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Hippo signalling maintains ER expression and ER+ breast cancer growth

Shenghong Ma[1], **Zhengming Wu**[1], **Feng Yang**[2], **Jianmin Zhang**[3], **Randy L. Johnson**[4], **Michael G. Rosenfeld**[2], **Kun-Liang Guan**[1,*]

[1]Department of Pharmacology and Moores Cancer Center, University of California San Diego, La Jolla, CA, USA.

[2]Howard Hughes Medical Institute, Department of Medicine, University of California San Diego, La Jolla, CA, USA.

[3]Department of Cancer Genetics & Genomics, Roswell Park Cancer Institute, Buffalo, NY, USA.

[4]Department of Cancer Biology, University of Texas, MD Anderson Cancer Center, Houston, TX, USA.

The Hippo pathway regulates cell growth and fate decision, organ size and tissue homeostasis, and its dysregulation contributes to tumorigenesis[1,2]. Britschgi et al. reported that the Hippo pathway kinases LATS1/2 promote ERα degradation in a manner independent of their kinase activity or downstream effectors YAP/TAZ[3]. Here we report that LATS1/2 are required to maintain ERα expression as LATS1/2 deletion abolishes *ESR1* mRNA and protein in breast, endometrium, and ovary cells in a manner dependent of LATS kinase activity and YAP/TAZ. Consistently, LATS1/2 deletion selectively inhibits growth of ERα$^+$, but not ERα$^-$, breast cancer cells, thus revealing an unexpected role of Hippo signaling in breast cancer and a functional crosstalk with ERα.

To investigate the Hippo pathway in breast cancer, we generated LATS1 and LATS2 kinase double knockout (dKO) in MCF-7 cells, a well-established ERα$^+$ human breast cancer cell line, through CRISPR-Cas9 genomic editing technique. The LATS1/2 deficient cells, verified by the lack of LATS1 and LATS2 proteins, were no longer responsive to Hippo upstream signals, including serum starvation or mevalonate metabolism blockage, thus rendered constitutive YAP/TAZ dephosphorylation and activation (Extended Data Fig. 1a,b). During the culture of LATS1/2 deficient MCF-7 cells, a significant growth retardation was observed (Extended Data Fig. 1c). The reduced growth in *LATS1/2* dKO cells was unexpected as LATS1/2 are generally known as tumor suppressors.

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[*]**Correspondence and requests for materials** should addressed to K.-L.G., kuguan@ucsd.edu.

Author Contributions

S.H.M. and K.-L.G. conceived the study with important advice from J.M.Z., R.L.J. and M.G.R.; S.H.M. performed the majority of the experiments and data analyses with help from Z.M.W. and F.Y.; The manuscript was written by S.H.M. and K.-L.G., with input from all the authors.

Methods. A full list of materials, methods, and experimental protocols can be found in the Supplementary Methods.

Competing interests

K.-L.G. is a co-founder of and has equity interest in Vivace Therapeutics. The other authors declare no competing interests.

Extended data and Supplementary information is available for this paper.

We set out to identify the factor(s) contributing to MCF-7 cell vulnerability to LATS1/2 deficiency. As the LATS1/2 kinase and their major targets YAP/TAZ have a pivotal role in transcriptional regulation, we performed RNA-sequencing. By gene ontology (GO) enrichment analysis of the differentially expressed genes, we found an enrichment in YAP target gene signature as well as apoptosis, and stress response in LATS1/2 deficient cells (Fig. 1a, Extended Data Fig. 1d, e). Interestingly, LATS1/2 deficiency robustly decreased *ESR1*, and ERα target gene signature (Fig. 1a, Extended Data Fig. 1d). In accord with the dramatic loss of ERα (Fig. 1b, c, Extended Data Fig. 1f), LATS1/2 knockout abolished E_2-induced transcription of ERα target genes *TFF1*, *GREB1*, and *FOXC1* (Fig.1d). The above observation is surprising as Britschgi *et al*. reported that LATS shRNA knockdown reduced ERα protein, and posited that LATS1/2 stimulated ERα degradation via a direct binding[3]. To test whether the transient incomplete LATS depletion by Britschqi et al vs the stable complete LATS knockout in our experiments might contribute to the opposite effects on ERα, we examined additional ERα+ breast cancer cell lines T47D and ZR-75-1 using transient LATS1/2 knockout cell pool and found that LATS1/2 were similarly required to maintain ERα expression (Fig. 1e). Furthermore, LATS1/2 knockdown with shRNA sequences same as those used by Britschgi et al. did not affect expression of ERα or YAP/TAZ target gene CTGF (Extended Data Fig. 1g, h), suggesting that partial depletion of LATS1/2 is insufficient to activate YAP/TAZ in MCF-7 cells. Consistent with the functional redundancy of LATS1 and LATS2, single deletion neither increased CTGF nor reduced ERα (Extended Data Fig. 1g, h).

We further examined the presence of Hippo-ERα axis in broader biological systems using organoid culture derived from ERα expressing tissues, including mammary, endometrium, and fallopian tube[4–6]. Deletion of *Lats1/2* by infection with Cre containing adenovirus in breast organoids derived from $Lats1^{fl/fl}/Lats2^{fl/fl}$ mice increased YAP/TAZ nuclear localization and target genes *Ctgf* and *Cyr61* expression (Fig. 1f–h). Importantly, *LATS1/2* deletion also reduced ERα mRNA and protein. Similar results, reduction of ERα expression by *LATS1/2* deletion, were observed in organoids derived from endometrium and fallopian tube (Extended Data Fig. 1i–n). Taken together, these data demonstrate that LATS1/2 are essential for *ESR1* expression in ER^+ breast cancer cells as well as normal cell types in physiologically relevant systems.

Britschgi et al.[3] also reported that LATS overexpression reduced ERα in a manner independent of the kinase domain. However, we failed to detect a reduction of ERα protein when LATS1 was overexpressed in the same cell type MCF-7 and T47D (Fig. 1i). We tested whether LATS kinase activity is required to maintain ERα by reintroduction of Lats2 wild-type or kinase dead mutant (K/R) into the LATS1/2 KO cells. Only the wild type LATS2, but not the kinase dead mutant, rescued ERα expression (Fig. 1j–l), suggesting the effect observed in our study is kinase activity dependent.

As YAP/TAZ are the best-known substrates and functional effectors of LATS kinases[7,8], we asked whether YAP/TAZ activation might mediate the LATS deficiency-induced ERα downregulation. Overexpression of the constitutively active mutant YAP(5SA) or TAZ(4SA) strongly reduced ERα mRNA and protein in MCF7, T47D, and ZR-75-1 breast cancer cells (Fig. 1m, n, Extended Data Fig. 2a–c), indicating that YAP/TAZ hyperactivation is sufficient

to inhibit ERα expression. YAP/TAZ bind to TEAD family transcription factor to induce gene expression[1,2]. We found that the TEAD binding defective YAP(5SA/94A) could not repress ERα whereas the WW domain deletion mutants, which still retain transcription activity[9], inhibited ERα expression (Fig. 1m, n, Extended Data Fig. 2d), suggesting that YAP/TAZ act via TEAD dependent transcription to inhibit ERα expression. Double deletion of both YAP and TAZ, but not single deletion of either, moderately increased the expression of ERα and target genes *TFF1* and *GREB1* (Extended Data Fig. 2e–g). Importantly, the LATS1/2 deficiency-induced ERα downregulation was completely blunted by concomitant depletion of YAP/TAZ (Fig. 1o), indicating that LATS1/2 act through YAP/TAZ to modulate ERα expression. We investigated ERα regulation *in vivo*. ERα repression was also observed in mammary tissue in the MMTV-rtTA/TRE-TAZ^{4SA} mice (Fig. 1p), in which the constitutively active TAZ(4SA) was induced by doxycycline (dox).

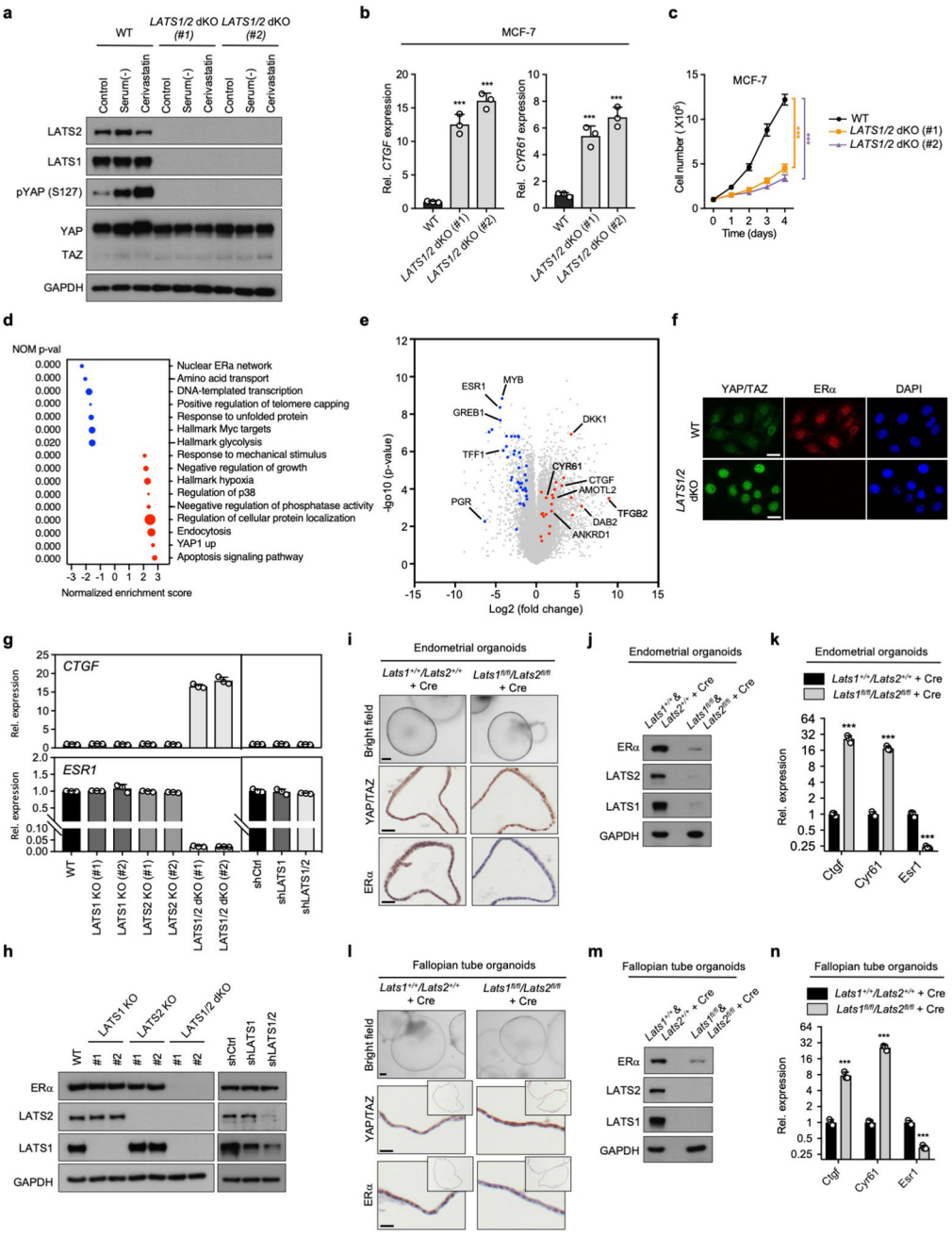
We then asked whether inhibition of LATS1/2 without removing the protein by manipulating Hippo upstream components could have a similar effect on ERα. NF2 is a key upstream regulator required for LATS activation[10,11]. NF2 knockout by three independent sgRNAs abolished phosphorylation of both LATS1 and YAP, and importantly also reduced ERα mRNA and protein without affecting LATS1 protein level (Extended Data Fig. 2h, i). Collectively, the above data establish a previously unrecognized crosstalk between the Hippo pathway and ERα, as well as an essential role (both necessary and sufficient) of YAP/TAZ in mediating the effect of LATS to maintain ERα expression.

Next, we investigated the function of Hippo-ERα axis in breast cancer cells. Consistent a positive role of ERα in breast cancer cell growth, LATS1/2 deletion reduced cell growth of the ERα$^+$ MCF7, T47D, and ZR-75-1 cells (Fig. 2a). In contrast, LATS1/2 deletion had little effect on growth of ERα$^-$ breast cancer cells (Fig. 2a). To test whether ERα mediates the LATS1/2 effect, cell growth in response to estradiol E_2 was measured. E_2 treatment increased proliferation in wild-type MCF-7 cells but this effect was blunted in *LATS1/2* dKO cells (Fig. 2b). MCF-7 cells were sensitive to growth inhibition by the ERα inhibitor 4-Hydroxytamoxifen (4-OHT) (Fig. 2c). However, the LATS1/2 deficient cells, which had little ERα expression, were no longer sensitive to inhibition by 4-OHT. LATS1/2 depletion suppressed anchorage independent growth (Fig. 2d–f). Importantly, reintroduction of ERα restored colony forming ability in *LATS1/2* dKO cells, indicating that ERα downregulation is the main effector mediating LATS1/2 deficiency-induced growth inhibition. To assess the role of LATS1/2 depletion in tumour formation, we performed xenograft experiments. The wild-type MCF-7 cells showed substantial xenograft growth in immune-deficient mice, whereas *LATS1/2* dKO cells grew poorly (Fig. 2g, h, Extended Data Fig. 3a). LATS1/2 deficient tumours showed impaired proliferation and increased apoptosis (Extended Data Fig. 3b–d). Collectively, these results show that *LATS1/2* deletion inhibits MCF-7 cell growth by reducing ERα.

In summary, we show that LATS1/2 are required to maintain ERα expression via YAP/TAZ inhibition, thereby promoting ERα$^+$ breast cancer cell growth. As such, the functions of LATS1/2 and YAP/TAZ in ERα$^+$ breast cancer cells are drastically different from most other cancer cell types where LATS1/2 and YAP/TAZ normally function as a tumor suppressors and oncogenes, respectively[1,12]. Notably, two recent reports suggested a tumor promoting

function of LATS in intestine by maintaining Wnt signaling[13,14]. We posit that ERα represents an important functional output of the Hippo pathway and this hypothesis has important implication given the broad physiological functions of ERα. Further study is required to elucidate how YAP/TAZ repress *ESR1* gene transcription. Hormone therapy by inhibiting ERα is the mainstay treatment for ER^+ breast cancer. Given that robust reduction of ERα by LATS1/2 inactivation, our data suggest a potential therapeutic strategy by targeting Hippo signaling for $ERα^+$ breast cancer, particularly for endocrine resistant breast cancers.

Extended Data



Extended Data Fig. 1 |. LATS1/2 are required to maintain ERα and target gene expression.

a, Impaired YAP phosphorylation in *LATS1/2* double knockout (dKO) cells. Wild-type (WT) and two MCF-7 *LATS1/2* dKO clones were serum starved or treated with 1μM cerivastatin (1 hour), and subjected to immunoblot analysis.

b, Increased YAP/TAZ transcriptional activity in *LATS1/2* dKO cells. qPCR of YAP/TAZ target genes *CTGF* and *CYR61*.

c, LATS1/2 deficiency inhibits MCF-7 cell growth.

d, YAP signature and estrogen receptor signature are among the top upregulated and down regulated gene sets in *LATS1/2* dKO cells, respectively. Gene enrichment analysis of LATS1/2 deficient (n = 3) and WT (n = 3) MCF-7 cells. Circle size represent the relative gene numbers in each set. Red, enriched in LATS1/2 deficient cells; blue, enriched in WT cells.

e, Opposite effects of *LATS1/2* deletion on the YAP signature genes (red) and estrogen response signature genes (blue) in MCF-7 cells. Significance, false discovery rate-adjusted p-value; Magnitude of difference, fold change; n=3 independent samples.

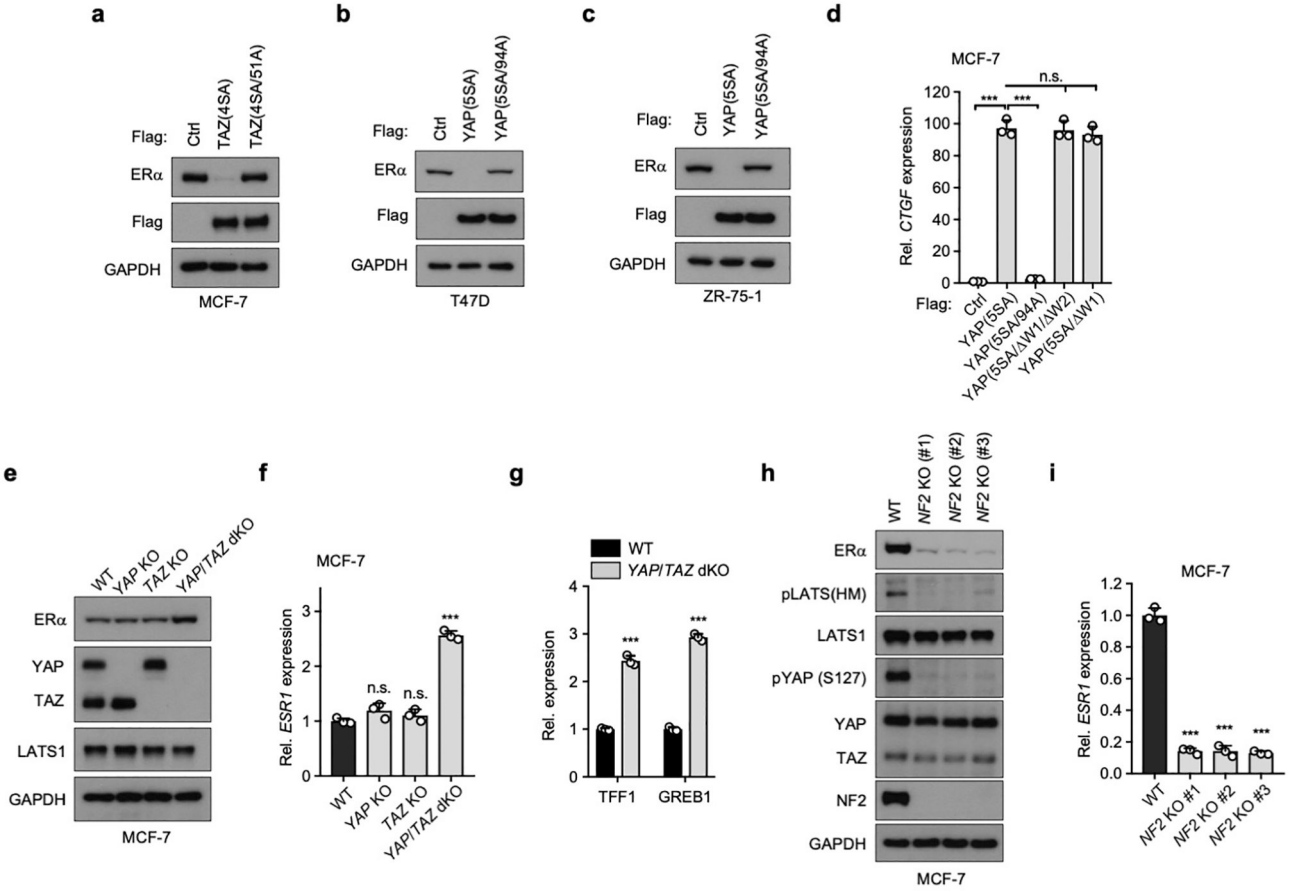
f, YAP/TAZ and ERα localization and intensity in WT or *LATS1/2* dKO MCF-7 cells. Scale bar, 20 μm.

g, h, Redundancy of LATS1 and LATS2 in regulating YAP activity and ERα expression. MCF-7 clones with different sgRNA targeting LATS1, LATS2, or both were subjected to qPCR analysis for *CTGF* and *ESR1* (**g**) or immunoblot (**h**).

i-k, *Lats1/2* deletion reduces ERα in endometrial organoids. Organoids derived from the endometrial tissues of $Lats1^{+/+}/Lats2^{+/+}$ and $Lats1^{fl/fl}/Lats2^{fl/fl}$ mice were infected with Cre-encoding adenovirus and subjected to immunohistochemistry (**i**), immunoblot (**j**), and qPCR analysis (**k**). Scale bar, 100 μM for bright-field, 30 μM for IHC.

l-n, *Lats1/2* deletion reduces ERα in fallopian tube organoids. Organoids derived from the fallopian tube tissues of $Lats1^{+/+}/Lats2^{+/+}$ and $Lats1^{fl/fl}/Lats2^{fl/fl}$ mice were infected with Cre-encoding adenovirus and subjected to immunohistochemistry (**l**), immunoblot (**m**) and qPCR analysis (**n**). Scale bar, 200 μM for bright-field, 25 μM for IHC staining.

***$P < 0.001$; mean + s.d.. See Supplementary Fig .1 for gel source data.



Extended Data Fig. 2 |. YAP/TAZ mediates Hippo signaling to repress *ESR1* expression

a, Repression of ERα level by TAZ. MCF-7 cells transduced with a control vector, Flag-TAZ(4SA) cDNA or Flag-TAZ(4SA/54A) cDNA were subjected to immunoblot. TAZ(4SA) is a constitutively active mutant with mutation of the four LATS phosphorylation sites while TAZ(4SA/54A) is defective in TEAD binding.

b, **c**, YAP reduces ERα level in additional ER^+ breast cancer cell lines. T47D (**b**) and ZR-75-1 (**c**) cells transduced with a control vector, flag-YAP(5SA) or Flag-YAP(5SA/94A) were analyzed by immunoblot.

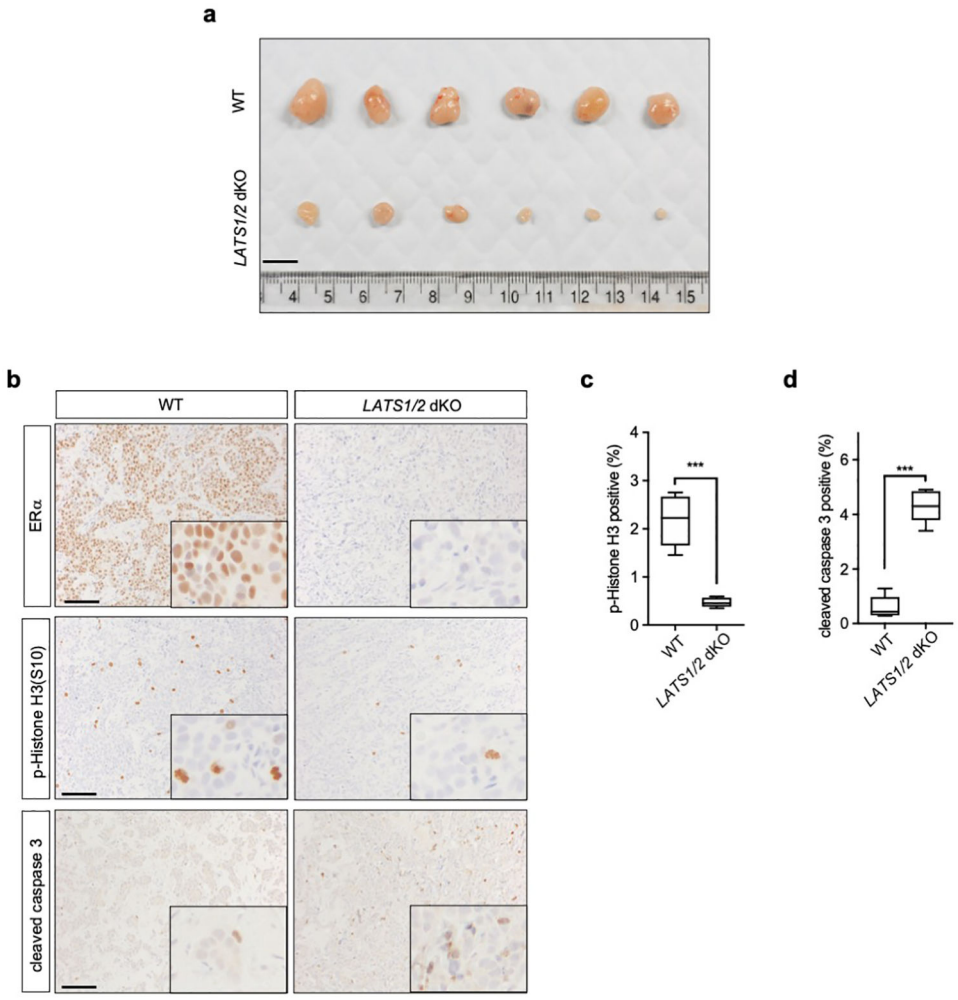
d, YAP TEAD-binding domain, but not WW domain, is essential for *CTGF* induction.

e, **f**, YAP and TAZ have redundant role in repressing ERα expression. WT, YAP KO, TAZ KO or YAP/TAZ dKO MCF-7 cells were subjected to immunoblot (**e**) or qPCR for *ESR1* (**f**).

g, YAP/TAZ dKO increases expression of ERα target gene *TFF1* and *GREB1.*

h, **i**, NF2 deficiency decreases ERα expression, YAP phosphorylation, LATS1 phosphorylation without affecting LATS1 protein. Wild-type (WT) or three independent clones of NF2 null MCF-7 cells were subjected to immunoblot (**h**) or qPCR (**i**).

***$P < 0.001$; mean + s.d., n.s., not significant. See Supplementary Fig .1 for gel source data.



Extended Data Fig. 3 |. *LATS* deletion inhibits ER$^+$ breast cancer cell growth.

a, Image representative of six biologically independent xenografts for Fig. 2h. Scale bar, 10 mm.

b-d, LATS1/2 deficiency inhibits tumor cell proliferation and promotes apoptosis in vivo. Representative images (**b**) and analysis (**c**, **d**) of ERα, p-Histone H3, and cleaved caspase3 immunostaining in LATS1/2 deficient and wild-type MCF-7 xenograft. Scale bar, 100 μM. Box plots indicate median and interquartile range.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability.

The RNA sequencing data are available in Gene Expression Omnibus database with the accession number GSE134615; all other data supporting the findings of this study are available from the corresponding author upon request.

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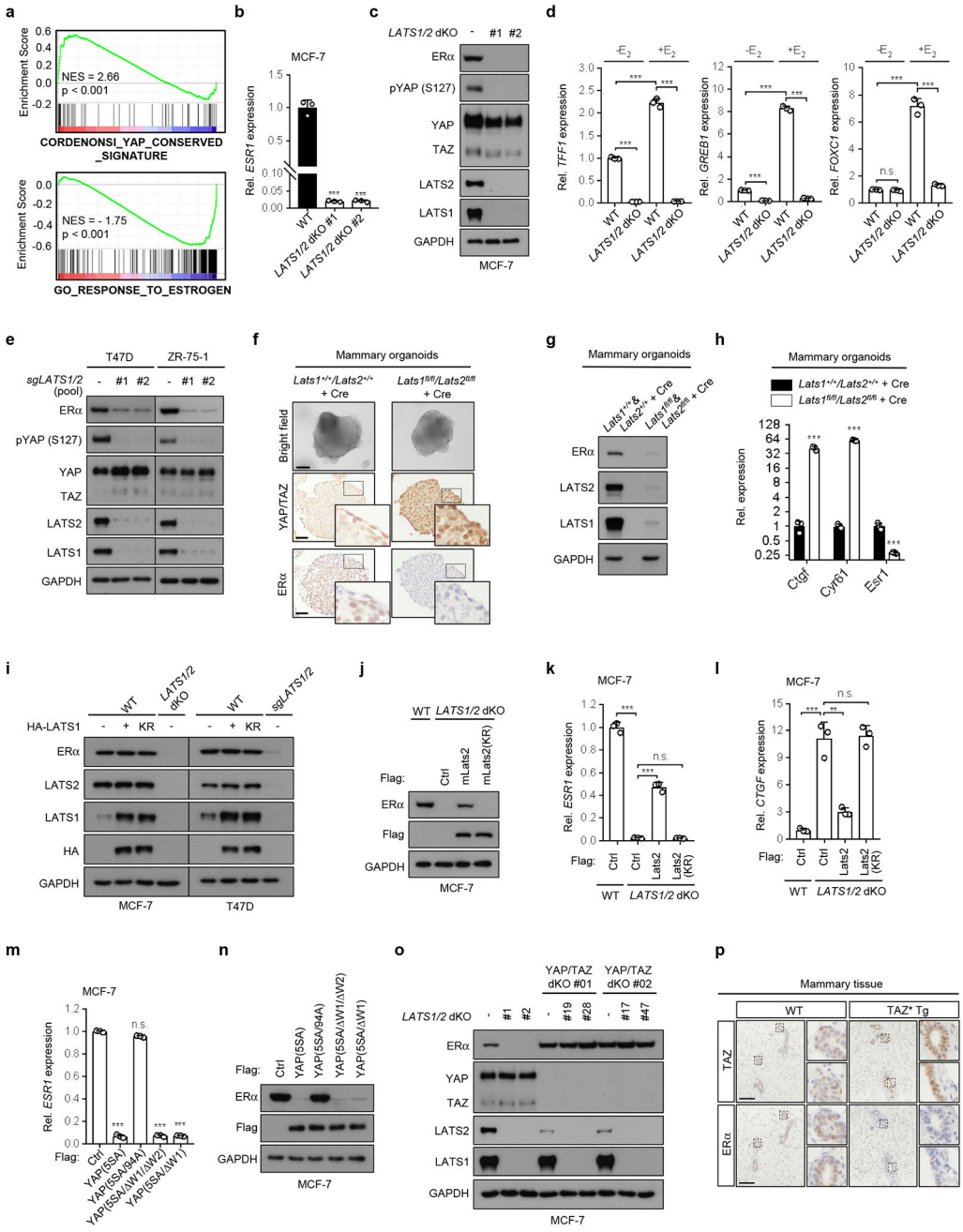

Fig. 1 |. LATS is essential to maintain ER expression.

a, LATS1/2 deletion causes an enrichment of YAP gene signature and depletion of ERα gene signature. **b**, **c**, *LATS1/2* dKO downregulates *ESR1* mRNA (**b**) and ERα protein (**c**) in MCF-7 cells. Two independent *LATS1/2* dKO MCF-7 clones were shown.

d, *LATS1/2* dKO inhibits ERα target genes. E_2 treatment (1 nM E_2 for 45 min).

e, LATS1/2 deficiency downregulates ERα. T47D and ZR-75-1 cells with lentivirus-mediated CRISPR deletion of *LATS1/2* (*sgLATS1/2*) were subjected to immunoblot analysis with indicated antibodies.

f-h, Deletion of *Lats1/2* activates YAP/TAZ and downregulates *ESR1* expression in mammary organoids. Organoids derived from the mammary tissues of $Lats1^{+/+}/Lats2^{+/+}$ and $Lats1^{fl/fl}/Lats2^{fl/fl}$ mice were infected with Cre-encoding adenovirus and subjected to immunohistochemistry (**f**), immunoblot (**g**) and qPCR analysis (**h**). Scale bar, 50 μm.

i, *LATS1* overexpression does not decrease ERα protein. HA-LATS1 or kinase dead mutant (KR) was expressed in WT or *LATS1/2* dKO cells of MCF-7 or T47D.

j-l, Expression of Lats2 wild-type, but not the kinase-dead mutant, rescued ERα expression in the *LATS1/2* dKO cells. Immunoblot (**j**) or qPCR for *ESR1* (**k**) and *CTGF* (**l**).

m, **n**, TEAD-binding is required for YAP mediated *ESR1* reduction. MCF-7 cell transduced with a control vector, Flag-YAP(5SA), Flag-YAP(5SA/94A), Flag-YAP(5SA/ΔW1ΔW2), or Flag-YAP(5SA/ΔW1) were subjected to qPCR for *ESR1* (**m**) or immunoblot (**n**).

o, YAP/TAZ mediates the ERα reduction by LATS1/2 deficiency. Numbers denote different cell clones.

p, TAZ transgene reduces ERα in vivo. Immunohistochemical staining was performed on mammary tissues from control and MMTV-rtTA TRE-TAZ^{4SA} transgenic (TAZ* Tg) mice. Scale bar, 100 μm.

***$P < 0.001$, n.s., not significant, Two-sided t-test or ANOVA; mean + s.d.. See Supplementary Fig .1 for gel source data.

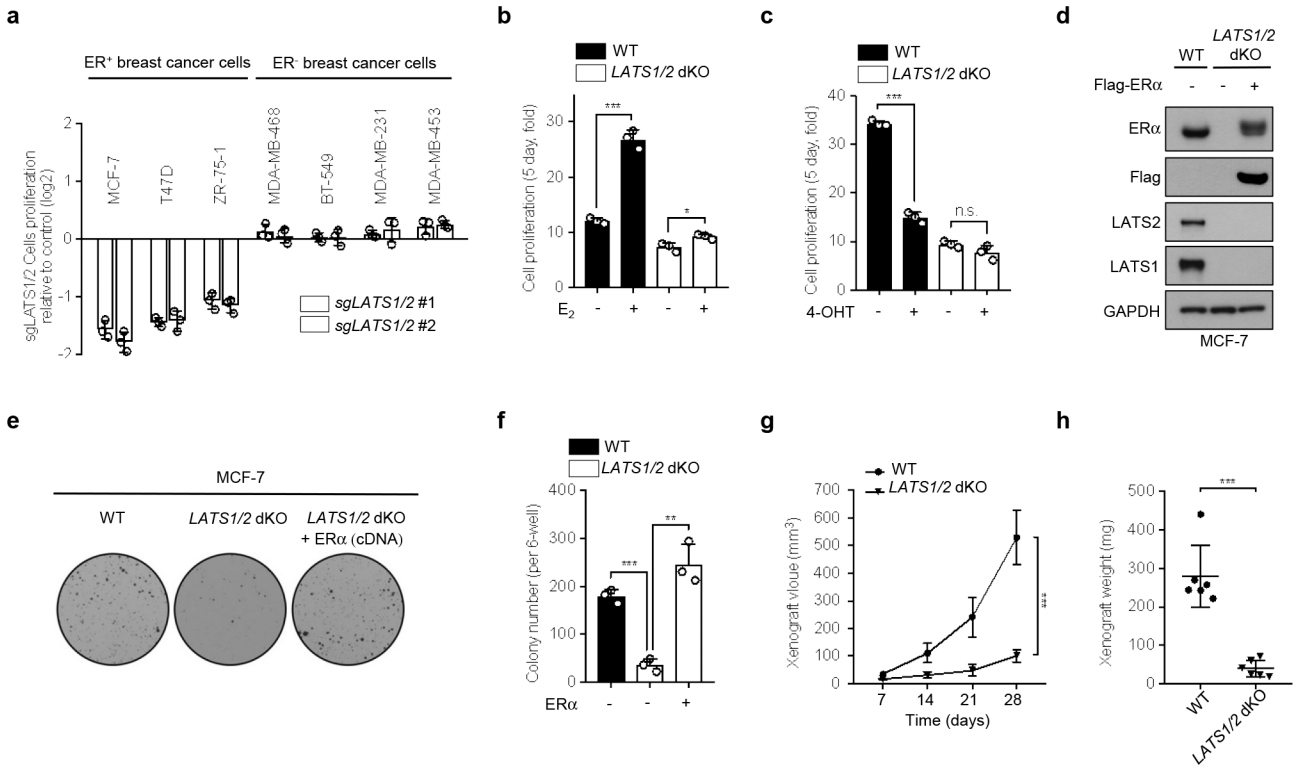

Fig. 2 |. LATS knockout inhibits ER$^+$ breast cancer cell growth by abolishing ERα expression.

a, LATS1/2 deletion inhibits growth of ERα positive, but not ERα negative, breast cancer cells. Cells infected with lentivirus encoding CRISPR-cas9 sgRNA targeting both LATS1 and LATS2 (sgLATS1/2) or control vector were grown for 4 days. n=3 independent samples. Two different set of guide sequences targeting *LATS1/2* were used and labelled as #1 and #2.

b, LATS1/2 is required for estrogen response. Wild-type and *LATS1/2* dKO MCF-7 cells were cultured in steroid-free media with or without 0.1 nM E_2 added for 5 days.

c, LATS1/2 deficient MCF-7 cells are insensitive to growth inhibition by 4-OHT. Wild-type and *LATS1/2* dKO MCF7 cells were cultured in absence or presence of 0.5 μM 4-OHT for 4.5 days.

d, Re-expression of ERα in *LATS1/2* dKO MCF-7 cells. Wild-type, *LATS1/2* dKO, and *LATS1/2* dKO with Flag-ERα re-expressing cells were subjected to immunoblot analysis.

e, **f**, Ectopic expression of ERα rescues the growth defect caused by *LATS1/2* knockout. Soft agar colony formation of wild-type (WT) and *LATS1/2* dKO MCF-7 cells, and *LATS1/2* dKO Cells re-expressing ERα (**e**). The colonies were stained with crystal violet for quantification (**f**).

g, LATS1/2 deficiency reduces in vivo xenograft growth. Nude mice were injected with wild-type or *LAST1/2* dKO MCF-7 cells and tumor growth was measured at the indicated times.

h, Tumour weight on day 28 from (**g**). See Supplementary Fig .1 for gel source data.