Addicted to Autophagy:

Ph+ B-ALL May Acquire Imatinib-resistance and Enhanced Malignancy

through a Highly-active Autophagy Pathway

by

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ABSTRACT

The majority of chronic myeloid leukemia (CML) and some of acute lymphocytic leukemia (ALL) cases are associated with possessing the BCR-Abl fusion protein from an oncogenic translocation, resulting in a constantly active form of Abl and rapid proliferation. CML and ALL cells that possess the BCR-Abl fusion protein are known as Philadelphia chromosome positive (Ph+). Currently, Imatinib (selective Abl inhibitor) is used as therapy against CML and ALL. However, some patients may have malignancies which show resistance to Imatinib.

Previous work displays that the transformation of progenitor B cells with the v-Abl oncogene of Abelson murine leukemia virus results in cell cycle progression, rapid proliferation, and potentially malignant transformation while preventing any further differentiation. Progenitor B cells transformed with the temperature-sensitive form of the v-Abl oncogene have served as a model to study cellular response to Imatinib treatment. After some manipulation, very few cells were forced to progress to malignancy, forming tumor in vivo. These cells were no long sensitive to v-Abl inactivation, resembling the Imatinib resistant ALL.

Autophagy is the process by which proteins and organelles are broken-down and recycled within the eukaryotic cell and has been hypothesized to play a part in cancer cell survival and drug-resistance. LC3 processing is a widely accepted marker of autophagy induction and progression. It has also been shown that Imatinib treatment of Ph+

leukemia can induce autophagy. In this study, we examined the autophagy induction in response to v-Abl inactivation in a Ph+-B-ALL cell model that shows resistance to Imatinib. In particular, we wonder whether the tumor cell line resistant to v-Abl inactivation may acquire a high level of autophagy to become resistant to apoptosis induced by v-Abl inactivation, and thus become addicted to autophagy.

Indeed, this tumor cell line displays a high basal levels of LC3 I and II expression, regardless of v-Abl activity. We further demonstrated that inhibition of the autophagy pathway enhances the tumor line's sensitivity to Imatinib, resulting in cell cycle arrest and massive apoptosis. The combination of autophagy and Abl inhibitions may serve as an effective therapy for BCR-Abl positive CML.

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Introduction

CML, ALL, BCR, Abl

The majority of chronic myeloid leukemia (CML) and some cases of acute lymphoblastic leukemia (ALL) are two forms of cancer which originate from the same genetic modification of stem cells in the bone marrow[1-5]. CML is distinguishable from ALL in that the cancer-causing modification occurs in myeloblasts, causing proliferation of mature and precursor granulocytes[1]. In ALL, lymphoblasts acquire the genetic modification, which is typified by proliferating immature B cells[1].

The genetic modification which gives rise to these two forms of cancer is an oncogenic translocation which yields a hybrid chromosome known as the Philadelphia chromosome. The formation of the Philadelphia chromosome occurs when the q34 region of chromosome 9 is substituted at the q11 region of chromosome 22[2-4]. Therefore, denotation for this translocation is t(9;22)(q34;q11). Most importantly, this translocation juxtaposes the Abl (Abelson) gene from chromosome 9 with the BCR (breakpoint cluster region) gene from chromosome 22 (see Figure 1)[2-4].

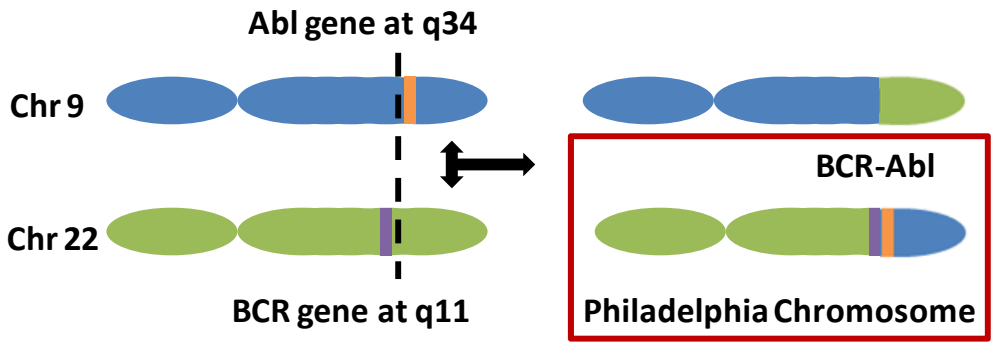


Figure 1. Schematic of the oncogenic translocation which results in the Philadelphia chromosome.

Cells possessing the Philadelphia chromosome produce a fusion protein known as BCR-Abl. Although the function of the normal BCR gene product is not clear[6], the Abl gene product (c-Abl) has been shown to have a nuclear function and a cytoplasmic function as a tyrosine kinase[5]. DNA damage causes translocation of cytoplasmic Abl to the nucleus, where it is activated and plays roles in cell cycle arrest and the induction of apoptosis[3-5]. However, cytoplasmic Abl has a contrasting function in which it, upon phosphorylation, is a transducer of growth factor signaling which results in the positive regulation of cell proliferation, differentiation and adhesion[1-6].

The BCR-Abl fusion protein localizes solely in the cytoplasm since the region of Abl containing the nuclear localization signal is replaced by the translocation. Therefore, the DNA damage-repair and apoptosis inducing functions of BCR-Abl are ablated, resulting in genome instability[7,11]. BCR-Abl's oncogenic properties are expanded by the fact

that it is constitutively active and does not require any extrinsic or intrinsic signals for activation[8-10]. The result is enhanced cell proliferation, survival and tumor transformation[2,5-7].

Currently, the first line of treatment for BCR-Abl positive CML and ALL is a selective Abl-inhibitor known as Imatinib (also known as Gleevec or STI571). Imatinib acts as an antagonist for the kinase domain of BCR-Abl, preventing its enzymatic function as an activator of pro-proliferation/survival pathways[12-16]. Imatinib-resistance is a major problem for patients with Philadelphia chromosome positive (Ph+) ALL in that 70% of patients develop resistance within 36 months of treatment[17-19]. Following the development of resistance, the disease state in these patients progresses rapidly and aggressively[18]. Characterization of these tumors is essential for the development of therapy targeted to these aggressive cancers.

Abelson murine leukemia virus (Ab-MuLV) can transform many cell types *in vitro* through the production of the v-Abl protein[20]. The C-Abl proto-oncoprotein is the normal cellular homolog to v-Abl. V-Abl resembles BCR-Abl in that it is also constitutively active, does not participate in the induction of apoptosis or DNA damage-repair, and causes rapid cell proliferation[5,20,21]. Given the similarities between BCR-Abl and v-Abl, the Abelson leukemia virus has served as a useful tool in the understanding of oncogenic transformation and tumor induction in Ph+ malignancies.

Interestingly, *in vivo*, in v-Abl transforms only partially differentiated B cells (pro- or pre-B cells) in which only the heavy chains have been rearranged[21]. Oncogenic transformation of Pre-B cells by v-Abl is characterized by three phases: 1) an initial phase of proliferation, also known as primary transformation; 2) a crisis phase that is characterized by erratic growth patterns and massive apoptosis and; 3) the cells which survive the crisis phase acquire some alterations (explained later) which allow them exit crisis and emerge as fully transformed malignancies[25,26]. A temperature-sensitive form of Ab-MuLV (ts- Ab-MuLV) has been developed via site-directed mutagenesis of Ab-MuLV using sequence information from temperature-sensitive mutants of a closely-related oncogene[24], which allows delineation of mechanistic steps in oncogenic transformation as well as the sensitivity and responses of these cells to v-Abl inactivation.

Infection of Pre-B cells with ts-Ab-MuLV produces an inducible model of v-Abl activity in that, at lower temperatures (33°), v-Abl is active and able to function as an oncoprotein while at higher temperatures (39°), v-Abl is inactivated. By transforming progenitor B cells with the temperature-sensitive version of Ab-MuLV, an inducible model of ALL has been developed. More specifically, holding these model cells at the v-Abl-inactivating temperature mimics treatment of ALL with Imatinib, causing cell cycle arrest and differentiation[22,23].

In this study, the tumorigenic potential of v-Abl transformation was examined in pre-B cells harvested from severe combined immunodeficient (SCID) mice which also are defective in the catalytic subunit of the DNA-damage-repair protein known as DNA-dependent protein kinase (DNA-PKcs). These SCID cells were then infected with ts-Ab-MuLV for transformation. Additionally, these cells are being used, by other members of our group, as a model to study the role of defective recombination in oncogenic transformation. To develop Imatinib-resistant cell lines from this model, the cells were subjected to the v-Abl-inactivating temperature for differing amounts of time, and then returned to the v-Abl-permissible temperature. Following v-Abl-inactivation, the parental cell line (called S9) remains in G1 arrest indefinitely, even after the reactivation of v-Abl.

However, a small number of cells were found to escape the G1 arrest, return to the cell cycle and, therefore, progress to an Imatinib-resistant-like state of malignancy. Further testing of these new cell lines with Imatinib confirmed their resistance to terminal cell cycle arrest. These lines were also found to form tumor *in vivo*. One of these malignant lines, called 3-4-1, will be the focus of this study.

Autophagy

Autophagy is the process by which proteins and organelles are broken-down and recycled within the eukaryotic cell. This can occur in a nutritionally-insufficient environment as a means of creating metabolic substrates from within, or when long-lived proteins or organelles are damaged or redundant as a means of removal[27]. All in all, the overall goal of autophagy is hypothesized to be the maintenance of cellular homeostasis. Insufficient autophagic degradation of proteins may lead to cell death[27,28]. However, abnormal autophagy has also been hypothesized to play a part in oncogenesis, cancer cell survival and drug-resistance[27-29].

Autophagy involves the selective degradation of proteins via lysosomes in two different ways[30,31]. In chaperone-mediated autophagy, proteins that are coupled with a lysosomal-targeting region are removed from the cytoplasm when they are recognized by a specific chaperone and shuttled to lysosomes for internalization and degradation[30,31]. The second form of autophagy is known as macroautophagy (to be referred to as autophagy for the remainder of this study) involves the encasement of the protein to be degraded by a double-membrane (isolation vesicle) vesicle known as an autophagosome[27-31].

Once the autophagosome fully encloses the protein target, it fuses with a lysosome, making the internal autophagosomal environment acidic to the point of degrading all contents[27-31]. Then these degraded contents are released to the cytoplasm for use in cellular metabolism[27-31]. As

research ensues, more and more players on the autophagy pathway are being uncovered and characterized. Some essential steps and components of this pathway have been previously described and following is an explanation of various processes (see Figure 2).

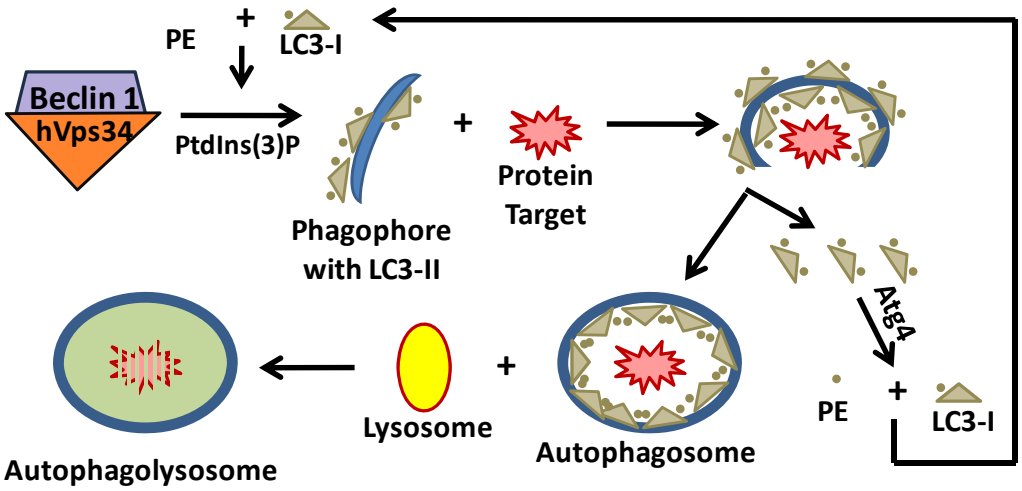


Figure 2. Diagram of the events that occur in the autophagic degradation of long-lived proteins in the cytoplasm.

The initiation of autophagy relies on the complex consisting of hVps34 (the PI3 kinase, more on PI3K later) with Beclin-1 (also known as Atg [autophagy-related gene] 6). In order for this complex to form, Beclin-1 must be released from its inhibitory complex with Bcl-2 upon Bcl-2 activation[32-35]. After binding with Beclin-1, hVps34 modifies lipids from the endoplasmic reticulum (ER) to form PtdIns(3)P (phosphatidylinositol 3-phosphate) [34,35]. These lipids are essential for the lipidation (via conjugation to PE [phosphatidylethanolamine]) of LC3-I (microtubule-associated light chain 3) into its further-lipidated form, LC3-II, which can

then associate with the growing autophagosome[34,35]. The association of LC3-II to the growing autophagosome is essential to the progression of autophagy[34,35].

The premature (open) autophagosome is known as a phagophore and its nucleation site is known as the phagophore assembly site (PAS) [27-36]. The growing phagophore recruits LC3-II on the inner concave and outer convex membrane as it grows and is thought to play a role in the shaping of the double membrane into a vesicle. Once the autophagosome is fully formed and closed, it fuses with a lysosome and degrades the protein target within as well as the LC3-II that was associated with the inner membrane. The LC3-II that was associated with the outer membrane is deconjugated from PE, transforming it back into LC3-I so it can be reused in the formation of another autophagosome[27-36]. Deconjugation of PE from LC3-II is carried out by ATG4[36].

LC3 processing is a widely accepted marker of autophagy induction and progression[37-40]. This can be accomplished with anti-LC3 antibodies and observation of the relative levels of LC3-I and –II as autophagy progresses. Autophagy is not considered complete until the autophagosome has fused with a lysosome and the outer-membrane-associated LC3-II is converted back to LC3-I.

Upregulation of the autophagy pathway can render cells resistant to apoptosis in a nutritionally insufficient or DNA-damaging environment or in

response to other stressors. This is indicative by studies in which tumor cells induce autophagy in the presence of chemotherapies or radiation therapies[41-46]. Interestingly, it has been shown that Imatinib treatment of BCR-Abl+ leukemia can induce autophagy[47-49]. Inferred from this finding, we speculate that 3-4-1 cells, which show resistance to v-Abl inactivation, somehow may exploit autophagy as a means of surviving Abl-inhibition.

The emerging implications of the autophagy pathway in cancer cell metabolism and survival has prompted an explosion of investigations to unravel autophagy induction and modulation. As the body of knowledge regarding autophagy grows, it has become evident that many cellular pathways are involved in the modulation of autophagy. Currently, the major pathways to note are AMPK, mTOR, p53 and PI3K. Although the complex interplay and balance between these pathways and autophagy is still not entirely understood, the key points regarding their links to v-Abl transformation and known control over autophagy will be explained below.

AMPK, mTORC1, p53 and PI3K Pathways

The AMP-activated protein kinase (AMPK) pathway is stimulated by elevated AMP:ATP ratios, acting as a sensor for low glucose levels and metabolic stress[50]. Activated AMPK enhances the oxidation of fatty acids and glucose metabolism while preventing the synthesis of fatty acids, proteins, cholesterol and glycogen (see Figure 3). Activation of AMPK,

required for kinase activity, depends upon phosphorylation at T172 by AMP[51]. AMP not only promotes phosphorylation of AMPK at T172, but also has the ability to stabilize this phosphorylated state[52]. High concentrations of ATP have been shown to prevent AMPK activity by competitively binding to the enzyme's active site, preventing downstream pathway progression[53].

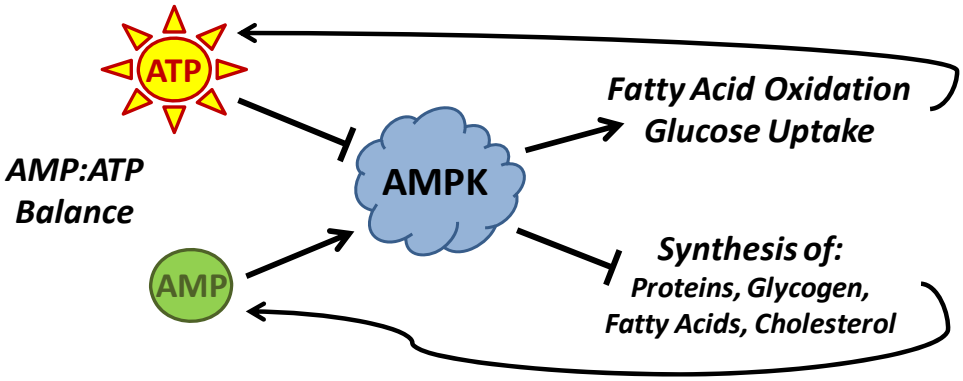


Figure 3. Role of AMPK as a cellular-energy-sensor.

Harhaji-Trajovic *et al*. have shown that AMPK-activation occurred just prior to induction of autophagy in cisplatin-treated cells, which results in reduction of p70S6 kinase phosphorylation by mammalian target of Rapamycin (mTOR)[54]. The mTOR protein, in combination with Raptor and GβL, form a complex known as mTOR complex 1 (mTORC1)[55-57]. The active mTOR complex can phosphorylate and activate S6 kinase (also known as p70S6K) which, in turn, can positively regulate ribosomal biogenesis and cell growth as well as inhibit autophagy[57].

AKT (also known as PKB [protein kinase B]), is phosphorylated and activated by PIP3 (product of PI3K class-I function, described below). AKT has been shown to play roles in metabolism, proliferation and survival. Furthermore, activation of mTOR relies on AKT to phosphorylate the mTOR-inhibitor known as TSC2 (tuberous slcerosis complex 2), which removes its suppressive actions on mTOR[69,70]. All in all, expression of v-Abl leads to proliferation by enhancing PI3K activation, leading to AKT activation and, therefore, reduction of p53 activation (see figure 6).

Research to elucidate the interactions among AMPK, mTOR and autophagy have further uncovered that nutrient-deprivation-mediated activation of AMPK inhibits mTORC1 which, in turn, inhibits cell growth and allows for the induction of autophagy until adequate growth factors are restored (see Figure 4)[57]. Furthermore, AMPK-silencing (via siRNA) prevented the induction of drug-induced autophagy[54] and silencing of mTOR (via siRNA) alone lead to autophagy initiation[54].

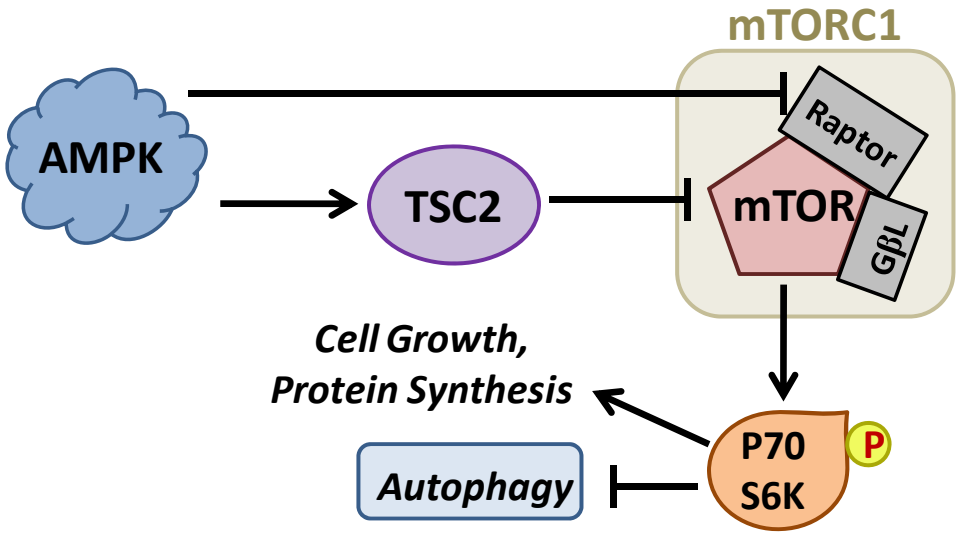


Figure 4. Interactions between AMPK and mTOR and their role in induction of autophagy.

One of the common denominators that have been shown to regulate both the AMPK and mTOR pathways is p53. P53 is a nuclear tumor suppressor protein that plays a positively-regulatory role in multiple pathways such as senescence, cell death and autophagy. P53 has been found to be mutated in the majority of cancers and its oncogenic effects have been notoriously explored[58-65]. Upon phosphorylation, p53 can leave the nucleus and act in the cytoplasm on the mentioned pathways in an indirectly-activating manner as described below.

Tasmedir *et al* has shown that p53 can have dual functionality in the activation of autophagy which may be dependent on the initial cause of its phosphorylation (i.e. oxidative stress, DNA damage, nutrient-deprivation etc.)[63]. In some cases, activated p53 has been shown to

induce autophagy by activating AMPK which, in turn, inhibits mTORC1. The inhibition of mTOR leads to a reduction of phosphorylated S6K which will allow for autophagy to ensue. Conversely, cytoplasmic-phosphorylated-p53 has also been shown to inhibit the induction of autophagy as demonstrated by this group's experiments in p53-null mice in which p53 degradation prompted by ubiquitination somehow allowed autophagy to proceed (see Figure 5).

They hypothesize that p53 may act as an endogenous repressor of autophagy in some cases and an inducer of autophagy in others. It can be imagined that one path may be cytoprotective while the other might lead to apoptosis, depending on the stimulus which prompts the induction of inhibition of autophagy. However, there is no general consensus describing this dichotomy and the factors which contribute to the cell's decision to adopt one of these two paths remain to be discovered.

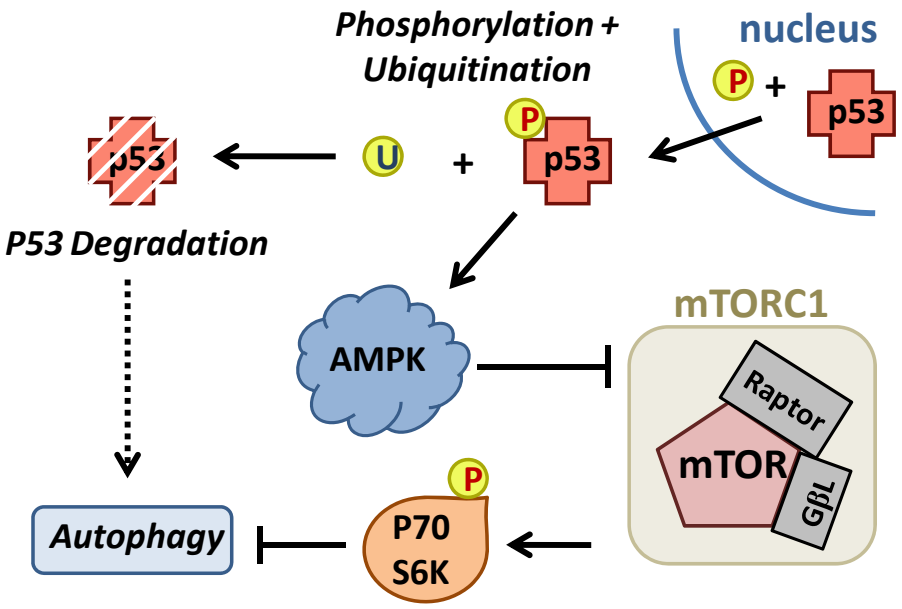


Figure 5. Schematic of the dual roles that p53 can play in the modulation of autophagy.

It is important to note the INK4A/ARF (CDKN2A) locus and its links to v-Abl transformation through interplay with the p53 pathway. $P19^{ARF}$ (ARF) is one of the products of the CDKN2A gene and acts as a tumor suppressor by enhancing p53 activity, through the inhibition of p53's inhibitor (MDM2), in the presence of oncogenic stress as a means of inducing cell cycle arrest or apoptosis in cells that have accumulated damage[98]. Furthermore, malignant transformation by v-Abl is either accompanied by reduced expression of ARF, or an inactivating mutation in p53 as a means of overcoming the crisis phase (v-Abl transformation phase 2; described earlier) induced by oncogene expression[99,100]. An intact p53 pathway or increased expression of ARF was shown to prevent

complete transformation by v-Abl through induction of cell cycle arrest and apoptosis[99,100]. Additionally, ARF has been indicated to positively regulate autophagy[75-77].

This additional potential role of p53 adds a new level of convolution to the motives of p53 in a v-Abl-transformed cell. Activated/cytoplasmic p53 can act as a tumor suppressor by inducing autophagy and inhibiting cell cycle progression (via inhibition of the mTOR pathway). Conversely, Tasmedir *et al* describe that activated p53 can inhibit autophagy by unknown means in cells that commit to this connection between p53 and autophagy. In this case, activation of p53 must be reduced or p53 must be mutated or degraded in order for autophagy to ensue.

An additional pathway that was shown to be entangled in this AMPK-mTOR-p53-autophagy web is the PI3K pathway. The PI3K (phosphatidylinositol 3-kinase) family of enzymes adds phosphate groups to phosphatidylinositol compounds. They are separated into classes depending upon their substrates and stimuli to perform their kinase activities. At the cell membrane, receptor-binding to growth factors (such as insulin or IGF-1) activate PI3K class-I_A to convert membrane-bound PIP2 (phosphatidylinositol-4,5-phosphate) into PIP3 (phosphatidylinositol-3,4,5-phosphate) which can leave the membrane. Additionally, BCR-Abl transformation has been shown to increase PI3K-I_A activity, leading to accumulation of PIP3 at the membrane[93-97]. PIP3 is then able to recruit and activate AKT (see Figure 6)[66-68].

The PI3K pathway has a direct role in autophagy which is through the actions of a class-III PI3K known as Vps34 (vacuolar protein sorting 34). Vps34 functions as a PI3-kinase by processing PtdIns(3)P (phosphatidylinositol 3-phosphate) from lipids supplied by the ER (endoplasmic reticulum) which must be present for the induction of autophagy (Figure 2). Vps34 is also essential for the conjugation of PE (phosphatidylethanolamine) to LC3-I, converting it to LC3-II so that it may associate to and facilitate the growing autophagosome. It has also been shown that universal-PI3K-inhibiting drugs can inhibit autophagic processing[66-68] and synergize with Imatinib in the treatment of Ph+ leukemias[44].

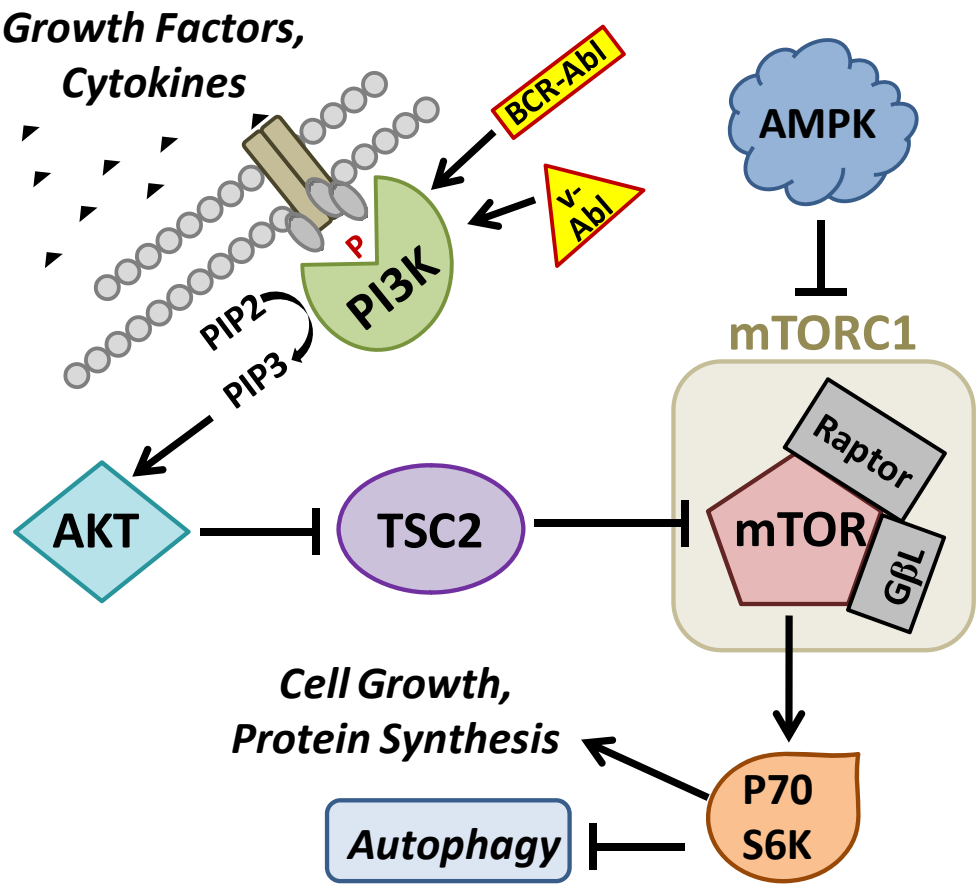


Figure 6. V-Abl/PI3K activity and its effects on autophagy via the mTOR pathway.

The enlightening findings on autophagy of many research groups in last decade have shown that many diseases (such as cancer, neurodegenration, cardiac disease and diabetes) may possess an altered autophagic process and use this alteration for cytoprotection in the presence of therapies[71-74]. Given that 3-4-1 cells have been perturbed to acquire Imatinib-resistance, it is reasonable to wonder whether an altered

v-Abl/PI3K pathway and, therefore, an altered autophagy pathway, is one of the mechanisms by which these cells survive drug-treatment.

As previously mentioned, Imatinib has been found to induce autophagy: since Imatinib inhibits v-Abl, the PI3K pathway is down regulated which reduces the activation of AKT, which then inhibits the mTOR pathway, leading to induction of autophagy (see Figure 6). Cancer cell survival in the absence of v-Abl activity (either by Imatinib treatment or v-Abl inactivation via temperature shift) could be achieved through compensation in the PI3K, AKT or autophagy pathways. Given the potential link between the malignancy of 3-4-1 and autophagy, this study will explore various players in the autophagy pathway, as well as PI3K and AKT, and their modulation in the presence and removal of Imatinib. We hypothesize that 3-4-1 may possess an altered autophagy pathway and exploits this abnormality to enhance drug-resistance and perpetuate its survival.

Methods

Cell Culture and ts-Ab-MuLV

S9 and 3-4-1 cells were cultured in medium consisting of RPMI 1640 with 10% fetal bovine serum, 1mM L-glutamine, 100 U/ml streptomycin, 100ug/ml penicillin and 50 uM β–mercaptoethanol. Cells were maintained at the v-Abl-permissible temperature (33°C) in a 5%CO_2,

air-humidified incubator. V-Abl was inactivated by holding cells at the v-Abl-inactivating temperature (39°C) for 48 hours. V-Abl reactivation was accomplished by returning cells to 33°C for 24 hours.

Drug Treatments

All stock drugs were prepared in DMSO. Final DMSO concentration in drug treated samples and control samples were 0.1%. AMPK-inhibitor (Compound C) and pan-PI3K-inhibitor (LY294002) were both obtained from CalBiochem/EMD Biosciences and applied to cells at a concentration of 10 uM for 24 hours at 33°C. Imatinib (STI571) was purchased from LC laboratories and applied to cells at a concentration of 1 uM for 48 hours at 33°C for inhibition of Abl. Re-activation of Abl in Imatinib-treated cells was accomplished by removing the drug-containing media, washing in PBS, and re-plating in Imatinib-free media for an additional 48 hours at 33°C. Doxorubicin was purchased from Sigma-Aldrich and applied to cells at 40 ng/ml for 24 hours at 33°C to serve as an apoptosis control for western blot analysis.

Cell Cycle, ROS, Doubling, and Apoptosis Analyses

Cells for cell cycle analysis were porated and stained in a solution containing 50 ug/ml propidium iodide, 1 mg/ml RNAse A, 0.1% Triton-X and 0.62% sodium citrate. Stained cells were subjected to flow cytometry on a FACSCalibur (BD) and fluorescence intensity was analyzed with an FL2-A histogram using Cell Quest Pro software (also BD).

ROS was measured by staining cells with the fluorescent dye DCFDA (dichlorofluorescein diacetate, from Sigma-Aldrich) at 2.5 uM per 10^6 cells, according to the manufacturer's protocol. Stained cells were subjected to flow cytometry on a FACSCalibur (BD) and fluorescence intensity was analyzed with an FL1-H histogram using Cell Quest Pro software (also BD).

Doubling was assessed using a vital dye known as CFSE (carboxyfluorescein succinimidyl ester, from Invitrogen) at a final working concentration of 2 uM per 10^6 cells, according to the manufacturer's protocol. Stained cells were subjected to flow cytometry on a FACSCalibur (BD) and fluorescence intensity was analyzed with an FL1-H histogram using Cell Quest Pro software (also BD).

Apoptosis analysis was accomplished by double-staining cells with 50 ug/ml propidium iodide (red fluorescence) and 50 uM FITC-conjugated Annexin-V protein (green fluorescence) per 10^6 cells. Stained cells were subjected to flow cytometry on a FACSCalibur (BD) and red vs. green positive fluorescent staining was analyzed with an FL1-H vs. FL2-A dot plot using Cell Quest Pro software (also BD).

Western Blot Detection of Various Proteins

The cells were lysed in RIPA buffer supplemented with 2% Triton-X as suggested by Tanida *et al* (2008) and Kimura *et al* (2009). Additionally, washed cells were suspended in lysis buffer and passed through a 20 gauge syringe 20 times, and then incubated on ice for 30 minutes to

complete lysis. The lysates were not centrifuged to maintain the membrane fraction in the lysate suspension and, therefore, all of the membrane-associated proteins (i.e. LC3-II).

Equal amounts of protein from each sample was separated by SDS-PAGE and transferred on ice to PVDF membrane. Membranes were incubated with their respective primary antibodies overnight at 4°C. PARP, caspase-3, phosphorylated-p53, p21, phosphorylated-AKT, and AKT antibodies were purchased from Cell Signaling. XIAP antibody was purchased from Abcam. LC3, GAPDH, Atg4, and p19ARF antibodies were purchased from Sigma-Aldrich. Actin antibody was purchased from Santa Cruz Biotechnology. The Membranes were then incubated in the appropriate peroxidase-conjugated secondary antibody (either goat-anti-rabbit or goat-anti-mouse, both from BioRad) for one hour at room temperature. Specific bands corresponding to each protein assessed was detected using enhanced chemiluminescence reagents (Pierce brand carried by Thermo Scientific). Luminescent signal was transferred to autoradiography film (Santa Cruz Biotechnology) and developed.

Electron Microscopy

S9 and 3-4-1 cells were incubated at 39°C for 48 hours to inactivate v-Abl, then returned to 33°C for 72 hours to re-activate v-Abl. These cells were then fixed and imaged using transmission electron microscopy at the Life Sciences EM Facility at Arizona State University. The transmission electron microscope was a Philips CM12, operated at 80 kV. Images

were captured with a Gatan model 791 camera, and analysis of images was accomplished with Gatan Digital Micrograph software version 3.9.1.

Results

3-4-1 is Imatinib-Resistant (Panels A and A-2)

The experiments in Panel A were to determine S9 and 3-4-1 cell responses to the inactivation and reactivation of v-Abl using the temperature-sensitive feature of ts-Ab-MuLV. We analyzed their cell cycle profile and doubling relative to the parental cell line (S9) while using temperature to inactivate and re-activate v-Abl. At 33°C, v-Abl is active and cells remaining at this temperature are the control cells. Treatment with Imatinib is mimicked by shifting the cells to 39°C, at which v-Abl is inactivated, for 48 hours. Then, to assess the effects of v-Abl reactivation, the cells were shifted back to the v-Abl-permissive temperature (33°C) for 24 hours.

Cell Cycle. The cell cycle profiles of all samples were determined via permeabilization and propidium iodide staining and subsequent flow cytometric analysis. Propidium iodide directly stains DNA and, therefore, can indicate DNA content at different wavelengths. This data can then be interpreted to the accumulation of DNA during the different phases of the cell cycle. The M1 region of the histogram represents the apoptotic population of the sample, M2 represents cells in G_0/G_1, M3 represents

cells in S phase, M4 represents cells in the G_2/M transition, and M5 shows aneuploidy. S9 cells show G_1 arrest upon v-Abl inactivation. This G1 arrest is maintained, even after v-Abl reactivation. However, 3-4-1 cells show a slight G_1 arrest when v-Abl is inactivated, followed by a robust re-entry into the cell cycle upon v-Abl reactivation. 3-4-1's ability to re-enter the cell cycle immediately following v-Abl inactivation is a clear sign of Imatinib-resistance.

Doubling. The rate of cell doubling was assessed using a vital dye known as CFSE (carboxyfluorescein succinimidyl ester) which incorporates into the cell membrane and, therefore, is diluted with every cell division. Fluorescent intensity was measured via flow cytometry. As cells divide/double, the fluorescent intensity of the histogram peak will shift to the left (lower fluorescent intensity). The samples from cells in which v-Abl was inactivated and reactivated (purple peaks) was overlaid with the peak acquired from the control cells for comparison. S9 cells showed a reduction in doubling in both v-Abl inactivated and reactivated samples in comparison to the control rate of doubling. 3-4-1 cells show less doubling upon v-Abl inactivation. When v-Abl was reactivated, 3-4-1 cells doubled faster than control cells. These results correlate with their respective cell cycle profiles.

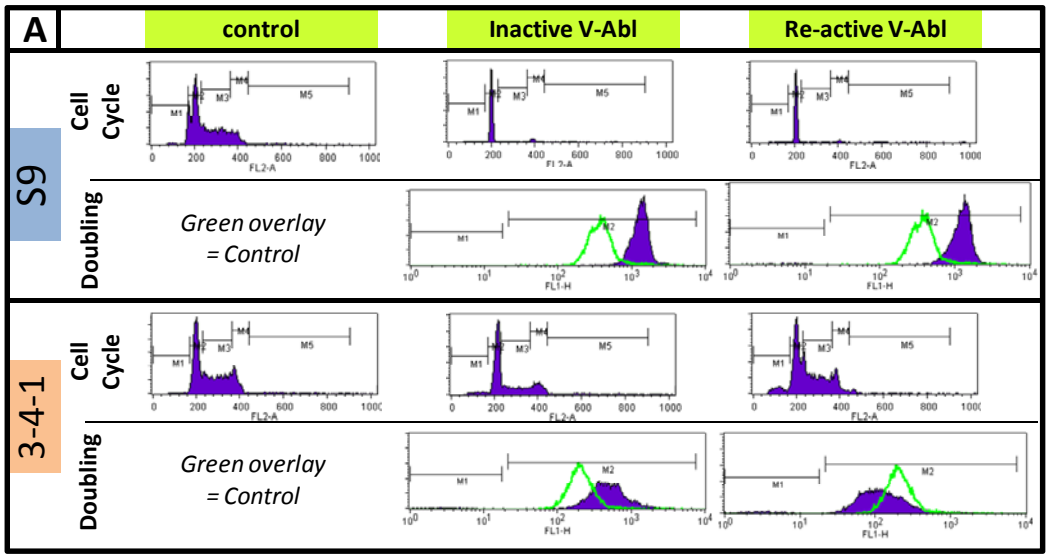
Imatinib Treatment. The experiments in Panel A-2 were to establish if 3-4-1 cells are indeed resistant to Imatinib, S9 and 3-4-1 cells were treated with 0.1 uM of Imatinib, in 0.1% DMSO as a carrier, for 48

hours to inhibit v-Abl. V-Abl reactivation was accomplished by washing the Imatinib-treated cells in PBS, then replacing the cells into Imatinib-free media for 48 more hours. Then, these samples were analyzed for cell cycle through PI-staining followed by flow cytometry. The data obtained for each phase of the cell cycle was normalized against the control (DMSO only) cells and represented in the histogram as percentages of the control, so the 100% mark of each bar is also the control (untreated) for that sample.

S9 and 3-4-1 cells both show G1 arrest when treated with Imatinib (first 2 blue bars). Furthermore, much of the G1 arrest in both cell lines remains after Imatinib is removed (first 2 red bars). Imatinib treatment dramatically reduces S phase in both cell lines (middle 2 blue bars). Removal of Imatinib allows the S phase in both cell lines to recover in a similar fashion (middle 2 red bars). The G2 phase of the cell cycle is where the two cell lines differ dramatically. Treatment of Imatinib reduces the G2 populations in both cell types (last 2 blue bars). However, when Imatinib is removed (last 2 red bars), the G2 population of S9 cells remains relatively unchanged where as the G2 population of 3-4-1 cells is restored to over 125% of the control cells.

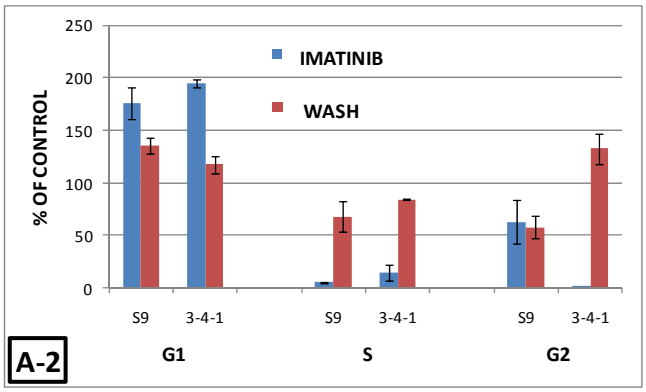
This data parallels the findings shown in Panel A, which indicate that 3-4-1 cells are able to robustly re-enter the cell cycle following attenuated growth-inhibition by v-Abl inactivation (either by temperature inactivation or chemical-inhibition via Imatinib). Given that the 3-4-1 cell

cycle shows some arrest in the presence of Imatinib, it is important to note that this cell line is only *somewhat* resistant to Imatinib in that the cell cycle does not progress until Imatinib is removed from the cells. However, v-Abl reactivation allows for 3-4-1 to robustly re-enter the cell cycle as seen in Panels A and A-2. In Panel A-2, the G2 population after Imatinib-removal (last 2 red bars) show that S9 cells maintain a similarly low number of cells as during Imatinib treatment. However, the 3-4-1 cells a dramatic increase in G2 population after Imatinib-removal (red bar furthest to the right). This increase in G2 population is indicative of cell cycle re-entry.



Panel A and A-2: *3-4-1 is Imatinib-resistant.* **A:** Left column is basal v-Abl activity, middle column is inactive v-Abl and right column is re-activated v-Abl. Top half of panel is S9 cells and bottom half of panel is 3-4-1 cells. Each cell type has been subjected to 2 assays: 1) cell cycle

analysis via propidium iodide staining and 2) doubling assay via CFSE staining. **A-2:** S9 and 3-4-1 cells were treated with 1uM Imatinib for 48 hours (blue bars), followed by washing cells and replacement of Imatinib-free media for 48 hours (red bars). Cell cycle analysis (showing only G1, S and G2 phases) via PI-staining and data is normalized as a percent of control (untreated/DMSO only) cells.



3-4-1 Has an Altered Autophagy Pathway (Panels B and C)

Immunoblotting and primary antibodies specific to various players in the autophagy pathway were used to determine autophagy progression and relative levels of autophagy-related-protein expression in S9 and 3-4-1 cell lines at 4 conditions: 1) control cells constantly maintained at the v-Abl-permissive temperature (+); 2) Cells that were held at the v-Abl inactivating temperature for 48 hours (-); 3) cells that were held at the v-Abl-inactivating temperature for 48 hours, then returned to the v-Abl-permissive temperature for 24 hours (++); and 4) cells that were treated with 40 ng/ml of Doxorubicin for 24 hours (DOX). Doxorubicin is a DNA-damaging agent to which both cell lines show sensitivity in the form of cell cycle arrest and partial apoptosis. Doxorubicin-treated cells were included

to serve as a DNA-damage/apoptosis control. Cell lysates were prepared as described by Tanida *et al* (2008) in order to maximize autophagy proteins that may be associated with the autophagosomes in the cytoplasm.

3-4-1 shows elevated expression of autophagy-enhancing proteins (Panel B).

LC3-I/II turnover is a notorious marker for autophagy progression. Both cell lines show dramatically different levels of autophagy as indicated by the presence of LC3-I and –II at the different levels of v-Abl activity. As seen in Figure 2, autophagy is a cyclic phenomenon in which LC3-I is converted to LC3-II as the autophagosome grows but, following lysosomal degradation of the protein target, LC3-II is recycled back to LC3-I for re-use in the formation of another autophagosome.

In S9 cells, LC3 turnover from LC3-I to –II can be seen as v-Abl goes from a basally active state to inactivation. When v-Abl is active, S9 cells have some LC3-I and little, if any, LC3-II, meaning that these cells have a low level of basal autophagy and, therefore, a low level of LC3 conversion. Therefore, an active autophagy pathway can be shown by LC3-I and –II levels in which LC3-II indicates autophagosome formation, but LC3-I indicates the readiness to form autophagosomes and the continuation of the autophagy cycle following target protein degradation.

When v-Abl is inactivated, S9 cells accumulate LC3-II, but have little to no detectable LC3-I, meaning that the recycling of LC3-II to –I is

inhibited and, therefore, the autophagy pathway is not permitted to continue as a cycle. The conversion of LC3-I to –II is expected upon v-Abl inactivation and cell cycle arrest, as the autophagy pathway should be inhibited in this situation (see Figure 6).

When v-Abl is reactivated in S9 cells, the effects of prolonged cell cycle arrest reduced levels of both LC3-I and –II to equally low levels, suggesting a correlation that the autophagy pathway is inhibited as long as cell cycle progression is inhibited. A possible explanation for this reduction on both forms of LC3 could be that the LC3-I precursor was consumed during the autophagosome formation during v-Abl inactivation and was converted to LC3-II. The inability of S9 to replenish LC3-I levels, when v-Abl is inactive or reactivated, could indicate that LC3 recycling is inhibited or that the synthesis of LC3 precursors is at a low level in S9.

However, 3-4-1 cells have high levels of both LC3-I and –II, which is a marker for high autophagic processing. Interestingly, the increased level of both LC3I and II appeared not to be influenced by v-Abl activation status, indicating an enhanced autophagy level. The lack of visible processing of LC3-I, to –II, and recycling could mean that 3-4-1 fails to up regulate autophagy according to v-Abl activation. However, the high levels of both LC3 forms make it difficult to discern LC3 conversion, if any. When taking into consideration that v-Abl inactivation/Imatinib-treatment is able to somewhat inhibit cell cycle progression in 3-4-1 cells (Panels A and A-2), this LC3 data suggests that 3-4-1 cells have a high level of

autophagy, regardless of cell cycle progression. However, since we did not explore any possible direct relationship between cell cycle and autophagy, this correlation remains speculation. This may suggest that 3-4-1 cells have been selected for high levels of autophagy in the presence of v-Abl inactivation.

As previously mentioned, the ATG4 protein acts in autophagy by converting LC3-II back to LC3-I at the conclusion of autophagy. Increased ATG4 protein levels can indicate an increased autophagy pathway. Interestingly, expression of ATG4 in 3-4-1 was abolished when v-Abl was inactivated and remained at undetectable levels, even upon reactivation. All the while, S9 maintained a constant level of ATG4 expression.

Although this seems contradictory to all of the previously mentioned results, Rzymski *et al* (2010) identified ATG4 as a target of oxidative degradation by cellular ROS[36]. As to be shown in Panel G, 3-4-1 cells have an elevated accumulation of ROS upon v-Abl inactivation, which is somewhat maintained when v-Abl is reactivated. Given the correlation between the elevated ROS and reduction of ATG4 in 3-4-1 when v-Abl is inactivated, the potential for ATG4 to be degraded by ROS in these cells was of interest.

To further investigate this possibility in 3-4-1 cells, we repeated this experiment with the addition of NAC (N-acetylcysteine, Sigma-Aldrich). NAC is an antioxidant which can reduce free radicals such as ROS. However, the addition of NAC did not increase the amount of ATG4

visualized by Immunoblot (data not shown). This suggests that the lack of ATG4 in 3-4-1 cells is not a result of oxidative degradation but, rather, is the result of some other cause.

Conversely, the lack of ATG4 in 3-4-1 cells may indicate incomplete autophagy. This would also mean that 3-4-1 cells might have an accumulation of damaged/redundant proteins and/or organelles/mitochondria. Therefore, the lack of ATG4 may be the cause of the elevated ROS seen in 3-4-1 as a result of erroneous protein accumulation.

3-4-1 shows reduced expression of autophagy-inhibiting proteins (Panel C).

$P19^{ARF}$ (p19 alternate reading frame, also referred to as ARF) and smARF (small mitochondrial ARF) have been indicated to positively regulate autophagy[75-77]. ARF is a tumor suppressor and the locus from which it originates is mutated in 40% of human cancers[75]. The normal function of ARF is known to activate p53 by inhibiting its inhibitor Mdm2, which leads to cell cycle arrest and apoptosis[76]. The shorter form of ARF, smARF, lacks the Mdm2-binding domain and, therefore, does not lead to p53 activation. This shorter form of ARF is a product of translation from an internal methionine in the ARF transcript[76]. Rather, smARF is known localize to the mitochondria, lowering mitochondrial membrane potential and potentially affecting the integrity and function of mitochondria[77]. In addition, smARF is usually expressed proportionally to ARF and has been

shown to induce massive autophagy to the extent of leading to caspase-independent cell death[76].

Panel C contains western blot data representative of ARF and smARF protein levels in S9 and 3-4-1 cell in response to v-Abl activity. In S9 cells, ARF and smARF are not expressed until v-Abl reactivation. However, in 3-4-1 cells, high levels of ARF and smARF are expressed at all conditions of v-Abl activity. As previously mentioned, smARF and ARF can positively regulate autophagy while ARF can also activate p53 (leading to cell cycle arrest or apoptosis). The high levels of ARF and smARF expression in 3-4-1 cells correlate with the signs of an increased autophagy pathway (implicated by LC3-I and –II expression in Panel B). Additionally, enhanced ARF can lead to p53 activation and, ultimately, cell cycle arrest and apoptosis.

Although the cell cycle analyses of 3-4-1 cells showed limited cell cycle arrest upon v-Abl inactivation (by both holding at the inactivating temperature and treatment with Imatinib), ARF and smARF levels seem unaffected, regardless of v-Abl activity. Since high levels of ARF have been found in tumors with mutated p53, the regulation of p53 by ARF may be lost in 3-4-1 cells. It is possible that the p53 protein in 3-4-1 cells has acquired an inactivating mutation or is expressed at low levels, which would prevent cell cycle arrest by ARF over-expression. A loss of or mutation in the p53 protein could be one of the ways that 3-4-1 cells

survive such high levels of ARF. Activated-p53 protein levels were investigated and discussion is below.

P21 accumulation in the cytoplasm is known to lead to cell cycle arrest (modulated by p53) and has recently been shown to positively regulate autophagy in nutrient-rich conditions, possibly through AMPK-mediated modulation of HDAC (histone deacetylase) activity which is crucial in controlling p21 expression[78]. Given that p21 can cause cell cycle arrest, we expected to see increased levels of this protein in S9 cells upon v-Abl inactivation and re-activation, which would correlate with the cell cycle data seen in Panel A. However, S9 showed little to no expression of p21 regardless of v-Abl activity which is contradictory to the cell cycle arrest data seen in Panel A. The lack of p21 in S9 cells could be a result of rapid p21 degradation given that previous work by our group showed that treating S9 cells with a proteasome inhibitor restored p21 protein levels.

3-4-1 cells had an upregulation of p21 upon v-Abl inactivation, which does not correlate with the limited cell cycle arrest upon this same experimental condition (seen in Panel A). Although, given that phosphorylated p53 is necessary for the production of p21, the presence of p21 in 3-4-1 cells is not expected at all because of the lack of activated p53 in this cell line. The presence of p21 in 3-4-1 is contradictory to the cell cycle analyses and expression of other proteins.

However, there are a few possible explanations for the contradictory p21 expression seen in 3-4-1. First, it is possible that the degradation pathway, which may be the cause for reduction of p21 in S9 (mentioned above), may be defective in 3-4-1, allowing an accumulation of p21. This potential defect in protein degradation might also be linked to the increased/defective autophagy pathway also observed in this tumor line. Conversely, Xia *et al* (2011) recently explained that p21 is not necessary for cell cycle arrest in some cell lines and, additionally, may play antiapoptotic roles. It is possible that the 3-4-1 cell line may have an augmented p21/cell cycle arrest/apoptosis relationship as well.

One explanation for the difference in p21 seen between S9 and 3-4-1 cells could be related to the higher levels of autophagy in 3-4-1 cells. As previously mentioned, p21 can positively feed into the autophagy pathway[78]. It is possible that 3-4-1 cells have a much higher level of p21 because of its potential utilization in autophagy in this tumor line. However, we can only speculate as to the cause of this seeming contradiction of p21 expression levels between the non-malignant and malignant tumor lines and realize that further investigation is needed to clarify this anomaly.

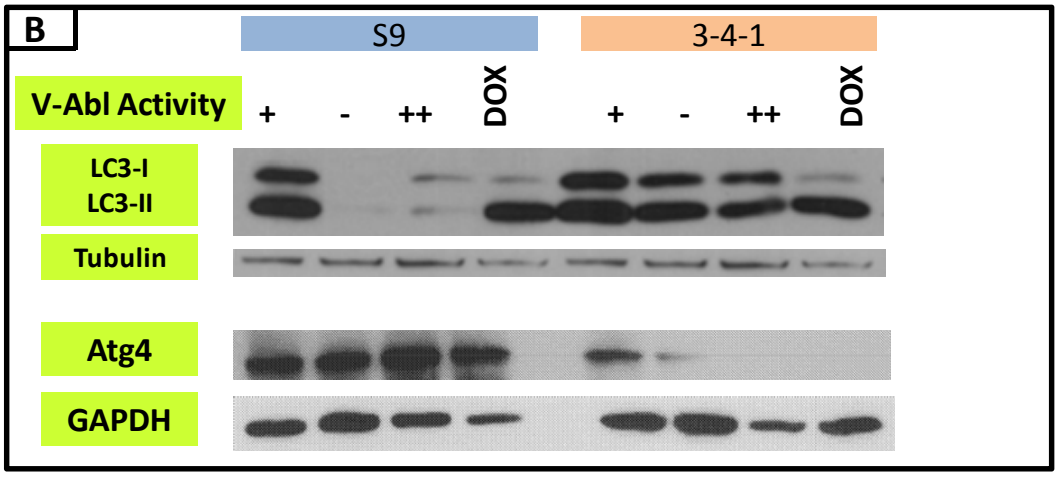
As mentioned earlier, cytoplasmic p53 has been shown to inhibit autophagy by a mechanism which still remains to be understood. However, p53 must be phosphorylated in order for it to translocate from the nucleus to the cytoplasm[58-65]. As previously mentioned, activated p53

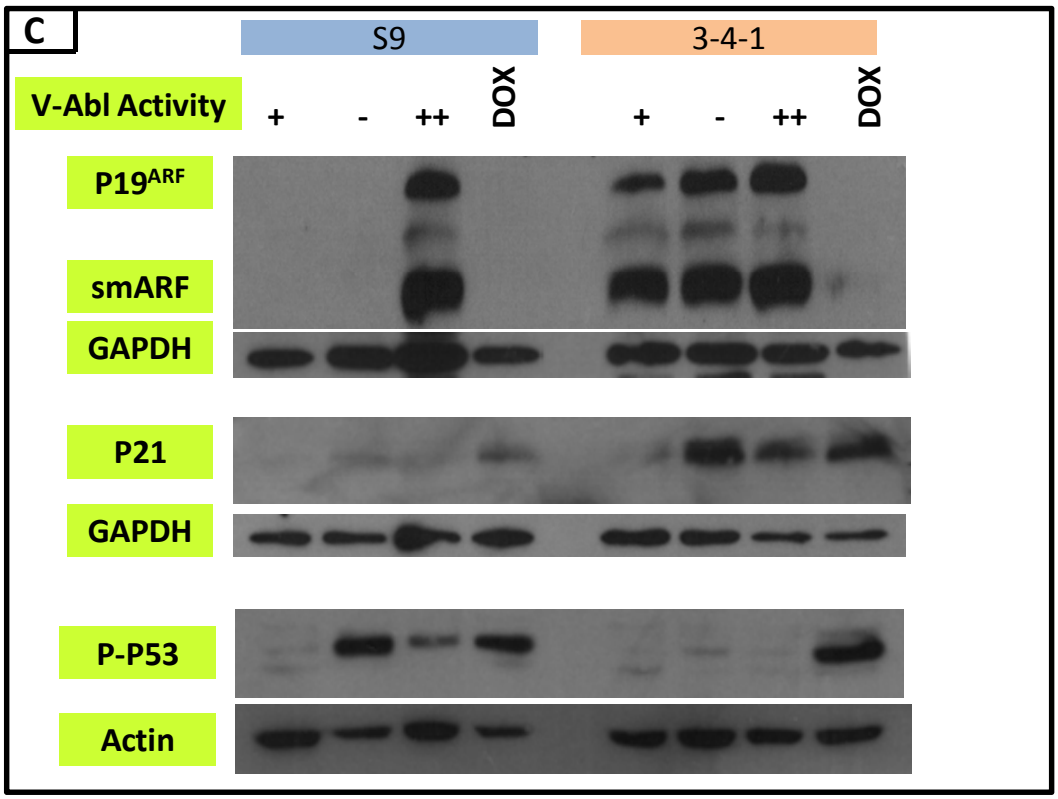
can either enhance autophagy and cell cycle arrest, or inhibit autophagy as described by Tasmedir *et al*. The determinants which direct tumor cells to use either a p53-enhanced or p53-inhibited autophagy pathway is still not clear. It is also important to be reminded that that ARF expression can activate p53 (if p53 is normal, not mutated) upon oncogenic stress, leading to cell cycle arrest and apoptosis.

While S9 shows increased activation of p53 upon v-Abl inactivation and reactivation, 3-4-1 had no detectable levels of P-p53, regardless of v-Abl activity. The high levels of P-p53 in 3-4-1 cells are contradictory to the high level of ARF expression (since ARF can activate p53). This suggests that 3-4-1 cells have adapted the p53-independent autophagy pathway in which autophagy is induced in a manner that is independent of the PI3K-I_A/AMPK/AKT/mTOR pathways (see Figure 5) and the presence of p53 actually inhibits the induction of autophagy. If this is the case, the lack of activated p53 in 3-4-1 cells could further explain the enhanced autophagy pathway witnessed in this tumor line.

Modulation of v-Abl activity had no effect on the expression of total and phosphorylated AKT (data not shown). The unaltered expression and activation of AKT correlates with the possibility that 3-4-1 may have adopted the p53-independent autophagy pathway. As previously mentioned, AKT can be phosphorylated and activated by PI3K class-I activity, leading to the inhibition the AMPK pathway and, eventually, positive regulation of autophagy (see Figure 6). If these cells utilized the

p53-dependent autophagy pathway, AKT activation would be increased in correlation with an increase in autophagy. However, both S9 and 3-4-1 cells have consistent expression and activation levels of AKT. Additionally, according to the previously discussed western blot data, 3-4-1 cells do show signs of upregulated autophagy and a lack of activated p53. Taken together, this data suggests that 3-4-1 cells have adopted the p53-independent autophagy pathway (see Figure 5).





Panels B and C: Immunoblots of autophagy-related proteins in S9 and 3-4-1 cell lines in response to basally active v-Abl (+), inactivated v-Abl (-), reactivated v-Abl (++), and 40 ng/ml Doxorubicin (DOX).

3-4-1 is sensitive to AMPK and PI3K inhibition (Panel D)

As previously mentioned the AMPK and PI3K pathways can modulate autophagic induction and processing (see Figure 6). It was also been suggested by many groups (explained above) that tumor cells often have an upregulated autophagy pathway which might be a route of survival in the presence of oncogenic stress or chemotherapies. To determine the involvement of these pathways in S9 and 3-4-1, we evaluated the cell cycle responses to inhibition of the AMPK and PI3K.

Given that 3-4-1 cells have an enhanced autophagy pathway, which seems independent of v-Abl activation status, we suspect S9 and 3-4-1 cells to exhibit different responses to AMPK and PI3K inhibition.

For the following experiment, we treated S9 and 3-4-1 cells for 24 hours with 0.1% DMSO (dimethyl sulfoxide, the drug carrier), 10 uM of Compound C (an AMPK-inhibitor) in 0.1% DMSO, or 10 uM of LY294002 (a PAN-PI3K-inhibitor) in 0.1% DMSO. The treated cells were then analyzed for cell cycle (as previously described) and the AMPK-inhibitor-treated cells were also analyzed for apoptosis through staining with FITC(fluorescein isothiocyanate)-conjugated Annexin-V protein and PI, followed by flow cytometric analysis.

Cell Cycle. Inhibition of AMPK caused G1 and G2 arrest (peaks in M2 and M4 regions, respectively) in both S9 and 3-4-1. However, the G1 arrest in 3-4-1 cells was coupled with massive apoptosis as indicated by the peak in the M1 region. This apoptotic population was not seen in S9 cell, suggesting that 3-4-1 have an enhanced sensitivity to AMPK-inhibition. These results are similar to the cell cycle reaction to treatment with the PI3K inhibitor (apoptosis and G1 arrest). This suggests that 3-4-1 also have an enhanced sensitivity to PI3K-inhibition.

Apoptosis (Annexin V + PI). Annexin V protein had been shown to bind specifically to phosphatidylserine, which has been shown to be expressed on the surface of early apoptotic cells, although its cellular function is not yet known[79,80]. By conjugating this protein to FITC, its

presence on the cell membrane can be assayed using flow cytometry on the FL1 channel. In panel D, cells that stain positive for Annexin-V binding will present in the top-right and bottom-right quadrants.

Since the cells are not permeabilized prior to staining with Annexin-V or PI, the PI will only be able to stain the DNA in cells that have a porated cell membrane which allows the entrance of PI into the cell. Disrupted cell membranes are a sign of later stages of apoptosis and necrosis. Since the fluorescence of PI is analyzed on the FL2 channel of the flow cytometer, the cells that stain positive for PI (which is positive for cell membrane disruption and, therefore, later apoptosis/necrosis) will display in the top-left and top-right quadrants.

More simply put, the bottom-left quadrant represents healthy cells which will be unstained by both dyes. The bottom-right quadrant represents early apoptotic cells, which have an in-tact cell membrane but express phosphatidylserine on the cell surface. The top-right quadrant represents late apoptotic cells, which have been double-stained by both dyes, meaning that these cells have progressed beyond expression of phosphatidylserine on the cell surface to membrane disruption (complete apoptosis). Finally, the top-left panel represents dead or necrotic cells, which have been stained by PI only, meaning that they have foregone the expression of phosphatidylserine on the cell surface and continued directly to membrane disruption.

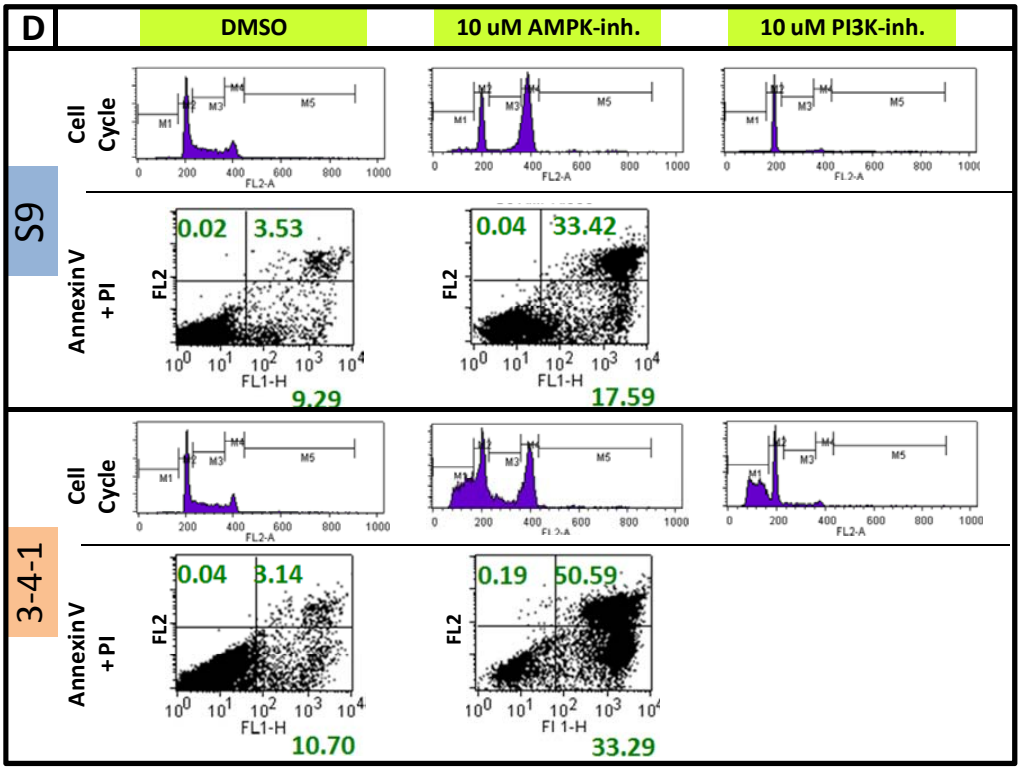
S9 cells showed some enhanced apoptosis when treated with the AMPK inhibitor. More specifically, early apoptosis was increased by about 8% and late apoptosis was enhanced by about 30%. However, Compound C-mediated-apoptosis in 3-4-1 cells extremely enhanced. Early apoptosis was increased by about 23% and late apoptosis was increased by about 47%. This data strengthens the previously mentioned cell cycle findings that 3-4-1 cells have an enhanced sensitivity to AMPK-inhibition.

Recall the previous explanation of AMPK and its positive modulation of the autophagy pathway. One explanation for the massive apoptosis seen in 3-4-1 cells following AMPK-inhibition could be that these cells are, somehow, reliant on the autophagy pathway to the point that inhibition of autophagy results in massive death. This finding coincides with the previous groups that found the autophagy pathway up-regulated in tumor cells when treated with radiation or medicinal therapies[64-69]. The notable finding in this study is that 3-4-1 shows signs of enhanced autophagy at all times, even when no perturbations are inflicted. This may indicate that 3-4-1 tumor cells rely on autophagy for survival in general or, in other words, these cells are addicted to the autophagy pathway.

Vucicevic, L. *et al*. (2009) recently found that induction of apoptosis through treatment with Compound C can occur in a mechanism independent of the AMPK pathway itself. They found that Compound C can elevate cellular accumulation of ROS to the point of causing cell death

via oxidative stress. Given that 3-4-1appears to have an intrinsic higher level of ROS (Panel A), their sensitivity to compound C may be due to an excessive amount of ROS generated inside the cells to cause demise of the cells. To test this hypothesis, we used an antioxidant scavenger to see if it could rescue these cells from the massive apoptosis upon treatment with Compound C.

To investigate the presence of this phenomenon in 3-4-1 cells, we also tried a co-treatment of 10 uM Compound C and 3 mM N-acetylcysteine (NAC) for 24 hours in S9 and 3-4-1 cells. The addition of the antioxidant did not rescue the 3-4-1 cells from Compound C-induced apoptosis, nor did it reduce the accumulation of cellular ROS (data not shown). This finding indicates that the apoptosis induced in 3-4-1 cells, when treated with Compound C, is related to AMPK-inhibition and not due to an accumulation of cellular ROS. Given the necessity of AMPK to the autophagy pathway, the possible addiction of 3-4-1 cells to the autophagy pathway seems more plausible in that inhibition of autophagy by AMPK-inhibition kills these tumor cells.



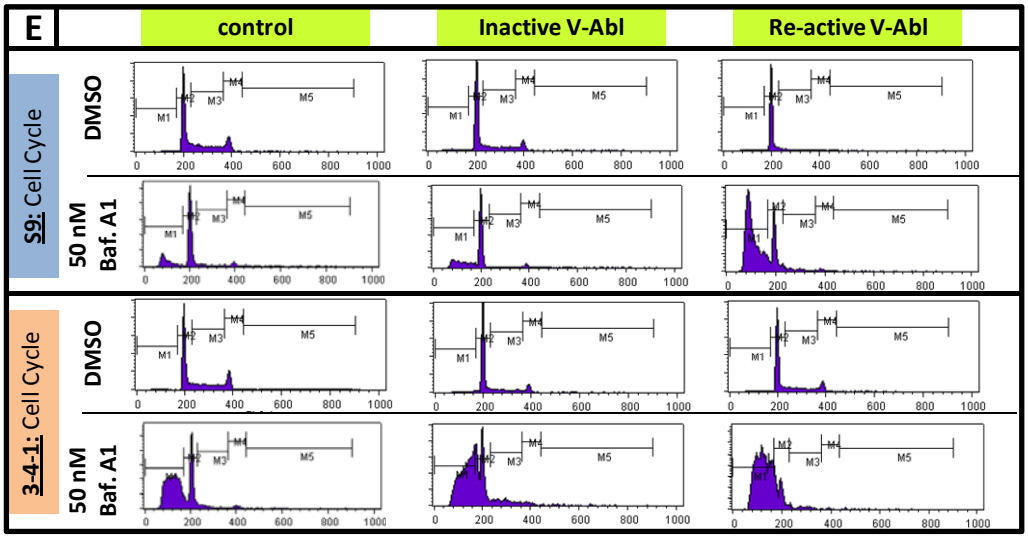
Panel D: 3-4-1 is sensitive to AMPK and PI3K inhibition. Rows 1 and 3 indicate the cell cycle analyses of S9 and 3-4-1 cells, respectively, treated with 0.1% drug carrier (DMSO), 10 uM Compound C (AMPK-inhibitor, or 10 uM LY294002 (PI3K-inhibitor) for 24 hours. The DMSO and Compound-C treated samples were further analyzed for apoptosis via Annexin-V and PI double-staining (S9 in row 2 and 3-4-1 in row 4).

Autophagy Inhibition Leads to Massive Apoptosis in 3-4-1 (Panel E)

Taking all of the data explained above into consideration, it is apparent that 3-4-1 cells are somehow addicted to autophagy for survival. If this is the case, the mere inhibition of autophagy itself should yield a

fatal blow to the 3-4-1 tumor line. To explore this possibility, we used an autophagy-inhibiting drug known as Bafilomycin A1. Bafilomycin A1 is an antibiotic that is a specific inhibitor of vacuolar-type H+-ATPase[82]. This drug inhibits autophagy by prevention the acidification of lysosomes and preventing lysosomal fusion with autophagosomes[83]. Cell cycle was analyzed in S9 and 3-4-1 cells in which v-Abl was active, inactivated, and reactivated (as in Panel A) with and without treatment with 50 nM of Bafilomycin A1.

S9 cells show some sensitivity to Bafilomycin A1 when v-Abl is reactivated as indicated by the apoptotic population in region M1 of the cell cycle analysis. However, 3-4-1 cells show that Bafilomycin A1 induces apoptosis even when v-Abl is normally activated. The percentage of apoptotic 3-4-1 cells is exacerbated when v-Abl is inactivated in the presence of Bafilomycin A1. Furthermore, v-Abl reactivation in the presence of Bafilomycin A1 leads to total annihilation of 3-4-1 cells. This data suggests that 3-4-1 cells somewhat rely on the autophagy pathway to survive v-Abl inactivation and reactivation. More importantly, 3-4-1 cells might require autophagy to maintain survival or Imatinib-resistance.



Panel E: Autophagy inhibition leads to massive apoptosis in 3-4-1. Cell cycle analysis of S9 (rows 1 and 2) and 3-4-1 (rows 3 and 4) cells while v-Abl is active (column 1), inactive (column 2) and reactivated (column 3) in the absence (DMSO: rows 1 and 3) and presence of 50 nM Bafilomycin A1 (rows 2 and 4).

Analysis of Apoptosis Pathway (Panel F)

We evaluated the apoptosis-related-proteins present in S9 and 3-4-1 as a means of determining the potentially cytocidal effects of v-Abl inactivation and reactivation.

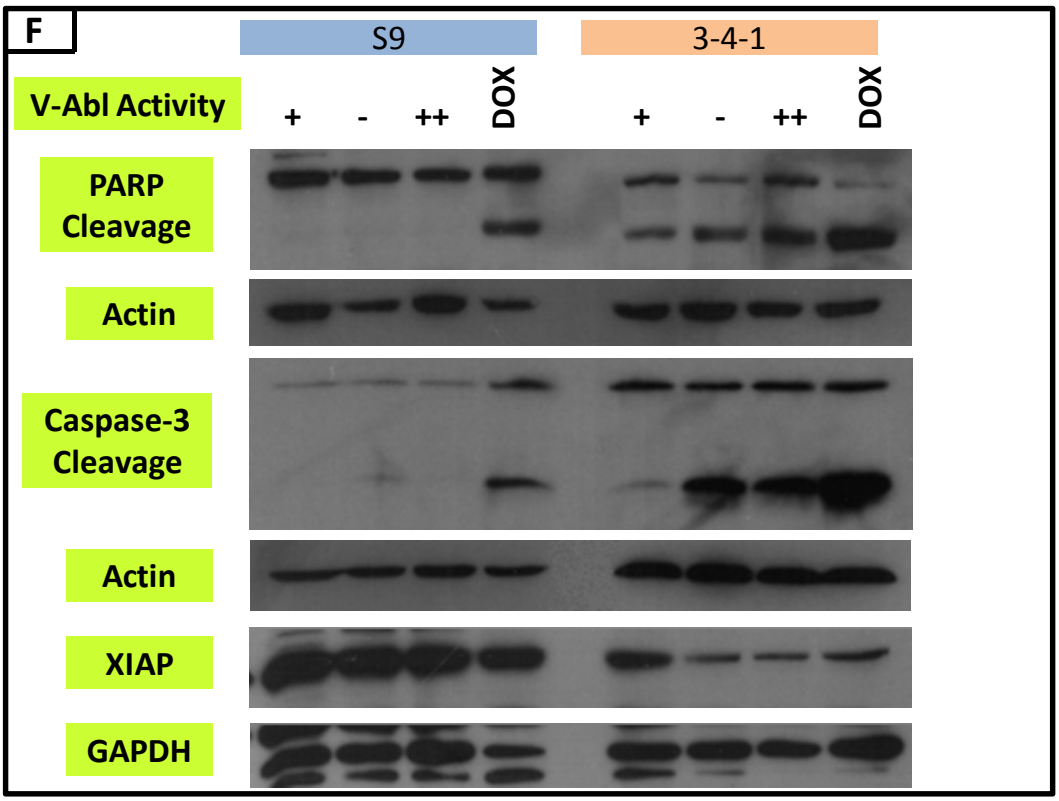
Immunoblotting and antibodies specific to apoptosis-associated proteins (PARP, caspase-3, and XIAP) revealed the apoptotic profiles of S9 and 3-4-1 when v-Abl is active (+), Inactive (-), reactive (++), and in the presence of 40 ng/ml of Doxorubicin (apoptosis control). PARP [poly(ADP-ribose) polymerase] is a protein involved with programmed cell

death stimulated by metabolic-, chemical-, or radiation-induced damage. PARP has been shown to be cleaved by activated caspase-3 upon the induction of apoptosis[84]. Caspase-3 is a member of the caspase family of enzymes that, upon activation, initiate a cascade of cleavage events which lead to apoptotic cell death. This cascade originated at the cell membrane, where caspase-8 is activated by death receptors, which is followed by the cleavage (activation) of other caspases terminating at caspase-3 cleavage which directly leads to apoptosis[85]. Although caspase-3 activation was previously thought to stimulate apoptosis in an irreversible manner, XIAP (X-linked inhibitor-of-apoptosis protein) has recently been shown to directly inhibit caspase-3 and be down regulated by the activation of caspase-3[86].

S9 shows no signs of apoptosis induction regardless of v-Abl activity. However, 3-4-1 shows apoptosis at all levels of v-Abl activity, even in the control cells (as indicated by PARP cleavage). The reduction in XIAP correlates with the increase in caspase-3 activation as activated caspase-3 inhibits XIAP expression. This apoptosis in 3-4-1 is contradictory to the results in Panel A which indicate that 3-4-1 has more active cell cycle and doubling than S9 when v-Abl is inactivated and reactivated.

As previously mentioned, v-Abl transformation begins with primary transformation (characterized by proliferation), followed by crisis (indicated by erratic cell growth and massive apoptosis), resulting in the surviving

cells becoming fully transformed. One possibility is that the signs of apoptosis seen in 3-4-1 cells could be remnants of the crisis phase of v-Abl transformation. More specifically, 3-4-1 may have escaped complete eradication during the crisis phase of v-Abl transformation by adopting a high level of autophagy as a means of survival or, in other words, an up-regulated autophagy pathway may have help these cells to remain in the crisis phase of transformation indefinitely. This theory of 3-4-1 autophagy-addiction/dependence accounts for the massive apoptosis induced when components of the autophagy pathway (AMPK, PI3K and lysosomal acidification) are inhibited. Furthermore, the high levels of apoptosis in 3-4-1, observed by western blot (Panel F) supports the possibility that 3-4-1 are, indeed, in some form of crisis independent of v-Abl activity.



Panel F: 3-4-1 shows signs of apoptosis. Lysates from S9 and 3-4-1 cells when v-Abl is basally active (+), inactive (-), reactivated (++), and treated with 40 ng/ml for 24 hours (DOX) were subjected to immunoblot and probed for PARP, caspase-3 and XIAP proteins.

This finding suggests that although 3-4-1 can escape v-abl inactivation, there is still a fraction of cells are susceptible to apoptosis. So, whatever mutation allows the cells to bypass the cell cycle arrest imposed by v-abl inactivation may also make some cells vulnerable to cell death. Yet, the death of these cells had little consequence on the whole population as a substantial number of cells continue grow despite some casualty. Thus, 3-4-1- cells are continuously undergoing selection for survival, in which some succeed and become more malignant whereas

others could be selected out because of acquired mutation may not be permissive for survival.

3-4-1 has an accumulation of ROS and abnormal mitochondria (Panel G)

ROS accumulation. Levels of total ROS (reactive oxygen species in the form of H_2O_2) was measured with the fluorescent dye DCFDA (dichlorofluorescein diacetate) which directly binds to ROS in the cell cytoplasm, then analyzed using flow cytometry. The fluorescent intensity of the resulting peak on the histogram will shift to the right with accumulation of ROS. The purple peaks are the ROS in cells in which v-Abl has been inactivated and reactivated. The green overlay placed atop the purple peaks is the ROS of the control cells for comparison. An accumulation of ROS can be correlated with metabolic activity (as a product of oxidative phosphorylation) or oxidative stress.

S9 cells show a decreased ROS accumulation when v-Abl is inactivated, and remains decreased even when v-Abl is reactivated. This could be indicative of lower metabolic activity or oxidative stress. 3-4-1 cells show an elevated level of ROS when v-Abl is inactivated, then remains still slightly elevated upon v-Abl reactivation. This could be interpreted as an increase in metabolic activity or oxidative stress. These results correlate with the doubling and cell cycle results (Panel A), which

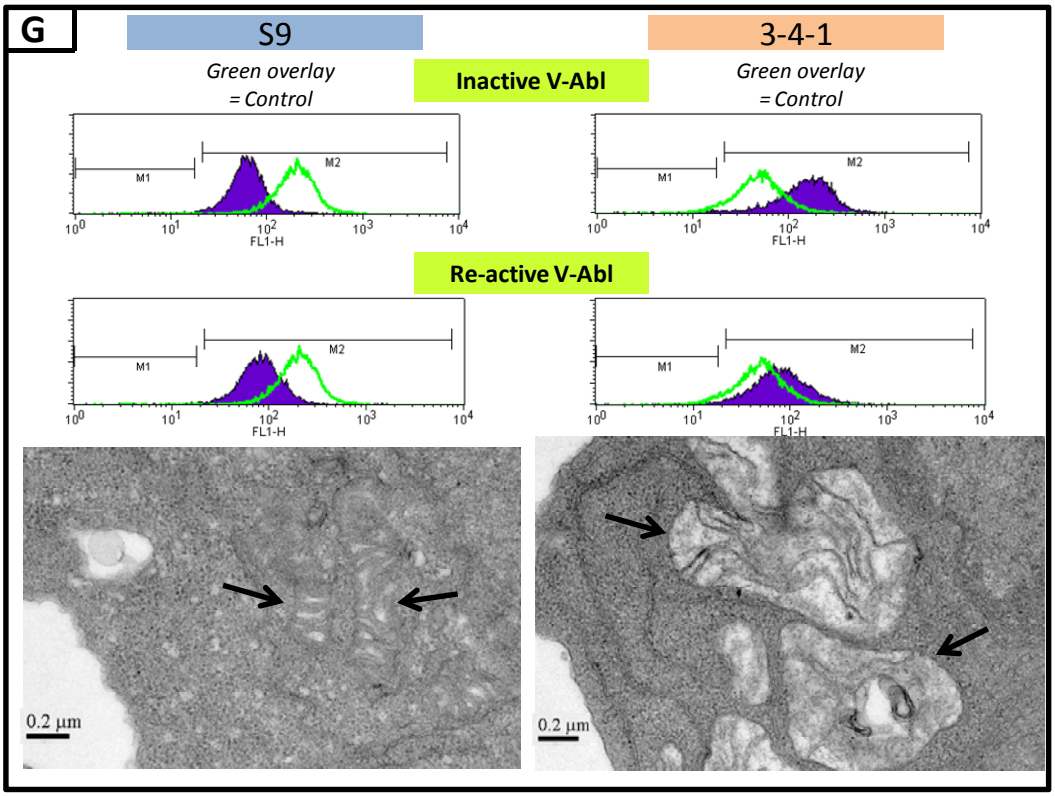
all point to 3-4-1's ability to overcome terminal cell cycle arrest upon v-Abl inactivation, which is synonymous to Imatinib-resistance.

TEM. S9 and 3-4-1 cells were held at the v-Abl-inactivating temperature for 48 hours, then returned to the v-Abl-permissible temperature for 72 hours, then analyzed via TEM (transmission electron microscopy) to assess any potential structural difference between these two cell lines as a result of v-Abl reactivation. Since 3-4-1 is a model for Imatinib-resistance, this data may uncover any structural differences between Imatinib-sensitive and-resistant cells.

The majority of cancer cells have been shown to have an accumulation of mitochondria which possess pleomorphisms such as distorted cristae and swollen matrix[87] when analyzed via electron microscopy. Furthermore, Xu *et al*. (2005) have examined the mitochondria in human leukemia cells, have observed these same pleomorphisms, and described that these structural characteristics caused these cancer cells to be inadequate in mitochondrial respiration and more reliant on glucose uptake[88].

The electron micrographs shown in panel G display that 3-4-1 cells also possess mitochondrial abnormalities such as swelling, disorganized cristae, and increased electron density (visible by the increased contrast in the image). The mitochondria in S9 appear to be smaller and fully-enclosed by the mitochondrial membrane, with a more regular organization of cristae. This finding further strengthens the claim that the

3-4-1 cell line has tumorigenic qualities and might explain some of the autophagic tendencies of this cell line. Furthermore, the accumulation of ROS in 3-4-1 upon the inactivation and re-activation of v-Abl may be a result of the abnormal mitochondria seen in the TEM image. However, it is not clear whether the ROS accumulation in 3-4-1 is a result of the abnormal mitochondria or vice versa.



Panel G: 3-4-1 has an accumulation of ROS and abnormal mitochondria. S9 and 3-4-1 cells were subjected to ROS assay (via DCFDA staining) upon v-Abl inactivation and re-activation. Green overlay is the ROS accumulation in control cells with basally-active v-Abl. TEM image from S9 and 3-4-1 cells that were held at the v-Abl-inactivating temperature for

48 hours, then returned to the v-Abl permissive temperature for 72 hours. Arrows indicate mitochondria.

Discussion

As previously mentioned, both S9 and 3-4-1 cells were derived from pre-B cells that were harvested from SCID mice and transduced with ts-Ab-MuLV. After v-Abl-activity perturbation, the 3-4-1 line diverged from the S9 line with its ability to re-enter the cell cycle, mimicking Imatinib-resistance. The Imatinib-resistance status of 3-4-1 was further verified by inactivating and reactivating v-Abl, and showing 3-4-1's ability to re-enter the cell cycle, double, and accumulate ROS (Panel A). Given this data, we can be reassured that the 3-4-1 cell line is a model for Imatinib-resistant-Ph^+ B-ALL.

We have shown that these Imatinib-resistant 3-4-1 cells express high levels of autophagy-related proteins (Panel B: LC3-I and –II, $p19^{ARF}$, p21,), low levels of autophagy-inhibiting (Panel C: phosphorylated-p53), are sensitive to the inhibition of enzymes that are crucial to the regulation of autophagy (Panel D) and are sensitive to direct inhibition of autophagy (Panel E). Additionally, contradicting expression levels of p21 and ATG4 must be noted. 3-4-1 was expected to have little to no expression of p21 given the lack of activated p53 detected. Furthermore, p21 is an enforcer of cell cycle arrest and, since 3-4-1 is unable to properly arrest upon v-Abl inactivation, we did not anticipate the p21 expression seen in 3-4-1 cells

(Panel C). Additionally, since 3-4-1 seems to possess an enhanced autophagy level (as indicated by the elevated levels of LC3-I and –II), we expected these cells to also express elevated levels of ATG4 as this protein is essential for the completion of autophagy and recycling of LC3-II back to –I.

Given these findings alone, it is reasonable to wonder if the ability of 3-4-1 to resist Imatinib treatment is somehow related to an over-active or impaired autophagy pathway. We were questioning whether 3-4-1 used autophagy as a means of producing metabolic substrates when faced with Imatinib-induced starvation.

However, the findings in Panel F contradicted this previous hypothesis in that, although 3-4-1 cells show signs of proliferation and cell cycle progression, they also show signs of apoptosis. We began to wonder how cells can proliferate and die at the same time. Furthermore, microscopic examination of 3-4-1 cells revealed no difference in viability than S9 cells. In fact, 3-4-1 cells consistently grow to confluency more rapidly than S9.

Various groups that also investigate Ph+ B-ALL[89-92], have turned their attention to ARF expression. Mullighan *et al* (2008) have found that 50-80% of all human Ph+B-ALL have deletions in ARF. Furthermore, Williams *et al* (2008) report that B-cell progenitors respond to the oncogenic stress of BCR-Abl transduction by expressing ARF. As a tumor suppressor, ARF expression leads to apoptosis when induced by

oncogenic stress unless the locus harbors an inactivating deletion or mutation.

Interestingly, Signer *et al* (2008) showed that all B-cell progenitors up to, but not including, pre-B cells do not express ARF at all. He and his group later go on to show (2010) that the ability of a Ph^+ B-cell progenitor to develop into the disease-state of leukemia is dependent upon ARF expression. BCR-Abl transduction in the early B-cell progenitors HSC (hematopoietic stem cells), CLP (common lymphoid progenitors), and pro-B cells (progenitor B cells) does lead to expression of ARF, but at low enough levels that a subset of the transduced cells survive the tumor suppression abilities of ARF and continue on to initiate disease.

Contrastingly, they also note that pre-B cells did not initiate disease after BCR-Abl transduction because it lead to an over-expression of ARF and, therefore, massive apoptosis. They go on to stake the claim that the ability of the B cell lineage to initiate Ph^+ B-ALL is developmentally regulated, disproving the previous belief that inactivation of ARF was a prerequisite for oncogenic-Abl transformation. It is important to note here that these groups did not investigate Imatinib-resistance in their studies.

Parallel to Signer's findings, the S9 cells that our group previously transduced with v-Abl via ts-Ab-MuLV did not reach a malignant disease state and, furthermore, have little to no expression of ARF and smARF until v-Abl reactivation (see Panel C). This can coincide with the fact that S9 maintains cell cycle arrest indefinitely after v-Abl reactivation,

eventually leading to apoptosis. In contrast to the findings of these other groups, S9 cells are an established tumor line, that is non malignant, which was transformed with v-Abl while these other groups studies primary pro- and pre-B cells transduced with BCR-Abl. Upon transformation, S9 cells did increase their expression of ARF initially as a response to the oncogenic stress of v-Abl. Then, to establish itself as tumor, the transformed S9 population probably underwent a selection for low levels of ARF expression as a means of survival.

Also, in parallel with William's, Mullighan's and Signer's findings, 3-4-1 cells do have an enhanced expression of ARF at all levels of v-Abl activity given that they are constantly suffering from the oncogenic stress of expressing the BCR-Abl gene. The elevated ARF expression on 3-4-1 may also account for the expression of apoptosis-related proteins seen in Panel F.

Although 3-4-1 cells do express higher levels of ARF and show signs of apoptosis, this cell line also managed to progress to the malignant disease state (by forming tumors *in vivo*) and beyond by acquiring Imatinib-resistance (as previously mentioned). Additionally, this cell line is of pre-B cell lineage and has enhanced proliferative and cell cycle re-entry abilities when v-Abl is reactivated (see Panel A). These phenomena are contradictory to Signer's findings. Somehow, 3-4-1 has high levels of active ARF but is still able to proliferate and cause disease. An explanation for this might be that 3-4-1 cells express elevated ARF as a

sign of oncogenic stress, but are able to survive as a result of an inactivating/deleting p53 mutation. This would also account for the high levels of apoptosis-related proteins seen in this cell line. Accordingly, it can be speculated that the inactivation of ARF in 3-4-1 cells would further enhance its tumorigenic potential.

Given that the 3-4-1 cell line is an apparent anomaly, we wonder what is responsible for this malignant line to exist. Two factors that were not accounted for in the previously-mentioned studies were examined here: Imatinib-resistance and enhanced autophagy. It is possible that the v-Abl-perturbation leading to the creation of 3-4-1 is responsible for this cell line's adoption of and seemingly addiction to autophagy as a means of survival in the presence of Imatinib and, luckily, also in the presence of ARF over-expression.

Recall that activated-p53 have potentially dual roles in autophagy. Another possibility is that 3-4-1's lack of activated-p53 expression (see Panel B) could mean that this cell line adopted the alternate p53-autophagy pathway as described by Tasmedir *et al* in which activated/cytoplasmic-p53 acts as an inhibitor of autophagy and not an inducer (see Figure 6). The decision to adopt this pathway could be a result of forced Imatinib-resistance, autophagy, or, perhaps, enhanced expression of ARF.

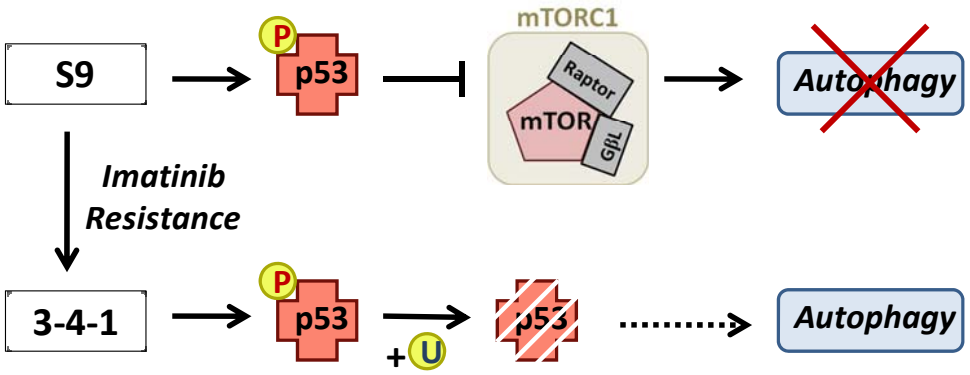


Figure 7. 3-4-1 may utilize the alternate p53-signaling pathway to favor autophagy-progression.

All in all, we are potentially faced with a question of causality. Given that S9 and 3-4-1 cells are both established tumor lines, but only 3-4-1 is malignant and Imatinib-resistant, leads us to believe that the acquisition of malignancy and Imatinib-resistance may be the results of the same initial cause (i.e. a mutation). It is possible that the perturbations of 3-4-1 to acquire Imatinib-resistance forced for the selection of cells that harbored an inactivating or deleting p53 mutation. Following this potential mutation, 3-4-1 cells were able to survive and proliferate regardless of the oncogenic stress of v-Abl activity/inactivation/re-activation and the high levels of expressed ARF.

The combination of contradictory phenomena in the 3-4-1 cell line can serve as a useful tool for further understanding of Imatinib-resistant Ph+ B-ALL. However, the series of events that occur for this cell line to acquire its various survival tactics remains to be understood. More specifically, it would be of great importance to decipher if any one of these

3 characteristics is the key to this cell's malignancy as a potential target for future therapies.

REFERENCES

1. Kasper, D. L. *et al*. (2004) Malignancies of lymphoid cells: clinical features, treatment and prognosis of specific lymphoid malignancies. *Harrison's principles of internal medicine*. 16th edition: chapter 19.
2. Kurzrock, R. *et al*. (2003) Philadelphia chromosome-positive leukemias: from basic mechanisms to molecular therapeutics. *Ann Intern Med*. 138(10):819- 830.
3. Yoshida, K. *et al*. (2002) c-Abl tyrosine kinase regulates the human Rad9 checkpoint protein in response to DNA damage. *Mol Cell Biol.* 22(10): 3292-3300.
4. Yoshida, K. (2007) Regulation for nuclear targeting of the Abl tyrosine kinase in response to DNA damage. *Adv Exp Med Biol.* 604: 155-165.
5. Zou, X. *et al*. (1999) Signaling pathways activated by oncogenic forms of Abl tyrosine kinase. *J Biol Chem*. 274(26): 18141-18144.
6. Melo, J. V. (1996) The molecular biology of chronic myeloid leukemia. *Leukemia.* 5: 751-756.
7. Szczylik, C. *et al*. (1991) Selective inhibition of leukemia cell proliferation by BCR-ABL antisense oligodeoxynucleotides. *Science.* 253: 562-265.
8. Pasternak, G. *et al*. (1998) Chronic Myelogenous leukemia: molecular and cellular aspects. *J Cancer Res Clin Oncol.* 124(12): 643-660.
9. Million, R. P. *et al*. (2000) The Grb2 binding site is required for the induction of chronic myeloid leukemia-like disease in mice by the BCR/Abl tyrosine kinase. *Blood*. 96(2): 664-670.
10. Puil, L. *et al*. (1994) BCR-Abl oncoproteins bind directly to activators of the Ras signaling pathway. *EMBO J.* 13(4): 764-773.
11. Shafman, T. *et al*. (1997) Interaction between ATM protein and c-Abl in response to DNA damage. *Nature*. 387(6632): 520-523.
12. Jabbour, E. *et al*. (2007) Current and emerging treatment options in chronic myeloid leukemia. *Cancer*. 109(11):2171-2181.
13. Kimura, S. *et al*. (2006) New tyrosine kinase inhibitors in the treatment of chronic myeloid leukemia. *Curr Pharm Biotech*. 7(5):371-379.
14. Gambacorti-Passerini, C. *et al*. (2008) Part I: Milestones in personalized medicine – Imatinib. *Lancet Onc*. 9(600): 600.

15. Druker, B. J. *et al*. (2000) Lessons learned from the development of an Abl tyrosine kinase inhibitor for chronic myelogenous leukemia. *J Clin Invest*. 105(1): 3-7.

16. Desininger, M. W. *et al*. (2003) Specific targeted therapy of chronic myelogenous leukemia with Imatinib. *Pharmacol Rev*. 55(3): 401-423.

17. Kantarijan, H. *et al*. (1988) Characteristics of accelerated disease in chronic myelogenous leukemia. *Cancer*. 61(7): 1441-1446.

18. Gambacorti-Passerini, C. *et al*. (2003) Molecular mechanisms of resistance to Imatinib in Philadelphia-chromosome-positive leukemias. *Lancet Oncol*. 4(2): 75-85.

19. Talpaz, M. *et al*. (2006) Dasatinib in Imatinib-resistant Philadelphia chromosome-positive leukemias. *N Engl J Med*. 354(24):2531-2541.

20. Faderi, S. *et al*. (1999) Chronic myelogenous leukemia: biology and therapy. *Annals Int Med*. 131(3): 207-219.

21. Rudoplh, C. *et al*. (2005) Cytogenetic characterization of a BCR-Abl transduced mouse cell line. *Cancer Gen Cytogen*. 161(1):51-56.

22. Jacobsen, E.A. *et al*. (2006) Growth, differentiation, and malignant transformation of pre-B cells mediated by inducible activation of v-Abl oncogene. *J Immunol*. 176(11):6831-6838.

23. Franco, D. *et al*. (2009) Accessibility of chromosomal recombination breaks in nuclei of wild-type and DNA-PKcs-deficient cells. *DNA Rep*. 8(7): 813-821.

24. Engelman, A. *et al*. (1987) Isolation of temperature-sensitive Abelson virus mutants by site-directed mutagenesis. *Proc Natl Acad Sci*. 84: 8021-8025.

25. Unnikrishnan, I. *et al*. (2003) Absence of p53 complements defects in Abelson Murine Leukemia Virus signaling. *J Virol*. 77(11): 6208-6215.

26. Zimmerman, R. S. *et al*. (2008) Changes in p19ARF localization accompany apoptotic crisis during Pre-B-cell transformation by Abelson Murine Leukemia virus. *J Virol*. 82(17): 8383-8391.

27. Komatsu, M. *et al*. (2010) Selective autophagy regulates various cellular functions. *Genes to Cells*. 15: 923-933.

28. Andrew, R. J. *et al*. (2010) Autophagy mediates the mitotic senescence transition. *Genes Dev*. 23: 798-803.

29. Yogalingam, G. *et al*. (2008) Abl kinases regulate autophagy by promoting the trafficking and function of lysosomal components. *J Biol Chem*. 283(51): 35941-35953.

30. Maiuri, M. C. *et al*. (2007) Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nature Rev*. 8: 741-752.
31. Kundu, M. *et al*. (2005) Macroautophagy versus mitochondrial autophagy: a question of fate? *Cell Death Diff*. 12: 1484-1489.
32. Ioannis, M. *et al*. (2010) Regulation of the Autophagic machinery in human neutrophils. *Eur J Immunol*. 40: 1461-1472.
33. Ichimura, Y. *et al*. (2008) Structural basis for sorting mechanism of p62 in selective autophagy. *J Biol Chem*. 283(33):22847-22857.
34. Kabeya, Y. *et al*. (2000) LC3 , a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J*. 19(21): 5720-5728.
35. Noda, N. *et al*. (2008) Structural basis of target recognition by Atg8/LC3 during selective autophagy. *Genes to Cells*. 13: 1211-1218.
36. Rzymski, T. *et al*. (2010) Regulation of autophagy by ATF4 in response to severe hypoxia. *Oncogene*: 1-12.
37. Barth, S. *et al*. (2010) Autophagy: assays and artifacts. *J Pathol*. 221:117-124.
38. Tanida, I. *et al*. (2008) LC3 and autophagy. *Methods in Molecular Biology*. 445(4): 77-89.
39. Tanida, I. *et al*. (2005) Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. *Autophagy*. 1(2): 84-91.
40. Kimura, S. *et al*. (2009) Monitoring autophagy in mammalian cultured cells through the dynamics of LC3. *Methods in Enzymol*. 452(1): 1-12.
41. White, E. *et al*. (2009) The double-edged sword of autophagy modulation in cancer. *Clin Can Res*. 15(17): 5308-5316.
42. Oh, M. *et al*. (2008) Inhibition if histone deacetylase 1 induces autophagy. *Biochem Biophys Res*. 369: 1179-1183.
43. Ko, K. *et al*. (2009) Autophagy inhibition enhances apoptosis induced by ginsenoside Rk1 in hepatocellular carcinoma cells. *Biosci Biotechnol Biochem*. 70(10): 2183-2189.
44. Amaravaldi, R. K. (2007) The roles of therapy-induced autophagy and necrosis in cancer treatment. *Clin Cancer Res*. 13(24): 7271-7279.
45. Pivtoraiko, V. N. *et al*. (2010) Low-dose Bafilomycin attenuates neuronal cell death associated with autophagy-lysosome pathway dysfunction. *J Neurochem*. 114: 1193-1204.
46. Mammucari, C. *et al*. (2008) Downstream of AKT: FoxO3 and mTOR in the regulation of autophagy in skeletal muscle. *Autophagy*. 4(4): 524-526.
47. Bellodi, C. *et al*. (2009) Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in Philadelphia chromosome-positive cells, including primary CML stem cells. *J CLIN INVEST*. 119(5): 1109-1123.

48. Kamitsuji, Y. *et al*. (2008) The Bcr-Abl kinase inhibitor INNO-406 induces autophagy and different modes of cell death execution in Bcr-Abl-positive leukemias. *Cell Death Diff.* 15(11): 1712-1722.
49. Mishima, Y. *et al*. (2008) Autophagy and autophagic cell death are the next targets for the elimination of the resistance to tyrosine kinase inhibitors. *Cancer Sci*. 99(11): 2200-2208.
50. Lindsley, J. *et al*. (2004) Nutrient sensing and metabolic decisions. *Comp. Biochem. And Physiol*. 139: 543-559.
51. Hawley, SA. *et al*. (1996) Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. *J. Biol. Chem*. 271: 27879-27887.
52. Davies, SP. *et al*. (1995) 5' –AMP inhibits dephosphorylation as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. *FEBS Lett*. 377: 421-425.
53. Adams, J. *et al*. (2004) Intrasteric control of AMPK via the gamma1 subunit AMP allosteric regulatory site. *Protein Sci*. 13: 155-156.
54. Harhaji-Trajovic, L. *et al.* (2009) AMPK-mediated autophagy inhibits apoptosis in cisplatin-treated tumour cells. *J. Cell. Mol. Med.* 13: 3644-3654.
55. Efeyan, A. *et al*. (2009) mTOR and cancer: many loops in one pathway. *Curr Op Cell Biol*. 22: 18.
56. D'Antona, G. *et al*. (2010) Branched-chain amino acid supplementation promotes survival and supports cardiac and skeletal muscle mitochondrial biogenesis in middle-aged mice. *Cell Metabol*. 12: 362-372.
57. Alexander, A. *et al*. (2010) ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. *PNAS*. 107(9): 4153-4158.
58. Balaburski, G. M. *et al*. (2010) p53 and ARF: unexpected players in autophagy. *Trends Cell Biol*. 20: 363-369.
59. Wilkinson, S. *et al*. (2010) Autophagy: an adaptable modifier of tumorigenesis. *Curr Op Gen & Dev*. 20: 57-64.
60. Pimkina, J. *et al*. (2009) ARF, autophagy and tumor suppression. *Autophagy*. 5(3): 397-399.
61. Bellodi, C. *et al*. (2009) Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in Philadelphia chromosome-positive cells, including primary CML stem cells. *J Clin Cancer Res*. 119(5): 1109-1123.
62. Feng, Z. *et al*. (2005) The coordinate regulation of the p53 and mTOR pathways in cells. *Proc Natl Acad Sci*. 102: 8204-8209.

63. Tasdemir, E. *et al*. (2008) Regulation of autophagy by cytoplasmic p53. *Nat Cell Biol*. 10: 676-687.
64. Levine, B. *et al*. (2008) p53: the Janus of autophagy? *Nat Cell Biol*. 10: 637-639.
65. Morselli, E. *et al*. (2008) Mutant p53 protein localizes in the cytoplasm and inhibits autophagy. *Cell Cycle*. 7(19): 3056-3061.
66. Lindsley, J. E. *et al*. (2004) Nutrient sensing and metabolic decisions. *Comp Biochem Physiol*. 139(B): 543-559.
67. Wong, K. *et al*. (2010) Targeting the PI3K signaling pathway in cancer. *Curr Op Gen & Dev*. 20: 87-90.
68. Shamji, A. F. *et al*. (2000) Partitioning the transcriptional program induced by Rapamycin among the effectors of the Tor proteins. *Curr Biol*. 10: 1574-1581.
69. Shamji, A. F. *et al*. (2003) Integration of growth factor and nutrient signaling: implications for cancer biology. *Mol Cell*. 12: 271-280.
70. Bozulic, L. *et al*. (2009) PIKKing on PKB: regulation of PKB activity by phosphorylation. *Curr Op Cell Biol*. 21: 256-261.
71. Abedin, MJ. *et al.* (2007) Autophagy delays apoptotic death in breast cancer cells following DNA damage. *Cell Death Differ*. 14: 500-510.
72. Carew, JS. *et al.* (2007) Targeting autophagy augments the anticancer activity of the histone deacetylase inhibitor SAHA to overcome Bcr-Abl-mediated drug resistance. *Blood*. 110: 312-322.
73. Paglin, S. *et al*. (2001) A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res*. 61: 439-444.
74. Codogno, P. *et al*. (2005) Autophagy and signaling: their role in cell survival and cell death. *Cell Death Differ*. 2: 1509-1518.
75. Itahana, K. *et al*. (2008) ARF in the mitochondria: the last frontier? *Cell Cycle*. 7(23): 3641-3646.
76. Reef, S. *et al*. (2006) A short mitochondrial form of p19ARF induces autophagy and caspase-independent cell death. *Mol cell*. 22: 436-475.
77. Reef, S. *et al*. (2007) The autophagic inducer smARF interacts with and is stabilized by the mitochondrial p32 protein. *Oncogene*. 26: 6677-6683.
78. Yang, P. *et al*. (2010) Inhibition of autophagy enhances anticancer effects of Atorvastatin in digestion malignancies. *Cancer Res*. 70(19): 7699-7709.
79. Koopman, G. *et al*. (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood*. 84(5): 1415-1420.
80. Vermes, I. *et al*. (1995) A novel assay for apoptosis – flow cytometric detection of phosphatidylserine expression on early

apoptotic cells using fluorescein labeled Annexin V. *J Immunol Methods*. 184(1): 39-51.
81. Vucicevic, L. *et al*. (2009) AMPK-activated protein kinase-dependent and –independent mechanism underlying in vitro antiglioma action of compound C. *Biochem Pharmacol*. 77: 1684-1693.
82. van Schalkwyk, D. A. *et al*. (2010) Inhibition of plasmodium falciparum pH regulation by small molecule indole derivatives results in rapid parasite death. *Biochem Pharmacol*. 79(9): 1291-1299.
83. Klionsky, D. J. *et al*. (2008) Does Bafilomycin A1 block the fusion of autophagosomes with lysosomes? *Autophagy*. 4(7): 849-950.
84. Boulares, A. H. *et al*. (1999) Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis: caspase-3 resistant PARP mutant increases rates of apoptosis in transfected cells. *J Biol Chem*. 274(33): 22932-22940.
85. Fulda, S. (2010) Evasion of apoptosis as a cellular stress response in cancer. *Int J Cell Biol.* 2010: 1-6.
86. Bratton, S. B. *et al*. (2002) XIAP inhibition of caspase-3 preserves its association with the Apaf-1 apoptosome and prevents CD95-and Bax-induced apoptosis. *Cell Death Diff*. 9: 881-892.
87. Arismendi-Morillo, G. (2009)Electron microscopy morphology of the mitochondrial network in human cancer. *Int J Biochem & Cell Biol.* 41: 2062-2068.
88. Xu, R. *et al*. (2005) Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. *Cancer Res*. 65: 613-621.
89. Williams, R. T. *et al*. (2008) The Ink4a-ARF (CDKN2A/B) locus in hematopoiesis and BCR-ABL-induced leukemia. *Cold Spring Harb Symp Quant Biol.* 73: 461-467.
90. Signer, R. A. J. *et al*. (2008) Aging and cancer resistance in lymphoid progenitors are linked processes conferred by p16Ink4a and ARF. *Genes Dev*. 22(22): 3115-3120.
91. Mullighan, C. G. *et al*. (2008) Failure of CDKN2A/B (INK4a/B ARF)-mediated tumor suppression and resistance to targeted therapy in acute lymphoblastic leukemia. *Genes Dev*. 22(11): 1411-1415.
92. Signer, R. A. J. *et al*. (2010) Immature B-cell progenitors survive oncogenic stress and efficiently initiate Ph+ B-acute lymphoblastic leukemia. *Blood*. 116: 2522-2530.
93. Kharas, M. G. *et al*. (2005) ABL oncogenes and phosphoinositide 3-kinase: mechanism of activation and downstream effectors. *Cancer Res*. 65: 2047-2053.

94. Steelman, L. S. *et al*. (2004) JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. *Leukemia*. 18: 189-218.
95. Brown, B. I. *et al*. (2008) Novel molecular and cellular therapeutic targets in acute lymphoblastic leukemia and lymphoproliferative disease. *Immunol Res*. 42: 84-105.
96. Sallmyr, A. *et al*. (2008) Genomic instability in myeloid malignancies: increased reactive oxygen species (ROS), DNA double strand breaks (DSBs) and error-prone repair. *Caner Lett*. 270: 1-9.
97. McCubrey, J. A. (2008) Targeting survival cascades induced by activation of Ras/Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/SAT pathways for effective leukemia therapy. *Leukemia*. 22: 708-722.
98. Sherr, C. J. *et al*. (2006) Divorcing ARF and p52: an unsettled case. *Nat Rev.* 6: 663-673.
99. Unnikrishnan, I. *et al*. (2003) Absence of p53 complements defects in Abelson Murine Leukemia Virus signaling. *J Virol*. 77(11): 6208-6215.
100. Zimmerman, R. S. *et al*. (2008) Changes in p19ARF localization accompany apoptotic crisis during Pre-B-cell transformation by Abelson Murine Leukemia virus. *J Virol*. 82(17): 8383-8391.
101. Xia, M. *et al*. (2011) p21 does not protect cancer cells from apoptosis induced by nongenotoxic p53 activation. *Oncogene*. 30: 346-355.