The role of phosphorylation in non homologous end joining

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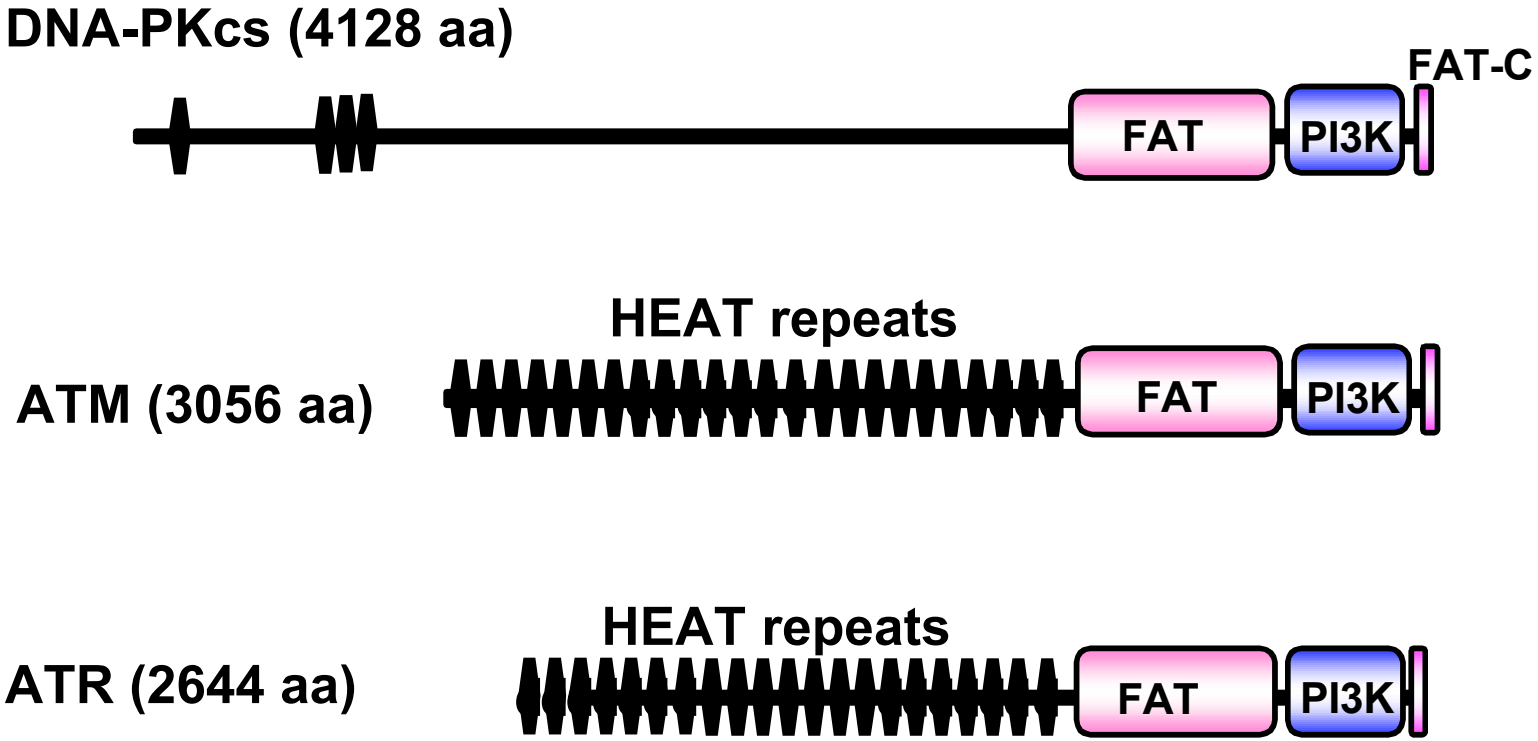
Phosphatidyl Inositol-3 kinase-like protein kinases (PIKKs)



Large polypeptides ~ 300-450 kDa (2600-4100 aa)
Serine/threonine protein kinases
Phosphorylate substrates on SQ/TQ motifs
Inhibited by wortmannin (IC_{50} ~100 nM) and caffeine (~1 mM)
Involved in the cellular response to DNA damage

Functions of PIKKs

DNA-PKcs:

Catalytic subunit of the DNA-dependent protein kinase (DNA-PK)

Repair of DNA double strand breaks (DSBs)

ATM:

Ataxia telangiectasia mutated

Activation of cell cycle checkpoints in response to DSBs

ATR:

ATM- and Rad3-related

Activation of cell cycle checkpoints in response to collapsed replication forks and bulky lesions (UVC)

DNA double strand breaks, DSBs:

Caused by ionizing radiation:
X-rays, γ-rays, cosmic radiation

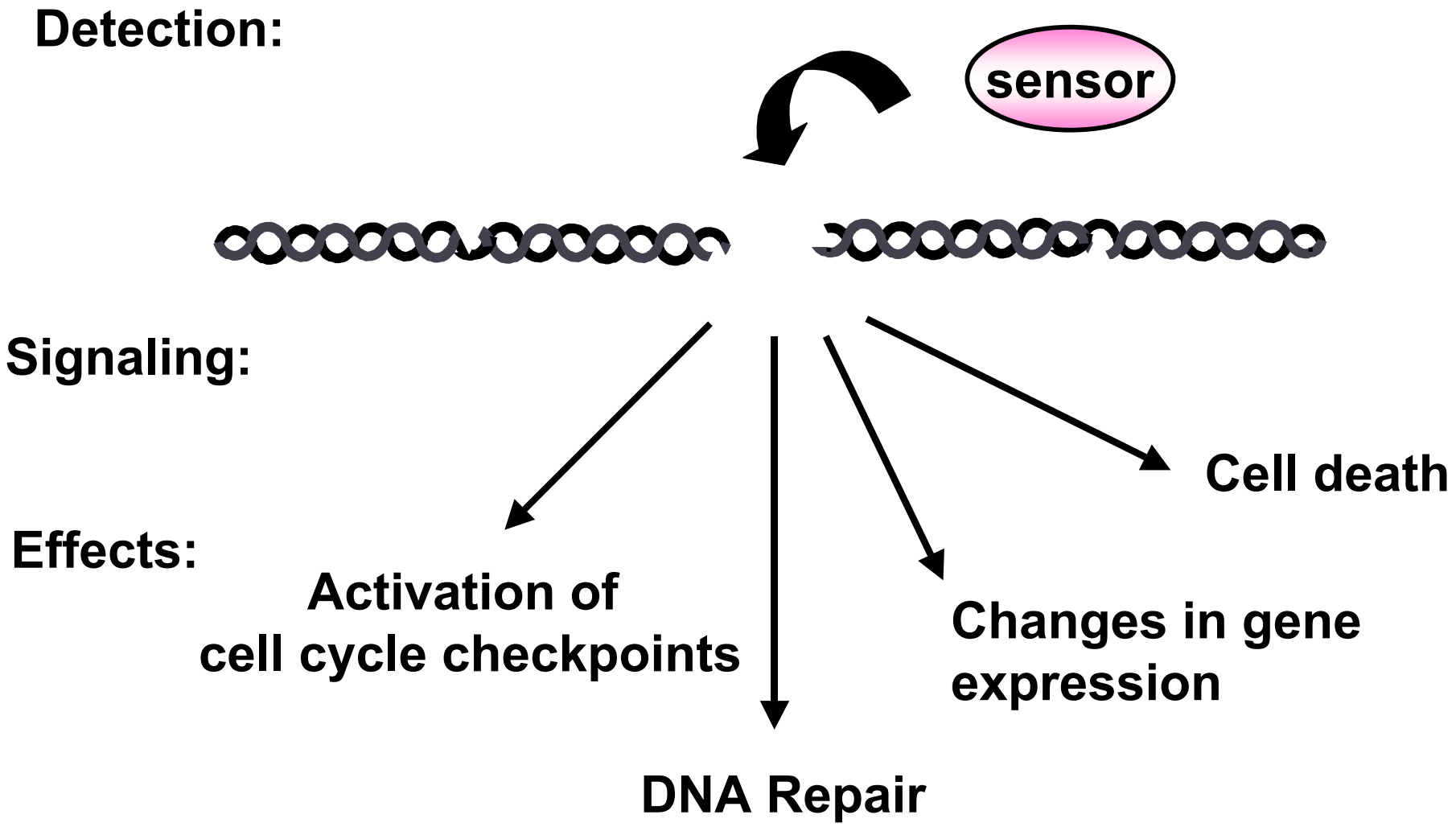
Topoisomerase poisons:
etoposide, camptothecin, doxorubicin

Collapsed replication forks

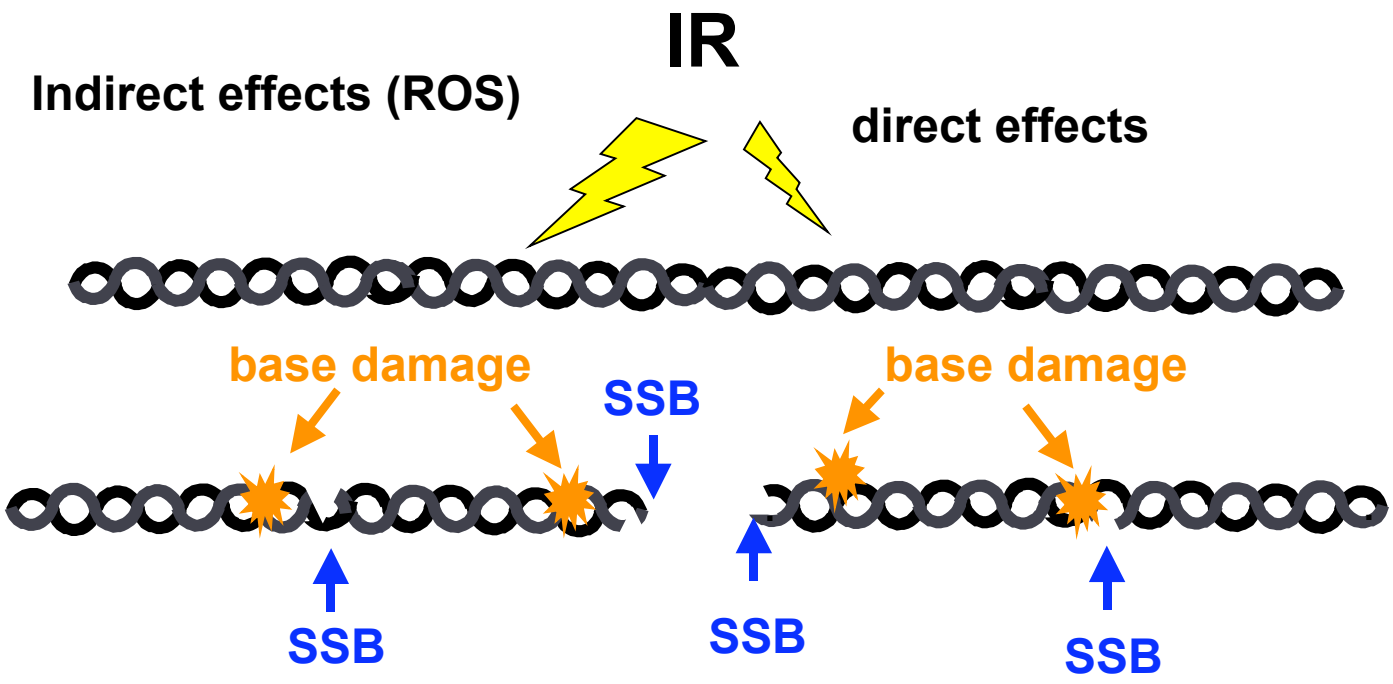
Reactive oxygen species

Introduced by RAG genes in V(D)J recombination and AID in Class Switch Recombination

Cellular Responses to a DSB



Mechanism of IR-induced DNA damage:

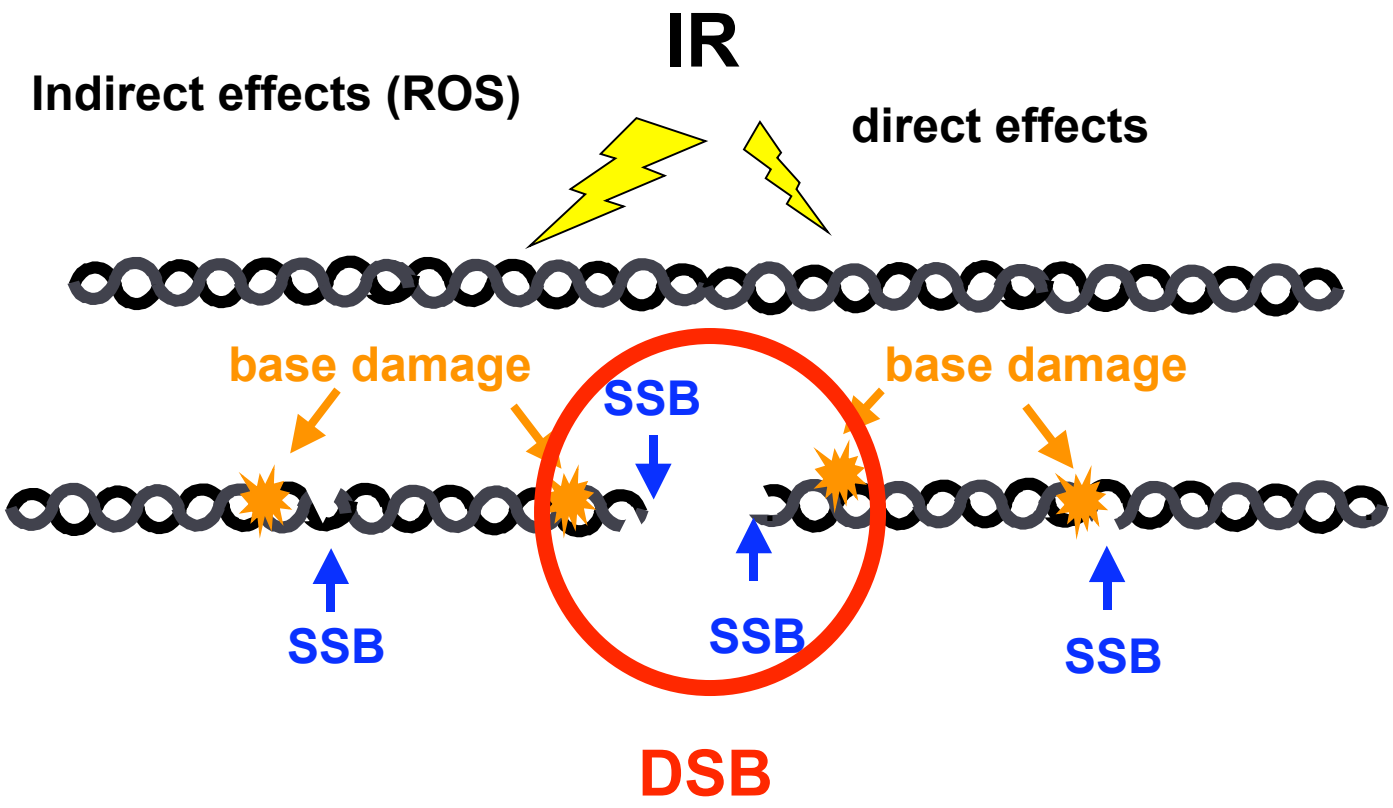


IR induces complex DNA damage

Damage to bases

Production of single strand breaks (SSBs)

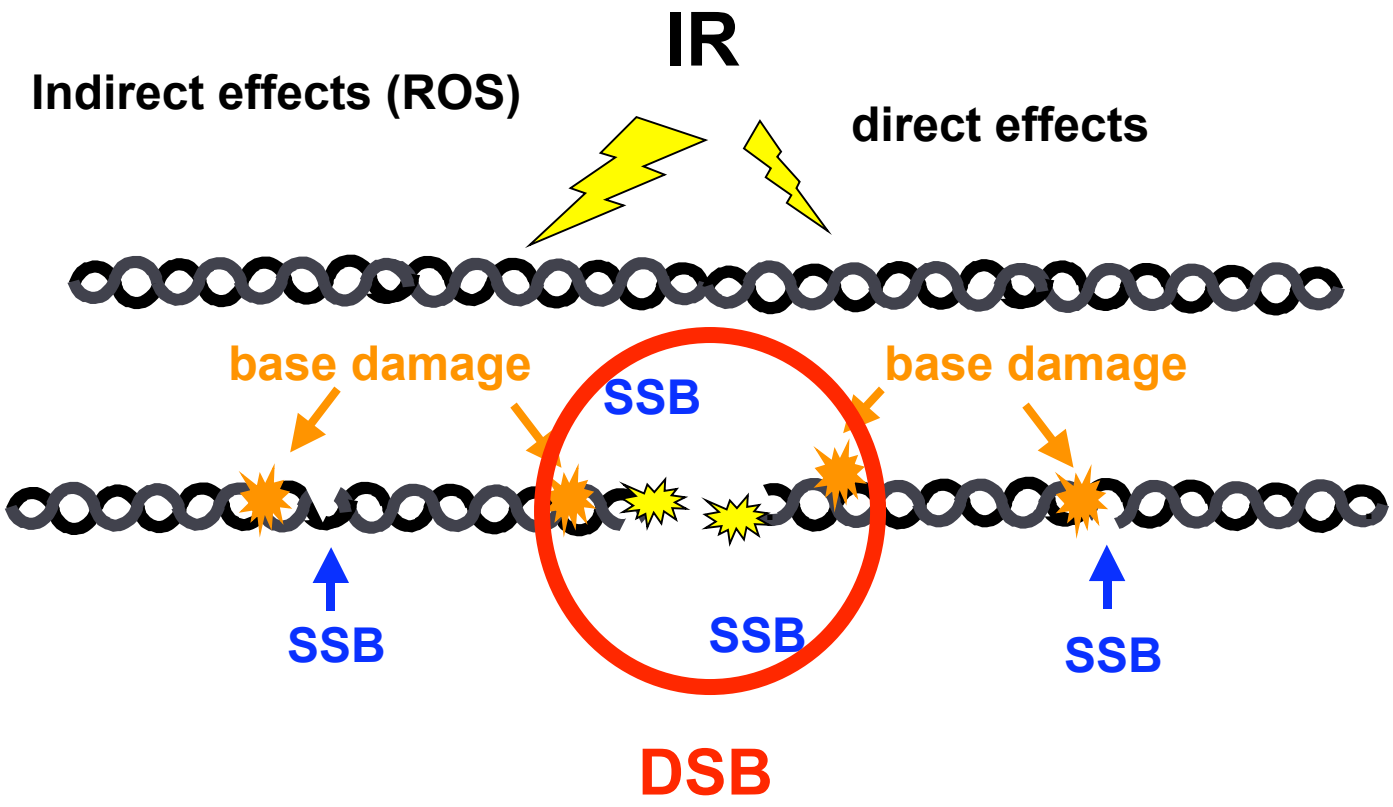
Mechanism of IR-induced DNA damage:



DNA double strand break (DSB):

2 SSBs on opposite strands within 10-20 bp

Mechanism of IR-induced DNA damage:



DNA double strand break (DSB):
2 SSBs on opposite strands within 10-20 bp

Ends SSBs frequently contain non-ligatable ends

Human cells contain two pathways for the repair of DSBs

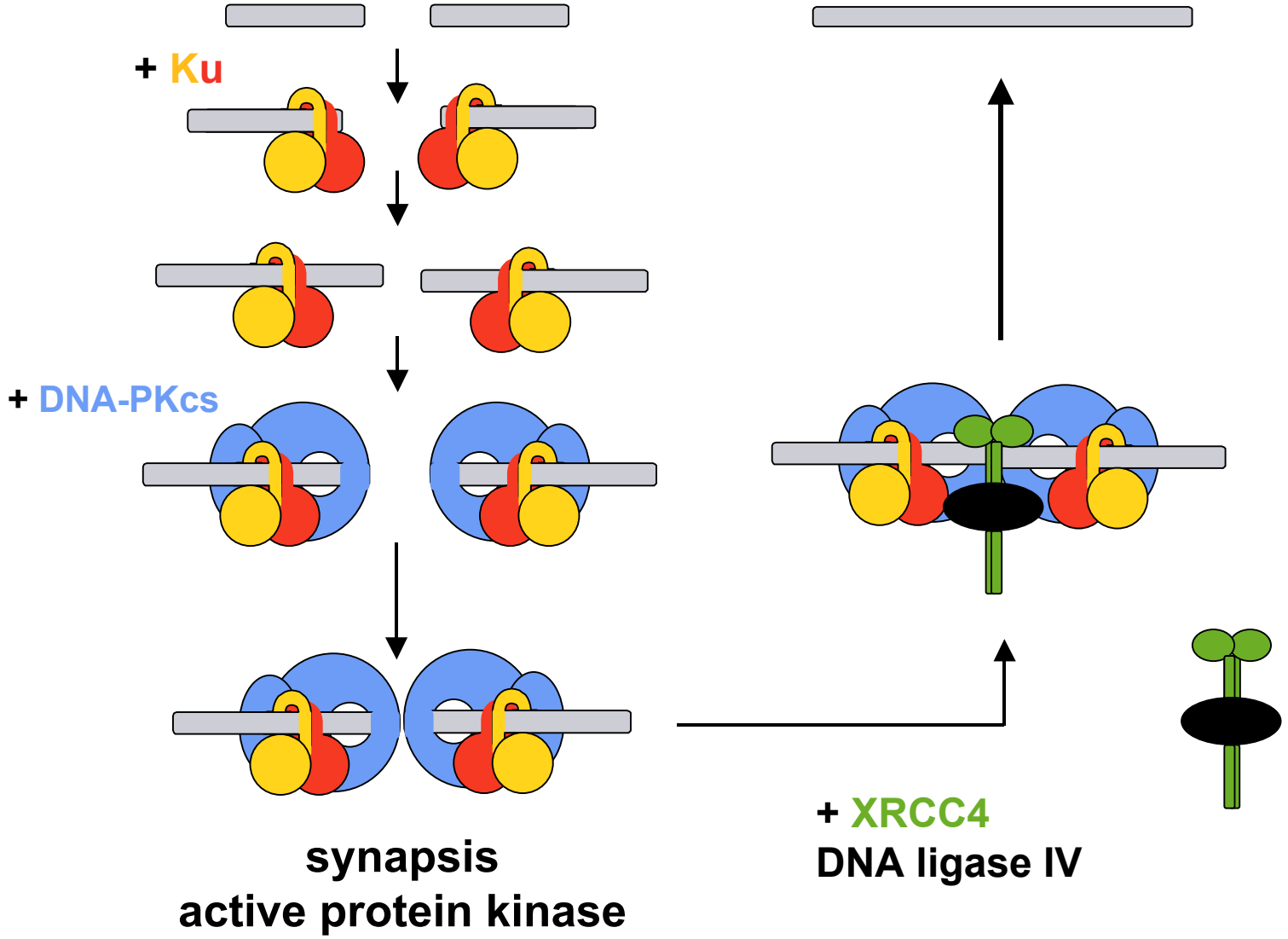
Homologous recombination repair (HRR):

- Mre11-Rad50-Nbs1 (*Xrs2* in yeast), RPA, Rad51, Rad52, XRCC2, XRCC3, BRCA2, BRCA1
- Predominant pathway in yeast
- Active in late S and G2
- Requires undamaged DNA template, accurate repair

Nonhomologous endjoining (NHEJ):

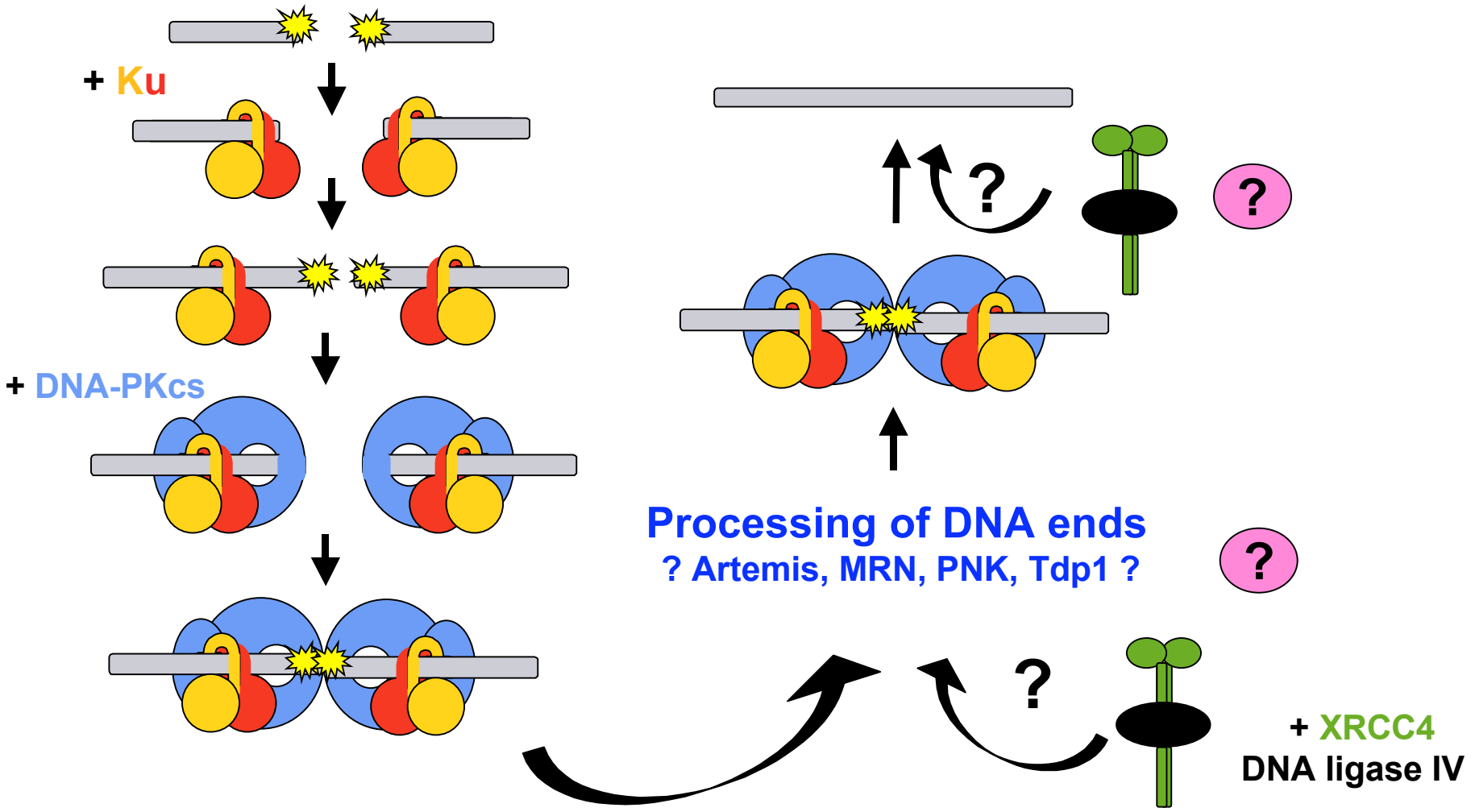
- DNA-PKcs, Ku70/80, XRCC4, DNA ligase IV
- Also Artemis, Tdp1, PNK, DNA polymerase μ
- Major pathway in human cells for repair of IR-induced DSBs
- Active throughout the cell cycle, predominant in G0, G1
- Does not require DNA template, inaccurate repair
- Required for V(D)J recombination and class switch recombination

A Simple Model for Nonhomologous Endjoining



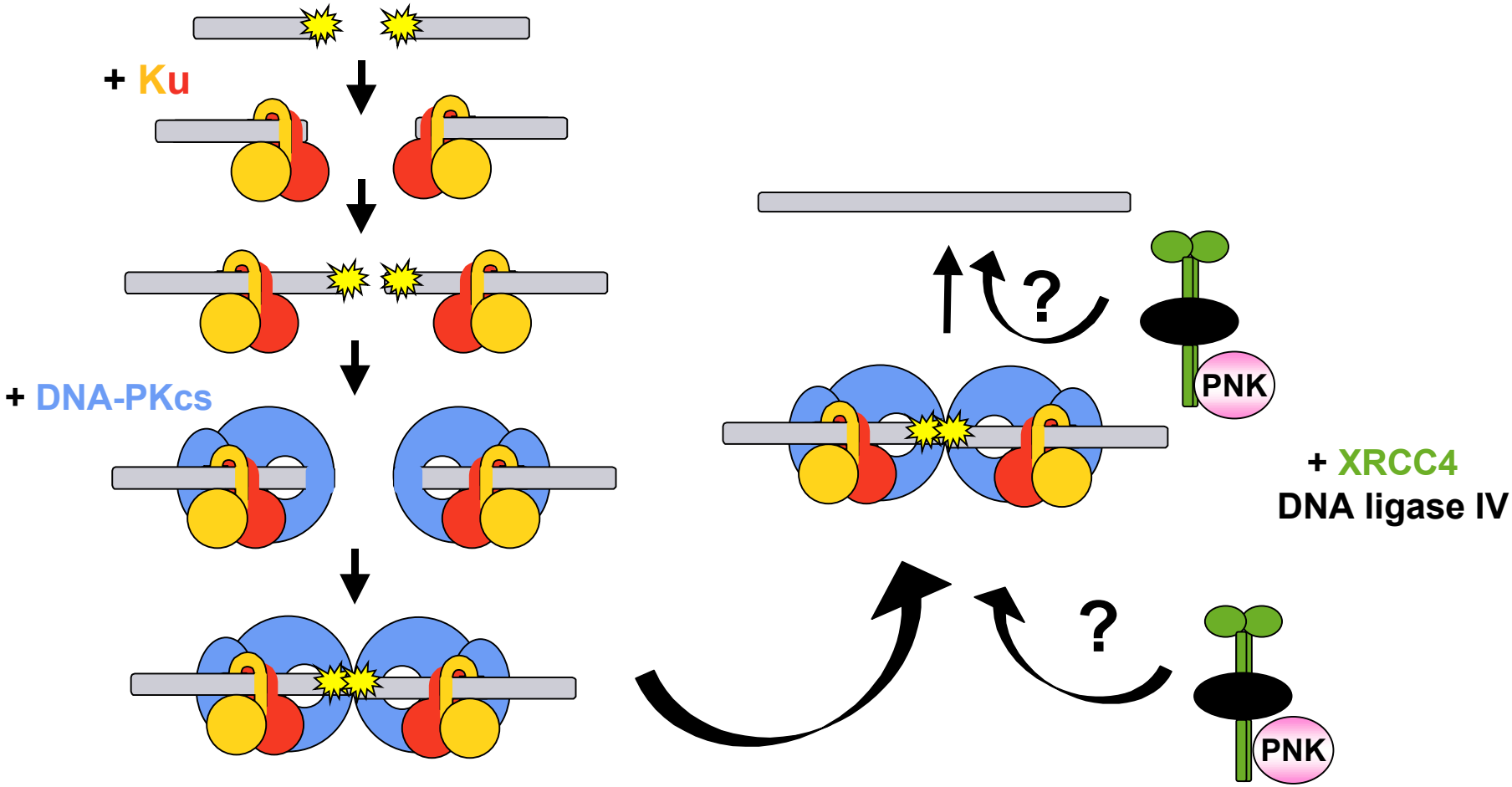
IR-induces non-ligatable DNA ends: ✸
3'-phosphates and 3'-phosphoglycolates

How and when are non ligatable DNA ends removed?



Candidate processing enzymes: Polynucleotide kinase PNK

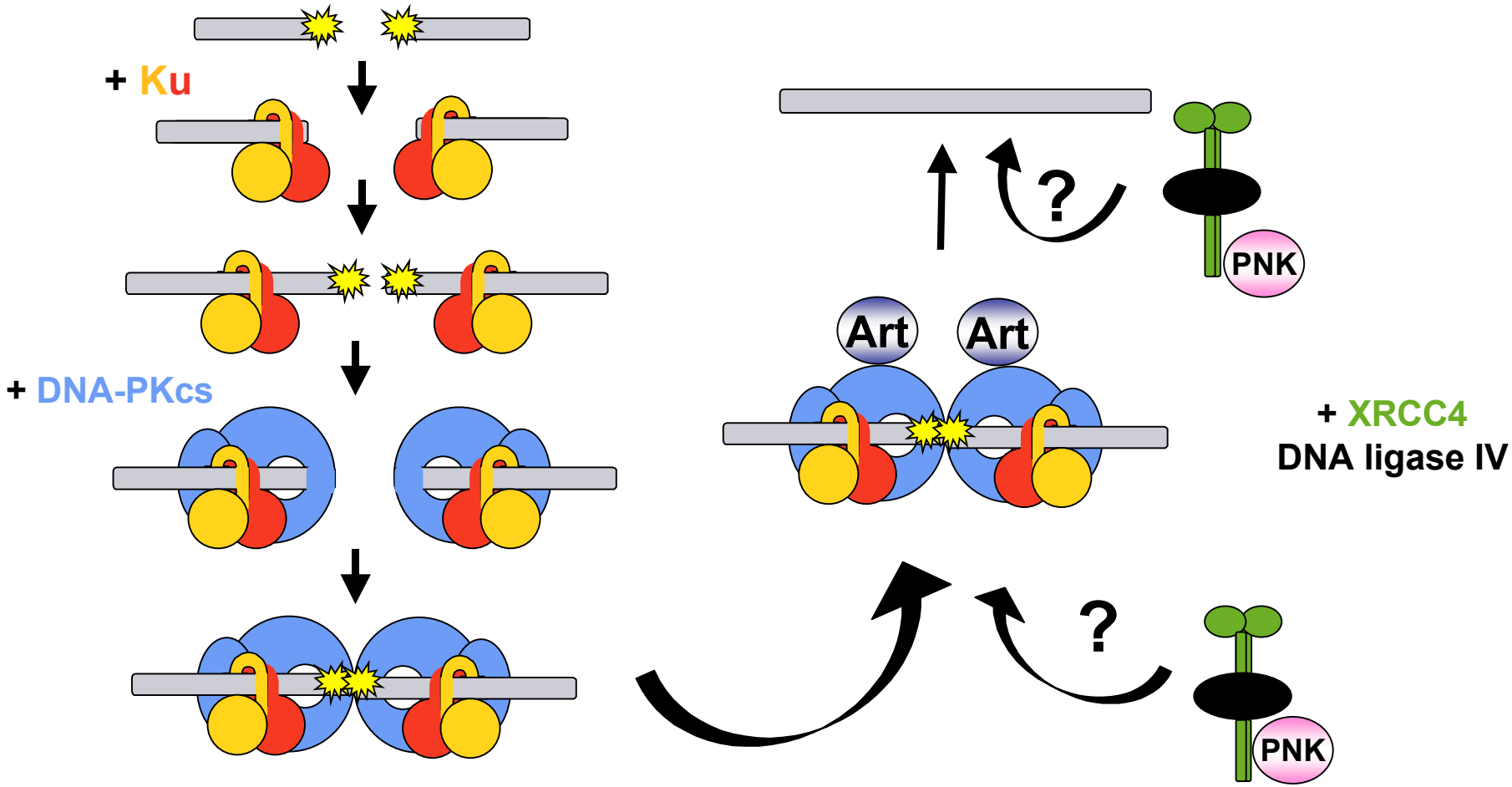
Removes 3'-phosphates and adds 5'-phosphates to DNA
(Karimi-Busheri et al, 1999)
Interacts with XRCC4 (requires CK2 phosphorylation) (Koch et al, 2004)



Candidate processing enzymes: Artemis

Nuclease, interacts with DNA-PKcs (Ma and Lieber, 2002)

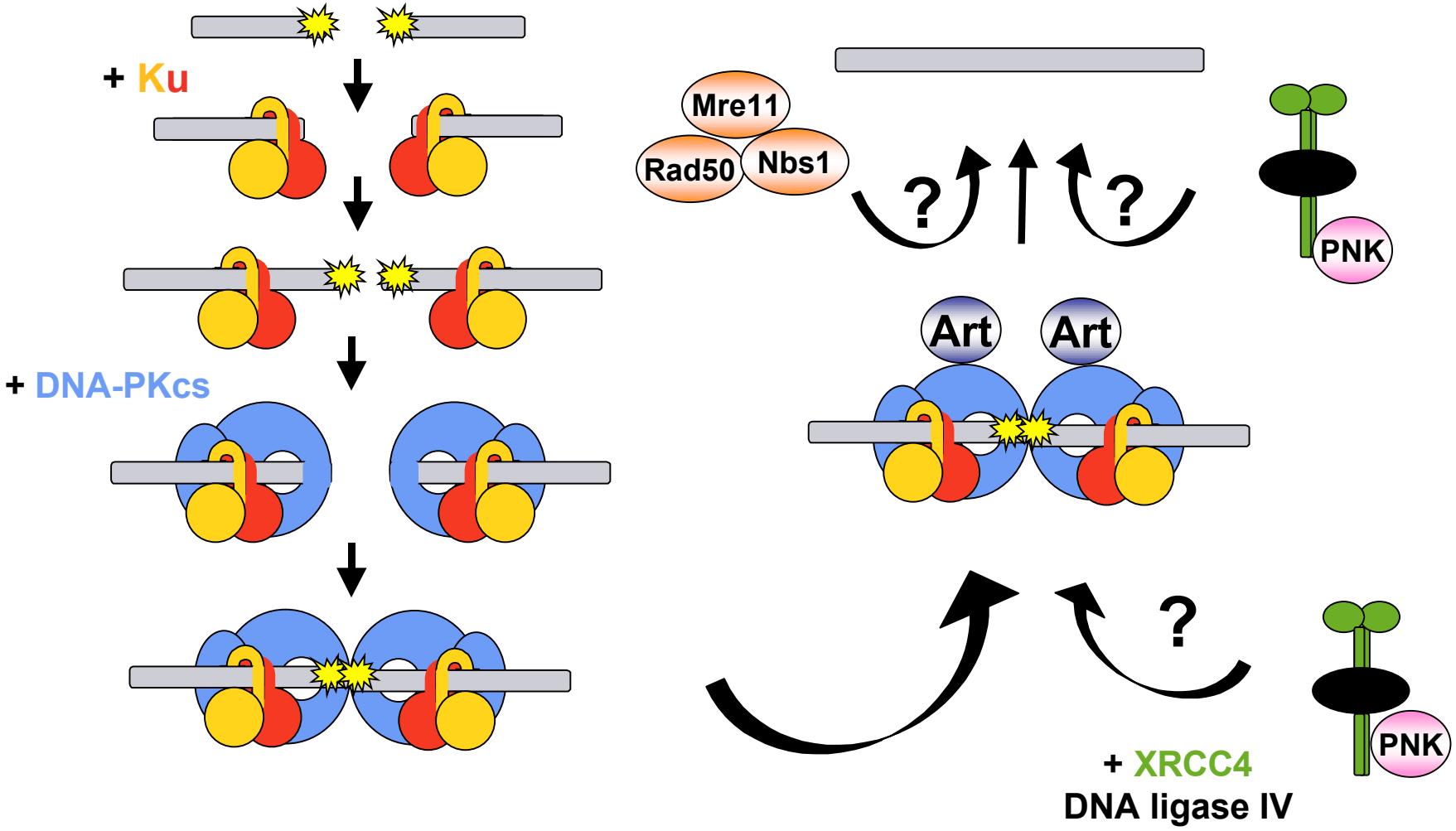
Mutations in Artemis lead to radiation sensitivity and immune deficiency (RS-SCID) (Moshous et al, 2001)



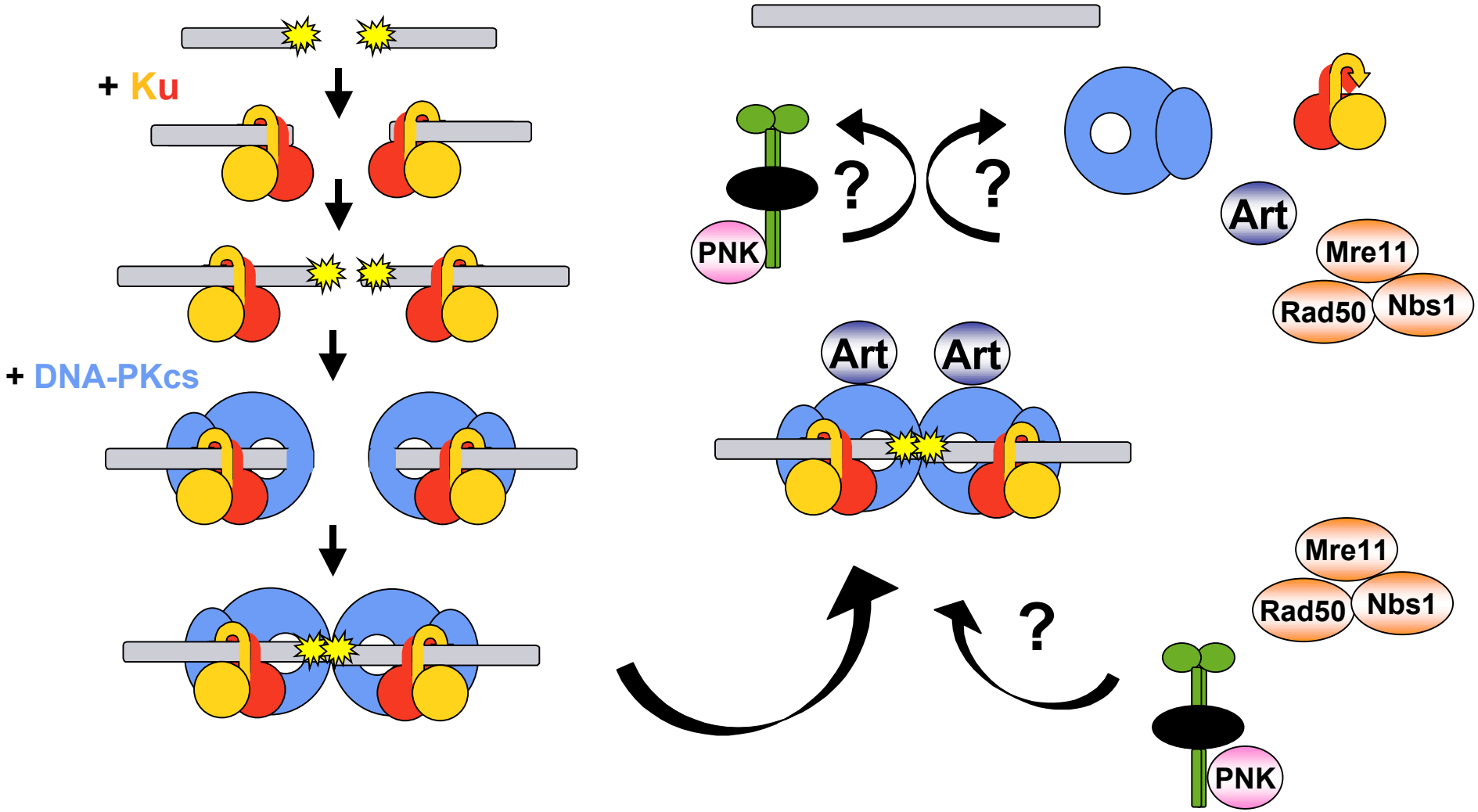
Candidate processing enzymes: MRN complex

Mre11, Rad50, Nbs1 Mre 11 nuclease activity

Required for NHEJ in yeast but mammalian cells = ?



When and how are the proteins released?

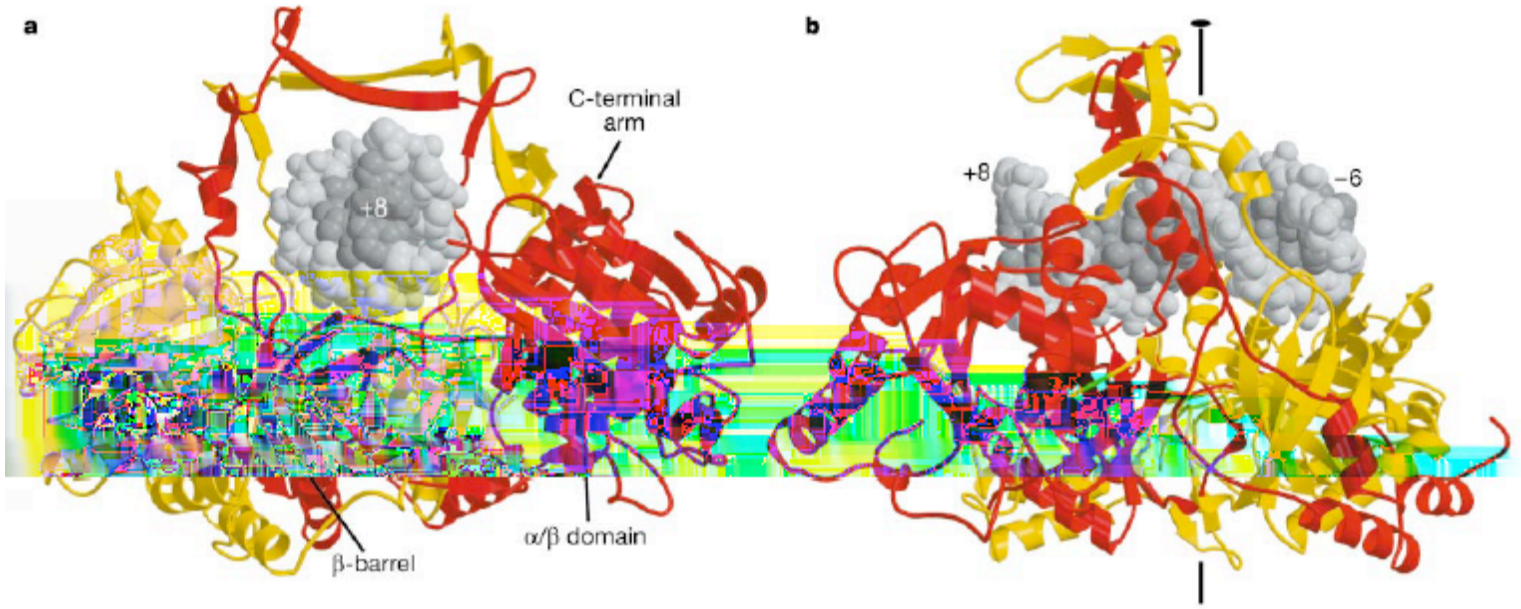


Structure of Ku70/80 heterodimer

Ku70/80 dimer threads onto DNA at DSB - encircles DNA

Options for release:

proteolysis, push or back off, nucleolytic cleavage?



Walker et al, Nature, 412, 607-614 (2001)

Role of phosphorylation in NHEJ:

- DNA-PK is a serine/threonine protein kinase
- Protein kinase activity is inhibited by wortmannin
- Cells that lack DNA-PKcs or Ku are radiosensitive and defective in DSB repair
- Wortmannin radiosensitizes cells and inhibits DSB repair
- DNA-PKcs null cells containing DNA-PKcs with an inactivating mutation in the kinase domain are radiation sensitive and defective in DSB repair

(reviewed in Lees-Miller and Meek, 2003)

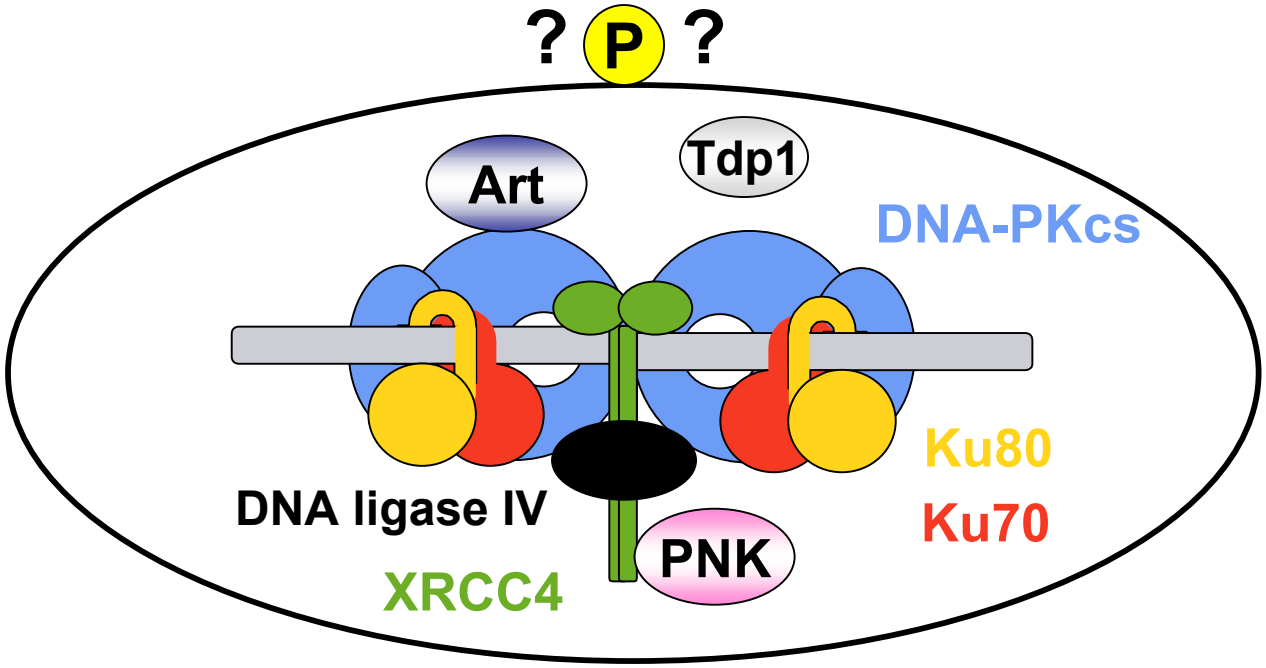
Experimental:

- The protein kinase activity of DNA-PK is required for NHEJ

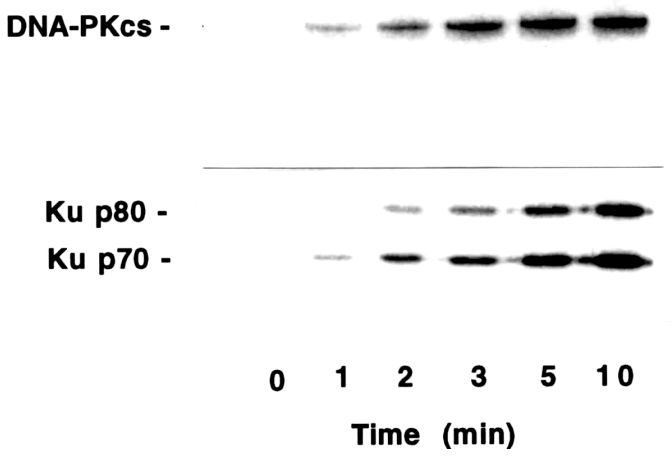
- DNA-PK is only active when bound at a DNA DSB

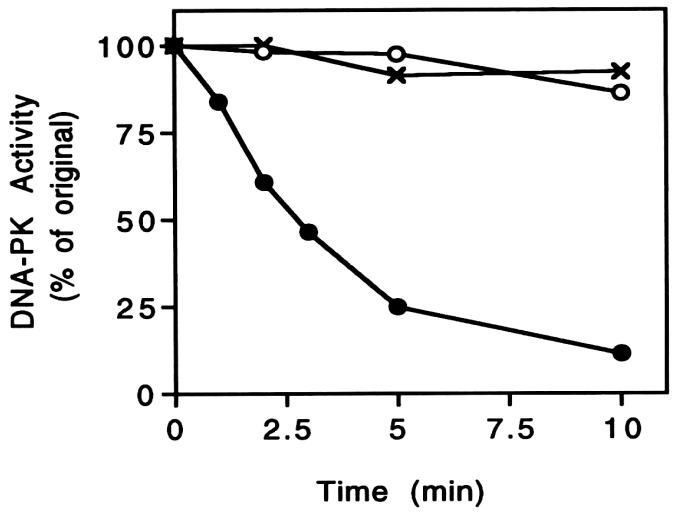
Hypothesis:

DNA-PK substrates such as Ku70, Ku80, DNA-PKcs, XRCC4 and DNA ligase IV, as well a processing enzymes such as PNK, Tdp1 or Artemis are possible physiological substrates

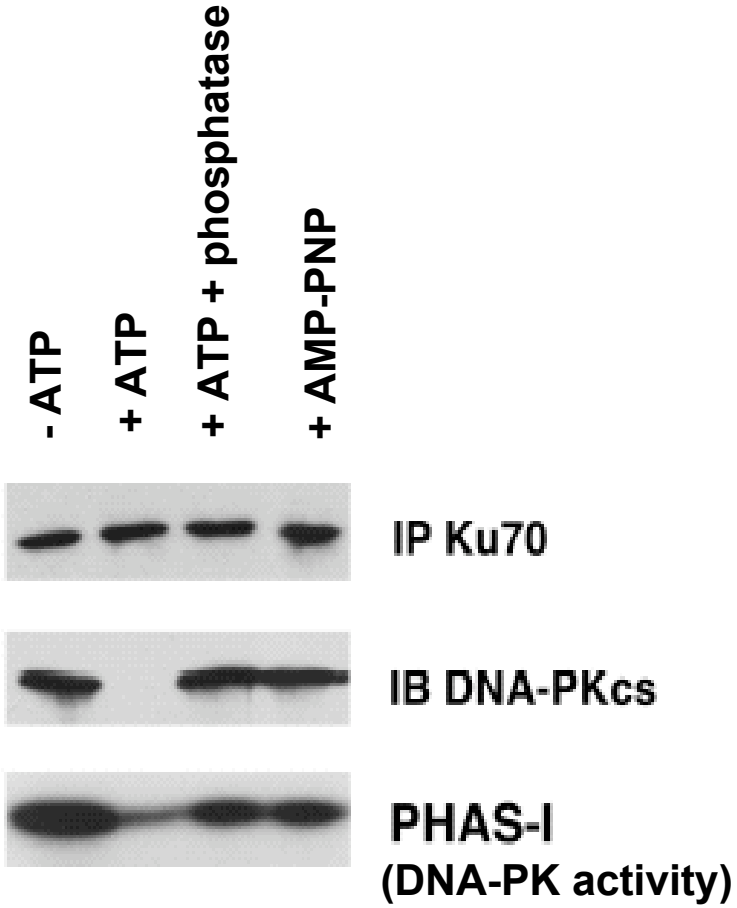


Autophosphorylation of DNA-PK correlates with loss of protein kinase activity and dissociation of DNA-PKcs + Ku





Chan and Lees-Miller, JBC, 1996



Douglas et al, JBC, 2001

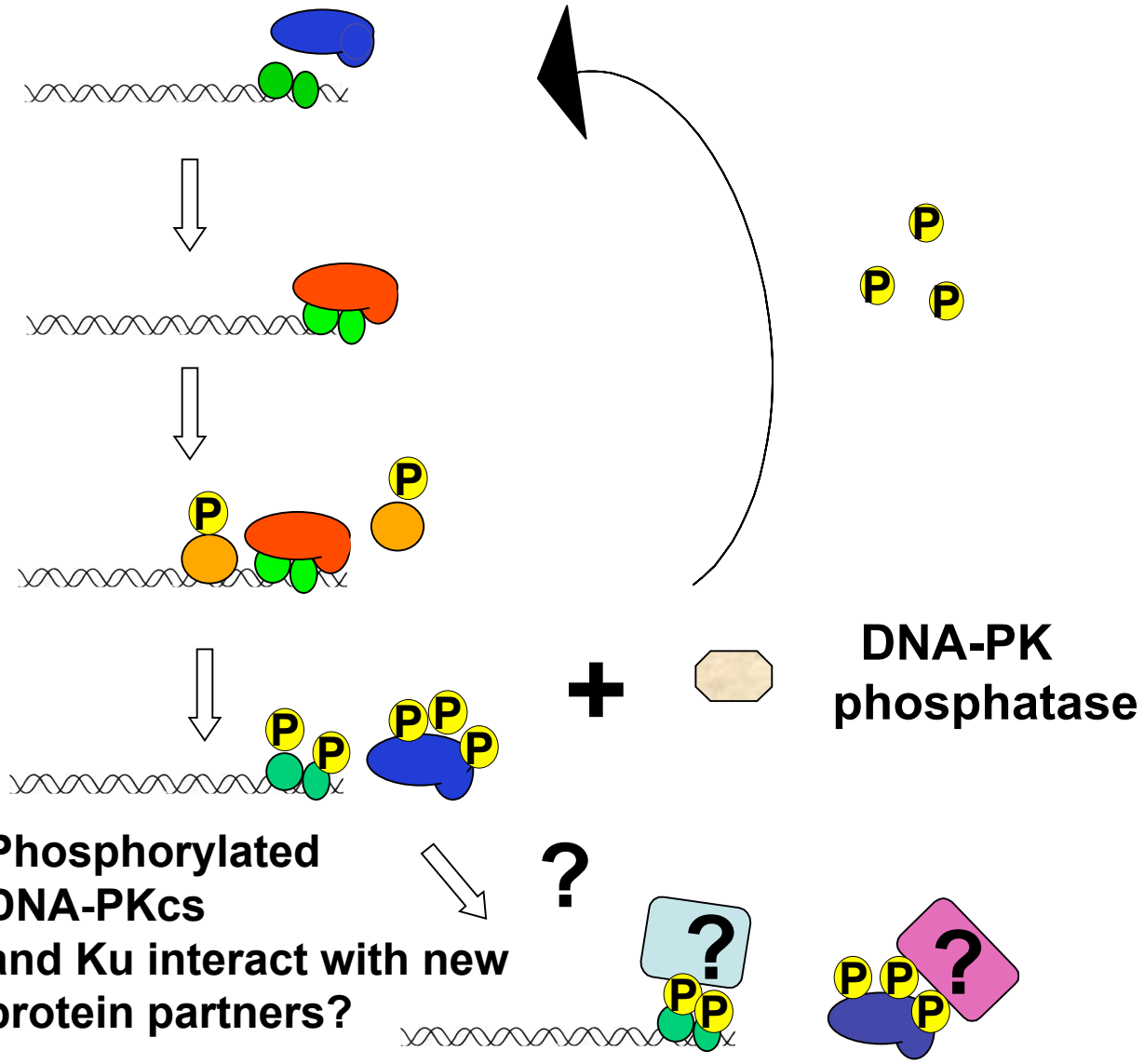
Model for regulation of DNA-PK by reversible protein phosphorylation.

DNA-PKcs and Ku assemble at a DNA double-strand break

Active DNA-PK complex is formed.

DNA-PK phosphorylates its substrates.

DNA-PK autophosphorylates, inactivates itself and dissociates.



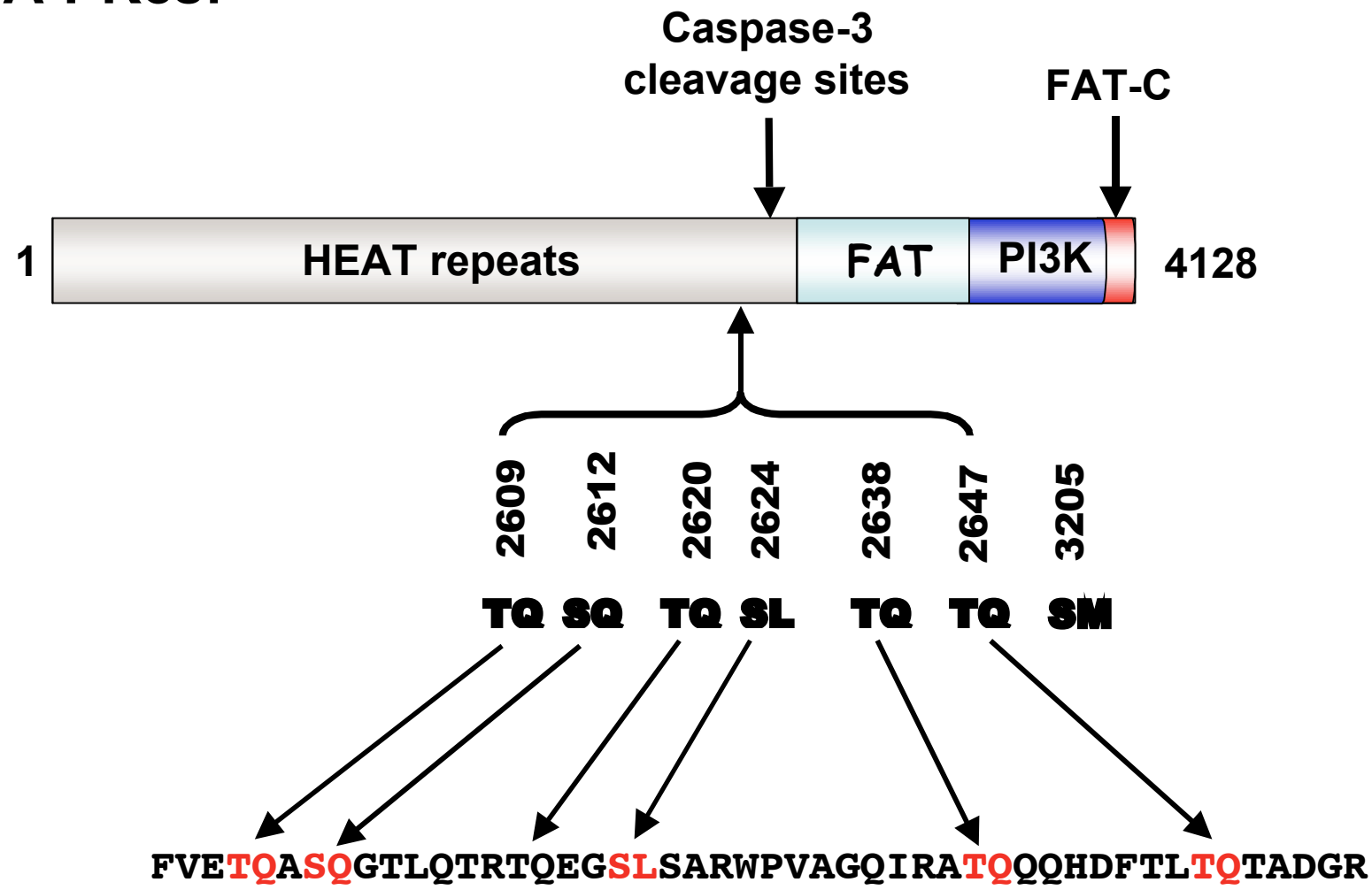
Identification of phosphorylation sites in DNA-PKcs and its putative substrates:

- Phosphorylation in vitro using purified substrates
- SDS PAGE
- Tryptic digestion
- HPLC
- MALDI-TOF
- Radiochemical sequencing (Edman Degradation)
- MS-MS

Generation and characterization of phosphospecific antibodies

Expression of phosphorylation mutants in null cell lines and effect on DSB repair and V(D)J recombination

DNA-PKcs:



Identification of seven in vitro phosphorylation sites in DNA-PKcs

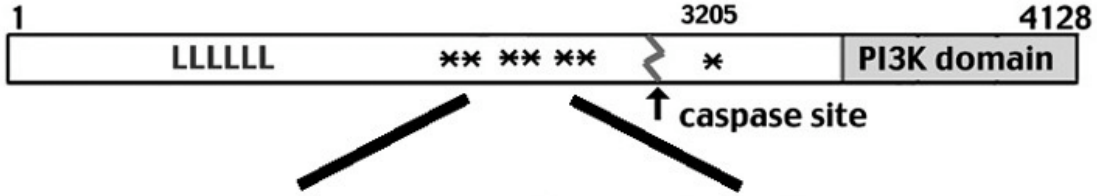
(Douglas et al, Biochem J, 2002)

DNA-PKcs autophosphorylation sites are highly conserved between human, mouse, dog, horse, chicken and *Xenopus*:

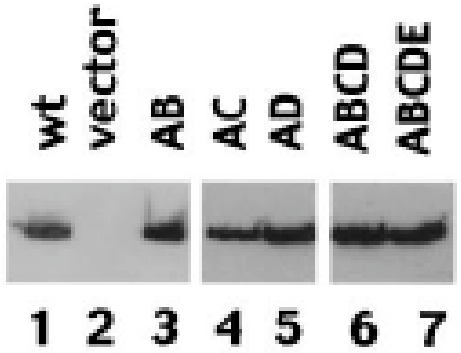
Douglas et al, Biochem J, 2002

Kathy Meek: Michigan State University

Generate single and multiple phosphorylation mutants (S or T to A) in full length human DNA-PKcs and stably express in DNA-PKcs$^{-/-}$ rodent cells (V3/CHO):



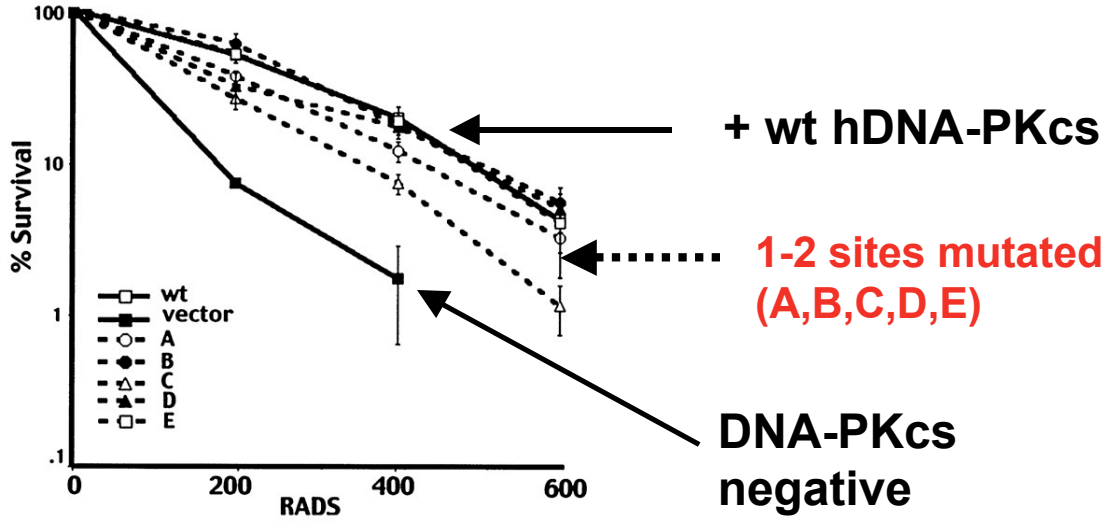
	2609	2612	2620	2624	2638	2647
	TQ	SQ	TQ	SL	TQ	TQ
site	"A"	"E"	"B"		"C"	"D"
mutant						
A	AQ	SQ	TQ	SL	TQ	TQ
B	TQ	SQ	AQ	AL	TQ	TQ
C	TQ	SQ	TQ	SL	AQ	TQ
D	TQ	SQ	TQ	SL	TQ	AQ
E	TQ	AQ	TQ	SL	TQ	TQ
AB	AQ	SQ	AQ	AL	TQ	TQ
AC	AQ	SQ	TQ	SL	AQ	TQ
AD	AQ	SQ	TQ	SL	TQ	AQ
ABCD	AQ	SQ	AQ	AL	AQ	AQ
ABCDE	AQ	AQ	AQ	AL	AQ	AQ

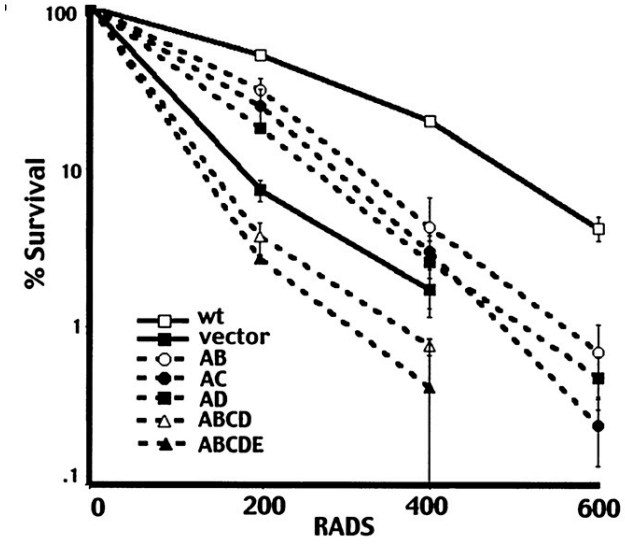


Ding et al, MCB, 2003

Clonogenic survival assays in stably transfected V3 cells (DNA-PKcs null) complemented with wt or autophosphorylation mutant DNA-PKcs:

A: T2609A (TQ)
B: T2620A (TQ)
S2624A (SL)
C: T2638A (TQ)
D: T2647A (TQ)
E: S2612A (SQ)





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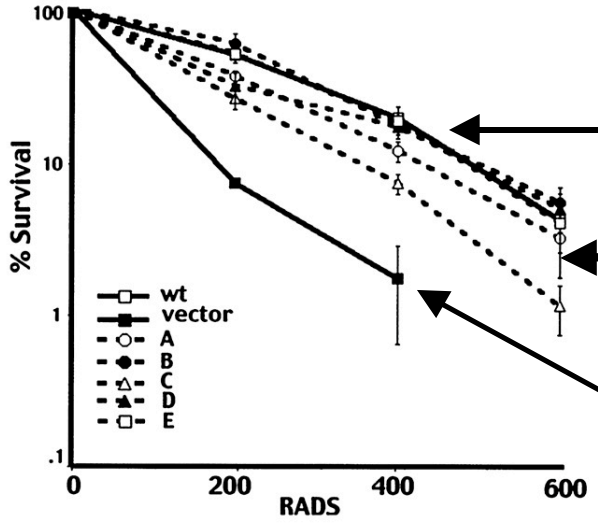
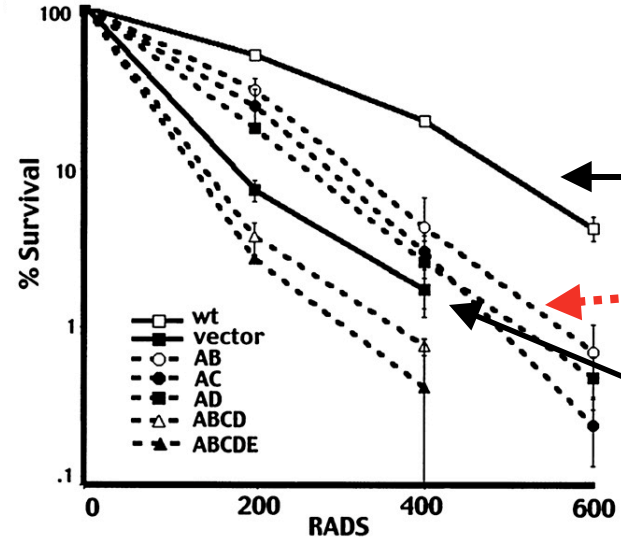


+ wt hDNA-PKcs

1-2 sites mutated (A,B,C,D,E)

DNA-PKcs negative



+ wt hDNA-PKcs

2-4 sites mutated (AB,AC,AD)

DNA-PKcs negative

Ding et al, MCB, 2003

Clonogenic survival assays in stably transfected V3 cells (DNA-PKcs null) complemented with wt or autophosphorylation mutant DNA-PKcs:

A: T2609A (TQ)
B: T2620A (TQ)
S2624A (SL)
C: T2638A (TQ)
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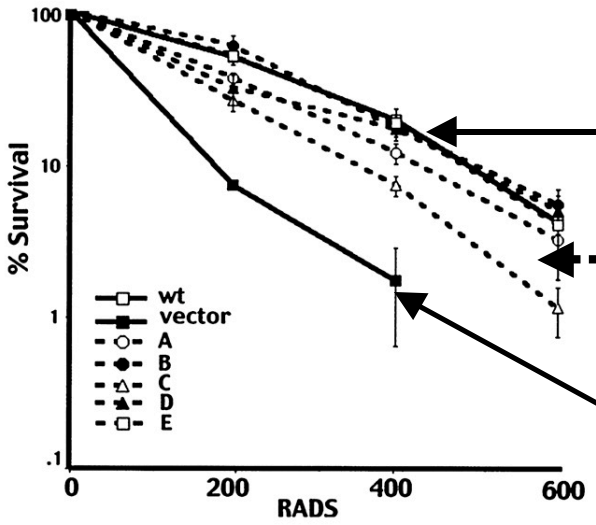
E: S2612A (SQ)

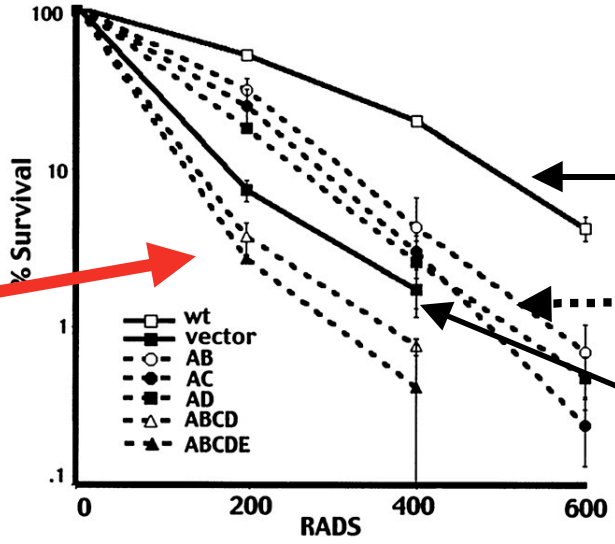


+ wt hDNA-PKcs

1-2 sites mutated (A,B,C,D,E)

DNA-PKcs negative

Cells expressing DNA-PKcs with 5 or 6 sites mutated (ABCD, ABCDE) are more radiosensitive than cells expressing no DNA-PKcs at all.



+ wt hDNA-PKcs

2-4 sites mutated (AB,AC,AD)

DNA-PKcs negative

Ding et al, MCB, 2003

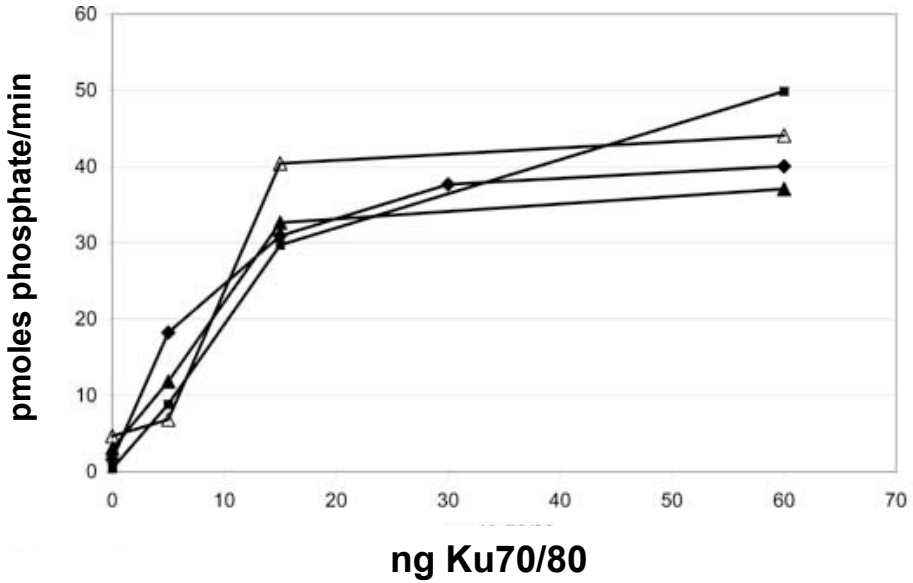
What is the basis of radiation sensitivity in the DNA-PKcs autophosphorylation mutant cells?

Purified proteins:

Are they defective protein kinase activity ?

Are they defective DNA DSB repair in an in vitro DNA end joining assay?

Purified wt, A6, and D6-DNA-PKcs from V3 cells and assayed for DNA-PK kinase activity:

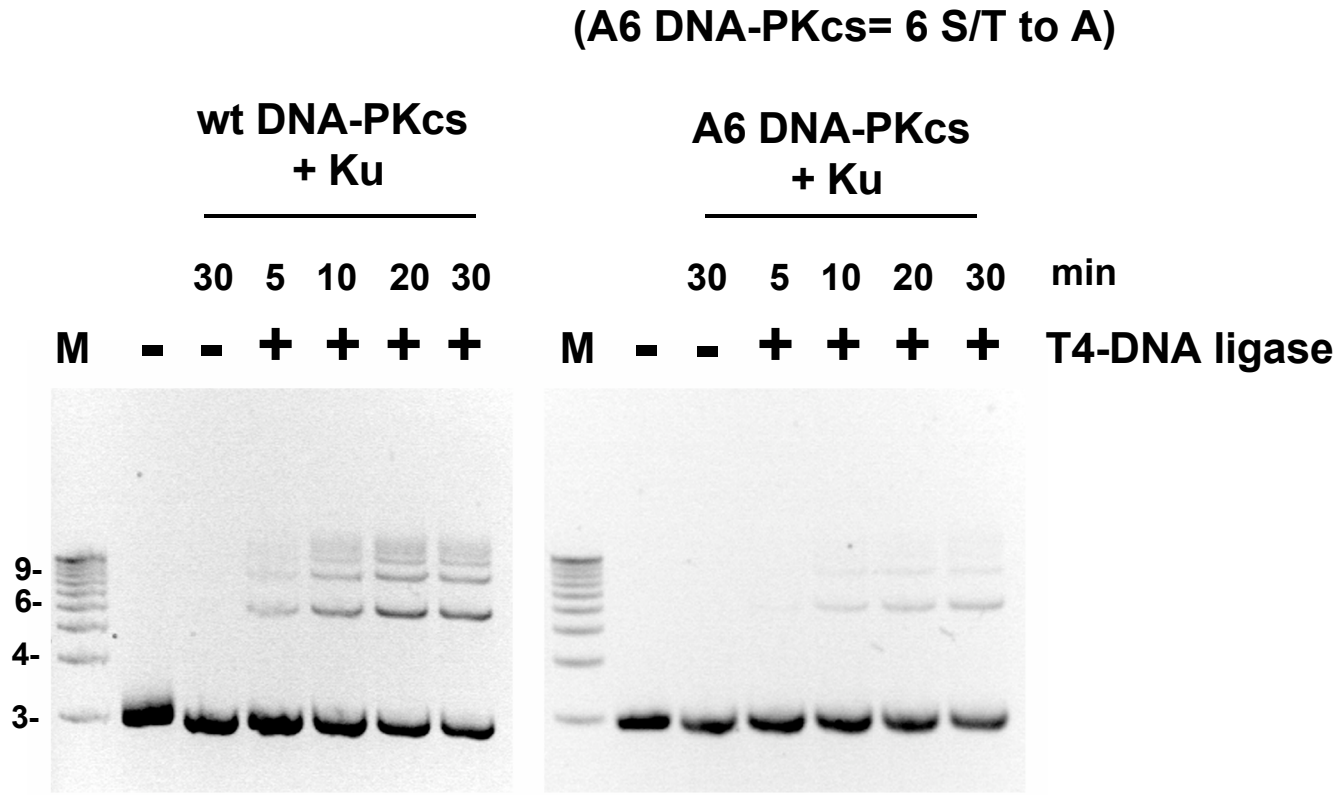


Stimulation of DNA-PKcs protein kinase activity by Ku

Purified DNA-PKcs containing S/T-A mutations at all 6 phosphorylation sites is identical to wt DNA-PKcs in a variety of biochemical activity assays.

Block et al, NAR, 2004

T4-DNA ligase end joining assay with purified Ku plus purified wt and autophosphorylation mutant DNA-PKcs:

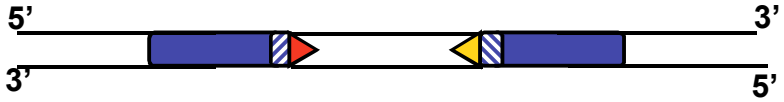


DNA-PKcs autophosphorylation mutants A6 has impaired ability to support DNA end joining mediated by T4 DNA ligase (Block, Yu et al, NAR, 2004) or XRCC4-DNA ligase IV (Reddy et al, 2004).

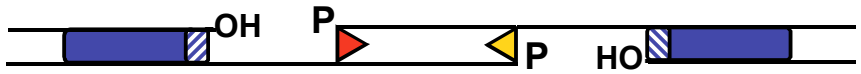
V(D)J Recombination: site specific rearrangement of IgG genes

Coding regions (blue)

Recombination signal sequences (RSS) red and yellow

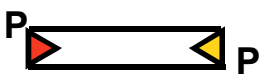


Rag1/Rag2: single strand break 5' of RSS



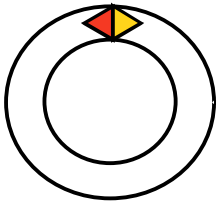
- Formation of DNA hairpins on coding ends:

RSS sequences removed



- DNA hairpin ends opened
- Processing of DNA ends
- Religation of DNA ends

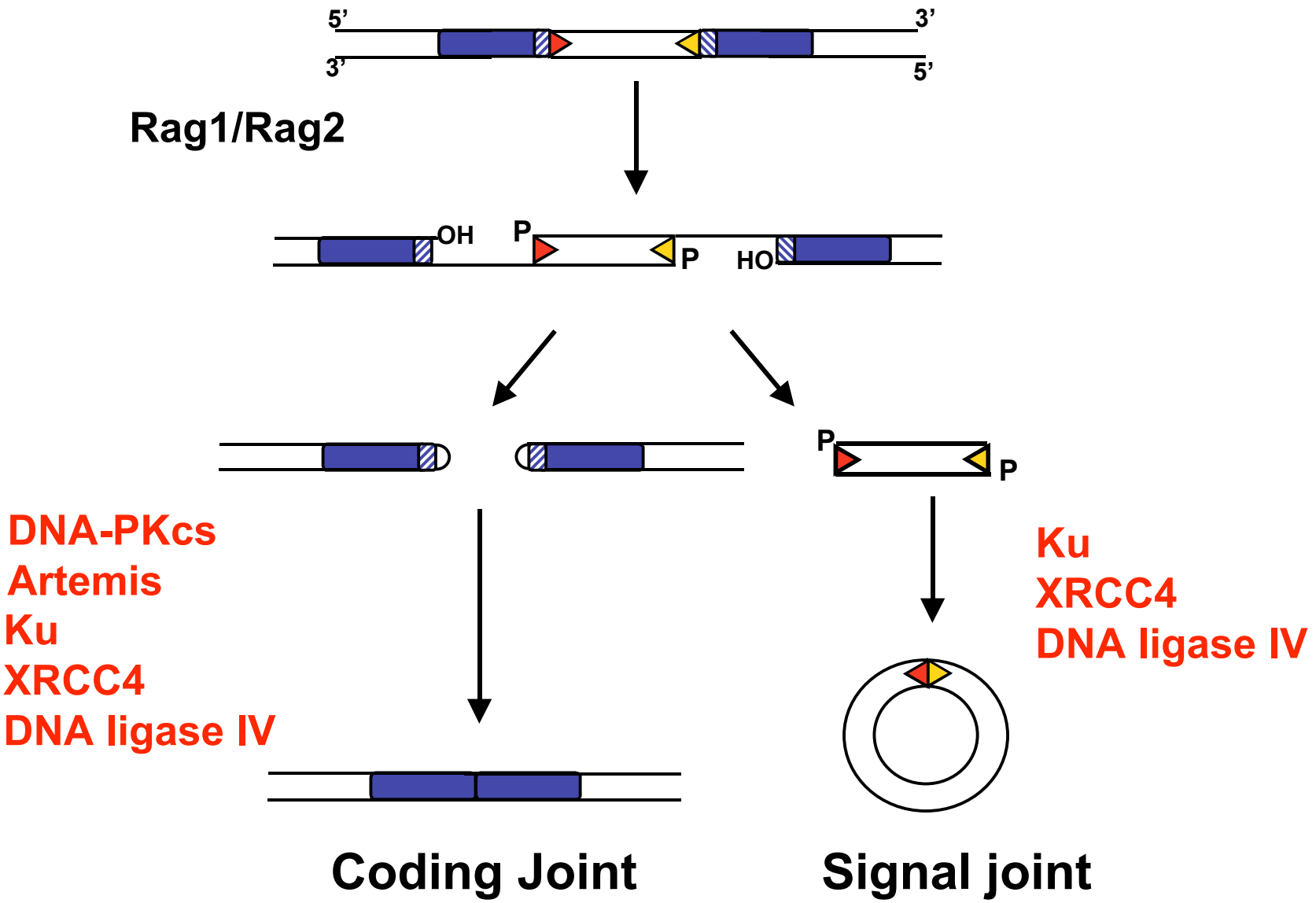
RSS sequences ligated



Coding Joint

Signal joint

Requirement for NHEJ in V(D)J Recombination:

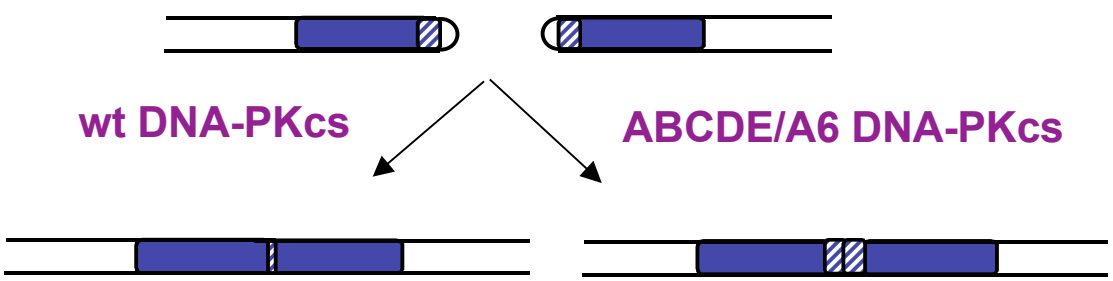


V(D)J recombination defects in autophosphorylation mutant DNA-PKcs:

TABLE 2. Coding joints mediated by mutant ABCDE have minimal nucleotide loss from joined coding ends[a]

DNA-PKcs	No. of sequences	No. of bases lost/joint	% Complete ends (no. complete/ total no.)	% SSH[b] (no. with SSH/ total sequences)	% P segments (no. of P segments/ no. complete ends)
Wild type	61	4.61	30 (37/122)	44 (27/61)	27 (10/37)
ABCDE	28	1.43	70 (39/56)	0 (0/27)	25 (10/40)
S/T→D	25	3.08	38 (19/50)	52 (13/25)	26 (5/19)
RAGS only	16	14.69	41 (13/32)	31 (5/16)	92 (12/13)

DNA-PKcs	No. of bases lost/joint
Wild type	4.61
ABCDE	1.43
S/T→D	3.08
RAGS only	14.69



Coding joints are very rarely formed in cells expressing the A6 autophosphorylation defective mutant

Of those that were formed: less nucleotides were lost from either side of the DSB in the ABCDE/A6 mutant than in cells expressing wt-DNA-PKcs

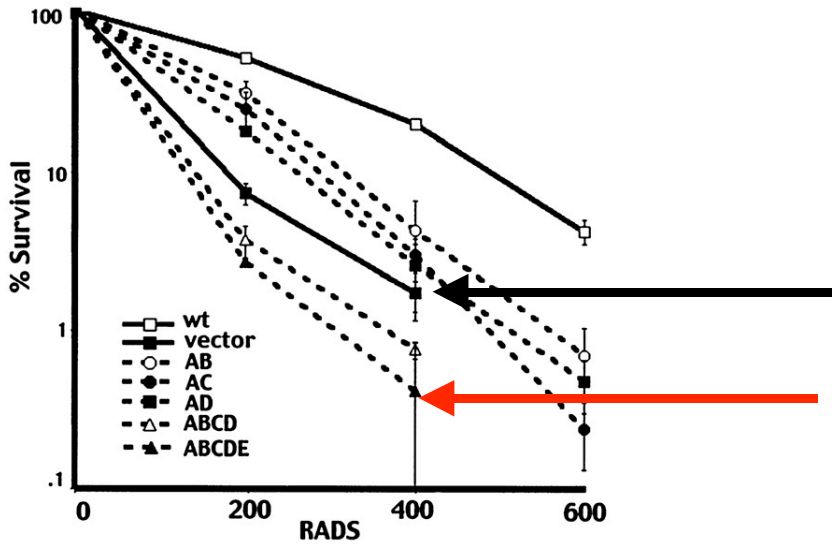
D6 (aspartic acid mutant: phosphorylation mimic) closer to wt

Ding et al, MCB, 2003

Summary so far:

- Identified a cluster of autophosphorylation sites in DNA-PKcs (2609-2648)
- Cells expressing DNA-PKcs in which S or Ts in the cluster of sites is changed to A are more radiosensitive than cells expressing no DNA-PKcs at all.
- Purified DNA-PKcs containing mutations at the cluster of phosphorylation sites has "normal" protein kinase activity in vitro but is inefficient at supporting DNA end joining in in vitro assays: T4-DNA-PK phosphorylation dependent assay (Block et al, 2004) and XRCC4-DNA ligase IV assay (Reddy et al, 2004).
- Less nucleotide loss from DSB ends in cells expressing ABCDE/A6 mutant
- Questions:
- Why is autophosphorylation defective DNA-PKcs defective at supporting end joining?
- Why are autophosphorylation defective cells more radiosensitive?
- Does autophosphorylation defective DNA-PKcs remain at DNA ends as predicted by the in vitro model?
- Are there additional sites of autophosphorylation? (yes)

Why are cells expressing alanine in place of S or T at 5 or 6 phosphorylation sites more radiosensitive than cells expressing no DNA-PKcs at all?



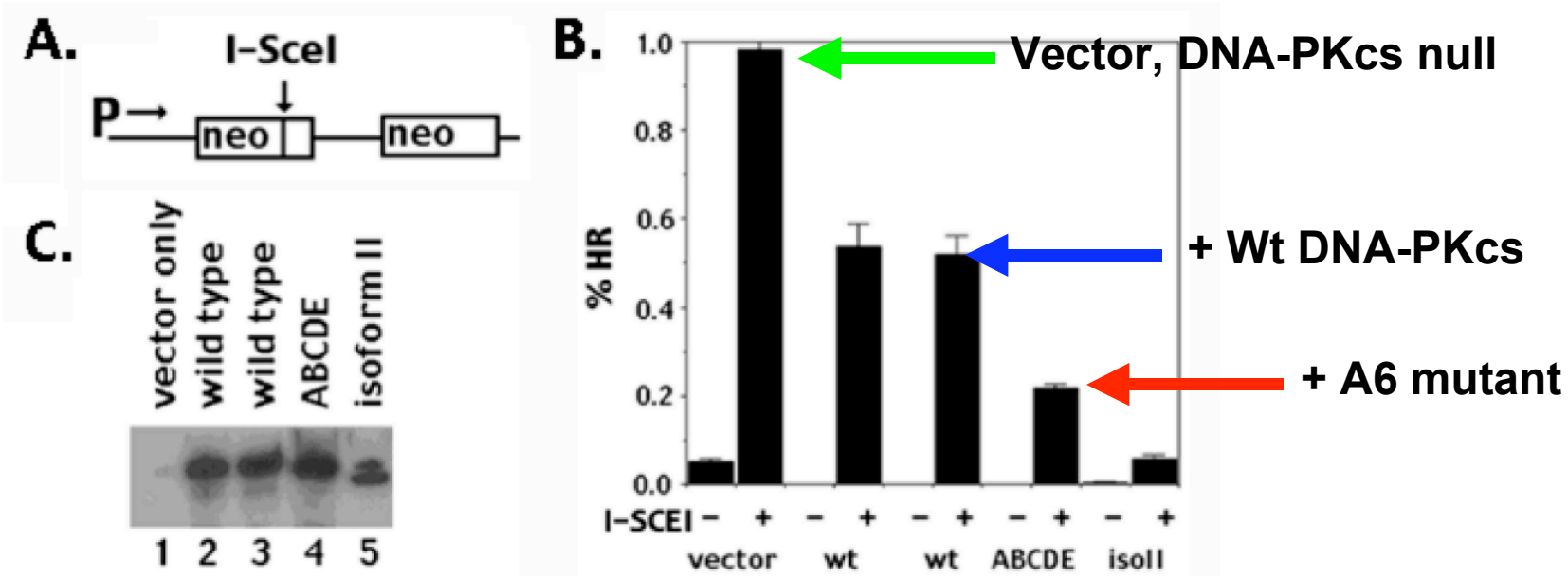
V3 (no DNA-PKcs)

Phosphorylation mutant ABCDE (A6)

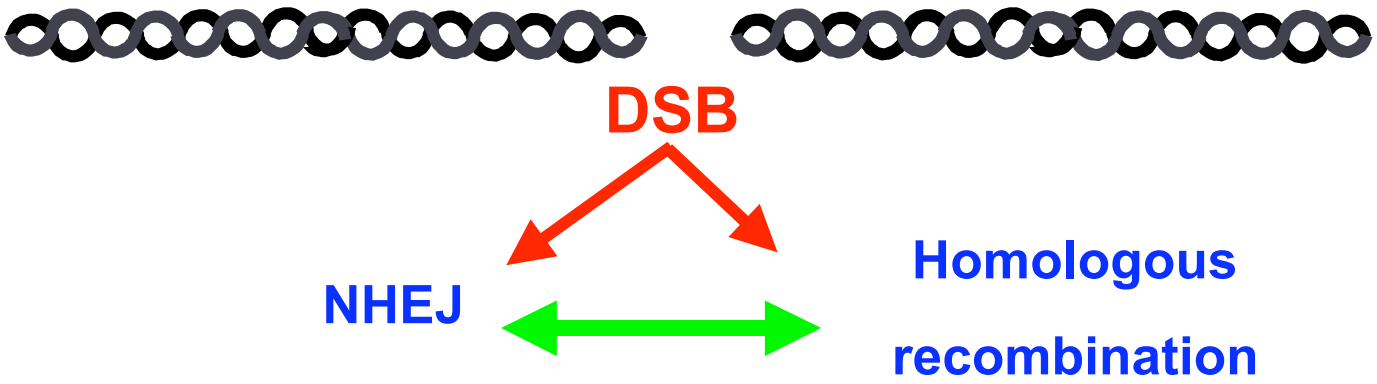
Kathy Meek/Jac Nickoloff:

Measure HRR in DNA-PKcs-phosphorylation defective cells

Methods:
DNA-PKcs null cells transfected with integrated HR substrate that contains two non-functional neomycin resistance genes. The first is non-functional because of a frameshift mutation that is co-incident with the restriction site for the homing endonuclease I-SceI. The second is nonfunctional because it lacks a promoter. A DSB is induced in the first neo gene by transient expression of I-SceI. This DSB is repaired by HR/gene conversion such that both copies of the neo gene are retained.
Production of G418 resistant clones is a direct measure of successful HR.



Convery et al, PNAS, 2005



No DNA-PKcs	inactive	Rate set to “100%”
+ wt DNA-PKcs	active	Rate = 50% (Convery et al, 2005)
+ Phos-defective	inactive	Rate = 20% (Convery et al, 2005)
+ DNA-PK inhibitors	inactive	Attenuated (Allen et al, 2003)

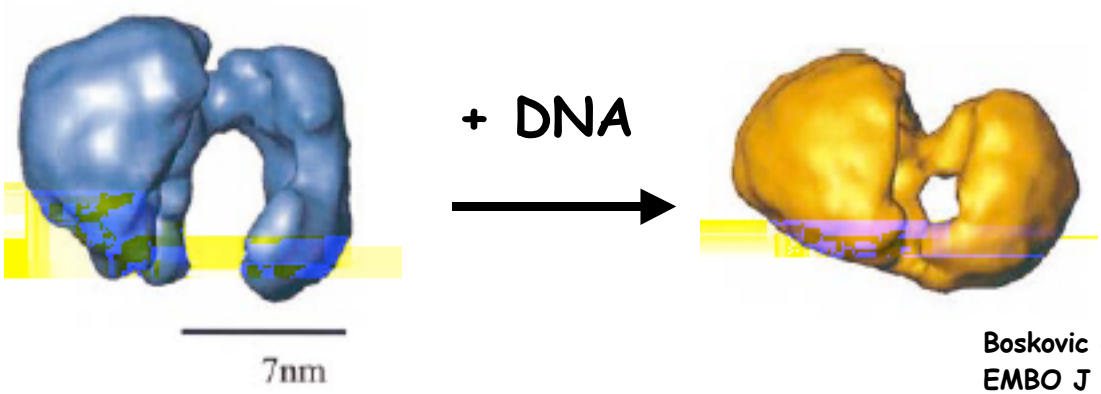
Inability of DNA-PKcs to undergo autophosphorylation results in inhibition of both NHEJ and HR

Mechanism??

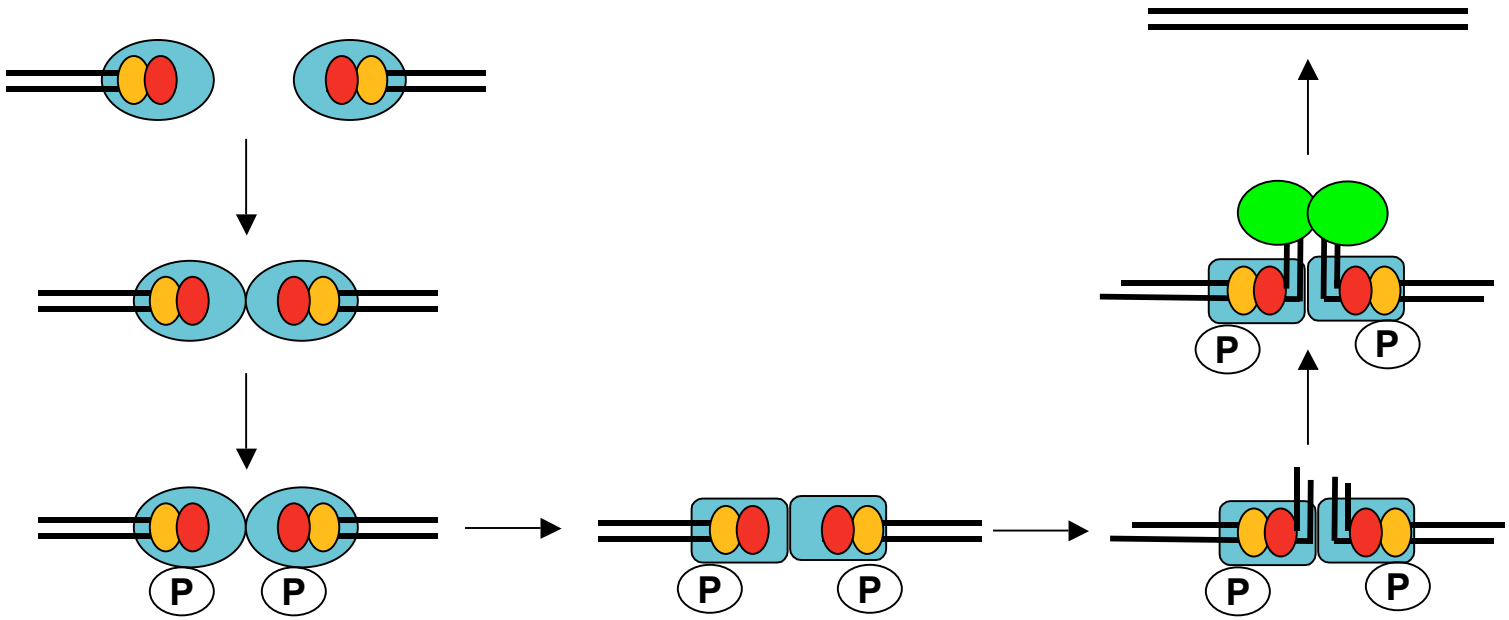
ABCDE/A6 mutant blocks access to DNA ends?

Autophosphorylation of DNA-PKcs is required to allow the ligase and other processing enzymes e.g.nucleases access to the DNA ends?

What is the role of DNA-PKcs autophosphorylation?



Boskovic et al
EMBO J 2003: 22, 5875-5882,



autophosphorylation

conformational change? or dissociation???

"remodelling" of DNA ends

Phosphorylation of other DNA-PK substrates:

Ku:

XRCC4:

DNA-ligase IV:

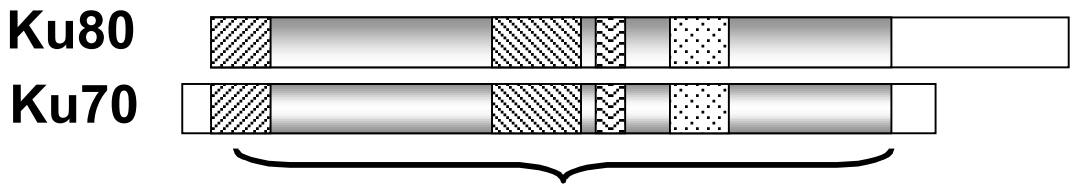
Artemis:

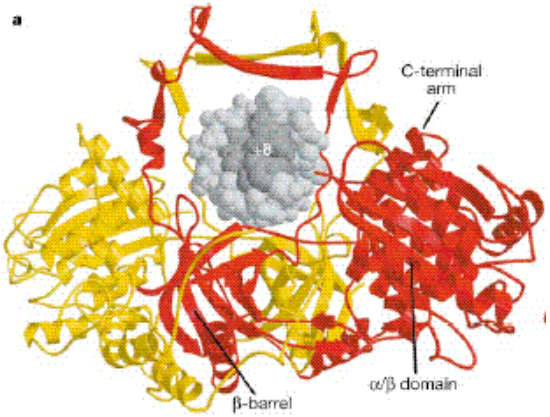
Tdp1:

PNK:

Ku heterodimer:

Conserved DNA binding core (Gell and Jackson, 1999)





Walker et al, Nature, 2001

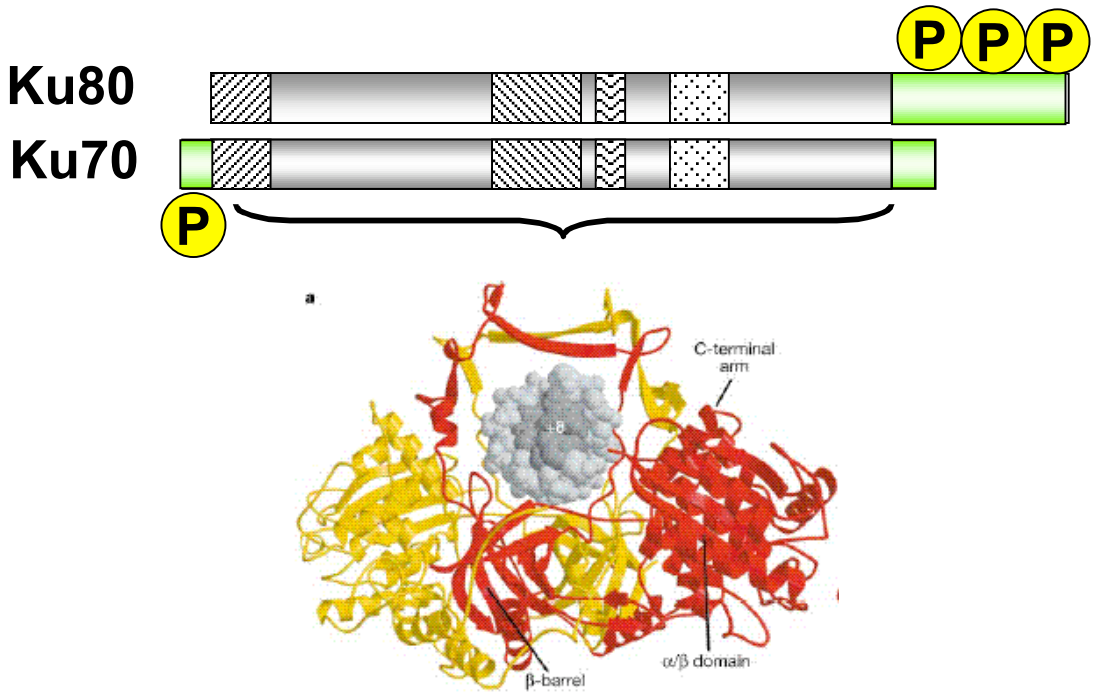
Ku heterodimer:

Conserved DNA binding core (Gell and Jackson, 1999)

Unique N terminal (Ku70) and C-terminal (Ku70 and 80) domains

DNA-PK phosphorylation sites:

Ku70 ser6; Ku80 ser577, 580 and thr715 (Chan et al, 1999)



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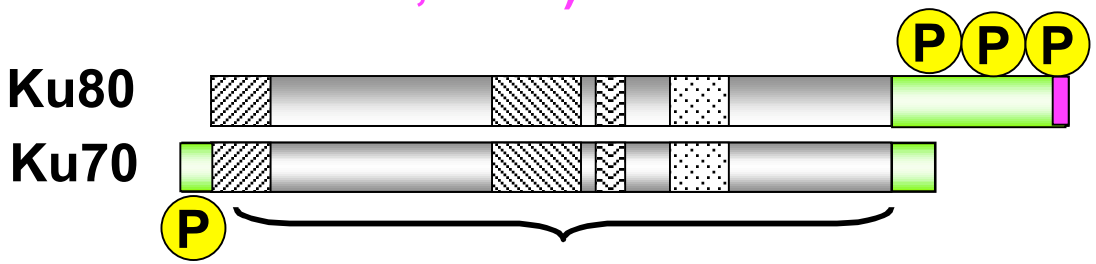
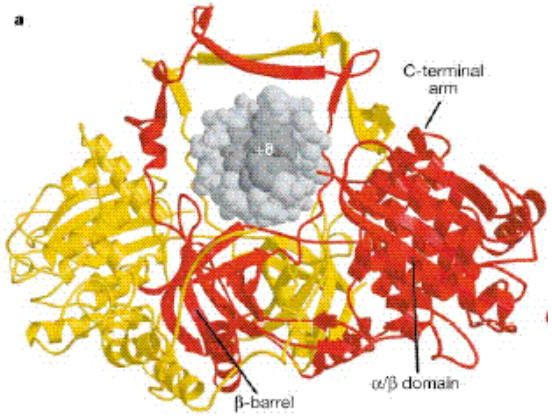
C terminal 12 aa Ku80 required for interaction with DNA-PKcs (Gell and Jackson, 1999)





Walker et al, Nature, 2001

Phosphorylation of other DNA-PK substrates:

Ku: DNA-PK sites identified: Ku70: ser 6; Ku80: ser577, ser580, thr715 (Chan et al, 1999)
Not required for NHEJ or V(D)J recombination (Douglas et al, 2005)

XRCC4:

DNA Ligase IV:

Artemis:

Tdp1:

PNK:

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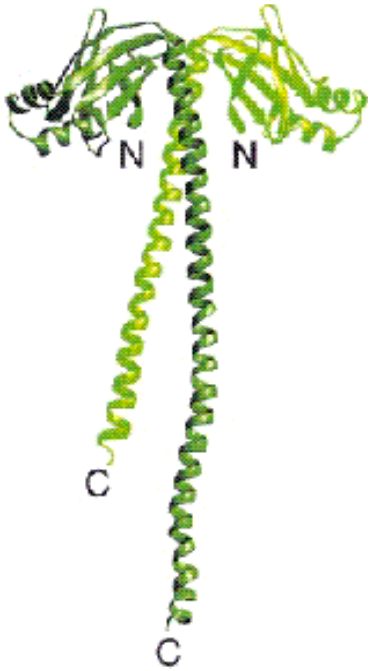
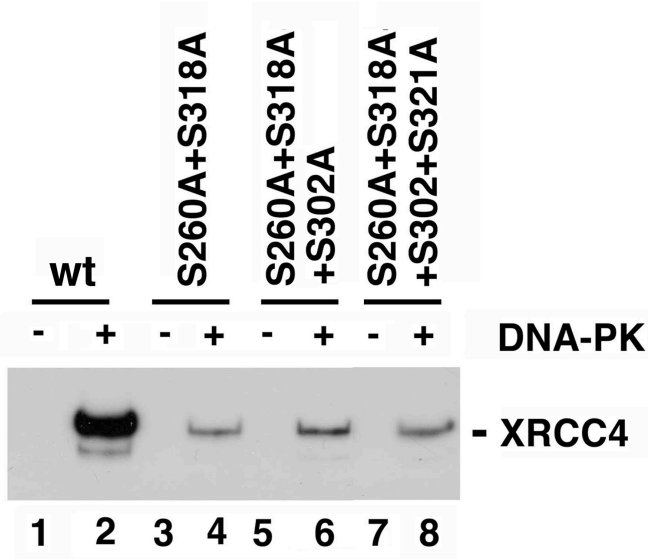


Structure of amino acids 1-203 (Junop et al, 2000)

XRCC4 is phosphorylated by DNA-PK in vitro



Serine 260 and serine 318 are the major in vitro DNA-PK phosphorylation sites in XRCC4; also 6 minor sites: all in C terminal 130 amino acids

Phosphorylation at these sites is not required for NHEJ or V(D)J recombination

Yu et al, 2003

Phosphorylation of other DNA-PK substrates:

Ku: DNA-PK sites identified: Ku70: ser 6; Ku80: ser 577, 580, thr 715 (Chan et al, 1999)
Not required for NHEJ (Douglas et al, 2005)

XRCC4: DNA-PK sites identified ser 243 and 318
Not required for NHEJ (Yu et al, 2003)

DNA Ligase IV:

Artemis:

Tdp1:

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Phosphorylation of other DNA-PK substrates:

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Not required for NHEJ (Douglas et al, 2005)

XRCC4: DNA-PK sites identified ser 243 and 318
Not required for NHEJ (Yu et al, 2003)

DNA Ligase IV: Phosphorylation not required for NHEJ (Wang et al, 2003)

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Tdp1:

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Phosphorylation of other DNA-PK substrates:

Ku: DNA-PK sites identified: Ku70: ser 6; Ku80: ser 577, 580, thr 715 (Chan et al, 1999)
Not required for NHEJ (Douglas et al, 2005)

XRCC4: DNA-PK sites identified ser 243 and 318
Not required for NHEJ (Yu et al, 2003)

DNA Ligase IV: Phosphorylation not required for NHEJ (Wang et al, 2003)

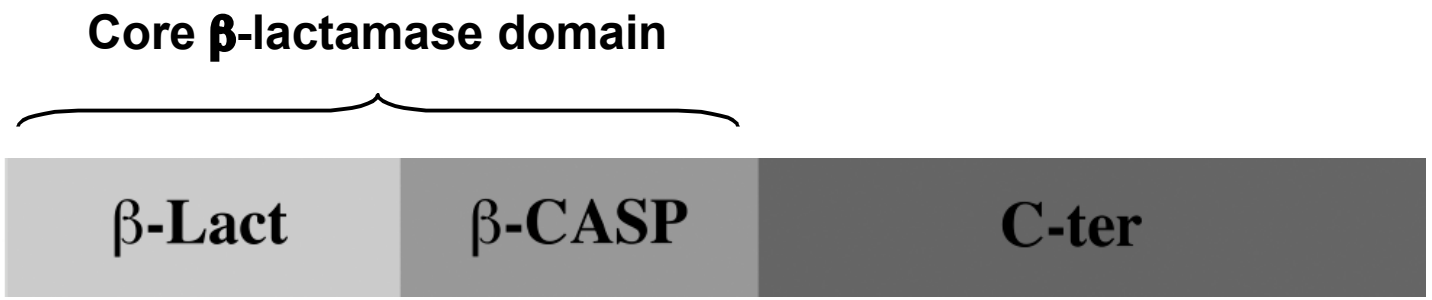
Artemis:

Tdp1:

PNK:

Artemis

78 kDa 692 amino acid protein
5'-3' exonuclease activity



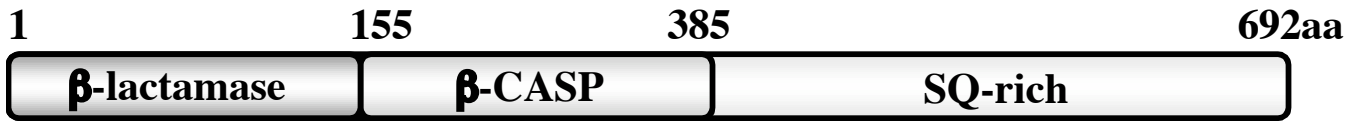
Interacts with DNA-PKcs and is phosphorylated by DNA-PKcs in vitro (Ma and Lieber, 2002)

Interaction with DNA-PKcs confers 5'-3' nuclease activity towards ssDNA and DNA hairpin opening activity

Artemis defective cells (RS-SCID) unopened DNA hairpins during coding joint formation in V(D)J recombination

Le Deist et al, Immunological Reviews 200, 142-155 (2004)

Artemis




```
  1 MSSFEGQMAE YPTISIDRFD RENLRARAYF LSHCHKDHMK GLRAPTLKRR LECSLKVYLY
 61 CSPVTKELLL TSPKYRFWKK RIISIEIETP TQISLVDEAS GEKEEIVVTL LPAGHCPGSV
121 MFLFQGNNGT VLYTGDFRLA QGEAARMELL HSGGRVKDIQ SVYLDTTFCD PRFYQIPSRE
181 ECLSGVLELV RSWITRSPYH VVWLNCKAAY GYEYLFTNLS EELGVQVHVN KLDMFRNMPE
241 ILHHLTTDRN TQIHACRHPK AEEYFQWSKL PCGITSRNRI PLHIISIKPS TMWFGERSRK
301 TNVIVRTGES SYRACFSFHS SYSEIKDFLS YLCPVNAYPN VIPVGTTMDK VVEILKPLCR
361 SSQSTEPKYK PLGKLKRART VHRDSEEEDD YLFDDPLPIP LRHKVPYPET FHPEVFSMTA
421 VSEKQPEKLR QTPGCCRAEC MQSSRFTNFV DCEESNSESE EEVGIPASLQ GDLGSVLHLQ
481 KADGDVPQWE VFFKRNDEIT DESLENFPSS TVAGGSQSPK LFSDSDGEST HISSQNSSQS
541 THITEQGSQG WDSQSDTVLV SSQERNSGDI TSLDKADYRP TIKENIPASL MEQNVICPKD
601 TYSDLKSRDK DVTIVPSTGE PTTLSSETHI PEEKSLLNLS TNADSQSSSD FEVPSTPEAE
                    661 LPKREHLQYL YEKLATGESI AVKKRKCSLL DT
```

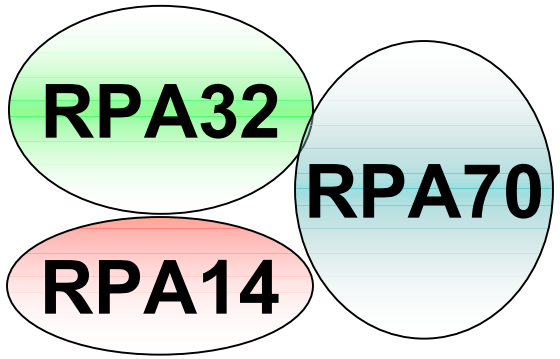
Ten potential DNA-PK/ATM/ATR phosphorylation sites in Artemis:

7 in C-terminal ~200 amino acids:

S516, S534, S538, S548, S553, S562, and S645

The same substrate can be phosphorylated by different PIKKs in response to different DNA damaging agents

RPA: replication protein A
involved in DNA replication, multiple repair pathways

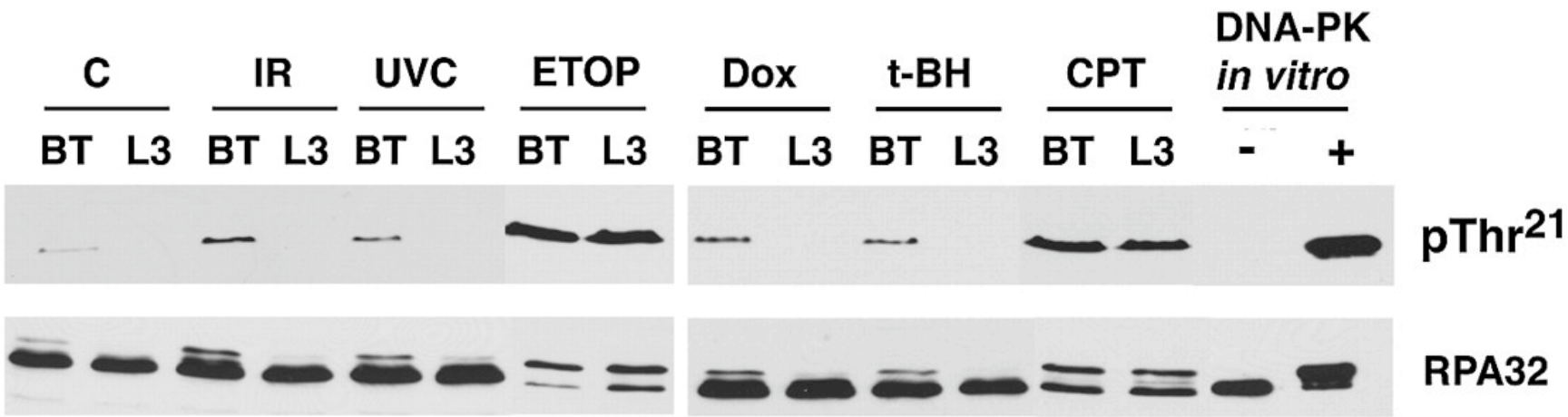


DNA damage induced phosphorylation of RPA:

RPA32 phosphorylated on threonine 21 in vitro by DNA-PK, ATM and ATR

IR induced phosphorylation of RPA32-Thr21 is ATM dependent (absent in ATM negative L3 cells)

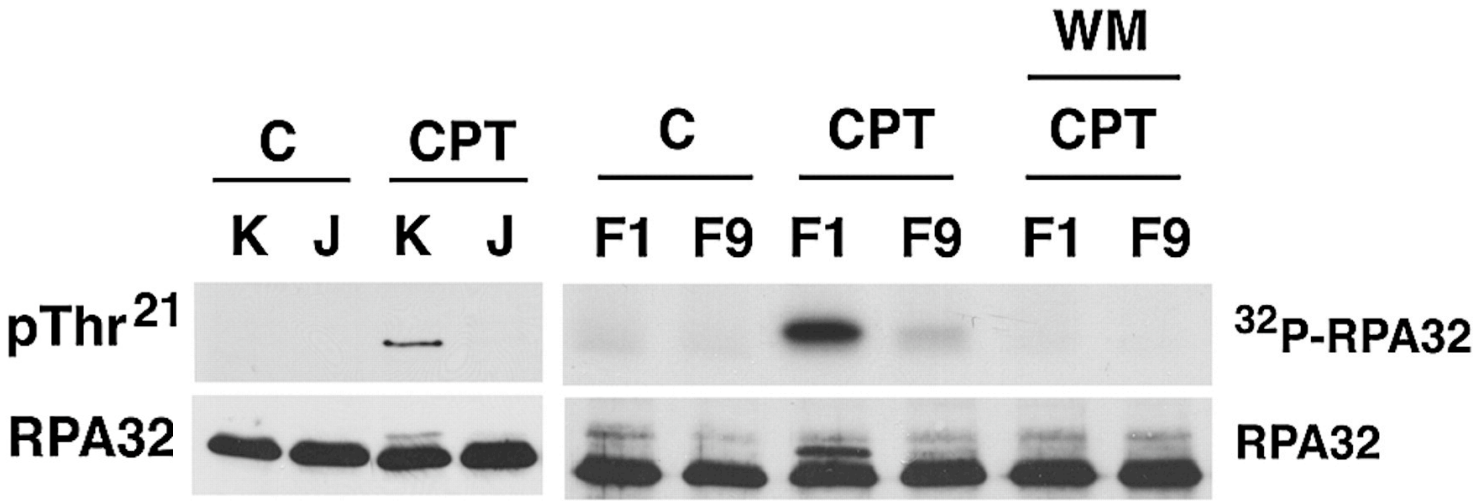
Phosphorylation in response to etoposide and camptothecin is not ATM-dependent



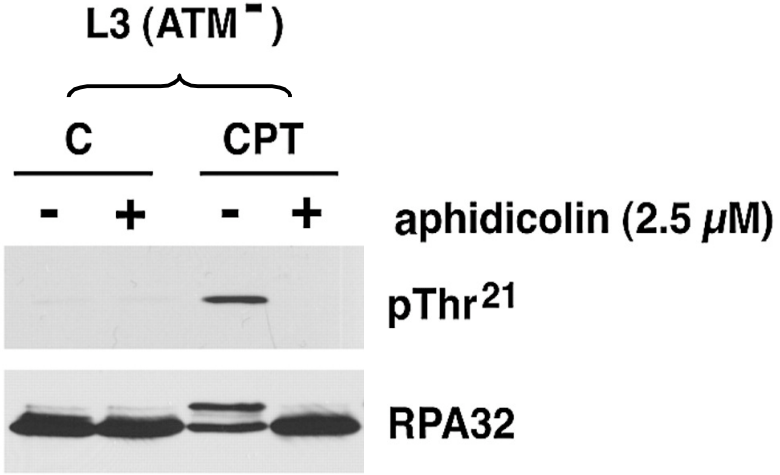
RPA32-thr21 phosphorylation is DNA-PK dependent in response to camptothecin

J= M059J, lacks DNA-PKcs

F1 = M059J reconstituted DNA-PKcs/chromosome 8



Camptothecin induced phosphorylation of RPA32-Thr21 is blocked by aphidicolin (requires entry into S phase



Summary

Autophosphorylation of DNA-PKcs is important for NHEJ - possibly by autophosphorylation-dependent dissociation and/or "remodelling" of DNA ends prior to ligation and/or processing

DNA-PK mediated phosphorylation of Ku, XRCC4 and DNA ligase IV is NOT required for NHEJ

Artemis is phosphorylated by DNA-PK and ATM in vitro and is phosphorylated in vivo in response to DNA damage

Histone H2AX is phosphorylated in response to IR;
ATM and DNA-PK act redundantly to phosphorylate H2AX
(as observed by Stiff et al, 2004)

RPA32 is phosphorylated on threonine 21 in vivo in a DNA-PK dependent manner in response to camptothecin.

The Lees-Miller Lab

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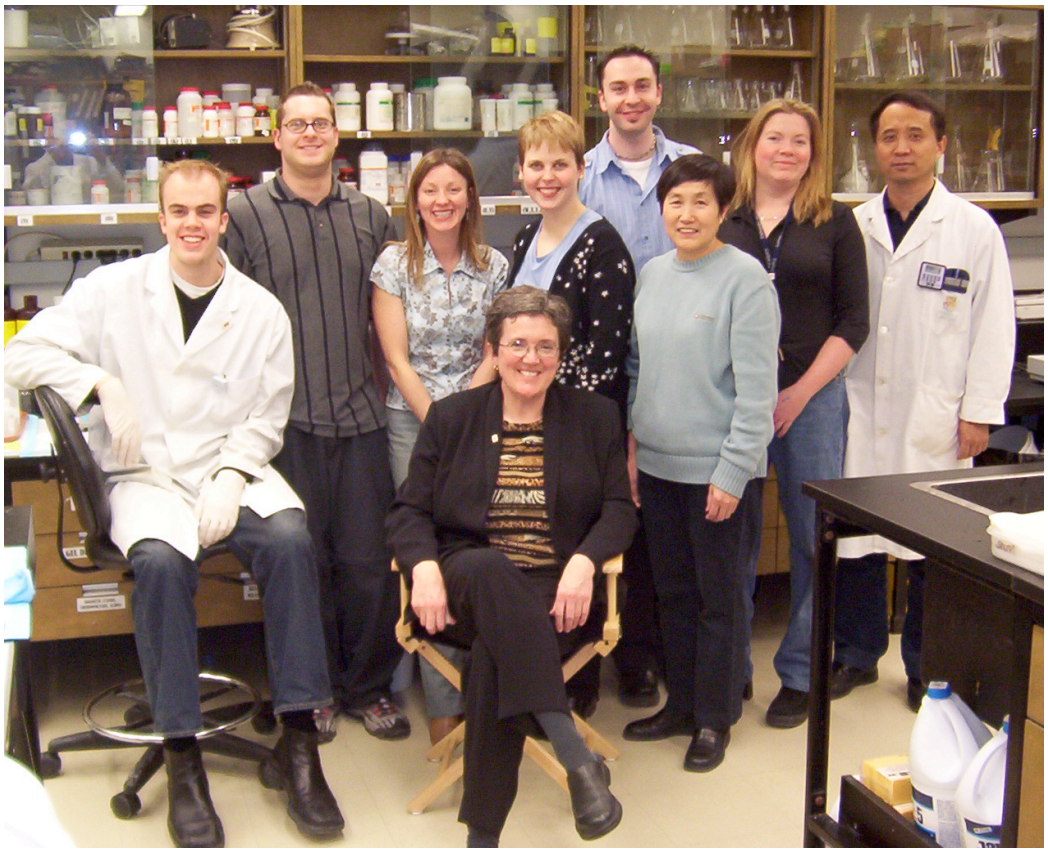
Ruiqiong Ye
Marina Siponen
Dr Pauline Douglas
Dr Yaping Yu
Dennis Merkle, PhD
Wesley Block, PhD
Dr Barry Phipps
(Doug Chan, PhD)



Collaborators:

Dr Kathy Meek (DNA-PKcs mutants)
Dr Penny Jeggo (Artemis)
Dr Nick Morrice (mass spec)
Dr Jac Nickoloff (HR assays)
Dr Graeme Smith (KuDos Pharmaceuticals)

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National Cancer Institute of Canada / Institut national du cancer du Canada

UNIVERSITY OF CALGARY

CIHR IRSC Canadian Institutes of Health Research / Instituts de recherche en santé du Canada

Alberta Cancer Board

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Alberta Heritage Foundation for Medical Research