Induction of Multiple Alternative Mitogenic Signaling Pathways Accompanies Emergence of Slowly Growing Drug-Tolerant Cancer Cells

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Abstract

Drug resistance continues to be a major obstacle to curing cancer. Resistance can evolve from a subpopulation of cancer cells that initially survive drug treatment and then gradually form a pool of slowly growing drug-tolerant cells. Several studies have pinpointed activation of a specific bypass pathway that appears to provide the critical therapeutic target for preventing drug tolerance. Here we take a systems-biology approach using proteomics and genomics to examine the development of drug tolerance to EGFR inhibitors in EGFR-mutant lung adenocarcinoma cells and BRAF inhibitors in BRAF-mutant melanoma cells. We found that there are numerous alternative mitogenic pathways that become activated in both cases, including YAP, STAT3, IGFR1, and phospholipase C (PLC)/protein kinase C (PKC) pathways. Our results suggest that an effective therapeutic strategy to prevent drug tolerance will need to take multiple alternative mitogenic pathways into account rather than focusing on one specific pathway.

Introduction

There has been much interest recently to decipher the non-genetic mechanisms where some cancer cells enter a reversible drug-tolerant state in response to treatment and continue to slowly grow\(^1\). Time-lapse microscopy studies have demonstrated that drug-tolerant tumor cells are not totally quiescent and continue to cycle during drug treatment, albeit at a much slower rate\(^2,3\). These slowly cycling cells have been shown to give rise to a diverse set of stably resistant clones that can harbor the same genetic alterations observed in resistant human tumor samples\(^4,5\).

Several research efforts have focused on identifying alternative signaling mechanisms by which drug-tolerant tolerant cancer cells can ‘bypass’ the mitogenic block generated by targeted therapeutics such as EGFR or BRAF inhibitors. For example, inhibition of mutant-EGFR in lung adenocarcinoma cells or mutant-BRAF in thyroid cancer cells were shown to induce secretion of IL6 and subsequent activation of STAT3, and in both cases blocking STAT3 function largely prevented the emergence of drug-tolerant cells\(^6,7\). Inhibition of mutant EGFR in lung adenocarcinoma cells has been shown to cause increased expression of FGFR3, and inhibition of FGFR3 was shown to block the formation of drug-tolerant cells\(^8\). Additionally, inhibition of mutant EGFR with the third-generation inhibitor osimertinib in lung adenocarcinoma cells has been shown by one group to cause up-regulation of the tyrosine kinase receptor AXL\(^9\), and another group to cause up-regulation of the mitotic protein kinase AURKA\(^10\). In both cases, the studies found that inhibition of their target of interest prevented the emergence of drug tolerant cells.

One of the primary motivations for each of the studies described above has been to discover a therapeutic strategy that could be used in the clinic to block the development of drug-tolerant cells. However, disruption of a single bypass mitogenic signaling may not fully eliminate drug-tolerant cells if there is activation of other alternate growth signaling pathways that remain intact. In this study, we set out to determine the entire scope of mitogenic signaling pathways that are upregulated in the response to the blockade of mutant EGFR and mutant BRAF signaling.
Results

Numerous transcriptional alterations are shared between EGFR-mutant lung cancer cells during EGFR inhibition and BRAF-mutant melanoma cells during BRAF inhibition.

Transcriptional changes are critical to a cancer cell’s ability to tolerate targeted therapy; however, it is unclear if changes are common between different cancer types and inhibitors. We performed RNA-seq on EGFR-mutant PC9 NSCLC cells treated with the EGFR inhibitor getinib, and BRAF-mutant SKMEL28 melanoma cells treated with the BRAF inhibitor dabrafenib to investigate the scope of transcriptional changes in each system, and to uncover shared mechanisms of drug tolerance. Differential expression analysis revealed 2,179 differentially expressed genes (DEGs) (1,524 up-regulated and 655 down-regulated) in PC9 cells treated with getinib for 3 days, and 2,219 DEGs (1,674 up-regulated and 545 down-regulated) in PC9 cells treated with getinib for 9 days, compared to untreated controls. In SKMEL28 cells cultured in dabrafenib, 4,926 genes (2,431 up-regulated and 2,495 down-regulated) were differentially expressed at day 3, and 4,581 (2,463 up-regulated and 2,118 down-regulated) genes were differentially expressed at day 9, compared to untreated controls. Additionally, we found 24 Hallmark gene sets were significantly enriched in 9-day getinib-treated PC9 cells, and 13 Hallmark gene sets were significantly enriched in 9-day dabrafenib-treated SKMEL28 cells, compared to untreated cells when using a strict cut-off of FDR < 0.05. Interestingly, 11 Hallmark gene sets were significantly enriched in both drug-treated cell lines.

We performed unbiased, hierarchical clustering on the 544 genes (422 up-regulated and 122 down-regulated) we identified as significantly altered in both getinib- and dabrafenib-treated cells (Fig. 1a, b). Narrowing our focus to genes that have a log2-fold change of at least 2.0, we found alterations in 150 genes that are shared between cell lines (Fig. 1c). Unique changes occur in each cell line, with 93 genes significantly altered in PC9 cells treated with gefitinib, and 523 genes significantly altered in SKMEL28 cells treated with dabrafenib. A very small number of genes showed significant changes in opposite directions between cell lines. 18 genes showed significant upregulation in PC9 cells and significant downregulation in SKMEL28 cells, whereas 5 genes showed significant upregulation in SKMEL28 cells, and significant downregulation in PC9 cells (Fig. 1c).

Principal component analysis (PCA) revealed distinct separation of gefitinib-treated and untreated PC9 cells by the first principal component, which explained over 74% of the variance (Fig. 1d). Interestingly, when we performed PCA on SKMEL28 cells in dabrafenib, the first principal component did not completely separate samples on treatment status alone (Fig. 1e).
These findings suggest that many transcriptional changes are shared between different cancer types adapting to various targeted therapies, although the exact timing of these changes may be unique to each cell line and drug combination.

Proteomic and phospho-proteomic alterations accompany the development of drug tolerance

We next sought to investigate proteomic and phospho-proteomic changes involved in the development of drug tolerance by performing Reverse Phase Protein Array (RPPA) analysis on PC9 cells cultured in gefitinib for 0, 3, 6, and 9 days. Of the 471 antibodies included in the panel, we saw significant changes in 305 protein and/or phospho-protein levels at one or more time points during gefitinib treatment. We performed unbiased, hierarchical clustering to visualize changes in the 55 phospho-proteins that we identified as significantly altered in gefitinib-treated cells (Fig. 2a). We integrated our transcriptomic and proteomic data sets by comparing the log2-fold change of mRNA transcripts to differences in protein levels, each in 9-day gefitinib-treated versus untreated PC9 cells. We observed a positive correlation of the 336 genes/proteins featured in both data sets (Fig. 2b). The labeled points show both a significant increase in protein level from RPPA analysis and a log-fold change of >2 in our RNA-seq data set. Fibronectin was selected from these points to illustrate the large increases in both protein (Fig. 2c) and transcript abundance (Fig. 2d). These significant changes observed in numerous protein and phosphoprotein levels in gefitinib-treated PC9 cells complement prior transcriptomic studies and reveal potential mechanisms by which cancer cells continue to cycle in targeted therapy.

Targeted inhibition of mutationaly activated oncogenes induces sustained mTOR-pathway suppression.

We began investigating shared effects of inhibiting mutationaly activated oncogenes in both EGFR-mutant lung cancer cells and BRAF-mutant melanoma cells by analyzing changes in gene expression at 3-, 6-, and 9-days following addition of the EGFR inhibitor gefitinib or the BRAF inhibitor dabrafenib, compared to untreated controls. Using gene set enrichment analysis (GSEA), we found that mTOR-related gene sets were more enriched in untreated controls relative to gefitinib and dabrafenib-treated cells (Fig. 3a, b). Transcript levels of indicated mTOR-related genes such as RPS6, EIF4G1,
and EEF2K, showed significant decreases in PC9 cells in gefitinib and SKMEL28 cells in dabrafenib at multiple time points (Fig. 3c, d).

We then examined individual protein and phospho-protein levels of mTOR pathway members. RPPA analysis revealed that total protein levels of mTOR do not significantly change, but phosphorylated mTOR levels decreased during gefitinib treatment of PC9 cells (Fig. 3e). Downstream targets of mTOR including eiF4E, p70-S6K, and S6 showed significant decreases in the amount of phosphorylated protein and total protein (Fig. 3e). When immunoblotting was performed on SKMEL28 cells in dabrafenib to complement protein array results obtained in PC9 cells treated with gefitinib. We observed a significant decrease in phospho-S6 within 24 hours of dabrafenib treatment (Fig. 3f). Taken together, these results indicate treated susceptible cells

with corresponding targeted therapies results in a sudden, sustained decrease in mTOR signaling.

**Targeted inhibition of mutationally activated oncogenes induces IGF-pathway activation.**

To better understand how cells continue to proliferate despite a crash in mTOR signaling, we began to search for alternate mitogenic growth pathways that were upregulated in drug-treated cells. GSEA revealed that genes associated with the Insulin-like growth factor (IGF) signaling cascade are significantly up-regulated in PC9 cells treated with gefitinib (Fig. 4a) and SKMEL28 cells treated with dabrafenib for 9 days, relative to untreated controls, (Fig. 4b). In gefitinib- (Fig. 4d) or dabrafenib-treated cells (Fig. 4e), we observed significant increases in individual transcript levels of various IGF-related genes such as IGFBP3, IGFBP5, IGFBP7, and most notably IGF1R. Total protein levels of IGFRB did not appear to significantly change during 9-day gefitinib exposure of PC9 cells (Fig. 4c). However, we observed significant increases in the amount of phosphorylated IGF1R (Y1135/6) in PC9 cells treated with gefitinib for 9 days (Fig. 4c). We also observed significant increases in IGFBP3 at days 3 and 6 of gefitinib treatment (Fig. 4c), coinciding with the increase observed in the earlier IGFBP3 transcript levels. These results obtained in multiple cancer contexts expand on the previous work implicating IGF1R in anti-EGFR drug tolerance.

**Targeted inhibition of mutationally activated oncogenes induces PLC/PKC signaling.**
We observed increased phosphoinositide-specific phospholipase C (PLC) activity in drug treated cells. PLC is often a key player in transmembrane signaling. GSEA performed using genes associated with GPCR signaling revealed strong enhancement in gefitinib-treated PC9 cells relative to untreated controls (Fig. 5a). Further GSEA studies performed on SKMEL28 cells showed a strong enrichment of genes associated with the regulation of phospholipase activity in the drug-treated group (Fig. 5b). Transcript levels of several genes associated with increased PLC/PKC signaling such as \textit{ADCY6, GNAL, GNAS, PDE1C,} and \textit{PLCG1} showed significant increases in PC9 cells in gefitinib (Fig. 5d) and SKMEL28 cells in dabrafenib (Fig. 5e) at multiple time points. Phosphorylation of PLCG2 at tyrosine 759 is a marker of the active state, and increased levels were observed in PC9 cells at days 6 and 9 of gefitinib treatment (Fig. 5c). Additionally, we observe increase in PKCA and phospho-PKCA/B (T638/6431) in gefitinib-treated PC9 cells. Together, these findings indicate activation of the PKC/PLC signaling pathway upon gefitinib or dabrafenib treatment, which to our knowledge is the first time this has been documented.

**Targeted inhibition of mutationaly activated oncogenes induces STAT3 activation and YAP activation.**

We also saw activation of two pathways previously observed by others. Our GSEA studies revealed that STAT3 signaling was significantly enriched in PC9 treated with gefitinib (Fig. S1a) or SKMEL28 cells treated with dabrafenib (Fig. S1b) for 9 days when compared to untreated controls. Transcript levels of STAT3-associated genes including \textit{JAK1} and \textit{BCL6} were also significantly increased in both gefitinib-treated PC9 cells (Fig. S1d) and dabrafenib-treated SKMEL28 cells (Fig. S1e). At days 3 and 9 of gefitinib treatment, total protein levels of STAT3 did not differ significantly from untreated PC9 cells (Fig. S1c). STAT3 is considered active when phosphorylated at tyrosine 705, which induces dimerization and nuclear translocation. Interestingly, we observed a significant increase in the amount of phosphorylated STAT3 (Y705) following 9 days of gefitinib treatment (Fig. S1c). From these results, we conclude that increased STAT3 signaling accompanies the emergence of drug tolerance in various contexts.

We also observed activation of YAP signaling. GSEA studies show a strong enrichment of genes associated with YAP transcriptional signature in PC9 cells treated with gefitinib (Fig. S2a) or SKMEL28 treated with dabrafenib (Fig. S2b), when compared to untreated controls. Total YAP protein levels do not significantly change in PC9 cells during 9-day treatment with dabrafenib (Fig. S2c). YAP phosphorylated at serine 127 is sequestered in the cytoplasm. Interestingly, we observed a significant decrease in level of phospho-YAP (S127) at each time point during gefitinib treatment (Fig. S2c). Indeed, transcript levels of numerous YAP-related genes are significantly higher in
drug-treated cells compared to untreated controls of both PC9 (Fig. S2d) and SKMEL28 (Fig. S2e). Proteomic data also revealed increased protein levels of PAI-1 (Fig. S2c), a common YAP-associated protein, in PC9 cells treated with gefitinib for 3, 6, and 9 days. These results suggest YAP signaling is induced upon targeted blockade in oncogene-dependent cells.

**Single-cell RNA-seq reveals simultaneous upregulation of markers of multiple alternate mitogenic signaling pathways.**

To determine whether the activation of the multiple alternate mitogenic signaling pathways we observed in bulk experiments (i.e. RNA-seq, RPPA) occurred simultaneously in individual cells, or occurred in separate sub-populations, we performed single-cell RNA seq on PC9 cells in gefitinib and SKMEL28 cells in dabrafenib for 0, 24, 48, or 72 hours. Gene-gene plots showed select marker genes for IGF and STAT3 signaling (Fig. 6a), IGF and YAP signaling (Fig. 6b), IGF and PKC/PLC signaling (Fig. 6c) were simultaneously upregulated in PC9 cells in gefitinib for 72 hours compared to untreated controls. Additionally, we observed simultaneous upregulation of the same pathways in SKMEL28 cells in dabrafenib for 72 hours, compared to untreated SKMEL28 cells (Fig. 6d-f). The simultaneous upregulation of multiple marker genes indicates that the enriched pathways are simultaneously up-regulated in single cells, rather than being up-regulated in different sub-populations prior to pooled analysis.

**Autophagy accompanies the development of drug-tolerance and can be disrupted with hydroxychloroquine to reduce the number of drug-tolerant cells.**

To investigate potential survival mechanisms utilized by cells in targeted therapy, we investigate the role of autophagy during the development of drug tolerance. We observed enrichment of the GO gene set “Selective Autophagy” in PC9 cells treated with gefitinib for 9 days, compared to untreated controls (Fig. S3a). Increased transcript levels of the common autophagy marker ULK1 were observed in gefitinib-treated PC9 cells (Fig. S3b). scRNA-seq analysis of ULK1 and a second marker, GABARAPL1, also showed up-regulation in gefitinib-treated PC9 cells (Fig. S3d). Additionally, a common protein marker used to assess autophagy, LC3A/B was increased in gefitinib-treated PC9 cells, when measured by RPPA (Fig. S3c). Direct visualization of autophagic flux was performed with the CYTO-ID live cell autophagy detection kit. Increased fluorescent signal, corresponding to the increase in autophagosomes critical for autophagy, was observed in gefitinib-treated PC9 cells (Fig. S3e). PC9 cells in regular media showed little to no evidence of autophagy.
To determine if disrupting autophagy leads to fewer drug tolerant cells, we supplemented gefitinib treatment with hydroxychloroquine. This anti-malarial drug is widely available and has been previously used to disrupt autophagy by lowering lysosomal pH, thereby blocking the key step of lysosome-autophagosome fusion\(^{11}\). Hydroxychloroquine does not exert any growth effects on PC9 cells in regular media (Fig. 7a), but significantly reduces the number of surviving PC9 cells when combined with gefitinib (Fig. 7b). From these results, it seems autophagy is induced upon targeted therapy exposure, and using hydroxychloroquine co-treatment is an effective way to reduce the number of drug tolerant cells.

**Disruption of ATG5 results in decreased autophagic flux but does not enhance gefitinib killing of PC9 cells.**

To validate the utility of blocking autophagy in cells developing drug tolerance, we disrupted the critical ATG5-ATG12 interaction via CRISPR-induced mutations in exon 2 of ATG5\(^{12}\). In unmodified PC9 cells, immunoblotting revealed the 55 kDa ATG5-ATG12 complex, while the CRISPR modified clones we generated appear to only have free ATG5 (Fig. 7c). The ATG5-ATG12 complex is required for autophagy, and indeed, these clones showed a decreased ability to undergo autophagy compared to parental PC9 cells (Fig. 7d). Similar to the results observed with chloroquine co-treatment, CRISPR-ATG5 cells did not show any growth differences when compared to control PC9 cells in regular media (Fig. 7e). Interestingly, contrary to the results observed with chloroquine co-treatment, CRISPR-ATG5 cells survived gefitinib treatment at a higher rate than control PC9 cells (Fig. 7f). Although autophagy appears to be induced by the presence of targeted therapy, and chloroquine co-treatment lead to fewer drug-tolerant cells, genetic studies cast doubt on autophagy's importance in the development of drug-tolerance.

**Cells display features of senescence during the development of drug-tolerance.**

Cancer cells surviving challenging environments have been shown to display features of cellular senescence, a process which overlaps with autophagy. PC9 cells were subjected to X-gal staining, a common method used to assess senescence-associated beta-galactosidase (SA-\(\beta\)-gal) activity, a feature unique to senescent cells. We observed the characteristic blue-green colorimetric change resulting from SA-\(\beta\)-gal-mediated cleavage of X-gal in PC9 cells treated with gefitinib, and SKMEL28 cells treated with dabrafenib (Fig. S4a). GSEA revealed an enrichment of a senescence-associated gene set in PC9 cells treated with gefitinib compared to untreated controls (Fig. S4b). Increases in normalized transcript abundance levels of several senescence-associated genes such as \(CDKN1A\) (Fig. S4c), and \(CDKN2B\) (Fig. S4d) are
observed in PC9 cells treated with gefitinib for 3 and 9 days, compared to untreated PC9 cells. scRNA-seq analysis of CDKN2A and CDKN1B, also showed up-regulation in gefitinib-treated cells PC9 cells (Fig. S4e).

We co-treated PC9 cells in gefitinib with multiple “senolytic” drugs to determine if selectively targeting cells displaying features of cellular senescence would reduce the number of drug tolerant cells. The first compound, navitoclax (ABT-263) is a BCL-2 family protein inhibitor that has been shown to selectively eliminate senescent cells\textsuperscript{13}. Navitoclax did not appear to alter the number of PC9 cells when cultured in regular media (Fig. S5a). However, when added to gefitinib media, navitoclax appeared to significantly reduce the number of surviving cells (Fig. S5b). Rather than broadly targeting BCL-2 family proteins, A1331852 is a purported BCL-XL-specific inhibitor that showed no significant impact on PC9 growth in regular media (Fig. S5c). However, similar to navitoclax, co-treatment with A1331852 significantly reduced the number of PC9 cells surviving gefitinib (Fig. S5d). From these results, we can conclude that inhibitors targeting BCL-2 family proteins, specifically BCL-XL, significantly reduce the number of drug tolerant cells.

Disruption of BCL-XL does not result in enhanced killing of PC9 cells by gefitinib.

To validate the utility of disrupting BCL-XL in cells developing drug tolerance, we used CRISPR/cas9 to create a BCL-XL knockout PC9 cell line (Fig. S5e). There were slightly fewer CRISPR-BCL-XL cells compared to unmodified PC9 cells when cultured in regular growth media for 3 days (Fig. S5f). However, contrary to the results observed with navitoclax and A1331852 co-treatments, CRISPR-BCL-XL cells exhibited no differences in survival during gefitinib treatment compared to unmodified PC9 cells (Fig. S5g). Although senescence appears to be induced by the presence of targeted therapy, and co-treatment with senolytic compounds lead to fewer drug-tolerant cells, genetic studies cast doubt on the exact mechanism by which drug tolerance is blocked.

Discussion

The ability of cancer cells to tolerate lethal doses of targeted inhibitors and form resistant tumors is a widely recognized hinderance to the long-term treatment of many cancer types. Currently, we do not fully understand how oncogene-addicted cells continue to proliferate in the presence of drugs designed to inhibit their oncogenic signaling dependencies. In this study, we sought to identify growth signaling pathways that were activated in drug-tolerant cells.

Through a combination of transcriptomic and proteomic studies, we observed increased signaling via the phospholipase C (PLC)/protein kinase C (PKC) signaling pathway. PKC’s involvement in regulating
cellular proliferation, survival and apoptosis is well documented\textsuperscript{14}. Recently, PLC/PKC signaling has been linked with chemotherapeutic resistance in NSCLC, through inhibition of caspase activation\textsuperscript{15}. Studies in B-cell lymphoma models suggest PKC mediates chemoresistance via stabilization of anti-apoptotic BCL-XL, and that small-molecule PKC inhibitors can be used to restore chemosensitivity\textsuperscript{16}. To our knowledge, we are the first to propose that activation of PKC signaling may contribute to the drug-tolerant phenotype observed in slow-growing cells adapting to targeted TKIs.

In addition to PLC/PKC, we revealed activation of several alternate mitogenic signaling pathways including: IGF1R, STAT3, and YAP, that appear to accompany the emergence of drug-tolerance. Interestingly, upon review of the literature we found individual examples of each pathway’s activation. IGFBP3-mediated IGF1R activation was first observed in erlotinib-treated PC9 cells\textsuperscript{1} and a similar mechanism was also described in BRAF(V600E) melanoma cells adapting to targeted BRAF inhibition\textsuperscript{17}. Separate investigations of EGFR inhibition in NSCLC\textsuperscript{6} and ovarian cancer\textsuperscript{18} models identified activation of STAT3 as being critical to the drug-tolerant state. More recently, YAP activation was observed in drug-tolerant PC9 cells co-treated with EGFR and MEK inhibitors\textsuperscript{19}, and several other cell line/anti-EGFR therapy combinations\textsuperscript{20}. Despite many of these studies being conducted in highly similar contexts, each concludes by promoting the benefit of pharmacological inhibition of their individual proposed mechanism of drug tolerance. Our study is the first to observe activation of several diverse pathways in cancer cells adapting to targeted EGFR or BRAF inhibition.

Our observation that drug-tolerant cells activate multiple alternate growth signaling pathways raises an interesting question: are these pathways simultaneously upregulated in each surviving cell, or are sub-populations of cells each strongly upregulating a single pathway such that multiple pathways appear upregulated when cells are pooled prior to bulk analyses? The scRNA-seq data we presented supports the former scenario and suggest that the adaptive response to a molecular targeted therapy is more complicated than a switch to a single “bypass” growth signaling pathway. Although co-activation of two alternate signaling pathways has been proposed\textsuperscript{21}, the overwhelming majority of studies focus solely on a secondary pathway driving drug tolerance. The full complement of changes that accompany the emergence of drug tolerance seems largely underestimated, which may explain why dual-targeting strategies have not made a significant clinical impact.

This activation of multiple alternate growth signaling pathways is likely an important cellular response to the sudden and sustained crash in mTOR signaling that we observed in drug-treated cells. Cell division in targeted therapy was once thought to be a simple case of incomplete inhibition, or re-activation of MAPK growth signaling\textsuperscript{22}. However, our proposal of alternate signaling pathways becoming activated is in agreement with more recent studies showing that MAPK-reactivation does not occur in BRAF(V600E) melanoma cells that cycle slowly in vemurafenib\textsuperscript{3}. We speculate that drug-tolerant cells compensate for a blockade in mTOR signaling by activating alternate growth signaling pathways to continue cell cycle signaling despite the presence of targeted therapy.
Not only have we and several others observed slow proliferation in \textit{EGFR}- and \textit{BRAF}-mutant cells lines treated with targeted therapies\textsuperscript{2}, but slow cell cycle appears to be a highly conserved feature of drug tolerant cells. Studies in pathogenic yeast have shown inhibition of TORC1 growth signaling induces fungal persistence\textsuperscript{23,24}. Similarly, studies in bacteria have highlighted the importance of a switch to a dormant, or slow growth state to enable antibiotic persistence\textsuperscript{25}.

In our study, we also observed activation of several cellular mechanisms that promote survival and are associated with slow growth, such as autophagy and senescence. Similar to our findings, second-generation EGFR-inhibitors have been shown to induce autophagy in NSCLC cells, and functional studies show combination with chloroquine enhances cell killing\textsuperscript{26}. Although we also observed chloroquine's ability to enhance gefitinib-killing of PC9 cells, our CRISPR-mediated disruption of ATG5-ATG12 association casts some doubts on the actual mechanism of increased cell death. We were initially surprised to observe a greater number of drug-tolerant ATG5-modified cells, however, functional genetic studies performed in BRAF(V600E) melanoma cell lines also showed that increased resistance - rather than increased cell death – occurred when mechanisms promoting autophagy were disrupted\textsuperscript{27}. In fact, RNAi-rescue experiments in that study showed that induction of autophagy was critical for BRAF inhibitor-induced cell death\textsuperscript{27}. Conflicting results obtained with pharmacological agents on the one hand and genetic disruption methods on the other suggests that off-target pharmacological effects are likely to be involved in some cases of strategies proposed to block drug-tolerance.

Autophagy and senescence have overlapping regulatory mechanisms and have been proposed to function together to promote chemoresistance by modulating tumor dormancy\textsuperscript{28}. We observed features of cellular senescence in NCSLC cells following EGFR inhibition and melanoma cells following BRAF inhibition. Other groups have shown a senescent-like state in response to EGFR inhibition\textsuperscript{19,29}. Additionally, we and others have reported benefits of co-treating gefitinib-tolerant PC9 cells with “senolytic” compounds to increase cell killing\textsuperscript{30}. However, our genetic studies against the pharmacological target casts doubt on whether these drugs are acting through their intended mechanisms. Although BCL-XL inhibitors have been used to enhance the efficacy of EGFR TKIs\textsuperscript{31}, our BCL-XL CRISPR knockouts survive at a higher rate, suggesting off-target drug effects may be a concern worthy of future investigation. Moreover, it should be noted that although we observed fewer drug-tolerant cells both when using the YAP inhibitor CA3, and a YAP1-knockout cell line, the greater killing efficiency observed with CA3 may indicate that some fraction of the effects is due to off-target mechanisms. In studies that use CRISPR knockout cell lines to validate drug targets\textsuperscript{19}, it is still worth performing side-by-side comparisons to quantify how much of the drug-killing is due to the disruption of the target versus off-target effects. Taken together, we propose that these observations should serve as a cautionary tale for studies using pharmacological inhibitors without complete genetic validation studies. Additionally, as numerous off-target effects are associated with RNAi methods\textsuperscript{32,33}, the lack of rescue assay should be considered when validating therapeutic targets of dual-treatment strategies.
A major concern of dual-treatment strategies is the buildup of toxic ROS, which triggers apoptosis\(^34\). This is particularly relevant due to the emerging role ROS-mediated cell death signaling likely has in drug-tolerant cells\(^35\). We and other groups have observed the ability of the ROS scavenger N-acetylcysteine to not only enhance drug-tolerance, but to blunt the effects of co-treatment strategies designed to target drug tolerant persisters\(^36\). These findings further highlight the importance of supporting any proposed co-treatment strategies with appropriate genetic validation studies to ensure any observed increase in persister cell death is not simply due to drug combinations resulting in lethal ROS toxicity.

To conclude, although we identified involvement of PLC/PKC signaling in the ability of drug tolerant cells to continue slow proliferation, our finding of numerous alternate mitogenic growth signaling pathways becoming activated simultaneously has made us hesitant to propose a treatment strategy. Rather, our study uncovers novel aspects of persister cell biology and provides a new framework for understanding how cells adapt to targeted therapy. With this in mind, we reason future strategies aimed to block the drug-tolerant phenotype will need to exploit on mechanisms other than targeting a single growth signaling pathway in order to elicit durable, long term responses in cancer patients receiving targeted therapies.

**Methods**

**Cell culture**

PC9 cells (obtained from Sigma) were cultured in RPMI (Thermo) supplemented with 10\% FBS (v/v) (Gemini) and 1\% Penicillin-Streptomycin (Thermo). SKMEL28 cells (obtained from ATCC), were cultured in MEM (Thermo) supplemented with 10\% FBS (v/v) and 1\% Penicillin-Streptomycin. Drug-naive, early passage PC9 and SKMEL28 cells were clonally expanded and cryopreserved for drug tolerance studies. Cell lines were maintained at 37°C in 5\% CO\(_2\). Media changes were performed every 2-3 days.

To generate early drug tolerant cells, PC9 and/or SKMEL28 cells were plated on 100 mm plates (1 x 10\(^5\) cells/plate) and allowed to attach for 16 hours. Media was replaced with fresh growth media containing gefitinib (Selleck Chemicals) (5 \(\mu\)M) or dabrafenib (Selleck Chemicals) (5 \(\mu\)M) for PC9 or SKMEL28 cells respectively. Cells were cultured for the indicated amount of time and media changes were performed every 2-3 days.

**Drug Co-Treatment Studies**

For chloroquine co-treatment studies, PC9 cells were plated in 96 well plates at 5 x 10\(^4\) cells/well in regular growth media (RPMI supplemented with 10\% FBS (v/v) and 1\% Penicillin-Streptomycin) and
allowed to attach for 16 hours. Media was replaced with regular growth media with or without gefitinib (5 μM), containing either chloroquine (Neta Scientific) (25 μM) or DMSO. Cells were treated for 6 days (with media changes performed every 3 days) and then quantified via CyQUANT Direct Cell Proliferation Assay (Thermo).

For senolytic co-treatment studies, PC9 cells were first plated in 96 well plates at 1 x 10^4 cells/well, in regular growth media (RPMI supplemented with 10% FBS (v/v) and 1% Penicillin-Streptomycin) and allowed to attach for 16 hours. Media was replaced with regular growth media with or without gefitinib (5 μM) containing either ABT263 (12.5 μM), A1331852 (12.5 μM), or DMSO as a control. Cells were grown for 3 days and then quantified via CyQUANT Direct Cell Proliferation Assay (Thermo).

**RNA Preparation and Bulk RNA-Seq**

PC9 cells were treated with gefitinib (5 μM), and SKMEL28 cells with dabrafenib (5 μM) for 3 and 9 days (with media changes performed every 3 days). Cells were cultured in regular media for 3 days as untreated controls. Cell plating was staggered such that RNA from 3-day treated, 9-day treated, and untreated control samples was extracted from cells simultaneously, using RNeasy Mini Kit (QIAGEN). RNA concentration was measured with a Qubit 4 Fluorometer and associated reagents (Thermo). Library preparation was performed using the KAPA mRNA HyperPrep Kit (Roche). An Agilent Bioanalyzer was used to verify the RNA concentration and assess RNA sample quality. Samples were multiplexed and sequencing was performed with 76 bp, single-end reads to an estimated depth of approximately 10 million reads, using the Illumina NextSeq500. A total of 18 samples were sequenced, three time points in triplicate, across two cell lines.

**Bulk RNA-seq Data Analysis and Visualization**

FASTQ files for each cell line were individually quantified using the Salmon pipeline (version 1.2.1) according to the developer's instructions. The most up-to-date reference transcriptome assembly at the time of analysis (GRCh38.p13, release 33) was obtained directly from Gencode. Salmon quantification files were imported directly into RStudio (version 1.1.456) running R (version 3.6.1) using the tximport package (version 1.14.2).

Differential gene expression and transcript abundance was determined using the DESeq2 pipeline (version 1.26.0). Significant gene expression was determined by DESeq2 Wald statistic with a padj < 0.05. Heatmaps were generated with the ComplexHeatmap package (version 2.2.0) using r-log (regularized log)
transformed DESeq2 data. Bar plots of the mean normalized transcript abundance with error bars representing the standard error of the mean were generated using the *ggpubr* package (version 0.3.0).

Gene Set Enrichment Analysis was performed in R using the *fgsea* package (version 1.12.0). Genes with a padj < 0.05 were ranked based on Wald statistic and 1,000 permutations were performed for each analysis. GSEA plots were made using the *clusterProfiler* package (version 3.14.3).

**Single-Cell RNA-Seq**

PC9 cells were treated with gefitinib, and SKMEL28 cells with dabrafenib for 24, 48, and 72 hours, and regular media for 24 hours as an untreated control. Plating and dosing were staggered such that sample collection from each time point could be performed on the same day. Upon collection, cell media was removed, and cells were briefly washed with DPBS (Gibco). Cells were incubated at 37°C for 4 minutes in 1 mL of TrypLE (Gibco) to fully detach. 5 mL of growth media was used to neutralize the TrypLE and cells were spun at 300 x G for 5 minutes. Cell pellets were resuspended in 1 mL cold DPBS and cell counting was performed using a Nexelcom Cellometer.

Single-cell suspensions were simultaneously subjected to 3’ single-cell RNA-sequencing, using the 10x Chromium platform (10x Genomics). According to the manufacturer's recommended protocols for the Single Cell 3’ Reagent Kit v2 (10x Genomics), cells samples were loaded into the Single Cell A chip (10x Genomics) with a recovery target of ~3,000 cells. Single Cell 3’ gel beads and emulsion oil (both from 10x Genomics) were added to the Single Cell A chip, and a single reaction (for each cell line time course) was run on the 10x Controller to generate single-cell gel beads in emulsions (GEMS). Once GEMs were generated, reverse transcription reactions generated individually barcoded cDNA. cDNA cleanup was performed with Silane DynaBeads (Thermo Fisher) and then subjected to 12 amplification cycles, according to 10x Genomics protocol recommendations. Post-cDNA amplification reaction cleanup was performed with SPRIselect beads (Beckman Coulter). Quantification of post-amplification cDNA was performed with Bioanalyzer High Sensitivity chip. Pooled libraries were then sequenced on the Illumina Hi-Seq 4000 platform using paired-end sequencing with dual indexing, as recommended by the 10X Genomics protocol.

**scRNA-seq Data Pre-processing**

The CellRanger 3.0.1 analysis pipeline was used to process Chromium single-cell RNA-seq output to align reads, generate feature-barcode matrices. Feature-barcode matrices for each time point were imported
individually and merged using the R package “Seurat” (version 3.0)\textsuperscript{37,38}. Each cell line and targeted therapy combination was processed individually. Cells with low features (less than 750 UMI counts), potential doublets (cells containing greater than 6000 UMI counts), and dead/dying cells (over 20% mitochondrial-derived UMI counts) were filtered out prior to analysis. Following these quality control processes, a total of 10,403 single PC9 cells and 10,891 single SKMEL28 cells, were used in downstream analyses. Normalization and variance stabilization was performed using SCT transformation\textsuperscript{39}.

**scRNA-seq Analysis**

Several dimensionality reduction methods were used to explore our single-cell RNA-seq. Principal component analysis was performed using the built-in “RunPCA” function in the Seurat package. Scatter plots of the PCA analysis were generated using the “DimPlot” command, specifying “pca” as the reduction method. Similarly, tSNE analysis was performed using the built-in “RunTSNE” function of the Seurat package. tSNE analysis was performed using principal components 1-13, which were deemed significant when evaluating elbow plots of the top 40 principal components. Finally, UMAP reduction was performed using the python-based “umap-learn” package. This was performed in R using the “reticulate” package and the “RunUMAP” command built into Seurat version 3. The number of neighbors was set to 15 (for PC9 cells) and 50 (for SKMEL28 cells). The number of epochs used was 500, and the minimum distance was set to 0.3 for each cell line. The first 13 principal components were used. Generation of clusters and identification of top cluster markers was performed using the built-in functions of the Seurat package.

Cluster marker genes were identified using the Seurat command “FindAllMarkers” with a logfc threshold of 0.25 (only included genes with an increase in transcript count) and an adjusted p-value of < 0.01.

Single-cell RNA-seq gene-gene relationships were visualized using the python-based tool, MAGIC\textsuperscript{40}. scRNA-seq counts were imported, normalized, and transformed using the default parameters. MAGIC was run on the data using the default values (knn=5, knn_max=3*knn, decay=1, t=3). Gene-gene relationships were plotted, with each point colored by time in drug.

**Reverse Phase Protein Array Analysis**

PC9 cell plating and drug time courses were staggered such that cells were pelleted simultaneously, prior to RPPA analysis. For each sample, three 100 mm plates were pooled prior to pelleting to ensure enough bulk material was collected. Cell pellets were immediately stored at -80 C and shipped on dry ice to the
RPPA Core Facility at MD Anderson Cancer Center. Protein extraction, reverse phase protein array, and quality control was performed on a total of 12 samples (untreated = 3, 3-day gefitinib = 3, 6-day gefitinib = 3, and 9-day gefitinib = 3) by the RPPA Core Facility. Heatmaps of normalized, log2 values were generated with the complexHeatmaps package in R. The scatter plot of RPPA and RNA data was generated using the differences in the mean of normalized linear log2 centered values between 9-day gefitinib-treated and untreated PC9 cells. Log-fold change values for RNA transcripts were generated using DESeq2. Boxplots of protein levels were generated with ggpubr, using the mean, log2-centered values for each time point.

Antibodies and immunoblotting

The following antibodies were used for immunoblotting: Phospho-S6 Ribosomal Protein Ser235/236 Rabbit mAb (CST, #4858), Phospho-S6 Ribosomal Protein Ser240/244 Rabbit mAb (CST, #5364), BCL-xL (CST #2764), ATG5 (CST #12994) Beta-Actin Rabbit mAb (CST, #4970), and Anti-rabbit IgG HRP-linked Antibody (CST #7074). The antibodies were diluted 1:1000 for immunofluorescence and immunoblotting.

Cells were detached by briefly washing with DPBS and incubating at 37 C for 4 minutes in 1 mL of TrypLE. 5 mL of growth media was used to neutralize the TrypLE and cells were spun at 300 x G for 5 minutes. Cells pellets were placed on ice and lysed with RIPA buffer (Thermo) containing Halt Protease and Phosphatase Inhibitor (Thermo). Cell lysate was quantified using Bradford assay (Bio-Rad) prior to addition of Bolt Reducing Agent (500 mM DTT) and LDS (Thermo). Samples were separated on 4-12% Bolt Bis-Tris Plus Gels (Thermo) and subsequently transferred to a nitrocellulose membrane using the iBlot2 transfer module (Thermo). The blot was washed with TBST (Bio-Rad) and then blocked with 5% w/v BSA (Sigma) for 60 minutes at RT. After a brief wash with TBST, blots were incubated with primary antibody (1:1000) overnight at 4 C. The following day, blots were washed with TBST 4 times and then incubated with HRP-linked secondary antibody (1:5000) at RT for 1-2 hours. Following another 4 washes with TBST, immunoreactive bands were detected using the Clarity ECL substrate (Bio-Rad).

CRISPR studies

gRNA sequences (Supplemental Table 1) were designed and ordered as custom DNA oligos (Thermo). gRNA components were annealed and cloned into the lentiCRISPRv2 plasmid (Addgene ##52961), according to the instructions from the Zhang Lab41. lentiCRISPRv2, along with helper plasmids VSVG (Addgene #8454), and psPAX2 (Addgene #12260), were transfected into 293T cells using ProFection
Mammalian Transfection System (Promega). Packaged lentivirus was harvested and transduced into PC9 cells (5 x 10^4 cells/well in 6 well plates). Following 9-day selection in regular growth media supplemented with puromycin (5 μg/mL), surviving cells were plated in 96 well plates (at 0.5 cells/well) to generate clonal colonies. Single-cell colonies were verified via inspection with a microscope and cells were expanded, with media being changed every 3 days. Expanded colonies were passaged from 96 well to 24 well plates, and eventually expanded in 100 mm dishes prior to cryopreservation and use in future studies.

ATG5 and BCL-XL CRISPR-modified cell lines, as well as control PC9 cells, were plated in 96 well plates at 5 x 10^4 cells/well in regular growth media and allowed to attach for 16 hours. Media was replaced with growth media with or without gefitinib (5 μM). Cells were grown for 3 days and then quantified via CyQUANT™ Direct Cell Proliferation Assay (Thermo) to assess differences in cell numbers between control and CRISPR-modified PC9 cell lines.

**Visualizing autophagy and senescence**

To visualize autophagy or senescence in cells developing drug tolerance, PC9 cells were plated in 6-well plates at 1.15 x 10^4 cells/well in regular growth media and allowed to attach for 16 hours. Media was replaced with regular growth media with or without gefitinib (5 μM). Media was replenished every 2-3 days. At the indicated time in drug, cells were stained to visualize autophagy or senescence. Autophagic flux was visualized using the CYTO-ID Autophagy Detection Kit (ENZO, #ENZ-51031-0050). Staining was performed according to the manufacturer's instructions. Representative brightfield, DAPI, and GFP images were captured for each well and channels were merged using ImageJ.

Senescence was visualized using the commercially available Cellular Senescence Activity Assay (Enzo, #ENZ-KIT129-0120) at each indicated time point, according to the manufacturer's instructions. Representative brightfield images were captured for each well.

To visualize the effects on autophagy of the ATG5 CRISPR-modification, relative to parental PC9 cells, each cell line was plated at 7.5 x 10^5 cells/well in 6 well plates in regular media and allowed to attach for 16 hours. Negative control cells were grown in regular growth media and positive control cells were grown starvation media consisting of normal growth media supplemented with rapamycin (500 nM) and chloroquine (10 μM), according to the manufacturer's instructions. Regular growth media was replaced with starvation media 16 hours prior to imaging to induce autophagic signal. Autophagy was visualized using the CYTO-ID Autophagy Detection Kit (ENZO, ENZ-51031-0050). Staining was performed according to the manufacturer's instructions. Representative brightfield, DAPI, and GFP images were captured for each well and channels were merged using ImageJ.
Data Availability

Raw FASTQ files for bulk and single-cell RNA-seq studies, along with processed data files are available for download from the Gene Expression Omnibus (GSE162045).

Declarations

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Author Contributions

F.C. and S.P. together planned the project, interpreted data, and wrote the manuscript. Additionally, F.C. performed experiments and bioinformatic analysis.

References


Figures

Figure 1
Numerous transcriptional alterations are shared throughout the early development of drug tolerance. a Heatmap of shared, significantly altered genes in PC9 cells in gefitinib at 0, 3, and 9. b Heatmap of shared, significantly altered genes in SKMEL28 cells in dabrafenib at 0, 3, and 9 days. c Scatter plot of log-fold change values for significantly altered genes (adj. p-value < 0.05) in PC9 cells treated with gefitinib and SKMEL28 cells treated with dabrafenib. Each point represents an individual gene, and its color corresponds to the status in each cell line. d PCA plot of PC9 cell samples with each point representing a unique sample, colored by time spent in gefitinib (UT = untreated control). The first principal component (PC1) is associated with 74% of the variance and separates samples based on presence or absence of drug. e PCA plot of SKMEL28 cells with each point representing a unique sample, colored by time spent in dabrafenib (UT = untreated control). The first principal component (PC1) is associated with 65% of the variance.
Figure 2

Proteomic alterations during development of drug tolerance. a Heatmap showing unsupervised clustering of all significantly altered (p-value < 0.05) phospho-proteins probed by RPPA. Columns represent the average (n = 3) for each indicated time point, and row colors correspond to the z-score. b Scatter plot comparing the log-fold change values of mRNA (day 9 versus untreated) versus the change in protein (day 9 versus untreated) quantified by RPPA, in PC9 cells in gefitinib. Several members that are strongly upregulated in both data sets are labelled. c RPPA quantification of fibronectin protein in PC9 cells in gefitinib for various times (UT = untreated). d Bar chart of the normalized transcript abundance levels of...
the fibronectin gene (FN1) in PC9 cells in gefitinib for 0 (UT), 3, and 9 days. c,d Data are represented as the mean ± SEM (n = 3). Two-tailed Student’s t test were used for comparisons between two groups (untreated vs time point) for statistical analyses. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Targeted therapy induces sustained mTOR suppression in drug-tolerant cells. a GSEA NES scores indicate the Reactome gene set “mTOR signaling” is enriched in untreated PC9 cells when compared to PC9 cells treated with gefitinib for 9 days. b GSEA NES scores indicate the Hallmark gene set “PI3K/AKT/mTOR signaling” is enriched in untreated SKMEL28 cells when compared to SKMEL28 cells treated with dabrafenib for 9 days. c,d Normalized transcript abundance levels of several genes associated with mTOR signaling including RPS6, EIF4E, EIF4EG1, and EEF2K in PC9 cells in gefitinib (c) or SKMEL28 cells in dabrafenib (d) for 0 (UT), 3, or 9 days. e RPPA quantification of proteins and phospho-proteins associated with mTOR signaling, including mTOR, phospho-mTOR (S2848), eIF4E, eIF4E (S209), p70-S6K1, phospho- p70-S6K (T389), S6, and phospho-S6 (S240/S244) from PC9 cells in gefitinib for 0, 3, 6, or 9 days. f Western blot analysis of phospho-S6 (S235/236) and phospho-S6 (S240/244) from untreated SKMEL28 (UT) and SKMEL38 cells in dabrafenib (D) for 24 hours (top) or 72 hours (bottom). c,d Data are represented as the mean + SEM (n = 3). Two-tailed Student’s t test were used for comparisons between two groups (untreated vs time point) for statistical analyses. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns = P > 0.05.
Figure 4

Targeted therapy induces activation of the IGF signaling pathway in drug-tolerant cells. a GSEA NES scores indicate the gene set “Targets of IGF1 and IGF2 Up” is significantly enriched in PC9 cells treated with gefitinib for 9 days, relative to untreated PC9 cells. b GSEA NES scores indicate the gene set “Targets of IGF1 and IGF2 Up” is significantly enriched in SKMEL28 cells treated with dabrafenib for 9 days, relative to untreated SKMEL28 cells. c RPPA quantification of proteins and phospho-proteins associated with IGF signaling, including IGFRb, phospho-IGF1R (Y1135/6), and IGFBP3, from PC9 cells in gefitinib for 0, 3, 6, or 9 days. d,e Normalized transcript abundance levels of several genes associated with IGF
signaling including IGF1R, IGFBP3, IGFBP5, and IGFBP7 in PC9 cells in gefitinib (d) or SKMEL28 cells in dabrafenib (e) for 0 (UT), 3, or 9 days. d,e Data are represented as the mean + SEM (n = 3). Two-tailed Student’s t test were used for comparisons between two groups (untreated vs time point) for statistical analyses. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns = P > 0.05.

Figure 5

Targeted therapy induces activation of PLC/PKC signaling in drug-tolerant cells. a GSEA NES scores indicate the Reactome gene set “Signaling by GPCR” is significantly enriched in PC9 cells treated with...
gefitinib for 9 days, relative to untreated PC9 cells. b GSEA NES scores indicate the GO gene set “Regulation of Phospholipase Activity” is significantly enriched in SKMEL28 cells treated with dabrafenib for 9 days, relative to untreated SKMEL28 cells. c RPPA quantification of proteins and phospho-proteins associated with PLC/PKC signaling, including phospho-PLC-gamma2 (Y759), PKCa, and PKCa/b (T638/641), from PC9 cells in gefitinib for 0, 3, 6, or 9 days. d,e Normalized transcript abundance levels of several genes associated with PLC/PKC activity including ADCY6, GNAL, GNAS, PDE1C, and PLCG1 in PC9 cells in gefitinib (d) or SKMEL28 cells in dabrafenib (e) for 0 (UT), 3, or 9 days. d,e Data are represented as the mean + SEM (n = 3). Two-tailed Student’s t test were used for comparisons between two groups (untreated vs time point) for statistical analyses. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns = P > 0.05.
scRNA-seq analysis reveals markers of several alternate mitogenic signaling pathways are simultaneously upregulated during the development of drug tolerance. a,d Markers of IGF signaling (IGF1R or IGFBP7) and JAK/STAT signaling (STAT3 or JAK1) are simultaneously upregulated in single PC9 cells treated with gefitinib (a) or single SKMEL28 cells treated with dabrafenib (d). b,e Markers of IGF signaling (IGF1R or IGFBP7) and YAP signaling (CYR61 or VIM) are simultaneously upregulated in single PC9 cells treated with gefitinib (b) or single SKMEL28 cells treated with dabrafenib (e). c,f Markers of IGF signaling (IGF1R or IGFBP7) and PLC/PKC signaling (GNAS) are simultaneously upregulated in single PC9 cells treated with gefitinib (c) or single SKMEL28 cells treated with dabrafenib (f).
Figure 7

Pharmacologic, but not genetic disruption of autophagy results in increased gefitinib killing. a Quantification of PC9 cells in normal media treated with DMSO or 25 μM Chloroquine. b Quantification of PC9 cells in gefitinib media treated with DMSO or 25 μM Chloroquine. a,b Data are represented as the mean + SD (n = 10). Two-tailed Student’s t test were used for comparisons between two groups for statistical analyses. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns = P > 0.05. c Western blot
analysis of ATG5 protein from parental PC9 cells (PC9 Ctrl.) or clonal populations (C1-C8) of CRISPR-ATG5 PC9 cells. d Autophagic flux in parental PC9 cells (PC9 Ctrl.) and CRISPR-ATG5 PC9 cells in normal growth media and autophagy-inducing starvation media, visualized via CYTO-ID Autophagy detection kit.
e Quantification of parental PC9 cells and CRISPR-ATG5 PC9 cells (Clone 2) normal media. f Quantification of parental PC9 cells and CRISPR-ATG5 PC9 cells (Clone 2) gefitinib media. e,f Data are represented as the mean + SD (n = 8). Two-tailed Student’s t test were used for comparisons between two groups for statistical analyses. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns = P > 0.05.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigures.docx