PSMG2-controlled proteasome-autophagy balance mediates the tolerance for MEK-targeted therapy in triple-negative breast cancer

Graphical abstract

Highlights

- Hyperactivated AKT pathway confers MEK inhibitor resistance in TNBC

- Proteasome blockade promotes autophagy-mediated PDPK1 degradation

- Activation of autophagy sensitizes TNBC to MEK inhibition

- The combination of MEK and proteasome inhibitors synergistically suppresses TNBC growth

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In brief

By means of high-throughput screening, Wang et al. show that a proteasome blockade sensitizes TNBCs to MEK-targeted therapy through autophagy-mediated degradation of PDPK1. The combination of MEK and proteasome inhibitors synergistically suppresses tumor proliferation in vitro and in vivo.

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PSMG2-controlled proteasome-autophagy balance mediates the tolerance for MEK-targeted therapy in triple-negative breast cancer

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SUMMARY

Although the MAPK pathway is aberrantly activated in triple-negative breast cancers (TNBCs), the clinical outcome of MEK-targeted therapy is still poor. Through a genome-wide CRISPR-Cas9 library screening, we find that inhibition of PSMG2 sensitizes TNBC cells BT549 and MB468 to the MEK inhibitor AZD6244. Mechanistically, PSMG2 knockdown impairs proteasome function, which in turn activates autophagy-mediated PDPK1 degradation. The PDPK1 degradation significantly enhances AZD6244-induced tumor cell growth inhibition by interrupting the negative feedback signals toward the AKT pathway. Consistently, cotargeting proteasomes and MEK with inhibitors synergistically suppresses tumor cell growth. The autophagy inhibitor chloroquine partially relieves the PDPK1 degradation and reverses the growth inhibition induced by combinatorial inhibition of MEK and proteasome. The combination regimen with the proteasome inhibitor MG132 plus AZD6244 synergistically inhibits tumor growth in a 4T1 xenograft mouse model. In summary, our study not only unravels the mechanism of MEK inhibitor resistance but also provides a combinatorial therapeutic strategy for TNBC in clinics.

INTRODUCTION

Triple-negative breast cancer (TNBC), which is pathologically negative for estrogen receptors, progesterone receptors, and human epidermal growth factor receptor-2, accounts for 15%–20% of all breast carcinomas.1 Compared with other subtypes, the typical characteristics of TNBC are earlier age of onset, greater metastatic potential, faster relapse, and lower survival rates.1,2 The lack of receptors makes it unresponsive to endocrine therapy and HER2-targeted therapy. To date, chemotherapy, radiotherapy, and surgery remain the standard of care for TNBC. Although some early-stage patients benefit from these canonical treatments, the overall clinical prognosis is still poor.3–7

Fortunately, targeted therapy and precision medicine have shown promising outcomes in the clinical treatment of TNBC for decades. Several regimens targeting PARP-1, EGFR, FGFR2, and VEGF have been developed, either as a single agent or in combination with chemotherapy.8–10 Despite exciting efficacy in the preclinical and early-stage clinical studies, a clear benefit from these regimens cannot be confirmed in large-cohort clinical trials. Therefore, the identification of novel targets and the development of efficient combinatorial regimens are highly desired for improving long-term outcomes, especially in cases where surgery is not an option. The mitogen-activated protein kinase (MAPK) pathway was reported as one of the most frequently hyperactivated pathways in human breast cancer.11 Aberrant activation of the MAPK pathway results in resistance to chemotherapeutics, promotion of immune evasion, reduction of overall survival time, and high risk of recurrence in patients with TNBC.12–15 In this linear kinase signaling cascade, small molecules targeting MEK1/2 have been well researched and developed by pharmaceutical companies.16 These MEK inhibitors (MEKis) have shown promising efficacy in colorectal cancer, non-small cell lung cancer, and anaplastic thyroid cancer.17–19 One of them (trametinib), as a single agent or in combination with the BRAF inhibitors, has been approved by the US FDA to treat BRAF-mutated melanoma.20
However, these MEKis, either alone or combined with paclitaxel, show modest antitumor activity in TNBC clinical trials. A potential explanation reported by scientists is that the rapidly activated compensatory pathway confers drug resistance to these single-agent therapies. Therefore, illumination of these resistance mechanisms will pave the way for the optimization of novel combination regimens.

RESULTS

Screening MEK inhibitor synthetic lethal targets in TNBCs

To identify the compensatory pathway and the potential resistance-driver (or synthetic lethal) genes, we carried out a genome-wide CRISPR-Cas9 knockout library screening in TNBC model cell lines that do not respond to MEKis. We first assessed the drug sensitivity of three TNBC cell lines (BT549, MB468, and MB231) to the selective MEKis selumetinib (AZD6244) and trametinib by short-term cell viability assay in vitro (Figures S1A and S1B). The data showed that MB231 cells were sensitive to AZD6244 and trametinib, whereas BT549 and MB468 cells barely responded to AZD6244 (up to 10 μM) or trametinib (up to 1 μM). In addition, the long-term response of TNBC cells to AZD6244 determined by clonogenic assay showed that BT549 and MB468 cells could tolerate AZD6244 up to 1 μM (Figure S1C). The western blot probing the activities of the MAPK pathway indicated that the signaling was sufficiently blocked by AZD6244 at 1 μM (Figure S1D). These results indicated that BT549 and MB468 cells could serve as model cell lines for synthetic lethal gene screening. Then, we performed lentivirus-based CRISPR-Cas9 library screening on BT549 cells with the optimized concentration of AZD6244 (1 μM). After infection with the GeCKO v.2 library, BT549 cells were split into three parts. One group was directly applied to single-guide RNA (sgRNA) sequencing and labeled as DMSO-Day0. The other two groups, labeled as DMSO-Day7 and AZD-Day7, were treated with DMSO and AZD6244, respectively. The cells were collected and subjected to next-generation sequencing (NGS) of the sgRNA sequences on day 7 (Figure 1A).

The sequencing data were analyzed using the procedures shown in Figure S1E. In principle, the synthetic lethal genes should be downregulated in the AZD6244-treated group compared with the DMSO-treated group. We compared the sgRNA data of DMSO-Day7 and AZD-Day7, 1,649 genes were significantly downregulated in the AZD-Day7 group. In the scatter diagram, the color and size of the dots indicate the number of detected sgRNAs for each gene and the down recurrence (sgRNA_{down}/sgRNA_{total}), respectively. (C) The upset diagram shows the numbers of overlapping genes that may drive MEK inhibitor resistance under different criteria. (D) The scatterplot shows that 52 final candidate genes potentially confer tolerance to MEK inhibitors in our model cells. According to the log2 FC, the top 10 genes are marked with different colored dots and gene symbols.
downregulated in the AZD-Day7 group (Figure 1B). By checking against the transcriptome dataset of BT549 (GEO: GSE112365), we determined 684 genes that were efficiently expressed in our model cell line (reads per kilobase per million mapped reads [RPKM] > 10). To evaluate the clinical relevance, we then characterized the expression levels of these genes in the transcriptome datasets obtained by bulk sequencing (BioProject: PRJNA553096) and single-cell sequencing (GEO: GSE161529).
Figure 3. PSMG2 knockdown downregulated PDPK1/AKT signaling

(A) The scatterplot shows the correlation between proteasome and all 50 hallmark gene sets in the two single-cell transcriptome datasets (GEO: GSE75688 and GEO: GSE11838). The x axis and y axis represent the correlation coefficients of proteasome and hallmark gene sets in the GEO: GSE11838 and GEO: GSE75688 datasets, respectively. The size and color of the points represent the \(-\log_{10} p\) value in the GEO: GSE11838 and GEO: GSE75688 datasets, respectively. The points of the top 5 signal pathways in the comprehensive correlation are marked with different colored outer circles.

(B) The scatterplot shows the high correlation between mTOR signaling and proteasome in two independent single-cell transcriptomic datasets (GEO: GSE75688 and GEO: GSE11838). The pink triangles represent the GEO: GSE75688 dataset, and the blue dots represent the GEO: GSE11838 dataset.

(C) After vector (pLKO.1) or PSMG2 shRNA (shPSMG2) transfection, BT549 and MB468 cells were treated with AZD6244 (1 μM) for 2 or 24 h, and then the phosphorylation of ERK in the MAPK pathway and the phosphorylation and total protein of AKT and PDPK1 in the upstream mTOR pathway were detected by western blot. Loading control, β-actin.
The results indicated that 57 genes were recurrently upregulated in tumors (for tissue, adjusted p value ($p_{adj}$) < 0.01, log2FC > 0.5, and for cells, $p_{adj}$ < 0.01, log2FC > 0.2) compared with the normal tissue (or cells) (Figure 1C). To identify the genes driving MEKi tolerance, we also excluded the housekeeping genes, which may independently cause cell death rather than cooperating with AZD6244. To this end, we compared the sgRNA sequencing data of DMSO-Day0 and DMSO-Day7 with the algorithm packages MAGeCK and BAGEL. The significantly downregulated genes (p < 0.05, fitness score <0) in the DMSO-Day7 group were defined as the housekeeping genes (Figure S1F). After excluding these genes, we finally identified 52 candidates that potentially conferred tolerance of MEKi in our model cells (Figure 1C).

**Inhibition of PSMG2 promotes the efficacy of MEK inhibitors**

To verify the function of identified genes in MEKi tolerance, we knocked down 10 genes ranking at the top of 52 candidates and evaluated the cell proliferation in the presence or absence of AZD6244 by clonogenic assay in BT549 and MB468 cells (Figures 1D and 2A). The knockdown efficiency was confirmed by qRT-PCR determining mRNA expression levels of the indicated genes individually (Figures S2A and S2B). The results showed that only PSMG2 (proteasome assembly chaperone 2) gene knockdown could inhibit cell proliferation along with AZD6244 in both drug-resistant TNBC cell lines (Figure 2A). The heterodimer that consisted of PSMG2 and PSMG1 was reported as a chaperone complex that promotes the assembly of 20S proteasome.22 Heterodimer blockade decreases proteasome activity and subsequently suppresses the ubiquitin-proteasome system (UPS)-mediated protein degradation.23 To verify it, we detected the ubiquitination level in the total lysis of TNBC model cells with PSMG2 knockdown or MG132 (a proteasome inhibitor) treatment (Figures 2B and 2C), followed by quantification with Image Lab software (Figure S2C). The results showed that PSMG2 knockdown did promote the whole-cell ubiquitination level in BT549 and MB468. Similar to the result of PSMG2 knockdown, the upregulation of total ubiquitination was also observed in the cells treated with MG132. To verify whether or not the UPS mediates AZD6244 tolerance, we measured the proliferation of TNBC cells in the presence of AZD6244, MG132, or their combination. Consistent with the result of PSMG2 knockdown plus AZD6244, the inhibitory efficiency of the combination regimen was superior to that of either single-agent treatment in BT549, MB468, SUM159, and HCC1937, as well as two MEKi-sensitive cell lines, MB231 and SUM149 (Figures 2D, 2E, and S2D–S2G). We then evaluated the synergy of the combination regimens in a proliferation assay with careful drug titration. The combination index indicated that MG132 and AZD6244 could synergistically inhibit TNBC cell growth (Figures 2F, S2H, and S2I; Table S1). To further confirm that UPS mediates the synergistic inhibition, we carried out a functional rescue assay. PSMG2 was ectopically expressed in TNBC model cell lines with and without MG132 and AZD6244. The results showed that the synergistic inhibition conferred by the combination regimens was partially alleviated by overexpression of PSMG2 (Figures 2G–2I). Moreover, the efficacy of this combinatorial regimen was further verified with the clinically available MEKi trametinib and the proteasome inhibitor bortezomib. Similarly, the combination of trametinib and bortezomib, rather than single-drug treatment, significantly suppressed the growth of MEKi-resistant cells BT549 and MB468 (Figures S2J and S2K). Taken together, these results imply that PSMG2 knockdown potently sensitizes TNBCs to MEKi by impairing the UPS.

**PSMG2 knockdown dampens AKT signaling**

To unravel the mechanism of UPS-regulated drug resistance, we explored the potential signaling pathways related to the proteasome by calculating the correlation coefficients with TNBC transcriptomic datasets (GEO: GSE75688, GEO: GSE11838, TCGA-TNBC, and BioProject: PRJNA553096). By robust rank aggregation (RRA) algorithm analysis,24 we found that the mTOR pathway is the most proteasome-correlated signature among all 50 hallmark gene sets in single-cell (Figure 3A) and bulk transcriptomic profiles (Figure S3A). This conclusion was further confirmed by correlation R-score analysis (Figures 3B and S3B), implying that mTOR signaling is the most recurrent pathway under the regulation of the proteasome.

Previous studies have shown that the mTOR pathway plays a pivotal role in the development of resistance to chemotherapy and targeted therapy in breast cancer.25,26 In line with this, we also found that the activity of the mTOR upstream kinase AKT in MEKi-sensitive cell lines is much lower than that in MEKi-resistant cell lines (Figure S3C). We then detected the expression and phosphorylation levels of key kinases composing this pathway in BT549 and MB468 cells with PSMG2 knockdown and AZD6244 treatment. The results showed that suppression of PSMG2 inhibited the phosphorylation of AKT but did not disturb the total AKT protein level (Figure 3C). The suppression of PSMG2 downregulated the phosphorylation and total level of PDPK1, implying...
that PDPK1 was degraded. Consistently, phosphorylation of S6RP and 4EBP1, the downstream components of the AKT/mTOR pathway, was suppressed in the TNBC cells with PSMG2 knockdown (Figure S3D). Similar to PSMG2 knockdown, MG132 and bortezomib could also suppress the total PDPK1 protein level and the phosphorylation of AKT (Figures 3D and S3E). Here, p21 protein served as an internal control to ensure the efficacy of MG132 and bortezomib. Thereby, we speculated that PSMG2 knockdown inhibited the AKT signaling pathway by reducing total PDPK1 protein levels and thus blocking its downstream kinases, such as AKT.

To verify this speculation, we knocked down PDPK1 with shRNA pools and tested the level of p-AKT by western blot. The results showed that the level of p-AKT decreased upon PDPK1 knockdown.
Figure 5. PDPK1 was degraded by autophagy in TNBC cells

(A) BT549 and MB468 cells were treated with different concentrations of autophagy activator (rapamycin) for 24 h, and then PDPK1 and autophagy-related proteins were detected by western blot. Loading control, β-actin.

(B and C) Clonogenic assays of BT549 and MB468 cells treated with different concentrations of AZD6244 and LiCl (6 mM) for 10–14 days.

(D) After vector (pLKO.1) or PSMG2 shRNA (shPSMG2) transfection, BT549 and MB468 cells were treated with CQ (40 μM) for 8 or 12 h, and then PDPK1 and autophagy-related proteins were detected by western blot. Loading control, β-actin.

(E) After treatment with or without MG132 (2 μM) for 24 h, BT549 and MB468 cells were treated with CQ (40 μM) for 8 or 12 h, then PDPK1 and autophagy-related proteins were detected by western blot. Loading control, β-actin.

(F) Confocal microscopy images show the merged subcellular co-localization images of PDPK1 and LAMP1 of BT549 and MB468 cells. BT549 and MB468 cells treated with MG132 (2 μM) or rapamycin (1 μM) for 24 h or transfected with PSMG2 shRNA (shPSMG2) and treated with or without CQ (40 μM) for 12 h. Nucleus and LAMP1 were stained with DAPI (blue) and Alexa Fluor 594-conjugated wheat germ agglutinin (red), respectively. PDPK1 was stained with Alexa Fluor 488 combined with wheat germ agglutinin (green). White arrows indicated the co-localization between PDPK1 and LAMP1. The scale bar represents 40 μm.

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knockdown without affecting the level of AKT protein (Figure 3E). Interestingly, the feedback activation of ERK was repeatedly observed in the knockdown of PSMG2 and PDPK1 (Figures 3C and 3E). A potential explanation supported by previous reports is that the MAPK and AKT pathways compensate for each other in targeted therapy of multiple cancer types.\(^{27} \) Actually, we did observe the potent activation of AKT upon AZD6244 treatment in BT549, MB468, MB231, and SUM149 cells (Figure 3F). We then investigated the functional contribution of the PSMG2-PDPK1 signaling axis to MEKi tolerance. Similar to the result of PSMG2 knockdown, PDPK1 knockdown, combined with AZD6244, could also inhibit cell proliferation in long-term clonogenic assays followed by quantification (Figures 3F and 3G). To further confirm this observation, we performed functional rescue experiments. Overexpression of PDPK1 in PSMG2-knockdown cells could efficiently compensate for the inhibition of p-AKT induced by PSMG2 knockdown (Figure 3H). More importantly, the overexpression of PDPK1 attenuated the cell growth suppression induced by the combination treatment of PSMG2 knockdown and AZD6244 (Figures 3I–3K). On top of that, we reblocked the pathway with AKT inhibitor (AKTi)-1/2 and found that the cell growth was again suppressed (Figure 3G).

In addition, we also tested whether direct inhibition of AKT kinase may sensitize the TNBCs to MEKi. The results showed that the combination of AKTi-1/2 and AZD6244 synergistically suppressed cell proliferation in BT549, MB468, SUM159, MB231, and SUM149, whereas either AKTi-1/2 or AZD6244 alone showed only a marginal inhibition (Figures S3H–S3K). Interestingly, the synergy of AKTi-1/2 plus AZD6244 seems to be less than that of MG132 plus AZD6244 (Figures 2F and S3L; Table S1). A potential explanation is that the AKTi may induce rebound activation of its own signaling, which dampens the efficacy in sensitizing MEKi.\(^{28–30} \) To validate this speculation, we compared the capabilities of AKT inhibition between these two combinations. Unsurprisingly, the restoration of p-AKT was observed in TNBC cells treated with AKTi-1/2 plus AZD6244, whereas no visible p-AKT rebound was observed up to 72 h after the initial response to MG132 plus AZD6244 treatment (Figures S3M and S3N). These results suggested that MG132 plus AZD6244 is superior to AKTi-1/2 plus AZD6244 in terms of TNBC growth inhibition. Taken together, these results indicate that blockade of PSMG2 inhibits the proteasome, which in turn downregulates the AKT pathway by decreasing the total protein level of PDPK1.

### Blockade of PSMG2 activates autophagy of TNBCs

The UPS is the predominant machinery for intracellular protein degradation in eukaryotic cells. In general, the impairment of the proteasome leads to the accumulation of ubiquitinated proteins.\(^{31–33} \) Contradictorily, our previous results indicated that blockade of the proteasome (PSMG2 knockdown or MG132 treatment) resulted in a decrease (but not increase) in total PDPK1 protein level (Figures 3C and 3D). To investigate the mechanism underlying this paradox, we evaluated the transcriptional and translational efficiency of PDPK1 upon PSMG2 knockdown by mRNA qRT-PCR and ribosome-bound mRNA qRT-PCR, respectively. The results showed that no significant alterations in PDPK1 were observed at either the mRNA level (Figure 4A) or the ribosome-bound mRNA level (Figure 4B). These results highlighted the possibility that protein degradation rather than protein generation accounted for PSMG2 knockdown-mediated PDPK1 decrease. Since our data excluded the possibility of UPS-mediated PDPK1 degradation, we then sought an alternative pathway. Protein degradation in mammalian cells is controlled by a reciprocal balance between the UPS and the autophagy-lysosomal system (ALS).\(^{14,30} \) To explore whether the balance was under the control of PSMG2, we detected the autophagy-related proteins in our model cells. Compared with the control group, PSMG2 knockdown or proteasome inhibitor treatment increased the LC3-II:I ratio and the accumulation of lysosomal-associated membrane protein 1 (LAMP1), indicating that autophagy is activated (Figures 4C–4E). To further confirm it, we visualized the ultrastructure of cells by transmission electron microscopy (TEM). Upon PSMG2 knockdown, we observed a series of canonical phenomena of autophagy, including a large number of autophagosomes, dilation of the endoplasmic reticulum (ER), and shrinkage (malformation) of mitochondria (Figure 4F). We then validated these phenotypes at the molecular level. Not surprisingly, ATF4 and phosphorylation of eIF2α increased in BT549 and MB468 cells with PSMG2 knockdown (Figure 4G). These data suggest that PSMG2 plays a pivotal role in controlling the compensatory balance between the UPS and the ALS in our TNBC model cells.

### PDPK1 degradation is mediated by autophagy

We then sought to make clear whether the degradation of PDK1 was mediated by PSMG2-controlled autophagy. We first measured the alteration of PDPK1 level upon treatment with rapamycin and lithium chloride (canonical autophagy activators). The results showed that both rapamycin and lithium chloride poiently downregulated the level of PDPK1 (Figures 5A and 5A). Consistently, the combination of AZD6244 and lithium chloride synergistically suppressed the proliferation of BT549 and MB468 (Figures 5B, 5C, and 5D). Then, chloroquine (CQ; an autophagy inhibitor) was applied to block the autophagy, and PDK1, along with autophagy markers, was probed by western blot. The results showed that the downregulation of PDPK1 induced by MG132 treatment or PSMG2 knockdown was partially alleviated by the addition of CQ in BT549 and MB468 cells (Figures 5D and 5E). Meanwhile, the alteration of autophagy markers indicated that CQ potently suppressed autophagy. To further confirm that the degradation was mediated by ALS, we visualized the PDPK1 along with LAMP1, a canonical lysosome marker. The results showed that a relatively specific co-localization between PDPK1 and LAMP1 was observed at the subcellular scale in BT549 and MB468 (Figure 5F). We then evaluated the lysosome-localized PDPK1 levels in cells treated under the indicated conditions under the low-magnification

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(G) Clonogenic assays of BT549 and MB468 cells treated with or without different concentrations of AZD6244 and CQ (0.5 μM) for 10–14 days after PSMG2 shRNA (shPSMG2) transfection.

(H and I) The results of the clonogenic assays (G) were quantified by ImageJ. The quantitative results are presented as the mean ± SD (n = 4 technical replicates); *p < 0.05, ***p < 0.001, ****p < 0.0001; one-way ANOVA.
Figure 6. MEK inhibitors and proteasome inhibitors synergistically inhibited tumor progression in vivo

(A) Clonogenic assays of 4T1 cells treated with different concentrations of AZD6244 or MG132 for 10–14 days.

(B) The relative synergy of different concentrations of drug combinations (expressed as log10 of CI value) in 4T1 cells.

(C) 4T1 cells were treated with or without MG132 (2 μM) for 24 h and the related proteins were detected by western blot. Loading control, β-actin.

(D) 4T1 cells were treated with or without MG132 (2 μM) for 24 h and the autophagy-related proteins were detected by western blot. Loading control, β-actin.

(E) 4T1 cells were treated with different concentrations of rapamycin for 24 h, and then PDPK1 and autophagy-related proteins were detected by western blot. Loading control, β-actin.

(F) After treatment with or without MG132 (2 μM) for 24 h, 4T1 cells were treated with CQ (40 μM) for 8 or 12 h, then PDPK1 and autophagy-related proteins were detected by western blot. Loading control, β-actin.

(G) BALB/c mice were subcutaneously inoculated with 4 × 10^5 4T1 cells on day 0. Tumor-bearing mice (n = 8 mice) were treated with various drugs when tumor volume achieved = 100 mm^3. Details of treatment are provided in the STAR Methods.

(H) The average tumor growth curves for mice treated with PBS (control), AZD6244, MG132, and AZD6244 plus MG132. The quantitative results are presented as the mean ± SD (n = 8 mice). *p < 0.05; one-way ANOVA.

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view (Figures S4C and S4D). Consistent with the results of the western blot above, fluorescence intensities of co-localized PDPK1 were attenuated by treatment with MG132 and rapamycin, as well as PSMG2 knockdown. CQ restored the co-localized PDPK1 intensity, which was downregulated by PSMG2 knockdown. This conclusion was further confirmed by quantifying the proportion of cells with or without the PDPK1/LAMP1 co-localization (Figures S4E and S4F).

To functionally validate the synthetic lethality between MEKi and PSMG2-mediated activation of autophagy, we performed a rescue assay in our model cell lines. Long-term cell growth inhibition by AZD6244 was detected in BT549 and MB468 cells with PSMG2 knockdown in the presence or absence of CQ. The proliferation of TNBC cells with PSMG2 knockdown was suppressed by AZD6244 at 0.5 and 1 μM, which was significantly relieved by the addition of 0.5 μM CQ (Figures 5G–5I). All these results indicated that MEKI and PSMG2 knockdown significantly suppressed TNBC cell proliferation, which was driven by ALS-mediated PDPK1 degradation.

MEK inhibitor and MG132 combination suppresses TNBC tumor growth

We then validated this hypothesis in a murine TNBC cell line, 4T1. The cells were characterized in vitro. Short-term and long-term proliferation assays indicated that 4T1 cells could tolerate AZD6244 up to 1 μM (Figures S5A and S5B). These cell proliferation results were endorsed by the molecular-level detection of ERK phosphorylation (Figure S5C). Similar to that in the human TNBC cell lines, MG132 synergized the AZD6244-induced inhibition of cell proliferation by blockade of the UPS in 4T1 (Figures 6A, 6B, and S3D; Table S1). Notably, the blockade of the UPS also promoted the degradation of PDPK1 through activation of autophagy (Figures 6C and 6D). Similar degradation of PDPK1 was observed in 4T1 cells treated with rapamycin (Figure 6E). The degradation could be partially reversed by CQ (Figure 6F). Moreover, the fluorescence intensities of PDPK1 were also attenuated by treating 4T1 cells with MG132 and rapamycin (Figures S5E–S5G). In summary, 4T1 cells recapitulated the synergistic inhibition between MEKI and UPS-ALS balance-mediated suppression of PDPK1 in human TNBC cells.

Encouraged by the results in vitro, we then investigated the efficacy of combination regimens in a mouse model xenografted with 4T1. In detail, the mice xenografted with 4T1 on the flanks were randomly divided into four groups when tumors grew to around 100 mm³, and then they were treated with PBS, AZD6244, MG132, or AZD6244 plus MG132 every other day (Figure 6G). Tumor volumes were recorded every 2 days, and growth curves were profiled (Figure 6H). At the end of treatment (day 24), tumor volumes and weights were determined after dissection (Figure 6I). The results showed that both AZD6244 alone and MG132 alone exhibited marginal tumor suppression efficacy, which was significantly boosted by the combination regimens. To explore the molecular mechanism underlying this functional assay in vivo, we probed for PDPK1 along with autophagy markers (LC3) by immunohistochemistry (IHC). The results showed that PDPK1 levels in the MG132 alone- or AZD6244 plus MG132-treated groups were significantly lower than those in the PBS- or AZD6244 alone-treated groups. In line with these data, the staining intensity of LC3 showed an opposite pattern (Figures 6J and 6K). In conclusion, our data indicated that the synergistic inhibitory effects of MEKI and proteasome blockade are driven by ALS-mediated PDPK1 degradation in TNBC.

DISCUSSION

Although MEKI has shown impressive efficacy in multiple types of cancer, such as melanoma, non-small cell lung cancer (NSCLC), etc., TNBC patients cannot obtain long-term benefits from it due to acquired and/or primary drug tolerance. The mechanism of developing acquired resistance in MEKI-sensitive cells has been documented, showing that MEK inhibition results in the activation of the AKT pathway by releasing the negative feedback loop of RTKs. On the other hand, the activation of autophagy blocks by activating autophagy, which leads to drug resistance.44,45 On one hand, cells recycle organelles or macromolecules to provide an energy supply or building blocks by activating autophagy, which leads to drug resistance.44,45 On the other hand, the activation of autophagy potently enhances the efficacy of antitumor drugs. For example, autophagy induced by cysteamine and nano-c60 sensitizes doxorubicin-resistant cells to doxorubicin.46–48 Moreover, the activation of autophagy enhances the antitumor efficiency of bevaczumab in lung cancer cells.49 However, the molecular mechanism underlying these observations is still largely unknown. Our study found that inhibition of proteasome function may potently activate the autophagy pathway that promotes PDPK1 degradation. The loss of the key kinase PDPK1 dampened the AKT signaling pathway as well as cell survival. Thus, autophagy induced by blocking proteasomes sensitizes TNBC cells to MEKI. Our conclusion provides an insightful mechanism...
for this autophagy-mediated efficacy promotion in targeted therapy. Activation of the RTK/PI3K/AKT axis through the negative feedback loop is well documented as the mechanism of MEKi resistance in multiple cancer types. Thus, co-targeting the AKT and MAPK pathways was regarded as a promising strategy for tumor therapy. However, the combination regimens with AKTi and MEKi showed limited efficacy in terms of tumor regression in clinics. Previous studies have shown that AKTi rebounds to activate its own signaling, impairing the efficacy of MEKi. We observed the rebound restoration of p-AKT in the cells treated with AKTi-1/2 plus AZD6244 but not in those treated with MG132 plus AZD6244 (Figures S3M and S3N). This suggests that degradation of PDPK1 promoted by proteasome blockade physically interrupts the negative feedback signals toward the downstream kinase AKT. Actually, we compared the inhibitory efficacies of cell proliferation between AKTi plus MEKi and proteasome inhibitors (MG132) plus MEKi in a head-to-head pattern (Figures 2D, 2E, S2D–S2G, and S3H–S3K). Although both combination regimens clearly suppressed the proliferation of TNBC model cells, the synergy of the proteasome inhibitor (MG132) plus MEKi was much better than that of AKTi plus MEKi (Figures 2F and S3L). This indicated that a proteasome inhibitor plus MEKi is superior to AKTi plus MEKi in terms of TNBC growth inhibition.

In summary, our research not only revealed important mechanistic insights into MEKi resistance but also has significant therapeutic implications for TNBC in clinics. It provides a potential combination regimen of proteasome inhibitors and MEKis for TNBC therapy, which surmounts the poor effect of AZD6244 single agent or a proteasome inhibitor single agent.

**Limitations of the study**

The reciprocal regulation between the ALS and the UPS has been well documented. ER stress was reported as a potential mechanism mediating the cross talk between proteasome and autophagy systems. Although our study also found that autophagy activation was accompanied by ER stress (Figures 4F and 4G), the detailed molecular mechanism, including other ER stress-independent pathways, needs to be investigated in the future. Recently, increasing evidence has shown that autophagy could be either a bulk degradation system or a selective degradation system. Compared with non-selective autophagy, selective autophagy, which is mediated by specific receptors, eliminates damaged organelles and aggregated proteins without disruption of other necessary cellular components. In addition to PDPK1, which proteins are also targeted by this proteasome blockade-elicited selective autophagy needs to be further investigated in the future.

**STAR METHODS**

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

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

H.S. and J.J. supervised the project, conceived and designed the experiments, analyzed the data, and reviewed and edited the manuscript. X.W. performed the experiments in vitro and in vivo, analyzed the data, generated the final figures, and co-wrote the original manuscript. J.Y. performed all bioinformatics analysis, generated the related diagrams, and co-wrote the original manuscript. X.L. performed the experiments in vitro and co-wrote the original manuscript. D.L. designed the experiments in vitro and revised the manuscript. Y.L. performed the preliminary in vitro experiments and analyzed the data. Y.W. and L.C. provided technical support and visualized western blot results. T.L., X.J., L.S., and X.Y. participated in the revision of the manuscript. All authors read and approved the final paper.

DECLARATION OF INTERESTS

All authors declare they have no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) | Cell Signaling Technologies | Cat# 4370S; RRID: AB_2315112 |
| p44/42 MAPK (ERK1/2) (137F5) rabbit mAb | Cell Signaling Technologies | Cat# 4695S; RRID: AB_390779 |
| Akt (pan) (C67E7) Rabbit mAb | Cell Signaling Technologies | Cat# 4691L; RRID: AB_915783 |
| Phospho-Akt (Ser473) (D9E) XP Rabbit mAb | Cell Signaling Technologies | Cat# 4060L; RRID: AB_2315049 |
| PDK1 (D4Q4D) Rabbit mAb | Cell Signaling Technologies | Cat# 130375; RRID: AB_2798095 |
| Phospho-PDK1 (Ser241) Antibody | Cell Signaling Technologies | Cat# 3061 s; RRID: AB_2161919 |
| LAMP1 (D2D11) XP Rabbit | Cell Signaling Technologies | Cat# 9091T; RRID: AB_2687579 |
| SQSTM1/p62 (D6M5X) Rabbit mAb | Cell Signaling Technologies | Cat# 23214; RRID: AB_2798858 |
| LC3A/B (D3U4C) XP Rabbit mAb | Cell Signaling Technologies | Cat# 12741S; RRID: AB_2617131 |
| Mouse Anti-Ubiquitin | Cell Signaling Technologies | Cat# 3936; RRID: AB_331292 |
| Phospho-S6 Ribosomal Protein (Ser240/24) mAb | Cell Signaling Technologies | Cat# 5364; RRID: AB_10694233 |
| S6 Ribosomal Protein (54D2) Mouse mAb | Cell Signaling Technologies | Cat# 2317; RRID: AB_2238583 |
| Anti-4E-BP1, phospho (Thr37/Thr46) | Cell Signaling Technologies | Cat# 2855; RRID: AB_560835 |
| 4E-BP1 (53H11) Rabbit mAb | Cell Signaling Technologies | Cat# 9644; RRID: AB_2097841 |
| p21 Waf1/Cip1 (12D1) Rabbit | Cell Signaling Technologies | Cat# 2947; RRID: AB_823586 |
| Phospho-eIF2alpha (Ser51) Rabbit mAb | Cell Signaling Technologies | Cat# 3398; RRID: AB_2096481 |
| eIF2alpha (D7D3) XP Rabbit mAb | Cell Signaling Technologies | Cat# 3398; RRID: AB_10692650 |
| ATF-4 (D4B8) Rabbit mAb | Cell Signaling Technologies | Cat# 11815; RRID: AB_2610625 |
| Anti-PSMG2 (PAC-2) | Abcam | Cat# ab172909;RRID: AB_2923324 |
| Anti-PDK1 | Abcam | Cat# ab186870; RRID: AB_2923325 |
| Anti-PDK1 | Abcam | Cat# ab234064; RRID: AB_2923326 |
| Anti-LAMP1 | Abcam | Cat# ab208943; RRID: AB_2923327 |
| Anti-β-actin | ZsBio | Cat# TA-09; RRID: AB_2636897 |
| Alexa Fluor 594TM Donkey anti-Rabbit IgG (H + L) | Invitrogen | Cat# A-21207; RRID: 141837 |
| Alexa Fluor 488TM Goat anti-Mouse IgG (H + L) | Invitrogen | Cat# A-11001; RRID: 2534069 |

### Chemicals, peptides, and recombinant proteins

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| AZD6244 | Selleck | S1008; Cat# 606143-52-6 |
| Trametinib | Selleck | S2673; Cat# 871700-17-3 |
| MG132 | Selleck | S2619; Cat# 1211877-36-9 |
| Bortezomib | Selleck | S1013; Cat# 179324-69-7 |
| Rapamycin | Selleck | S1039; Cat# 53123-88-9 |
| Puromycin | Selleck | S7417; Cat# 58-58-2 |
| AKT1-1/2 | Selleck | S7776; Cat# 612847-09-3 |
| Chloroquine | Sigma-Aldrich | Cat# 50-63-5 |
| Lithium chloride | Sigma-Aldrich | Cat# 7447-41-8 |
| Bacterialidin | InvivoGen | Cat# ant-bi-1 |
| Poly-L-lysine | Sigma-Aldrich | Cat# 26124-78-7 |
| Sodium butyrate | Sigma-Aldrich | Cat# 156-54-7 |
| MTS | Promega | Cat# G3582 |
| SUPERase In™ RNase Inhibitor | Life Technologies | Cat# AM2694 |
| Sephacryl S400 spin column chromatography | GE Healthcare | Cat# 27514001 |

(Continued on next page)
**Critical commercial assays**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| iQ™ SYBR® Green Supermix | BIO-RAD | Cat# 172-S121 |
| ReverAid First Strand cDNA Synthesis kit | Thermo Fisher | Cat# K1622 |
| RiboZero kit | Illumina | Cat# MRZH11124 |
| Human CRISPR Knockout Pooled Library (GeCKO v2) | addgene | Cat# 1000000049 |

**Deposited data**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| BT549 CRISPR-Cas9 sgRNA sequencing data | http://www.ncbi.nlm.nih.gov/geo/ | GEO: GSE206748 |
| TCGA Breast Cancer (TCGA-BRCA) cohort | UCSC Xena: http://xena.ucsc.edu/ | TCGA-BRCA |
| Bulk transcriptome sequencing data of TNBC | https://www.ncbi.nlm.nih.gov/geo/ | BioProject: |
| Single-cell transcriptome sequencing data of TNBC | http://www.ncbi.nlm.nih.gov/geo/ | GEO: GSE161529 |
| Single-cell transcriptome sequencing data of TNBC | http://www.ncbi.nlm.nih.gov/geo/ | GEO: GSE11838 |
| Single-cell transcriptome sequencing data of TNBC | http://www.ncbi.nlm.nih.gov/geo/ | GEO: GSE75688 |

**Experimental models: Cell lines**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| BT549 | The National Collection of Authenticated Cell Cultures (Shanghai, China) | Cat# TCHu 93; RRID: CVCL_1092 |
| MB468 | The National Collection of Authenticated Cell Cultures (Shanghai, China) | Cat# TCHu136; RRID: CVCL_0419 |
| MB231 | The National Collection of Authenticated Cell Cultures (Shanghai, China) | Cat# TCHu227; RRID: CVCL_0062 |
| 4T1 | The National Collection of Authenticated Cell Cultures (Shanghai, China) | Cat# SCSP-5056; RRID: CVCL_0125 |
| HEK-293T | The National Collection of Authenticated Cell Cultures (Shanghai, China) | Cat# GNHu 43; RRID: CVCL_1926 |
| HCC1937 | The National Collection of Authenticated Cell Cultures (Shanghai, China) | Cat# TCHu148; RRID: CVCL_0290 |
| SUM159 | State Key Laboratory of Biotherapy (Chengdu, China) | RRID: CVCL_5423 |
| SUM149 | State Key Laboratory of Biotherapy (Chengdu, China) | RRID: CVCL_3422 |

**Experimental models: Organisms/strains**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse: BALB/c | Beijing Huafukang Biotechnology Co. Ltd. | Cat# JAX:000651; RRID: IMSR_JAX:000651 |

**Oligonucleotides**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Primers in shRNA plasmid construction, see Table S2 | This paper | N/A |
| Primers in overexpression plasmid construction, see Table S3 | This paper | N/A |
| Primers for RT-PCR, see Table S4 | This paper | N/A |

**Recombinant DNA**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Plasmid: cPPT-puromycin-PDPK1 | This paper | N/A |
| Plasmid: cPPT-puromycin-PSMG2 | This paper | N/A |

**Software and algorithms**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| R script for analysis | This paper | https://github.com/jingyu9603/Crispr-Cas9_TNBC |
| R (Version 4.0.2) | R Core | https://www.r-project.org/ |
| Kallisto (Version 0.46.2) | Bray et al., 2016 | https://pachterlab.github.io/kallisto/ |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hubing Shi (shihb@scu.edu.cn).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This article includes all data generated or analyzed during this study and is summarized in the accompanying tables, figures, and supplemental materials. All sequencing data have been deposited into the NCBI -Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE206748). The accession number (GEO: GSE206748) is listed in the key resources table. The R scripts used for data analysis and visualization in this study have been uploaded to Github (https://github.com/jingyu9603/Crispr-Cas9_TNBC). 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

Cell lines
The TNBC cell lines BT549, MB468, MB231, HCC1937, and 4T1 were purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China). HEK-293T cell line was purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China). SUM149 and SUM159 cells were obtained from the State Key Laboratory of Biotherapy of Sichuan University (Chengdu, China). MB468, MB231, and SUM149 cells were cultured in a humidified of 5% CO2 at 37°C in DMEM (Gibco) medium supplementing with 10% fetal bovine serum (FBS) (NATOCOR) and 1% penicillin-streptomycin (100 μg/mL, Hyclone). BT549, HCC1937, SUM159, and 4T1 cells were cultured in a humidified of 5% CO2 at 37°C in RPMI (Gibco) medium supplementing with 10% fetal bovine serum and 1% penicillin-streptomycin. All cell lines were confirmed mycoplasma negative by end-point PCR.

Mouse models
Five- to six-week-old weeks female BALB/c mice were obtained from Beijing Huafukang Biotechnology Co. Ltd. (Beijing, China). The animals were housed and maintained under specific pathogen-free conditions in facilities and treated humanely throughout the studies. All animal experiments were performed according to the protocols approved by the Ethics Review Committee of Animal Experimentation of Sichuan University. All animals were allowed to acclimate in the animal facility with access to food and water ad libitum for one week before manipulation.
4T1 cell lines were derived from a mammary tumor in BALB/cfC3H mice as previously described. Then the mice were inoculated subcutaneously with 4 × 10⁵ 4T1 cells on the left and right flanks. Tumor volumes were measured with the caliper every two days and calculated using the following formula: tumor volume (mm³) = (length × width²)/2. Body weights and tumor weights were measured by the electronic balance once every two days. When the average tumor size reached ~ 100 mm³, the mice were randomly assigned to four groups of four mice each group. The control cohort received PBS, and treatment cohorts received either AZD6244 (10 mg/kg, dissolved in 4%DMSO, 30%PEG300, and 5%Tween-80 in distilled water), MG132 (2 mg/kg, dissolved in 5%DMSO, 40%PEG300, and 5%Tween-80 in distilled water), or the combinations. Then these were injected once every two days via intraperitoneal injection. At the end of treatment, mice were euthanized according to Institutional Animal Care and Use Committee guidelines. Tumors were weighed, collected, and fixed in formalin for IHC.

**METHOD DETAILS**

**Cell viability assay**
Cells were seeded into 96-well plates at a density of 3000 or 5000 cells per well, then treated with the drug on the following day. Then, cells were incubated for 72 h, and the viability was measured using MTS (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) following the manufacturer’s recommendations. Relative survival in the presence of indicated drugs was normalized to DMSO after subtracting background, and the survival curve was protracted by GraphPad Prism 8.3 software.

**Clonogenic assay**
Cells were seeded into 12-well plates at a density of 1500 or 3000 cells per well and incubated for 24 h. The next day, cells were transfected lentiviral or treated with the inhibitors. Medium and drugs were replaced every 2 days. Colonies were fixed in 4% paraformaldehyde and stained with 0.05% crystal violet after 10–14 days. Finally, quantitative analysis was performed by Image J software.

**Lentivirus production**
Co-transfect the packaging plasmids pMD.G, RSV-REV, pMDLg/p, and the target plasmid into HEK-293T cells to prepare lentiviral preparations. Treat the culture plates with polyline 10 min before seeding the cells. 1 × 10⁷ HEK-293T cells were seeded onto 75cm² culture plates 1 day before transfection. The fresh medium was replenished 4 h before transfection. The mixture containing the target plasmids and packaging plasmids was transfected into HEK-293T cells with Calcium Phosphate Transfection. The medium was changed to the fresh medium containing 10 mM sodium butyrate and 20 mM HEPES after 12–15 h of transfection. After another 8 h, it was changed to the fresh medium containing 20 mM HEPES, and incubated for 16–18 h. Finally, the supernatant containing lentivirus was collected, filtered through a 0.45µm PVDF filter membrane (Millipore) to remove cells, aliquoted, and stored at −80°C. Cells were seeded into 6-well or 12-well plates, and infections were implemented in the presence of 5 µg/mL polybrene. Following transduction, the target cells were treated with 2 µg/mL puromycin for 24 h to select cells that stably express the target plasmid. Knockdown primers used to construct the vector are listed in Table S2. Overexpression primers used to construct the vector are listed in Table S3.

**Western blot assay**
Cells were lysed in RIPA buffer (Millipore) in the presence of phosphatase inhibitor and protease inhibitor (cocktail). Cell lysate was quantified with Pierce® BCA Protein Assay Kit (Thermo Fisher). Then it was normalized, denatured (98°C), and resolved by SDS gel electrophoresis on 10–12% Tris-Glycine gels. Cell lysate was separated on an SDS-polyacrylamide gel and transferred to a PVDF membrane (Millipore). Proteins were transferred to PVDF membranes (Millipore). Subsequently, a series of routine operations such as antibody incubation and exposure were then carried out.

**Transmission electron microscopy (TEM)**
The cells were prefixed with 3% glutaraldehyde in 0.1 M PBS and stored at 4°C until embedding. Then, the samples were postfixed in 1% osmium tetroxide (GP18456, Leica) for 1 h at 4°C. They were dehydrated through a graded series of acetone (30%–100%). After dehydration, they were infiltrated for a longer time and embedded (GP18010, Beijing Zhongjingkeyi Technology Co., Ltd) in Epon812. Serial sections were cut using an ultramicrotome (Leica). The ultrathin sections were cut with a diamond knife and stained with uranyl acetate (GS02624, Beijing Zhongjingkeyi Technology Co., Ltd.) and lead citrate (GZ02616, Beijing Zhongjingkeyi Technology Co., Ltd.). Sections were examined with JEM-1400-Flash Transmission Electron Microscope. The experiment was completed with the assistance of the Lilai Biomedical Experimental Center in Chengdu, China.

**Immunofluorescence (IF) staining**
Adding a coverslip into the 12-well plates and grow cells in culture media overnight. After treatment, cells were fixed with 4% paraformaldehyde for 20 min or 100% methanol for 5–10 min at room temperature. Then, cells were permeabilized with 0.2% Triton X-100 for 10 min and blocked for 1 h with 3% BSA for 60 min at room temperature. Stain with the primary antibody in 40 µl of 1%BSA-PBS overnight at 4°C by forming a drop on the coverslip. The next day, staining with Alexa Fluor 488-conjugated or Alexa Fluor 647-conjugated secondary antibodies and visualized using an inverted fluorescence microscope.
594-conjugated secondary antibody for 1 h at RT in 1% BSA-PBS. Finally, mounting slides with anti-fading DAPI. Fluorescence images were captured with the same fluorescent parameters using confocal microscopy (Nikon).

**Immunohistochemistry staining (IHC)**

To prepare the tumor samples for IHC staining, the tumor pieces were fixed with 10% formalin followed by paraffin embedding. Tumor sections of 4 μm thickness were mounted on glass slides for IHC staining as described previously. For PDPK1, LC3 immunohistochemistry staining, the slides were deparaffinized, incubated in 3% hydrogen peroxide, and antigen retrieval was performed in EDTA (pH = 9.0) or citrate (pH = 6.0) for 15 min in the pressure cooker. The slides were rinsed in PBS at RT and incubated with primary antibodies at 4°C overnight. After rinsing with PBS, the slides were incubated with appropriate HRP-conjugated secondary antibodies at room temperature for 45 min. Finally, the slides were incubated with DAB (3, 3'-diaminobenzidine) for visualization, washed in tap water, counterstained with hematoxylin, dehydrated in ethanol, and mounted with media. Images were captured with the same parameters by an ortho fluorescence microscope (Nikon). Integrated optical density (IOD), area, and mean density of cell (IOD/Area) were calculated using Image-Pro Plus 6.0.

**Quantitative reverse transcription PCR**

RNA extraction and purification: total RNA was extracted from cells using Trizol, following the manufacturer’s protocol. As described, ribosome-bound mRNAs were extracted and purified with slight modifications. In detail, the 10 cm² dish of adherent cell culture was placed on ice and washed once with cold PBS. The dish was quickly immersed in liquid nitrogen and rapidly moved to ice. The cells were scraped with 400 μL cold lysis buffer (20 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 150 mM NaCl, 1% Triton X-100, 1 mM DTT, 0.1% NP40, 25 U/mL DNase I, and 0.1 mg/mL cycloheximide) and incubated for 10 min on ice. The lysate was clarified by centrifugation for 10 min at 20,000 g at 4°C. Then, 7.5 μL RNase I (100 U/μL) was added to 300 μL of supernatant, and then the mixture was incubated at room temperature for 45 min and mixed gently. Next, we stopped the nuclease digestion with 10 μL SUPERase*InTM RNase inhibitor and chilled samples for at least 30 min on ice. The Sephacryl S400 spin column chromatography was used to purify the ribosome-RNA complexes. Then, the ribosomal protected RNA was extracted with Trizol, and purified with the RiboZero kit (Illumina; MRZH11124). The RNA samples were separated in the 15% urea gel by electrophoresis, and then the 25–35 nt fragment was extracted from the gel. Finally, the mRNA fragments were eluted in 400 μL nuclease-free water, 40 μL of 5 M ammonium acetate, and 2 μL of 10% SDS for at least 2 h, then precipitated with isopropanol.

0.5–1 μg of RNA was used to synthesize cDNA with the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative PCR was performed using Universal SYBR Green Supermix (Bio-Rad). ACTIN was used as the reference gene for relative quantification. Primers used for qPCR are listed in Table S4.

**Genome-wide CRISPR/Cas9 library screening**

The sgRNA pooled library and the Cas9 plasmid were purchased from Addgene. The lentiviral particles were obtained via the above-mentioned lentiviruses production method. Cells were seeded into the cell culture dish and gradient concentration of blasticidin or puromycin to treat cells on the following day. And the minimum lethal concentration of the cell was obtained. The concentration of blasticidin is 5 μg/mL for 7 days, and puromycin is 0.5 μg/mL for 5 days. Construct the cancer cell lines with constitutive Cas9 expression. Cells were seeded into the cell culture dish. The next day, cells were transduced with Cas9 lentivirus particles in the presence of 2 μg/mL protamine. After transduction, the target cells were treated with 5 μg/mL blasticidin for 7 days to select cells that stably express the Cas9 plasmid.

Stable Cas9-expressing cells were transduced with the lentiviral sgRNA library at a multiplicity of infection (MOI) of 0.3 and an average of 500-fold coverage of the library. Since the library contains 123,411 sgRNAs, at least 2 × 10⁶ cells were infected to achieve 500-fold coverage. Cells were selected in puromycin (0.5 μg/mL) for 5 days after 24 h of infection. When the cells have multiplied to 6 × 10⁸, the cells are divided into three groups. One group of cells were directly harvested (labeled as DMSO-Day0), and the other two groups were treated with DMSO (labeled as DMSO-Day7) or 1 μM AZD6244 (labeled as AZD-Day7) for 7 days, respectively. Finally, cells were harvested and snap-frozen in liquid nitrogen to extract genomic DNA (gDNA) using Phenol-Chloroform. Using genomic DNA as a template, the target product is obtained through two rounds of PCR for high-throughput sequencing.

**RNA-seq data analysis**

The collection of the transcriptome data sets for TNBC: the bulk sequencing (bulk) data of TNBC is the transcriptome sequencing data of TNBC biopsy (normal and tumor tissues) collected from West China Hospital downloaded from GEO (https://www.ncbi.nlm.nih.gov/geo/); the TCGA Breast Cancer (TCGA-BRCA) cohort downloaded from UCSC Xena (http://xena.ucsc.edu/) and selected TNBC samples data from it as the TCGA-TNBC data set; three single-cell sequencings (scRNA) data sets (GSE161529, GSE11838, and GSE75688) were downloaded from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/); the transcriptome expression level of the BT549 cell line can be obtained from the GSE112365 data set, which is also downloaded from the GEO database.

Genes expressed in TNBC tumor tissues were screened at the level of bulk and scRNA sequence, respectively. For the bulk-seq level, Kallisto (v0.46.2) was used for aligning raw data (PRJNA553096) with human transcriptome (GRCh38), and DESeq2 (v1.26.0) was used for differential analysis to evaluate the genes that significantly changed between tumor tissue and normal tissue.
The genes with adjusted p value < 0.01 and log2FC > 0.5 are significantly up-regulated in tumor tissues. For the scRNA-seq level, we downloaded 8 scRNA-seq profiles from GEO (GSE161529) which include normal epithelial cells of 4 normal patients and total breast cells of 4 patients with TNBC. Firstly, the epithelial cell data was screened by Seurat (v3.2.2) and SciBet (v1.0), and the differentially expressed genes between tumor and normal epithelial cells were identified by the Wilcoxon Rank Sum test. Genes matched the following conditions are significantly up-regulated in tumor epithelial cells: adjust p value < 0.01 and log2FC > 0.2.

**CRISPR screen data analysis**

Using the MAGeCK (v0.5.9.4) count function to obtain the sgRNA read counts of different screening groups (DMSO-Day0, DMSO-Day7, and AZD-Day7), and then using the MAGeCK test function to compare the data normalized by the non-targeting control guides in our CRISPR screen data (AZD-Day7 vs DMSO-Day7, DMSO-Day7 vs DMSO-Day0). Based on the comparison results of AZD-Day7 and DMSO-Day7 groups, genes that matched the following conditions were considered to be significantly down-regulated in the AZD6244 treatment group: 1) p value less than 0.05, 2) total identified sgRNA reads in DMSO-Day7 more than those in AZD-Day7 (log2FC < 0), 3) the identified sgRNA sequences for each gene not less than 3, and 4) more than half of the sgRNA sequences targeting indicated gene were down-regulated (sgRNA_{down}/sgRNA_{total} >50%). The BF value (Bayes Factor) of DMSO-Day7 and DMSO-Day0 was calculated by BAGEL (v 0.91) software. The BF value greater than 0 indicated that the gene is essential. To maintain the consistency of visualization, all BF values were multiplied by –1 to get the fitness score. The housekeeping genes were identified by combining the results of MAGeCK (p value <0.05, fitness score <0). Therefore, the non-housekeeping genes were screened out (p value >0.05 or fitness score >0).

**Signaling pathway analysis**

To explore the potential signaling pathways correlated to the proteasome, GSVA (v1.34.0) was used to calculate the enrichment score of all hallmark gene sets and proteasome at bulk-seq (TCGA-TNBC, BioProject: PRJNA553096) and scRNA-seq levels (GEO: GSE75688 and GEO: GSE11838). Pearson’s correlation between the proteasome and all 50 hallmark gene sets was calculated based on enrichment score by Hmisc (v4.4–2). In the two single-cell transcriptome data sets (GEO: GSE11838 and GEO: GSE75688), the hallmark gene sets were sorted according to correlation, respectively. Then, the robust rank aggregation algorithm (RobustRankAggreg v1.1) was used to aggregate the two sorts. Finally, the top 5 signal pathways were screened out. Similar to the single-cell data analysis method, the correlation was calculated, and the top 5 signal pathways were screened out in two bulk sequencing data sets (TCGA-TNBC, BioProject: PRJNA553096).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data are presented as mean ± SD unless otherwise stated. Statistical analyses were performed using GraphPad Prism 8 software. Significance was determined by a two-tailed Student’s t test or one-way analysis of variance (ANOVA). Details of the statistical analyses can be found in the main text and figure captions. Statistical significance thresholds were set at *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns, not significant.