Stk40 deletion elevates c-JUN protein level and impairs mesoderm differentiation

Received for publication, February 8, 2019, and in revised form, May 7, 2019. Published, Papers in Press, May 15, 2019, DOI 10.1074/jbc.RA119.007840

Jing Hu1, Shuang Li1, Xiaozhi Sun1, Zhuoqing Fang9, Lina Wang1, Feng Xiao5, Min Shao9, Laixiang Ge5, Fan Tang1, Junjie Gu1, Hongyao Yu1, Yueshuai Guo8, Xuejiang Guo8, Bing Liao5,11, and Ying Jin1,3,2

From the 1Basic Clinical Research Center, Renji Hospital and Shanghai Key Laboratory of Reproductive Medicine, Department of Histoembryology, Genetics and Developmental Biology, Shanghai Jiao Tong University School of Medicine, 227 South Chongqing Road, Shanghai 200025, China; 2CAS Key Laboratory of Tissue Microenvironment and Tumor, Shanghai Institute of Nutrition and Health, CAS Center for Excellence in Molecular Cell Science, Shanghai Institutes for Biological Sciences, University of Chinese Academy of Sciences, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200032, China; 3School of Life Science and Technology, ShanghaiTech University, 100 Haise Road, Shanghai 201210, China; and 4State Key Laboratory of Reproductive Medicine, Department of Histology and Embryology, Nanjing Medical University, Nanjing 211166, China

This work was supported by National Natural Science Foundation of China Grants 31871373, 31730055, 31301015, and 31200980; Ministry of Science and Technology of China Grant 2016YFA010100; and Strategic Priority Research Program Grant XDB19020100 of the Chinese Academy of Sciences, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200032, China, 4CAS Key Laboratory of Tissue Microenvironment and Tumor, Shanghai Institute of Nutrition and Health, CAS Center for Excellence in Molecular Cell Science, Shanghai Institutes for Biological Sciences, University of Chinese Academy of Sciences, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200032, China, 3School of Life Science and Technology, ShanghaiTech University, 100 Haise Road, Shanghai 201210, China, and 4State Key Laboratory of Reproductive Medicine, Department of Histology and Embryology, Nanjing Medical University, Nanjing 211166, China

Edited by Joel M. Gottesfeld

Mesoderm development is a finely tuned process initiated by the differentiation of pluripotent epiblast cells. Serine/threonine kinase 40 (STK40) controls the development of several mesoderm-derived cell types, its overexpression induces differentiation of mouse embryonic stem cells (mESCs) toward the extraembryonic endoderm, and Stk40 knockout (KO) results in multiple organ failure and is lethal at the perinatal stage in mice. However, molecular mechanisms underlying the physiological functions of STK40 in mesoderm differentiation remain elusive. Here, we report that Stk40 ablation impairs mesoderm differentiation both in vitro and in vivo. Mechanistically, STK40 interacts with both the E3 ubiquitin ligase mammalian constitutive photomorphogenesis protein 1 (COP1) and the transcriptional regulator proto-oncogene c-Jun (c-JUN), promoting c-JUN protein degradation. Consequently, Stk40 knockout leads to c-JUN protein accumulation, which, in turn, apparently suppresses WNT signaling activity and impairs the mesoderm differentiation process. Overall, this study reveals that STK40, together with COP1, represents a previously unknown regulatory axis that modulates the c-JUN protein level within an appropriate range during mesoderm differentiation from mESCs. Our findings provide critical insights into the molecular mechanisms regulating the c-JUN protein level and may have potential implications for managing cellular disorders arising from c-JUN dysfunction.

Gastrulation is critical for early mammalian embryonic development, and one of its major tasks is to generate a mesodermal layer between the endoderm and ectoderm. Mesoderm development initiates with differentiation of pluripotent epiblast cells into the primitive streak, which then segregates into the mesoderm layer (1–3). This delicate process is coordinated by multiple key signaling pathways to ensure the correct formation of mesodermal tissues. Among them, bone morphogenesis protein (BMP)3 and WNT signaling pathways play profound roles to orchestrate mesoderm development (4–6). For instance, Wnt3-null mouse embryos lack the primitive streak and mesoderm formation (4). Moreover, WNT signaling is required for the generation of embryonic stem cell (ESC)-derived mesoderm, particularly for the expression of genes related to primitive streak formation and gastrulation in vivo (5). Furthermore, induced differentiation of pluripotent stem cells toward the mesodermal fate in monolayer cultures by manipulating WNT and BMP signaling pathways has been achieved in vitro (2, 3, 7), highlighting the decisive roles of the two pathways in mesoderm development. However, the molecular mechanisms regulating their activities in mesoderm formation remain incompletely understood.

The transcriptional regulator proto-oncogene c-Jun (c-JUN), encoded by Jun gene, acts as a subunit of the activating protein 1 (AP-1) family of transcription factors. By forming a homodimer or heterodimer with other members of the AP-1 family, c-JUN plays important roles in regulating cell proliferation as well as cell migration and oncogenic transformation (8–10). Forced expression of c-JUN in mouse ESCs (mESCs)

This is an open access article under the CC BY license.

3 The abbreviations used are: BMP, bone morphogenesis protein; STK40, serine/threonine kinase 40; c-JUN, transcriptional regulator proto-oncogene c-Jun; T, Brachyury; AP-1, activating protein 1; ESC, embryonic stem cell; JNK, c-JUN N-terminal kinase; UPS, ubiquitin–proteasome system; COP1, mammalian constitutive photomorphogenesis protein 1; GO, gene ontology; KO, knockout; CHIR, CHIR99021; MEF, mouse embryonic fibroblast; co-IP, coimmunoprecipitation; CHX, cycloheximide; Dox, doxycycline; DDB1, DNA damage–binding protein-1; TCF, T-cell factor; mESC, mouse ESC; HM, high mobility group; CEBPβ; CCAAT-enhancer–binding protein; β-HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; qRT-PCR, quantitative real-time PCR; TMT, tandem mass tag; ACN, acetonitrile; CDS, coding sequence; GST, glutathione S-transferase; DAPI, 4′,6-diamidino-2-phenylindole; DET1, de-etiolated-1.

This is an open access article under the CC BY license.
activated endoderm lineage–related transcriptional factors (Gataa4/Gata6) with the concomitant repression of Brachyury (T), a mesodermal marker gene (11), whereas the loss of c-JUN function in zebrafish inhibited ventral mesoderm induction (12), implying that the precisely balanced c-JUN level is crucial for mesoderm differentiation. A previous study showed that phosphorylated c-JUN (serine 63 and serine 73) could interact with the HMG-box transcription factor T-cell factor 4 (TCF4) to form protein complexes containing c-JUN, β-catenin, and TCF4 (13). A later study by Gan et al. (12) clearly showed that c-JUN is involved in the canonical WNT signaling, dependent on its phosphorylation. Moreover, multiple E3 ligases that add ubiquitin molecules on c-JUN have been unveiled, such as FBW7 (14), ITCH (15), and mammalian constitutive photomorphogenesis protein 1 (COP1) (16). COP1 is a RING-finger E3 ubiquitin ligase. It acts through two distinct regulatory mechanisms, either as an E3 ligase or an adaptor to recruit substrates to DET1-Cullin4 ubiquitin ligase complexes, mediating ubiquitination and degradation of target proteins, such as c-JUN, E26 transformation-specific (ETS) (17), and ETS variant (ETV) (18). c-JUN can bind COP1 directly through a conserved consensus sequence, VP(D/E), located at its C terminus (19). The mutation of the VP sequence into AA disrupts the association between c-JUN and COP1 (16, 19). Remarkably, Cop1 deficiency stimulates cell proliferation by means of elevating c-JUN protein levels in vivo during embryogenesis, and Cop1-hypomorphic mice are tumor-prone, in line with the inverse correlation between COP1 and c-JUN proteins in human prostate cancers (20). Therefore, the proper amount of c-JUN is essential for early embryo development, and the ubiquitin–proteasome system (UPS) acts as a potential pathway to reduce c-JUN protein levels.

Serine/threonine kinase 40 (Stk40) was originally identified as a direct target gene of OCT4, a pluripotency-associated transcription factor, in mESCs by our laboratory (21). Stk40-null mice die at the perinatal stage (22). Further studies unveiled that Stk40 deletion leads to disorders in adipogenesis (23), myogenesis (24), and erythropoiesis (25). Interestingly, all of these involved cell types are of mesodermal origin. Therefore, we speculated that Stk40 might participate in the control of early mesoderm development in mouse embryos. Recently, human STK40 was identified as a pseudokinase lacking the ATP-binding property (26). The same study also reported the interaction between STK40 and E3 ligase COP1 in human cells, although the functional consequence of the STK40–COP1 interaction remains unclear.

To address the question of whether STK40 plays a role in mesoderm development, we modified a previously published protocol for mesoderm induction from both wildtype (WT) and Stk40-knockout (KO) mESCs by activating BMP and WNT signaling sequentially (27) and discovered that the loss of Stk40 increased the steady-state level of c-JUN proteins and impeded mesoderm differentiation. Moreover, STK40 could facilitate COP1–c-JUN complex formation to regulate c-JUN protein levels and ensure proper mesoderm differentiation. Taken together, the current study uncovers an important role and regulatory mechanism of STK40 in the control of mesoderm differentiation from pluripotent cells.
Stk40 deletion impairs mesoderm differentiation

A

B

C

D

E

F

G

H

J. Biol. Chem. (2019) 294(25) 9959 –9972
Stk40 deletion impairs mesoderm differentiation

Figure 2. Stk40 deletion attenuates the WNT signaling activity during mesoderm differentiation from ESCs. A, the heat map of differentially expressed genes between WT and Stk40 KO cells. B, GO analyses of down-regulated and up-regulated genes, respectively, in Stk40 KO cells from the data sets of RNA-Seq in A. The DAVID method was applied to GO analyses, and enrichment levels of selected GO terms (p value < 0.05) are marked by −log10(p value). Missing values are shown as black boxes. C and D, the heat map of the mesoderm-related genes (C) and WNT signaling–related genes (D), which were down-regulated in Stk40 KO cells compared with WT cells at mesoderm differentiation day 3. Fold change levels of selected genes are marked by log2-fold change (p value < 0.05). TGF, transforming growth factor; MAPK, mitogen-activated protein kinase.

Figure 1. Stk40 deletion impairs mesoderm differentiation from ESCs. A, a schematic illustration of the in vitro mesoderm differentiation procedure. B, qRT-PCR analysis of mRNA levels of marker genes at indicated time points during mesoderm differentiation from WT and Stk40 KO mESCs. Data were normalized to the expression of Actb and are represented as -fold changes relative to those in undifferentiated ESCs. Error bars denote the means ± S.D. (n = 4 independent experiments); Student’s t test: *, p < 0.05; **, p < 0.01; ***, p < 0.001. C, representative Western blot analysis of STK40 protein levels during the differentiation process. DAPI was used to label the nuclei (blue). Scale bars represent 50 μm. E, representative results of immunofluorescence staining of OCT4 (green) and NANOG (red) in WT and Stk40 KO cells at the undifferentiated stage or differentiation days 1, 2, and 3. DAPI was used to label the nuclei (blue). Scale bars represent 50 μm. F, representative results of immunofluorescence staining of T (red) in WT and Stk40 KO cells at differentiation day 3. One colony is presented in the upper panel, and the lower panel shows colonies at the lower magnification. DAPI was used to label the nuclei (blue). Scale bars represent 50 μm. Similar results were obtained in at least three independent experiments.

To explore how STK40 could be associated with the WNT signaling pathway, we employed mass spectrometric analysis to find proteins with distinct levels between the WT and Stk40 KO mESCs. Data were chosen primarily due to their convenience in use as a cellular system. Interestingly, we found that AP-1 targets, such as DNMT1, CEBPβ, TGM2, FOSL2, APLS1, and JUNB, had higher protein amounts in Stk40 KO mESCs than in WT mESCs. AP-1 transcription factors, encompassing c-JUN, JUNB, JUND, c-FOS, and FRA-1, are known to control cellular proliferation, transformation, and death (8, 9, 33). Among AP-1 transcription factors, c-JUN plays critical roles in...
a broad range of developmental events in a dosage-dependent manner (33), and it was previously reported to participate in the modulation of canonical WNT signaling (12). Hence, we tested whether there was a difference in the c-JUN protein levels between WT and Stk40-KO MEFs. Indeed, the c-JUN protein level was obviously higher in Stk40-KO MEFs than in WT cells (Fig. 3C). To learn whether the c-JUN protein level would also be higher in Stk40-KO cells during mesoderm differentiation of ESCs, we measured both phosphorylated (Ser-63/Ser-73) and total c-JUN protein levels at 3, 6, 12, and 24 h post-CHIR addition after differentiation for 2 days. Consistently, higher total c-JUN protein levels were detected in Stk40-KO cells than in WT cells at all time points tested (Fig. 3D), although Stk40 KO did not alter the transcript level of c-Jun (Fig. 3F). In addition, the levels of phosphorylated c-JUN at serine residues 63 and 73 were also higher in Stk40-KO cells than in WT cells at most time points examined. However, there was not a discerned difference in protein levels of phosphorylated c-JUN N-terminal
kinases (JNKs) (Fig. 3D), which have been reported to phosphorylate c-JUN within its N terminus (34). Despite simultaneous increases in both total and phosphorylated c-JUN protein amounts, ratios of phosphorylated c-JUN/total c-JUN proteins for their relative levels at different time points decreased in Stk40-KO cells for both Ser-63 and Ser-73 phosphorylation as compared with that in WT cells post-CHIR treatment (Fig. 3E). These results indicate that STK40 could modulate the level of c-JUN proteins at a posttranscriptional level.

Given that WNT signaling was enriched in genes down-regulated by Stk40 KO (Fig. 2B) and that c-JUN was reported to participate in the regulation of WNT signaling (12), we conducted the TOP/FOPFlash luciferase reporter assay to determine the role of c-JUN in controlling WNT signaling activity and found that overexpression of c-JUN or its phospho-dead (SS-AA) mutant from serine to alanine at residues Ser-63/Ser-73 in 293FT cells reduced WNT signaling activity in the presence of CHIR (Fig. 3G), suggesting that ectopic expression of c-Jun could suppress the WNT signaling activity independently of c-JUN phosphorylation at Ser-63/Ser-73.

The aforementioned findings implied that STK40 might modulate WNT signaling activities through controlling c-JUN protein levels during mesoderm differentiation. To address this issue, we established a doxycycline (Dox)-induced c-Jun overexpression system in the GFP-Bry ESCs. Addition of Dox induced overexpression of c-JUN in c-Jun sequence—containing virus-infected cells but not in empty vector virus—infected cells (Fig. 3H). At differentiation day 3, the proportion of GFP-positive cells was lower in cells overexpressing exogenous c-Jun as compared with that in cells without exogenous c-Jun (Fig. 3I). Therefore, our results indicate that an abnormally high level of c-JUN has a suppressive effect on mesoderm differentiation from ESCs.

**STK40 interacts with c-JUN and facilitates the formation of c-JUN and COP1 protein complexes**

It is known that c-JUN proteins can be degraded through the UPS by several E3 ligases, such as COP1 (35), MEKK1 (36), FBW7 (37), and ITCH (38). Of note, STK40 was recently reported to bind to COP1 directly, possibly serving as a COP1 adaptor to recruit substrates (26). Hence, we anticipated that STK40 might control the c-JUN protein level by modulating the interaction between COP1 and c-JUN to promote c-JUN degradation. To test this hypothesis, we first validated the interaction between STK40 and COP1 as well as between STK40 and c-JUN by GST pulldown and communoprecipitation (co-IP) assays, respectively. GST-STK40 specifically bound to His-c-JUN and His-COP1 in vitro, respectively (Fig. 4A). As a negative control, GST alone was not able to associate with either His-c-JUN or His-COP1. We next examined which region(s) of STK40 was responsible for its interaction with c-JUN or COP1. To this end, we generated FLAG-tagged expression vectors containing the coding sequences for the three truncated forms of STK40: the lack of the N terminus (∆N), the lack of C terminus (∆C), and the kinase homolog domain (PK) (Fig. 4B). Then, HA-tagged c-Jun expression vectors were cotransfected into 293FT cells in combination with FLAG-tagged WT or truncated STK40 expression vectors. The lack of the C terminus on STK40 abrogated its interaction with c-JUN, whereas the absence of the N terminus augmented the association between STK40 and c-JUN (Fig. 4C). This observation suggested that the C terminus of STK40 was required for its interaction with c-JUN, whereas the N-terminal region might have a negative impact on the interaction between STK40 and c-JUN. Similarly, the interaction between STK40 and COP1 was abrogated by the deletion of the C-terminal region. However, their interaction was not affected by the absence of the N-terminal region of STK40 (Fig. 4D).

Posttranslational modifications could regulate protein interactions, and phosphorylation is perhaps one of the most studied modifications with respect to modulating binding affinity of proteins. To learn how the N-terminal region of STK40 affected its interaction with c-JUN, we examined phosphorylation sites within this region through prediction using the web tool DISPHOS (www.dabi.temple.edu/disphos) (39). Among the four predicted residues, the serine at residue 6 (Ser-6) had the highest scores (Fig. 4E). Hence, we investigated whether STK40 mutants of phospho-dead (S6A, the mutation from serine to alanine) and phosphomimic (S6E, the mutation from serine to glutamic acid) could impact the interaction between STK40 and c-JUN by co-IP assays. We found that the S6A STK40 mutant displayed an obviously stronger association with c-JUN than did WT STK40. In contrast, the S6E STK40 mutant had a similar interaction with c-JUN.

*Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.*
weaker interaction with c-JUN compared with WT STK40 (Fig. 4F). The result suggests that the phosphorylation of the N-terminal Ser-6 could interfere with the interaction between STK40 and c-JUN.

To address the question of whether STK40 could modulate the formation of COP1 and c-JUN complexes to control the c-JUN protein level, co-IP assays were conducted to determine the binding intensity between COP1 and c-JUN in the presence and absence of exogenous STK40, respectively. Overexpression of HA-tagged Stk40 enhanced the interaction between COP1 and c-JUN in 293FT cells (Fig. 4G, right panel, lane 4 versus lane 3). COP1 was previously shown to bind the VP motif of c-JUN, and a mutation of VP to AA disrupted the direct interaction between COP1 and c-JUN (16, 35). Consistently, our co-IP assay results showed that c-JUN(AA) could not interact with COP1 (Fig. 4G, right panel, lane 5). Interestingly, in the presence of exogenous STK40, a relatively weak interaction between COP1 and c-JUN(AA) was detected (Fig. 4G, right panel, lane 6).
STK40 deletion impairs mesoderm differentiation

panel, lane 6). Therefore, we propose that STK40 could enhance the interaction between COP1 and c-JUN.

**STK40 promotes c-JUN protein degradation partially through COP1**

Considering that Stk40 ablation led to elevated levels of c-JUN proteins and that STK40 facilitated the interaction between COP1 and c-JUN, we predicted that STK40 might accelerate c-JUN protein degradation. To test this idea, we blocked protein synthesis by cycloheximide (CHX) treatment to analyze c-JUN protein stability in WT and Stk40-KO MEFs. As expected, the protein stability of c-JUN was higher in Stk40-KO MEFs than in WT MEFs (Fig. 5A). Consistently, the reintroduction of Stk40 into Stk40-KO MEFs reduced steady-state levels of c-JUN protein in a Stk40 dosage-dependent manner (Fig. 5B). In contrast, the stability and amount of COP1 proteins were not affected by Stk40 expression levels (Fig. 5, A and B). These results suggest that STK40 participates in the control of c-JUN protein stability.

To gain experimental evidence that COP1 is involved in STK40-mediated control of c-JUN protein levels, we silenced the expression of Cop1 in MEFs using an shRNA interference approach. Western blotting results showed that knockdown of Cop1 increased c-JUN protein levels in both WT and Stk40-KO MEFs and that the c-JUN protein level was highest in Stk40-KO cells expressing the Cop1 shRNA, although there was a lower-fold change in c-JUN protein levels between Cop1-knockdown and control MEFs in the absence of Stk40 (Fig. 5C, lane 6 versus lane 2) than that in the presence of Stk40 (Fig. 5C, lane 5 versus lane 1). Of note, overexpression of Stk40-GFP fusion proteins decreased the amount of c-JUN proteins in Stk40-KO MEFs (Fig. 5C, lane 4 versus lane 2). However, the rescue effect of Stk40 overexpression was attenuated when Cop1 was silenced (Fig. 5C, lane 8 versus lane 6 and lane 8 versus lane 4), suggesting that STK40-mediated control of c-JUN protein level is, at least partially, dependent on COP1.

To determine the role of COP1 in the control of c-JUN protein amounts and mesoderm differentiation from ESCs, we silenced Cop1 expression by two sets of shRNAs specifically targeting Cop1, respectively, in the GFP-Bry ESCs, which were induced to mesoderm differentiation as described in Fig. 1A. At day 3 of differentiation, GFP-positive cells were analyzed by flow cytometry. The high efficiency of Cop1 knockdown was validated by Western blot analysis. Cells expressing Cop1 shRNAs had markedly higher c-JUN protein levels than control cells (Fig. 5D). Compared with cells expressing the control shRNA, the percentage of GFP-positive cells decreased dramatically in cells expressing Cop1 shRNAs (Fig. 5E). Therefore, COP1 plays an important role in tightly controlling c-JUN protein levels during mesoderm differentiation.

**Stk40 depletion impairs mesoderm differentiation and leads to c-JUN protein accumulation in vivo**

To determine whether STK40 participates in mesoderm development in vivo, we conducted whole-mount immunofluorescence staining of mouse embryos at the gastrulation stage (E7.0) when T-positive cells migrate from the posterior to anterior region to establish mesodermal and endodermal layers (30, 40). Antibodies against T and SOX2 were employed for the whole-mount staining to mark the mesodermal and ectodermal cells, respectively. Altogether, 10 WT and 17 Stk40-null embryos were subjected to immunostaining assays. Confocal imaging analyses demonstrated that T-positive cells distributed similarly in WT and Stk40 KO embryos, but the signal intensity of T was drastically reduced in Stk40-KO embryos. Statistically, eight of 10 WT and five of 17 Stk40-KO embryos displayed normal T signal intensities, whereas two of 10 WT and 12 of 17 Stk40-null embryos showed weak T signals. These observations suggest that STK40 is required for the proper differentiation of pluripotent cells into T-positive cells at the gastrulation stage. As a control, Stk40 ablation did not affect signal intensities of SOX2 staining significantly (Fig. 6A).

Due to the ubiquitous function of c-JUN in many cell types, we posited that STK40 might modulate c-JUN protein levels in a variety of tissues. We compared c-JUN protein levels in the limb, liver, and head between WT and Stk40-KO embryos at E13.5 and found that c-JUN protein levels were higher in the limb, liver, and head from Stk40-KO embryos than in WT counterparts, whereas there was no difference in the level of COP1 proteins in the limb, liver, and head between WT and Stk40-KO embryos (Fig. 6B). Therefore, STK40 might control the c-JUN protein level in multiple organs/tissues of mouse embryos.

**Discussion**

C-JUN is expressed in a wide range of cell types in developmental processes and is essential for normal mouse development (41). Moreover, high c-JUN abundance shows a tight correlation with the tumorigenesis of several cell lines (9, 42). Thus, it is of significance to address the question of how c-JUN levels are modulated. Here, we report that STK40 could be an important new regulator of c-JUN protein amount. Additionally, our study reveals that STK40 might control c-JUN protein turnover through modulating the formation of COP1 and c-JUN complexes. Functionally, we show that this STK40–COP1–c-JUN axis plays a role in mesoderm differentiation (Fig. 7).

Our previous studies showed that Stk40 ablation in mice leads to developmental disorders in multiple mesoderm-derived cell types (22–25). Thus, we speculated that STK40 might be involved in mesoderm development. Utilizing an in vitro model of mesoderm induction from mESCs, we explored the function of Stk40 in mesoderm differentiation at both the molecular and cellular levels. By analyzing the RNA-Seq data from WT and Stk40-KO cells at mesoderm differentiation day 3, we found that expression levels of some WNT family members were lower in Stk40-ablated cells than in WT cells, and some of these WNT family members are known to play critical roles in mesoderm development (4, 6, 43). Therefore, attenuated WNT signaling might be responsible for the impaired mesoderm differentiation caused by Stk40 KO. c-JUN has previously been reported to bridge TCF and Dvl, and its N-terminal phosphorylation is likely required in the canonical WNT signaling pathway. Functionally, c-JUN contributes to the induction of ventral mesoderm in zebrafish (12). In this study, we revealed that both phosphorylated and total c-JUN protein...
levels were elevated upon Stk40 ablation. However, ratios of the phosphorylated (Ser-63 or Ser-73)/total c-JUN levels were lower in Stk40 KO cells than in WT cells during mesoderm differentiation from ESCs, providing a possible explanation for the reduced WNT signaling activity in Stk40-deleted cells. Moreover, overexpression of c-Jun suppressed WNT signaling activity and decreased the percentage of T-positive mesoderm-like cell populations at day 3 of ESC differentiation. In line with
Stk40 deletion impairs mesoderm differentiation

Figure 6. Stk40 deletion impairs mesoderm development and enhances c-JUN abundance in vivo. A, representative whole-mount immunofluorescence staining images of T (green) and SOX2 (red) in WT and Stk40-KO embryos at E7.0. A normal T–staining image in a WT embryo and a weak T–staining image in a Stk40-KO embryo are shown. DAPI was used to label the nuclei (blue). Scale bars represent 250 μm. B, protein levels of COP1, STK40, and c-JUN in the limb, liver, and head of WT and Stk40-KO embryos at E13.5 were analyzed by Western blotting. The arrowhead indicates the specific signal of STK40. β-actin was used as a loading control. Samples were collected from three WT (WT-1, WT-2, and WT-3) and three Stk40-KO embryos (KO-1, KO-2, and KO-3), respectively.

Figure 7. The schematic illustration of how STK40 contributes to mesoderm induction from mESCs. STK40 facilitates the association between COP1 and c-JUN, likely leading to proteasomal degradation of c-JUN, and the proper amount of c-JUN ensures mesoderm differentiation. In the absence of Stk40, mesoderm induction from mESCs is impaired at least partially due to c-JUN protein accumulation and WNT signaling inhibition. Ub, ubiquitin.
our finding, Liu et al. (11) reported that overexpression of c-Jun in mESCs leads to the activation of certain endodermal marker genes with the concomitant repression of T. Collectively, we propose that the appropriate level of c-JUN, especially its phosphorylation state, participates in the control of mesoderm differentiation, probably through modulating the WNT signaling pathway.

COP1 was previously shown to recruit c-JUN to a multisubunit ubiquitin ligase complex containing DNA damage-binding protein-1 (DDB1), Cullin 4A, and regulator of Cullins-1 (ROC1) rather than acting as an E3 ligase to catalyze c-JUN ubiquitination directly (16). Here, we unveiled that overexpression of Stk40 enhanced the interaction between COP1 and c-JUN. The mutation of the VP motif of c-JUN abrogated the interaction between COP1 and c-JUN(AA). However, in the presence of exogenous STK40, the interaction between COP1 and c-JUN(AA) could be detected. Subsequently, c-JUN might be ubiquitinated by the multisubunit ubiquitin ligase complex (DET1–DDB1–Cullin 4A–RBX1) and degraded through the UPS. Moreover, we found one component of the ubiquitin ligase complex, DET1, in proteins potentially interacting with STK40 from a public database (BioPlex 2.0 data set) (44), supporting the possibility that STK40 might recruit the ubiquitin ligase complex to down-regulate c-JUN protein levels. Nevertheless, we could not exclude the possibility that COP1 might serve as an E3 ligase to add ubiquitin molecules on c-JUN directly in our context. More studies should be conducted to address the question of how STK40 precisely modulates c-JUN protein stability through COP1.

Taken together, our study suggests that STK40 promotes COP1 and c-JUN to form complexes, hence maintaining c-JUN proteins at an appropriate level and taking part in the regulation of mesoderm development (Fig. 7). The molecular mechanism described here not only extends our understanding of how COP1 regulates c-JUN protein stability but also uncovers STK40 and COP1 as new regulators for mesoderm differentiation. Additionally, given the well-established roles of c-JUN in cancers, our finding may provide potential therapeutic targets to treat diseases.

Experimental procedures

Animals

Animals were raised under the condition described previously and performed according to the guidelines approved by the Shanghai Jiao Tong University School of Medicine (22). The genotype of WT ([Stk40]+/+) and Stk40-KO ([Stk40]−/−) mice was determined as described previously (22).

Cell culture and differentiation

mESCs were derived from WT and Stk40-KO blastocysts (22) as described previously (45). The ESCs were maintained on inactivated MEF feeders in N2B27 medium with 2 mM GlutaMAX (Gibco), 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol (Sigma), 1000 units/ml leukemia inhibitory factor (Millipore), 3 μM CHIR99021, and 1 μM PD0325901 (STEMCELL Technologies) (46). Cell colonies were digested into single cells for regular passaging using Accutase (STEMCELL Technologies). For induced mesoderm differentiation from mESCs, cells were passaged onto gelatin-coated cell culture plates to remove feeder cells prior to the initiation of differentiation. After one passage, ESCs were dissociated with Accutase, and 1.5 × 10^6 cells were seeded in 1 well of 6-well plates precoated with gelatin and maintained in N2B27 medium with addition of 1% KnockOut Serum Replacement (Gibco), 0.1% BSA (Gibco), and 10 ng/ml BMP4 (R&D Systems) for 2 days. Then, the medium was replaced by a DMEM-based medium with 15% KnockOut Serum Replacement, 1 μM CHIR99021 (STEMCELL Technologies), and 0.5% DMSO (Sigma) for 1 additional day (27).

MEFs were generated from E13.5 mouse embryos and cultured in medium consisting of DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), Plat-E (Cell Biolabs) and 293FT (Invitrogen) cell lines were cultured in 293FT medium consisting of DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), 2 mM GlutaMAX (Gibco), 1 mM pyruvate (Gibco), and 0.1 mM nonessential amino acid (Gibco).

Stk40 deletion impairs mesoderm differentiation

For Stk40 and Cop1 knockdown assays, the DNA encoding sequences of shRNA specific to Stk40 and Cop1 were cloned into the plKO.1 plasmid, respectively. Sense sequences for Stk40, Cop1, and control RNAi were as follows: Stk40 shRNA-1, 5′-GGACCCCATCGGATAACTAT-3′; Stk40 shRNA-2, 5′-TGATACCGAGTACTCTT-3′; Cop1 shRNA-1, 5′-CCTTGGTATAACGACATAT-3′; Cop1 shRNA-2, 5′-GACAAATGGGGCATGCTAGA-3′; control shRNA, 5′-GTGCGCTGCTGGTGCCAAC-3′.

For overexpression assays, cDNA sequences of Stk40 or Stk40-GFP were inserted into pMXs plasmid. For inducible overexpression assays, the cDNA sequence of c-Jun was inserted into the pLVX-Tight-puro vector, and the Tet-On advanced inducible gene expression system was established according to the manufacturer’s user manual (Clontech). Viral packaging and transduction were performed as described previously (24).

RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA utilizing oligo(dT)_{18} and ReverTra Ace reverse transcriptase (Toyobo). qRT-PCR was performed using the ABI PRISM 7900 Fast Real-Time PCR system (Applied Biosystems) and SYBR Premix Ex Taq (Takara). The primer sequences for qRT-PCR are provided in Table S1.

RNA-Seq

Total RNA was extracted from WT and Stk40-0KO cells collected at mesoderm differentiation day 3 using TRIzol reagent. Sequencing libraries were prepared according to Illumina’s instructions. Paired-end RNA-Seq of 2 × 150-bp reads were sequenced on the Illumina HiSeq X Ten. Salmon software was used to calculate the samples’ transcripts per million and raw counts (47). DESeq2 (48) was then used to identify differentially expressed genes with the following setting: adjusted p value < 0.05. RNA-Seq raw data were submitted to
Stk40 deletion impairs mesoderm differentiation

GEO (accession number GSE125877), and processed data are provided in Table S2.

**Protein extraction and Western blot analysis**

Total protein extracted from cells or mouse tissues was prepared with radioimmune precipitation assay lysis buffer (1 mM EDTA, 1% Nonidet P-40, 50 mM Tris (pH 7.5), 150 mM NaCl, and 10% glycerol), and protein concentrations were determined using the Pierce BCA Protein Assay kit (Thermo Fisher) following the manufacturer’s instructions. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). Membranes were incubated with specific primary antibodies, and the antibody–protein complexes were visualized by horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and Pierce ECL Western Blotting Substrate (Thermo Fisher). Primary antibodies used for Western blotting are provided in Table S3.

**Mass spectrometry**

The mass spectrometric analysis was performed as described previously (49).

**Digestion and TMT labeling**—WT and Stk40-KO MEF samples were lysed using urea lysis buffer (8 u urea, 75 mM NaCl, 50 mM Tris (pH 8.2), 1% (v/v) EDTA-free protease inhibitor, 1 mM NaF, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and 1 mM phenylmethylsulfonyl fluoride), measured by the Bradford assay (50), reduced with 5 mM DTT (56 °C, 25 min), alkylated in 14 mM iodoacetamide for 30 min in the dark, and quenched by DTT. The protein mixtures were diluted in 50 mM Tris-HCl (pH 8.8), digested by Lys-C overnight at 25 °C, desalted using an Oasis HLB 1-ml Vac cartridge (Waters), and subjected to TMT six-plex labeling. After TMT labeling, all samples were combined and lyophilized.

**Strong cation-exchange fractionation**—The peptide mixture was fractionated using a cation ion-exchange column (2.1-mm internal diameter × 20 cm packed with Poros 10 S, Dionex, Sunnyvale, CA) with an UltraMate® 3000 HPLC system at a flow rate of 200 µl/min using buffer A (7 mM KH₂PO₄ (pH 2.65) and 30% ACN) and buffer B (7 mM KH₂PO₄, 350 mM KCl (pH 2.65), and 30% ACN) in the following gradient: 0–8% B in 0.1 min, 8–20% B for 15.9 min, 20–40% B for 20 min, 40–100% B for 1 min, 100% B for 5 min, 100–0% B in 1 min, 0% B for 25 min. Fractions were collected every 1.5 min.

**LC-MS³ analysis**—29 fractions were separated by a reverse-phase microcapillary column (0.075 × 150 mm, Acclaim® PepMap100 C₁₈ column, 3 µm, 100 A; Dionex) using 2% ACN and 0.5% acetic acid (buffer A) and 80% ACN and 0.5% acetic acid (buffer B) under a 222-min gradient (3% buffer B for 10 min, 3–5% buffer B for 3 min, 5–20% buffer B for 167 min, 20–36% buffer B for 15 min, 36–100% buffer B for 1 min, 100% buffer B for 7 min, 100–3% buffer B for 1 min, 3% buffer B for 18 min) and analyzed using an LTQ Orbitrap Velos (Thermo Finnigan, San Jose, CA) with a higher-energy collisional dissociation MS³ method.

**Protein identification and quantification**—The raw files were processed with MaxQuant software (version 1.2.2.5) using the UniProt mouse protein database (55,269 sequences). A common contaminants database was also included for quality control. The reverse strategy was used to estimate the false discovery rate. Except for TMT labels, carbamidomethyl (Cys) was set as fixed modification. Variable modifications were oxidation (Met) and acetyl (protein N terminus). The site, peptide, and protein false discovery rates were all set to 0.01. Protein quantification was calculated by combining MaxQuant identification results with a local modified Libra algorithm (49). For the identification of differentiated expressed proteins among groups, the cutoffs of -fold change and p value (Student’s t test) were set to 1.5 and 0.05, respectively.

The MS proteomics data have been deposited to the ProteomXchange Consortium (www.proteomexchange.org) via the PRIDE partner repository with the data set identifier PXD012579. The analyzed data are provided in Table S4.

**Immunoprecipitation and GST pulldown assays**

The full-length coding sequence (CDS) of Stk40, c-Jun, and Cop1 were cloned into the pcDNA 3.0 vector, respectively. The truncated forms and mutants of Stk40 and mutated c-Jun were also cloned into the pcDNA 3.0 vector, respectively. 293FT cells were seeded at 2 × 10⁶ cells in 6-cm dishes on day 1, and 2 μg of expression vectors each or an empty vector were transfected with Lipofectamine 2000 reagent according to the manufacturer’s instructions (Thermo Fisher Scientific) on day 2. After 24 h, cells were washed in the culture dish with cold PBS three times. Whole-cell lysates were prepared in co-IP buffer (50 mM Tris-HCl (pH 7.4) with 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitor mixture (Selleck)) and incubated with anti-FLAG M2 beads (Sigma) overnight at 4 °C. Then, beads were washed three times and eluted by 2× loading buffer for 10 min at 95 °C. For GST pulldown assays, the full-length Stk40 CDS was cloned into the pGEX-4T-1 plasmid, and the full-length c-Jun and Cop1 CDSs were cloned into the pET-30a(+) vector, respectively. GST and His fusion proteins were expressed in the BL21 strain and purified according to the manufacturers’ instructions (GE Healthcare and Novagen). GST pulldown assays were performed as described previously (21). The samples from immunoprecipitation and GST pulldown were analyzed by Western blotting.

**Flow cytometric analysis**

GFP-Bry mESCs were infected with lentiviral particles that express shRNA. After puromycin selection (1 µg/ml), the remaining cells were subjected to induced differentiation toward mesoderm, and both GFP-positive and -negative cells were analyzed by flow cytometry.

**Immunofluorescence staining**

ESCs and differentiated cells were fixed with 4% paraformaldehyde at room temperature for 10 min, and immunostaining was performed as described previously (24). The mouse embryos were collected at E7.0, and whole-mount immunostaining was performed according to the iDISCO protocol (51). Images were captured using a confocal microscope (Leica TCS SP8). Primary antibodies used for immunostaining are provided in Table S3.
Protein stability assays with CHX

CHX (Sigma-Aldrich) was dissolved in DMSO at a concentration of 100 mM. Cells were cultured with CHX at a concentration of 50 μM for the indicated time. Then, cells were collected and subjected to Western blotting to visualize protein levels.

TOP/FOPFlash assays

8×TOPFlash or 8×FOPFlash and pRL-TK vectors (Promega) in combination with or without the WT or mutant (SSA) c-Jun expression vector were transfected into 293FT cells with Lipofectamine 2000 reagent according to the manufacturer’s instructions (Thermo Fisher Scientific). pRL-TK serves as a control to normalize the transfection efficiency. Twenty-four hours later, cells were treated with CHIR99021 (1 μM) for an additional 6 h and then lysed for luciferase assays. Luciferase activities were evaluated using a Dual-Luciferase Assay kit (Promega) according to the manufacturer’s instructions. TOP/FOPFlash ratios are represented as mean ± S.D. (n = 3).

Statistical analysis

Data are presented as the mean ± S.D. from at least three independent experiments or samples. Statistical significance was analyzed by two-tailed Student’s t test and is shown as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Author contributions—J. H., B. L., and Y. J. conceptualization; J. H. formal analysis; J. H., H. Y., and B. L. investigation; J. H., S. L., F. X., L. G., F. T., J. G., H. Y., Y. G., X. G., and B. L. software; J. H. formal analysis; J. H., H. Y., and B. L. investigation; J. H., B. L., and Y. J. conceptualization; J. H. supervision.

Acknowledgment—We thank Dr. Gordon M. Keller for kindly providing the GFP-Bry mouse embryonic stem cell line.

References

1. Kojima, Y., Tam, O. H., and Tam, P. P. (2014) Timing of developmental events in the early mouse embryo. Semin. Cell Dev. Biol. 34, 65–75 CrossRef Medline
2. Gadue, P., Huber, T. L., Nostro, M. C., Kattman, S. J., and Keller, G. (2005) Germ layer induction from embryonic stem cells. Exp. Hematol. 33, 955–964 CrossRef Medline
3. Murry, C. E., and Keller, G. (2008) Differentiation of Embryonic Stem Cells to Clinically Relevant Populations: Lessons from Embryonic Development. Cell 132, 661–680 CrossRef Medline
4. Liu, P., Wakamiya, M., Shea, M., Albrecht, U., Behringer, R. R., and Bradley, A. (1999) Requirement for Wnt3 in vertebrate axis formation. Nat. Genet. 22, 361–365 CrossRef Medline
5. Lindsley, R. C., Gill, J. G., Kyba, M., Murphy, T. L., and Murphy, K. M. (2006) Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. Development 133, 3787–3796 CrossRef Medline
6. Kelly, O. G., Pinson, K., and Skarnes, W. C. (2004) The Wnt co-receptors Lrp5 and Lrp6 are essential for gastrulation in mice. Development 131, 2803–2815 CrossRef Medline
7. Wang, L., and Chen, Y. (2016) Signaling Control of Differentiation of Embryonic Stem Cells toward Mesoderm. J. Mol. Biol. 428, 1409–1422 CrossRef Medline
8. Angel, P., and Karin, M. (1991) The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim. Biophys. Acta 1072, 129–157 Medline
9. Shaulian, E., and Karin, M. (2002) AP-1 as a regulator of cell life and death. Nat. Cell Biol. 4, E131–E136 CrossRef Medline
10. Eferl, R., and Wagner, E. F. (2003) AP-1: a double-edged sword in tumorigenesis. Nat. Rev. Cancer 3, 859–868 CrossRef Medline
11. Liu, J., Han, Q., Peng, T., Meng, M., Wei, B., Li, D., Wang, X., Yu, S., Yang, J., Cao, S., Huang, K., Hutchins, A. P., Liu, H., Kuang, J., Zhou, Z., et al. (2015) The oncogene c-Jun impedes somatic cell reprogramming. Nat. Cell Biol. 17, 856–867 CrossRef Medline
12. Gan, X. Q., Wang, J. Y., Xi, Y., Wu, Z. L., Li, Y. P., and Li, L. (2008) Nuclear Dvl, c-Jun, β-catenin, and TCF form a complex leading to stabilization of β-catenin–TCF interaction. J. Cell Biol. 180, 1087–1100 CrossRef Medline
13. Nateri, A. S., Spencer-Dene, B., and Behrens, A. (2005) Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development. Nature 437, 281–286 CrossRef Medline
14. Wei, W., Jin, J., Schlissel, S., Harper, J. W., and Kaelin, W. G. (2005) The v-Jun point mutation allows c-Jun to escape GSK3-dependent recognition and destruction by the Fbw7 ubiquitin ligase. Cancer Cell 8, 25–33 CrossRef Medline
15. Gao, M., Labuda, T., Xia, Y., Gallagher, E., Fang, D., Liu, Y. C., and Karin, M. (2004) Jun turnover is controlled through INK-dependent phosphorylation of the E3 ligase Itch. Science 306, 271–275 CrossRef Medline
16. Wertz, I. E., O’Rourke, K. M., Zhang, Z., Dornan, D., Arnott, D., Deshaies, R. J., and Dixit, V. M. (2004) Human de-etiolated-1 regulates c-Jun by assembling a CUL4A ubiquitin ligase. Science 303, 1371–1374 CrossRef Medline
17. Vitari, A. C., Leong, K. G., Newton, K., Yee, C., O’Rourke, K., Liu, J., Phu, L., Vij, R., Ferrando, R., Couto, S. O., Mohan, S., Pandita, A., Hongo, I. A., Arnott, D., and Wertz, I. E., et al. (2011) COP1 is a tumour suppressor that causes degradation of ETS transcription factors. Nature 474, 403–406 CrossRef Medline
18. Baert, J., Monte, D., Verreman, K., Degen, C., Coutte, L., and De Launoit, Y. (2010) The E3 ubiquitin ligase complex component COP1 regulates PEA3 group member stability and transcriptional activity. Oncogene 29, 1810–1820 CrossRef Medline
19. Uljon, S., Xu, X., Durzynska, I., Stein, S., Adelantam, G., Marto, J. A., Pear, W. S., and Blacklow, S. C. (2016) Structural basis for substrate selectivity of the E3 ligase COP1. Structure 24, 687–696 CrossRef Medline
20. Migliorini, D., Bogaerts, S., Defever, D., Vyas, R., Denecker, G., Radaelli, E., Zwiolska, A., Depaepe, V., Hocheplied, T., Skarnes, W. C., and Marine, J. C. (2011) COP1 constitutively regulates c-Jun protein stability and functions as a tumor suppressor in mice. J. Clin. Investig. 121, 1329–1343 CrossRef Medline
21. Li, L., Sun, L., Gao, F., Jiang, J., Yang, Y., Li, C., Gu, J., Wei, Z., Yang, A., Lu, R., Ma, Y., Tang, F., Kwon, S. W., Zhao, Y. L., Li, J., et al. (2010) Stk40 links the pluripotency factor Oct4 to the Erk/MAPK pathway and controls extra-embryonic endoderm differentiation. Proc. Natl. Acad. Sci. U.S.A. 107, 1402–1407 CrossRef Medline
22. Yu, H., He, K., Li, L., Sun, L., Tang, F., Li, R., Ning, W., and Jin, Y. (2013) Deletion of Stk40 protein in mice causes respiratory failure and death at birth. J. Biol. Chem. 288, 5342–5352 CrossRef Medline
23. Yu, H., He, K., Wang, L., Hu, J., Gu, J., Zhou, C., Lu, R., and Jin, Y. (2015) Stk40 represses adipogenesis through translational control of CCAAT enhancer-binding proteins. J. Cell Sci. 128, 2881–2890 CrossRef Medline
24. He, K., Hu, J., Yu, H., Wang, L., Tang, F., Gu, J., Ge, L., Wang, H., Li, S., Hu, P., and Jin, Y. (2017) Serine/threonine kinase 40 (Stk40) functions as a novel regulator of skeletal muscle differentiation. J. Biol. Chem. 292, 351–360 CrossRef Medline
25. Wang, L., Yu, H., Cheng, H., He, K., Fang, Z., Ge, L., Cheng, T., and Jin, Y. (2017) Deletion of Stk40 impairs definitive erythropoiesis in the mouse fetal liver. Cell Death Dis. 8, e2722 CrossRef Medline
26. Durzynska, I., Xu, X., Adelantam, G., Ficarro, S. B., Marto, J. A., Siz, P., Uljon, S., and Blacklow, S. C. (2017) STK40 is a pseudokinase that binds the E3 ubiquitin ligase COP1. Structure 25, 287–294 CrossRef Medline

Stk40 deletion impairs mesoderm differentiation

8. Angel, P., and Karin, M. (1991) The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim. Biophys. Acta 1072, 129–157 Medline
9. Shaulian, E., and Karin, M. (2002) AP-1 as a regulator of cell life and death. Nat. Cell Biol. 4, E131–E136 CrossRef Medline
27. Chal, J., Oginuma, M., Al Tanoury, Z., Gobert, B., Sumara, O., Hick, A., Bousson, F., Zidouni, Y., Mursch, C., Moncuquet, P., Tassy, O., Vincent, S., Miyarani, A., Bera, A., Garnier, J. M., et al. (2015) Differentiation of pluripotent stem cells to muscle fiber to model Duchenne muscular dystrophy. *Nat. Biotechnol.* **33**, 962–969 CrossRef Medline

28. Kazanskaya, O., Glinka, A., de Barco Barrantes, I., Stannek, P., Niehrs, C., and Wymeersch, F. J., Huang, Y., Kleinjung, J., Oginuma, M., Al Tanoury, Z., Gobert, B., Sumara, O., Hick, A., Bousson, F., Zidouni, Y., Mursch, C., Moncuquet, P., Tassy, O., Vincent, S., Miyarani, A., Bera, A., Garnier, J. M., et al. (2015) Differentiation of pluripotent stem cells to muscle fiber to model Duchenne muscular dystrophy. *Nat. Biotechnol.* **33**, 962–969 CrossRef Medline

29. Gadue, P., Huber, T. L., Paddison, P. J., and Keller, G. (2006) Wnt and TGF-β signaling are required for the induction of an *in vitro* model of primitive streak formation using embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 16806–16811 CrossRef Medline

30. Gouti, M., Delile, J., Stamataki, D., Wymeersch, F. J., Huang, Y., Kleinjung, J., Wilson, V., and Briscoe, J. (2017) A gene regulatory network balances neural and mesoderm specification during vertebrate trunk development. *Dev. Cell* **41**, 243–261.e7 CrossRef Medline

31. Gouti, M., Delile, J., Stamataki, D., Wymeersch, F. J., Huang, Y., Kleinjung, J., Wilson, V., and Briscoe, J. (2017) A gene regulatory network balances neural and mesoderm specification during vertebrate trunk development. *Dev. Cell* **41**, 243–261.e7 CrossRef Medline

32. Hartl, M., Bader, A. G., and Bister, K. (2003) Molecular targets of the oncogenic transcription factor jun. *Curr. Cancer Drug Targets* **3**, 41–55 CrossRef Medline

33. Joehum, W., Passegué, E., and Wagner, E. F. (2001) AP-1 in mouse development and tumorigenesis. *Oncogene* **20**, 2401–2412 CrossRef Medline

34. Dérijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) INK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76**, 1025–1037 CrossRef Medline

35. Bianchi, E., Denti, S., Catena, R., Rossetti, G., Polo, S., Gasparian, S., Pugniano, S., Rogge, L., and Pardi, R. (2003) Characterization of human constitutive photomorphogenesis protein 1, a RING finger ubiquitin ligase that interacts with Jun transcription factors and modulates their transcriptional activity. *J. Biol. Chem.* **278**, 19682–19690 CrossRef Medline

36. Xia, Y., Wang, J., Xu, S., Johnson, G. L., Hunter, T., and Lu, Z. (2007) MEKK1 mediates the ubiquitination and degradation of c-Jun in response to osmotic stress. *Mol. Cell. Biol.* **27**, 510–517 CrossRef Medline

37. Nateri, A. S., Riera-Sans, L., Da Costa, C., and Behrens, A. (2004) The ubiquitin ligase SCFβTrCP antagonizes apoptotic JNK signaling. *Science* **303**, 1374–1378 CrossRef Medline

38. Fang, D., and Kerppola, T. K. (2004) Ubiquitin-mediated fluorescence complementation reveals that Jun ubiquitinated by Itch/AIP4 is localized to lysosomes. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 14782–14787 CrossRef Medline

39. Iakoucheva, L. M., Radivojac, P., Brown, C. J., O’Connor, T. R., Sikes, J. G., Obradovic, Z., and Dunker, A. K. (2004) The importance of intrinsic disorder for protein phosphorylation. *Nucleic Acids Res.* **32**, 1037–1049 CrossRef Medline

40. Downs, K. M., and Davies, T. (1993) Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. *Development* **118**, 1255–1266 Medline

41. Johnson, R. S., van Lingen, B., Papaioannou, V. E., and Spiegelman, B. M. (1993) A null mutation at the c-jun locus causes embryonic lethality and retarded cell growth in culture. *Genes Dev.* **7**, 1309–1317 CrossRef Medline

42. Herschman, H. R. (1991) Primary response genes induced by growth factors and tumor promoters. *Annu. Rev. Biochem.* **60**, 281–319 CrossRef Medline

43. Huelser, J., Vogel, R., Brinkmann, V., Erdmann, B., Birdmeyer, C., and Birchmeier, W. (2000) Requirement for β-catenin in anterior-posterior axis formation in mice. *J. Cell Biol.* **148**, 567–578 CrossRef Medline

44. Huttlin, E. L., Bruckner, R. J., Julio, A., Cannon, J. R., Baltzer, K., Colby, G., Gebreab, F., Gygi, S. M., Parzen, H., Szpyt, J., Tam, S., Zarraga, G., Pontano-Vaites, L., Swapup, S., et al. (2017) Architecture of the human interactome defines protein communities and disease networks. *Nature* **545**, 505–509 CrossRef Medline

45. Li, S., Xiao, F., Zhang, J., Sun, X., Wang, H., Zeng, Y., Hu, J., Tang, F., Gu, J., Zhao, Y., Jin, Y., and Liao, B. (2018) Disruption of OCT4 ubiquitination increases OCT4 protein stability and ASH2L-B-mediated H3K4 methylation promoting pluripotency acquisition. *Stem Cell Rep.* **11**, 973–987 CrossRef Medline

46. Ying, Q. L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P., and Smith, A. (2008) The ground state of embryonic stem cell self-renewal. *Nature* **453**, 519–523 CrossRef Medline

47. Patro, R., Duggal, G., Love, M. I., Iriarraz, R. A., and Kingsford, C. (2017) Salmon provides fast and bias-aware quantification of transcript expression. *Nat. Methods* **14**, 417–419 CrossRef Medline

48. Love, M. I., Huber, W., and Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550–550 CrossRef Medline

49. Wen, F. P., Guo, Y. S., Hu, Y., Liu, W. X., Wang, Q., Wang, Y. T., Yu, H. Y., Tang, C. M., Yang, J., Zhou, T., Xie, Z. P., Sha, J. H., Guo, X., and Li, W. (2016) Distinct temporal requirements for autophagy and the proteasome in yeast meiosis. *Autophagy* **12**, 671–688 CrossRef Medline

50. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254 CrossRef Medline

51. Renier, N., Wu, Z., Simon, D. J., Yang, J., Ariel, P., and Tessier-Lavigne, M. (2014) iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging. *Cell* **159**, 896–910 CrossRef Medline