

**Roles of *Wsb1* and *Ksr1* in Growth of Mammary Tumor Cells**

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## ABSTRACT

The RAS protein is crucial in signaling pathways that regulate cellular proliferation. Tumors can form when this normal growth regulation breaks down due to defects in signaling mechanisms. The *Chaos3* mouse model has a point mutation in the *Mcm4* gene, a DNA replication licensing factor, which triggers replicative stress and genome instability, ultimately resulting in tumor formation. It was previously seen that two genes, *Wsb1*, a E3 ubiquitin ligase, and *Ksr1*, a kinase suppressor of RAS, were commonly deleted in *Chaos3* tumors. In this project, I used mammary tumor cells from the *Chaos3* mouse model to study the effect of re-introducing RAS-regulatory factors. In order to accomplish this, stable *Chaos3* mammary tumor cell lines were created with the random integration of ectopic transgenes (complementations) that corresponded to endogenous genes deleted in these cells. I confirmed successful insertions through RT-qPCR, immunofluorescence, and western blotting. I also analyzed DNA replication (through EdU labeling), growth curves, and the amount of active RAS. I found that KSR1 and a fragment of WSB1 both resulted in fewer replicating cells, but had different effects on RAS. Future research should involve replicating these preliminary experiments to elucidate potential involvement of the *Ksr1* and *Wsb1* genes in mammary tumor formation.

## INTRODUCTION

Cellular replication is controlled by a complex network of signaling pathways that ensures cells proliferate only at specific times [5]. This helps to maintain genome integrity through the strict regulation of DNA replication and chromosomal division, as well as recognition and repair of DNA damage. Genomic DNA of a eukaryotic cells is replicated once during the S-phase of the cell cycle [4]. For replication to occur, pre-replicative complexes, with the ORC, Cdc6, MCM2-7, and Cdt1 proteins, are key factors. These complexes and proteins are regulated so that the licensing proteins, that allow an origin of replication to begin DNA replication at that site, occurs during late M to early G1 phase, but before S phase, in order to prevent re-replication of genomic regions [2]. Importantly, DNA replication stress can occur when this DNA replication is compromised by disruptions to replication fork progression [2]. This replication stress might then trigger cell cycle arrest and senescence. When this normal growth regulation breaks down due to defects in these signaling mechanisms or DNA repair, cells can transform and ultimately form tumors. Therefore, further investigation of these cell cycle regulation pathways could aid in controlling abnormal growth and tumorigenesis.

The maintenance protein complex (MCM 2-7) constitutes the replicative helicase core. Down regulation of MCM (minichromosomal maintenance protein complex) proteins leads to replication stress because MCM is a key part of the pre-replicative complex and the formation of replication forks. MCM2-7 plays an important role in genome replication initiation as it is the replication licensing factor and presumptive replicative helicase [17]. In this study, I utilize a mouse model harboring the *Chaos3* point mutation in the *MCM4* gene, which destabilizes the MCM2-7 replicative helicase [19]. This causes a pan-reduction in mRNA and protein levels of

the other MCM subunits. In *Chaos3* cells, we observe increased replicative stress and genomic instability as well as the formation of mammary tumors with gene expression profiles closely resembling mature human mammary luminal cell signatures [19].

Previously, my lab found that tumor cells derived from *Chaos3* mice are frequently deficient for *Nf1* expression. *Nf1* encodes neurofibromin-1, a GTPase-activating protein (GAP) that hydrolyses RAS-bound GTP to GDP [20]. Thus, it negatively regulates RAS, a cell growth activator, by stimulating its GTPase activity and pushing it to the inactive state [19]. It was seen that the levels of activated RAS were much higher in *Chaos3* mammary tumor cells deleted for *Nf1* [19]. This hyperactive RAS can cause overactive signaling in cell growth and division, which can lead to excessive cell proliferation and tumorigenesis. Recent work, however, showed that deleting *Nf1* in *Chaos3* mice did not increase the rate of tumor formation, suggesting that NF1 is not the driver of these mammary tumors (McNairn, unpublished).

We previously found that the genes *Wsb1* and *Ksr1* are co-deleted with *Nf1* in *Chaos3* mammary tumors (Figure 12) and human breast cancer [19]. *Wsb1* is an E3 ubiquitin ligase with seven WD repeats and contains a SOCS box at the C-terminus [13]. WSB1 is observed to play a role in overcoming oncogene-induced senescence, promote tumor metastasis, and enhance proliferation [1] [12] [13]. Another gene, *Ksr1*, a kinase suppressor of RAS, is a scaffolding protein. It plays a regulatory role in the oncogenic RAS-RAF-MAPKs signaling, and it has been seen that breast cancer patients with high KSR1 had better disease free and overall survival [21]. KSR1 positively regulates the RAF/MEK/ERK cascade upstream of RAS to elicit responses such as

proliferation, differentiation, and cell survival [9]. However, it is unknown how the deletion of *Wsb1*, *Ksr1*, and *Nf1* in *Chaos3* mice regulates tumorigenesis.

RAS was one of the first proteins identified with the ability to regulate this cell growth [5].

Constitutively activated RAS proteins contribute to the deregulation of cell growth, programmed cell death, and new blood-vessel formation [16]. RAS functions as a relay switch for signal transduction positioned downstream of cell surface receptor tyrosine kinases and upstream of a cytoplasmic cascade of kinases like mitogen-activated protein kinases (MAPKs) (Figure 11) [3].

In untransformed cells, RAS localization is governed by its post-translational modifications [5].

RAS proteins bound to GTP are in their active forms and can activate downstream targets. But, if they are bound to GDP, they are inactive and fail to interact with these effectors. Evidently, the activity of RAS proteins is controlled by the ratio of bound GTP to GDP [3]. In tumors, mutations compromise the GTPase activity of RAS, which prevents GTPase-activating proteins (GAPs) from promoting hydrolysis of GTP on RAS and causes RAS to accumulate in the GTP-bound, active form [5]. Too much of this active RAS can cause neoplastic transformation.

Since *Wsb1* and *Ksr1* are usually deleted along with *Nf1*, I hypothesize that they may play a role in the formation of the mammary tumors. This project aimed to examine the effects of complementing the deletion of the *Wsb1* and *Ksr1* genes in *Chaos3* mouse mammary tumor cells in order to observe the effect on cell growth and RAS activity. To accomplish this experiment, I created stable cell lines with either the *Wsb1*, *Ksr1*, or *GFP* gene complemented. I confirmed the presence of the genes in the cells through RT-qPCR, western blotting, and immunofluorescence labeling. I then analyzed the growth rate of each cell line as well as the number of replicating

cells through EdU labeling. Additionally, active RAS pulldown detected the levels of active RAS. This research will help to further study the novel contributions of *Ksr1* and *Wsb1* to cell cycle regulation and proliferation. This study may also advance the understanding of the regulation of the RAS pathway and its effects on mammary tumor formation. Future understanding of the regulation of the RAS pathway could allow us to identify if *Wsb1* and *Ksr1* are genes that can be targeted for tumor therapy.

## **MATERIALS AND METHODS**

### Cell Lines and Cell Culture

To start the project, I utilized HEK293T as well as 22168, 21253, and 22418 mammary tumor cell lines from *Chaos3* mouse mammary tumors. The 22168 and 21253 have homozygous deletions for *Ksr1*, *Wsb1*, and *Nf1*, while 22418 only has heterozygous deletions. Cells were grown in complete media: DMEM (Dulbecco's Modification of Eagle's Medium with 4.5g/L glucose & L-glutamine without sodium pyruvate) with added MEM Non-Essential Amino Acids, Penicillin Streptomycin, Sodium Pyruvate, and 10% of either Fetal Bovine Serum or Bovine Calf Serum (iron supplemented). Cells were grown at 37°C in 5% CO<sub>2</sub>. All freshly thawed cells were allowed to attach to the plates overnight. To culture HEK293T cells, tissue culture plates were coated with 0.2% (Porcine) gelatin in PBS in order to enhance adhesion.

### Cell Splitting/Passaging

In order to passage cells, I began by pre-warming the complete media in a 37°C water bath. Plates were maintained in a 37°C incubator with 5% CO<sub>2</sub>. After removing the media from the plates, they were washed twice with PBS. Trypsin was added and the plate was placed back into

the incubator for a few minutes in order to detach the cells from the surface. Complete media was added to inactivate trypsin. The appropriate dilution or number of cells were transferred per the experimental requirements.

### Generation of Stable Cell Lines Using Lentivirus

#### -Production of Lentiviral Plasmids with *Ksr1*, *Wsb1*, and GFP Complements

To produce lentivirus to transduce *Ksr1* or *Wsb1* plasmids into target cell lines, I first cloned these genes into lentiviral plasmids. The *Ksr1* and *Wsb1* plasmids were each digested with Kpn1 and Xme1 restriction enzymes, respectively, in order to form sticky ends. After filling in the ends to produce blunt ends and purifying the cut bands, the FUGW lentiviral plasmids, were cut with the Sma1 restriction enzyme. The ends of the empty vector plasmid, FUGW, were dephosphorylated so that they would not ligate to themselves. T4 ligase was used to ligate the *Ksr1* and *Wsb1* genes into separate FUGW lentiviral plasmids. After selecting for ampicillin resistance, the successful plasmids were verified by PCR and purified. A FUGW GFP lentiviral plasmid was also obtained as a control.

#### -Electroporation into Cells to Create Lentivirus

The purified plasmids were electroporated into HEK293T cells, which would produce the lentivirus.  $5 \times 10^6$  HEK293T cells were suspended in 100 $\mu$ L of Ingenio Electroporation Solution per nucleofection. The cells were transferred into an electroporation cuvette along with a pMD2.G packaging plasmid, a pCAG-Eco envelope plasmid, and the indicated lentiviral vectors. The Nucleofector's Q-001 program was used for electroporation through the Ingenio Electroporation Kit (Mirus). The transfected cells were plated in indicated media and grown for

48-72 hours before viral production was quantified. I confirmed the production of lentivirus by harvesting RNA from the HEK293T media, producing cDNA, and then running PCR. Packaging of functional lentivirus could be confirmed through visibility of the GFP signal in the HEK293T cells that were producing the lentivirus.

#### Infection of Tumor Cells with Lentivirus

I collected the media of the HEK293T cells containing the virus 48-72 hours after electroporation. Virus-containing media was concentrated by passage through Millipore centrifugal concentration tubes and sterilized through by passage through a 0.45µm PES filter. After collecting the three lentiviruses produced by the HEK293T cells, I infected each of them into three *Chaos3* mammary tumor cells lines: 22168, 21253, and 22418. HEK293T cells were also infected as a control. In order to select for successful infection, 500µg/ml of zeocin selection was used until colonies formed and visibility of GFP signal was looked for in the cell lines infected with the *GFP* lentivirus.

#### Generation of Stable Cell Lines using TransIT-LTI

In addition to cell lines with lentiviral infection, I also created stable cells lines through TransIT-LTI. To create stable cell lines of the *Chaos3* mammary tumors with the gene complementation, I linearized the *GFP*, *Wsb1*, and *Ksr1* plasmids through REnzyme Digest with SmaI restriction enzyme and the CutSmart Buffer. PCR cleanup (Qiagen) was then performed.  $3 \times 10^5$  cells were plated 24-48 hours prior to transfection. I then used the TransIT-LTI in OptiMem serum free media to transfect the cell lines. 500µg/ml of zeocin selection was used until colonies formed.

### Reverse transcription polymerase chain reaction (RT-qPCR)

In order to complete RT-qPCR, I extracted RNA from the cell lines with the E.Z.N.Z Total RNA Kit I. Cells were lysed and centrifuged with 70% ethanol, washed with RNA buffers, and eluted with DEPC water. Concentrations of the purified RNA were measured using NanoDrop. cDNA was synthesized from 500ng of the RNA using the iScript cDNA synthesis kit (Bio-Rad) to reverse transcribe the purified RNA into cDNA.

I performed RT-qPCR using 2x iTaq and primers for *GAPDH* (housekeeping reference gene), *Wsb1*, *Ksr1*, and *Nfl*. Assays were run on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Each sample was run in triplicate wells in a 96 well plate, from which mean Ct values were obtained. RT-qPCR data was analyzed using the  $2^{-\Delta\Delta CT}$  method where  $\Delta\Delta CT = [(T_{\text{sample}} - T_{\text{GAPDH}}) - (T_{\text{GFP control}} - T_{\text{GFP control GAPDH}})]$  [14]. Data analysis was performed with the Bio-Rad CFX Manager software (Bio-Rad).

### Immunofluorescence

I performed immunofluorescence labeling to identify ectopic FLAG-WSB1 and FLAG-KSR1. Coverslips with cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature. The coverslips were blocked for an hour with PBS, 5% goat serum, and 0.3% tritonX-100. After washing with PBS, coverslips were incubated with a FLAG primary antibody (DYKDDDDK Tag DGW5B Rabbit mAb, cell signaling) in antibody dilution buffer (1% BSA and 0.3% triton-100, in PBS) at 4°C overnight. After washing three times with 0.15 tritonX-100 in PBS, they were labeled with a secondary Anti Rabbit IgG Alexa594-linked antibody. The coverslips were stained with DAPI and mounted onto slides for analysis. The Olympus BX51 microscope was

used. Cells were observed using the 20x objective. They were detected and imaged through the Olympus cellSens Standard software.

### Western Blot Analysis

I also ran a western blot in order to detect the presence of the FLAG-WSB1 and FLAG-KSR1 proteins. Whole cell lysis was performed on each cell line, through which they were lysed with IP lysis buffer (150mM NaCl in EDTA with 1x protease inhibitor) and sonicated (3x 10 seconds, 25% amplitude). A SigmaAldrich MG132 Protease Inhibitor (1:1000) was also added to the IP lysis buffer in some experiments. The lysed cells were mixed with 3x SDS loading buffer (with 2-mercaptoethanol) and boiled. Each sample was then loaded into a 10-well precast gradient gel (4-20%) or a 12% gel. It was run with loading buffer and a precision plus protein dual color ladder at 150V for 80 minutes. Protein was then transferred to a methanol activated PVDF membrane at 1A for 45 minutes. The membrane was blocked with 5% milk in TBST for 1 hour and incubated with the FLAG primary antibody overnight at 4°C. After washing three times in TBST for 5 minutes, it was labeled with the secondary Anti Rabbit IgG HRP linked antibody in 5% milk for an hour. The membrane was washed three times in TBST for 5 minutes and labeled with Millipore Luminata Classico Western HRP Substrate. After washing with TBS, it was stripped of antibody using the Thermo Scientific Restore Plus Western Blot Stripping Buffer by incubating it for 10 minutes at room temperature. The buffer was removed and the blot was washed with TBS. It was then blocked for 1 hour and labeled with Actin as a control.

### EdU Incorporation Detection (Click Chemistry)

Cells grown on coverslips were pulse labeled with 10 $\mu$ M EdU for 30 minutes. The coverslips were fixed in 4% Paraformaldehyde for 10 minutes at room temperature. After washing with PBS, the cells were permeablized with 0.3% Triton X-100 in PBS for 15 minutes, followed by washing in 1% BSA in PBS. The 'click' reaction staining was performed by placing the cells in a 10mM (+)-sodium-L-ascorbate, 0.1mM AlexaFlur 594 Azide, and 2mM CuSO<sub>4</sub> cocktail for 30 minutes at room temperature. After washing in 0.5% Tween20 in 1% BSA in PBS, nuclei were counterstained with DAPI and mounted onto slides. The percentage of EdU positive cells were compared to the total number of DAPI stained cells. Again, they were analyzed using the Olympus BX51 microscope. Cells were observed using the 20x objective. They were detected and imaged through the Olympus cellSens Standard software.

### Measuring Growth of Cell Lines

I analyzed the growth of each complemented cell line expressing GFP, FLAG-WSB1, or FLAG-KSR1. 1x10<sup>5</sup> cells were plated and the number of cells were recorded every 2-4 days. 1x10<sup>5</sup> cells were replated. The growth was normalized to the number of days in between each growth count.

### Active RAS Pulldown

I used the Thermo Scientific Active RAS Pull-down and Detection Kit in order to measure levels of active RAS. Cells were washed with ice cold TBS. After adding 1x lysis/binding/wash buffer, they were scraped down, vortexed, and kept on ice for 5 minutes. After centrifugation at 6000xg at 4°C for 15 minutes, the supernatant was saved. A BCA Protein Assay was run (with Diluted Albumin (BSA) Standards and by adding a BCA working reagent to each sample) to measure the

concentration of each sample on the NanoDrop. GTPYS and GDP controls were created by using the GFP lysate and adding 0.5M EDTA pH 8.0 as well as either 10mM GTPYS or 100mM GDP. After incubation at 30°C for 15 minutes with agitation, the reaction was terminated by placing the sample on ice, adding 1M MgCl<sub>2</sub>, and vortexing. For the affinity precipitation of activated RAS, Glutathione resin was added to spin cups in collection tubes, and this was centrifuged at 6000xg for 30 seconds. GST-Raf1-RBD was added to the spin cups along with 500µg of each sample (unknowns and controls). After sealing the tubes with parafilm and vortexing, the samples were incubated at 4°C for 1 hour with gentle rocking. They were then centrifuged, transferred to new tubes, and washed with 1x lysis/binding/wash buffer three times. In new tubes, reducing sample (2x SDS Sample Buffer with 2-mercaptoethanol) was added to the spin cups. This was vortexed, incubated at room temperature for 2 minutes, centrifuged for 2 minutes, and the solutions were saved. After heat eluting for 5 minutes at 100°C, proteins were separated by SDS-PAGE and transferred to the membrane. The membrane was then blocked with 3% BSA in TBS for 1 hour. After rinsing with 0.05% Tween 20 in TBS (TBST) for 5 minutes, it was incubated in Anti-RAS antibody in 3% BSA with 0.1% NaN<sub>3</sub> in TBS overnight at 4°C. The membrane was washed five times in TBST and incubated with anti-mouse IgG-HRP-conjugate in TBST containing 5% nonfat dry milk for 1 hour at room temperature. It was washed with TBST five times, incubated with immobilon crescendo western HRP substrate (chemiluminescent), and exposed to x-ray for band detection.

## **RESULTS**

### *Chaos3* Mammary Tumor Cells Express Low Levels of *Wsb1* and *Ksr1*

Since both *Wsb1* and *Ksr1* were commonly deleted in *Chaos3* mammary tumors, I wanted to see

how these genes played a role in mammary tumor formation. For my study, three cell lines were created from 3 *Chaos3* mammary tumors: 22153, 22148, and 22168. The 22168 and 21253 tumors had homozygous deletions for *Ksr1*, *Wsb1*, and *Nf1*, while 22418 had heterozygous deletions. I analyzed RNA expression of WSB1 and KSR1 in these cell lines in order to establish a baseline of expression in the tumor cell lines with these gene deletions. Previous studies in the lab have shown a high correlation in *Wsb1* and *Ksr1* expression in the tumors as both were either increased or decreased in expression when compared to non-tumor cells. I used RT-qPCR to show that *Wsb1* expression was very low and that *Ksr1* expression was undetectable in all three cell lines (Figure 1).

#### Unsuccessful Creation of Stable *Chaos3* Mammary Tumor Cell Lines through Lentivirus

##### -Successful *Ksr1* and *Wsb1* Lentiviral Plasmid Creation and Transfection of HEK293T Cells

To introduce the *Wsb1*, *Ksr1*, and *GFP* genes permanently into the *Chaos3* mammary tumor cell lines, I chose to use lentiviral transduction. After cloning these genes into lentiviral vector plasmids, I performed restriction digests of purified plasmid DNA to ensure that the genes were inserted in the correct orientation. They were cut with the XhoI enzyme in order to identify a 0.75kb band size, which indicated that the gene was inserted into the FUGW lentiviral plasmid correctly.

I used electroporation for the purpose of inserting the lentiviral plasmids into HEK293T cells along with envelope and packaging plasmids. Electroporation is the use of high-voltage electric shocks to introduce DNA into cells [15]. It is popular for standard gene transfer because it can be used with most cell types, yields a high frequency of both stable transformation and transient

gene expression, and requires fewer steps than other techniques [15]. The presence of GFP signal in the infected HEK293T cells indicated that transduction was successful in this control (figure not shown). The presence of the *Wsb1* and *Ksr1* transgenes in the lentiviral preps, containing RNA, was also verified using RT-qPCR, indicating some virus was present. After electroporating the HEK293T cells with the lentiviral plasmids, I isolated purified lentivirus from the media of the cells. I then extracted the RNA of this purified lentiviruses and ran gel electrophoresis using *Ksr1* and *Wsb1* specific primers. This confirmed the presence of the *Ksr1* gene in the *Ksr1* lentivirus and *Wsb1* gene in the *Wsb1* lentivirus, confirming that the lentiviral plasmids were successfully created.

#### -Unsuccessful Infection of Lentivirus into Chaos3 Mammary Tumor Cells

The lentiviruses were ready to infect the cell lines due to their ability to insert the desired genes with high efficiency. After I purified the three lentiviruses from the media of HEK293T cells, I infected each of the three *Chaos3* mammary tumor cells lines. After a few days of infection, there was a weak GFP fluorescence signal in the GFP complementation cell lines. Since this infection was unsuccessful for the *GFP* lentivirus, it was likely unsuccessful for the *Wsb1* and *Ksr1* lentiviruses as well. Additionally, many cells died during selection.

#### Successful Creation of Stable Chaos3 Mammary Tumor Cell Lines through TransIT-LTI

Since the lentiviral method of transduction of the complement genes did not work, I transfected *GFP*, *Wsb1*, and *Ksr1* DNA expression plasmids into the tumor cells to form stable cell lines [11]. However, only the 22168 cell line survived zeocin selection for integration. These cells were picked for single colonies, grown, and further selected for zeocin resistance. Six 22168

stable cell lines resulted from selection: GFP-1, GFP-5, WSB1-D2, WSB1-B3, KSR1-A2, and KSR1-B4.

### *Wsb1* and *Ksr1* Genes Successfully Inserted into “Complementation” *Chaos3* Tumor Cell Lines

Since stable cell lines were created with *GFP*, *Wsb1*, and *Ksr1* genes, I analyzed them for the presence of these genes/proteins using three techniques. First, RT-qPCR data showed relative expression levels of the WSB1 and KSR1 complementation cell lines. RT-qPCR allowed analysis of gene expression through mRNA transcript levels. Using this method, each sample Cq data was normalized with respect to the *GAPDH* housekeeping gene and to the control sample (geometric mean of GFP-1 and GFP-5).

Previously research in the lab using transient transfections of *Wsb1* was used as a proof of concept that showed an increase in both *Wsb1* and *Ksr1* levels. Figure 2 showed positive results that the desired genes were present in each of the cell lines. The WSB1 cell lines showed a huge increase in *Wsb1* expression, and a small increase in *Ksr1* expression. The KSR1 cell lines showed a huge increase in *Ksr1* expression, and a small decrease in *Wsb1* expression. *Nf1* was slightly increased in the KSR1-A2 and WSB1-B3 cell lines, slightly decreased in the WBS1-D2 cell line, and unchanged in the KSR1-B4 cell line. This showed that adding back either *Wsb1* or *Ksr1* into stable cell lines increased the expression of each of these genes in their appropriate complementation cell lines.

Next, I used GFP fluorescence in order to identify the number of cells with the genes present compared to the number of total cells. Figure 3 showed bright field and fluorescent images of the

GFP-1 and GFP-5 complementation cell lines. GFP-1 showed a higher percentage of green cells compared to the GFP-5 cell line. Since this shows that more cells contain the GFP gene, GFP-1 is a better GFP control than GFP-5. This proved, again, that the *GFP* genes were inserted successfully into the GFP-1 cell line and that a sufficient amount of GFP+ cells are present.

I also observed immunofluorescence of the FLAG epitope tag in the two *Wsb1* and two *Ksr1* complementation *Chaos3* mammary tumor cell lines (Figure 4). Immunofluorescence allows for assessment of cellular localization [10]. This was done in order to confirm, again, that the *Wsb1* and *Ksr1* genes were successfully inserted into these cell lines as their proteins could be identified by the FLAG epitope tag. The signal was present in the WSB1-D2 and KSR1-A2 cell lines, but absent in the WSB1-B3 and KSR1-B4 cell lines. It showed the WSB1-D2 and KSR1-A2 to be more reliable complementation cell lines and WSB1-B3 and KSR1-B4 to be unreliable.

Finally, I performed western blotting in complementation *Chaos3* mammary tumor cell lines to detect levels of WSB1 and KSR1 protein (Figure 5). The FLAG epitope tag, which is present on both WSB1 and KSR1 proteins, would be identified if either protein was present in the complementation cell line. This was seen through the presence of a band on the blot. The protein levels were compared to Actin, a housekeeping protein. GFP-1, GFP-5, and 293T-ctrl did not show a band, as expected, due to their role as negative controls without the insertion of either of these genes. Therefore, these proteins were not present. Bands were only seen in the KSR1 cell lines, proving that the *Ksr1* gene was inserted and the KSR1 protein is produced in the two KSR1 cell lines. 293T-WSB1 was used as a positive control, and was expected to show a band. However, no band was present. There were also no bands for both of the WSB1 cell lines,

suggesting the gene may not be inserted or the protein was not expressed. This may be due to a technical problem or it may be that the protein is expressed but degraded, and therefore not seen on the gel. Figure 6 shows a second western blot that was performed with the addition of a protease inhibitor in order to prevent ubiquitinated protein from degradation. In this blot, I again saw the presence of KSR1 and an absence of 293T-ctrl, 293T-wsb1, and WSB1-B4. However, a band was present this time for WSB1-D2, suggesting that the WSB1 protein may be degraded in the cell line, and only a fragment of the gene with the FLAG tag is present in the cell line.

### WSB1 and KSR1 Changed the Growth Rate of *Chaos3* Mammary Tumor Cell Lines

Afterwards, I analyzed how the gene complementations affected the replication and growth rate of the cell lines. To observe replicating cells, EdU labeling Click Chemistry was performed on the 22168 GFP-1, WSB1-D2, and KSR1-A2 cell lines. EdU uses pulse labeling of replicating cells with nucleoside analogs followed by microscopic observation as a visual method to analyze DNA replication (S phase) and label dividing cells [10]. This process uses 5-ethynyl-2'-deoxyuridine (EdU), a thymidine analogue that can be incorporated *in vivo* and detected using Click reaction [18]. EdU detection is easy to use because it requires no heat or acid treatment to denature the DNA and is compatible with multiple probes for fluorescence immunochemistry [6]. The EdU detection procedure uses a copper (I) catalyzed click reaction chemistry to covalently couple an azide modified fluorescent dye to incorporated EdU to form a stable triazole ring [7].

The number of EdU labeled cells were compared to the total number of (DAPI stained) cells in Figure 7 and Figure 8. This number was represented as a percentage. 730 cells were analyzed for

GFP-1, 831 for WSB1-D2, and 1249 for KSR1-A2. There was a significant difference between GFP and WSB1 cells ( $p=0.0022$ ), showing that WSB1 complementation cell lines had fewer replicating cells. There was also a significant difference between GFP and KSR1 cells ( $p=0.0462$ ), showing that KSR1 complementation cell lines had fewer replicating cells. There was no significant difference between WSB1 and KSR1 cells ( $p=0.0839$ ), showing that they both had less replication than GFP, but were similar to each other.

I next analyzed proliferation and population doubling. In order to observe the growth of each cell line, I plated  $10^5$  cells of each 22168 complementation cell line and recorded the growth 2-4 days later.  $10^5$  cells were replated and this was repeated every 2-4 days. The total number of cells was calculated by subtracting the starting number of cells from the counted amount and adding this to the previous day's cell count. Figure 9 showed exponential trend lines for each cell line's growth. GFP-1 grew the least, WSB1-D2 grew the most, and KSR1-A2 was in the middle. This shows that the WSB1 and KSR1 genes resulted in an increase in growth.

### WSB1 and KSR1 Affect Active RAS

Finally, after looking at the effect of *Wsb1* and *Ksr1* on growth and replication, it was desired to perform active RAS pulldown in order to compare growth to RAS. Active-RAS pulldown enables selective enrichment and detection of GTP-bound RAS GTPase through a specific protein interaction with the Raf1 protein-binding domain. It allows determination of the GTP-bound levels to detect how much active RAS is present [8]. Levels of active RAS were measured for each *Chaos3* complementation cell line. The darker the band meant that there was a greater amount of active RAS in the cell line. In the Active RAS blot in Figure 10, GTPYS was a

positive control that activated all the RAS to show the total amount of RAS (in GFP). Therefore, this band was the most intense. GDP was a negative control that inactivated all of the RAS, and therefore showed a very faint band. I focused on the reliable cell lines only. KSR1-A2 and GFP-1 showed dark bands, indicating a lot of active RAS. WSB1-D2 showed a slightly less intense band, indicating that it had less active RAS.

The Total RAS Blot showed the total amount of RAS in each cell line. Any low intensity of total RAS may be due to less confluency and a lower starting number of cells. In this blot, WSB1-D2 had the darkest band, indicating that they had the highest amount of total RAS (both active and inactive). GFP-1 had the next most intense band, followed by KSR1-A2. The GTPYS and GDP controls were not run in this blot in Figure 10.

I quantified the intensity levels of active RAS for each *Chaos3* “complementation” cell line compared to the amount of total RAS in that cell line from the blot in Figure 10. This was graphed as a percentage of active RAS in Figure 11. The percent of active RAS was averaged between two GFP cell lines, two WSB1 cell lines, and two KSR1 cell lines. KSR1 had a higher percent of active RAS than the GFP did. WSB1 had a similar to lower percent of active RAS than the GFP did.

## **DISCUSSION**

Since previous research showed deletions of both *Wsb1* and *Ksr1* in many of *Chaos3* mammary tumors, I wanted to see if and how these genes might play a role in mammary tumor formation. Previous results and Figure 1 showed a high correlation between *Wsb1* and *Ksr1* expression and

that both levels of *Wsb1* and *Ksr1* were downregulated in the original *Chaos3* mammary tumor cell lines before complementation (22168, 21253, and 22418) when compared to non-tumor forming cells. Since past research in the lab showed an increase in both *Wsb1* and *Ksr1* expression due to transient transfections of *Wsb1*, it is likely that these two genes may be co-regulated or regulate the another. I desired to test this by transfecting these genes through more permanent stable cells lines in order to analyze the effects of the genes *Wsb1* and *Ksr1* on *Chaos3* mammary tumors.

I created separate cell lines with each of these gene complementations. However, when creating complementations through lentivirus infection, the lentivirus failed to infect the HEK293T control cells as well as the *Chaos3* tumor cells lines. It was confirmed that the genes were inserted into the lentiviral plasmids in the correct orientation due to the occurrence of a 0.75kb band when cut with the XhoI enzyme. Additionally, when the RNA was extracted from all three types of lentiviruses, *Ksr1* and *Wsb1* specific primers confirmed the expression of these genes. The lentiviruses were seen to be successfully constructed due to the GFP signal seen in the HEK293T cells that they were created in, however, these may have been dead cells. The failure of the lentivirus may have been due to a problem during infection. There was a weak GFP fluorescence signal in the cells infected with the *GFP* lentivirus. This may be due to the fact that most of the cells died, possibly due to infection conditions being too harsh. It could also be due to a low number of lentivirus purified or none at all. Although an envelope plasmid more specific to mice, pCAG-Eco, was tried instead of psPAX2 used previously, this did not help to insert the lentivirus into the cells either. The lentivirus, if present, may have had trouble infecting the cell lines due to low titer. HEK cells could also be the problem if they were infected with

mycoplasma and resulted in the low production of lentivirus. The transfection protocol will need to be troubleshoot if attempted again in the future.

Of the three cell lines, 22168, 22418 and 21253, six stable “complementation” cell lines were created only for 22168: GFP-1, GFP-5, WSB1-D2, WSB1-B3, KSR1-A2, and KSR1-B4. In order to confirm the presence of the gene inserts, three techniques were used.

First, RT-qPCR data confirmed the presence of *Wsb1* expression in WSB1 cell lines and *Ksr1* expression in KSR1 cell lines. Additionally, there was evidence that *Wsb1* increases the expression of *Ksr1*. These genes may be co-regulated; however, it is unclear how they directly or indirectly affect one another since *Ksr1* decreases *Wsb1* expression. It is possible that *Wsb1* upregulates *Ksr1*, which then uses negative feedback to downregulate *Wsb1*. There was also variation in the increase and decrease in *Nf1* in the cell lines, leading it to be unclear if *Nf1* is included in the regulation pathway by either of these genes.

Second, I used GFP fluorescence to confirm that GFP-1 was a more reliable control than GFP-5 due to a greater number of GFP+ cells. Further, immunofluorescence of the FLAG epitope tag demonstrated that *Wsb1* was present in the WSB1-D2 cell line and that *Ksr1* was present in the KSR1-A2 cell line. The weak signal in WSB1-B3 and KSR1-B4 cell lines suggested the absence of the genes. Although it could be due to an error in the staining procedure, due to loss of fluorescence, or due to a low number of transfected cells, it could not confirm the presence of the genes in the cells.

Third, the two western blots confirmed the KSR1 protein in both *Ksr1* cell lines. Additionally, the smaller than full sized WSB1-D2 band indicated that the protein was present but degraded, and only a fragment of the gene with the FLAG tag was expressed in this cell line. The absence of the WSB1-B3 protein may also be due to degradation, but its presence could not be confirmed. The lack of the positive control 293T-WSB1 may also be due to degradation or a technical error.

Given these three experiments, the WSB1-D2, KSR1-A2, and GFP-1 cell lines are the only ones that showed consistent evidence of the presence of the *Wsb1*, *Ksr1*, and *GFP* genes respectively. Therefore, these cell lines would be most reliable to use for further analysis into growth, replication, and active RAS. GFP-5, WSB1-B3, and KSR1-B4 would be removed from future analysis due to inefficient or lack of expression of their respective genes.

I then analyzed the WSB1 and KSR1 complementations for changes in growth compared to the GFP complementation cell lines. EdU labeling showed that both the WSB1 and KSR1 complementation cell lines had a significantly lower percent of replicating cells than the GFP complementation cell lines. When analyzing growth curves of each cell line, both WSB1 and KSR1 resulted in greater growth compared to GFP.

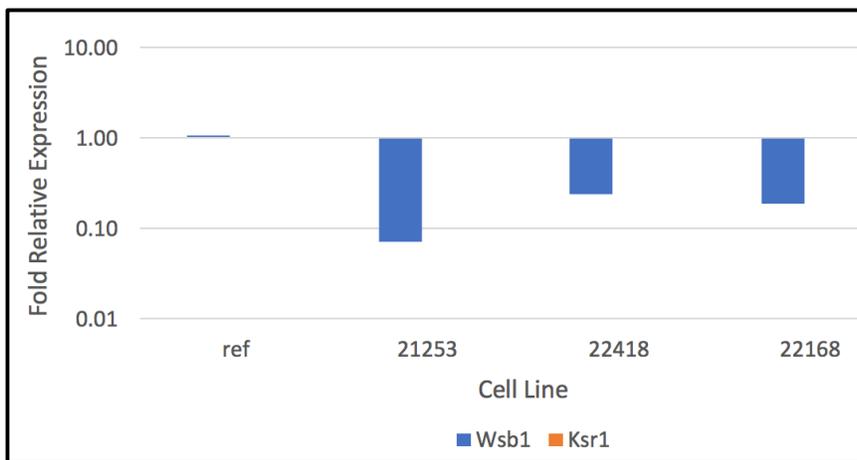
I also analyzed the WSB1 and KSR1 complementation cell lines for their effects on active RAS compared to the GFP complementation cell lines. I saw that the KSR1-A2 had a higher percent of active RAS than GFP-1. However, the WSB1-D2 had a similar to lower percent of active RAS than the GFP-1.

To put all the data together, some conclusions can be made about effects of complementing the genes *Wsb1* and *Ksr1* in *Chaos3* mouse mammary tumor cells. Since WSB1 is likely to have been partially degraded in the WSB1-D2 cell lines, as seen by the western blot, we cannot conclude that the full WSB1 protein was in the cell line. Rather we can evaluate effects of just a fragment of this gene. The fragment of the *Wsb1* gene caused a lower percent of replicating cells than GFP, grew faster than GFP, and had a similar/lower percent of active RAS compared to GFP. The *Ksr1* gene caused a lower percent of replicating cells than GFP, grew faster than GFP, and had higher percent of active RAS compared to GFP. This is contradictory to the commonly known role of *Ksr1* as a negative regulator of RAS. Therefore, the effect of complementing with either KSR1 or a fragment of WSB1 was that it caused less cells to replicate, but increased growth. This growth could be due cells replicating very quickly, despite the fact that a fewer number of cell are replicating. Since both genes had different effects on RAS, it is likely that these effects on growth and replication are due to some factor outside of active RAS. It is necessary to repeat experiments in order to confirm and solidify these results.

One limitation of this study was the lack of sufficient replication for experiments. Future research would replicate these EdU, cell growth, and active RAS pulldown for the 22168 complementation cell lines. Repeating these experiments with more technical as well as biological replicates would allow for more accurate and precise results. Additionally, it would be interesting to be able to create the other two complementation cell lines, 22418 and 21253, in order to see if *Wsb1* and *Ksr1* insertions affect those cell lines similarly to the 22168 cell line.

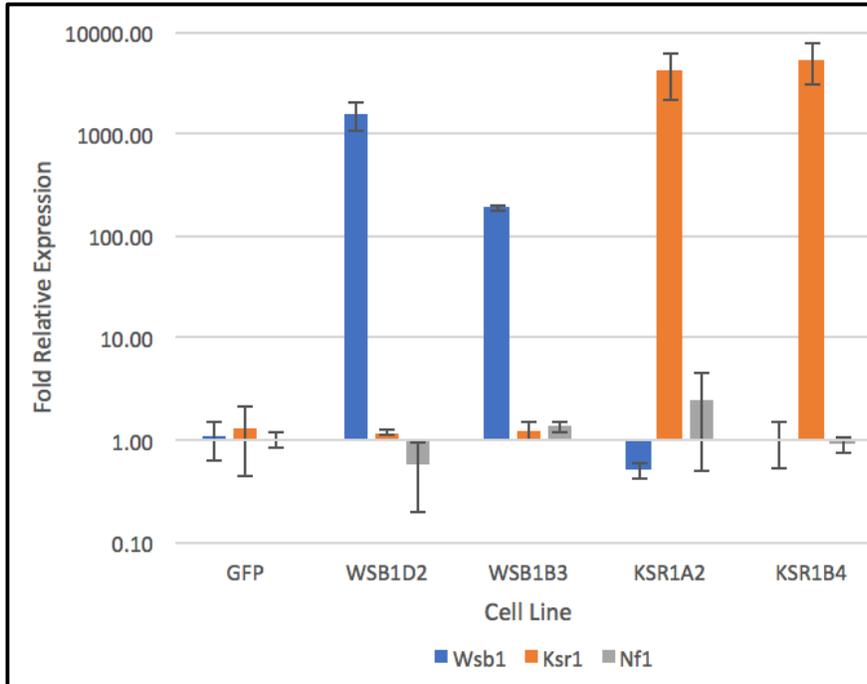
These findings help to learn more about the role of the *Wsb1* and *Ksr1* genes in *Chaos3* mammary tumors. The results suggest that the WSB1 (fragment) and KSR1 proteins may play a role in cell proliferation and may affect RAS in ways contradictory to their known roles. Because these genes increase cell growth, we can do further studies to see if inhibiting these genes will slow down cell growth and help to treat tumor formation. Since these mammary tumors have tumor gene expression profiles closely resembling mature human mammary luminal cell signatures, this knowledge, along with future research, may allow us to identify if *Wsb1* and *Ksr1* are genes that can be targeted to fight tumors.

## FIGURES AND TABLES



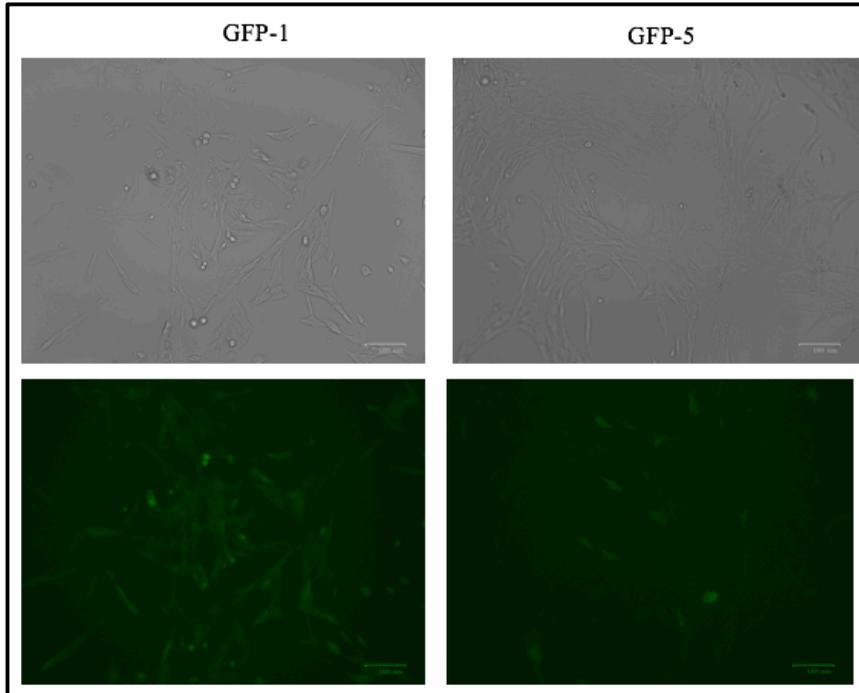
**Figure 1: Relative *Wsb1* and *Ksr1* Levels in *Chaos3* Mammary Tumor Cell Lines**

Three cell lines were created from *Chaos3* mammary tumors: 21253, 22418, and 22168. RT-qPCR data showed the expression of *Wsb1* and *Ksr1* in these cell lines. The expression levels were relative to a reference cDNA. Each cell line was run in triplicates (three technical replicates) and the average was graphed.



**Figure 2: *Wsb1*, *Ksr1*, and *Nf1* Levels in Complementation *Chaos3* Mammary Tumor Cell Lines**

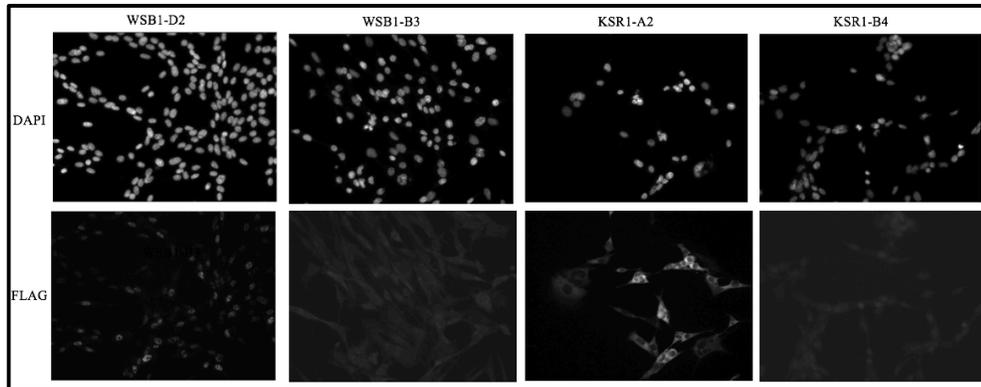
Six stable cell lines were created with gene complementation. RT-qPCR data showed the relative expression of *Wsb1*, *Ksr1*, and *Nf1* in each of the six 22168 cell lines. The data for GFP-1 and GFP-5 cell lines were geometrically averaged together (geometric mean). The expression levels are relative to expression levels of *GAPDH* and the GFP geometric mean. Each cell line was run in triplicates (three technical replicates) and the average of two biological replicates were graphed.



**Figure 3: Fluorescent Imaging of GFP Complementation *Chaos3* Mammary Tumor Cell**

**Lines**

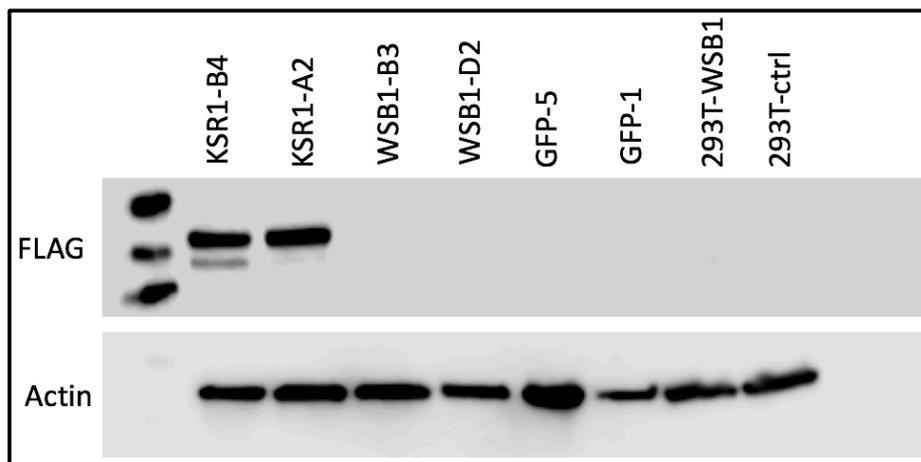
After the 22168 cell lines were grown out, levels of GFP fluorescence were observed in the GFP-1 and GFP-5 complementation cell lines for the presence of GFP+ cells. These images are a representation of 5 pictures taken of each cell line.



**Figure 4: Immunofluorescence of the FLAG Epitope Tag in WSB1 and KSR1**

**Complementation *Chaos3* Mammary Tumor Cell Lines**

Immunofluorescence was performed on the 22168 KSR1-A2, KSR1-B4, WSB1-D2, and WSB1-B3 cell lines. This was done to identify the presence of the FLAG epitope tag in both the *Wsb1* and *Ksr1* genes that were inserted into these cell lines. The DAPI stained DNA showed the total number of cells present, while the antibody bound only to the epitope tag in the protein.



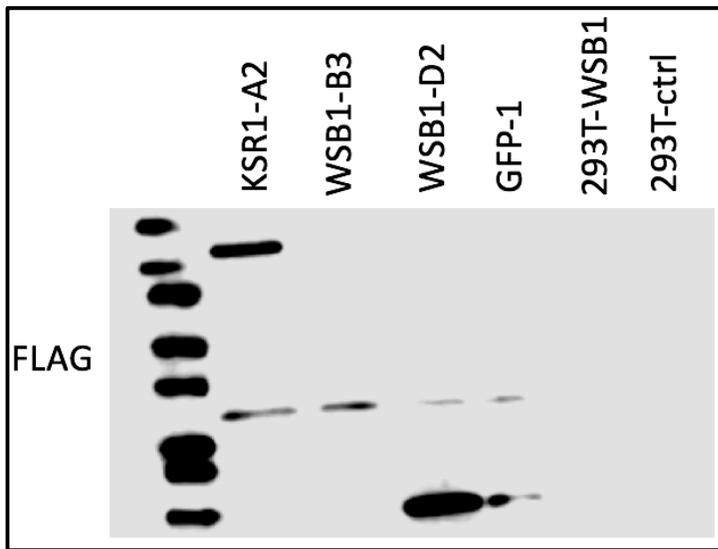
**Figure 5: Western Blotting for WSB1 and KSR1 Protein in Complementation *Chaos3***

**Mammary Tumor Cell Lines**

The FLAG epitope tag, which is present in both WSB1 and KSR1 ectopically expressed protein, helped to identify the presence of these protein in each complementation cell line. The presence

of a band indicated that the WSB1 or KSR1 protein was present in that cell line. GFP-1, GFP-5, and 293T-ctrl were used as negative controls. 293T-WSB1 was used as a positive control.

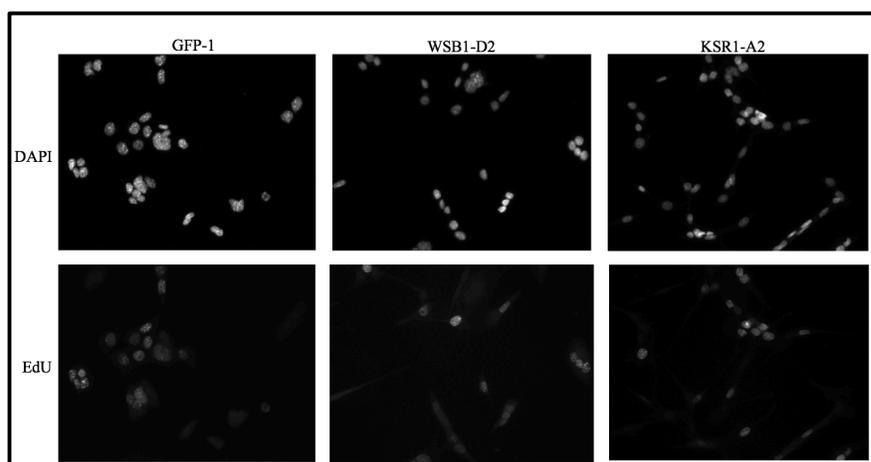
Protein levels were compared to Actin.



**Figure 6: Western Blotting for WSB1 and KSR1 Protein in Complementation *Chaos3***

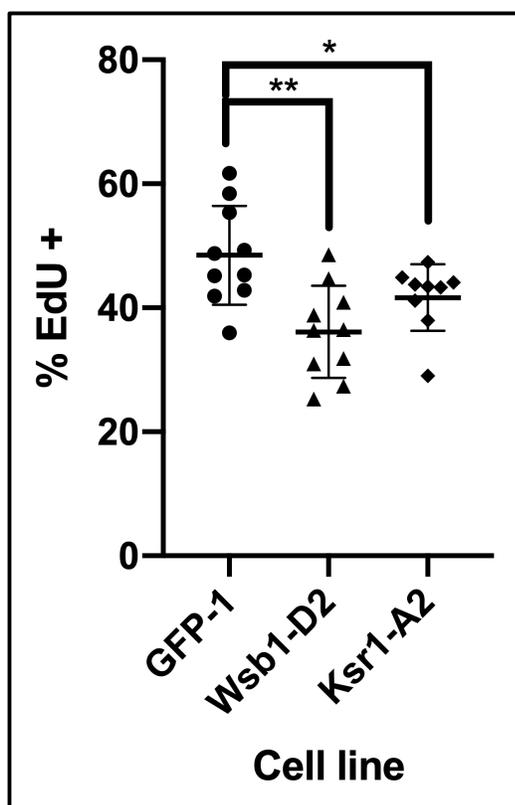
#### **Mammary Tumor Cell Lines with a Protease Inhibitor**

The FLAG epitope tag, which is present in both WSB1 and KSR1 ectopically expressed protein, helped to identify the presence of these protein in each complementation cell line. A protease inhibitor was added in order to prevent ubiquitinated protein from degradation. The presence of a band indicated that the WSB1 or KSR1 protein was present in that cell line. GFP-1, GFP-5, and 293T-ctrl were used as negative controls. 293T-WSB1 was used as a positive control.



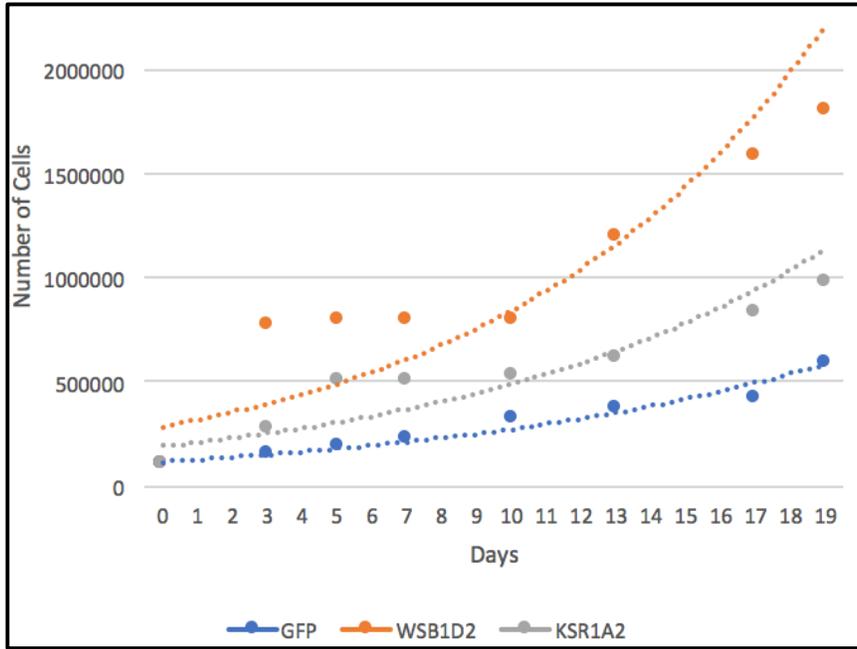
**Figure 7: EdU Labeling of Complementation *Chaos3* Mammary Tumor Cells**

EdU labeling Click Chemistry was performed on the 22168 GFP-1, KSR1-A2, and WSB1-D2 cell lines. This was done to identify the number of cells that were replicating. The DAPI stained DNA showed the total number of cells present, while the EdU stained only the replicating cells.



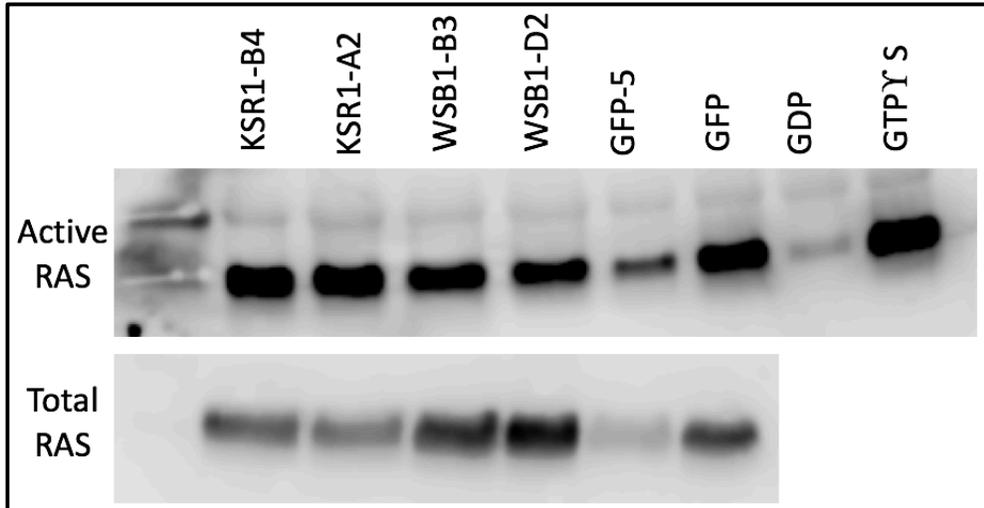
**Figure 8: EdU Labeling of Complementation *Chaos3* Mammary Tumor Cells**

EdU labeling Click Chemistry was performed on the 22168 GFP-1, KSR1-A2, and WSB1-D2 cell lines. This EdU stain identified the number of cells that were replicating. The number of EdU labeled cells were compared to the total number of (DAPI stained) cells. This number was represented as a percentage. 730 cells were analyzed for GFP-1, 831 for WSB1-D2, and 1249 for KSR1-A2. There was a significance difference between GFP-1 and WSB1-D2 cells ( $p=0.0022$  through an unpaired two-tailed T test) as well as between GFP-1 and KSR1-A2 cells ( $p=0.0462$  through an unpaired two-tailed T test). There was no significant difference between WSB1-D2 and KSR1-A2 cells ( $p=0.0839$  through an unpaired two-tailed T test).



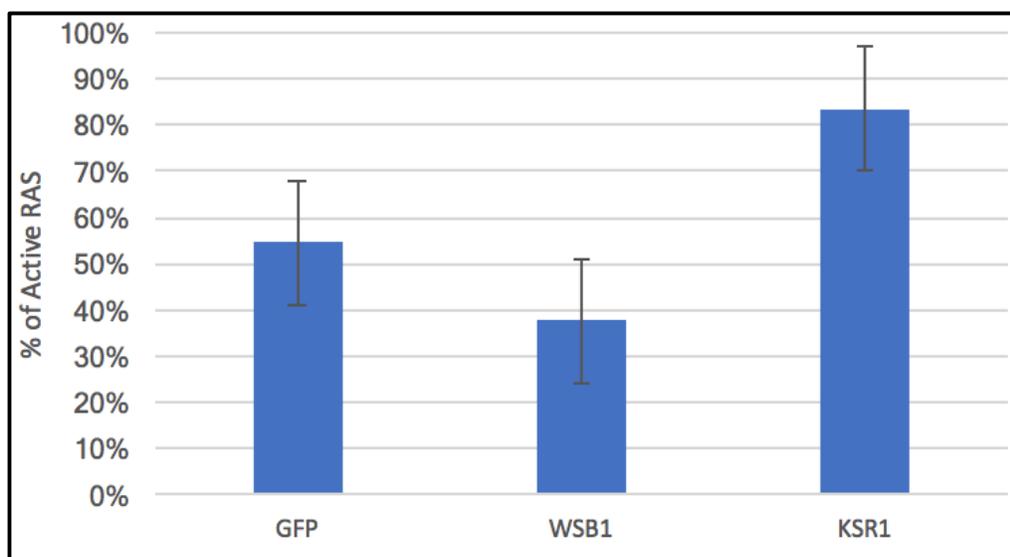
**Figure 9: Growth Curve for Complementation *Chaos3* Mammary Tumor Cell Lines**

$10^5$  cells of each 22168 GFP-1, WSB1-D2, and KSR1-A2 cell line were plated and growth was recorded 3 days later.  $10^5$  cells were re-plated and this was repeated six more times every 2-4 days. The total number of cells was calculated by taking the number of cells counted that day, subtracting the starting number of cells, and adding this to the total number of cells from the previous recorded day. An exponential trend line was drawn for each cell line.



**Figure 10: Active RAS Pulldown of Complementation *Chaos3* Mammary Tumor Cells**

Levels of active RAS were measured for each *Chaos3* complementation cell line. The darker the band, the greater amount of active RAS was present in the cell line. In the “Active RAS” blot, GTPYS was a positive control that activated all the RAS and showed the total amount of RAS (in GFP). GDP was a negative control that inactivated all the RAS. The “Total RAS” blot showed the total amount of RAS in each cell line. The GTPYS and GDP controls were not run in this blot.



**Figure 11: Percentage of Active RAS in “Complementation” *Chaos3* Mammary Tumor Cells**

Levels of active RAS were measured for each *Chaos3* complementation cell line relative to the amount of total RAS in that cell line. Intensity quantifications were taken from active RAS pulldown blot. This percentage was averaged for the two GFP cell lines, two WSB1 cell lines, and two KSR1 cell lines. The percentage was used to tell the percent of RAS in the cell line that is active (GTP bound). Error bars represent standard deviation from the mean, N=2.

## ACKNOWLEDGEMENTS

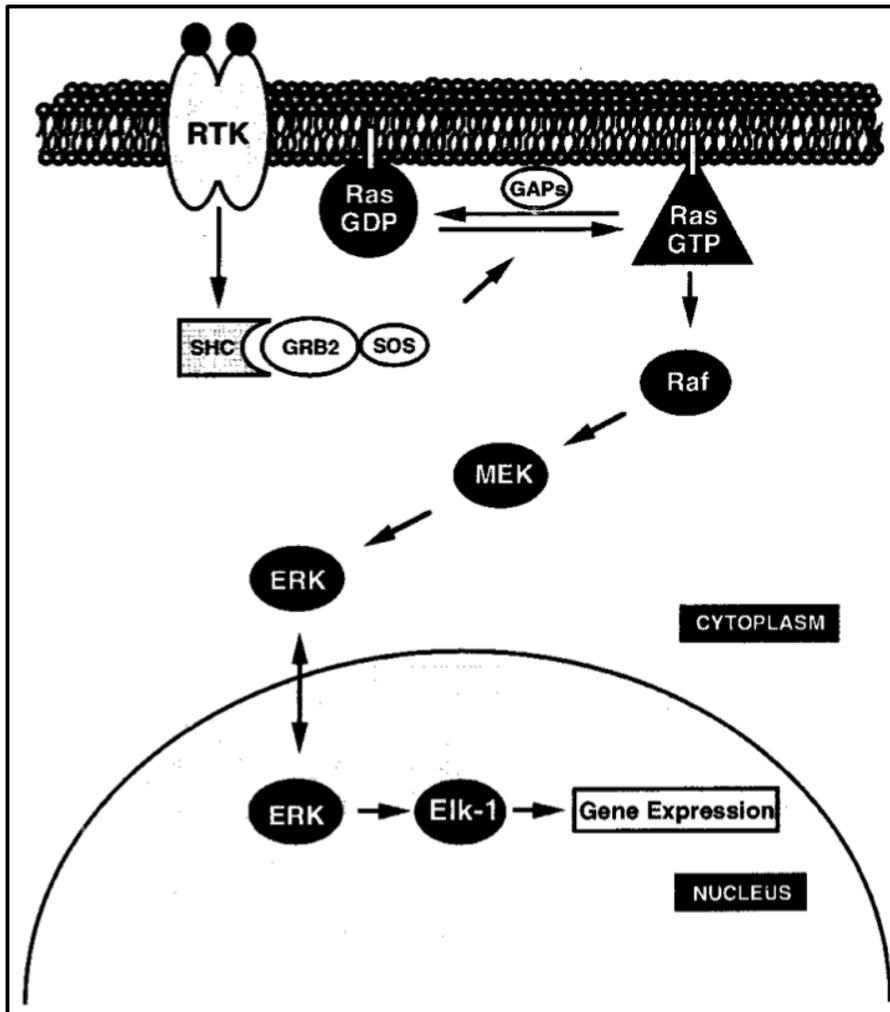
I would like to thank Dr. John Schimenti, Dr. Adrian McNairn, and the rest of the Schimenti lab for their support and guidance during my years working in this lab.

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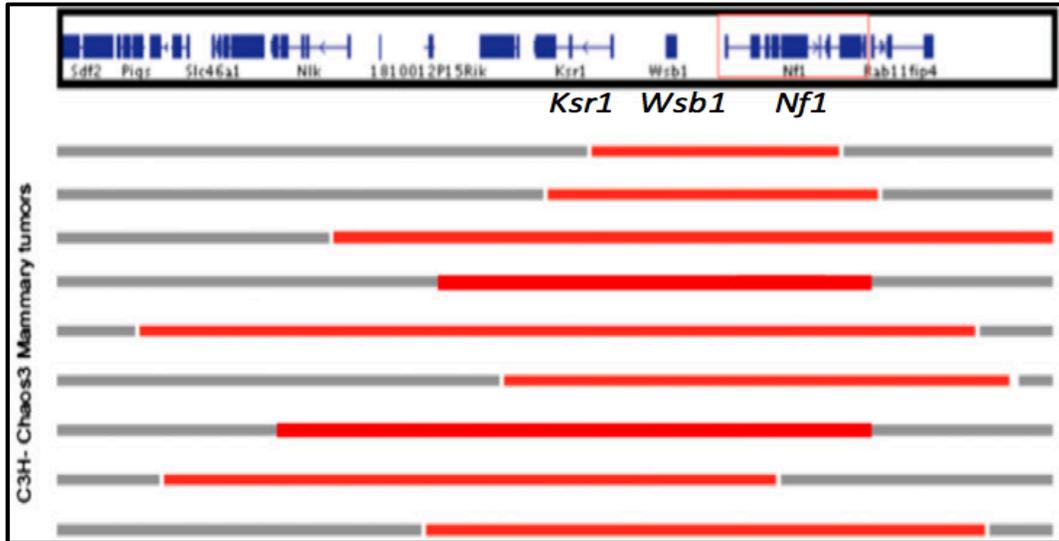
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## APPENDIX



**Figure 11: RAS Regulation**

RAS is a GDP/GTP-related binary switch that regulates signal cascades into the cytoplasm. RAS functions downstream of receptor tyrosine kinases (RTKs) and upstream of a cascade of serine/threonine kinases (Raf, MEK, MAPK). It can activate this pathway when it is bound to GTP, but not when it is bound to GDP. [3]



**Figure 12: Deletions in *Chaos3* Mammary Tumors**

All nine *Chaos3* mammary tumors show chromosome 11 deletions (red) overlapping portions of *Nf1*, *Wsb1*, and *Ksr1*. [19]

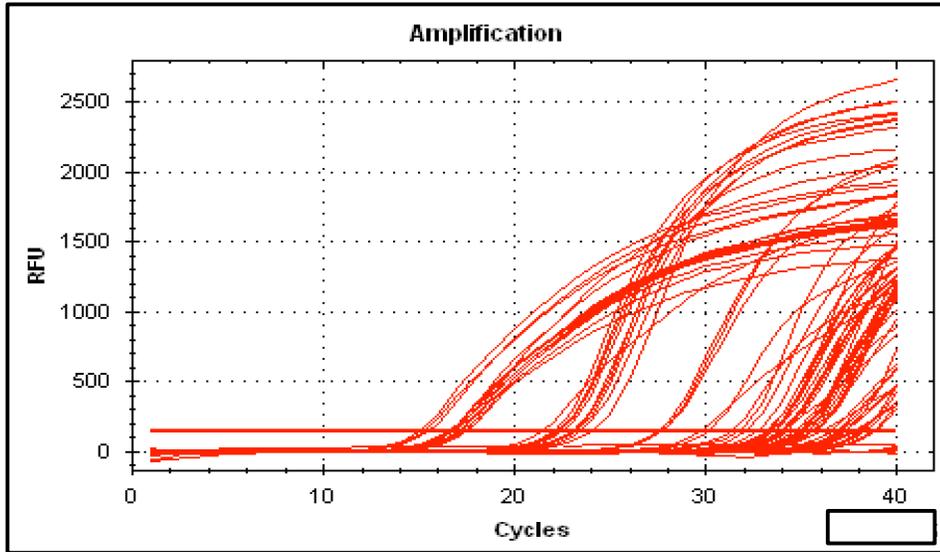


AM_mKSR1_qF1	ATCAATAAGACTAGGCAGATCGC
AM_mKSR1_qR1	ATCCCAAACAGCCCGAAG
MsNF1ex47F1	CACTTCTGGAACAAAACCTGC
MsNF1ex48R1	AGTTAGAGTTGAAGTTGAGTCCG
MsNF1ex27F2	GTGGTCCCTTGTTCTCAGTG
MsNF1ex28R2	AGCTGGTAGAGTAAATGCCG
MsNF1ex8F3	TGGCCCCTACAAATCATTCTC
MsNF1ex9R3	TGCTTCCGTAAACTGTCCAG
MmGAPDH_F1	CTTTGTCAAGCTCATTCCTGG
MmGAPDH_R1	TCTTGCTCAGTGTCCCTGC
AM_WSB1_qF1	CTAAGAGTGTGGGACCTGAAAG
AM_WSB1_qR1	GGAAAACCTGCTTTACTGGCG

**Table 1: Primer sequences for RT-qPCR for *Ksr1*, *Wsb1*, and *Nfl* Gene Expression**

	Wsb1	Ksr1	Nfl
GFP	1.00	1.00	1.00
WSB1D2	1675.06	0.56	0.80
WSB1B3	310.83	0.46	1.00
KSR1A2	0.65	2778.33	3.76
KSR1B4	0.84	3468.27	0.92

**Table 2: Ct Values for RT-qPCR on “Complementations” *Chaos3* Mammary Tumor Cell Lines**



**Figure 14: Amplification plot for RT-qPCR of “Complementations” *Chaos3* Mammary Tumor**

This amplification graph indicates that the qPCR was successful.