Lats2 is critical for the pluripotency and proper differentiation of stem cells

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Differentiation is a highly controlled process essential for embryonic and adult development. Moreover, disruption of proper differentiation is often associated with human diseases, including cancer. We analyzed the involvement of the tumor-suppressor Lats2 in mouse embryonic stem cell (mESC) pluripotency and differentiation, and report that mESCs lacking Lats2 are unable to sustain stemness and are not able to initiate and coordinate developmental transcriptional programs. Lats2 $-/-$ mESCs retain bivalent 'poised' chromatin marks on developmental genes and exhibit germ layer ambiguity both *in vitro* and *in vivo*. Importantly, in coordinating proper germ layer specification, Lats2 engages in a feedback loop with another tumor suppressor, p53.

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Embryonic stem cells (ESCs) are unique in that they self-renew and harbor the potential to differentiate into all adult cell types (characteristics of 'stemness'). A delicately balanced network of transcription factors is crucial for both ESC immortality and pluripotency. Highlighting the importance of transcription is the discovery that overexpression of a distinct set of transcription factors suffices to reprogram differentiated adult cells into induced pluripotent stem cells (iPSCs).¹ It is noteworthy that disabling the tumor-suppressor p53 improves the efficiency of iPSC production.^{2–6}

Deregulation of genes important for maintaining stemness is also invariably implicated in cancer.⁷ In fact, oncogenic transformation often hijacks and distorts developmental transcriptional wiring. Following hand in hand with transcriptional alterations, formation of bivalent chromatin domains characteristic of stem cells facilitates tumorigenesis.⁸ Notably, undifferentiated tumors bearing transcriptional signatures indicative of stemness generally predict worse prognosis.⁹ In a complementary manner, tumor suppressors often promote differentiation.¹⁰

The Hippo cascade is a highly conserved tumor-suppressor pathway,¹¹ whose inactivation can result in dramatic overgrowth (in flies) or tumors (in mammals).¹² The Ser/Thr kinases Lats2 and Lats1 act as central mediators of the Hippo activity by negatively regulating the pathway's downstream effectors Yap and its paralog Taz. Phosphorylation of Yap by the Lats kinases expels Yap from the nucleus and targets it for proteasomal degradation.¹³ Yap function affects numerous tumor-associated attributes, such as cell contact inhibition, mechanosensing and proliferation.¹⁴ During development, Yap modulates stem cell behavior¹⁵ and preimplantation cell

lineage.¹⁶ To complicate matters, there is mounting evidence for Lats-independent Yap functions.^{14,17} Moreover, Lats2 has a spectrum of functions that extend beyond Yap regulation, including maintenance of genome stability, induction of apoptosis, cell cycle and tetraploidy checkpoint control and inhibition of cell migration.¹⁸

One striking example of this is Lats2 interaction with components of the p53 pathway. Lats2 expression is positively regulated by p53,¹⁹ and the *Lats2* gene is a direct transcriptional target of p53.²⁰ Furthermore, Lats2 binds and inhibits the negative regulator of p53, Mdm2, whereas it promotes the nuclear localization and transcriptional functionality of the proapoptotic p53 coactivator ASPP1.^{20,21} These interactions serve to augment the p53 transcriptional response, defining a p53–Lats2 positive feedback loop.

We now report that Lats2 $-/-$ ESCs are compromised both in their ability to maintain pluripotency and their ability to respond effectively to differentiation triggers. The differentiation defect is associated with a failure of Lats2-deficient ESCs to resolve bivalent chromatin modifications in response to differentiation signals. Inability of Lats2 $-/-$ ESCs to sustain a coordinated epigenetic program hampers transcriptional induction of differentiation-specific genes, resulting in unbalanced differentiation into the three germ layers both *in vitro* and *in vivo*. Moreover, during normal ESC differentiation, Lats2 expression is selectively induced in a p53-dependent manner. We propose that during the differentiation process, Lats2 is recruited to the p53 pathway to coordinate proper transcriptional reprogramming.

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Abbreviations: ESC, embryonic stem cell; iPSCs, induced pluripotent stem cells; AP, alkaline phosphatase; SKY, spectral karyotyping; BF, brightfield; EB, embryoid body; ECM, extracellular matrix; RA, retinoic acid; ChIP, chromatin immunoprecipitation; TSS, transcription start site; Alb, albumin; NT, non-treated; RA-D4, 4 days retinoic acid treated; VP, Verteporfin

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Results

Lats2^{-/-} ESCs are less pluripotent and differentiate ineffectively. Lats2^{-/-} mice are embryonic lethal, death occurring at ~E10.5–E12.5.^{22,23} To investigate the role of Lats2 in early development, we generated several independent Lats2^{-/-} ESC clones, as well as matched wild-type (WT) control clones, by intercrossing C57BL/6 mice heterozygous for a Lats2-null allele (Lats2^{+/-}). Unless otherwise indicated, all experiments were performed with three independent WT clones and three independent Lats2^{-/-} clones.

Colonies of WT and Lats2^{-/-} ESCs were indistinguishable morphologically (Figure 1a). However, significant differences between the two genotypes became apparent when we compared the activity of alkaline phosphatase (AP). Staining intensity of AP activity, a biomarker of undifferentiated stem cells, was quantified in a high-throughput manner (Supplementary Figure S1). As seen in Figure 1b (left panel), the average AP intensity of Lats2^{-/-} ESC colonies was significantly lower than that of WT ESC colonies. Moreover, unlike the case for WT ESCs, only a rather small fraction of Lats2^{-/-} ESCs could give rise successfully to AP-positive colonies (Figure 1b, right panel), implying a defect in self-renewal capacity. Together, these results indicate that

Lats2 is required for sustaining full ‘stemness’ and self-renewal potential in the absence of differentiation signals.

Lack of Lats2 has been associated with a variety of chromosome instability phenotypes.^{20,22,23} We therefore asked whether, in addition to pluripotency defects, Lats2^{-/-} ESCs might be susceptible to genomic anomalies. To that end, WT and Lats2^{-/-} ESC clones were propagated to high passage number (passage > 20). FACS analysis was performed to determine ploidy and replication kinetics. WT ESC clones retained a uniform, diploid cell cycle distribution; in contrast, Lats2^{-/-} clones acquired aberrant, excessive DNA content (Figure 1c and Supplementary Figure S2A); in addition, they displayed a slightly higher fraction of cells in the S phase (Supplementary Figure S2B). Fluorescent spectral karyotyping (SKY) analysis revealed that although late-passage WT ESCs maintained normal chromosome numbers, Lats2^{-/-} ESCs exhibited near-tetraploid genomes, with the number of copies of different individual chromosomes varying from 2 to 5 (Figure 1d and Supplementary Figure S2D). In view of the tendency of Lats2^{-/-} clones to develop aneuploidy, we performed all subsequent experiments on early passage cultures (passage < 12), when both genotypes still retained a normal karyotype.

Figure 1 Lats2^{-/-} ESCs are less pluripotent and tend to become aneuploid. (a) Representative WT and Lats2^{-/-} ESCs cells visualized by brightfield (BF) microscopy at $\times 4$ magnification. (b) Three WT and three Lats2^{-/-} independent ESC clones were seeded in 96-well tissue culture plates at 200 cells/well (a total of 18 wells per each clone). After 5 days, staining of colonies for alkaline phosphatase (AP) activity was performed and quantified as described in Supplementary Materials and Methods. Left panel: quantification of mean AP staining intensity per colony. Right panel: amount of AP-positive colonies per 200 single cells seeded. Green and red regions of plots represent upper 75 and lower 25 percentile, respectively. Whiskers represent maximum and minimum values. For each genotype, values were collected and averaged from all 54 wells of the three corresponding clones. (c) Passage 20 ESCs were fixed, stained with propidium iodide and subjected to FACS analysis of cell cycle distribution. (d) Passage 20 ESCs were processed for SKY analysis

Lats2^{-/-} ESCs and Lats2^{-/-} embryos execute differentiation programs aberrantly and inefficiently.

Embryoid body (EB) formation is often used as a means to initiate spontaneous differentiation of ESCs to the three germ layers. EBs are three-dimensional multicellular spheroids that mimic patterning in the early developing embryo.²⁴ At 5 days following induction of EB formation, a marked difference in size was observed between WT and Lats2^{-/-} EBs (Figure 2a, left panels); Lats2^{-/-} EBs were approximately twice the diameter of WT EBs and appeared more dense than WT EBs. In most WT EBs, differentiation of exterior cells to primitive endoderm and the resultant ring of extracellular matrix (ECM) could be visualized by phase contrast microscopy. In contrast, most Lats2^{-/-} EBs did not exhibit definitive basement membrane delineation. By 12 days of EB development, both WT and Lats2^{-/-} EBs reached maximum size. However, whereas WT EBs retained a uniform distribution of mass, Lats2^{-/-} EBs exhibited a very irregular internal bulk. Histological sections of EBs at day 18 revealed that WT EBs displayed the expected architectural features: a thin, even layer of endoderm external to a regular basement membrane. In contrast,

Lats2^{-/-} EBs displayed an aberrant architecture with thick, uneven endoderm and a discontinuous, irregular basement membrane (Figure 2a, right panels). To further characterize the WT and Lats2^{-/-} EBs, we performed immunofluorescent staining of Foxa2, an endoderm marker. Whereas WT EBs consistently exhibited a clearly delineated endodermal layer (Figure 2b), the endoderm of individual Lats2^{-/-} EBs revealed either massive hyperproliferation or hypoproliferation (Figure 2b, right and middle panels, respectively). Hence, Lats2^{-/-} EBs develop irregularly, with abnormal architecture and aberrant germ layer distribution and size.

Oct4 is a key component of the ESC transcriptional circuitry, and is essential for maintenance of the undifferentiated, pluripotent state.²⁵ Induction of differentiation, for example by retinoic acid (RA), elicits rapid shutoff of Oct4 expression. Indeed, within 3 days of RA treatment, Oct4 protein and RNA became barely detectable in WT ESCs (Figures 3a and b, respectively). In contrast, Lats2^{-/-} ESCs displayed a dual defect in regulation of Oct4 expression. On one hand, basal Oct4 levels in the undifferentiated state were almost twofold lower in Lats2^{-/-} ESCs than in

Figure 2 Lats2^{-/-} ESCs produce defective embryoid bodies. (a) Left panels: brightfield microscopy photo of EBs at day 5 (D5); magnification $\times 10$. Middle panels: brightfield microscopy photo of EBs at day 12 (D12); magnification $\times 10$. Right panels: H&E-stained section of EBs at day 18 (D18); magnification $\times 4$. (b) Immunohistochemical staining of EBs at day 18. Foxa2 is a marker for endoderm. Nuclei were visualized with DAPI. Magnification $\times 10$. Two representative fields from Lats2^{-/-} EBs (fields A and B) are shown

WT ESCs. On the other hand, *Lats2*^{-/-} ESCs failed to efficiently shut off *Oct4* expression in response to differentiation signals, and retained detectable *Oct4* even after 6 days of RA treatment (RA-D6). Similarly, the expression of other pluripotency markers such as *Esrrb*, *Klf4* and *Rex1* was also significantly lower in *Lats2*^{-/-} ESCs than in WT ESCs (Figure 3c). In addition, similar to *Oct4*, expression of *Klf4* and *Rex1* was retained at elevated levels in RA-treated *Lats2*^{-/-} ESCs. Notably, the impaired ability of *Lats2*^{-/-} ESCs to maintain basal expression of pluripotency genes was also apparent when the different ESC strains were propagated in 2i medium that retains ESCs in a more 'naive' state (Supplementary Figure S1B).

A complex network of transcription and epigenetic factors maintain ESCs in a pluripotent state by repressing genes required for germ layer specification. Once differentiation commences, such genes are activated in a lineage-specific manner, thus driving induction of germ layer-specific transcription programs and cell-fate determination.²⁶ As expected, RA treatment of WT ESCs for 6 days led to efficient induction of genes representative of all three germ layers:²⁷ *Pax6* (ectoderm), *Flk1* (mesoderm) and *Gata6* (endoderm) (Figure 3d). However, induction of these genes was severely compromised in *Lats2*^{-/-} ESCs. Hence, *Lats2* deficiency leads to a generalized defect in differentiation into all three germ layer fates.

To exclude the possibility that the differentiation defect of *Lats2*-deficient ESCs was specific to RA treatment, we subjected cells to a hepatocyte differentiation protocol. WT ESCs rapidly decreased expression of *Sox2* mRNA and subsequently reduced *Oct4* expression (Figure 4a). This sequential decrease in *Sox2* followed by *Oct4* is characteristic of cells undergoing mesendodermal differentiation.²⁶ In contrast, despite low basal *Sox2* mRNA, no further reduction was observed in *Lats2*^{-/-} ESCs, even after 26 days. Repression of *Oct4* at day 26 was also partly compromised in *Lats2*^{-/-} ESCs. Similarly, induction of progenitor and mature hepatocyte genes was impaired in the *Lats2*^{-/-} ESCs. Notably, although the hepatoblast transcription factor HNF4a was partly upregulated in *Lats2*^{-/-} ESCs (Figure 4b), albumin, indicative of mature functional hepatocytes, was not induced at all and remained at basal predifferentiation levels. The inability of *Lats2*^{-/-} ESCs to complete differentiation into mature hepatocytes was validated by albumin immunostaining (Figure 4c). Albumin-positive WT cells clustered in groups adjacent to regions of spontaneously differentiated beating cardiomyocytes, a phenomenon previously reported to occur during *in vitro* hepatocyte differentiation.^{28,29} In contrast, *Lats2*^{-/-} cells displayed only background albumin staining (Figure 4c), and no beating cells were observed. Hence, *Lats2* is necessary for the full

Figure 3 *Lats2*^{-/-} ESCs execute differentiation programs inefficiently. (a) Cells were harvested before treatment (NT), or treated for 3 (RA-D3) or 6 (RA-D6) days with retinoic acid. Lysates were subjected to western blot analysis with the indicated antibodies. (b) Cells were treated as in (a), and relative levels of *Oct4* mRNA were assessed by qRT-PCR analysis. *P*-value relates to WT versus *Lats2*^{-/-} in each condition, wherein **P* < 0.1; ***P* < 0.05; and ****P* < 0.01. (c) Cells were treated as in (a), and mRNA levels of indicated genes were assessed by qRT-PCR analysis. *P*-values are as in (b). (d) Cells were harvested before treatment, or treated for 6 days with retinoic acid. Expression of the indicated transcripts was determined by qRT-PCR. 'Fold induction' relates to expression in RA-treated cells relative to levels of the same transcript in NT samples. *P*-values are as in (b).

Figure 4 *Lats2*^{-/-} ESCs are unable to differentiate to mature hepatocytes. (a and b) ESCs were subjected to a hepatocyte differentiation protocol. RNA was isolated from day 5 (D5), day 18 (D18) and day 26 (D26) cultures, and subjected to qRT-PCR analysis with primers specific for the indicated genes. (c) ESCs were subjected to a hepatocyte differentiation protocol. Cells were fixed on day 18 and immunostained with antibodies specific for albumin (Alb). Nuclei were stained with DAPI. Magnification $\times 10$. Dashed line denotes region of beating cells

execution of differentiation programs with different germ layer specifications.

Teratomas are benign tumors characterized by an intrinsic ability to yield all three germ layers. Indeed, when injected into nude mice, WT ESCs generated teratomas harboring differentiated cells of all three lineages (Figure 5a). In contrast, although *Lats2*^{-/-} ESCs gave rise to teratomas of comparable size, these teratomas consistently tended to differentiate into only one or two germ layers per injection, with no preference for any particular germ layer specification. Germ layer inclination was unpredictable, as even replicate injections of an identical clone generated teratomas with different lineage imbalances (Figure 5a). Hence, *Lats2*^{-/-} ESCs are not inherently biased toward a particular differentiation scheme, but rather have a global defect in negotiating complex differentiation programs.

To test this notion in a more physiological setting, we turned to *in vivo* embryogenesis. As mentioned above, *Lats2*^{-/-} embryos die at day E10.5–E12.5 because of neuronal²² or cardiac²³ differentiation defects. We therefore compared gene expression patterns between WT and *Lats2*^{-/-} embryos obtained at day E8.5, before any phenotypic difference was observed.^{22,23} Analysis of RNA isolated from whole embryos revealed that at this stage, when embryonic germ layer differentiation commences, *Lats2*^{-/-} embryos retained a nearly twofold excess of Oct4 expression (Figure 5b). It is also noteworthy that compared with littermate WT embryos, *Lats2*^{-/-} embryos exhibited a pronounced imbalance of ectoderm/endoderm differentiation (Figure 5b). These observations are consistent with our *in vitro* data and support the notion that *Lats2* is essential for proper coordination of balanced differentiation into the different lineages.

Lats2^{-/-} ESCs retain bivalent chromatin markers.

Epigenetic markings play critical roles in regulating the expression of developmental genes.³⁰ In ESCs, many key developmental genes are associated with bivalent chromatin, decorated simultaneously with both repressive histone H3 Lys27 trimethylation (H3K27me3) and activating histone H3 Lys4 trimethylation (H3K4me3) marks.³¹ To determine whether WT and *Lats2*^{-/-} ESCs differ in their ability to manage their epigenetic landscape, we performed chromatin immunoprecipitation (ChIP) analysis of H3K4me3, H3K27me3 and total H3 on ESCs before and after differentiation with RA. In the undifferentiated state, WT and *Lats2*^{-/-} ESCs displayed similar patterns of these modifications (Figure 6a, left panel): both cell types presented high levels of H3K4me3 and low levels of H3K27me3 adjacent to the transcription start sites (TSS) of the pluripotency genes *Oct4* and *Nanog*. Moreover, in both undifferentiated WT and *Lats2*^{-/-} ESCs, activating and repressive histone marks were simultaneously present near the TSS of the genes encoding the ectodermal transcription factors Pax6 and Sox21, indicative of their bivalent state. Hence, in the basal state we did not detect substantial differences in epigenetic landscape between the two cell types. However, within 4 days of RA treatment, marked differences became apparent (Figure 6a, right panel). As expected, WT ESCs significantly diminished H3K4me3 and augmented H3K27me3 near the TSS of *Oct4* and *Nanog*. In addition, the bivalency at the Pax6 and Sox21 loci was resolved into high H3K4me3 and low H3K27me3. In contrast, *Lats2*^{-/-} ESCs exhibited marked epigenetic defects upon exposure to differentiation signals. Specifically, *Lats2*^{-/-} ESCs sustained relatively high levels of H3K4me3 and low levels of H3K27me3 on pluripotency genes. Importantly, *Lats2*^{-/-} ESCs retained bivalency on Pax6 and Sox21, indicating that they remained frozen in their poised state, unable to either extinguish pluripotency or commit to a specific differentiation program.

Lats2 expression is induced during differentiation in a p53-dependent manner.

Given the impact of *Lats2* on embryogenesis and embryonic SC biology, we next explored the regulation of *Lats2* gene expression in these cells. As shown in Figure 6b, *Lats2* mRNA was markedly induced upon treatment of WT ESCs with RA. *Lats2* transcription is positively regulated by p53 through direct binding of p53 to the *Lats2* promoter.²⁰ We therefore utilized p53^{-/-} ESCs to investigate the p53 dependence of *Lats2* induction. Remarkably, whereas basal *Lats2* mRNA levels were not significantly affected by p53 depletion, RA-induced *Lats2* upregulation was strongly compromised in p53^{-/-} ESCs (Figure 6b). Both *Lats2* and *Lats1* act in the Hippo pathway; however, RA elicited only a mild change in *Lats1* expression that was unaffected by depletion of either p53 or *Lats2* (Supplementary Figure S3). Hence, p53 specifically transactivates *Lats2* during ESC differentiation.

In addition to *Lats2*, the canonical p53 targets *p21* and *PUMA* were also induced by RA treatment in WT ESCs (Figure 6c), indicative of generalized p53 activation during ESC differentiation. Interestingly, exposure of undifferentiated ESCs to the p53-activating drug Nutlin-3 strongly upregulated *p21* and *PUMA* (Figure 6c) but not *Lats2* (Figure 6d).

Figure 5 *Lats2*^{-/-} ESCs fail to differentiate into all three germ layers. (a) Histological sections of teratomas, stained with H&E. Two representative teratomas (1 and 2) from two independent *Lats2*^{-/-} clones are presented. *Denotes ectoderm; **mesoderm; and ***endoderm. Magnification $\times 20$. (b) Total embryo RNA was extracted from day E8.5 WT and *Lats2*^{-/-} embryos. mRNA levels of the indicated genes were assessed by qRT-PCR. Bars represent average and S.D. of three embryos from each genotype. 'RNA (relative levels)' and *P*-value relate to WT versus *Lats2*^{-/-}, wherein ***P* < 0.05; ****P* < 0.01; and NS is not statistically significant

Figure 6 *Lats2* expression affects developmental chromatin landscape and is induced during differentiation in a p53-dependent manner. (a) Cells were harvested before treatment (NT) or treated for 4 days with retinoic acid (RA-D4). Chromatin was immunoprecipitated with total H3, H3K4me3 or H3K27me3 antibodies. Immunoprecipitated DNA was quantified by qPCR, employing primers spanning the transcription start sites (TSS) of the indicated genes. Values were normalized to total H3, and then converted to Log₂ scale. (b) Cells were harvested before treatment (NT), or treated for 2 (RA-D2) or 6 days (RA-D6) with retinoic acid. *Lats2* mRNA was quantified by qRT-PCR. (c and d) Cells were harvested before treatment (NT), or treated for 4 days with retinoic acid (RA-D4). Before harvesting, cells were mock-treated or treated with 25 μ M Nutlin-3 overnight. RNA was isolated and subjected to qRT-PCR analysis with primers specific for (c) *p21*, *PUMA* or (d) *Lats2*

In contrast, in differentiating cells (RA-D4), *Lats2* was prominently further upregulated upon p53 activation by Nutlin-3 (Figure 6d), whereas *p21* and *PUMA* were not (Figure 6c). This implies a distinct mode of regulation of the *Lats2* gene during ESC differentiation. Specifically, it suggests that in undifferentiated cells, *Lats2* may be actively prevented from responding to p53, becoming 'unlocked' only in response to differentiation signals.

Regulation of Yap and p53 transcriptional targets by differentiation and *Lats2* deletion. Next, we wished to explore the signaling pathways affected downstream to *Lats2* during differentiation. As mentioned previously, *Lats2* has well-documented functions in the Hippo pathway, upstream to Yap and Taz,³² and also affects the p53 pathway through inhibition of Mdm2.²⁰

As predicted from the canonical Hippo signaling, *Lats2* $-/-$ ESCs displayed augmented, although mild, basal expression levels of the Yap targets *CTGF*, *Cyr61* and *Birc5*³³ (Figure 7a, compare NT *Lats2* $-/-$ with NT WT), confirming that *Lats2* can curtail Yap activity in this system. Although RA treatment caused a marked upregulation of *CTGF* mRNA, other Yap targets were not consistently induced during differentiation (Figure 7a). Together, these observations suggest that Yap transcriptional activity probably does not play a substantial role in the RA response of these cells.

To further evaluate the involvement of Yap in ESC differentiation, we employed the Yap inhibitor Verteporfin (VP).³⁴ As expected, VP strongly decreased *CTGF* expression in both WT and *Lats2* $-/-$ ESCs (Figure 7b). Importantly, VP did not rescue the differentiation defect of *Lats2* $-/-$ ESCs, as assessed by its effect on *Oct4* expression (Figure 7c). Similarly, the failure of *Lats2* $-/-$ ESCs to upregulate

differentiation-specific genes, such as *Gata6*, was also not effectively relieved by VP treatment. The inability of VP to restore proper differentiation capacity to *Lats2* $-/-$ ESCs substantiates the assertion that Yap hyperactivation is probably not the main driver of the differentiation defect of these cells.

Lats2 is required for optimal p53 activation in response to a variety of stresses.³⁵ We therefore hypothesized that *Lats2* might not only be transcriptionally regulated by p53 in ESCs (Figure 6b), but might also facilitate p53 activation during differentiation of these cells. Treatment with RA led to a gradual prominent increase in p53 protein levels (Figure 8a, left panels), in line with our observation that p53 target genes were upregulated under those conditions (Figure 6c). Importantly, ESCs lacking *Lats2* were less capable of fully activating p53 during differentiation; this was apparent in p53 protein levels (Figure 8a, right panels) as well as in p53 target gene transcriptional activation, as exemplified by *p21* and *PUMA* (Figures 8b and c). In that regard, *Lats2* $-/-$ ESCs mimicked to some extent the defect of p53-null ESCs. Our data support a scenario in which *Lats2* operates via p53-mediated mechanisms during differentiation, and compromised p53 activation at least partially contributes to the differentiation defect of *Lats2* $-/-$ ESCs.

Discussion

In this study, we report that ESCs lacking *Lats2* are both less able to sustain pluripotency and less capable of responding efficiently to differentiation signals. Thus, when exposed to differentiation triggers, *Lats2* $-/-$ ESCs retain bivalent 'poised' chromatin marks on developmental genes, and stem cell-like epigenetic marks on pluripotency genes.

Figure 7 Regulation of Yap transcriptional targets by differentiation and *Lats2* deletion. (a) Cells were harvested before treatment (NT), or treated for 4 days with retinoic acid (RA-D4). RNA was extracted and subjected to qRT-PCR analysis with the indicated primers. (b and c) Cells were harvested before treatment (NT), or treated for 6 days with retinoic acid (RA-D6). Before harvesting, cells were mock-treated or treated with 10 μ M Verteporfin (VP) for 8 h. RNA was extracted and subjected to qRT-PCR analysis with the indicated primers

Figure 8 Regulation of p53 and its transcriptional targets by differentiation signals and Lats2 deletion. **(a)** Cells were harvested before treatment (NT), or treated for 3 (RA-D3) or 6 days (RA-D6) with retinoic acid. Protein lysates were subjected to western blot analysis with the indicated antibodies. **(b and c)** Cells were harvested before treatment (NT), or treated for 2 (RA-D2) or 6 days (RA-D6) with retinoic acid. RNA was extracted and subjected to qRT-PCR analysis with the indicated primers

This epigenetic ambiguity may underlie the inability of Lats2^{-/-} ESCs to execute efficiently the transcriptional programs required for proper differentiation. As bivalency presumably maintains a permissive chromatin structure, simultaneously poised for differentiation-specific gene expression while preserving pluripotency, we propose that Lats2-dependent resolution of bivalency and maintenance of epigenetic order might be critical to avoid ‘confused’ developmental messages. Indeed, as reported recently, transient reduction of Lats1 and Lats2 in early preimplantation embryos traps cells in a conflicting trophectoderm–inner cell mass (TE–ICM) developmental impasse.^{16,36} We now show that lack of Lats2 causes cells to express contradictory lineage-specific transcripts also subsequent to the first TE–ICM cell lineage specification; in ESCs originating from the ICM as well as in embryos at later developmental stages (E8.5) (Figure 5b). In all cases, absence of Lats2 gives rise to discordant developmental processes.

Upon differentiation, bivalent H3K4me3/H3K27me3 marks are resolved to monovalency in tune with establishment of lineage-specific gene expression programs.³⁷ Interestingly, ESCs deficient for H3K27 methylation display inappropriate expression of conflicting lineage-specific genes,³⁸ reminiscent of the defect we observe in Lats2^{-/-} ESCs. It is noteworthy that some differentiated cells such as multipotent neural and hematopoietic stem cells and embryonic fibroblasts also possess bivalent marks,^{38,39} suggesting that bivalency might better represent plasticity, rather than pluripotency. Moreover, it was recently demonstrated that precursor cancer SCs are plastic cell populations that can transition from epithelial to mesenchymal identity by regulating bivalent/monovalent marks on developmental

genes.^{40,41} The related aspects of the Lats2^{-/-} phenotype may not be coincidental, as one of the hallmarks of defective Hippo signaling is enhanced EMT.⁴²

Although Lats2 is best recognized for its role as a negative regulator of Yap in the Hippo pathway, our data strongly suggest that it is not solely in this capacity that Lats2 maintains the proper chromatin landscape and transcriptional performance of ESCs. Yap overexpression has been reported to augment stemness.^{15,43} More recently, it has been suggested that Yap may actually play a positive role in hepatocyte differentiation.⁴⁴ In that regard, the phenotypes described here for Lats2^{-/-} ESCs (failure to maintain stemness and inability to differentiate into mature hepatocytes) are contrary to those observed in response to Yap hyperactivation. Importantly, inhibition of Yap activity failed to rescue the transcriptional defect of Lats2^{-/-} ESCs (Figure 7c). Furthermore, if disabling Yap were the primary role of Lats2 in differentiation and development, then one might expect Lats1 and Lats2 to behave similarly. However, this does not appear to be the case. Thus, although Lats2 is essential for embryonic development,^{45,46} Lats1 knockout mice are viable.⁴⁷ Moreover, as shown here (Figure 6a), whereas Lats2 expression is strongly induced during differentiation, Lats1 expression is not. Lastly, we show that Lats1 expression in differentiating ESCs is affected neither by p53 levels nor p53 activity, both of which strongly affect ESC differentiation.^{48–50} Hence, our findings reflect a unique role for Lats2 in the delicate balance of signals required for proper differentiation.

Intriguingly, Yap and Taz appear to possess divergent roles in mouse as compared with human cells. Whereas Yap can regulate mouse ESC self-renewal⁵¹ and increase the efficiency of mouse iPSC generation,⁵² it does not appear to have such role in human ESCs⁵³ or in human iPSC generation.⁵⁴ The opposite holds for Taz: although Taz mediates human ESC renewal⁵⁵ and iPSC reprogramming,⁵⁴ it does not appear to have a similar role in mouse ESCs.⁵⁵ The reason for this intriguing apparent switch of tasks remains unclear. Yet, despite the reportedly more prevalent contribution of Yap in the mouse setting, lack of Yap inhibition does not seem to underlie the defects that we observe in Lats2^{-/-} ESCs.

We propose that during differentiation, Lats2 functions primarily via p53-mediated mechanisms. p53 is a major driving force for ESC differentiation. ESCs lacking p53 are less prone to spontaneous differentiation⁵⁶ but also more impervious to RA-driven differentiation.⁵⁷ Furthermore, p53^{-/-} progenitor cells have an increased tendency toward aneuploidy, particularly at higher passages.⁵⁸ The similarities between the impact of p53 loss and Lats2 loss on ESCs, along with the mutual cross-regulation of these two tumor suppressors, strengthen the conjecture that Lats2 and p53 work hand in hand to oversee proper differentiation.

Additional mechanisms for Lats2 function in ESC differentiation are plausible, and are not mutually exclusive with the proposed Lats2–p53 axis. Indeed, two very recent studies demonstrate critical roles for Lats proteins and the Hippo signaling in coordinating crosstalk with other pathways during differentiation of mammalian adipocytes⁵⁹ and in fly retina.⁶⁰ In addition, Lats2 might phosphorylate a subset of epigenetic machinery proteins, or even directly bind and phosphorylate

chromatin itself. Either way, the end result is that Lats2-deficient ESCs remain suspended in differentiation limbo, unable to retain pluripotency or to fully commit to mature cell types.

In sum, the Hippo and p53 tumor-suppressor pathways have well-documented roles in controlling proliferation, survival and genomic integrity. Recent evidence, including the present study, also implies a central role for those tumor suppressors in the regulation of stem and progenitor cells, and loss of their function in such cells may be a key event in the initiation of many cancers. We propose that Lats2, a constituent of both Hippo and p53 pathways, plays a particularly critical role in regulating ESC pluripotency and differentiation.

Materials and Methods

Isolation and culture of mESCs. C57BL/6 Lats2 +/− (5821, Jackson Laboratory, Mt Desert Is., ME, USA) mice were time mated. E2.5 morulas were isolated from the uteri of female mice and allowed to attach to irradiated MEF feeder layers. Subsequently, 2i medium (media recipes listed in Supplementary Materials and Methods) was changed daily. After colonies were established, ESCs were acclimated and maintained in mES medium. For differentiation experiments, feeders were depleted by 30–45 min of incubation on 0.1% gelatin, followed by gentle aspiration of purified ESCs. For RA-induced differentiation, feeder-depleted cells were seeded on gelatin and transferred to differentiation medium containing 1 μM RA. RA was replenished daily. EBs were generated using the hanging drop method (660 feeder-depleted cells/20 μl) on the lid of a tissue culture dish and then transferred to plastic Petri dishes in differentiation media. For teratoma formation in mice, 1.5 × 10⁶ cells were resuspended in PBS with 30% Matrigel. Hind quarters of 8-week-old CD1 nude mice (Harlan Labs, Indianapolis, IN, USA) were injected subcutaneously. Mice were killed 4 weeks after injection. All mouse procedures were performed under approval of the Institutional Animal Care and Use Committee (IACUC no. 06131209).

Immunofluorescence. EBs were fixed in 4% paraformaldehyde (PFA) for 30 min at RT and resuspended in 4% agarose. Agarose plugs were fixed for another 2 h in 4% PFA and embedded in paraffin. Slides were then stained for H&E or subjected to IHC, using standard procedures. Differentiating cells were grown on 12 mm coverslips and fixed with ice-cold methanol for 20 min at 4°C. Samples were then incubated for 1 h with primary antibodies (Foxa2, ab40874 or albumin, ab106582; Abcam, Cambridge, UK), washed and then incubated with secondary antibody and DAPI (5 μg/ml final) for 40 min in the dark.

Additional Materials and Methods are detailed in Supplementary Materials and Methods.

Conflict of Interest

The authors declare no conflict of interest.

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- Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663–676.
- Hong H, Takahashi K, Ichisaka T, Aoi T, Kanagawa O, Nakagawa M *et al*. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature* 2009; **460**: 1132–1135.
- Sarig R, Rivlin N, Brosh R, Bornstein C, Kamer I, Ezra O *et al*. Mutant p53 facilitates somatic cell reprogramming and augments the malignant potential of reprogrammed cells. *J Exp Med* 2010; **207**: 2127–2140.
- Kawamura T, Suzuki J, Wang YV, Menendez S, Morera LB, Raya A *et al*. Linking the p53 tumour suppressor pathway to somatic cell reprogramming. *Nature* 2009; **460**: 1140–U1107.
- Zhao Y, Yin XL, Qin H, Zhu FF, Liu HS, Yang WF *et al*. Two supporting factors greatly improve the efficiency of human iPSC generation. *Cell Stem Cell* 2008; **3**: 475–479.
- Marion RM, Strati K, Li H, Murga M, Blanco R, Ortega S *et al*. A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. *Nature* 2009; **460**: 1149–U1119.
- Suva ML, Riggi N, Bernstein BE. Epigenetic reprogramming in cancer. *Science* 2013; **339**: 1567–1570.
- Orkin SH, Hochedlinger K. Chromatin connections to pluripotency and cellular reprogramming. *Cell* 2011; **145**: 835–850.
- Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A *et al*. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet* 2008; **40**: 499–507.
- Pomerantz JH, Blau HM. Tumor suppressors: enhancers or suppressors of regeneration? *Development* 2013; **140**: 2502–2512.
- Oren MaYa (ed.). *The Hippo Signaling Pathway and Cancer*. 1st edn. Springer Science & Business Media: New York, 2013.
- Pan D. The hippo signaling pathway in development and cancer. *Dev Cell* 2010; **19**: 491–505.
- Zhao B, Li L, Tumaneng K, Wang CY, Guan KL. A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF(beta-TRCP). *Genes Dev* 2010; **24**: 72–85.
- Dupont S, Morsut L, Aragona M, Enzo E, Giullitti S, Cordenonsi M *et al*. Role of YAP/TAZ in mechanotransduction. *Nature* 2011; **474**: 179–U212.
- Lian I, Kim J, Okazawa H, Zhao J, Zhao B, Yu J *et al*. The role of YAP transcription coactivator in regulating stem cell self-renewal and differentiation. *Genes Dev* 2010; **24**: 1106–1118.
- Nishioka N, Inoue K, Adachi K, Kiyonari H, Ota M, Ralston A *et al*. The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. *Dev Cell* 2009; **16**: 398–410.
- Zhou DW, Conrad C, Xia F, Park JS, Payer B, Yin Y *et al*. Mst1 and Mst2 maintain hepatocyte quiescence and suppress hepatocellular carcinoma development through inactivation of the Yap1 oncogene. *Cancer Cell* 2009; **16**: 425–438.
- Visser S, Yang X. LATS tumor suppressor: a new governor of cellular homeostasis. *Cell Cycle* 2010; **9**: 3892–3903.
- Kostic C, Shaw PH. Isolation and characterization of sixteen novel p53 response genes. *Oncogene* 2000; **19**: 3978–3987.
- Aylon Y, Michael D, Shmueli A, Yabuta N, Nojima H, Oren M. A positive feedback loop between the p53 and Lats2 tumor suppressors prevents tetraploidization. *Genes Dev* 2006; **20**: 2687–2700.
- Aylon Y, Ofir-Rosenfeld Y, Yabuta N, Lapi E, Nojima H, Lu X *et al*. The Lats2 tumor suppressor augments p53-mediated apoptosis by promoting the nuclear proapoptotic function of ASPP1. *Genes Dev* 2010; **24**: 2420–2429.
- McPherson JP, Tamblin L, Elia A, Migon E, Shehabeldin A, Matysiak-Zablocki E *et al*. Lats2/Kpm is required for embryonic development, proliferation control and genomic integrity. *EMBO J* 2004; **23**: 3677–3688.
- Yabuta N, Okada N, Ito A, Hosomi T, Nishihara S, Sasayama Y *et al*. Lats2 is an essential mitotic regulator required for the coordination of cell division. *J Biol Chem* 2007; **282**: 19259–19271.
- Murry CE, Keller G. Differentiation of embryonic stem cells to clinically relevant populations: Lessons from embryonic development. *Cell* 2008; **132**: 661–680.
- Shi G, Jin Y. Role of Oct4 in maintaining and regaining stem cell pluripotency. *Stem Cell Res Ther* 2010; **1**: 39.
- Thomson M, Liu SJ, Zou LN, Smith Z, Meissner A, Ramanathan S. Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. *Cell* 2011; **145**: 875–889.
- Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA* 2000; **97**: 11307–11312.
- Heo J, Factor VM, Uren T, Takahama Y, Lee JS, Major M *et al*. Hepatic precursors derived from murine embryonic stem cells contribute to regeneration of injured liver. *Hepatology* 2006; **44**: 1478–1486.
- Pal R, Khanna A. Role of hepatocyte-like cells in the differentiation of cardiomyocytes from mouse embryonic stem cells. *Stem Cells Dev* 2005; **14**: 153–161.
- Meissner A. Epigenetic modifications in pluripotent and differentiated cells. *Nat Biotechnol* 2010; **28**: 1079–1088.
- Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J *et al*. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* 2006; **125**: 315–326.
- Avruch J, Zhou D, Fitamant J, Bardeesy N, Mou F, Barrufet LR. Protein kinases of the Hippo pathway: regulation and substrates. *Semin Cell Dev Biol* 2012; **23**: 770–784.
- Dong J, Feldmann G, Huang J, Wu S, Zhang N, Comerford SA *et al*. Elucidation of a universal size-control mechanism in Drosophila and mammals. *Cell* 2007; **130**: 1120–1133.
- Liu-Chittenden Y, Huang B, Shim JS, Chen Q, Lee SJ, Anders RA *et al*. Genetic and pharmacological disruption of the TEAD-YAP complex suppresses the oncogenic activity of YAP. *Genes Dev* 2012; **26**: 1300–1305.

35. Aylon Y, Yabuta N, Besserglick H, Buganim Y, Rotter V, Nojima H *et al*. Silencing of the Lats2 tumor suppressor overrides a p53-dependent oncogenic stress checkpoint and enables mutant H-Ras-driven cell transformation. *Oncogene* 2009; **28**: 4469–4479.
36. Lorthongpanich C, Messerschmidt DM, Chan SW, Hong W, Knowles BB, Solter D. Temporal reduction of LATS kinases in the early preimplantation embryo prevents ICM lineage differentiation. *Genes Dev* 2013; **27**: 1441–1446.
37. Bernstein BE, Mikkelsen TS, Xie XH, Kamal M, Huebert DJ, Cuff J *et al*. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* 2006; **125**: 315–326.
38. Azuara V, Perry P, Sauer S, Spivakov M, Jorgensen HF, John RM *et al*. Chromatin signatures of pluripotent cell lines. *Nat Cell Biol* 2006; **8**: 532–U189.
39. Roh TY, Cuddapah S, Cui KR, Zhao KJ. The genomic landscape of histone modifications in human T cells. *Proc Natl Acad Sci USA* 2006; **103**: 15782–15787.
40. Ohm JE, Baylin SB. Stem cell chromatin patterns: an instructive mechanism for DNA hypermethylation? *Cell Cycle* 2007; **6**: 1040–1043.
41. Chaffer CL, Marjanovic ND, Lee T, Bell G, Kleer CG, Reinhardt F *et al*. Poised chromatin at the ZEB1 promoter enables breast cancer cell plasticity and enhances tumorigenicity. *Cell* 2013; **154**: 61–74.
42. Cordenonsi M, Zanconato F, Azzolin L, Forcato M, Rosato A, Frasson C *et al*. The Hippo transducer TAZ confers cancer stem cell-related traits on breast cancer cells. *Cell* 2011; **147**: 759–772.
43. Tamm C, Bower N, Anneren C. Regulation of mouse embryonic stem cell self-renewal by a Yes-YAP-TEAD2 signaling pathway downstream of LIF. *J Cell Sci* 2011; **124**(Pt 7): 1136–1144.
44. Septer S, Edwards G, Gunewardena S, Wolfe A, Li H, Daniel J *et al*. Yes-associated protein is involved in proliferation and differentiation during postnatal liver development. *Am J Physiol Gastrointest Liver Physiol* 2012; **302**: G493–G503.
45. Yabuta N, Okada N, Ito A, Hosomi T, Nishihara S, Sasayama Y *et al*. Lats2 is an essential mitotic regulator required for the coordination of cell division. *J Biol Chem* 2007; **282**: 19259–19271.
46. McPherson JP, Tamblyn L, Elia A, Migon E, Shehabeldin A, Matysiak-Zablocki E *et al*. Lats2/Kpm is required for embryonic development, proliferation control and genomic integrity. *EMBO J* 2004; **23**: 3677–3688.
47. St John MAR, Tao WF, Fei XL, Fukumoto R, Carcangiu ML, Brownstein DG *et al*. Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. *Nat Genet* 1999; **21**: 182–186.
48. Jain AK, Allton K, Iacovino M, Mahen E, Milczarek RJ, Zwaka TP *et al*. p53 regulates cell cycle and microRNAs to promote differentiation of human embryonic stem cells. *PLoS Biol* 2012; **10**: 2.
49. Lin TX, Chao C, Saito S, Mazur SJ, Murphy ME, Appella E *et al*. P53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat Cell Biol* 2005; **7**: 165–U180.
50. Li MM, He YL, Dubois W, Wu XL, Shi JX, Huang J. Distinct regulatory mechanisms and functions for p53-activated and p53-repressed DNA damage response genes in embryonic stem cells. *Mol Cell* 2012; **46**: 30–42.
51. Tamm C, Bower N, Anneren C. Regulation of mouse embryonic stem cell self-renewal by a Yes-YAP-TEAD2 signaling pathway downstream of LIF. *J Cell Sci* 2011; **124**: 1136–1144.
52. Lian I, Kim J, Okazawa H, Zhao JG, Zhao B, Yu JD *et al*. The role of YAP transcription coactivator in regulating stem cell self-renewal and differentiation. *Gene Dev* 2010; **24**: 1106–1118.
53. Chia NY, Chan YS, Feng B, Lu XY, Orlov YL, Moreau D *et al*. A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity. *Nature* 2010; **468**: 316–U207.
54. Qin H, Blaschke K, Wei G, Ohi Y, Blouin L, Qi ZX *et al*. Transcriptional analysis of pluripotency reveals the Hippo pathway as a barrier to reprogramming. *Hum Mol Genet* 2012; **21**: 2054–2067.
55. Varelas X, Sakuma R, Samavarchi-Tehrani P, Peerani R, Rao BM, Dembowy J *et al*. TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal. *Nat Cell Biol* 2008; **10**: 837–848.
56. Qin H, Yu T, Qing T, Liu Y, Zhao Y, Cai J *et al*. Regulation of apoptosis and differentiation by p53 in human embryonic stem cells. *J Biol Chem* 2007; **282**: 5842–5852.
57. Lin T, Chao C, Saito S, Mazur SJ, Murphy ME, Appella E *et al*. p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat Cell Biol* 2005; **7**: 165–171.
58. Armesilla-Diaz A, Bragado P, Del Valle I, Cuevas E, Lazaro I, Martin C *et al*. p53 regulates the self-renewal and differentiation of neural precursors. *Neuroscience* 2009; **158**: 1378–1389.
59. An Y, Kang Q, Zhao Y, Hu X, Li N. Lats2 modulates adipocyte proliferation and differentiation via Hippo signaling. *PLoS One* 2013; **8**: e72042.
60. Jukam D, Xie B, Rister J, Terrell D, Charlton-Perkins M, Pistillo D *et al*. Opposite feedbacks in the Hippo pathway for growth control and neural fate. *Science* 2013; **342**: 1238016.

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