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RUNX1 Mediated Epigenetic Control of Tumor Suppression

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RUNX1 Mediated Epigenetic Control of Tumor Suppression

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Abbreviations

AML – Acute Myeloid Leukemia

BSA – Bovine Serum Albumin

DMSO – Dimethyl Sulfoxide

CAS-9 – CRISPR Associated Protein-9

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

dTAG – Degradation Tag

E-Cadherin – Epithelial Cadherin

EMT – Epithelial Mesenchymal Transition

PAGE – Polyacrylamide Gel Electrophoresis

PBS – Phosphate Buffered Saline

RIPA – Radioimmunoprecipitation Assay

RUNX1 – Runt Related Transcription Factor 1

Snail - SNAI1

Slug – SNAI2

TBST – Tris Buffered Saline and Tween

ZO-1 Zonula Occludens-1/Tight Junction Protein 1

Abstract

Runt related transcription factor 1 (RUNX1) is a tumor suppressor gene well understood for its role in acute myeloid leukemia[1]. It also is known to have a role in breast cancer, however which is a context in which comparatively less is known about its function[2]. Currently, one of the key facts known about the relationship between RUNX1 and breast epithelium cells is that the downregulation of RUNX1 is responsible for an epithelial mesenchymal transition (EMT) in these cells[3]. This is hypothesized to be a crucial first step in tumorigenesis, as it results in cells separating from one another and gaining the ability to move to other parts of the body[4]. The aim of this study was to degrade RUNX1 using the ubiquitin mediated degradation tag (dTAG) system in MCF10A non-cancerous mammary epithelial cells and observe the epigenetic effects on several genes also implicated in EMT. These genes included epithelial cadherin (E-cadherin), and tight junction protein 1 (ZO-1), genes involved in intercellular adhesion, vimentin, an intermediate filament and marker of EMT, and claudin, a gene known to be lost when breast cells become cancerous[5-9]. Additionally, the genes SNAI1 (Snail) and SNAI2 (Slug), and ZEB-1 all of which are transcriptional repressors of E-Cadherin, were examined due to their relationship to E-Cadherin[10, 11]. Two of these proteins, vimentin, and claudin-1, were found to have changes in expression that reflected what would be expected of them when a tumor suppressor gene was degraded. Specifically, vimentin expression increased, and claudin-1 expression decreased. Contrary to what was initially expected however, expression of both ZO-1 and E-Cadherin increased after RUNX1 degradation, while expression of Snail, ZEB-1, and Slug decreased. This suggests that perhaps that while RUNX1 does appear to contribute to EMT, the mechanism may be more complex, and removal of this gene alone may not be enough for a complete transition.

Additionally, there are many pathways that induce EMT, and these may not be the critical components driving EMT upon RUNX1 loss.

Introduction

The human genome is an incredibly complex network of connections that all function to maintain homeostasis and keep a cell working with others as one whole organism. This means however that a small change in a single gene's expression can have a ripple effect throughout the rest of the genome, resulting in a significant phenotypic change. If these effects occur via altering the expression of a gene, they are considered epigenetic[12]. This means they do not affect the genetic code itself, only the degree of expression.[12]. Epigenetic modification is the mechanism by which cells within the same body that all contain the exact same genetic code can have entirely different phenotypes[12]. Different cell types upregulate and downregulate different genes that alter their structure and function. One of the primary ways this occurs is through DNA methylation[13]. This is the addition of a methyl group to DNA, usually functioning to suppress the gene[13]. These kinds of modifications can be just as significant as changes in the genome itself, and there is far less literature on them so studying them is an important part of understanding our genetic makeup.

The protein of interest in this study is Runt related transcription factor 1 (RUNX1). It is a relatively well understood tumor suppressor gene, implicated in multiple different types of cancers[14]. RUNX1 is known primarily for its role in acute myeloid leukemia (AML), in which a translocation of the gene results in the formation of the mutant AML1-ETO protein[1]. Along with this however, a decrease in the expression RUNX1 protein has been implicated in cases of breast

cancer[2], though understanding of the RUNX1's effect in this area is currently far less developed than its effect on leukemia. Despite this, it is currently understood that in healthy mammary epithelial cells, the direct consequence of the loss of RUNX1 is an epithelial mesenchymal transition[4] (EMT). This is the process by which the cells separate from one another and gain the ability to invade other parts of the body[15]. This can be a necessary process, as is the case in embryonic development and wound healing, but if left unregulated, an EMT is a key early step in carcinogenesis[15].

The key tool that will be used to understand the epigenetic effects of this protein is the degron protein degradation system[16]. This is a method of targeting a specific protein for immediate degradation to determine the direct consequences of its absence[16]. This system works by inducing ubiquitination of a protein of interest. When ubiquitin is added to a protein by ubiquitin ligase, it signals to the cell to bring the protein to a proteosome for degradation into its constituent amino acids[17]. This is a normal mechanism in most cells, used to break down unusable proteins, but it can be exploited to target a specific protein in the lab for degradation. This is done by introducing a sequence known as $FKBP12^{F36V}$ into the protein's genetic code by a CRISPR-Cas9 protocol[16]. Ubiquitin mediated protein degradation is then activated by a small molecule designated degradation tag (dTAG), that can form a complex with both $FKBP12^{F36V}$, and ubiquitin ligase[16]. This results in the ubiquitin mediated degradation of the protein of interest by the cell's own machinery.

This degradation system makes it possible to observe the effects the removal of a specific protein confers on the rest of the genome. This is relevant to this study, because it becomes possible to observe some of the effects RUNX1 has on the genome that allow it to function as a tumor suppressor. Because of this it becomes useful to observe changes in expression of other

proteins in the cell, specifically those implicated in epithelial cell adhesion, since it is likely that these proteins are involved in the mechanism by which RUNX1 removal induces EMT.

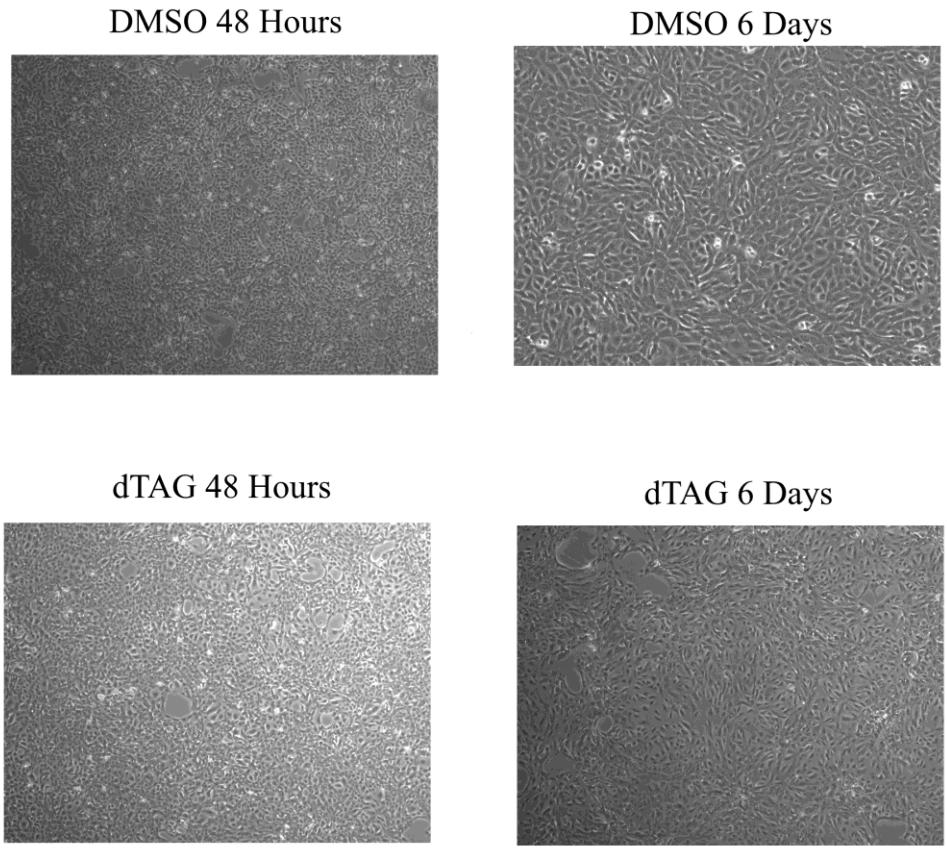


Figure 1 RUNX1 1Degradation in MCF10A cells results in a mesenchymal-like morphology. Cells treated with dTAG do not have as much adherence to one another as normal epithelial cells do, which indicates a mesenchymal phenotype.

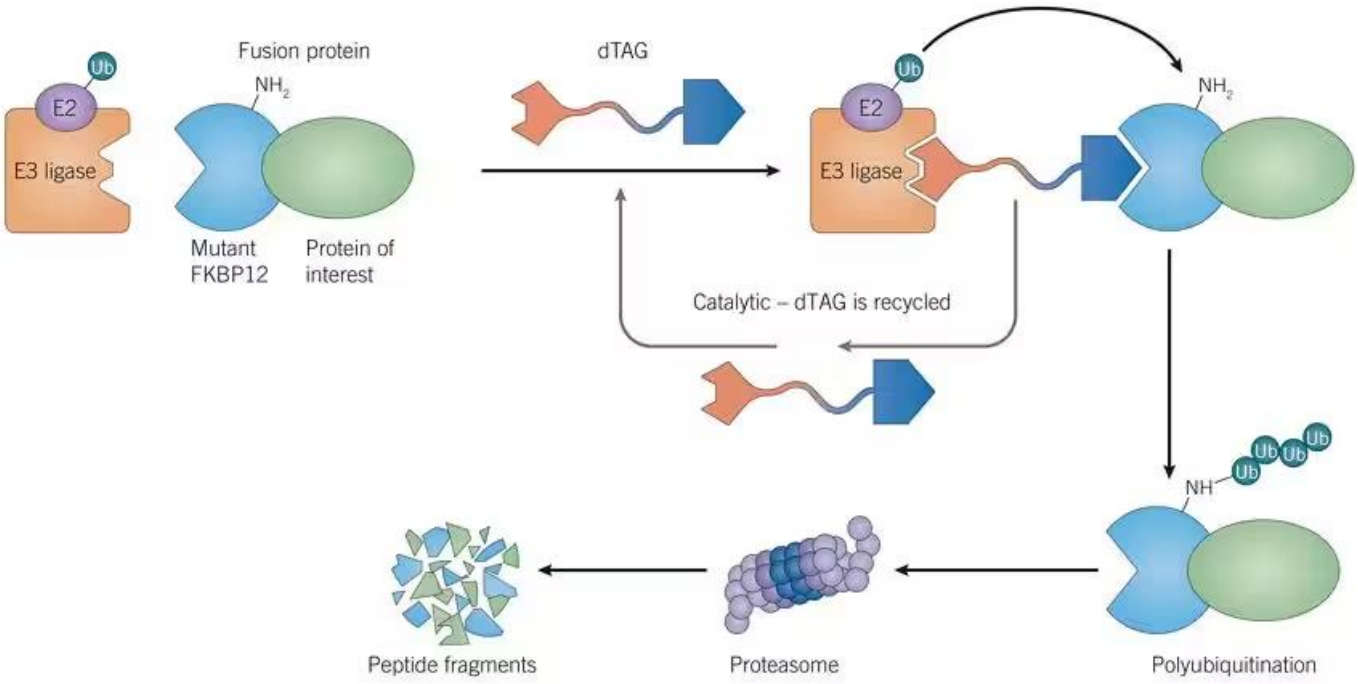


Figure 2 dTAG Mechanism of action. The dTAG molecule functions as a ligand for both the mutant FKBP12, and ubiquitin ligase. If FKBP12 is added to the protein of interest via CRISPR-CAS9, it will be ubiquitinated and targeted for degradation in a proteasome. (Tocris.com)

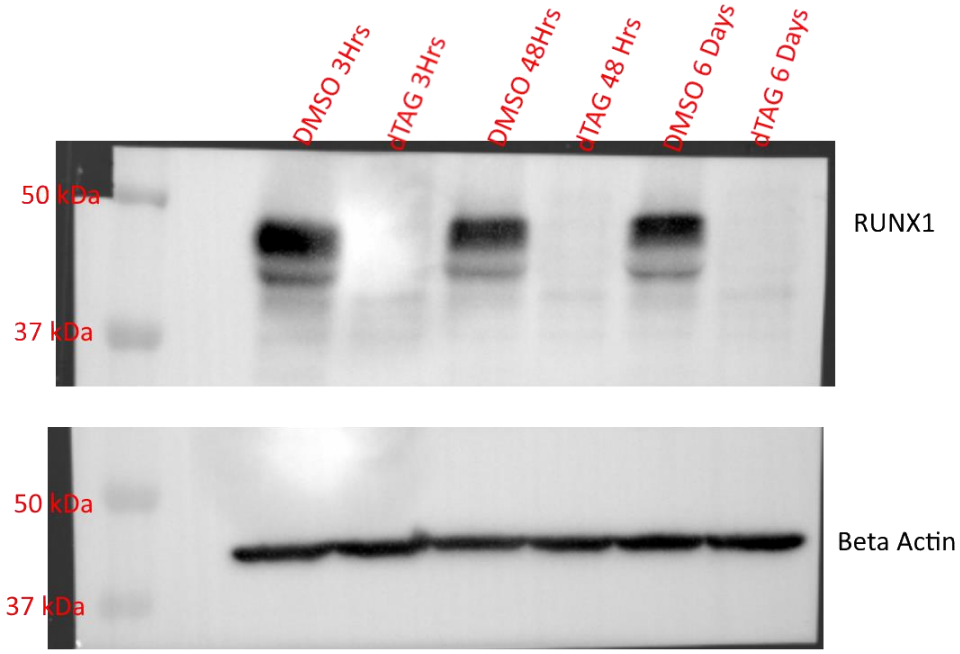


Figure 3 western blot gel 1. Stained for RUNX1, and β-actin as a loading control, as well as molecular weight standards.

One key protein that is likely to play a role in this transition is Epithelial Cadherin (E-Cadherin). This is another tumor suppressor gene, since it has its primary function in forming the junctions between cells that keep them adherent to one another[5]. Because of this, it was expected that it would be downregulated when RUNX1 is removed[5]. Lacking this key protein would be a potential explanation for why the cells would become less adherent to one another, as is the case in an EMT. In addition, transcriptional repressors of E-Cadherin may also undergo changes in expression during this experiment. Three of these genes are known as Slug, Snail[10], and ZEB-1[18]. Because these genes are connected directly to E-Cadherin expression, there is a good chance that their activity may be involved in any pathway that results in a change in expression for E-Cadherin, which, again would likely be related to the EMT. This would mean that an increase in expression for each of these genes in the absence of RUNX1 would be hypothesized, since they are repressing the gene responsible for maintaining cell adhesion.

Another protein that may be implicated in this transition is Tight junction protein 1 or Zonula Occludens-1 (ZO-1). This is yet another protein involved in the formation of the junctions between epithelial cells[9]. It at first may appear like it would be expected to decrease in transcription in the absence of RUNX1, however this may not be entirely the case as ZO-1 is known to relocate during an EMT[7]. In healthy cells it is usually found embedded in the membrane, however when an EMT occurs, it migrates into the cell's cytoplasm and nucleus, likely playing a role in transcriptional regulation[7]. Because of this, we may observe a wider range of effects on it when RUNX1 is degraded compared to E-Cadherin, since it has a far less understood function when it moves further inside the cell. An increase in expression could still occur, which would indicate that this gene can have two opposite functions based on where it is located.

Claudin-1 functions similarly to ZO-1 in that it also composes part of the tight junctions between epithelial cells[19]. This protein is expressed in healthy breast epithelial cells, though it is known to significantly decrease or even cease expression entirely in breast cancer cells[6]. This gives two reasons that RUNX1 degradation should cause a decrease in its expression. Both since a decrease in expression is associated with breast cancer, and since it is involved in cell adhesion. Similar to E-Cadherin, the loss of an adhesion protein is a likely mechanism by which the previously observed EMT may occur.

Finally, vimentin is a useful marker as to whether a cell is undergoing an EMT[8]. It is an intermediate filament that composes the cytoskeleton of the cell, but it is found primarily in mesenchymal cells, and not epithelial cells[8]. This means that an increase in expression of this gene would be expected, as it would be a very strong indicator of a mesenchymal phenotype, confirming that the expected transition is likely occurring.

The link between RUNX1 and breast cancer is not very well understood, so this presents an opportunity to further our knowledge about how this disorder occurs. The primary goal of this study will be to determine what effects occur on the expression of the previously mentioned genes as a result of RUNX1 degradation. This will tell a lot about what specifically RUNX1 regulates. If these epigenetic regulators can then be traced back to the EMT, that would bring our understanding much closer to getting the full picture on how breast tumorigenesis occurs. Similarly, because RUNX1 is a tumor suppressor, and we are observing its removal from noncancerous cells, the observed epigenetic modifications could show what specifically RUNX1 is doing to allow the epithelial cells to maintain cohesion with their neighboring cells. This is likely a key naturally occurring guardrail against tumorigenesis, so understanding how it works could potentially be critical to understanding the normal functions disrupted within cells by

cancer. These are all critical factors that could lead to better treatments as well as improved diagnostic and preventive capabilities for this incredibly harmful disease.

Methods

Cell Culture

The process began with MCF10A mammary epithelial cells at 100% confluence. Six groups of about one million of these cells were plated in a 100mL dish (Corning) along with 10mL media composed of 500ml Dulbecco's Modified Eagle Medium: F12 (Corning) with 25mL 5% horse serum (Gibco), 500 µL of 10µg/ml human insulin, 100 µL of 20ng/ml human epidermal growth factor (Pepro Tech), 50µL of 100ng/ml cholera toxin, 5mL PenStrep (Sigma Aldrich) and 5mL L glutamine (Caisson Labs). These cells were allowed to grow normally for 24 hours before any treatment was performed. After a day of unimpeded growth, cells were treated either with a dimethyl sulfoxide (DMSO) blank or 500nM dTAG (Tocris Bioscience). This initial date of treatment was considered day 0, with cells intended to be harvested after 3 hours, on day 2, and on day 6. These intervals were chosen to give an idea of the immediate, intermediate, and long-term consequences of the degradation of RUNX1. Cells were then harvested and analyzed via western blot 3 hours after initial treatment, the procedure for which is described in the next section. On day 2, or when about 8 million cells were present, yet another harvest was performed using the exact same method described below. In addition, at this time, cells remaining to be harvested after 6 days were split once again into new plates of about 500k cells. They were then allowed to divide normally again, until day 4, at which point they were treated once more with their respective treatment (dTAG or DMSO) and given 10mL fresh

media. Finally, on day 6, the last of the samples were harvested to be used in further experiments.

Cell Harvest and Western Blot

This is the test performed on cells after their harvest at the respective 3 hour, 2 days and 6 days of treatment to determine the change in protein expression[20]. Firstly, cells were centrifuged for 3 minutes at 1500rpm and washed with phosphate buffered saline (PBS). Harvested cells were then treated with 400µl of 1x radioimmunoprecipitation assay buffer (RIPA) with 1 protease inhibitor pellet (Thermo Fischer), and 1µL/mL MG132 proteasome inhibitor. The samples were then sonicated (QSonica Q700), two steps that together destroy the cell membrane and expose the cell's contents through chemical and physical means, respectively. The now lysed samples were loaded onto an 8% polyacrylamide gel and separated using polyacrylamide gel electrophoresis (PAGE). The resulting gel was then transferred to a PVDF membrane (Thermo Scientific) overnight, allowing for immunoblotting. The membranes were blocked using a 25mL a solution of 5% bovine serum albumin (BSA) or 5% milk which they were rinsed in for an hour before being washed 3 times for 5 minutes with a solution of tris buffered saline and tween (TBST). Next, the membrane was washed in its respective primary antibody (Cell Signaling) overnight. The type of antibody used to blot for each protein can be found in table 1. The next day, after removal of the primary antibody and another 3 washes for 5 minutes with TBST, the secondary antibody was added (Cell Signaling). Again, the specific antibodies used can be found in table 1. Blots were performed for each desired protein of interest mentioned previously (E-cadherin, ZO-1, ZEB-1, Snail, Slug, claudin and vimentin), as well as β-actin, which was used as a reference point with which to compare changes in expression. β-actin was chosen for this reference point as it is almost always expressed constitutively This makes it a consistent marker

of the cell's protein expression to measure more variable proteins against [21]. The membrane was left in this solution for one hour before a final wash with TBST. The membrane was then stained with LumiGLO (Cell Signaling) and imaged using a Chemidoc XRS+ imaging system (Bio-Rad). Analysis of these samples was performed using FIJI software to quantify the resulting western blot images. The values obtained from Fiji were normalized with those of β-actin, which allowed for the quantitative comparison of expression for each protein of interest in cells both with and without treatment.

Protein of Interest	Primary Antibody	Secondary Antibody	Supplier
E-Cadherin	Rabbit monoclonal antibody	Goat anti rabbit w/ horseradish peroxidase	Cell Signaling, Catalogue #9782
Snail	Rabbit monoclonal antibody	Goat anti rabbit w/ horseradish peroxidase	Cell Signaling, Catalogue #9782
Slug	Rabbit monoclonal antibody	Goat anti rabbit w/ horseradish peroxidase	Cell Signaling, Catalogue #9782
ZEB-1	Rabbit monoclonal antibody	Goat anti rabbit w/ horseradish peroxidase	Cell Signaling, Catalogue #9782
ZO-1	Rabbit monoclonal antibody	Goat anti rabbit w/ horseradish peroxidase	Cell Signaling, Catalogue #9782
Claudin-1	Rabbit monoclonal antibody	Goat anti rabbit w/ horseradish peroxidase	Cell Signaling, Catalogue #9782
Vimentin	Rabbit monoclonal antibody	Goat anti rabbit w/ horseradish peroxidase	Cell Signaling, Catalogue #9782
β-actin	Mouse monoclonal antibody	Goat anti mouse w/ horseradish peroxidase	Cell Signaling, Catalogue #3700

Table 1 Summary of antibodies used for western blotting.

Results and Discussion

The primary objective of this study was to characterize the effects of RUNX1 loss in breast cancer cells. While somewhat consistent patterns were observed in the expression of most of the observed genes, many of the findings were not consistent with what was hypothesized. This means that the observed results potentially challenge the notion that RUNX1 loss is enough on its own to completely result in an EMT.

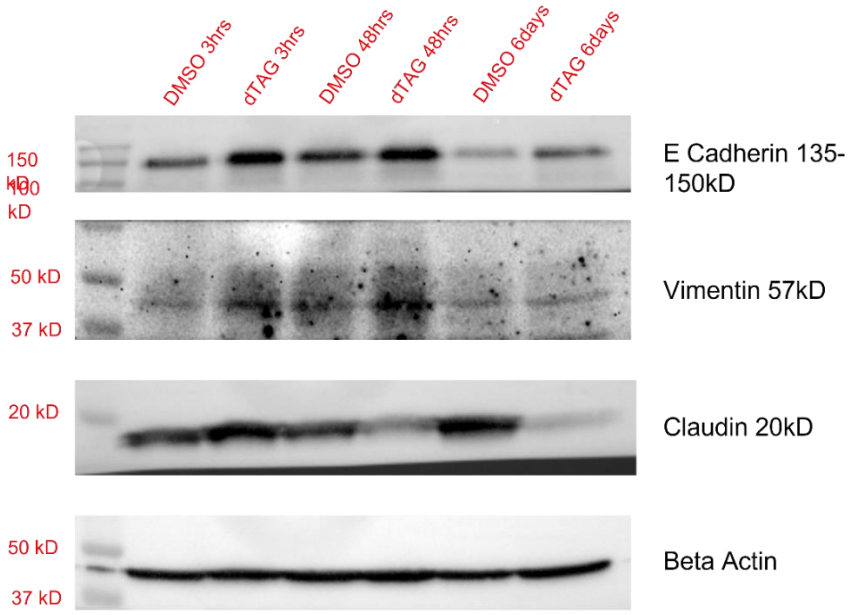
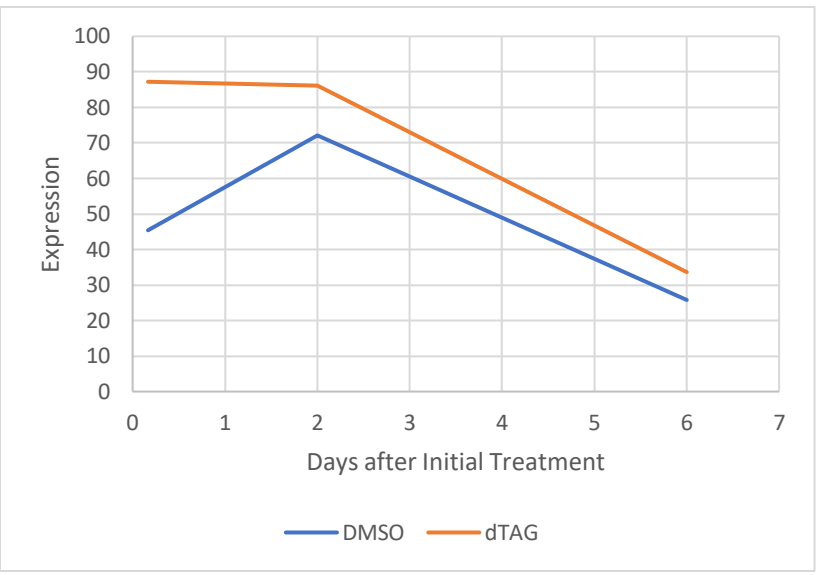


Figure 4 western blot gel 4. Stained for E-cadherin, vimentin, claudin, and β-actin as a loading control, as well as molecular weight standards.



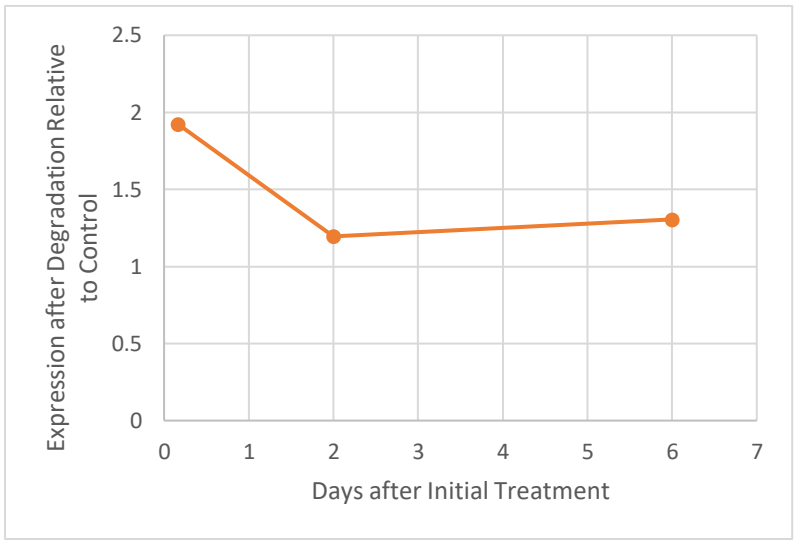
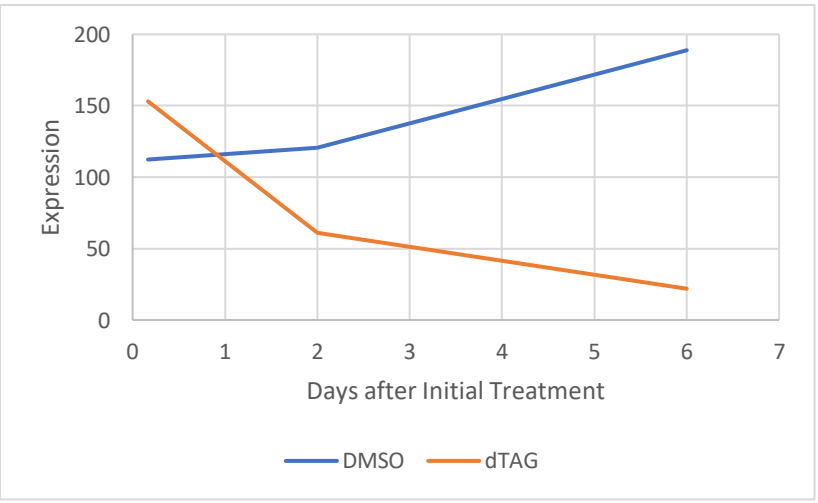


Figure 5 E-cadherin expression after RUNX1 degradation. Western blot performed with rabbit, followed by goat anti rabbit antibodies. Western blots were quantified using Fiji software. Expression of E-cadherin both with and without dTAG was calculated relative to β-actin, and values shown above were obtained by calcuating the change in expression between both β-actin adjusted values relative to one another for each point in time.



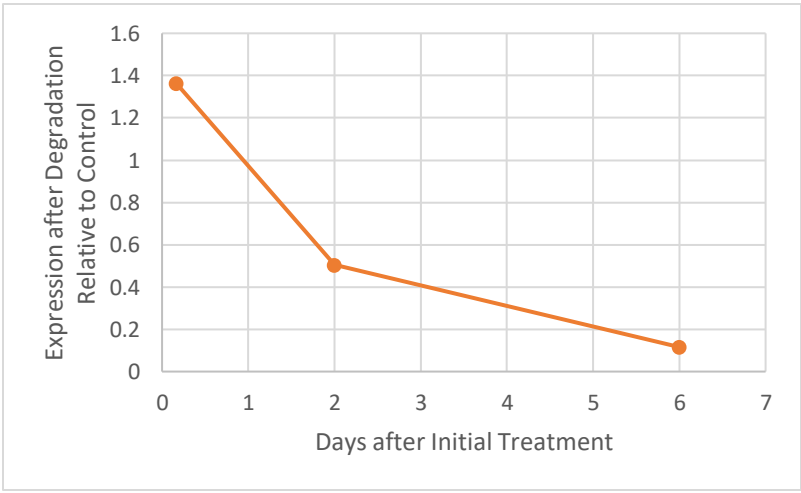
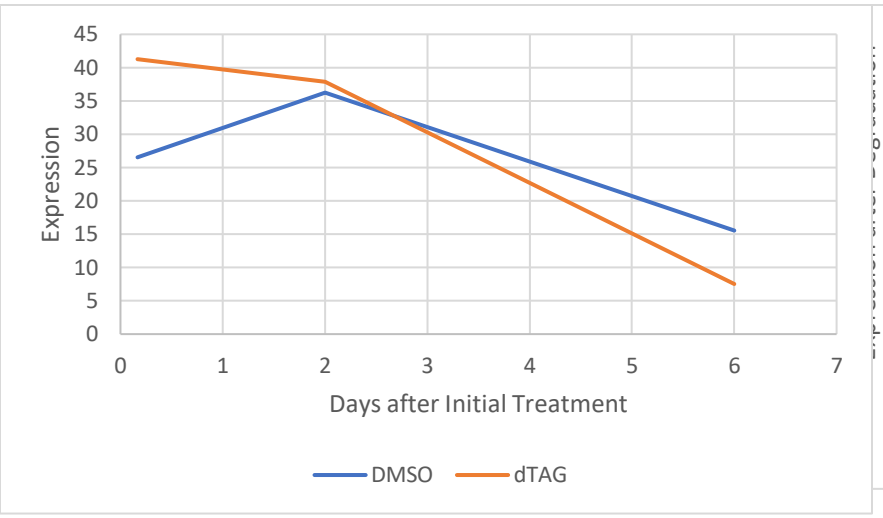


Figure 6 Claudin-1 expression after RUNX1 degradation. Western blot performed with rabbit, followed by goat anti rabbit antibodies. Western blots were quantified using Fiji software. Expression of claudin-1 both with and without dTAG was calculated relative to β-actin, and values shown above were obtained by calcuating the change in expression between both β-actin adjusted values relative to one another for each point in time.



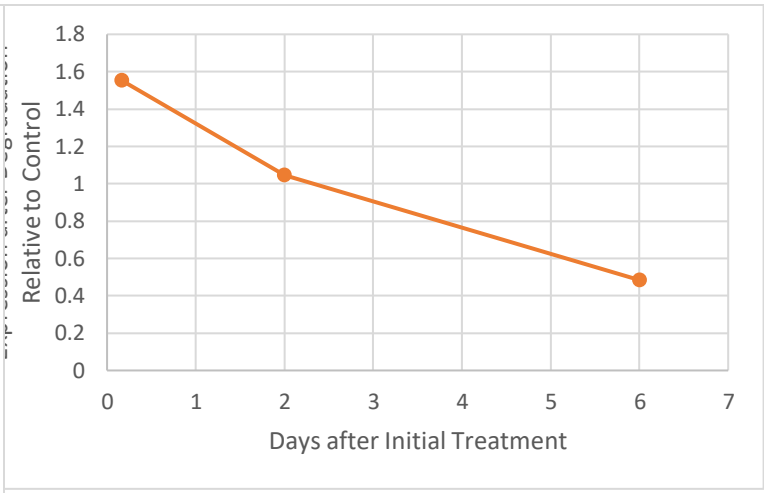


Figure 7 Vimentin expression after RUNX1 degradation. Western blot performed with rabbit, followed by goat anti rabbit antibodies. Western blots were quantified using Fiji software. Expression of vimentin both with and without dTAG was calculated relative to β-actin, and values shown above were obtained by calcuating the change in expression between both β-actin adjusted values relative to one another for each point in time.

The proteins observed in figure 4 together give a picture of how the cells show signs of EMT, while not appearing to completely transition. Firstly, vimentin, a protein indicative of a mesenchymal phenotype was indeed found to increase in expression immediately after dTAG treatment. This was only up until 6 days however, at which point it did fall back down to the baseline DMSO expression level. The second gene to show a change in expression that was consistent with what was hypothesized was Claudin-1. It was found to immediately increase in expression after 3 hours, but with time it showed a significant gradual decrease, consistent with its depletion observed in cases of breast cancer. Together, these two genes indicate that these cells did indeed likely undergo some level of EMT after RUNX1 degradation. The eventual

decrease in vimentin expression observed could however indicate that a full transition does not occur in every cell in the sample after RUNX1 knockdown.

The main protein that gave unexpected results in this study was E-Cadherin. As a tumor suppressor and cell adhesion protein, we would expect it to decrease in expression when RUNX1 is degraded. This is why it is so strange that it had a distinct and consistent increase in expression compared to normal cells at all timepoints. There are a couple of potential explanations for this. Loss of E-cadherin is known to contribute to EMT, so it is possible that some of the cells less affected by dTAG may have expressed higher E-cadherin as a reactionary mechanism when their neighbors began undergoing transition. A second possibility is that, again, what is observed is not a complete EMT and instead, RUNX1 loss is only one part of what is needed for a complete transformation of the cell. In this experiment we may only have been able to initiate the process of transition, but more changes in the genome may need to occur before we observe the hypothesized effects.

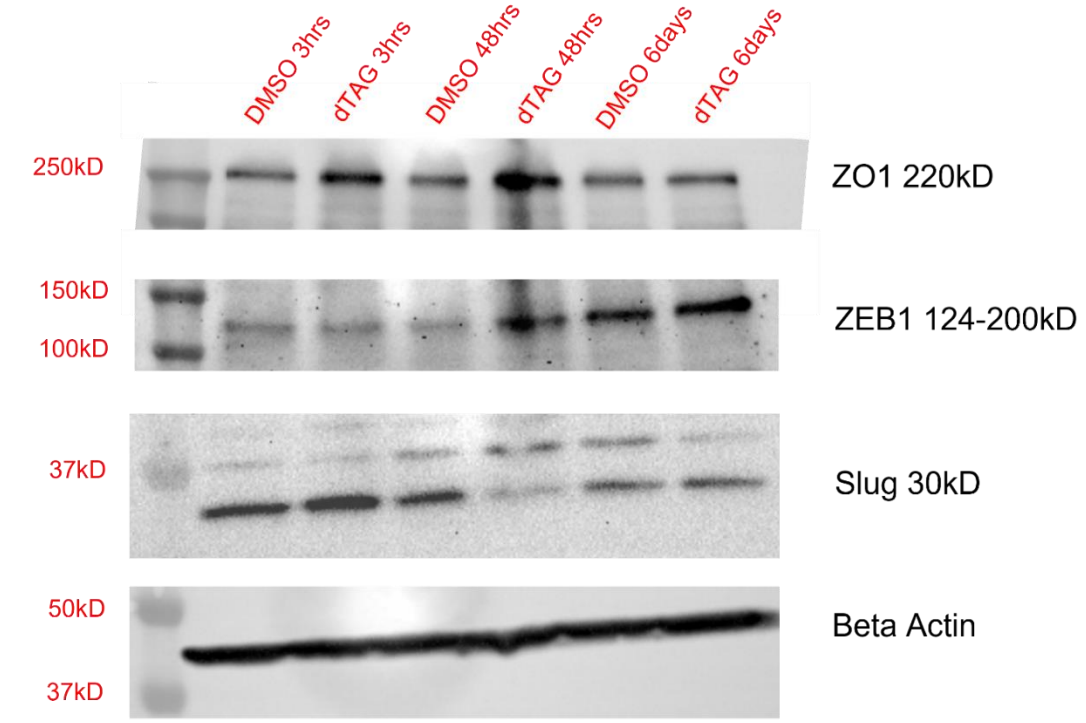


Figure 8 western blot gel 2. Stained for ZO-1, ZEB1, Slug, and β-actin as a loading control, as well as molecular weight standards.

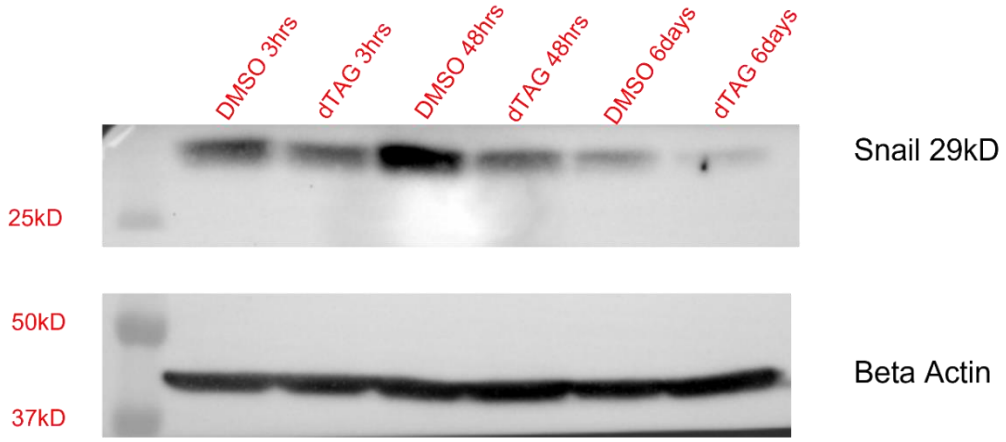
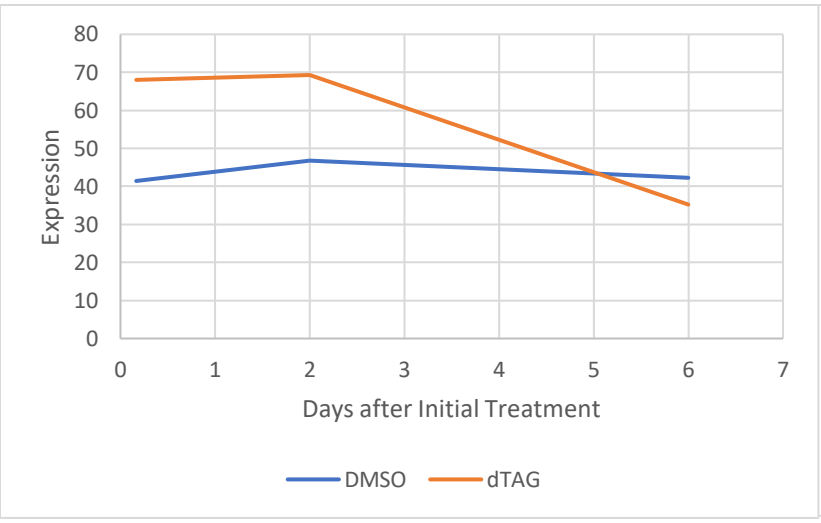


Figure 9 western blot gel 3. Stained for Snail, and β-actin as a loading control, as well as molecular weight standards.



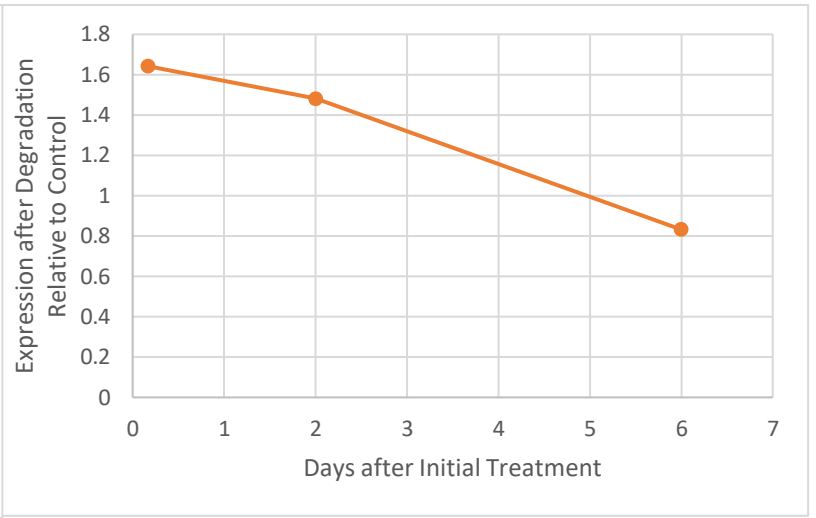
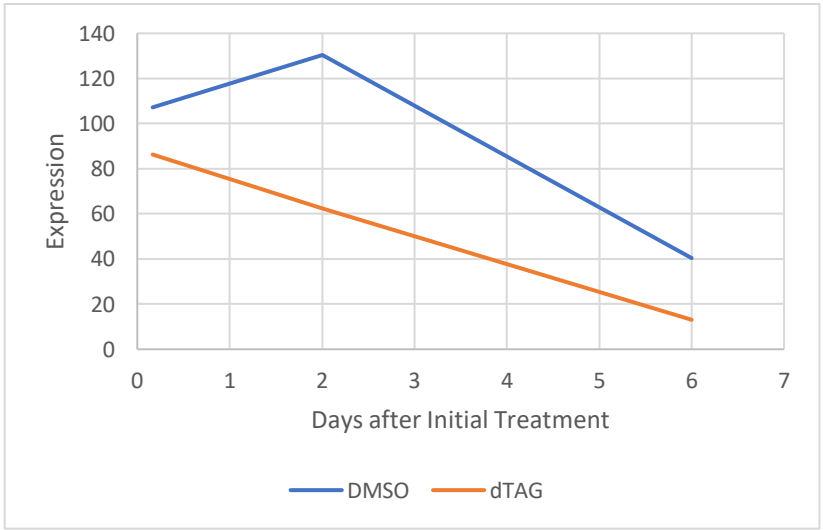


Figure 10 ZO-1 expression after RUNX1 degradation. Western blot performed with rabbit, followed by goat anti rabbit antibodies. Western blots were quantified using Fiji software. Expression of ZO-1 both with and without dTAG was calculated relative to β-actin, and values shown above were obtained by calcuating the change in expression bet ween both β-actin adjusted values relative to one another for each point in time.



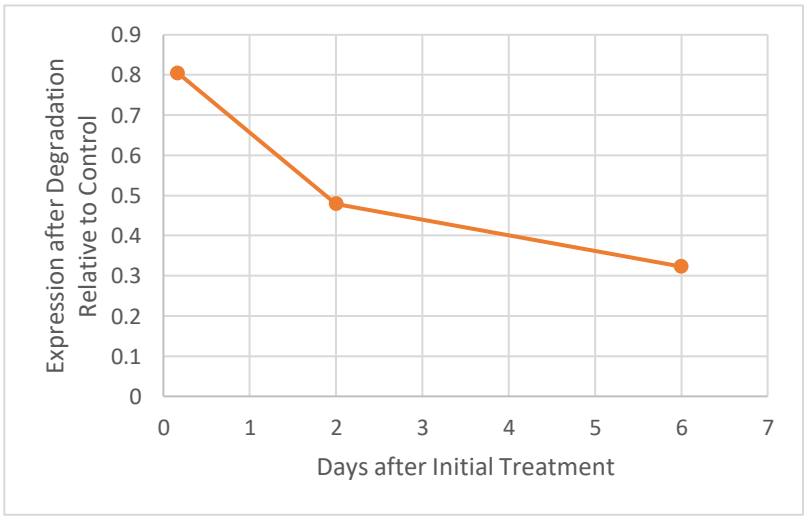
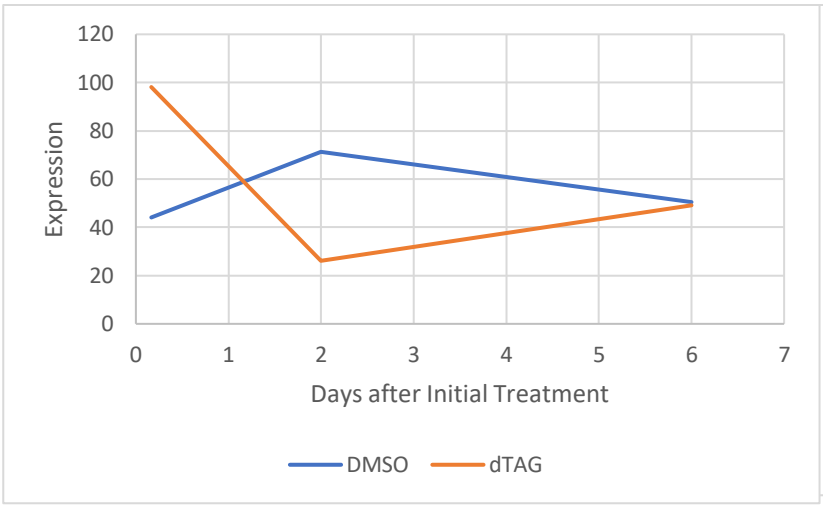


Figure 11 Snail expression after RUNX1 degradation. Western blot performed with rabbit, followed by goat anti rabbit antibodies. Western blots were quantified using Fiji software. Expression of Snail both with and without dTAG was calculated relative to β-actin, and values shown above were obtained by calcuating the change in expression between both β-actin adjusted values relative to one another for each point in time.



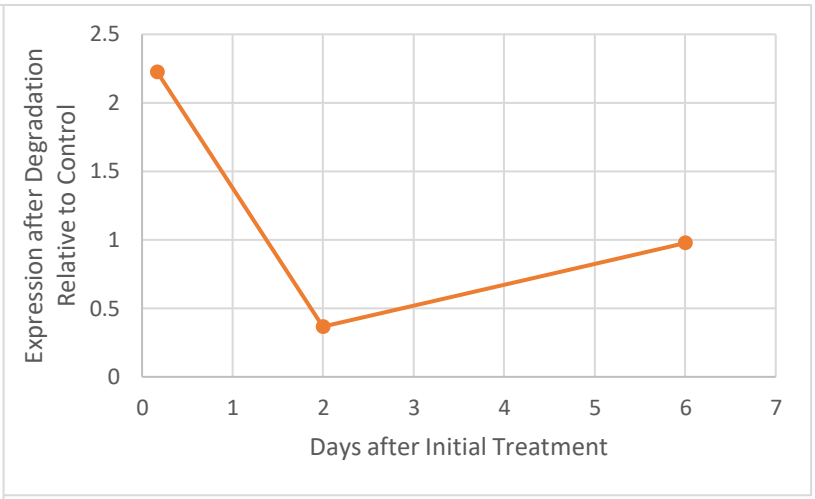
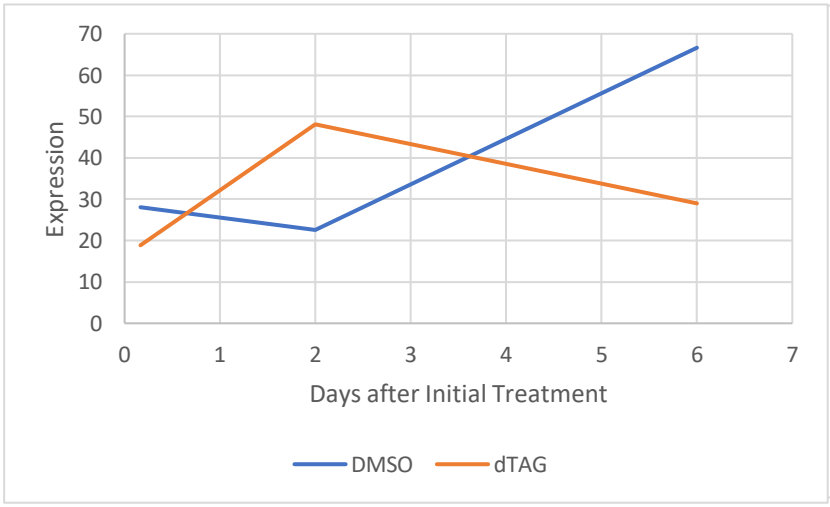


Figure 12 Slug expression after RUNX1 degradation. Western blot performed with rabbit, followed by goat anti rabbit antibodies. Western blots were quantified using Fiji software. Expression of Slug both with and without dTAG was calculated relative to β-actin, and values shown above were obtained by calcuating the change in expression between both β-actin adjusted values relative to one another for each point in time.



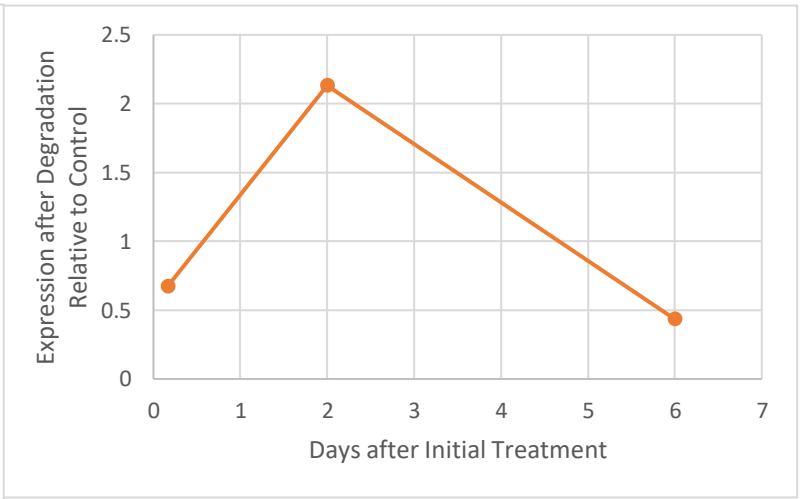


Figure 13 ZEB-1 expression after RUNX1 degradation. Western blot performed with rabbit, followed by goat anti rabbit antibodies. Western blots were quantified using Fiji software. Expression of ZEB-1 both with and without dTAG was calculated relative to β-actin, and values shown above were obtained by calcuating the change in expression between both β-actin adjusted values relative to one another for each point in time.

Another odd finding was that the regulators of E-cadherin, Snail and Slug both appeared to be themselves downregulated when RUNX1 was removed. This again goes contrary to what was expected, as these proteins should have increased expression as repressors of E-Cadherin. Additionally, slug at first showed the expected increase in expression in the absence of RUNX1 before significantly decreasing and then returning about to normal, while Snail was consistently expressed less at every time point when RUNX1 was degraded. Other than ZEB-1, which did not have a consistent pattern in its change in expression, the results obtained from its repressors are consistent with the findings for E-cadherin. This indicates that it is likely that the upregulation of E-cadherin caused by RUNX1 knockdown was a result of Snail and Slug downregulation. This is still unusual, however. As previously mentioned, each of these genes was expected to have an opposite reaction than was observed. This again, may be explained by the EMT being a complex process that RUNX1 loss is only a single part of. It is possible that if given more time, or if more changes to the genome were to occur, we may see some more consistent results with a more pronounced EMT.

Yet another interesting finding was the result of the western blots for ZO-1. This cell junction protein too was found to increase in expression rather than decrease, before eventually decreasing to slightly lower than normal values. This could be an initial compensation for the loss of RUNX1, explaining the initial increase and subsequent decrease in expression. This could also be explained by the alternate role that ZO-1 is known to take on during the EMT. This is because it migrates into the nucleus and has a relatively uncharacterized effect on transcription[7]. Perhaps its activity as a transcription factor is being upregulated, rather than its activity in adhesion. This could potentially mean that RUNX1 functions to keep ZO-1 on the membrane, and when it localizes in the nucleus, it in some way contributes to the EMT, rather than

preventing it. This leads to the question of whether or not ZO-1 itself has an alternate effect on EMT if it moves to the nucleus, or if it functions only in tumor suppression and adhesion.

Conclusion

The findings of this project, while they initially may seem somewhat unusual, have served to emphasize the complexity of the genome. While RUNX1 is strongly tied to EMT, the results with respect to E-Cadherin and its repressors suggest one of two things. Either more damage must be done to the genetic code for a complete transition to occur, or the cells have some sort of defensive mechanism at play to attempt to upregulate E-Cadherin when an undesired mesenchymal transition initially begins to occur. Because of this, it would be useful for future studies to attempt to identify other factors that together with RUNX1 degradation can lead to a more pronounced EMT. Specifically, what else is needed to result in E-Cadherin downregulation rather than upregulation, since this is likely another key guardrail alongside RUNX1 itself that must be overcome for cells to become cancerous. Another study that would yield interesting results could be one that analyzes these same genes with differing concentrations of dTAG to ensure that the majority of the cells are undergoing the transition. Additionally, further study on ZO-1 may yield interesting results. A study in which this gene, as well as RUNX1 was degraded and compared to cells with only RUNX1 degraded could give information about this gene's relatively unknown role during EMT. Would the cells have an even more pronounced EMT as a result of the loss of this adherence protein, or would they instead remain closer to an epithelial phenotype due to the loss of ZO-1 function within the nucleus? If the latter is the case, then a study of ZO-1's effect as a transcription factor would also further characterize the interactions at play that lead to tumorigenesis.

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