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TITLE: Deficient BIM Expression as a Mechanism of Intrinsic and Acquired
Resistance to Targeted Therapies in EGFR-Mutant and ALK-Positive Lung Cancers

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14. ABSTRACT

This project has made very good progress. We have now unveiled an even larger role for defective apoptosis in the emergence of resistance. We have been very successful in our ability to generate cell lines derived from patient biopsies both before treatment and at the time of resistance. We are now using these patient-derived cell lines to assess BIM levels and apoptotic response to next-generation inhibitors. The capacity to develop cell lines from patient biopsies was published in December, 2014 in *Science*. We have used these models to develop effective combinations to overcome the defect in apoptosis which has led to a CTEP-sponsored clinical trial combining AZD9291 and ABT-263. Our research has expanded beyond just assessing BIM, and is also focusing on the role of a diminished apoptotic response as clones develop resistance to targeted therapies. Although BIM is one such mechanism, it is not the only one. This research has uncovered a novel, unexpected connection between EMT, low BIM, and resistance to targeted therapies. Two manuscripts (one under review at *Nature Communications* and one to be submitted) have been written this year describing our findings.

15. SUBJECT TERMS
BIM, apoptosis, targeted therapy, BCL-XL, kinase, EGFR, ALK, lung cancer

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1. INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

In lung cancers that have oncogene-addiction to a specific kinase, inhibition of that kinase often leads to cell growth arrest and apoptosis. For example, *EGFR* mutant and *EML4-ALK* lung cancers have been proven highly sensitive to the corresponding specific tyrosine kinase inhibitors (TKIs). Although cancers with these genetic abnormalities often respond to the appropriate targeted therapy, there is marked heterogeneity in degree of clinical benefit. We hypothesized that that some cancers are poised to undergo apoptosis following treatment, whereas others are not; and expression of the critical pro-apoptotic protein BIM in pre-treatment biopsies may distinguish patients who have impressive, durable responses from those who have weak, transient responses. We originally aimed to assess *EGFR* mutant and *EML4-ALK* lung cancer specimens to determine if low basal BIM expression predicts a poorer clinical outcome to TKIs.

Since the inception of this research program, our findings have led us to appreciate the fundamental importance of diminished apoptosis for the evolution of resistance to targeted therapies. We have now determined that *EGFR* mutant cancers will have different apoptotic responses to 2nd and 3rd line targeted therapies depending on "how" the cancer became resistant (please see Figure 1 below for a description of the different models for how cancers become resistant). These fundamental findings have been put together for publication and are now under review at *Nature Medicine* and are the basis for a new clinical trial that is about to begin (ABT-263 + AZD9291 in *EGFR* mutant lung cancers (clinicaltrials.gov NCT02520778)). We have aligned the aims of this proposal with this promising avenue of investigation.

We have studied this process in detail in *EGFR* mutant cancers that develop the T790M resistance mutation under the selective pressure of gefitinib. We now have 2 models for how cancers become resistant to targeted therapies:

1) Pre-existing model: In this model, cells with acquired resistance to EGFR inhibitors exist prior to treatment and are simply selected by treatment.

2) Persister-evolution model: In this model, cancer cells that initially survive therapy are selected by drug treatment. **These cells do not undergo apoptosis.** During weeks and months of therapy, these cells can develop mutations and become fully resistant. However, the tumors that evolve from these drug tolerant cells continue to have an apoptotic defect.
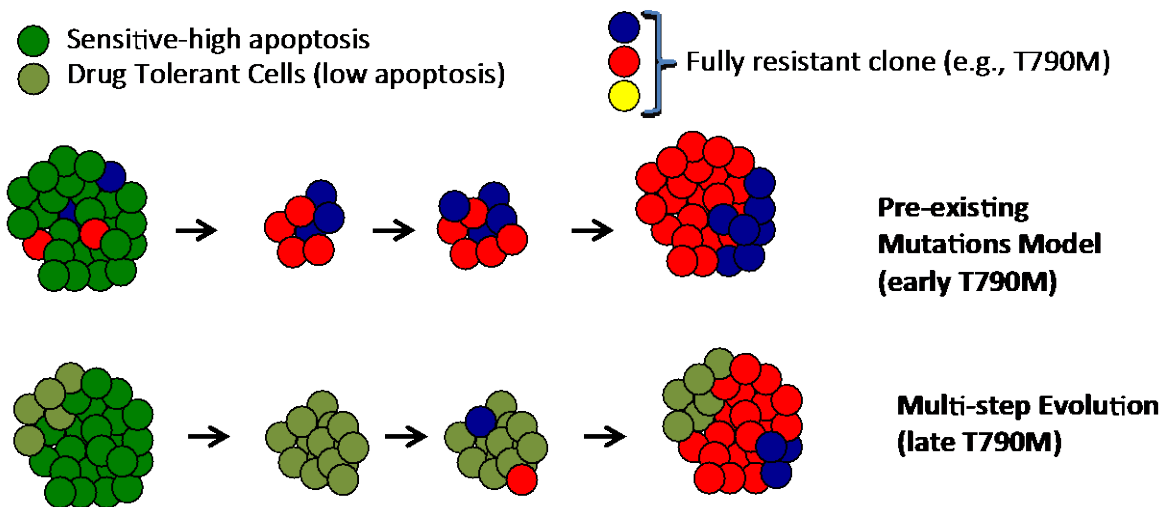
Figure 1. Two models of the development of acquired resistance to EGFR inhibitors

We continue to leverage our robust repeat biopsy program that routinely biopsies patients upon the development of resistance. This effort is led by my co-PI, Lecia Sequist. We are now able to cultivate cell cultures from these resistant cancers which allow us to not only to measure BIM levels, but also to functionally determine if these cancers have an impaired apoptotic response to next-generation inhibitors.

2.KEYWORDS: Provide a brief list of keywords (limit to 20 words).

BIM, apoptosis, targeted therapy, BCL-XL, kinase, EGFR, ALK, lung cancer

3.OVERALL PROJECT SUMMARY: This project has made very good progress. As noted above, we have now unveiled an even larger role for defective apoptosis in the emergence of resistance. We are now using patient-derived cell lines to assess BIM levels and apoptotic response to next-generation inhibitors. The capacity to develop cell lines from patient biopsies was published in December, 2014 in *Science*. We have used these models in Aim 3 to develop effective combinations to overcome the defect in apoptosis which has led to a CTEP-sponsored clinical trial combining AZD9291 and ABT-263.

Our research has expanded beyond just assessing BIM, and is also focusing on the role of a diminished apoptotic response as clones develop resistance to targeted therapies. Although BIM is one such mechanism, it is not the only one. We are now also trying to identify the other mechanisms as well.

Aim 1: Validate BIM as a biomarker that predicts outcome in patients treated with EGFR and ALK inhibitors.

Task 1:

Current objectives: obtain biopsies from 100 *EGFR* mutant lung cancer patients and 60 *ALK*-translocated lung cancer specimens prior to TKI treatment.

Summary of Results, Progress and Accomplishments: Please see the Progress Report by Lecia Sequist.

Subtasks.

1A. Current objectives: Perform BIM IHC and RNA ISH on approximately 100 pre-treatment *EGFR* mutant lung cancer specimens. These slides will be processed from paraffin-embedded tissue.

Summary of Results, Progress and Accomplishments: Please see the Progress Report by Lecia Sequist.

1B. Current objectives: Perform BIM IHC and RNA ISH on approximately 60 pre-treatment *ALK*-translocated lung cancer specimens. These slides will be processed from paraffin-embedded tissue.

Summary of Results, Progress and Accomplishments: Please see the Progress Report by Lecia Sequist.

1C. Current objectives: Prospectively analyze progression free survival (PFS) in relationship with BIM expression, as determined by IHC and RNA ISH.

Summary of Results, Progress and Accomplishments: As mentioned above, the publication of the EURTAC trial results has performed a prospective analysis on clinical outcome as it relates to BIM expression. We will not be performing a redundant analysis in *EGFR* mutant lung cancer samples. We will pursue efforts of performing a prospective analysis on *ALK* mutant lung cancers to determine if BIM expression is predictive of progression-free survival, response rate, and overall survival in this genetic subset. However, we now know that crizotinib is not a highly potent inhibitor. Therefore, this analysis will be performed on tumor samples from patients that go on to treatment with 2nd and 3rd line ALK inhibitors. We are now focusing on determining if cancers with acquired resistance to first-line targeted therapies will exhibit decreased apoptosis

Aim 2: Determine if BIM expression is lost in cancers that develop resistance to targeted therapies.

Task 2:

Current Objectives: Interrogate approximately 50 *EGFR* mutant lung cancers pre- and post-treated matched specimens that have acquired resistant to TKI and approximately 30 *ALK*-

translocated lung cancers pre- and post-treated matched specimens that have acquired resistance to TKI.

Summary of Results, Progress and Accomplishments: Please see the Progress Report by Lecia Sequist.

Subtasks.

2A. Current objectives: Make *EGFR* mutant cell lines resistant to the EGFR TKI gefitinib (cell-lines commercially available from ATCC or acquired from the Center for Molecular Therapeutics at Mass General Hospital Cancer Center).

Summary of Results, Progress and Accomplishments: We have been very successful in our ability to generate cell lines derived from patient biopsies both before treatment and at the time of resistance (Please see Table below in Subtask 2D). Since these cells are developed directly from biopsies from patients after the development of resistance in the clinic, we believe that they are more clinically relevant than cultivating resistance in vitro using established cell lines. When we originally wrote this grant, we did not expect to be as successful in developing cell lines from biopsies, so this subtask was to compensate for the lack of the patient-derived models. However, we have been markedly more successful than we originally conceived as described in Subtask 2D and 2E. Moving forward, we will use cell lines derived from patients at the time of resistance. However, below we list the cell lines that we used to generate resistance in vitro that we will continue to study.

2B. Current objectives: Use commercially available *ALK* translocated cell lines to generate resistant clones to the ALK TKI crizotinib

Summary of Results, Progress and Accomplishments: As mentioned above, we have been very successful in our ability to generate cell lines derived from patient biopsies both before treatment and at the time of resistance (Please see Table below in Subtask 2E). For this reason, we are no longer generating resistance to TKIs in commercially-available cell lines (since there only a few such lines). Moving forward, we will only generate resistance in cell lines derived from patients before treatment or use cell lines derived from patients at the time of resistance.

2C. Current objectives: Determine if BIM levels are reduced in resistant cell lines compared to parental cell lines, and whether resistant lines have a reduced apoptotic response to second-line targeted therapies.

Summary of Results, Progress and Accomplishments: We generated two gefitinib-resistant *EGFR* mutant NSCLC PC9 cells (PC9-GR2 and PC9-GR3) until the T790M EGFR gatekeeper mutation arose. Although both cell lines developed T790M, there was a marked difference in the time required to develop resistance, with GR2 and GR3 lines developing in 6 and 24 weeks, respectively (Figure 2A). Treatment of PC9-GR2 and GR3 with the third generation EGFR inhibitor WZ4002 induced robust apoptosis in the PC9-GR2 cells, while apoptosis was reduced in PC9-GR3 cells (Figure 2B). Assessment of BIM levels in these cells revealed that the GR3

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levels had reduced BIM, thereby explaining their reduced responsiveness to the 3rd generation EGFR TKIs (Figure 2C). Furthermore, tumor regressions were observed in PC9-GR2 xenografts, while tumor progression was observed in PC9-GR3 xenografts (data not shown).
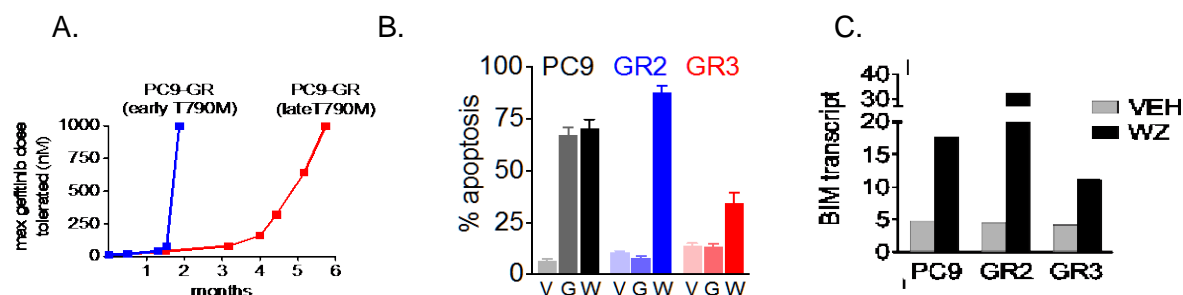

Figure 2. The evolution of the development of the T790M resistance mechanism dictates apoptotic response and BIM expression to 3rd generation EGFR inhibitor. A) PC9 parental cells were cultured in escalating concentrations of gefitinib as tolerated until fully resistant. PC9-GR2 resistant cells developed resistance more rapidly than PC9-GR3 cells. B) PC9-GR3 cells have decreased apoptotic response to the 3rd generation EGFR inhibitor WZ4002 compared to PC9-GR2 and parental cells. Cells were treated with 1uM gefitinib (G), 1uM WZ4002 (W), or vehicle (V) for 72 hours and apoptosis was determined by annexin staining. C) PC9-GR3 cells show reduced BIM mRNA expression when treated with WZ4002.

We determined that the late T790M-emerging PC9-GR3 resistant cells represent cancers that emerge from drug tolerant persister cells, whereas the early T790M-emerging PC9-GR2 resistant cells represent pre-existing T790M cells (Figure 1). Since drug tolerant cells are characterized by their inability to die following treatment with TKI, it appears that this "diminished apoptotic" state persists in the cells even after the development of T790M. Thus, the "path" to T790M impacts the apoptotic response to subsequent EGFR inhibitors.

2D. Current objectives Make approximately 10 patient-derived resistant cell line models by establishing cell lines from patients with treatment-naïve *EGFR* mutant lung cancers, and exposing them in vitro in increasing concentrations of EGFR TKI.

Summary of Results, Progress and Accomplishments: As noted above, we have had great success is establishing cell lines from patient specimens both before treatment and at the time of resistance to therapy. For this reason, we will no longer be generating resistance in pre-treatment patient-derived cell lines. Below is the table of resistant models that we have generated directly from patient biopsies.

Cell Line	Method	Drug	Number of models	Resistance Mechanism(s)
PC9	Increasing Dose	Gefitinib	3	T790M(2), Sustained ERK act. (1)
HCC827	Increasing Dose	Gefitinib	1	MET Amplification
H3255	Increasing Dose	Gefitinib	1	T790M
H1975	Increasing Dose	Dacomitinib	2	EMT
MGH119	Increasing Dose	Gefitinib	1	T790M

MGH119-GR*	Increasing Dose	WZ4002	5	Sustained Akt act., EMT
MGH119-GR*	High Initial Dose	WZ4002	In process	Unknown
MGH119	High Initial Dose	WZ4002	2	Sustained ERK and Akt act.
MGH121	Increasing Dose	WZ4002	5	EGFR C797S (1), Sustained ERK (2), Unknown (2)
MGH134	Increasing Dose	WZ4002	4	Unknown
MGH134	High Initial Dose	WZ4002	5	Unknown
MGH141	Increasing Dose	WZ4002	3	Unknown
MGH164	Increasing Dose	WZ4002	4	EMT
MGH707	High Initial Dose	CO-1686	In process	Unknown

 * MGH119-GR is the gefitinib resistant (T790M+) model derived from MGH119 pt

2E. Current objectives: Make approximately 10 patient-derived resistant cell line models by establishing cell lines from patients with treatment-naïve *ALK* translocated lung cancers, and exposing them *in vitro* to increasing concentrations of ALK TKI

Summary of Results, Progress and Accomplishments: As noted above, we have had great success is establishing cell lines from patient specimens both before treatment and at the time of resistance to therapy. For this reason, we will no longer be generating resistance in pre-treatment patient-derived cell lines. Below is the table of resistant models that we have generated directly from patient biopsies.

Patient derived Cell line	Patient relapsed on	Oncogene	ALK genetics	known mechanism of resistance
MGH006-1A	untreated	EML4-ALK Var 1	WT	
MGH026-1A	untreated	EML4-ALK Var 3	WT	
MGH039-1A	untreated	EML4-ALK Var 1	WT	
MGH048-1A	untreated	EML4-ALK Var 1	WT	
MGH064-1B	untreated	EML4-ALK Var 2	WT	
MGH010-1A	Crizotinib	EML4-ALK Var 1	WT	SRC hit
MGH021-2C	Crizotinib	SQSTM1-ALK	G1269A	G1269A
MGH022-2A	Crizotinib	EML4-ALK Var 2	WT	no hit in screen
MGH025-1B	Crizotinib	EML4-ALK Var 1	WT	EGFR, SRC
MGH044-1B	Crizotinib	EML4-ALK Var 2	WT	EGFR, SRC
MGH045-1B	Crizotinib	EML4-ALK Var 1	L1196M	L1196M+EGFR, SRC
MGH045-2A	Crizotinib	EML4-ALK Var 1	L1196M 50%	
MGH051-1B	Crizotinib	EML4-ALK Var 3	WT	
MGH065-1C	Crizotinib	EML4-ALK Var 1	WT	
MGH065-1B	Crizotinib	EML4-ALK Var 1	WT	
MGH073-2B	Crizotinib	EML4-ALK Var 3	WT	
MGH075-3D	Crizotinib	EML4-ALK Var 2	WT	
MGH021-5A	LDK	SQSTM1-ALK	G1202R	G1202R

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MGH034-2A	LDK	EML4-ALK Var 5	WT	MEK activating mutation
MGH049-1A	LDK	EML4-ALK Var 1	WT	EGFR, SRC
MGH023-2A	LDK	EML4-ALK Var 1	F1174C	F1174C
MGH051-2C	LDK	EML4-ALK Var 3	G1202R	
MGH089-1	LDK	EML4-ALK Var 1	WT	
MGH092-1	LDK	EML4-ALK Var 1	G1202del	
MGH075-2E	LDK	EML4-ALK Var 2	WT	
MGH056-1G	Alectinib	EML4-ALK Var 1	I1171T	I1171T
MGH084-1D	Criz/Alectinib/LDK		I1171N, C1156Y	

2F. Current objectives: Determine if patient-derived cell lines collected at time of progression on first-line therapy have varying degrees of apoptotic response to 2nd and 3rd generation EGFR or ALK inhibitors.

Summary of Results, Progress and Accomplishments: As noted above, we have had great success at generating cell lines derived directly from patients at the time of progression. There are now several new EGFR and ALK inhibitors that can overcome resistance to 1st generation EGFR and ALK inhibitors. We have been using these aforementioned cell lines to determine if a subset of these resistant cancers has a diminished apoptotic response to 2nd and 3rd generation inhibitors. Indeed, we have found this to be the case and this observation has led to a novel therapeutic approach described in Aim 3.

2G. Current objectives: Determine if any of these acquired resistant cell line models and patient-derived resistant models have common, already identified major resistant mechanisms (such as EGFR *T790M*) and whether they have a depressed apoptotic response to second-line targeted therapies.

Summary of Results, Progress and Accomplishments: We have examined seven cell lines derived from *EGFR* mutant NSCLC patient tumors at the time of clinical progression due to T790M acquired resistance while on the 1st generation EGFR inhibitor gefitinib. When treated with 2nd or 3rd generation EGFR inhibitors, three cell lines showed robust apoptosis towards WZ4002, while four had a diminished apoptotic response (Figure 3A). Intriguingly, the four cell lines with the lowest apoptotic response to WZ4002 corresponded to patients with the longest duration of response to first-generation EGFR inhibitor therapy (Figure 3B). This is consistent with cancers developing resistance via the multi-step evolution shown in Figure 1.
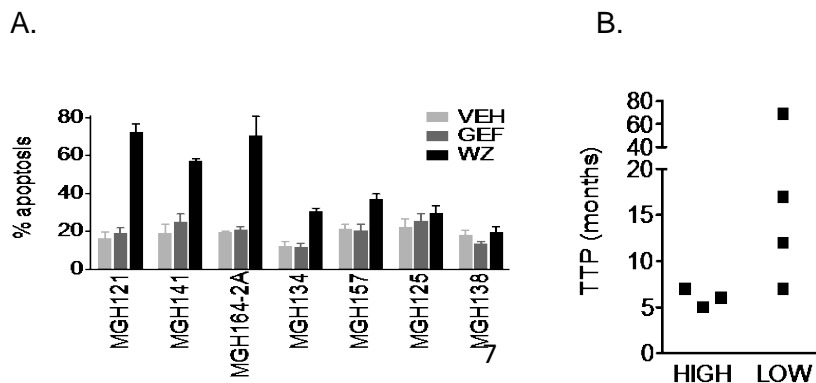
A.

B.

2H. Current objectives: In acquired resistant models that do not have common major resistant mechanisms, determine if low BIM is a primary major resistant mechanism.

Summary of Results, Progress and Accomplishments: Recently, we and others have found that *EGFR* mutant NSCLCs that have an epithelial to mesenchymal (EMT) phenotype associate with resistance to EGFR inhibitor therapy, both in the upfront setting and in the acquired resistance setting. These findings have spurred new efforts to uncover the molecular mechanisms that make EMT *EGFR* mutant NSCLCs refractory to EGFR inhibitors. **This research has uncovered a novel, unexpected connection between EMT, low BIM, and resistance to targeted therapies.** Given that low BIM expression was shown to confer upfront resistance to EGFR inhibitors in the EURTAC trial, and that we have seen EMT transition to play a role in resistance, we sought to uncover any potential link between the two. In order to identify potential modifiers of BIM expression, we interrogated the cancer cell line encyclopedia (CCLE), to investigate gene expression data of ~19,000 genes in 857 solid tumor cancer cell lines. With this data set, we performed an unbiased expression analysis of the genes to determine their relationship with BIM. We identified the EMT marker Vimentin as the strongest associated gene amongst ~19,000, being highly inversely correlated with BIM. Additionally, several other epithelial and mesenchymal markers were among the most co- and differentially expressed, respectively. Furthermore, when we analyzed the cancers with the lowest BIM expression throughout the collection of solid tumor cell lines, those cancers had almost uniformly high Vimentin expression (Figure 4A). To corroborate these findings, we searched other deposited databases in Oncomine ®. Analysis of several other solid tumor cancer cell line and solid tumor databases confirmed the striking relationship between mesenchymal cancers and BIM expression (Figure 4B and 4C). To determine whether mesenchymal status and BIM were also inversely correlated specifically in lung cancers, we re-analyzed these data sets. Again, we found low BIM to be strongly associated with mesenchymal status (Figure 4D).
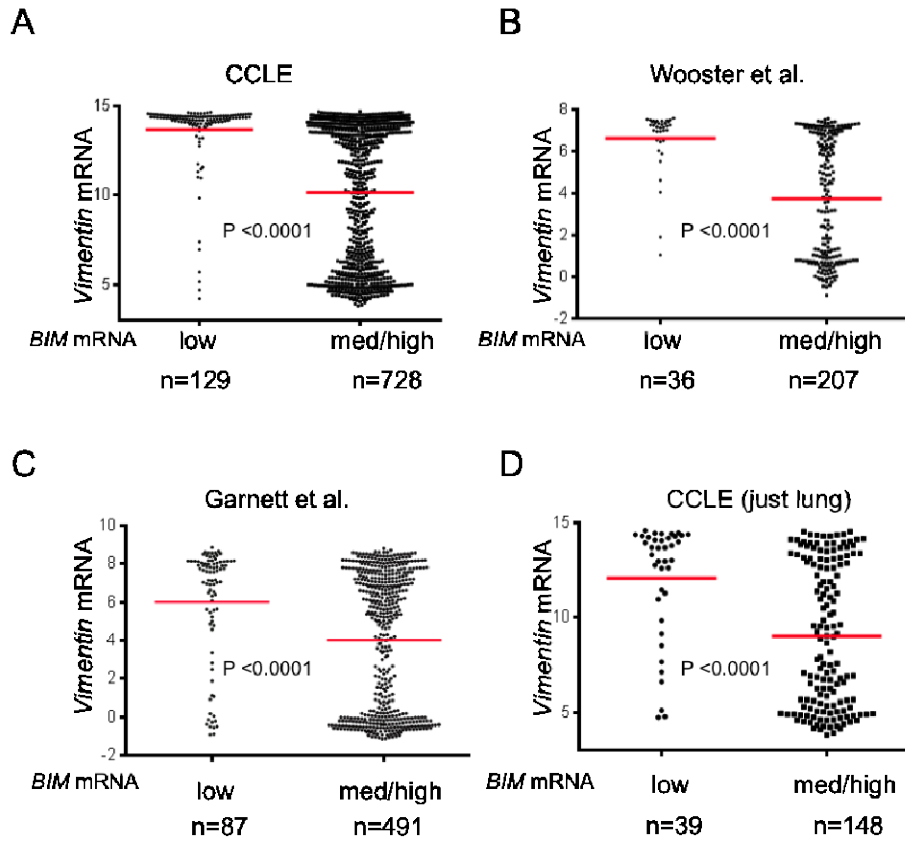
Figure 4. Low BIM-expressing cancers exhibit high Vimentin expression consistent with epithelial to mesenchymal transition. A-D). Expression analysis of the relationship between BIM and Vimentin between four independent expression datasets confirms the inverse relationship.

Since both EMT and low BIM expression associate with resistance to EGFR inhibitors, we sought to determine the relationship between EMT and BIM specifically in *EGFR* mutant NSCLC. We induced EMT in the *EGFR* mutant NSCLC cell lines H1975 and H827 via chronic exposure to recombinant TGF Beta. The TGF Beta-treated clones, H1975T and H827T, underwent characteristic changes attributed to EMT, including upregulation of ZEB1, and downregulation of E-Cadherin, and elevated Vimentin (Figure 5A), as well as distinct morphological changes. Both clones exhibited reduced signaling of EGFR and the downstream effectors PI3K and MEK/ERK following EGFR inhibitor treatment (Figure 5B). In combination with the reduced BIM expression in both H1975T and H827T cells, both clones had a significantly depressed apoptotic response to EGFR inhibitor compared to the parental cells (Figure 5C). Interestingly, the decreases in BIM occurred at the RNA level in both TGF-Beta-treated clones (Figure 5D), suggesting a potential transcriptional alteration may mediate changes in BIM expression in these EMT-associated resistant clones.
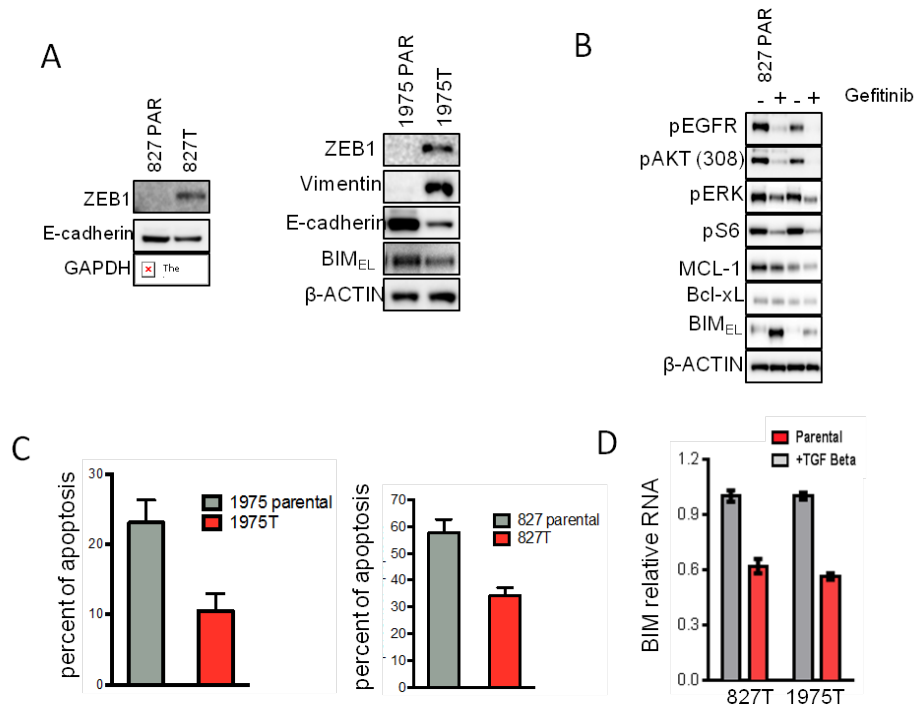
Figure 5. Induction of EMT by TGF beta in *EGFR* mutant NSCLC results in reduced apoptosis and BIM expression. A) Treatment of the parental cell lines H827 and H1975 with TGF beta causes induction of EMT as indicated by increased ZEB1 and Vimentin and decreased E-cadherin. B) Treatment with the 1st generation EGFR inhibitor gefitinib causes reduced EGFR signaling in both H827 parental and EMT induced lines. C) Induction of EMT causes reduced apoptotic response towards gefitinib. D) Induction of EMT causes reduced expression of BIM mRNA.

We next sought to induce EMT by utilizing ER-*TWIST* plasmids that conditionally express TWIST in the presence of 4-hydroxytamoxifen (4-OH). We were able to induce EMT by this method in two *EGFR* mutant NSCLC cell lines, HCC4006 and H1975, as evidenced by increased ZEB1 and decreased E-cadherin (Figure 6A). As in the TGF-Beta models of EMT, induction of EMT led to a sharp decrease in BIM$_{EL}$ expression at the protein level (Figure 6A) and at the RNA level (Figure 6B), and consistently associated with depressed apoptosis following EGFR inhibitor treatment (Figure 6C), despite similar signaling shutdown between the ER-*TWIST* active cells and the controls (Figure 6D), consistent with the TGF-beta-treatment experiments. This depressed apoptosis correlated with poorer response to EGFR inhibitor in long-term cell viability assays (Figure 6E).
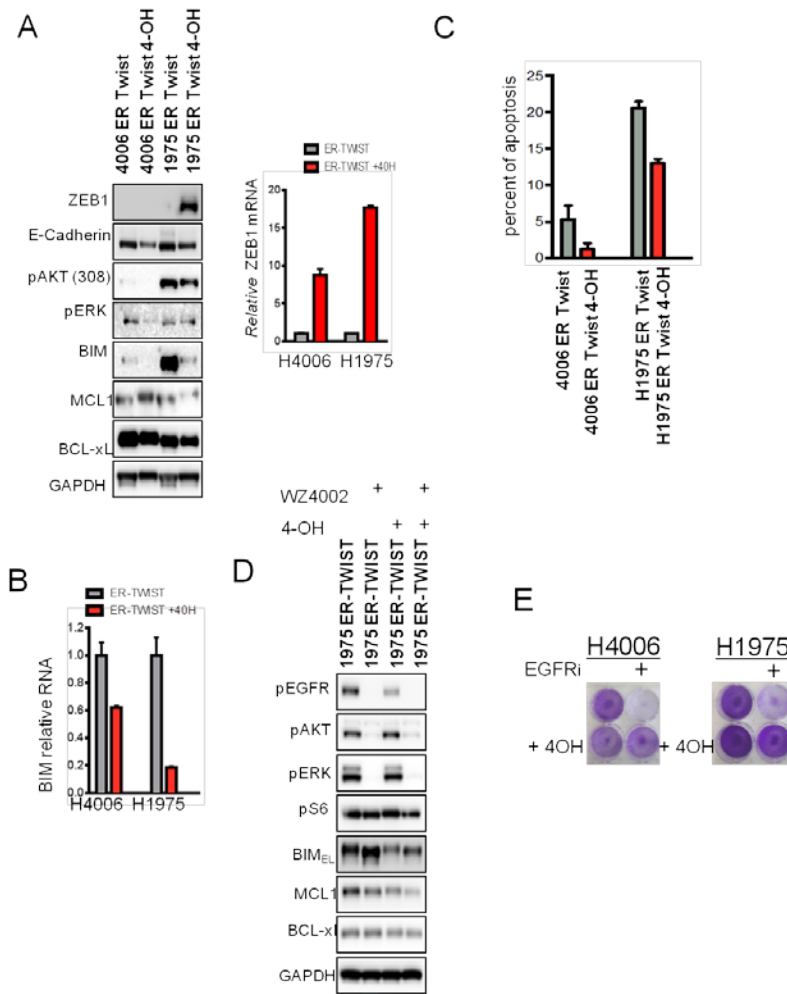
Figure 6. Induction of EMT by over-expression of TWIST in *EGFR* mutant NSCLC results in reduced apoptosis and BIM expression. A) Conditionally-expressed TWIST in the cell lines HC406 and H1975leads to a decrease in BIM protein and an increase in ZEB1 mRNA. B) BIM levels are also reduced at the mRNA level. C) Induction of EMT by over-expression of TWIST causes reduced apoptotic response towards gefitinib. D) Gefitinib treatment of TWIST over-expressing lines successfully reduces EGFR signaling. E) EMT induction by TWIST over-expression leads to prolonged survival following gefitinib treatment.

Resistance occurs through EMT in *EGFR* mutant lung cancers. We studied a series of three resistant clones from the H1975 *EGFR* mutant NSCLC cell line made resistant to an EGFR inhibitor. Two resistant clones underwent EMT and one clone that did not (Figure 7A). Consistent with our previous data and the gene expression analysis, the two clones that underwent EMT had low BIM expression at the protein and RNA level (Figure 7A and 7B), consistent with the TGF-Beta data, ER-*TWIST* data .However, the third clone that did not undergo EMT had similar levels of BIM$_{EL}$ as the parental cells. As expected, the low BIM expressing resistant clones were resistant to apoptosis (data not shown).
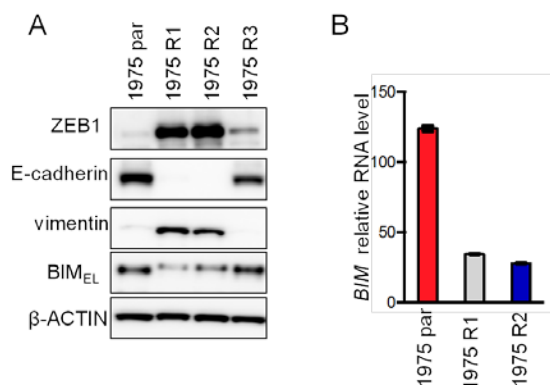
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Figure 7. EGFR mutant NSCLC cell lines that become resistant to EGFR inhibitor exhibit epithelial to mesenchymal transition and reduced BIM levels. A) Three resistant clones were generated by treating H1975 parental cells with increasing concentrations of gefitinib. Two resistant clones underwent EMT as indicated by increased ZEB1 and Vimentin and decreased E-cadherin. B) BIM mRNA is reduced in the two gefitinib-resistant clones that underwent EMT.

2I. Current objectives: To expand our studies of the role of EMT and BIM in apoptotic response, we propose to look at the expression of both BIM and EMT markers in a larger panel of *EGFR* mutant patient-derived cell lines resistant to first-line TKI therapy.

Summary of Results, Progress and Accomplishments: We have developed a collaboration with the NCI to explore the relationship between acquired resistance and EMT/BIM. We will send 90 *EGFR* mutant patient-derived cell lines that were generated at the time of progression on first-line EGFR therapy. RNA sequencing will be performed on these samples to determine the expression levels of EMT markers and BIM.

Aim 3: Assess novel therapeutic strategies for cancers with low BIM expression that aim to increase BIM and thereby enhance response.

Task 3:

Current Objectives: Interrogate 200 female NU/NU mice for this task.

Summary of Results, Progress and Accomplishments: Please see below in section on Subtasks.

Subtasks.

3A. Current objectives: Determine if demethylase inhibitors increase free BIM and if co-treatment with demethylase inhibitors and TKIs re-sensitize low BIM expressing resistant cancers, identified previously and in Aim 2, to apoptosis in vitro.

Summary of Results, Progress and Accomplishments: These studies have not yet been initiated. We have focused significant efforts for objective 3D. Those results have been very impressive, leading to a clinical trial sponsored by CTEP and they have occupied more effort than we initially planned.

3B. Current objectives: Determine whether there are alterations at the BIM promoter causing epigenetic silencing at the BIM locus in resistant lung cancer models identified previously and in Aim 2.

Summary of Results, Progress and Accomplishments: In response to the observation that BIM levels were changing at the RNA level in the lung cancers in the mesenchymal state, and that there are a number of well characterized transcriptional repressors that are upregulated

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following EMT, we looked for potential transcriptional modifiers of BIM in mesenchymal cancers. Using the SwissREgulon portal and the Eukaryotic promoter database we searched for mesenchymal genes that could regulate BIM uniquely in mesenchymal cancers. We identified that the master EMT regulator and transcriptional repressor ZEB1 could potentially bind the BIM promoter. We next identified data archived in the ENCODE project of whole genome CHIP-SEQ of ZEB1 in the HepG2 kidney cell line. We subsequently identified ZEB1 binding sites in the BIM promoter. These data proved that ZEB1 could bind, and presumably suppress, BIM in at least a subset of ZEB1-expressing cancer cell lines. To determine where ZEB1 was binding in the *EGFR* mutant NSCLCs that undergo EMT, we performed whole genome ChIP-SEQ of ZEB1 in the H1975 Parental and H1795 R2 cells. This analysis identified 9,744 ZEB1 binding sites in the 1975 R2 cells. BIM was the 6th ranked gene in respect to the largest number of ZEB1 binding sites in the 1975 R2 cells. These data demonstrate ZEB1 is a novel regulator of BIM through direct binding of the BIM promoter in cells including *EGFR* mutant NSCLCs that undergo EMT. These findings support the hypothesis that EMT-related EGFR inhibitor resistance involves depressed apoptosis through depressed levels of BIM.

Based on these data, we next stably suppressed ZEB1 by short hairpin (sh) RNA in the 1975 R2 model. Consistently, knockdown of ZEB1 led to de-repression of BIM, at both the protein and RNA level. Knockdown of ZEB1 also de-repressed levels of E-cadherin in these cells, affirming the role of ZEB1 in EMT. Importantly, knockdown of ZEB1 led to re-sensitization of 1975 R2 cells to EGFRi (data not shown). Altogether, these data indicate EGFR mutant NSCLCs that undergo EMT repress BIM through ZEB1-mediated transcriptional repression, and its de-repression can re-sensitize EMT-associated resistant cells to EGFRi.

3C. Current objectives: Determine whether agents that work by reversal of epigenetic silencing (e.g.demethylase inhibitors) lead to de-repression of BIM in these cancers.

Summary of Results, Progress and Accomplishments: These studies have revealed that demethylase inhibitors de-repress BIM expression in a subset of cancers. We are currently aiming to understand the underlying mechanisms that will identify which cancers are regulated in this manner
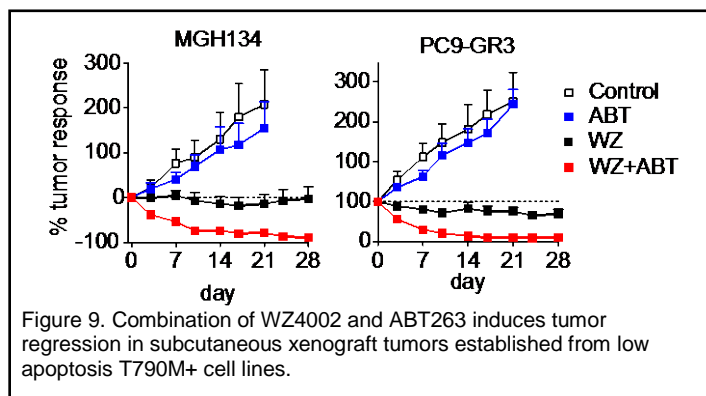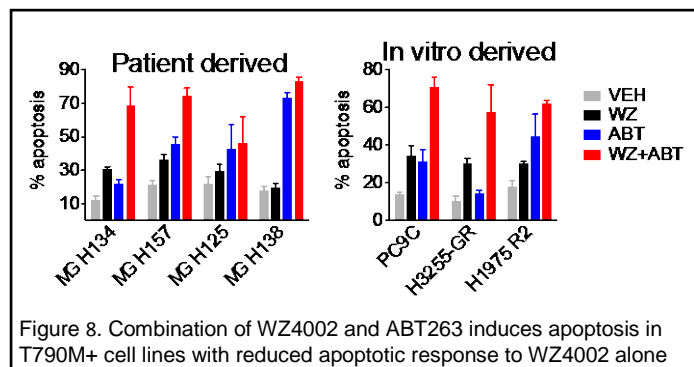
3D. Current objectives: Determine whether the amount of free BIM can be maximized in low BIM expressing resistant cancer models identified previously and Aim 2, by addition of ABT-263, through immunoprecipitation analysis of Bcl-2 family member proteins.

Summary of Results, Progress and Accomplishments: We have determined that free BIM can be enhanced by addition of ABT-263.

3E. Current objectives: Determine whether BH3 mimetics sensitize low BIM expressing lung cancers identified previously and Aim 2 to TKIs to apoptosis.

Summary of Results, Progress and Accomplishments: We sought to identify therapeutic strategies that could enhance the apoptotic response to third generation EGFR inhibitors. We have previously reported a drug screening strategy for identifying effective drug combinations for cancers with acquired resistance to targeted therapies. We screened four low-apoptosis T790M patient-derived cell lines (MGH134, MGH157, MGH125, and MGH138) to identify drugs that increased E_{max} when combined with WZ4002. The BCL-XL/BCL-2 inhibitor

ABT263/navitoclax was shown to induce a robust apoptotic response in combination with WZ4002 in both T790M patient-derived cell lines and in in vitro generated T790M resistant cell lines (Figure 8). In vivo, WZ4002 and navitoclax in combination induced regressions in MGH134 and PC9-GR3 xenograft tumor models (Figure 9).

Figure 8. Combination of WZ4002 and ABT263 induces apoptosis in T790M+ cell lines with reduced apoptotic response to WZ4002 alone

BH3 mimetics are effective anti-cancer drugs that work by reducing the apoptotic thresholds of cells by liberating BIM protein from complexes with the anti-apoptotic proteins BCL-2 and BCL-xL. We also treated the low BIM expressing clones H1975 R1 and H1975 R2 with ABT-263 in combination with WZ4002 to determine whether this would restore the apoptotic response. Indeed, combination treatment with ABT-263 in the two low BIM expressing clones was able to restore the apoptotic response to EGFR inhibition (data not shown).

Figure 9. Combination of WZ4002 and ABT263 induces tumor regression in subcutaneous xenograft tumors established from low apoptosis T790M+ cell lines.

These findings are the basis for a new clinical trial combining ABT263 with AZD9291. Our data was presented to a national committee of lung cancer experts convened by NCI-CTEP and was chosen from several potential ideas as a promising concept. A clinical trial proposal was designed by these experts (including Dr. Engelman) and presented to the NCI Investigational Drug Steering Committee, who voted to proceed with the trial. Hence, a national CTEP-sponsored trial will be rolling out in 2015. This trial is the direct result of the studies supported by this DOD award.

3F. Current objectives: Use the pharmaceutical strategy that does in fact re-sensitize low BIM expressing cancers to TKIs, and treat these cancers with this combination in vivo by xenografting female NU/NU mice.

Summary of Results, Progress and Accomplishments: As described above, we have tested the combination of ABT263 + WZ4002 in xenograft models of in vitro acquired resistance (PC9) as well as in a patient-derived cell line from resistant tumor (MGH134) and demonstrated that this combination induces dramatic tumor regressions (Figure 9).

4.KEY RESEARCH ACCOMPLISHMENTS:

- During this year, we have continued to surpass our initial goals of obtaining pre-treatment and post-treatment biopsy specimens for both *EGFR* mutant and *ALK*-translocated tumors.
- We have successfully optimized our methods to establish cell lines taken from patient-derived tumors taken at the time of progression. This has allowed us to expand the number of both in vitro and xenograft models for laboratory use.
- Intrinsic loss of apoptotic response appears to be a distinct mechanism contributing to acquired resistance and not mutually exclusive with commonly clinically observed genetic clinical mechanisms of resistance such as T790M (manuscript under review).
- Cancers that evolve from drug tolerant persisters likely maintain an apoptotic defect when they subsequently genetic mutations leading to resistance (manuscript under review).
- Cancers with T790M and suppression of apoptotic response may be less sensitive to subsequent therapy with next generation irreversible EGFR inhibitors
- Cancers with loss of apoptotic response can be resensitized to TKI treatment by use of BH3 mimetics
- **New clinical trial combining ABT263 with AZD9291 is opening based on these results**

5.CONCLUSION:

We have established that a subset of resistant cancers that developed T790M have decreased apoptotic response to irreversible EGFR inhibitors in vitro and in vivo. The changes in these cells that underlie the loss of apoptotic response may involve BIM in some cases but overall appears to be more complex. We have determined that the combination of BH3 mimetics plus irreversible EGFR inhibitors is effective against many of these cancers in preclinical studies. These data have provided rationale for planned upcoming clinical trials investigating the combination of navitoclax (ABT263) with irreversible EGFR inhibitors.

6.PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Two manuscripts describing this data have been written:

"Dynamic evolution of resistance to EGFR blockade from drug tolerant cancer cells"

     -Under review at *Nature Medicine*

"Epithelial-mesenchymal transition antagonizes EGFR inhibitors in *EGFR* mutant NSCLCs by suppressing BIM"

7.INVENTIONS, PATENTS AND LICENSES:

Nothing to report

8.REPORTABLE OUTCOMES:

Two manuscripts describing the combination of ABT263 and irreversible EGFR inhibitors have been written. One is under review at *Nature Medicine* and one will be submitted. These data are the basis of an upcoming clinical trial

9.OTHER ACHIEVEMENTS: Nothing to report

10.REFERENCES: Nothing to report

11.APPENDICES: Nothing to report