Regulation of Aurora A activity during checkpoint recovery

Yan Zhou

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Biology Education Centre, Uppsala University, and Cell and Molecular Biology Department at Karolinska Institutet
Supervisors: Assist. Prof. Arne Lindqvist and Doctor student, M.Sc. Elvira Hukasova
External opponent: Ph.D Himjyot Jaiswal
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Verifying the setup by IF quantification on auto-phosphorylated AurA

A new setup to study Aurora A activation

A setup is established on western blot to study AurA phosphorylation in checkpoint recovery

Intracellular calcium release may regulate AurA activation

The role of calcium levels for AurA phosphorylation and Plk1 activity during checkpoint recovery

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Materials and Methods

Antibodies and reagents

Cell line and cell culture

Cell synchronization

Western blotting

Immunofluorescence (IF)

Live cell imaging

Acknowledgements
Abstract

Cell division requires accurate DNA replication and cells develop checkpoint mechanisms to ensure the correct passage of the genetic material. Cells arrest by a checkpoint when DNA damage is found. After the checkpoint is silenced, the cell cycle can be resumed. Polo-like kinase 1 (Plk1) and Aurora A kinase (AurA) are both important regulators for checkpoint recovery. The question how AurA is activated was studied by many researchers, but the exact mechanism stays unclear.

We developed a new setup to study AurA activation during checkpoint recovery. Quantitative immunofluorescence of fixed cells as well as a FRET probe that monitors Plk1 activity in time-lapse filming were applied in this study as indirect readouts of Aurora A activation. The result suggests that a Plk1-AurA feedback loop exists during checkpoint recovery. It can also be concluded that the inhibition of Cdk1 reduces Plk1 and AurA activity during checkpoint recovery. We also investigated the effect of calcium interfering drugs on AurA activation but no conclusive result was obtained.

Keywords: AurA activation, G2 checkpoint, checkpoint recovery, Time-lapse FRET microscopy.
Introduction

G2 /M checkpoint and Checkpoint recovery

Cell cycle is the process that allows a cell to divide. It starts from the end of the latest mitosis (M), through a comparably long interphase which includes Gap 1 (G1), Synthesis (S), Gap 2 (G2) to the next M phase. In G1 phase cells start to grow and prepare for DNA duplication; in S phase DNA is replicated; in G2 phase cells continue to; in the end, M phase allows cells to divide (Cooper 2000).

Cells have developed complex mechanisms to ensure that mitosis processes correctly, and many kinases are involved in mitosis. Well-known cell cycle regulators are cyclin-dependent kinase complexes (cyclin-CDK), and each complex is formed with two proteins, cyclin and CDK. Each cyclin-CDK activity peaks in different stages of the cell cycle. The most important regulator for entering mitosis is cyclin B-CDK1. In the end of G2 phase, it is activated by the phosphatase cell division cycle25 (Cdc25) and then triggers the event of entering mitosis (Ford 1999). Other cyclin-CDK complexes are involved in cell cycle regulation. Cyclin A-CDK2 is required in S phase and it is reported that the cyclin A-CDK2 regulates Cdk1 and Cdc25 phosphatases (Shree 2004). Cyclin D-CDK4 and cyclin D–CDK6 activity peak during the G1/S transition and they are key regulators of the G1/S transition (Hunter 1994). Cyclin E-CDK2 activity peaks in G1 and G1/S transition (Hinds 1992).

Due to the complexity of the genome in a eukaryotic cell, the consequence of a small mistake in DNA replication can be fatal to the cell. To ensure that the genetic material is correctly passed to the next generation, the cell develops a special mechanism called checkpoint to ensure an accurate completion of each cell cycle phase. Currently four checkpoints have been identified in the whole cell cycle, G1/S, G2/M, intra-S and spindle assembly checkpoint, which all function in different stages of the cell cycle. In this study we focus on the G2/M checkpoint (Lindqvist 2009).

Before entry into mitosis, the G2 checkpoint controls if damaged DNA is present (Kastan 2004). With DNA damage, the cell arrests in G2 and cannot enter mitosis until the DNA damage is repaired. When the cell finds that DNA double-strand is broken, Ataxia telangiectasia mutated (ATM) and Rad3-related kinase (ATR) are activated, and they target Chk2 and Chk1, respectively. Chk1 and Chk2 both inhibit Cdc25 phosphorylation, and hence, inhibit the de-phosphorylation of CDK so that the cell cannot enter mitosis. In this way, cells are forced to stay in G2 without entering mitosis.

Checkpoint recovery is a process when a checkpoint is silenced and the cell cycle can be resumed. When cells are arrested by a checkpoint, entering mitosis does not become possible until checkpoint signaling kinases (Chk1 and Chk2) are deactivated (Bartek 2003). The cell cycle is regulated differently during checkpoint recovery compared to unperturbed mitotic entry. Specifically, Aurora A and Plk1 are essential for checkpoint recovery but not for unperturbed mitotic entry (Lindqvist 2009).
Aurora A activation

Aurora A (AurA) is a serine/threonine kinase which is a key regulator for mitotic entry, activated before mitosis (Hannak 2001). It colocalizes on centrosomes together with γ-tubulin during G2 phase and associates with spindle poles (Carmena 2009). AurA localizes to centrosomes in the end of S phase. In G2 and M phase, the concentration of AurA is highest at the centrosome (Fukuda 2005), and the activity of AurA keeps high until the early G1 phase of the next cell cycle when it will be degraded (Hannak 2001).

Several studies have shown different regulators of AurA activation. The first identified and the most well studied AurA co-factor is Targeting protein for Xklp2 (TPX2). The binding of TPX2 to AurA, changes the conformation of AurA, and protects the threonine 288 residue on AurA to form a restricted access for phosphatase (the function of phosphatase is to reverse the phosphorylation of kinases) and provides a better binding conformation of AurA for other functional substrate (Bayliss 2003). TPX2 is the key structure to recruit AurA to spindle microtubules (MT) (De luca 2008, Kufer 2002). Another AurA activator is Human Enhancer of Filamentation 1 (HEF1) which colocalize with AurA on centrosomes in G2 phase, and it activates AurA (Pugacheva 2005). It was shown that enhanced endogenous calcium signal rapidly promotes AurA activation, both in vivo and in vitro without requiring of secondary messengers (Plotnikova 2010). Calcium-dependent calmodulin (CaM) binds to different motifs on AurA to regulate AurA activation, and two primary regions in AurA N- and C- termini were shown to allow the CaM-AurA interaction (Plotnikova 2010). The general opinion is that phosphorylation of threonine 288 (T288) is the key to activate AurA (Walter 2000). Nucleophosmin/B23 (NPM) colocalize with AurA on centrosomes in G2 and coimmunoprecipitate with AurA. In addition to phosphorylation of threonine 288, which is the most well recognized AurA auto-phosphorylation site, NPM promoted AurA phosphorylation on Serine 89. In conclusion, NPM was shown to activate AurA at centrosomes by an alternative pathway in G2 phase where AurA activity starts (Roboutier 2012).

Release of calcium ions from intracellular endoplasmic reticulum was reported to be able to activate AurA without involving second messengers (Plotnikova 2010). The authors indicated that activation of AurA is regulated by calcium dependent calmodulin (CaM), which binds to N- and C- termini of AurA. Calmodulin is reported to induce dimerization or oligomerization in the presence of calcium (just as Centrosomal protein 192 (Cep192), which is the co-factor of AurA to promote the oligomerization of AurA may promote auto-phosphorylation (Joukov 2010)), thus it is possible that increased intracellular calcium levels would bring Aurora A molecules close to each other, which in turn would enable them to phosphorylate and therefore activate each other.

However, until now only one article demonstrated that increasing cytoplasmic calcium levels by treating cells with thapsigargin (calcium uptake inhibitor) can regulate AurA activation without secondary messengers. Moreover, a treatment with BAPTA-AM, a calcium chelator
drug, reduces AurA T288 auto-phosphorylation. To be noticed, the cells used in this study overexpress AurA (Plotnikova 2010) and no cell cycle connection is illustrated in the paper.

Not only one, but several AurA activators were reported to regulate AurA activation in different residues of AurA in G2 on centrosomes, and the explanation could be that interfering with AurA localization on centrosomes will eventually lead to interference with AurA activation. The complex regulator network of AurA activation in checkpoint recovery, until now, is not clear (Macůrek 2009). In this study, we initially want to study whether calcium signal is an alternative pathway to activate AurA in checkpoint recovery.

**Kinases regulate by AurA in mitotic entry**

It is reported that the target of AurA for mitotic entry is Polo-like kinase 1 (Plk1), which is a serine/threonine kinase, locates in centrosomes. AurA targets Plk1 to promote mitotic entry (Macůrek 2008). Its well-studied function in cell cycle includes the regulation of centrosome maturation and other mitotic activities (Petronczski 2008).

CDC25B belongs to CDC25 family, and it activates CDC2 which is required for entering mitosis. It stays in cytoplasm during G2 phase, and relocates to nuclear when the cell enters mitosis (Morgen 2007). CDC25B is activated by AurA in centrosomes to promote G2/M transition (Dutertre 2004).

Aurora B (AurB) is a serine/threonine kinase with similar molecular structure as AurA but different functions. Unlike AurA, AurB localizes first in chromosomes in prophase and then move to centromere in prometaphase and metaphase (Adams 2001). Its activity peaks from metaphase till the end of mitosis (Fu 2007). Recently, AurB has been reported to activate Plk1 in Drosophila and it is suggested that this pathway is conserved in human species (Carmena 2012).

**Förster Resonance Energy Transfer**

Förster Resonance Energy Transfer (FRET) is an energy transfer mechanism that happens between molecules of energy donor and acceptor (Truong 2001). The donor molecule in its excited state can transfer energy to the acceptor molecule when these two molecules are linked through dipole-dipole coupling (Helms 2007).

In this study a FRET-probe that monitors Plk1 activity was used. It consists of two fluorophores, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), that are joined by a linker sequence and a consensus sequence for Plk1. Plk1 can recognize this sequence and phosphorylate it. The phosphorylation causes a change in a localization of CFP and YFP (conformational change) which leads to a low resonance energy transfer between YFP and CFP (low FRET, color-coded as yellow) (Tramier 2006, Sahoo 2011).
Results

Intracellular calcium release may regulate AurA activation

To investigate whether calcium signaling can influence endogenous AurA activation from late G2 to early mitosis, where AurA has clear functions, we performed experiments in an osteosarcoma cancer cell line (U2OS). We used both synchronized mitotic cell population and unsynchronized cells that were treated with thapsigargin or BAPTA-AM; DMSO was used as a control. Results obtained from two independent experiments for a mitotic population were not consistent. In one experiment (Figure 1, Mitotic) BAPTA-AM addition reduced AurA T288 auto-phosphorylation in mitosis while addition of thapsigargin had no clear effect, indicating that calcium may be important to sustain AurA activity in mitosis. The second experiment (Figure 1, Mitotic*) showed that both calcium drugs had no evident effect on AurA phosphorylation. In the experiments with unsynchronized cells and G2 cells (Figure 1) perturbation of endogenous calcium signaling did not lead to a clear effect on AurA auto-phosphorylation. In both experiments, the phosphorylated AurA signal from unsynchronized cells was barely visible, probably reflecting that AurA activity peaks in mitosis. From the obtained western blot results it is hard to conclude how calcium signaling affects AurA activation in G2 and mitosis. There is a possibility that averaging of the cell population masks changes that happen on the individual cell level.

![Thapsi TH] BAPTA [-] DMSO [+]

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Figure 1 Perturbation of intracellular calcium level may affect AurA activation. U2OS cells were unsynchronized, or synchronized in G2/M phase by 2.5 mM thymidine for 24 hours and released for 12 hours with addition of 250ng/ml nocodazole. Cells were incubated for 15 minutes with 5 μM thapsigargin, 50 μM BAPTA-AM or 1 μM DMSO. Cells were lysed and analyzed by western blot using pAurA T288 antibody. Unit of molecular weight is kilodalton. * indicates that this result is obtained by repeated western blot.

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Since all approaches to study AurA activation by calcium signaling did not lead to conclusive results (initial immunofluorescence, IF, and time lapse FRET cell filming, data not shown), there was a need for a better set up that would allow for studying AurA activation in the early hours before mitosis.

To investigate how calcium signaling influences AurA activation during checkpoint recovery on a single cell level, U20S-cyclin B-eYFP cells were treated with etoposide (a topoisomerase II inhibitor, which leads to cell arrest with DNA damage in G2 phase) to introduce DNA damage to cells. Cells were then treated with the checkpoint kinase inhibitor caffeine to allow entry into mitosis and ensure an enrichment of cells in either late G2 or mitosis. Finally, cells were treated with a 15 minutes pulse of thapsigargin, BAPTA-AM, or DMSO (as a control). Cells were then fixed and examined by immunofluorescence, using antibodies against auto-phosphorylated AurA, γ-tubulin (centrosome marker) and DAPI (nuclear stain) (Figure 2). The U20S-CycB-eYFP cells express an endogenously tagged cyclin B, which was used as a cell cycle marker; in the G1 phase of the cell cycle cyclin B is almost not visible in a cell, while in early G2 phase it starts to appear in the cytoplasm but is excluded from the nucleus. In late G2 cyclin B enters the nucleus and in early mitosis cyclin B can be seen in the entire cell. As expected, T288 phosphorylated AurA could be detected mainly on centrosomes, with the strongest staining present in or shortly before mitosis (Figure 2). In the cells treated with BAPTA-AM (Figure 2), the auto-phosphorylated AurA signal in late G2 and M phase appears weaker than in control cells, indicating that depletion of intracellular calcium levels could influence AurA activation. Unfortunately, the auto-phosphorylated AurA (T288) signal after thapsigargin addition was not conclusive (data not shown). AurA auto-phosphorylation is rapidly increasing in late G2, making it very difficult to find comparable cells in an unsynchronized population. We therefore thought to find a situation where AurA is initially activated in a large proportion of the cells. Since AurA is essential for resumption of the cell cycle after recovery from a DNA-damage induced checkpoint in G2 (checkpoint recovery), we therefore reasoned that forced recovery from a DNA-damage checkpoint in G2 may lead to a synchronous activation of AurA and allow for testing of the functional consequences of calcium perturbation on checkpoint recovery.
Figure 2 Intracellular calcium release may affect AurA activation. U2OS-CycB-eYFP cells were cultured with 1 µM etoposide for 16 hours to introduce DNA damage, with 8 hours addition of 5 mM caffeine. Cells were incubated for 15 minutes with 5µM thapsigargin, 50 µM BAPTA-AM or 1 µM DMSO. Cells were fixed and investigated by immunofluorescence using antibodies for γ-tubulin, pAurA T288 and nuclear stain DAPI.

A setup is established on western blot to study AurA phosphorylation during checkpoint recovery

With checkpoint inhibitors treatment after DNA damage occurs, cells start to enter mitosis. To obtain a reliable set up during this mitotic entry period, and to study the effect of fast calcium signaling on AurA activation, we first decided to perform the experiment on western blot. U2OS cells were synchronized with etoposide. After introducing DNA damage with etoposide, cells were forced into mitosis with UCNO1 or caffeine. Samples from different time points were collected (Figure 3). Caffeine is an ATM/ATR kinase inhibitor, while UCNO1 is a Chk1 inhibitor. When DNA damage occurs, cells activate ATM/ATR kinases, which in turn activate Chk1, to stop the cell cycle and repair the damaged DNA (Poehlmann 2011). Since UCNO1 inhibits Chk1, which is downstream of ATM/ATR, cells should enter mitosis earlier with UCNO1 treatment than after caffeine treatment. Mitotic cells were collected, lysed and analyzed with western blot using antibodies against phosphorylated Histone H3 and auto-phosphorylated AurA. Phosphorylated histone H3 signal represents the amount of mitotic cells and auto-phosphorylated AurA signal represents the AurA phosphorylation at T288. From Figure 3, it can be seen that the phosphorylated Histone H3 signal increases when the drug treatment time increases, indicating that more cells enter mitosis. The auto-phosphorylated AurA levels also increase proportionally to the amount of mitotic cell. In UCNO1 treatment mitotic cells start to accumulate from the second hour, while in caffeine treatment the accumulation begins at the forth hour. Therefore, it can be concluded that 40 hours etoposide treatment followed by 2 hours UCNO1 is the setup where AurA activation can be monitored.
Figure 3 Western blot setup to study kinase activity before mitotic entry and in checkpoint recovery. DNA damage was introduced to U2OS cells by 2 µM etoposide for 40 hours. Cells were pushed into mitosis by adding 5 mM caffeine or 3 µM UCNO1. 250 ng/ml nocodazole were added to the cells to arrest them in M phase. Cells were collected, lysed and analyzed by western blot, using antibodies against pAurA (T288) and pHiston H3. The unit of molecular weight is kilodalton.

Verification of the setup by immunofluorescence quantification on auto-phosphorylated AurA

To verify the efficiency of the setup, we performed IF experiment on U2OS cells followed by quantification based on auto-phosphorylated AurA and γ-tubulin fluorescence signal intensity. Figure 4A shows representative G2 cells during checkpoint recovery or with only DNA damage; it can be observed that with the effect of UCNO1, AurA has stronger signal on centrosomes. To quantify the signal on the centrosomes, we measured the intensity of the strongest pixel in the γ-tubulin channel and estimated the background by subtracting the median pixel value in a 5 x 5 pixel rectangle surrounding the centrosome (Figure 4B), the same measurements were performed in the phosphorylated AurA channel. After measuring the centrosomal intensities in U2OS cells, higher AurA phosphorylation was found in cells treated with UCNO1, compared to the control (Figure 4C, lower part), in which all the cells appeared to have low AurA phosphorylation. From Figure 4 it can be concluded that after UCNO1 addition cells have a stronger signal of pT288-AurA on the centrosomes. Therefore it is possible to use the setup with etoposide induced DNA damage followed by 2 hours UCNO1 to study AurA activation.
Figure 4 Verification of the mitotic entry setup. IF quantification of auto-phosphorylated AurA. A. An example shows representative cells from two experimental conditions: etoposide induced DNA damage only, or with DNA damage and then forced into mitosis by UCNO1. Cells were fixed and investigated by immunofluorescence using antibodies for γ-tubulin, pT288-AurA and nuclear stain DAPI; B. An example shows how the fluorescence intensity is measured. Signal intensity (lightest dots marked by yellow rectangle) was measured with Image J, and the data were collected and shown in the form of Figure C; C. U2OS cells were synchronized by DNA damage with 2 µM etoposide for 40 hours. 3 µM UCNO1 was added to the cells for 2 hours to force them enter mitosis. Control cells were treated with 2 µM etoposide only. Cells were fixed and investigated by immunofluorescence using antibodies for γ-tubulin, pT288-AurA and nuclear stain DAPI. The left figure is a representative picture of the cells in each condition. The graph shows IF quantification of auto-phosphorylated AurA in both condition.

Testing the checkpoint recovery setup on live cells with the addition of calcium interfering drugs

To study the effect of calcium signaling on checkpoint recovery in live cells, we applied fluorescence live cell microscopy. U2OS cells stably expressing a FRET-based probe fused to histone H2B (here not to detect FRET, but to identify mitotic cells) were arrested in G2/M phase by addition of DNA damaging agent etoposide. Caffeine or UCNO1 were used to inhibit DNA-damage signaling, and cells were followed by time-lapse fluorescence microscopy. In the control group, cells were treated with only caffeine or UCNO1; in the
experimental group, thapsigargin, BAPTA-AM (Plotnikova 2010) or BI 2536 (Plk1 inhibitor), which should inhibit checkpoint recovery (Van Vugt 2004), were added together with caffeine or UCNO1 to the cells. As shown in Figure 5, both thapsigargin and BAPTA-AM treated cells did not enter mitosis. The fact that most cells died under these conditions indicates that calcium signaling is essential for the cell survival and cells cannot tolerate a long-term perturbation of intracellular calcium levels, both after caffeine and UCNO1 addition. After addition of UCNO1 (Control in Figure 5B, lower graph), cells start to enter mitosis in the second hour of filming, which is faster compared to the eighth hour in caffeine control, (Figure 5A, lower graph), consistent with the western blot experiment in Figure 3. Since a long-term exposure to the calcium drugs was fatal for the cells, the result is not supportive enough to conclude that calcium signaling has an effect on activation of AurA.

A

Caffeine

B

UCNO1

Figure 5 Perturbation of calcium signaling is fatal to cell survival. U20S cells expressing H2B-Plk1-FRET probe were treated with 1µM etoposide for 24 hours. Cells were forced into mitosis by addition of 5 mM caffeine or 3 µM UCNO1, 50 mM BAPTA-AM, 5 µM thapsigargin or 10 mM BI2536 were added to the cells separately. Cells were filmed at 37°C for 15 hours.

A comparison of the effects of NCS and etoposide on the dynamics of checkpoint recovery

The observation that few mitotic cells can be detected after UCNO1 treatment lead to a suspicion that etoposide treatment may be too harsh for cells to recover from DNA damage
(see Figure 5, few cells enter mitosis). Live cell data from other group members indicated that checkpoint recovery after giving DNA-damage with Neocarzinostatin (NCS), a radiomimetic drug, may be more efficient compared to etoposide (data not shown). Therefore, we chose to perform parallel experiments with either NCS or etoposide to induce DNA damage, followed with UCNO1 to force cells into mitosis, and compare the effect of these two drugs in the checkpoint recovery setup.

To determine if phosphorylated AurA can be detected with both setups, we repeated the analysis performed in Figure 4. The result obtained from fixed cells showed that both setups are effective, since after inducing DNA damage (control group), cells were in interphase with low levels of phosphorylated AurA on centrosomes. Moreover, after addition of UCNO1, an increase of phosphorylated AurA can be detected on the centrosomes in both setups (Figure 6A). We then wanted to test if both setups are suitable for use in live cell experiments.

A newly published protocol developed in the lab made it possible to monitor kinase activity throughout the cell cycle (including our interest checkpoint recovery) by analyzing FRET ratio change (Hukasova 2012). For our experiments we used an available FRET-probe for Plk1 activity (Macůrek 2008). The role of Plk1 is important, since it is essential for checkpoint recovery from a DNA-damage induced arrest in G2 (van Vugt 2004). Moreover, Plk1 is a direct target of AurA to promote checkpoint recovery (Macůrek 2008), indicating that studying Plk1 activity could function as an indirect measurement of AurA activation. In total, applying the protocol by Hukasova et al. to study Plk1 activity in checkpoint recovery appears interesting, hence we first tested live cell imaging with U2OS cells stably expressing a FRET-based probe that responds to Plk1 activity (U2OS-Plk1-FRET). We treated U2OS-Plk1-FRET cells with either etoposide or NCS and monitored the FRET change of single cells after UCNO1 addition. Figure 6B shows an example of a time-lapse sequence of a single cell showing the inverted false-colored FRET-ratio. Plk1 activation occurs gradually before a cell enters mitosis. In mitosis Plk1 is fully active, which is reflected through yellow false coloring of the cell. To study Plk1 activity just before cells enter mitosis, U2OS-H2B-Plk1 cells were treated with NCS (24 hours) or etoposide (40 hours) and forced into mitosis. The filming started 2 hours after UCNO1 addition. The obtained result (Figure 6C, left) shows that after NCS treatment cells enter mitosis earlier (during the first 2 hours after UCNO1 addition) and generally more cells enter mitosis, possibly due to a better synchronization. In etoposide treatment cells enter mitosis later (approx. 5 hours after UCNO1 addition) and fewer cells enter mitosis (Figure 6C, right). Since a checkpoint recovery set up with the use of NCS appears more time-efficient and apparently is less harmful to the cells, more cells enter mitosis quite synchronously at early time points, we chose to use NCS and not etoposide for the next experiments.
Figure 6 Verification of the mitotic entry set up by time-lapse imaging of Plk1-FRET probe cells. A. U2OS cells were treated for 24 hours with 1 µM NCS or 2 µM etoposide to introduce DNA damage. Cells were forced to enter mitosis by UCNO1 addition for 2 hours. Cells were investigated by IF using pAurA and γ-tubulin. The figure shows the fluorescence intensity of pAurA and γ-tubulin signals by addition of two drugs, NCS and etoposide. B. The figure shows an example of inverted false-colored FRET ratio of a single cell passing through mitosis. The scale 1 to 3 next to the right part of Figure 6 shows the FRET ratio scale, which means, a high FRET (violet) is when probe is not phosphorylated, whereas low FRET (yellow) refers to a phosphorylated probe. C. U2OS cells expressing Plk1-FRET probe were treated for 24 hours with 1 µM NCS or 2 µM etoposide to introduce DNA damage. Cells were forced to enter mitosis by UCNO1 (UCNO1 were cultured with cells in the entire filming period and the filming started 2 hours after UCNO1 addition) and were filmed in 37°C for 9 hours. In control, cells were only subjected to DNA damage. FRET ratio change curve shows how FRET ratio value changes through the whole filming, to get a clearer vision of mitotic cells, part of FRET ratio curves were erased after cells enter mitosis.

Inhibition of Cdk1 activity reduces Plk1 activity in checkpoint recovery

Since the study using drugs affecting calcium levels were impractical (with long term effect of calcium drugs, cells all died in the end of filming), we decided to use the setup to investigate which other cell cycle regulators could affect AurA activation during checkpoint recovery.
Two major kinases that are activated during mitosis are cyclin dependent kinase 1 (Cdk1) and Aurora B (Medema 2011). To study whether reducing Cdk1 and Aurora B activity can affect Plk1 activity during checkpoint recovery we performed live cell experiments and illustrated the result with FRET ratio change analysis and cumulative index of mitotic cells. Here RO 3306 is a Cdk1 inhibitor (Vassilev 2006) and ZM 447439 is an Aurora B inhibitor (Kasuboski 2011). Figure 7A shows a comparison between FRET ratios observed for different treatments: UCNO1 with RO 3306, UCNO1 with ZM 447439 and UCNO1. It demonstrates that the reduction of Cdk1 activity also inhibited Plk1 activity, while inhibition of Aurora B activity did not affect slightly enhanced Plk1 activity.

Figure 7B shows that the reduction of Aurora B activity did not initially affect mitotic entry; the number of mitotic cells during the first 2 hours was almost the same as for NCS UCNO1 control, later the number of mitotic cells slightly decreased. The result indicates that Aurora B might affect checkpoint recovery but seems not to influence Plk1 activity; reduction of Cdk1 activity affects mitotic entry much stronger, which indicates that Cdk1 could be an upstream regulator of Plk1.
Inhibit ion of Cdk1 activity influence Plk1 activity during checkpoint recovery. U2OS-FRET-Plk1 cells were treated with 4 nM NCS to bring DNA-damage to cell, followed by UCNO1 treatment. At the same time of UCNO1 addition, Cdk1 inhibitor RO 3306 and Aurora B inhibitor ZM 447439 were added to the experimental groups, while control groups were without drugs (with only DNA damage or only with cells which were forced into mitosis). Cells were filmed for 10 hours starting when UCNO1 was added; in the figure above only the first 5 hours of filming are shown. A. Analysis of a FRET ratio change in various conditions. B. Analysis of cumulative index of mitotic cells. Each dot represents one mitotic cell. The graph shows how many cells enter mitosis at each time point.

Inhibition of Aurora B or Cdk1 activity influences AurA phosphorylation during checkpoint recovery

To study whether inhibition of Cdk1 and Aurora B activity affects AurA phosphorylation in checkpoint recovery, we performed fixed cell experiments and quantified the levels of phosphorylated AurA (T288) on centrosomes. Fluorescence intensity was analyzed in G2 cells. Figure 8 illustrates that inhibition of Cdk1 activity leads to a decreased amount of cells in which AurA phosphorylation is detectable. Nevertheless, when comparing fluorescence signal intensities on a single cell level, the pAurA signals in two cells in RO 3306 treatment are slightly higher compared to the UCNO1 treatment alone. Inhibition of Aurora B activity enhanced AurA phosphorylation on a single cell level (comparing cells with UCNO1 and ZM 447439 treatment). Also reduced Aurora B activity promotes AurA phosphorylation, which can be observed in a larger cell population as compared to UCNO1 control. The conclusion from Figure 8 is that inhibition of Aurora B activity seems to promote AurA phosphorylation, and it is known that AurA activates Plk1 to promote checkpoint recovery (Macůrek 2008), hence, the hypothesis for the relationship between Aurora B and Plk1 can be made: inhibition of Aurora B activity may promote Plk1 activity. However it does not fit with the conclusion from Figure 7 that demonstrates that Aurora B inhibition does not affect Plk1 activity.
Figure 8 Inhibition of Aurora B or Cdk1 activity influence AurA phosphorylation during checkpoint recovery. U2OS cells were treated with 4 nM NCS to lead to DNA damage, followed by 2 hours UCNO1 treatment to force cells overcome the checkpoint and enter mitosis. At the time of UCNO1 addition, Cdk1 inhibitor and Aurora B inhibitor were added to the experimental groups, while control groups were without drugs (only with DNA damage or only with cells which were forced into mitosis). Cells were fixed with cold methanol and IF with auto-phosphorylated AurA and γ-tubulin antibodies was performed.

Inhibition of CDK activity reduces Plk1 activity during checkpoint recovery

To study whether inhibition of general cyclin dependent kinase (CDK) activity affects Plk1 activity during checkpoint recovery, live cell experiment was performed on U2OS-FRET-Plk1 cell. Results were obtained by FRET ratio analysis and quantification of cumulative index of mitotic cells. In this set of experiment, roscovitine is used as CDK inhibitor (Peng 2011), while BI 2536 (Lénárt 2007) is used as Plk1 inhibitor. As expected, addition of BI2536 decreased the FRET-ratio change (Figure 9A). Comparing the FRET ratios in Figure 9A it can be seen that the addition of roscovitine blocks the Plk1 activity more efficiently than BI 2536. This suggests that CDK activity is essential for Plk1 activation during checkpoint recovery. From Figure 9B, few cells enter mitosis after addition of both roscovitine and BI2536, indicating that both CDK activity and Plk1 activity are important for checkpoint recovery.
Figure 9 Inhibition of CDK activity influences Plk1 activity during checkpoint recovery. U2OS-FRET-Plk1 cells were treated with 4 nM NCS to bring DNA-damage to cells, followed by UCNO1 treatment. At the time of UCNO1 addition, CDK inhibitor roscovitine and Plk1 inhibitor BI 2536 were added to the experimental groups, while control groups were without drugs (only with DNA damage or only with cells which were forced into mitosis). Cells were filmed for 10 hours starting when UCNO1 was added; in the figure above only the first 5 hours of filming are shown. A. Analysis of a FRET ratio change in various conditions. B. Analysis of cumulative index of mitotic cells. Each dot represents one mitotic cell. The graph shows how many cells enter mitosis at each time point.
Inhibition of CDK or Plk1 activity reduces AurA phosphorylation during checkpoint recovery

To study whether inhibiting general CDKs and Plk1 activity can affect AurA activity during checkpoint recovery, an IF experiment was performed on U2OS cells. Plk1 is reported to be activated by AurA in checkpoint recovery (Macůrek 2008), and we want to confirm whether Plk1 can regulate AurA activation during checkpoint recovery after DNA-damage. Results were obtained by analyzing fluorescence intensities of phosphorylated AurA and γ-tubulin on centrosomes of cells subjected to DNA damage and subsequently treated with UCNO1. Inhibiting CDK or Plk1 activity completely blocks phosphorylated AurA fluorescence signal (Figure 10), indicating that CDK and Plk1 activity are necessary to induce AurA activity on centrosomes.

![Figure 10](image.png)

**Figure 10 Inhibition of CDK or Plk1 activity affects AurA phosphorylation during checkpoint recovery.** U2OS cells were treated with 4 nM NCS to lead to DNA damage, followed by 2 hours UCNO1 treatment to force cells exit checkpoint and enter mitosis. At the time of UCNO1 addition, roscovitine and BI 2536 were added to the experimental groups, while control groups were without drugs (only with DNA damage or only with cells which were forced into mitosis). Cells were fixed with cold methanol and analyzed in IF using antibodies for auto-phosphorylated AurA, γ-tubulin. Data for quantification were collected from synchronized cells. Cells with duplicated centrosomes were selected for quantification.

Interfering with intracellular calcium levels may inhibit Plk1 activity in checkpoint recovery

We next wondered if we could see an effect of temporal perturbation of calcium signaling with this setup. Apparently, cells do not survive under long-term interference of intracellular calcium, but we reasoned that we may see an effect on Plk1 activity or AurA auto-phosphorylation if we look shortly after addition of calcium interfering drugs. To study the
effect of altering intracellular calcium signaling on Plk1 activity during checkpoint recovery, live cell experiments on U2OS-Plk1-FRET cells were performed and the results were obtained with FRET ratio change analysis. Due to a lack of mitotic cells, cumulative index of mitotic cells is not shown in this series of experiment. Unfortunately, despite some initial Plk1 activation, cells treated with UCNO1 did not enter mitosis, making comparisons difficult. From figure 11, the addition of thapsigargin to cells (the graph marked with NCS UCNO1 Thapsigargin) showed enhanced Plk1 activity before thapsigargin addition, but after 15 minutes thapsigargin treatment Plk1 activity was turned off, the same with BAPTA-AM group where Plk1 activity appeared to be suppressed. Examining the 4 graphs in Figure 11, we found that the drug effect varied among experiments. For instance, if compare the first two hours of filming, the 3 conditions (except NCS control) were the same because all of them were treated with UCNO1 after NCA-induced DNA damage. The observation indicates that interfering with endogenous calcium level may reduce Plk1 level activity during checkpoint recovery, but in the future similar experiments need to be repeated and the drug effect variation should be avoided as much as possible.

Figure 11 Altering endogenous calcium signaling may affect Plk1 activity in checkpoint recovery. U2OS-FRET-Plk1 cells were treated with 4 nM NCS to bring DNA-damage to cell, followed by UCNO1 treatment throughout the entire filming period. Calcium drugs (thapsigargin and BAPTA-AM) were added after 1:45 hours filming for 15 minutes to the experimental groups, while in control groups cells were with only DNA damage or with DNA damage and UCNO1. Cells were filmed for 10 hours started when UCNO1 was added, however in the figure above, only the first 5 hours of filming were shown. The graph shows analysis of FRET ratio change in various conditions.
Interfering with intracellular calcium levels may inhibit AurA phosphorylation during checkpoint recovery

To study whether the perturbation of intracellular calcium levels can affect AurA activity during checkpoint recovery, we performed IF experiment in U2OS cells using NCS to induce DNA damage. UCNO1 was added to cells for 2 hours; calcium interfering drugs were added into cells for the last 15 minutes of UCNO1 treatment. Afterwards cells were fixed and analyzed by IF with fluorescence intensity quantification. The NCS control group had some cells with AurA phosphorylation, indicating that the DNA damage might not have been very efficient. The hypothesis is that we should observe enhanced pAurA signal upon thapsigargin treatment in comparison to the cells with only UCNO1 addition. Similarly, the BAPTA-AM treatment should weaken phosphorylated AurA signal intensity. However, the real observation is that the addition of both thapsigargin and BAPTA-AM slightly decreased the levels of pAurA (Figure 12). This indicates that the altering of endogenous calcium levels may affect AurA activity, but simply boosting intracellular calcium levels may not be sufficient to activate AurA during checkpoint recovery. Further experiments need to be performed to assess how calcium could regulate AurA activation in G2 phase.

Figure 12 Altering intracellular calcium signaling may influence AurA phosphorylation during checkpoint recovery. U2OS cells were treated with 4 nM NCS to lead to DNA damage, followed by 2 hours UCNO1 treatment to force cells exit checkpoint and enter mitosis. Calcium drugs (thapsigargin and BAPTA-AM) were added to the experimental groups after 1:45 hours of UCNO1 for 15 minutes, while control groups were with only DNA damaged cells or with DNA damaged cells and UCNO1. Cells then were fixed with cold methanol and processed by IF using antibodies for auto-phosphorylated AurA and γ-tubulin; the data for quantification were collected from synchronized cells in G2 /M phase. In the graph, some of pAurA fluorescence intensity, with NCS UCNO1 Thapsi, and NCS UNCO1 BAPTA conditions, were much higher than 100. However, to be able to compare with previous fluorescence intensity figures, the visible range of y-axis was set.
in between -20 to 100. In fact, some pAurA signal (fluorescence intensity larger than 100) were not shown in the figure above, but they were counted in the statistics analysis (shown as % numbers in Figure 12)

Discussion

A new setup to study Aurora A activation

As shown in Figure 1, it is difficult to study AurA phosphorylation with western blot performed on unsynchronized cells. This is because very few cells are in late G2 or mitosis, where Aurora A is phosphorylated. In a synchronized mitotic cell population a signal can be detected. However, it is difficult to synchronize cells well enough to have only late G2 and no mitotic cells. Since Aurora A phosphorylation is much stronger in mitosis compared to in G2, it is difficult to conclude whether the signal on western blot comes from the G2 cells. The checkpoint recovery setup in Figure 3 is therefore more suited to study the initial Aurora A activation, since AurA activity was observed to stay low before the addition of checkpoint inhibitors and was enhanced after treating with checkpoint inhibitors. The setup is simple with minimal time consumption, especially when 40 hours etoposide treatment was replaced with 24 hours NCS treatment.

Due to the limit of fixation and antibody choices, AurA and pAurA (T288) could not be studied in the same sample in one experiment. For future experiments, more suitable combinations of AurA and pAurA antibodies could be used to monitor un-phosphorylated and phosphorylated AurA, making it possible to correlate protein levels to phosphorylation.

In an ideal situation, all cells in the checkpoint recovery setup enter mitosis in the end. But in my experiments, the mitotic cell population is maximally 40-50%. The non-mitotic cell population may influence the results. Figure 7-10 showed that a variation on how well cells arrested exists when NCS was used to induce DNA damage to cells. Thus, timings and the percentage of cells entering mitosis cannot be directly compared between experiments. Instead, every experiment needs to be compared to its own controls.

Four adjusted methods were applied in this study to monitor AurA activation during checkpoint recovery. With the western blot setup described in Figure 3, the average phosphorylation in a population of cells can be monitored; the setup using immunofluorescence described in Figure 8 can be used to quantify mitotic kinases specially on centrosomes; time-lapse microscopy can be used to monitor functional effects as mitotic entry; in the end, analyzing FRET-Plk1 activity can be combined with time lapse microscopy to monitor AurA activity since Plk1 is the downstream target of AurA. Thus, by combining several methods, I can indirectly monitor Aurora A activation and the functional effect on checkpoint recovery.
The role of calcium levels for AurA phosphorylation and Plk1 activity during checkpoint recovery

It was reported that raising endogenous calcium levels can rapidly promote AurA activation in mammalian cells (Plotnikova 2010). However, whether calcium levels regulate endogenous Aurora A activity in G2 and mitosis stays unclear. The time lapse microscopy in Figure 5 confirmed the importance of intracellular calcium signals to cell survival, but the conclusion of whether disturbing calcium signaling regulates auto-phosphorylation of AurA is not clear. Since Aurora A activity was decreased after both enhancing and suppressing calcium levels, the decrease may be due to a general stress that blocked cell cycle progression and later induced cell death.

Mitotic entry kinases regulate AurA phosphorylation during checkpoint recovery

Aurora B is known as a protein that is mostly activated during mitosis to regulate kinetochore-microtubule attachment, and therefore, the spindle assembly checkpoint (Hauf 2003). Due to different functions and activation periods in the cell cycle, Aurora B is unlikely to be involved in checkpoint recovery, which is a process that occurs before mitosis. However, recent data has indicated that Aurora B may function together with AurA to activate Plk1 in G2 (Archambault 2012). The result from the Plk1-FRET analysis (Figure 7A) suggests that before cells enter mitosis, inhibiting Aurora B did not influence Plk1 activity and that the addition of ZM has no effect in G2 phase. Aurora B is most active at centrosomes near kinetochores after entering mitosis (Lampson 2004, Lampson 2011), while AurA is activated on centrosomes before mitosis (Portier 2007), suggesting that these two kinases have separate functions. However, as reported I detect phosphorylated Aurora A on centrosomes and Plk1 activity in the nucleus, where Aurora B is localized. Clearly more studies are needed to explain this paradox (Macůrek 2009). In Figure 8, the observation was that inhibiting Aurora B appeared to promote AurA phosphorylation, which does not fit into previous knowledge. The experiment may be repeated and if the result remains the same, this can be a hint that there may be some alternative regulation mechanism between AurA and Aurora B, and bioinformatics method may be applied to screen for the potential regulator.

From the result of Figure 7 to 10, it is clear that compared with inhibiting only Cdk1, inhibiting general CDK activity leads to larger reducing effect of AurA auto-phosphorylation and Plk1 activity. It suggests that in addition to Cdk1, other CDKs may also regulate AurA and Plk1 activity during checkpoint recovery. Even though Cdk2 has much lower activities than Cdk1 in G2 and M phases (Bashir 2005), it may still influence G2 /M progression, especially if Cdk1 activity is absent. However, the exact interplay between Cdk1 and Cdk2 is still lacking. The result in my study suggests that there is a possibility that Cdk1 and Cdk2 both regulate AurA in G2. An alternative explanation could be that Roscovitine inhibits Cdk1 more efficiently than RO-3306 in our experimental conditions. Future experiment plan is to investigate whether Cdk2 regulate AurA phosphorylation in checkpoint recovery. Selective Cdk2 inhibitor (such as SU 9516) (Lane 2001) may be utilized in new experiments (IF and
live cell filming) and the result from SU 9516 can be compared with the result from general CDK inhibitor Roscovitine and from Cdk1 inhibitor RO 3306, to see whether there will be difference by inhibiting different CDKs on Aurora A activation.

Previous research has shown that Plk1 acts as an upstream regulator of AurA (Hanisch 2006, Luca 2006). The conclusion of Figure 10 showed that Plk1 activity is necessary for Aurora A activation and positive regulates AurA activity on centrosomes, suggesting that Plk1 is an upstream regulator of AurA during checkpoint recovery. Compared with the conclusion in a previous study (Macůrek 2008) that AurA regulates Plk1 activity to accelerate checkpoint recovery, we can conclude that Plk1 could work as both upstream and downstream regulator of AurA during checkpoint recovery. Thus, a feedback loop may exist between Plk1 and AurA. To confirm the existence of such a feedback loop, further experiments should be performed.
## Materials and Methods

### Antibodies and reagents

#### Cell culture reagents

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<th>Company</th>
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</thead>
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<td>0.05% Trypsin EDTA</td>
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<td>Dulbecco’s modified eagles medium, DMEM +Glutamax, +4.5g/L D-Glucose, +Pyruvate (DMEM)</td>
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<td>Dulbecco’s Phosphate Buffered Saline, DPBS, -CaCl₂, -MgCl₂ (PBS)</td>
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<td>Hyclone®, Thermo Scientific™</td>
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#### Antibodies

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<tr>
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<td>Cell signaling Technology®</td>
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#### Drugs

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Cell culture

Human osteosarcoma U2OS and U2OS-Plk1-FRET cells were grown in DMEM with Glutamax supplemented with 6% FBS, 1% P/S. Cells were cultured in 37°C, 5% CO₂ in HERA Cell® 240i CO₂ incubator (Thermo®).

Before time-lapse imaging DMEM culture medium was replaced by Leibovitz’s L-15 medium and culture dishes were sealed with PARAFILM® M sealing film to block direct contact of cells with CO₂-abundant environment of the incubator.

Cell synchronization

U2OS cells were synchronized in G2 phase by treatment with 2.5 mM thymidine for 24 hours and released for 12 hours with addition of 250 ng/ml nocodazole.

Western blotting

Cells were collected from culture dishes, centrifuged for 5 minutes (700 rcf) and the supernatant was discarded. The pellet was lysed with Laemmli Sample Buffer (BIO-RAD®) with additional 2-Mercaptoethanol (SIGMA®) in 20:1 proportion. Usually cell pellet from a confluent 10 cm culture dish requires 50 µl of sample buffer to prepare a lysate. The lysate was incubated in 95 °C for 5 minutes (or longer time, if needed) and if not loaded on gel immediately frozen in -20°C for further use.

The samples were loaded 10-20 µl per well (depending on the size of the well, the concentration of the sample) in 4-15 % Mini-PROTEAN® TGX™ Gels (BIO-RAD®). Gel electrophoresis was performed in electrophoresis buffer (25 mM Tris, 192 mM Glycine + 0.1 % SDS, PH 8.3) initially 15 minutes at 90 V, to allow samples pass through the separation gel, and then 30-40 minutes at 120 V.

Immun-Blot® PVDF Membrane (BIO-RAD®) was used for protein transfer. PVDF membrane was activated for 5 minutes in methanol. Protein transfer was performed in transfer buffer (25 mM Tris, 192 mM Glycine with 20 % methanol, PH 8.3) at 120 V for 1 hour (alternatively, 30 V, overnight) until all bands were transferred from gel to membrane.

The membrane was blocked in blocking solution (Tris-Buffered Saline with 0.1% Tween 20 (TBST) and 5% Bovine serum albumin (BSA)), for 1 hour at room temperature (alternatively, overnight in 4°C).

Primary antibodies (dilution 1:1,500 to 1:2,000 in TBST) in blocking solution were incubated with membrane for 1 hour at room temperature (or overnight 4°C), and washed with TBST for 3 x 10 minutes.
Secondary antibodies (Bio-Rad® anti-rabbit HRP conjugate, 1:10,000) in blocking solution were incubated with membrane for maximum 1 hour at room temperature in dark and washed with TBST for 3 x 10 minutes.

Membrane was developed with 1:1 Bio-Rad® Immun-Star™ HRP Luminol/Enhancer and Bio-Rad® Immun-Star™ HRP Peroxide Buffer (usually each membrane needs 1-2 ml mixed buffer) for 1-5 minutes and filmed.

**Immunofluorescence (IF)**

Cells were fixed and permeabilized with -20°C methanol for 2 minutes, washed 2 x 5 minutes with PBS (or TBS), blocked 1 hour in room temperature or overnight at 4°C in TBST, 2 % BSA. Cells were then incubated with primary antibodies in blocking solution for 1 hour in room temperature or overnight at 4°C in 2%BSA in TBST. Samples were washed 3 x 10 minutes in TBST, and then incubated with corresponding secondary antibodies for maximum 1 hour in room temperature, washed 2 x 3 minutes with TBST, stained with DAPI for 5 minutes and quickly washed 2 x 3 minutes with TBS and processed for microscopy (or kept in TBS until further use).

**Live cell imaging**

Cells were filmed at 37 °C in CO₂ independent media (L-15) on a DeltaVision Spectris Deconvolutional Microscope (applied precision) with appropriate time interval between images, usually 20 minutes in this study. Films were analyzed by monitoring FRET ratio change (detailed protocol see Hukasova 2012).
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I am very thankful to my supervisor Assistant Professor Arne Lindqvist for giving me the opportunity to practice in your outstanding and super happy group. During the period in your lab, your intelligence and very useful feedback helped me to gain tons of knowledge. Thanks for your excellent guide, so I can follow you to find the right path to do research; for encouraging me when I was disappointed by failure. Thanks for investing your valuable time on me.

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References


