# Breaking the Senescence: Inhibition of ATM Allows S9 Cells to Re-Enter Cell Cycle

by

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# ABSTRACT

The Philadelphia chromosome in humans, is on oncogenic translocation between chromosomes 9 and 22 that gives rise to the fusion protein BCR-Abl. This protein is constitutively active resulting in rapid and uncontrolled cell growth in affected cells. The BCR-Abl protein is the hallmark feature of chronic myeloid leukemia (CML) and is seen in Philadelphia-positive (Ph+) acute lymphoblastic leukemia (ALL) cases. Currently, the first line of treatment is the Abl specific inhibitor Imatinib. Some patients will, however, develop resistance to Imatinib.

Research has shown how transformation of progenitor B cells with v-Abl, an oncogene expressed by the Abelson murine leukemia virus, causes rapid proliferation, prevents further differentiation and produces a potentially malignant transformation. We have used progenitor B cells transformed with a temperature-sensitive form of the v-Abl protein that allows us to inactivate or re-activate v-Abl by shifting the incubation temperature. We are trying to use this line as a model to study both the progression from pre-malignancy to malignancy in CML and Imatinib resistance in $Ph^+$ ALL and CML. These progenitor B cells, once v-Abl is reactivated, in most cases, will not return to their natural cell cycle. In this they resemble $Ph^+$ ALL and CML under Imatinib treatment. With some manipulation these cells can break this prolonged $G_1$ arrested phenotype and become a malignant cell line and resistant to Imatinib treatment.

Cellular senescence can be a complicated process requiring inter-play between a variety of players. It serves as an alternate option to apoptosis, in that the cell loses proliferative potential, but does not die. Treatment with some cancer therapeutics will induce senescence in some cancers. Such is the case with Imatinib treatment of CML and $Ph^+$ ALL. By using the S9 cell line we have been able to explore the possible routes for breaking of prolonged $G_1$ arrest in these $Ph^+$ leukemias. We inhibited the DNA damage sensor protein ataxia telangiectasia mutated (ATM) and found that prolonged $G_1$ arrest in our S9 cells was broken. While previous research has suggested that the DNA damage sensor protein ataxia-telangiectasia mutated (ATM) has little impact in CML, our research indicates that ATM may play a role in either senescence induction or release.

## DEDICATION

I would like to dedicate this to my grandfather, James Ivy Dixon, Sr. He taught me to always be true to myself, follow my dreams, and keep my "eyes on the prize". Thank you grandfather, for teaching me that there is nothing that I cannot do if I work for it. I would also like to thank my older sister and brother, Pat and Jimmy for their encouragement. My mother, for setting the bar for my success so high. Thank you to all of my friends who supported me in my ambition. I would also like the thank my committee for their knowledgeable advice. And to Jen, thank you my love, for getting me past that final barrier – my own complacency. You pushed me to complete this journey, without ever realizing it.

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## Introduction

### CML, ALL, BCR-Abl

Leukemia is a type of cancer of the blood or bone marrow characterized by an abnormal increase of white blood cells. The term leukemia includes a spectrum of diseases. Two of which are chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL). CML is a clonal disease where cells of the myeloid lineage will undergo unregulated clonal expansion. This disease progresses through three different phases – a chronic phase, an accelerated phase, and finally blast crisis.[7,10] In ALL it is immature lymphoblasts become malignant and continuously multiply in the bone marrow, crowding out normal, healthy cells. The disease time course is quite short in ALL, with death occurring in weeks if left untreated. This type of myeloproliferative disease is associated with a characteristic genetic abnormality called the Philadelphia (Ph+) chromosome. This chromosome is the result of a reciprocal translocation between the long arms of chromosomes 9 and 22. Specifically, the q34 region of chromosome 9 is substituted at the q11 region of chromosome 22, denoted as t(9;22)(q34;q11). This oncogenic translocation places the Abelson (Abl) gene from chromosome 9 beside the breakpoint cluster region (BCR) gene from chromosome 22 giving rise to the fusion protein BCR-Abl (Figure 1).

The manner and reason for this translocation are currently unknown. The resultant fusion protein, BCR-Abl, is found in >90% of CML and ~ 30-35% of ALL patients, and has been determined to play a causal role in CML.[7,10] The BCR-Abl protein functions as a constitutively active tyrosine kinase, interacting with multiple downstream actors. The results of these interactions include altered cellular adhesion, inhibition of apoptosis, activation of mitogenic signaling, and defective DNA repair.[2] Recent advancements in targeted therapy have led to the use of a BCR-Abl tyrosine kinase inhibitor called Imatinib (Gleevec, Novartis). Imatinib works by sitting in the ATP-binding site of the Abl portion of BCR-Abl, maintaining it in its inactive form. (FIGURE 2) Imatinib will inhibit cell proliferation and tumorigenesis without inducing apoptosis.[2] The use of imatinib greatly improved prognosis, response rate, and overall patient survival. Some patients however, will develop resistance to imatinib, so eradication remains elusive. While research has provided alternate treatments for Imatinib-resistant CML, most of these therapies still affect the same fusion protein, BCR-Abl. Prognosis in CML and $Ph^+$ ALL is quite poor in patients resistant to Imatinib. Even in patients that respond well initially to Imatinib, treatment is not curative as BCR-Abl transcripts can still be detected in these patients.

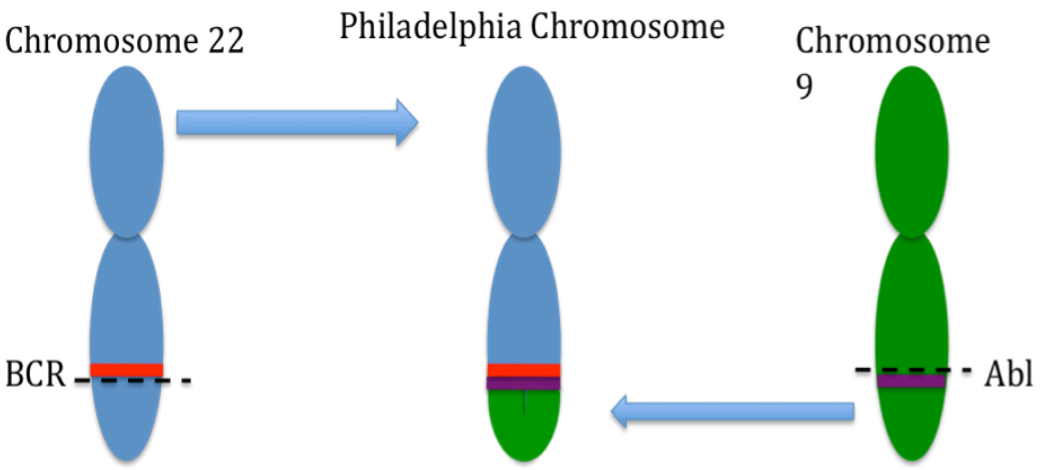



**Figure 1. Schematic of the Philadelphia Chromosome**

**ATM, p53, mTOR pathways**

Ataxia-telangiectasia mutated is a ~370kDa protein that plays a central role in the DNA damage response. A member of the phosphatidylinositol-3 kinase-related kinase (PIKK) family, ATM is activated in response to double-strand break (DSB) damage. ATM also plays a role in non-homologous end joining (NHEJ).[4] ATM can be specifically activated upon DNA DSB damage due to ionizing radiation, replication errors, general toxic drugs, or by-products of metabolism. Initially, ATM is activated upon DNA damage, being recruited to the DSB site by the MRN complex (Mre11, RAD50, and NBS1). At the site of DNA damage ATM autophosphorylates then proceeds to affect its downstream targets, one of which is p53 (Figure 3). Mutation of ATM leads to the autosomal recessive disorder ataxia-telangiectasia (A-T). This disease is characterized by cerebellar ataxia (gross lack of coordination due to

cerebellum dysfunction) and oculocutaneous telangiectasia (small dilated blood vessels in the eyes). Individuals with A-T also have symptoms of growth retardation, pre-mature aging, hypersensitivity to ionizing radiation, and an increase in lymphoma tumorogenesis.[24] ATM can function as an upstream regulator of a number of proteins and is this involved in several cellular processes including cell cycle arrest, DNA repair and apoptosis.

Because ATM plays such an integral role in DNA damage repair and tumorigenesis, some researchers have explored the possible role the ATM gene has in CML. The initial thought was that ATM might have some effect in the transformation from the chronic phase of CML to blast crisis, due to the association between the complex karyotypes of blast crisis and possibly defective DNA double-strand break repair and interaction between Abl and ATM.[33]

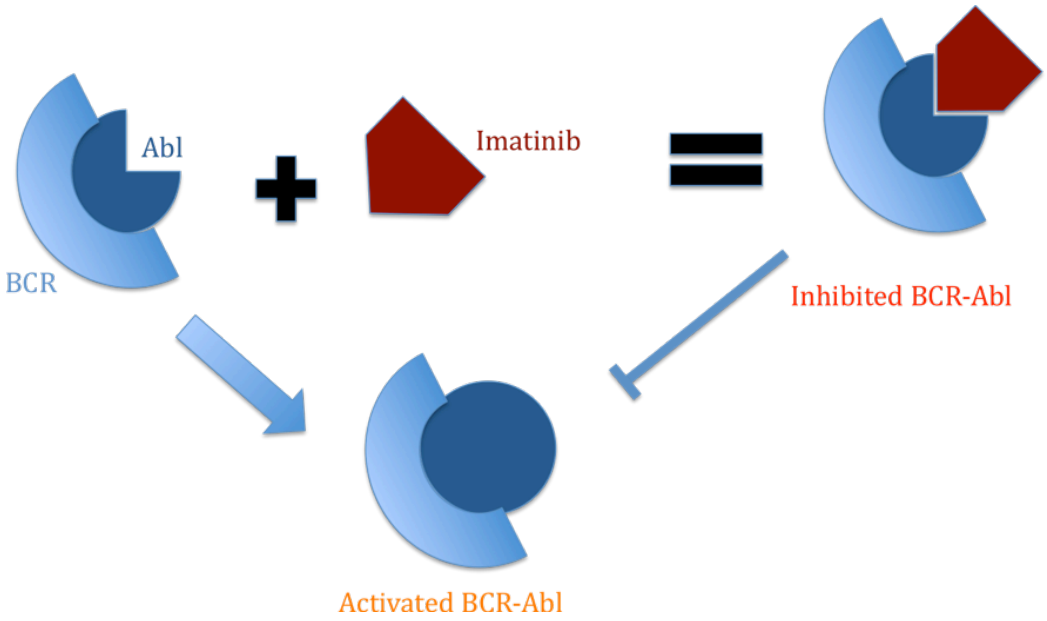



**Figure 2. Imatinib Mechanism of Action**

P53 plays a pivotal role in tumor surveillance and tumorogenesis. As a master regulator, p53 possesses the ability to signal simple cell cycle arrest or push further to apoptosis. In cell cycle arrest, p53 can signal the continued arrest at the $G_1$/S phase through p21, which can act as an inhibitor of CDK2/cyclin E. Prolonged activation of p53 leads to apoptosis through a variety of possible proteins, including Bax, NOXA, P53AIP1, and PIDD[42]. P53 has the ability to activate a diverse variety of genes. Some of these genes modulate DNA repair, cellular senescence, differentiation, angiogenesis, longevity, and oxidative stress, just to name a few.[8] ATM serves as an upstream regulator of p53 through activation of CHK2. This activation (of p53), is one step in the process of cell cycle arrest due to DNA DSB damage. While p53 halts the cell cycle at $G_1$, via p21, it will

also up-regulate proteins to address the DNA damage directly through repair. If repair of the DNA damage is unsuccessful then p53 is instrumental in initiating apoptosis.

One downstream target of p53 is mTOR (mammalian target of rapamycin resistance). It is through mTOR that p53 works to control cellular senescence and quiescence. Quiescence is the temporary halting of the cell cycle, a period in which division does not take place. During this stage cells can be resting or attempting to repair damaged DNA. Quiescence is contrasted to senescence, in which cells have lost the ability to divide. A senescent state may be activated in cells that are receiving conflicting growth/death signals, or that have severely shortened telomeres. Depending on further signaling, the cell can revert back to a quiescent state, proceed to apoptosis, or even enter into a state known as autophagy (self-eating). It is coming out now in literature that these three states are closely inter-related, and p53 sits nearly at the center.

The serine/threonine protein kinase, mTOR is a member of the PIKK family and plays a role in the regulation of autophagy, cell growth, cell proliferation, cell survival, and protein synthesis and transcription (Hay, Beevers, Bandhakavi). mTOR has an important role in the induction of cellular senescence by p53 and has even been linked to DNA damage repair (DDR) through ATM. It has been suggested that the ability of p53 to induce senescence or quiescence is linked to inhibition of mTOR. Korotchkina, et al., determined that cells normally fated for senescence

could be reverted back to quiescence through p53 regulation of mTOR.[27] The group determined that, in quiescent cells, p53 would inhibit mTOR ensuring the quiescent state, but if mTOR was activated that quiescence would be converted to senescence. In senescent cells, p53 caused cycle arrest without inhibiting mTOR and thus ensured senescence. If mTOR was inhibited in these cells, then quiescence could be restored.

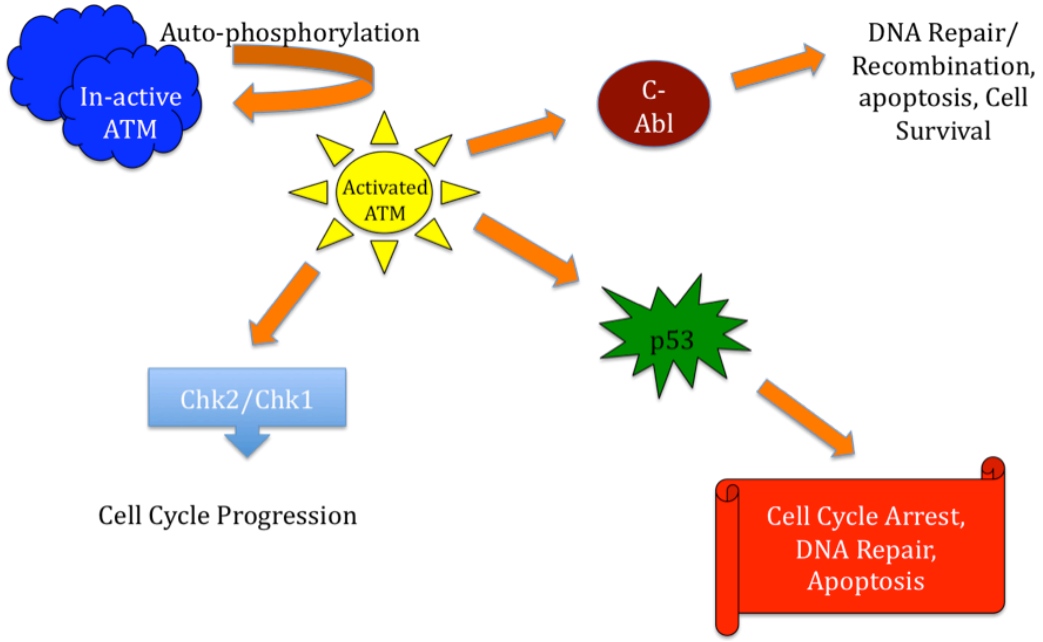



**Figure 3. Pathway for ATM Regulation of Cell Cycle Arrest, DNA Repair, and Apoptosis**

### P38 and p27

Another potential activator of p53 is p38MAPK (mitogen-activated protein kinase). There are four different p38 family members (α, β, γ, and δ), the most prevalent and important of which is p38α. A different gene

encodes each member, and expression varies in different tissue types, however p38α is ubiquitously expressed in most tissue types at significant levels. Activation of p38 occurs through phosphorylation on both a threonine and a tyrosine residue in the activation loop of the protein. How these phosphorylation events occur is dependent upon the activating pathway. In the canonical pathways MAP2Ks will phosphorylate p38 on threonine 180 and tyrosine 182. Alternate paths toward p38 activation have also been described. The cascade leading to p38 activation can start with a number of signals including UV irradiation, osmotic shock, cytokines, and ROS. P38 activation by ROS is noted as being through canonical activation via MKK3 or MKK6.[34] This activation has downstream effects on p53, and can lead to premature senescence. P38 has also been linked to ATM activation through regulation of oxidative stress.[20, 28,35]

Another protein that has been linked to cellular senescence is the Cdk inhibitor $p27^{Kip1}$. $P27^{Kip1}$ is a member of the Cip/Kip family of cyclin dependent kinase (Cdk) inhibitors along with $p57^{Kip2}$, and $p21^{Cip1}$. $P27^{Kip1}$ functions as an upstream regulator of Cdk4 and Cdk2 complexes. It is through the regulation of Cdk4 and Cdk2 complexes that $p27^{Kip1}$ is able to arrest cells at $G_1$. How $P27^{Kip1}$ regulates Cdk2 and Cdk4 is still being discussed in literature. Evidence has come out indicating that while $p27^{Kip1}$ can inhibit Cdk2 complexes, regulation of Cdk4 complexes may be through activation.[6] Members of the Kip/Cip family, in general, are

portrayed as being potent inhibitors of cyclin-Cdk complexes and $p27^{Kip1}$ is reported to be active in quiescent cells but not in cycling cells. The inhibitory potential of $p27^{Kip1}$ is dependent on the cellular context.[5, 6] For example, Cip/Kip proteins adopt specific tertiary structure upon binding to other proteins. This conformational flexibility may modify the folding of the Cip/Kip proteins and modulate their ability to inhibit cyclin-CDK complexes.[5] This is likely why members of Cip/Kip are able to interact with so many different partners. Regulation of $p27^{Kip1}$ is through phosphorylation and protein-protein interactions. The phosphorylation of $p27^{Kip1}$ can alter its affinity for different Cdk complexes, other proteins and their subcellular localization.[5] Research has indicated that treatment with Imatinib will cause an increase in $p27^{Kip1}$ expression and that the nuclear accumulation of $p27^{Kip1}$ is in part responsible for the anti-proliferative activity of Imatinib.[7]

**S9 – Senescent Pre-B Cell, Cell Line**

Our lab is currently exploring the use of a cell line of Pre-B cells harvested from severe combined immunodeficient (SCID) mice. This cell line also has a defect in the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), a DNA damage repair protein. After harvesting these cells were transformed by infection with a temperature sensitive Abelson murine leukemia virus (ts-Ab-MuLV). This temperature sensitivity conferred a constitutively active v-Abl in the S9 cell line that could be

inactivated by shifting the cells from the permissive temperature (33°C) to the non-permissive temperature (39°). Initially created to monitor V(D)J recombination, these cells were found to arrest at $G_1$ phase when v-Abl was inactivated (shifted to 39°C) as expected. However, when v-Abl is reactivated the cells do not return to their normal cell cycle. Instead they enter a state of prolonged $G_1$ arrest. Some cells were able to escape the $G_1$ arrest, returning to the cell cycle. When grown in culture, these cells have been found to be resistant to cell cycle arrest and to arrest induced by Imatinib. This goes along with their transformation to a malignant cell line. The purpose of this study was to determine what the cause of the temperature-induced prolonged $G_1$ arrest of these S9 cells in an effort to see if the S9 cell line could serve as a model for $Ph^+$ ALL.

## Materials and Methods

### *Cell Culture*

S9, A70, and SB 1.1 cells were cultured in RPMI 1640 media with 10% fetal bovine serum, 1mM L-glutamine, 100 U/ml streptomycin, 100ug/ml penicillin, and 50uM β-mercaptoethanol. S9 cells were maintained at 33°C (v-Abl permissive temperature) in 5% $CO_2$. V-Abl inactivation was accolplished by incubating S9 cells at 39°C in 5% $CO_2$ for two days. Re-activation of v-Abl was through shifting S9 cells back to the permissive temperature (33°C). A70 and SB 1.1 cells were maintained at

37°C in 5% $CO_2$. A70 is a pre-B cell line that has been transformed with Ab-MuLV. SB 1.1 is also a Ab-MuLV transformed pre-B cell line, however this line was derived from a DNA-PK knockout mouse and has a defective DNA-PK. Because A70, and SB 1.1 cells did not have a temperature-sensitive v-Abl, inhibition of v-Abl was accomplished through chemical inhibition with STI571 (Imatinib).

***Drug Treatments***

To determine what effect different inhibitors had on senescence in S9 cells we treated them with either pan-PI3K-inhibitor, LY294002, or ATM inhibitor KU55933 (both from CalBiochem/EMD). LY294002 was applied to cells at a concentration of 10μM, and KU55933 at a concentration of 8μM for 48hrs at 39°C then cells were washed in PBS and re-incubated in either fresh control media or fresh media containing LY294002 or KU55933 for another 48hrs. Alternate S9 v-Abl inactivation and v-Abl inactivation in A70, and SB 1.1 was accomplished through treatment with 1μM STI571 (LC Laboratories) for 48hrs. As with the other inhibitors, after 48hrs the STI571 was washed off and the cells re-incubated in control media. All drugs were prepared in DMSO with a final DMSO concentration of 0.1%. Control media was prepared with 0.1% DMSO to mirror drug treatment DMSO concentrations.

***Cell Cycle Analysis***

Flow cytometry was run on a FACSCaliber, (BD Biosciences) according to the following: 250μl of cell suspension was centrifuged at 1500rpm for 5 minutes at 4°C. Supernatant was removed and cells were re-suspended with equal amounts of RNAse A (2mg/ml in 1.12% sodium citrate) and Propidium Iodide (100ug/ml in 0.2% triton-x 100/0.1% sodium citrate). The FACSCaliber recorded a total of 10,000 events. Fluorescence intensity was analyzed with a FL2-A histogram using Cell Quest Pro software (BD Biosciences).

***Western Blot Protein Detection***

Cells were counted, washed in cold PBS, then lysed in lysis buffer and incubated on ice for 30 minutes. Equal amounts of protein lysate from each sample (~ 2million cells or 65ng total protein as determined by Bradford assay) were separated via SDS-PAGE then transferred to PVDF membrane while on ice. Membrane were blocked in 5% BSA in tris-buffered saline with 5% Tween 20 (TBST) then incubated with primary antibody overnight at 4°C.Phospho-p53, p53, phospho-p38, p38, and p27 antibodies were purchased from Cell Signaling. GAPDH was purchased from Sigma –Aldrich, and Actin from Santa Cruz Biotechnology. After primary antibodies, membranes were incubated at room temperature with an appropriate peroxidase-conjugated secondary antibody (anti-rabbit, anti-mouse or anti-goat) for one hour. Primary antibodies were used at 1:1000 dilutions, while secondary antibodies were at 1:5000 dilutions.

Detection of specific bands was by using enhanced chemiluminescent reagents from Pierce (Thermo Scientific). Luminescent signal was transferred to blue autoradiography film (Santa Cruz Biotechnology) and developed.

## Results

### ATM Inhibition Allows S9 cells to Re-enter cell cycle After v-Abl Reactivation

The first experiment performed was to see if inhibition of certain pathways could break the senescence phenotype in S9 cells and get them to re-enter the cell cycle. We used two different PI3K kinase inhibitors to determine if their disruption could release S9 cells from $G_1$ arrest. We treated S9 cells with two different PI3K inhibitors while shifting the cells up to the non-permissive temperature. The cell cycle was analyzed via flow cytometry after two days of v-Abl inactivation (at 39°), and every day following re-activation.

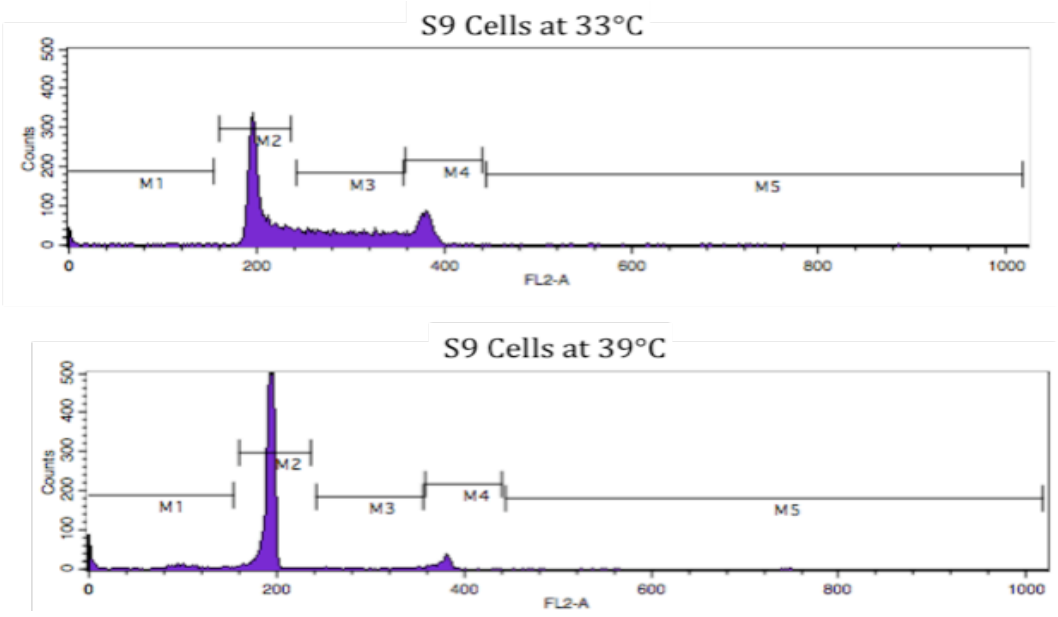



**Figure 4. Flow cytometry of S9 cells with v-Abl active (top) or inactive (bottom).**

*Cell Cycle.* Cell cycle profiles for each drug were determined by permeabilization and propidium iodide (PI) staining. Because PI stains the DNA directly, DNA content can be measured at different wavelengths. This information can then be extrapolated to the accumulation of DNA in different phases of the cell cycle. The inhibitors used were LY294002, and KU55933. The first, LY294002 is a PI3K inhibitor that acts reversibly and has been shown to block PI3 kinase-dependent Akt phosphorylation and kinase activity. The second is a small molecule inhibitor specific for ATM and is called KU55933. Figures 4 & 5 represents an example of the output from our flow cytometric analysis of the PI staining of S9 cells under treatment with LY294002 and KU55933. The M1 region represents cells undergoing apoptosis. M2 is cells in $G_0/G_1$, M3 is cells in S phase, and M4 represents cells in $G_2$. Aneuploid cells are seen in M5. While treatment with LY294002 offered no real change in $G_1$ arrest after v-Abl reactivation, treatment with KU55933 displays a robust re-entry into the cell cycle within 2 days after v-Abl re-activation (Figure 6). From this we determined that specific inhibition of ATM was able to release the $G_1$ cycle arrest and allow S9 cells to re-enter the cell cycle.

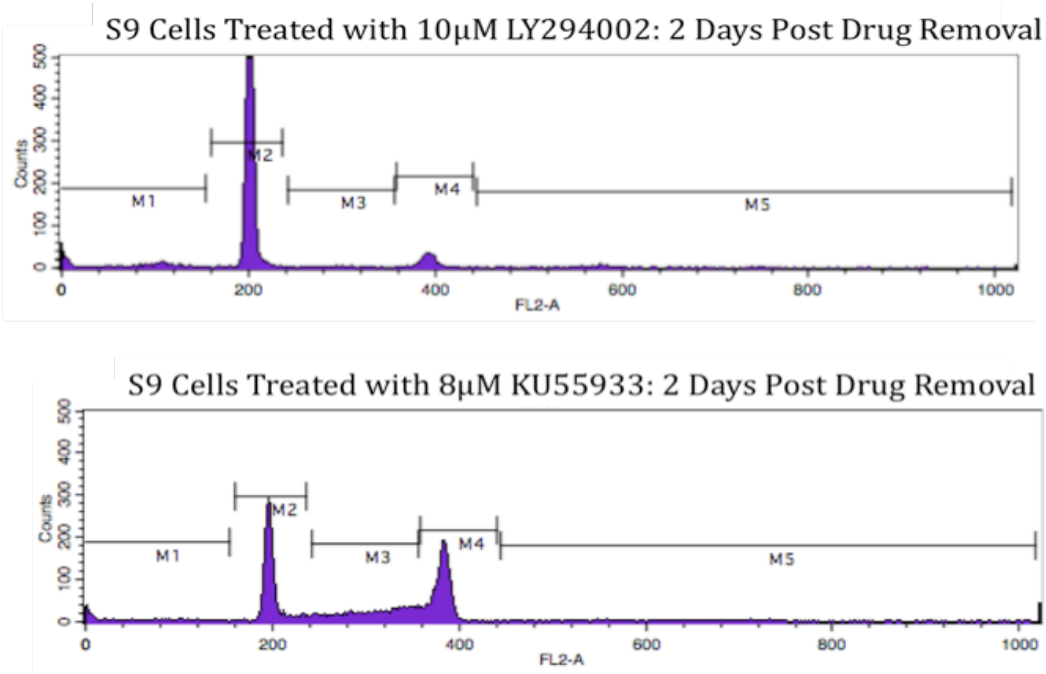



**Figure 5. Flow cytometry of S9 cells after v-Abl re-activation. Inhibition of PI3K (top), or inhibition of ATM (bottom).** S9 cells were treated with either LY294002 (PI3K inhibitor) or KU55933 (ATM inhibitor) and shifted to 39°C for 48hrs, washed in PBS, and re-incubated with treated media at 33°C to re-activate v-Abl. Results shown are 48 hours after v-Abl re-activation.

### Prolonged $G_1$ Arrest of S9 Not Dependent upon mTOR

Studies have indicated that there is a relationship between the PI3K kinase mTOR and cellular senescence.[17] Korotchkina, et al. have recently shown that mTOR can have an effect in the decision between senescence and quiescence in p53-arrested cells. The group used nutlin-3a to transiently induce p53 in normally quiescent or in senescent-prone cells. They found that the quiescent cells could be forced into senescence, while the senescent cells were pushed toward

reversible quiescence and the state of mTOR (active or inactive) seemed to be at the center of this.

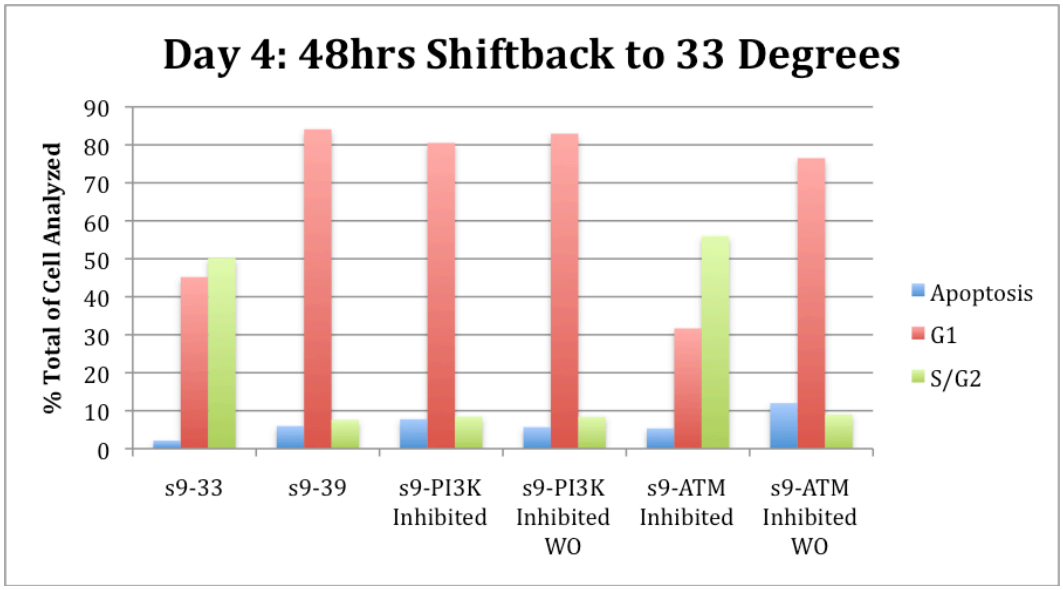



**Figure 6. ATM inhibition allows S9 cells to re-enter the cell cycle.** S9 cells were shifted up to 39°C to in-activate v-Abl for 48hrs. One set was PI3K inhibited, another was ATM inhibited. After 48hrs of v-Abl inactivation, cells were washed with PBS, then incubated in either treatment media (PI3K Inhibited, ATM Inhibited) or in control media (PI3K Inhibited WO [washed off], ATM inhibited WO [washed off]). As controls, untreated S9 cells were shifted to 39°C for 48hrs, washed, and shifted back to 33°C or kept at 33°C.

Our S9 cells enter into a senescent state when v-Abl is reactivated and do not return to their normal growth cycle. The reason for this senescence is currently unknown. We tried inhibiting mTOR via treatment with rapamycin to see if this senescent phenotype was the result of mTOR induction. Rapamycin is an immunosuppressant drug used to prevent rejection in organ transplants. Rapamycin works by binding to cytosolic

FK-binding protein 12 (FKBP12), then the rapamycin-FKBP12 complex binds to mTOR complex 1 (mTORC1) inhibiting the cell's response to interlukin-2 (IL-2), thereby blocking the activation of both T and B cells. In our experiments we tested to see if by shutting down mTOR with rapamycin we could force our normally senescent S9 into quiescence. We treated S9 cells with 1μM rapamycin and shifted up to the non-permissive temperature for 2 days then washed in PBS and shifted back to the permissive temperature for 2 days. Upon shift back the cells were either re-incubated in media with 1μM rapamycin or untreated media. Cell cycle was analyzed via flow cytometry after 2 days at the non-permissive temperature and 2 days after v-Abl reactivation. After 2 days of v-Abl inactivation all cells were arrested at $G_1$ regardless of the treatment. Two days after v-Abl reactivation S9 cells were still arrested at the $G_1$ phase (Figure 7). Unlike ATM inhibition, inhibition of mTOR did not release the cellular senescence.

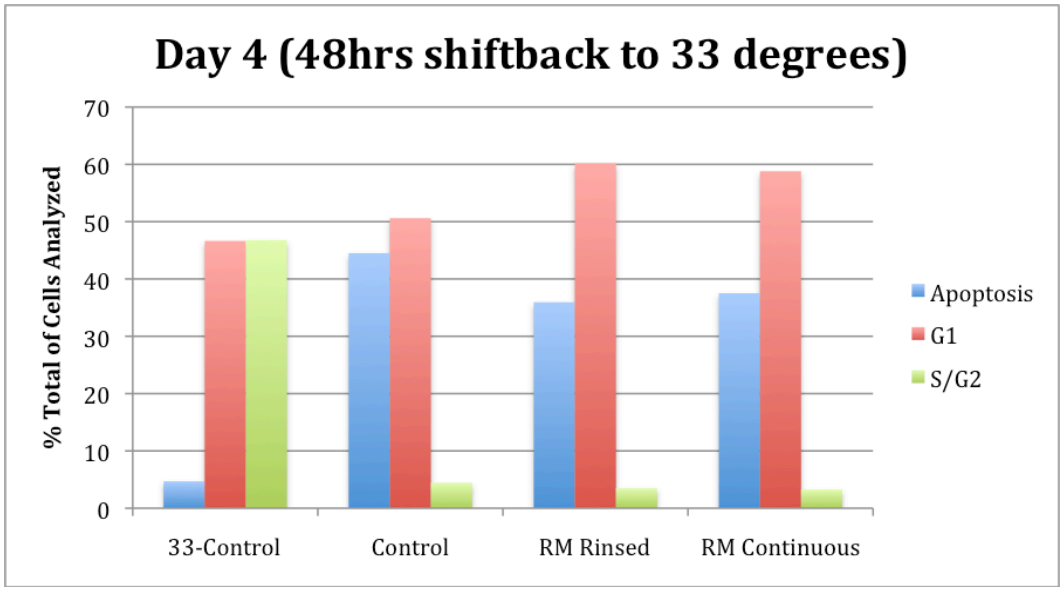
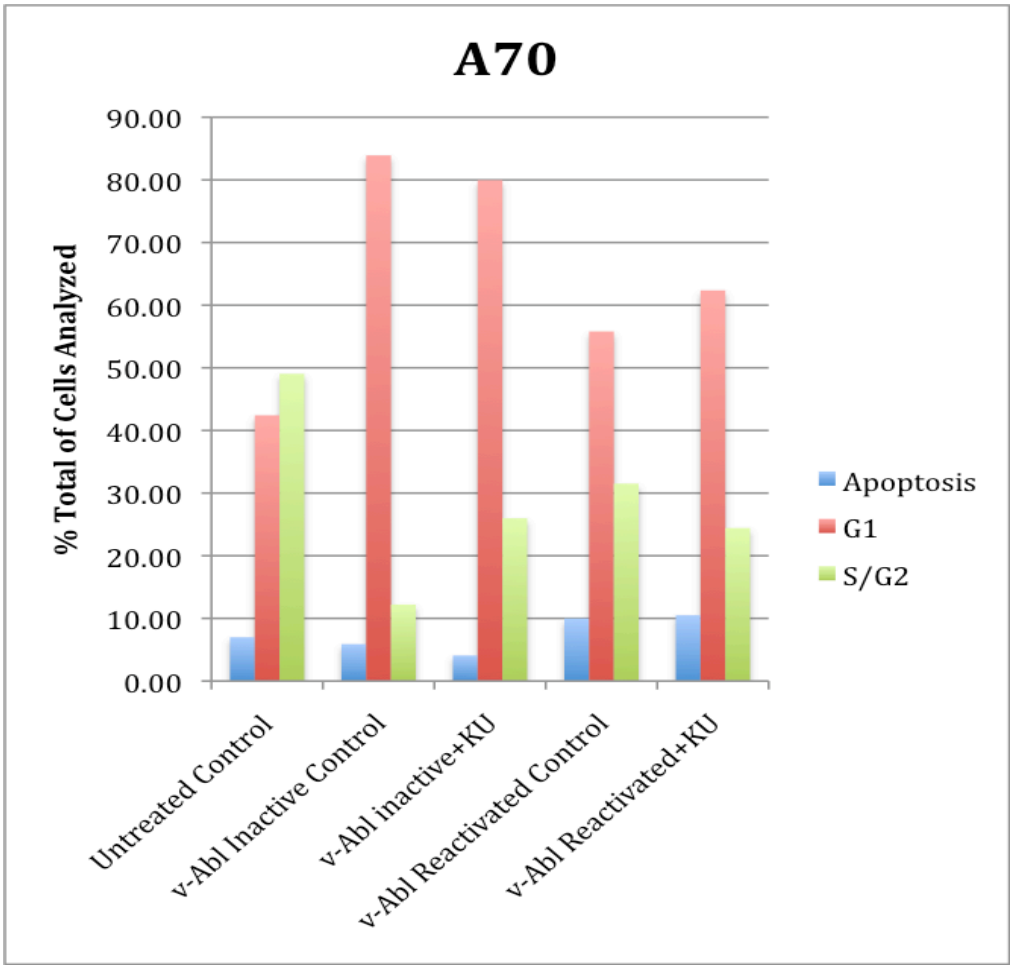



**Figure 7. Prolonged $G_1$ arrest in S9 cells is not mTOR dependent.** S9 cells were either treated with rapamycin or left untreated and shifted up to 39°. Cells were treated with rapamycin for 48hrs then washed in PBS, re-incubated in control media (RM Rinsed), or rapamycin treated media (RM Continuous) at 33 degrees.

**Prolonged $G_1$ arrest phenotype unique to our S9 cell line**

We also attempted to see if this condition was unique to our S9 cells or if it could be seen in similar B cell lines. We used the scid cell line SB 1.1 with the v-Abl transformed pre-B cell line A70 as a control. While both of these cell lines are immortalized, the v-Abl that they are transformed with is not temperature sensitive. Therefore we had to inactivate v-Abl using the BCR-Abl inhibitor STI571 (commercially known as imatinib, marketed as Gleevec). First we treated the cells with 1μM STI for 2 days to inactivate v-Abl. We also treated some cells with 8μM KU55933. After two days the cells were washed in PBS and re-incubated in either fresh media or media containing KU55933 for another two days.

Cell cycle analysis was performed after two days of v-Abl inactivation and two days after v-Abl reactivation. As seen in Figure 8, both A70 and SB 1.1 arrest with STI treatment and return to their cycle after STI is removed. ATM inhibition does not seem to have much if any significant effect on cycle arrest or return.



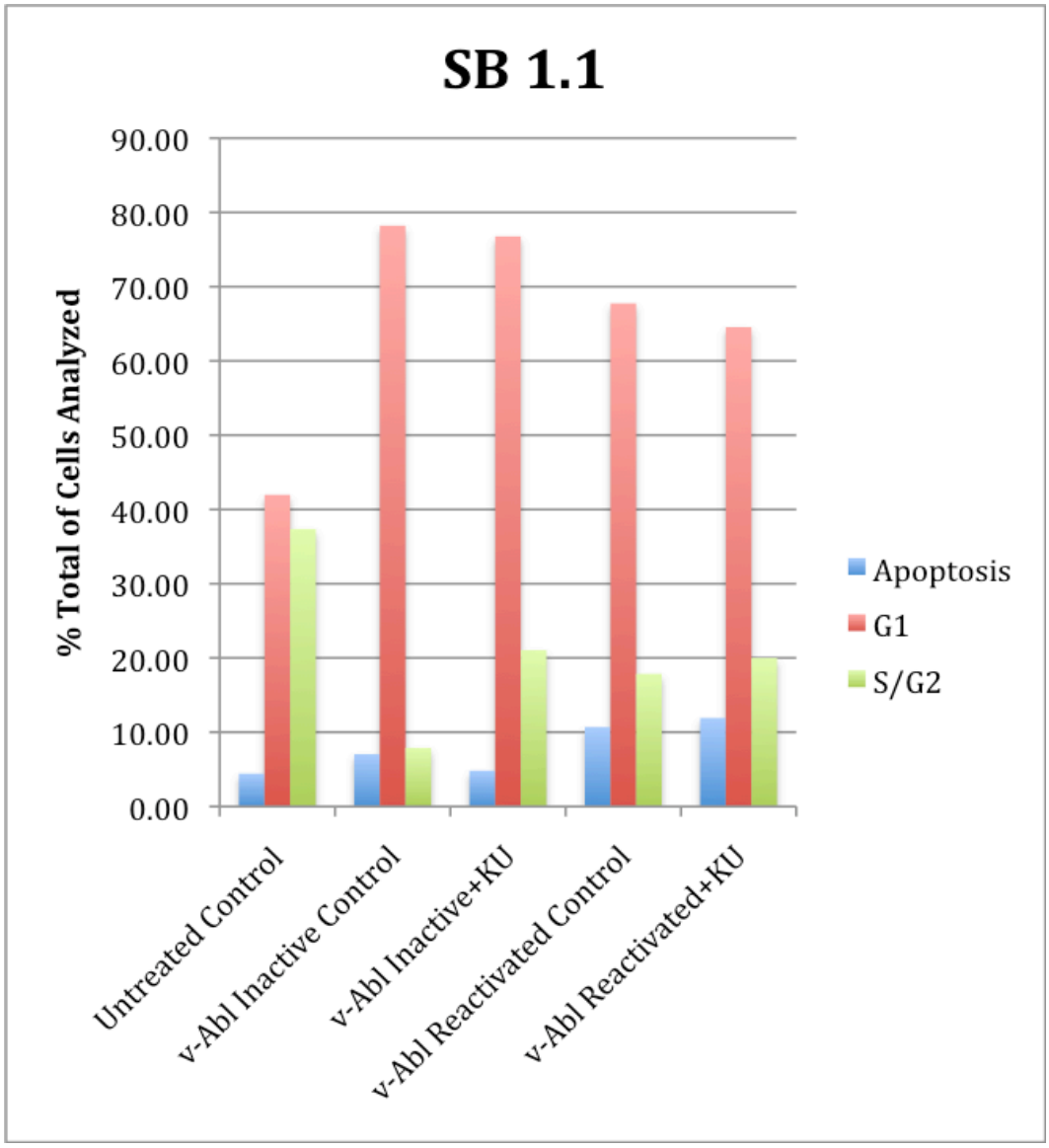



**Figure 8. Prolonged $G_1$ Arrest Unique to S9 Cells.** A70 (WT) and SB 1.1 (SCID) cells had their v-Abl inactivated by treatment with STI571 (Imatinib) for 48hrs then re-activated by washing the imatinib off and re-incubating the cells in control media. One set of cells had ATM inhibited through treatment with KU55933. ATM inhibition was continued throughout the experiment.

**Prolonged $G_1$ Arrest of S9 is Temperature Dependent**

Our S9 cell line mimics Imatinib treatment through shifting the cells to the non-permissive temperature, thus inactivating v-Abl. In order to determine if the senescence seen was unique to the temperature-sensitive mutant we inhibited v-Abl using imatinib at the permissive temperature for 48hrs, then washing the drug off and examining the cells' cycle recovery 48hrs after drug removal. Imatinib inactivated S9 cells arrested at the $G_1$ phase after two days treatment with 1μM Imatinib. Once the drug was washed off S9 cells quickly returned to their cell cycle (Figure 9).

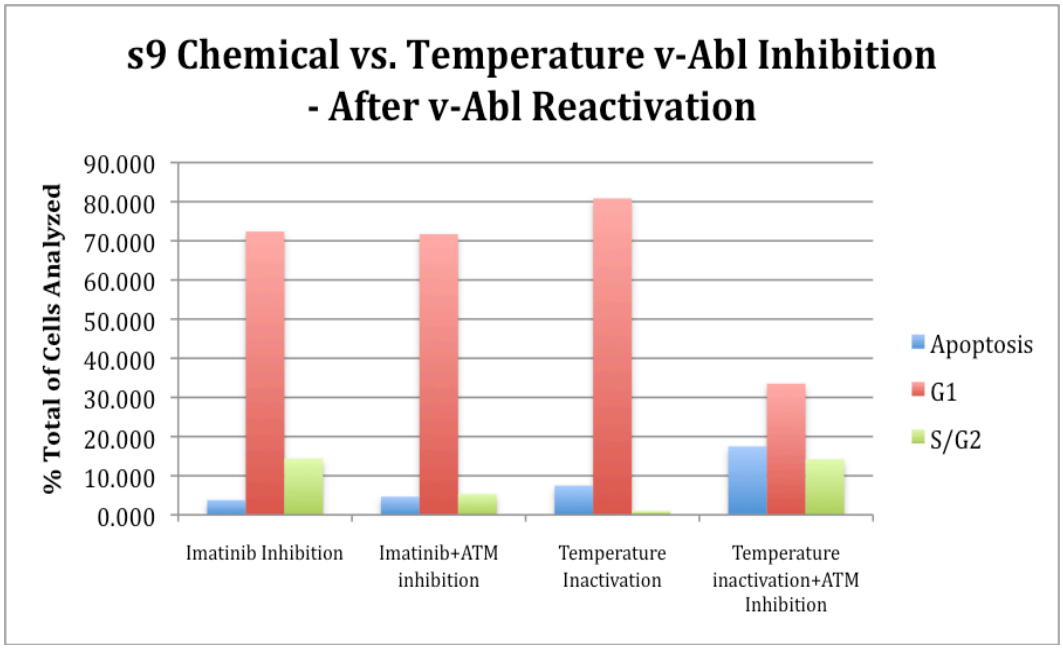



**Figure 9. Senescence in S9 Cells is Temperature Dependent.** Comparison of Imatinib inactivated v-Abl to v-Abl inactivation through temperature shift in S9 cells with or without ATM inhibition.

**Senescence in S9 is dependent on p27 activation**

Through analysis of the cell cycle we have seen that senescence in S9 cells is dependent on how v-Abl is inactivated. When v-Abl is inactivated through temperature shifting the cells fail to re-enter the cell cycle upon v-Abl re-activation, unless ATM is inhibited. If v-Abl inactivation is from treatment with Imatinib, then S9 cells can re-enter the cell cycle regardless of ATM inhibition. (Figure 9) ATM is a serine/threonine kinase that functions predominately as a DNA damage sensor with many downstream targets including p53. To determine how ATM was able to render temperature shifted S9 cells in senescent state but not cells whose v-Abl was chemically inhibited, we looked at the protein profiles of S9 cells under both treatment conditions. Protein lysates were collected and prepared according to the method previously mentioned. Samples were collected two days after v-Abl inactivation and two days after v-Abl re-activation.

The first protein examined was p53. P53 is a major downstream target for ATM, and plays a leading role in $G_1$ checkpoint arrest. Phosphorylation of p53 by ATM has been shown to contribute to cell cycle arrest at $G_1$ through p21 (Reinhardt). Phosphorylation of p53 is the critical step in cell cycle arrest and in apoptosis. It has been known for years that p53 serves as a 'Master regulator', being a deciding factor between cell cycle arrest and apoptosis. Mutation or inactivation of p53 is found in over 50% of cancers. Additionally, p53 works with mTOR to induce cell

senescence or quiescence, thus its potential involvement with our S9 cells was of particular interest.

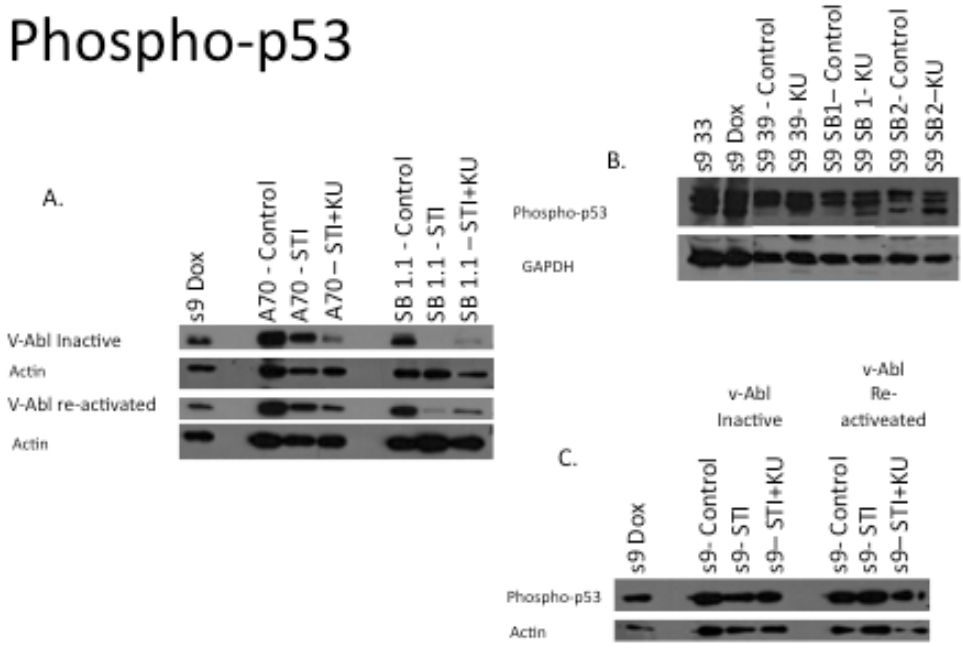



**Figure 10. Western blots:** A: A70 and SB 1.1 with v-Abl inactive (top) and re-activated (bottom); B: S9 cells with ATM inhibited (KU), v-Abl inactive (39), re-active for 1 day (SB 1), 2 days (SB2); C: S9 treated with Imatinib alone, or Imatinib+ KU55933.

Cells shifted from the non-permissive temperature and back again have a similar level of p53 phosphorylation regardless of ATM inhibition (Figure 10 - B). This characteristic is seen after 1, and 2 days of v-Abl re-activation. This general pattern (similar levels of p53 phosphorylation) is also seen in S9 cells treated with imatinib or imatinib and KU55933, both at two days of v-Abl inactivation and two days after re-activation. From

this we can gather that regardless of the method of v-Abl inactivation, p53 will be phosphorylated, though not necessarily by ATM (when ATM is inhibited).

Researchers have found evidence-linking ATM to p38MAPK and ROS signaling[23]. As a response to stress stimuli, p38 will be phosphorylated to help activate downstream targets effecting cell differentiation and apoptosis. Since our S9 cells, when shifted to their non-permissive temperature will arrest at $G_1$ but do not necessarily undergo apoptosis we wanted to see if p38 signaling was intact in this cell line. S9 cells maintain some level of p38 phosphorylation at all times. When v-Abl is inactivated through a temperature shift, inhibition of ATM increases phosphorylation over controls (Figure 11). This holds true even when v-Abl is reactivated (cells shifted back to 33°C). When S9 cells are treated with imatinib, p38 phosphorylation increases in treated cells (Imatinib+KU and imatinib alone) when v-Abl is inactivated. This increase does not seem to continue once v-Abl is re-activated.

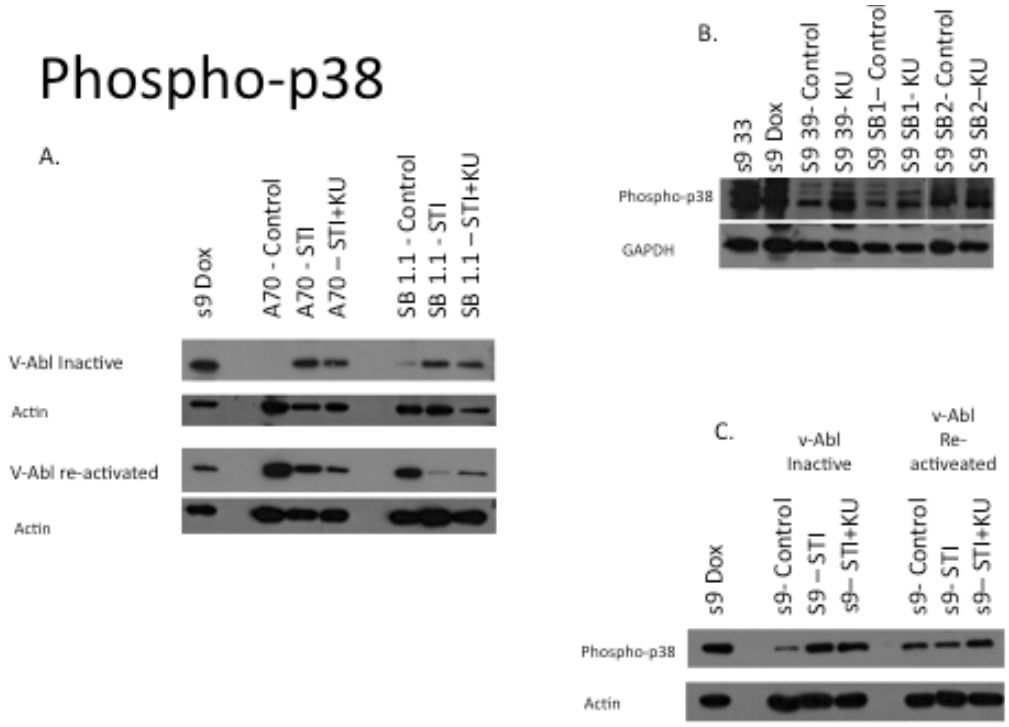



**Figure 11. Western blots of A70, SB 1.1 and S9.** A: v-Abl inactive (top), v-Abl re-activated (bottom); B: ATM inhibited (KU), cell shifted (v-Abl inactive), or shifted back (SB, v-Abl Re-active); C: Cell treated with Imatinib, or Imatinib+KU 55933 (ATM inhibited).

$P27^{Kip1}$ is tied to $G_1$ cycle arrest and is similar in DNA sequence and function to $p21^{Cip1}$. This cell cycle progression inhibitor works by binding to targets such as Cyclin D or Cdk2 and inhibiting their activity thus holding the cell in the $G_1$ phase[5]. Treatment of CML cells with imatinib has been shown to increase levels of $p27^{Kip1}$ [7]. We thought it would be interesting to see if the increase in $p27^{Kip1}$ could be seen in our S9 cells with imatinib treatment or with temperature shift. When S9 cells are treated with imatinib, as is expected $p27^{Kip1}$ is expressed after two days of imatinib treatment. We examined this in the A70 and SB 1.1 cells for consistency. When v-Abl is inactivated via temperature shift $p27^{Kip1}$ is not activated (Figure 12). It seems that the prolonged $G_1$ arrest displayed by

S9 cells upon temperature dependent inactivation of v-Abl is dependent on or related to $p27^{Kip1}$ activation.

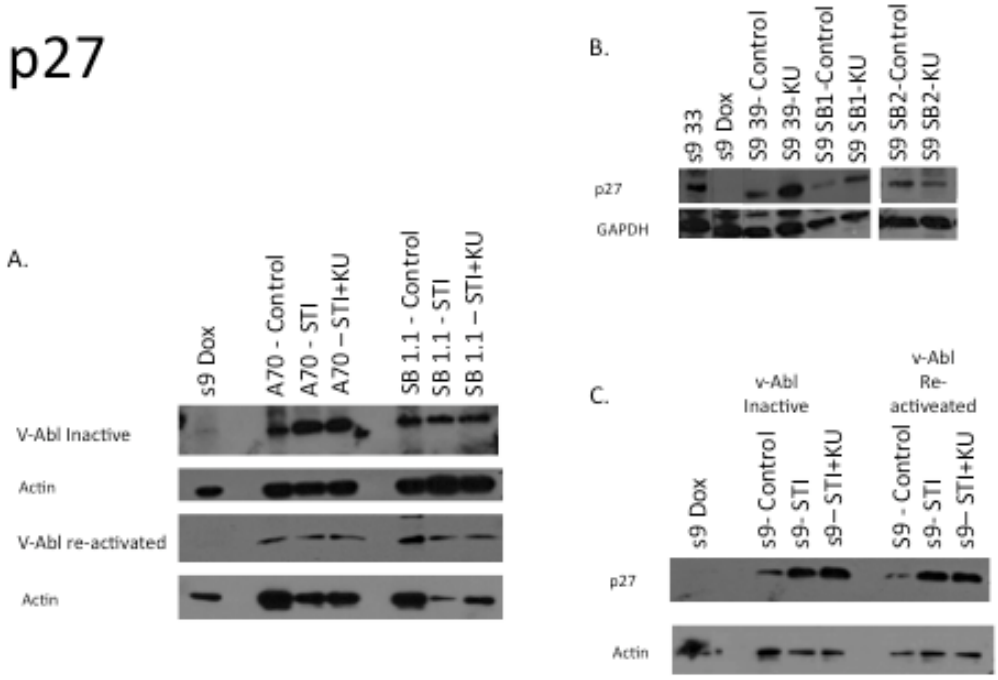



**Figure 12. Western blots of A70, SB 1.1 and S9**. A: v-Abl inactive (top), v-Abl re-activated (bottom); B: ATM inhibited (KU), cell shifted (v-Abl inactive), or shifted back (SB, v-Abl Re-active); C: Cell treated with Imatinib, or Imatinib+KU 55933 (ATM inhibited).

## Discussion

The S9 cell line was initially created in order to study the dynamics of V(D)J recombination. Derived from pre-B cells harvested from SCID mice, then transduced with ts-Ab-MuLV, S9 cells will arrest at $G_1$ when v-

Abl is turned off. Once v-Abl is turned back on, by shifting the cells from the non-permissive to the permissive temperature, the $G_1$ arrest continues. The molecular biology behind this continued arrest is a matter we are still working out. Looking at it from a cancer research perspective, S9 cells seem to mimic Imatinib treatment by exiting the cell cycle when shifted, but they do not apoptosis. Treatment of $Ph^+$ leukemia with Imatinib does not actually drive the cancer into apoptosis, rather it forces them into a cellular senescence that with treatment continues, until some cancer cells develop a resistance. The difference in this scenario is that the leukemia is stuck at $G_1$ only until treatment is halted or resistance is developed. S9 cells will continue their arrest even after Abl has been turned back on. S9 cells that break the prolonged $G_1$ arrest phenotype on their own will progress into a malignancy that is resistant to Imatinib treatment and v-Abl inactivation. Given these observations, we think that S9 cells can offer a possible glimpse into the events surrounding the development of Imatinib resistance in CML and $Ph^+$ ALL. First, we needed to determine the molecular mechanisms behind the observed senescence in the temperature sensitive S9 cells.

We first sought to determine, what, if anything could break S9 cells free from their senescence. We treated S9 cells with a pan-PI3K inhibitor and an inhibitor of ATM and found that ATM inhibition of S9 cells at the time of v-Abl inactivation would permit re-entry into the cell cycle after v-Abl re-activation. So far this re-entry seems to be dependent on ATM

inhibition only at the time of v-Abl inactivation, with little to no effect seen if ATM is inhibited only at re-activation or inhibition is discontinued at v-Abl re-activation (data not shown). Inhibition of PI3K kinases in general does not have the same effect.

ATM is heavily involved in the DDR pathway and plays a role in V(D)J recombination. Research has shown that ATM can function directly in the repair of chromosomal DNA double-strand breaks. During lymphocyte antigen receptor generation, ATM maintains the DNA ends in repair complexes.[8] ATM has been linked to regulation of c-Abl and retinoblastoma (Rb) protein.[28, 35] Activation of ATM was thought to only be through DNA damage, however, recently it has been noted that oxidative stress can also activate ATM even in the absence of DNA damage.[20, 37,47] With all that it has been linked to in previous research it is conceivable that ATM could be activating a downstream target that is able to hold the cells in an arrested state even after v-Abl reactivation. Because ATM sits upstream of proteins Chk2 and p53, and their downstream targets have a direct affect on senescence, it is not completely unexpected that ATM inhibition might be able to break the senescence phenotype in S9 cells.

It has been long known that ATM is an upstream activator of p53. Activation of p53 has been linked to quiescence (temporary cycle arrest), senescence (permanent cycle arrest), apoptosis, and DNA repair. When v-Abl is inactive it is likely that stress levels in the cells can increase and thus p53 would be activated to help initiate DNA repair and cycle arrest.

However the phosphorylation of p53 in S9 cells does not seem to be entirely dependent on ATM. Phosphorylation levels of p53 in S9 cells are not significantly higher or lower than the controls regardless of how v-Abl is inactivated. This may differ from the A70 and SB 1.1 cell lines used for comparison. Both of these lines have slightly lower p53 phosphorylation, though ATM inhibition does not seem to make a significant difference in phosphorylation. This could indicate that a change in p53 in S9 or in a p53 related pathway. According to our protein analysis, p53 is still phosphorylated in S9 cells, even when ATM has been inhibited. This points toward p53 being activated through something other than ATM.

One thing that can activate p53 is oxidative stress. The start of this stress can be signaled through one of the MAPKs. One of the more active signal transducers is p38. This protein is ubiquitously expressed at significant levels in most cell types.[13] In our experiments the phosphorylation of p38 was high in A70, SB 1.1, and S9 cells when v-Abl was inactivated with Imatinib treatment, though ATM inhibition did not have any significant effect on p38 phosphorylation under this treatment. This is in contrast to p38 phosphorylation in S9 cells when v-Abl is inactivated through a temperature shift. In the temperature shift experiment, ATM inhibition effectively increases p38 phosphorylation. It is noted that p38 phosphorylation is high in the S9 cell line normally, so it may be more correct to say that phosphorylation of p38 is low in temperature inactivated S9 cells. Once v-Abl is reactivated in S9 cells,

p38 phosphorylation levels do go down regardless of how v-Abl was inactivated. Since it is known that oxidative stress can activate p53, it is possible that p38 could be activating p53 in these cells and not ATM. This would indicate that v-Abl inactivation is somehow increasing the levels of p38 phosphorylation.

Korotchkina, et al., found evidence intricately linking p53 and the PIKK mTOR to cellular the decision over quiescence or senescence. Through their studies they found that p53 pushed cells toward senescence by failing to inhibit mTOR, and mTOR causing the senescence seen. To determine if mTOR might be somehow causing the senescence in S9 cells we tried inhibiting mTOR by treating the cells with rapamycin in an experiment mirroring the one used for ATM inhibition. Inhibition of mTOR did not release cellular senescence in S9 cells upon v-Abl re-activation. So activation of mTOR was not causing the senescence seen.

One major difference between our S9 cells and leukemia cells bearing the Ph chromosome is that inactivation of v-Abl in S9 cells can be accomplished through shifting to a non-permissive temperature. We wanted to see if v-Abl inactivation would cause senescence in non-temperature sensitive cell lines, so we repeated the experiment with v-Abl transformed cells A70, and SB 1.1, using SB 1.1 as a DNA-PK knockout line and A70 as a wild type. While treatment with the Abl inhibitor Imatinib did induce cycle arrest, this arrest was not permanent and the cells quickly re-entered the cycle upon Imatinib removal. ATM inhibition had only a

minor effect in the cells, allowing slightly less cycle arrest 48 hours after v-Abl inactivation and slight slowing of the return to the cell cycle.

Next we tried inhibiting v-Abl in S9 cells using Imatinib instead of via temperature shift. Imatinib did arrest the cells as expected. What was not expected was the re-entry into the cell cycle after Imatinib removal. Inhibition of v-Abl through Imatinib would not cause senescence in S9 cells. ATM inhibition had little significant effect on the re-entry. Inhibition of v-Abl with Imatinib did not produce as complete an arrest as temperature shifting usually does. All of these factors would indicate that there is a difference between inhibiting v-Abl and simply turning it off as is done in S9 cells that are shifted.

Armed with the knowledge that temperature dependent v-Abl inactivation was intrinsically different than chemical dependent v-Abl inactivation we sought to find what proteins are different between the two types of inactivation. It has been noted by some researchers that $p27^{Kip1}$ is activated in Imatinib treated cells derived from CML patients. $P27^{Kip1}$ is a cyclin dependent kinase (Cdk) inhibitor that can bind and prevent the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes. This inhibitory effect allows p27 some control over cell cycle progression at $G_1$. $P27^{Kip1}$ is a member of the Cip/Kip family that includes $p57^{Kip2}$ and $p21^{Cip1/Waf1}$. In addition to DNA sequence and structural similarity, all members of this family are able to bind to different classes of cyclin and Cdk molecules.[5] Borriello, et al., investigated the relationship between $p57^{Kip2}$ and the

BCR-Abl kinase inhibitor Imatinib and found that $p57^{Kip2}$ would accumulate in $Ph^+$ cells prior to $p27^{Kip1}$ accumulation and thus may be a downstream effector of Imatinib.[7]

Since $p27^{Kip1}$ was involved in $G_1$ arrest we looked at the levels of $p27^{Kip1}$ in S9 cells treated with Imatinib vs. cells shifted to the non-permissive temperature. The $p27^{Kip1}$ levels in cells treated with Imatinib are higher than those of cells not treated. Also, inhibition of ATM in shifted cells is generally higher than in non-ATM inhibited cells. The only exception to this in the S9 cells shifted back to 33°C for two days. In these cells, $p27^{Kip1}$ is slightly higher in the controls than in the ATM inhibited cells. Analysis of the $p27^{Kip1}$ levels in the A70 and SB 1.1 cells indicated that Imatinib treatment could raise $p27^{Kip1}$ levels in cells. Interestingly, the $p27^{Kip1}$ levels decrease only a little when v-Abl is re-activated in Imatinib treated cells.

From this information we can conclude that Imatinib inactivation of v-Abl will cause $G_1$ arrest in cells possibly through the activation of $p27^{Kip1}$ and independent of ATM inhibition. Also, temperature induced inactivation of v-Abl in our S9 cells causes a permanent $G_1$ arrest that is not through $p27^{Kip1}$ but can be abrogated by inhibition of ATM. Inhibition of ATM at the time of temperature induced v-Abl inactivation starts a cascade that allows $p27^{Kip1}$ to be activated in these cells to levels that will allow them to re-enter the cell cycle after v-Abl re-activation. This seems counter-intuitive in that $p27^{Kip1}$ is normally described as an inhibitor of cell cycle

progression. The activation of p27 $^{Kip1}$ with ATM inhibition is only seen during v-Abl inactivation and concludes within 48 hours of v-Abl reactivation. This is different than cells treated with Imatinib. Imatinib treated S9 cells have higher p27 $^{Kip1}$ levels throughout treatment with ATM inhibitor KU55933 and do not depend on v-Abl inactivation, or this is not abated with v-Abl reactivation.

In S9 cells a temperature shift from permissive to non-permissive temperature will inactivate v-Abl causing a permanent cell cycle arrest. This cell cycle arrest can be overcome through inhibition of ATM during v-Abl inactivation and has downstream repercussions on p27 $^{Kip1}$ activation. In the treatment of CML and ALL $Ph^+$, what this means is that ATM may be allowing the malignant cells to re-enter the cell cycle. This is important because while Imatinib can be used to treat CML and ALL $Ph^+$, resistance to Imatinib can develop. Additionally, if treatment is halted, then the condition returns. ATM may be how these malignant cells are able to return. Further examination of the senescent phenotype in S9 cells may allow us to determine more key players in the senescence seen. If this senescence can be recapitulated in $Ph^+$ leukemia, then there is hope that the disease may finally be eradicated.

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