Targeting the mRNA endonuclease CPSF73 inhibits breast cancer cell migration, invasion, and self-renewal

UBE3D regulates CPSF73 in a conserved way as is found for its yeast homolog

UBE3D stabilizes CPSF73 by protecting it from ubiquitin-mediated degradation

UBE3D regulates breast cancer cell migration and invasion through CPSF73

The level of CPSF73 correlates with breast cancer cell self-renewal properties

Inefficient 3' end processing and transcription termination
Dysregulated gene expression
Reduced breast cancer cell migration, invasion and self-renewal
Targeting the mRNA endonuclease CPSF73 inhibits breast cancer cell migration, invasion, and self-renewal

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SUMMARY
Cleavage by the endonuclease CPSF73 and polyadenylation of nascent RNA is an essential step in co-transcriptional mRNA maturation. Recent work has surprisingly identified CPSF73 as a promising drug target for inhibiting the growth of specific cancers, triggering further studies on understanding CPSF73 regulation and functions in cells. Here, we report that a HECT-like E3 ligase, UBE3D, participates in stabilizing CPFS73 protein by preventing its ubiquitin-mediated degradation by the proteasome. Depletion of UBE3D leads to CPSF73 downregulation, a pre-mRNA cleavage defect, and dysregulated gene expression in cells. UBE3D dysfunction or chemical inactivation of CPSF73 inhibited migration and invasion as well as stem cell renewal phenotypes in vitro in triple-negative breast cancer cells. In addition, genetic overexpression of CPSF73 promoted breast cancer stemness and knocking down CPSF73 inhibited stem cell renewal properties. Together, our findings indicate that targeting the pre-mRNA processing nuclease CPSF73 has potential for breast cancer therapy.

INTRODUCTION
Precursor mRNA (pre-mRNAs), which are the primary products resulting from the transcription of protein-coding genes, undergo a series of chemical modifications to form the mature mRNA that is exported to the cytoplasm and translated into the corresponding protein. Key mRNA processing steps include 5’ end capping, removal of introns by splicing, cleavage of mRNA at the 3’ end, and the addition of a long chain of adenine nucleotides known as the poly(A) tail. RNA processing has long been recognized as a means to regulate gene expression, and the abnormal processing of pre-mRNA through alternative splicing and alternative polyadenylation can also initiate cancer and drive tumor progression (Bonnal et al., 2020; Gruber and Zavolan, 2019). These studies make targeting mRNA processing an intriguing anti-cancer strategy (Desterro et al., 2020).

Cleavage and polyadenylation of nascent transcripts is a fundamental process for eukaryotic mRNA maturation and the production of different mRNA isoforms. Through this mechanism, all animal cell mRNAs except those encoding histones receive a 3’ poly(A) tail which protects the mRNA from degradation (Chen and Shyu, 2011), aids in the export of the mature mRNA to the cytoplasm (Stewart, 2019), and binds proteins involved in initiating translation (Moore and von Lindern, 2018). The processing is carried out by machinery that recognizes conserved sequence elements around the cleavage site and is composed of 15 core proteins consisting of the poly(A) polymerase and four complexes (Ren et al., 2020; Sun et al., 2020; Zhang et al., 2020): Cleavage and Polyadenylation Specificity Factor (CPSF), Cleavage Stimulation Factor (CstF), and mammalian Cleavage Factors I (CFIm) and II (CFIIm). Abnormal mRNA 3’ end profiles found in cancer are caused at least in part by changes in the expression levels of components of the cleavage and polyadenylation machinery (Abadi et al., 2019; Chen et al., 2021; Komin et al., 2021; Lin et al., 2021).

A critical component of the processing complex is the essential endonuclease CPSF73. Cleavage by CPSF73 is necessary to provide a substrate for poly(A) polymerase. Without CPSF73’s cleavage activity, mRNAs cannot be polyadenylated and released from the site of transcription for export to the cytoplasm. Cleavage by CPSF73 is also necessary for the termination of transcription that defines gene boundaries, and thus inhibits transcriptional interference at downstream genes. Several studies have associated CPSF73 activity with cancer phenotypes (reviewed by (Liu and Moore, 2021)). It has been proposed as a...
promising biomarker for cancer outcomes (Ning et al., 2019), and its activity is inhibited by the tumor suppressor protein CSR1 but activated by IncRNA ASC9 (Luo et al., 2019; Zhu et al., 2009). Furthermore, small molecule inhibitors of CPSF73 have therapeutic potential for treating specific cancers, inflammatory diseases, and protozoan infections (Liu and Moore, 2021).

Given the central role of CPSF73 for mRNA processing and its implications in therapeutics, it is important to define how it is regulated in order to completely decode its function in cells. Our previous studies showed that Ysh1, the yeast CPSF73 homolog, is a target for ubiquitin-mediated proteasomal degradation. Mechanistically, ubiquitination of Ysh1 is modulated by the presence of Ipa1, an essential protein that physically interacts with Ysh1 and Mpe1, another cleavage/polyadenylation complex subunit (Costanzo et al., 2016; Hill et al., 2019). Mutation of Ipa1 causes the degradation of Ysh1 by the proteasome (Lee et al., 2020), defects in mRNA 3' end processing in vitro, lengthening of mRNAs in vivo, and inefficient transcription termination downstream of poly(A) sites (PAS) and at snoRNA genes (Costanzo et al., 2016; Pearson et al., 2019). However, the post-translational regulation of CPSF73 levels in human cells has not been documented. Interestingly, two independent quantitative proteomics screens identified the human Ipa1 homolog, UBE3D, as a CPSF73 interactor (Hein et al., 2015; Huttlin et al., 2017), and CPSF73 was found to be part of the ubiquitome (Chen et al., 2014). Based on these observations, we hypothesized that the mechanism by which Ipa1 regulates polyadenylation may be conserved in humans.

To test our hypothesis, we generated a UBE3D KO line in HEK293 cells and found that among the cleavage/polyadenylation core factors, CPSF73 protein, but not its mRNA, was specifically downregulated. CPSF73 is less stable in UBE3D-depleted cells, and the degradation of CPSF73 utilized the ubiquitin-proteasome pathway. CPSF73 downregulation mediated by UBE3D depletion caused 3' end processing defects in both HEK293 and the triple-negative breast cancer MDA-MB-231 cell lines. Additional functional studies showed CPSF73 dysfunction mediated by either UBE3D depletion or chemical inhibition resulted in the reduction of breast cancer cell migration, invasion, and self-renewal. Furthermore, direct knockdown of CPSF73 mRNAs effectively suppressed breast cancer cell self-renewal while overexpression of CPSF73 promoted it, highlighting the potential of inhibiting mRNA 3' end processing in breast cancer therapeutics.

RESULTS
UBE3D regulates CPSF73 at the post-translational level
UBE3D is a HECT-like E3 ligase that has been implicated in cell cycle regulation (Hundley et al., 2021; Koblumaki et al., 2005), age-related macular degeneration (Huang et al., 2015; Xia et al., 2020), the inflammatory condition of aggressive juvenile periodontitis (Offenbacher et al., 2016), and alterations in the fatty acid profile of muscle (Rovadoscki et al., 2018), but its functional role in mammalian cells has not been determined. Although UBE3D was identified as a CPSF73 interactor in HEK293 and HeLa cells (Hein et al., 2015; Huttlin et al., 2017), how CPSF73 might be regulated by UBE3D is not known. We generated a HEK293 UBE3D knockout (KO) cell line using CRISPR-Cas9 technology (Ran et al., 2013). UBE3D depletion in the KO cell line was verified both at the genomic level (Figure S1A) and protein level (Figure 1A). To test if CPSF73 was specifically regulated by UBE3D as we observed with their homologs in yeast, we tested the protein levels of core cleavage/polyadenylation (C/P) factors in UBE3D KO cells. CPSF73, but not other C/P subunits, was decreased dramatically in UBE3D KO cells (Figure 1B), and this regulation was also observed in UBE3D knockdown cells (Figures S1B and S1C). Furthermore, the CPSF73 level was rescued by overexpression of UBE3D protein in KO cells (Figure 1B), suggesting that UBE3D is important for maintaining the CPSF73 protein level in human cells. These data also indicate that UBE3D is the likely human homolog of Ipa1 and that its function is conserved across eukaryotes.

To further understand the level at which UBE3D regulated CPSF73, we measured the CPSF73 mRNA levels in the UBE3D wild-type (WT), KO, and addback cells and observed no change among the groups (Figure 1C), suggesting that UBE3D depletion does not affect transcription or stability of CPSF73 mRNAs and that the decrease in CPSF73 protein might be owing to post-translational regulation. To test this hypothesis, we employed a cycloheximide chase assay (Kao et al., 2015) to analyze the stability of CPSF73 in UBE3D WT and KO cells. Compared to WT cells, the depletion of UBE3D accelerated CPSF73 degradation after blocking protein translation with cycloheximide (Figure 1D), verifying the post-translational regulation of CPSF73 by UBE3D. To assess whether the ubiquitin-proteasome pathway was involved in CPSF73 degradation as it is for the yeast homolog Ysh1 (Lee et al., 2020), we treated the UBE3D KO cells with the proteasome-specific inhibitor MG132 and the ubiquitin-activating enzyme (E1) inhibitor TAK-243 (Hyer et al.,
followed by examining the levels of CPSF73. Western blot results (Figures 1E and S1D) showed that CPSF73 increased in UBE3D KO cells upon MG132 or TAK-243 treatment. Together, these results indicate that UBE3D stabilizes CPSF73 in human cells by preventing its ubiquitin-mediated proteasomal degradation.

**UBE3D loss causes mRNA readthrough and global suppression of gene expression**

Given that CPSF73 inactivation or depletion inhibits pre-mRNA cleavage and causes significant readthrough transcription downstream of PASs and histone mRNA cleavage sites in metazoan cells (Eaton et al., 2018; Pettinati et al., 2018; Ross et al., 2020), we asked whether loss of UBE3D, which causes
CPSF73 down-regulation, would affect mRNA 3’ end processing and transcription termination. We used previously described primers to monitor cleavage of ACTB or MYC transcripts in vivo by RT-qPCR of total RNA reverse transcribed with random hexamers (Davidson et al., 2014; Eaton et al., 2018). These primer pairs detect unspliced transcripts (US) or RNA-containing sequence that spans the PAS (Span) (Figure 2A). When there is a cleavage defect at the PAS, more “Span” products are expected after normalization to “US” control. For both genes, the accumulation of RNA spanning the PAS indicated a significant reduction of PAS cleavage in UBE3D KO cells, with an increase of 2.9-fold for ACTB and 3.6-fold for MYC. When UBE3D expression was restored in KO cells, cleavage of both transcripts was rescued (Figure 2A).

To test the global effect of UBE3D depletion on transcription termination, we prepared RNA-seq libraries from WT and UBE3D KO cells and analyzed the data by DoGFinder (Wiesel et al., 2018), a software for the discovery and quantification of readthrough transcripts from RNA-seq. UBE3D KO caused 445 intergenic regions to exhibit an increase in the downstream of Gene-containing transcripts (DoGs) and 328 to show a decrease compared to WT (Figure 2B). We next asked if the changes in the level of DoGs were owing to changes in the expression levels of upstream genes. Correlation analysis showed that the majority of genes associated with an increase in DoGs generally did not show a corresponding increase in expression (Figure S2), indicating that the likely reason for DoG accumulation was the defect in 3’ end processing. Furthermore, many of the DoGs that are increased in KO cells are suppressed when UBE3D is added back (Figure 2C). The reads maps of representative genes are presented in Figure 2D. The reads downstream of histone gene HIST2H2BC11 and non-histone gene SNRPA were strongly increased in UBE3D KO samples and decreased with UBE3D addback. These results show that, consistent with the concomitant CPSF73 degradation, UBE3D KO causes readthrough transcription at mRNA encoding genes.

As noted above, some DoGs showed decreased expression upon UBE3D loss. Many of the genes associated with decreased DoGs have a corresponding decrease in gene expression (Figure S2, lower left quadrant), an outcome that would be predicted if the transcription of these genes was decreased. Only 6 genes showed decreased DoGs with increased gene expression. Curiously, the majority of genes with decreased DoGs have no change in gene expression. Our analysis cannot discriminate the underlying reasons for these outcomes and whether they are direct or indirect consequences of UBE3D KO. Some genes may be poorly transcribed in the UBE3D KO cells, leading to the decrease in DoGs compared to UBE3D + cells, but the cell compensates by increasing the stability of the mRNAs of these genes so that the steady-state level remains the same.

Differential gene expression analysis of RNA-seq data in UBE3D KO samples showed that UBE3D KO led to a global downregulation of gene expression compared to WT (Figure 2E), and a global gene upregulation was observed when we expressed UBE3D protein in the KO cells (Figure 2F). The genes downregulated in KO cells shared a high overlap with genes upregulated by UBE3D addback (Figure 2G), indicating that UBE3D was responsible for the gene expression changes.

**UBE3D depletion inhibits migration and invasion of breast cancer cells through down-regulating CPSF73**

CPSF73 has been shown to correlate with different cancers and is considered a biomarker for prognosis as well as a target for cancer treatment (Liu and Moore, 2021). CPSF73 knock-down reduced the migration of fibroblasts and triple-negative breast cancer cells (TNBC) (Mitra et al., 2018), a highly aggressive breast cancer subtype (Rakha and Ellis, 2009) in wound-healing assays in vitro. In addition, Kaplan Meier plots showed that a higher expression level of either UBE3D or CPSF73 correlates with a worse overall survival rate in patients with breast cancer (Figures S3A and S3B), indicating the clinical relevance of regulating UBE3D and CPSF73 levels. To test whether UBE3D also regulates CPSF73 in breast cancer cells, we tried to make UBE3D KO lines with the triple-negative breast cancer cell line MDA-MB-231 but could not recover cells with the knockout. Instead, we constructed two inducible UBE3D shRNA lines using MDA-MB-231 cells and a control cell line using shRNA targeting luciferase (shLUC). UBE3D immuno-staining showed a successful, doxycycline-inducible knockdown (KD) of UBE3D as well as a corresponding decrease in CPSF73 protein compared to the luciferase shRNA (Figure S3C). RT-qPCR showed that the CPSF73 mRNA level in UBE3D KD samples was not significantly changed (Figure S3D), indicating that similar to HEK293 cells, the regulation of CPSF73 by UBE3D in triple-negative cancer cells is also at its protein level.
Figure 2. *UBE3D* KO leads to impaired 3' end processing, transcriptional readthrough of PASs, and dysregulated gene expression

(A) Analysis of ACTB and MYC 3' end processing in *UBE3D* WT and KO cells. The diagram depicts the relative positions of unspliced (US) and PAS spanning (Span) amplicons. Span values are expressed relative to *UBE3D* WT cells after normalizing to US RNA levels from each gene. Error bars show the mean ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (Student’s t test, unpaired, two-tailed) from two biological replicates

(B) Volcano plot of DoGs in *UBE3D* WT versus *UBE3D* KO cells showing the log2-fold change in DoG expression compared to the log10 *P*adj value for each gene. A total of 2954 genes were identified with associated DoGs. Log2 fold change cutoff, 0.5; *P*adj =value cutoff, 0.05.

(C) Overlap analysis of DoGs upregulated in KO and DoGs downregulated in *UBE3D* addback samples.

(D) Reads maps of the non-histone mRNA SNRPA and histone mRNA HIST2H2BC11 show increased transcriptional readthrough into intergenic regions after *UBE3D* KO and suppression of the DoGs after *UBE3D* expression in the KO cells (KO + U). Traces for the individual replicates are shown in different shades.

(E) Volcano plot of differential gene expression in *UBE3D* KO cell versus WT cells. Log2 fold change cutoff, 1.0; *P*adj =value cutoff, 0.05.

(F) Volcano plot of differential gene expression in *UBE3D* addback cells versus KO cells. Log2 fold change cutoff, 1.0; *P*adj =value cutoff, 0.05.

(G) Overlap analysis of genes downregulated in KO and genes upregulated in *UBE3D* addback samples. For all panels, error bars show the mean ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (Student’s t test, unpaired, two-tailed).

*UBE3D* KO leads to impaired 3' end processing, transcriptional readthrough of PASs, and dysregulated gene expression.
Next, we tested the effect of downregulating UBE3D on mRNA 3' end processing and transcription termination in breast cancer cells. RT-qPCR showed a 1.5-fold or greater increase in unprocessed ACTB and MYC transcripts in the UBE3D KD cells upon Dox induction compared to the sh.LUC control, and no increase in cells without Dox (Figure 3A), demonstrating that UBE3D loss caused a processing defect.

UBE3D KD in MDA-MB-231 cells caused 280 genes to be downregulated and 113 genes to be upregulated.

Figure 3. UBE3D depletion inhibits breast cancer cell motility in a CPFS73-dependent manner

(A) Analysis of mRNA 3' end processing efficiency of ACTB and MYC transcripts in MDA-MB-231 after UBE3D KD from two biological replicates, as described in Figure 2A.

(B) GO term analysis of the genes downregulated in MDA-MB-231 UBE3D KD samples was performed using Metascape.

(C) Migration and invasion assessments in MDA-MB-231 cells upon UBE3D KD. Representative images of the migrated and invaded cells. Scale bar = 200 μm.

(D) Quantification of migratory and invasive cells upon UBE3D KD.

(E) CPSF73 overexpression in UBE3D KD cells was determined by Western blot.

(F and G) CPSF73 overexpression reversed the UBE3D KD-mediated MDA-MB-231 migration and invasion defects. Scale bar = 200 μm. For all panels, error bars show the mean ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (Student’s t test, unpaired, two-tailed).
Gene ontology (GO) analysis of the 113 genes upregulated in UBE3D KD samples showed that the two most enriched terms were in the transmembrane receptor protein serine/threonine kinase signaling pathway (Figure S3F), while the GO terms of the 280 downregulated genes revealed that cell mobility related gene sets, including extracellular matrix organization, wound healing, regulation of cell adhesion, were the most significantly enriched (Figure 3B), suggesting that UBE3D KD might induce a defect in migration and invasion. Moreover, while wild-type MDA-MB-231 cells are spindle-shaped, the UBE3D KD cells show a more compact epithelial-like morphology (Figure S4A). Scratch wound healing and Transwell assays were used to determine how UBE3D affected cell motility. UBE3D KD inhibited MDA-MB-231 migration and slowed the closure of the scratch wound (Figure S4B). Similarly, UBE3D KD significantly reduced the migration of MDA-MB-231 cells in Transwell assays (Figures 3C and 3D). In addition, Transwell invasion assays showed that UBE3D KD also markedly impaired the ability of MDA-MB-231 cells to move through the extracellular matrix, a process critical for cancer metastasis (Figures 3C and 3D). Inhibition of migration and invasion was not a consequence of reduced proliferation, as UBE3D KD had no effect on cell growth (Figure S4C).

The chemical JTE-607 was recently identified as a specific inhibitor of CPSF73 that acts by blocking RNA access to the nuclease’s active site (Liu and Moore, 2021; Ross et al., 2020). It also inhibits the growth of acute myeloid leukemia (AML) and Ewing’s sarcoma cancer, without a strong effect on most other types of cancer (Ross et al., 2020; Tajima et al., 2010; Uesato et al., 2006). As described above, the depletion of CPSF73 slowed breast cancer migration, and we wanted to know whether catalytic inhibition of CPSF73 would also affect the motility of breast cancer cells. We determined that the IC50 of JTE-607 against MDA-MB-231 (Basal B type TNBC) or MDA-MB-468 (Basal A type TNBC) cells were both greater than 50 μM (Figures SSA and S6D). Consistent with CPSF73 as a target, JTE-607 at a 2.5 μM concentration inhibited the processing of both ACTB and MYC transcripts (Figure 4A and S6A). Interestingly, the spindle-like morphology of MDA-MB-231 was also changed with the JTE-607 treatment (Figure S5B) in a way similar to what we observed with UBE3D KD. Scratch wound healing (Figure S5C) and Transwell migration (Figures 4B and 4C) assays revealed the inhibition of migration, and the Transwell invasion assay showed a significant reduction of invasiveness (Figures 4B and 4C). In addition, similar results were observed in MDA-MB-468 cells with JTE-607 in Transwell migration and invasion assay (Figures S6B and S6C). Thus, catalytic inhibition of CPSF73 is sufficient to alter these breast cancer cell phenotypes.

Three-dimensional (3D) cultures of tumor cells such as MDA-MB-231 can more accurately recapitulate the in vivo microenvironment than 2D growth and are considered a better model to evaluate drug effectiveness, as cells grown in this way can be altered in their sensitivity to potential cancer drugs (Fang and Eglen, 2017; Gunness et al., 2013; Pinto et al., 2020). To further evaluate the inhibitory potential of JTE-607 in tumor progression, we treated cells with JTE-607 in 3D culture, in which cells are embedded in a 10% Matrigel. Branching structures formed by cancer cells in 3D culture are associated with tumor invasion and aggressive phenotypes (Kenny et al., 2007). JTE-607 significantly reduced the formation of these structures in MDA-MB-231 cells (Figure 4D). We only observed invasive branching in the MDA-MB-231 line, but not in the MDA-MB-468 cells (data not shown). To test the effectiveness of JTE-607 in inhibiting tumor cell proliferation in 3D cultures, we assayed the cell numbers by the CellTiter-Glo 3D Cell Viability Assay and calculated an IC50 value of 24.6 μM in MDA-MB-231 and an IC50 value of 15.1 μM in MDA-MB-468, respectively (Figures S5D and S6D), suggesting that basal TNBC cells in 3D cultures are sensitized to JTE-607 treatment. The formation of spheres when cancer cells are embedded in 50% Matrigel is an indication of the self-renewal ability of the cells, a defining property of cancer cell stemness that affects metastasis, drug resistance, and cancer recurrence (Bahmad et al., 2018). Both MDA-MB-231 and MDA-MB-468 cells treated with JTE-607 were defective in spheroid formation (Figures 4E and S6E). These results demonstrate that the catalytic inhibition of CPSF73 suppressed the invasive and self-renewal phenotypes of triple-negative breast cancer cells.
CPSF73 expression level correlates to breast cancer cell self-renewal properties

Given the essential roles of cancer stem cells in breast cancer oncogenesis and metastasis, next, we explored the impact of direct manipulation of CPSF73 expression on breast cancer cell stemness. We made three inducible CPSF73 KD MDA-MB-231 cell lines (Figure 5A) and assayed the self-renewal properties after Dox-inducible knockdown. Our results showed that directly reducing CPSF73 mRNA levels inhibited breast cancer cell self-renewal, as determined by 3D sphere formation in Matrigel (Figures 5B and 5C). In contrast, when we overexpressed CPSF73 in MDA-MB-231 cells (Figure 5D), the self-renewal efficiency was enhanced, suggesting that CPSF73 overexpression can drive the cancer cell self-renewal (Figures 5E and 5F). Neither knockdown nor overexpression of CPSF73 affected proliferation in 2D culture (Figure S7), indicating that these manipulations are mostly affecting the stem cell population. These findings are consistent with our results in MDA-MB-231 cells treated with JTE-607. Given the fact that self-renewal is an essential property of cancer stem cells, our results support the value of future studies on...
the potential of CPSF73 and other mRNA 3'end processing factors as targets to suppress stem cell self-renewal and reduce TNBC metastasis.

**DISCUSSION**

The essential endonuclease CPSF73, a protein highly conserved from archaea to single-cell and metazoan eukaryotes, plays a central role in the 3' end processing of mRNA. UBE3D was reported as one of the CPSF73's interactors in human cells (Chen et al., 2014; Huttlin et al., 2017). In this study, we show that loss of UBE3D specifically decreases the level of CPSF73 but not that of other C/P proteins and this occurs at the post-translational level. We also demonstrated that targeting CPSF73 through UBE3D depletion or chemical inhibition disrupted mRNA 3' end processing and transcription termination, and surprisingly, inhibited breast cancer cell migration, invasion, and tumor sphere formation, which are properties that contribute to metastasis and relapse. Moreover, decreasing the CPSF73 mRNA level repressed sphere formation, while increasing the level enhanced it. Our findings support a previously unappreciated role for CPSF73 in promoting breast cancer cell stemness and suggest that understanding CPSF73's regulation may provide insights into new treatment avenues.

In our study, we found that CPSF73 downregulation on UBE3D depletion led to processing defects and increased gene readthrough transcripts. Poor 3' end processing will negatively affect the mRNA output of the upstream gene, in agreement with our observation that many genes showed decreased expression.

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**Figure 5.** CPSF73 expression level correlates with breast cancer cell self-renewal properties

(A) CPSF73 knockdown by three independent inducible shRNAs in MDA-MB-231 cells. (B and C) CPSF73 knockdown effectively inhibited MDA-MB-231 sphere formation in Matrigel. Spheres with a diameter over 50 μm were counted and quantified from three replicates. Representative images are shown. Scale bar = 200 μm. (D) Doxycycline-inducible overexpression of CPSF73 in MDA-MB-231 cells. (E and F) CPSF73 overexpression (OE) promoted MDA-MB-231 sphere formation in Matrigel. Spheres with a diameter over 50 μm were counted and quantified from three replicates. Representative images are shown. Scale bar = 200 μm. For all panels, error bars show the mean ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (Student’s t test, unpaired, two-tailed).
when CPSF73 was depleted. In addition to the direct consequences of poor 3’ end processing, CPSF73 dysfunction causes readthrough transcription as demonstrated here and in other studies (Eaton et al., 2018; Ross et al., 2020). Readthrough transcription can interfere with downstream gene transcription through promoter interference of genes arranged in tandem or alternatively, promote aberrant expression by generating RNA chimeras (Proudfoot, 2016). For convergent genes, it can trigger RNAi pathways or lead to RNA Polymerase II collisions (Proudfoot, 2016). Elongation into intergenic regions has been shown to remodel genome 3D organization and switch heterochromatin regions to chromatin permissive for transcription factor binding, and in this way, alter transcription programs (Heinz et al., 2018; Hennig et al., 2018). All of these consequences of poor processing and termination could contribute to the changes we have observed in cancer cell phenotypes when CPSF73 function is hindered.

The cell has found ways to naturally regulate CPSF73 activity at multiple levels—by recruiting it to different types of mRNA 3’ end processing complexes (reviewed in (Liu and Moore, 2021)), by limiting its association with the 3’ ends of transcribed genes, as has been observed after heat shock (Cardiello et al., 2018), by blocking its nuclear localization (Zhu et al., 2009), or by altering the level of its mRNA. Regarding the latter, the HIV-1 Tat protein increases CPSF73 mRNA levels to regulate both viral and cellular gene expression (Calzado et al., 2004), and the eukaryotic translation initiation factor eIF4E promotes the processing and nuclear export of mRNAs of CPSF73 and other CPSF subunits and consequently increases their protein levels (Davis et al., 2019). At the protein level, CPSF73 is subject to modification by SUMO and ubiquitin (Chen et al., 2014; Vethantham et al., 2007), but the consequences of such modifications have not been investigated except in yeast, where we showed that Ipa1 stabilizes Ysh1, the CPSF73 homolog, by inhibiting its ubiquitin-mediated proteasomal degradation (Lee et al., 2020). Our work with UBE3D, the Ipa1 counterpart, demonstrates that this regulatory mechanism is conserved.

Cancer cells frequently increase their transcriptional rate and the resulting stress on the pre-mRNA-processing machinery is a point that can be exploited therapeutically (Naro et al., 2021). The potential of this strategy is evident from a recent study showing that JTE-607, a specific CPSF73 inhibitor, suppressed the proliferation of specific types of cancer cells (Ross et al., 2020). JTE-607 was identified as an immuno-suppressive agent over two decades ago and progressed to clinical studies with healthy human volunteers (Kakutani et al., 1999). In the study by Ross et al., profiling of cell viability across 92 cell lines of diverse lineages showed that AML and Ewing’s sarcoma are the cell lines most sensitive to JTE-607 (Ross et al., 2020). Curiously, in comparison to these two cancers, the growth of the triple-negative cancer cell lines, MDA-MB-231 and MDA-MB-468, was relatively insensitive to JTE-607. However, we investigated the possibility that JTE-607 might have effects on cancer properties other than proliferation, such as metastasis and self-renewal. Indeed, we found that a low, non-cytotoxic dose of JTE-607 suppressed triple-negative breast cancer cell migration and invasion in both 2D and 3D cultures. These properties are indicative of a cancer cell’s ability to metastasize, which is the main cause of death in patients with cancer. JTE-607 also repressed tumor sphere formation in vitro, which is a measurement of the percentage of cancer stem cells in a tumor cell population. These cells can self-renew as well as differentiate into the cells that form the bulk of a tumor and are responsible for tumor initiation, chemical, and radiation resistance, metastasis, and relapse, all of which are properties that lead to therapy failure and mortality (Desai et al., 2019; Du et al., 2019; Shibata and Hoque, 2019; Yang et al., 2020; Zhou et al., 2021). Depletion of CPSF73 by shRNA-mediated knockdown or by interfering with UBE3D also supported the important role of CPSF73 in maintaining these cancer cell properties. In summary, our findings argue that the inhibition of pre-mRNA 3’ end processing, a fundamental cellular function, can be not only a druggable node for inhibiting cancer cell proliferation, but also for slowing cancer metastasis and recurrence.

Limitations of the study
This study described the conserved regulation of CPSF73 by UBE3D in human cells and revealed CPSF73 as a target for inhibiting triple-negative breast cancer migration, invasion, and self-renewal. In UBE3D KO cells, the increased DoGs can be interpreted by the depletion of CPSF73 while the decreased DoGs might be regulated by other pathways which are unclear. CPSF73 inhibition leads to gene readthrough, gene expression dysregulation, and alteration of triple-negative breast cancer cell properties. However, which mRNA expression changes or readthrough transcripts are mediating the output phenotypes have not been defined. Further work is necessary to comprehensively decode how an mRNA cleavage defect regulates breast cancer cell behavior. Additionally, whether targeting CPSF73 in vivo is sufficient to inhibit breast cancer growth and metastasis is unknown.
STAR+METHODS
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Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104804.

ACKNOWLEDGMENTS
The authors acknowledge the Tufts University High-Performance Compute Cluster (https://it.tufts.edu/high-performance-computing) which was utilized for computational analyses reported in this article. We thank Rebecca E. Batorsky at Tufts Technology Services for help with RNA-seq data processing. We also thank all members of the Moore laboratory for discussions and comments on the article. This work was supported by the National Science Foundation grant MCB1244043 and the NIH grant R01 GM101010-01A1 to C. Moore.

AUTHOR CONTRIBUTIONS
H.L. and C.L.M. conceived the study. D.H. performed sequence data analysis. H.L. conducted laboratory experiments. H.L. and C.L.M. drafted and edited the article. All authors contributed to the interpretation of data and approved the final article. C.L.M. provided supervision and funding acquisition.

DECLARATION OF INTERESTS
The authors declare no competing interests.

Received: January 20, 2022
Revised: May 26, 2022
Accepted: July 15, 2022
Published: August 19, 2022

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal anti-UBE3D | Abnova | Cat#PAB21883; RRID: AB_10966255 |
| Rat monoclonal α-Tubulin Antibody | Thermo Fisher Scientific | Cat#MA1-80189; RRID: AB_2210200 |
| Mouse monoclonal anti-GAPDH antibody (G-9) | Santa Cruz Biotechnology | Cat#sc-365062; RRID: AB_10847862 |
| Rabbit polyclonal anti-CPSF160 | Bethyl | Cat#A301-580A; RRID: AB_1078859 |
| Rabbit polyclonal anti-CPSF100 | Bethyl | Cat#A301-581A; RRID: AB_1078861 |
| Mouse monoclonal anti-CPSF73 antibody (C-3) | Santa Cruz Biotechnology | Cat#sc-39001 |
| Mouse monoclonal anti-CPSF4 antibody (D-1) | Santa Cruz Biotechnology | Cat#sc-390516 |
| Mouse monoclonal anti-FIP1L1 antibody (C-10) | Santa Cruz Biotechnology | Cat#sc-398392; RRID: AB_2811203 |
| Mouse monoclonal anti-Symplekin antibody (G-6) | Santa Cruz Biotechnology | Cat#sc-398897; RRID: AB_2811202 |
| Mouse monoclonal anti-WDR33 antibody (D-1) | Santa Cruz Biotechnology | Cat#sc-374466; RRID: AB_10988720 |
| Mouse monoclonal anti-CstF64 | C. Macdonald |        |
| Mouse monoclonal anti-CstF50 | Bethyl | Cat#A301-250A; RRID: AB_890586 |
| Mouse monoclonal anti-NUDT21 antibody (2203C3) | Santa Cruz Biotechnology | Cat#sc-398897; RRID: AB_2811202 |
| Rabbit polyclonal anti-Cpf11 antibody (A-7) | Santa Cruz Biotechnology | Cat#sc-515669 |
| Rabbit polyclonal anti-hClnp1 | Invitrogen | Cat#AAS02642C |
| Rabbit polyclonal anti-PAP antibody (H-300) | Santa Cruz Biotechnology | Cat#sc-32915; RRID: AB_2159213 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Polybrene | EMD Millipore | Cat#TR-1003-G |
| Puromycin | InvivoGen | Cat#ant-pr-1 |
| Doxycycline | Fisher Scientific | Cat#10592-13-9 |
| Cycloheximide | EMD Millipore | Cat#AC357420010 |
| MG-132 | Sigma-Aldrich | Cat#74790-5MG |
| TAK-243 | MedChem Express | Cat#HY-100487 |
| Chloroquine Diphosphate | Fisher Scientific | Cat#AAJ6445914 |
| JTE-607 | Tocris | Cat#5185 |
| Sulforhodamine B | Fisher Scientific | Cat#3520-42-1 |
| Crystal Violet | Thermo Scientific | Cat#548-62-9 |
| BSA solution | Sigma-Aldrich | Cat#9048-46-8 |
| EGF | R&D Systems | Cat#236-EG-200 |
| FGF | R&D Systems | Cat#233-FB-025 |
| B-27 | Life Technologies | Cat#17504044 |
| Matrigel | VWR | Cat#47743-718 |
| Fugene | Promega | Cat#E2311 |
| **Critical commercial assays** | | |
| SURVEYOR assay | Transgenomic | Cat#706025 |
| Monarch® Total RNA Miniprep Kit | New England BioLabs | Cat#T20105 |
| LunaScript® RT Super-Mix Kit | New England BioLabs | Cat#E30105 |
| Luna® Universal qPCR Master Mix Kit | New England BioLabs | Cat#M3003L |
| CellTiter-Glo Luminescent Cell Viability Assay | Promega | Cat#G7572 |

*Continued on next page*
### Deposited data

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HEK293 UBE3D KO RNA-seq | This paper | GEO GSE189746 |
| MDA-MB-231 UBE3D KD RNA-seq | This paper | GEO GSE189723 |

### Experimental models: Cell lines

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HEK-293            | ATCC   | CRL-1573   |
| HEK-293, UBE3D_KO  | This paper | N/A |
| MDA-MB-231         | ATCC   | CRM-HTB-26 |
| MDA-MB-231, UBE3D_KD#1 | This paper | N/A |
| MDA-MB-231, UBE3D_KD#2 | This paper | N/A |
| MDA-MB-231, UBE3D_KD#3 | This paper | N/A |
| MDA-MB-231, CPSF73_KD#1 | This paper | N/A |
| MDA-MB-231, CPSF73_KD#2 | This paper | N/A |
| MDA-MB-231, CPSF73_KD#3 | This paper | N/A |
| MDA-MB-231, mCherry_OE | This paper | N/A |
| MDA-MB-231, CPSF73_OE | This paper | N/A |
| MDA-MB-468         | ATCC   | HTB-132    |

### Oligonucleotides

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| sgRNAs for UBE3D KO | This paper (Table S1) | N/A |
| shRNAs for inducible UBE3D KD | This paper (Table S1) | N/A |
| shRNAs for inducible CPSF73 KD | This paper (Table S1) | N/A |
| Primers for SURVEYOR assay | This paper (Table S1) | N/A |
| CPSF73 qPCR primers | This paper (Table S1) | N/A |
| UBE3D qPCR primers | This paper (Table S1) | N/A |
| ACTB US primers | This paper (Table S1) | N/A |
| ACTB Span primers | This paper (Table S1) | N/A |
| MYC US primers | This paper (Table S1) | N/A |
| MYC Span primers | This paper (Table S1) | N/A |

### Recombinant DNA

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Constitutive human shUBE3D#1 | Sigma-Aldrich | Cat#TRCN0000146557 |
| Constitutive human shUBE3D#2 | Sigma-Aldrich | Cat#TRCN0000146775 |
| Constitutive scramble shRNA control | Addgene | Plasmid#1864 |
| Inducible human shUBE3D#1 | This paper | N/A |
| Inducible human shUBE3D#3 | This paper | N/A |
| Inducible shLUC (LT3GEPIR) | Addgene | Plasmid#111177 |
| UBE3D Human Tagged ORF Clone | ORIGENE | Cat#RC210924 |
| CPSF73 Human Tagged ORF Clone | ORIGENE | Cat#RC205834 |
| pSpCas9(Bb)-2A-GFP | Addgene | Plasmid#48138 |
| pSpCas9(Bb)-2A-GFP-UBE3D #3 | This paper | N/A |
| Inducible human shCPSF73#1 | This paper | N/A |
| Inducible human shCPSF73#2 | This paper | N/A |
| Inducible human shCPSF73#3 | This paper | N/A |
| pSBtet-mCherry | This paper | N/A |
| pSBtet-CPSF73 | This paper | N/A |

(Continued on next page)
RESOURCES AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Claire L. Moore (claire.moore@tufts.edu).

Materials availability

- Plasmids generated in this study will be made available upon request to the lead contact.
- Edited HEK293 cells and MDA-MB-231 cells generated in this study will be made available upon request to the lead contact.

Data and code availability

- All datasets generated or analyzed during this study are included in the published article. Detailed datasets supporting the current study are available from the lead contact upon request.
- RNA-seq data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- This paper does not report original code. Custom code is available from the lead contact upon request.
- 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 and cell culture

The human embryonic kidney HEK293 and triple-negative breast cancer MDA-MB-231 and MDA-MB-468 cell lines used in this study were cultured in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (10,000 U/mL). HEK293FT cells were grown in Dulbecco’s modified Eagle medium (DMEM) with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 1 mM MEM Non-Essential Amino Acids and 1% penicillin/streptomycin. All cells were grown at 37°C with 5% CO2.

Plasmids, viral infection, overexpression and stable cell line generation

The LT3GEPIR vector (Fellmann et al., 2013) used for constructing inducible shRNAs was purchased from Addgene. Human shRNAs against UBE3D (shUBE3D#1, ATTCAATCACAAACTGTCTGA; shUBE3D#2, TTTTATTATGACTTCACCGCAG; shUBE3D#3, TTTGATTAAACATTGAAATCAG) or CPSF73 (shCPSF73#1, TTGAATTTCTAATTCTTCCACA; shCPSF73#2, TAATATTAGGAATTCACTTCCACA; shCPSF73#3, TAATACATGATCTTCCACTTTC) were integrated into LT3GEPIR vector for inducible expression. shUBE3D#2 did not work for knockdown (data not shown). The small hairpin RNA sequence targeting luciferase was used as control (Fellmann et al., 2013). To generate lentiviruses, HEK293FT cells were co-transfected with the viral plasmid of interest and packaging and envelope plasmids using Fugene (Promega, E2311). Virus-containing supernatants were collected at 48 h after transfection. Target cells were infected with 0.45 μM-filtered viral supernatants in the culture medium supplemented with 8 μg/mL polybrene for 24 h. After 24 h, virus-containing medium was removed, and cells were grown in serum-containing medium for 24 h. Lentivirus-infected MDA-MB-231 cells were continuously selected by puromycin (2 μg/mL). For inducing shRNA expression, doxycycline...
was added into the medium at final concentration of 1.5 μg/mL and cells were monitored by fluorescence microscope for GFP expression. The knockdown of target protein was confirmed by immunoblot and RT-qPCR analysis.

For overexpression, plasmids encoding the open reading frames of UBE3D (NM_198920.3) and CPSF73 (NM_016207.4) were purchased from Genscript. pSBtet-GP was a gift from Eric Kowarz (Addgene plasmid # 60,495). Human CPSF73 (NM_016207.4) were subcloned into pSBtet-GP following the strategy described by Kowarz et al.19 to produce pSBtet-CPSF73. pCMV(CAT)T7-SB100 was used as a helper vector to supply the Sleeping Beauty transposase. pCMV(CAT)T7-SB100 was a gift from Zsuzsanna Izsvak (Addgene plasmid # 34,879). Each pSBtet plasmid was mixed 9:1 with helper plasmid in Opti-MEM (Gibco, 31,985-062). Transfection was performed using Lipofectamine 3000 (Invitrogen, L3000001) according to the manufacturer’s instructions. Cells which had successfully integrated the Sleeping Beauty cassette were selected using puromycin (2 μg/mL). Successful selection was confirmed by monitoring the percentage of GFP-positive cells. To induce overexpression, cells were cultured in fresh growth media with 1.5 μg/mL doxycycline.

Method details
CRISPR/Cas9 knockout
Knocking out (KO) of UBE3D in HEK293 cells was performed using the CRISPR/Cas9 genome editing system as previous described (Ran et al., 2013). The guide RNA sequence (AAAGGATCACCACGCGAAGGG) against human UBE3D was designed using CHOPCHOP software (http://chopchop.cbu.uib.no/) and cloned into pSpCas9(BB)-2A-GFP (Addgene plasmid ID: 48,138). HEK293 cells were transfected using Fugene, and GFP-positive cells were sorted by FACS and isolated into single cell clones of UBE3D knockout cells. The knockout efficiency was confirmed by SURVEYOR assay as reported (Ran et al., 2013) and immunoblot analysis.

Gene expression analysis by RT-qPCR
The extraction and purification of total RNA was performed using Monarch Total RNA Miniprep Kit (New England BioLabs, T2010S) according to the manufacturer’s protocol. RNA concentration and quality was determined using the NanoDrop 2000 spectrophotometer before downstream processing. Reverse transcription of isolated RNA was performed using the LunaScript RT Super-Mix Kit (New England BioLabs, E3010S) according to the manufacturer’s instructions. qPCR was performed on a CFX96 Touch Real-Time PCR Detection System in 96-well plates with the primers listed in Table S1. cDNA was added to the Luna Universal qPCR Master Mix Kit (New England BioLabs, M3003L) in a total reaction volume of 10 μL, and qPCR carried out according to the manufacturer’s instructions. Target gene expression was normalized to that of human RPL13A. Comparative 2^DDCt methods were used for the quantification of qPCR results.

Western blotting
Protein was extracted from whole cells by lysis in RIPA buffer (Thermo Fisher Scientific, 89,900) supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, 87,786). Cells were lysed on ice for 10 min and then homogenized by vortexing. Lysates were cleared by centrifugation at 12,000 x g for 15 min at 4°C. Supernatants were collected and total protein content quantified by BCA assay (Thermo Fisher Scientific, 23,227). Lysates were normalized by total protein content and prepared for western blotting with the addition of 4X SDS Sample Buffer (+355 mM β-mercaptoethanol) and heated to 95°C for 10 min. Lysates (20-80 μg) were resolved on 10% SDS gels and transferred to PVDF membranes. Total protein staining was performed using Revert Total Protein Stain (LI-COR, 926-11021). Membranes were blocked for 5 min using EveryBlot Blocking Buffer (Bio-Rad, 12,010,020). All primary antibodies were diluted in EveryBlot Blocking Buffer and incubated with membrane overnight at 4°C. Membranes were washed three times in TBS +1% TWEEN 20 and incubated with HRP (HorseRadish Peroxidase) labeled secondary antibodies (1:10,000) for 1h at room temperature. Chemiluminescence signal was captured using the Syngene Imager and the band intensity was quantified using ImageJ.

Cycloheximide chase analysis
UBE3D KO cells were seeded at a density of 5 × 10^5 cells per well in a 6-well plate and grown in DMEM medium. On the day of the experiment, the medium was replaced with fresh DMEM medium containing 100 μg/mL cycloheximide and incubated for the times indicated. DMSO-treated cells served as control. Cells were lysed with RIPA buffer and immunoblots performed as described above. The images of the blots were quantified using ImageJ. CPSF73 expression was normalized to GAPDH and the 0 h time point. The relative expression of CPSF73 in the time course was plotted by GraphPad 8.0.
Treatment with ubiquitin and proteasome inhibitors

UBE3D KO cells were seeded at a density of 5 × 10^5 per well in a 6-well plate and grown in DMEM medium. On the day of the experiment, the medium was replaced with fresh medium containing MG132 (proteasome inhibitor) and TAK-243 (ubiquitin E1 ligase) with the concentrations indicated. Vehicle-treated cells served as control. Cells were lysed with RIPA and immunoblots performed as described above. The images of the blots were quantified using ImageJ. CPSF73 expression was normalized to α-Tubulin and untreated samples. The relative expression of CPSF73 was plotted by GraphPad 8.0.

RNA-sequencing Total RNA from HEK293 cells was prepared using TRIzol Reagent (Invitrogen, 15,596,018) according to manufacturer’s protocol. Total RNA samples were submitted, and poly(A) selected RNAs were reversed transcribed to cDNA using random primers and used for 150bp paired end sequencing by Genewiz, Inc. For MDA-MB-231 cells, total RNA samples depleted of rRNA were converted to cDNA using random primers and 75bp paired-end stranded sequencing was done by Realseq, Inc. Three biological replicates were prepared for each sample.

Differential gene expression analysis and functional annotation Raw reads were trimmed by Cutadapt (Martin, 2011), and the quality of clean reads was evaluated with FastQC and MultiQC programs. Trimmed reads were aligned to the hg38 annotation of human genome using STAR v2.5.2b (Dobin et al., 2013). DESeq2 (Love et al., 2014) was used to analyze differential gene expression. The raw read counts were normalized using reads per kilobase of transcript per million mapped reads (RPKM). The significance of differential expression was estimated for each gene using the Wald test and then corrected for multiple comparisons using the Benjamini and Hochberg procedure (Padj). Visualization of the generated data was achieved using ggplot2 (Gómez-Rubio, 2017) and EnhancedVolcano (Blighe et al., 2022). GO enrichment analyses were performed using Metascape (https://metascape.org) (Zhou et al., 2019).

Gene readthrough analysis downstream of Genes (DoG) transcripts were identified using DoGFinder (Wiesel et al., 2018). Differential expression analysis of normalized read counts in DoG regions was performed using DESeq2, with shrinking of log2 fold changes using ashr (Stephens, 2017), significance testing using the Wald test, and multiple-comparison correction using Benjamini and Hochberg. Visualization of the generated data was achieved using ggplot2 (Gómez-Rubio, 2017) and EnhancedVolcano (Blighe et al., 2022). Eulerr (Larsson, 2020) was used to generate proportional Venn diagrams for overlap analysis. Normalized reads maps were visualized using Jbrowse (Buels et al., 2016).

Accession numbers RNA-seq data have been submitted to NCBI under the following GEO accession numbers: for UBE3D KO HEK293 samples, accession no.GEO: GSE189746; for UBE3D KD MDA-MB-231 samples, accession no.- GEO: GSE189723.

Transwell migration and invasion assays For the transwell migration assays, MDA-MB-231/MDA-MB-468 cells were digested using trypsin/EDTA, and cells (4 × 10^4 cells) were suspended with 200 µL DMEM medium and seeded onto the upper chamber of x 24-well transwell (8 µm aperture). The lower chamber of the transwell is filled with 500 µL of DMEM medium, and the transwell incubated at 37 °C for 16 h. For migratory cell staining, transwell inserts were removed from 24-well plates and washed twice with PBS, followed by scraping off non-invaded cells on the top of the membrane with a cotton swab. The migrated cells were fixed with ice-cold methanol for 15 min and then stained with 0.2% crystal violet for 20 min. After washing with water, five pictures for each group were taken randomly under an inverted microscope. The cell numbers in each field were counted using ImageJ. Relative cell migration activities were expressed as the fold change over respective controls. Data were analyzed by GraphPad Prism 8.0. For the invasion assay, cells (8 × 10^4 cells) were plated on the top of chambers (Corning 3422, USA) covered by Matrigel (ITEM: Corning Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix, LDEV-Free, 5mL, Corning 356,230) according to the manufacturer’s instructions. Briefly, Matrigel was thawed at 4 °C overnight and diluted (1:8) in cold DMEM, and 80 µL of the diluted Matrigel placed into the upper chamber of 24-well transwell (8 µm aperture) and incubated with the transwell at 37 °C for 30 min. The remaining steps of cell seeding and crystal violet staining were the same as for the migration assay.
3D invasion assay
Growth Factor Reduced BD Matrigel (140 μL) was plated on the bottom of the 24-well plate and the plate placed in the incubator for 30 min. MDA-MB-231/MDA-MB-468 cells (3 x 10^4 cells) were added into serum-free medium DMEM/F12 supplemented with B-27 supplement (Life Technologies, 17,504,044), 0.6% BSA, 20 ng/mL EGF (R&D Systems, 236-EG-200), 10 ng/mL FGF (R&D Systems, 233-FB-025), and 1% penicillin/streptomycin. Matrigel was added to the cells to a final concentration of 10% and the cells were plated on the top of the Matrigel layer. After cells were incubated at 37 °C for 45 min, the indicated concentrations of JTE-607 were added to the cells. Pictures were taken after 72 h of incubation and cell branches in each colony are counted.

Sphere forming assay
Single MDA-MB-231/MDA-MB-468 cells (2000 cells per well) were resuspended in serum-free DMEM/F12 medium supplemented with B-27 supplement, 0.6% BSA, 20 ng/mL EGF, 10 ng/mL FGF, and 1% penicillin/streptomycin. Matrigel was added into the cells at a volume ratio of 1:1. Cells in medium with 50% Matrigel were plated onto a 24-well ultra-low attachment plate (Corning). Covering medium and JTE-607 were refreshed every three days. After 10 days of culture, the number of spheres larger than 50 μm was counted under an inverted microscope.

Cell proliferation assay
Stable cell lines with inducible UBE3D/CPSF73 shRNAs or CPSF73 overexpression were seeded at 50,000/well in 12-well plates and treated with Dox (1.5 μg/mL) for the indicated days. Cell number was determined using Sulforhodamine B (SRB) as reported (Vichai and Kirtikara, 2006). Briefly, culture medium was removed from cell monolayers and cells were washed once with sterile PBS, followed by cell fixation with cold 10% (W/V) TCA (trichloroacetic acid) overnight at 4 °C. Cells were stained by 0.4% SRB (Sigma) in 1% acetic acid, after which cells were washed with 1% acetic acid and air dried. SRB dye was dissolved in the same amount (400 μL) of 10 mM Tris buffer (pH 10.5) in each well. The absorption of SRB in each well was read at 492 nm in a microplate reader.

Wound healing assay
MDA-MB-231 WT and UBE3D KD Cells were plated in a 6-well plate. After 12–24 h cultivation, cells formed a confluent monolayer and scratches were performed using a 200 μL tip, and culture medium was replaced with 100 μL fresh complete medium with or without Dox. The wound was monitored under the fluorescence microscope at the start of the experiment (t = 0 h) and after 8, 16, or 24 h of incubation. For testing the effect of JTE-607 treatment on cell migration, JTE-607 was added into the cells after the scratch at the concentration indicated.

Cell viability assay after JTE-607 treatment
For the cell viability assay in 2D cultures, MDA-MB-231/MDA-MB-468 cells were cultured in DMEM medium with 10% FBS and seeded at a density of 5 x 10^3 per well in 96-well plates for 24 h, and then treated with JTE-607 at the concentrations indicated. After 72 h, cell numbers were determined using SRB assay as described above. For the cell viability assay in 3D cultures, MDA-MB-231/MDA-MB-468 cells were culture in DMEM/F12 supplemented with B-27 supplement, 0.6% BSA, 20 ng/mL EGF, 10 ng/mL FGF, and 1% penicillin/streptomycin. A density of 5 x 10^3 cells was seeded in a 96-well ultra-low attachment plate (Corning), ATP was analyzed using CelTiter-Glo 3D Cell Viability Kit (Promega, G9681) in accordance with the manufacturer's protocol. Briefly, an equal volume of CellTiter-Glo reagent was added into each well, and the cells were incubated at room temperature in the dark. Luminescence was measured in a microplate reader.

QUANTIFICATION AND STATISTICAL ANALYSIS
Data is presented as mean, ± SD or SEM, unless otherwise stated. Two or three independent biological replicates have been performed for each experiment. Data are statistically analyzed in GraphPad Prism Software 8.0 using Student’s t test. Detailed methods and p values are described in figure legends and individual method sections.