The human intra-S checkpoint response to UV-induced DNA damage

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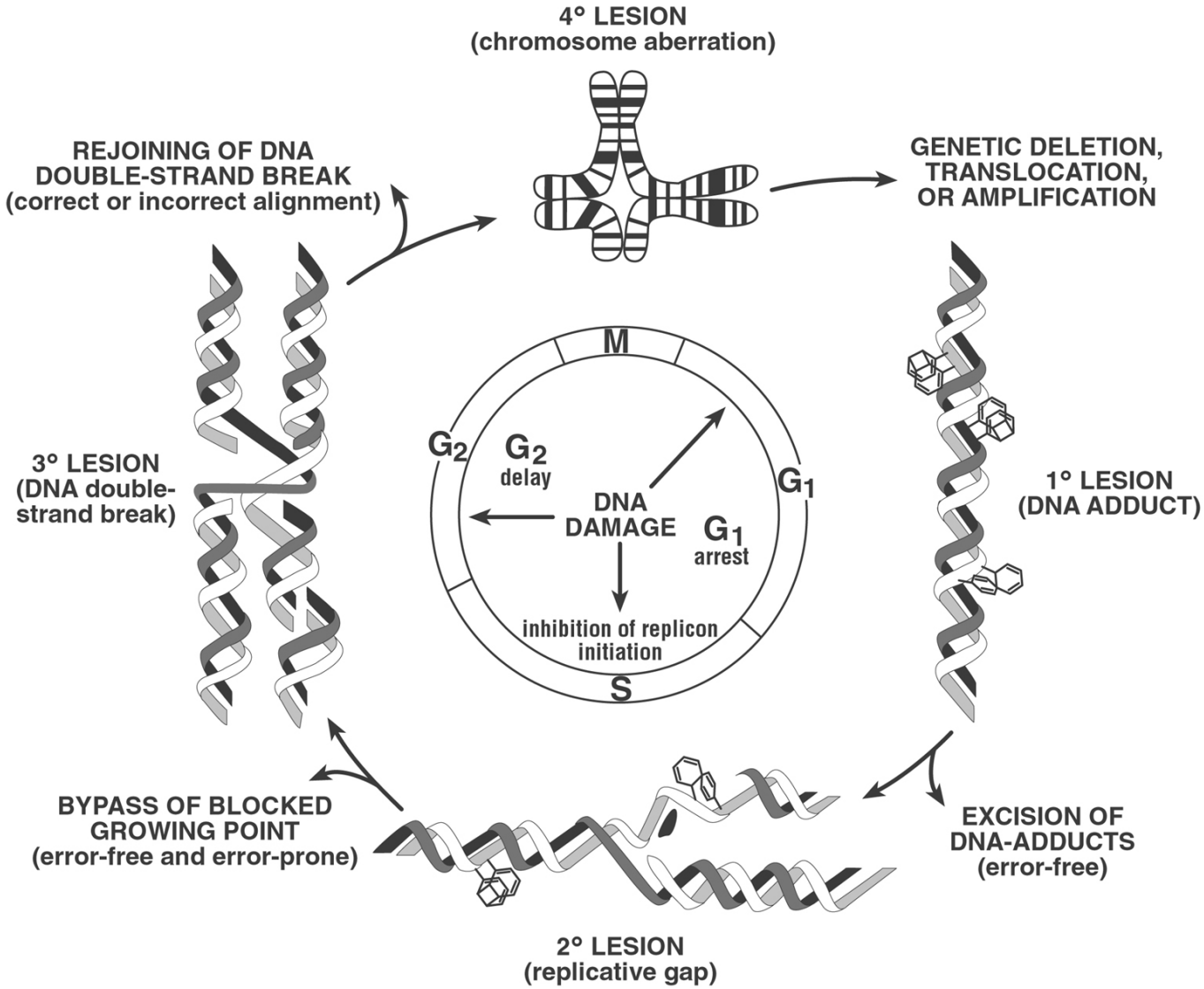
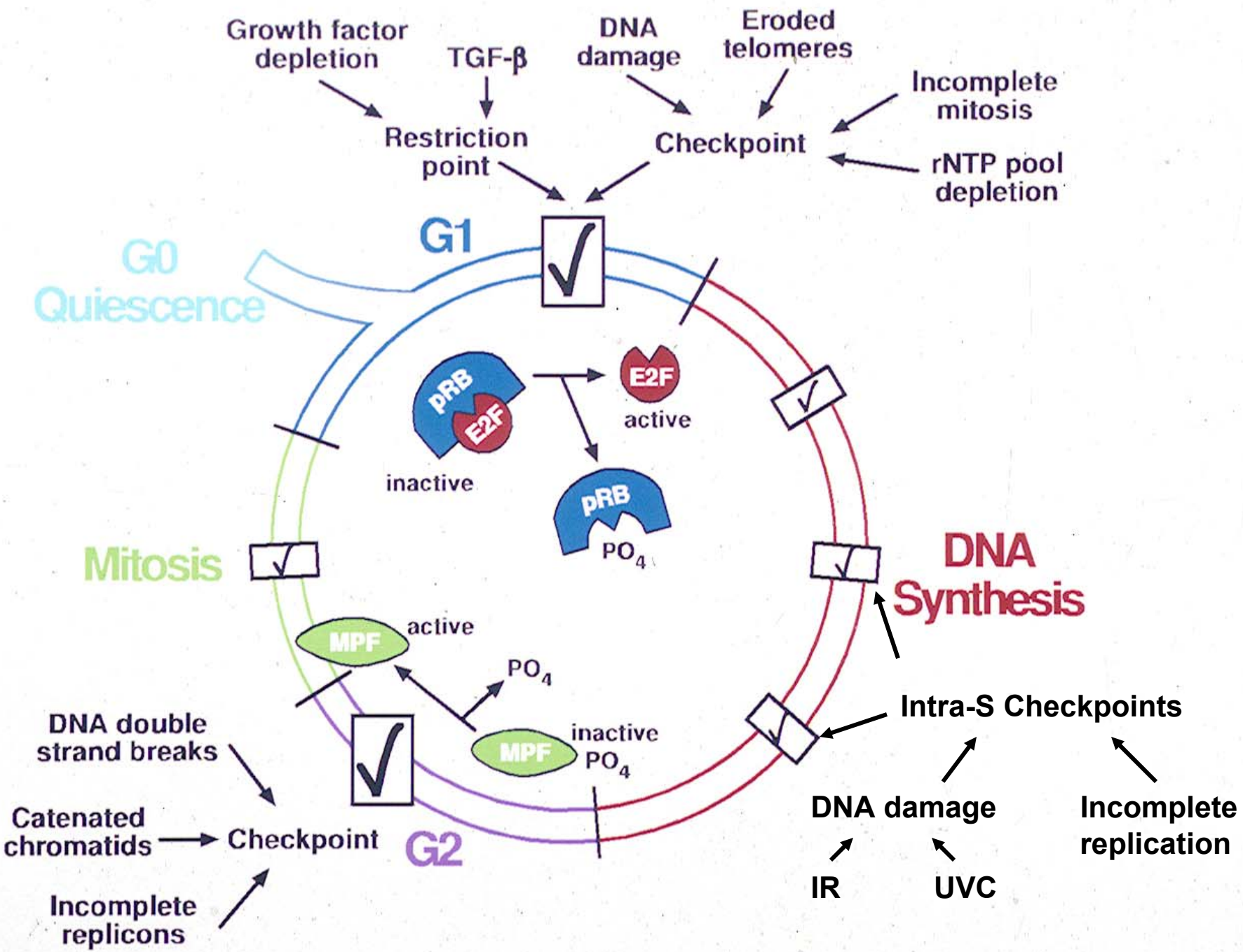
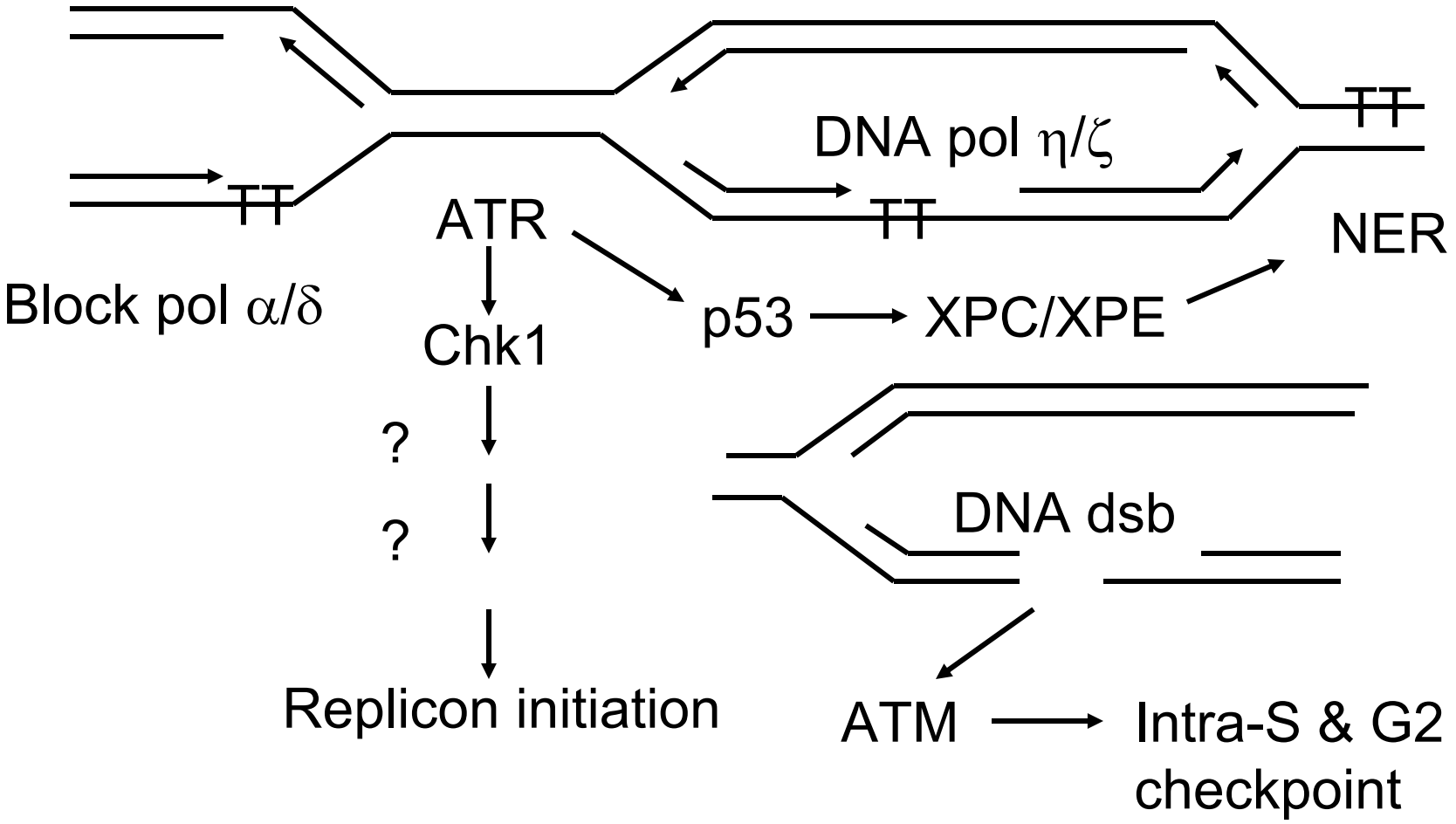
Evolution of DNA damage during the cell cycle




Growth factor depletion
TGF-β
DNA damage
Eroded telomeres
Incomplete mitosis
rNTP pool depletion
Restriction point
Checkpoint
G1
G0 Quiescence
pRB
E2F
inactive
E2F
active
pRB
PO4
Mitosis
DNA Synthesis
MPF
active
PO4
MPF
inactive
PO4
DNA double strand breaks
Catenated chromatids
Checkpoint
Incomplete replicons
G2
Intra-S Checkpoints
DNA damage
Incomplete replication
IR
UVC

Mechanisms and consequences of inhibition of DNA replication by UVC

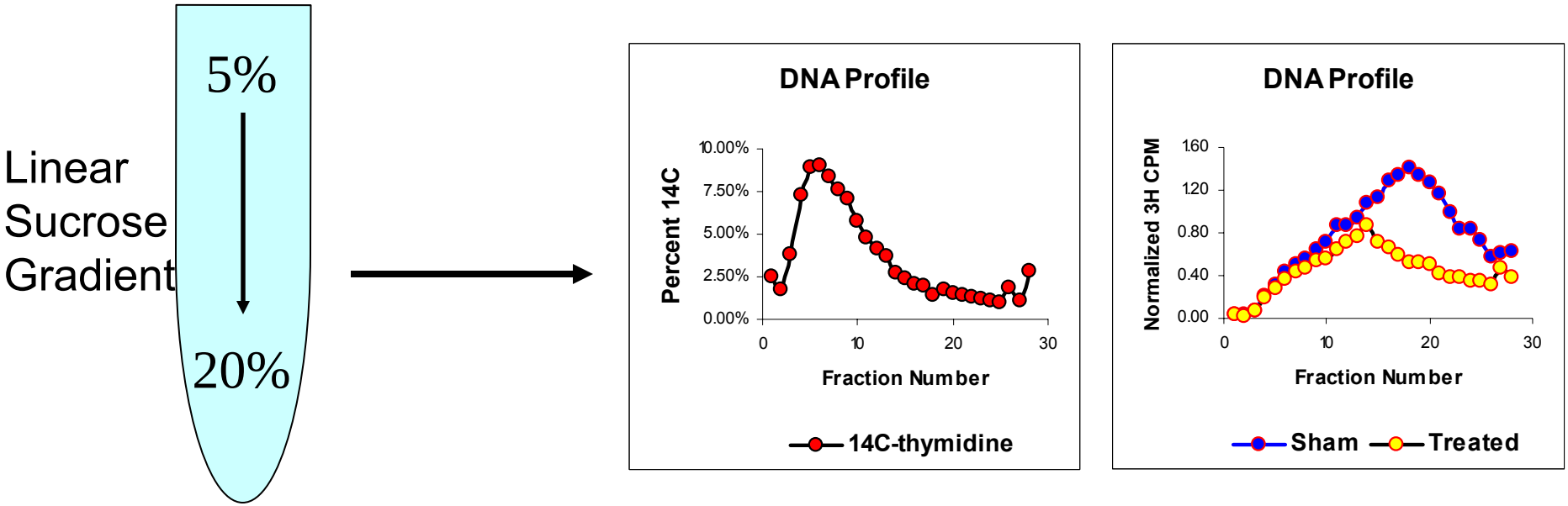


Velocity Sedimentation

Separates DNA replication intermediates based on size and is used to quantify the inhibition of DNA synthesis in classes of nascent DNA molecules.

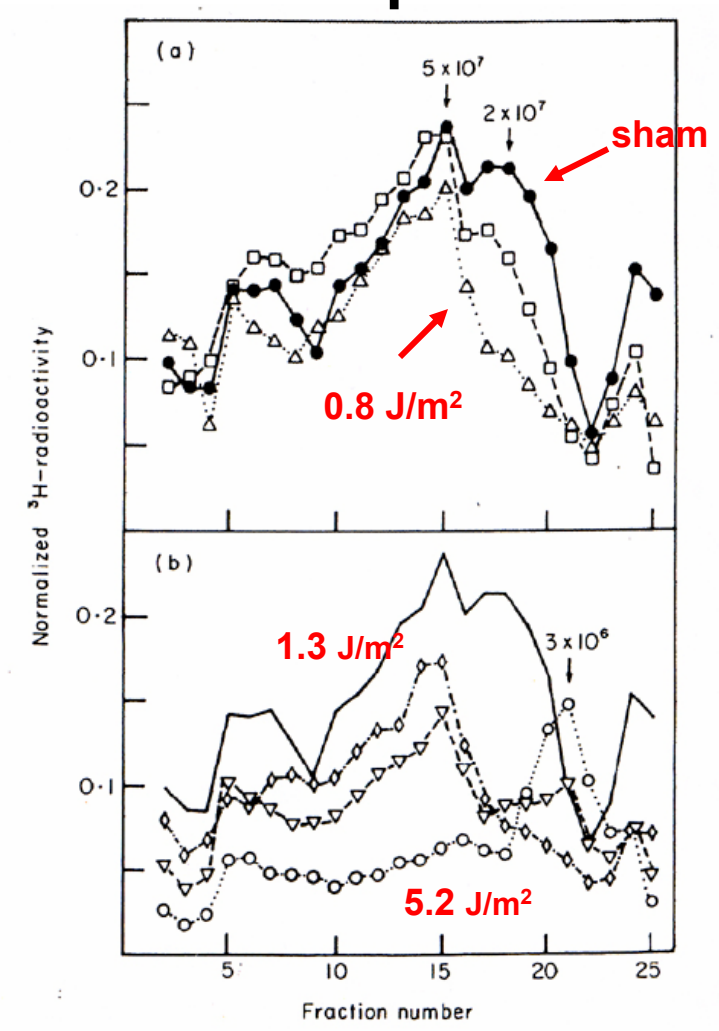
•Cells are irradiated, incubated for 30 minutes, and pulse-labeled with ^{3}H-thymidine. Cells are harvested and lysed on top of a linear sucrose gradient. Nascent DNA molecules are separated by centrifugation.

•Bulk DNA is pre-labeled with ^{14}C-thymidine for at least one population doubling.

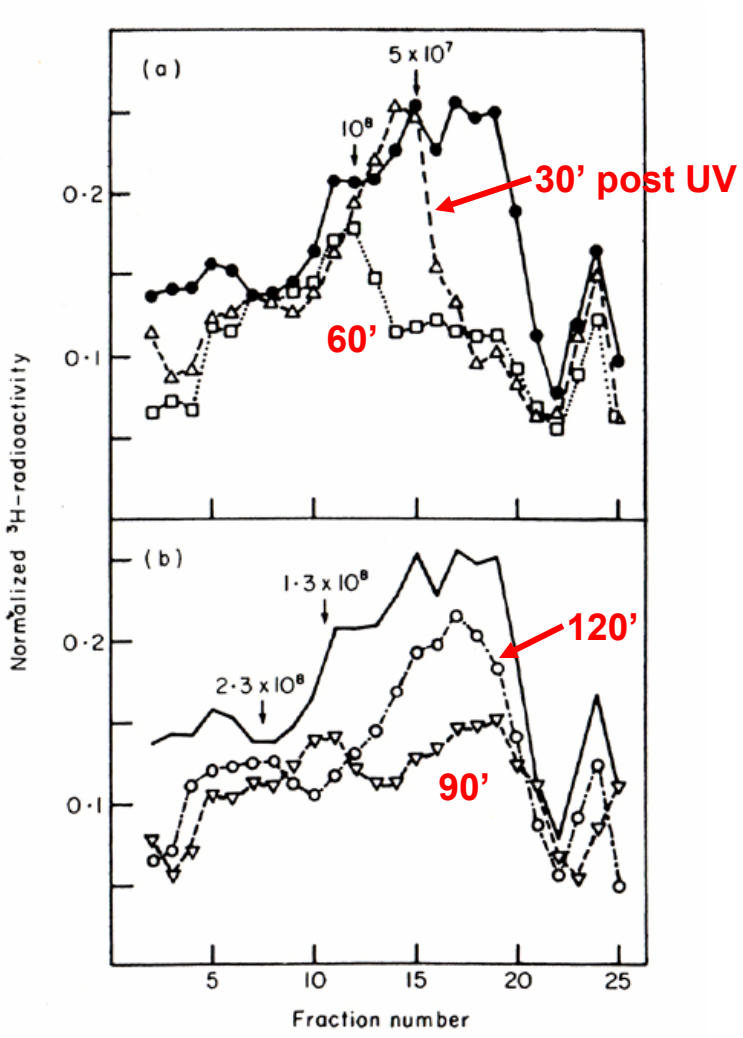


Dose-response and time-course for inhibition of DNA replication in UVC-treated human fibroblasts

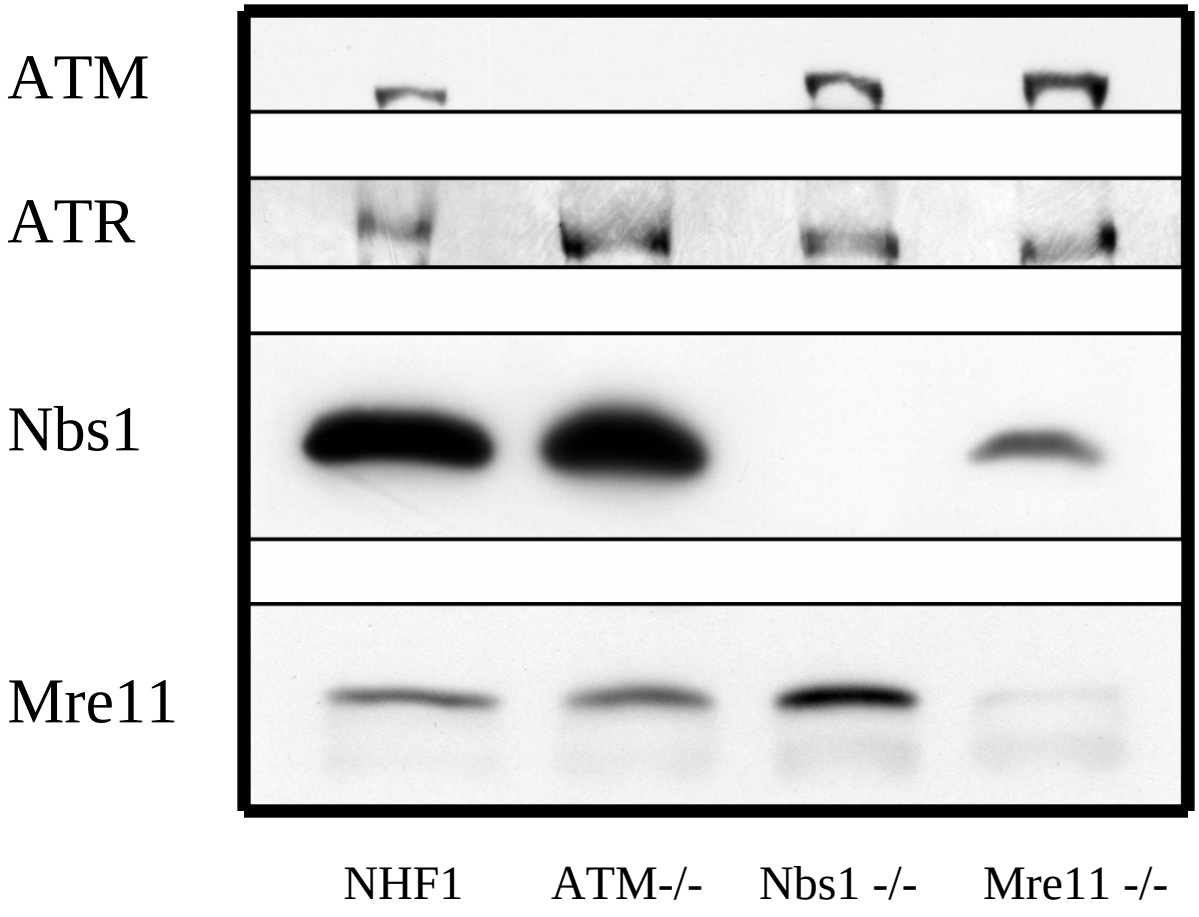
Dose: 30’ post UVC



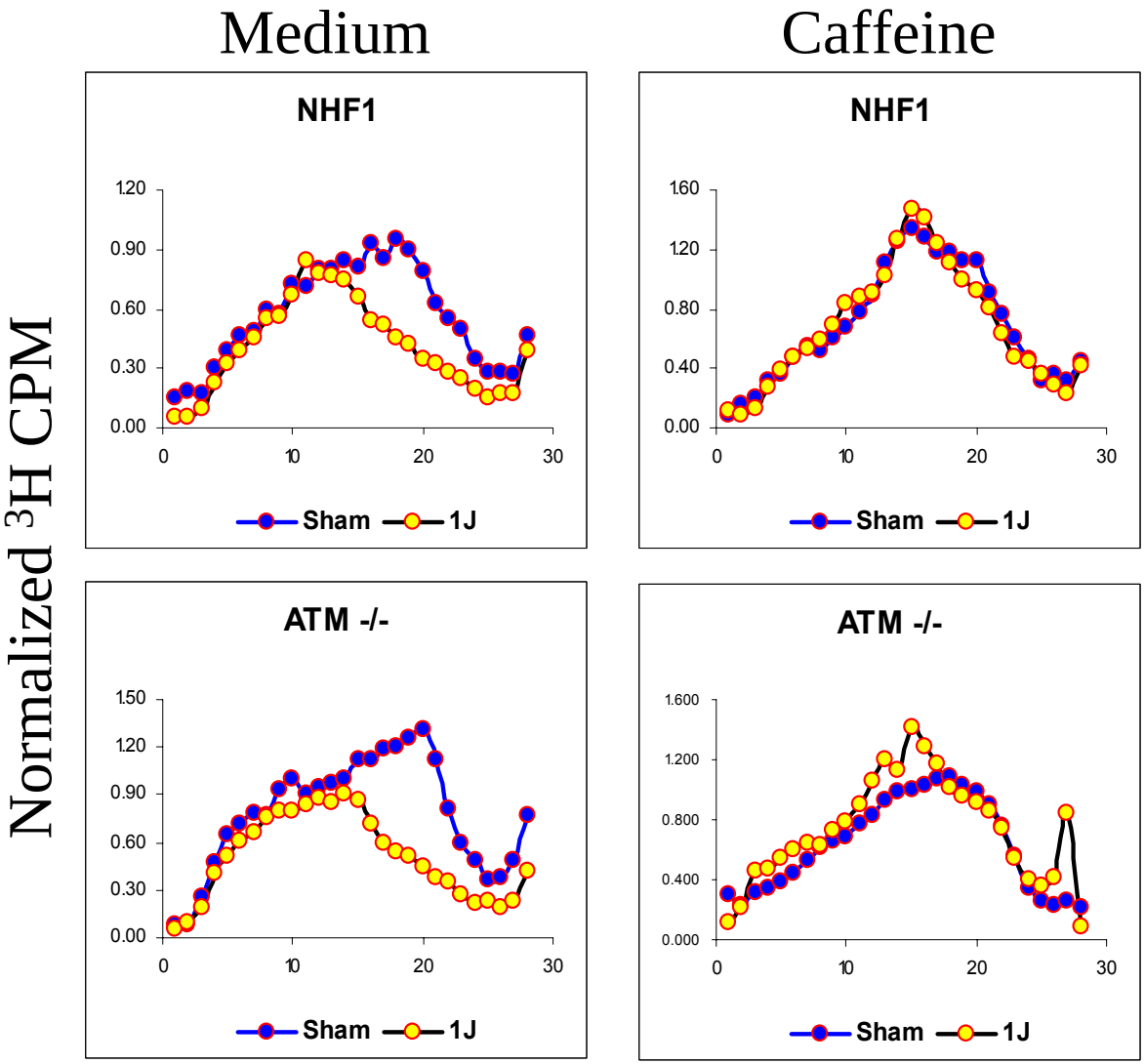
Time: 0.8 J/m²



Telomerized cell lines from patients with genetic instability syndromes.



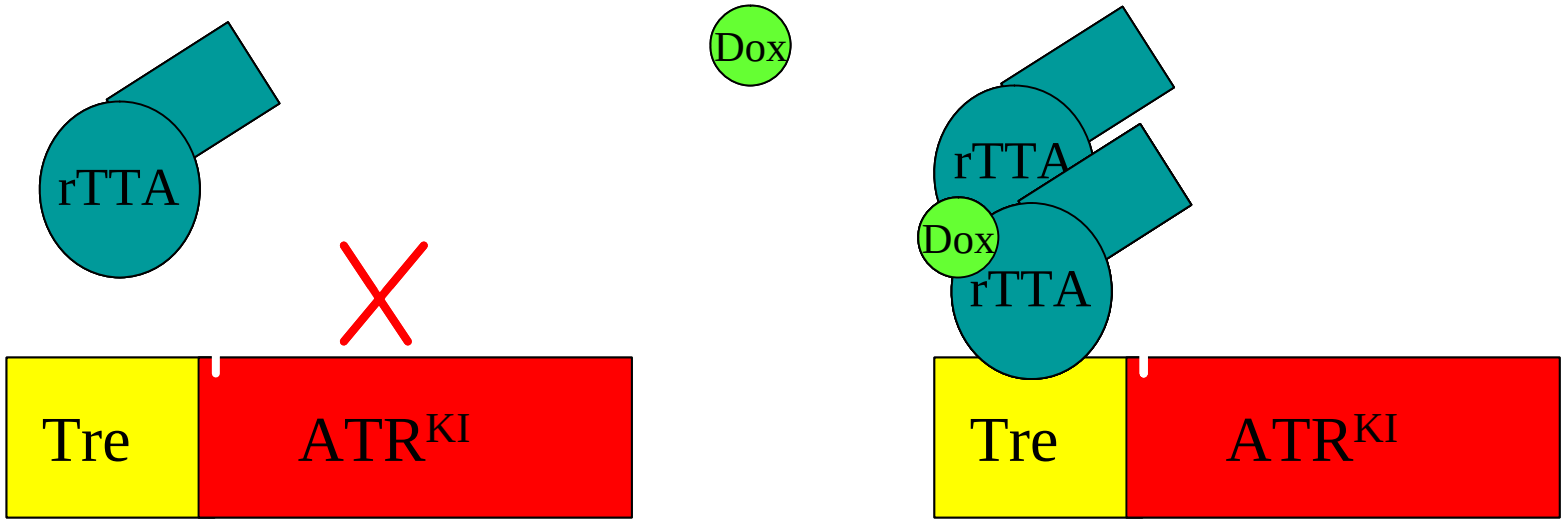
ATM is not required for the UVC-induced inhibition of replicon initiation; caffeine reverses the effect

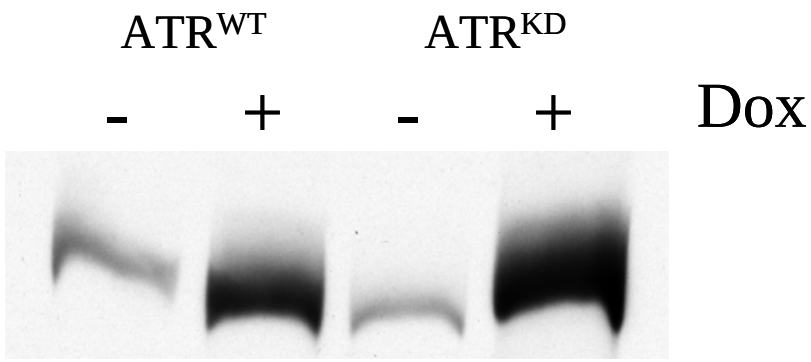


ATR (AT-and rad3-related)

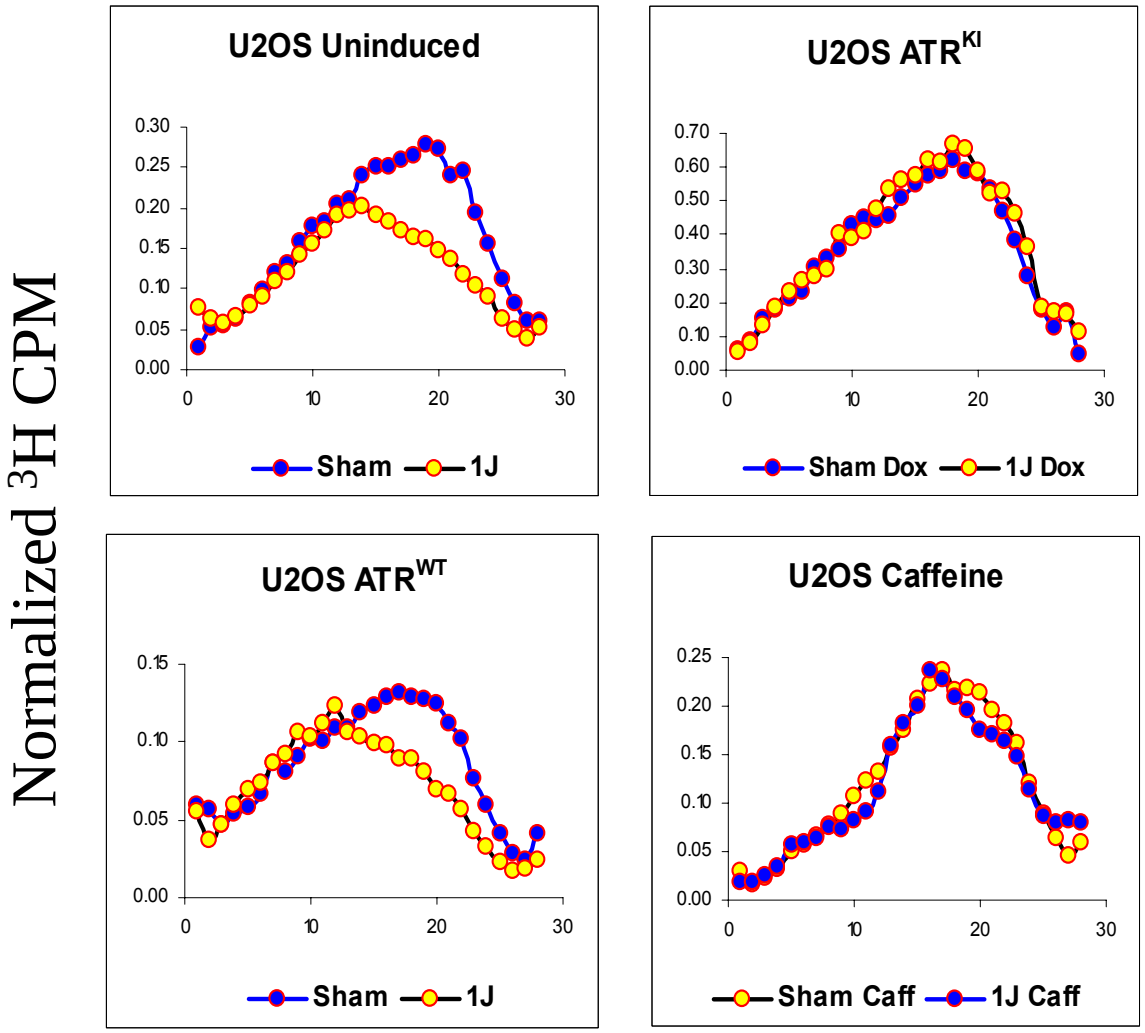
- Member of the PI-3-kinase-related kinase family
- Essential gene product
  - ATR -/- mice die by E8.5
  - Cultured blastocysts display proliferation defects and mitotic catastrophe
- Overexpression of kinase-inactive ATR (ATR^{KI}) allele
  - Renders cells hypersensitive to DNA damaging agents
  - Abrogates the Decatenation, Replication, and G2 DNA Damage Checkpoints.

Tet-on Inducible System





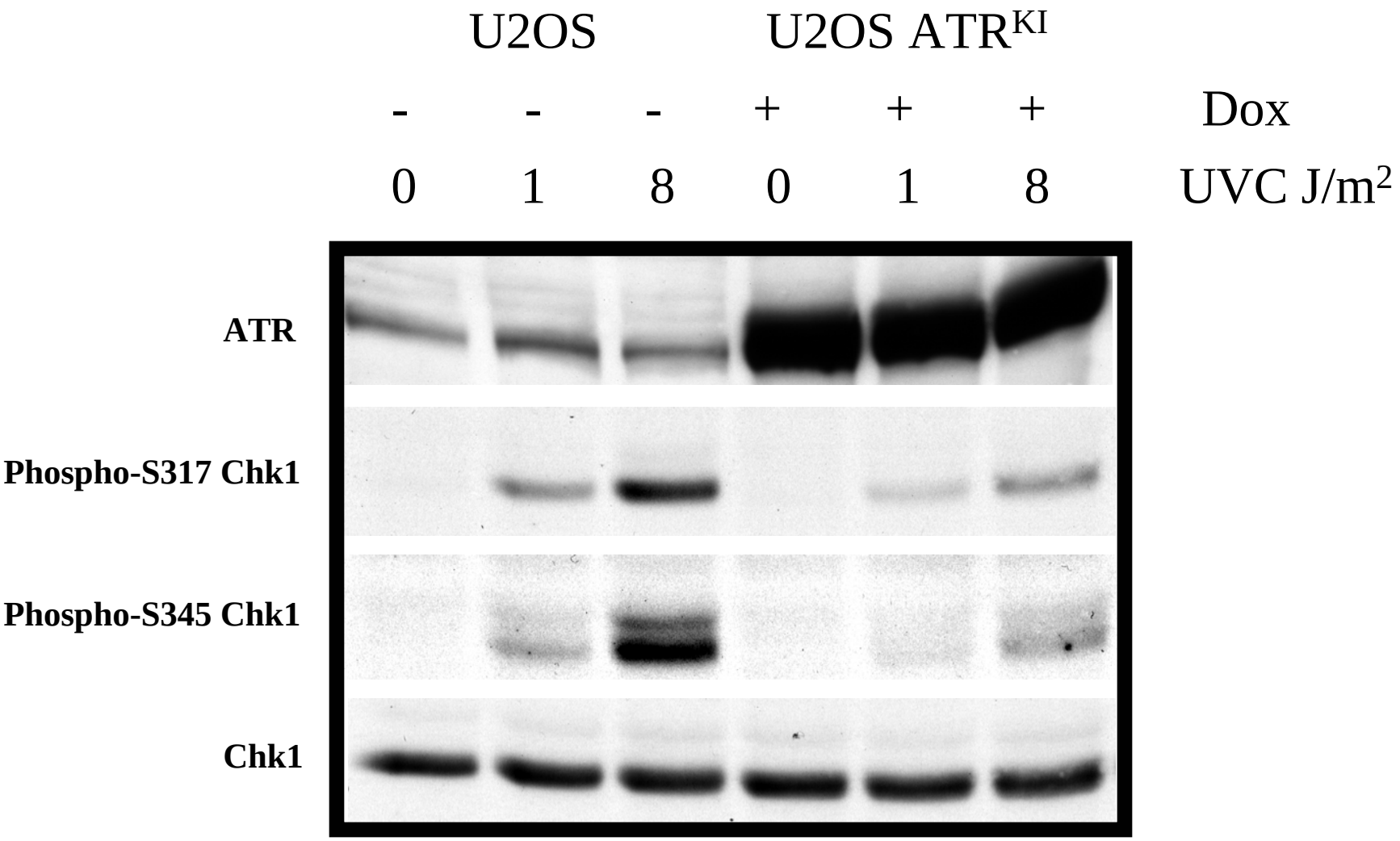
Overexpression of ATR^{KI} abrogates the UVC-induced intra-S Checkpoint



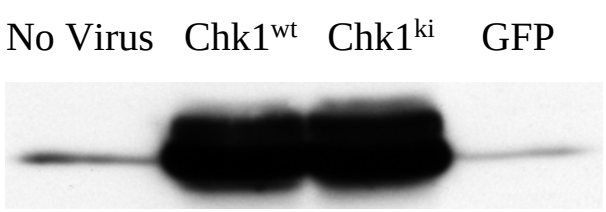
Chk1

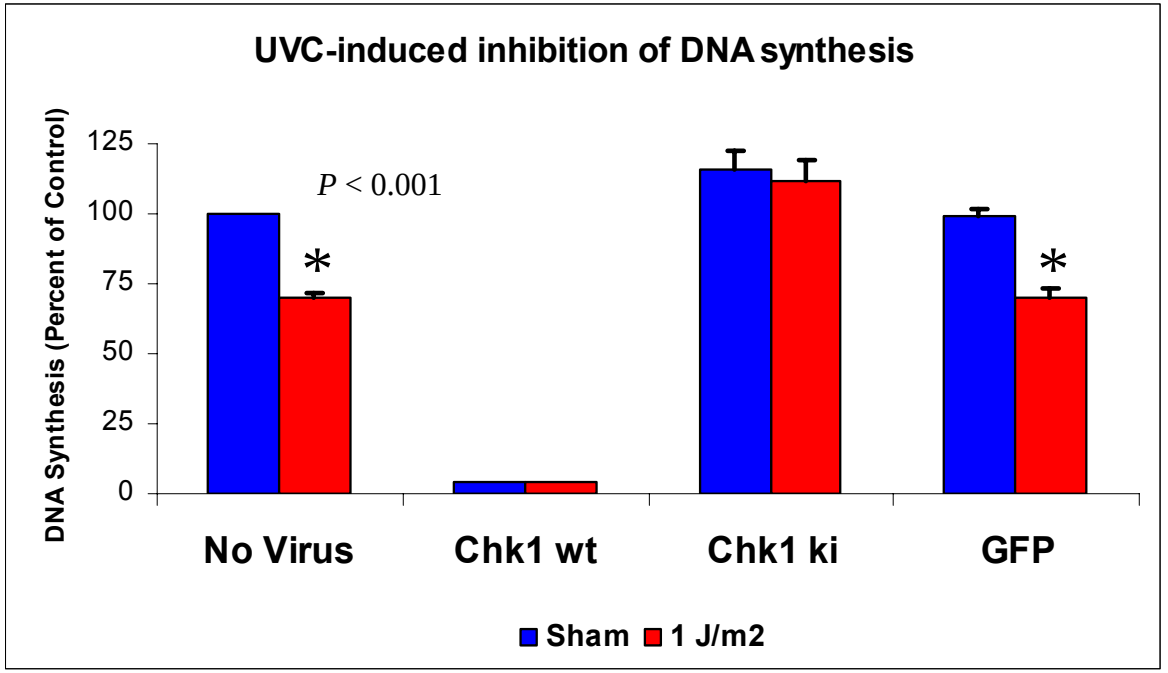
- Essential gene product
  - Chk1 -/- mice die by E6.5
  - Cultured blastocysts display proliferation defects
- Effector Kinase
  - Required for Replication, and G2 DNA Damage checkpoints.
  - Sensitive to UCN-01 (7-hydroxystaurosporine)

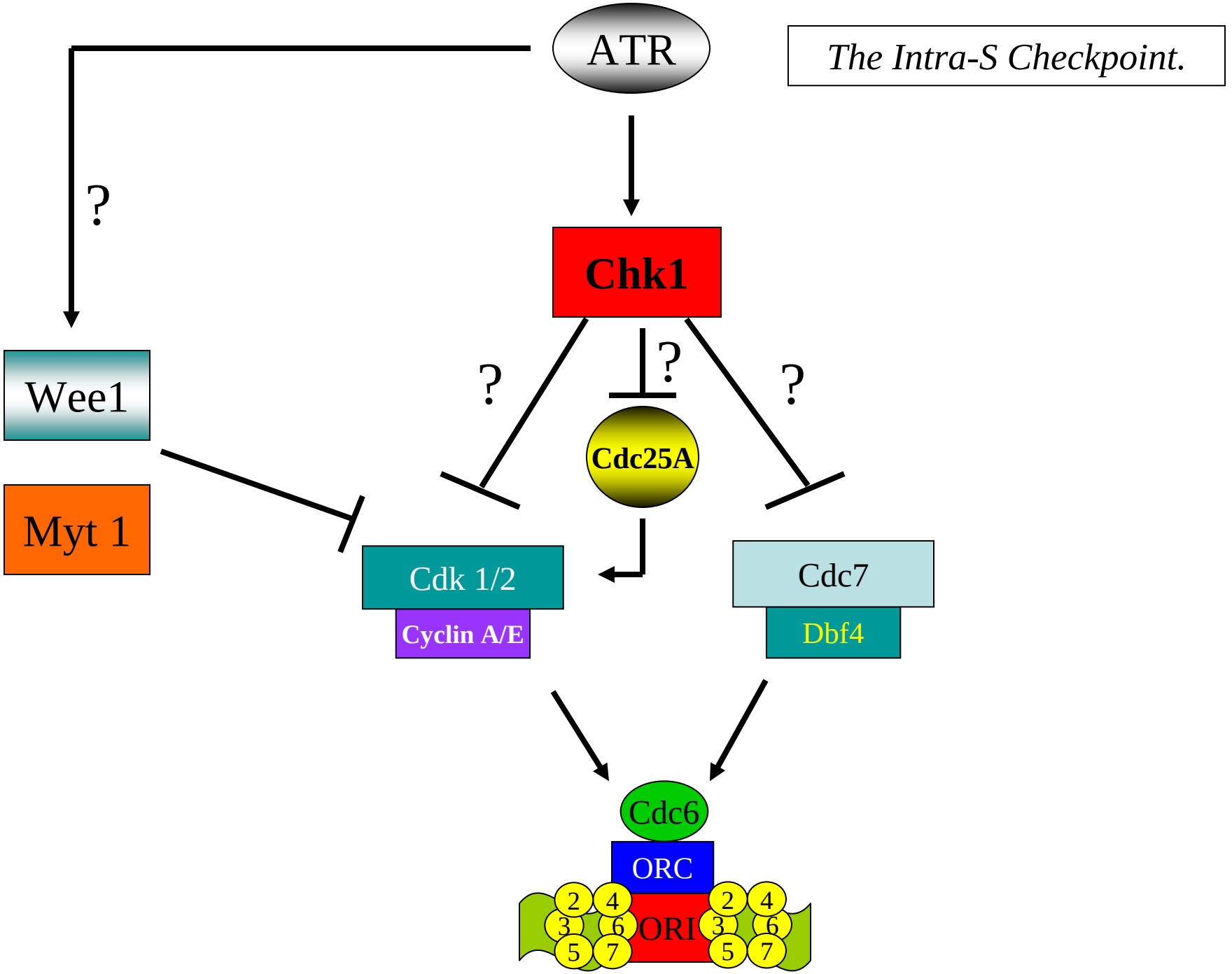
Chk1 phosphorylation following UVC irradiation is dependent on ATR



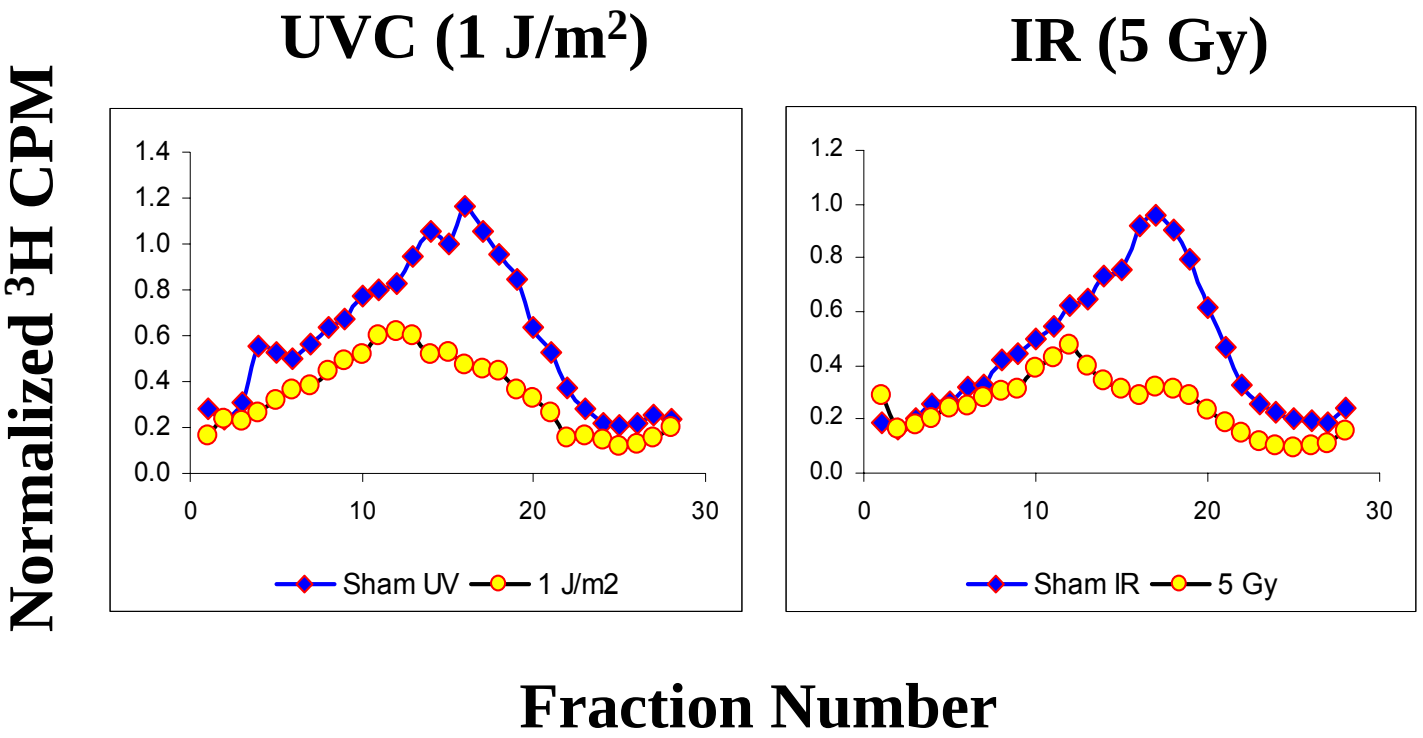
Over-expression of Chk1^{ki} overrides the UVC-induced intra-S checkpoint



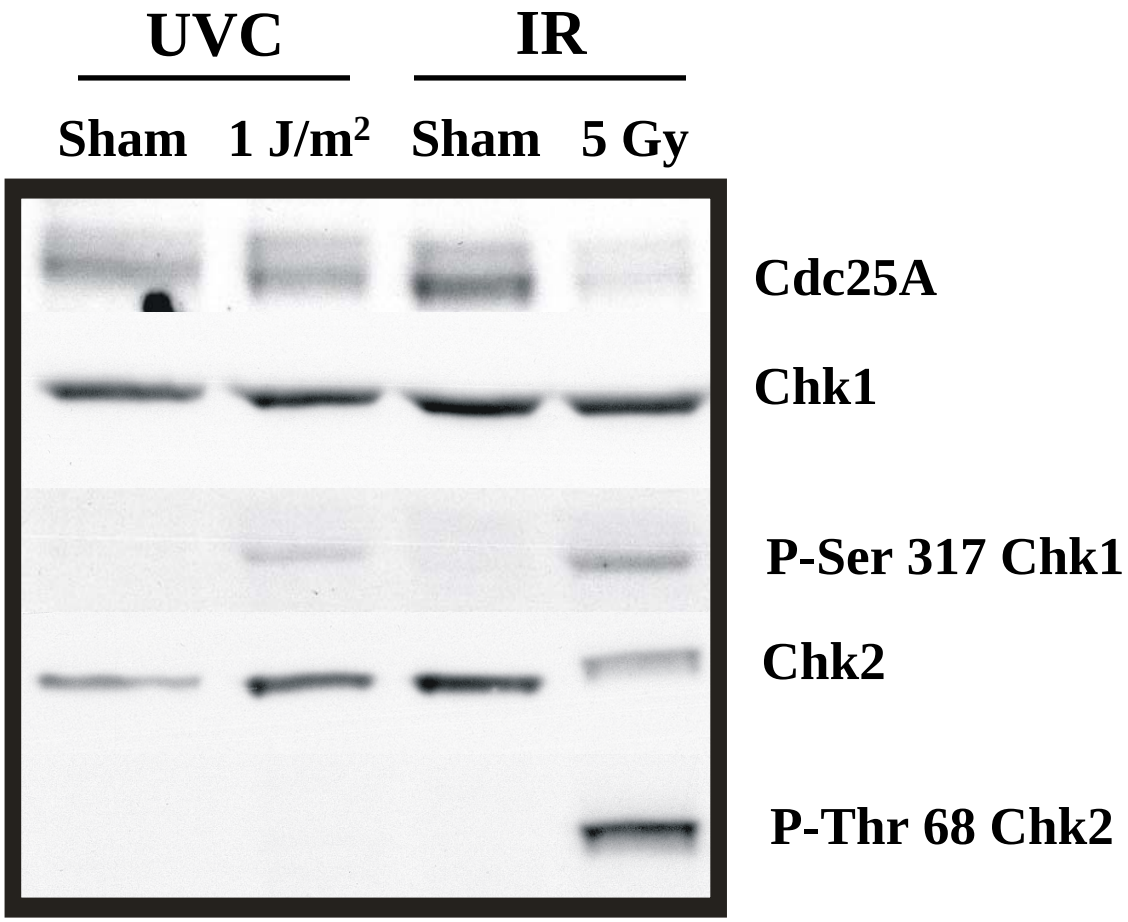



ATR
The Intra-S Checkpoint.
?
Chk1
Wee1
?
?
?
Myt 1
Cdc25A
Cdk 1/2
Cyclin A/E
Cdc7
Dbf4
Cdc6
ORC
2
4
3
6
5
7
ORI
2
4
3
6
5
7

Effective doses for UVC- and IR-induced inhibition of replicon initiation

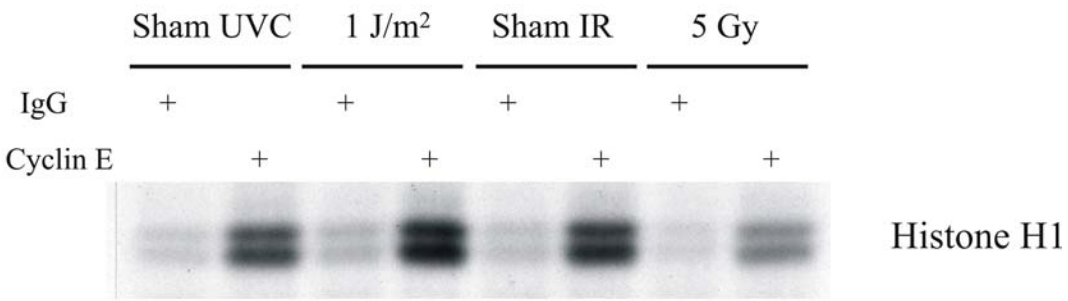


Cdc25A is not degraded under conditions that activate the UVC-induced intra-S checkpoint

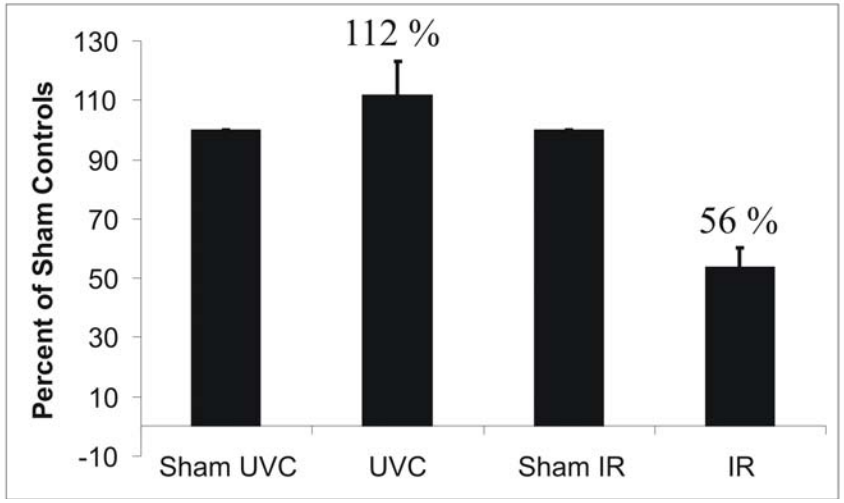


Cyclin E/Cdk2 kinase is not inhibited in UVC-damaged fibroblasts

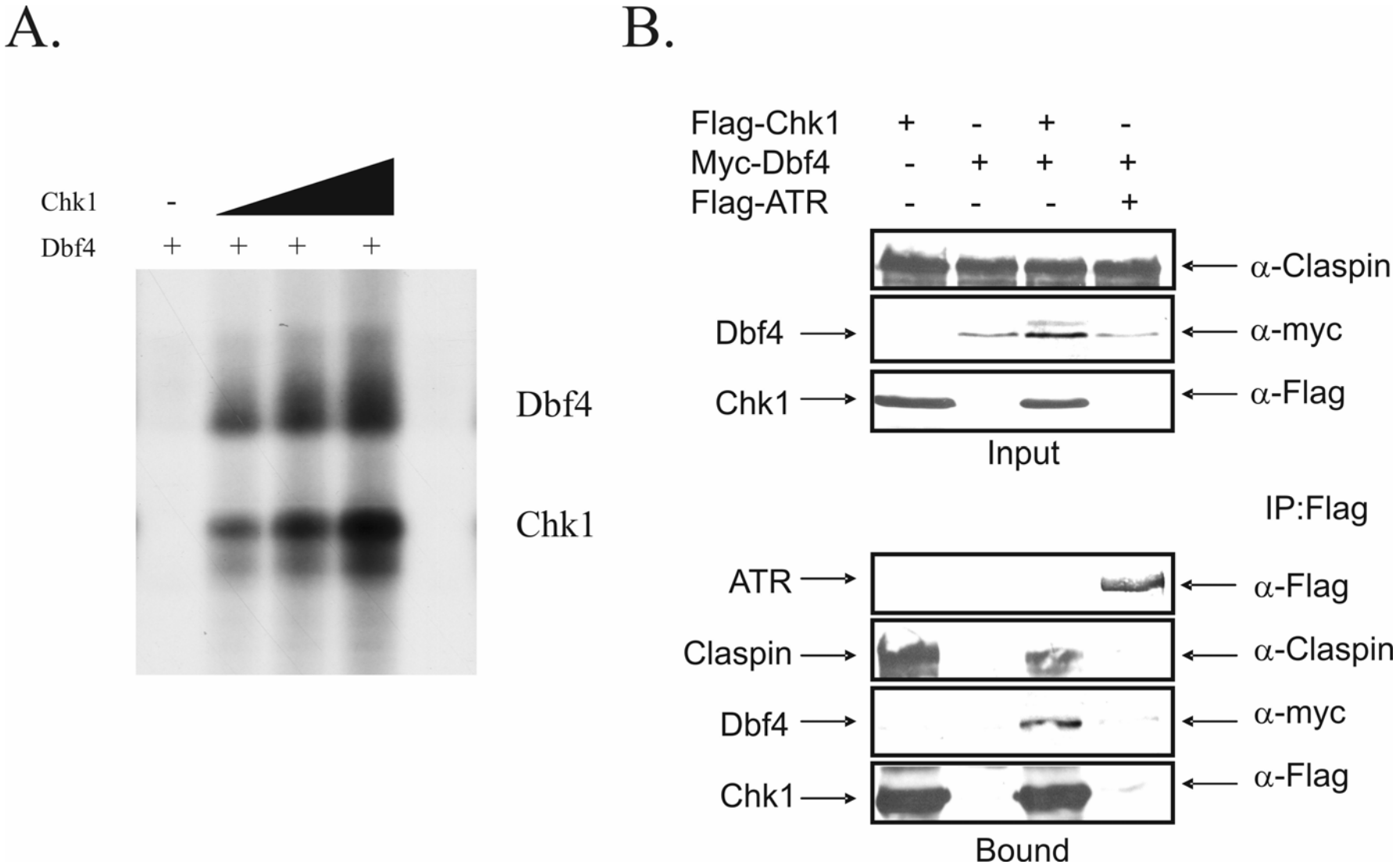
A.



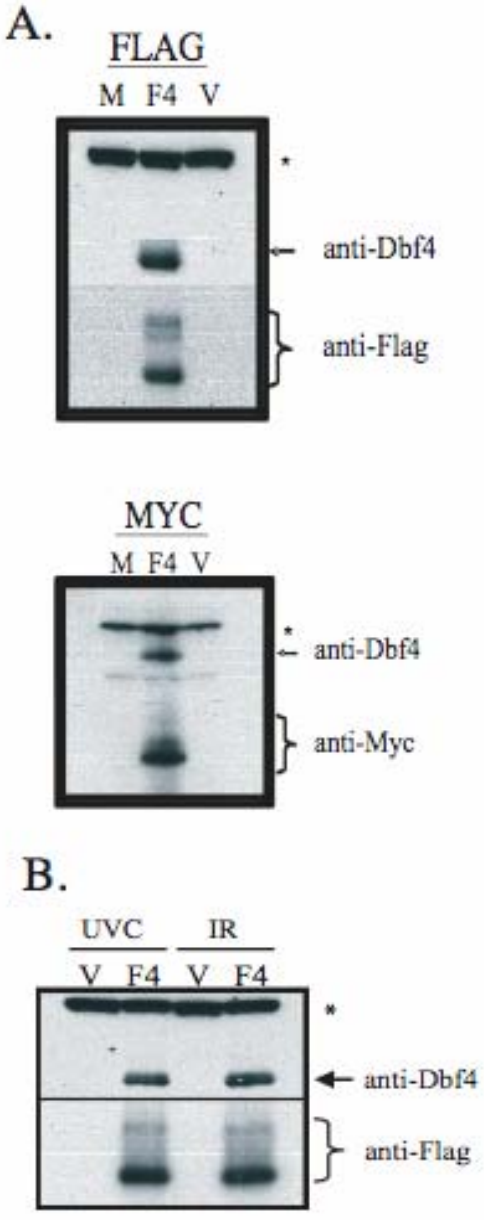
B.

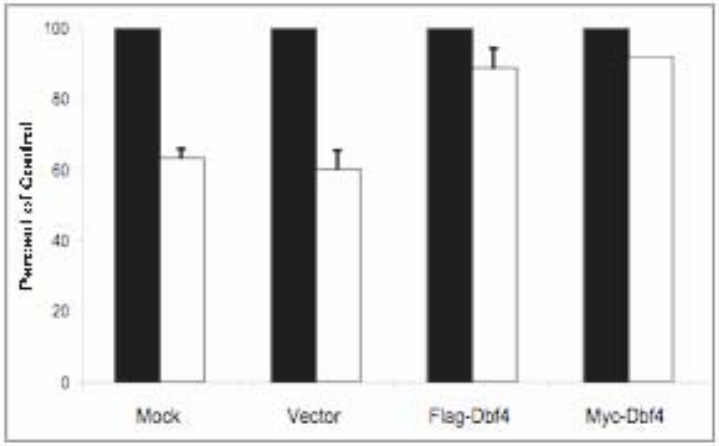


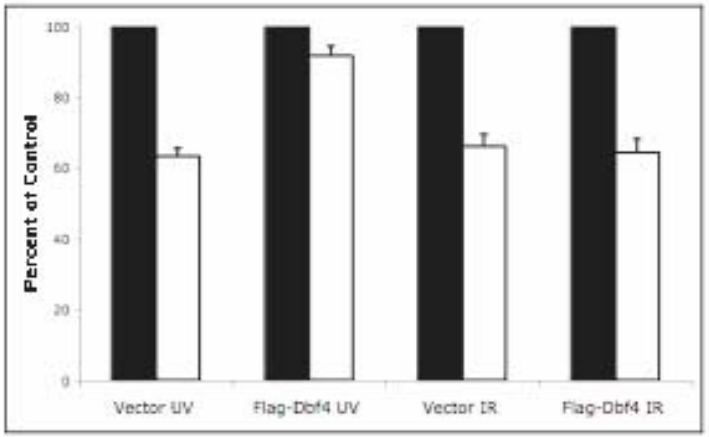
Chk1 phosphorylates Dbf4 in vitro and interacts with Dbf4 in vivo



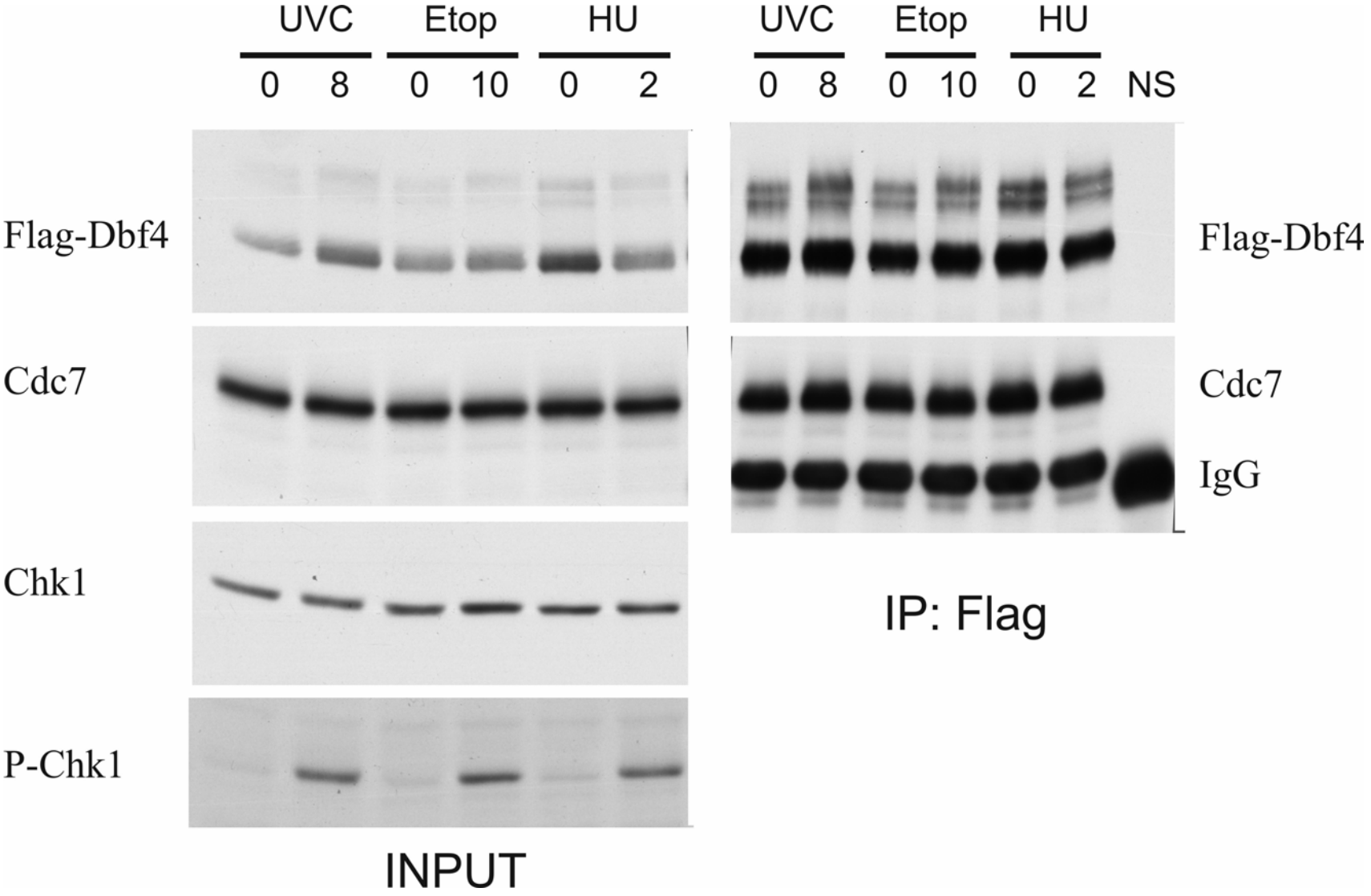
Over-expression of Flag- or Myc-tagged Dbf4 attenuates intra-S checkpoint response to UVC but not IR





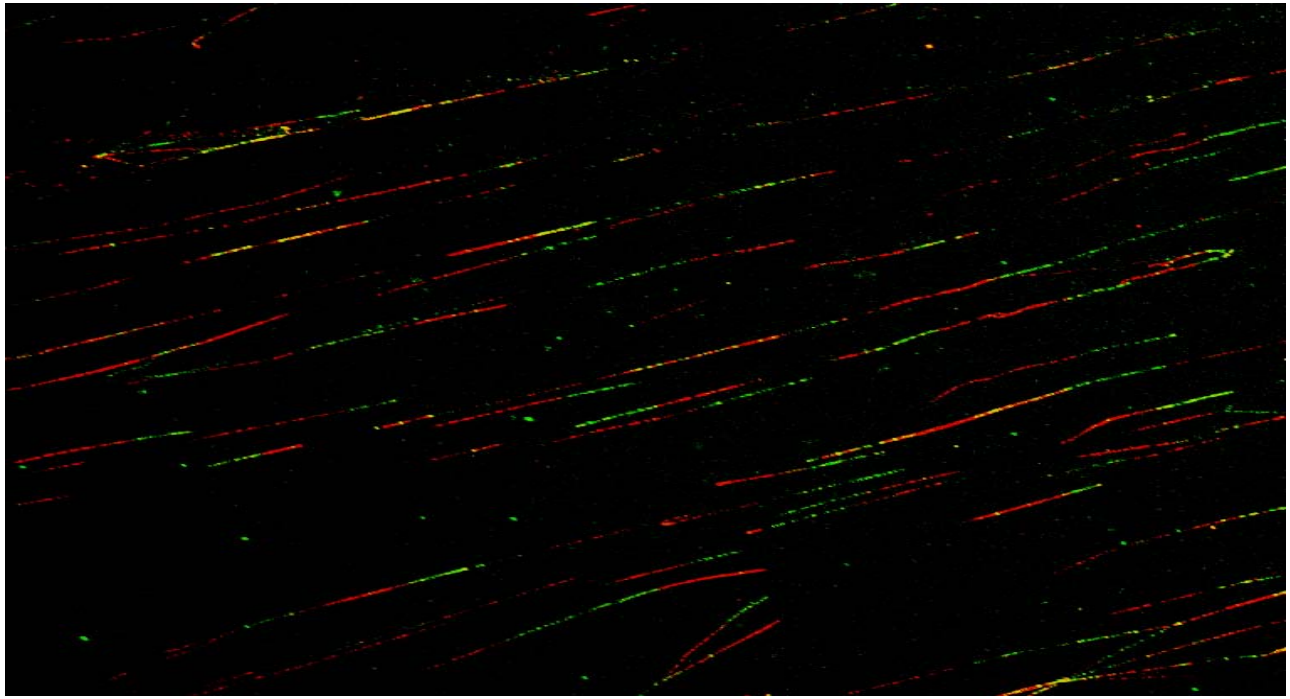


Over-expression of Flag-Dbf4 does not block Chk1 activation after UVC and Flag-Dbf4/Cdc7 interaction is not affected by UVC



Add 10 μM IdU,
10 min
(to label active
replication units)

Add 10 ml of reserved medium
containing 100μM CldU,
20 min
(ongoing DNA synthesis, and the firing
of new replication origins)

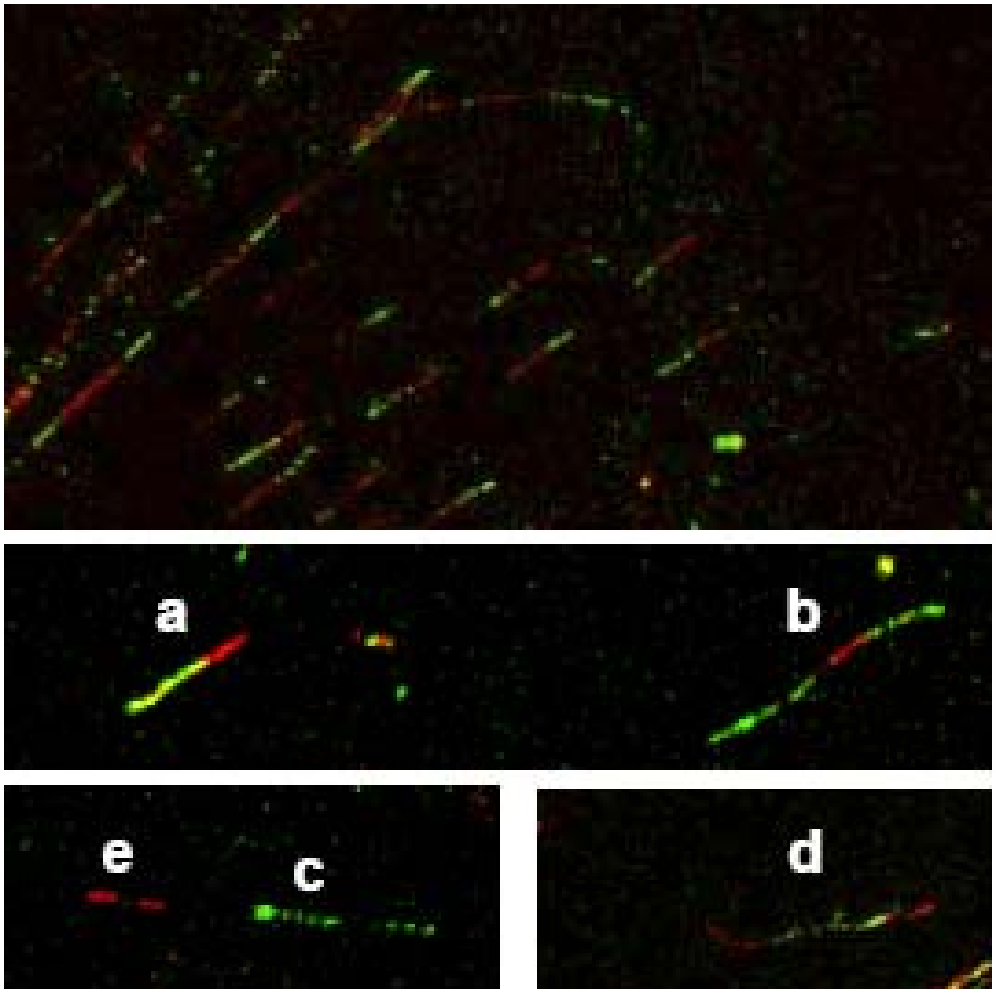


Cells released
with trypsin,
washed with PBS

Resuspend to
100 – 200
cells per μl.

Take 2 μl of cell solution and molecular comb the DNA

Labelling patterns of DNA fibers reveal replicon dynamics



Log Population
of HeLa Cells

↓

Add 10 mM IdU,
10 min

↓

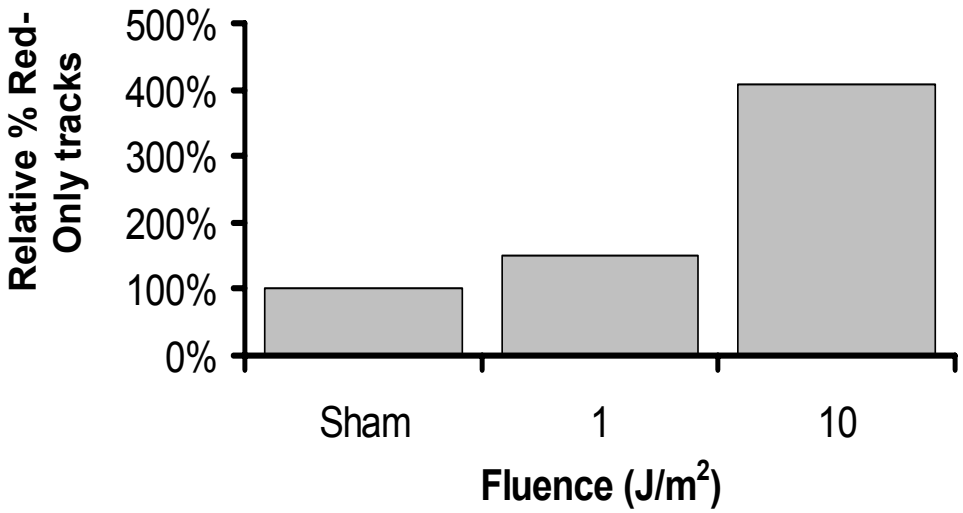
+/- UVC
(0, 1, 10 J/m^2)

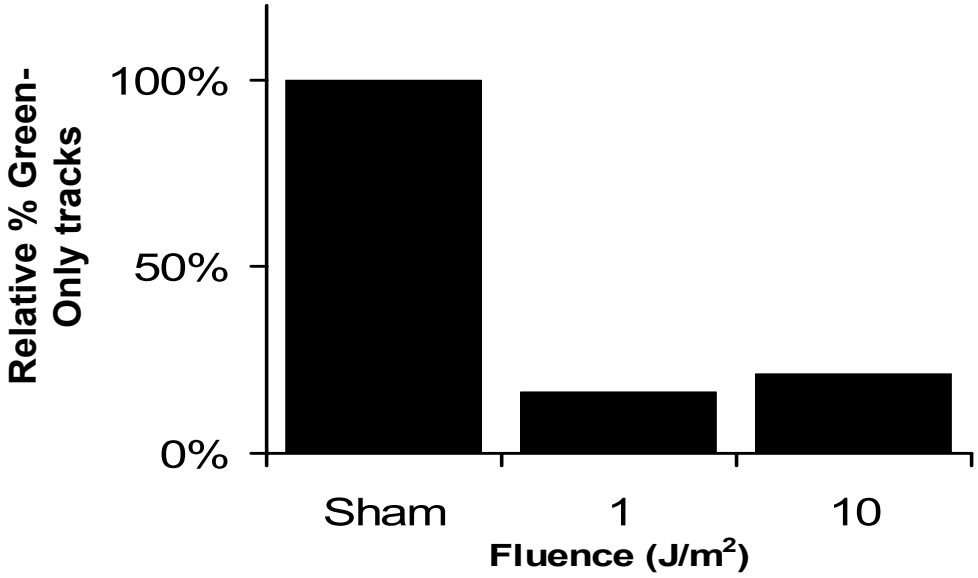
↓

Add 10 ml of reserved medium
containing 100 mM CldU, 20 min

↓

Count new origins (CldU-only tracks)
and IdU-only tracks





Log Population
of HeLa Cells

↓

+/- Caffeine
(added 30 min prior to UV)

↓

Add 10 mM IdU,
10 min

↓

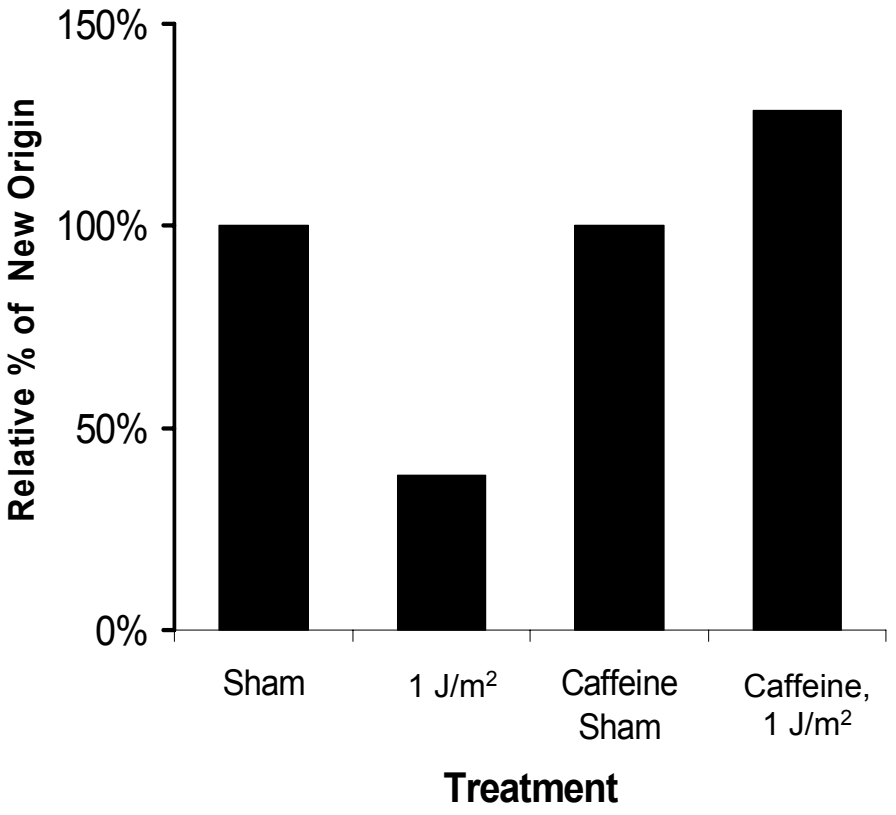
+/- UVC
(0, 1 J/m^2)

↓

Add 10 ml of reserved medium
containing 100 mM CldU, 20 min

↓

Count new origins (CldU-only tracks)



+/- Knockdown of ATR or CHK1

↓

Log Population of HeLa Cells

↓

Add 10 mM IdU, 10 min

↓

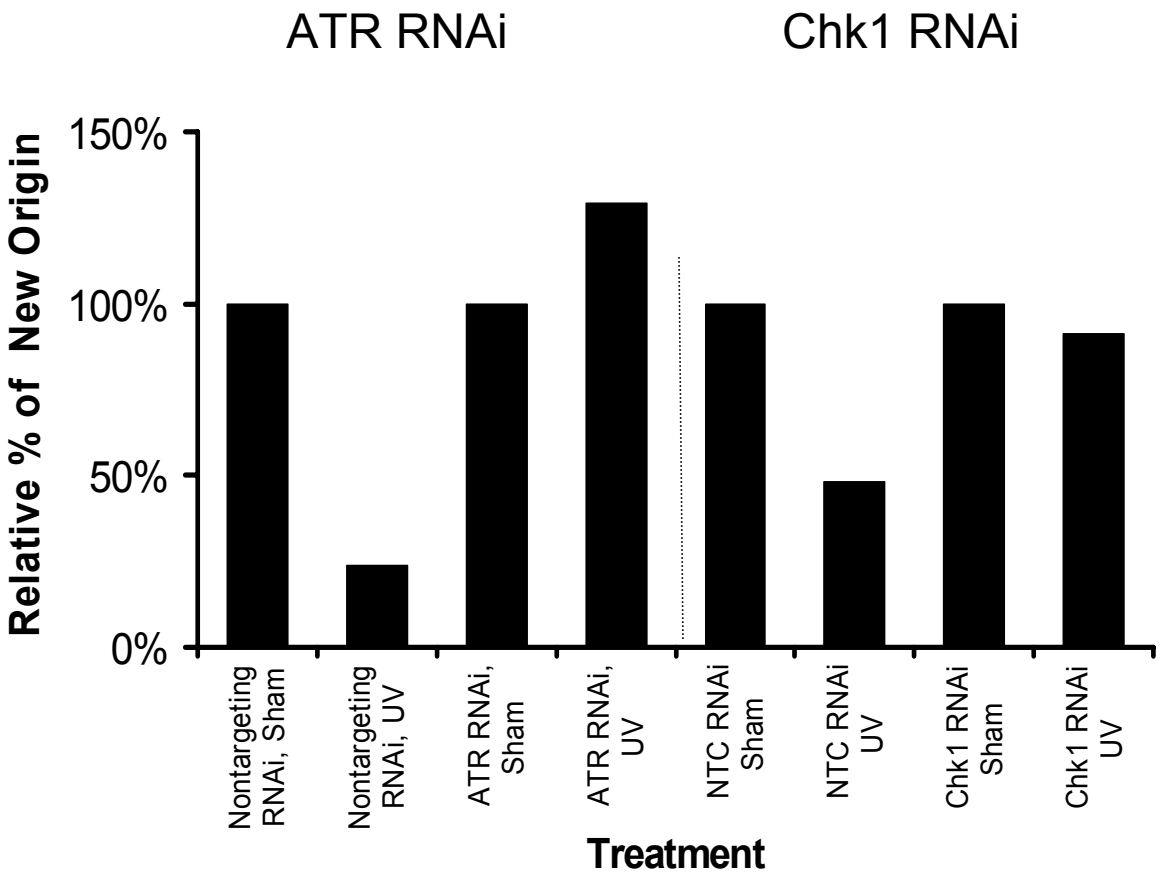
+/- UVC (0, 1J/m^2)

↓

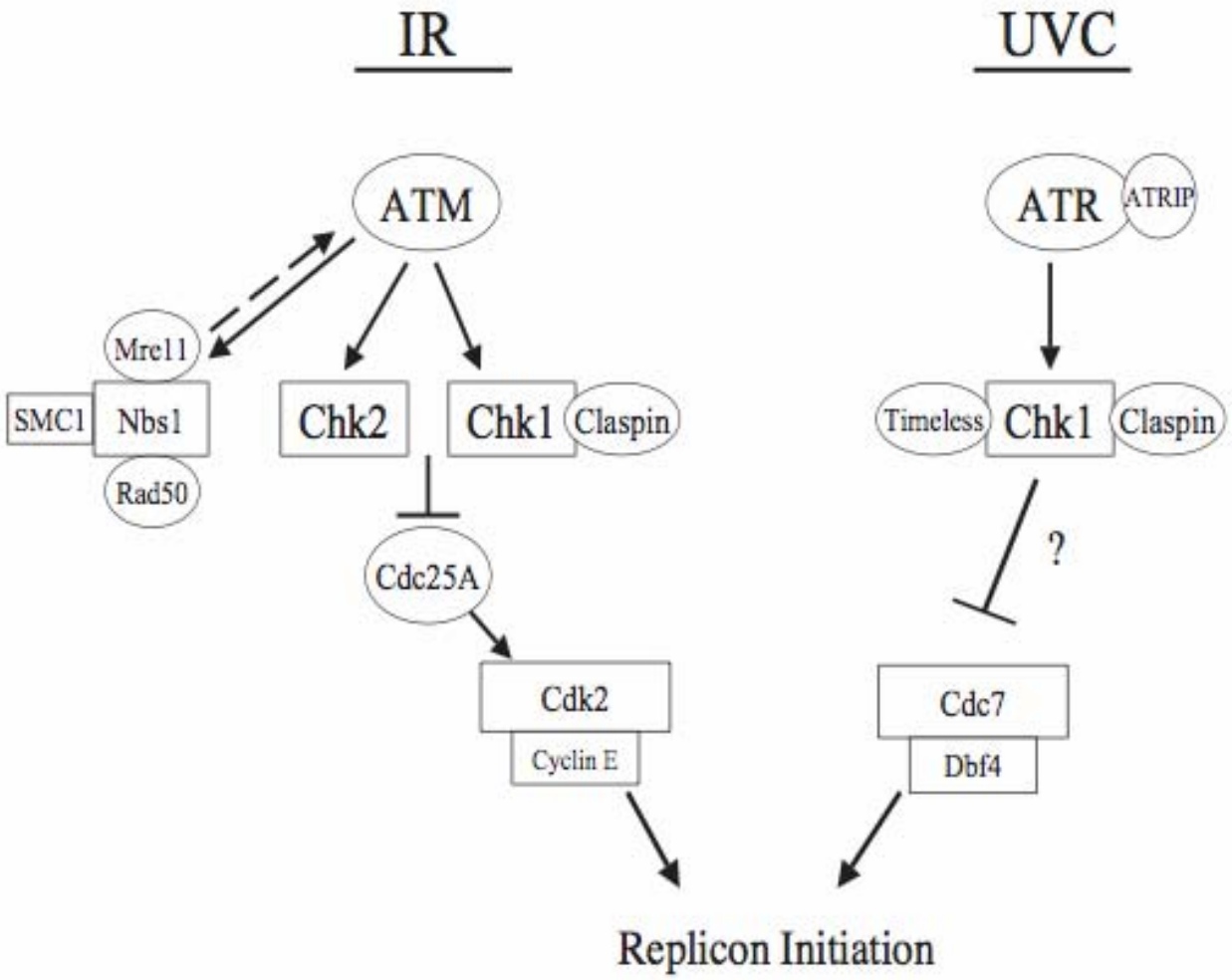
Add 10 mls of reserved media containing 100mM CldU, 20 min

↓

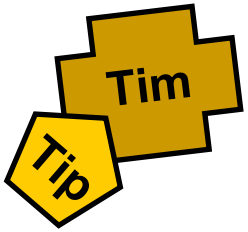
Count new origins (CldU tracks)



Human S Checkpoints



Role of Timeless and Timeless-interacting protein (Tipin) in Checkpoint Activation

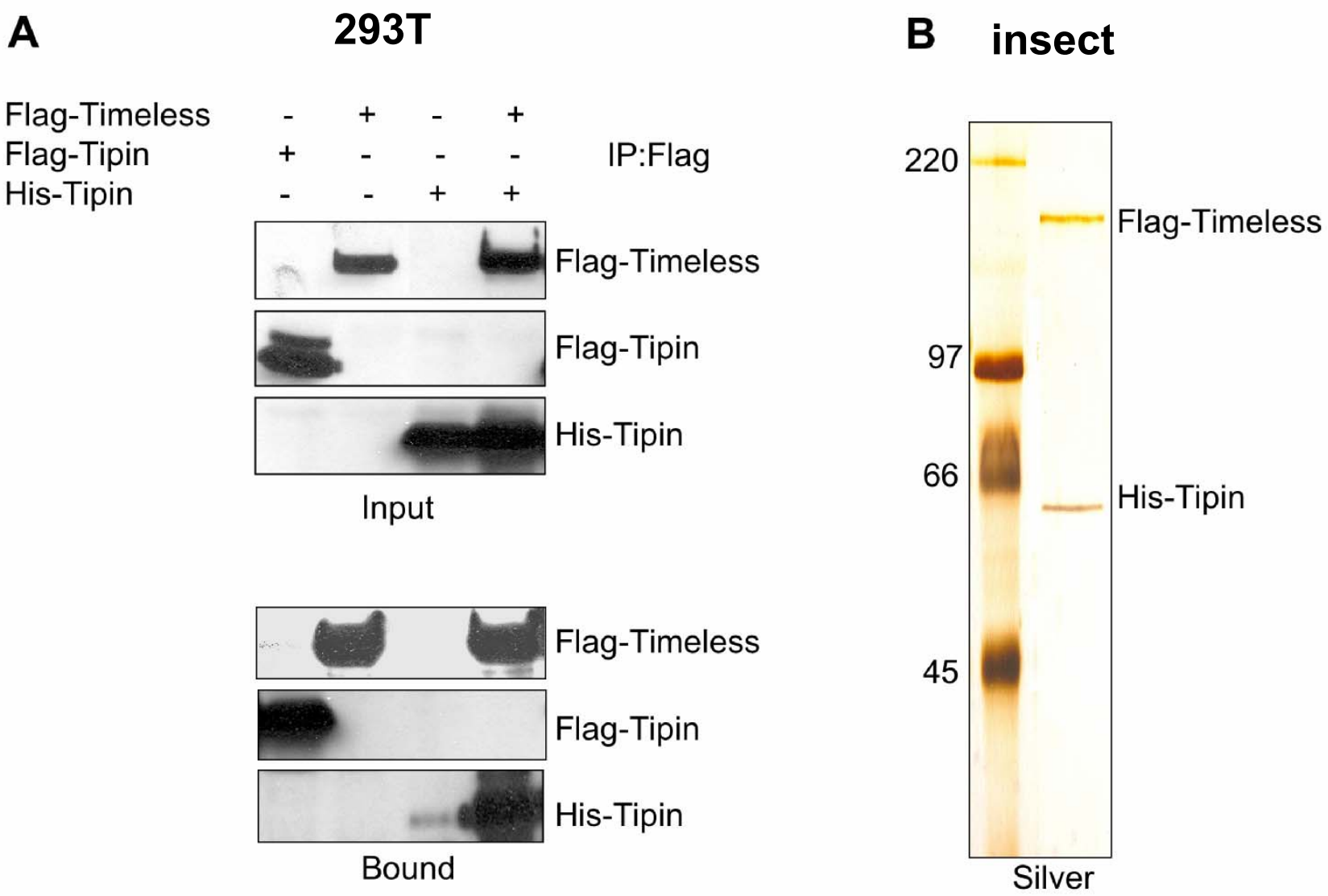


Timeless (Human) is required for activation of Chk1 and inhibition of DNA synthesis by low dose UVC (Ünsal-Kaçmaz et al., 2005, *Mol Cell Biol).*

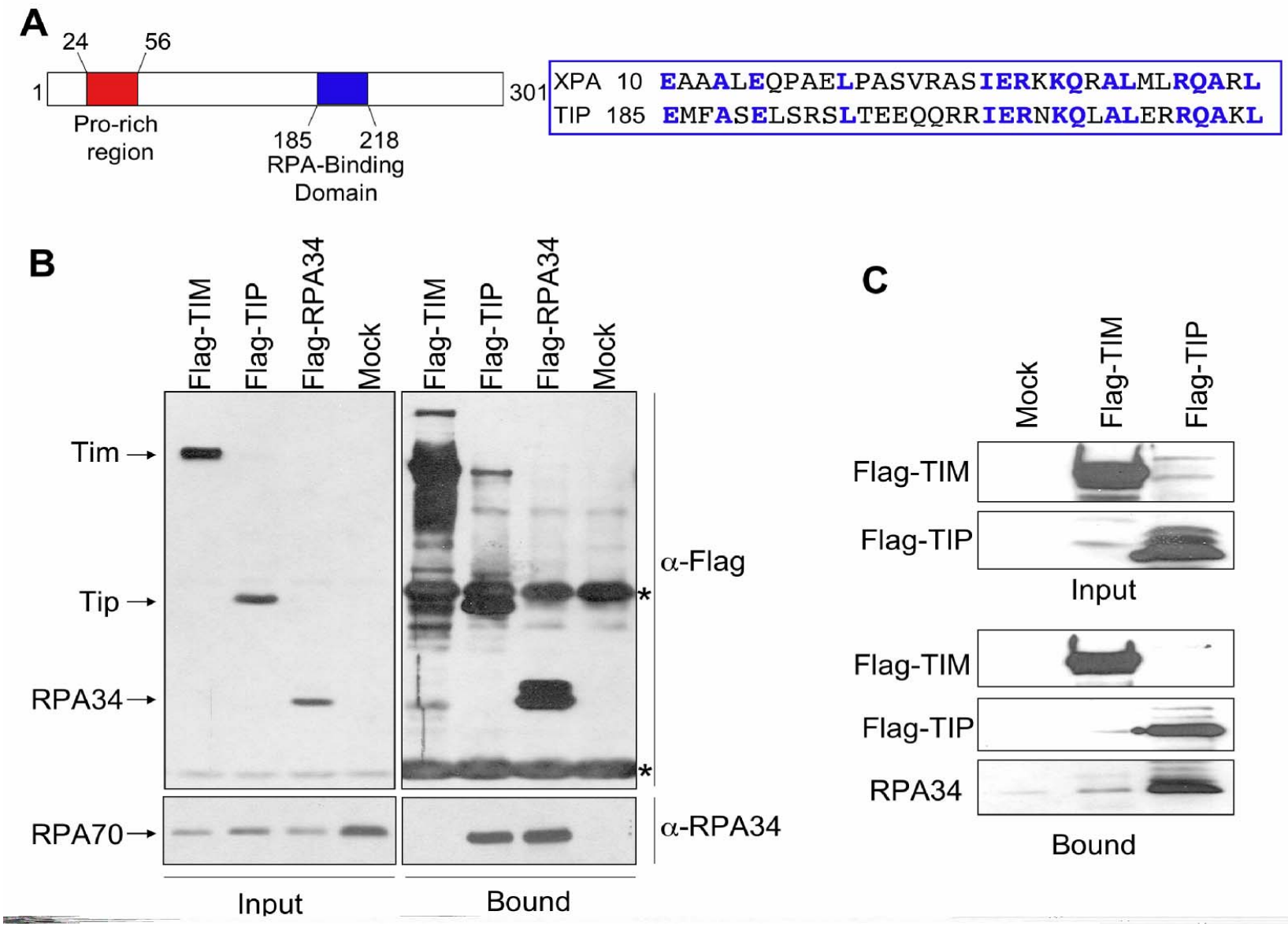
Timeless-interacting protein (Tipin) discovered in yeast two-hybrid analysis of murine Timeless (Gotter, 2003, *J Mol Biol*)

Timeless and Tipin are homologs of *S. pombe* **Swi/Swi3 which serves as a replication fork protection complex (e.g. Noguchi et al., 2004, *Mol Cell Biol*)**

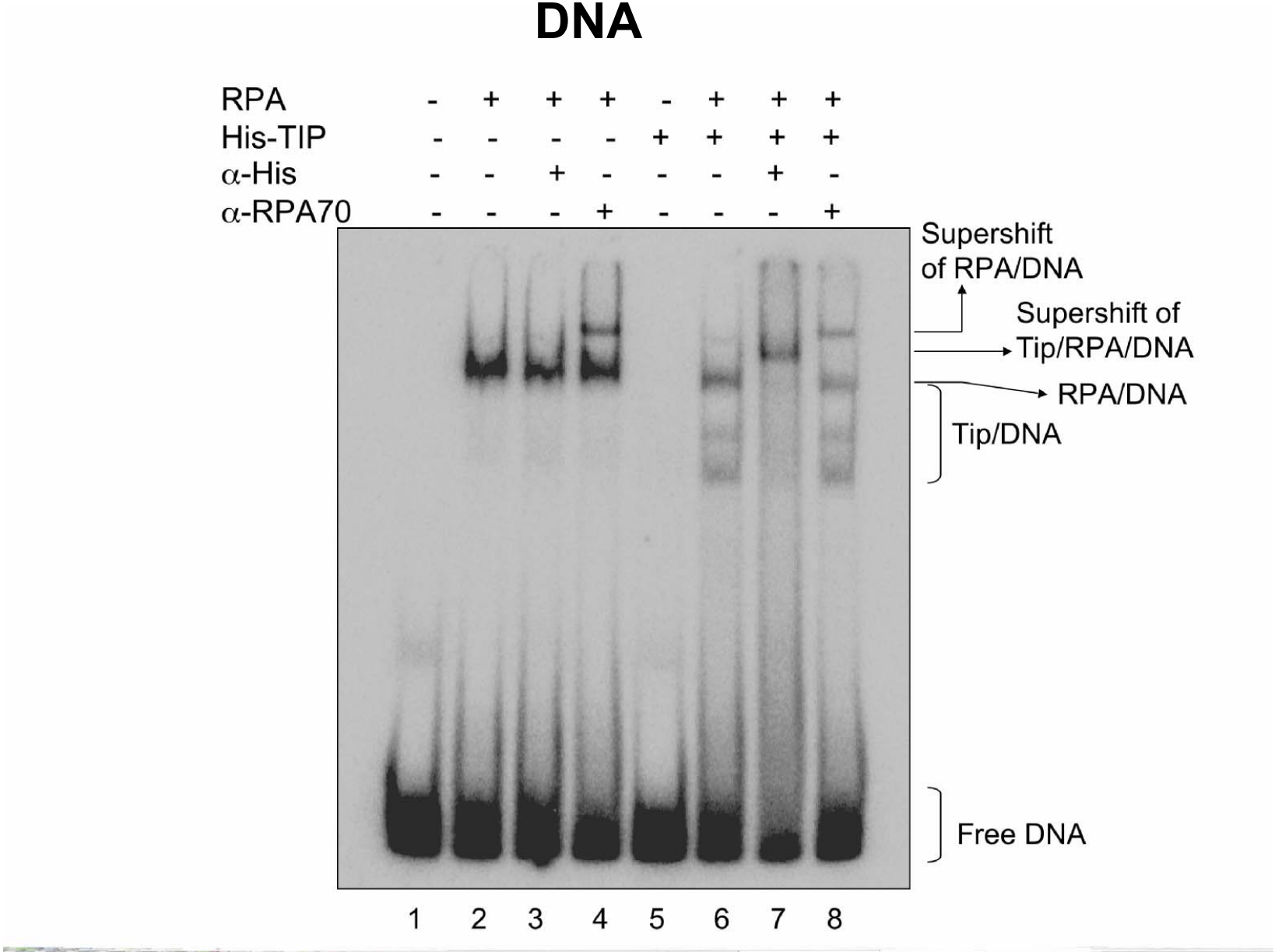
Timeless and Tipin form a complex in human and insect cells

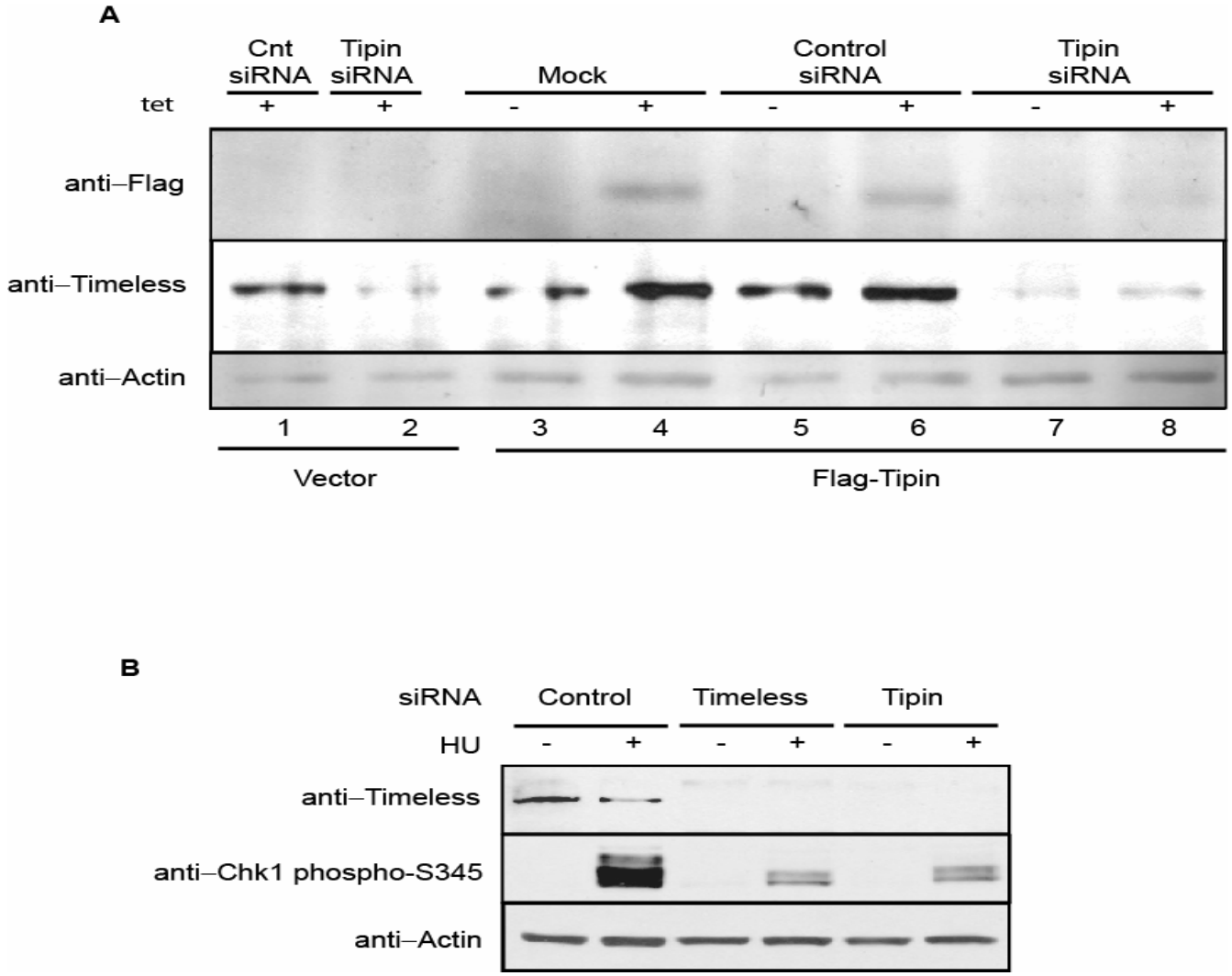


Tipin has an RPA-binding domain similar to that seen in XPA, and binds to RPA34 in human cells



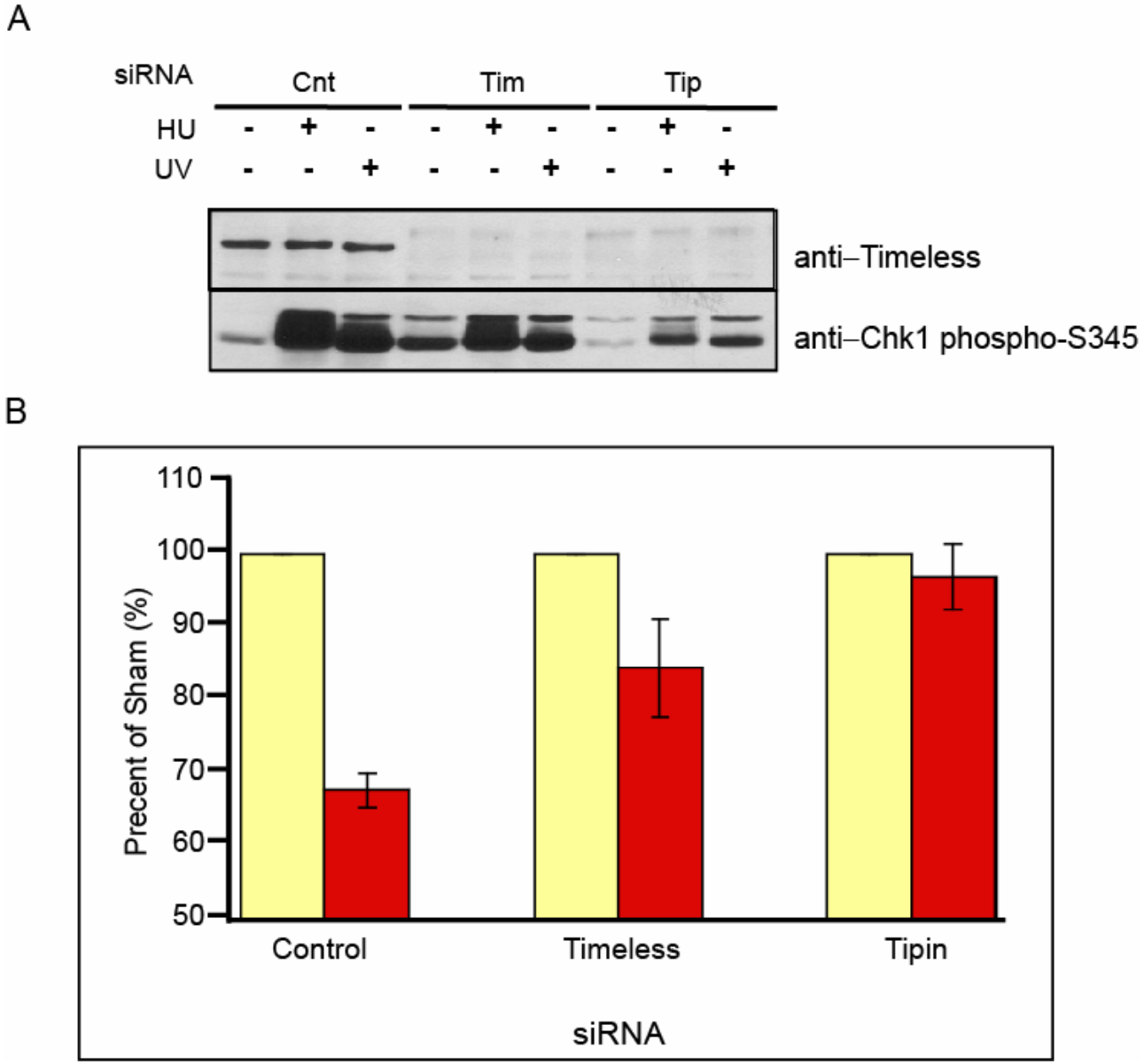
Tipin does not bind DNA directly but interacts with RPA/DNA complexes; RPA appears to load Tipin onto DNA

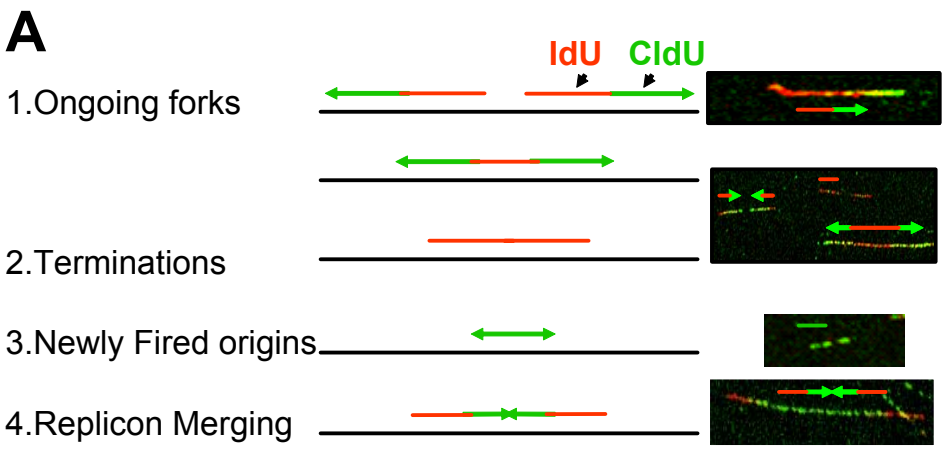


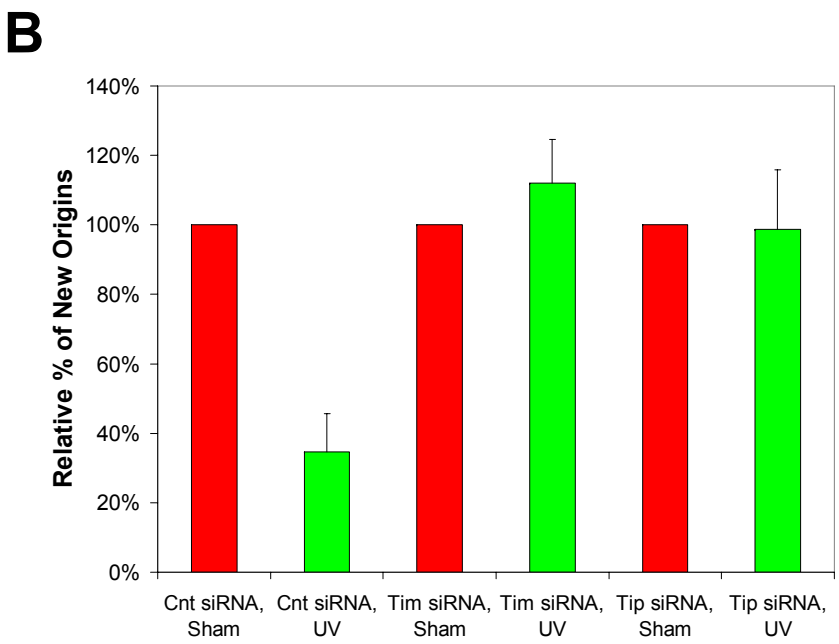


Knockdown of Tipin reduces expression of Timeless and inhibits activation of Chk1 in response to replication stress

Knockdown of Timeless and Tipin attenuates intra-S checkpoint response to UVC




A
IdU
CldU
1.Ongoing forks
2.Terminations
3.Newly Fired origins
4.Replicon Merging


B
Relative % of New Origins
140%
120%
100%
80%
60%
40%
20%
0%
Cnt siRNA, Sham
Cnt siRNA, UV
Tim siRNA, Sham
Tim siRNA, UV
Tip siRNA, Sham
Tip siRNA, UV

UVC inhibits DNA chain elongation; knockdown of Timeless inhibits DNA chain elongation; knockdown of Tipin reverses the UVC-induced inhibition of chain elongation.

Red tracks[a]				
siRNA[c]	**Mean μm**	**S.D. (n)**	**B/A**	**p-value**
A. NTC	5.3	2.9 (n=166)		
B. Tim	2.7	1.9 (n=225)	0.52	<0.0001
A. NTC	5.3	2.9 (n=166)		
B. Tipin	4.7	2.4 (n=201)	0.89	0.57
A. Tipin	4.7	2.4 (n=201)		
B. Tim	2.7	1.9 (n=225)	0.58	<0.0001

Green tracks[b]				
siRNA[c]	**Mean μm**	**S.D. (n)**	**B/A**	**p-value**
A. NTC, Sham	5.8	3.0 (n=146)		
B. NTC, 2.5 J/m^2	2.9	2.0 (n=139)	0.49	<0.0001
A. Tim, Sham	2.6	1.5 (n=158)		
B. Tim, 2.5 J/m^2	2.2	1.1 (n=165)	0.82	0.153
A. Tipin, Sham	3.8	2.4 (n=162)		
B. Tipin, 2.5 J/m^2	4.6	2.6 (n=161)	1.20	0.721

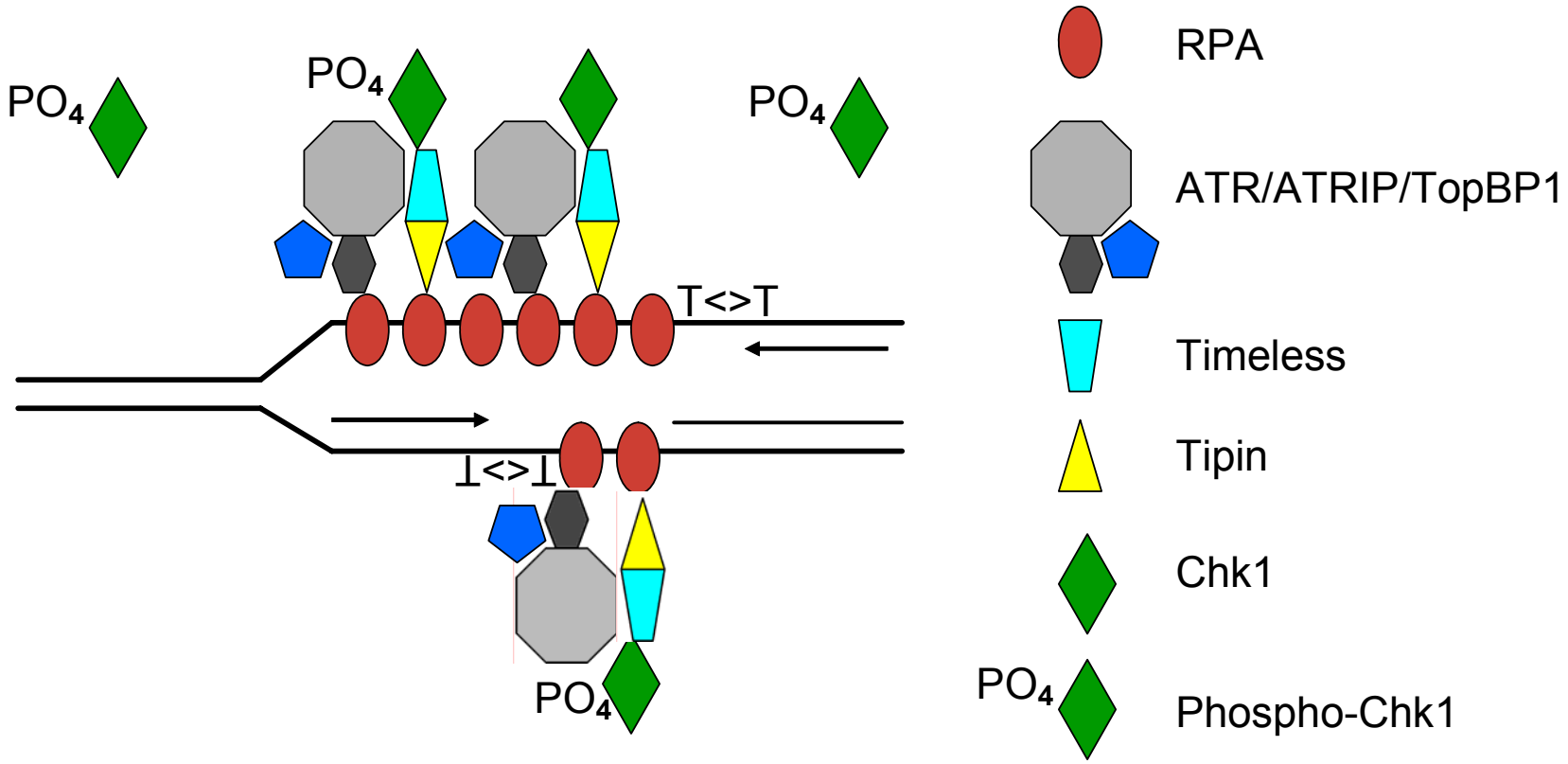
Analysis of DNA fibers reveals separation of function between Timeless and Tipin

Knockdown of either protein attenuates intra-S checkpoint response to UVC

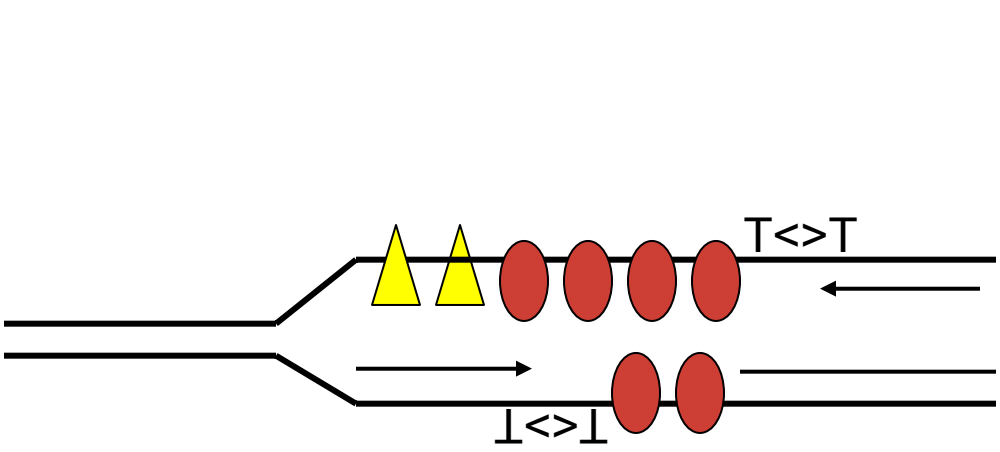
Knockdown of Timeless inhibits DNA chain elongation in undamaged cells; knockdown of Tipin reverses this effect suggesting that Tipin may be responsible for the inhibition

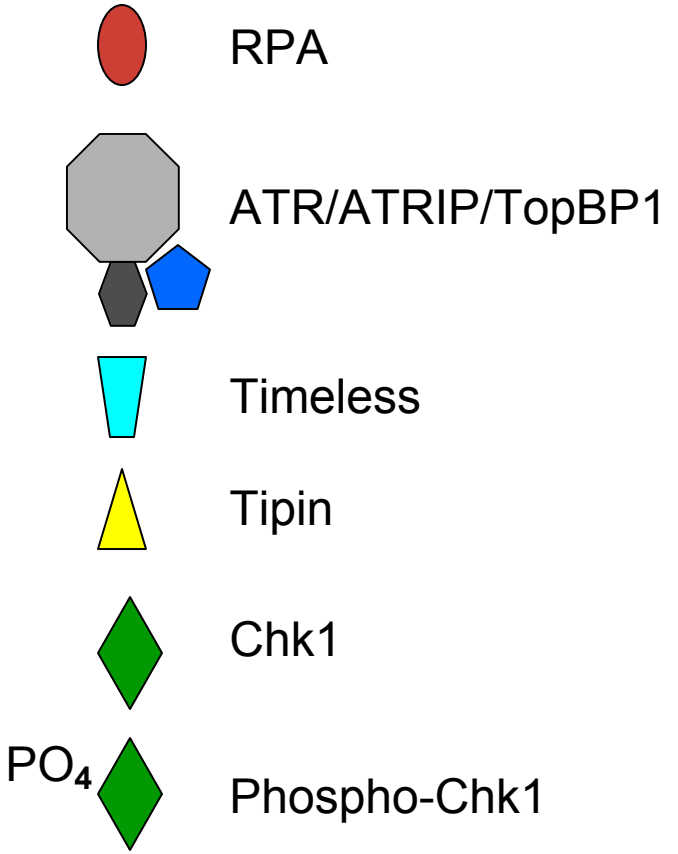
Knockdown of Tipin reversed the UVC-induced inhibition of DNA chain elongation indicating that the inhibition of chain elongation includes active checkpoint signaling (see Wang et al. (2004) *Nucl. Acids Res*)

Tim/Tipin complex brings Chk1 to ATR/ATRIP/TopBP1 at stalled replication forks

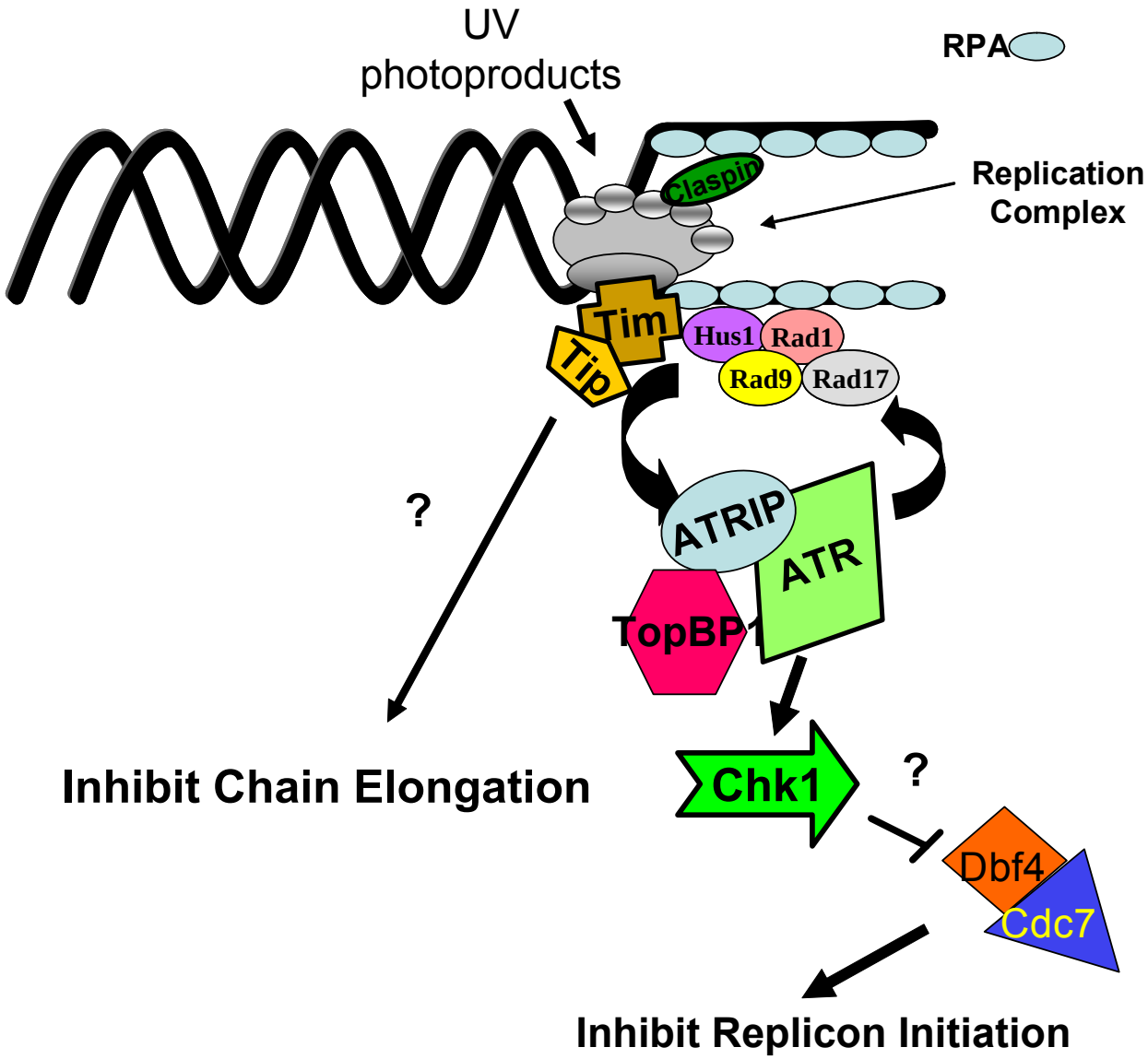


RPA loads Tipin onto DNA; this may inhibit DNA chain elongation





Intra-S Checkpoint Response to UVC



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- Hisao Masai
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