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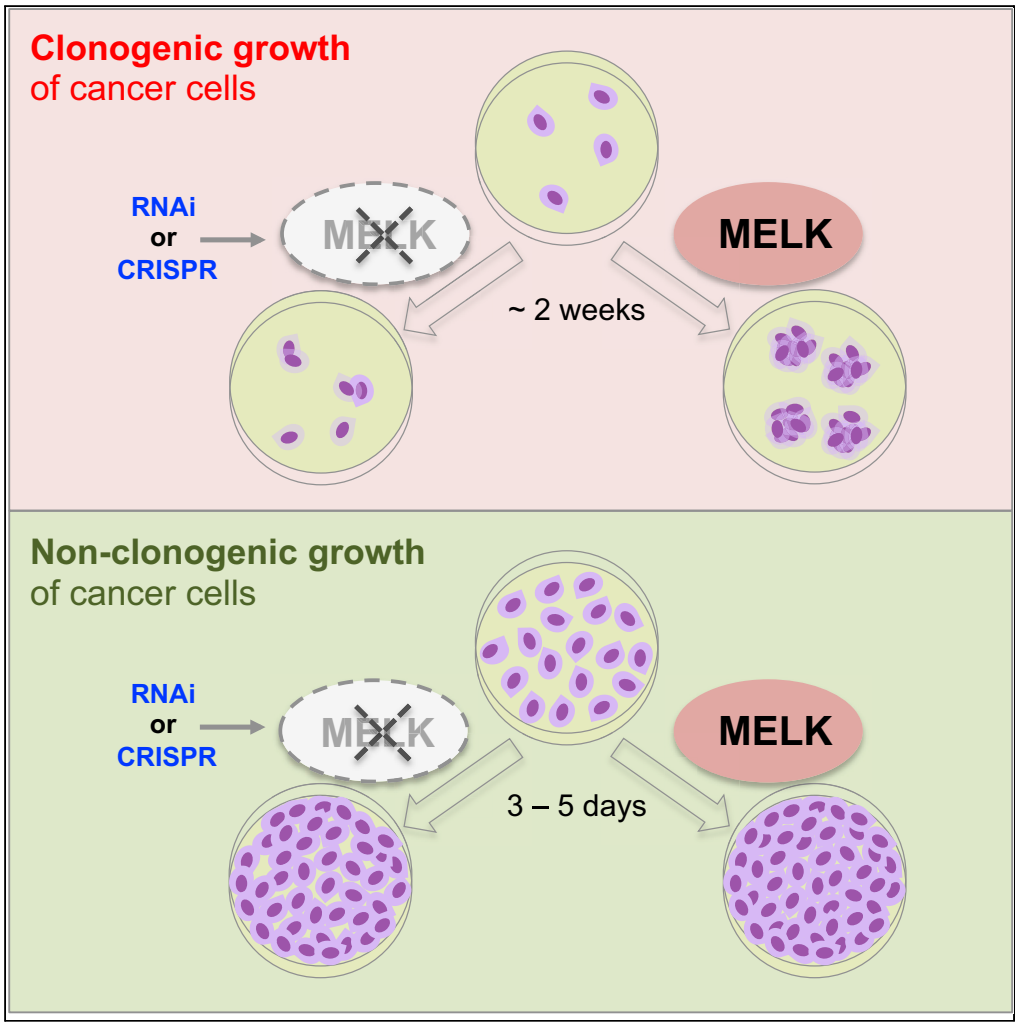

Yubao Wang, Ben B. Li, Jing Li, Thomas M. Roberts, Jean J. Zhao

yubao_wang@dfci.harvard.edu (Y.W.)
jean_zhao@dfci.harvard.edu (J.J.Z.)

HIGHLIGHTS

Inhibiting MELK expression compromises clonogenic growth of cancer cells

MELK depletion minimally affects non-clonogenic cell growth

MELK depletion by RNAi or CRISPR has similar effects on cell growth

Cancer cell dependency on MELK is similar to that on classic oncogenes

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A Conditional Dependency on MELK for the Proliferation of Triple-Negative Breast Cancer Cells

Yubao Wang,[1,2,*] Ben B. Li,[1,2,3] Jing Li,[1] Thomas M. Roberts,[1,2] and Jean J. Zhao[1,2,3,4,*]

SUMMARY

The role of maternal and embryonic leucine zipper kinase (MELK) in cancer cell proliferation has been contentious, with recent studies arriving at disparate conclusions. We investigated the *in vitro* dependency of cancer cells on MELK under a range of assay conditions. Abrogation of MELK expression has little effect under common culture conditions, in which cells are seeded at high densities and reach confluence in 3–5 days. However, MELK dependency becomes clearly apparent in clonogenic growth assays using either RNAi or CRISPR technologies to modulate MELK expression. This dependency is in sharp contrast to that of essential genes, such as those encoding classic mitotic kinases, but is similar to that of other oncogenes including MYC and KRAS. Our study provides an example demonstrating some of the challenges encountered in cancer target validation, and reveals how subtle, but important, technical variations can ultimately lead to divergent outcomes and conclusions.

INTRODUCTION

Maternal and embryonic leucine zipper kinase (MELK) is overexpressed in multiple cancers, including breast cancer (Gray et al., 2005; Wang et al., 2014), and is frequently included in gene sets used to predict the risk of breast tumor recurrence (Parker et al., 2009; Van De Vijver et al., 2002; Van't Veer et al., 2002). Notably, in breast tumors, MELK is most highly overexpressed in triple-negative breast cancer (TNBC) (Wang et al., 2014), a subgroup that lacks expression of hormone receptors or human epidermal growth factor receptor 2 (HER2) and is extremely aggressive with few effective therapies.

To investigate the functional role of MELK, we and others previously used an inducible short hairpin gene knockdown approach and found that MELK knockdown suppresses TNBC cell growth (Wang et al., 2014; Touré et al., 2016). In addition, hormone receptor-positive breast cancer cells and untransformed cells are insensitive to MELK depletion, indicating a selective dependency of TNBC cells on MELK (Wang et al., 2014; Touré et al., 2016). Moreover, recent studies using RNA interference (RNAi) techniques have found MELK to be a promising target in other cancer types, including melanoma (Janostiak et al., 2017), prostate cancer (Jurmeister et al., 2018), high-risk neuroblastoma (Guan et al., 2018), adrenocortical carcinoma (Kiseljak-Vassiliades et al., 2018), chronic lymphocytic leukemia (Zhang et al., 2018), and diffuse intrinsic pontine glioma (Meel et al., 2018).

Despite these studies suggesting an important role for MELK in cancer, the validity of MELK as a cancer target was recently challenged. Gene editing of MELK, mediated by transducing Cas9-expressing cells with guide RNA-encoding lentivirus, was found to have little effect on cancer cell proliferation (Lin et al., 2017). Specifically, lentiviral transduction of MELK-targeting guides abrogated MELK protein expression in TNBC lines (CAL-51, MDA-MB-231) and a melanoma cell line (A375), but did not alter cancer cell growth in any of these cases (Lin et al., 2017). In addition, the authors noted that multiple high-throughput genetic screens had not identified MELK as a potential cancer target (Hart et al., 2015; Marcotte et al., 2012, 2016). The authors further derived MELK knockout clones of cancer cells and demonstrated that these clonal cells grew normally both in cell culture and in xenograft mouse models (Giuliano et al., 2018; Lin et al., 2017).

Consistent with these findings, our laboratories and those of our collaborators also generated clones featuring CRISPR-mediated MELK knockout in MDA-MB-468 cells, a TNBC line that is highly sensitive to inducible short hairpin RNA (shRNA)-mediated knockdown of MELK (Touré et al., 2016; Wang et al., 2014). Intriguingly, the MELK CRISPR knockout clones have similar response as the parental cells to five MELK-targeting shRNAs (Huang et al., 2017), raising a number of possibilities including (1) growth

[1]Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA 02215, USA

[2]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA

[3]Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA

[4]Lead Contact

*Correspondence: yubao_wang@dfci.harvard.edu (Y.W.), jean_zhao@dfci.harvard.edu (J.J.Z.)

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inhibition caused by multiple independent shMELK is due to off-target effects or (2) certain functional, shMELK-sensitive MELK isoforms may be present in the knockout cells but remain unaffected by CRISPR reagents. Notably, the MDA-MB-468 cell line harbors gene amplification of MELK and a splice mutation in the MELK gene (Figure S1; Barretina et al., 2012), bringing further challenges in data interpretation.

These conflicting observations not only initiated a debate into whether MELK is indeed a cancer target, but also more generally contributed to broader questions on how best to identify and validate cancer targets. In this instance, one possibility is that these particular disparities stem from differences in RNAi and CRISPR-Cas9 technologies. However, these studies utilized shRNA and single guide RNA (sgRNA) of multiple sequences, and the results have been independently reproduced. Moreover, a comparison between RNAi and CRISPR screens in cancer cell lines reveals consistency regarding cancer gene dependencies (McDonald et al., 2017). Therefore, we hypothesize that these disparities may originate from fundamental differences in the target validation assays, rather than the choice of genetic tools.

We examined in depth the experimental procedures of the studies concerned (Wang et al., 2014; Touré et al., 2016, Hart et al., 2015: Lin et al., 2017; Marcotte et al., 2012, 2016) and noted that among other technical variations there are considerable differences in assay formats between studies that identified MELK as a target and those that showed the contrary or did not find MELK as potential target. Specifically, the former studies (Wang et al., 2014; Touré et al., 2016) tested MELK dependency using clonogenic growth assays, which examined the ability of single cells to proliferate into colonies (Franken et al., 2006; Puck and Marcus, 1956), whereas the latter studies (Hart et al., 2015; Lin et al., 2017; Marcotte et al., 2012, 2016) primarily employed common cell growth assays, wherein cells are seeded at relatively high density to allow them to reach confluence within 3–5 days for analysis or are sub-passaged if necessary. In this study, we demonstrate that this subtle technical variation leads to dramatic differences in experimental outcomes—that is, MELK is required for clonogenic growth of TNBC cells regardless of how its expression is abrogated, but the effects of its abrogation are largely negligible under the common "short-term, high-density" culture conditions.

RESULTS

RNAi Study Reveals MELK Dependence for Clonogenic Cell Growth

We evaluated whether the outcome of proliferation assays testing MELK dependence is influenced by different assay formats, using inducible RNAi technology to modulate MELK expression. We confirmed that efficient MELK knockdown occurred as early as 1 day after doxycycline induction (Figure 1A), supporting the suitability of this approach for studying functional effects in short-term assays. In addition, MELK knockdown efficiency is comparable between triple-negative (BT549) and estrogen receptor-positive (ER+, MCF7) cells (Figure 1B). In both cell lines, MELK knockdown did not cause any significant effects on cell growth when cells were seeded at a density such that they reached confluence in 3–5 days (Figure 1C). In the TNBC cell line, however, a growth inhibitory effect of shMELK appeared with decreasing plating density and became clearly apparent under long-term (11-day) clonogenic conditions (Figure 1C, left column). In contrast, ER+ breast cancer cells were substantially more resistant to MELK knockdown, even under long-term clonogenic conditions (Figure 1C, right column). These findings demonstrate that determination of MELK requirement for proliferation is strongly dependent on the assay conditions.

Identifying MELK Guide Sequences for Efficient Gene Editing

We next attempted to confirm these RNAi-based findings using CRISPR/Cas9-mediated gene editing approaches, to rule out any potential artifacts conferred by RNAi technology. A lack of understanding of the biology of MELK isoforms (UniProt Consortium, 2016; Figure 2A) brings challenges to the rational design of guide sequences. However, the kinase domain of MELK constitutes the prioritized target, given its location at the amino terminus as well as its essential role for MELK functions. We evaluated the seven sgRNA sequences used in the previous study by Lin et al., five of which target early exons encoding MELK kinase domain and two of which target the kinase-associated domain 1 (KA1) residing in the carboxyl terminus (Lin et al., 2017).

We cloned these guide sequences into an all-in-one lentiCRISPR vector (Sanjana et al., 2014; Shalem et al., 2014) and generated lentiviral particles. Target cells were transduced twice with freshly prepared virus, and 7 days after initial transduction, they were tested for MELK expression by immunoblotting.

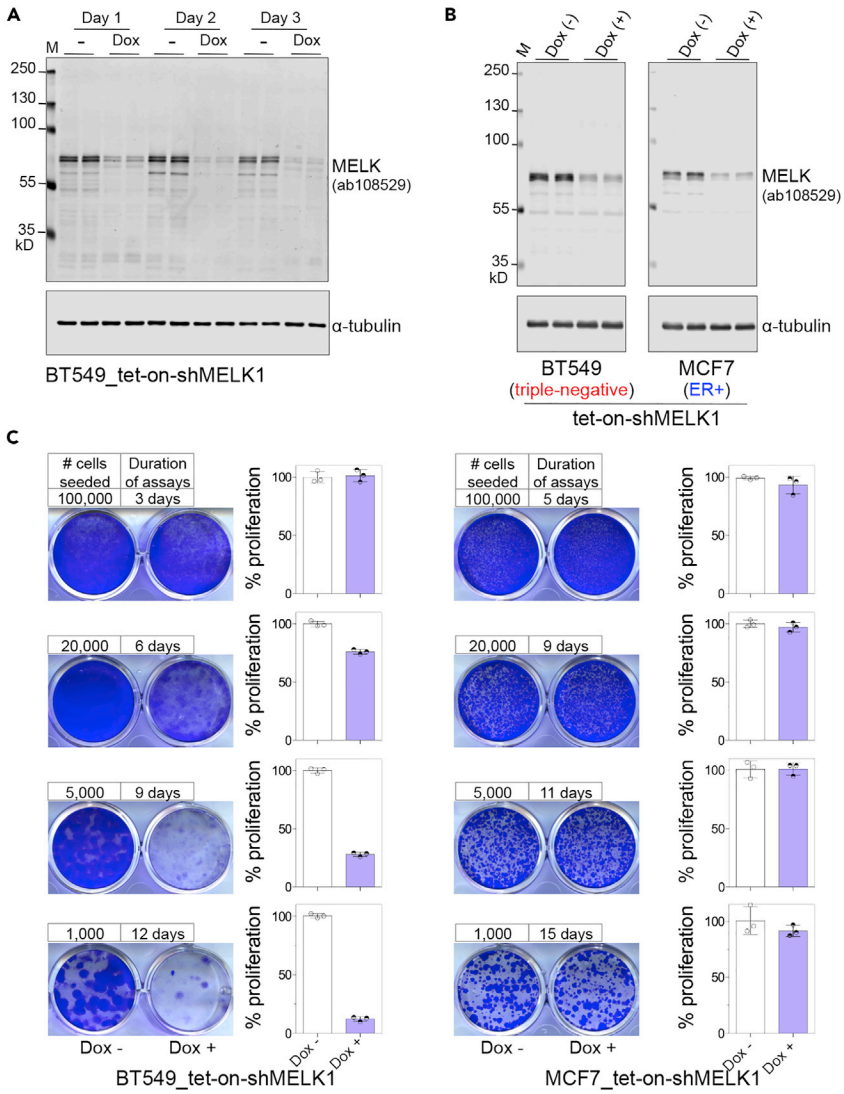

Figure 1. Doxycycline-Inducible Knockdown of MELK Impairs the Clonogenic Growth of TNBC Cells

(A) Fluorescent western blot analysis of MELK in BT549 cells that were stably transduced with tet-on-shMELK1 (Wang et al., 2014). Cells were either left untreated or treated with doxycycline (100 ng/mL) for the indicated time periods. The images were acquired via Odyssey CLx Infrared Imaging System (LI-COR Biosciences). Note that the first lane (M) was loaded with PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific, cat# 26619), which have near-infrared fluorescence (molecular weight of the markers indicated).

(B) Fluorescent western blot analysis of MELK in BT549 and MCF7 cells that were stably transduced with tet-on-shMELK1. Cells were either left untreated or treated with doxycycline (100 ng/mL) for 2 days before lysate preparation and immunoblotting.

(C) Cells were seeded in 12-well plates at the indicated densities and were either left untreated or treated with doxycycline (100 ng/mL). Upon harvest, cells were fixed and stained with crystal violet. The staining was then extracted for quantification of cell growth (mean ± SD; n = 3).

Notably, we found that different target sequences had distinctly varied effects on the protein abundance of MELK (Figure 2B), assayed by two antibodies raised against epitopes in the amino or carboxyl terminus of MELK. We also noted the same effects of guide sequences in another cancer cell line (Figure S2A). Among the seven guide sequences targeting MELK, sg_6 caused the greatest reduction in MELK expression (Figures 2B and S2A). Using sg_6, we further demonstrated that its effect on MELK protein abundance lasted until at least 11 days after transduction (Figure S2B), indicating the suitability of this tool for testing clonogenic cell growth.

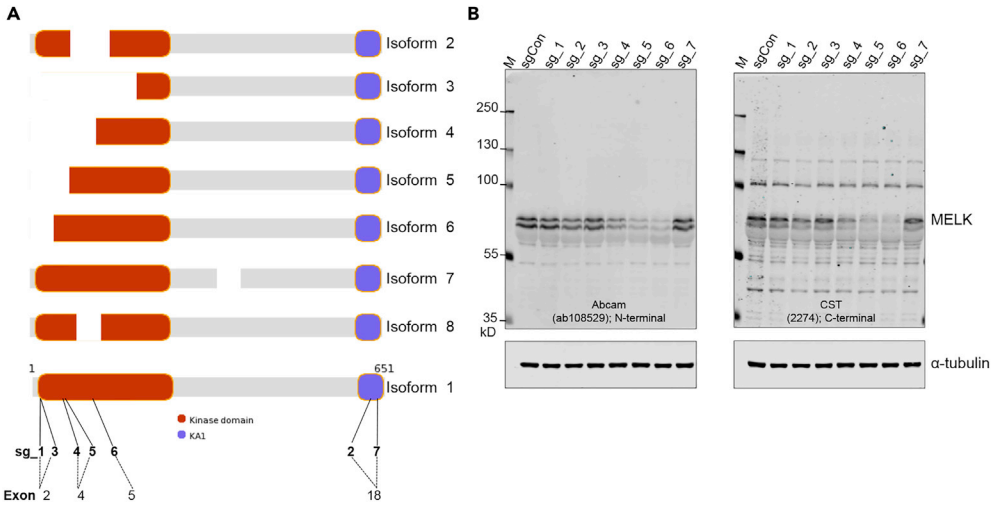

Figure 2. Identifying Guide Sequences for Efficient Gene Editing of MELK

(A) Schematic diagram of human MELK transcripts. The longest full-length one is isoform 1, shown at the bottom. The target locations of seven guide sequences (Lin et al., 2017), as well as the exon number of the MELK gene, are indicated. (B) Fluorescent western blot analysis of MELK in MDA-MB-231 cells infected with control or MELK-targeting lentiCRISPR. Cells were harvested 7 days after infection. Total cell lysates were resolved on freshly cast 8% SDS-PAGE, transferred onto nitrocellulose membranes. Membranes were incubated with the indicated primary antibodies against MELK. The images were acquired using the Odyssey CLx Infrared Imaging System. Note that among the total seven guide sequences, sg_6 caused the most efficient reduction of MELK protein abundance.

CRISPR/Cas9-Mediated Gene Editing Reveals a Role of MELK for the Clonogenic Growth of Cancer Cells

After identifying a guide sequence (sgMELK_6) that can efficiently reduce MELK protein abundance, we proceeded with subsequent experiments to examine whether MELK depletion elicits any functional consequences.

We infected cells with virus encoding all-in-one lentiCRISPR (Figure 3A), confirmed efficient genomic editing (Figure 3B), and observed an apparent decrease of MELK protein level via the use of multiple independent anti-MELK antibodies (Figure 3C). Concurrently, cells were seeded at a relatively high density (50,000 cells per well, 12-well plates) for short-term cell proliferation assays and at a low density (500 cells per well) for clonogenic growth assays. Consistent with our RNAi-based findings, little growth reduction was observed in 3-day assays, whereas approximately 80% growth inhibition was observed under clonogenic growth conditions in 10-day colony formation assays (Figure 3D). Consistent with this observation, we also used the dual-vector lentiCRISPR platform (Lin et al., 2017) and found a similar inhibition on clonogenic cell growth by MELK gene editing (Wang et al., 2018).

Based on these findings, we reasoned that cells introduced with MELK guide would likely "drop out" during culture under the same conditions. The lentiGuide vector (LRG2.1) encodes GFP (Lin et al., 2017; Tarumoto et al., 2018), providing an excellent tool for measuring the depletion or enrichment of sub-population of cells. Despite a lack of antibiotic-resistant gene on LRG2.1 for selection, we were able to achieve an efficient decrease of MELK protein abundance in cells infected with MELK guide, as evidenced by fluorescent immunoblotting (Figure 4B). After Cas9-expressing MDA-MB-231 cells were introduced with lentiGuide, the cells were mixed with parental cells (GFP negative) and seeded for clonogenic assays (Figure 4A). Using this protocol, we found that cells expressing MELK guide were depleted 3-fold more than cells transduced with lentivirus encoding control sgRNA (Figure 4C), indicating a compromised fitness of MELK-edited cells under the clonogenic culture conditions.

CRISPR/Cas9-Mediated Editing of Essential Genes and Oncogenes

We next compared the effects of MELK gene editing with those of essential genes, particularly classic mitotic kinases that more often scored in genome-wide functional screens (Hart et al., 2015; Marcotte

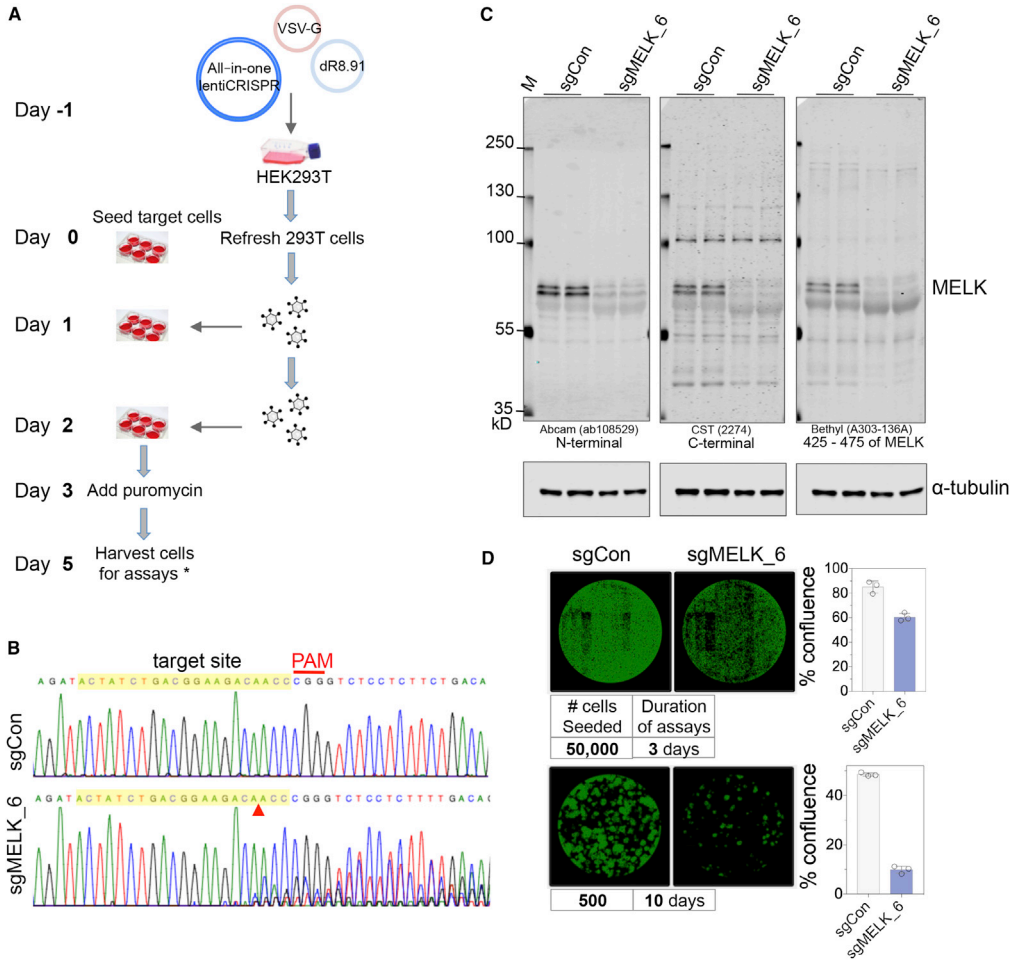

Figure 3. CRISPR/Cas9-Mediated Gene Editing of MELK in TNBC Cells

(A) Workflow of lentiCRISPR-mediated gene editing in cancer cells. Asterisk denotes that following the treatment of puromycin (1.5 μg/mL; 48 hr), all uninfected cells died, whereas the viability or proliferation status of infected cells were unaffected by puromycin selection.

(B) Sanger sequence traces of PCR products that amplify exon 5 of MELK. The predicted cutting site (red triangle) and protospacer adjacent motif (PAM) are indicated. The target site of sgMELK_6 is highlighted yellow.

(C) Fluorescent western blot analysis of MELK in MDA-MB-231 cells infected with control or MELK-targeting lentiCRISPR (harvested 11 days after infection). Information about MELK antibodies (vendor, catalogue number, and position of the used peptide immunogen) is indicated.

(D) lentiCRISPR-mediated gene editing of MELK suppresses the clonogenic growth of MDA-MB-231 cells. Four days after initial virus infection, cells were harvested and seeded in 12-well plates. Cell proliferation was measured by Celigo Imaging Cytometer (Nexcelom Bioscience) on indicated days post plating (mean ± SD; n = 3). The whole-well images filled in with a green color indicate cell confluence.

et al., 2012, 2016). CRISPR-Cas9-mediated editing of PLK1 or AURKB yielded modest loss of protein expression (Figures 5A, S3A, and S3B) but had dramatic suppression of cell growth under both regular and clonogenic culture conditions (Figures 5B and S3C). These observations suggest that reliance of cells on expression of essential genes is evident independent of assay conditions.

We further asked whether the requirement of MELK for clonogenic growth is a unique feature of this gene, or whether it is a characteristic shared with other (proto-)oncogenes. To this end, we performed a side-by-side comparison of MELK gene editing with that of KRAS and MYC in MDA-MB-231 cells, a cell line that harbors KRAS-activating mutation G13D and is sensitive to the depletion of KRAS (Kopp et al., 2014; approximately 30% inhibition of cell growth 7 days after transfection of KRAS-targeting small interfering RNA) or MYC (Kessler et al., 2012; approximately 80% inhibition of clonogenicity following viral

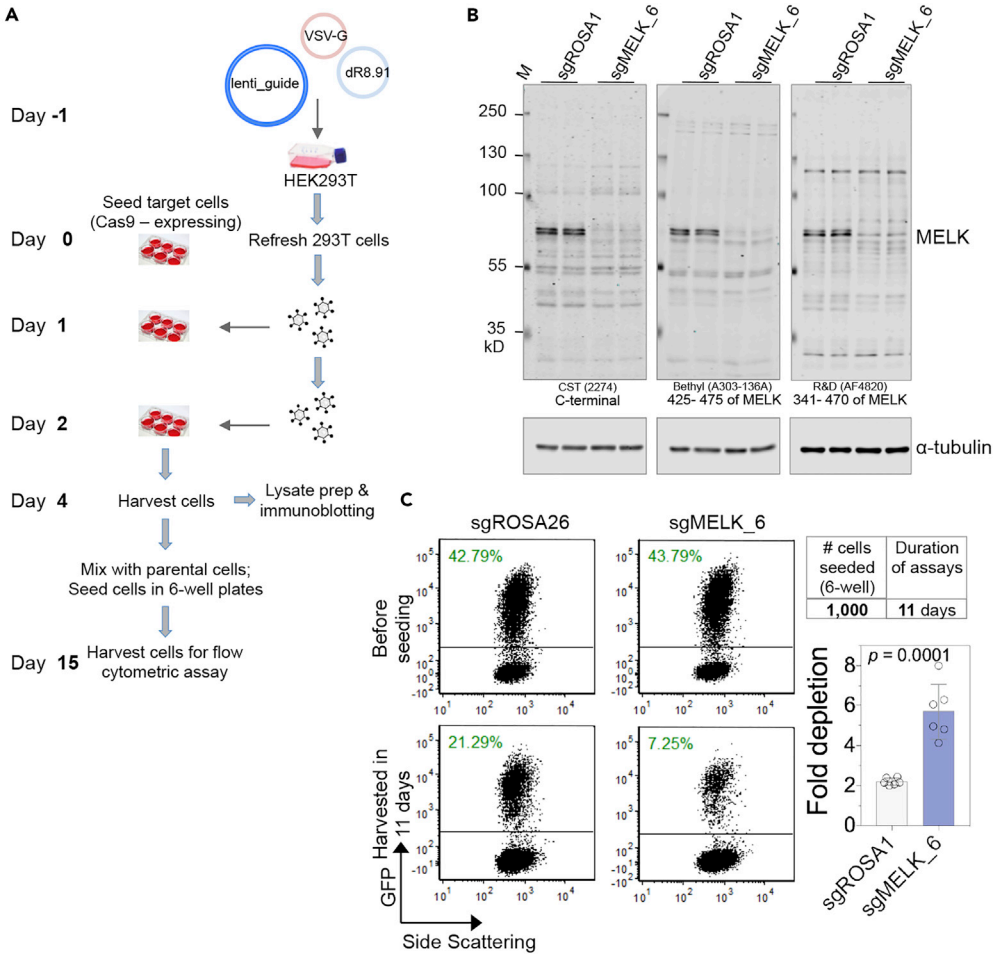

Figure 4. Depletion of Cancer Cells following CRIPSR/Cas9-Mediated Gene Editing of MELK

(A) Workflow of the experimental procedures. Note that the cells (MD-MB-231) express Cas9 and that the lentiGuide vector encodes GFP.

(B) Fluorescent western blot analysis of MELK in Cas9-expressing MDA-MB-231 cells infected with control or MELK-targeting lentiGuide. Cells were harvested 4 days after the initial virus infection. Information of MELK antibodies is indicated.

(C) Cas9-expressing MDA-MB-231 cells were infected with GFP-encoding lentiGUIDE that targets ROSA or MELK. Four days after the first (of total two) infection, cells were harvested and mixed at 1:1 ratio with uninfected cells (MDA-MB-231-Cas9). Part of the cells was fixed for flow cytometric analysis (top panel). The remaining cells were seeded in 6-well plates (1,000 cells per well) and harvested in 11 days for measuring the percentage of GFP+ cells (bottom panel). The quantification of fold depletion from six duplicates is shown in the histogram (right).

transduction of shMYC). Similar to MELK, KRAS or MYC gene editing also caused cell growth inhibition that is largely dependent on the initial plating density (Figure 5C). Consistent with these genetic studies, a recent study found that spheroid assays have been found to be required for the majority of KRAS G12C cancer cell lines to show sensitivity to a covalent inhibitor of KRAS G12C, whereas under regular short-term monolayer culture conditions only a minority of G12C mutant lines are sensitive to the treatment (Janes et al., 2018).

Correlation between Gene Dependency and Self-Expression

Having made consistent observations between RNAi- and CRISPR/Cas9-mediated perturbation of MELK, we next analyzed existing large-scale datasets to identify any potential pattern of MELK dependency. We analyzed the gene dependency scores from Project Achilles and gene expression values from the Cancer Cell Line Encyclopedia among the hundreds of cancer cell lines that were scored in both datasets (Figure 6A) (Broad Institute; Barretina et al., 2012; Tsherniak et al., 2017). In Project Achilles, gene dependency scores were generated from a genome-scale RNAi screen that was performed across 501 cancer cell lines,

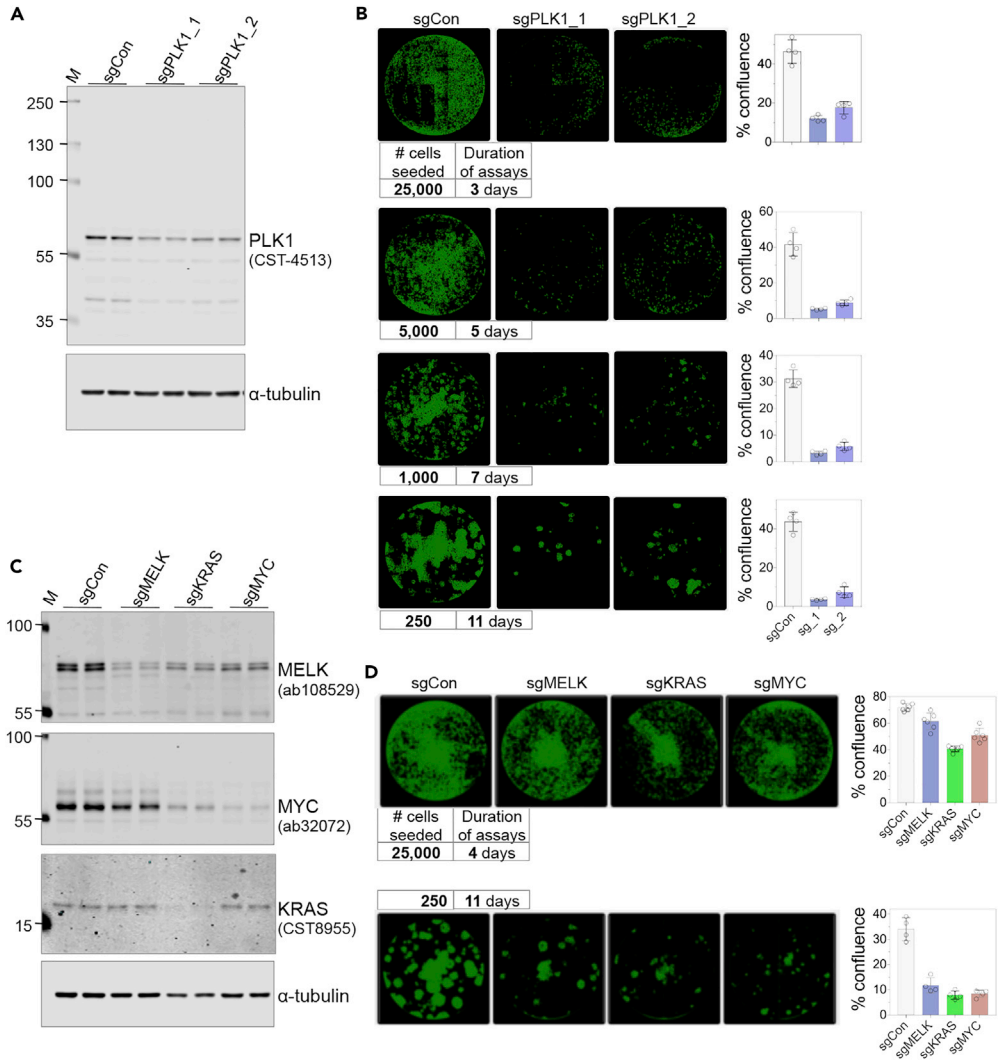

Figure 5. CRISPR/Cas9-Mediated Gene Editing of Potential Cancer Targets in TNBC Cells

(A) Fluorescent western blot analysis of PLK1 in MDA-MB-231 cells infected with control or all-in-one lentiCRISPR targeting PLK1. Note that cells were harvested 5 days after the initial infection.

(B) lentiCRISPR-mediated gene editing of PLK1 suppresses the growth of cancer cells, largely independent of assay formats. Four days after initial virus infection, cells were harvested and seeded in 24-well plates. Cell proliferation was measured by Celigo Imaging Cytometer (Nexcelom Bioscience) on indicated days post plating (mean ± SD; n = 4). The whole-well images filled in with a green color indicate cell confluence.

(C) Fluorescent western blot analysis of lysates that were harvested from MDA-MB-231 cells infected with all-in-one lentiCRISPR vectors targeting control sequence (sgCon) or those of MELK, KRAS, or MYC. Cells were lysed 4 days after initial infection.

(D) Cells were harvested 3 days after initial all-in-one lentiCRISPR infection and seeded in 24-well plates at the indicated densities. Cell proliferation was measured by cell confluence.

using a computation model to distinguish between on- and off-target effects of RNAi (Tsherniak et al., 2017). Despite the practice of cell culture at a high seeding density, the long-term cell culture (at least 28 days or 16 population doublings) combined with the quantitative readout of the screen enables the comparison of gene dependence among different cancer cell lines.

Using the Cancer Cell Line Encyclopedia, we focused on its gene expression data since genetic mutations and gene amplifications of MELK are quite rare in cancer. Interestingly, by analyzing the data from the two data sets, we found a moderate, but statistically significant, negative correlation between MELK

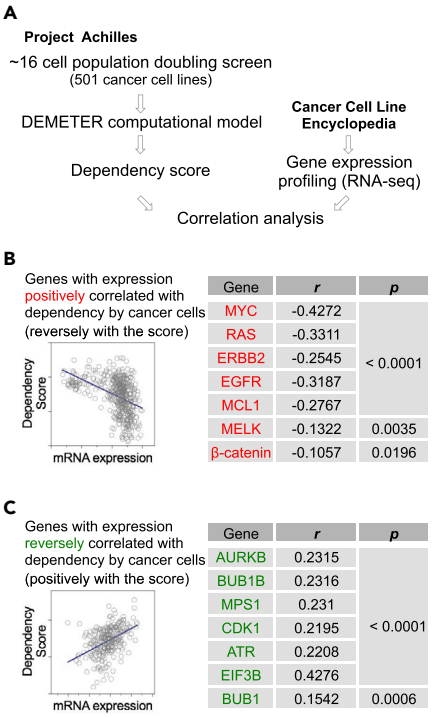

Figure 6. Correlation between MELK Expression and Cancer Cell Dependence on MELK

(A) Workflow of data analysis. Genome-wide RNAi screen was performed in 501 cancer cell lines, followed by DEMETER computational analysis for exclusion of off-target effects of RNAi (Tsherniak et al., 2017). The dependency score values were then subject to correlation analysis with gene expression, a database generated by Cancer Cell Line Encyclopedia (Barretina et al., 2012).

(B and C) Analysis of indicated genes for the correlation between cancer cell dependency scores and gene expression. The left plots demonstrate exmples where expression of a given gene is reversely (B) or positively (C) correlated with the scores of cancer cell dependence on the gene (each dot represents one cancer cell line). The right tables include correlation values for the indicated genes. Pearson correlation coefficient (*r*) is shown, with p values denoting the statistical significance test for Pearson correlation (GraphPad Prism 7). Note that greater depletion of cells expressing shRNAs targeting specific genes causes more negative dependency scores, indicating greater dependency of cancer cells on these genes.

dependency scores and its gene expression (Figure 6B). This is indicative of cells with higher expression of MELK having greater dependence (and more negative dependency scores). We further used the same approach and analyzed well-established (proto-)oncogenes, including MYC, KRAS, ERBB2, EGFR, MCL, and β-catenin. Similar to MELK, the dependency scores of these oncogenic drivers were all negatively correlated with their expression levels (Figure 6B).

In contrast, the expression levels of genes encoding classic mitotic kinase (e.g., AURKB, CDK1, MPS1, BUB1, BUB1B) or components of essential cellular machinery (e.g. EIF3B), were positively correlated with dependency scores (Figure 6C). This is consistent with the notion of these serving as essential genes, whereby cell lines with low expression of such genes—due to either genomic or epigenetic alterations—are likely more sensitive to gene knockdown. Indeed, a previous study documented the CYCLOPS (copy number alterations yielding cancer liabilities owing to partial loss) phenomenon, and found that CYCLOPS genes are enriched for components of fundamental cellular processes such as RNA splicing and proteasome-mediated protein degradation (Nijhawan et al., 2012). Together, these analyses suggest that MELK behaves in a more similar manner to an oncogenic factor than an essential gene.

DISCUSSION

Driven by the recently arising controversy on the validity of MELK as a cancer target, we utilized both inducible RNAi and lentiCRISPR tools for modulating the expression of MELK, and investigated the impact of gene expression perturbation under a range of assay conditions. Our study demonstrates that MELK is required for clonogenic proliferation of TNBC cells but that its abrogation has negligible effects under regular culture conditions that allow cells to reach confluency after short-term culture. This is in contrast to essential genes, such as classic mitotic kinases, whose necessity for cell proliferation is observable independent of such assay conditions. Notably, such a role of MELK for cancer cell growth was also observed for oncogenes including MYC and mutant KRAS. Furthermore, analysis of gene dependency across hundreds of cancer cell lines revealed that MELK dependency has a drastically different pattern from that of essential genes, but is similar to that of established oncogenic drivers.

Is MELK a Viable Cancer Target?

The functional dependence on MELK has been reported in models representing an array of tumor types (reviewed by Settleman et al., 2018), and more recently in a few others including prostate cancer (Jurmeister

et al., 2018), adrenocortical carcinoma (Kiseljak-Vassiliades et al., 2018), chronic lymphocytic leukemia (Zhang et al., 2018), and diffuse intrinsic pontine glioma (Meel et al., 2018). These studies, including our earlier report of MELK as a target for TNBC (Wang et al., 2014), all used RNAi as the genetic approach. The conclusions of these studies were recently questioned because of the potential off-target effects of RNAi and upon the observations that, in other studies, CRISPR/Cas9-mediated gene editing had no appreciable impacts on tumor cell growth (Giuliano et al., 2018; Lin et al., 2017). We believe that our current findings underline the importance of assay design and technical considerations in cancer target validation efforts and begin to explain and reconcile some of the disparities in the current literature concerning the role of MELK in cancer cell proliferation.

Our study lends confidence toward continued investigation of MELK modulation as a potential approach for cancer therapy. The role of MELK for clonogenic growth of TNBC cells was evidenced by the use of three genetic tools: doxycycline-inducible shRNA, all-in-one lentiCRISPR, and dual-vector lentiCRISPR. We also found that both the functional reliance on MELK by our experimental cell models and MELK dependency across hundreds of cancer cell lines (Tsherniak et al., 2017) is surprisingly similar to established oncogenes, and in contrast to essential genes. These data support the notion of MELK dependence by specific types of tumor cells.

Nevertheless, a number of critical issues need to be addressed before anti-MELK strategies are considered for clinical investigation. For MELK-targeting approaches, their magnitude of efficacy *in vivo* remains a key question. Will perturbing MELK activity or expression effectively decrease tumor burden or improve response to existing therapies? An inherent demand of these studies is the availability of MELK-targeting methods with sufficient potency and selectivity. Directions for future investigation may include the construction of cell models with inducible gene editing of MELK and development of MELK inhibitors with desired potency and pharmacokinetic features.

Given the widespread utility of small molecules in cancer research and treatment, we summarize MELK-targeting compounds that were recently developed or identified from compound library screens (Table 1). Among these studies, one interesting strategy is to find MELK as an off-target of drugs that are either approved or in clinical development, and to leverage the information on scaffold and chemical groups for further design and optimization (Edupuganti et al., 2017; Klaeger et al., 2017).

RNAi versus CRISPR: Which Is the Right Choice?

Our study uses both RNAi and CRISPR approaches in examining MELK dependency. From this direct comparison, we hope to provide some insights into the choice of genetic tools for perturbing gene expression in cancer biology studies.

With regard to the efficiency of targeting gene expression, it is tempting to term RNAi as a "knockdown" and CRISPR as a "knockout" technique. Our study, however, fails to tell which tool excels, but does indicate that CRISPR is not equal to gene knockout, at least in the context of using non-clonally-derived, pooled populations of cells generated from lentiviral transduction of a single guide sequence and antibiotic selection. This is consistent with the occurrence of in-frame mutations during CRISPR/Cas9-mediated gene editing (Koike-Yusa et al., 2014). Another feature of CRISPR, similar to RNAi, is the unpredictability on gene editing effect. It is common to observe that some guides are completely ineffective in altering target protein abundance (Figures 2 and S3B). The observation might be explained by the possibility that certain loci remain inaccessible to the gene editing machinery. As such, our studies indicate that neither tool is able to entirely overcome the deficiencies of the other, but that the two tools—CRISPR and RNAi—are likely to be complementary, especially in the settings of studying gene function in pooled population of cells.

In summary, we provide evidence—based on both RNAi and CRISPR tools—that MELK is required for clonogenic cell growth. This feature, together with the observed pattern of MELK dependency among hundreds of cancer cell lines, points toward MELK as an oncogenic kinase. We expect the current study to contribute to a valuable, and necessary, discussion about how best to design target validation assays and evaluate the fitness of such assays for their designed purposes.

Limitations of the Study

The current study focuses on MELK in MDA-MB-231, a cell line that was used in both our previous RNAi-based study (Wang et al., 2014) and two recent ones that leveraged the tool of CRISPR/Cas9-mediated gene editing

Compound	Biochemical IC_{50} (nM)[a]	Reference	Description
OTSSP167	0.41	Chung et al., 2012	Highly potent but unselective
	0.5	Huang et al., 2017	
		Klaeger et al., 2017	
NVS-MELK8a	2	Touré et al., 2016	Highly selective; inhibiting TNBC cell growth
	11.9	Huang et al., 2017	
17	3 ± 0.8	Edupuganti et al., 2017	Inhibiting TNBC cell growth
HTH-01-091	10.5	Huang et al., 2017	Low potency in TNBC cells
PF-3758309	~30	Klaeger et al., 2017	An inhibitor of PAK4
Nintedanib	43	Klaeger et al., 2017	A multi-kinase inhibitor approved for idiopathic pulmonary fibrosis
	~100	Edupuganti et al., 2017	
BI-847325	~100	Klaeger et al., 2017	An MEK and aurora kinase inhibitor

Table 1. MELK Inhibitors

[a]The biochemical assays vary in the use of different forms of MELK recombinant protein (such as full-length versus kinase domain only), substrates, and readouts.

(Giuliano et al., 2018; Lin et al., 2017). Although we believe that the current study solves some of the discrepancies among these different observations, it does not explain how MELK knockdown still compromises cell growth in clonal MELK-null MDA-MB-468 cells (Huang et al., 2017). Although the phenotype was considered to evidence off-target effects of a total of five independent shMELKs, data interpretation can be challenged by the MELK gene amplification status in this cell line, a situation that tends to introduce difficulties in creating homozygous MELK-null clonal cells by CRIPSR technique. Nevertheless, we expect that if given sufficient time and selection pressure, MELK-resistant clones could be generated from parental cancer cells that have MELK dependence, similar to previous observations for Kras (Mou et al., 2017). It would be interesting to study factor(s) substituting for or forming synthetic lethal interactions with MELK.

Another limitation of the current study concerns the genetic tool used for MELK knockdown. The constitutive expression of both Cas9 and guide RNA in cells transduced with all-in-one lentiCRISPR limits the ability to examine MELK dependency in established tumors *in vivo*. Further study of MELK as a cancer target would necessitate the development of models whereby efficient MELK knockdown can be triggered by inducible expression of Cas9 and guide sequences.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods and three figures and can be found with this article online at https://doi.org/10.1016/j.isci.2018.10.015.

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AUTHOR CONTRIBUTIONS

Y.W., B.B.L., T.M.R., and J.J.Z. designed the experiments. Y.W. and J.L. conducted the experiments. Y.W., B.B.L., T.M.R., and J.J.Z. wrote the paper.

DECLARATION OF INTERESTS

Y.W. and J.J.Z. are inventors on patent applications WO2014110163, WO2015073509, WO2016141296, and WO2016141279. The other authors declare no completing interests.

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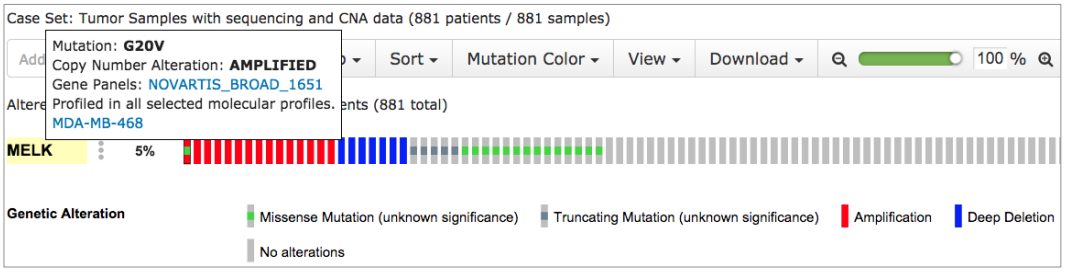
Supplemental Information

A Conditional Dependency on MELK for the Proliferation of Triple-Negative Breast Cancer Cells

Yubao Wang, Ben B. Li, Jing Li, Thomas M. Roberts, and Jean J. Zhao

A



B

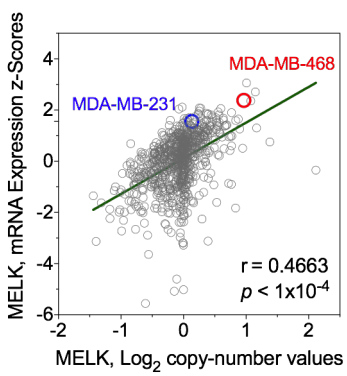


Figure S1. Genetic Alteration of MELK Gene in Human Cancer Cell Lines, related to Figure 3 and 4.

(A) Status of MELK genetic alteration in human cancer cell lines. The amplification, deletion, and/or mutation occur in 5% human cell lines (total 881 lines tested; Barretina et al., 2012). The oncoprint visualization was derived from the inquiry of MELK gene in the database of Cancer Cell Line Encyclopedia (Barretina et al., 2012), performed at cBioPortal for Cancer Genomics (www.cbioportal.org; Cerami et al., 2012; Gao et al., 2013). Note that MDA-MB-468, one of TNBC lines we previously used for tet-on inducible MELK knockdown (Wang et al., 2014) harbors a splice site mutation (G20V) and gene amplification of MELK. Given that multiple cutting derived from CRISPR guides targeting amplified loci causes DNA damage response and consequently gene-independent inhibition of cancer cell growth (Aguirre et al., 2016; Munoz et al., 2016), MDA-MB-468 is considered less suitable for assessing MELK function using CRISPR/Cas9-mediated gene editing.

(B) Expression of MELK (assayed by microarray) versus its copy number (log_2) in cancer cell lines. The data were generated by Cancer Cell Line Encyclopedia (CCLE) (Barretina et al., 2012) and downloaded from cBioPortal for Cancer Genomics. Note that MDA-MB-231 cell lines does not have MELK gene amplification, and have a relatively high level of MELK expression.

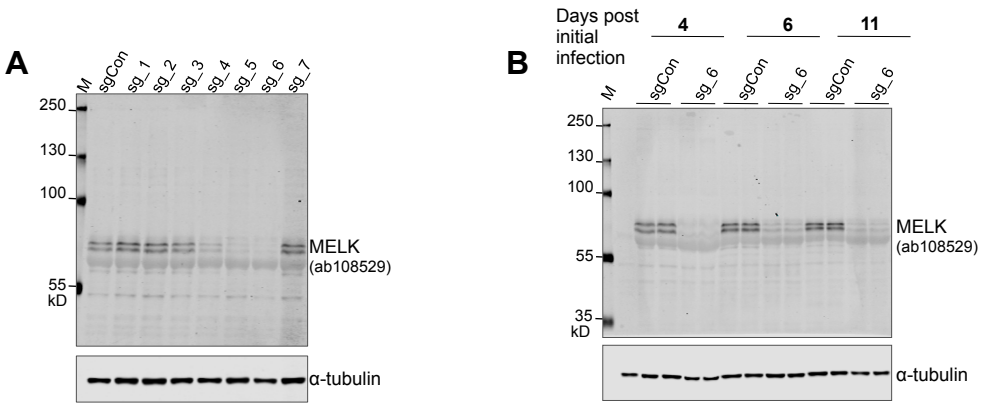


Figure S2. Identifying Guide Sequences Targeting MELK, related to Figure 2.

(A) Fluorescent western blotting analysis of MELK in MDA-MB-468 cells infected with control or MELK-targeting lentiCRISPR. Cells were harvested seven days post infection. Total cell lysates were resolved on 8% SDS-PAGE, transferred onto nitrocellulose membranes. Membranes were incubated with anti-MELK (ab108529, Abcam). The images were acquired via Odyssey CLxinfrared imaging system (LI-COR Biosciences). Note that in this cell line, sg_6 is the most effective guide sequence in reducing MELK protein abundance.

(B) Examining the efficiency of gene editing in cells harvested at different time points after lentiCRISPR infection.

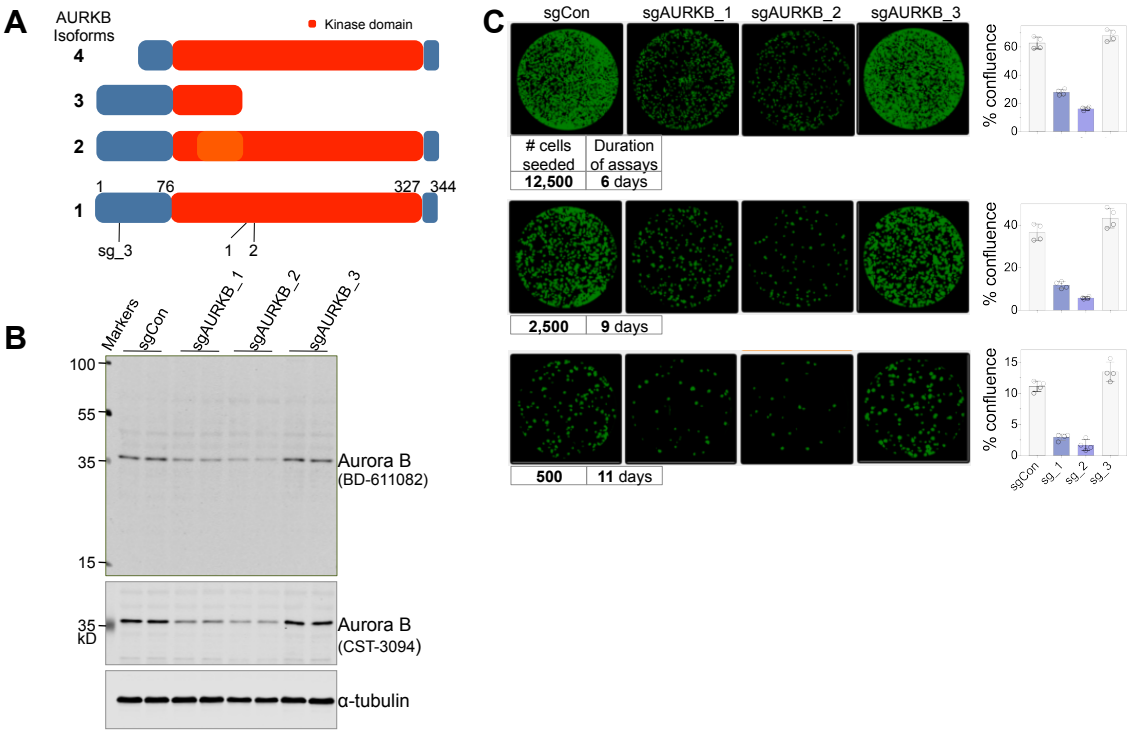


Figure S3. LentiCRISPR-mediated Editing of AURKB in TNBC Cells, related to Figure 5.

(A) Schematic diagram of human AURKB transcripts. The longest full-length one is isoform 1, shown at the bottom. The target locations of three guide sequences are indicated.

(B) Fluorescent western blotting analysis of AURKB in MDA-MB-468 cells infected with control or lentiCRISPR targeting AURKB (harvested five days after initial infection). Information of AURKB antibodies is indicated

(C) LentiCRISPR-mediating gene editing of AURKB suppresses the growth of cancer cells in a manner that is largely independent of the duration of cell growth assays. Four days after the initial virus infection, cells were harvested and seeded in 24-well plates. Cell proliferation was measured by Celigo Image Cytometry (Nexcelom Bioscience) on indicated days post plating (mean ± SD; n=4). The whole-well images filled in with a green color indicate cell confluence.

TRANSPARENT METHODS

List of antibodies and reagents for immunoblotting

	SOURCE	IDENTIFIER	DESCRIPTION
PageRule Plus Prestained Protein Ladder	ThermoFisher Scientific	26619	Generating near-infrared signal in fluorescence immunoblotting.
Anti-MELK	Abcam	ab108529	Rabbit monoclonal (EPR3981)
	Cell Signaling	2274	Rabbit polyclonal
	Bethyl	A303-136A	Rabbit polyclonal
	R&D	AF4820	Sheep polyclonal
Anti-AURKB	Cell Signaling	3094	Rabbit polyclonal
	BD Biosciences	611082	Mouse monoclonal (6)
Anti-PLK1	Cell Signaling	4513	Rabbit monoclonal (208G4)
Anti-RAS	Cell Signaling	8955	Rabbit monoclonal (D2C1)
Anti-MYC	Abcam	ab32072	Rabbit monoclonal (Y69)
Anti-α-tubulin	Cell Signaling	3873	Mouse monoclonal (DM1A)
Anti-rabbit IgG	Life Technologies	A21109	Secondary antibody; conjugated with Alexa Fluor 680
Anti-mouse IgG	Rockland	610-145-121	Secondary antibody; conjugated with DyLight 800
Anti-Sheep IgG	Jackson ImmunoResearch	713-655-147	Secondary antibody; conjugated with Alexa Fluor 790

Cell Culture

Human breast cancer cell lines (BT549, MDA-MB-231, MDA-MB-468, and MCF7) were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gemini Bioproducts) and 1% penicillin/streptomycin (Invitrogen). For cells stably introduced with tetracyclin-inducible shRNAs, Tet System Approved FBS (Clontech, cat# 631106) was used.

HEK293T cells were cultured in DMEM (Invitrogen) with 10% FBS and 1% penicillin/ streptomycin.

Construction of all-in-one lentiCRISPR vectors

Cloning of lentiCRISPR targeting MELK, PLK1, AURKB, KRAS, or MYC was performed in the backbone of all-in-one lentiCRISPR v2 vector (a gift from Feng Zhang, Addgene plasmid # 52961; Sanjana et al., 2014). Briefly, forward and reverse oligos (synthesized at Eton Bioscience) were mixed and annealed, ligated with BsmBI-digested lentiCRISPR backbone, and then transformed into competent E.coli (Stbl3). Following overnight incubation in warm room, single bacterial colonies were transfered into 50 µl sterile water, 2 ul of the suspension was then used as template for PCR using U6 primer and individual reverse oligo as primers. Positive colonies were subject to culture for midiprep of plasmids (Qiagen). Plasmids were verified by sequencing using U6 primer (Eton Bioscience). The sequence of oligos is listed in the following table.

Guide RNA sequences	
sgCon	GAGCTGGACGGCGACGTAAA
sgMELK_1	ATGAATTACATGAAACTATT
sgMELK_2	AACCCGATGTGGTGGGTATC
sgMELK_3	TATGAATTACATGAAACTAT
sgMELK_4	TCAATCTCCGTTTTGATCCG
sgMELK_5	CCGGATCAAAACGGAGATTG
sgMELK_6	CTATCTGACGGAAGACAACC
sgMELK_7	AGCGGCTTAAGGGCGATGCC
sgAURKB_1	ATTCTAGAGTATGCCCCCCG
sgAURKB_2	GCTCCTTGTAGAGCTCCCCG
sgAURKB_3	GCTCTTTCCGGAGGACTCGC
sgPLK1_1	AGCCAAGCACAATTTGCCGT
sgPLK1_2	TACCTACGGCAAATTGTGCT
sgKRAS	AACATCAGCAAAGACAAGAC
sgMYC	GCCGTATTTCTACTGCGACG

Lentivirus Packaging and Infection

One day prior to transfection (day 0), HEK293T cells were seeded in T-25 tissue culture flasks (2.5 - 3 million cells seeded). On day 1, 4 µg DNA (2 µg vector DNA, 1.5 µg pCMVdR8.91, and 0.5 µg pMD2-VSVG) and 12 µl polyethylenimine (PEI; homemade from powder purchased from Polysciences, cat # 23966-2) were each diluted in PBS, mixed, and added to cells following 15 min incubation at room temperature. The medium was refreshed on day 2. On day 3 and 4, viral supernatant was collected and filtered through 0.45-µm membranes, supplemented with polybrene at the final concentration of 8 µg/ml (Millipore, cat# TR-1003-G), and then freshly added to target cells.

Target cells were seeded in 6-well plates one day after HEK293T transfection. On the next day, after removal of old medium, 1.5 - 2 ml fresh viral supernatant collected was added to the cells. The infection was repeated on the following day. Two days after the initial infection, cells were refreshed with medium containing puromycin (1.5 μg/ml). After two days of puromycin selection, all uninfected cells (set as a control) are expected to die while infected cells appear as healthy as normally cultured cells.

PCR and sequencing

Genomic DNA was extracted using a PureLink Genomic DNA Mini Kit (Invitrogen, #K1820-01), and used as template for PCR. NovaTaq™ Hot Start DNA Polymerase (EMD Millipore, #71091) was used to amplify exon 5 of human MELK, with the following primers: Forward, 5'-CCTTACTCGGTTCCATTCCCT-3'; reverse, 5'-AGGTATGACTGGAGCAACAACA-3'. The forward primer was also used for Sanger sequencing (Eton Bioscience).

Immunoblotting

Cells were rinsed with PBS and then lysed in 1x SDS-PAGE sample buffer (typically 200-400 μl used for one well of cells that were seeded one day earlier at the density of 4×10^5 per well of a 6-well plate). Lysates were boiled for 5 min before loading on 8% for detection of MELK, MYC, PLK1, or α-tubulin, or 12% SDS-PAGE for detection of AURKB or KRAS. PageRuler™ Plus Prestained Protein Ladder (ThermoFisher Scientific, cat# 26619) was loaded (1 μl in the total volume of 20 μl SDS-PAGE sample buffer) (Note that the total six blue-prestained recombinant proteins fluoresce in the 700 nM channel of Odyssey Imaging System). Nitrocellulose membrane with protein transferred was blocked with 5% non-fat milk and was then incubated with primary antibodies overnight at 4°C. After washing, the membrane was incubated with fluorophore-conjugated secondary antibodies for one hour at room temperature. The membrane was then washed and scanned with an Odyssey CLxinfrared imaging system (LI-COR Biosciences). Antibodies are listed in a separate table.

Cell growth assays

After cells were harvested and re-suspended in medium, cell counting was performed with the Countess Automated Cell Counter (Life Technologies). Cell viability, determined via trypan blue dye exclusion, is expected to be higher than 90% in all assays. A high concentration of cell suspension was first made (e.g. 5×10^4 cell per ml), and then diluted serially (e.g. 5-fold

dilutions involve mix 1 ml concentrated cells with 4 ml medium). Cells were subsequently seeded in multi-well plates (typically, 1 and 0.5 ml per well for 12- and 24-well plates respectively).

For colongenic assays, each well was replenished with medium five days after seeding. Cell proliferation was measured by calculating cell confluence, which was performed via scanning whole wells with Celigo Image Cytometry (Nexcelom Bioscience). Alternatively, at the endpoints of assays, cells were fixed with formalin and subsequently stained with crystal violet. The stained plates were scanned before the staining was extracted by 10% acetic acid with absorbance measured.

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