LMTK2: A Novel Drive of Endocrine Resistance in ERα+ Breast Cancer

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ABSTRACT

Breast cancer is the most common female malignancy in the U.S. and the leading cause of cancer-related deaths in women worldwide. Nearly 80% of breast tumors express estrogen receptor alpha (ERα) which drives tumor progression. While CDK4/6 inhibitors in combination with endocrine therapy improve overall survival in the metastatic setting and disease-free survival in the adjuvant setting, many patients eventually relapse/progress with resistant forms of disease. In the post-CDK4/6 inhibitor setting, second line endocrine therapy responses are limited, highlighting the need for alternative treatment approaches. Through a genome-wide CRISPR knockout screen, we identified Lemur Tail Kinase 2 (LMTK2) as the top hit exhibiting synthetic lethality in the presence of the tamoxifen metabolite, endoxifen, in both endocrine-sensitive and -resistant cell line models. We further studied multiple other endocrine resistant models and identified that LMTK2 mRNA and protein expression were both increased in ERα cell lines resistant to estrogen deprivation, tamoxifen, fulvestrant, abemaciclib and palbociclib, as well as in endocrine resistant patient derived models. We then studied LMTK2 in patient samples and identified copy number gain/amplification in 67% of ERα+ human metastatic breast tumors, while only 1% of primary tumors harbored these genomic alterations. From a prognostic standpoint, elevated tumoral LMTK2 expression was associated with worse recurrence-free survival and overall survival in early-stage breast cancer. Through proteomic studies, we identified the MET-PI3K-AKT pathway as overactivated in LMTK2 overexpressing cells. Unlike activating *PIK3CA* mutations that drive AKT signaling, LMTK2 was found to employ an alternative mechanism to activate AKT signaling by inhibiting PP1α, an AKT-inhibitory phosphatase. Further, LMTK2 overexpression was sufficient to drive endocrine and CDK4/6 inhibitor resistance *in vitro* and resulted in dramatic increases in AKT and ERα phosphorylation and activity. While knockout of LMTK2 in treatment naïve ERα+ breast cancer cells did not alter AKT phosphorylation/activity nor endocrine/CDK4/6 inhibitor responsiveness, attempts to knockout LMTK2 in endocrine/CDK4/6 inhibitor-resistant models failed, further confirming its essentiality in this setting. In summary we have uncovered LMTK2 as a novel biomarker of endocrine/CDK4/6 inhibitor resistance that is mechanistically linked to the AKT pathway. LMTK2 may be a novel drug target for the effective treatment of resistant and advanced forms of ERα+ breast cancer.

LMTK2 IS A TOP HIT IN A GENOME-WIDE CRISPR KNOCKOUT SCREEN

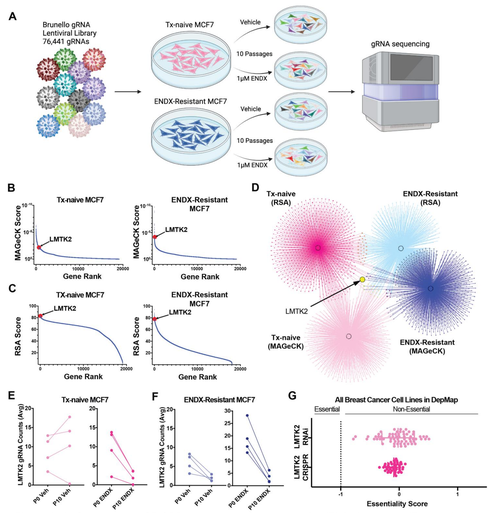


Figure 1: A) Genome-wide CRISPR knockout screen in endoxifen (ENDX)-sensitive (Tx-naïve) and -resistant MCF7 cells analyzed with MAGeCK **(B)** or RSA **(C). D)** Venn network plot of top candidates in which LMTK2 was the only common hit between both cell lines and analysis methods. **D)** Average gRNA counts at indicated passage and treatment in Tx-naïve **(E)** and ENDX-resistant **(F)** cell lines. **G)** Essentiality scores for LMTK2 in all breast cancer cell lines included in the DepMap RNAi and CRISPR screen databases.

LMTK2 EXHIBITS AMPLIFICATION AND COPY NUMBER GAIN IN METASTATIC BREAST TUMORS

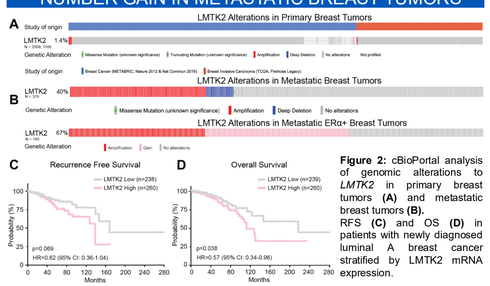


Figure 2: cBioPortal analysis of genomic alterations to *LMTK2* in primary breast tumors **(A)** and metastatic breast tumors **(B).** RFS **(C)** and OS **(D)** in patients with newly diagnosed luminal A breast cancer stratified by LMTK2 mRNA expression.

LMTK2 IS ROBUSTLY EXPRESSED IN BREAST CANCER CELLS, INCLUDING ENDOCRINE RESISTANT MODELS

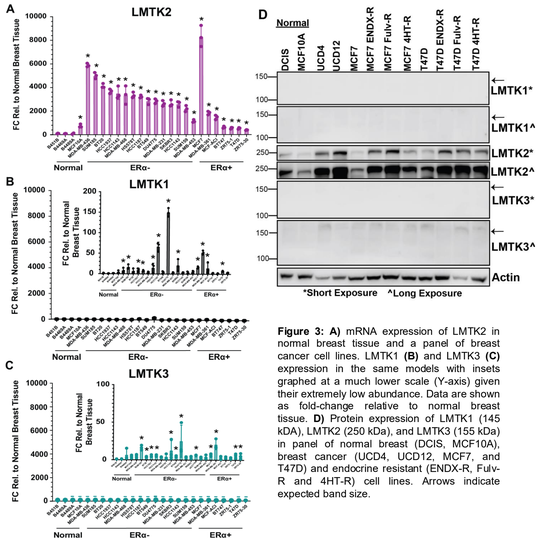


Figure 3: A) mRNA expression of LMTK2 in normal breast tissue and a panel of breast cancer cell lines. LMTK1 **(B)** and LMTK3 **(C)** expression in the same models with insets graphed at a much lower scale (Y-axis) given their extremely low abundance. Data are shown as fold-change relative to normal breast tissue. **D)** Protein expression of LMTK1 (145 kDA), LMTK2 (250 kDa), and LMTK3 (155 kDa) in panel of normal breast (DCIS, MCF10A), breast cancer (UCD4, UCD12, MCF7, and T47D) and endocrine resistant (ENDX-R, Fulv-R and 4HT-R) cell lines. Arrows indicate expected band size.

LMTK2 KNOCKDOWN KILLS RESISTANT CELLS

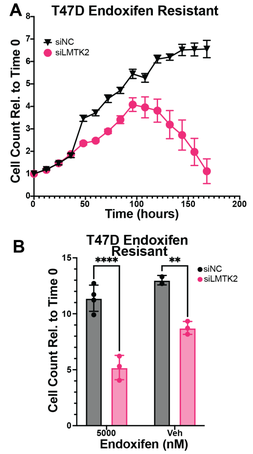
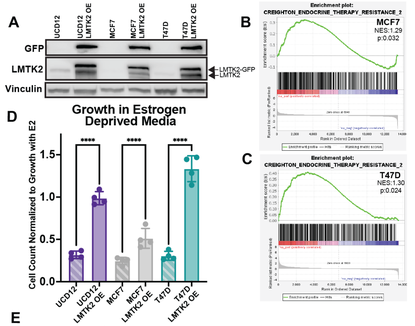


Figure 5: A) Quantification of T47D endoxifen-resistant cells over time following transfection with control or LMTK2 targeting siRNAs. Data are normalized to cell counts at time 0. **B)** Cell counts at day 5 following siRNA transfection and treatment with vehicle or 5000 nM endoxifen for 5 days.

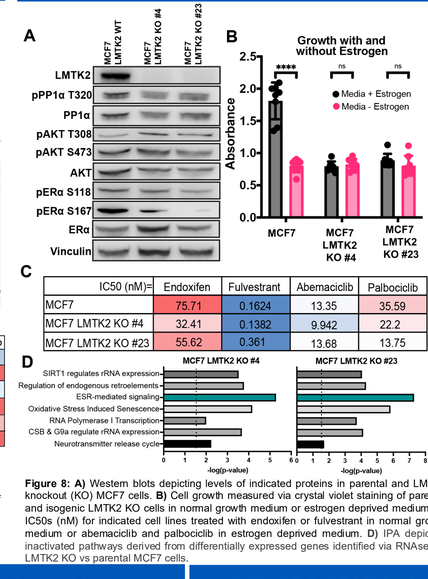
LMTK2 OVEREXPRESSION INDUCES ENDOCRINE AND CDK4/6i RESISTANCE



IC50 (nM)=	Endoxifen	Tamoxifen	Fulvestrant	Abemaciclib	Palbociclib
MCF7	75.71	297	0.1624	13.35	35.59
MCF7 LMTK2 OE	290.6	1,570	11.25	>1,000	>1,000
T47D	633	460.3	3.212	6.584	51.09
T47D LMTK2 OE	3,898	3,898	16.26	>1,000	>1,000
UCD12	53.86	142.1	11.16	68.94	355.2
UCD12 LMTK2 OE	2,499	>10,000	>500	>1,000	>1,000

Figure 6: A) Western blot showing expression levels in parental cell lines and their derivative GFP tagged LMTK2 overexpressing (OE) models. GSEA analysis of differentially expressed genes identified via RNAseq of LMTK2 overexpressing vs parental MCF7 **(B)** and T47D **(C)** cells. **D)** Relative cell proliferation rates of indicated cell lines in estrogen (E2) deprived medium normalized to cell growth in estrogen containing medium. **E)** IC50s (nM) for indicated cell lines treated with endoxifen, tamoxifen or fulvestrant in normal growth medium or abemaciclib and palbociclib in estrogen deprived medium.

LMTK2 KNOCKOUT DECREASES ESTROGEN SIGNALING



IC50 (nM)=	Endoxifen	Fulvestrant	Abemaciclib	Palbociclib
MCF7	75.71	0.1624	13.35	35.59
MCF7 LMTK2 KO #4	32.41	0.1382	9.942	22.2
MCF7 LMTK2 KO #23	55.62	0.361	13.68	13.75

Figure 8: A) Western blots depicting levels of indicated proteins in parental and LMTK2 knockout (KO) MCF7 cells. **B)** Cell growth measured via crystal violet staining of parental and isogenic LMTK2 KO cells in normal growth medium or estrogen deprived medium. **C)** IC50s (nM) for indicated cell lines treated with endoxifen or fulvestrant in normal growth medium or abemaciclib and palbociclib in estrogen deprived medium. **D)** IPA depicting inactivated pathways derived from differentially expressed genes identified via RNAseq of LMTK2 KO vs parental MCF7 cells.

LMTK2 KNOCKDOWN ENHANCES THE EFFICACY OF MULTIPLE ENDOCRINE THERAPIES

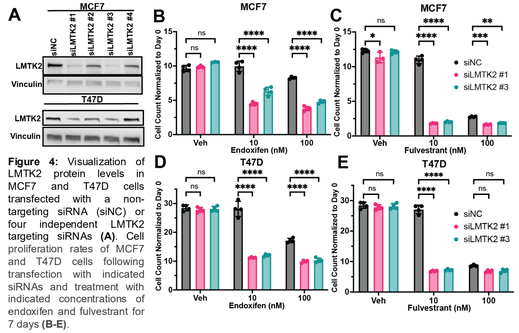


Figure 4: Visualization of LMTK2 protein levels in MCF7 and T47D cells transfected with a non-targeting siRNA (siNC) or four independent LMTK2 targeting siRNAs **(A)**. Cell proliferation rates of MCF7 and T47D cells following transfection with indicated siRNAs and treatment with indicated concentrations of endoxifen and fulvestrant for 7 days **(B-E)**.

LMTK2 ACTIVATES AKT AND ERα SIGNALING

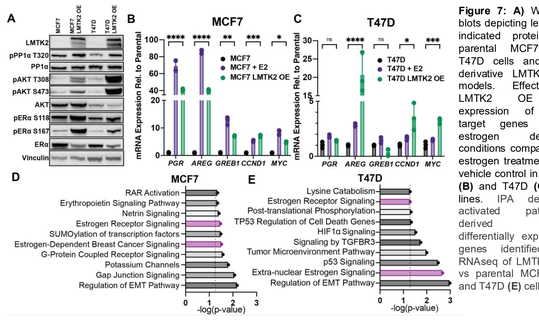


Figure 7: A) Western blots depicting levels of indicated proteins in parental MCF7 and T47D cells and their derivative LMTK2 OE models. Effects of LMTK2 OE on expression of ERα target genes under estrogen deprived conditions compared to estrogen treatment and vehicle control in MCF7 **(B)** and T47D **(C)** cell lines. IPA depicting activated pathways derived from differentially expressed genes identified via RNAseq of LMTK2 OE vs parental MCF7 **(D)** and T47D **(E)** cells.

SUMMARY

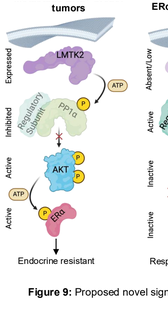


Figure 9: Proposed novel signaling axis.

FUTURE DIRECTIONS

- Identify the mechanism(s) by which LMTK2 drives AKT and ERα phosphorylation.
- Identify direct substrates of LMTK2 via chemical genetics methods.
- Characterize the phospho-proteome of LMTK2 KO and OE cell models relative to parental controls.
- Identify other mechanisms by which LMTK2 induces endocrine/CDK4/6i resistance and tumor progression.
- Characterize the impact of LMTK2 expression on treatment response *in vivo*.
- Develop LMTK2 inhibitors and characterize them as novel therapeutics.

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