

Kinome screening for regulators of the estrogen receptor identifies LMTK3 as a new therapeutic target in breast cancer

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Therapies targeting estrogen receptor α (ER α , encoded by *ESR1*) have transformed the treatment of breast cancer. However, large numbers of women relapse, highlighting the need for the discovery of new regulatory targets modulating ER α pathways^{1–5}. An siRNA screen identified kinases whose silencing alters the estrogen response including those previously implicated in regulating ER α activity (such as mitogen-activated protein kinase and AKT). Among the most potent regulators was lemur tyrosine kinase-3 (LMTK3), for which a role has not previously been assigned. In contrast to other modulators of ER α activity, LMTK3 seems to have been subject to Darwinian positive selection, a noteworthy result given the unique susceptibility of humans to ER α ⁺ breast cancer. LMTK3 acts by decreasing the activity of protein kinase C (PKC) and the phosphorylation of AKT (Ser473), thereby increasing binding of forkhead box O3 (FOXO3) to the *ESR1* promoter. LMTK3 phosphorylated ER α , protecting it from proteasomal degradation *in vitro*. Silencing of LMTK3 reduced tumor volume in an orthotopic mouse model and abrogated proliferation of ER α ⁺ but not ER α [–] cells, indicative of its role in ER α activity. In human cancers, LMTK3 abundance and intronic polymorphisms were significantly associated with disease-free and overall survival and predicted response to endocrine therapies. These findings yield insights into the natural history of breast cancer in humans and reveal LMTK3 as a new therapeutic target.

More than two-thirds of breast tumors express ER α (ref. 2), and patients with ER α ⁺ disease respond to antiestrogens (tamoxifen), estrogen withdrawal (aromatase inhibitors) and direct targeting of the receptor (fulvestrant)¹. The introduction of these treatments has had a profound impact on patient survival⁶. However, resistance to these therapies is common, and *in vitro* evidence points to the role of ER α phosphorylation³ in the development of endocrine resistance^{4,5}. To identify kinases that regulate ER α activity, we performed

a whole human kinome siRNA screen using expression of the estrogen-responsive *TFF1* gene, encoding trefoil factor-1, as a read-out for altered ER α activity in the presence of estradiol (E2 (ref. 7)) (Supplementary Fig. 1). We identified five genes whose knockdown resulted in a >100% increase in *TFF1* expression and 16 genes whose knockdown reduced *TFF1* expression by <50% (Fig. 1a). Two further independent replicate screenings confirmed these findings (data not shown). The identification of the kinases mitogen-activated protein kinase-3 (MAPK3) and AKT, which phosphorylate ER α at Ser118 and Ser167, respectively^{8–12}, confirmed the screen could successfully identify regulators of estrogen-responsive gene expression.

We subsequently measured the expression of two other ER α regulated genes (*PGR*, encoding progesterone receptor, and *GREB1*, encoding growth regulation by estrogen in breast cancer-1)¹³ and two control genes (*GAPDH*, encoding glyceraldehyde 3-phosphate dehydrogenase, and *MCL1*, encoding myeloid cell leukemia sequence-1). Two of the five genes (*LATS2* (encoding large tumor suppressor, homolog 2) and *CCRK* (encoding cell cycle-related kinase)) whose downregulation upregulated *TFF1* also upregulated *PGR* or *GREB1* >100% (group A, upregulated), and three of the 13 kinases (*TYRO3* (encoding protein tyrosine protein kinase receptor 3), *LMTK3* and *KSRI* (encoding kinase suppressor of ras 1)) that downregulated *TFF1* expression were also able to downregulate the expression of both *PGR* and *GREB1* >50% (group B, downregulated) (Supplementary Table 1), whereas the expression of *GAPDH* and *MCL1* did not change, indicating that the effects were E2 treatment dependent.

To prioritize among the kinases whose silencing downregulated the activity of ER α , we asked whether any of the candidate proteins showed evidence of positive selection as measured by analyzing changes in synonymous versus nonsynonymous genomic alterations. It is well established that humans and the great apes (especially chimpanzees) differ in their susceptibilities to epithelial neoplasms, including breast cancer^{14–18} possibly resulting from recent evolutionary events reflected in the adaptive profile of genes that have a regulatory role in estrogenic signaling. Of those genes that we found to

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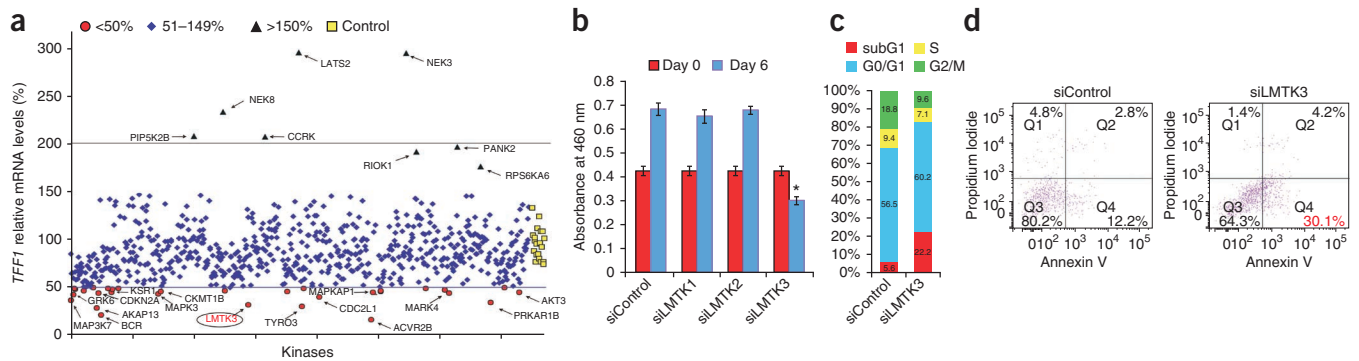


Figure 1 High-throughput siRNA screen identifies kinases modulating ER α transcriptional activity. **(a)** Relative mRNA levels of *TFF1* expression for each kinase in MCF-7 cells transfected with a pool of two different siRNAs per gene followed by E2 treatment (100% = *TFF1* mRNA expression after E2 treatment alone). $P < 0.05$. **(b)** Effects of LMTK3 silencing on MCF-7 cell proliferation. * $P < 0.001$ (Student's *t* test). siControl, non targeting siRNA. **(c)** The proportion of MCF-7 cells in subG₁ phase (apoptosis) after treatment with LMTK3 siRNA or with a control siRNA. **(d)** Representative cytograms and quantification of apoptosis upon LMTK3 silencing or treatment with a control siRNA. Error bars represent s.d. of two separate experiments in triplicate.

regulate ER α , only LMTK3 has been subject to recent Darwinian positive selection compared to its chimpanzee ortholog (**Supplementary Table 2**). Further, LMTK3 silencing consistently inhibited the expression of estrogen-regulated genes potentially (**Supplementary Fig. 2a**

and **Supplementary Fig. 3**), whereas transfection of LMTK3 in ER α -positive breast cancer cell lines (MCF-7 and ZR-75-1) resulted in opposite effects, as measured by quantitative RT-PCR analysis. (**Supplementary Fig. 2b**).

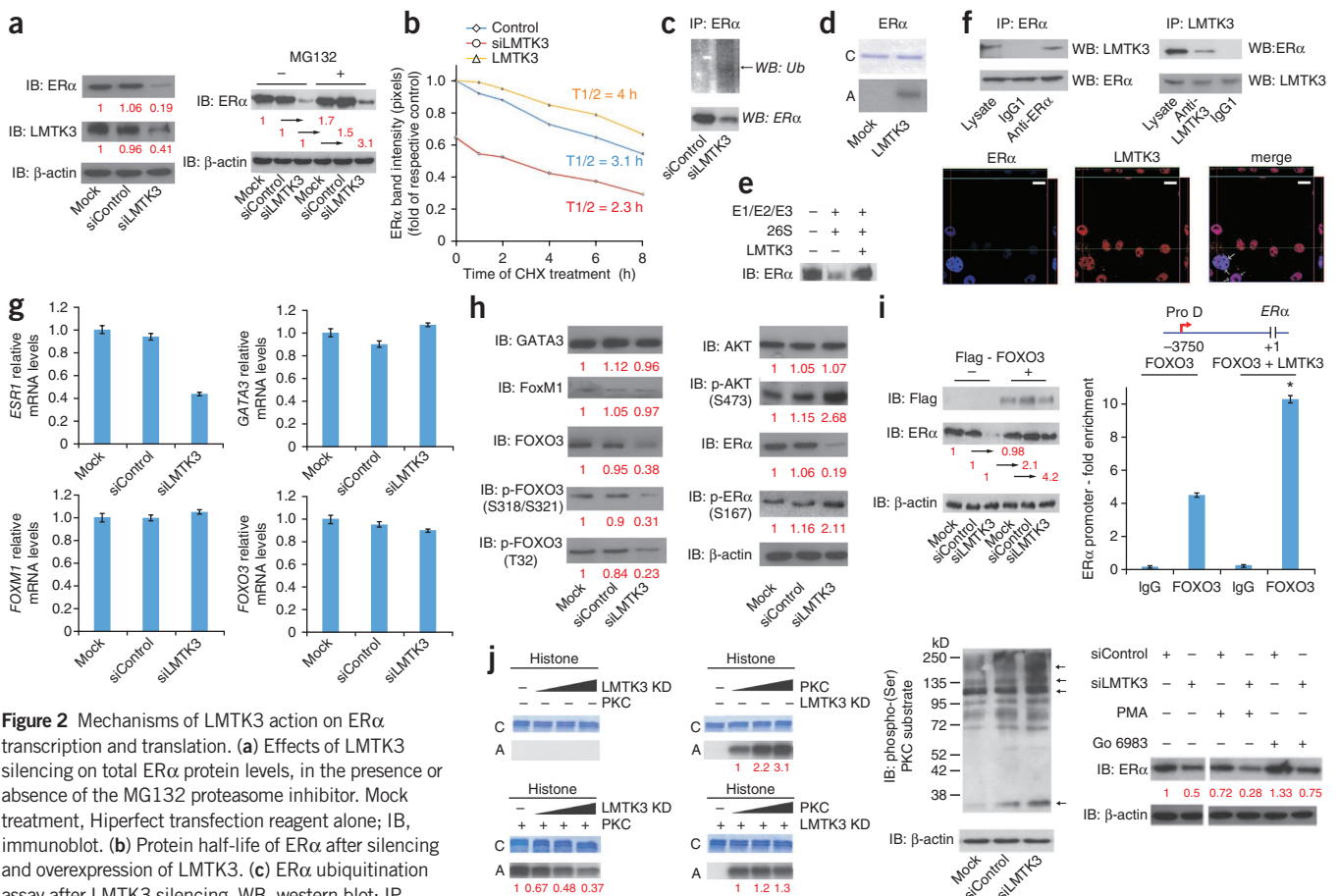


Figure 2 Mechanisms of LMTK3 action on ER α transcription and translation. **(a)** Effects of LMTK3 silencing on total ER α protein levels, in the presence or absence of the MG132 proteasome inhibitor. Mock treatment, Hipertext transfection reagent alone; IB, immunoblot. **(b)** Protein half-life of ER α after silencing and overexpression of LMTK3. **(c)** ER α ubiquitination assay after LMTK3 silencing. WB, western blot; IP, immunoprecipitation. **(d)** *In vitro* phosphorylation of ER α by LMTK3. C, Coomassie; A, autoradiogram. **(e)** *In vitro* degradation assay of ER α . E1/E2/E3 are ubiquitin ligases; 26S is the proteasome fraction. **(f)** Coimmunoprecipitation and coimmunofluorescence of ER α and LMTK3. Scale bar, 50 μ m. **(g)** Gene expression of *ESR1*, *GATA3*, *FOXO3* and *FOXM1* after LMTK3 silencing. **(h)** Effects of LMTK3 silencing on FOXO3, p-FOXO3 Ser318/Ser321, p-FOXO3 Thr32, p-AKT Ser473 and p-ER α Ser167 protein levels. In red is the fold change compared to the mock-treated samples relative to actin loading control. **(i)** Left, effects of FOXO3 overexpression on ER α protein levels. Right, effects of LMTK3 overexpression on FOXO3 binding to the *ESR1* promoter. **(j)** Left, *in vitro* kinase assays examining the effect of LMTK3 phosphorylation on the catalytic activity of PKC. Middle, effects of LMTK3 silencing on the ability of PKC to phosphorylate its substrates *in vivo*. Right, effects of LMTK3 silencing on ER α protein amounts in the presence of a PKC activator (PMA) or a PKC inhibitor (Go 6983). Error bars represent s.d. of two independent experiments in triplicate.

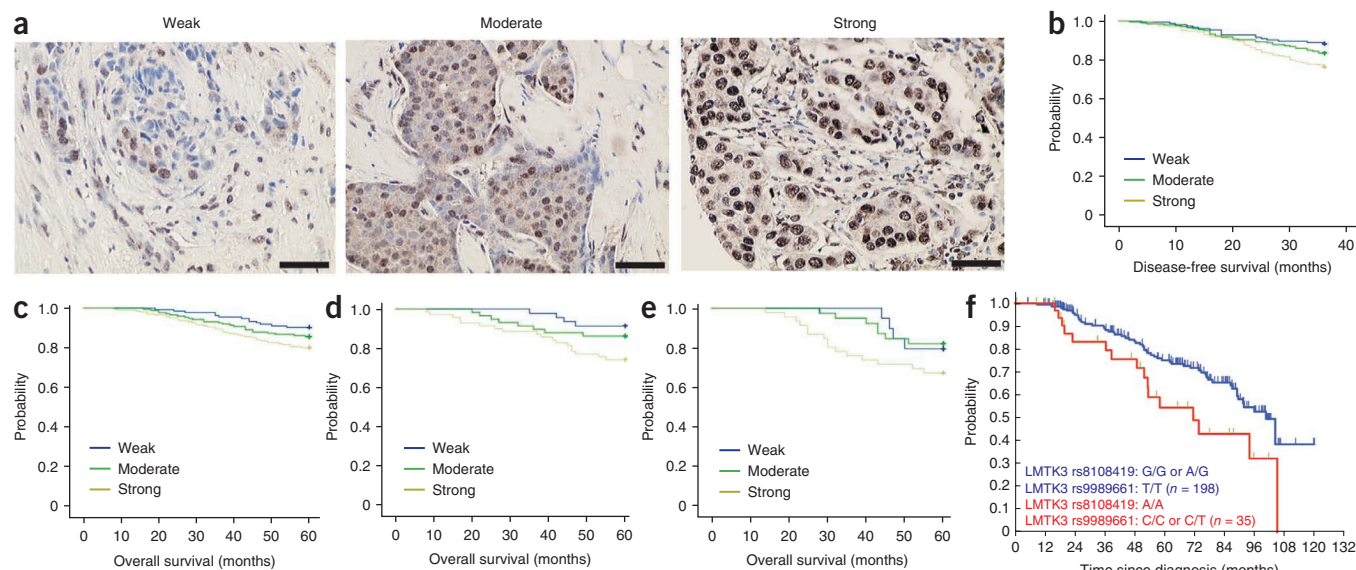


Figure 3 Association of LMTK3 expression and germline polymorphisms with clinical outcome. (a) Representative images of different immunohistochemistry staining intensities are shown. LMTK3 staining: weak (H score = 0–25); moderate (H score = 26–134); strong (H score = 135–300). H score: histology score, a method of assessing the extent of nuclear immunoreactivity. Original magnification, 200 \times ; scale bars, 100 μ m. (b–e) Kaplan-Meier plots showing the association between LMTK3 expression and disease-free survival ($P = 0.012$) (b), overall survival ($P = 0.033$) (c), response to endocrine therapy ($P = 0.039$) (d), response to chemotherapy ($P = 0.184$) (e). (f,g) Kaplan-Meier plots demonstrating the association between two LMTK3 polymorphisms and overall survival ($P = 0.017$) (f) and disease-free survival ($P = 0.002$) (g).

LMTKs are a family of serine-threonine-tyrosine kinases^{19–21}. A function has not been ascribed to LMTK3, although screens have suggested a putative role in the β -catenin pathway²² and leukemic cell survival²³. We found that only LMTK3 isoform knockdown, and not LMTK1/2 isoform knockdown, inhibited the activity of an estrogen-regulated luciferase reporter, whereas LMTK3 did not alter *GAPDH* or *MCL1* expression (Supplementary Figs. 2c–e, 4 and 5). In addition, knockdown of LMTK3, but not LMTK1 or LMTK2, inhibited MCF-7 human breast adenocarcinoma E2-dependent cell growth (Fig. 1b), accompanied by accumulation of cells in the sub-G1 phase (Fig. 1c,d). We obtained similar results in other ER α^+ cell lines and saw no effects in ER α^- cells (Supplementary Fig. 6). Taken together, these data indicate that LMTK3 is a regulator of ER α activity.

To establish the mechanisms of LMTK3 regulation of ER α in MCF-7 cells, we next examined ER α expression. ER α protein amounts were reduced 80% by LMTK3 knockdown (Fig. 2a). ER α amounts were higher in the presence versus absence of proteasome inhibitors (Fig. 2a), and the ER α half-life was reduced after LMTK3 knockdown, whereas LMTK3 overexpression stabilized ER α (Fig. 2b). There was an increase in ER α ubiquitination after LMTK3 knockdown (Fig. 2c). Moreover, phosphorylation of ER α by LMTK3 (as suggested by data in Fig. 2d) protected ER α from *in vitro* proteasomal degradation (Fig. 2e), and LMTK3 and ER α were able to interact *in vivo* (Fig. 2f). Together, these data indicate that LMTK3 regulates ER α by phosphorylating and protecting it from proteasomal degradation. Next, we wished to understand the contribution of LMTK3 to *ESR1* transcription. We observed that LMTK3 knockdown reduced expression of *ESR1* mRNA (Fig. 2g). ER α expression is regulated by GATA binding protein 3 (encoded by *GATA3* (ref. 24)), FOXO3

(refs. 25–27) and forkhead box M1 (encoded by *FOXO1* (ref. 28)), as well as by ER α regulating its own expression²⁹. LMTK3-targeting siRNA (LMTK3 siRNA) did not affect mRNA levels of these genes (*FOXO3*, *GATA3* and *FOXO1*) (Fig. 2g); however FOXO3 protein abundance was reduced 70%, and FOXO3 phosphorylation was reduced relative to total FOXO3 abundance (Fig. 2h). Overexpression of FOXO3 partially rescued the LMTK3 siRNA-mediated decrease in ER α (Fig. 2i), whereas chromatin immunoprecipitation confirmed that overexpression of LMTK3 increased binding of FOXO3 to the *ESR1* promoter (Fig. 2i). As it has already been described that AKT phosphorylates and inhibits FOXO3 by promoting its degradation³⁰, we examined the effects of LMTK3 silencing on AKT. We found no changes in total AKT abundance, but we did see an increase in phosphorylated cytoplasmic AKT (on Ser473), suggesting that LMTK3 siRNA-induced FOXO3 downregulation is regulated via AKT (Fig. 2h). Notably, we observed an increased phosphorylation of ER α at Ser167, despite decreased total ER α amounts, as a result of activated AKT, as previously described⁸ (Fig. 2h). As protein kinase C (PKC) activity has been implicated in ER α protein degradation³¹ and in decreased *ESR1* transcription via activation of AKT and inhibition of FOXO3 (ref. 32), we examined the effects of LMTK3 on PKC. *In vitro* kinase assays indicated that LMTK3 inhibits the ability of PKC to phosphorylate histone (Fig. 2j), whereas the use of a specific phospho-serine PKC substrate antibody showed that LMTK3 silencing increased the ability of PKC to phosphorylate a number of substrates (Fig. 2j). In addition, inhibition of PKC, using the Go 6983 inhibitor³³, partly rescued the downregulation of ER α protein induced by LMTK3 silencing, whereas concurrent treatment with a PKC activator (PMA)³⁴ and LMTK3 siRNA resulted in further degradation of

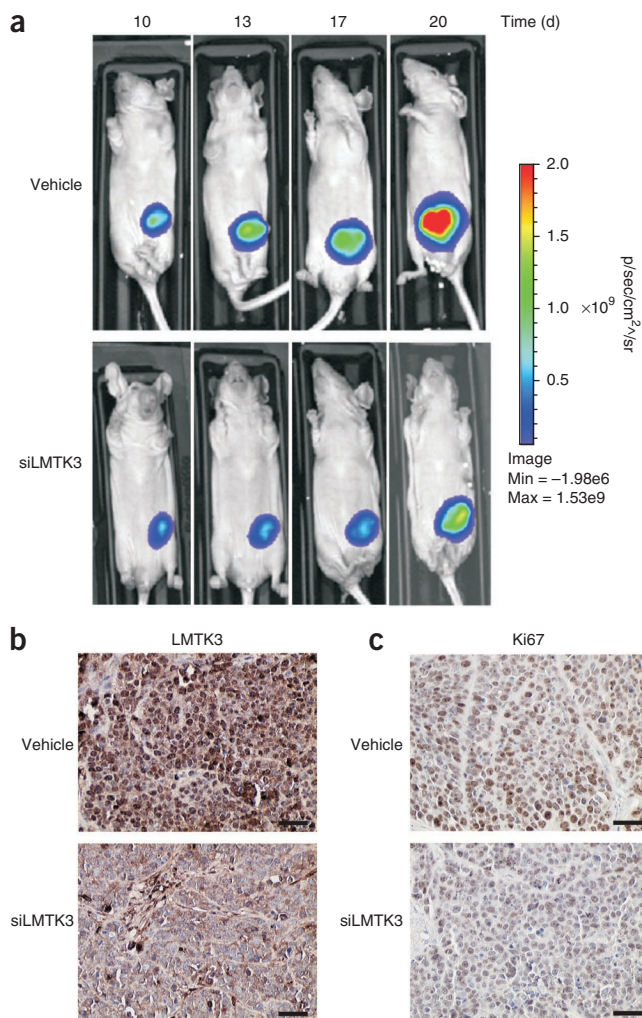


Figure 4 Tumor growth inhibition by *in vivo* LMTK3 siRNA in an orthotopic mouse model. (a) Bioluminescent images of a representative mouse for each group ($n = 8$) ($P < 0.01$). Tumor volume (in mm^3) was used for statistical analysis. Vehicle, PBS alone. (b,c) Histological analysis of LMTK3 (b) and Ki67 (c) expression in representative tumor tissue sections of LMTK3 siRNA-treated versus vehicle-treated tumors. Original magnification, 200 \times . Scale bars, 100 μm .

transcription or translation of LMTK3. We found only five out of 227 subjects¹² (Supplementary Table 3) with a methylated *LMTK3* gene, suggesting that methylation is not a prevalent mechanism in the control of *LMTK3* expression in this context. In this cohort, we then examined polymorphisms that could underlie the differential levels of *LMTK3* in people with cancer. We found that two intronic polymorphisms (see Online Methods) were independently associated with disease-free survival and overall survival, suggesting functionally relevant polymorphisms. Individuals harboring the *LMTK3* rs8108419 GG or AG and the *LMTK3* rs9989661 TT alleles were at a lower risk of developing tumor recurrence, which is our reference with a relative risk = 1 compared to patients carrying the *LMTK3* rs8108419 AA and *LMTK3* rs9989661 CT or CC alleles who have an increased risk (relative risk = 2.44; confidence interval: 1.40–4.25) ($P = 0.002$; Supplementary Table 4). Overall survival was associated with combined analyses of risk of these two polymorphisms ($P = 0.017$; Fig. 3f,g and Supplementary Table 4). In multivariate analyses, *LMTK3* polymorphisms were an independent prognostic factor for both disease-free survival and overall survival (Supplementary Table 4). Next, to investigate the effects of LMTK3 knockdown on breast tumor xenograft growth, we injected naked LMTK3 siRNA, diluted in PBS, into pre-established human MCF-7 breast carcinoma tumors grown in nude mice. *In vivo* bioluminescence imaging of the xenografted tumors showed that loss of LMTK3 protein expression, observed by immunohistochemistry, leads to a significant decrease in tumor growth (Fig. 4 and Supplementary Table 5, $P = 0.024$).

The majority of human breast tumors express ER α , and individuals with ER α^+ disease usually respond to endocrine therapies. Endocrine resistance is a major problem, highlighting a need for understanding the mechanisms of ER α action and the development of new therapeutic agents. By performing a kinome siRNA screen to identify new proteins modulating ER α transcriptional activity, combined with evolutionary and mechanistic analyses, we have established a role for LMTK3 in regulating ER α in breast cancer. We propose a model where LMTK3 regulates the stability and activity of ER α at the mRNA level, via down-regulation of PKC catalytic activity resulting in less phosphorylated AKT (Ser473) that stabilizes FOXO3, which in turn leads to increased ER α transcriptional activity, and at the protein level, directly by phosphorylating ER α and protecting it from proteasomal degradation (Supplementary Fig. 10). Notably, LMTK3 expression was down-regulated by E2 and upregulated in response to tamoxifen, revealing a feedback loop between LMTK3 and ER α (Supplementary Fig. 11).

The demonstration that expression of and polymorphisms in *LMTK3* are associated with clinical outcome and response to endocrine therapy in breast cancer, in combination with our *in vivo* studies, suggests clinical and translational relevance. Although presumably all proteins must have been positively selected for their biochemical functions at some time in the past, very few show evidence of such adaptive evolution⁴⁰. It is relevant that LMTK1 and LMTK2 are not positively selected for between humans and chimpanzees; rather, they are well conserved. Positive selection has been operational on human LMTK3 (in a region containing no recognized conserved kinase domains (data not shown), which may have altered

ER α (Fig. 2j). These data further imply that the effects of LMTK3 on ER α are both directly (*ESR1*) and indirectly (ER α protein) mediated via PKC signaling.

Our findings indicate that LMTK3 is key in regulating ER α activity. To confirm these data in primary breast cancer, we used immunohistochemistry to determine LMTK3 abundance (Fig. 3a and Supplementary Fig. 7) in 613 breast cancer samples³⁵. High nuclear LMTK3 expression was associated with a significantly shorter disease-free survival time ($P = 0.01$) and overall survival time ($P = 0.03$) (Fig. 3b,c). LMTK3 abundance was also predictive of response to endocrine therapy ($P = 0.04$) (Fig. 3d) but did not predict response to adjuvant chemotherapy ($P = 0.18$) (Fig. 3e). To further investigate the potential involvement of LMTK3 in the development of tamoxifen resistance, we analyzed the effects of LMTK3 silencing in tamoxifen-resistant cell lines (BT-474, MLET5 and LCC9)^{36–38}. Tamoxifen alone slightly affected baseline levels of cell growth, whereas addition of LMTK3 siRNA increased the growth inhibitory effects of tamoxifen, and the expected elevated levels of phosphorylated ER α and its major oncogenic co-activator amplified in breast cancer 1 (AIB1) (ref. 39) were decreased, (Supplementary Fig. 8). In addition, LMTK3 was also essential for E2-induced growth, as silencing of LMTK3 impeded cell proliferation in the presence of E2 (Supplementary Fig. 9).

The significant associations of LMTK3 expression with clinical outcome led us to test whether methylation might have a role in

the characteristics of human versus chimpanzee LMTK3. Although the selective pressure that drove this adaptive event is at present unclear, an evolutionary tradeoff may have led to increased human susceptibility to breast cancer. Most humans we examined had the 'protective' TT allele, whereas the less-susceptible nonhuman primates lacked the protective TT allele as a result of selective pressure to counter possible deleterious effects of sequence changes to human LMTK3 (**Supplementary Table 6**). Further investigation of chimpanzee LMTK3 may yield insights into the natural history of breast cancer in humans versus chimpanzees. Together, our data reveal LMTK3 as a potential biomarker of response to endocrine therapy in breast cancer and highlight its potential as a therapeutic target.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

G.G. and J.S. conceived of the study, initiated, designed, supervised and conducted most of the experiments and wrote the manuscript. W.M., S.A., H.-J.L., C.T.-S. and R.C.C. contributed to manuscript editing. G.G. and J.J. performed the kinase screening. A.F., B.A.S., A.P., L.C. and H.Z. performed *in vitro* experiments (including proliferation assays, quantitative RT-PCR and FACS). A.F. and J.S. performed the immunohistochemistry scoring. D.Y., W.Z. and H.J.L. generated the single-nucleotide polymorphism data. W.M. produced all the evolutionary data. A.R.G. and I.O.E. performed the statistical analysis of the clinical data. All authors discussed the results, conceived further experiments, commented on the manuscript and approved the final submitted version.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

High-throughput siRNA screening. The human kinase siRNA Set Version 3.0 library (Qiagen) targeting 691 kinases and kinase-related genes was used. The library was supplied in a 96-well format and contained a pool of two individual siRNAs per well, targeting two different sequences for each gene. MCF-7 cells (American Type Culture Collection) were maintained in phenol red-free medium with 10% charcoal-stripped serum (DSS) 48 h before experimentation. Cells were plated in 24-well plates and transfected with siRNA (Qiagen) (final concentration 20 nM) using the Human Kinase siRNA Set Version 3.0 library and HiPerfect reagent according to the manufacturer's instructions (Qiagen). At 48 h after transfection, cells were treated with vehicle (ethanol) or E2 (10 nM) for 24 h, and cells were collected following RNA extraction and cDNA synthesis. Quantitative RT-PCR analysis to examine the expression of *TFPI* and *GAPDH* (endogenous control) was performed for each well (kinase gene). Next, the *TFPI* gene expression after silencing each kinase individually was calculated in relation to *GAPDH* expression; screening was performed in duplicate.

Evolutionary analysis. Positive selection on the protein-coding regions of *LMTK3* was detected by use of molecular evolution algorithms that characterize the relative proportion of nonsynonymous (replacement) nucleotide substitutions as compared to synonymous (silent) nucleotide substitutions in the kinase coding sequences. (The *LMTK3* rs8108419 GG or AG and the *LMTK3* rs9989661 TT alleles were not examined in this manner, as these regions are exclusively intronic.) All kinases shown in our screen to modulate ER α , as well as the isoforms *LMTK1* and *LMTK2*, were examined for evidence of sequence-level positive selection between human and chimpanzee orthologs using Li93 software (a kind gift from W. Hsiung-Li). Both whole coding sequence and subsection sliding windows were examined. Only *LMTK3* showed evidence of positive selection ($P < 0.005$).

Candidate polymorphisms and genotyping. Candidate *LMTK3* polymorphisms were chosen with the assistance of the Ensembl program (<http://www.ensembl.org/>) using two main criteria. First, the polymorphism had to have some degree of likelihood of altering the function of the gene in a biologically relevant manner. The rs8108419 polymorphism is located in intron 2 of the

LMTK3 gene, whereas the rs9989661 polymorphism is located in intron 15 of the *LMTK3* gene. Intron polymorphisms can change gene transcription levels by alternative splicing or by affecting binding of a transcription factor. Second, the frequency of the polymorphism had to be sufficient that its impact in clinical outcome would be meaningful on a population level (above 10% allele frequency). Genomic DNA was extracted from microdissected tissue specimens using the QIAamp kit (Qiagen). *LMTK3* polymorphisms (rs8108419 and rs9989661) were tested by the PCR restriction fragment length polymorphism (PCR-RFLP) technique. Briefly, forward primer 5'-ATTCCACCACTCCC TCCAG-3' and reverse primer 5'-GACCCTGCAGTGCCTCAC-3' for rs8108419 and forward primer 5'-GGGCCTTCCCAAGTGGTT-3' and reverse primer 5'-ATCCAAGCCTGGGGTGAG-3' for rs9989661 were used for PCR amplification; PCR products were digested by the restriction enzyme BsrD1 (rs8108419) or Btscl1 (rs9989661), and alleles were separated on 4% NuSieve ethidium bromide-stained agarose gel (Lonza Rockland). Samples were obtained with approval from the Riverside Ethics Committee with appropriate informed consent from the subjects.

In vivo tumorigenicity assay in nude mice bearing orthotopic breast cancer xenografts. Bioluminescent MCF-7 breast cancer cell lines (PRECOS) were injected into the mammary fat pad of nude mice (PRECOS). When tumors reached an approximate volume of 100–200 mm³ (day 15), mice were randomly assigned to different groups ($n = 8$, each group) to receive intratumoral injections of 10 μ g *in vivo*-modified *LMTK3* siRNA or control siRNA (Qiagen). Three intratumoral injections were repeated every 3 d (50 μ l volume per injection), and mice were killed 3 d after the last injection. Tumor growth was monitored with caliper measurements, and bioluminescent imaging was performed 24 h before dosing and 72 h after dosing. After the mice were killed, primary tumors were excised, weighed and formalin fixed. Samples were paraffin embedded, cut at 3 μ m and H&E stained for histological evaluation of target proteins expression. This study was conducted under the UK Home Office Licence number PPL 40/2962.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.