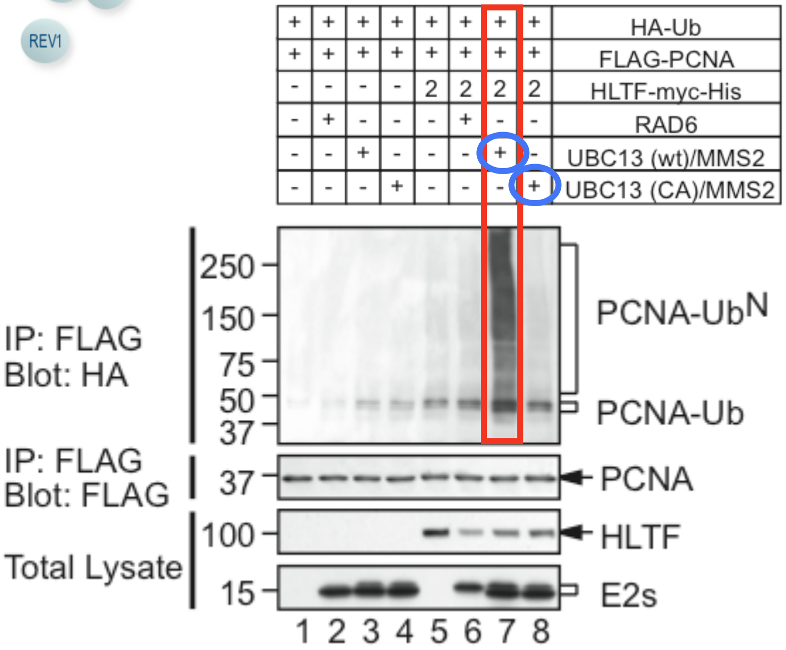
SHPRH



	1	2	3	4	5	6	7	8	9
Ub	+	+	+	+	+	+	+	+	+
PCNA	-	+	+	+	+	+	+	+	+
SHPRH	-	-	-	-	-	+	+	+	+
hRAD6	-	-	+	-	-	-	+	-	-
hUBC13(wt)/hMMS2	-	-	-	+	-	-	-	+	-
hUBC13(CA)/hMMS2	-	-	-	-	+	-	-	-	+

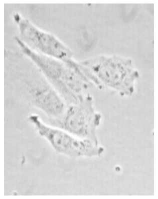
Motegi et al., JCB (2006) 175:703

HLTF



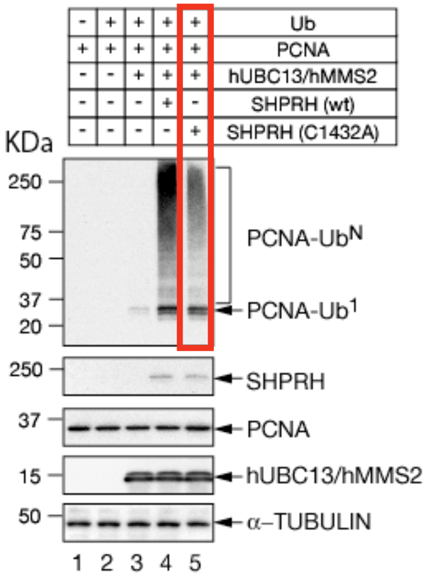
	1	2	3	4	5	6	7	8
HA-Ub	+	+	+	+	+	+	+	+
FLAG-PCNA	+	+	+	+	+	+	+	+
HLTF-myc-His	-	-	-	-	2	2	2	2
RAD6	-	+	-	-	-	+	-	-
UBC13 (wt)/MMS2	-	-	+	-	-	-	+	-
UBC13 (CA)/MMS2	-	-	-	+	-	-	-	+

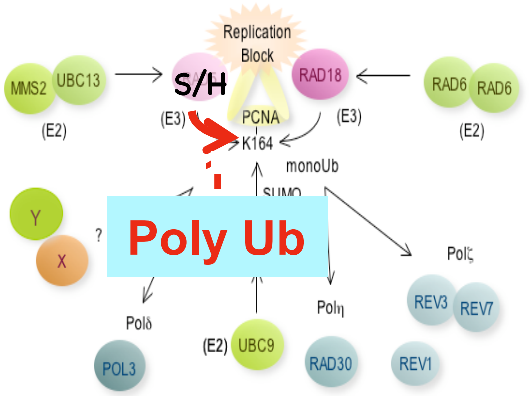
Motegi & Liaw et al., PNAS (2008) 105:12411

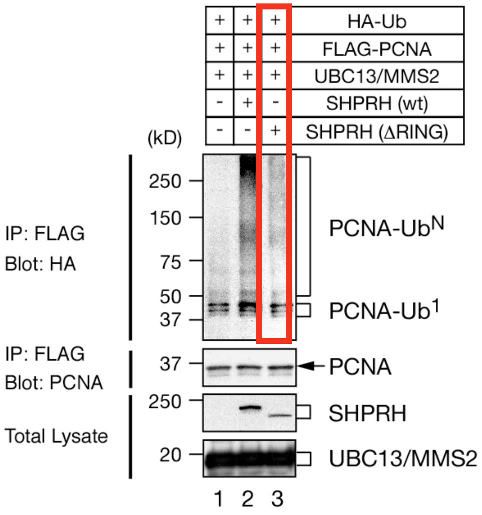


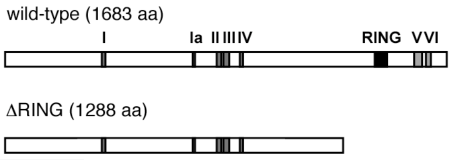
The Ring Domain in SHPRH and HLTF is Important for the Poly-Ubiquitination of PCNA

SHPRH

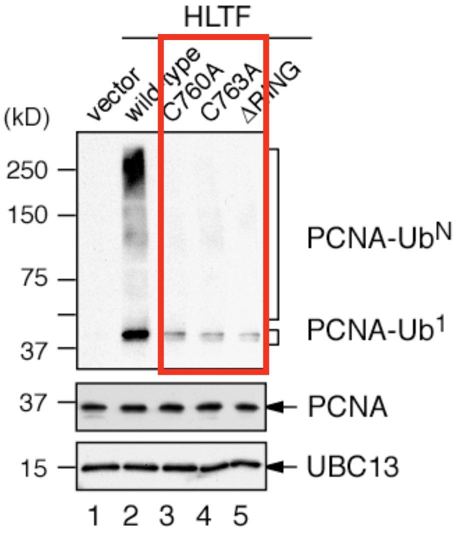


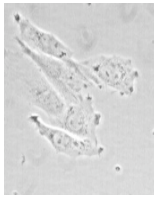




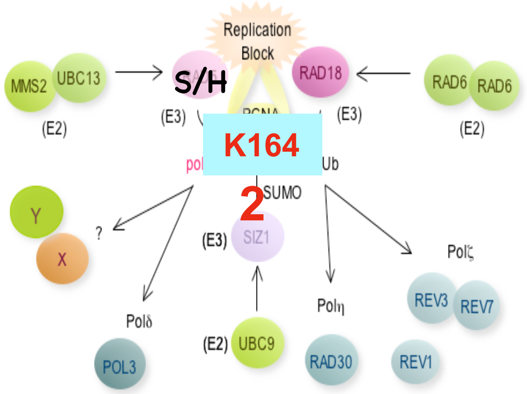


HLTF



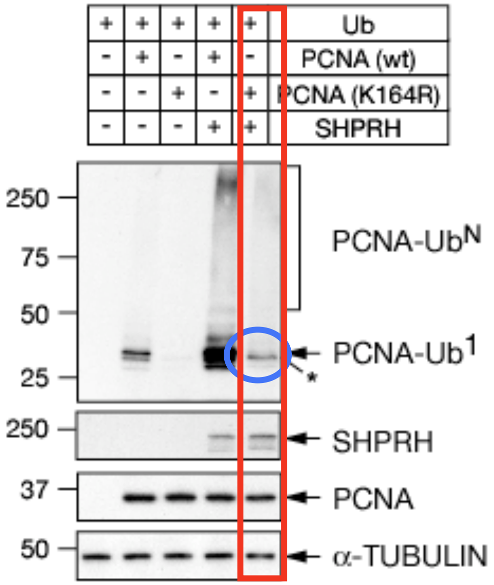


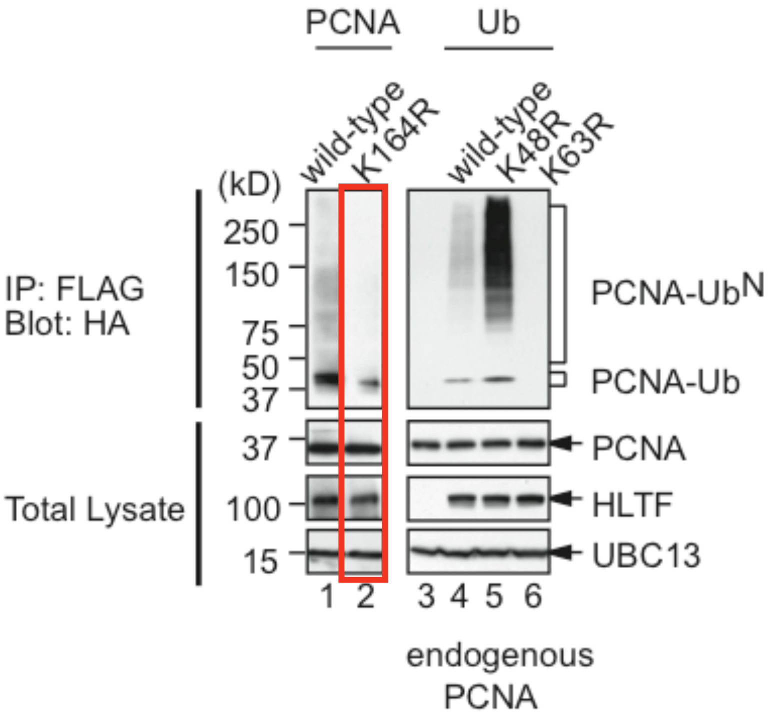
SHPRH/HLTF Ubiquitinate K164 of PCNA *in vivo*

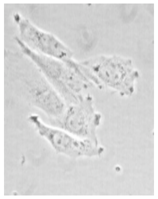


SHPRH

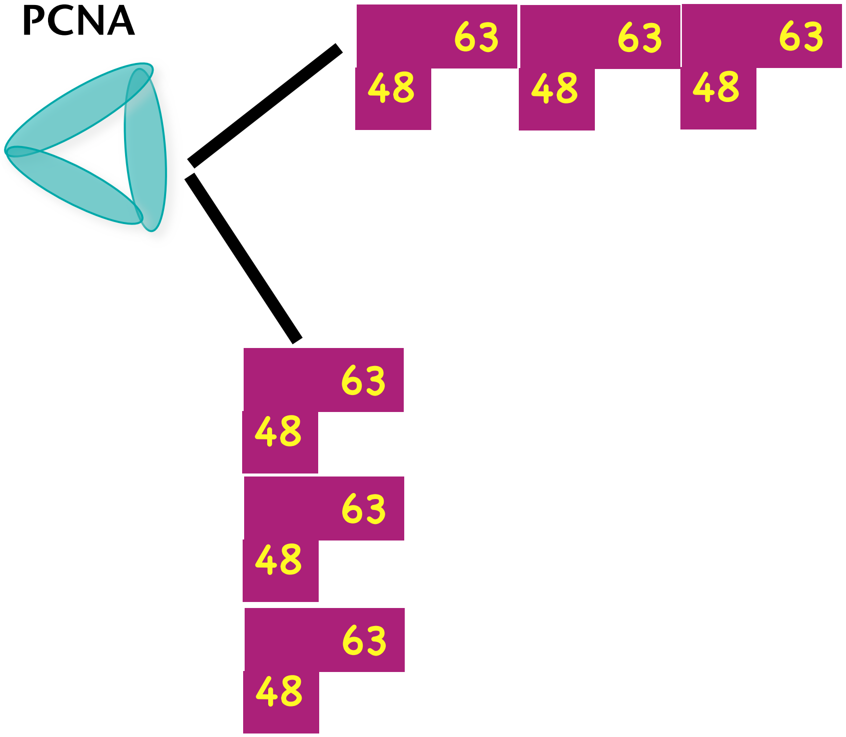
HLTF

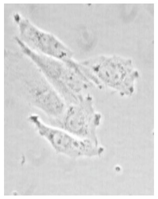




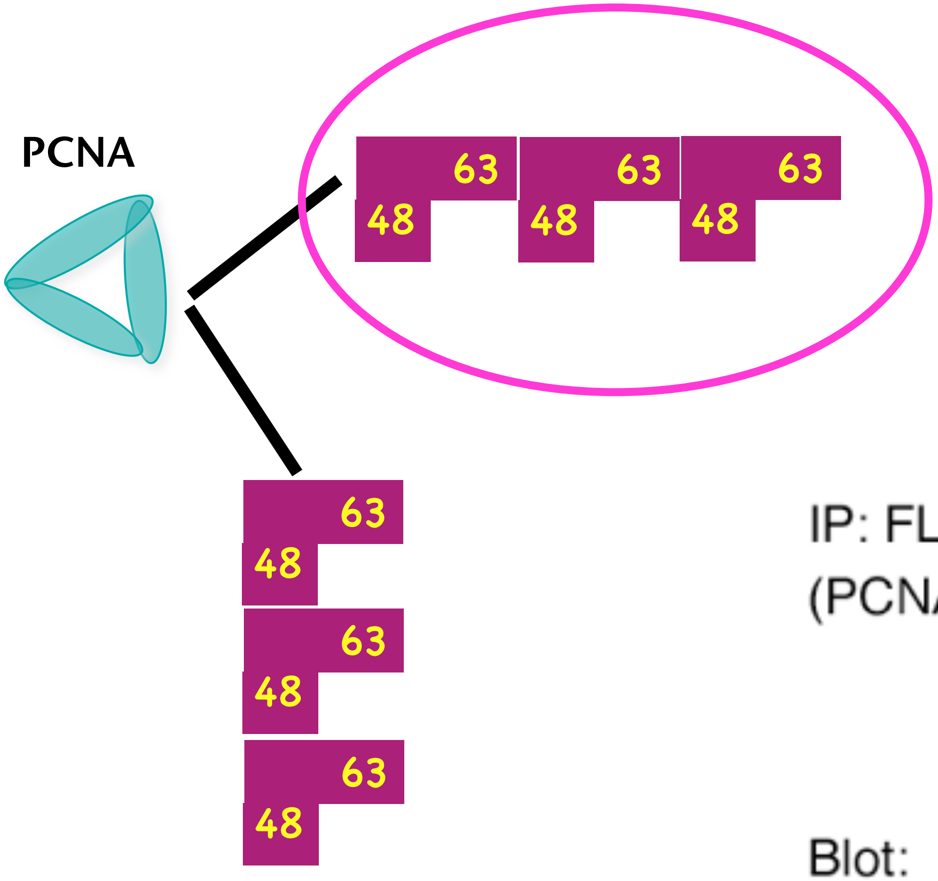


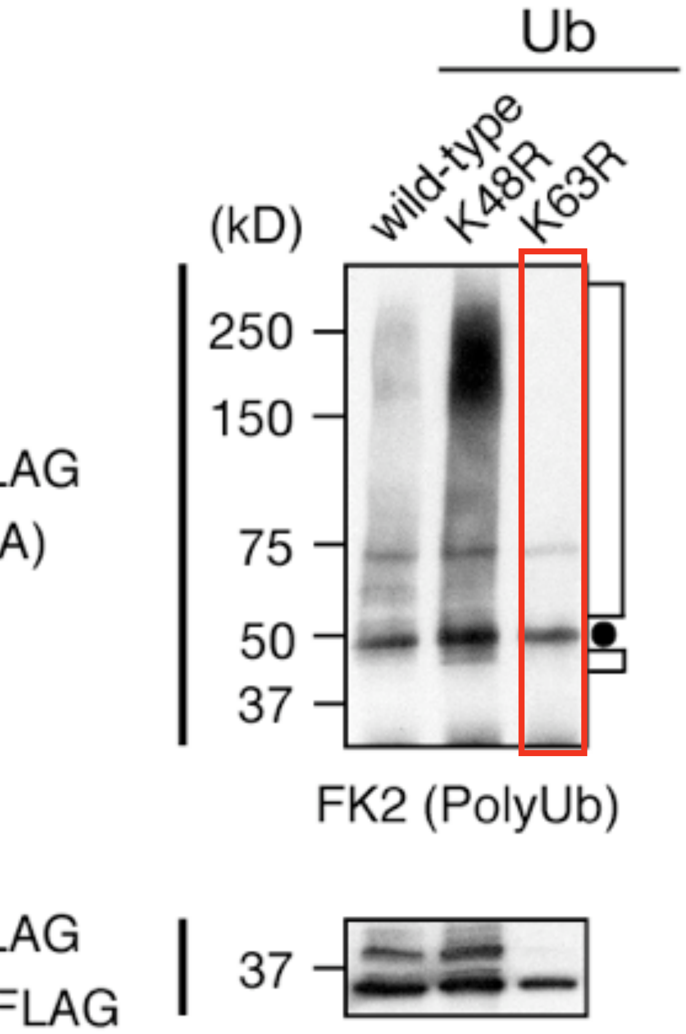
SHPRH Ubiquitinates PCNA through K63 Chain

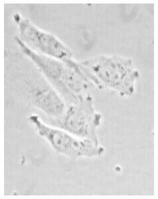




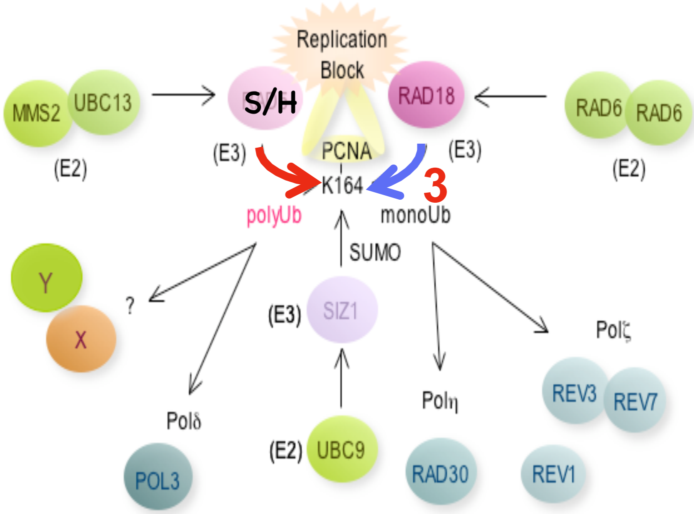
SHPRH/HLTF Ubiquitinates PCNA through K63 Chain

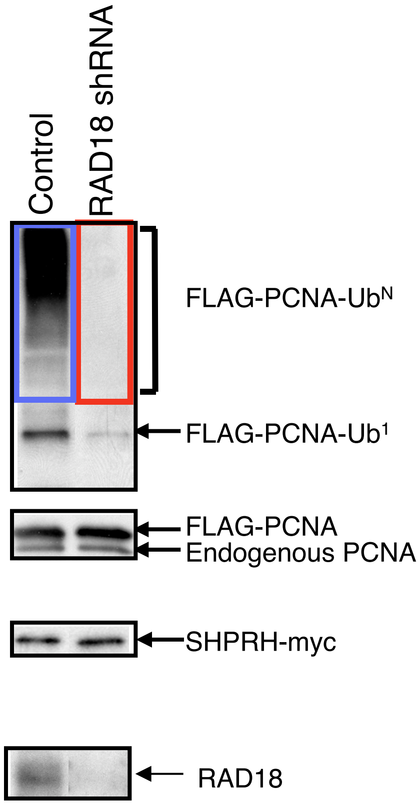






PCNA PolyUb is Dependent on RAD18





IP: FLAG (PCNA)
Blot: HA (Ub)

FLAG-PCNA-Ub^N
FLAG-PCNA-Ub^1

IP: FLAG (PCNA)
Blot: PCNA

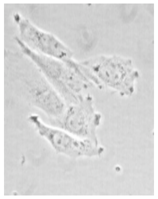
FLAG-PCNA
Endogenous PCNA

Total Lysate
Blot: myc (SHPRH)

SHPRH-myc

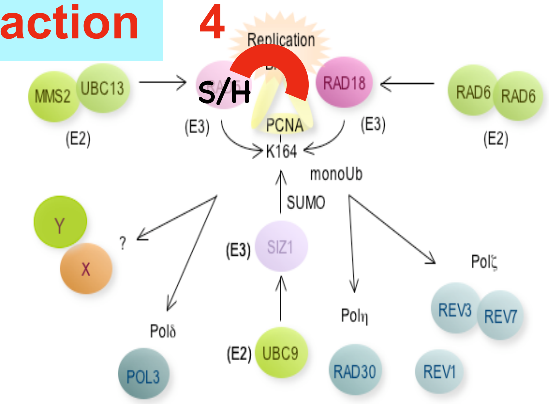
Total Lysate
Blot: RAD18

RAD18



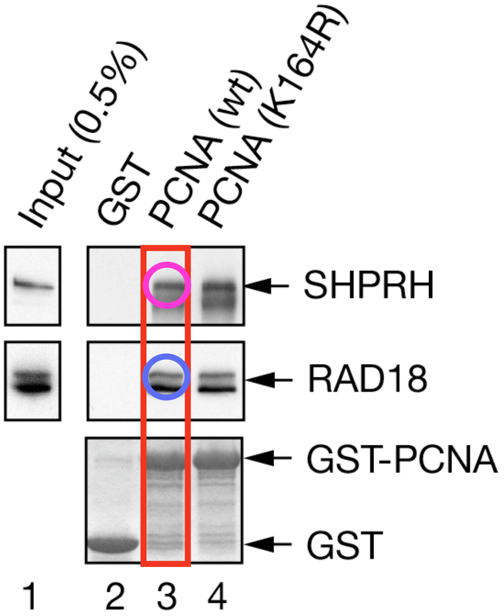
SHPRH/HLTF Physically Interact with PCNA *in vivo*

Interaction 4

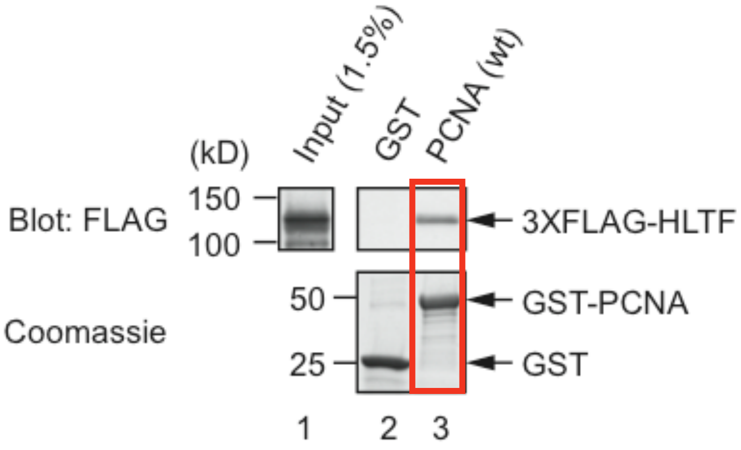


SHPRH

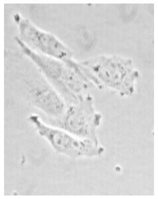
HLTF



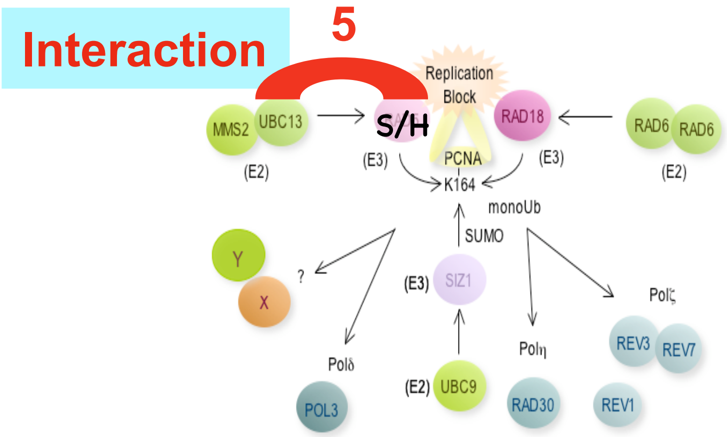
GST-Pull Down
(GST: PCNA/WB: SHPRH or RAD18)



GST-Pull Down
(GST: PCNA/WB: HLTF)

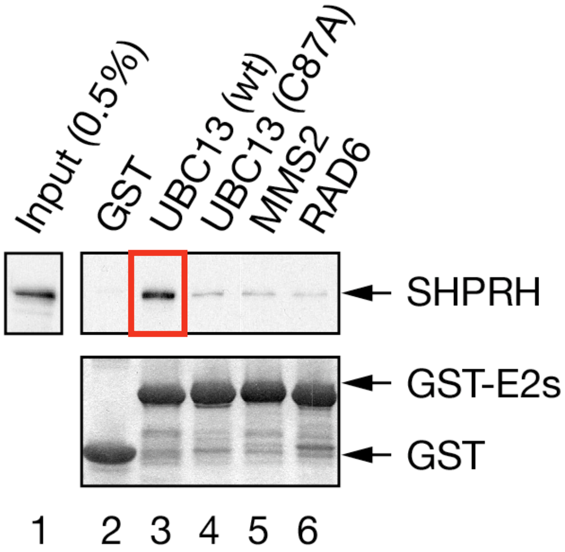


SHPRH/HLTF Physically Interact with UBC13

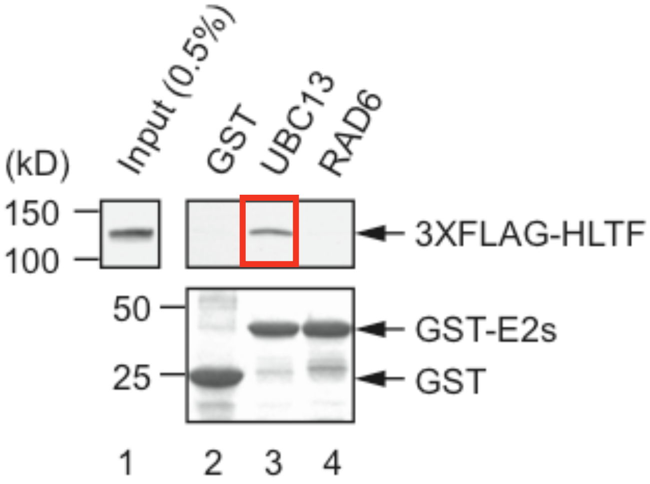


SHPRH

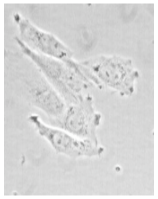
HLTF



GST-Pull Down
(GST: E2s / WB: SHPRH)

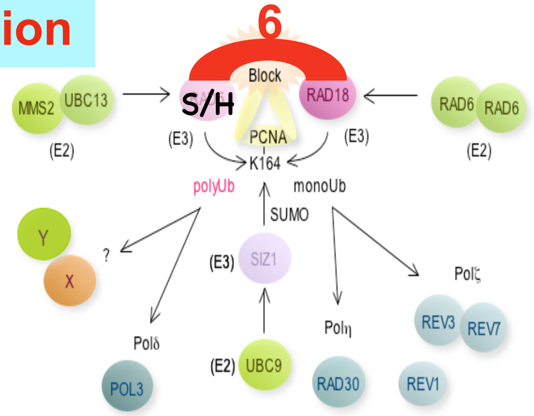


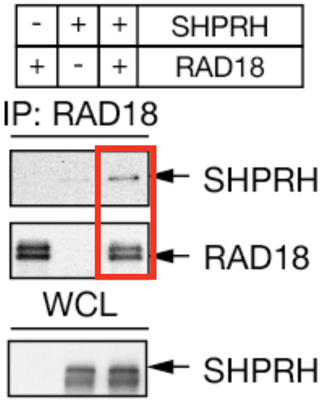
GST-Pull Down
(GST: E2s / WB: HLTF)



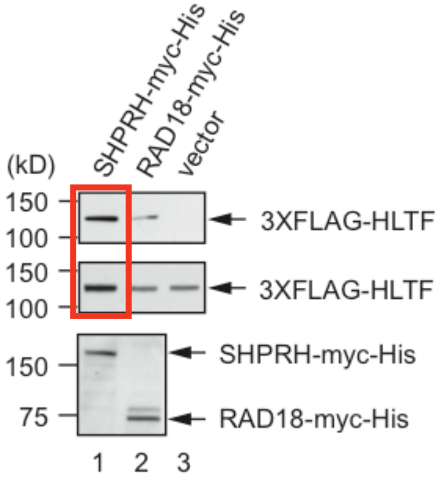
Physical Interaction between RAD18, SHPRH, HLTF

Interaction

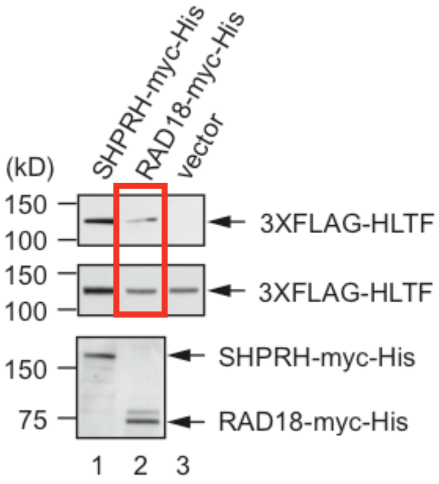




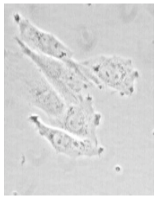
SHPRH-RAD18



SHPRH-HLTF



HLTF-RAD18

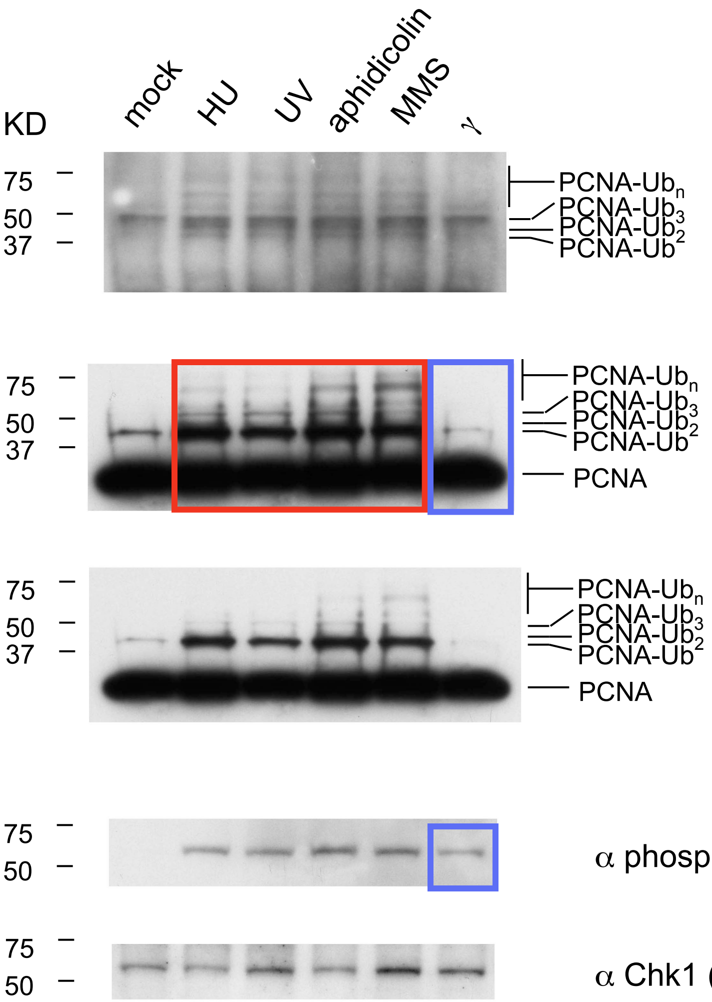


PCNA PolyUb is Induced by DNA Damage Stalling DNA Replication

IP with α-PCNA Ab

↓

WB



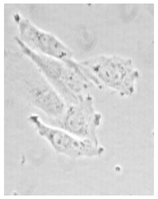
α-Ub Ab

α-PCNA Ab

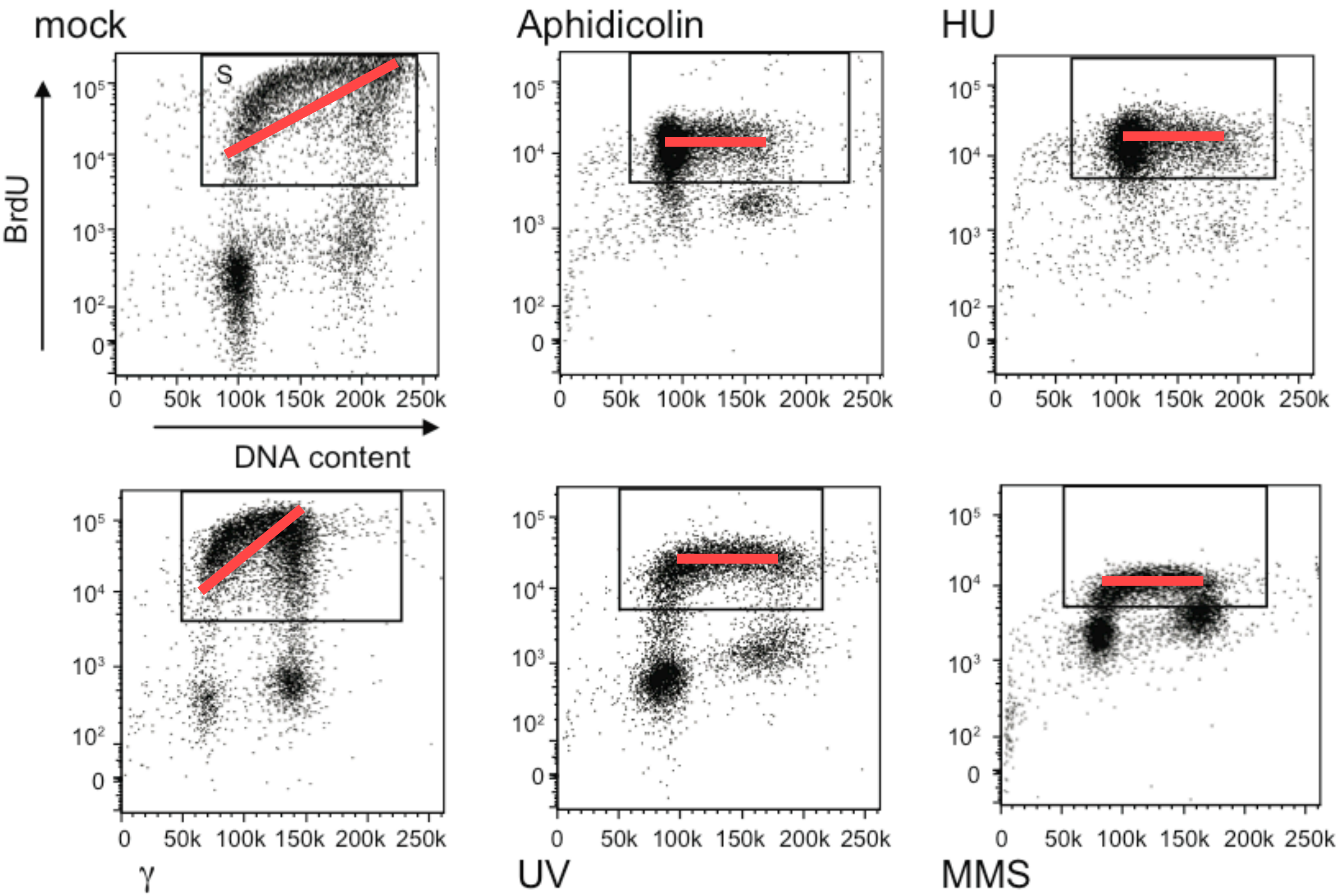
α-PCNA Ab short exposure

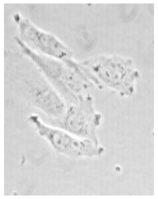
α phospho-Chk1 (Ser345)

α Chk1 (G4)

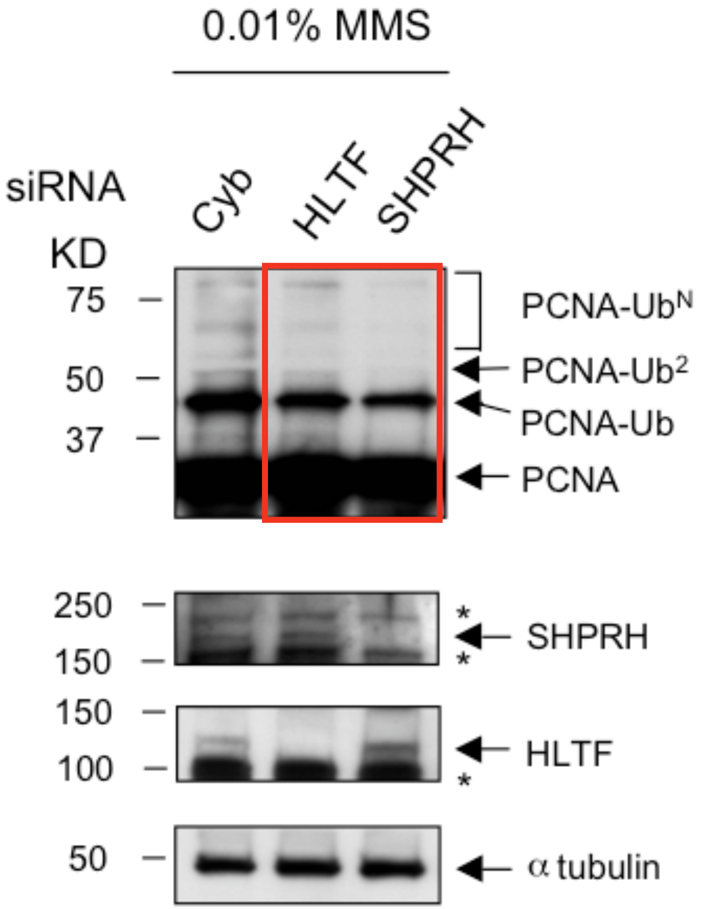


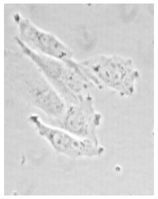
PCNA PolyUb is Induced by DNA Damage Stalling DNA Replication





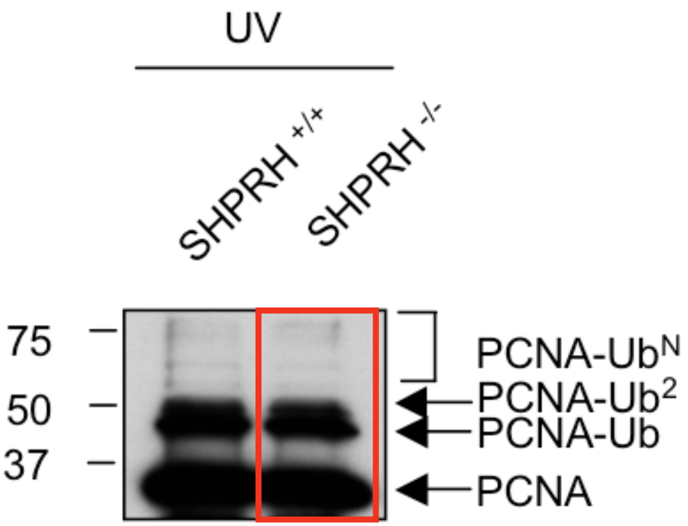
Reduction of SHPRH or HLTF by siRNA Reduced MMS-induced PCNA Polyubiquitination



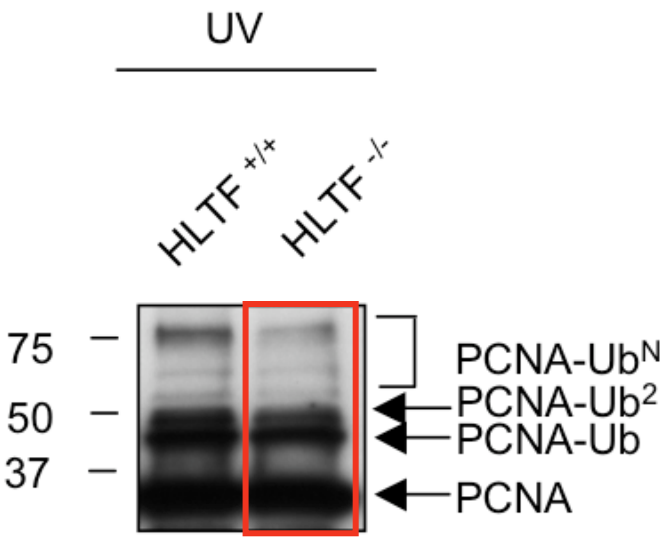


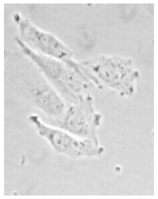
SHPRH$^{-/-}$ or HLTF$^{-/-}$ MEFs Showed Reduction of DNA Damage Induced PCNA Polyubiquitination

SHPRH

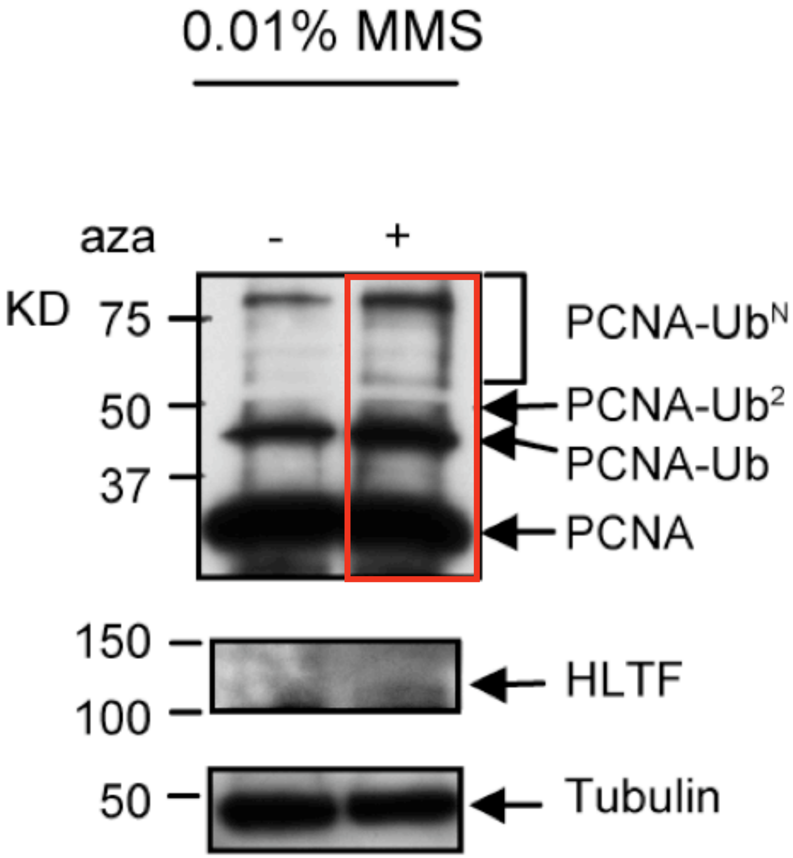


HLTF



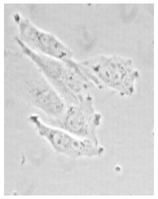


Restoration of HLTF expression in SW480 Induced MMS-induced PCNA Polyubiquitination



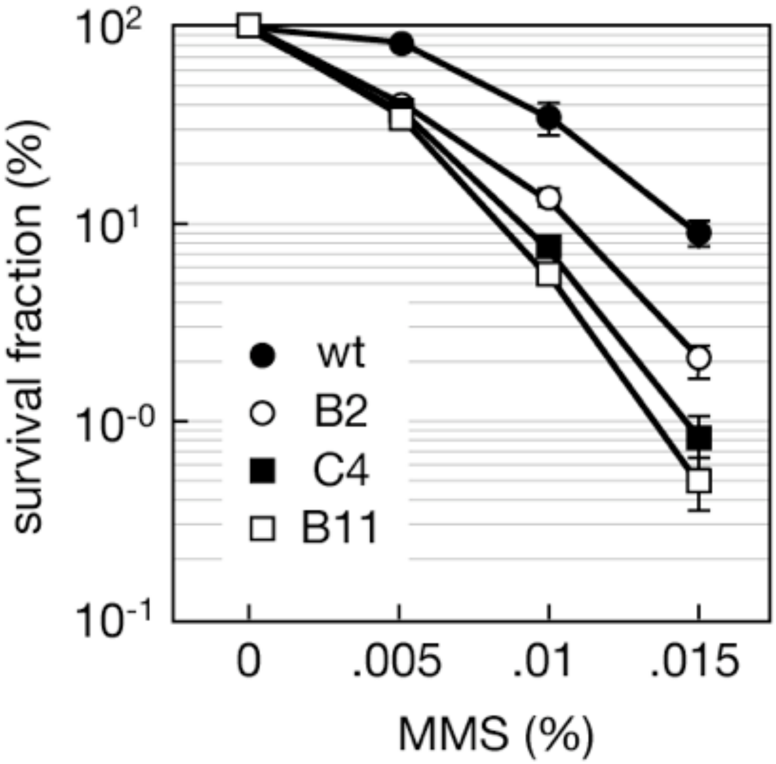
SW480 is a cell line from Colon Cancer Patient and does not express HLTF due to the methylation of its promoter.

Aza; Azacytidine.

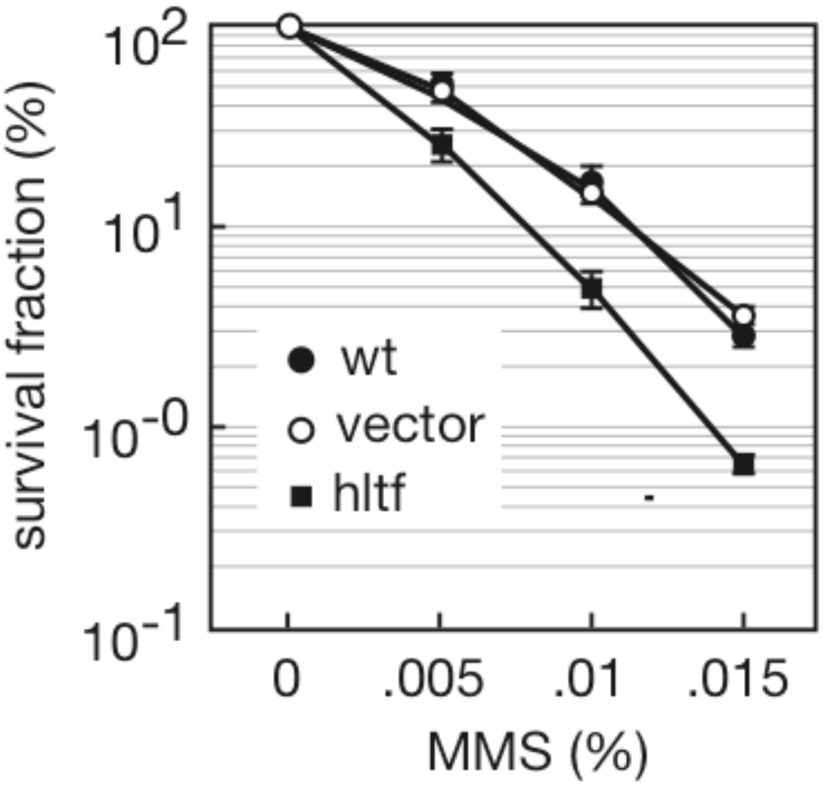


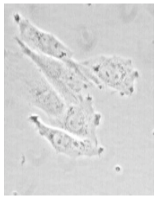
Reduced Expression of SHPRH or HLTF by shRNA Makes HCT116 Cells Sensitive to MMS

SHPRH

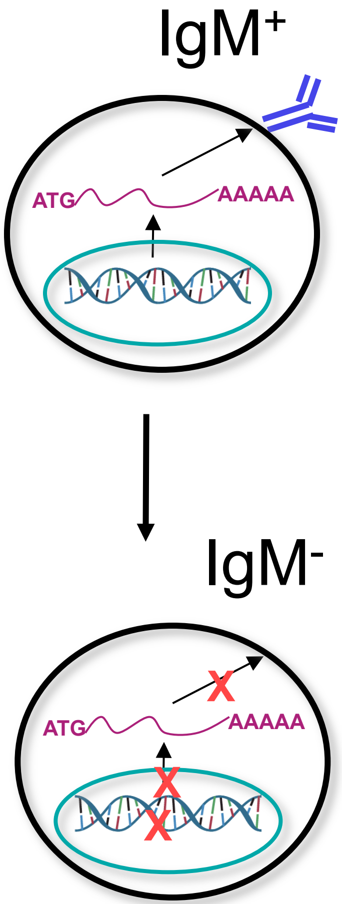


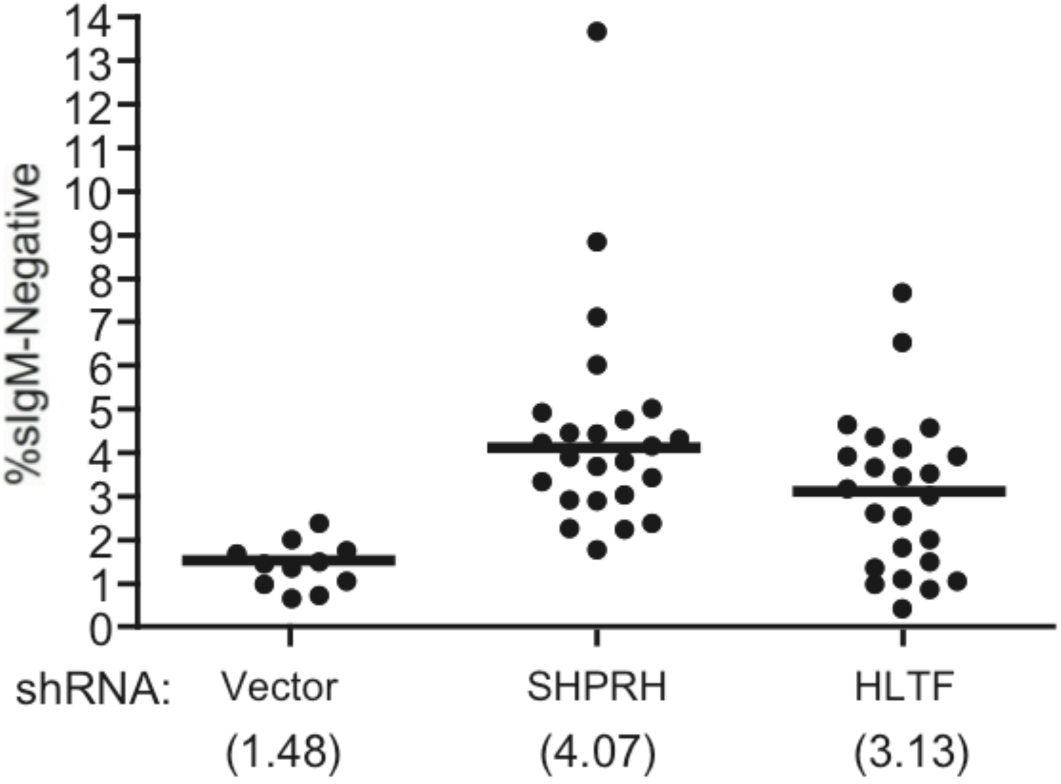
HLTF

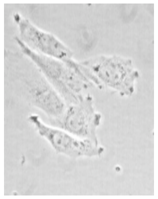




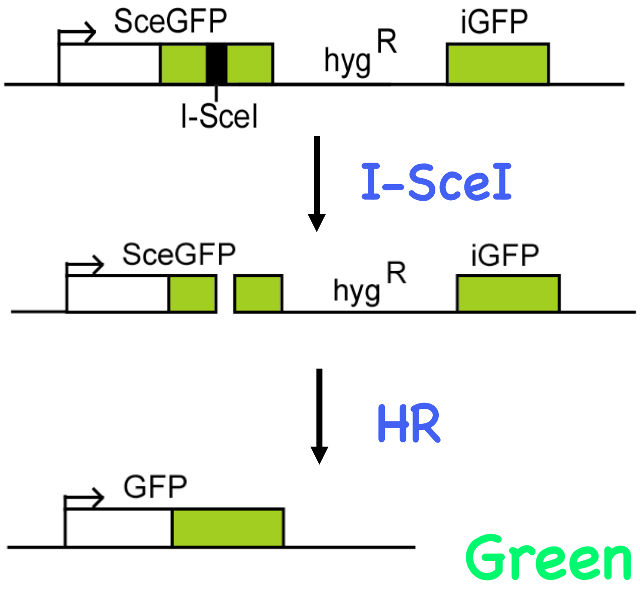
Reduction of SHPRH or HLTF Expression by shRNA increase Mutagenesis







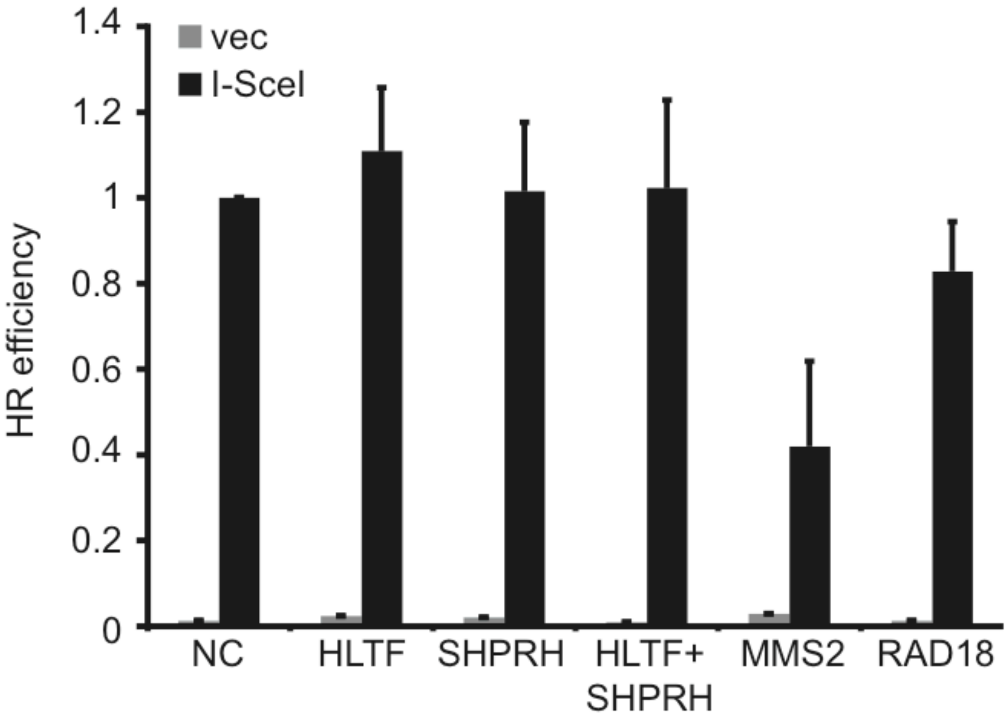
Reduction of SHPRH or HLTF Expression by siRNA does not Affect DSB-induced HR Frequency

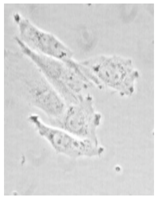


I-SceI

HR

Green





MMS Treatment Induces Chromosomal Breaks in SHPRH Knockdown Cells or HLTF$^{-/-}$ MEFs

WT or KD HCT116(SHPRH)

WT or KO MEFs (HLTF)

↓

Treat with 0.01% MMS (2 hrs)

↓

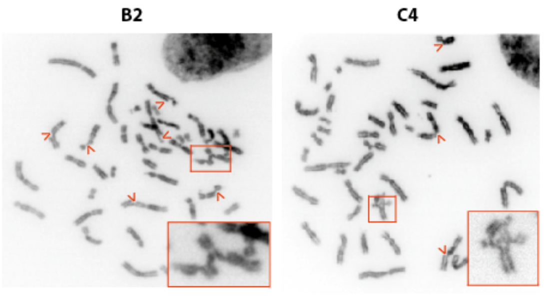
Stain with DAPI

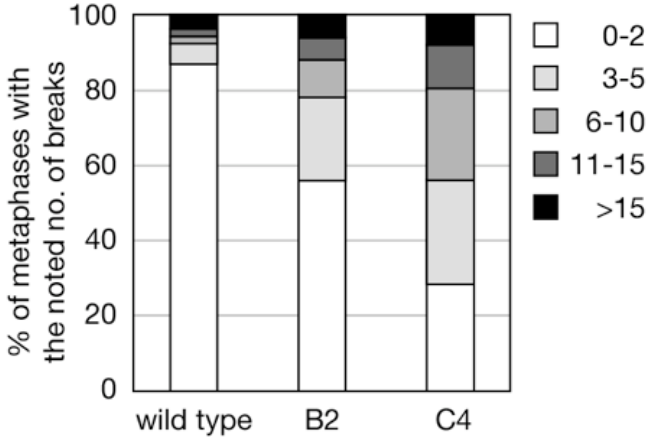
↓

Observe chromosomes

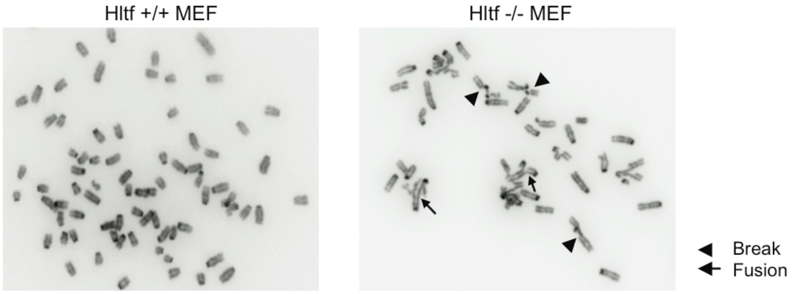
under the microscope

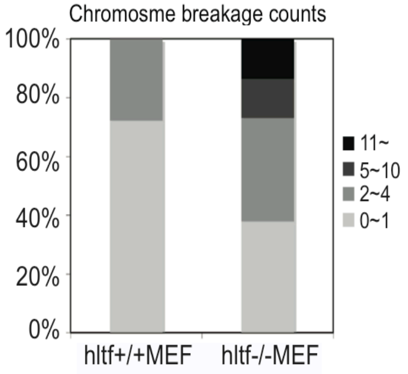
SHPRH

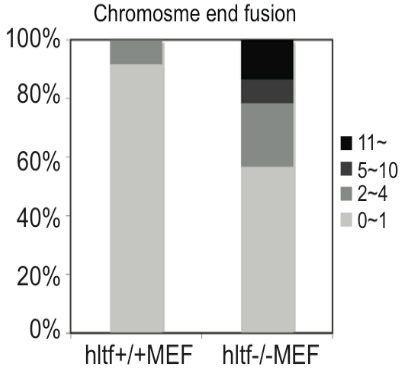




HLTF







Conclusions

1. SHPRH and HLTF Promote PCNA Polyubiquitination.

2. SHPRH and HLTF Physically Associates with PCNA, RAD18, and UBC13.

3. Lower Expression of SHPRH and HLTF Reduced Endogenous PCNA PolyUb.

4. DNA Damaging Agents Except γ-ray Enhances PCNA Polyubiquitination suggesting PCNA PolyUb is Induced by Stalled Replication Forks.

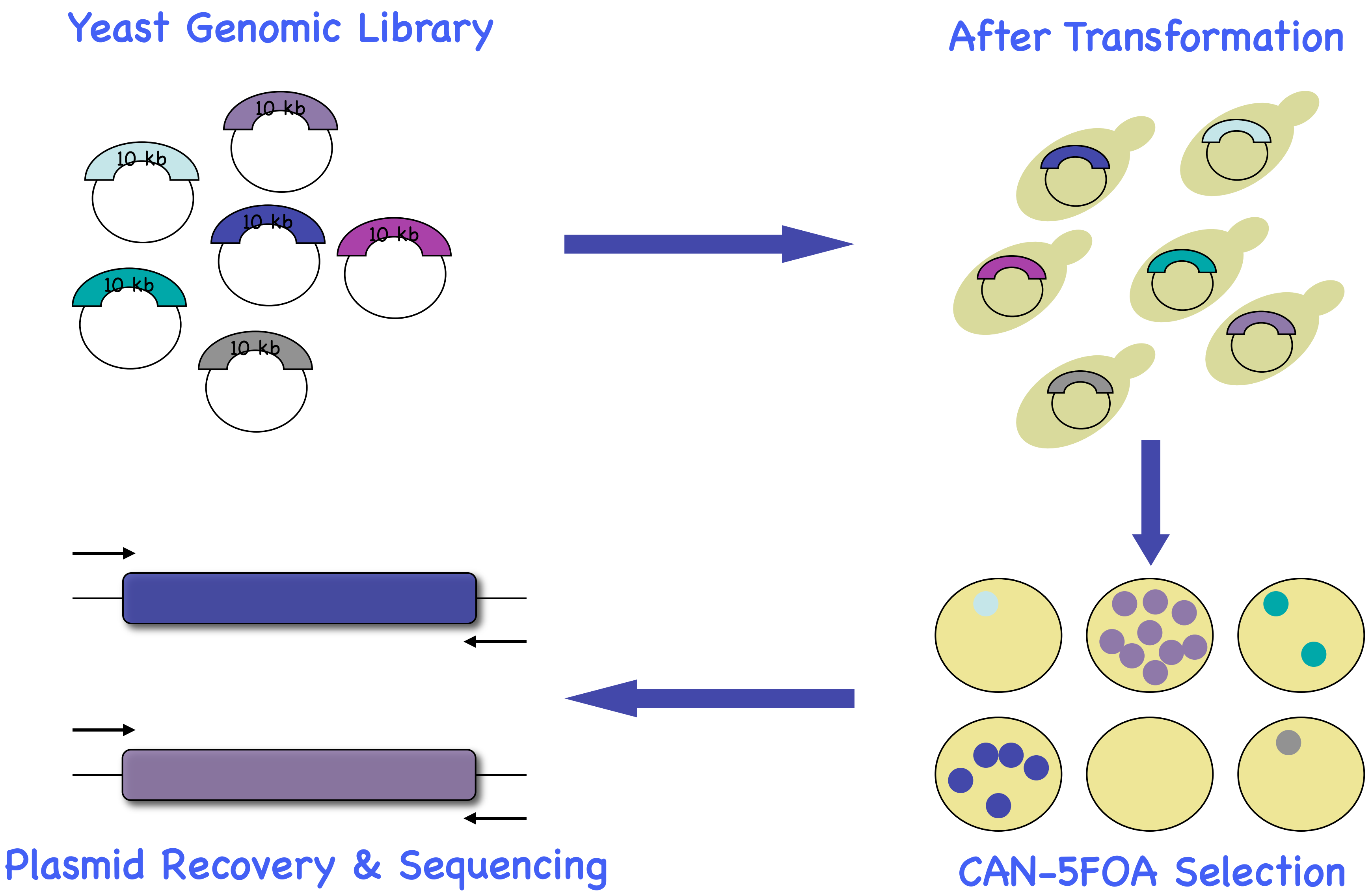
5. Reduced Expression of SHPRH and HLTF Enhances Mutagenesis.

6. SHPRH and HLTF Suppress MMS-induced Genome Instability.

7. SHPRH and HLTF are not Involved in DSB-induced Homologous Recombination.

* *SHPRH* and *HLTF* are Functional Human Orthologs of Yeast *RAD5*.

Screening of Genes Enhancing GCR



Yeast Genomic Library

After Transformation

CAN-5FOA Selection

Plasmid Recovery & Sequencing

Over-Expression Screen:

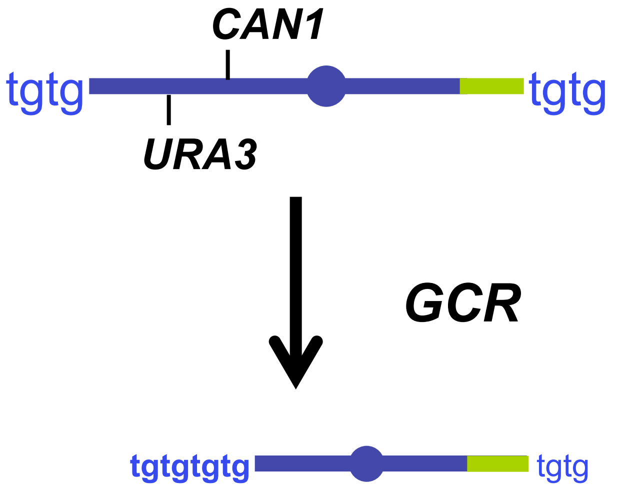
Which genes, when over-expressed, increase GCR?

YIL001W : Hypothetical Open Reading Frame
YIR003W : Hypothetical Open Reading Frame
SGN1 : Cytoplasmic RNA Binding Protein

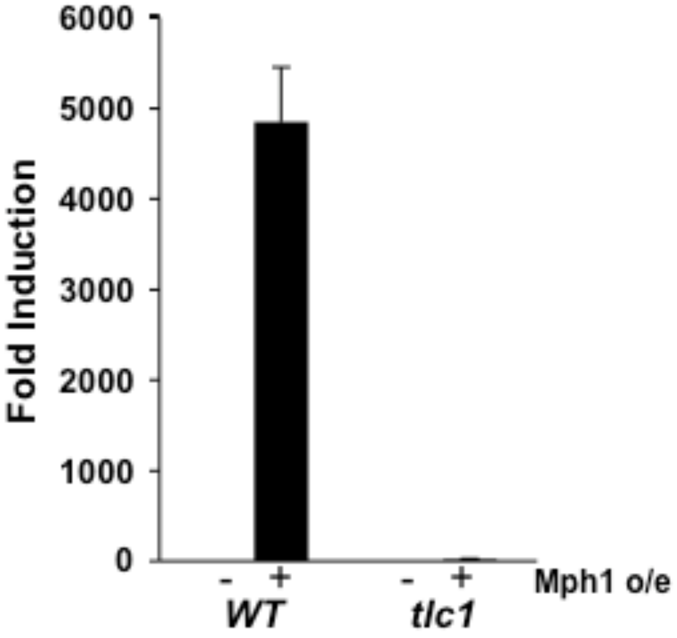
MPH1 : Member of the DEAH family of helicases, functions in an error-free DNA damage bypass pathway that involves homologous recombination, mutations confer a mutator phenotype. Putative Fanconi Anemia M protein in yeast.

Banerjee et al., JCB (2008) 181:1083

Mph1-directed GCRs were de novo Telomere Addition and dependent on Telomerase and yKu70

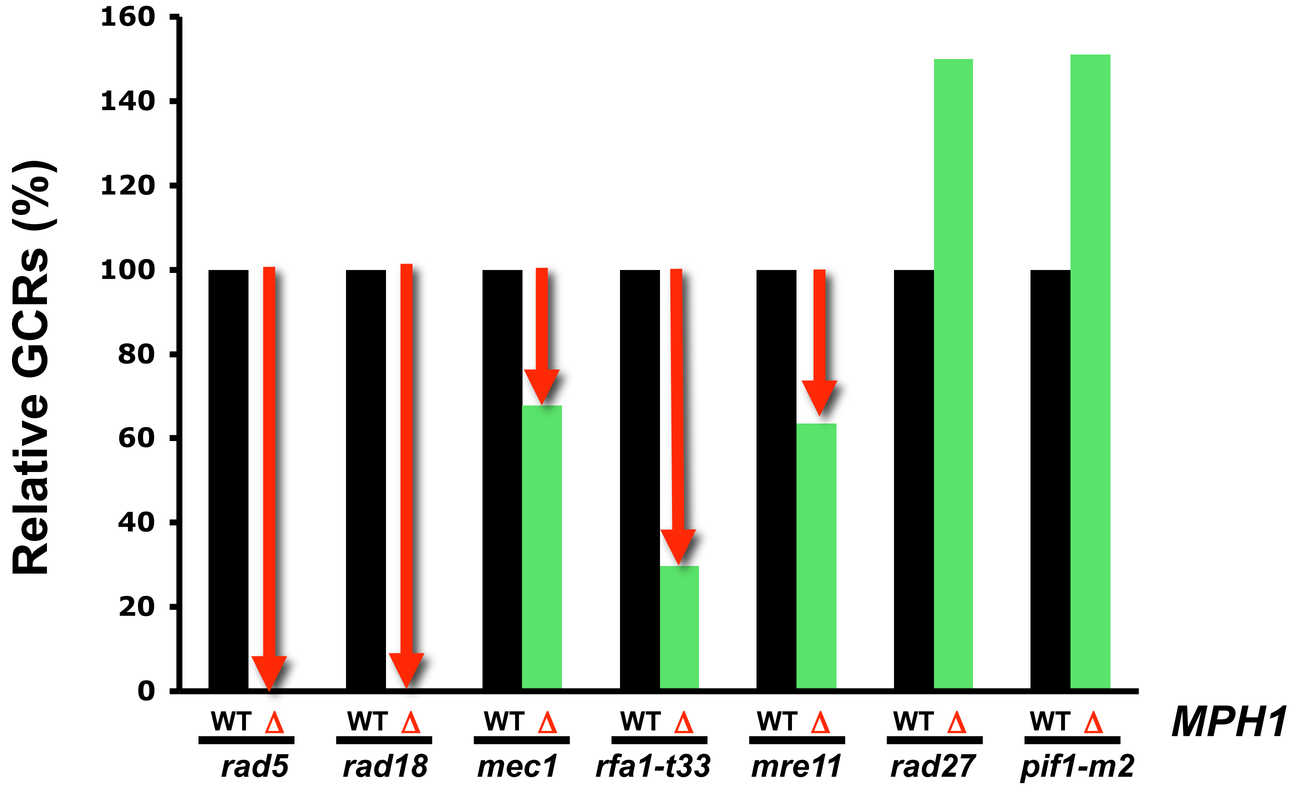


terminal deletion with
***de novo* telomere addition**

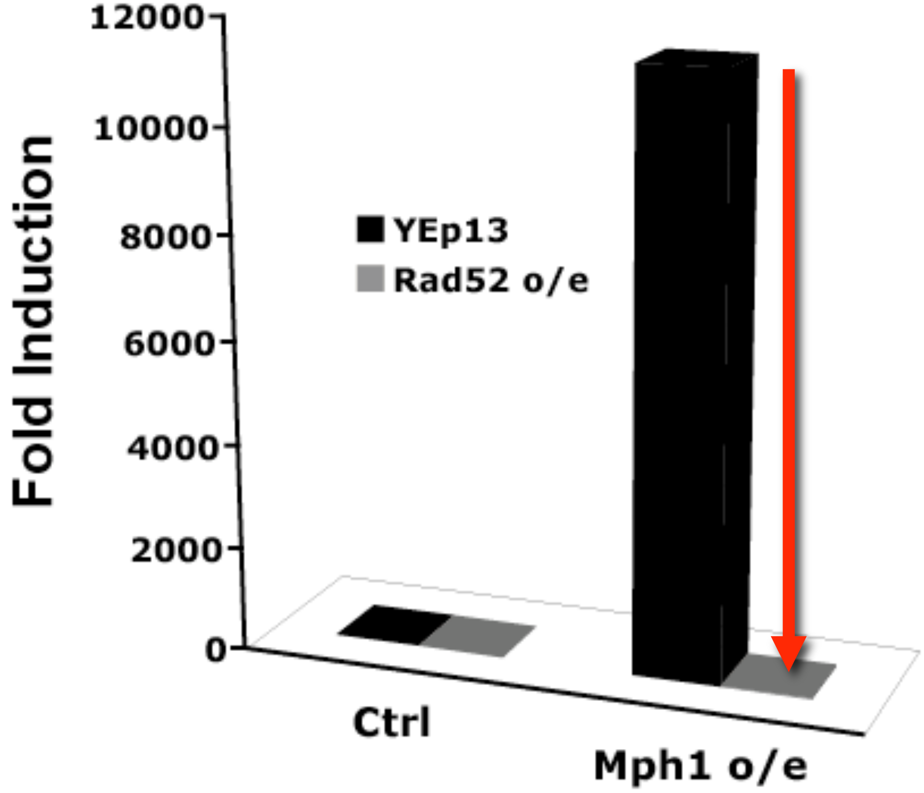


TLC1 is RNA subunit of Telomerase

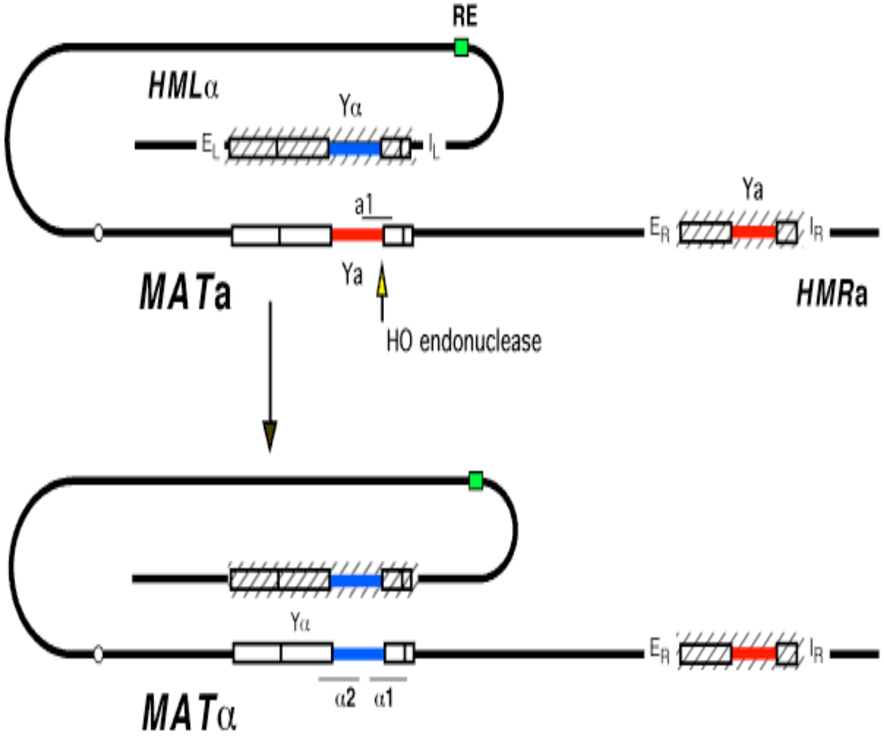
The Mutation in MPH1 Reduced GCRs in Some GCR Mutator Strains

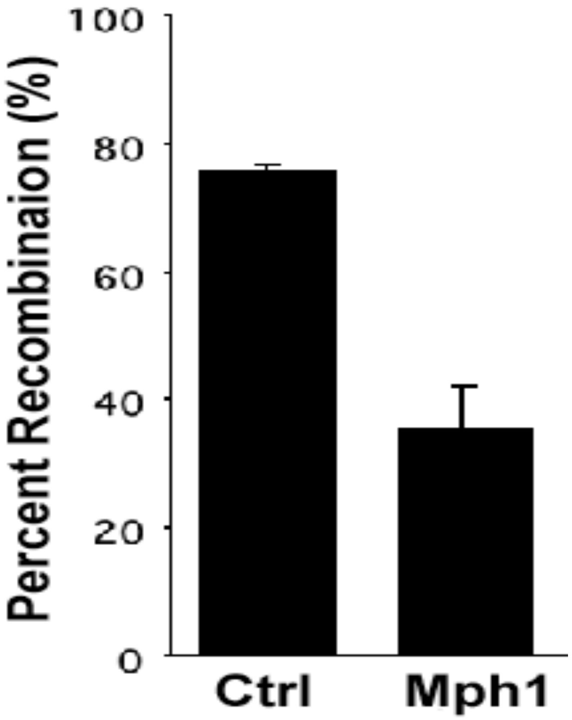


Co-overexpression of Rad52 Reduced Mph1-directed GCRs

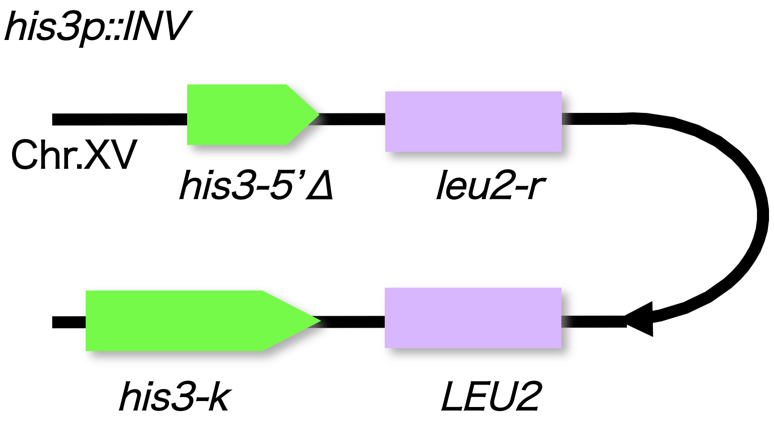


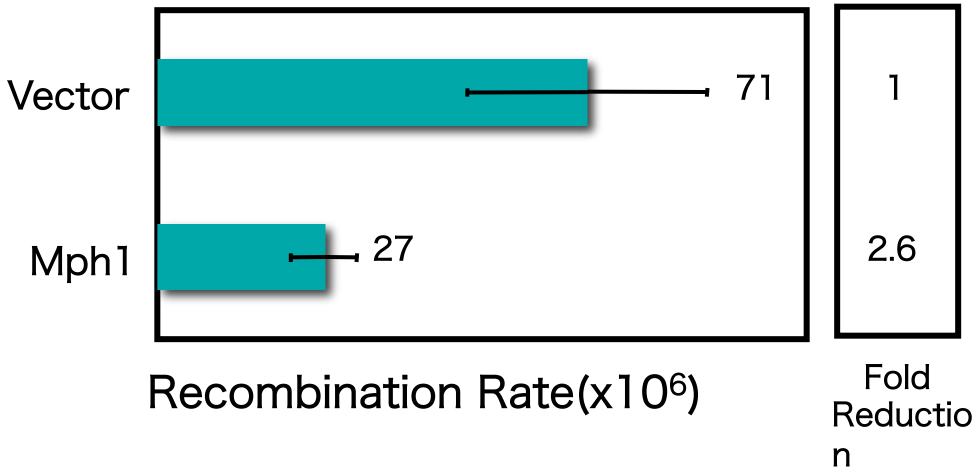
Excess Mph1 Inhibits Homologous Recombination



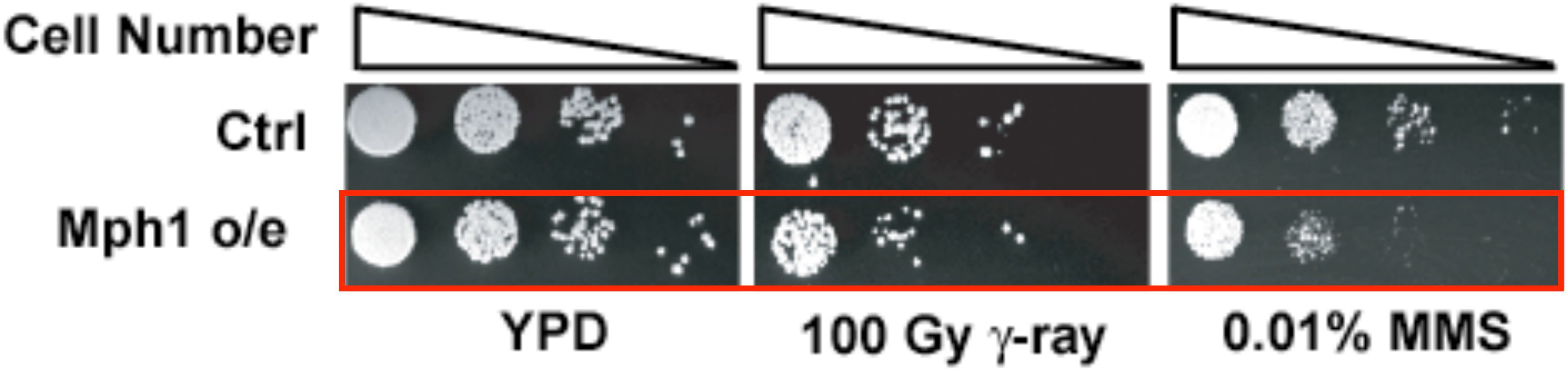


Excess Mph1 Inhibits Homologous Recombination

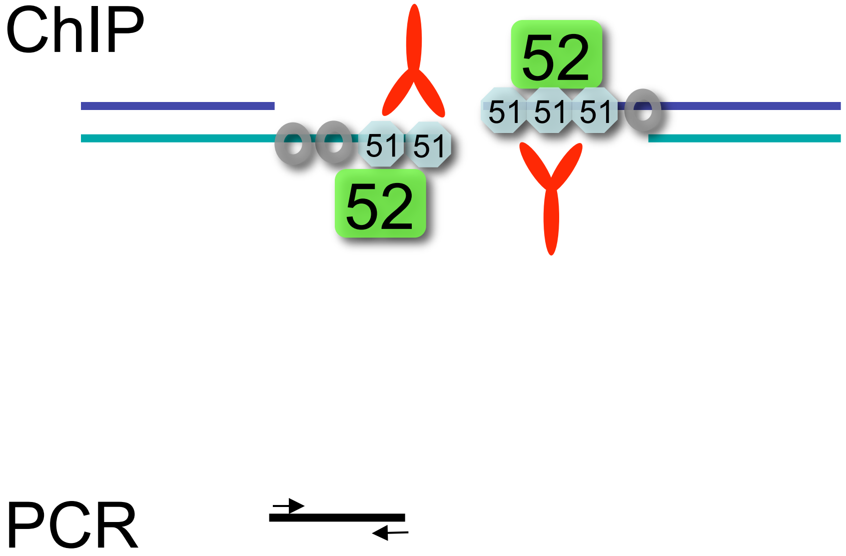


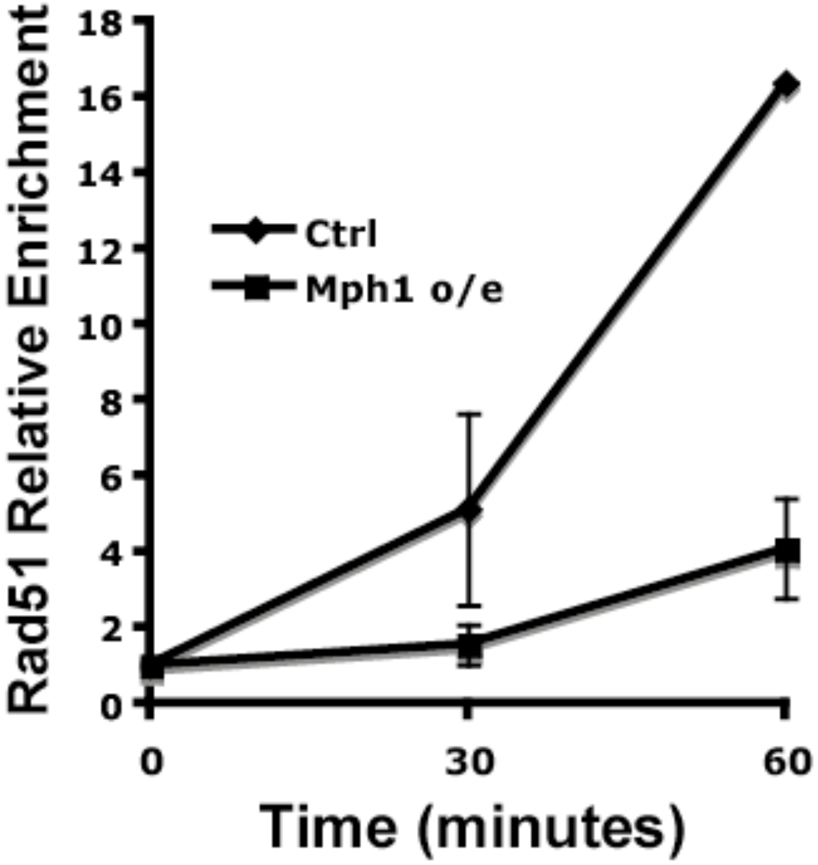


Excess Mph1 Makes Yeast More Sensitive to DNA Damaging Agents Treatments

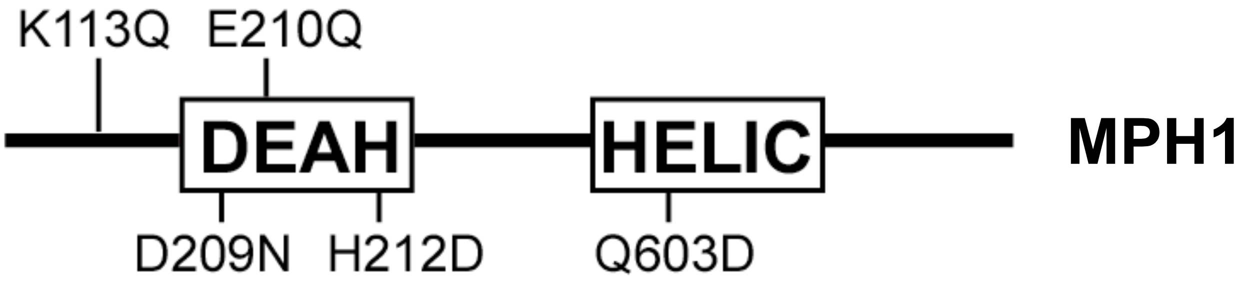


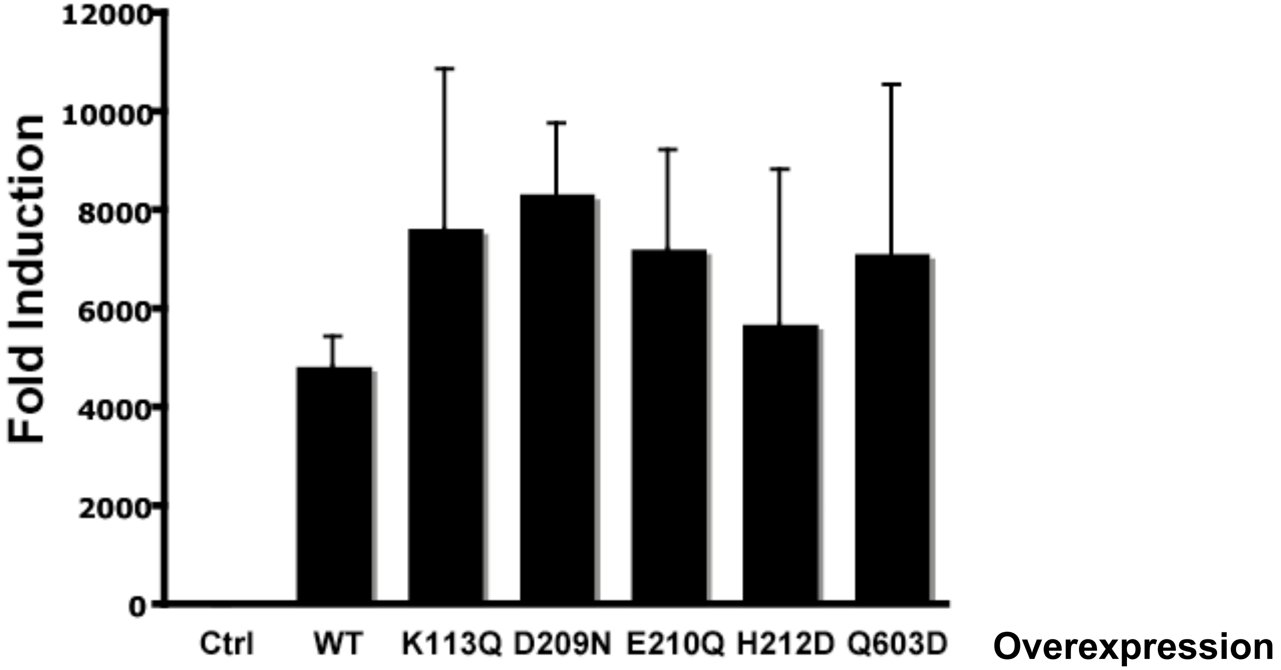
Excess Mph1 Slows Down Rad51 Recruitment to Double Strand Breaks



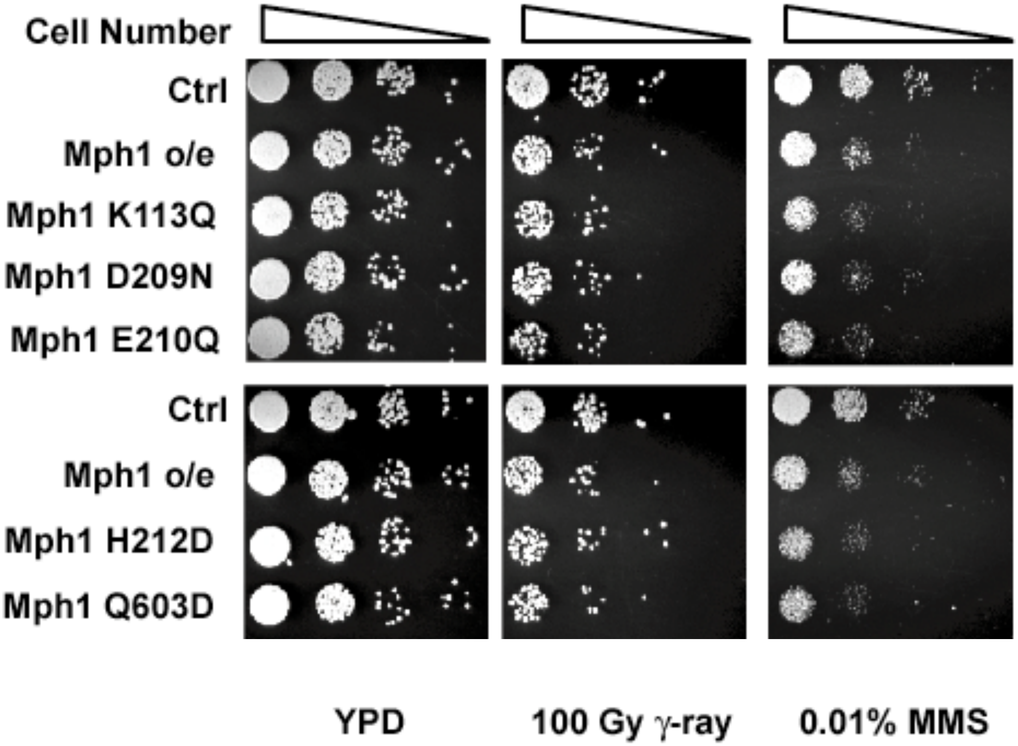


Mph1-directed GCRs do not Require Mph1's ATPase/Helicase Activities

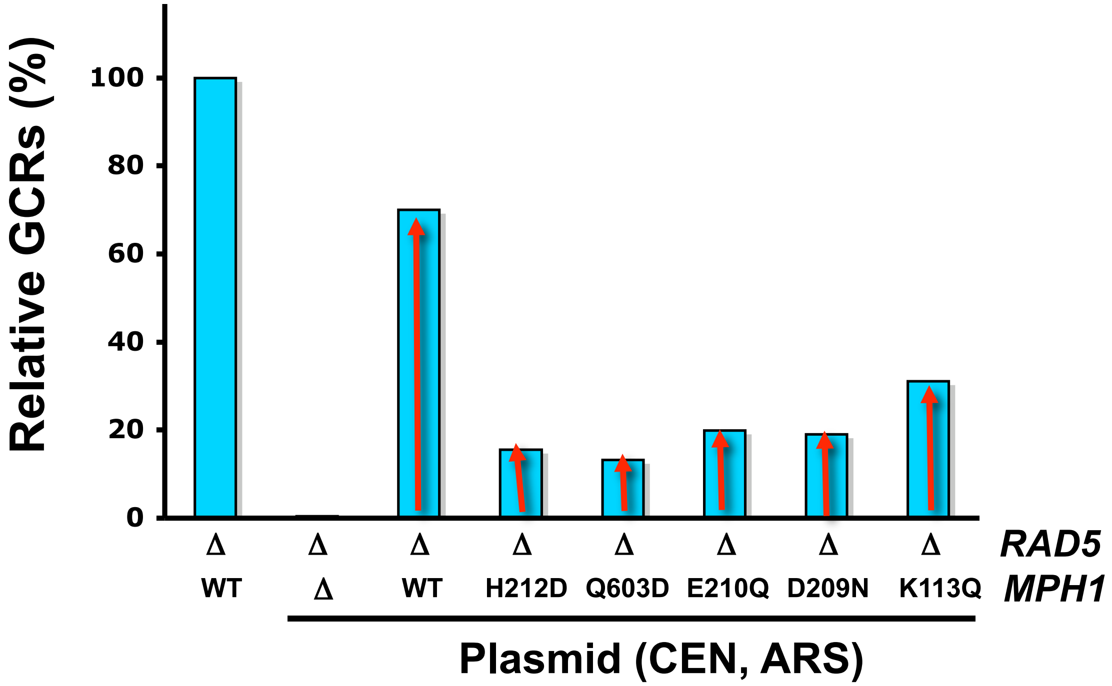




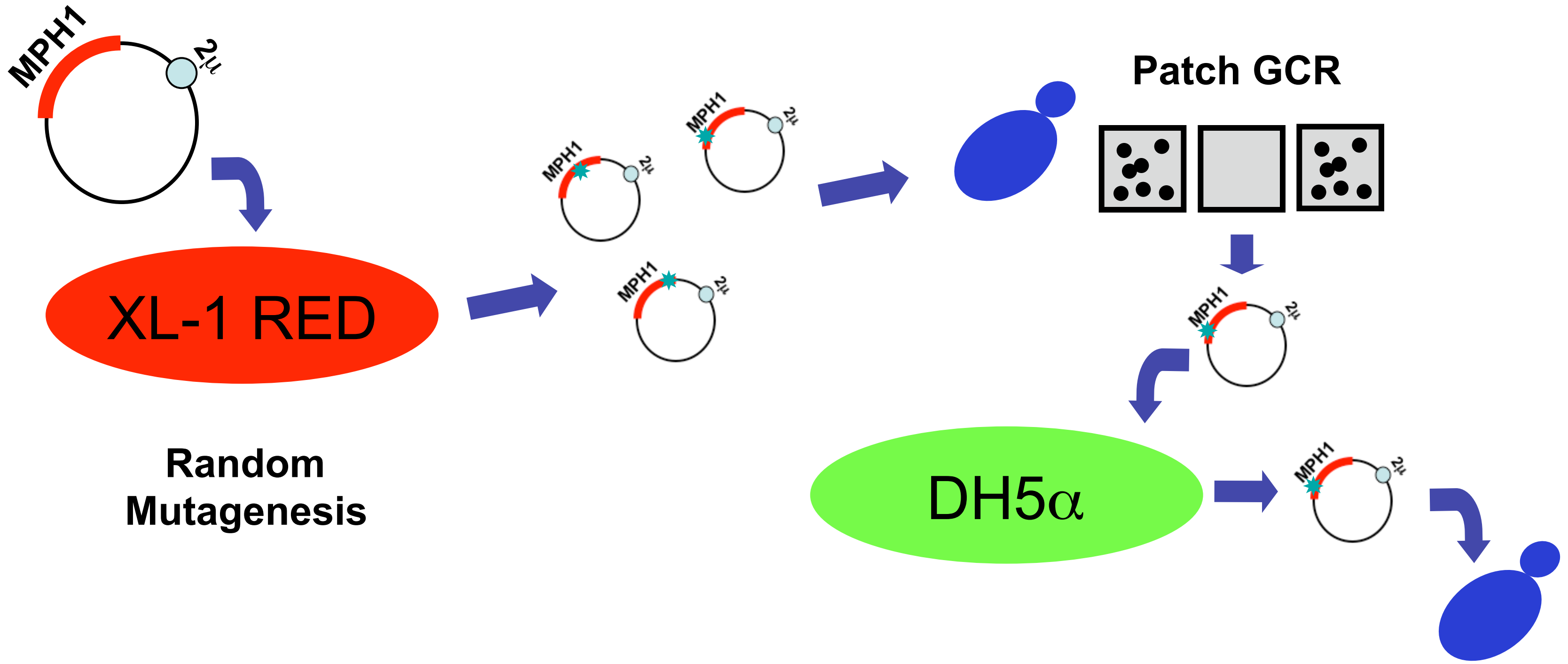
Excess ATPase/Helicase Defective MPH1 Sensitize Yeast to DNA Damaging Agents



Mph1-directed GCRs in the rad5 strain Require other function besides Mph1's ATPase/Helicase Activities



The C-terminus of Mph1 is Important for GCR Formation

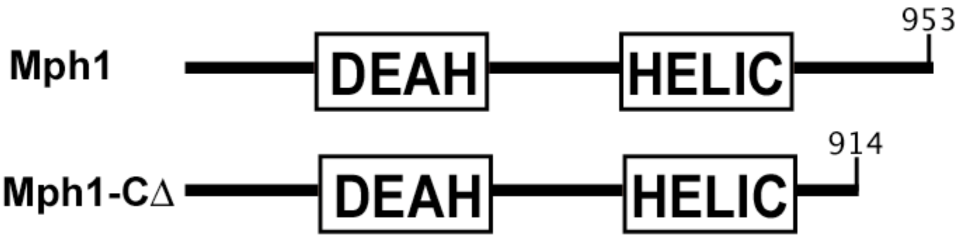


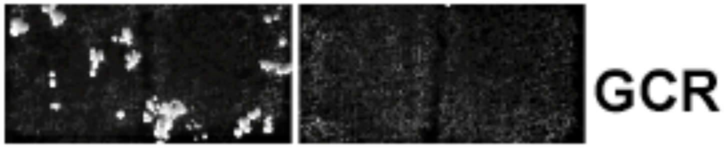
XL-1 RED

Random Mutagenesis

Patch GCR

DH5α

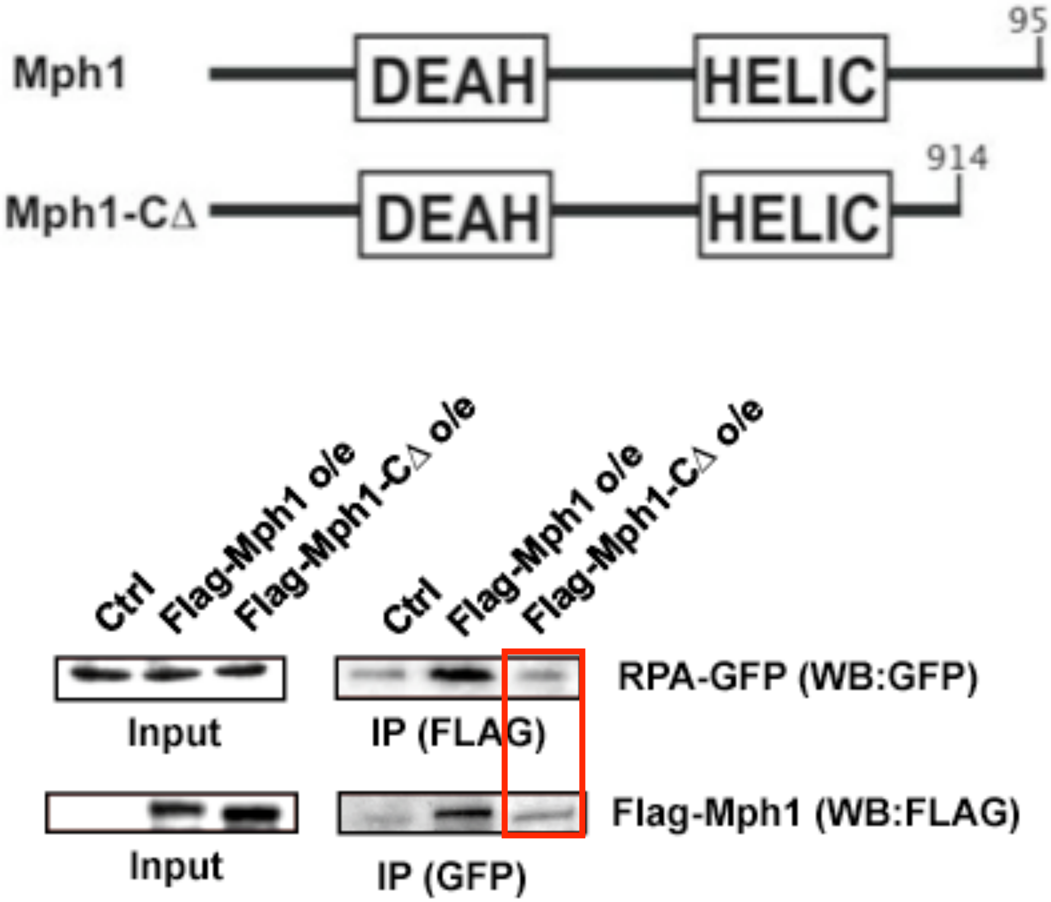




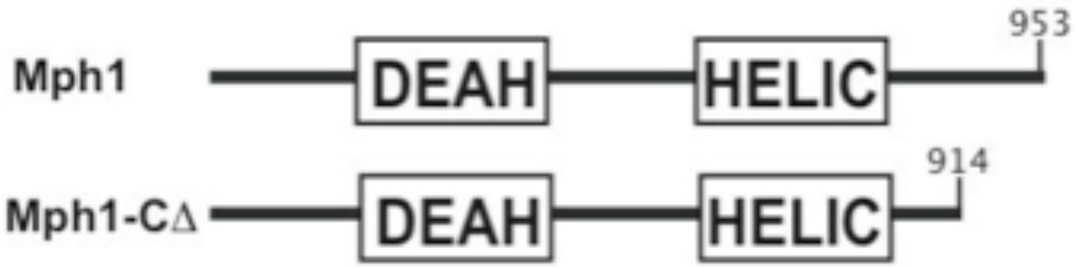
Flag-Mph1 overexpression

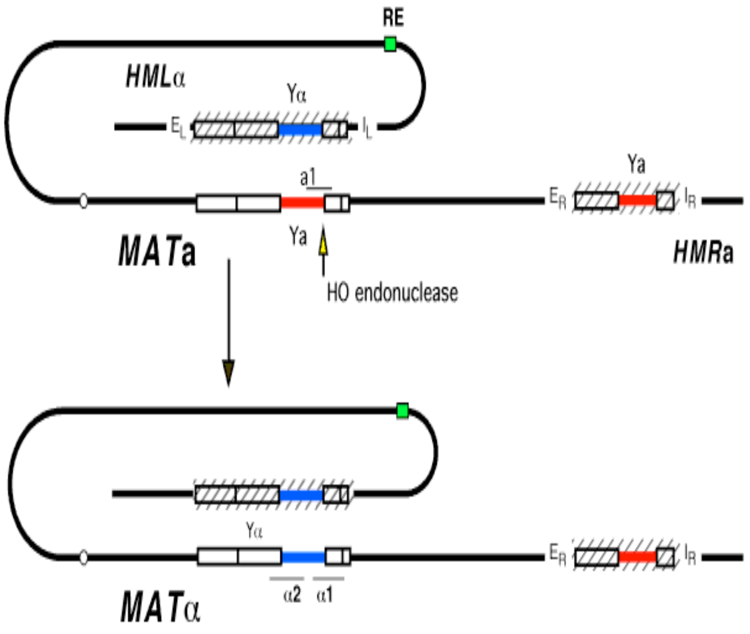
Flag-Mph1-CΔ overexpression

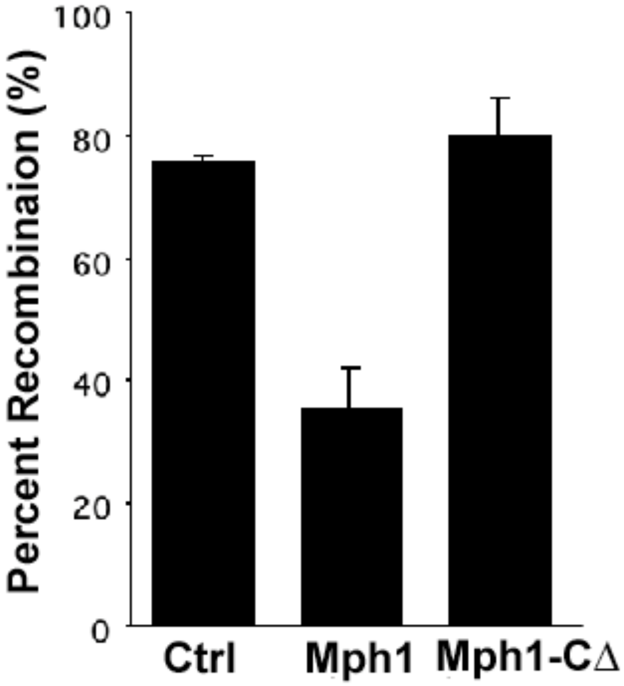
The C-terminus of Mph1 Interacts with RPA



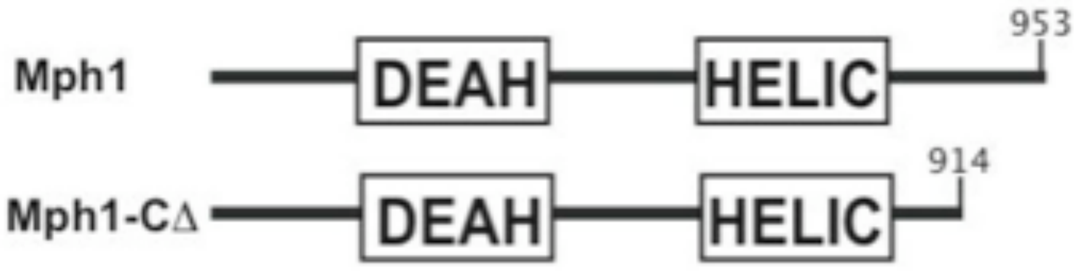
Excess CΔ-Mph1 dose not Cause Defects in Homologous Recombination

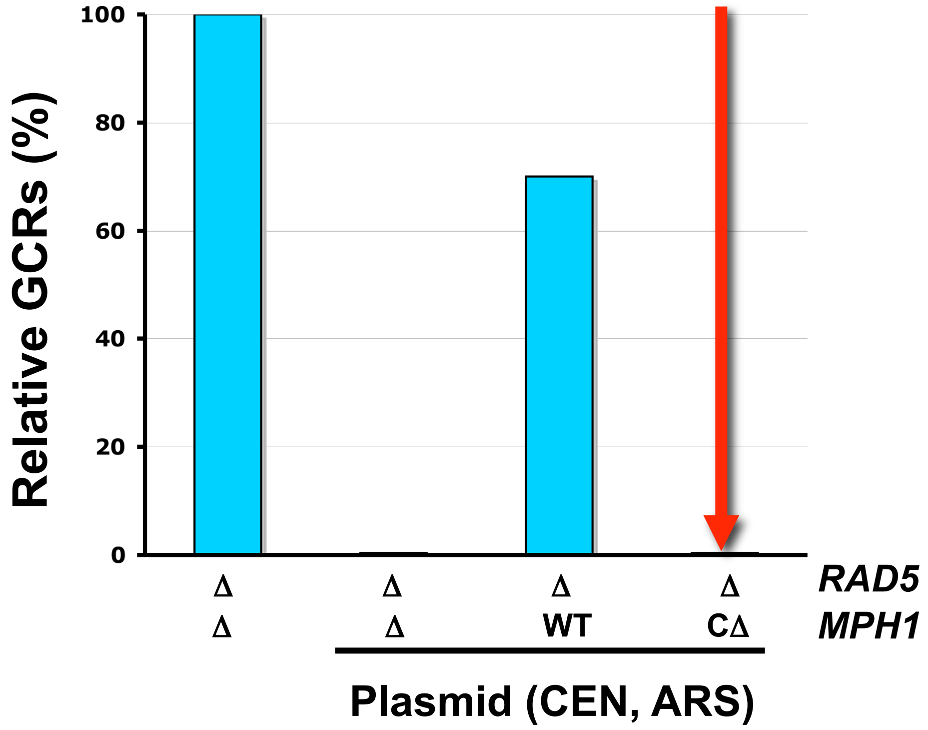




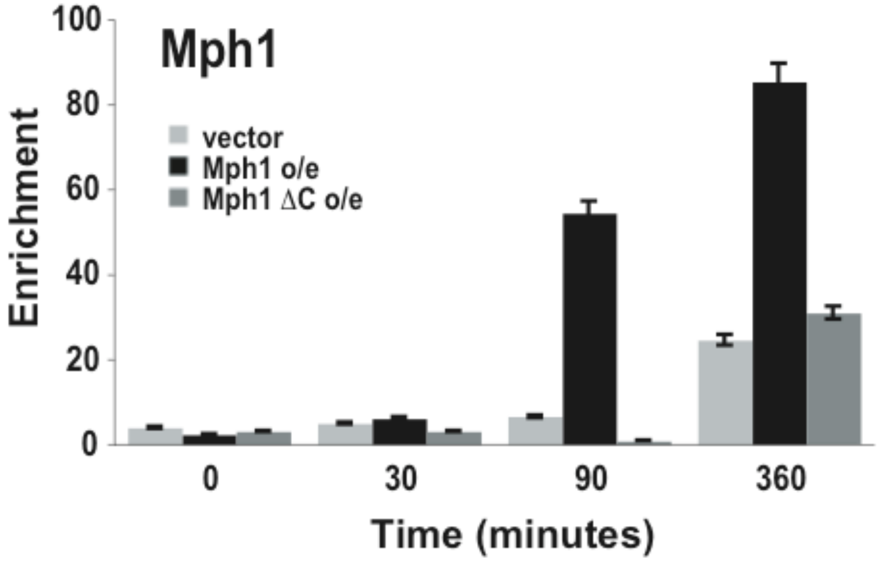


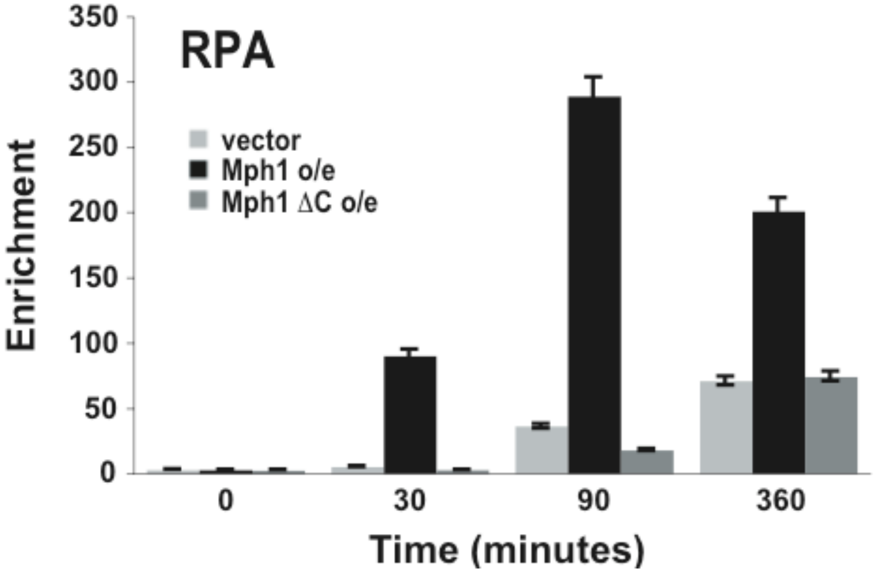
The CΔ-Mph1 Mutation Abolished Mph1's GCR-Promoting Activity in the rad5 Strain

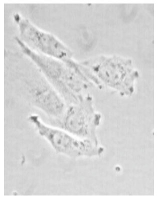




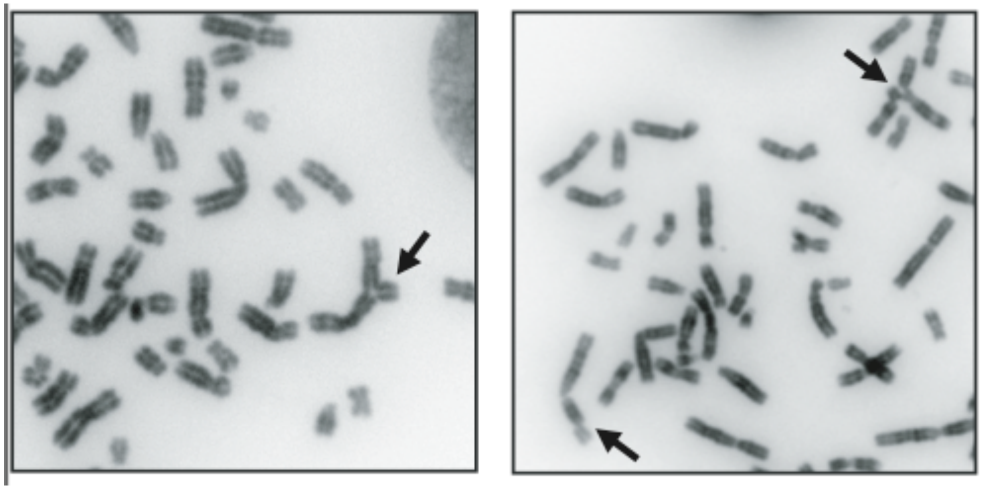
Excess Mph1 Stabilizes RPA at Double Strand Breaks (ChIP)

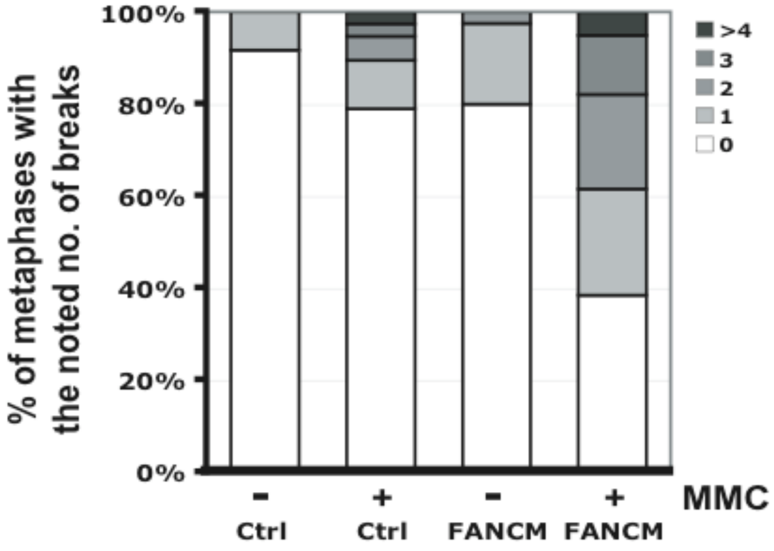






Mammalian FANCM Overepxression Enhances More Genomic Instability upon MMC Treatment





Conclusions

1. Mph1 overexpression enhances GCR formation.
2. *MPH1* deletion reduces GCR formation in *rad5*, partially in *mec1, rfa1-t33, mre11.*
3. Excess Mph1 blocks DSB induced & spontaneous HR.
4. Excess Mph1 blocks Rad51 recruitment to DSB.
5. Excess Mph1 localizes at DSB and stabilizes RPA at DSB.
6. Mph1's helicase/DEAH motifs are dispensable for GCR promoting activity.
7. The GCR promoting activity of Mph1 is resided in the C-terminus that interacts with RPA.
8. FANCM overexpression in HCT116 cells enhances more Chromosome breaks upon MMC treatment.

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