Role of p31^{COMET} Phosphorylation in Regulating the Mitotic Checkpoint Silencing in Human Cancer Cells

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▪ Background ▪

- The transition from metaphase to anaphase is tightly regulated by the spindle assembly checkpoint (SAC).
- SAC activation causes the inhibition of the anaphase promoting complex (APC) by a protein complex called MCC.
- p31^{comet} is a protein complex that assists in the silencing of the SAC and can promote aneuploidy through its collaboration with Mad2.
- Phosphorylation of the p31^{comet} weakens its interactions with Mad2.
- Conflictingly, only about half the number of p31^{comet} are phosphorylated when SAC is turned on as a result of nocodazole treatment HeLa cells.

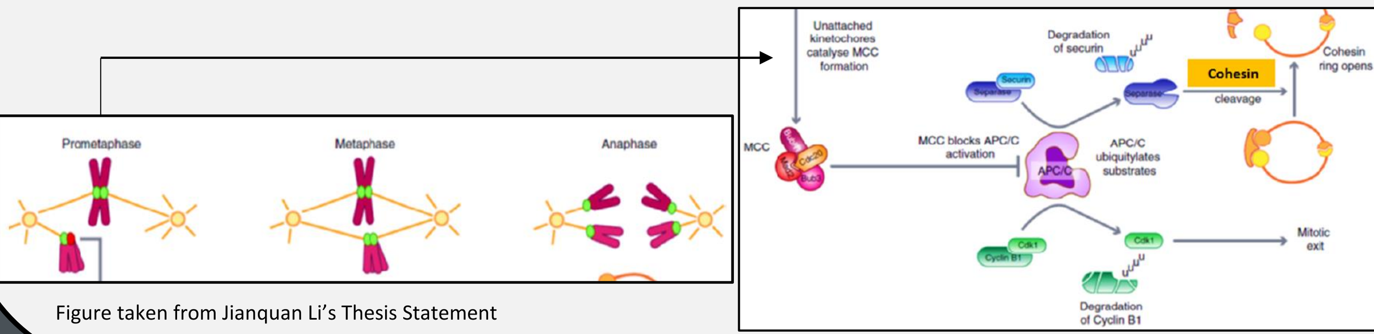


Figure taken from Jianquan Li's Thesis Statement

▪ Aims ▪

- To use an mGFP-p31 fusion protein to investigate the role of phosphorylation of p31^{comet} in the SAC pathway.
- Make pCMV-AN-p31^{comet} –mGFP plasmid DNA to express GFP tagged at the C terminus of p31^{comet} with the aim of producing a sequenced p31^{comet} containing flanking restriction sites.

▪ Methods ▪

HeLa cells were cultured and maintained in penicillin containing DMEM medium, and they were split every 2-3 days once 65-75% confluence was reached.

⇩

After splitting the cells, they were moved to an incubator at 37°C which was supplied with 5% CO_2.

⇩

Cells went through protein purification with 4 kinase inhibitors, DNAse and RNAse.

⇩

Samples were then run on a 10% protein gel.

⇩

Samples were transferred to a membrane from the protein gel through western blotting.

⇩

Phosphorylated and non-phosphorylated p31^{comet} was then detected in the scanned membranes.

Figure A –

IPTG is a drug to induce p31 in the bacterial plasmid. We have tried different concentrations of it to find out which concentration can induce the most p31^{comet}.

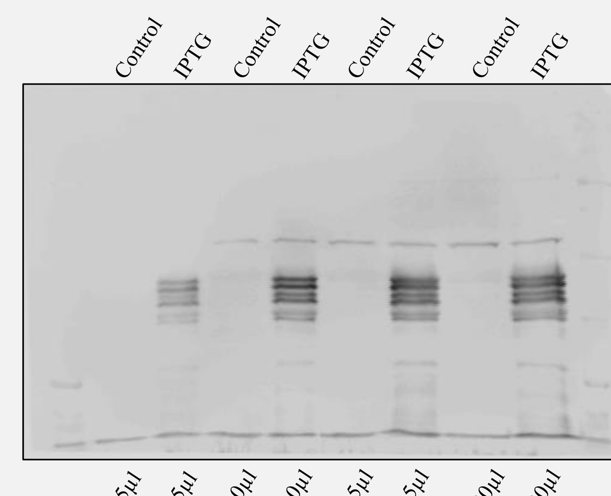


Figure B –

How p31^{comet} phosphorylation can be detected on phos-tag gel.

Lane 1 - Control;
Lane 2 - Hela cells treated with Nocodazole which arrests cells in mitosis;
Lane 3 - samples in Lane 2 treated with lambda Phosphatase, which could dephosphorylate p31.
GAPDH is a loading control; **Histon3S10** shows which stages the cells are.
The upper band in Nocodazole treated cells is the phosphorylation form of p31.
Therefore, about half of the p31 is phosphorylated in mitosis.

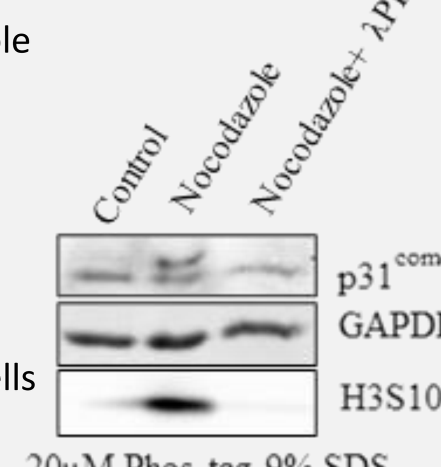


Figure C -

- We tested whether his-p31 can be phosphorylated in Hela extract on phos-tag gel (a gel to identify phosphorylation of p31).
- We expected that if the His-p31 can be phosphorylated, the band of His-p31 should be higher than the non-phosphorylated ones.
- **Lane 1 -** His-p31+Hela extract for 1 hour;
- **Lane 2 -** His-p31+Hela extract +ATP for 30 minus;
- **Lane 3 -** His-p31+Hela extract +ATP for 1 hour;
- The left panel was stained with a p31 antibody, which showed two bands but no migration differences.
- The right panel was stained with a His antibody, which also showed no differences.
- Therefore, maybe the induced His-p31 is not the same as the endogenous p31, or His-p31 cannot be phosphorylated in Hela extracts.

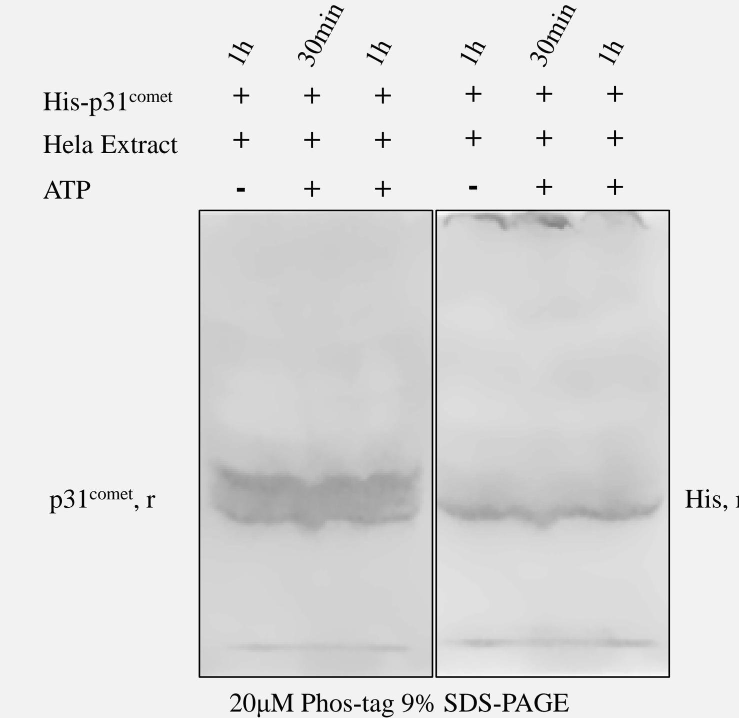
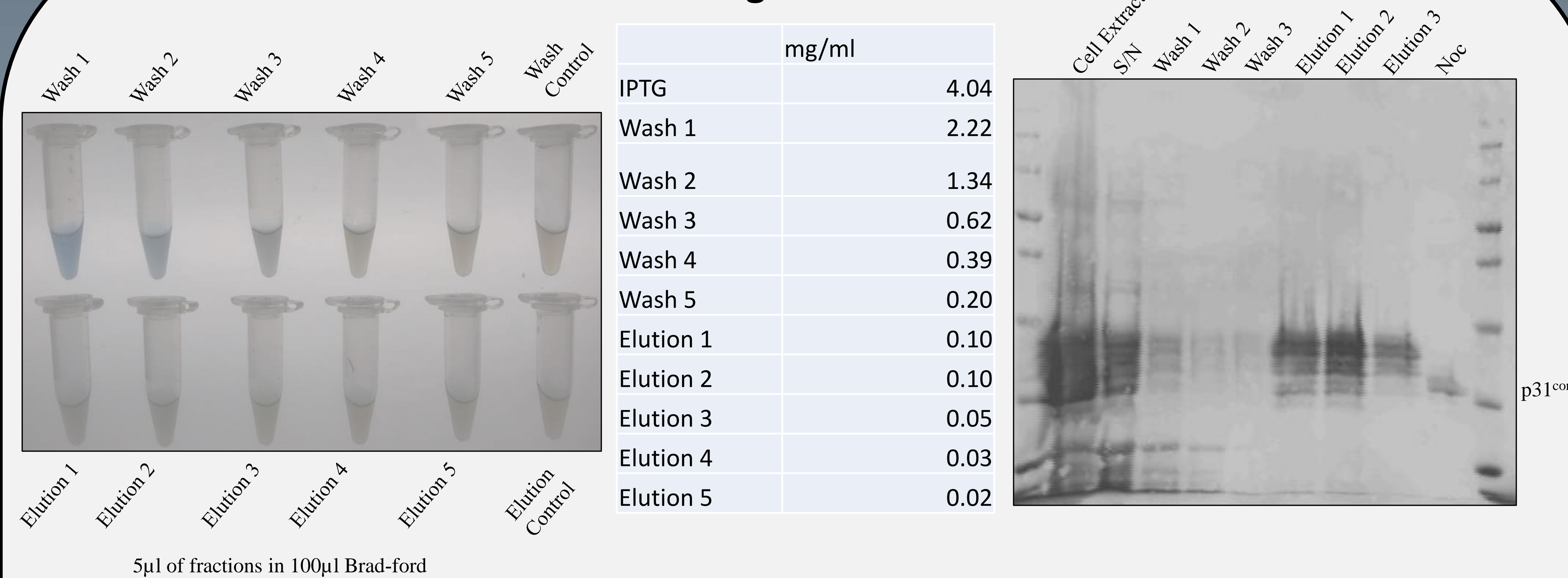


Figure D -



	mg/ml
IPTG	4.04
Wash 1	2.22
Wash 2	1.34
Wash 3	0.62
Wash 4	0.39
Wash 5	0.20
Elution 1	0.10
Elution 2	0.10
Elution 3	0.05
Elution 4	0.03
Elution 5	0.02

- **(Left Picture)** – The darker the blue-ish color, the more proteins that are present.
  - First row is washed by wash buffer, which gets rid of the non-specific proteins.
  - Second row is washed with elution buffer which breaks bonds between the antibody and the beads.
- **(Middle Picture)** - A table denoting the amount of p31^{comet} protein left after every wash.
  - IPTG is used as a control
- **(Right Picture)** - The samples on the left picture were run on a protein gel and these are the results that were produced.
  - The concentration of protein that is seen increases in "Elution 1" because the elution buffer is 10x more concentrated with imidazole than the wash buffer.
  - After all the washes we were able to purify the p31^{comet} from all the other proteins.

▪ Discussion ▪

- The level of p31^{comet} is low during interphase, but it starts to increase in prophase and reaches very high level at prometaphase and anaphase.
- P31^{comet} is localized mainly in the nucleus and somewhat around the nuclear envelope during prophase.
- After the nuclear envelope breaks down, p31^{comet} gets distributed throughout the whole cell.
- The levels of p31^{comet} is low during interphase and high during metaphase and prometaphase and then decreases again in telophase were substantiated by western blot results.
  - Suggesting that p31^{comet} levels are cell cycle regulated.

Figure E – Western Blot Result of elevated p31^{comet} in HeLa Cells After Nocodazole Treatment

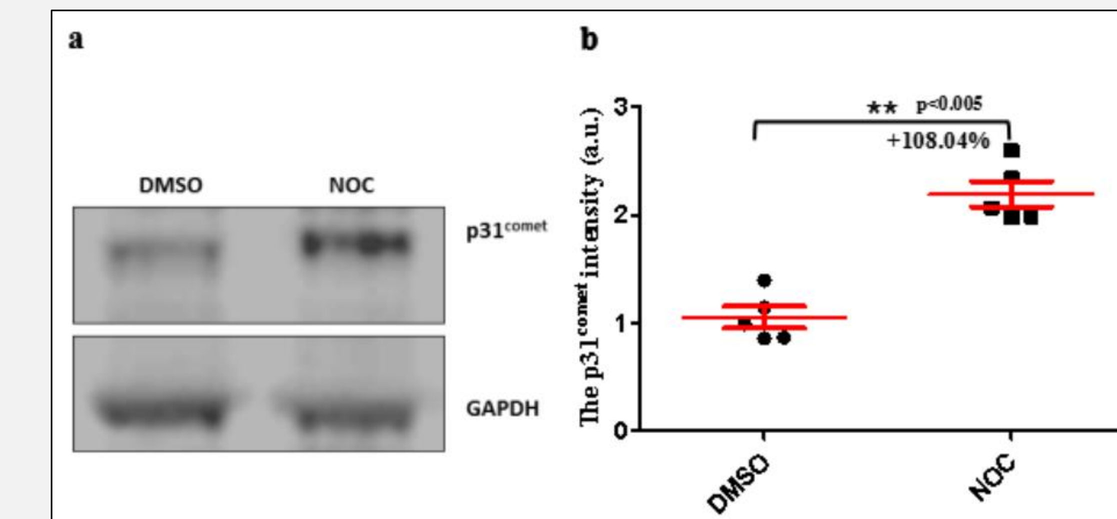


Figure taken from Jianquan Li's Thesis Statement

- After nocodazole treatment, high level of p31^{comet} was detected during prometaphase.
- The high accumulation of the protein is caused by new protein synthesis.
  - We know this because, after nocodazole treatment, increased level of p31^{comet} can be stopped by treating the cells with cycloheximide.
- The decline of p31^{comet} from cells that are released from nocodazole treatment can be prevented by MG132, but not leupeptin.
  - This p31^{comet} degradation is probably mediated by the ubiquitin-proteasome pathway.
- p31^{comet} contains 2 degradation motifs which are the D-box and KEN-box.
  - These are signature motifs that are targeted by the APC/C E3 ligase dependent proteolysis pathway.
- This suggested that if we inhibit the APC/C ligase pathway we would get an increased amount of p31 but unfortunately it was not evident in the results.

▪ Conclusion ▪

- To sum it all up, more experiments need to take place to see how the APC/C contributes to p31^{comet} degradation as they decline after nocodazole treatment release.
- The results also depict that the level of p31^{comet} increases after cullin-1 siRNA treatment.
  - This proposes that SCF E3 ligase ubiquitin – mediated proteolysis might be the contributing factor in the degradation of p31^{comet} as Cullin-1 is the essential core protein of SCF E3 ligase.
- Therefore, the degradation process of p31^{comet} needs to be further investigated.

▪ Acknowledgments ▪

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