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Title: **Smad4 regulates the nuclear translocation of Nkx2-5 in cardiac differentiation**

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Short title: **Smad4 regulates the nuclear localization of Nkx2-5**

1 **Abstract**

2 Bmp plays an important role in cardiomyocyte differentiation, but the function of Smad4 in
3 Bmp signaling remains elusive. Here, we show that disruption of the *Smad4* gene in cardiac
4 progenitors expressing *Sfrp5* led to embryonic lethality with hypoplastic heart formation.
5 Although the expression of *Nkx2-5* is regulated by Bmp signaling, expression of *Nkx2-5* was
6 weakly detected in the mutant heart. However, the nuclear translocation of Nkx2-5 was
7 impaired. Expression of *CK2* or *PPI1*, which could alter the phosphorylation status of the NLS
8 of Nkx2-5, was not affected, but Nkx2-5 was found to bind to Smad4 by
9 co-immunoprecipitation experiments. Introduction of *Smad4* into cells derived from *Smad4*
10 conditional knockout embryonic hearts restored the nuclear localization of Nkx2-5, and
11 exogenous Nkx2-5 failed to translocate into the nucleus of *Smad4*-depleted fibroblasts. These
12 results suggest that Smad4 plays an essential role in cardiomyocyte differentiation by
13 controlling not only transcription but also the nuclear localization of Nkx2-5.

1 Introduction

2 The heart is the first organ to establish a blood circulatory system required for the subsequent
3 development of other organs. Decades of research have revealed that intricate signaling
4 networks, including those involving members of the Wnt, Bmp, and Fgf pathways, play key
5 roles in heart development because perturbation of these secreted factors has been shown to
6 result in a wide range of defects in embryonic and adult hearts ¹⁻⁵. Understanding how
7 signaling networks regulate heart development is critical for elucidating mechanisms
8 underlying congenital heart diseases and for developing novel approaches in regenerative
9 cardiovascular medicine.

10 Bmp signaling is involved in fate decisions of multiple cell lineages of
11 cardiomyocytes through the transcriptional regulation of cardiac-specific transcription factors,
12 such as GATA4, Mef2c, and Nkx2-5 ⁶⁻¹³. Bmp signaling has been reported to directly regulate
13 transcription of the *Nkx2-5* gene, which encodes one of the earliest and most important
14 cardiac transcription factors ^{14,15}, given the presence of Smad binding sites in the enhancer
15 region of the *Nkx2-5* gene ¹³. Smad4 is a common-mediator Smad (Co-Smad). Once the Bmp
16 receptor is activated by ligand binding, Smad1/5/8 is phosphorylated and forms a complex
17 with Smad4. This complex then translocates into the nucleus and regulates the transcription of
18 target genes, suggesting that Smad4 plays a central role in Bmp signaling.

19 *Smad4*-deficient mouse embryos die by E7.5 due to impaired gastrulation, suggesting
20 that Smad4 is required for mesodermal formation ¹⁶. An analysis of cardiomyocyte-specific
21 *Smad4*-KO mice revealed that Smad4 functions in the proliferation and differentiation of
22 cardiomyocytes, as reflected in the thinner ventricles in both trabecular and compact hearts ¹⁷.
23 However, the function of Smad4 during the process of cardiomyocyte differentiation remains
24 unknown.

25 Wnt proteins are secreted proteins that play a key role in differentiation during the
26 development of many organs, including the heart ¹⁸⁻²⁰. In a previous study, we reported that
27 Sfrp5 (a decoy receptor of the Wnt ligand) is a marker of cardiac progenitor cells which form
28 the first heart field (FHF) and later contribute to the left ventricle (LV), both atria, sinus
29 venous (SV), and a part of the outflow tract (OFT), but not to the right ventricle (RV) ²¹. Thus,

1 mice that express Cre in *Sfrp5*-expressing cells could serve as a tool to generate mice that
2 specifically lack the *Smad4* gene in cardiac progenitor cells.

3 In the present study, we provide evidence that Smad4 forms a complex with Nkx2-5
4 to control the nuclear localization of Nkx2-5, and that this in turn promotes the differentiation
5 of cardiac progenitors and heart morphogenesis. To examine specific functions of Smad4 in
6 cardiogenesis, we generated a *Smad4*-conditional knock-out (*Smad4*-cKO) model by crossing
7 *Smad4*^{flxed/flxed} mice with *Sfrp5*-Cre mice. *Smad4*-cKO mice exhibited severe cardiac
8 hypoplasia with inhibited myocardial cell differentiation due to the down-regulation of
9 *Nkx2-5* expression and loss of nuclear localization of Nkx2-5. These findings suggest that, in
10 addition to a mediator of Bmp signaling, Smad4 could be responsible for cardiac
11 differentiation by binding to Nkx2-5 and regulating its nuclear localization.

14 Results

15 Smad4 is required for proper cardiogenesis

16 In order to explore the function of Smad4 in cardiomyocyte differentiation, we disrupted the
17 *Smad4* gene in *Sfrp5*-expressing cardiac progenitor cells. Specifically, *Sfrp5*^{Cre/Cre}; *Smad4*
18 ^{del/+} mice were crossed with *Smad4*^{flf} mice²¹. Compared to the littermate controls (*Sfrp5*^{Cre/+};
19 *Smad4*^{del/+}), *Smad4*-cKO mice (*Sfrp5*^{Cre/+}; *Smad4*^{del/del}) were embryonic lethal at around E8.5,
20 with a hypoplastic heart tube and edema of the pericardium (Fig. 1A). The looping process
21 has already begun but stopped in the middle of the process in the mutant hearts
22 (Supplementary Fig. S1). Histological analysis showed that both trabecular and compact
23 myocardial layers in the mutant heart were much thinner than those in the control. This
24 suggests that Smad4 is required for heart development.

25 Since Bmp signaling regulates many genes involved in myocardial differentiation, we
26 assessed the expression of downstream target genes by RT-PCR to gain insight into the
27 potential cause of the hypoplastic heart in *Smad4*-cKO embryos. Transcription factors
28 required in the myocardial differentiation process, including *Nkx2-5*, *Gata4*, and *Mef2c*, as
29 well as myocardial components, such as *Troponin-I* and *Anf*, were down-regulated in
30 *Smad4*-cKO hearts relative to control hearts. The expression of FHF markers, including *Hcn4*,

Tbx5, and *Sfrp5*, was decreased, and the expression of SHF markers, such as *Tbx1* and *Islet1*, were unaffected or increased, in *Smad4*-cKO hearts relative to control hearts (Fig. 1B). Immunofluorescent staining of cTnT also revealed impaired cardiac differentiation in *Smad4*-cKO hearts (Fig. 1C). These observations suggested that Bmp signaling through Smad4 is required for proper cardiogenesis by regulating the expression of cardiac-specific transcription factors and/or cardiomyocyte-specific components.

Smad4 regulates the expression and nuclear localization of Nkx2-5

Since many genes encoding cardiac-specific transcription factors, including *Nkx2-5*, are down-regulated in *Smad4*-cKO hearts, and *Nkx2-5* has been suggested to be regulated by Bmp signaling through Smad-binding regions²², we hypothesized that lack of *Nkx2-5* function might explain the impaired cardiac differentiation in *Smad4*-cKO embryos. In a previous study, *Nkx2-5*, a homeobox transcription factor, was reported to be a key factor in heart progenitor specification and differentiation by regulating the transcription of multiple cardiac-specific genes, including *Gja5* (Cx40), *Mef2c*, *Hey2*, *Nppa* (ANF), and *Isl1*¹⁵. Since Smad4 is required for *Nkx2-5* gene expression as it binds its upstream enhancer region *in vivo*¹³, we expected that *Nkx2-5* expression would be low or absent in *Smad4*-cKO hearts. However, *Nkx2-5* was expressed, albeit weakly (Fig. 1B), but detectable in *Smad4*-cKO hearts by *in situ* hybridization (ISH) (Fig. 2A). To confirm whether the *Nkx2-5* protein was translated, immunofluorescent staining was performed and revealed that *Nkx2-5* protein was weakly expressed (Fig. 2B, 2C). Interestingly, *Nkx2-5* was present mainly in the nuclei of control heart cells, while they were found outside the nuclei of *Smad4*-cKO heart cells (Fig. 2B, 2D), suggesting that the loss of Smad4 prevented the nuclear localization of *Nkx2-5*.

Impaired nuclear localization of Nkx2-5 in Smad4-cKO hearts is not a result of alterations in the phosphorylation status of Nkx2-5

The nuclear translocation of a transcription factor is often determined by the phosphorylation state of its NLS domain. *Nkx2-5* contains a highly conserved NLS domain, and phosphorylation of serine 163 (Ser163) by Casein kinase 2 (CK2), a ubiquitously expressed and constitutively active Ser/Thr protein kinase²³, is required for nuclear translocation. On

1 this basis, we hypothesized that CK2 expression could be induced by Bmp signaling through
2 Smad4 and examined this by qPCR and *in situ* hybridization. The expression level of *CK2α*
3 was decreased, but clearly present, in *Smad4*-cKO hearts relative to control hearts
4 (Supplementary Fig. S2A, S2B). We used *Csnk2a1* as a probe for the CK2 gene because the
5 CK2 catalytic subunit α, rather than its other regulatory subunits, is expressed dominantly in
6 the mouse embryonic heart ²⁴. Moreover, luciferase assays showed no activation of the
7 upstream region of the *CK2α* gene upon stimulation of Bmp signaling (Supplementary Fig.
8 S2C, S2D), suggesting that CK2 expression was not mainly regulated by Smad4.

9 We next determined whether excessive dephosphorylation of Nkx2-5 by the
10 β-isoform of protein phosphatase 1 (PP1β) might explain the impaired nuclear localization of
11 Nkx2-5. Since the regulatory subunit of PP1β, myosin phosphatase targeting subunit 1
12 (Mypt1), has been reported to be responsible for the nuclear exclusion of Nkx2-5 through
13 direct interaction ²⁵, we expected Mypt1 to be dominantly localized in the nuclei and thereby
14 prevent Nkx2-5 nuclear localization. However, in both control and *Smad4*-cKO hearts, Mypt1
15 was robustly expressed outside the nuclei (Supplementary Fig. S3). We also tested the
16 possibility that the expression of protein phosphatase 1 regulatory subunit 3c (*Ppp1r3c*), a
17 regulatory subunit of PP1 specifically expressed in the heart, might be increased in
18 *Smad4*-cKO hearts. Here too, no difference was noted in the level of expression of *Ppp1r3c*
19 by RT-PCR and ISH in *Smad4*-cKO and control hearts (Supplementary Fig. S4A, S4B),
20 suggesting that Smad4 is not essential for *Ppp1r3c* expression. Taken together, these findings
21 suggest that the phosphorylation state of the Nkx2-5 NLS might not be regulated by
22 Bmp/Smad4 signaling.

23 24 **Smad4 regulates Nkx2-5 nuclear localization via protein interaction**

25 Since phosphorylated Smad1/5/8 forms a complex with Smad4 to translocate into the nucleus
26 and regulate the transcription of target genes, we hypothesize that a protein complex
27 containing both Smad4 and Nkx2-5 might similarly be involved in the proper nuclear
28 translocation of Nkx2-5. Immunoprecipitation (IP) experiments using COS-7 cells
29 demonstrated an interaction between Smad4 and Nkx2-5 (Fig. 3A, 3B).

Smad4 has two functional domains, MH1 and MH2, both of which bind to distinct proteins, such as importins and exportins. To gain insight into whether Nkx2-5 binds to either of these domains, we performed additional IP experiments with MH1- or MH2-deleted Smad4. As shown in Figure 3B, the MH2 domain of Smad4 is responsible for binding to Nkx2-5 (Fig. 3B). These results indicate that Smad4 interacts with the MH-2 domain of Nkx2-5 to regulate Nkx2-5 nuclear translocation.

We then tested subcellular localization of Smad4 and Nkx2-5 by western blot analysis of subfractionated cellular compartments of COS-7 cells which were transfected with their expression vectors (Fig. 3C). Smad4 was found in both cytoplasm and nucleus, while Nkx2-5 was detected only in the nucleus, suggesting that nuclear distribution of Nkx2-5 is occurred not only in cardiomyocytes but also in the fibroblasts. This observation gave rise to a question if the distribution of Nkx2-5 is controlled by Smad4. We performed IP experiments with subcellular compartments (Fig. 3D). We found that Nkx2-5 forms a complex with full length of Smad4 in nuclei or MH2 domain in cytoplasm, suggesting that interaction of full length of Smad4, including MH1 domain for translocation and MH2 domain for binding to Nkx2-5, is required for nuclear localization.

Smad4 is essential for the nuclear localization of Nkx2-5

We further examined whether the introduction of Smad4 would be sufficient to restore the nuclear localization of Nkx2-5 by immunohistochemical analysis. *Smad4*-cKO heart cells which express exogenous Flag-tagged Smad4 showed a significant increase in the ratio of cells with Nkx2-5 localized in the nucleus (Fig. 4A, 4B), suggesting that Smad4 is required for the nuclear localization of Nkx2-5.

Since our results indicate that Smad4 seems required for the nuclear translocation of Nkx2-5, we next tested whether exogenous Nkx2-5 would localize to the nucleus of not only cardiomyocytes but also in other types of cells that express CK2 and Smad4, such as fibroblasts. In primary cultures of fibroblasts derived from *Smad4^{flf}* embryos transfected with an expression vector for *Nkx2-5*, Nkx2-5 was able to enter the nucleus. This suggests that the nuclear translocation of Nkx2-5 is not limited to a specific cell type. We also tested whether Smad4 is required for the nuclear translocation of Nkx2-5. After transfecting fibroblasts derived from *Smad4^{flf}* embryos with a Cre expression vector to abolish Smad4 expression, the

1 cells were further transfected with an expression vector for *Nkx2-5*. As shown in Figure 4C
2 and 4D, *Nkx2-5* failed to enter the nucleus in the absence of *Smad4* expression, suggesting
3 that *Smad4* is required for the nuclear localization of the *Nkx2-5*.

4 5 **Discussion**

6 In this study, we successfully generated mice that have a deletion of the *Smad4* gene in
7 cardiac progenitor cells by crossing *Sfrp5*-Cre mice with *Smad4^{flf}* mice. These mice showed
8 hypoplastic heart tubes with disruption of cardiomyocyte differentiation, suggesting that
9 *Smad4* is involved in cardiac development. Bmp/*Smad4* signaling has been suggested to
10 regulate the transcription of cardiac-specific transcription factors ⁸. We found that expression
11 of such transcription factors, especially those related to the FHF but not SHF, were
12 down-regulated in *Smad4*-cKO hearts (Fig. 1B). This suggests that disruption of the *Smad4*
13 gene in *Sfrp5*-expressing cells, which are predominantly cardiac progenitor cells for the FHF,
14 resulted in the downregulation of cardiac-specific genes and may explain the hypoplastic
15 heart tubes observed in *Smad4*-cKO embryos.

16 *Nkx2-5* transcription was not completely eliminated in *Smad4*-cKO hearts, suggesting
17 that Bmp signaling through *Smad4* is likely not required for the expression of *Nkx2-5*. Since
18 the heart tube itself was formed in *Smad4*-cKO embryos, Bmp/*Smad4* does not appear to be
19 required for cardiac cell fate decisions. One possible explanation is that the disruption of
20 *Smad4* was incomplete and residual *Smad4* could have activated the transcription of *Nkx2-5*.
21 However, *Sfrp5* is expressed in cardiac development before the *Nkx2-5* gene, and *Smad4*
22 disruption in the early epiblast using *Sox2*-Cre results in rudimentary heart formation and the
23 expression of cardiac-specific markers, including *Nkx2-5* ²⁶. Moreover, the loss of type1-Bmp
24 receptor in early mesoderm progenitors using *Mesp1*-Cre also results in the formation of a
25 hypoplastic heart ⁷. Thus, Bmp/*Smad4* signaling might not be a trigger for initial cardiac cell
26 specification.

27 Disruption of *Smad4* resulted in the failure of *Nkx2-5* to localize to the nucleus (Fig.
28 5) in both cardiomyocytes and fibroblasts. The NLS sequence in the homeobox of *Nkx2-5* is
29 required for nuclear translocation, which is controlled by regulating the phosphorylation state
30 of a serine residue near the NLS by CK2 and PP1 β ^{23,25}. In our experiments, however, the

1 expression of CK2 and PP1 β were not transcriptionally controlled by Bmp/Smad4. Since CK2
2 and PP1 β are extensively involved in fundamental metabolic processes, they may be
3 constitutively expressed ubiquitously to serve as housekeeping genes. This suggests that CK2
4 is likely always in a position to phosphorylate Nkx2-5, implying that phosphorylation of
5 Nkx2-5 is unlikely to be involved in cardiomyocyte fate decisions. Moreover, Smad4 is
6 ubiquitously expressed after the early definitive endoderm and mesoderm are formed ²⁷,
7 suggesting that the nuclear localization of Nkx2-5 should be inevitable once Smad4 is
8 synthesized. Consequently, nuclear localization might be a prerequisite for transcription
9 factors, such as Nkx2-5, to play their respective roles in cardiac differentiation.

10 Nkx2-5 interacted with the MH2 domain of Smad4. Smad4 is known to bind to
11 nuclear transporter proteins, such as Importin, via the MH1 domain, and bind to Exportin
12 (Chromosome Region Maintenance 1; CRM1) via its linker region ²⁸⁻³³. The MH2 domain,
13 however, is implicated in transcriptional activation and interactions with other Smad proteins
14 to form homo- and heteromeric complexes. This suggests that Nkx2-5 could behave similarly
15 to other Smad proteins with regard to its nuclear localization (Fig. 5). In the nucleus, we
16 speculate that the interaction between Smad4 and Nkx2-5 persists and allows for the
17 transcriptional regulation of genes involved in heart development. For instance, Nkx2-5
18 regulates its own continuous expression ^{13,34}. Moreover, GATA4 reportedly binds to an
19 upstream region of the *Nkx2-5* transcriptional start site and controls *Nkx2-5* expression in
20 cooperation with Smad1/4 ²². Some studies have also reported an interaction between GATA4
21 and Nkx2-5 ^{35,36}. Thus, after the Smad4-Nkx2-5 complex translocates into the nucleus, a
22 complex containing Smad4, Nkx2-5, and GATA4 may allow for the sustained expression of
23 Nkx2-5, thereby acting as a maintenance mechanism for mature cardiomyocytes.

24 Our results provide evidence that Smad4 is required for the transcription of several
25 cardiac-specific genes, despite the fact that initial tube formation occurs and residual Nkx2-5
26 expression remains in *Smad4*-cKO embryonic hearts. This suggests that while Bmp/Smad
27 signaling does not serve as a trigger for myocardial differentiation, it can promote cardiac
28 differentiation by controlling the nuclear localization of Nkx2-5 via Smad4. Hence, Smad4
29 could function not only as a mediator of Bmp signaling for the transcriptional regulation of

cardiac-specific genes but also as a regulator of the nuclear localization of Nkx2-5 to ensure proper cardiac differentiation.

Methods

Mice

The *Sfrp5*^{Cre/+}; *Smad4*^{del/del} mice with a C57Bl6 background were obtained by crossing *Sfrp5*^{Cre/Cre}; *Smad4*^{del/+} mice with *Smad4*^{floxed/floxed} mice, which carry the *Smad4* gene flanked by loxP recognition sites. Generation of the *Sfrp5*^{Cre/+} line was described previously²¹. The *Smad4*^{floxed/floxed} line was a gift from Dr. CX Deng³⁷. All procedures had local approval by the research ethics committee of Hiroshima University (A18-109-2) and conformed to the NIH Guide for the Care and Use of Laboratory Animals and all study carried out in compliance with the ARRIVE guidelines. Mouse tissues for all experiments were isolated after euthanizing mice by CO₂ asphyxiation.

Ex vivo rescue experiment

Hearts were dissected from *Smad4*-cKO embryos at E9.5. Cardiomyocytes were harvested by trypsinization with 0.1% (w/v) Trypsin (Nacalai Tesque, Kyoto, Japan, 32778-05) for 2 min, seeded onto 24 mm × 24 mm glass plates, and cultured in 6-well tissue culture plates. A plasmid containing *Smad4* cDNA (Flag-tagged) or an empty vector (pDEF) was transfected using 3.23% (w/v) Polyethylenimine (PEI Max, Cosmobio, Tokyo, Japan, 24765-2) on the following day. The immunofluorescent analysis was performed 24 hours after transfection to detect the cellular distribution of Nkx2-5.

Fibroblast cells were obtained from *Smad4*^{fl/fl} embryos using the same method described above and cultured in 6-well tissue culture plates. To delete *Smad4*, plasmids containing *Cre* cDNA were transfected into the fibroblasts twice using 3.23% (w/v) PEI.

Cell culture

COS-7 cells were maintained in DMEM (Life Technologies, Carlsbad, CA, 11995-065) containing 4.5 g/L D-glucose supplemented with 1 mM sodium pyruvate, 4 mM L-Glutamine,

and 10% (v/v) fetal bovine serum (CELLect, Kefar Sava, Israel, 2916754) at 37°C and 5% CO₂.

Transfection

COS-7 and fibroblast cells were transfected with expression plasmids, including Flag-tagged *Smad4*, *MH1*, or *MH2* (a gift from Dr. T. Imamura, Ehime University) and Myc-tagged *Nkx2-5* (a gift from Dr. Koshiba, Toyo University) using 3.23% (w/v) polyethyleneimine (PEI Max, Cosmobio, Tokyo, Japan, 24765-2) following the same steps described above for the rescue experiment.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed as described previously²¹. Embryos were dissected at E9.5 and fixed with 4% paraformaldehyde in PBS for durations of 1 h to overnight. After dehydration using a methanol series, embryos were rehydrated with 0.1% Tween 20 in PBS (PBST), bleached with 6% H₂O₂ in PBS, treated with Protease K, and then hybridized with digoxigenin (DIG)-labeled probes at 68°C overnight. Embryos were then washed with 5 × and 2 × SSC/50% formamide and PBST. To detect DIG-labelled probes, embryos were incubated with anti-DIG antibodies after treatment with blocking reagent for 1 h, and the signal was developed with BM purple AP substrate (Roche, Basel, Switzerland).

Real-time PCR

Real-time PCR was performed as previously described²¹. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA). Reverse transcription was performed with the ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). Real-time PCR was conducted using SYBR Premix Ex Taq II (Kapa Biosystems Inc., Wilmington, MA). Intensities of PCR products were measured and analyzed using Mini-Opticon (Bio-Rad, Hercules, CA). Amplification conditions were: 5 s at 95°C, 20 s at 60°C, and 15 s at 72°C for 49 cycles. *G3pdh* was amplified as an internal control. Primers used in the present study are shown in Supplementary Table S1.

Immunohistochemistry

Embryos were embedded in optimum cutting temperature (OCT) compound (Sakura Tissue-Tek, Tokyo, Japan, 4583) after fixation and cut into 10–14 μ m sections. After washing with PBS and blocking with 1% BSA in PBS for 1 h, sections were incubated with 500 \times diluted primary antibodies, including anti-Smad4 (Santa Cruz, Dallas, TX, sc-7966), anti-Mypt1 (Proteintech, Rosemont, IL, 22117-1-AP), anti-Flag (DYKDDDDK)-Tag (Sigma Aldrich, St. Louis, MO, F3165 or Cell Signaling Technology, Danvers, MA, 8146S), anti-Myc Tag (Cell Signaling Technology 2278S), anti-Nkx2-5 (Abcam, Cambridge, UK, ab35842 or ab97355), and anti-TnT (Thermo, Waltham, MA, MS-295-P0) at 4°C overnight. Sections were then incubated with 500 \times diluted secondary antibodies conjugated with Alexa Fluor 488 or 555 (Molecular Probes, Eugene, OR). Nuclei were stained with 4, 6-diamidino-2-phenylindole (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Nuclear and Cytoplasmic Protein Extraction

COS-7 cells, transfected expression vectors, were collected after 24hr, and then their nuclei and cytoplasm fractions were separated. Their containing proteins were harvested by using a NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, 78833) following the instruction protocols and stored at -80 °C for further use.

Western blotting

Cultured cells were washed with cold PBS and lysed on ice with Sample buffer (100 mM DTT, 50 mM Tris/HCl pH 8.0, and 0.1% (w/v) SDS). After centrifugation at 15,500 rpm for 10 min at 4°C, supernatant fractions were collected and analyzed by SDS-PAGE and immunoblotting.

Immunoprecipitation

Cultured cells were washed with cold PBS and lysed on ice with RIPA buffer (150 mM NaCl, 5 mM EDTA pH 8.0, 50 mM Tris/HCl pH 8.0, 1% (v/v) IGEPAL CA-630, 0.1% (w/v) SDS, and 0.5% (w/v) sodium deoxycholate) containing phenylmethylsulfonyl fluoride (PMSF, Tocris Bioscience, Bristol, UK) for 30 min. After centrifugation at 15,000 rpm for 10 min at

4°C, supernatants were collected and used for immunoprecipitation (IP). IP was performed with the Immunoprecipitation Starter Pack (GE Healthcare, Chicago, IL) according to the manufacturer's instructions, using 100 µl column resin conjugated with 5 µg anti-Flag antibody, 5 µg anti-Myc antibody, 5 µg anti-Smad4 antibody, 5 µg anti-Nkx2-5 antibody, or 100 µg IgG, and then analyzed by immunoblotting after SDS-PAGE. Signal detection was performed with the LuminoGraph I (ATTO, Amherst, NY, WSE-6100H-CSP) system.

Statistical analysis

Data are expressed as mean±SEM and were analyzed using one-way analysis of variance (ANOVA) with the Tukey–Kramer *post hoc* test for multiple comparisons or the nonparametric Mann-Whitney U test to compare two independent populations. Statistical analyses were performed using SPSS 20.0 software (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp. <https://www.ibm.com/analytics/spss-statistics-software>). **P*<0.05 was considered statistically significant.

Acknowledgments

We thank Dr. Fujii for his technical assistance and members of the Saga and Yoshizumi laboratories for their help. We also thank Dr. T. Imamura from Ehime University for sharing Flag-tagged *Smad4*, *MH1*, and *MH2*, Dr. Koshiba from Toyo University for the Myc-tagged *Nkx2-5* plasmid, and Dr. CX Deng from the National Institutes of Health for the *Smad4^{flf}* mice.

Funding

This study was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (16K08443). W. H. is financially supported by the Natural Science Foundation of Liaoning Province (2019-MS-386) and the Scientific Research Project of Liaoning Provincial Department of Education (JC2019007).

References

- 1 1 Srivastava, D. Making or breaking the heart: from lineage determination to morphogenesis. *Cell* **126**, 1037-1048, doi:S0092-8674(06)01150-0 [pii] 10.1016/j.cell.2006.09.003 (2006).
- 2 2 Rochais, F., Mesbah, K. & Kelly, R. G. Signaling pathways controlling second heart field development. *Circulation research* **104**, 933-942, doi:10.1161/CIRCRESAHA.109.194464 (2009).
- 3 3 Spater, D., Hansson, E. M., Zangi, L. & Chien, K. R. How to make a cardiomyocyte. *Development* **141**, 4418-4431, doi:10.1242/dev.091538 (2014).
- 4 4 Bruneau, B. G. The developmental genetics of congenital heart disease. *Nature* **451**, 943-948, doi:10.1038/nature06801 (2008).
- 5 5 Epstein, J. A. Franklin H. Epstein Lecture. Cardiac development and implications for heart disease. *N Engl J Med* **363**, 1638-1647, doi:10.1056/NEJMra1003941 (2010).
- 6 6 Brazil, D. P., Church, R. H., Suras, S., Godson, C. & Martin, F. BMP signalling: agony and antagonism in the family. *Trends Cell Biol* **25**, 249-264, doi:10.1016/j.tcb.2014.12.004 (2015).
- 7 7 Klaus, A., Saga, Y., Taketo, M. M., Tzahor, E. & Birchmeier, W. Distinct roles of Wnt/beta-catenin and Bmp signaling during early cardiogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 18531-18536, doi:10.1073/pnas.0703113104 (2007).
- 8 8 van Wijk, B., Moorman, A. F. & van den Hoff, M. J. Role of bone morphogenetic proteins in cardiac differentiation. *Cardiovascular research* **74**, 244-255, doi:10.1016/j.cardiores.2006.11.022 (2007).
- 9 9 Andree, B., Duprez, D., Vorbusch, B., Arnold, H. H. & Brand, T. BMP-2 induces ectopic expression of cardiac lineage markers and interferes with somite formation in chicken embryos. *Mechanisms of development* **70**, 119-131, doi:10.1016/S0925-4773(97)00186-X (1998).
- 10 10 Schlange, T., Andree, B., Arnold, H. H. & Brand, T. BMP2 is required for early heart development during a distinct time period. *Mechanisms of development* **91**, 259-270, doi:10.1016/S0925-4773(99)00311-1 (2000).
- 11 11 Zheng, M. *et al.* Bone morphogenetic protein2 enhances the expression of cardiac transcription factors by increasing histone H3 acetylation in H9c2 cells. *Mol Med Rep* **7**, 953-958, doi:10.3892/mmr.2013.1266 (2013).
- 12 12 Schultheiss, T. M., Burch, J. B. & Lassar, A. B. A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes & development* **11**, 451-462, doi:10.1101/gad.11.4.451 (1997).
- 13 13 Lien, C. L., McAnally, J., Richardson, J. A. & Olson, E. N. Cardiac-specific activity of an Nkx2-5 enhancer requires an evolutionarily conserved Smad binding site. *Dev Biol* **244**, 257-266, doi:10.1006/dbio.2002.0603 (2002).
- 14 14 Komuro, I. & Izumo, S. Csx: a murine homeobox-containing gene specifically expressed in the developing heart. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 8145-8149, doi:10.1073/pnas.90.17.8145 (1993).
- 15 15 Prall, O. W. *et al.* An Nkx2-5/Bmp2/Smad1 negative feedback loop controls heart progenitor specification and proliferation. *Cell* **128**, 947-959, doi:10.1016/j.cell.2007.01.042 (2007).

1 16 Sirard, C. *et al.* The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later
2 for anterior development of the mouse embryo. *Genes & development* **12**, 107-119,
3 doi:10.1101/gad.12.1.107 (1998).

4 17 Song, L. *et al.* Myocardial smad4 is essential for cardiogenesis in mouse embryos. *Circ. Res.*
5 **101**, 277-285, doi:10.1161/CIRCRESAHA.107.155630 (2007).

6 18 Gessert, S. & Kuhl, M. The multiple phases and faces of wnt signaling during cardiac
7 differentiation and development. *Circulation research* **107**, 186-199,
8 doi:10.1161/CIRCRESAHA.110.221531 (2010).

9 19 Rao, T. P. & Kuhl, M. An updated overview on Wnt signaling pathways: a prelude for more.
10 *Circulation research* **106**, 1798-1806, doi:10.1161/CIRCRESAHA.110.219840 (2010).

11 20 Marinou, K., Christodoulides, C., Antoniadis, C. & Koutsilieris, M. Wnt signaling in
12 cardiovascular physiology. *Trends Endocrinol Metab* **23**, 628-636,
13 doi:10.1016/j.tem.2012.06.001 (2012).

14 21 Fujii, M. *et al.* Sfrp5 identifies murine cardiac progenitors for all myocardial structures except
15 for the right ventricle. *Nature communications* **8**, 14664, doi:10.1038/ncomms14664 (2017).

16 22 Brown, C. O., 3rd *et al.* The cardiac determination factor, Nkx2-5, is activated by mutual
17 cofactors GATA-4 and Smad1/4 via a novel upstream enhancer. *The Journal of biological*
18 *chemistry* **279**, 10659-10669, doi:10.1074/jbc.M301648200 (2004).

19 23 Kasahara, H. & Izumo, S. Identification of the In Vivo Casein Kinase II Phosphorylation Site
20 within the Homeodomain of the Cardiac Tissue-Specifying Homeobox Gene Product
21 Csx/Nkx2.5. *Mol. Cell. Biol.* **19**, 526-536, doi:10.1128/mcb.19.1.526 (1999).

22 24 Lou, D. Y. *et al.* The alpha catalytic subunit of protein kinase CK2 is required for mouse
23 embryonic development. *Molecular and cellular biology* **28**, 131-139,
24 doi:10.1128/MCB.01119-07 (2008).

25 25 Ryan, T. *et al.* Myosin phosphatase modulates the cardiac cell fate by regulating the
26 subcellular localization of Nkx2.5 in a Wnt/Rho-associated protein kinase-dependent
27 pathway. *Circulation research* **112**, 257-266, doi:10.1161/CIRCRESAHA.112.275818 (2013).

28 26 Chu, G. C., Dunn, N. R., Anderson, D. C., Oxburgh, L. & Robertson, E. J. Differential
29 requirements for Smad4 in TGFbeta-dependent patterning of the early mouse embryo.
30 *Development* **131**, 3501-3512, doi:10.1242/dev.01248 (2004).

31 27 Luukko, K., Ylikorkala, A. & Makela, T. P. Developmentally regulated expression of Smad3,
32 Smad4, Smad6, and Smad7 involved in TGF-beta signaling. *Mechanisms of development* **101**,
33 209-212, doi:10.1016/s0925-4773(00)00556-6 (2001).

34 28 Pierreux, C. E., Nicolas, F. J. & Hill, C. S. Transforming growth factor beta-independent
35 shuttling of Smad4 between the cytoplasm and nucleus. *Mol. Cell. Biol.* **20**, 9041-9054,
36 doi:10.1128/mcb.20.23.9041-9054.2000 (2000).

37 29 Baas, R. *et al.* Quantitative Proteomics of the SMAD (Suppressor of Mothers against
38 Decapentaplegic) Transcription Factor Family Identifies Importin 5 as a Bone Morphogenic
39 Protein Receptor SMAD-specific Importin. *J. Biol. Chem.* **291**, 24121-24132,
40 doi:10.1074/jbc.M116.748582 (2016).

41 30 Braun, D. A. *et al.* Mutations in nuclear pore genes NUP93, NUP205 and XPO5 cause
42 steroid-resistant nephrotic syndrome. *Nat. Genet.* **48**, 457-465, doi:10.1038/ng.3512 (2016).

1 31 Xiao, Z., Latek, R. & Lodish, H. F. An extended bipartite nuclear localization signal in Smad4 is
2 required for its nuclear import and transcriptional activity. *Oncogene* **22**, 1057-1069,
3 doi:10.1038/sj.onc.1206212 (2003).

4 32 Baburajendran, N., Jauch, R., Tan, C. Y., Narasimhan, K. & Kolatkar, P. R. Structural basis for
5 the cooperative DNA recognition by Smad4 MH1 dimers. *Nucleic Acids Res.* **39**, 8213-8222,
6 doi:10.1093/nar/gkr500 (2011).

7 33 Makkar, P., Metpally, R. P., Sangadala, S. & Reddy, B. V. Modeling and analysis of MH1
8 domain of Smads and their interaction with promoter DNA sequence motif. *J. Mol. Graph.*
9 *Model.* **27**, 803-812, doi:10.1016/j.jmglm.2008.12.003 (2009).

10 34 He, A., Kong, S. W., Ma, Q. & Pu, W. T. Co-occupancy by multiple cardiac transcription factors
11 identifies transcriptional enhancers active in heart. *Proc. Natl. Acad. Sci. U. S. A.* **108**,
12 5632-5637, doi:10.1073/pnas.1016959108 (2011).

13 35 Jumppanen, M. *et al.* Synthesis, Identification, and Structure-Activity Relationship Analysis of
14 GATA4 and NKX2-5 Protein-Protein Interaction Modulators. *Journal of medicinal chemistry*
15 **62**, 8284-8310, doi:10.1021/acs.jmedchem.9b01086 (2019).

16 36 Kinnunen, S. M. *et al.* Cardiac Actions of a Small Molecule Inhibitor Targeting GATA4-NKX2-5
17 Interaction. *Scientific reports* **8**, 4611, doi:10.1038/s41598-018-22830-8 (2018).

18 37 Yang, X., Li, C., Herrera, P. L. & Deng, C. X. Generation of Smad4/Dpc4 conditional knockout
19 mice. *Genesis* **32**, 80-81 (2002).

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Figure legends

Figure 1. Disruption of the *Smad4* gene in *Sfrp5*-expressing cells leads to hypoplastic heart formation and impaired cardiac differentiation.

(A, B) Gross appearance (A) and Hematoxylin and Eosin staining (B) of sagittal sections of *Smad4* mutant (*Sfrp5*^{Cre/+}; *Smad4*^{del/del}) and littermate control (*Sfrp5*^{Cre/+}; *Smad4*^{del/+}) embryos dissected at E9.5. Scale bar=100 μ m. (C) Relative gene expression levels of key factors involved in cardiogenesis. n=9 and 7 for control and *Smad4*-cKO, respectively. Data are presented as mean \pm SEM. (D) Immunofluorescent staining for cTnT (green) in control and *Smad4* mutant embryos at E9.5. Samples were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) to visualize nuclei (blue). Scale bar=50 μ m.

Figure 2. Lack of nuclear localization of Nkx2-5 in *Smad4*-cKO embryonic hearts.

(A) Whole-mount ISH of control and *Smad4*-cKO embryos with a probe for *Nkx2-5* at E9.5. (B) Immunofluorescent staining of sagittal sections of control and *Smad4*-cKO embryos at E9.5 using anti-Nkx2-5 antibodies (red) and DAPI for detection of nuclei (blue). Arrows indicate the lack of nuclear localization of Nkx2-5. H, heart. Scale bar=100 μ m. (C and D) Percentage of Nkx2-5 protein distributing cell in control and *Smad4*-cKO embryonic hearts (C) and the percentage of cells showing nuclear localization of Nkx2-5 in control and *Smad4*-cKO embryonic hearts (D). Data were collected from 10 sections for each embryo (n=3 and 3 for control and *Smad4*-cKO, respectively). Data are presented as mean \pm SEM, ***P*<0.01 (Mann-Whitney U test).

Figure 3. Interaction between Nkx2-5 and Smad4 via the MH2 domain.

(A) Myc-tagged Nkx2-5 was co-immunoprecipitated with Flag-tagged Smad4 and detected using anti-Nkx2-5- and anti-Myc-antibodies. (B) Flag-tagged Smad4, and MH1- and MH2-deleted versions, were co-immunoprecipitated with Myc-tagged Nkx2-5 and detected using anti-Smad4 and Flag-antibodies. Nkx2-5 interacts with the Flag-tagged MH2 domain but not the MH1 domain. (C) Subcellular distribution of Flag-tagged Smad4 (upper) and Myc-tagged Nkx2-5 (lower) was determined by western blotting for extracts subcellular fractionated from cytoplasm or nuclei. (D) Interaction of Myc-Nkx2-5 to Flag-Smad4-full length, -MH1, or

-MH2 is tested by Co-immunoprecipitation of extracts subcellular fractionated from cytoplasm or nuclei.

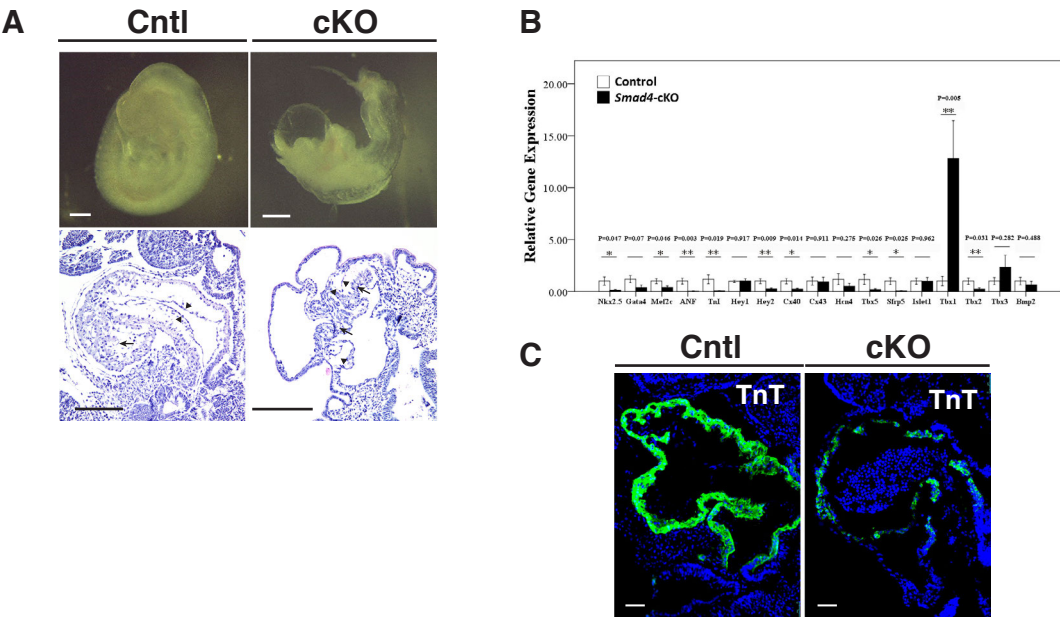
Figure 4. Introduction of *Smad4* rescues the nuclear localization of Nkx2-5 in mutant cells and depletion of the *Smad4* gene blocks the nuclear translocation of Nkx2-5 in fibroblast cells.

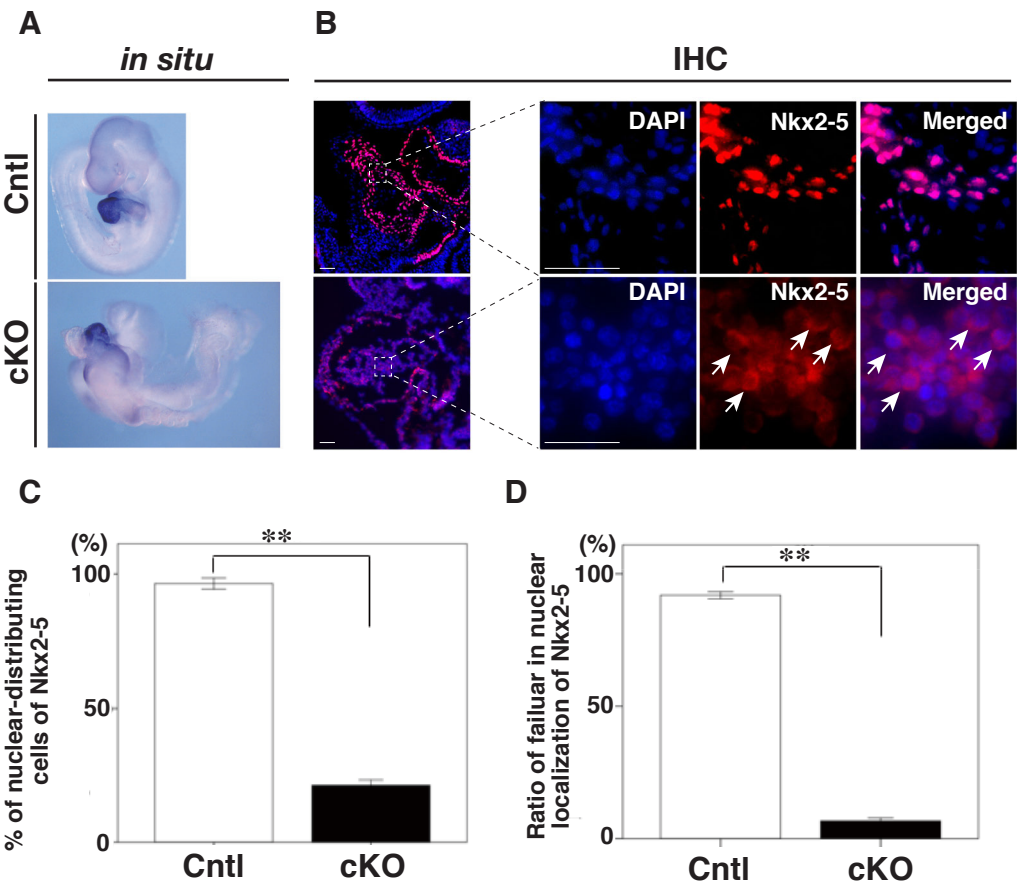
(A) Double immunohistochemistry with anti-Nkx2-5 (red) and anti-Flag (green) antibodies. Cells from *Smad4*-cKO embryonic hearts were transfected with an empty vector (pDEF) as a control (upper panels) or Flag-tagged Smad4 expression vector (lower panels). Arrows show the failure of Nkx2-5 to localize to the nucleus. DAPI was used to stain nuclei. (B) Percentage of cells showing nuclear localization of Nkx2-5. Nuclear localization of Nkx2-5 was significantly increased in the presence of Smad4, compared to cells transfected with empty vector. Data were collected from 20 slides for each experiment (n=4 and 4 for control and *Smad4*-cKO, respectively). Data are presented as mean±SEM. **P*<0.01 (Mann-Whitney U test). (C) Double immunohistochemistry with anti-Myc (red) and anti-Smad4 (green) antibodies. Fibroblast cells from *Smad4*^{ff} embryos were transfected with the Myc-Nkx2-5 expression vector and showed nuclear localization of Nkx2-5. After depleting Smad4 by transfecting fibroblasts with Cre expression vector twice, Nkx2-5 was no longer localized to the nucleus. DAPI was used to stain nuclei. (D) The proportion of non-nuclear localization of Nkx2-5 in control (transfected with Myc-Nkx2-5) and *Smad4*-cKO (transfected with Myc-Nkx2-5 after two rounds of Cre expression vector transfection) cells. Data were collected from 20 slides for each experiment (n=3 and 3 for control and *Smad4*-cKO, respectively). Data are presented as mean±SEM. ***P*<0.01 (Mann-Whitney U test).

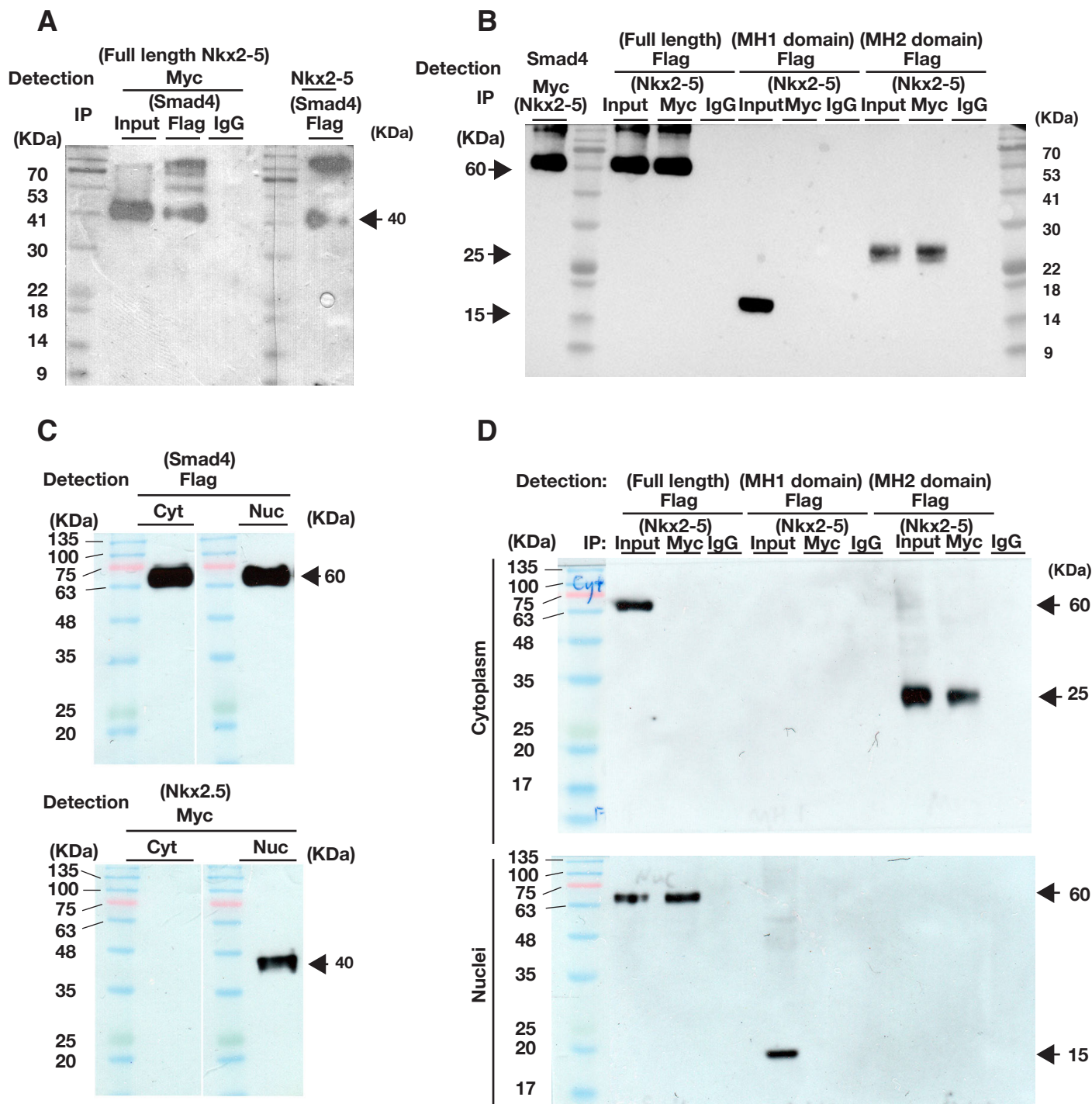
Figure 5. Schematic of Smad4 regulation of cardiac-specific gene expression and nuclear localization of Nkx2-5.

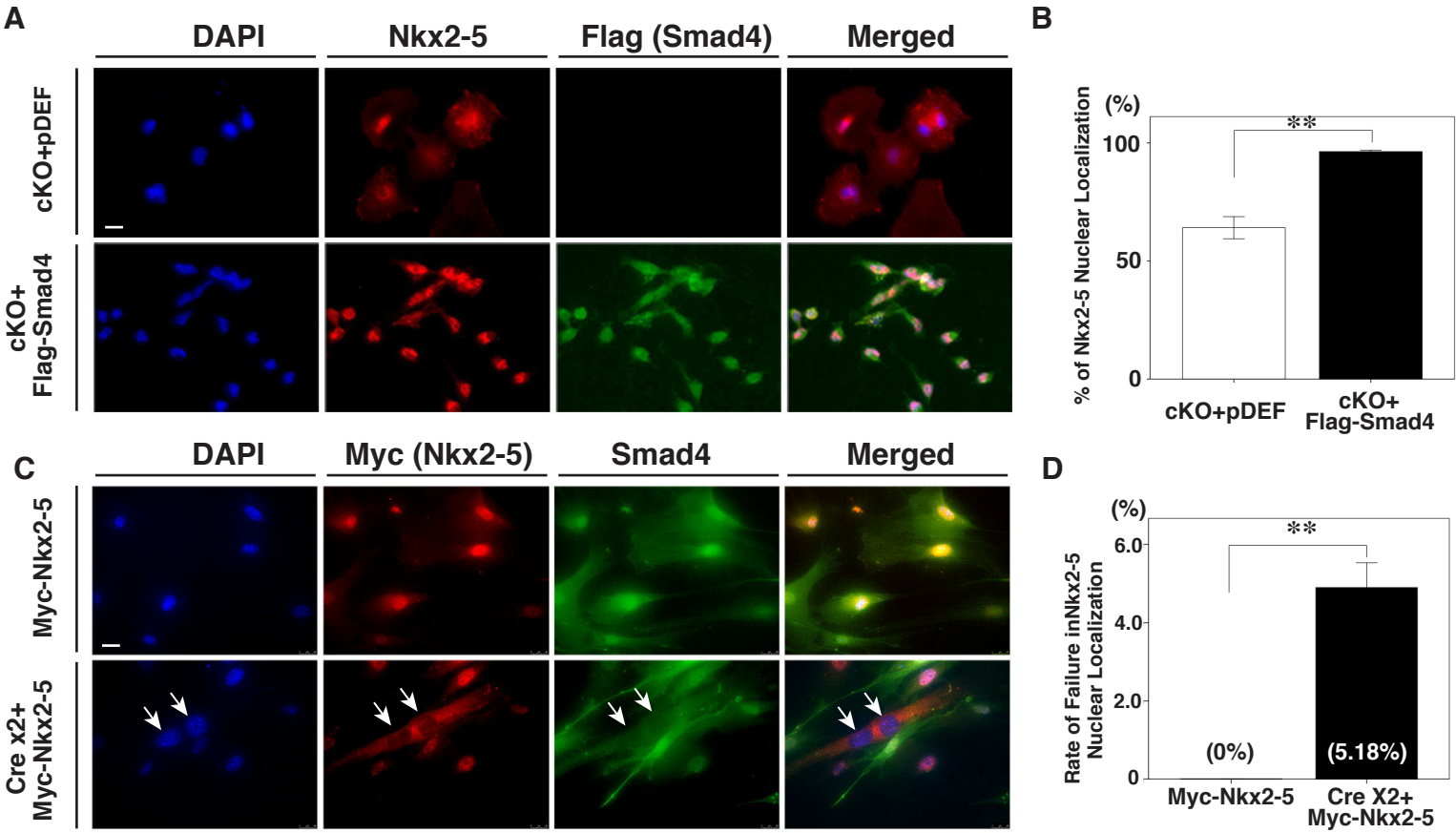
Bmp activates its receptor, which then phosphorylates the Smad1/5/8 complex. In turn, the Smad1/5/8 complex binds to Smad4, which subsequently translocates into the nucleus to enhance *Nkx2-5* transcription. Nkx2-5 protein is phosphorylated by CK2 and forms a protein complex with Smad4. This complex translocates into the nucleus and regulates *Nkx2-5*

- 1 transcription, thereby maintaining Nkx2-5 expression. Through this mechanism, Smad4 plays
- 2 a decisive role in cardiac development and myocardial differentiation.

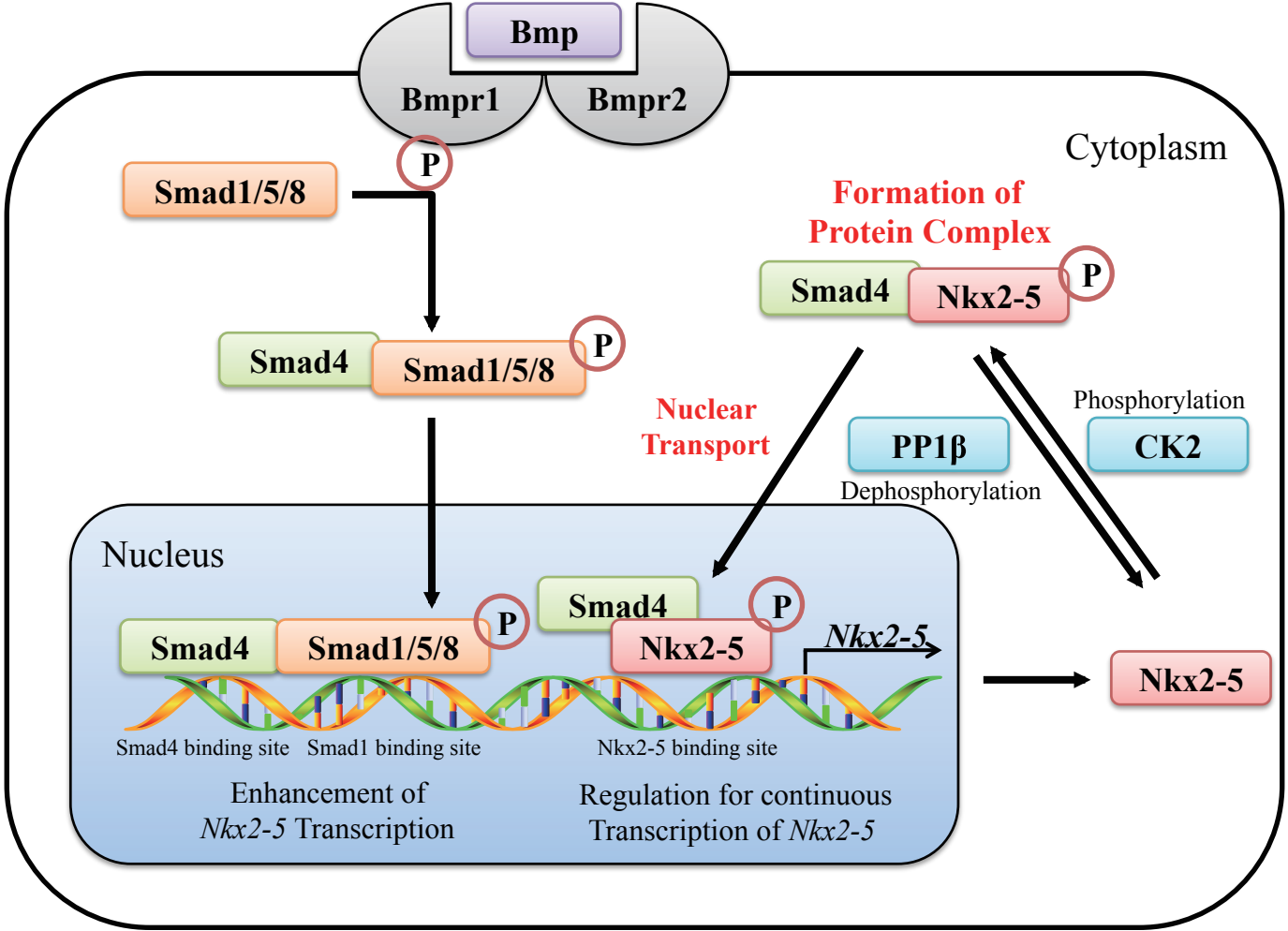








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Figure 5



Supplementary information

Supplementary Figure S1. Morphology of *Smad4*-cKO and littermate control embryos.

Compared to the littermate control (*Sfrp5*^{Cre/+}; *Smad4*^{del/+}), *Smad4*-cKO (*Sfrp5*^{Cre/+}; *Smad4*^{del/del}) hypoplastic heart tubes were formed with a clearly flawed looping shape in the severe phenotypes. Scale bar=200 μ m.

Supplementary Figure S2. *CK2* may not be a direct downstream target of *Smad4*.

(A) Relative expression of *CK2* in control and mutant embryonic hearts at E9.5 (n=9 and 7 for littermate control and *Smad4*-cKO, respectively). Data are presented as mean \pm SEM. **P*<0.05 (Mann-Whitney U test). (B) Whole-mount ISH analysis using a *CK2* antisense probe in control and *Smad4*-cKO embryos at E9.5. (C) Schematic of reporter plasmids used in the luciferase assay. The genomic region of the *CK2* gene, which contains prospective *Smad4*-binding sites upstream of the transcription starting site of *CK2*, was inserted into the Luc reporter vector, pGL-basic (*CK2* -Pst1-Luc and *CK2* -EcoR1-Luc). (D) Luciferase assay. In order to clarify the relationship between Bmp signaling and *CK2*, Alk6KR (dominant-negative form), Alk6QD (activated form), *Smad5*, and/or empty vector (pDEF) were transfected together with reporter plasmids into NIH3T3 cells as indicated. Statistical analysis was performed by one-way ANOVA, with Tukey–Kramer *post hoc* analysis for multiple comparisons. n=3 for each group. Data are presented as mean \pm SEM.

Supplementary Figure S3. Mypt1 expression is not affected in *Smad4*-cKO hearts.

Immunofluorescent staining using anti-Mypt1 (green) antibody on sagittal sections of control and *Smad4* mutant embryos. DAPI was used to stain nuclei.

Supplementary Figure S4. Bmp signaling via Smad4 may not be essential for *Ppp1r3c* expression.

(A) Relative expression of *Ppp1r3c* in littermate control and *Smad4*-cKO embryonic hearts at E9.5. n=9 and 7 for control and *Smad4*-cKO, respectively. Data are presented as mean±SEM. (Mann-Whitney U test). (B) Whole-mount ISH with the probe for *Ppp1r3c*. Signal was detected in the hearts of control and *Smad4*-cKO embryos.

Supplementary Figure S5. Raw images at different exposure times for Figure 3A.

The immunoblotted membrane was contacted with a film in 5 min (A), 1 min (B), or 30 sec (C). Then the film was developed and scanned.

Supplementary Figure S6. Raw images at different exposure times for Figure 3B.

The immunoblotted membrane was directly scanned for 1 min (A), 5 min (B), 10 min (C), or 20 min (D).

Supplementary Figure S7. Raw images at different exposure times for Figure 3C.

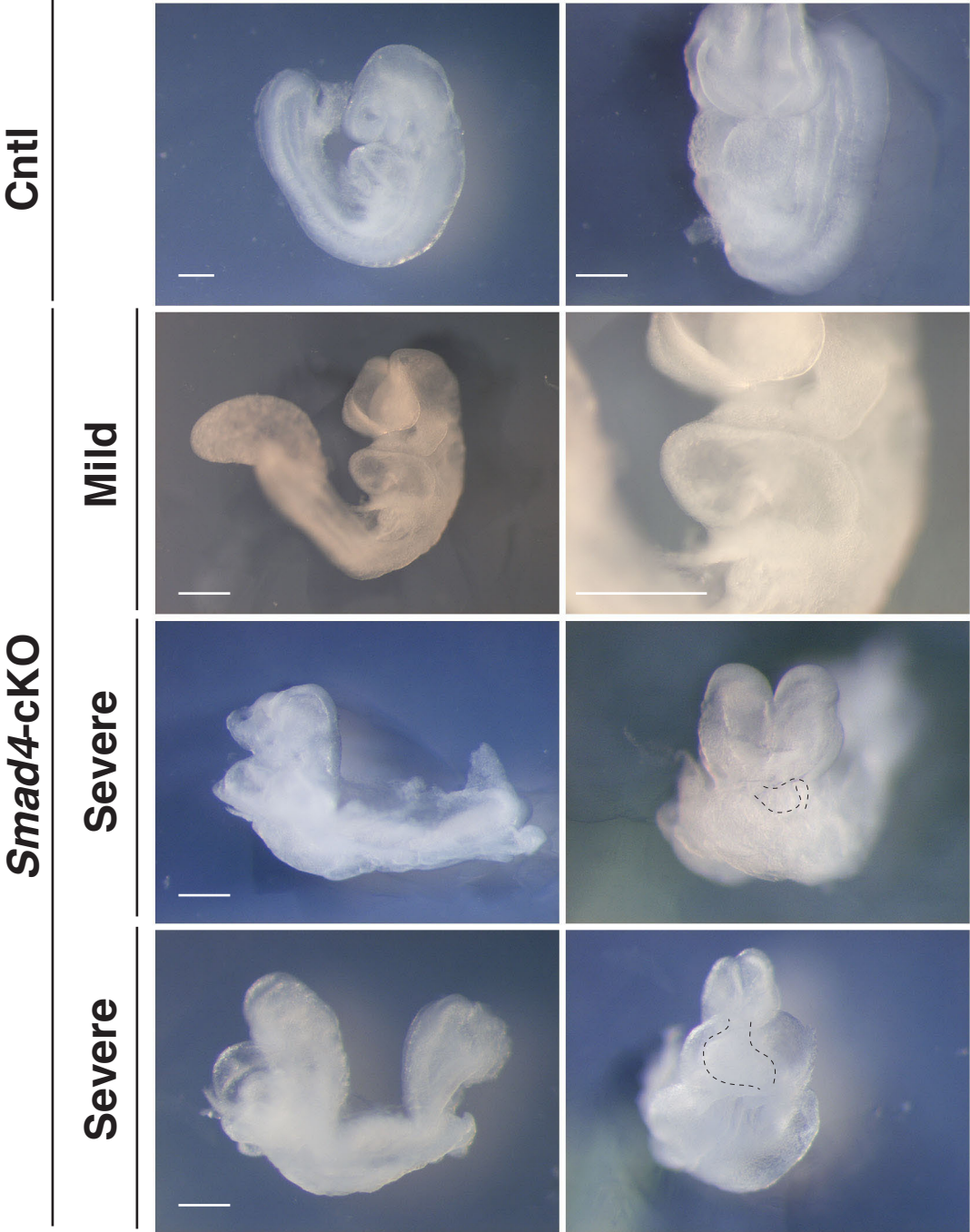
The immunoblotted membrane was directly scanned for 30 sec (A), 1 min (B), 3 min (C), or 5 min (D).

Supplementary Figure S8. Raw images at different exposure times for Figure 3D.

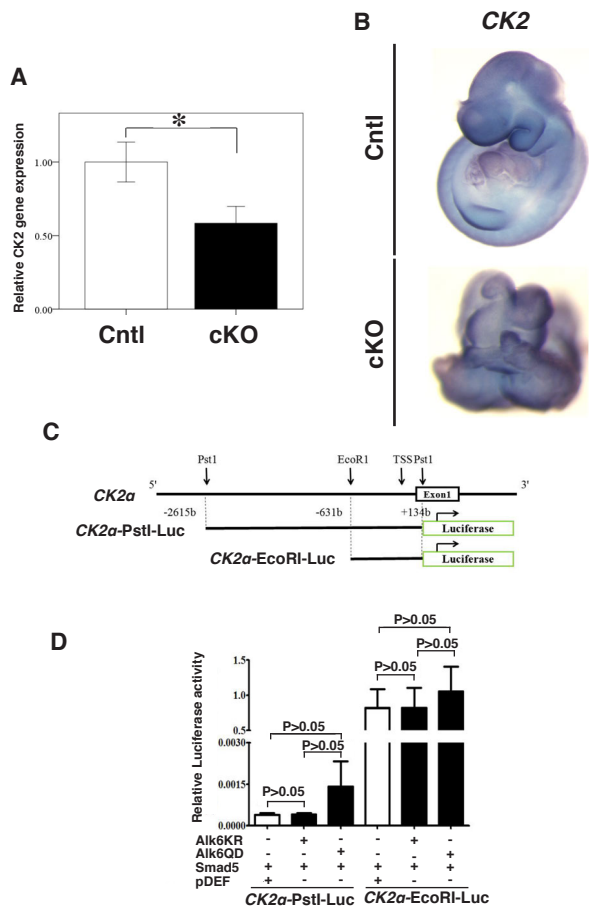
The immunoblotted membrane was directly scanned for 1 min (A), 2 min (B), or 3 min (C).

Supplemental Table S1. Primers used for Real-time PCR

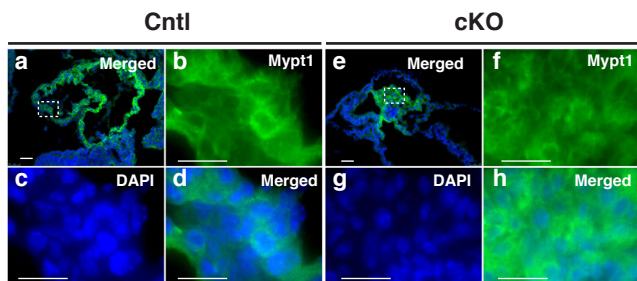
	Forward	Reverse
<i>Csnk2a1</i>	5'-ACACTGTTGTGAAGGACCAGG-3'	5'-CATCAGAAGGTAGATGGGGGT-3'
<i>Ppp1r3c</i>	5'-GCTAATGGGAGGATCTTCTGG-3'	5'-GGTCAAGTTCTCCACTCTCCC-3'
<i>Nkx2-5</i>	5'-GCTACAAGTGCAAGCGACAG-3'	5'-GGGTAGGCGTTGTAGCCATA-3'
<i>Hcn4</i>	5'-CTCAACTCAGGCGTCTTCAAC-3'	5'-CCACCGAAGTAGTAGCAGCAG-3'
<i>Mef2c</i>	5'-GTACACCGAGTACAACGAGC-3'	5'-CCTGTGTTACCTGCACTTGG-3'
<i>Gata4</i>	5'-TCTCACTATGGGCACAGCAG-3'	5'-CGAGCAGGAATTTGAAGAGG-3'
<i>Nppa</i>	5'-GAAGCAGCTGGATCTTCGTAG-3'	5'-TGTGTACAGTGCGGTGTCCAA-3'
<i>Tnni2</i>	5'-CCAGCACTGCTGCACAGCA-3'	5'-AGACATGGAGCCTGGGATG-3'
<i>Hey1</i>	5'-ACTTCCCCAGGGAATGTGTC-3'	5'-ATCGGAGTTTGGGGTTTCGG-3'
<i>Hey2</i>	5'-GCAGCAGTGATGACATCCTC-3'	5'-GCAGAATCTGCATGGGCAAAC-3'
<i>Gja5</i>	5'-TTCATCGTAGGCCAGTACCTC-3'	5'-CAGGTGGTAGAGTTCAGCCAG-3'
<i>Gja1</i>	5'-CTGTACTTGGCTCAGGTGTTC-3'	5'-CACCTCTCATCTTCACCTTGC-3'
<i>Tbx5</i>	5'-CTTCCCTACCAGCACTTCTCC-3'	5'-GTGTAGAGAACTCTGGGGGC-3'
<i>Sfrp5</i>	5'-TGTGCTCCAGTGACTTTGTGG-3'	5'-TCAGGTTGTCTAACTGTGGGC-3'
<i>Isl1</i>	5'-CACGACCAGTATATTCTGAGGG-3'	5'-TCTCTCCACCACATCGTGGTC-3'
<i>Tbx1</i>	5'-ACACCTGGCCGAGTACACTAC-3'	5'-ACTGTCTTTTCGAGGGTCCAC-3'
<i>Tbx2</i>	5'-CCTGCTAATGGACATCGTGGC-3'	5'-CAAGATGTGATTGGCTCGCAC-3'
<i>Tbx3</i>	5'-CATCGTCAGAGCCAACGATATC-3'	5'-CCTCAAACACTCTCATGGAC-3'
<i>Bmp2</i>	5'-CCAGGTTAGTGACTCAGAACAC-3'	5'-TCATCTTGGTGCAAAGACCTGC-3'
<i>G3pdh</i>	5'-ACCACAGTCCATGCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA -3'



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Supplemenatal figure 2

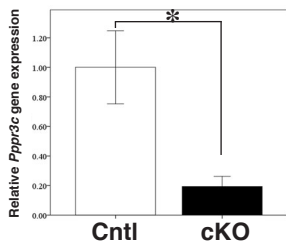


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Supplemental figure 3

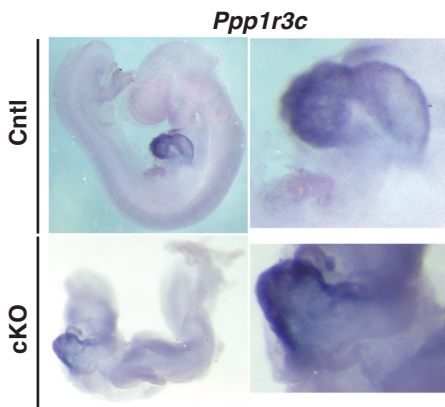


Hu et al.
Supplemenatal figure 4

A



B



A 5min B 1min C 30 sec



