Defining Cell Cycle-Specific Interactions of the pRB Tumor Suppressor

A Major Qualifying Project

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Abstract

The retinoblastoma protein is a known tumor suppressant that is inactivated in almost all forms of cancer. One of the main functions of pRB is to prevent excessive cell growth prior to proliferation by inhibiting cell cycle progression. While this function of pRB is mainly attributed to its binding to and regulation of the E2F family of transcriptional regulators, over 300 proteins have been identified as interactors with pRB but the relevance of most of these interactions remain unclear. Since mitotic defects are observed when pRB is depleted, we hypothesize that pRB makes crucial and functionally relevant interactions with proteins that have important roles during mitosis. One candidate protein, Aurora B kinase, has an important role in mitotic chromosome segregation and has previously been suggested to interact with pRB in asynchronous cells. My analysis indicates that pRB and Aurora B likely interact in mitotic cells. This interaction between pRB and Aurora B was confirmed through western blot analysis, however, it was inconclusive as to if the interaction was mitosis specific. The silver stain analysis identified multiple distinct bands representing proteins interacting with pRB during mitosis with limited or no interaction in the asynchronous population.

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Background

Functional role of pRB and its interactors

The retinoblastoma protein (pRB) is a well characterized transcriptional co-regulator, restricting cell cycle entry into S phase to prevent unregulated proliferation. More specifically, pRB binds to and inactivates E2F, another well-known transcriptional regulator, during G1 phase

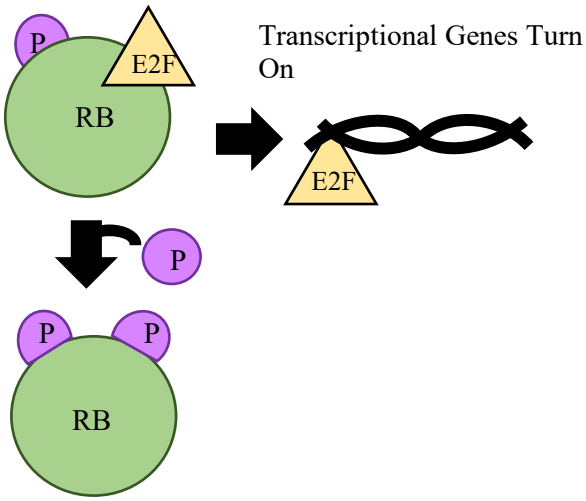

Figure 1. pRB/E2F pathway at the G1 checkpoint through phosphorylation

(Chew et al, 1998). The inactivation of pRB through hyperphosphorylation is key to the cells transition into mitosis as E2F is responsible for the regulation of cyclin dependent kinase expression as well as the expression of proteins vital for replication (Degregori et al., 1995). As seen in Figure 1, pRB is bound to E2F until the G1 checkpoint where pRB becomes hyperphosphorylated, releasing E2F. When released, E2F is active and induces the expression of genes needed to progress into S phase.

Although negative regulation of cell proliferation through binding to E2F is the most well-known function of pRB, there are other functions that should be recognized. As an abundant nuclear protein, pRB has the potential to bind and control other nuclear proteins, however many of these interactions have unknown cellular functions. Two examples of nuclear proteins with

known pRB interaction and an unknown function include Elf-1 and ATF-2. One protein, c-Abl, does have a known function when interacting with pRB. This nuclear tyrosine kinase binds to pRB when it is hypophosphorylated via the enzyme's catalytic domain. This inactivates the kinase. The interaction is lost when pRB is hyperphosphorylated and c-Abl is activated, similar to the E2F interaction. The ability for pRB to bind to and inactivate multiple proteins at a time indicates that pRB could have an effect in the downstream growth controlling pathways that is disrupted when pRB is inactivated at the incorrect stage of the cell cycle (Weinberg, 1995).

Of the over 300 proteins that bind to pRB, only a few are interactions that have a known purpose (Sanidas et al., 2019). A small subset of these proteins interacts with pRB regardless of its level of phosphorylation (Knudsen & Wang, 1996). This means that even though pRB is hyperphosphorylated during mitosis, it may still be interacting with other proteins. So, although 'inactivation' of pRB is said to be achieved through hyperphosphorylation, mitotic activity may prove that pRB isn't truly inactivated after all.

In general, pRB is extremely abundant in the cell, with many protein interactors identified. Although they may be listed, the functional protein interactions of pRB both in cancer research and cell biology are still unknown and require further study.

Disruption in function on cancer cells

Cancer cells are often characterized through excessive proliferation. When pRB is hyperphosphorylated through the entire cell cycle, it is unable to bind to E2F to regulate the rate of proliferation. pRB is most often hyperphosphorylated due to mutations or deletions in other proteins like p16 along with amplifications of cyclin dependent kinases that lead to increased phosphorylation. Although it is rare for pRB to actually be mutated or lost completely, some

aggressive cancers have done this successfully (Giacinti & Giordano, 2006). Due to how universal this is among cancer types, pRB is a focal point in the field of cancer research. Along with the hyperphosphorylation of pRB, mitotic defects are also a commonality among different types of cancers. Research has shown that there is a correlation between pRB inactivation and mitotic defects such as chromosome instability and aneuploidy (Dyson, 2016).

Cancer cells that exhibit chromosome instability (CIN) are often associated with pRB inactivation (Manning et al., 2010). In these cells, pRB is unable to regulate the progression of the cell cycle, including when the cells enter mitosis and reach certain check points. Additionally, a cascade of mitotic defects is common in CIN tumors. Some of these defects include moderate changes in centromere function, chromosome cohesion, and chromosome missegregation that decrease the fidelity of mitosis as a whole (Huang et al., 2018; Manning et al, 2010).

Aurora B Kinase

Aurora B kinase is member of a family of Aurora kinases that are responsible for mitotic progression. While all the members of the Aurora kinase family play an important role in mitosis, the member of interest in terms of pRB is Aurora B kinase. This protein is mainly

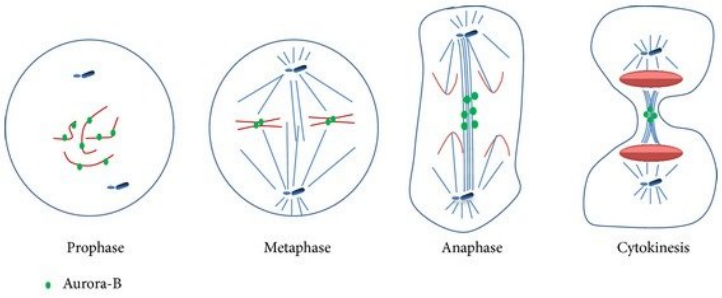

Figure 2. Schematic of Aurora B function in the cell during mitosis. Adapted with permission (Baldini et al., 2014).

responsible for centromere function, condensation, chromosomal attachment to kinetochores, the

alignment of chromosomes during metaphase, and cytokinesis (Willems et al., 2018). Along with these important functions, Aurora B is also responsible for correcting errors within the kinetochores. When kinetochores make aberrant attachments to microtubules, Aurora B is responsible for correcting the error before anaphase. If the error is not corrected the cell will experience aneuploidy and the formation of micronuclei, which are mitotic defects (Huang et al., 2018). Figure 2 depicts this function through marking Aurora B kinases in green and showing their localization in various phases of mitosis.

Depletion of pRB has shown to change the expression of certain mitotic proteins (Dyson, 2016). Aurora B kinase is one of these. It is regulated through phosphorylation and degradation, similar to pRB (Fu et al., 2007). However, the phosphorylated state of Aurora B is considered active whereas the phosphorylated state of pRB is considered inactive. The active form of Aurora B regulates its localization to the centromere and central spindles responsible for chromosome segregation and cytokinesis respectively (Honda et al., 2003). In hyperphosphorylated pRB cancer cells, depletion of Aurora B decreases the cells' fitness exponentially compared to pRB proficient cells. This correlation between pRB inactivation and Aurora B activity indicates a potential mitotic interaction between the two proteins (Oser et al., 2018). In addition, mitotic defects consistent with changes in Aurora B functions are observed in pRB inactivated cancer cells. Some of these defects include chromosome missegregation and lack of the error correction function within the kinetochores. Although these functions are disrupted in almost all CIN tumor cells, Aurora B expression either increased or remained the same (Huang et al., 2018).

In the paper by Sanidas et al. a list of proteins that interact with pRB was compiled through mass spectrometry. Aurora B was identified on this list. However, this study looked only

at an asynchronous population of cells, where the average number of cells actively going through mitosis is only about 2%. Since the majority of cells in an asynchronous population are in G1 phase, this study likely only identifies the most robust mitotic interactions due to the population type (Sanidas et al., 2019). The lack of mitotic analysis in this paper lead to the focus of the following study.

Objectives and Hypothesis

We hypothesized that there are proteins that interact with phosphorylated pRB and that these interactions may have been missed or underrepresented in earlier studies that explored interactions in G1 arrested or asynchronous cells (Sanidas et al., 2019). This could indicate changes specifically in mitosis because pRB mutations preventing E2F binding in G1 phase do not have the same mitotic defects (Wenzel & Singh, 2018). We suspect there to be interactions between phosphorylated pRB and proteins with specific functions in mitosis. Along with this, that protein interaction could potentially explain the mitotic defects we see when pRB is depleted. The progression to test the hypothesis was broken down into multiple steps. The first part of the study was to optimize an immunoprecipitation protocol for mitotic cells. The second part of the study was to determine if pRB had any binding partners strictly in mitosis. Although previous models suggest the hyperphosphorylation of pRB during mitosis, restricting binding partners, this is potentially not the case. The last part of the study was to determine if the mitotic proteins listed as interactors in the general cell cycle could be characterized as interactors in mitosis. It was hypothesized that pRB has distinct interactors during mitosis, one of which being Aurora B kinase.

Methods

Cell Culture

Human epithelial cells, RPE-1 cells, were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 50 ug/mL Penicillin and streptomycin cocktail.

Cell Treatment

To induce a uniform mitotic population, the cells were treated with Nocodazole at a concentration of 100 ng/mL in Dimethyl Sulfoxide (DMSO) for 16 hours.

Co-immunoprecipitation

Two lysis buffers were prepared and prechilled with sterile PBS, lysis 150 and lysis 250. The low salt lysis 150 buffer was comprised of 150 mM NaCl, 50 mM Tris-HCl pH 7.6, 2 mM EDTA, 10% glycerol, and 0.1% NP40. The high salt lysis 250 buffer contained the same reagents but with a concentration of 250 mM NaCl. Immediately before use, complete lysis 250 buffer was created by adding 1 tablet of protease inhibitor per 10 mL of buffer, 1 uL/mL of phosphatase cocktail II and 1 uL/mL phosphatase cocktail III. 3.0 x 10^7 cells were collected per population of cells, asynchronous and nocodazole induced mitotic arrested. The cells were washed with pre chilled PBS and resuspended in complete lysis 250. 5 uL/mL ethidium bromide was added to each cell suspension and were incubated on ice for 15 minutes. The samples were centrifuged at 13000 rpm for 5 minutes. A portion of the supernatant was saved as the input. The remaining supernatant was divided evenly into two tubes. Mouse IgG antibody was added to one tube and 4H1 antibody was added to the other. Both antibodies were used at the concentration

indicated as optimal by the manufacturer. The samples were incubated on the rocker at 4ºC for 1 hour. The beads were prepped at this time. 20 uL of protein G beads were added to two tubes. The beads were washed twice with 1 mL of complete lysis 250 followed by 1.0 mL of lysis 150 rocking at room temperature for 5 minutes. The beads were blocked in 1.0 mL of 1% BSA in TBS for 45 minutes. The previous wash cycle was repeated. After incubation, the cells extract was added to the beads and was incubated on the rocker at 4ºC for 1 hour. The supernatant was collected in a new tube for each sample. The beads were washed with 1.0 mL of lysis 150 followed by 1.0 mL lysis 250 for 5 minutes on the rocker. The beads were resuspended in 30 uL of sample buffer and boiled for 10 minutes at 95ºC. All samples were stored at -20ºC.

Western Blot

An 8% denaturing gel was made. Equal percentages of each sample prepared 1:1 with sample buffer, was loaded. 7 uL of dual colored molecular weight marker was loaded into the first well. The gel electrophoresis was run in 1x SDS-PAGE running buffer for 30 minutes at 90v. The voltage was turned up to 120v for 90 minutes. The transfer sandwich was presoaked in 1x transfer buffer and the PVDF blotting membrane was activated in methanol. The transfer sandwich was assembled and transferred for 90 minutes at 90v at 4oC. After the transfer, the membrane was blocked in 5% milk in TBST for 45 minutes. The primary antibody of interest was diluted according to manufacturer's specifications in 5% milk. The membrane was incubated in primary antibody overnight on a rocker at 4ºC. After incubation, the membrane was washed 3 times for 5 minutes on the rocker in 1x TBST. The secondary antibody was diluted according to manufacturer's specifications in 1x TBST. The washed membrane was incubated with secondary antibody at room temperature for 1 hour on the rocker. The membrane was

washed 3 times for 5 minutes on the rocker in 1x TBST. Chemiluminescent substrate was applied to the blot following the manufacturer's instructions. The blot was imaged using a CCD camera-based imager.

Silver Stain

An 8% denaturing gel was made. Equal percentages of each sample prepared 1:1 with sample buffer, was loaded. 7 uL of dual colored molecular weight marker was loaded into the first well. The gel electrophoresis was run in 1x SDS-PAGE running buffer for 30 minutes at 90v. The voltage was turned up to 120v for 90 minutes. The gel was washed twice for 5 minutes in Milli Q water. The gel was fixed twice for 15 minutes in a 30% ethanol: 10% acetic acid solution. The gel was sanitized for 1 minute in 50 uL sensitizer in 25 mL water. The gel was washed twice for 1 minute in Milli Q water. The gel was stained for 30 minutes in 0.5mL enhancer with 25 mL stain. The gel was washed twice for 20 seconds in Milli Q water. The gel was developed in 0.5 mL enhancer in 25 mL developer for 3 minutes or until bands appeared. The reaction was stopped in 5% acetic acid for 10 minutes.

Results

Optimization of co-immunoprecipitation

The first step of this project was to determine optimal conditions for pulling down pRB in cells arrested in mitosis using nocodazole. It was imperative to ensure that there was minimal nonspecific binding to pRB or chromatin being pulled down inadvertently. Figure 3 shows the successful pull down of sufficient amounts of pRB in both an asynchronous and mitotic populations of cells. The asynchronous cells have 2 distinct bands due to varying levels of

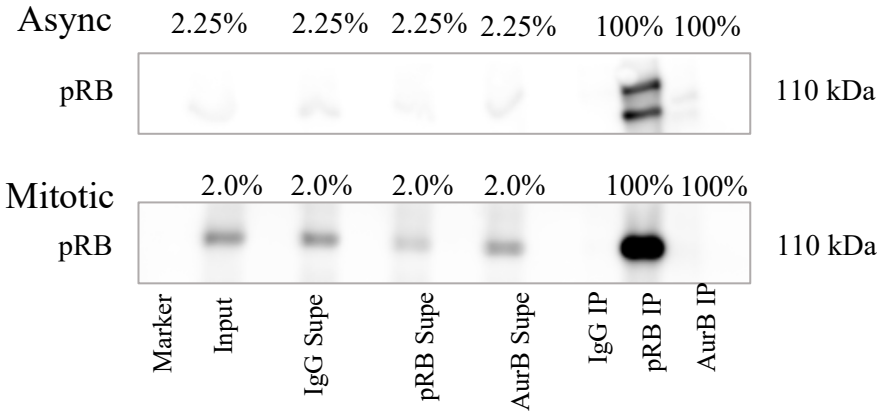

Figure 3. Western blots comparing pRB probed blots in asynchronous and mitotic cell populations

phosphorylation among the population, which was expected. The mitotic band is single and intense due to the uniform population of cells, which was also an expected result. The IgG control lanes are clean of protein in the 110 kDa mass which indicates that the control is working and there is no nonspecific association of pRB with the magnetic beads.

In order to accomplish further optimization of the coIP, ethidium bromide was added to buffer used to resuspend the cells. Ethidium bromide is an intercalating agent that gets in between stacked bases in the DNA double helix (Nguyen & Goodrich, 2006). Through adding it to the lysed cells, the affinity for proteins to be bound to chromatin and in turn be pulled down nonspecifically is minimized. To test the effectiveness of adding ethidium bromide, a single

population of cells were split and run in parallel, one with ethidium bromide and one without. In the western blot in Figure 6, the intensity of the bands seen when probing for Aurora B kinase was more intense without ethidium bromide compared to the samples with ethidium bromide. This means that there are more proteins being pulled down that may indirectly interact with pRB because both bind chromatin rather than being directly bound to pRB.

Lastly, blocking the magnetic beads with 1% BSA ensured that there was minimal to no nonspecific binding. This binds to open sites that are available for nonspecific binding of proteins. This was coupled with bead washes both vortexed and on the rocker for 5 minutes each. Through extending the wash times and adding a vortex step, proteins that are loosely bound through nonspecific binding are removed.

pRB has Distinct Interactors During Mitosis

After determining the optimal conditions for the co immunoprecipitation protocols, silver stains were used to compare the mitotic and asynchronous populations. As seen in Figure 4, the mitotic pRB IP on the right shows distinct bands below the 50 kDa weight mark that are not present in the asynchronous pRB IP. One of these bands is characteristic of Aurora B. In addition to the band that is potentially Aurora B, there are 2 other distinct regions that show bands in the mitotic population that are less intense or absent from the asynchronous population. From this data, it can be concluded that there are at least 2 additional proteins, aside from Aurora B, that

interact with pRB exclusively during mitosis. These proteins could be identified through mass spectroscopy or probing for specific proteins speculated to interact with pRB during mitosis.

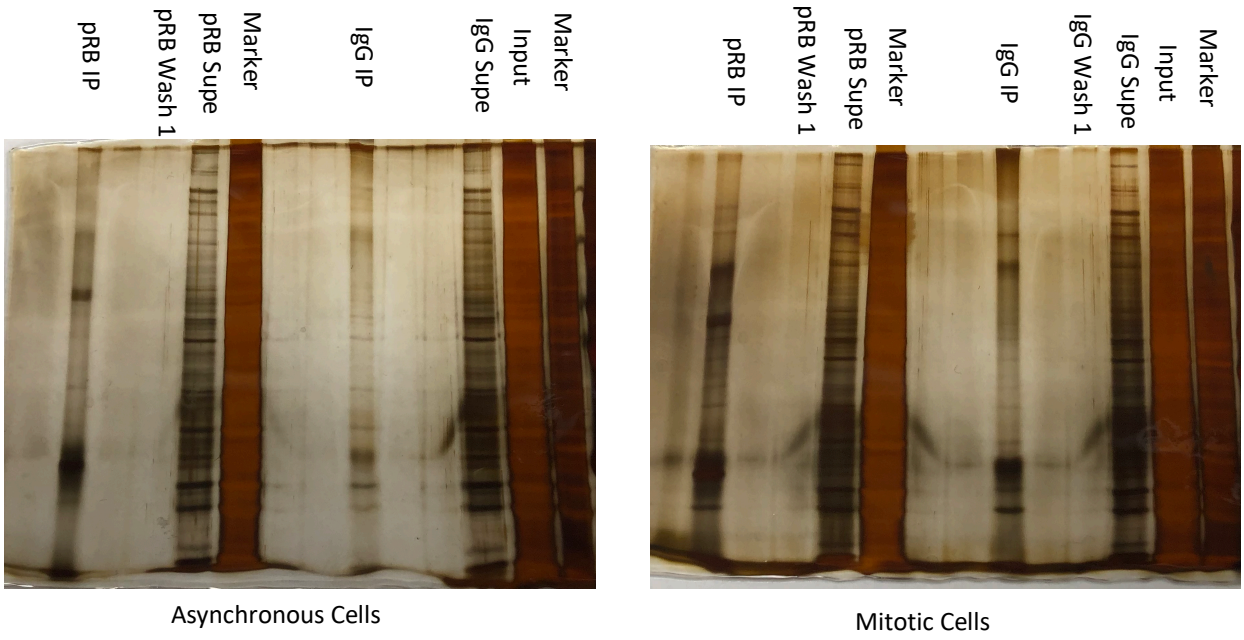

Figure 4. Silver stains of both the asynchronous and mitotic cell populations highlighting the distinct bands in the mitotic cells. The clean IgG control bands indicates assay optimization

Aurora B Kinase Interacts with pRB During Mitosis

The silver stains showed indication of multiple bands below 50 kDa, potentially matching the weight of Aurora B kinase. To determine if this relationship exists, western blots probing for

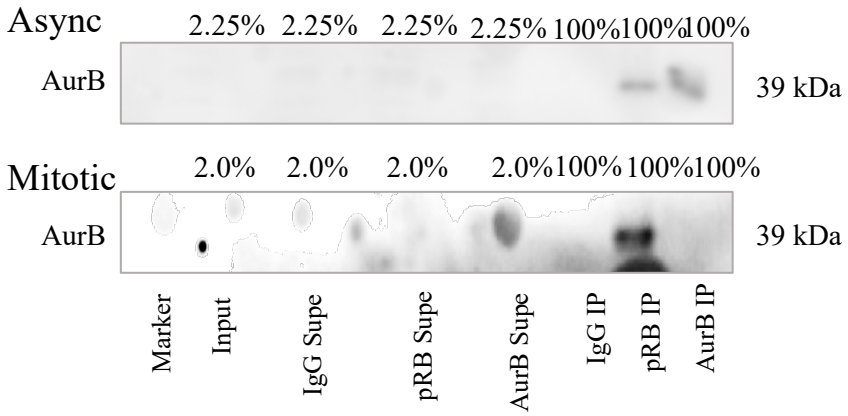

Figure 5. Western Blot comparing asynchronous and mitotic cell populations when probing for pRB and Aurora B. The percentages indicate the relative percentage of sample loaded in each assay

Aurora B were conducted. In the Figure 5, a band in the pRB IP at about 39 kDa is observed. This is characteristic of Aurora B. Due to Aurora B having various chromatin binding functions,

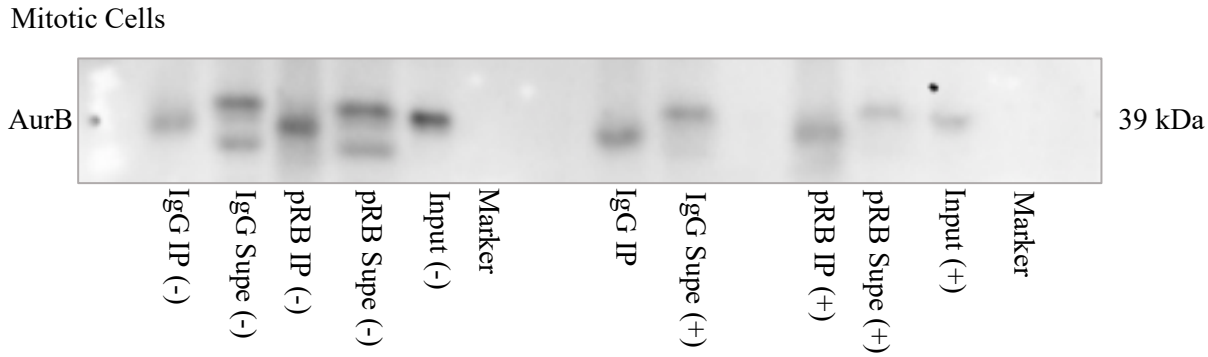

Figure 6. Western Blot comparing two mitotic samples, one with the addition of Ethidium Bromide (+) and one without Ethidium Bromide (-)

the co-IP in figure 5 was completed with ethidium bromide. Figure 6 shows the difference in band intensity between co-IPs conducted with and without the addition of ethidium bromide. Aurora B is pull down in sufficient quantities in both groups, however the bands are much more intense without ethidium bromide indicating some Aurora B is pulled down through just chromatin binding rather than direct pRB binding.

Discussion

The Co-IP protocol was optimized in several steps

It was imperative to first optimize the Co-IP protocol for pRB in order to get the most accurate results. Some problems that arose in the beginning included potential nonspecific binding and protein residues appearing in both washes. Along with this, the IgG control lane in the silver stains indicated proteins other than the light and heavy chains being pulled down with the magnetic beads. This was problematic because we did not expect any protein bands in the control lanes. The presence of these bands could indicate nonspecific binding to the beads and inadequate washes. In order to eliminate nonspecific binding, the beads were treated with 1% BSA. BSA binds to open potential binding partners to prevent the antibody from binding to a nonspecific site. Adding rigorous washes in stringent conditions through vortexing and prolonged incubation on the rocker also ensured that any proteins loosely bound to pRB were removed before the sample was collected.

There are unique and distinct pRB protein interactions in mitosis compared to asynchronous cells

Through silver stain analysis at least 3 distinct bands were identified on the mitotic IP compared to the asynchronous. This was repeated in replicate with the same results. Only 1 of the 3 distinct bands on the silver stain are at the molecular weight of Aurora B. This means that there are at least 2 additional mitotic protein interactors that regularly interacted with hyperphosphorylated pRB during mitosis. When these proteins are identified, they may also be linked to the mitotic defects we seen in cancer, similar to Aurora B.

Aurora B does interact with pRB during mitosis but is also present in the asynchronous cell population indicating the interaction may not be mitosis specific

This finding is the most unique finding from the study. We knew from the beginning that Aurora B had some interaction with pRB in the cell cycle. We were able to prove through repeatable experiments that the interaction is much more prevalent and intense in a uniform mitotic population of cells compared to an asynchronous population. However, we are unable to prove that the interaction is mitosis specific because it was seen in the asynchronous population of cells. This could be due to the 2% of cells that are going through mitosis at any given time in an asynchronous cell population. It is also possible that Aurora B and pRB interact at an additional point in the cell cycle, with lower activity compared to during mitosis.

Future Steps

Reverse Co Immunoprecipitation

The data gathered through the coIP pulling down pRB showed consistent interaction with Aurora B. However, Aurora B is a sticky protein that also binds right to the chromatin. The question becomes: is Aurora B really interacting with pRB or is it getting pulled down through chromatin interaction? Although the addition of ethidium bromide to the coIP functions to eliminate chromatin binding, the most effective way of proving the pRB/Aurora B interaction is real is through completing a reverse coIP. In this protocol, the Aurora B antibody, AIM-1, is used to pull down Aurora B along with the proteins it is interacting with. pRB would be probed for in a western blot. The western blot in Figure 6 includes the Aurora B IP. Since this blot was probed for Aurora B there should be an intense band in the same location as in the pRB band. This is not the case. This could be due to the antibody not being effective for immunoprecipitation or the concentration of antibody used not being sufficient since there was no recommended concentration for a co-IP. Due to this, we used 1 mg/mL to be consistent with the concentration used of both the IgG control and the 4H1 antibodies. The interaction is still proved to be existent through probing for Aurora B in pRB IP. This experiment was completed with a mitotic population of cells. Due to time constraints this reverse coIP was only completed once and would require further experimentation to gather results from repeatable experiments.

Mass Spectrometry

The ultimate goal of this experimentation was to complete mass spectrometry on the samples obtained to identify exactly which proteins were present pRB mitotic interactors without doing additional trial and error through western blots. Although this was not completed, the Co-

IP protocol has been optimized, making the samples clean enough to run the mass spec. This would be a valuable next step given that repeated trials yielded extremely similar results in the silver stains. This repeatability indicates that the bands that are expressed are legitimate pRB mitotic interactors that we have not identified yet.

Probe for additional interactors

The interaction between Aurora B and pRB was confirmed through probing the western blot with the specific Aurora B antibody. There are many more proteins that are both known mitotic proteins and have pRB interaction in the cell cycle. Some mitotic proteins that could be probed for are MAT1 and BCR as they are both kinases that have a role in transcription during mitosis (Sanidas et al., 2019). They have molecular weights of 36 kDa and 210 kDa respectively, which are regions on the silver stain with distinct bands in the mitotic cell population (Tassan et al., 1995; Denderen et al., 1989). Since Aurora B is 39 kDa, the band n the silver stain indicative of Aurora B could also contain MAT1. Although they are not confirmed to interact during mitosis specifically, this is something that can be tested through probing the pRB Co-IP with the specific antibodies for these mitotic proteins.

Future research question

While conducting both background and experimental research, many future research questions have been considered with one in particular sparking interest. We know pRB hyper phosphorylates to release E2F to allow for transcription. This pathway is disrupted in cancer contexts. Is the specific pRB protein interactions during mitosis also disrupted? Does this contribute to the cell characteristics we see in cancer?

If future research continues, it may be possible to identify the specific pRB protein interactions that occur in mitosis, shedding light on the binding mechanisms of hyperphosphorylated pRB as well as identifying a potential cascade of disrupted interactions in cancer cells that lead to mitotic defects.

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