ABL allosteric inhibitors synergize with statins to enhance apoptosis of metastatic lung cancer cells

Graphical abstract

Highlights

- ABL kinase allosteric inhibitors induce mitochondrial-mediated apoptosis
- HMG-CoA reductase inactivation synergizes with ABL allosteric inhibitors to induce apoptosis
- Isoprenoid pathway rescues cell survival upon cotreatment with statin and ABL001
- Cotreatment of ABL001 and simvastatin decreases metastases in mouse models

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In brief

Metabolic reprogramming in tumors is an adaptation that generates vulnerabilities that can be exploited for developing new therapies. Here Luttman et al. identify synergism between ABL allosteric inhibitors and lipophilic statins to impair metastatic lung cancer cell outgrowth and colonization, leading to increased survival in mouse models of advanced disease.
ABL allosteric inhibitors synergize with statins to enhance apoptosis of metastatic lung cancer cells

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SUMMARY

Targeting mitochondrial metabolism has emerged as a treatment option for cancer patients. The ABL tyrosine kinases promote metastasis, and enhanced ABL signaling is associated with a poor prognosis in lung adenocarcinoma patients. Here we show that ABL kinase allosteric inhibitors impair mitochondrial integrity and decrease oxidative phosphorylation. To identify metabolic vulnerabilities that enhance this phenotype, we utilized a CRISPR/Cas9 loss-of-function screen and identified HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway and target of statin therapies, as a top-scoring sensitizer to ABL inhibition. Combination treatment with ABL allosteric inhibitors and statins decreases metastatic lung cancer cell survival in vitro in a synergistic manner. Notably, combination therapy in mouse models of lung cancer brain metastasis and therapy resistance impairs metastatic colonization with a concomitant increase in animal survival. Thus, metabolic combination therapy might be effective to decrease metastatic outgrowth, leading to increased survival for lung cancer patients with advanced disease.

INTRODUCTION

Metabolic reprogramming is a hallmark of cancer and contributes to tumor development (Pavlova and Thompson, 2016). Oncogenic activation can increase the expression and activity of metabolic enzymes and transporters to meet the bioenergetic and biosynthetic needs of the cancer cell, thus creating metabolic vulnerabilities that might be exploited for emerging cancer therapies (DeBerardinis and Chandel, 2016; Dong et al., 2020; Jin et al., 2019; Pupo et al., 2019; Tarrado-Castellarnau et al., 2016). Among these dependencies is mitochondrial metabolism, which generates energy, regulates redox homeostasis, and provides key metabolites for macromolecule synthesis (Zong et al., 2016). While results from clinical trials evaluating the anticancer capability of drugs targeting mitochondrial metabolic pathways have shown potential benefits, the utility of these drugs is limited by the expression of transporters that facilitate import of these drugs into cancer cells, or toxicity associated with targeting mitochondrial metabolism not only in tumor cells but also in noncancerous tissue (Vasan et al., 2020).

Members of the Abelson (ABL) family of nonreceptor tyrosine kinases, ABL1 and ABL2, are activated downstream of diverse stimuli, including oncogenic drivers such as epidermal growth factor receptor (EGFR), human epidermal growth factor 2 (HER2), and Kirsten rat sarcoma virus (KRAS), and promote progression and metastasis of solid tumor types, including lung and breast cancer (Gu et al., 2016, 2020; Hoj et al., 2019; Plattner et al., 1999; Khatri et al., 2019; Wang et al., 2016). ABL kinases promote cancer cell growth, survival, adhesion, and migration depending on the cellular context (Greuber et al., 2013; Wang and Pendergast, 2015). Recently, a role for ABL kinases in the regulation of mitochondria function was shown in HER2 amplified breast cancer cells, as HER2 promoted ABL-mediated tyrosine phosphorylation of mitochondrial creatine kinase 1 (MtCK1), leading to increased cellular energy production through the mitochondrial phosphocreatine shuttle (Kurmi et al., 2018). These findings suggested that inhibition of ABL signaling may uncover additional metabolic vulnerabilities in tumor cells.

Lung cancer is the leading cause of mortality among cancers worldwide in part due to the lack of actionable targets and transient responses to current therapies (Herbst et al., 2018). Here, we show that ABL kinases regulate mitochondrial function and integrity in lung adenocarcinoma cells harboring EGFR and KRAS mutations, and we also show that inactivation of ABL kinases impairs oxidative mitochondrial metabolism. As ABL inhibition impairs mitochondrial oxidation, we sought to
determine whether targeting metabolic pathways could enhance sensitivity to ABL allosteric inhibitors by performing a CRISPR/Cas9 loss-of-function screen targeting 2,322 metabolic enzymes and transporters. This screen identified 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), a rate-limiting enzyme of the mevalonate (MVA) pathway and target of statin therapy, as a top-scoring sensitizer capable of potentiating cell death in the presence of sublethal doses of ABL allosteric inhibitors. Notably, we found that combination therapy of ABL kinase allosteric inhibitors with lipophilic statins impaired growth of clinically relevant therapy-resistant and brain-metastatic lung cancer cells in in vitro and in vivo mouse models. These results suggest new treatment avenues for lung cancer patients with advanced disease.

RESULTS

ABL kinase allosteric inhibitors regulate mitochondria function in lung cancer cells

We investigated whether inhibition of the ABL kinases could perturb mitochondrial function in lung adenocarcinoma cells with oncogenic mutations in EGFR, either sensitive to EGFR tyrosine kinase inhibitors (TKIs) (PC9) or TKI resistant (PC9 GR4), as well as KRAS mutant large cell lung carcinoma (LCC) H460 cells and KRAS mutant lung adenocarcinoma H358 cells. Lung cancer cells were analyzed for mitochondrial basal respiration, maximal respiration, and ATP production following treatment with ABL kinase inhibitors (Figures 1A–1D). For these studies, we used ABL allosteric inhibitors GNF5 and ABL001 (asciminib), which bind with high affinity to the unique myristate-binding pocket of the ABL kinase domain (Hoj et al., 2020). Moreover, treatment with ABL ATP-competitive inhibitor nilotinib (Deng et al., 2010; Zhang et al., 2010). Surprisingly, only the ABL allosteric inhibitors, but not nilotinib, markedly impaired mitochondrial basal respiration, maximal respiration, and ATP production at all time points evaluated (Figures 1A–1D and S1A–S1E). The inability of nilotinib to inhibit mitochondria function might be due to the lack of specificity of ABL ATP-competitive inhibitors, as these drugs inhibit multiple enzymes other than ABL in solid tumors, and/or the inability of nilotinib to disrupt interactions with specific ABL downstream targets, which we showed can be blocked by ABL allosteric inhibitors through binding to a distinct site in the ABL kinase domain (Hoj et al., 2020). Moreover, treatment with ABL ATP-competitive inhibitors, but not allosteric inhibitors, induces activation of the RAF-ERK pathway in diverse cancer cell types (Gu et al., 2016; Packer et al., 2011; Wang et al., 2016). Notably, genetic knockdown of ABL1 and ABL2 (shAA) demonstrated that depletion of the ABL kinases similarly decreased mitochondrial respiration and ATP production in EGFR and KRAS mutant lung cancer cells (Figures 1E and S2A). Thus, depletion of ABL kinases phenocopied the ABL allosteric inhibitors by decreasing mitochondria function in lung cancer cells.

Next we evaluated whether aberrant mitochondria function induced by treatment with ABL allosteric inhibitors was also observed following treatment with two current US Food and Drug Administration (FDA)-approved therapeutics for lung adenocarcinoma patients: gefitinib, an EGFR TKI, and docetaxel, a taxane chemotherapy (Reck and Rabe, 2017). Lung cancer cells were treated with half-maximal inhibitory concentration (IC50) drug doses to promote cell death as determined by dose-response assays (Table S1). Strikingly, mitochondrial function, as measured by basal and maximal respiration and ATP production, was greatly decreased following treatment with either GNF5 or ABL001 in lung cancer cells harboring EGFR or KRAS mutations (Figures 1A–1D) but was not impaired upon treatment with gefitinib or docetaxel in EGFR mutant PC9 parental cells and gefitinib-resistant PC9 GR4 cells (Figures 1A, 1B, S1D, and S1E). Additionally, treatment with docetaxel in KRAS mutant H460 and H358 lung cancer cells did not significantly impair mitochondria function (Figures 1C and 1D). Notably, while the ABL001 and GNF5 allosteric inhibitors markedly decreased mitochondrial function as measured by decreased mitochondrial respiration and impaired ATP production at all of the time points examined, treatment with gefitinib, nilotinib, and docetaxel failed to elicit these changes (Figures 1A–1D, S1D, and S1E). Next, we examined changes in basal and compensatory glycolysis in lung cancer cells following drug treatment. In contrast to nilotinib, gefitinib, and docetaxel which decreased basal and compensatory glycolysis in PC9 and PC9 GR4 cells, the ABL allosteric inhibitors did not inhibit glycolytic capacity in these cells (Figures S1F and S1G). Thus, ABL allosteric inhibitors impair mitochondria function.

To dissect the mechanism by which mitochondria function is impaired by ABL allosteric inhibitors, mitochondrial superoxide release was examined to identify changes in organelle integrity (Perillo et al., 2020; Zorov et al., 2014). We observed that mitochondrial reactive oxygen species (MitoROS) levels were increased upon GNF5 or ABL001 treatment, but not following gefitinib or docetaxel treatment, in EGFR mutant lung cancer cells sensitive or resistant to gefitinib therapy (Figures 1F and 1G). MitoROS levels were also increased in KRAS mutant H460 cells upon ABL allosteric inhibitor treatment, but not by docetaxel (Figure S2B). Thus, ABL allosteric inhibitors impair organelle integrity in lung cancer cells irrespective of the oncogenic driver (Figures 1F, 1G, and S2B). MitoROS levels were also increased following ABL1 and ABL2 knockdown in PC9 and H460 cells (Figures S2C and S2D). Analysis of mitochondria numbers following knockdown or pharmacologic inhibition of the ABL kinases did not show detectable changes in mitochondria numbers in PC9 and H460 cells, indicating that the decrease in the oxygen consumption rate (OCR) is not due to changes in mitochondrial density but rather mitochondria function (Figures S2E–S2H). Examination of changes in mitochondrial morphology by immunofluorescence staining with MitoTracker revealed that ABL001 caused a marginal, nonsignificant increase in mitochondrial network morphology, while the other drugs did not affect mitochondria length and width (Figures S2I and S2J).

Metabolically focused CRISPR/Cas9 loss-of-function screen identifies HMGCR inhibition with statin therapy as an apoptotic sensitizer to ABL allosteric inhibitors in lung cancer cells

Because inhibition of ABL kinases impairs oxidative mitochondrial metabolism, we sought to determine whether targeting additional metabolic nodes enhanced sensitivity to ABL...
inhibition. Thus, we employed a CRISPR/Cas9 loss-of-function screen targeting 2,322 metabolic enzymes and transporters (Lin et al., 2019) in the absence and presence of sublethal doses of the ABL allosteric inhibitor GNF5 that corresponded to a 20% loss in cell viability following a 3-day dose-response assay (Figure 2A). Library-transduced cells were puromycin selected and grown for 10 days prior to treatment. The cells were then exposed to either vehicle or GNF5 for 2 weeks, after which

Figure 1. ABL kinase allosteric inhibitors regulate mitochondria function in lung cancer cells

(A–D) Seahorse XF Analyzer Mito Stress Test analysis of mitochondrial basal respiration, maximal respiration, and ATP production as measured by changes in oxygen consumption rate (OCR). Cells were treated with IC50 doses of each drug (see Table S1) for 24 h (n = 3). Data are the mean ± SEM.

(E and G) Mitochondrial ROS (MitoROS) release as measured by FACS analysis of median fluorescent intensity (MFI) using MitoSOX probe. Cells were treated with IC50 doses for 24 h (n = 3). Data are the mean ± SEM. Statistical analysis was performed using one-way ANOVA and Tukey post hoc testing. *p < 0.05; **p < 0.01; ***p < 0.001. See also Figures S1, Figure S2, and Table S1.
DNA was extracted from cell samples and polymerase chain reaction (PCR) was used to amplify and index barcode chain reaction RNA (sgRNA) amplicons, and the composition of sgRNA pools was deconvoluted through deep sequencing. The screen was validated for known essential genes by comparing the final and initial sgRNA pools in the vehicle-treated screen as previously described (Lin et al., 2019). Depletion metrics for each sgRNA were determined by normalizing the relative abundance of each construct following GNF5 treatment to the construct quantity present in vehicle-treated cells. The three most depleted constructs per gene were averaged to produce a gene-level three score (TS) as previously detailed (Lin et al., 2019) (Table S2). TS scores were ranked, allowing for identification of genes that were specifically depleted or enriched in the GNF5-treated cell population (Figure 2B). We focused on the subset of depleted genes that fell below the inflection point of the curve to experimentally evaluate whether loss of the top 5% of deleted genes could potentiate the cell-killing effects of ABL allosteric inhibition (Table S2). Among these hits were complexes of the electron transport chain as well as metabolic enzymes and transporters that converged on metabolic nodes that regulate cholesterol synthesis and mobilization. We focused on targets that could be pharmacologically inhibited with FDA-approved drugs, and we identified HMGCR, the rate-limiting enzyme of the MVA pathway, as a top-scoring reactive sensitizer to cell death in the presence of low-dose GNF5 (Figures 2B and 2C). We selected HMGCR for further study because it was in the top 1% of depleted genes and is the target of statin therapies commonly prescribed for patients with high cholesterol (Karr, 2017). Statins have a highly tolerable pharmacokinetic profile and availability, making HMGCR an attractive target for combination therapy.

To validate the results of the screen, PC9 cells were treated with sublethal doses of GNF5 and two statins, simvastatin and fluvastatin. Following 72 h of combination treatment, over 90% of cells underwent cell death (Figure 2D). Similar results were seen when cells were cultured in colony formation assays (Figures 2E, 2F, S3A, and S3B). To validate the on-target effect of statins, shRNAs against HMGCR were transduced into PC9 lung cancer cells, and these cells were then treated with GNF5 (Figures S3C and S3D). HMGCR was partially knocked down, allowing for the cells to remain viable albeit with reduced pathway activity. HMGCR knockdown exhibited markedly decreased cell survival compared to control lung cancer cells following treatment with GNF5 (Figures S3C and S3D), in agreement with the pharmacologic data. Further, immunoblotting revealed dramatic induction of the apoptotic mediators cleaved poly (ADP-ribose) polymerase (PARP) and cleaved caspase-3 following 24 h of combination treatment with GNF5 and statins, which was consistent with identification of apoptotic cells by flow cytometry of Annexin V-stained lung cancer cells cotreated with GNF5 and statins (Figures 2G and 2H). These data reveal a treatment paradigm whereby HMGCR inhibition combined with ABL allosteric inhibition sensitizes cells toward apoptotic cell death.

ABL allosteric inhibitors preferentially synergize with statins to induce lung cancer cell death

To assess whether ABL allosteric inhibitors might preferentially synergize with statins in comparison to EGFR inhibitors and chemotherapeutic drugs, lung cancer cells were treated at equivalent sublethal doses below the IC50 value of each drug as determined with dose-response assays for each cell line. Notably, only the ABL allosteric inhibitors exhibited enhanced cell-killing effects upon combination with either simvastatin or fluvastatin in EGFR mutant cells sensitive to EGFR TKIs (PC9), resistant to gefitinib (PC9 GR4), or harboring metastatic tropism to the brain (PC9 BrM3) (Figures 3A–3C). Similar findings were observed in KRAS mutant H460 and H358 cancer cell lines (Figures 3D and 3E). The combination of ABL allosteric inhibitors with statins was found to be synergistic across cell lines as assessed by the Bliss formula for synergy (Demidenko and Miller, 2019), where a score of 1 indicates true synergy (Figure 3F).

Next, we sought to evaluate whether sensitization to statin treatment was specific to the ABL allosteric inhibitors or could also be induced by ABL ATP-site inhibitors. Cotreatment of PC9 GR4 and H460 cells with sublethal doses of nilotinib and either simvastatin or fluvastatin did not promote additive or synergistic decreases in cell viability (Figures S3E and S3F). These findings highlight the differential effects induced by ABL allosteric versus ATP-competitive inhibitors, and they support the notion that ABL allosteric inhibitors preferentially sensitize lung...
cancer cells to cell death in vitro by combination treatment with lipophilic statins.

There are conflicting in vitro reports indicating additive cell-killing effects of chemotherapy with high-dose statin therapeutics (Chou et al., 2019; Otahal et al., 2020). Thus, we treated lung cancer cells with IC50 doses of GNF5, ABL001, gefitinib, and docetaxel to determine whether treatment at higher than sublethal doses could enhance the cell-killing effects of these drugs. Again, we found that only ABL allosteric inhibitors could synergize with simvastatin and promote cleavage of PARP and caspase-3 in PC9 cells (Figures S4A–S4C). In this regard, clinical trial data have shown that statins in combination with various chemotherapies had either marginal or no effect on progression-free survival or overall survival in lung cancer patients (Han et al., 2011; Lee et al., 2017; Seckl et al., 2017). Together, these results show that in contrast to gefitinib and docetaxel, ABL allosteric inhibitors preferentially sensitize lung cancer cells to statins and dramatically inhibit cancer cell survival.

**MVA, but not cholesterol, rescues cell survival in lung cancer cells cotreated with statins and ABL allosteric inhibitors**

The MVA pathway catalyzes the conversion of acetyl-CoA to HMG-CoA, which is then converted by HMGCR into MVA (Figure 2C). MVA is required for the generation of cholesterol and isoprenoids among other end products. Rescue experiments were performed to identify whether MVA or the downstream metabolite cholesterol could reverse the cell-killing effect induced by low-dose simvastatin treatment in cells cotreated with sublethal doses of ABL allosteric inhibitors. Interestingly, only MVA, but not cholesterol, could rescue cell survival in PC9 GR4 and H460 lung cancer cells (Figures 4A and 4C). Consistent with these findings, the addition of MVA, but not cholesterol, prevented cleavage of PARP and caspase-3, suggesting that the sensitization of lung cancer cells to apoptosis induced by ABL allosteric inhibitors in combination with statin therapy is independent of cholesterol (Figures 4B and 4D).

The apoptotic cascade is mediated by interplay among BCL-2 family proteins composed of proapoptotic and antiapoptotic proteins (Warren et al., 2019). Following combination treatment with ABL allosteric inhibitors and statins, we observed that gene expression of the prosurvival factors BCL-2 and BCL-XL was downregulated, while expression of proapoptotic p53 upregulated modulator of apoptosis (PUMA) was increased (Figure 4E). Supplementation with MVA, but not cholesterol, restored gene expression back to baseline (Figure 4E). Changes in BCL-2 family member expression can elicit pore formation in the mitochondria, resulting in mitochondrial outer membrane permeabilization.
(MOMP), leading to cytochrome c release (Garrido et al., 2006). Subcellular fractionation revealed release of cytochrome c from the mitochondrial membrane fraction into the cytosol in response to cotreatment with ABL001 and simvastatin, which was reversed following the addition of MVA (Figure 4F). These data show that ABL001 and simvastatin combination therapy alters gene expression of BCL-2 family members, leading to permeabilization of the mitochondria, release of cytochrome c into the cytosol, and cleavage of caspase 3, and that these processes can be reversed by the addition of MVA.

**Apoptotic sensitization to statin therapy by ABL allosteric inhibitors requires inhibition of protein prenylation**

MVA is the precursor to farnesyl diphosphate (FPP), which can either be converted to geranylgeranyl diphosphate (GGPP) or cyclized to produce squalene for cholesterol production (Thurnher et al., 2012). Both FPP and GGPP are metabolites in the isoprenoid pathway required for protein prenylation, a posttranslational enzymatic modification that adds a prenylated motif to CAAX proteins such as the RAP1A GTPase (Konstantinopoulos and...
Papavassiliou, 2007). These modifications regulate protein localization to different cellular compartments, facilitate specific protein-protein interactions, and modulate protein stability. Since the downstream sterol metabolite cholesterol did not rescue cell survival, we investigated whether metabolites in the isoprenoid pathway were critical for sensitization to statin therapeutics. We found that the addition of GGPP preferentially rescued cell viability compared to FPP in PC9 GR4, PC9, and H460 cells co-treated with ABL001 and simvastatin (Figures 5A, S5A, and S5B).

Immunoblotting was performed to assess whether protein prenylation was altered following simvastatin treatment (Figure 5B). Simvastatin treatment increased levels of unprenylated RAP1A indicating inhibition of the geranylgeranylation pathway and induced a mobility shift in HDJ-2 signifying inhibition of the farnesylation pathway, both of which were reversed by the addition of the indicated prenylation metabolites (Figure 5B) (Lobell et al., 2002; Munoz et al., 2017). We next tested whether inhibition of either geranylgeranyl transferase (GGT) or farnesyl...
transferase (FT) could impact cell survival in a manner similar to simvastatin treatment. Survival of PC9 GR4, PC9, and H460 cells cotreated with ABL001 and a GGT-1 inhibitor (GGTI-298) was significantly impaired, but cell survival was only slightly decreased following the addition of the FT inhibitor (FTI-277) in the presence of ABL001 (Figures 5C, S5C, and SSD). Further, the nonadditivity observed for statin treatment and GGT and FT inhibition suggested that the synergizing effects of statins or GGTI-298 + FTI-277 in the presence of an ABL allosteric inhibitor operate through the same pathway. Immunoblotting confirmed that each inhibitor specifically suppressed its target pathway (Figure 5D). Collectively, these data reveal that inhibition of protein geranylgeranylation is sufficient to sensitize cells to ABL allosteric inhibitors, leading to enhanced intrinsic apoptosis.

Since we observed that oxidative metabolism was impaired following treatment with IC50 doses of the ABL allosteric inhibitors (Figure 1) and that combination therapy induced MOMP (Figure 4), we next investigated whether combination treatment with an ABL allosteric inhibitor and a statin affected mitochondrial metabolism and whether these effects might be due to changes in the protein prenylation pathway. To this end, we examined changes in mitochondrial respiration in cells cotreated with low doses of ABL001 and simvastatin and found that basal and maximal respiration as well as ATP production were decreased (Figure 5E). Notably, the addition of MVA restored mitochondrial respiration back to baseline; while treatment with FTI-277 had minimal effect, treatment with GGTI-298 negated the MVA rescue and caused mitochondrial respiration levels to decrease to a similar degree as to those observed following ABL001 and simvastatin treatment (Figure 5E). These data suggest that inhibition of the MVA pathway in combination with ABL kinase inhibition promotes cell death by impairing mitochondrial function.

**Combination therapy of ABL001 and simvastatin impairs metastatic tumor growth and increases animal survival in mouse models of lung cancer brain metastasis and gefitinib resistance**

Despite recent clinical successes with next-generation EGFR TKIs such as osimertinib, relapses often occur among patients harboring EGFR mutant non-small cell lung cancer cell (NSCLC). Moreover, patients harboring KRAS driver mutations have few tractable therapeutic options available (Kelly et al., 2018; Yang et al., 2019). Further, the ability of anticancer drugs to efficiently penetrate the blood-brain barrier (BBB) and reach therapeutic doses for lung cancer patients harboring brain metastases is limited. Thus, we chose to evaluate whether statins could synergize with ABL inhibitors in vivo to treat cancer cells seeded at distal sites following intracardiac injection in clinically relevant mouse models of brain metastasis and therapy resistance. We employed ABL001 as it has been shown to cross the BBB in preclinical mouse models and is currently in clinical trials for therapy-resistant patients with BCR-ABL+ chronic myeloid leukemia (Hoj et al., 2019; Hughes et al., 2019). Importantly, administration of ABL001 by oral gavage is well tolerated and does not induce weight loss in mice (Zhang et al., 2010). Pharmacokinetic data have shown that lipophilic statins can cross the BBB more readily than hydrophilic statins (Wood et al., 2010). In this regard, studies testing the ability of radiolabeled simvastatin to cross the BBB identified simvastatin-derived radioactivity in the rat brain following oral administration (Merck, 2020). Thus, we employed clinically relevant low doses of simvastatin and treated mice with 10 mg/kg simvastatin once a day, which is equivalent to doses used in humans (Barter, 2018; Björkhem-Bergman et al., 2011; Hu et al., 2012).

To determine whether combination treatment could impair brain-metastatic outgrowth, we used brain-metastatic PC9-BrM3 cells derived through serial rounds of intracardiac injection in athymic nude mice (Hoj et al., 2019). Previous studies have shown that following injection into the arterial circulation, brain-metastatic lung cancer cells extravasate into the brain parenchyma by day 6 postinjection (Kienast et al., 2010; Valiente et al., 2018). Thus, we performed bioluminescent imaging (BLI) on day 6 postintracardiac injection to stratify mice into treatment groups and began drug treatments on day 7 (Figure 6A). Mice were divided into four treatment groups: vehicle, ABL001, simvastatin, or a combination of ABL001 and simvastatin. We found that overall survival was significantly increased in mice harboring PC9-BrM3 brain metastases following combination treatment in comparison to vehicle, ABL001, or simvastatin alone (Figure 6B). Similar results were observed in mice harboring gefitinib-resistant PC9 cells (PC9 GR4), as ABL001 and simvastatin combination therapy extended overall survival (Figure 6C). Quantification of brain flux at day 27 revealed decreased disease burden in mice treated with both ABL001 and simvastatin compared to the vehicle or single drug treatment groups (Figures 6D and 6E). Immunofluorescence analysis of brain metastases for proliferation of the cell death marker cleaved caspase-3 (Figures 6F–6H). Notably, the effect of combination drug treatment on subcutaneous xenograft tumor growth was minimal in comparison to the vehicle or single treatment groups for PC9 GR4 cells injected subcutaneously into the flank (Figures S6A and S6B). Quantification of vehicle- and drug-treated mice harboring orthotopic lung tumors following intrathoracic injections showed that the combination of ABL001 and simvastatin elicited a decrease in tumor burden, but this did not reach statistical significance (Figures S6C–S6E). These findings are consistent with previous reports showing that the inhibitory effects of ABL inactivation on primary tumor growth are cell context dependent and that genetic and pharmacologic inhibition of the ABL kinases predominantly decreased spontaneous metastasis (Gil-Henn et al., 2013; Meirson et al., 2018). Together, these data reveal that combination treatment with both ABL001 and simvastatin impairs metastatic outgrowth of lung cancer cells by enhancing tumor cell death.

**DISCUSSION**

Metabolic reprogramming in tumors is an adaptation that allows cancer cells to meet enhanced bioenergetic needs, but metabolic dysregulation also generates vulnerabilities in cancer cells that can be exploited for the development of treatment strategies. Among these vulnerabilities is mitochondrial oxidative metabolism, as cancer cells are reliant on functional...
mitochondria for malignant transformation and growth (Vasan et al., 2020). Here we show that ABL allosteric inhibitors markedly decrease mitochondria function in lung cancer cells without affecting glycolysis. A CRISPR/Cas9 loss-of-function screen targeting metabolic enzymes revealed that HMGCR inactivation synergizes with ABL allosteric inhibitors at sublethal doses to induce metastatic lung cancer cell apoptosis, thereby revealing dual inactivation of the MVA pathway and ABL kinases as a strategy to augment apoptotic cell death and enhance therapeutic efficacy. Patients with lung cancer have the highest leading cancer-related mortality worldwide in part due to the lack of durable responses to current therapies resulting in metastatic and therapy-resistant disease progression (Herbst et al., 2018).

By targeting unique metabolic vulnerabilities of metastatic lung cancer cells, we uncovered combination treatment strategies using available ABL allosteric inhibitors with FDA-approved statins for the treatment of therapy-resistant and brain-metastatic lung cancer cells in preclinical mouse models.

Figure 6. Combination therapy of ABL001 and simvastatin promotes tumor cell apoptosis and increases survival in mouse models of lung cancer brain metastasis and gefitinib resistance

(A) Schematic showing experimental overview. PC9 BrM3 or PC9 GR4 cells labeled with luciferase-tomato (pFULT) were intracardially injected into mice on day 0. IVIS imaging of metastatic burden was performed on day 6, followed by equivalent stratification of mice into treatment groups based on tumor flux. Oral gavage treatment of drugs began on day 7, followed by weekly IVIS imaging until the experimental end point. Statistical analysis of overall survival was calculated using log-rank (Mantel-Cox) test and interactions below an adjusted p < 0.017 were deemed significant, accounting for three pairwise comparisons.

(B) Overall survival of mice injected intracardially with PC9 BrM3-pFULT cells treated with DMSO (n = 16), ABL001 (n = 19), simvastatin (n = 17), and the combination of ABL001 and simvastatin (n = 19).

(C) Overall survival of mice injected intracardially with PC9 GR4-pFULT cells treated with DMSO (n = 9), ABL001 (n = 9), simvastatin (n = 9), and the combination (n = 9).

(D) Representative images (day 27 postinjection) of mice intracardially injected with PC9 BrM3-pFULT cells.

(E) Quantitative analysis (day 30 postinjection) of the whole body metastatic index in mice injected intracardially with PC9 BrM3 cells and treated with DMSO (n = 16), ABL001 (n = 19), simvastatin (n = 17), and the combination (n = 19). Data are the mean ± SEM. (F) Immunofluorescence staining of brain tumors from PC9 BrM3 mice on day 35 stained with Ki67 (green), cleaved caspase 3 (c-C3) (red), and Hoechst 33342 (nuclei) (blue). Scale bars, 100 μm.

(G and H) Quantification of percent positive proliferative (G) and apoptotic (H) nuclei in tumor sections. Statistical analysis was performed using one-way ANOVA with Dunnett post hoc testing (n = 3). Data are the mean ± SEM. *p < 0.05. See Figure S6.
cholesterol depletion induces glioblastoma cell death (Villa et al., 2016). In contrast, we found that the synergistic interaction between ABL allosteric inhibitors and statins appears to be mediated by inhibition of protein prenylation and is independent of decreased cholesterol. Specifically, metabolic rescue of the geranylgeranylation pathway, but not cholesterol, was capable of rescuing cell survival in lung cancer cells cotreated with ABL001 and statins to an extent equivalent to MVA. Protein geranylgeranylation is required for processes such as protein and vesicular trafficking and cell proliferation (Wang and Casey, 2016). Multiple geranylgeranylated proteins might be targeted by statins in ABL-depleted cells. A recent report showed that lipophilic statins prevent membrane association of Rab11b, a small GTPase that regulates endosomal recycling and decreases breast cancer brain metastasis in mice (Howe et al., 2020). Among numerous substrates of the geranylgeranylation pathway are RAS-related GTPases, including members of the RAS and RHO-RAC families (Wang and Casey, 2016). Future studies are needed to assess whether decreased protein geranylgeranylation of specific targets mediates the decrease in lung cancer cell survival in vitro and in mouse models of metastasis following combination therapy with ABL001 and statins.

Previous reports have identified the potential of statins to function as anticancer agents; however, clinical trials using various chemotherapies in combination with statins have had either marginal or no effect on distant metastasis-free survival or overall survival in lung cancer patients with advanced disease (Han et al., 2011; Lee et al., 2017; Seckl et al., 2017). Retrospective analyses of various lung cancer patient cohorts have reported mixed findings regarding the impact of statin therapeutics on cancer-related mortality for patients taking statins at the onset of chemotherapy treatment (Cardwell et al., 2015; Khurana et al., 2007; Kuoppala et al., 2008; Wang et al., 2013). Our findings are consistent with clinical reports showing that statins added to first-line, standard-of-care chemotherapy do not impact the growth of primary lung adenocarcinoma tumors (Lee et al., 2017; Seckl et al., 2017) and suggest the potential use of ABL allosteric inhibitors in combination with statins for the treatment of advanced metastatic disease.

Whereas inactivation of ABL kinases impairs breast and lung cancer metastasis in mouse models (Gu et al., 2016, 2020; Hoj et al., 2019; Wang et al., 2016), clinical trials to treat breast and lung cancer patients with ABL ATP-site inhibitors have been ineffective in part due to targeting of multiple kinases other than ABL, possibly leading to paradoxical activation of cell survival pathways (Bauman et al., 2012; Chew et al., 2008; Cristofanilli et al., 2008; Dy et al., 2005; Johnson et al., 2003). Moreover, upon inhibiting the ABL kinases, the ATP-competitive inhibitors cause the ABL kinase SH3-SH2 cassette to remain in an open conformation while the allosteric inhibitors induce a closed conformation (Skora et al., 2013). In the open conformation, the SH3-SH2 domains can bind to downstream targets, but these domains are not accessible upon binding to ABL allosteric inhibitors. Notably, our recent work revealed that ABL allosteric inhibitors, but not ABL ATP-competitive inhibitors, disrupt the interaction between ABL2 and the HSF1 transcription factor (Hoj et al., 2020). This finding suggests that some protein-protein interactions dependent on distinct ABL protein conformations might be specifically disrupted by the binding of allosteric inhibitors to a unique site in the ABL kinase domain. Consistent with these findings, our work shows that the ABL allosteric inhibitors, which bind to the myristoyl-binding pocket in the C-lobe of the ABL kinase domain and are highly selective inhibitors of the ABL kinases, are capable of impairing mitochondria function in a manner similar to genetic inhibition of the ABL kinases, whereas the ATP-competitive inhibitors fail to impair mitochondrial function (Zhang et al., 2010). Thus, our findings support the potential use of ABL allosteric site inhibitors in combination with statins as a treatment strategy for lung cancer patients with advanced metastatic disease, including those patients with difficult-to-treat brain metastases or EGFR TKI resistance.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Animal models
  - Cell lines and cell culture
- **METHOD DETAILS**
  - Pharmacologic inhibitor treatment studies
  - Seahorse measurements
  - MitoSOX staining
  - MitoTracker staining
  - Pooled CRISPR screen
  - Screen analysis
  - Cell viability assay
  - Colony formation assays
  - Annexin V staining
  - Immunoblotting procedures
  - Real-time quantitative PCR
  - Intracardiac injections
  - Subcutaneous flank xenografts
  - Orthotopic lung injection
  - Immunofluorescence and confocal microscopy
  - DNA plasmids
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION

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DECLARATION OF INTERESTS
A.M.P. is a consultant and advisory board member for The Pew Charitable Trusts. A.M.P. holds patent number U.S. 9,931,342 B2 related to this work. All other authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Cleaved PARP        | Cell Signaling | Cat# 5625; RRID: AB_10699459 |
| Total PARP          | Cell Signaling | Cat# 9532; RRID: AB_659884 |
| Cleaved Caspase-3   | Cell Signaling | Cat# 9661; RRID: AB_2341188 |
| Caspase-3           | Cell Signaling | Cat# 9668; RRID: AB_2069870 |
| Phospho-CrkL (Tyr207) | Cell Signaling | Cat# 3181; RRID: AB_331068 |
| Cytochrome C        | Cell Signaling | Cat# 11940; RRID: AB_2637071 |
| beta-Catenin        | Cell Signaling | Cat# 8480; RRID: AB_11127855 |
| beta-Tubulin (D2N5G) | Cell Signaling | Cat# 15115; RRID: AB_2798712 |
| Ki67                | Cell Signaling | Cat# 9449; RRID: AB_2797703 |
| CRKL (C-20)         | Santa Cruz | Cat# sc-319; RRID: AB_631320 |
| GAPDH (6C5)         | Santa Cruz | Cat# sc-32233; RRID: AB_627679 |
| RAP1                | Santa Cruz | Cat# sc-398755; RRID: AB_2884025 |
| HMGCR               | Thermo Fisher | Cat# MAS-12748; RRID: AB_10982482 |
| HDJ2                | Kerafest | Cat# EST203; RRID: AB_2732803 |
| Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Thermo Fisher | Cat# A-11001; RRID: AB_2534069 |
| Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 633 | Thermo Fisher | Cat# A-21070; RRID: AB_2535731 |
| Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 | Thermo Fisher | Cat# A-11077; RRID: AB_141874 |
| Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L) | Jackson ImmunoResearch | Cat# 115-035-003; RRID: AB_10015289 |
| Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L) | Jackson ImmunoResearch | Cat# 115-035-144; RRID: AB_2307391 |

| **Chemicals, Peptides, and Recombinant Proteins** | | |
| GNF-5 (Abl kinase small molecule inhibitor) | Duke Small Molecule Synthesis Facility | N/A |
| ABL001 (Ascinib) | Duke Small Molecule Synthesis Facility | N/A |
| Simvastatin | Cayman | Cat# 10010344; CAS: 79902-63-9 |
| Gefinitib | Sigma-Aldrich | Cat# SML1657; CAS: 184475-35-2 |
| Fluvasstatin | Sigma-Aldrich | Cat# SML0038; CAS: 93957-55-2 |
| Mevalonolactone | Sigma-Aldrich | Cat# M4667; CAS: 674-26-0 |
| Cholesterol | Sigma-Aldrich | Cat# C3045; CAS: 57-88-5 |
| Geranylgeranyl pyrophosphate | Sigma-Aldrich | Cat# G6025; CAS: 6699-20-3 |
| Farnesyl pyrophosphate | Sigma-Aldrich | Cat# F6892; CAS: 13058-04-3 |
| FTI-277 | Tocris | Cat# 2407; CAS: 1217447-06-7 |
| GGTI-298 | Tocris | Cat# 2430; CAS: 1217457-86-7 |
| Docetaxel | LC Laboratories | Cat# D-1000; CAS: 114977-28-5 |
| Rotenone | Sigma-Aldrich | Cat# R8875; CAS: 83-79-4 |
| Antimycin A | Sigma-Aldrich | Cat# A8674; CAS: 1397-94-0 |
| Oligomycin | Sigma-Aldrich | Cat# 75351; CAS: 579-13-5 |

(Continued on next page)
## Critical Commercial Assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Seahorse XF96 V3 PS Cell Culture Microplates | Agilent | Cat# 101085-004 |
| Seahorse XF Cell Mito Stress Test Kit | Agilent | Cat# 103015-100 |
| MitoSOX Red Mitochondrial Superoxide Indication | Thermo Fisher | Cat# M36008 |
| MitoTracker Red CMXRos | Thermo Fisher | Cat# M7512 |
| Cell Titer Glo | Promega | Cat# G7571 |
| PE Annexin V Apoptosis Detection Kit I | BD Biosciences | Cat# 559925 |
| Cell Fractionation Kit | Cell Signaling | Cat# 9038 |
| Hoescht 33342 Solution | Thermo Fisher | Cat# 62249 |

## Deposited Data

- **PC9 cells transduced with LOF CRISPR/Cas9 metabolism library treated with vehicle or GNF5 sequencing files**
  
  This paper BioProject: PRJNA679091

## Experimental Models: Cell Lines

| Experimental Models: Cell Lines | SOURCE | IDENTIFIER |
|--------------------------------|--------|------------|
| PC9 | Valiente et al. (2014) | N/A |
| PC9 GR4 | Cortot et al. (2013) | N/A |
| PC9 BrM3 | Hoj et al. (2019) | N/A |
| H460 | Vicent et al. (2008) | N/A |
| H358 | ATCC | Cat# CRL-S807, RRID:CVCL_1559 |

## Experimental Models: Organisms/Strains

| Experimental Models: Organisms/Strains | SOURCE | IDENTIFIER |
|---------------------------------------|--------|------------|
| Mouse outbred athymic nu/nu | Jackson Laboratory | 007850; RRID:IMSR_JAX:007850 |

## Oligonucleotides

See Table S3 for a list of all primers and oligonucleotides

## Recombinant DNA & Plasmids

| Recombinant DNA & Plasmids | SOURCE | IDENTIFIER |
|----------------------------|--------|------------|
| pLKO-puro Non-Target shRNA Control | Sigma Mission TRC1 | SHC016-1EA |
| pLKO-puro shHMGCR 46448 | Sigma Mission TRC1 | TRCN00000046448 |
| pLKO-puro shHMGCR 46452 | Sigma Mission TRC1 | TRCN00000046452 |
| Scramble control shRNA | Gu et al. (2016) | N/A |
| ABL1 shRNA | Gu et al. (2016) | N/A |
| ABL2 shRNA | Gu et al. (2016) | N/A |

## Software and Algorithms

| Software and Algorithms | SOURCE | IDENTIFIER |
|-------------------------|--------|------------|
| PC9 cells transduced with LOF CRISPR/Cas9 metabolism library treated with vehicle or GNF5 code repository | This paper | https://doi.org/10.5281/zenodo.5530434 |
| Prism 6 and 8 | Graphpad | https://graphpad.com/scientific-software/prism |
| ImageJ | (Schneider et al., 2012) | https://imagej.nih.gov |
| RStudio | R Foundation for Statistical Computing | https://rstudio.com |
| Living Image | Perkin Elmer | https://perkinelmer.com |
| Knitr | Foundation for Open Access Statistics | https://yihui.org/knitr/ |
| bcSeq | Bioconductor | https://bioconductor.org/packages/release/bioc/html/bcSeq.html |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the corresponding author and Lead Contact, Ann Marie Pendergast (ann.pendergast@duke.edu).

Materials availability
All unique and stable reagents generated in this study are available from the Lead Contact upon completion of a Materials Transfer Agreement.

Data and code availability
- CRISPR dataset has been deposited at BioProject and is publicly available as of the date of publication. Accession number is listed in the key resources table.
- All original code has been deposited at Zenodo and is publicly available as of the date of publication. DOI is listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal models
All procedures involving mice were approved and performed following the guidelines of the IACUC of Duke University Division of Laboratory Animal Resources. All studies employed 8-12-week old age-matched female outbred athymic nu/nu mice (#007850; RRID: IMSR_JAX:007850) purchased from The Jackson Laboratory. The mice were maintained under pathogen-free conditions in the Duke Cancer Center Isolation Facility for immune-deficient mice.

Cell lines and cell culture
PC9 parental cells were a gift from Dr. Joan Massagué (Memorial Sloan Kettering Cancer Center, New York, NY, USA) (Valiente et al., 2014). PC9-GR4 (gefitinib-resistant) cells were a gift from Dr. Passi Jänne (Dana-Farber Cancer Institute, Boston, MA, USA) (Cortot et al., 2013). Large cell carcinoma (LCC) H460 cells were provided by Dr. Fernando Lecanda (University of Navarra, Pamplona, Spain) (Vicent et al., 2008). PC9-BrM3 cell lines were derived in the Pendergast laboratory by serial intracardiac injection as previously described (Hoj et al., 2019). Human H358 lung cancer cells were purchased from ATCC. Parental and derivative cell line pairs were subjected to short tandem repeat (STR) profiling through the Duke University DNA Analysis Facility Human cell line authentication (CLA) service to confirm their authenticity. Lung cancer cells were maintained in RPMI 1640 (Life Technologies) supplemented with 10% tetracycline-screened fetal bovine serum (FBS, Hyclone), 10 mM HEPES, 1 mM sodium pyruvate, and 0.2% glucose. H293T cells used for transfection and virus production were purchased from ATCC and were maintained in DMEM (Life Technologies) with 10% FBS (Corning). All cultures were maintained at 37°C in humidified air containing 5% CO2.

METHOD DETAILS

Pharmacologic inhibitor treatment studies
For experiments assessing effects of pharmacologic inhibitors in vitro (GNF-5, ABL001, Gefitinib, Docetaxel, Simvastatin, Fluvastatin, FTI-277, GGTI-298), drugs were dissolved in DMSO and the final concentration of DMSO in culture media did not exceed 0.1% v/v. Cholesterol was solubilized in 40% (2-hydroxypropyl)-β-cyclodextrin at room temperature, sterile filtered (0.45 μM) and stored at −20°C. MVA was resolved with 0.1M NaOH, followed by neutralizing with 0.1M HCL/1M HEPES. The ABL allosteric inhibitors GNF-5 and ABL001 were synthesized by the Duke University Small Molecule Synthesis Facility and validated by LC-MS and 1H-NMR, as well as cell-based assays. The following drugs used for in vitro analysis were purchased from: Cayman: Simvastatin (10010344); Sigma: Gefitinib (SML1657), Fluvarstatin (SML0038), Mevalonolactone (M4667), Cholesterol (C3045), Geranylgeranyl pyrophosphate (G6025), Farnesyl pyrophosphate (F6892); Tocris: FTI-277 (2407) and GGTI-298 (2430); LC Laboratories: Docetaxel (D-1000).

Seahorse measurements
Basal and maximal oxygen consumption rate and ATP production were measured using a Mito Stress test Kit and a Glycolytic Rate Kit was used to measure basal and compensatory glycolysis. Both kits used a XF96 Extracellular Flux Analyzer (Seahorse Bioscience) to measure changes according to manufacturer’s instructions. Cells were plated in XF96 plates at 10,000 cells per well on Day 0. Cells were treated on Day 1 with IC50 doses of GNF5, ABL001, gefitinib, docetaxel, and vehicle control. For rescue experiments, cells were treated with 2.5 μM ABL001, 1 μM simvastatin, 500 μM MVA, 5 μM GGTI-298, 12.5 μM FTI-277. On the day of the Seahorse run, media was aspirated and replaced with XF Assay Medium (Seahorse Bioscience) supplemented with 10 mM glucose, 1 mM...
pyruvate, and 2 mM glutamine supplemented with corresponding drug or vehicle. The plate was incubated in a non-CO2 incubator at 37°C for 1 hr to equilibrate. OCR measurements, taken every 6 min, were collected at baseline and after the sequential addition of oligomycin 1 μM (final concentration), FCCP 0.5 μM, and rotenone 0.75 μM + antimycin A 1.5 μM. Data were normalized to total nuclei count per well.

**MitoSOX staining**

MitoSOX was purchased from Thermofisher (cat. M36008). 100,000 cells were plated in six-well plates and treated with vehicle or IC50 doses of indicated drugs for 24 hr. Cells were stained with 5 μM MitoSOX resuspended in serum-free RPMI containing associated drug concentration in the dark for 10 mins in a 37°C 5% CO2 incubator. Cells were washed once with PBS and trypsinized followed by another wash in PBS and resuspended in 500 μL of PBS. The samples were analyzed using flow cytometer BD FACS Canto II. Gating strategy was defined using untreated/unstained cells. Analysis of flow cytometry data was performed with FlowJo v10.

**MitoTracker staining**

MitoTracker Red CMXRos was purchased from Thermofisher (cat. M7512). 100,000 cells were plated in six-well plates and treated with vehicle or IC50 doses of GNF5. Cells were stained with 100 nM MitoTracker resuspended in serum-free RPMI containing associated drug concentration in the dark for 30 mins in a 37°C 5% CO2 incubator. Cells were washed once with PBS and trypsinized followed by another wash in PBS and resuspended in 500 μL of PBS. The samples were analyzed using flow cytometer BD FACS Canto II. Gating strategy was defined using untreated/unstained cells. Analysis of flow cytometry data was performed with FlowJo v10.

**Pooled CRISPR screen**

PC9 cells were seeded into 12 six-well plates at 0.25e6 cells/well. A separate plate was also prepared for no puromycin and puromycin controls of non-transduced cells. Cells were transduced at a MOI of 0.2. 24 hours after viral transduction, cells were replated into puromycin-containing media. A sample was collected after 48 hours of puromycin exposure to confirm library coverage in the transduced population. Transduced cells were expanded in puromycin for a total of 10 days prior to drug introduction, at which point the transduced cell population was split into vehicle (DMSO) and GNF5 treatment conditions and maintained for up to two weeks. Cells were treated with 2 μM GNF5 which corresponded to 20%-30% loss in cell viability following a 3-day dose-response assay. Cells were counted and replated every 2 days with drug being replenished every day. At any given point during the screen, each replicate was represented by a minimum of 12E6 cells, sufficient to provide 1000x coverage of the library (~1000 cells per unique sgRNA). Samples of 25E6 cells were collected upon screen initiation, termination, and at weekly intervals. Following completion of the screens, DNA was extracted (DNeasy Blood & Tissue Kit, QIAGEN) and prepared for sequencing as previously described (Shalem et al., 2014).

**Screen analysis**

Deep sequencing was performed on an Illumina Nextseq platform (75 bp, paired-ended) to identify differences in library composition. All sequencing was performed by the Duke University genome sequencing facility. Barcoded reads were mapped to the guide RNA library using bcSeq (Lin et al., 2018) to obtain the counts for each guide RNA. Determinations of genetic essentiality and drug sensitization/resistance were made by evaluating differential guide compositions between the initial population and subsequent drug-treated and vehicle-treated cell populations. Briefly, the fractional representation (FR) for the guide reads within a sample was normalized to the total reads attributed to that sample. A direct comparison between two samples was represented by the quotient of the respective FRs in the log2 scale, which we term the depletion metric (DM). The guide-level DMs for each gene were then collapsed to gene-level scores by taking the average of the top three most depleted constructs resulting in a biased analysis focused on depleted genes. Genes represented by fewer than 5 guides per condition were excluded from analysis. In the 2,322-gene library, 7 genes (representing 0.3% of the total library) were excluded. Genetic essentiality was calculated by considering the depletion/enrichment of the vehicle-treated (DMSO) population over time (DMSO final / DMSO initial). Drug sensitization/resistance was calculated by considering the depletion/enrichment of the drug-treated population relative to the vehicle-treated population (Drug final / DMSO final). All depletion/enrichment effects are reported as log2 ratios. All analyses were conducted using the R statistical environment (https://www.r-project.org/) along with extension packages from the comprehensive R archive network (CRAN; https://cran.r-project.org/) and the Bioconductor project (Gentlemen et al., 2004). The analyses were carried out with adherence to the principles of reproducible analysis using the knitr package (Xie, 2016) for generation of dynamic reports and gitlab for source code management. The code for replicating the statistical analysis was made accessible through a public source code repository: https://doi.org/10.5281/zenodo.5530434. Because many metabolic genes are known to be essential to cellular viability (Hart et al., 2015), determining the effect of cell-essential genetic loss on apoptosis is difficult. To this point, a subset of essential metabolic genes will have lost representation in our screen before the 10-day puromycin selection period is over; our screen does not capture the effect of these genes (which represent a trivial fraction of our library) on apoptosis.
The remaining cell-essential genes are captured by the screen. Since our analysis normalizes the effect of gene knockout + drug treatment to gene knockout alone, the interpretation of these genes does not require additional correction, except that they necessarily suffer from reduced resolution.

**Cell viability assay**

Cells were seeded in white-walled clear bottom 96-well plates in triplicate at 3,000 cells per well. Each condition was run in triplicate wells each from three independent experiments and measured using CellTiter-Glo reagent (Promega). Plates were read on a Tecan Infinite M1000 Microplate Reader and results were analyzed in GraphPad.

**Colonies formation assays**

Cells were seeded in 12-well plates in duplicate at 1,000 cells per well for PC9 and PC9 GR4 cell lines and at 250 cells per well for H460 cells (n = 2). Cells were treated with 1 µM ABL001, 100 nM Simvastatin, or combination of ABL001 and Simvastatin for 1 week. Cells were then fixed with methanol and stained with crystal violet. Images were taken using an Epson scanner. 1 mL of 10% acetic acid was added per well and a plate reader was used to measure absorbance at 590 nm.

**Annexin V staining**

Annexin V staining was performed to determine the percentage of cells undergoing apoptosis. 100,000 cells were plated in six-well plates and treated with vehicle, 10 µM GNFS, 1 µM Simvastatin, 0.5 µM Fluvastatin or the combination for 24 hr. Upon collection, cells were trypsinized, washed twice with PBS, resuspended in 100 µL 1X Annexin V binding buffer (BD Biosciences) containing 5 µL Annexin V stain conjugated to APC (allophycocyanin) (BD Biosciences). Phosphatidylserine externalization was measured using APC-conjugated Annexin. Following a 15 min incubation at RT, the samples were analyzed using flow cytometer BD FACSCanto II. Gating strategy was defined using untreated/unstained cells. Analysis of flow cytometry data was performed with FlowJo v10.

**Immunoblotting procedures**

Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS) containing protease-phosphatase inhibitor cocktail (Cell Signaling). Cell suspensions were rotated at 4°C for 15 minutes followed by microcentrifugation to remove cell debris, and protein concentration was quantified using the DC Protein Assay (Bio-Rad). Equal amounts of protein were separated by SDS/PAGE and transferred onto nitrocellulose membranes using the Trans-blot Turbo Transfer system (Bio-Rad). Membranes were incubated with primary antibody overnight at 4°C, followed by 3 washes in 1×TBST and incubation with corresponding secondary antibody for 1 hr at room temperature. Blots were developed using SuperSignal West PLUS Chemiluminescent Substrate developing solution (Invitrogen) and imaged using either film or a ChemiDoc XR5+ imager (Bio-Rad). The following antibodies used for immunoblot analysis were purchased from: Cell Signaling: Phospho-CrkL (Tyr207) (3181L), beta-Tubulin (D21515) (151115S), cleaved PARP (5625), total PARP (9542), cleaved caspase 3 (9661), total caspase 3 (9668), cytochrome C (11940), beta-Catenin (8480); Thermostifer: HDJ2 (MAS-12748); Millipore Sigma: ABL1 (8E9) (MAB1130), ABL2 (6DS) (H00000275-M03); Santa Cruz: RAP1 (sc-398755), CRKL (C-20) (sc-319), GAPDH (6C5) (sc-32233); Jackson ImmunoResearch: Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L) (115-035-003), Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L) (115-035-144).

**Real-time quantitative PCR**

RNA was isolated from subconfluent monolayers of cancer cells using the RNeasy RNA isolation kit (QIAGEN), and cDNA synthesis was performed using oligo(dT) primers and M-MLV reverse transcriptase (Invitrogen). RT-qPCR was performed in triplicate wells using iTaq Universal SYBR Green Supermix (Bio-Rad). Primers used in this study were purchased from Sigma Aldrich. Analysis of real-time data was collected using a Bio-Rad CFX384 machine and CFX Maestro software. Expression levels of each gene were normalized to GAPDH control housekeeping genes using the ddCT algorithm. Primers sequences used: BBC3 Fwd (GACCTCAAGCAGCATGACGA), BBC3 Rev (AGAGTCCCATGATGAGTTGT), Bcl2 Fwd (GGTGGGGTCATGTGTGTGG), Bcl2 Rev (CGGTTCAGGCTGACAGTGT), Bcl2 Fwd (GGTGTTCTGATCTGGTGTTG), Bcl2 Rev (GGTGGGTATGCAATGACTGG), Bcl2 Fwd (GAGCCTGGTGGTGGTTGTTTCT), Bcl2 Rev (TCCATCTCGGATGTCGCT). Primer pairs for the genes of interest were purchased from Sigma Aldrich.

**Intracardiac injections**

All animal experiments were conducted in accordance with protocols approved by the Duke University Division of Laboratory Animal Resources Institutional Animal Care and Use Committee (IACUC). Cells were stably transduced with pFU-luciferase-Tomato (pFuLT) DNA prior to injection to allow for bioluminescent imaging (BLI) in vivo. We used 8-12-week old age-matched female athymic nu/nu mice for all studies (Jackson Laboratory). Mice were anesthetized with 5% isoflurane prior to injections. For all studies, 4x10^5 lung cancer cells suspended in 100 µL PBS were injected into the left cardiac ventricle with a 30-gauge needle. Animals were monitored until full recovery from anesthesia and were subsequently imaged weekly to both confirm proper anatomical injection and to monitor for progression of disease burden using an IVIS XR bioluminescent imager. The ABL allostERIC inhibitor ABL001 (Asciminib) was used for in vivo inhibition of the ABL kinases in tumor-bearing mice and was prepared as a suspension in sterile 0.5% methylcellulose/0.5% Tween-80 as described previously.
Mice were treated with either vehicle control or 100 mg/kg/qd ABL001 via oral gavage once per day. ABL001 was synthesized by the Duke University Small Molecule Synthesis Facility and validated by LC-MS and 1H-NMR. Simvastatin was purchased from Toronto Research Chemicals (cat. S485000) and dissolved in aqueous 2% dimethyl sulfoxide (DMSO), 30% polyethylene glycol 400 (PEG 400), and 5% Tween 80. Mice were treated with either vehicle control or 10 mg/kg/qd simvastatin. To account for potential interactions between the two drugs and solvents, mice were treated each morning with either simvastatin or vehicle control, and two hours later with either ABL001 or vehicle control via oral gavage. The presence of brain metastases was confirmed through in vivo BLI followed by isolation of brains for OCT or paraffin sectioning. Living Image software was used for analysis of BLI data.

Subcutaneous flank xenografts
Mice were injected subcutaneously with 1e6 PC9 GR4 cells re-suspended in growth factor reduced Matrigel matrix in the back right flank. Mice were stratified into treatment groups once the tumors reached an average of 50 mm³. The animals were drug treated for a total of 3 weeks and tumor volume was measured weekly by a caliper. At the end of the study, mice were euthanized, and tumors were extracted for image collection.

Orthotopic lung injection
PC9 pFuLT lung cancer cells were used for orthotopic lung injection into athymic nude mice. Mice were anesthetized with 4% isoflurane and placed in the left lateral decubitus position. PC9 pFuLT cells were re-suspended in PBS solution, and 50 μL of the cell suspension was injected percutaneously into the right lateral thorax at the lateral dorsal axillary line. The needle was quickly inserted approximately 6 mm into the thorax and quickly removed. Mice were turned to the right lateral decubitus position following tumor cell injection. Respiratory rate was monitored throughout the entire procedure, and mice were observed for 30 min until fully recovered. Body weights and wound healing was monitored weekly. Injected mice were treated with either vehicle (0.5% methylcellulose, 0.5% Tween-80), ABL001, Simvastatin, or combination of ABL001 and Simvastatin starting on day 6 post-injection for 36 days. Mice were monitored weekly for tumor growth via bioluminescent imaging (BLI) using the IVIS XR imaging system.

Immunofluorescence and confocal microscopy
Brains were perfused and fixed with 4% paraformaldehyde in PBS prior to extraction. Upon extraction, brains were rotated overnight (O/N) in 4% paraformaldehyde in PBS at 4°C followed by subsequent washes in PBS the following day. For OCT embedding, brains underwent surose protection in 15% surose in water at 4°C O/N rotation following by O/N rotation in 30% surose in water at 4°C before OCT embedding at –80°C. OCT sections were 10 μm thick. For paraffin embedding, brains were placed into 70% ethanol prior to paraffin embedding. Paraffin embedding was performed at the Duke University Immunohistopathology Core Facility sections were cut at 5 μm thick. OCT sections were thawed at room temperature for 15 minutes followed by acetone fixation. Paraffin sections were deparaffinized, rehydrated, and heat inactivated (BioCare Medical Decloaking Chamber). Both deparaffinized and OCT sections were then washed in PBS and blocked in 3% goat serum in PBS with 0.05% Tween-20 for one hour. Sections were incubated with primary antibodies in blocking solution overnight at 4°C in a humidified chamber at concentrations indicated below. Sections were then washed with PBS followed by incubation with the appropriate secondary antibody in blocking solution for one hour at room temperature. Sections were then washed with PBS, incubated with the nuclear stain, Hoechst33342, and washed again with PBS before mounting using aqueous mounting media (Dako-S3025). Antibodies for immunofluorescence and IHC experiments included: cleaved caspase 3 (Cell Signaling 9661) at 1:100 dilution, Ki67 (Cell Signaling 9449) at 1:200 dilution, tdTomato (Kerafast EST203) at 1:100 dilution. All images were captured on an Axio Imager D10 (Carl Zeiss) with a 20 x /0.75 EC Plan-Neofluar objective lens. Mitochondrial morphology analysis was performed by plating 75,000 H460 cells on glass coverslips in 12-well plates. Cells were treated with 100 nM MitoTracker Red CMXRos (Thermofisher cat. M7512) for 30 min, fixed (formaldehyde), permeabilized (Triton-X), stained with Hoechst 33342 and mounted. The slides were imaged using a Zeiss 880 inverted confocal Airyscan microscope with 63x oil objective. For all representative images in the manuscript, experiments were conducted at least twice, and had no repeatability issues. Mitochondrial morphology was determined using the tubeness and vesselness algorithms in Fiji. Mitochondrial fragmentation versus connectivity was determined by plotting length x width of several thousand mitochondrial from at least 10 cells across at least two independent experiments.

DNA plasmids
Sequences for shRNAs targeting the ABL kinases were as follows: scrambled shRNA (GGGTGTATGGGCTACTATAGAA); ABL1 shRNA (GGGTATGAGCTCGTACAGA); ABL2 shRNA (CTTATCTACCACACTGTA). Stable non-inducible shRNAs against non-target control (NTC) and HMGCR in the pLKO.1 vector were from the Sigma Mission TRC1 Lentiviral shRNA library and were obtained through the Duke Functional Genomics Shared Resource Facility. Sequences and Sigma clone identifiers for each of these shRNAs were as follows: HMGCR shRNA 46448 (CCGGGCAGTGAAGAGGAGCATTTTCGAGAAATGCGTCCTTTATCACACTGCTTTTG) and HMGCR shRNA 46452 (CCGGCCCTGCTCTATAATGGATATCAGGTATCAATTATAGCAGCAGGTTTTG).
QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism 7 and GraphPad Prism 9 software. Mouse numbers per group were determined through statistical power calculations where 10 mice per group allows for 90% power, at the unadjusted 0.05 two-sided level, to detect inter-group differences of 50% and assuming intra-group differences of 25%. For Kaplan-Meier survival curves, p values were calculated using log-rank (Mantel-Cox) testing. P values below an adjusted p < 0.017 were deemed significant accounting for 3 pairwise comparisons. Statistical analysis of tumor flux was evaluated by ANOVA followed by Fisher post hoc testing to calculate p values and those less than 0.05 were quantified as statistically significant. For comparisons between mouse groups of unequal size, the mean value and SEM were used to allow for statistical analysis by ANOVA. Bar graph data represent averages ± SEM.
Supplemental information

ABL allosteric inhibitors synergize with statins to enhance apoptosis of metastatic lung cancer cells

Jillian Hattaway Luttman, Jacob P. Hoj, Kevin H. Lin, Jiaxing Lin, Jing Jin Gu, Clay Rouse, Amanda G. Nichols, Nancie J. MacIver, Kris C. Wood, and Ann Marie Pendergast
Figure S1. ABL allosteric inhibitors preferentially impair mitochondria function in lung cancer cells. Related to Figure 1.

(A-E) Seahorse XF Analyzer Mito Stress Test analysis of mitochondrial basal respiration, maximal respiration, and ATP production as measured by changes in oxygen consumption rate (OCR) in the indicated lung cancer cells. Cells were treated with IC_{50} drug doses for each drug in cell line. Cells were treated for either 24 hr (A-C), 48 (D), or 72 hours (E) (n=3). Data are mean ± SEM.

(F-G) Seahorse XF Analyzer Glycolytic Rate analysis of basal and compensatory glycolysis in PC9 and PC9 GR4 cells. Cells were treated with IC_{50} doses for 24 hours (n=3). Data are mean ± SEM. Statistical analysis was performed using one-way ANOVA and Tukey post hoc testing *p<0.05, **p<0.01.
Figure S2. ABL inhibition impacts mitochondria function in lung cancer cells without altering mitochondria number. Related to Figure 1.

(A) Mitochondrial basal respiration, maximal respiration, and ATP production as measured by changes in oxygen consumption rate (OCR) from Seahorse XF Analyzer Mito Stress Test analysis for H460 cells harboring shRNAs against ABL1/ABL2 (shAA) and non-targeting control (shSCR) (n=3). Data are mean ± SEM.

(B) FACS analysis of mitochondrial ROS (MitoROS) using mitosox probe in H460 cells treated with IC50 doses for each drug for 24 hours (n=3). Data are mean ± SEM.

(C-D) MitoROS in H460 AA knockdown cells (C) or PC9 AA knockdown cells (D). Statistical analysis was calculated using unpaired two-tailed t test. *p<0.05

(E-F) Mitotracker staining to analyze mitochondria number in H460 cells with 15 μM GNF5 (E) or PC9 cells treated with 10 μM GNF5 (F) (n=3). Data are mean ± SEM.

(G-H) Mitotracker staining of H460 AA knockdown cells (G) or PC9 AA knockdown cells (H) (n=3). Data are mean ± SEM.

(I) Representative immunofluorescence images of H460 cells stained with Mitotracker and Hoescht 33342 following treatment with IC50 dose for each drug for 24 hours; scale bar 20 μm.

(J) Image quantification for Fig. S2I showing mitochondrial length × width plotted for thousands of mitochondria from >10 cells across at least two independent experiments. Changes were not statistically significant.
Figure S3. HMGCR inactivation sensitizes lung cancer cells to decreased colony formation and reduced cell viability in the presence of ABL allosteric inhibitor but not ATP-competitive inhibitor. Related to Figure 2.

(A) Representative images from colony formation assays of PC9 GR4 and H460 cells treated with ABL001 (1 μM), simvastatin (100 nM), and combination of ABL001 and simvastatin for 1 week.

(B) Quantification of absorbance reading for colony formation assays from Fig. S3A (n=2). Data are mean ± SEM.

(C) PC9 cells harboring two distinct shRNAs (#46448 and #46452) against HMGCR or non-targeted control (NTC) were treated with GNF5 for 72hr to assess cell viability. (n=2). Data are mean ± SEM.

(D) Immunoblotting for HMGCR and β-Tubulin shows knockdown of HMGCR protein with both shRNAs #46448 and #46452.

(E-F) Cell viability assays were used to examine sensitization of statin therapeutics to the ABL ATP-site inhibitor Nilotinib after 72 hr of co-treatment in the indicated lung cancer cells. Sublethal doses of Nilotinib (PC9 GR4, 5 μM; H460, 7.5 μM), simvastatin (PC9 GR4, 1 μM; H460 3 μM), and fluvastatin (PC9 GR4, 0.5 μM; H460 2 μM), were used and cell viability assays were performed after 72 hr with indicated drugs (n=3). Data are mean ± SEM. Statistical analysis was performed using one-way ANOVA and Tukey post hoc testing. *p<0.05, ** p<0.01
Figure S4. Combination treatment of subtherapeutic doses of statins with IC_{50} value dose for ABL allosteric inhibitors, Gefitinib, or Docetaxel, demonstrate preferential synergy with ABL allosteric inhibitors for decreased cell viability and induction of apoptotic markers. Related to Figure 3.

(A-B) Cell viability assays to evaluate sensitization of statin therapeutics to ABL allosteric inhibitors versus FDA-approved therapies at IC_{50} doses using 72 hr growth inhibition assays. Simvastatin was used at subtherapeutic doses (1 \( \mu \)M for PC9, PC9 GR4) (n=3). Data are mean ± SEM. Statistical analysis was performed using one-way ANOVA and Tukey post hoc testing. ** p<0.01, ***p<0.005, ****p<0.001

(C) Corresponding western blot analysis of cleaved PARP, total PARP, cleaved caspase 3, total caspase, and \( \beta \)-Tubulin in PC9 cells treated with IC_{50} doses of ABL001, Gefitinib, and Docetaxel combined with subtherapeutic doses of simvastatin (1 \( \mu \)M).
Figure S5. Inhibition of protein prenylation sensitizes lung cancer cells to ABL allosteric inhibitor. Related to Figure 5.

(A) Cell viability of PC9 cells treated with the combination of 5 μM ABL001 and 1 μM simvastatin supplemented with 500 μM MVA, FPP, or GGPP for 72 hours (n=3). Data are mean ± SEM.

(B) Cell viability of H460 cells treated with the combination of 7.5 μM ABL001 and 3 μM simvastatin supplemented with 500 μM MVA, FPP, or GGPP for 72 hours (n=3). Data are mean ± SEM.

(C) Cell viability of PC9 GR4 cells treated 5 μM ABL001 and 1 μM simvastatin, 500 μM MVA, 5 μM GGTI-298, 12.5 μM FTI-277 for 72 hr (n=3). Data are mean ± SEM.

(D) Cell viability of H460 cells treated with the combination of 7.5 μM ABL001, 3 μM simvastatin, 500 μM MVA, 3 μM GGTI-298, 3 μM FTI-277 for 72 hr (n=3). Data are mean ± SEM.

Statistical analysis was performed using one-way ANOVA and Tukey post hoc testing. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure S6. Combination therapy of ABL001 and simvastatin does not significantly impair growth of lung tumor xenografts implanted subcutaneously or orthotopically in the lung. Related to Figure 6.

(A) Mice were subcutaneously injected with PC9 GR4 cells and stratified into four treatment groups once tumors reached 50 mm³. Mice were treated for a total of 3 weeks and tumor volume was measured weekly. n=7 mice per group. Statistical analysis performed via one-way ANOVA. Quantification of tumors was normalized to the average flux on Day 1 of treatment for all tumors within each treatment arm; ns= not significant.

(B) Image of final tumors harvested from mice showing no appreciable change in tumor size of ABL001 + simvastatin combination treatment in comparison to vehicle or single treatment groups.

C) Timecourse quantification of PC9 tumor growth in athymic nude mice injected orthotopically via lung intrathoracic injection and treated with drugs as indicated. Dosing began 6 days post-injection and randomization of mice into treatment groups, followed by weekly bioluminescent imaging. Treatment continued for 36 days; n=9 mice per group. Statistical analysis performed via one-way ANOVA. Quantification of tumors was normalized to the average Day 0 flux for all tumors within each treatment arm; ns= not significant.

D) Quantification of tumor burden as measured via bioluminescent imaging on Day 42 post-intrathoracic injection of PC9 pFuLT-expressing tumor cells as in S6C. n=9 mice per group. Statistical analysis performed via one-way ANOVA. Tumor flux was normalized to the average Day 0 flux for all tumors within each treatment arm.

E) Representative images of mice injected orthotopically with PC9 pFuLT cells quantified in Figures S6C-D.
Table S1. IC<sub>50</sub> calculations for cell lines. Related to Figures 1-5.

| Cell Line | Drug      | Calculated IC<sub>50</sub> |
|-----------|-----------|-----------------------------|
| PC9       | GNF5      | 10 µM                       |
|           | ABL001    | 7.5 µM                      |
|           | Gefitinib | 15 nM                       |
|           | Docetaxel | 1 nM                        |
|           | Nilotinib | 2 µM                        |
|           | Simvastatin | 4 µM                    |
|           | Fluvastatin | 2 µM                    |
|           | FTI-277   | 50 µM                       |
|           | GGTI-298  | 20 µM                       |
| PC9 GR4   | GNF5      | 10 µM                       |
|           | ABL001    | 7.5 µM                      |
|           | Gefitinib | 200 nM                      |
|           | Docetaxel | 1 nM                        |
|           | Nilotinib | 2 µM                        |
|           | Simvastatin | 4 µM                    |
|           | Fluvastatin | 2 µM                    |
|           | FTI-277   | 50 µM                       |
|           | GGTI-298  | 20 µM                       |
| H460      | GNF5      | 15 µM                       |
|           | ABL001    | 15 µM                       |
|           | Docetaxel | 0.5 nM                      |
|           | Nilotinib | 2 µM                        |
|           | Simvastatin | 12 µM                   |
|           | Fluvastatin | 4 µM                    |
|           | FTI-277   | 15 µM                       |
|           | GGTI-298  | 15 µM                       |
| H358      | GNF5      | 15 µM                       |
|           | ABL001    | 15 µM                       |
|           | Docetaxel | 1 nM                        |
|           | Simvastatin | 12 µM                   |
|           | Fluvastatin | 4 µM                    |
| PC9 BrM3  | GNF5      | 10 µM                       |
|           | ABL001    | 7.5 µM                      |
|           | Gefitinib | 15 nM                       |
|           | Docetaxel | 1 nM                        |
|           | Simvastatin | 4 µM                    |
|           | Fluvastatin | 2 µM                    |
Table S3. List of primers and oligonucleotides. Related to STAR methods.

| Primer (Application) | Source | Oligo Sequence               |
|----------------------|--------|------------------------------|
| GAPDH-F (RT-qPCR)    | Sigma  | TGTGGGCATCAATGGATTTGG        |
| GAPDH-R (RT-qPCR)    | Sigma  | ACACCATGTATTCCGGGTTCAAT      |
| BBC3-F (RT-qPCR)     | Sigma  | GACCTCAACGCACAGTGACGAG       |
| BBC3-R (RT-qPCR)     | Sigma  | AGGAGTCCCATGATGAGTGT         |
| BCL2L1-F (RT-qPCR)   | Sigma  | GAGGTGTTGTTGACTTTTCTC        |
| BCL2L1-R (RT-qPCR)   | Sigma  | TCCATCTCGATTCAGTCCCT         |
| BCL2-F (RT-qPCR)     | Sigma  | GGTGGGCTATGTTGTGTTG          |
| BCL2-R (RT-qPCR)     | Sigma  | CGGTTCAGGTACTCAGTCATCC       |