Stk40 links the pluripotency factor Oct4 to the Erk/MAPK pathway and controls extraembryonic endoderm differentiation

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Self-renewal and differentiation of embryonic stem cells (ESCs) are controlled by intracellular transcriptional factors and extracellular factor-activated signaling pathways. Transcription factor Oct4 is a key player maintaining ESCs in an undifferentiated state, whereas the Erk/MAPK pathway is known to be important for ESC differentiation. However, the manner in which intracellular pluripotency factors modulate extracellular factor-activated signaling pathways in ESCs is not well understood. Here, we report identification of a target gene of Oct4, serine/threonine kinase 40 (*Stk40*), which is able to activate the Erk/MAPK pathway and induce extraembryonic–endoderm (ExEn) differentiation in mouse ESCs. Interestingly, cells overexpressing *Stk40* exclusively contribute to the ExEn layer of chimeric embryos when injected into host blastocysts. In contrast, deletion of *Stk40* in ESCs markedly reduces ExEn differentiation in vitro. Mechanistically, Stk40 interacts with Rcn2, which also activates Erk1/2 to induce ExEn specification in mouse ESCs. Moreover, Rcn2 proteins are specifically located in the cytoplasm of the ExEn layer of early mouse embryos. Importantly, knockdown of *Rcn2* blocks *Stk40*-activated Erk1/2 and ESC differentiation. Therefore, our study establishes a link between the pluripotency factor Oct4 and the Erk/MAPK signaling pathway, and it uncovers cooperating signals in the Erk/MAPK activation that control ExEn differentiation.

embryonic stem cells | Rcn2 | Ras | Gata6

The mouse embryo at the blastocyst stage contains three cell types, trophectoderm, primitive endoderm, and epiblast, each of which generates distinct cell lineages in the developing embryo. The trophectoderm is the first differentiated cell lineage of embryogenesis, and it generates ectoplacental cone, extraembryonic ectoderm, and trophoblast cells in the fetal placenta. The primitive endoderm, the second differentiated lineage, generates extraembryonic endoderm (ExEn), which contributes to the endoderm layer of the visceral and parietal yolk sacs (1–3). These ExEn lineage cells in the mammal provide nutritive supports and are crucial for cell-fate specification and axial-pattern initiation (4). Finally, epiblast cells are pluripotent cells that give rise to all of the fetal tissues and other extraembryonic tissues. Embryonic stem cells (ESCs) derived from the epiblast provide an excellent tool for study of the molecular networks that control mammalian development (5–7). They are capable of differentiating into all cell types of the three germ layers (ectoderm, mesoderm, and endoderm), including germ cells; they can also self-renew indefinitely in vitro. These unique features make ESCs an attractive resource in regenerative medicine (8). However, many obstacles exist on the road to the therapeutic use of ESC derivatives. In particular, our ability to drive ESC differentiation into a chosen fate is limited. Thus, understanding the mechanisms governing self-renewal and differentiation is an important step to overcoming the existing difficulties.

Transcriptional factor Oct4 is a critical maternal and embryonic protein found in the unfertilized egg, zygote, and all blastomeres until the morula stage. When the blastocyst forms, its expression is restricted to the inner cell mass, and it is absent in the trophectoderm (9). Deletion of *Oct4* in mice leads to an inability to generate pluripotent populations in the inner cell mass (10). In vitro, Oct4 is also highly expressed in undifferentiated ESCs, and it is down-regulated on differentiation. Knockdown of *Oct4* in ESCs triggers primitive endoderm as well as trophectoderm differentiation (11–13). Of note, Niwa et al.(13) found that up-regulation of *Oct4* expression causes differentiation of ESCs into primitive endoderm and mesoderm. These observations suggest that Oct4 at levels either higher or lower than normal could convert ESCs to primitive endoderm lineages. However, it is also possible that ESCs with different epigenetic settings might respond to the same level of Oct4 expression differentially. Although Oct4 is an essential regulator of cell fate, molecular mechanisms underlying its functions are not well-defined. Among the known Oct4 target genes, *Cdx2* and *Hand1* are transcription factors critical for trophectoderm development (13–15). However, less is known about the segregation of the epiblast and primitive endoderm within the inner cell mass of the blastocyst. The transcription factor Gata6 is required for the development of primitive endoderm lineages (3), whereas Nanog has been shown to prevent primitive endoderm differentiation by repressing expression of *Gata6* (16). However, the reason that Oct4 is implicated in the formation of the primitive endoderm and subsequent ExEn lineages remains elusive.

Here, we identify serine/threonine kinase 40 (*Stk40*) as a target gene of Oct4 and show that Stk40 is capable of activating the Erk/MAPK signaling pathway. Also, it induces ESC differentiation into ExEn lineages through Rcn2, a Ca^{2+} binding protein. Our study puts forward the concept that Oct4 suppresses expression of an important component of the Erk/MAPK signaling pathway to protect ESCs from ExEn differentiation.

Results

Oct4 Negatively Regulates *Stk40* Expression. To find new Oct4 target genes, we carried out chromatin immunoprecipitation (ChIP) assays using Oct4 antibody and identified a gene, *Stk40*. It encodes a protein of 435 amino acid residues with a serine/threonine

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kinase domain (Fig. 1*A*). Sequence orthologs of *Stk40* were found in a number of other organisms (Fig. S1*A*). Transcript and protein levels of Stk40 were up-regulated and maintained at high levels during the process of spontaneous embryoid-body (EB) formation (Fig. S1*B*). Moreover, proteins of Stk40 were found to distribute in both the nucleus and cytoplasm (Fig. S1*C*).

To test whether or not *Stk40* expression is regulated by Oct4, we used ZHBTc4 mouse ESCs in which *Oct4* expression is controlled by tetracycline (Tc) (13). On addition of Tc, *Oct4* expression was quickly silenced, and *Stk40* expression was substantially elevated but slowly (Fig. 1*B*). This suggests that *Stk40* expression was negatively controlled by Oct4 and that other factors might also be involved in this regulation (Fig. S1*D*). Moreover, a high level of luciferase activity was observed with an upstream 0.5-kb fragment (the putative *Stk40* promoter) in CGR8 mouse ESCs. However, the activity was significantly reduced when either a 2-kb or 4-kb upstream fragment was included (Fig. S1*E*), suggesting that an inhibitory component or components may exist within the 2-kb fragment. Strikingly, the repressive effect of the 2-kb fragment was completely abrogated by silencing *Oct4* expression in the ZHBTc4 cells (Fig. 1*C*), indicating that Oct4 is a factor that is likely responsible for repression of the reporter activity. Further analysis identified a conserved octamer motif (TTTTGCAT) positioned within the upstream 2-kb region of *Stk40*. Triple-point mutations in the octamer motif (mut, TTGTTCCT) abolished the inhibition of the 2-kb fragment on the reporter activity (Fig. 1*D*). Furthermore, we conducted ChIP assays with the Oct4 antibody, which revealed that Oct4 indeed associated with the *Stk40* regulatory sequence containing the octamer motif in a physiological context; it also associated with the promoter of *Rex1,* a known target gene of Oct4 (Fig. 1*E*). A similar result was obtained with an in vitro DNA-protein binding assay (*SI Text*). Concisely, Oct4 negatively regulates *Stk40* expression through its direct binding to the octamer motif located in the upstream 2-kb regulatory sequence.

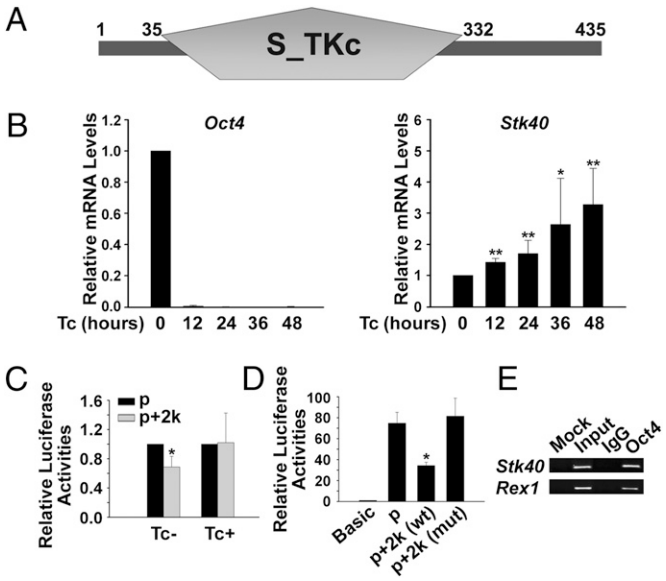

Fig. 1. *Stk40* is a direct target of Oct4 and is negatively regulated by Oct4. (*A*) The schematic diagram of the Stk40 protein showing a serine/threonine protein kinase domain (S_TKc) in the middle (aa35–332). (*B*) Analysis of *Oct4* and *Stk40* dynamic expression patterns in ZHBTc4 mouse ESCs treated with Tc by qPCR. All values are shown as means ± SD of results from three independent experiments. $*P < 0.05$; $**P < 0.01$. (*C*) Luciferase assays of the 2-kb upstream fragment of *Stk40* reporter in ZHBTc4 ESCs treated with or without Tc for 72 h. ZHBTc4 cells were pretreated with Tc for 24 h before transfection. p, 0.5 kb of putative promoter; p+2k, 2kb upstream regulatory sequence. $*P < 0.05$. (*D*) Luciferase assays of the 2-kb upstream fragment of *Stk40* reporter with or without mutations introduced in the octamer motif in CGR8 ESCs. p, promoter; wt, wild type; mut, mutant. Luciferase activities are shown as in *C*. $*P < 0.05$. (*E*) ChIP assays in CGR8 ESCs using rabbit IgG or the specific antibody against Oct4. The representative result of PCR amplification reactions of *Stk40* (*Upper*) or *Rex1* (*Lower*) is shown. Mock, no template; input, 10% of total genomic DNA from the nuclear extract.

Forced Expression of *Stk40* Induces ESC Differentiation into ExEn Lineages. To study the function of Stk40, we overexpressed *Stk40* in ESCs using an episomal expression system (17). ESCs overexpressing *Stk40* displayed differentiated morphology (Fig. 2*A* and Fig. S2*A*) with a few tightly compacted ESC colonies among the differentiated cells. Using colony-forming assays to evaluate the self-renewal ability of ESCs, we found that forced expression of *Stk40* resulted in a marked reduction in the number of undifferentiated colonies (Fig. S2*B*). Quantitative real-time PCR (qPCR) analysis showed that ExEn markers, including *Gata6*, *Gata4*, *Sox7*, *Laminin B1*, *Afp*, and *Ihh*, were substantially activated by *Stk40* overexpression (Fig. 2*B*). A modest increase in the expression of trophectoderm-lineage markers (*Cdx2* and *Hand1)* was also noticed. In contrast, we did not find marked changes in the expression of mesoderm and ectoderm markers. In pluripotency-related genes, transcript levels of *Oct4*, *Nanog*, and *Rex1* were reduced (Fig. S2*C*). Furthermore, immunostaining assays revealed evidently increased expression of ExEn markers (Dab2 and Gata4) and decreased expression of Nanog in *Stk40* overexpressed cells (Fig. S2*D*).

Wild-type ESCs spontaneously differentiated into cystic EBs containing heterogeneous cell types representing all three germ layers and an outer layer of ExEn cells when cultured in suspension (18). Remarkably, the outer layer of EBs aggregated from *Stk40* overexpressing ESCs was more prominent and thicker than that of control EBs (Fig. 2*Ca*, Fig. 2*Cb*, and Fig. S2*F*). Staining with H&E and the Dab2 antibody confirmed the identity of the cells in the expanded outer layer as ExEn lineages (Fig. 2*C c–f*). Furthermore, red dye (DiI)-labeled *Stk40* overexpressing cells specifically localized at the outer rim of chimeric EBs, whereas the vector-transfected control cells were distributed throughout the EBs (Fig. 2*D*) in chimeric EB-formation assays. This is consistent with a previously reported phenomenon that ExEn cells have an intrinsic property of surface positioning when mixed with undifferentiated ESCs in the EB-differentiation process (19).

In addition, the role of *Stk40* in the ExEn development under physiological conditions was investigated using $Stk40^{+/+}$ and $Stk40^{-/-}$ ESCs. Notably, Dab2 and Gata4 staining present in the outer layer of EBs of $Stk40^{+/+}$ ESCs was lost in EBs of $Stk40^{-/-}$ ESCs (Fig. 2*E*). Moreover, qPCR analysis revealed that induction of ExEn markers, *Gata6* and *Sox7,* but not markers of other germ layers was evidently impaired in EBs of $Stk40^{-/-}$ ESCs (Fig. 2*F* and Fig. S2*J*). Deletion of *Stk40* was validated by the complete lack of its protein product in $Stk40^{-/-}$ ESCs-derived EBs (Fig. S2*K*).

Finally, we generated chimeric embryos and compared the developmental potential of *Stk40* overexpressing cells (*Stk40*/E14Tg) with control cells expressing vector only (*Vector*/E14Tg) at E6.5 (embryo day) and E7.5. Both cell lines expressed a histone2B-GFP fusion protein for visualization. Strikingly, *Stk40*/E14Tg cells contributed to extra embryonic yolk sacs exclusively (Fig. 2*G* and Table S1). In contrast, more than 50% of *Vector*/E14Tg cells contributed to the embryonic portion. In addition, we generated chimeric embryos with E14Tg ESCs where *Oct4* expression was knocked down by specific RNAi oligos. Interestingly, cells transfected with *Oct4* RNAi oligos predominantly migrated to the ExEn layers, although a minority was found in the epiblast, probably because of the ineffective knockdown of *Oct4* in the cells (Fig. S2*N*, Fig. S2*O* , and Table S2). The results described above and in the *SI Text* indicate that Stk40 possesses an ability to induce ESCs to contribute to ExEn lineages.

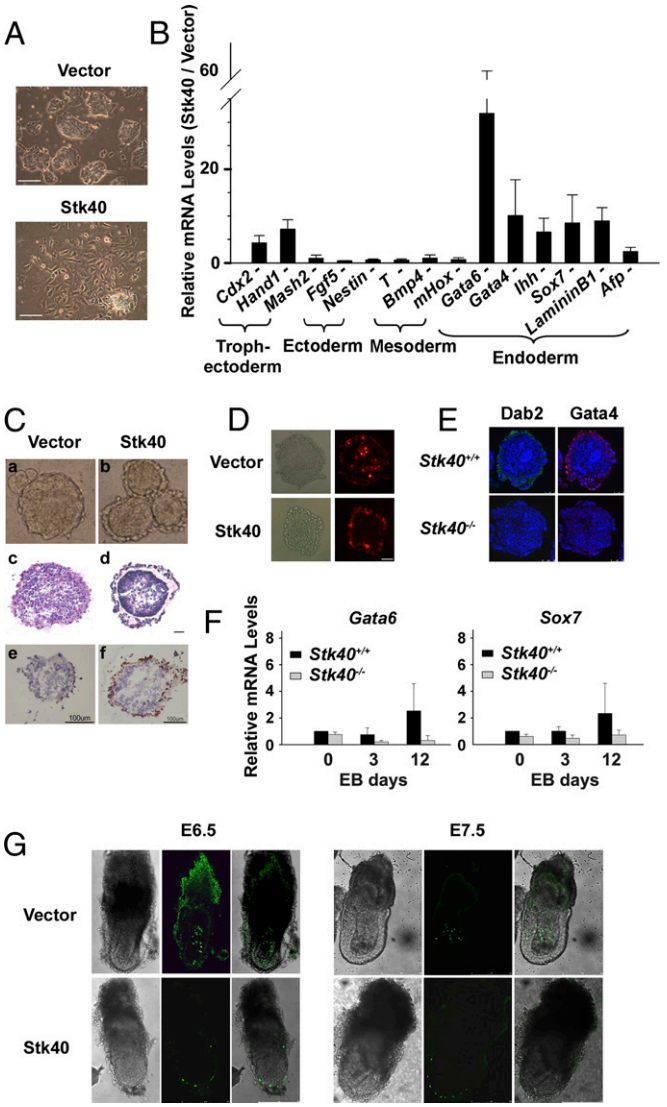

Fig. 2. Stk40 induces ESCs to differentiate into ExEn lineages. (*A*) Morphological changes in ESCs induced by *Stk40* overexpression. E14T ESCs were transfected with either pPyCAGIP (*vector* as a control) or pPyCAGIP-Stk40 (*Stk40*) and cultured for 10 days in the presence of puromycin. (Scale bar: 100 μm.) (*B*) Effect of Stk40 on expression of differentiation-related markers using qPCR. Data are shown as the ratio of values for *Stk40* overexpressing cells over control cells. All values are shown as means ± SD of results from three independent experiments. (*C*) Representative images showing expansion of ExEn-like cells in EBs aggregated from *Stk40* overexpressing ESCs. (*a* and *b*) Light microscopic images. (*c* and *d*) Hematoxylin/eosin-stained sections of EBs. (*e* and *f*) Costained with Dab2 antibody by the DAB (3,3′-Diaminobenzidine) method (brown signal). (Scale bar: 100 μm.) (*D*) Surface positioning of *Stk40*-induced differentiated ESCs in the chimeric EB formation assay. (Scale bar: 50 μm.) (*E*) Immunofluorescence staining of Dab2 and Gata4 in EBs from $Stk40^{+/+}$ or $Stk40^{-/-}$ ESCs. (Scale bar: 100 μm.) (*F*) Quantification of ExEn–marker expression by qPCR in EBs aggregated from $Stk40^{+/+}$ and $Stk40^{-/-}$ ESCs at the indicated time points. All values are shown as means ± SD of results from four independent experiments. (*G*) Contribution of injected *Stk40*/E14Tg cells to the ExEn lineage in E6.5 (*Left*) and E7.5 (*Right*) chimeric embryos. (Scale bar: 250 μm.)

Stk40 Induces ESC Differentiation Through Activation of the Erk/MAPK Pathway. Experimental evidence suggests that multiple signaling pathways including the Erk/MAPK, TGF-β, and PI3K pathways might be involved in ExEn development (4, 20–22). Using inhibitors of these pathways, we found that only the Mek1/2 inhibitor (U0126) abrogated *Stk40*-induced ESC differentiation, which was determined by both cell morphology and *Sox7* expression (Fig. 3*A* and Fig. S3*A*). This finding led to an assumption that forced expression of *Stk40* might induce ESC differentiation through activating the Erk/MAPK pathway. Furthermore, overexpression of a constitutively active mutated form of *Ras* for the Erk/MAPK pathway [H-Ras with G12V and T35S mutations (22) was termed *RasT35S* in this study] brought about differentiated morphology and high expression of *Gata6* in ESCs (Fig. 3*B* and Fig. S3*B*); this is consistent with a previously reported role of *Ras* in ExEn differentiation (22). Notably, coexpression of a dominant negative mutated form of *Ras* (*RasS17N*) and *Stk40* abolished *Stk40*-induced differentiation phenotypes (Fig. 3*B* and Fig. S3*B*), suggesting that active Ras is indispensable for Stk40 to induce differentiation. In addition, GST-RBD (Ras Binding Domain) pull-down assays showed that activated Ras was dramatically increased in *Stk40* overexpressed cells to a level comparable to that in *RasT35S*-expressed cells (Fig. 3*C*). Phosphorylation of Erk1/2 (pErk1/2) and Mek1/2 (pMek1/2) was also enhanced when *Stk40* or *RasT35S* was overexpressed, whereas U0126 abolished *Stk40*-activated pErk1/2 (Fig. 3*D* and Fig. S3*C*). Together, these data provide evidence that Stk40 is an activator of the Erk/MAPK pathway through which it induces ESC differentiation into the ExEn lineage.

Stk40 Associates with Rcn2 to Activate the Erk/MAPK Pathway. To understand how Stk40 activates the Erk/MAPK pathway, we searched for proteins associated with Stk40. Among candidate proteins identified by affinity chromatography and mass spectrometric analysis (Fig. S4*A*, column 2), we were particularly interested in an intracellular Ca^{2+} binding protein, Rcn2 (23), because it was recently reported to be highly expressed in the mouse ExEn lineage (24). Direct interaction between Stk40 and Rcn2 was verified by GST pull-down assays using bacterially expressed GST-Rcn2 and His-Stk40 in vitro (Fig. 4*A Top*). Moreover, the formation of protein complexes between Rcn2 and Stk40 in mouse ESCs was shown by coimmunoprecipitation

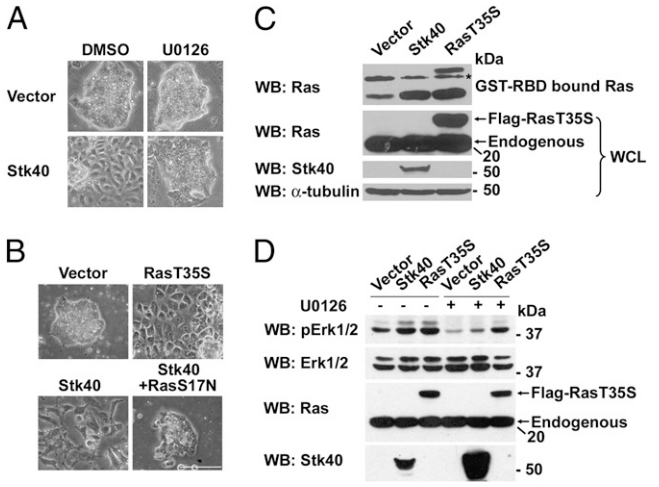

Fig. 3. Forced expression of *Stk40* induces ESC differentiation through activation of the Erk/MAPK pathway. (*A*) Phase–contrast images of *vector*- or *Stk40* overexpressed E14T cells treated with DMSO or the Mek1/2 inhibitor, U0126 (Magnification: ×20). (*B*) Phase–contrast images of E14T cells stably expressing *vector*, *Stk40*, or Ras constitutively active form (*RasT35S*) or coexpressing *Stk40* and Ras dominant negative form (*RasS17N*). (Scale bar: 100 μm.) (*C*) Western blot analysis of GST-RBD–bound activated Ras in E14T ESCs stably expressing *vector*, *Stk40*, or *RasT35S*. Asterisk, nonspecific bands recognized in GST-RBD–bound groups. (*D*) Western blot analysis of pErk1/2 in E14T cells stably expressing *vector*, *Stk40*, or *RasT35S* with or without U0126 treatment as indicated. WB, Western blot; WCL, whole cell lysate.

of endogenously expressed Rcn2 with ectopically expressed Flag-tagged Stk40 (Fig. 4*B*).

Interestingly, ESCs overexpressing *Rcn2* displayed morphology indistinguishable from that of *Stk40* overexpressed cells (Fig. 4*C*). Like *Stk40*-induced differentiation, expression of *Gata6* and *Sox7* was up-regulated in *Rcn2*-transfected cells (Fig. S4*B*). However, knockdown of *Rcn2* expression by specific RNAi prevented *Stk40* from inducing ESC differentiation and activating *Gata6* and *Sox7* expression (Fig. 4 *D* and *E*). In addition, like Stk40, Rcn2 was able to activate Erk1/2 (Fig. 4*F*). However, unlike Stk40, Rcn2 could not activate Ras. Nevertheless, knockdown of *Rcn2* expression substantially attenuated Stk40-activated pErk1/2 (Fig. 4*G*). Altogether, our data reveal that Rcn2 is sufficient to activate the Erk/MAPK pathway and is indispensable for Stk40 to induce ESC differentiation into ExEn lineages.

Rcn2 Is a Developmentally Regulated Protein Required for ExEn Differentiation. To determine the Rcn2 expression pattern, we first examined its expression levels during ESC differentiation in vitro. Transcript and protein levels of Rcn2 gradually increased along with the differentiation process of EBs (Fig. 5 *A* and *B*). Interestingly, *Rcn2* was also highly expressed in extraembryonic endoderm cells (Fig. S5*A*), which are derived from mouse blastocysts and retain properties of the primitive endoderm (25). During early mouse development in vivo, cytoplasmic Rcn2 proteins were readily detected in the endoderm layer of yolk sacs at E6.5 and E7.5 but not in embryos at E3.5 (Fig. 5*C*, Fig. S5*B*, and Fig. S5*C*). In parallel, nuclear Gata4 staining was also found in the ExEn cells. In contrast, Oct4 was exclusively expressed in the nucleus of epiblast cells. The specific expression of Rcn2 in ExEn cells of the embryo suggests its possible role in ExEn lineage specification. Indeed, knockdown of *Rcn2* markedly reduced induction of the ExEn markers during EB formation (Fig. 5*D*). In addition, our immunofluorescence study showed substantial enhancement of Rcn2 staining in *Stk40* overexpressing differentiated cells (Fig. S5*D*). This is consistent with our finding that *Stk40* overexpressing cells, which were injected into host blastocysts, expressed cytoplasmic Rcn2 in chimeric embryos (Fig. S5*E*). Collectively, our data suggest that Rcn2 might be an important protein in ExEn lineage specification in both ESC differentiation in vitro and early development in vivo.

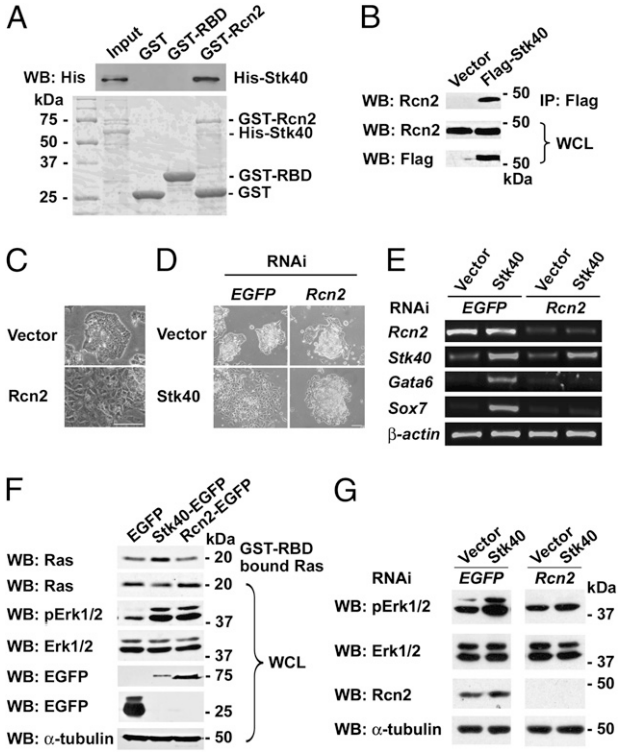

Fig. 4. Rcn2 is a Stk40-interacting protein implicated in *Stk40*-induced Erk1/2 activation and ExEn differentiation. (*A*) Rcn2 associates with Stk40 directly. (*Top*) Reaction products from GST pull-down assays were analyzed by Western blotting with anti-His antibody. (*Bottom*) Coomassie blue staining of SDS/PAGE gel containing proteins included in the reaction. The experiment was repeated three times. (*B*) Flag-tagged Stk40 forms protein complexes with endogenous Rcn2 in E14T ESCs. The representative result of three coimmunoprecipitation experiments is shown. (*C*) Overexpression of *Rcn2* induces ESC differentiation. Phase–contrast images of *vector*- or *Rcn2*-expressed E14T ESCs are shown. (Scale bar: 100 μm.) (*D*) Knockdown of *Rcn2* blocks *Stk40*-induced ESC differentiation. Phase–contrast images of *vector*- or *Stk40* over-expression in the stable *EGFP* or *Rcn2* RNAi E14T ESCs are shown. (Scale bar: 100 μm.) (*E*) Gene expression levels in the cells described in *D* were analyzed by RT-PCR. (*F*) Rcn2 can activate Erk1/2 but not Ras. The representative result of three independent Western blot analyses of GST-RBD–bound activated Ras and pErk1/2 in *EGFP*-, *Stk40-EGFP*–, or *Rcn2-EGFP*–expressed E14T cells is shown. (*G*) Western blot analysis reveals that knockdown of *Rcn2* abrogates *Stk40*-activated Erk1/2. The representative result of three independent experiments is shown.

Discussion

Primitive endoderm is the first cell lineage segregated from the inner cell mass at implantation, and the receptor tyrosine kinases (RTKs)-Ras-MAP kinase pathway is known to be required for this segregation (22, 26). Mutation of *Grb2*, an adaptor molecule downstream of several RTKs, results in loss of the primitive endoderm lineage in vivo and in vitro (27). Ras has also been shown to play a crucial role in initiation of ExEn differentiation in mouse ESCs (22). However, the manner in which transcription factors such as Oct4 and Nanog are integrated with the Erk/MAPK signaling to control pluripotency and lineage restriction has not been well explored. Our finding provides an example of genetic interaction between core-transcription factors and the Ras-MAPK signaling in ESCs. Suppression of *Stk40* expression could be one of the mechanisms through which Oct4 prevents pluripotent cells from differentiating. Moreover, *Stk40* could also be a common target of Oct4, Sox2, and Nanog. In fact, the genomic sequence of *Stk40* was also reported to associate with Oct4 as well as Sox2 and Nanog in a recent ChIP-chip report (28). The combinational control of Stk40 expression by at least three transcriptional factors could explain the phenomenon that Stk40 expression was gradually up-regulated when Oct4 expression was rapidly silenced, because Sox2 and Nanog expression was also gradually down-regulated during this process. Therefore, ESC differentiation is regulated by a controlled expression of the MAPK signaling-pathway components and is integrated to external cues through an Oct4-coordinated intrinsic transcriptional network.

Another significant advance of this study is the discovery of two proteins capable of activating the Erk/MAPK signaling pathway to induce ESC differentiation into the ExEn lineage. We found that Stk40 can activate the Erk1/2 through Rcn2. A recent microarray-based screening identified *Rcn2*, together with *Gata6* and *Dab2*, as a potential primitive endoderm-specific gene (24). Supporting this report, we found that cytoplasmic Rcn2 is up-regulated during ESC differentiation in parallel with Stk40, and it is expressed in the ExEn cells of early embryos in vivo. Although both proteins can activate Erk1/2 and induce ESC differentiation when overexpressed in ESCs, only Stk40 was able to activate Ras. This may explain our observation that *Stk40* exerts more efficient induction of ESC differentiation than *Rcn2*. This also implies that other not yet identified molecular mechanisms exist for activation of Ras by Stk40. However, knockdown of *Rcn2* was sufficient to block *Stk40*-induced activation of Erk1/2 and ESC differentiation, suggesting that Rcn2 functions downstream of Stk40. Fur-

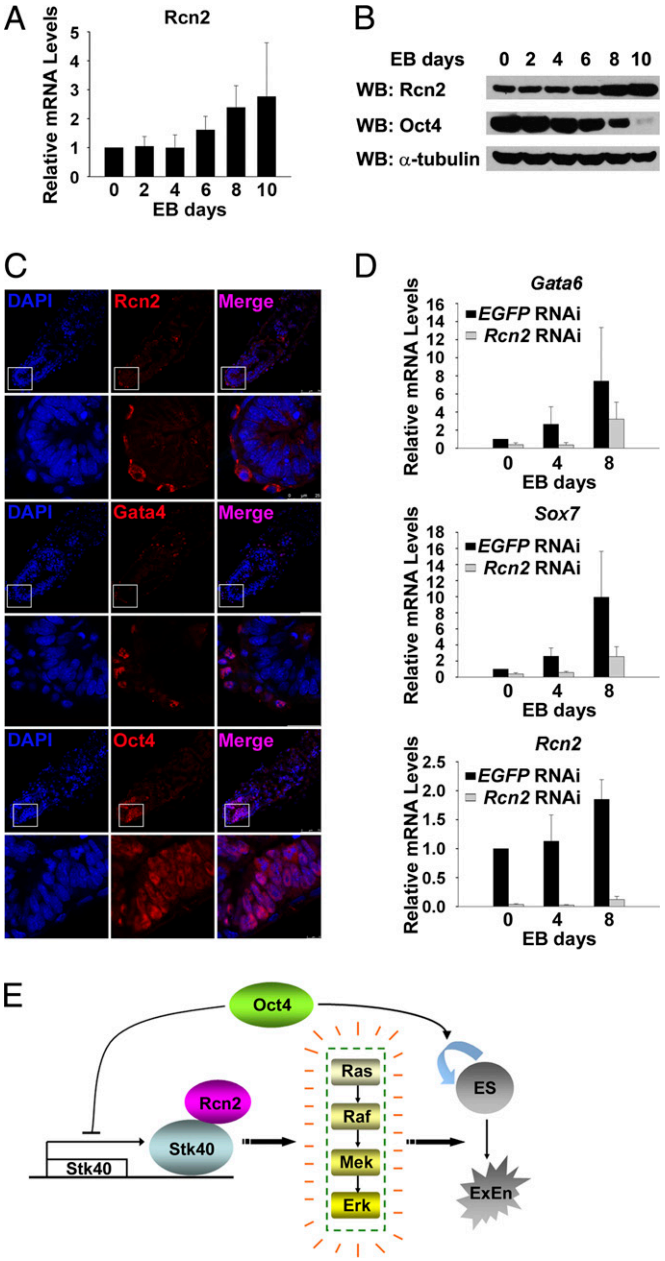

Fig. 5. Rcn2 is a developmentally regulated protein required for ExEn differentiation. (*A*) Quantification of dynamic *Rcn2* mRNA levels in EBs aggregated from E14T cells for indicated days. All values are shown as means ± SD of results from four independent experiments. (*B*) Western blot analysis of Rcn2 protein levels in the cells described in (*A*). A representative result of three independent experiments is shown. (*C*) Rcn2 proteins are detected in frozen sections of mouse embryos at E6.5. Immunofluorescent images of Rcn2, Gata4, and Oct4 proteins are shown. (Scale bar: 75 μm.) (*Inset*) Enlarged view at the distal part of ExEn layers covering the epiblast. (Scale bar: 25 μm.) (*D*) Knockdown of *Rcn2* by RNAi in E14T cells reduces induction of ExEn–marker expression during EB formation. Values of qPCR results from three independent experiments are shown as means ± SD. (*E*) A proposed model for the function of Stk40 in ExEn differentiation.

thermore, we found that expression of *Rcn2* RNAi could partially block *RasT35S*-mediated induction of *Gata6* expression in ESCs (Fig. S6 *A* and *C*). However, similarly to *Stk40*, *Rcn2*-mediated activation of *Gata6* expression was abrogated when *RasS17N* was coexpressed (Fig. S6 *B* and *D*). These observations suggest that Ras and Rcn2 might be functionally dependent on each other in this process. The incomplete blockage of *RasT35S*-activated *Gata6* expression could be explained by the possibility that there are additional factors associated with *Ras*-induced ExEn differentiation in addition to Rcn2. Currently, we do not know how Rcn2 expression is regulated. However, we speculate that Stk40 regulates the function or protein stability of Rcn2 posttranslationally based on our observations that Stk40 and Rcn2 interacted directly and that Rcn2 protein levels were elevated when *Stk40* was overexpressed. However, the exact mechanisms by which Stk40 and Rcn2 stimulate Erk1/2 remain unknown. Other factors in addition to Erk1/2 are most likely important in this process. Further investigation will greatly facilitate our interpretation of early embryogenesis at a molecular level.

In addition to the ExEn lineage, we also detected elevated expression of some trophectoderm marker genes after overexpression of *Stk40*, which is in agreement with a recent finding that inducible induction of Ras at low levels promoted expression of marker genes for both trophectoderm and primitive endoderm lineages (29). However, they also found that trophectoderm markers predominated at higher levels of Ras (29). Thus, it seems that the effect of Ras activation is dependent on developmental and cellular contexts as well as its activation levels. FGF stimulation of the Erk/MAPK signaling pathway has been considered to be the primary stimulus for undifferentiated ESCs to exit the self-renewal state and enter a state responsive to inductive signals for germ-layer segregation as well as blastocyst-lineage formation in vivo (30, 31). However, pluripotent embryonic cells at various stages of development would respond to the FGF/RAS/Erk1/2 signal differentially and develop into distinct lineages. Toyooka et al. (32) reported that an undifferentiated ESC culture is a heterogeneous population containing subpopulations corresponding to the inner cell mass, epiblast, and primitive ectoderm. Therefore, the lineage to which ESCs would differentiate in response to activation of the FGF/RAS/Erk1/2 cascade may be dependent on the epigenetic setting of ESCs. Furthermore, the activity level of the FGF/RAS/Erk1/2 cascade would also play an important role in cell-fate specification, because the balance among various signaling pathways, such as leukemia-inhibitory factor, TGFβ superfamily members, and FGF signal, would exert a great influence on the fate of cells at a specific developmental stage.

In conclusion, our data reveal that the ESC master regulator Oct4 suppresses expression of *Stk40*, which plays an active role in induction of ESC differentiation into ExEn cells through the Erk/MAPK signaling pathway, partially through its direct interaction with Rcn2 (Fig. 5*E*). The study provides insights into molecular mechanisms by which Oct4 maintains ESCs in an undifferentiated state and identifies two proteins important for ExEn development. Further studies will greatly expand our understanding of ESC self-renewal and lineage-specific differentiation.

Materials and Methods

E14T, CGR8, and ZHBTc4 mouse ESCs (Austin Smith) and F9 EC cells (ATCC, Rockville, MD, USA) were grown under feeder-free conditions as previously described (13, 33). ESCs were transiently transfected with Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. For stable cell lines, the cells were transfected by electroporation and selected with antibiotics.

Additional materials and methods as well as PCR primers are provided in the *SI Materials and Methods*.

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