

2011

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Hanna, Paula (2011) "Loss of Cul3 in Primary Fibroblasts," *PSU McNair Scholars Online Journal*: Vol. 5: Iss. 1, Article 15.
<https://doi.org/10.15760/mcnair.2011.25>

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Loss of Cul3 in Primary Fibroblasts

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Citation: Hanna, Paula. Loss of Cul3 in primary fibroblasts. Portland State University McNair Scholars Online Journal, Vol. 5, 2011: pages [39-51]



Loss of Cul3 in Primary Fibroblasts

Summer 2010 McNair Research

**Paula Hanna – McNair Scholar
Jeffrey Singer, PhD – Faculty Mentor
Final Research Paper for the McNair Scholars
Program**

Loss of Cul3 in Primary Fibroblasts

Abstract:

DNA holds the essence of life, and interpreting it can give us clues about cell dynamics. Using retrospective analysis of cell processes, such as protein production and degradation, we try to answer some basic questions, taking advantage of cells being the fundamental units of life. Disruption of these complex processes results in abnormality, dysfunction, and eventually cell death. Cul3 is an essential enzyme that regulates the levels of proteins that in turn regulate transitions between different cell cycle stages. Cell cycle is a description of progressive order of events that lead to cell division. When the Cul3 gene is knocked out in cells of living organisms, some cells undergo apoptosis, or programmed cell death. This research focuses on the role of Cul3 in regulation of cell cycle transitions with a particular interest in how cells that lose Cul3 enter into the apoptotic pathway. The experimental system utilizes a special allele of Cul3 that was constructed in the lab and contains flanking DNA signals for a recombinase called Cre. When expressed, the Cre recombinase knocks out the Cul3 gene. Cells that have lost the gene are then monitored and analyzed for DNA breaks. We introduced the Cre gene using retroviral infection of primary fibroblasts containing the Cul3 special allele. This virus also expresses a special form of green fluorescent protein that allows us to identify cells that lost the Cul3 gene. As part of the experimental design, controls, which are cells that cannot delete the Cul3 gene and viruses that only express the green indicator protein, are included.

Keywords:

Cul3 – apoptosis – primary fibroblasts – degradation – enzyme – knock out – Cre recombinase – retroviral infection

Introduction:

We study cells, taking advantage of them because they are the most basic units of life, to better understand the activity of science and appreciate the complexity of DNA, gene expression, and protein interactions. We do so through a reductionist lens to break them down into their elements and components that we can examine one at a time. Yet, with all our current advances in both science and technology, many aspects of cell dynamics and regulations remain a mystery. For instance, one basic questions we are trying to answer is how cells regulate their growth.

Cells divide in order to proliferate and they do so via cell cycle. Cell cycle is a series of phases or transitions that are driven by cell cycle promoter proteins. One type of such proteins is Cyclins. The cell cycle is also maintained under control by repressor proteins such as Cullins. It is the fine balance between cell cycle promoters and repressors that controls cell cycle progression.

The Singer lab has shown that Cul3 protein, which belongs to the family of repressor proteins, is essential for cell survival. This particular protein degrades cell cycle promoter protein, Cyclin E. A working model that the Singer Lab formulated is shown in Figure 1.

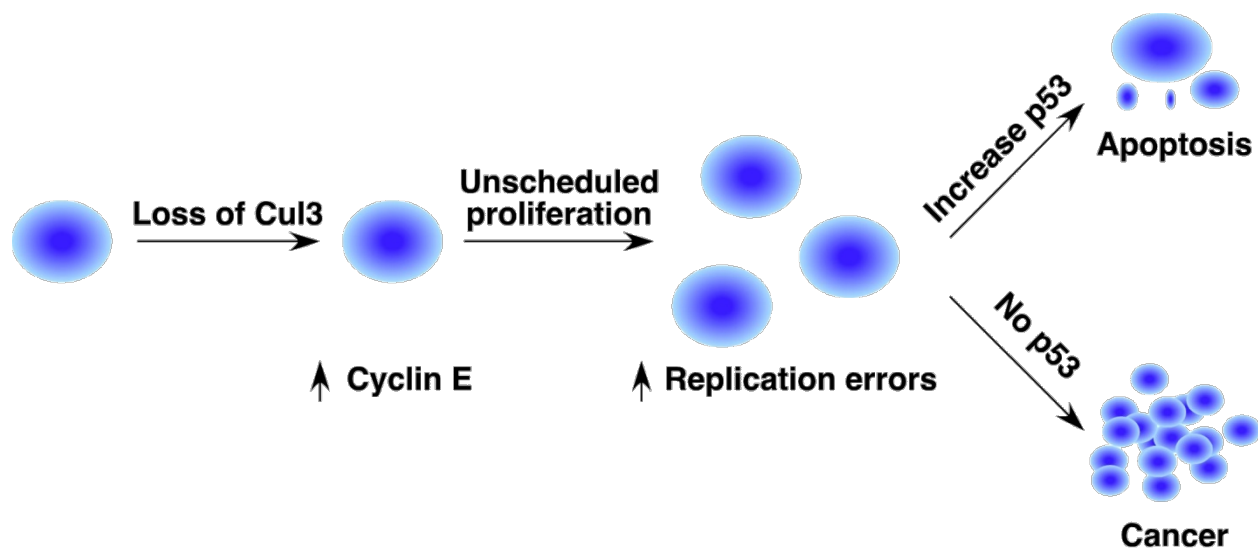


Figure 1. A working model that The Singer Lab formulated after Cul3 Loss in mice cells.

The loss of Cul3 protein leads to dramatic increase in Cyclin E levels which lead to uncontrolled cell division. Consequently, that leads to many replication or duplication errors, and depending on the presence or absence of a certain protein, called p53, apoptosis is induced or the cells become cancerous.

Apoptosis, or programmed cell death is a specific biochemical pathway that leads to the elimination of cells as a cell regulation mechanism. It is through apoptosis that the homeostasis of the body is conserved at all multi-cellular levels. Therefore, when abnormal cell functioning or atypical gene expression of vital proteins occurs, the cell commits suicide in order to eliminate the malfunctioning cells.

The primary goal of this research is to answer the question: Is apoptosis triggered by Cul3 loss which is induced by a gene knockout? And if so how?

Background Knowledge:

Section1: What triggers Apoptosis?

Apoptosis, or programmed cell death, can be triggered by many factors and different stimuli. Internal stimuli like DNA damage or cellular stress is one pathway that leads to apoptosis. The second pathway results from external stimuli, mediated by receptors that receive signals from neighboring cells. These are the most two common factors that lead to cell suicide. The mechanism by which the cell dies varies according to the types of the cell and stimuli. The p53-mediated cell death is perhaps the most familiar mechanism. The p53 gene gets activated, causing the transcription of the p53 protein, a tumor suppressor. This protein induces growth arrest or apoptosis depending on the stress that the cell experiences. A recent study has shown that over expression of Cyclin E protein during the cell cycle is linked to specific p53 mutations, which in turn could induce apoptosis (2). Another study has shown that another protein, the

ubiquitin ligase Itch, regulates apoptosis as well through controlling TXNIP protein level (7). TXNIP, or Thioredoxin interacting protein, is a regulatory factor that plays various roles within the cell. Perhaps the most important role of TXNIP is regulating the REDOX homeostasis within the cell and controlling tumor growth.

Section2: Why do we focus on Cul3?

Cyclin E regulates the G1 to S phase transition of the cell cycle. A recent study has shown that cells in G1 phase are more susceptible to death-inducing activity of wild-type p53 (6). Therefore, it is best to target cells within this transition period in order to gain an understanding of how the loss of Cul3 gene affects Cyclin E levels, and ultimately, cell cycle progression.

Section3: What are the results of Cul3 gene knockout? (3, 5)

Earlier studies in the Singer lab show that the knockout of the Cul3 gene results in early embryonic lethality. Further analysis revealed that Cul3 is not needed for all cellular processes since some cell types survived despite the lack of the gene. This observation is hypothesized to have been caused by the mis-regulation of cell cycle regulators and the “anti-proliferative” signals of p53.

Plan of Attack:

In order to approach the primary question of this research we needed to delete the Cul3 gene from mouse cells called fibroblasts. Figure 2 shows the Cul3 gene in the context of the genome. We used a very modern technology that utilizes a specifically engineered virus to introduce an enzyme called Cre recombinase that deletes Cul3 gene in fibroblasts. After the insertion of Cre recombinase, Cul3 gene is deleted.

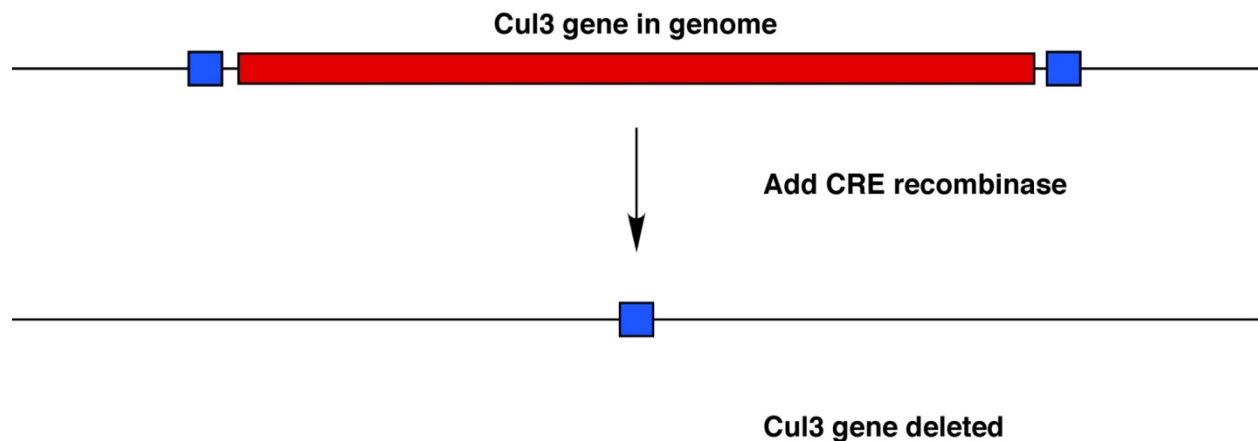


Figure 2. Cul3 gene in the context of the genome. Deletion sites are coded with the blue boxes shown. Notice that Cre recombinase causes the deletion of the whole gene and one deletion site, leaving the other site behind.

For the experimental design, we used the same design used by McEvoy et al. earlier (3). First, we constructed two groups; a control group that has a normal form of Cul3 gene and an experimental group that gets the floxed form. This floxed form has been treated with the Cre-recombinase enzyme that deletes Cul3 gene as shown in Figure 1. All of the floxed fibroblasts contained a transgene that expresses the Cre recombinase when the drug tamoxifen is added. Thus the addition of tamoxifen caused deletion of the Cul3 gene. Cell populations were then divided further into 3 groups; one for each tool we used to monitor Cre recombinase expression. Apoptotic effect was then monitored through terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling, or TUNEL labeling which stains apoptotic cells red.

Materials and Methods:

Plasmid vector isolation and purification. Three plasmid vectors, pBabe Cre, pBabe YFP, and pBabe Cre-eYFP were cultured, isolated and purified. The first two vectors are engineered for holding the Cre and YFP gene inserts, respectively. The third plasmid, however, is designed specifically to hold both gene inserts in order to act as a detecting tool. pBabe Cre expresses the Cre recombinase enzyme, while pBabe YFP expresses YFP protein that gives off green color when hit by UV light under microscope.

Cell Culture. Mouse embryonic fibroblasts, MEFs, were obtained from a stock supplied by the Singer lab.

Phoenix cells transfection and infection. Packaging cells were transfected by pBabe Cre, pBabe YFP, and pBabe Cre-eYFP. They were left to harvest for 2 days and were checked for fluorescence (only the cells that had YFP inserts).

Western blotting. Obtained proteins were separated by gel electrophoresis then transferred to a blotting membrane. This membrane is then incubated and treated with a primary antibody that is specific to our proteins of interest. A secondary antibody was then used to detect the primary antibody, and thereof, our proteins of interest.

Detection of apoptosis, and TUNEL labeling. In order to detect apoptosis, cells were labeled with terminal deoxynucleotidyltransferase-mediated dUTP-biotin that recognizes DNA breaks and nicks. Although this label does not directly indicate apoptosis, it's a good way to identify possible apoptotic cells.

Results and Explanations:

One of the challenges of this research was to monitor the expression of our enzyme, Cre recombinase. First we needed to introduce the Cre recombinase gene into fibroblasts through viral infection. Then we needed to monitor its expression and activity through three different techniques:

- First, the co-expression of YFP, which is a protein that fluorescence green when its exposed to UV light under the microscope,
- Second, Western blot analysis of CRE to ensure its presence, and
- Third, measurement of recombination activity through PCR and gel electrophoresis to ensure activity.

Figures 3, 4 and 5 show the viral cells that expressed Cre, YFP, and both, respectively.

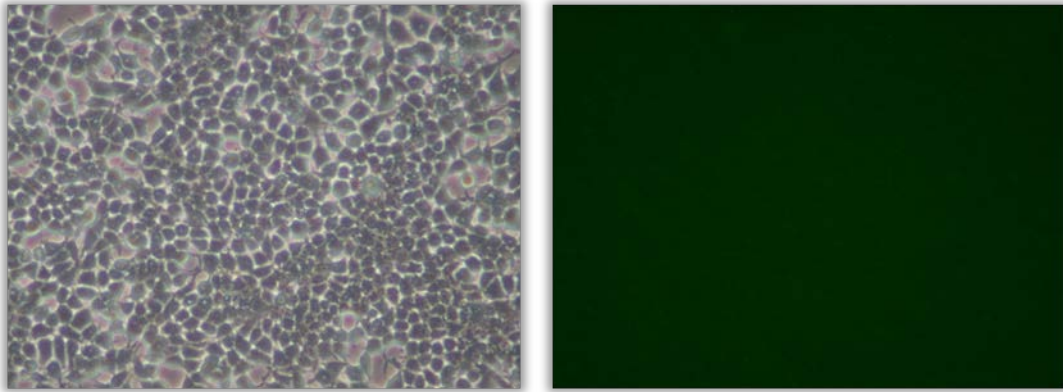


Figure 3. Cre recombinase plate shown under normal microscopy on the left and when UV light is shown upon it on the right.

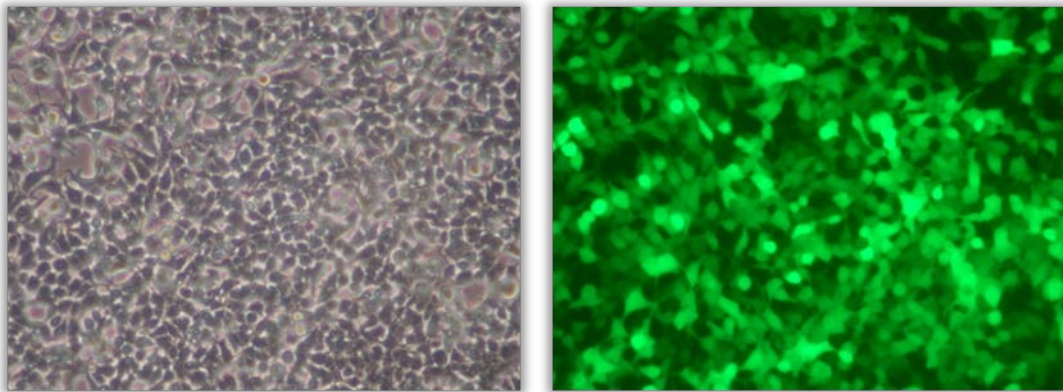


Figure 4. YFP plate shown under normal microscopy on the left and when UV light is shown upon it on the right.

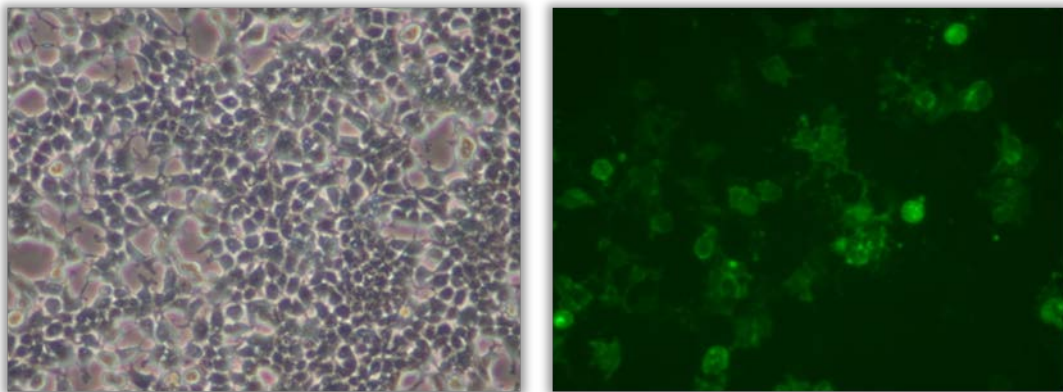


Figure 5. Cre-YFP plate shown under normal microscopy on the left and when UV light is shown upon it on the right.

In Figure 3, there are some growing cells and no fluorescence. That's because Cre recombinase doesn't fluorescence by itself under the microscope, i.e. it needs an indicator. In Figure 4, however, there is the YFP protein expressed in green color when visualized by UV light. Combining the two genes in one viral cell (Figure 5), we see some green fluorescence which is indicative of YFP, and since the cells are making YFP, they are probably making Cre too.

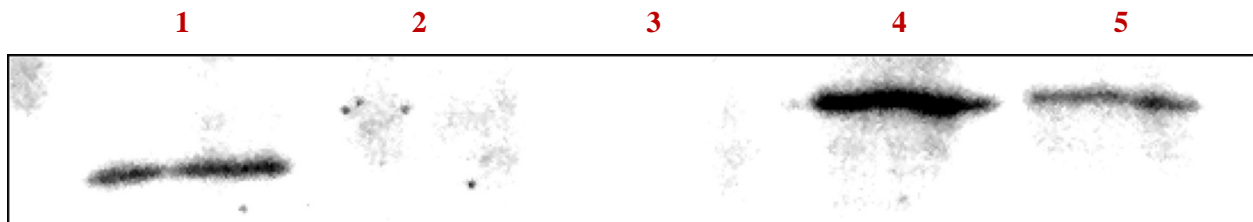


Figure 6. Western blotting. Lane 1: Cre Control. Lane 2: YFP Control. Lane 3: YFP. Lane 4: Cre. Lane 5: Cre and YFP.

Figure 6 shows the second technique used in this experiment, Western blotting. Western blot is an assay of proteins (or a protein blot). It checks for the presence or absence of specific proteins. In the first lane, there is Cre expressed as a control. In the 4th and 5th lanes Cre is expressed in the viral cells that have Cre by itself, and both Cre and YFP, respectively. Note that lanes 2 and 3 are not expressing Cre, simply because they did not receive the Cre plasmid.

Third technique we utilized is checking for the Cre recombination through PCR. PCR stands for polymerase chain reaction which is a machine that amplifies small pieces of DNA.

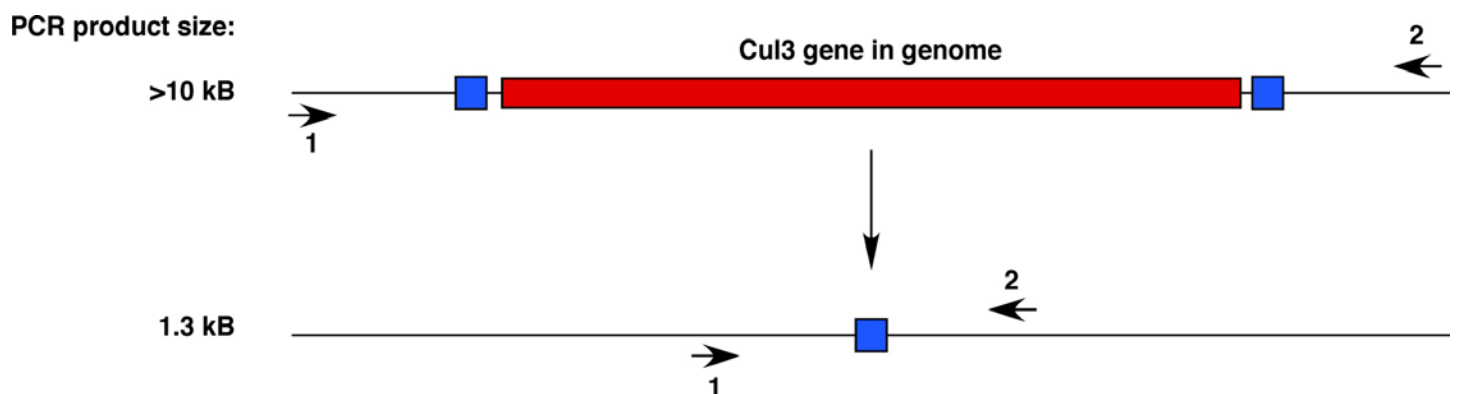


Figure 7. The upper cartoon shows genes before deletion, while the lower cartoon shows genes after treatment with Cre recombinase, i.e. deletion of Cul3 gene.

Figure 7 shows the construct that we expect to get out of PCR machine. The black arrows at the ends are the same PCR primers. The 10 kb size of the top construct is where recombination hasn't occurred. The DNA is too big for the PCR machine to process. When recombination occurs, however, the size is 1.3 kb. That's because the DNA construct is much smaller and can be processed by the PCR.

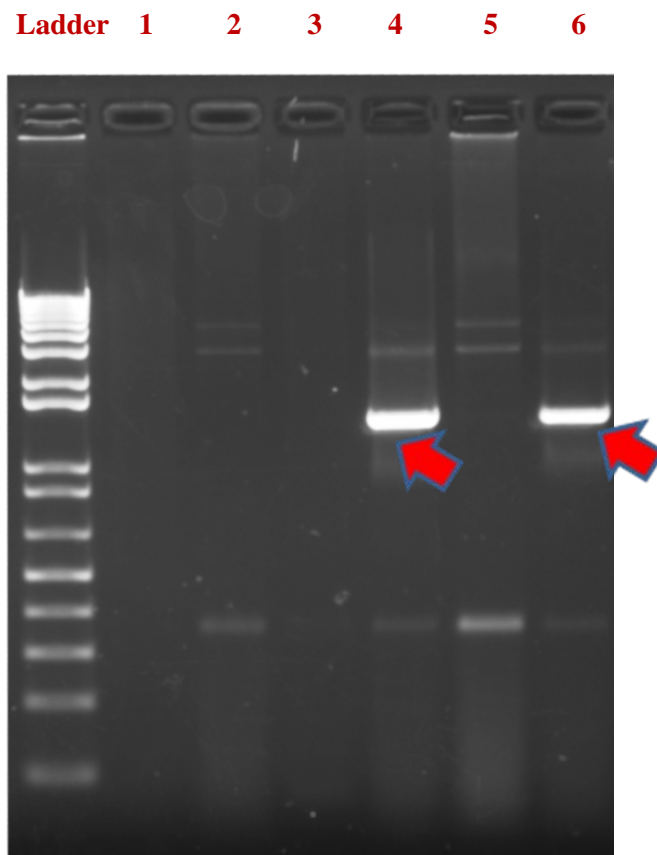


Figure 8. Gel electrophoresis of the DNA pieces amplified through PCR. Lane 1: Wild type fibroblasts + Cre. Lane 2: Wild type fibroblasts + YFP. Lane 3: Wild type fibroblasts + YFP-Cre. Lane 4: floxed fibroblasts + Cre. Lane 5: floxed fibroblasts + YFP. Lane 6: floxed fibroblasts + YFP-Cre.

Figure 8 shows the actual results of running gel electrophoresis, a technique to measure the size of DNA pieces. At the red arrows there are our 1.3 kb bands, shown only when viral Cre recombinase is used.

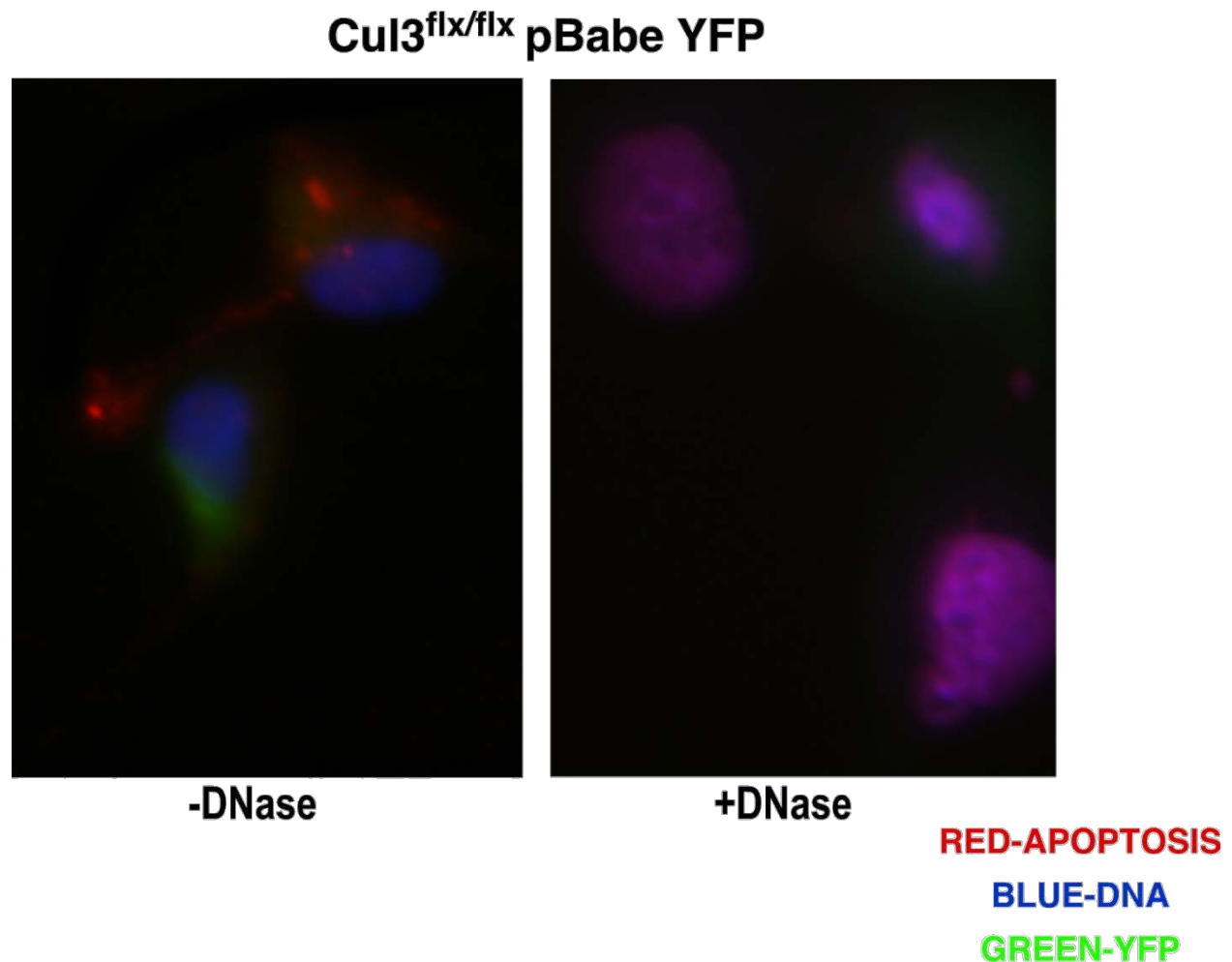


Figure 9. Floxed Fibroblasts infected with YFP under microscope without DNase (left) and with DNase (right).

Lastly, fibroblasts were infected with viral YFP under the microscope to check for apoptosis. Figure 9 shows fibroblasts that were infected with YFP gene. TUNEL assay uses DNase. DNase is a positive control that generates DNA strand breaks to show Apoptosis (RED color). In the left panel, the cells without DNase show clear distinct blue and green colors (DNA and YFP respectively). The red color is just background. In the right panel, however, there is purple color (or mixed red and blue) that indicates apoptosis in nucleus where we expected it (where breakages in DNA occurred). This Figure represents the controls for the experiment.

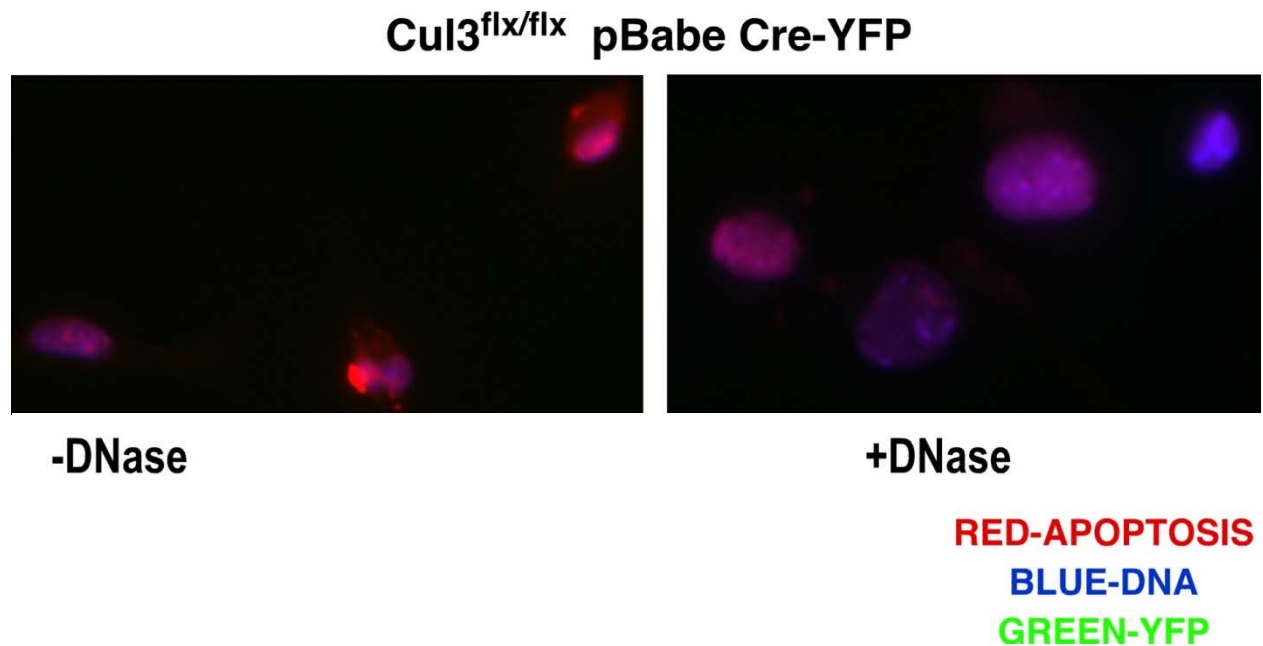


Figure 10. Floxed Fibroblasts infected with Cre-YFP under microscope without DNase (left) and with DNase (right).

Figure 10 shows fibroblasts infected with Cre recombinase and YFP. Both pictures show purple color which looks similar to +Dnase panel in Figure 9. This purple color indicates apoptosis and that is most likely due to the deletion of Cul3. What Figure 10 is interpreted to show is the apoptotic effect triggered by Cul3 loss which is induced by knocking out Cul3 gene.

Future studies:

First our short term goals are to optimize our experiments to allow for maximum efficiency, and analyze apoptotic assays of the mouse cells. Our long term goal is examine the dying cells to look at what could be happening in the background, other than apoptosis, that might have altered the results.

Acknowledgements:

I would like to thank Dr. Jeffrey Singer for his dedicated support and Dr. Justin Courcelle for his careful review of the manuscript.

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