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Serine Threonine Kinase Receptor-Associated Protein Deficiency Impairs Mouse Embryonic Stem Cells Lineage Commitment Through CYP26A1-Mediated Retinoic Acid Homeostasis

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ABSTRACT

Retinoic acid (RA) signaling is essential for the differentiation of embryonic stem cells (ESCs) and vertebrate development. RA biosynthesis and metabolism are controlled by a series of enzymes, but the molecular regulators of these enzymes remain largely obscure. In this study, we investigated the functional role of the WD-domain protein STRAP (serine threonine kinase receptor-associated protein) in the pluripotency and lineage commitment of murine ESCs. We generated *Strap* knockout (KO) mouse ESCs and subjected them to spontaneous differentiation. We observed that, despite the unchanged characteristics of ESCs, *Strap* KO ESCs exhibited defects for lineage differentiation. Signature gene expression analyses revealed that *Strap* deletion attenuated intracellular RA signaling in embryoid bodies (EBs), and exogenous RA significantly rescued this deficiency. Moreover, loss of *Strap* selectively induced *Cyp26A1* expression in mouse EBs, suggesting a potential role of STRAP in RA signaling. Mechanistically, we identified putative Krüppel-like factor 9 (KLF9) binding motifs to be critical in the enhancement of non-canonical RA-induced transactivation of *Cyp26A1*. Increased KLF9 expression in the absence of STRAP is partially responsible for *Cyp26A1* induction. Interestingly, STRAP knockdown in *Xenopus* embryos influenced anterior-posterior neural patterning and impaired the body axis and eye development during early *Xenopus* embryogenesis. Taken together, our study reveals an intrinsic role for STRAP in the regulation of RA signaling and provides new molecular insights for ESC fate determination. STEM CELLS *2018;36:1368–1379*

SIGNIFICANCE STATEMENT

Embryonic stem cells (ESCs) have an unlimited potential to self-renew and commit differentiation. Differentiation involves a fine-tune cascade of events promoted temporally and spatially by the multiple signal pathways. The findings of this study implicate that serine threonine kinase receptor-associated protein (STRAP) is essential for the mouse ESC lineages differentiation and early *Xenopus* embryo development. STRAP negatively regulates *Cyp26A1* transcript and loss of STRAP results in an accumulated level of CYP26A1 protein and subsequently interrupting the retinoic acid homeostasis. This provides a novel mechanistic basis of how RA signaling effectively induces ESC differentiation via STRAP.

INTRODUCTION

Retinoic acid (RA) synthesis from retinol (Vitamin A) follows a two-step enzymatic process. The first step is oxidation of retinol to retinaldehyde and is mainly controlled by the alcohol dehydrogenase (ADH) and retinol dehydrogenase (RDH) families [1–3]. The second step is controlled by members of the retinaldehyde dehydrogenase (RALDH) family that oxidize retinaldehyde to RA [4]. Cellular RA levels are degraded by cytochrome p450 family 26 (CYP26) enzymes that are responsible for the metabolism of active all-trans-RA to inactive polar metabolites [5, 6]. It has been reported that loss of CYP26 enzymes in zebrafish and mice results in severe phenotypes with caudal truncations, homeotic transformations, and embryonic lethality [7, 8].

Retinoid signals are required for the acquisition of germ layer subset specification in mouse ESCs [9]. RA promotes neural differentiation and plays diverse roles in regulating mesoderm commitment and hematopoietic

development in embryonic stem cells [10–12]. RA regulates the commitment of ES cells to form Pdx1+ pancreatic endoderm [13]. CYP26A1 activity regulates intracellular RA levels and transcriptional regulation of primary RA target genes during ES cell differentiation [14]. Conversely, RA itself regulates *Cyp26A1.* The proximal region of the murine *Cyp26A1* promoter contains an atypical G-rich element for regulating RA-induced expression [15, 16]. Besides the induced expression by exogenous RA, the regulation of *Cyp26A1* in ESC differentiation remains to be elucidated.

We have reported the identification of a novel WD40 domain-containing protein STRAP (serine threonine kinase receptor-associated protein), which is highly expressed in several human cancers and plays roles in promoting tumorigenicity as well as maintaining stemness of cancer-like cells [17, 18]. In this study, we found that *Strap* gene deletion negatively affects stem cell lineage commitment by attenuating RA signaling. Moreover, we found this reduced signaling is mediated, at least in part, by accumulated *Cyp26A1* expression, resulting in a potential increased RA metabolism. Mechanistically, we have shown that Krüppel-like factor 9 (KLF9) enhances *Cyp26A1* promoter activity in a non-RA-stimulated manner, which might account for the high *Cyp26A1* expression in *Strap* KO EBs. Interestingly, STRAP is also required for body axis and eye development as well as anterior-posterior neural patterning in early *Xenopus* embryogenesis. Overall, our work reveals a new role of STRAP as a novel regulator of ESC differentiation.

Materials and Methods

Generation of *Strap*+/– Mice

Vectors with zinc finger nucleases (ZFNs) targeting the *Strap* gene were electroporated into V6.5 ESCs. *Strap*+/– ESCs were then injected into C57BL/6 blastocysts to generate chimeric mice. The chimeras were backcrossed with C57BL/6 mice, and the *Strap*+/– offspring were subsequently intercrossed for ESC derivation. Primer sequences are included in Supporting Information. All animal experiments were carried out according to the guidelines for the care and use of laboratory animals of the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC).

Cell Culture

ESCs were cultured on irradiated mouse embryonic fibroblasts (MEFs) in ESC medium containing Knockout DMEM medium (Gibco, Grand Island, NY), 15% murine ESC defined FBS (Thermo Scientific, Waltham, MA), L-glutamine (Gibco), sodium pyruvate (Gibco), nonessential amino acids (Gibco), ß-mercaptoethanol (Gibco,), and mLIF (Millipore, Burlington, MA). Mouse ES-E14TG2a (E14) cells were kindly provided by Dr. Hao Jiang (University of Alabama at Birmingham). Strap+/+ and Strap–/– MEF cells were kindly given by Dr. Philippe Soriano (Icahn School of Medicine at Mount Sinai, New York). These cells originally isolated from wild-type(+/+) and homozygous null (–/–) ROSA71-*Strap* embryos at E9.5 [19].

EB Formation and Chemical Reagent Treatment

EBs were formed in EB medium (ESC medium without mLIF) in hanging drops for 48 hours. EBs were then cultured in 6-well ultra-low attachment plates. In some experiments, EBs were maintained in EB medium plus either Vitamin A (Sigma, St. Louis, MO) or RA (Sigma). To inhibit CYP26A1 function, the CYP26-inhibitor liarozole (50 μM; Santa Cruz Biotechnology, Dallas, TX), was added to the EB medium.

AP Staining

Alkaline phosphatase (AP) activity was measured using a commercial detection kit (Sigma). Images were captured using an Olympus microscope.

Flow Cytometry Analysis

Cells were collected at time points and fixed. The cell sediment was collected, incubated with RNase A (Sigma), and stained with propidium iodide (Sigma). Cell cycle distribution was evaluated using flow cytometry. To measure differentiation markers, CD133-PE (MACS, San Diego, CA), Gata4–488 (BD Biosciences, Billerica, MA), and Brachyury-PE (R&D Systems, Minneapolis, MN) antibodies were used to detect positive cells.

Immunofluorescence Staining

EBs were grown in a 12-well plate and were later fixed, permeabilized, and incubated with a rabbit anti-tubulin antibody (Cell Signaling Technology, Danvers, MA) followed by goat anti-rabbit Alexa Fluor488 antibody (Life Technologies, Carlsbad, CA). Images were captured using an Olympus microscope.

RNA Purification and quantitative PCR (qPCR) Assay

Total cellular RNA was extracted with TRIzol reagent (Life Technologies,). The reverse-transcribed products were used as the template for quantitative PCR. Primer sequences are included in the Supporting Information.

Microarray Analysis

Microarray was performed using the GeneChip 430 Mouse 2.0 Array from Affymetrix [20]. To define differentially expressed genes, we used a statistical threshold of log2FC >1 or <–1 and FDR-adjusted $p < .05$ to generate gene lists. The filtered data were computed by Gene Set Enrichment Analysis (GSEA) software (http://www.broad.mit.edu/gsea). The degree of enrichment is indicated by a normalized enrichment score (NES).

Western Blotting

Whole-cell lysates were prepared in RIPA buffer. Proteins were separated by 10% SDS/PAGE and probed with primary antibodies. Primary antibodies included: OCT4 c-Myc, NANOG (Cell Signaling Technology); STRAP (BD Transduction Labs); KLF9 (ThermoFisher); Sp1 and CYP26A1 (Santa Cruz Biotechnology); β-Actin (Sigma, A5316).

Cloning of the Murine *Cyp26A1* Promoter and Luciferase Assay

Genomic DNA was isolated from a C57BL/6 mouse. Based upon the location of the transcriptional start site, a 1,756-bp fragment from −1,636 to +120 in the 5′-flanking region of the *Cyp26A1* gene was generated by PCR. The PCR products for full-length and deleted fragments were digested with KpnI and SmaI, and subcloned into the pGL3-basic luciferase vector (Promega, Madison, WI). The Sp1 expression construct was a gift from Dr. Hans Rotheneder (University of Vienna, Austria). The KLF9 expression plasmid was kindly provided by Dr. Robert

Denver (University of Michigan, USA). Luciferase constructs with respective expression plasmids were transfected into cells. A β-galactosidase (β-Gal) plasmid was transfected to serve as an internal control.

Chromatin Immunoprecipitation Assay

Purification of sonicated nuclear lysates and immunoprecipitation (IP) were performed using an EZ-chromatin immunoprecipitation (ChIP) assay kit (Upstate Biotechnology, Lake Placid, NY). Sonicated DNA fragments were immunoprecipitated with the specific antibody. IgG was used as a negative control for IP. The DNA/protein complex was eluted using elution buffer and reversibly crosslinked.

Lentiviral Transduction

E14 ES cells were infected with STRAP or vector control lentivirus. The cells were then selected with puromycin. Stable STRAP knockdown cells were confirmed for STRAP expression by Western blotting.

Xenopus laevis

Xenopus embryos were obtained as described previously [21]. An antisense morpholino oligonucleotide (MO) was designed using Gene Tools and has the sequence: CCACTAGCGAGGGCTTCATGTCAAT. For in situ hybridization, 25 ng of the MO was co-injected with 0.2 ng RNA encoding the tracer β-Gal into the two-cell–stage embryos. The embryos were stained with the β-Gal substrate Red-Gal. The digoxygenin-labeled in situ probes were synthesized from the linearized pBSKS vector containing the indicated genes, and 0.5 μg/ml of the probes was used for in situ hybridization [22]. All animal protocols were approved by the University of Alabama Institutional Animal Care and Use Committee.

Statistical Analysis

All values were derived from at least three independent experiments and expressed as means ± s.d. Significant differences in mean values were evaluated using the Student's *t* test. In GSEA analysis Kolmogorov-Smirnov (K-S) statistics was used to test whether genes are "enriched" in the gene-set for the same phenotype.

Results

Strap Is Dispensable for Mouse ESCs Viability and Identity

STRAP had been reported to be essential for early mouse embryogenesis, as *Strap*-null embryos die at E9.5–10.5 (our unpublished data, 19). The lethality may be caused by defects in embryonic stem cell division, maintenance, or differentiation. To determine whether STRAP is required normal ESCs character, we first generated *Strap* heterozygous mice using ZFN technology [23, 24]. A pair of ZFNs specifically targeting introns 2 and 4 of the *Strap* locus were delivered into normal ECSs. Double stranded breaks were expected to occur at these two ZFN cleavage sites that resulted in deletion of exons 3 and 4 and no functional protein (Fig. 1A). The identity of *Strap* +/− ESC clones with targeted deletion was verified by DNA sequencing (Supporting Information Fig. S1). The confirmed *Strap* +/− ESCs were then injected into mouse blastocysts to generate heterozygous *Strap* KO mice. Intercrossing of *Strap* +/− mice resulted in no viable *Strap*−/− mice, indicating that removal of *Strap* led to embryonic lethality.

To derive wild-type (WT) and *Strap* KO ESCs, we cultured preimplantation blastocysts (E3.5) in ESC medium (Fig. 1B). Out of 27 ESC lines obtained by this method, 6 were *Strap* +/+, 13 were *Strap* +/−, and 8 were *Strap* −/− (Fig. 1C). Knockout ESCs exhibited normal morphology (Fig. 1B), cell cycle distribution (Fig. 1D), and growth rates (Fig. 1E) similar to the WT ESCs. Importantly, both WT and KO ESCs can be marked by alkaline phosphatase (AP)-positive colonies (Fig. 1F), indicating that loss of *Strap* has little effect on self-renewal potential of the undifferentiated embryonic stem cells.

To confirm the characteristics of the ESCs, we next determined the levels of several pluripotency markers, including OCT4, c-Myc and NANOG. Both the mRNA transcripts (data not shown) and the proteins of these genes were expressed at similar levels in WT and *Strap*-depleted ESCs (Fig. 1G). Taken together, our results suggest that *Strap* is not required for mouse ESC viability and identity.

Strap-Deficient ESCs Are Defective in Lineages Differentiation

Since deletion of *Strap* does not affect ESC proliferation or maintenance, the lethality of *Strap* knockout mice is probably caused by defects in ESC differentiation. To test this hypothesis, we examined cell differentiation in EBs derived from ESCs. Withdrawal of leukemia inhibitory factor (LIF) from the culture medium is the most frequently used method to induce EBs. Cells in such EBs consistently and spontaneously differentiate into lineages of all three embryonic germ layers [25–27].

At day 6 of differentiation, the number, size, and morphology of EBs derived from WT and *Strap* KO ESCs appear similar, indicating that *Strap* knockout does not affect early EB formation (Fig. 2A and Supporting Information Fig. S2A). Subsequently, WT EBs developed increasingly non-uniform shape and distinct regions with different cell density and compaction, implying formation of heterogeneous cell populations in the EBs (Fig. 2A, left panel). In contrast, *Strap* KO EBs maintained homogeneous cell population in size and shape (Fig. 2A, right panel), and the increase in EB diameter was also slowed drastically when compared with the WT EBs (Supporting Information Fig. S2A). Interestingly, the percentage of apoptotic cells in KO EBs is similar to that of WT EBs (data not shown), indicating that *Strap* knockout does not affect programmed cell death. Since cell cycle progression constitutes one of the critical rate-limiting steps during early ESC differentiation [28], we analyzed cell cycle of KO EBs. Despite reduced growth of EB at Day 10, the percentage of cells in each cell cycle phases remained similar in both WT and *Strap* KO EBs, indicating that no cell cycle transition defects occurred in *Strap* KO EBs (Fig. 2B). These results suggest that the absence of *Strap* does not influence cell cycle progression, but may result in defective cell differentiation programming.

To investigate whether the alteration of EB morphology truly reflects defects in cell differentiation, we assayed for the effect of depletion of *Strap* on the expression of lineage-specific genes directly. We cultured EBs over a 12-day period of differentiation and measured gene expression at four time

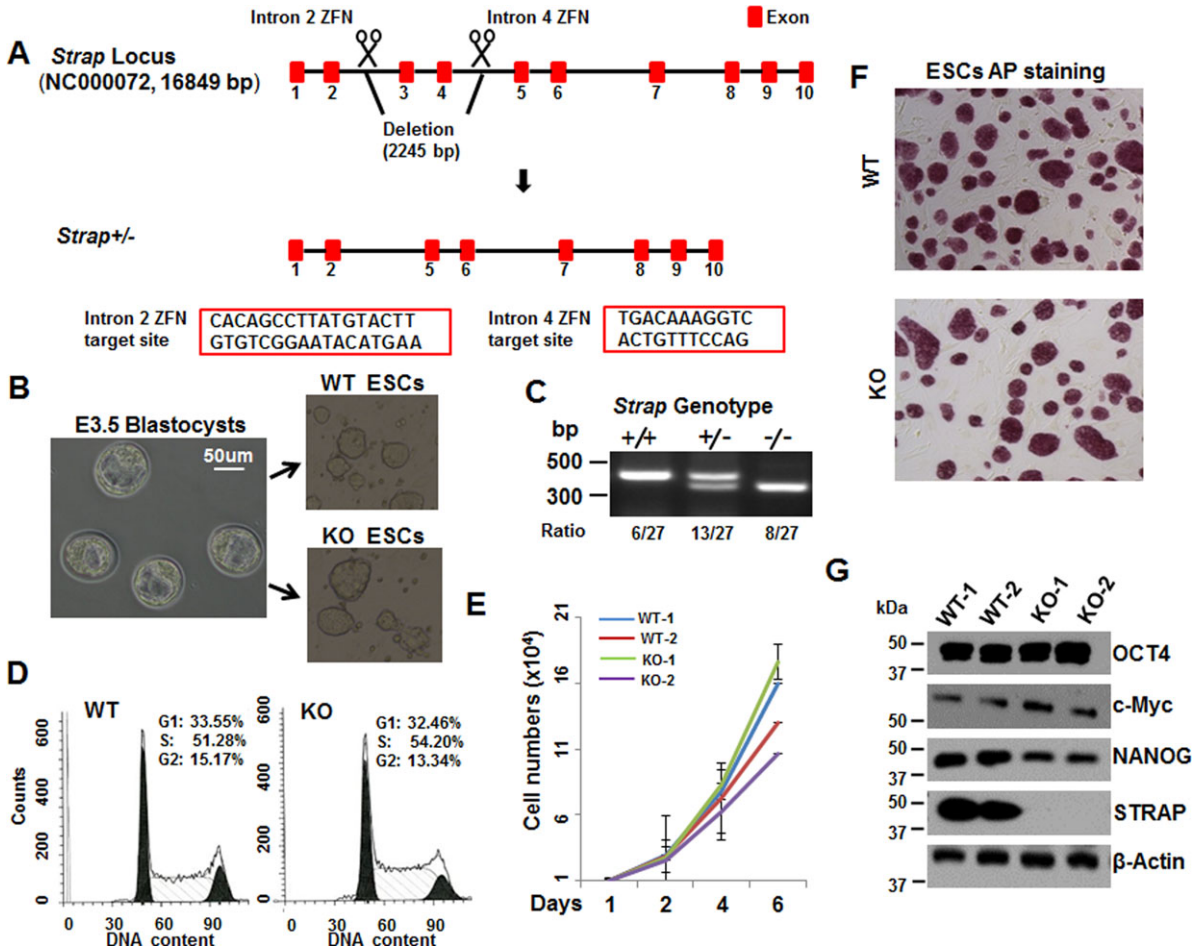

Figure 1. *Strap* depletion has no effect on the morphology or viability of ESCs. **(A)**: Schematic representation of the strategy used to generate *Strap* heterozygous mice. The *Strap* locus (from exons 1 to 10) is shown. Exons are shown as filled boxes and cutting positions of the two ZFNs are indicated. Details of targeted sequences are shown in boxes. **(B)**: Left panel, phase contrast image showing the morphology of isolated E3.5 blastocysts from *Strap*+/– intercrossings. Right panel, representative images of blastocyst-derived *Strap* WT and KO ESCs cultured on mouse embryonic fibroblasts. Image magnification ×40; scale bar = 50 μm. **(C)**: PCR genotyping of ESCs established from various blastocysts. **(D)**: Fluorescence-activated cell sorting analysis of WT and KO ESC lines. The percentages of cell populations at each cell cycle phase are displayed. **(E)**: Growth curve of two independent WT and KO ESC lines. A total of 1×10^4 ESCs were seeded in 12-well plates and cell numbers were counted every other day. **(F)**: Alkaline phosphatase staining of WT and KO ESC cells. **(G)**: Western blots of pluripotent markers and STRAP in the indicated ESCs. β-Actin was used as a loading control. Abbreviations: AP, alkaline phosphatase; ESCs, embryonic stem cells; KO, knockout; WT, wild-type; ZFNs, zinc finger nucleases

points that reflect: (a) ground-state ESCs (D0), (b) multipotent progenitors (D4), (c) lineage-associated progenitors (D6–8), and (d) differentiated cell lineages (D12) [29]. qPCR analyses indicates that expression of the pluripotent genes *Oct4*, *Nanog* and *Sox2* was reduced gradually to undetectable levels at D12 in either EBs (Supporting Information Fig. S2B). As expected, lineage-specific markers for ectoderm (*Nestin*, *Igf2*, *Pax6,* and *Vimentin*), mesoderm (*Twist*, *Pax3*, *Mef2c,* and *MyoD*), and endoderm (*Gata4*, *Gata6,* and *Sox17*) were increasingly expressed in WT EBs over the incubation period. In contrast, *Strap* KO EBs expressed these lineage-specific markers at low levels at all the time points tested (Fig. 2C–2E). These data demonstrate that *Strap* regulates lineage commitment during EB differentiation without affecting functional pluripotent genes. *Strap* may therefore participate in specific aspects of EB differentiation. We next estimated the percentage of germ layer cells in both WT and KO EBs. Single cells dissociated from stage-defined EBs were immunostained with antibodies against CD133 (ectoderm), Gata-4 (endoderm) and Brachyury (mesoderm) and then subjected to flow cytometry analysis. There was no autonomous differentiation in ESCs (data not shown) and percentages of the marker-labeled cells were significantly lower in KO-EBs than those in WT EBs at the indicated time points (Supporting Information Fig. S2C), indicating that STRAP deletion led to defects of all three lineages commitment.

Retinoic Acid Signaling Is Impaired in Ectoderm and Endoderm Committed *Strap*-KO ESCs

Embryonic cell differentiation is regulated by multiple signaling pathways. GSEA transcriptomics analyses on our MEFs microarray data indicate that the pathway with the highest NES is RA signaling (Fig. 3A, upper panel; NES 2.122, $p < .001$) compared with other multiple pathways with a significantly positive NES, including TGF-beta Signaling (NES 1.927, $p < .001$), Growth Factor Signaling (NES 1.588, $p < .001$), Wnt Signaling (NES 1.491, $p = .002$), and Cell Adhesion Signaling (NES 1.280,

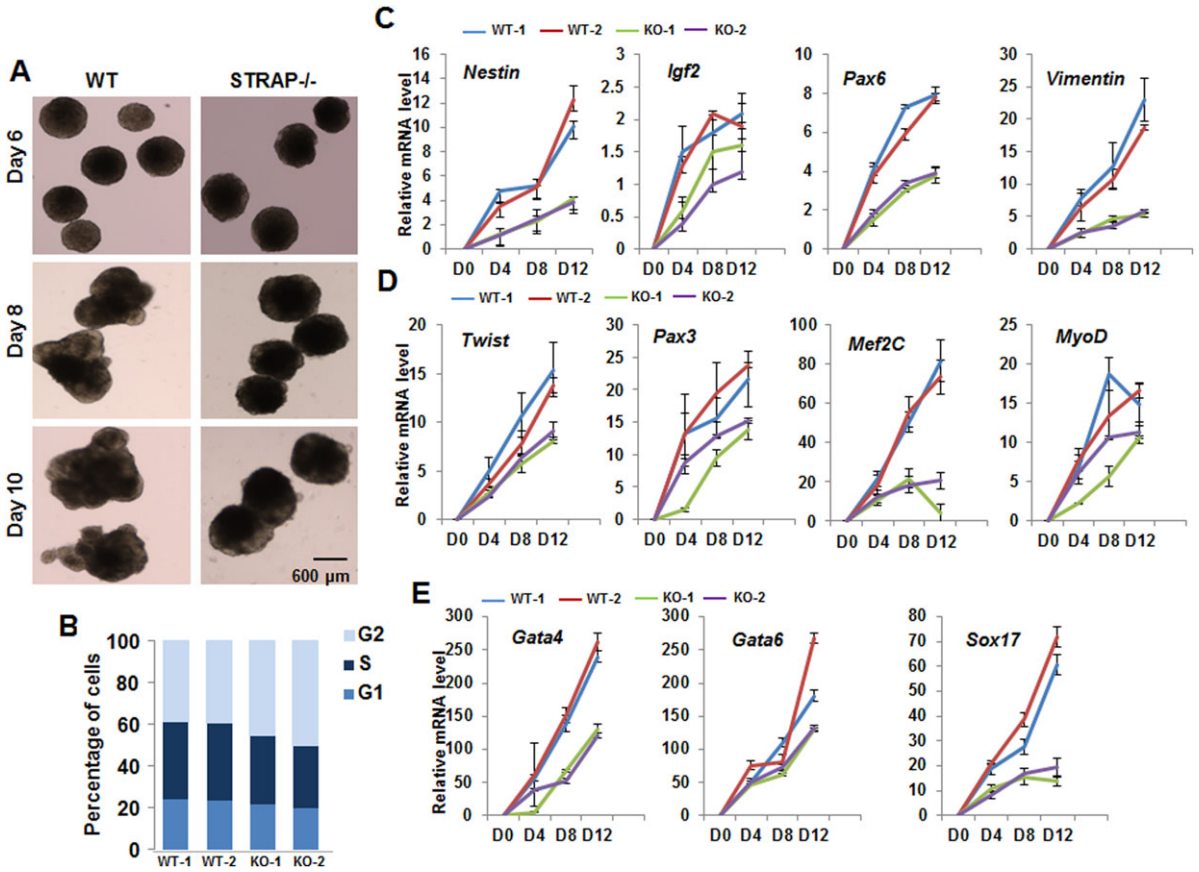

Figure 2. *Strap*-deficient embryoid bodies (EBs) are unable to differentiate into primary germ layers. **(A)**: Phase-contrast images of EB morphology during EB formation from *Strap* WT and KO embryonic stem cells (ESCs) at the indicated time points. Scale bar = 600 μm. **(B)**: The cell cycle distribution of D10 EBs from WT and KO ESC lines was analyzed by fluorescence-activated cell sorting. The percentages of cell populations at each cell cycle phase are labeled. **(C–E)**: qPCR analyses of lineage-specific gene' expression at Day 0, Day 4, Day 8, and Day 12 during EB formation of two independent WT and KO ESC lines. Error bars represent mean ± s.d. Each experiment was replicated at least three times. Abbreviations: KO, knockout; STRAP, serine threonine kinase receptor-associated protein; WT, wild-type.

p = .014). Many target genes of the RA signaling pathway as well as their activating components, such as RA receptors (*Rarα* and *Rarβ*), retinoid X receptors (*Rxrα* and *Rxrβ*), CREB binding protein (*Crebp*), and retinol-binding protein 1 (*Rbp-1*) were upregulated significantly in *Strap* KO MEFs. Nevertheless, the repressors of this pathway including the nuclear receptor-co-repressor-2 (*Ncor-2*) and Histone deacetylase 1 (*Hdac1*) are mainly downregulated (Fig. 3A, upper panel), and the canonical RA metabolic genes are not appreciably affected by genetic inactivation of *Strap* (Fig. 3A, bottom panel).

Based on our MEFs data, we explored the possibility that STRAP may regulate RA signaling during lineage-commitment of ESCs. To test this, we harvested EBs at 4-, 8-, and 10-days following incubation under differentiation conditions and performed qPCR analysis of a set of RA signature genes, including *Rarα, Rarβ, Crabp1, Hoxa10, Hoxb1, Stra6,* and *Stra8* [9, 30–32]. In contrast to the cultured MEFs (Supporting Information Fig. S3A), *Rarα, Rarβ, Crabp1*, and *Hoxb1* were significantly reduced in *Strap* KO EBs after LIF removal especially at Days 8–10, when compared with WT EBs (Fig. 3B, Supporting Information Fig. S3A). Since these genes are directly regulated by RA signaling at the transcription level, these results suggest that RA signaling is attenuated in *Strap* KO EBs during differentiation. In comparison, there is little change in the expressions of *Hoxa10* and *Stra8* in both WT and KO EBs, indicating the specificity of the effect of *Strap* on RA-regulated genes (Fig. 3B, Supporting Information Fig. S3A).

To verify that compromised RA signaling is indeed the key factor in impeding the expression of the above RA-responsive genes in *Strap* KO EBs, we performed the rescue assays by adding either 100 nM of Vitamin A (a precursor of RA) or 100 nM of all-trans RA to WT and *Strap* KO EBs at Day 6 and Day 8 under differentiation conditions. *Strap*-affected genes (*Rarα, Rarβ, Crabp1*, and *Hoxb1*) were analyzed by qPCR analysis. While treatment with DMSO had little effect on gene expression in WT or *Strap* KO EBs (Fig. 3C, Supporting Information Fig. S3C), exposure to Vitamin A or all-trans RA dramatically increased the levels of *Rarα, Rarβ, Hoxb1,* and *Crabp1* transcripts at day 8 in both WT and *Strap* KO EBs. Notably, the expression of these genes, with the exception of *Crabp1*, was rescued in *Strap* KO EBs to a similar level as their WT counterparts (Fig. 3C).

To further examine whether *Strap* deficiency affects RA signaling-induced biological function, we cultured EBs in floating conditions in the absence of LIF for 4 days. Then, these EBs were plated into 12-well plates for additional 8 days with the treatment of all-trans RA (100 nM) every

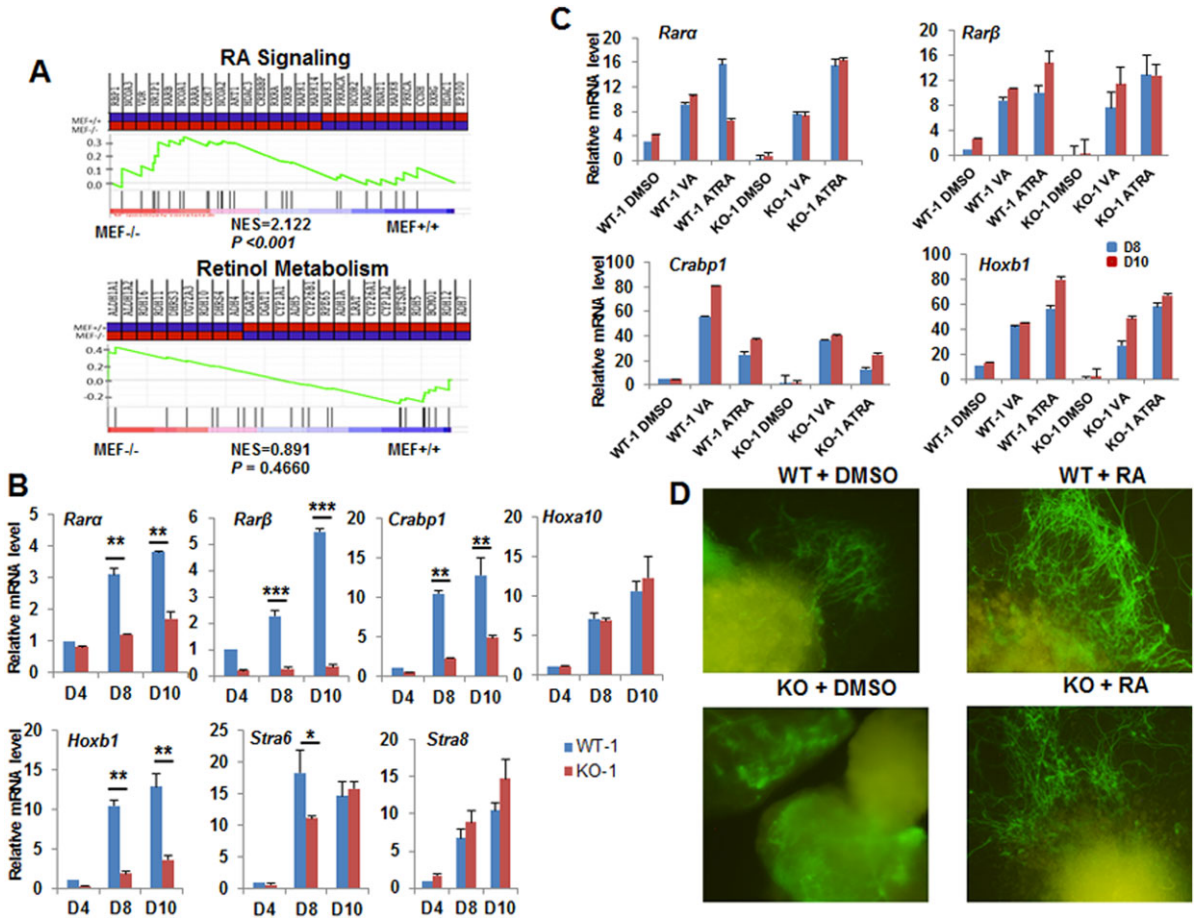

Figure 3. Endogenous RA signaling is inactivated during differentiation of *Strap* KO ESCs. **(A)**: Gene set enrichment analysis (GSEA) using gene expression profiles from *Strap* +/+ and *Strap* −/− MEFs. Representative gene expression patterns predicted RA signaling components were enriched in *Strap*−/− MEFs compared with Strap+/+ MEFs. **(B)**: RNA was extracted from EBs cultured without leukemia inhibitory factor (LIF) for various time points, and the transcript levels of RA-responsive genes were assessed by RT-qPCR. Bars represent mean ± s.d., $n = 3$, *, $p < .05$; **, $p, < .01$; ***, $p, < .005$, when compared with the corresponding control. **(C)**: RT-qPCR analyses of RA-specific gene expression at Day 8 and Day 10 during EB formation with VA or ATRA. DMSO was used as vehicle control. No significance between the two groups was observed. Bars represent mean ± s.d., $n = 3$. **(D)**: Free-floating EBs formed after 4 days of suspension culture with removal of LIF. Individual EBs were seeded into 12-well plates with either RA (100 μM) or DMSO. 12-old-day EBs were fixed and stained for anti-β-tubulin III (green) to illustrate neuronal growth. Image magnification ×40. Abbreviations: ATRA, all-trans retinoid acid; KO, knockout; MEFs, mouse embryonic fibroblasts; NES, normalized enrichment score; RA, retinoic acid; VA, vitamin A; WT, wild-type.

2 days. Immunofluorescence staining of the pan-neuronal marker β-tubulin III was performed at day 12 [33]. A low density of β-tubulin III-positive neuronal fibers was observed in WT EBs even without RA treatment, reflecting autonomous self-differentiation of neurons in a small percentage of cells in the WT EBs (Fig. 3D, left upper panel). Exposure of the EBs to RA induced an extensive network of β-tubulin III positive neurites extending from the central cell aggregates (Fig. 3D, right upper panel). Unlike WT EBs, *Strap* KO EBs did not harbor neurites in the absence of RA (Fig. 3D, left bottom panel). However, they responded to RA to extend neurites, though the density of neurites was less compared with that in the WT EBs (Fig. 3D, right bottom panel). These results imply that *Strap* is required for optimal response to RA signaling during neuronal differentiation in EBs.

In addition to neuronal cell differentiation, RA signals are also shown to regulate cell fate in other germ layers [9]. Therefore, we examined the expression of several germ layer markers by qPCR in EBs treated with RA for 6 days, starting at the day-4 in differentiation media. RA induced the expression of the ectodermal markers *Nestin* and *Pax6* and the endodermal markers *Gata4* and *Sox17* (Supporting Information Fig. S4A), but did not enhance the mesodermal markers *Pax3* and *Mef2C* (data not shown). The results were consistent with the finding that RA favors ectodermal and endodermal differentiation lineages [33, 34]. When comparing the WT and the *Strap* KO EBs, we observed that both responded to RA in similar ways, resulting in similar patterns and levels of marker expression (Supporting Information Fig. S4A). Taken together, our data indicate that STRAP is involved in the RA-mediated differentiation and *Strap* KO ESCs fail to commit to ectodermal and endodermal lineages mainly due to inactivated RA signaling. Since the *Strap* KO EBs responded to exogenous RA, it suggests that *Strap* KO impairs RA signaling probably through effects on the production of RA.

Enhanced Expression of CYP26A1 Contributes to the Reduction in Endogenous RA Signaling in *Strap* KO EBs

To test whether RA metabolic enzymes are being regulated by STRAP during development, we performed RT-qPCR using total RNAs extracted from 10-day-old cultured EBs. We did not observe appreciable changes in the transcripts of enzymes for RA biosynthesis, including retinol dehydrogenase 10 (*Rdh10*), aldehyde dehydrogenase 2 (*Raldh2*), and cellular RA binding protein 2 (*Crabp2*) in *Strap* KO EBs compared with those in WT EBs (Fig. 4A). Unlike reduced *Rarα* and *Rarβ* in *Strap* KO EBs (Fig. 3B), another RA receptor, *Rbp4* was not affected by *Strap* depletion (Fig. 4A). Interestingly, *Cyp26A1*, but not its paralog gene *Cyp26B1*, was strikingly enhanced in *Strap* KO EBs when compared with the corresponding WT EBs (Fig. 4A), implying that *Cyp26A1* may be a specific target of STRAP. Several other genes including retinol dehydrogenase 1 (*Rdh1*), retinaldehyde dehydrogenase 1 (*Raldh1*), and *Cyp26C1* were not detected by qPCR assays (data not shown). This suggests that different RA metabolic enzymes are selectively expressed during EB differentiation.

To determine the increase in CYP26A1 expression in *Strap*-KO EBs during differentiation, we analyzed the expression of *Cyp26A1* at different stages of EB differentiation. The level of *Cyp26A1* mRNA was low at day 6 in WT EBs and did not increase much at day 8 and 10. The *Strap* KO EBs consistently showed a much higher level of *Cyp26A1* mRNA from day 6 onward (Fig. 4B). We next measured dynamic patterns of CYP26A1 protein in the progress of differentiation by Western blot assays. We observed that CYP26A1 expression in WT EBs was detectable from day 6 and continuously expressed until D12 (Supporting Information Fig. S4B and Fig. 4C). Interestingly, downregulation of STRAP correlated with induced levels of CYP26A1 protein in EBs from day 8 of differentiation (Supporting Information Fig. S4B and Fig. 4C). To verify this observation in other cell models, we first established lentivirus-mediated stable shCtrl and shSTRAP E14 cells. The efficiency of STRAP knockdown in shSTRAP cells was greater than 80% compared with shCtrl cells (Fig. 4D) and reduced expression of STRAP was steadily maintained in KO EBs (Fig. 4E). We next cultured control and STRAP-KD EBs under the differentiation condition over a period of time. We observed that knockdown of STRAP in EBs induced the expression of CYP26A1 during the differentiation as compared with control EBs (Supporting Information Fig. S4C and Fig. 4E). Collectively, these data indicate that CYP26A1 could be a potential downstream target of STRAP and the absence of STRAP upregulates CYP26A1 through transcript level.

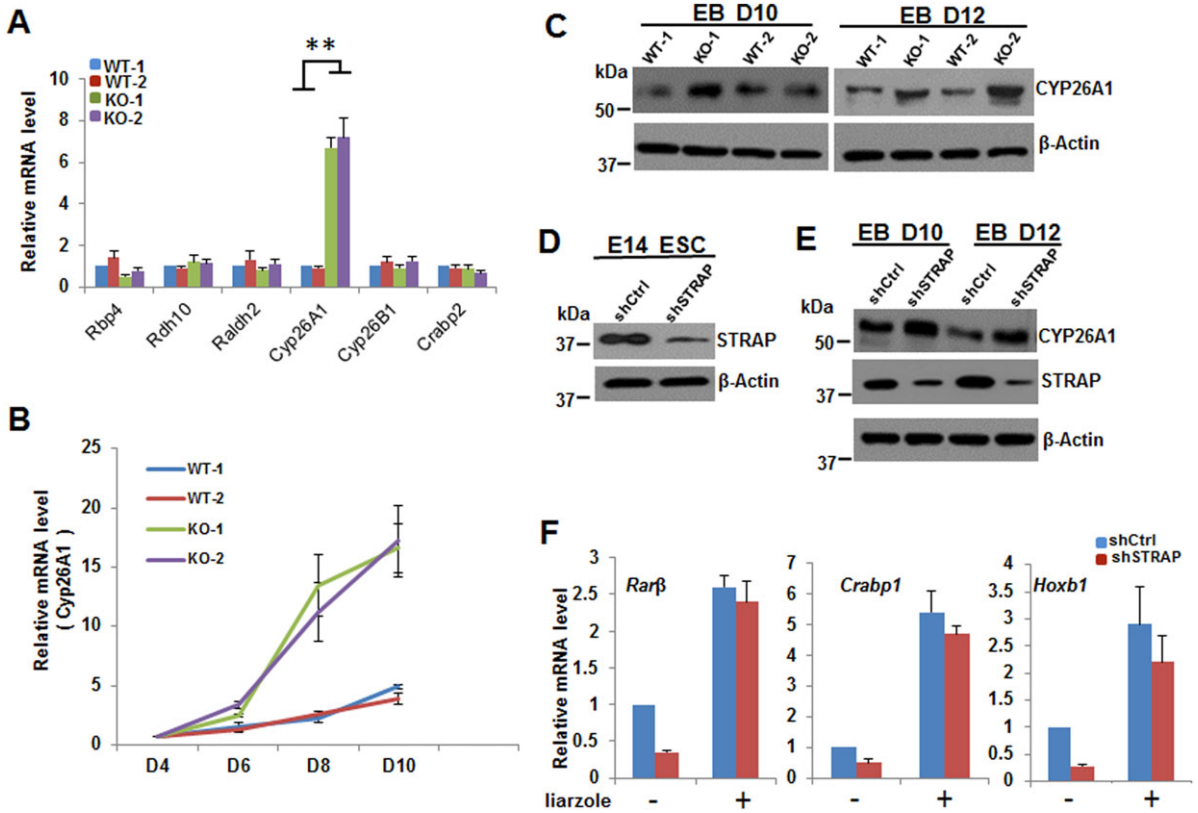

Figure 4. Loss of STRAP results in increased *CYP26A1* mRNA and protein levels during EB differentiation. **(A)**: RT-qPCR analyses of retinoic acid (RA) metabolic gene expression at Day 10 during EB formation. Bars represent mean ± s.d., $n = 3$, **, $p < .01$, when compared with the control. **(B)**: RT-qPCR analyses of *Cyp26A1* expression at Day 4, Day 6, Day 8, and Day 10 during EB formation. Each point represents mean ± s.d., $n = 3$. **(C)**: Western blot analyses of the level of CYP26A1 in the indicated EBs at Day 10 and Day 12. β-Actin was used as a loading control. **(D)**: Western blotting was used to determine the expression of STRAP protein in the control and STRAP knockdown E14 polyclonal cells. **(E)**: Levels of CYP26A1 and STRAP in EBs derived from shSTRAP and shCtrl clones (E14) were determined by Western blotting using β-Actin as a loading control. **(F)**: 8-day-old EBs were treated with CYP26A1 inhibitor, liarozole (50 μM), for 48 hours and total RNA from control and knockdown EBs was subjected to RT-qPCR analyses for detecting RA-targeted genes. No significance between the two groups was observed. Bars represent mean ± s.d., $n = 3$. Abbreviations: EBs, embryoid bodies; ESCs, embryonic stem cells; KO, knockout; STRAP, serine threonine kinase receptor-associated protein; WT, wild-type.

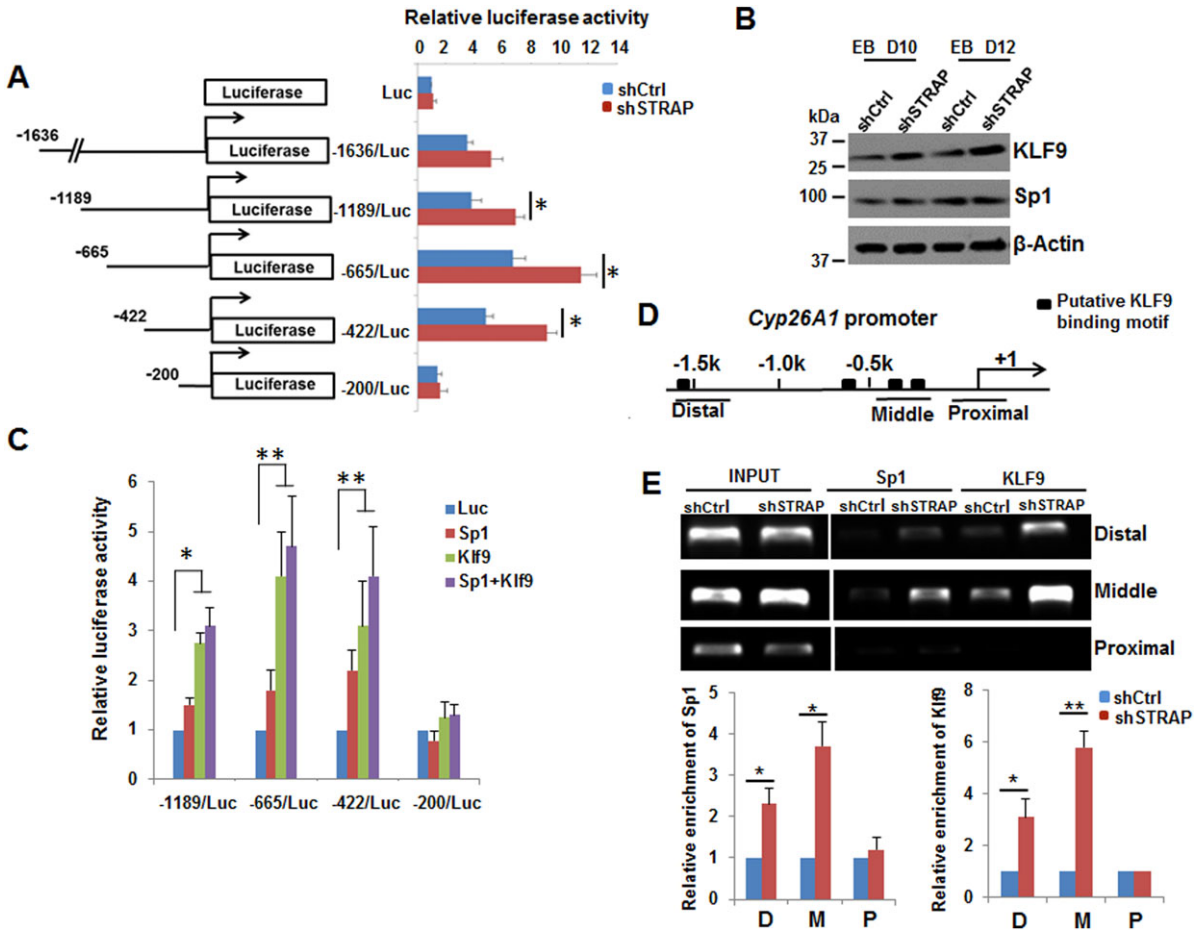

Figure 5. KLF9 and Sp1 coordinately regulate *Cyp26A1* transcription in STRAP-depleted EBs. **(A)**: Either E14 control or shSTRAP cells were transfected with the indicated *Cyp26A1* luciferase reporters. Luciferase activity was normalized to β-Gal activity and presented as mean ± s.d., $n = 3$, *, $p < .05$, when compared with the corresponding control. **(B)**: Western blots of total cell lysates from indicated EBs with STRAP knockdown were analyzed for KLF9 and Sp1 expression. **(C)**: E14 WT cells were co-transfected with the indicated *Cyp26A1* promoter reporter as well as Sp1 or KLF9 expression plasmids. Luciferase activity was normalized to β-Gal activity and presented as mean ± s.d., $n = 3$, *, $p < .05$; **, $p < .01$. **(D)**: The murine *Cyp26A1* promoter showing the positions of potential KLF9 binding sites (black boxes) and the transcription start site (TSS). Black lines indicate the amplified regions for ChIP primers. **(E)**: ChIP assays were performed from cells mentioned above using anti-KLF9 or anti-Sp1 antibody. PCR amplification was performed within the target region of the Cyp26A1 promoter. Results are expressed as percentages of immunoprecipitated DNA compared with total input DNA. *, $p < .05$; **, $p < .01$, when compared with the corresponding control. Abbreviations: EBs, embryoid bodies; KLF9, Krüppel-like factor 9; STRAP, serine threonine kinase receptor-associated protein.

If higher expression of CYP26A1 by Strap KO were indeed responsible for reduced RA signaling in EBs, we would expect that blocking CYP26A1 function would rescue the RA signaling. To test this hypothesis, we applied the CYP26 inhibitor liarozole (50 μM) [35] for 48 hours and analyzed levels of the RA-regulated genes *Rarβ, Crabp1,* and *Hoxb1.* While the expression of these genes were downregulated in the absence of STRAP, inhibition of CYP26 elevated the expression of these genes to the levels similar to that in WT EBs (Fig. 4F). Taken together, these results indicate that upregulation of CYP26A1 in the absence of STRAP leads to impaired RA signaling in the EBs, thereby preventing efficient lineage differentiation.

Krüppel-like Factor 9 and Sp1 Coordinate to Regulate *Cyp26A1* Transcription in EBs

Since we observed that knockout or shRNA-mediated downregulation of STRAP results in enhanced *Cyp26A1* mRNA levels, it is likely that STRAP regulates transcription of *Cyp26A1* during EB differentiation. Therefore, we constructed a set of luciferase-reporters containing either full-length (–1,636 to +120 bp) or a series of successive 5′ deletion of *Cyp26A1* promoter (Fig. 5A). Luciferase activities were measured in WT or *Strap* shRNA E14 cells transfected with these reporters. In control cells, STRAP-induced promoter activity was maximum with −665 bp construct. Moreover, in *Strap*-shRNA cells, increased luciferase activities were observed in the same set of reporters and activity was vanished when only −200 bp of promoter sequence was present (Fig. 5A). The data suggest that STRAP may control the availability of the transcription factors (TFs) or regulate the affinity of these factors to *cis*-regulatory element(s) in the promoter to influence *Cyp29A1* transcript levels.

To identify putative TFs that may be involved in the regulation of *Cyp26A1* expression, we used MatInspector online tool (http://www.genomatic.de/matinspector) to compute TF binding scores on murine *Cyp26A1* promoter (–1,700 to +120 bp).

Of the candidate TFs, Sp/KLF family members frequently scored high (>85) on the list. As KLF9 has been shown to promote Cytochrome P450 2D6 expression in mouse liver [36], we asked whether the *Klf* genes, *Klf2, Klf7,* and *Klf9* with top ranking scores were expressed in the EBs, and whether their expression was affected by STRAP. Expression of *Klf9*, but not of *klf2* or *klf7*, was greatly induced by *Strap* depletion during EB differentiation (Supporting Information Fig. S5A). High expression of KLF9 protein was observed in *Strap* KO EBs (Fig. 5B, Supporting Information Fig. S5B), and this occurred concurrently with enhanced CYP26A1 protein (Fig. 4D). This result implies that KLF9 levels are regulated by STRAP, and the protein might in turn control *Cyp26A1* expression. To further investigate this, we co-transfected KLF9 expression vector with the luciferase reporter constructs containing different deletion fragments of *Cyp26A1* promoter into E14 cells (Fig. 5C). Since Sp1 and KLF9 compete for the same binding sites in the target gene promoter [37], we co-transfected expression vectors for Sp1 either alone or with KLF9. KLF9 induced the luciferase activity from −665 and −422 vectors in comparison to the control vector (>3-fold) (Fig. 5C). Unexpectedly, the activation was further increased in the presence of Sp1, implying KLF9 significantly activated the Cyp26A1 promoter and Sp1 had an additive effect.

To further determine the mechanism of KLF9-mediated control of *Cyp26A1* expression, we identified four putative KLF9 binding motifs (C/GCCC) within the promoter [38]. Among them, one is located in the distal region from the transcription start site (TSS), two are located upstream of the −200 bp sequence (middle region), and the fourth one is in between (Fig. 5D). To test whether these sites mediate the binding of KLF9/Sp1 to the endogenous *Cyp26A1* locus, we performed chromatin immunoprecipitation (ChIP) assays using anti-KLF9 and anti-Sp1 antibodies followed by qPCR. As revealed in Figure 5E, higher occupancy of Sp1 and KLF9 (> 3.5-fold and >5-fold, respectively) was detected on the middle region with two KLF9 putative binding sites in shSTRAP 10-day-old EBs compared with their corresponding shCtrl. In contrast, minimal signals with proximal and low signals with distal DNA fragments were detected, suggesting that KLF9 and Sp1 associate preferentially with the middle region of *Cyp26A1* promoter. Collectively, these studies indicate that loss of STRAP results in KLF9/Sp1 recruitment to specific regions of the *Cyp26A1* promoter that are important for its expression.

STRAP Affects Neural Patterning During Early *Xenopus* Development

It is well documented that RA signaling regulates anterior-posterior neural patterning as well as formation of the eyes in *Xenopus.* We thus use this system to address whether STRAP similarly controls AP neural patterning and eye development through RA signaling. We first analyzed the expression pattern of *Strap* by in situ hybridization. At gastrula stages, *Strap* RNA was seen in the mesodermal tissues surrounding the entire blastopore (Fig. 6A, panel a). Its expression was subsequently enriched in the neural plate during neurulation (Fig. 6A, panel b). At the end of the neurula stages, *Strap* transcripts were detected both in the brain and the spinal cord regions and in the migrating neural crest cells (Fig. 6A, panel c). This pattern persisted to the tailbud and the tadpole stages, with additional signals seen in the eyes, the otic vesicles, and the pronephros ducts (Fig. 6A, panels d and e). Many sites of *Strap* expression overlapped with those of other RA signaling components [39–41], suggesting that *Strap* may modulate RA signaling during *Xenopus* development.

To investigate the endogenous function of STRAP, we designed antisense morpholino oligonucleotides (MO) against *Strap.* Injection of the *Strap*-MO into the marginal zone (mesodermal precursors) led to interference with gastrulation movements (data not shown), whereas injection of the *Strap*-MO into the animal region (ectodermal cells) resulted in reduction of the head structure, malformation of the eyes, decreased melanocytes, and reduced body axis (Fig. 6B). These phenotypes are similar to those with RARα and RARβ knockdowns [39, 42]. To ascertain that the morphant phenotypes were indeed induced by *Strap* knockdown, we performed a rescue experiment using a Myc-tagged STRAP construct that cannot be targeted by the MO. Co-expression of the Myc-*Strap* RNA with the MO greatly rescued the morphant defects, so that both the length of the body axes and the formation of the eyes were restored to a large extent (Fig. 6C). The data imply that the effects of the *Strap* MO are specific and that *Strap* is required for body axis and eye development in early *Xenopus* embryogenesis.

To further analyze the defects induced by *Strap*-MO, we performed in situ hybridization (ISH) of a panel of neural markers. *Otx2* is a marker for the forebrain and the midbrain. Engrailed (En) is a midbrain-specific gene. *Krox20* is expressed in the hindbrain. *HoxD4* and *HoxB9* are both spinal cord markers, whereas *Pax6* is expressed in the spinal cord, the diencephalon, and the eye fields. We injected the *Strap*-MO together with a lineage tracer β-Gal gene into one cell of the 2-cell stage embryos. The embryos were collected at late neural stages. ISH was then performed on the embryos, with the un-injected side as an internal control for marker expression. As shown in Figure 6D, the neural markers were expressed symmetrically on both sides in control. However, in embryos injected with *Strap*-MO, the markers were shifted posteriorly on the injected side relative to the control side, and the expression of *Krox20* and *Pax6* in the eye field was also reduced by *Strap* knockdown, similar to when RA signaling was impaired via knockdown or inhibition of its signaling components [39, 41, 43]. The results are thus consistent with the idea that STRAP may positively regulate RA signaling to influence anterior-posterior neural patterning.

Based on our cell culture studies, we hypothesize that *Strap* may regulate RA signaling via its control of expression of RA metabolic enzymes. We thus examined the expression of several RA metabolic enzymes in control and *Strap* morphant embryos by RT-qPCR. We observed a specific enhancement of *Cyp26C1* expression when Strap was knocked down, but the levels of *Cyp26A1*, *Cyp26B1* and *Crabp2* were not altered significantly (Fig. 6E). The data suggest that Strap inhibits *Cyp26C1* expression to affect neural patterning during early *Xenopus* development.

Discussion

Tissue-specific cell fate during development is determined by multiple signals, such as WNT, BMP/SMAD, FGF/MAPK, Hippo,

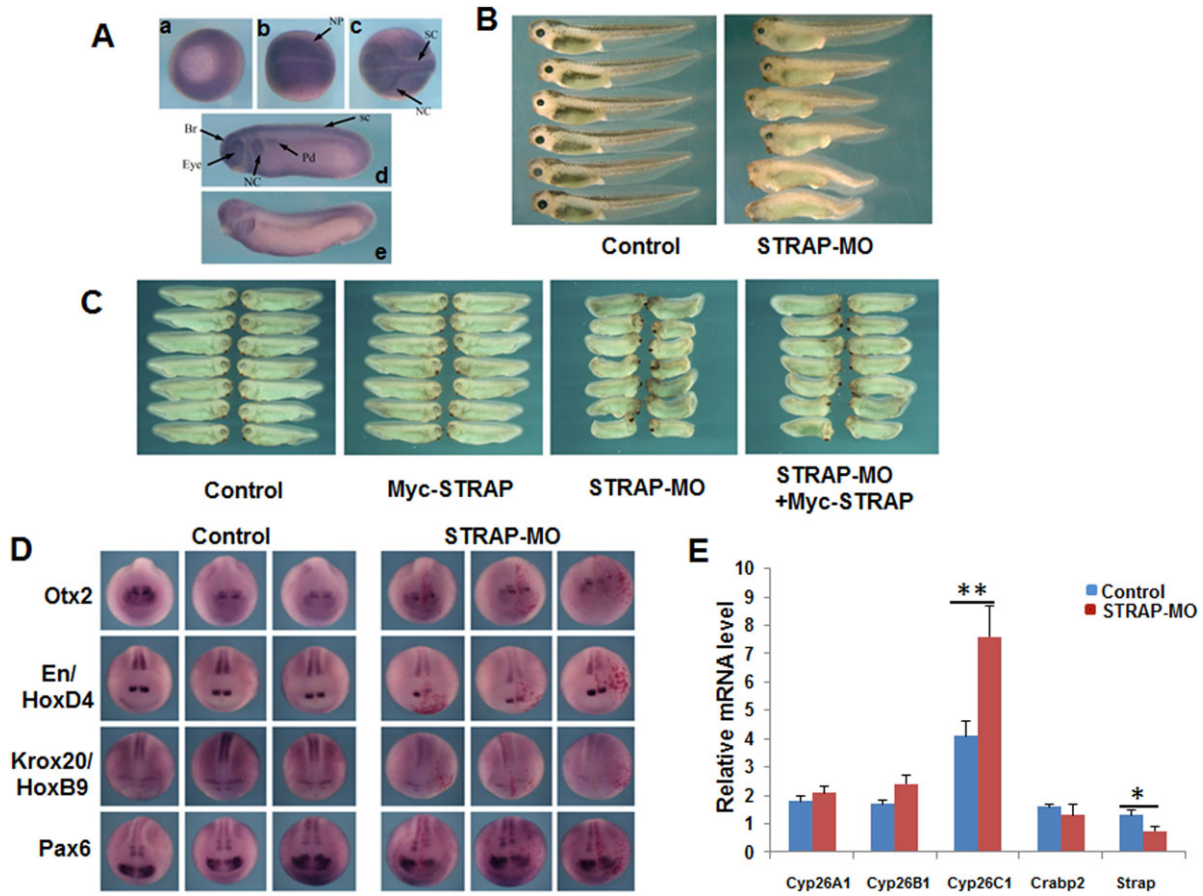

Figure 6. STRAP knockdown influences anterior-posterior neural patterning and impairs the body axis and eye development in early *Xenopus* embryogenesis. **(A)**: *Strap* transcript was detected in the entire blastopore (**Aa**), early neurulation (**Ab**), late neurula stage (**Ac**), and the tailbud and the tadpole stages (**Ad** and **Ae**, respectively). **(B)**: Fifty nanograms of *Strap*-MO was injected into the animal regions of two-cell-stage embryos and resulted in defects in the tadpole stage. **(C)**: *Strap* RNA (0.20 ng) was injected alone or with 25 ng *Strap*-MO unilaterally into two-cell- stage embryos. The embryos were stained with the β-Gal substrate Red-Gal. **(D)**: Fifty nanograms *Strap*-MO was injected unilaterally into two-cell- stage embryos. The embryos were examined at neurula stages 17–18 by ISH for neural markers. **(E)**: Fifty nanograms *Strap*-MO was injected into the animal regions of two-cell-stage embryos. Animal caps were dissected at blastula stage 9 and cultured until neurula stages 19–20. RNA was then extracted for RT-qPCR analysis of marker expression. Bars represent mean ± s.d., $n = 3$. *, $p < .05$; **, $p < .01$, when compared with the control. Abbreviations: MO, morpholino oligonucleotide; STRAP, serine threonine kinase receptor-associated protein.

NF-κB, JAK/STAT, and RA pathway [43]. Numerous studies have focused on the function of RA signaling in vertebrate development; however, its upstream regulation during ESC differentiation is largely unexplored. Based on gene expression profiling, we detected that endogenous RA signaling was attenuated in *Strap*-KO EBs (Fig. 3A, 3B). Importantly, this impaired signaling was restored when Vitamin A or all-trans RA was added back to the *Strap*-KO EB culture (Fig. 3C), suggesting that STRAP is indeed an effector of RA-regulated ESC differentiation. Notably, our study shows that RA could rescue the deficiency in ectodermal and endodermal lineages but not mesodermal commitment in KO-EBs. It could be due to two reasons: (a) loss of RA signaling is not a major trigger for the failure of mesodermal differentiation upon STRAP deletion, and (b) other signaling pathway(s) may be activated in RA-exposed KO-EBs and neutralize its effect.

We initially examined the expression of RA-metabolic genes in *Strap*-KO EBs during differentiation. *Rdh10* and *Raldh2*, representing two key enzymes for RA synthesis, were analyzed with qRT-PCR and appeared to be similar in KO and WT EBs (Fig. 4A). *Crabp2* was also not affected by the absence of *Strap* (Fig. 4A). Additionally, we observed that the levels of two RA nuclear receptors, *Rarα* and *Rarβ*, were reduced in KO EBs compared with WT EBs (Fig. 3B) and were dramatically rescued by exogenous RA (Fig. 3C). These results suggest that RA receptor activity was attenuated by the low potency of RA signaling and not a receptor deficiency. CYP26A1 is the critical RA-degrading enzyme and *Cyp26A1* has also served as a well-known RA-responsive gene. Paradoxically, we found that CYP26A1 mRNA and protein levels was increased in KO cells during differentiation (Fig. 4A–4D), implying that higher expression of CYP26A1 might result in the RA degradation. These results are in agreement with the data from *Xenopus* model that loss of STRAP induces defective RA-signaling-induced phenotype probably through the upregulation of *Cyp26C1* (Fig. 6E). Together, this supports the notion that the RA degradation pathway might be regulated by different p450 family members during evolution. Our future work will determine the mechanism of how STRAP regulates the p450 family during the *Xenopus* development. Overall, our data suggest

that STRAP regulates mouse ESC differentiation through CYP26A1-mediated RA signaling.

To understand how STRAP controls Cyp26A1 expression, we used luciferase and ChIP assays (Fig. 5). We showed that STRAP depletion led to increased expression of KLF9, which binds to *Cyp26A1* promoter to increase its expression. KLF9 is a well-known transcriptional regulator, although its function during development is not well studied. KLF9 collectively associates with either transcriptional co-activators or co-repressors to influence the expression levels of genes [44, 45]. Our previous studies demonstrated that STRAP negatively modulates Sp1-dependent transcription [46], suggesting an important role of STRAP in regulating the binding of TFs to cis-acting sequences. Here, we observed that both KLF9 and Sp1 bound to the *Cyp26A1* locus, perhaps due to the high sequence identity shared by these two proteins (Fig. 5B, 5E).

In *Xenopus*, RA signaling has been shown to regulate anterior-posterior neural patterning as well as eye development. We have observed that knockdown of *Strap* affects AP neural identity and eye formation, phenotypes generated by RARα and RARß depletion. The shift of regional neural markers posteriorly is consistent with a reduction in RA signaling in *Xenopus.* Studies of RA metabolic enzymes show that expression of Cyp26C1 is specifically upregulated by strap knockdown. Thus, our in vivo data are consistent with the in vitro embryonic stem cell results, and both point to a *Strap*-dependent inhibition of Cyp26 expression, which it turn controls RA signaling during early embryonic differentiation and patterning.

Conclusion

Collectively, we show that RA signals are required for the ESC lineage commitments and that STRAP positively regulates this progress via specifically involving in RA signaling. These findings expand our understanding of the molecular mediators of RA signaling, and provide a novel mechanistic basis for how RA signaling effectively induces ESC differentiation via STRAP.

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Author Contributions

L.J. and C.C.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; K.M.P.: data analysis and interpretation, manuscript writing; A.D., L.M.J., and T.V.: collection and/or assembly of data; J.L.N.: data analysis and interpretation; P.K.D.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of the manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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