

# Regulation of Cell Motility via pRB

By Thomas Perry

## **Abstract**

Previous data in the Manning Lab indicates that human cells with depleted levels of pRB tumor suppressor protein may have a higher motility than other cells. Cancer cells possessing a higher motility are more able to enter the circulatory system and metastasize, and pRB is frequently lost in many cancer types. The goal of this project is to determine whether cells with depleted pRB have a different level of motility than cells with sufficient pRB. This was tested using assays that specifically looked at the effects on individual cells and the ability of cells to move *en masse*. Cell density is known to impact cell motility. To mitigate this as a potential variable, data was controlled so that cells with or without pRB from populations of similar density were compared. Differential behavior at high and low densities was also compared. My data indicates that pRB- deficiency does not alter cell motility at the population level but instead increases the motility of a subpopulation of individual cells. Further investigation is needed to determine the factors that influence this stochastic behavior.

## **Introduction**

### Regulation of Cell Cycle Entry

The Retinoblastoma protein (pRB), is coded for by the RB1 gene in humans, and is a well characterized regulator of the cell cycle. During the normal course of the cell cycle, pRB is hypophosphorylated during most of G1 phase, activating it to bind to and repress E2F transcription factors that drive the cell cycle (Narasimha et al., 2014). When a cell is about to

leave G1 phase and enter S phase, the cyclin E-cdk2 complex hyperphosphorylates pRB, causing it to release E2F, which then promotes transcription of genes

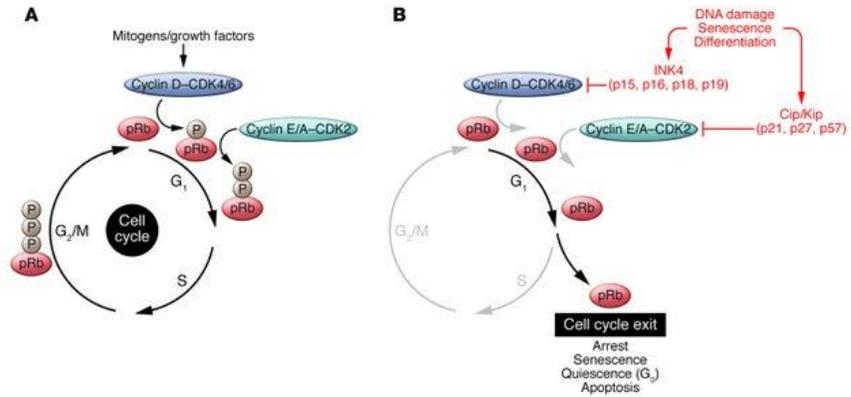


Figure 1) Regulation of pRB through the cell cycle (Sachdeva et al., 2012).

necessary for G1-S checkpoint transition (Narasimha et al., 2014). pRB then spends the rest of the cell cycle hyperphosphorylated until exiting mitosis, at which point the PP1 phosphatase dephosphorylates it and the cyclin D-cdk4/6 complex immediately hypophosphorylates it, returning it to suppressing the E2F transcription factor (Narasimha et al., 2014). This is

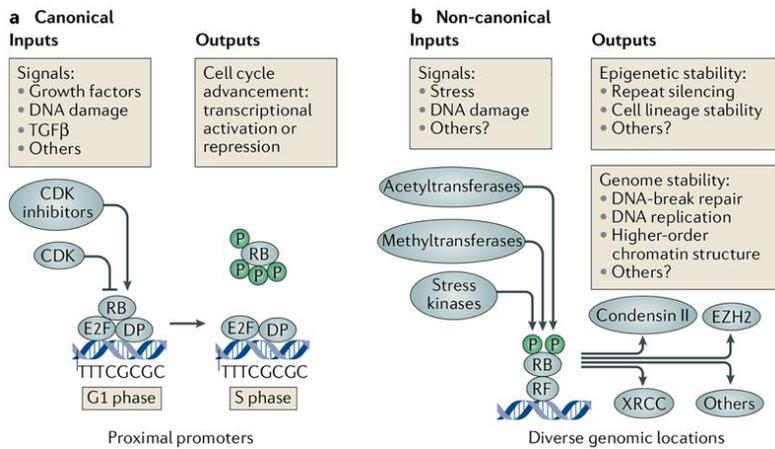


Figure 2) Summary of the canonical and some known non-canonical pRB pathways (Dick et al., 2018).

visualized in Figure 1. The role of pRB in regulating the G1/S phase transition is essential in restraining improper cell proliferation and loss of this function permits uncontrolled growth seen in cancer cells. pRB also has multiple non-canonical functions aside from its tumor suppressive role in regulating the cell cycle, some of which may be independent of pRB phosphorylation state and/or regulating tumor suppression in their own right; however, few of these additional functions have been explored in depth (Dick et al., 2018, Figure 2).

## pRB Status in Cancer

While the RB1 gene is lost or mutated in select cancers, like retinoblastoma which it is named for (Burkhart et al., 2008) and small cell lung cancer, in most other cancer contexts the RB1 gene remains intact and instead function of the pRB protein is compromised by constitutive phosphorylation. Functional inactivation of pRB can occur when Cyclin/CDK activity is enhanced by amplification or overexpression, or when p16, a negative regulator of CDK, is deleted or silenced (Figure 1). Such inactivation has been implicated in the formation of tumors.

## Regulation of Cell Motility

In addition to regulation of progression from G1 to S phase, multiple studies have implicated loss of pRB function in increased invasion and metastasis. These studies experimentally show that pRB plays a role in restraining metastasis in tumors arising from various tissues, including colon cancer, thyroid carcinomas, and neurons in the developing mouse cortex (Burkhart et al., 2008, Tsujii et al., 2007, Ziebold et al., 2003, Ferguson et al., 2005). As one example, pRB has been linked to increased expression of cyclooxygenase 2 (COX-2). COX-2 is an enzyme involved in prostaglandin synthesis, a set of hormones with varying effects. COX-2 overexpression has in turn been implicated in increased motility and invasion of breast cancer cells (Burkhart et al., 2008, Singh et al., 2005). This experiment was performed by counting the number of cells passing through a PET membrane for cell migration and the number of cells passing through a matrigel coated membrane for invasion. Together these data suggests that pRB may regulate cell motility non-cell autonomously: by increasing the production of hormones that promote motility, one pRB-deficient cell may influence the motility of other cells in a shared environment.

## pRB and the Actin Cytoskeleton

The Singh study indicates that pRB loss may make cancer cells more invasive but does not explore if this is due to a change in the baseline ability of cells to move without barriers blocking movement, nor does it determine how COX2 overexpression led to this increase in motility of breast cancer cells (Singh et al., 2005). In an independent series of studies,

researchers suggest that pRB may also impact cell-autonomous regulation of cell motility.

Migration and invasion are complex behaviors that are both thought to correlate with metastatic potential in a cancer context. Both behaviors require coordination of the actin cytoskeleton in response to external stimuli to promote advancement of the cell's leading edge, and to lay down focal adhesions for force generation (Figure 3). One study has suggested that pRB regulates the cell's response to extracellular factors that affect cell motility. While pRB loss is insufficient on its own to overcome the contact inhibition that prevents cells from proliferating in a crowded environment, transcription growth factor-beta 1 (TGF- $\beta$ 1) protein enforces constant levels of non- and hypo- phosphorylated levels of pRB until the G1-S checkpoint regardless of cell density (d'Alessandro et al., 2018, Brugarolas et al., 1998, Hneino et al., 2009). In this way, loss or hypophosphorylation of pRB may alter a cell's response to TGF- $\beta$ 1 (Figure 3). Other studies indicate that anchoring proteins like cadherins and integrins, which have previously been

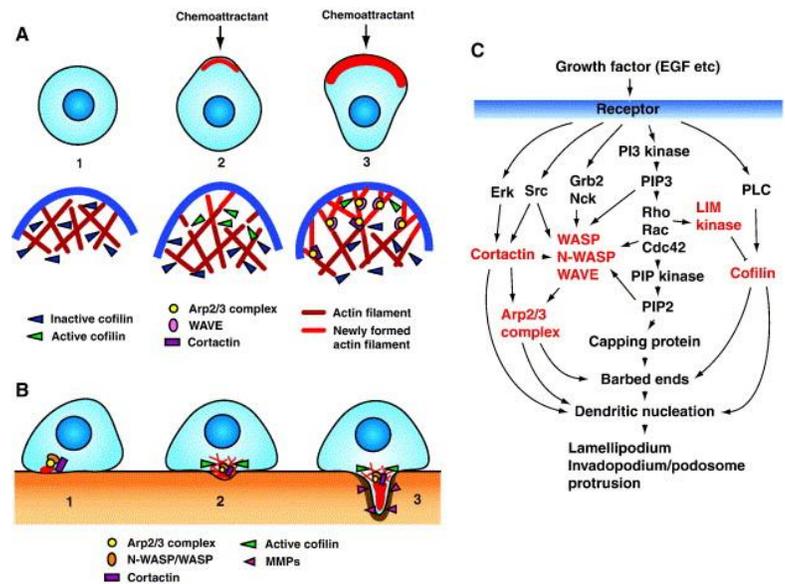


Figure 3) Diagram showing how the actin cytoskeleton plays a role in motility, invasion, and the complexity of its regulation (Yamaguchi et al., 2007).

implicated in increased cell motility and metastasis. Both cadherins and integrins are regulated by members of the pRB/E2F pathway. Cadherins are proteins that bind cells to one another, while integrins bind cells to the extracellular matrix (Jeanes et al., 2008, Engel et al., 2015). pRB loss has been correlated to down regulation of E cadherin expression, loosening the bonds between cells, It has also been confirmed to cause cells to switch the type of integrin they express on their surface, giving them a higher binding affinity to a different type of extracellular matrix (Engel et al, 2015). Together, this may allow cells to leave their normal location, and bind themselves to another location, which would increase the chances of metastasis.

While pRB has been shown to interact with several components of the cell motility machinery, and loss of pRB is linked to increased motility in cancer contexts, this role for pRB is not fully understood and it remains unclear if pRB primarily impacts cell autonomous or non-cell autonomous mechanisms of cell motility. This project will attempt to explore this unknown by studying human retinal pigment epithelium cells *in vitro* and characterizing how the cells move, both individually and as a population. Finding an answer, should there be a difference between pRB-sufficient and pRB-depleted cells, would have the potential to lead to the discovery of new targets to limit metastasis in cancer.

## **Results**

### *Cell Motility*

To determine if there are intrinsic differences in how cells lacking the pRB tumor suppressor move, long term, live cell, high resolution fluorescence imaging was performed. Human Retinal pigment epithelial cells (RPE) expressing a Histone 2B protein tagged with a red fluorescent protein (RFP-H2B) were used to identify and track cell nuclei (Nowotshcin et al.,

2009). Cells were depleted of pRB using siRNA transfection 48 prior to the experiment. This depletion approach has been validated to result in >80% depletion of the pRB protein (Manning et al., 2014). Control and pRB-depleted cells were then imaged every 5 minutes for at least 6 hours (Mercadante et al., 2019). The freely available open-source CellProfiler software was used to identify individual nuclei and monitor their movement in the x, y coordinates at each timepoint (Kamentsky et al., 2011). Movies were made, nuclei identified, and motility assessed for a minimum of 150 cells for each of 5 biological replicates. For each replicate, control cells were also assessed.

Save for one instance, each replicate had at least 150 cells in the population, and each replicate had a high variance in the motility of individual cells within the population. Across all replicates, the average velocity of pRB-depleted cells appears slightly less than control cells; however, the difference is not statistically significant, with a two-sample student's t-test returning a p-value of 0.33 (Figure 4).

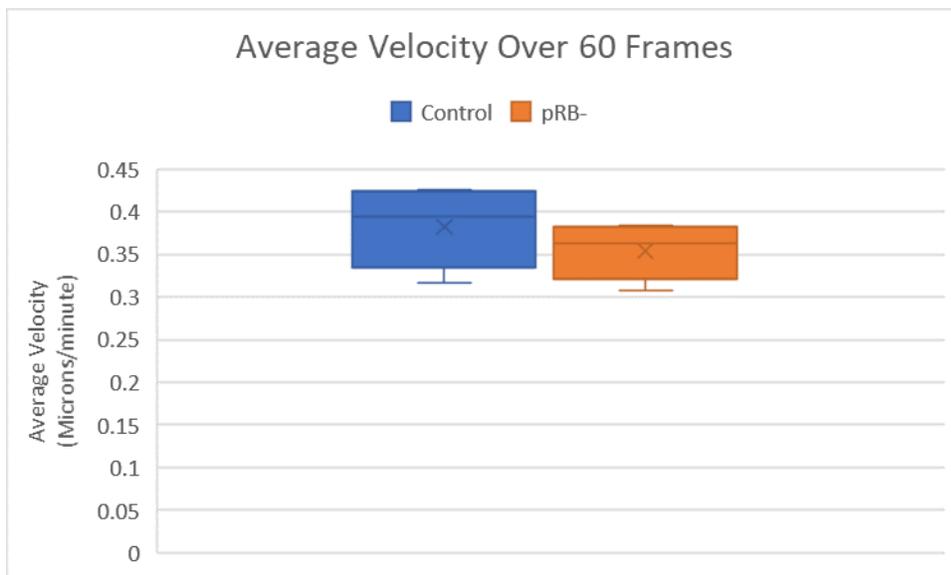


Figure 4) Average velocity of all cells in frame over the course of 60 frames. N=5. P-value of difference is 0.33 by two sample student's t-test Error bars represent maximum and minimum values for each condition.

To determine if cell density influences motility significantly and is a variable that should be controlled for, I compared the average velocity of all cells across 5 frames in each well to the average density of those frames (Figure 5). While many of the lower density pRB-depleted wells had a lower average instantaneous velocity, these wells were also at the extreme low end of cell density. Wells of pRB-depleted cells with confluency similar to that of control wells showed little change in average instantaneous velocity.

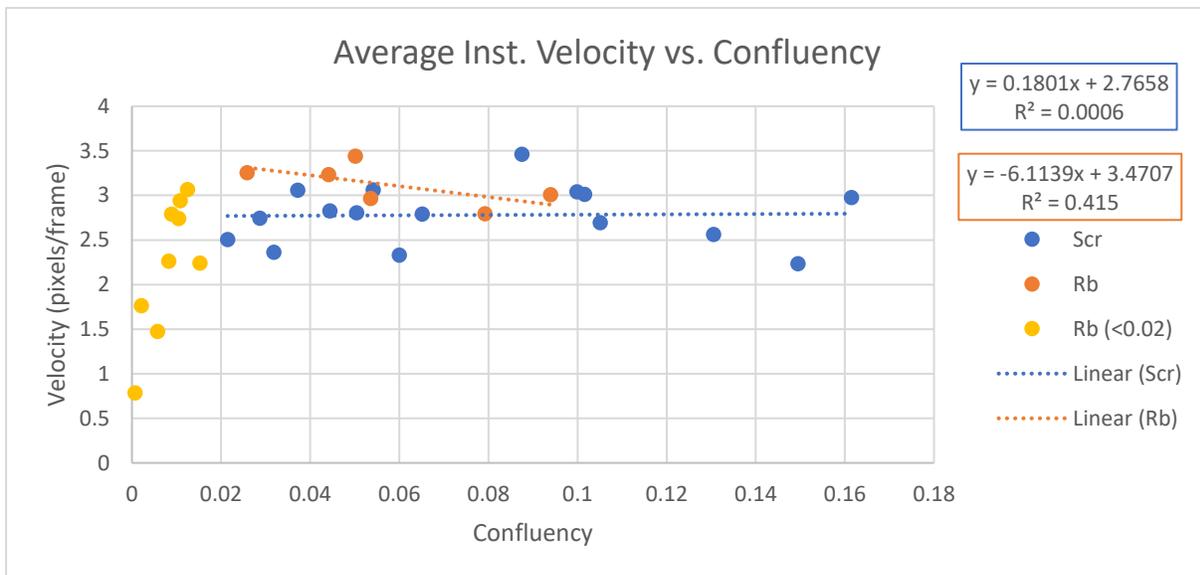


Figure 5) Average instantaneous velocity of all cells in frame vs cell density of the same frame. Colored boxes denote derived equations for trendlines, counting only wells with confluency higher than 0.02.

Interestingly, while a histogram (Figures 6 and 7) of 100 random cells chosen from populations of normal and pRB depleted cells with similar cell densities indicates that most cells in the two

groups have relatively similar average velocities, a subset of cells with a higher average velocity appear in the pRB depleted population. In addition, a histogram of the average linear velocities (the calculated velocity using final displacement from start point to end point, as opposed to the total distance traveled by the cell, Figure 8) indicates that pRB depleted cells have a higher linear velocity on average. However, checking the velocities of all the cells in relation to the maximum speed recorded by any cell in each condition (Figures 7 and 9) reveals that the two populations have a similar distribution, in both undirected and linear velocities, backing up the data in figures 4 and 5.

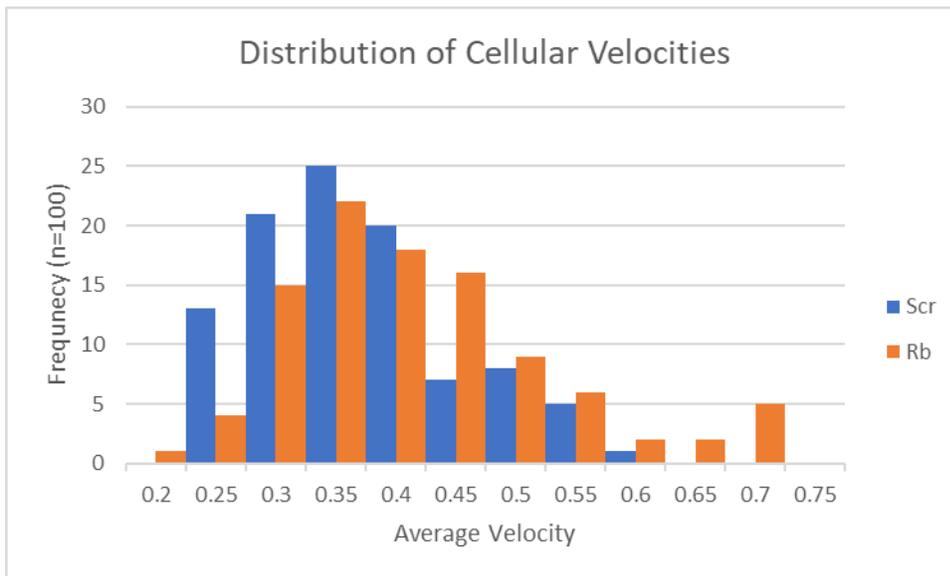


Figure 6) Histogram of average cellular velocities of 100 randomly selected cells. Groups represented were deliberately chosen because they had similar densities.

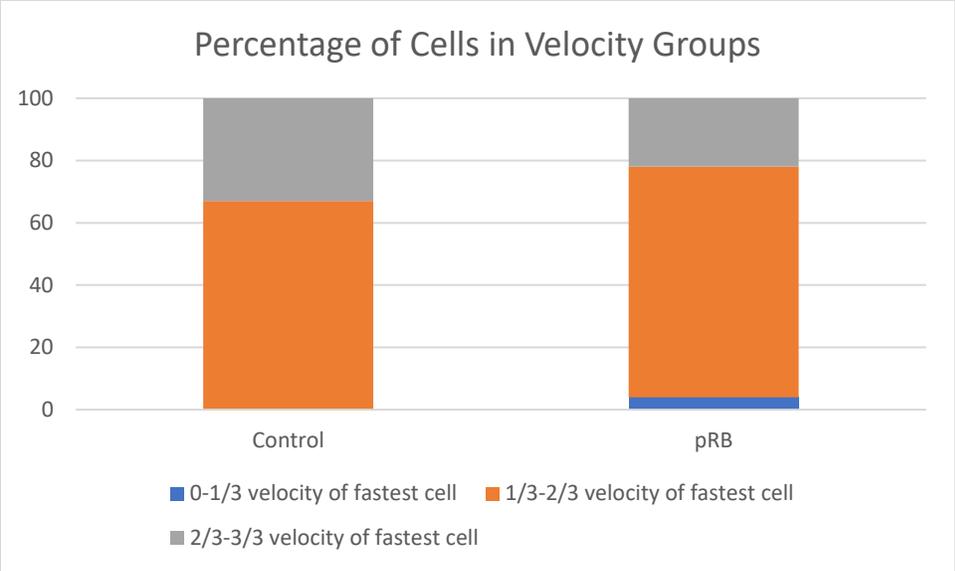


Figure 7) Percentages of cells in groups based on maximum velocity of cells in condition.

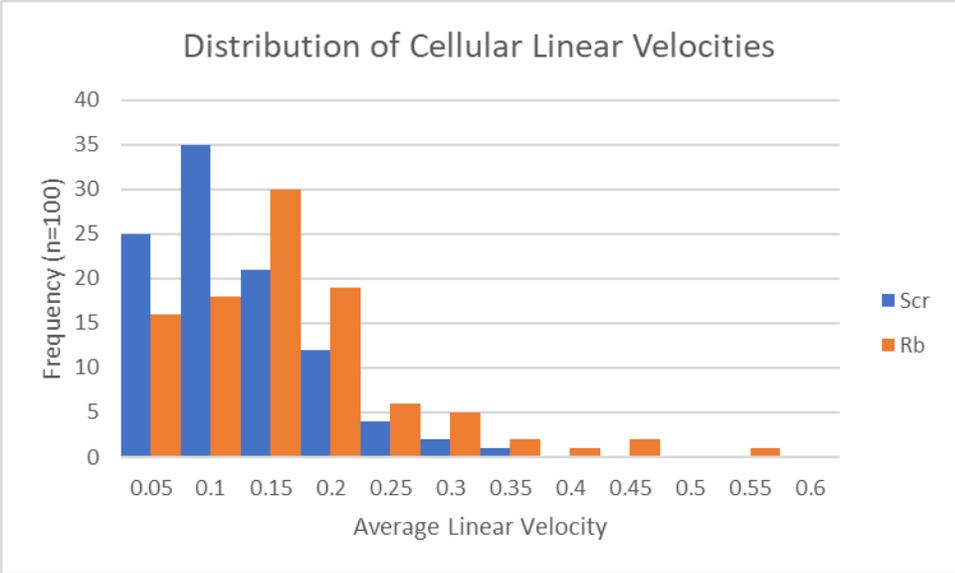


Figure 8) Histogram of average cellular linear velocities of 100 randomly selected cells. Groups represented were deliberately chosen because they had similar densities.

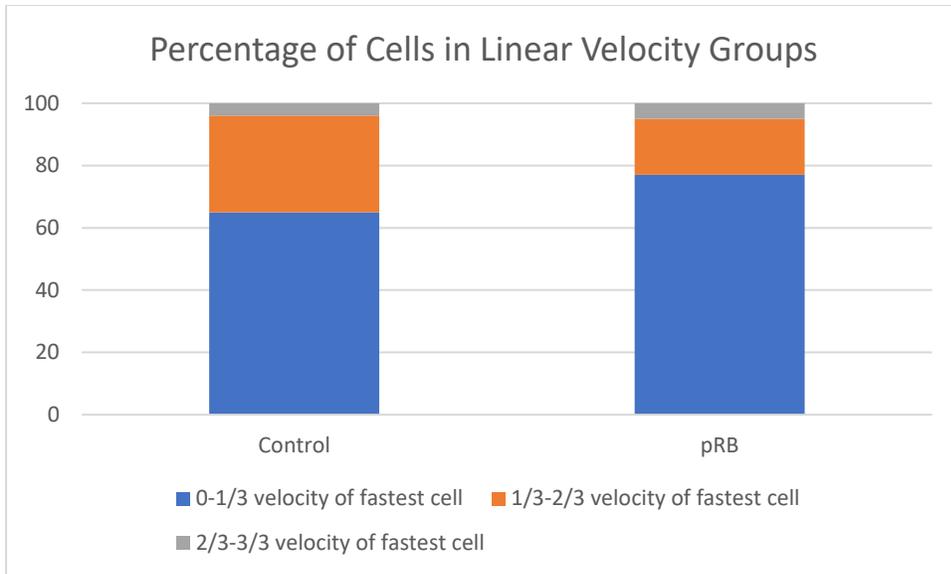


Figure 9) Percentages of cells in groups based on maximum linear velocity of cells in condition.

### Cell Motility as a Tissue

After observing the effects of pRB depletion on the motility of individual cells, an attempt was made to determine whether cells as a collective tissue responded differently to a lack of pRB. This was done using long term, live cell, high resolution phase contrast and fluorescence imaging. RPE cells expressing a doxycycline-

activated RFP transcript and doxycycline-activated shRNA inhibition of pRB synthesis (Figure 10) were grown to form a confluent monolayer, with half the wells being treated with doxycycline; the activatable RFP transcript served as a visual confirmation that pRB-

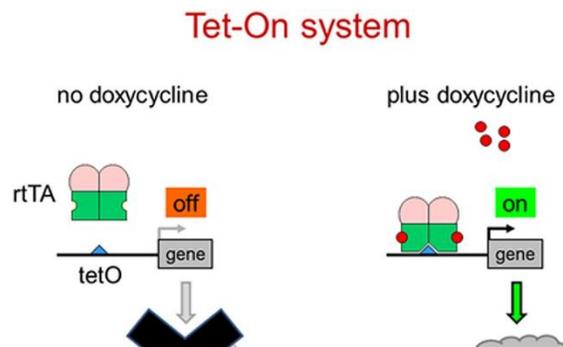


Figure 10) Visual representation of the doxycycline activated system used in the scratch assay (Das et al., 2016).

specific shRNA is expressed and knockdown had occurred. Wells were then treated with doxorubicin to prevent them from dividing further, ensuring that no movement is occurring due to proliferation. The bottom of each well was then scratched manually using a pipette tip. Imaging was performed over the course of 48 hours (Liang et al., 2007, representative images in figure 11). Each well was imaged x times, and each image was then analyzed using the analysis software NIS Elements to measure the width of the imaged wound in pixels. Measurements were then converted to microns to determine the distance the gap closed in each period and calculate rates of bulk cell movement (Figure 12). Images from a single well were averaged, and the average width was compared between wells at each timepoint.

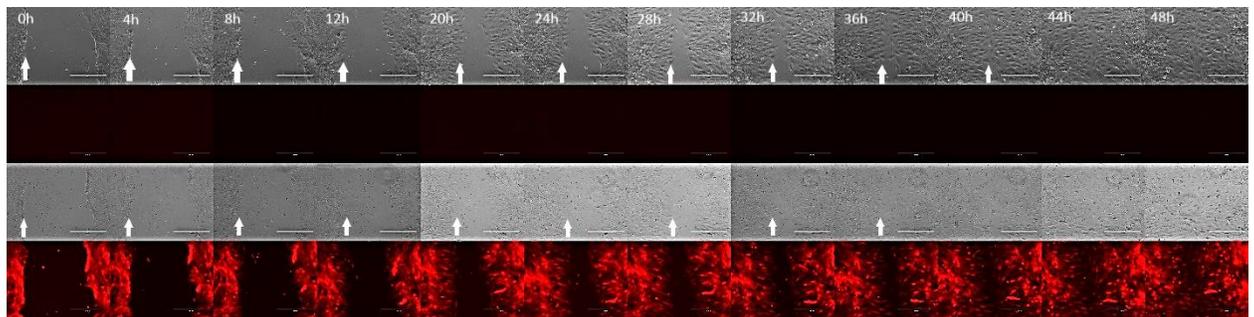


Figure 11) Representative images of wound healing over time, at hours 0, 4, 8, 12, 20, 24, 28, 32, 36, 40, 44, and 48 after scratch, from left to right (indicated by timestamps). Top; phase contrast and red fluorescent images of pRB-sufficient cells. Bottom; phase contrast and red fluorescent images of pRB-depleted cells. Images are oriented so that the scratch wound is centered in the field of view. White arrows indicate the left leading edge of each closing wound.

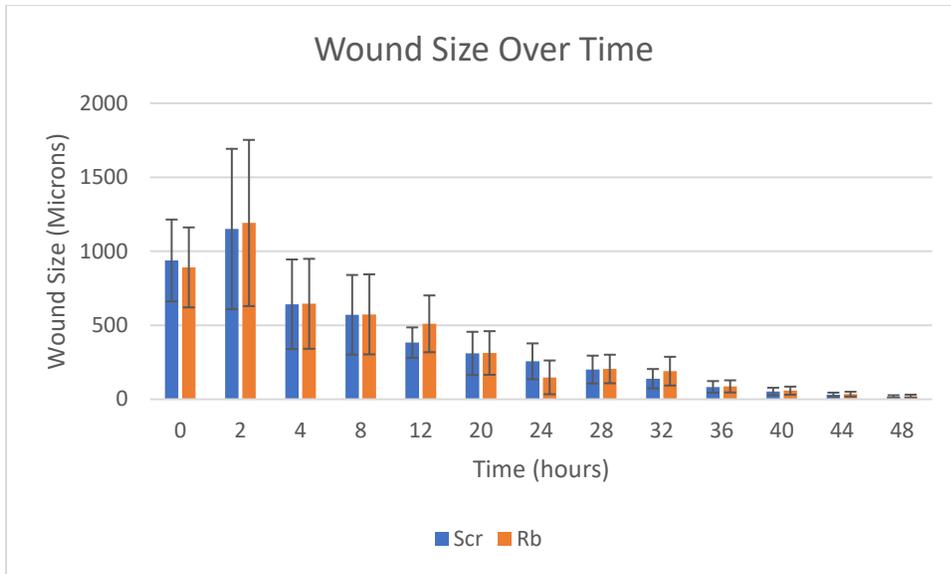


Figure 12) Wound size over time, +/- doxycycline for pRB depletion. Error bars are standard deviation between replicates.

The data indicates that there is no significant difference between pRB sufficient and depleted cells for wound healing; a one-way ANOVA test between pRB-sufficient and pRB-depleted cells across all timepoints returned a P-value of 0.97, showing little to no variance between the two groups. An additional one-way ANOVA test for the wound closure rate between pRB-sufficient and pRB-depleted cells across all timepoints also returned no significance, with a P-value of 0.99 (Figure 13).

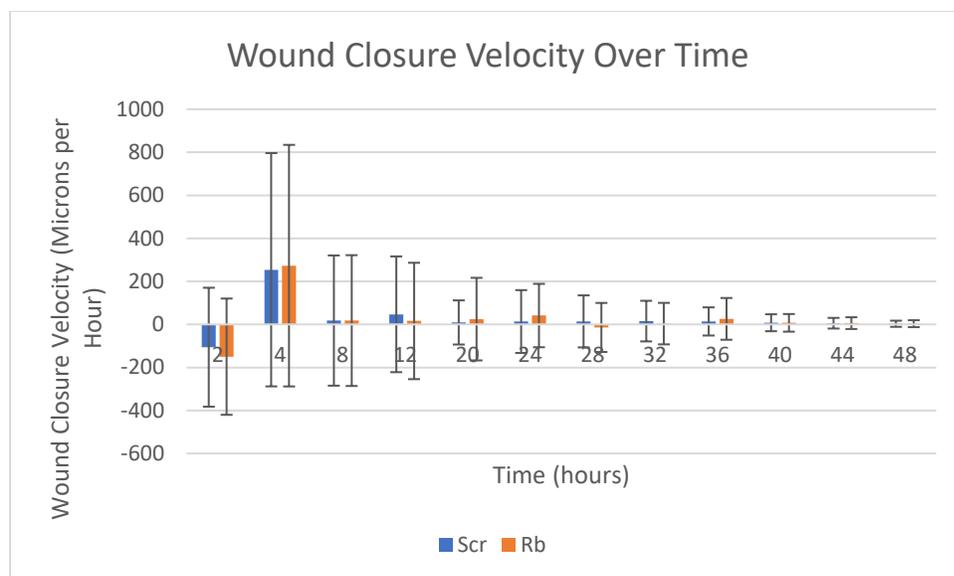


Figure 13) Rate of wound closing over time, +/- doxycycline for pRB depletion. Error bars are standard deviation between technical replicates.

## Discussion and Future Directions

The data indicates that there is no population level difference in cell motility following pRB loss. This argues against a non-cell autonomous role for pRB in regulation of cell movement. Under similar conditions, pRB-depleted cells on average do not have a statistically significant difference in motility from pRB-sufficient cells. This is true for average motility in single cell assays, regardless of cell density, and for the cells acting as a group in the scratch assay. A caveat of this interpretation is that the individual cell motility assays was primarily focused on measuring motility in subconfluent populations of cells. Our most confluent population reached only xx confluency. Under this condition both control and pRb deficient cells remain highly motile and we cannot say if populations of pRB deficient cells might exhibit differential motility in more confluent contexts, as would be found in a tissue. The scratch assay also had technical limitations. Since doxorubicin was used to prevent cell proliferation and

ensure that any movement was purely a result of cell motility, the cells needed to be treated at a specific time before performing the assay; too soon and there would be too few cells to form a monolayer, too late and the number of cells available to close the wound would not be consistent between treatments. Also, as evidenced by the large error bars within individual timepoints between technical replicates, this assay is extremely sensitive to both the uniformity and reproducibility of the initial scratch wound, such that slight variations may preclude accurate assessment of wound closure rates.

In contrast, a histogram of cell velocities show a greater number of cells with a high velocity in pRB-depleted populations compared to pRB-sufficient populations, and putting those values in proportion to each condition's own maximum showed the two populations actually had similar distributions, suggesting that pRB loss does increase the overall range of velocities that within a cell population. As described above, a limitation in the motility assay is the difficulty in effectively controlling the density of cells in the well. The density of cells was controlled by counting the cells in solution using a hemocytometer to determine the concentration of cells and diluting the solution when it was plated. While this does allow for general "high" and "low" density wells, this method is not perfect enough to ensure that there are highly similar densities between treatments within a replicate; in one instance, the "high" density pRB-depleted well contained a similar density of cells as the "low" density control well in the same replicate. For this reason, actual densities were calculated with imaging software and comparative populations assessed accordingly. Nevertheless, these results suggest that pRB-dependent regulation of cell motility is likely cell autonomous. That only some cells in the pRB-depleted population exhibit increased motility additionally suggests that pRb loss is not sufficient for increased motility but

instead cooperates with other intrinsic and extrinsic behavior (like cell density) to influence cell movement.

Given pRB's regulatory role in both canonical and non-canonical pathways, it is possible that certain cells lose control of the motility regulatory functions, causing them to behave with a much higher activity than other cells. This might arise as the loss of pRB nullifies the G1-S phase checkpoint, allowing a cell to proceed through the cell cycle even if there is existing DNA damage. It might also occur as a result of pRB not performing one of its functions as a genetic regulator; its loss has been linked to increased aneuploidy/polyploidy, and it interacts with members of the DNA polymerase complex, potentially inducing further DNA damage (Huang et al., 2015). This potential DNA damage is stochastic, limited to a single cell and its descendants, which would explain any one cell's increased motility. This loss of regulation might also impact the contact inhibition that causes cells to stop moving once surrounded by other cells. To test this hypothesis it would be necessary to synchronize cells at defined stages of the cell cycle, or to induce DNA damage and monitor cell motility of these cells independent of pRB status.

Our work shows that, for human RPE cells, there appears to be no difference in the base motility of pRB-depleted cells from normal cells. This experiment was done *in vitro*, allowing data to be collected on how cells moved on their own. However, in the body, cells are surrounded by an extracellular matrix created by themselves and all the cells around them, which eliminated many mechanics that might have also had a role, such as any regulation by pRB of integrins, which specifically bind cells to that matrix; given that the cells in this experiment at most formed a confluent monolayer, cadherins may have also had a greater part to play. Further experiments might use culture dishes coated to simulate an extracellular matrix to test these possibilities. Other experiments in other cell lines have confirmed that pRB loss leads to

increased invasion potential; our data does not contradict that, as the cells did not interact with one another except in the scratch assay, and then it was done to test the loss of contact inhibition. Further experiments might use an invasion assay, which measures the rate at which cells travel through pores in a membrane over a certain period. This assay is often used to quantify the rate at which tumor cells invade bodily tissues, which is a key step in a tumor metastasizing. This would allow us to confirm that RPE cells share the same increased invasion potential found in other cell lines.

## **Methods**

### *Scratch Assay Protocol*

The cells used were Retinal Pigment Epithelium (RPE) cells with a switch inducible by doxycycline that suppresses the synthesis of pRB and triggers the synthesis of red fluorescent protein (RFP). They were cultured in a 10 cm culture plate using Dulbecco's Modified Eagle Medium (DMEM) before using trypsin to transfer them to a 15 mL conical tube, where their concentration was counted on a hemocytometer. 0.5 mL of DMEM medium containing ~200,000 cells/mL was plated into each well of a 12-well plate that, on the bottom, had a line drawn down the center of each row of wells using a marker, then another line ¼" above and below that line. To half of the wells, 0.5 µL of 2 mg/mL doxycycline to suppress the synthesis of pRB. The plate was then incubated at 37°C, 95% humidity, and 5% CO<sub>2</sub> for 36 hours to allow for depletion of pRB as the cells proliferated, before the medium was removed and replaced with fresh medium. The doxycycline was reapplied to the pRB-depleted wells, and 0.5 mL of 100µg/mL doxorubicin to suppress cell proliferation, before the plate incubated at 37°C, 95% humidity, and 5% CO<sub>2</sub> for a further 12 hours. A P200 pipette tip was used to scratch the bottom of each well, taking care to

make the scratch as linear and a consistent width as possible, and using a fresh pipette tip for each well. Using the marker as a guide, the plate was then imaged using phase contrast microscopy and red fluorescent microscopy with a 4X objective, taking four images per well. More images were taken in the same locations every four hours for the next 48 hours.

### *Cell Motility Assay Protocol*

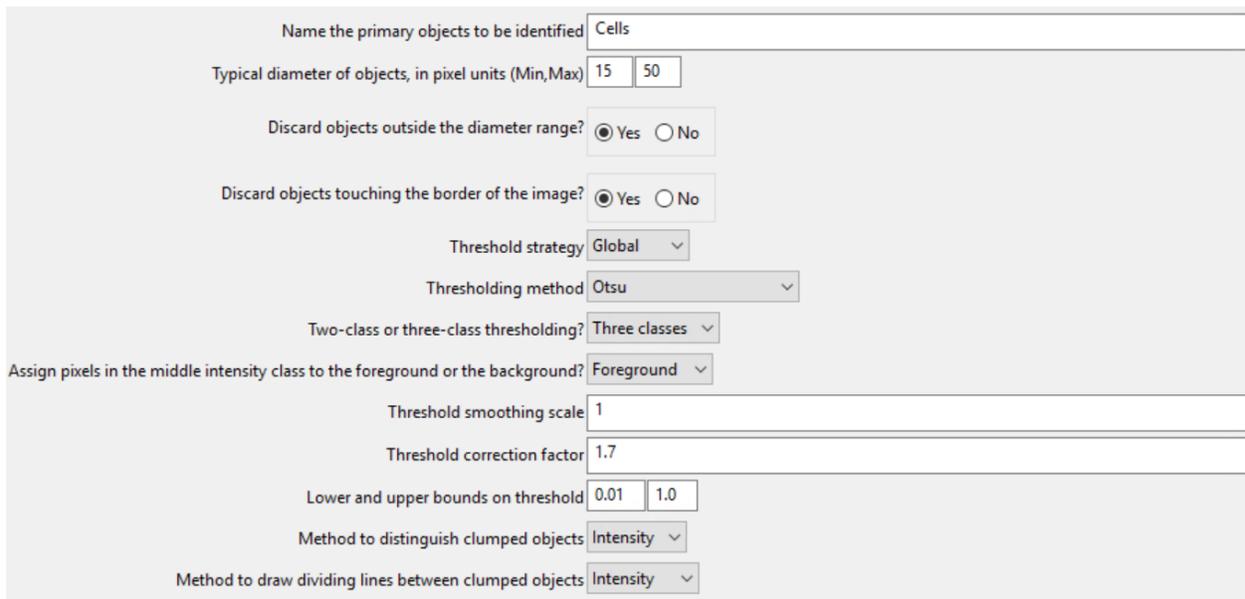
The cell motility assay used the same type of cells as the scratch assay, except they expressed RFP without having to suppress the synthesis of pRB, allowing them to be imaged using red fluorescence imaging. 0.5 mL of DMEM medium containing ~100,000 cells/mL was plated into two wells in a 12-well plate. 0.5 mL of DMEM medium containing ~250,000 cells/mL was plated into another two wells in the same plate. siRNA was transfected into one well of each cell density before incubating at 37°C, 95% humidity, and 5% CO<sub>2</sub> for 36 hours to deplete pRB. The plates were then imaged using red fluorescent microscopy using a 10X at four different coordinates per well. Images were taken every five minutes for the next six hours.

### *Cell Motility Pipeline Optimization*

To maximize the number of cells tracked for each condition, CellProfiler had to be optimized to pick up each cell without counting them as two objects or overlapping cells as one object. Metadata was extracted from the file name of each image for the purposes of frame grouping and analysis. Images were then grouped based on their well and XY location within the well so that CellProfiler could analyze them separately and for the full length of the experiment.

Once images were grouped, CellProfiler automatically converted them to grayscale images, and identified objects by contrasting their intensity against the background. To prevent the software from identifying groups of cells as a single object, the size of several cells was

measured as 15 to 50 pixels to exclude objects outside that range, or any objects touching the border of the image, before using an otsu three class threshold against the background to find the outline of cells; objects in the middle intensity class were considered part of the foreground. The thresholding smoothing scale was set to 1.7, while the correcting factor was set to 1. To distinguish between clumped objects, the intensity strategy was used, which found multiple peaks within a single object to classify them as individual cells.



The screenshot displays the 'Thresholding' settings in CellProfiler. The primary object to be identified is 'Cells'. The typical diameter of objects is set to a range of 15 to 50 pixels. Both 'Discard objects outside the diameter range?' and 'Discard objects touching the border of the image?' are set to 'Yes'. The 'Threshold strategy' is 'Global', the 'Thresholding method' is 'Otsu', and 'Two-class or three-class thresholding?' is set to 'Three classes'. The middle intensity class is assigned to the foreground. The 'Threshold smoothing scale' is 1, and the 'Threshold correction factor' is 1.7. The lower and upper bounds on the threshold are 0.01 and 1.0, respectively. Both the 'Method to distinguish clumped objects' and the 'Method to draw dividing lines between clumped objects' are set to 'Intensity'.

Figure 14) Screenshot of cell tracking settings in CellProfiler.

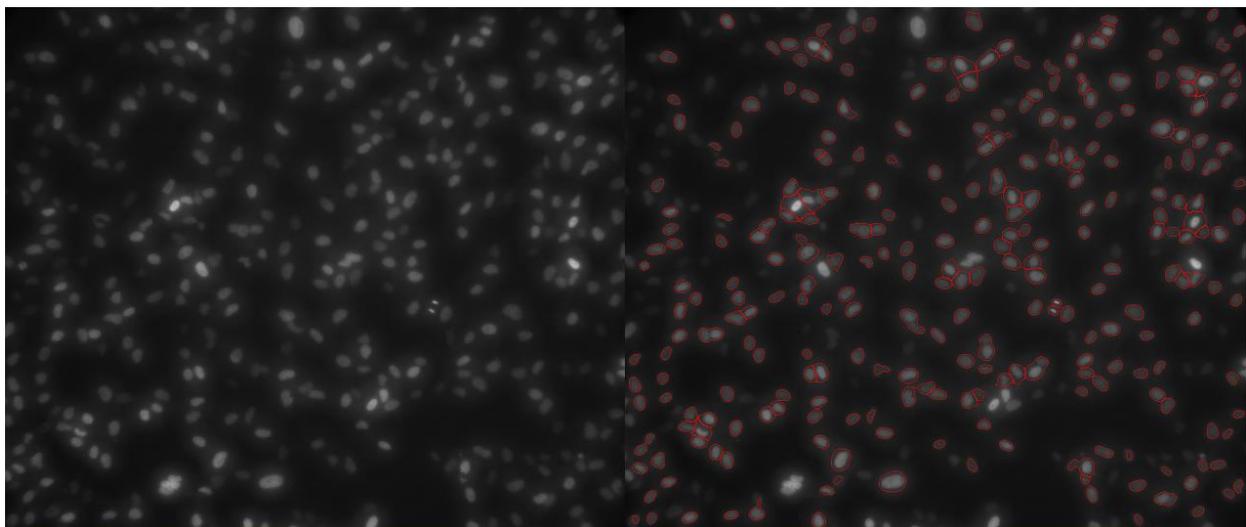


Figure 15) Representative image of CellProfiler outlining identified objects.

To track objects from frame to frame, the overlap method was used, which determined whether objects in one frame were the same as in the next by how much overlap they had. CellProfiler automatically calculates the area taken up by objects as part of this step; however, it does not report that data unless the pipeline requires it to, so this additional step was added for data acquisition.

### *Scratch Assay Analysis*

The images obtained during the scratch assay did not have a high contrast between the cells themselves and the wound; this prevented CellProfiler from distinguishing between the two, even when using an extremely forgiving threshold. The scratch assay was thus analyzed manually using NIS elements. The distance between the two fronts of the scratch at the vertical center of the image was measured using the linear distance tool, which output a pixel distance; comparing this pixel distance to that of the scale bar present in the image allowed the wound size to be measured in microns. This was repeated once for each image taken during the assay, with four images taken per scratch, and six biological replicates per condition.

## **Citations**

Burkhart, Deborah L, and Julien Sage. "Cellular mechanisms of tumour suppression by the retinoblastoma gene." *Nature reviews. Cancer* vol. 8,9 (2008): 671-82. doi:10.1038/nrc2399

Das, A.T., Zhou, X., Metz, S.W., Vink, M.A. and Berkhout, B. (2016), Selecting the optimal Tet-On system for doxycycline-inducible gene expression in transiently transfected and

stably transduced mammalian cells. *Biotechnology Journal*, 11: 71-79.

doi:10.1002/biot.201500236

Dick, Frederick A et al. "Non-canonical functions of the RB protein in cancer." *Nature reviews. Cancer* vol. 18,7 (2018): 442-451. doi:10.1038/s41568-018-0008-5

Ebata, Takahiro et al. "Functions of the Tumor Suppressors p53 and Rb in Actin Cytoskeleton Remodeling." *BioMed research international* vol. 2016 (2016): 9231057.

doi:10.1155/2016/9231057

Engel, B E et al. "THE RETINOBLASTOMA PROTEIN: A MASTER TUMOR SUPPRESSOR ACTS AS A LINK BETWEEN CELL CYCLE AND CELL ADHESION." *Cell health and cytoskeleton* vol. 7 (2015): 1-10. doi:10.2147/CHC.S28079

Ferguson KL, McClellan KA, Vanderluit JL, McIntosh WC, Schuurmans C, Polleux F, Slack RS *EMBO J.* 2005 Dec 21; 24(24):4381-91.

Huang, Paul H et al. "RB in DNA repair." *Oncotarget* vol. 6,25 (2015): 20746-7. doi:10.18632/oncotarget.5234

Hneino M, Bouazza L, Bricca G, Li J, Y, Langlois D: Density-Dependent Shift of Transforming Growth Factor-Beta-1 from Inhibition to Stimulation of Vascular Smooth Muscle Cell Growth Is Based on Unconventional Regulation of Proliferation, Apoptosis and Contact Inhibition. *J Vasc Res* 2009;46:85-97. doi: 10.1159/000142612

James Brugarolas, Roderick T. Bronson, Tyler Jacks; p21 Is a Critical CDK2 Regulator Essential for Proliferation Control in Rb-deficient Cells . *J Cell Biol* 20 April 1998; 141 (2): 503–514. doi: <https://doi.org/10.1083/jcb.141.2.503>

Jeanes, A et al. “Cadherins and cancer: how does cadherin dysfunction promote tumor progression?.” *Oncogene* vol. 27,55 (2008): 6920-9. doi:10.1038/onc.2008.343

Joseph d'Alessandro, Lauriane Mas, Laurence Aubry, Jean-Paul Rieu, Charlotte Rivière and Christophe Anjard. 2018. Collective regulation of cell motility using an accurate density-sensing system. *J. R. Soc. Interface*. 15:20180006 <http://doi.org/10.1098/rsif.2018.0006>

Kamentsky L, Jones TR, Fraser A, Bray M, Logan D, Madden K, Ljosa V, Rueden C, Harris GB, Eliceiri K, Carpenter AE (2011). Improved structure, function, and compatibility for CellProfiler: modular high-throughput image analysis software. *Bioinformatics* 2011/ doi. PMID: 21349861 PMCID: PMC3072555

Liang, C., Park, A. & Guan, J. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc* 2, 329–333 (2007). <https://doi.org/10.1038/nprot.2007.30>

Manning AL, Navarro-Serer B, Childers EP, Hermance NM, Mercadante D. Aurora A inhibition limits centrosome clustering and promotes mitotic catastrophe in cells with supernumerary centrosomes. *Oncotarget*. 2019;10(17):1649-1659. Published 2019 Feb 26. doi:10.18632/oncotarget.26714

Mercadante, D. L., Crowley, E. A., Manning, A. L. Live Cell Imaging to Assess the Dynamics of Metaphase Timing and Cell Fate Following Mitotic Spindle Perturbations. *J. Vis. Exp.* (151), e60255, doi:10.3791/60255 (2019).

Natsuki MATSUSHITA, Sachi MATSUSHITA, Satoshi HIRAKAWA & Shigeki HIGASHIYAMA (2013) Doxycycline-Dependent Inducible and Reversible RNA Interference

Mediated by a Single Lentivirus Vector, *Bioscience, Biotechnology, and Biochemistry*, 77:4, 776-781, DOI: 10.1271/bbb.120917

Narasimha, Anil M et al. "Cyclin D activates the Rb tumor suppressor by mono-phosphorylation." *eLife* vol. 3 e02872. 4 Jun. 2014, doi:10.7554/eLife.02872

Nowotschin, Sonja et al. "Dual transgene strategy for live visualization of chromatin and plasma membrane dynamics in murine embryonic stem cells and embryonic tissues." *Genesis* (New York, N.Y. : 2000) vol. 47,5 (2009): 330-6. doi:10.1002/dvg.20500

Sachdeva, Uma M., and Joan M. O'Brien. "Understanding PRb: toward the Necessary Development of Targeted Treatments for Retinoblastoma." *Journal of Clinical Investigation*, vol. 122, no. 2, Jan. 2012, pp. 425–434., doi:10.1172/jci57114.

Singh, B., Berry, J. A., Shoher, A., Ramakrishnan, V., Lucci, A. "COX-2 overexpression increases motility and invasion of breast cancer cells". *International Journal of Oncology* 26.5 (2005): 1393-1399.

Tsujii M, Kawano S, DuBois RN *Proc Natl Acad Sci USA*. 1997 Apr 1; 94(7):3336-40.

Yamaguchi, Hideki, and John Condeelis. "Regulation of the Actin Cytoskeleton in Cancer Cell Migration and Invasion." *Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1773, no. 5, 2007, pp. 642–652., doi:10.1016/j.bbamcr.2006.07.001.

Ziebold U, Lee EY, Bronson RT, Lees JA *Mol Cell Biol*. 2003 Sep; 23(18):6542-52.