

A BMP4-p38 MAPK signaling axis controls ISL1 protein stability and activity during cardiogenesis

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SUMMARY

During development, cells respond rapidly to intra- and intercellular signals, which induce signaling cascades regulating the activity of transcription factors at the transcriptional and/or post-translational level. The transcription factor ISL1 plays a key role in second heart field development and cardiac differentiation, and its mRNA levels are tightly regulated during cardiogenesis. Here, we show that a BMP-p38 MAPK signaling axis controls ISL1 protein function at the post-translational level. BMP-mediated activation of p38 MAPK leads to ISL1 phosphorylation at S269 by p38, which prevents ISL1 degradation and ensures its transcriptional activity during cardiogenesis. Interfering with p38 MAPK signaling leads to the degradation of ISL1 by the proteasome, resulting in defects in cardiomyocyte differentiation and impaired zebrafish and mouse heart morphogenesis and function. Given the critical role of the tight control of ISL1 activity during cardiac lineage diversification, modulation of BMP4-p38 MAPK signaling could direct differentiation into specific cardiac cell subpopulations.

INTRODUCTION

Dynamic interplay between signaling and transcription factor networks ensure the tight spatial and temporal control of cardiac progenitor cell (CPC) specification, expansion, and differentiation during heart morphogenesis (Galdos et al., 2017; Vincent and Buckingham, 2010). After specification of the cardiac mesoderm triggered by activin/Nodal and bone morphogenic protein (BMP) signals, BMP, WNT, and fibroblast growth factor (FGF) signals play specific roles in the migration, expansion, and differentiation of the distinct CPC subpopulations. The differentiation of the first wave of cardiac mesodermal cells, the first heart field, which forms the initial heart tube, is induced by BMP2, non-canonical WNT, and FGF8 signals. In parallel, canonical WNT/ β -catenin, FGF8/10 and SHH signals promote the expansion of the second heart field (SHF) progenitor cells. These cells are later added to the arterial and venous poles of the heart tube allowing its continuous growth and complex morphogenetic patterning, a process controlled by the non-canonical WNT ligands, WNT5A and WNT11, as well as by BMP2/4/7 signals (Galdos et al., 2017; Vincent and Buckingham, 2010).

At the molecular level, all these signals induce signaling cascades, which converge in the nucleus to regulate the activity of various transcription factors and epigenetic regulators at the transcriptional and/or post-translational level. For example, the TAK1/p38 MAPK signaling pathway induced by Bmp signals promotes cardiac differentiation

by upregulating the expression of the key cardiac transcription factors *Gata4* and *Nkx2-5* (Monzen et al., 1999), whereas p38 α -dependent MEF2C phosphorylation induces MEF2C translocation in the nucleus (Hernandez-Torres et al., 2008).

The transcription factor ISL1 orchestrates a complex gene regulatory network driving SHF development and ISL1+ CPC differentiation and *Isl1*-deficiency results in loss of all structures derived from the SHF, including the right ventricle, the outflow tract, and large portions of the atria (Cai et al., 2003; Caputo et al., 2015; Gao et al., 2019). Despite the important role of ISL1 in heart development, it remains poorly understood how its transcriptional activity is regulated.

Here, we show that p38-mediated phosphorylation of ISL1 at S269 induced by BMP signaling controls ISL1 protein stability and activity during cardiogenesis.

RESULTS

ISL1 is phosphorylated by p38 MAPK at serine 269

Two different isoforms of ISL1 exist: ISL1 α (predominant) and ISL1 β (Ando et al., 2003). Unlike ISL1 β , ISL1 α is mainly phosphorylated, but the exact mechanism and function of ISL1 phosphorylation remain unknown. To identify ISL1 phosphorylation sites, we mutated all serines and threonines within the 255–279 region, lacking in ISL1 β (Figure 1A), to alanine to prevent phosphorylation. Only the S269A mutant protein migrated as a single band with



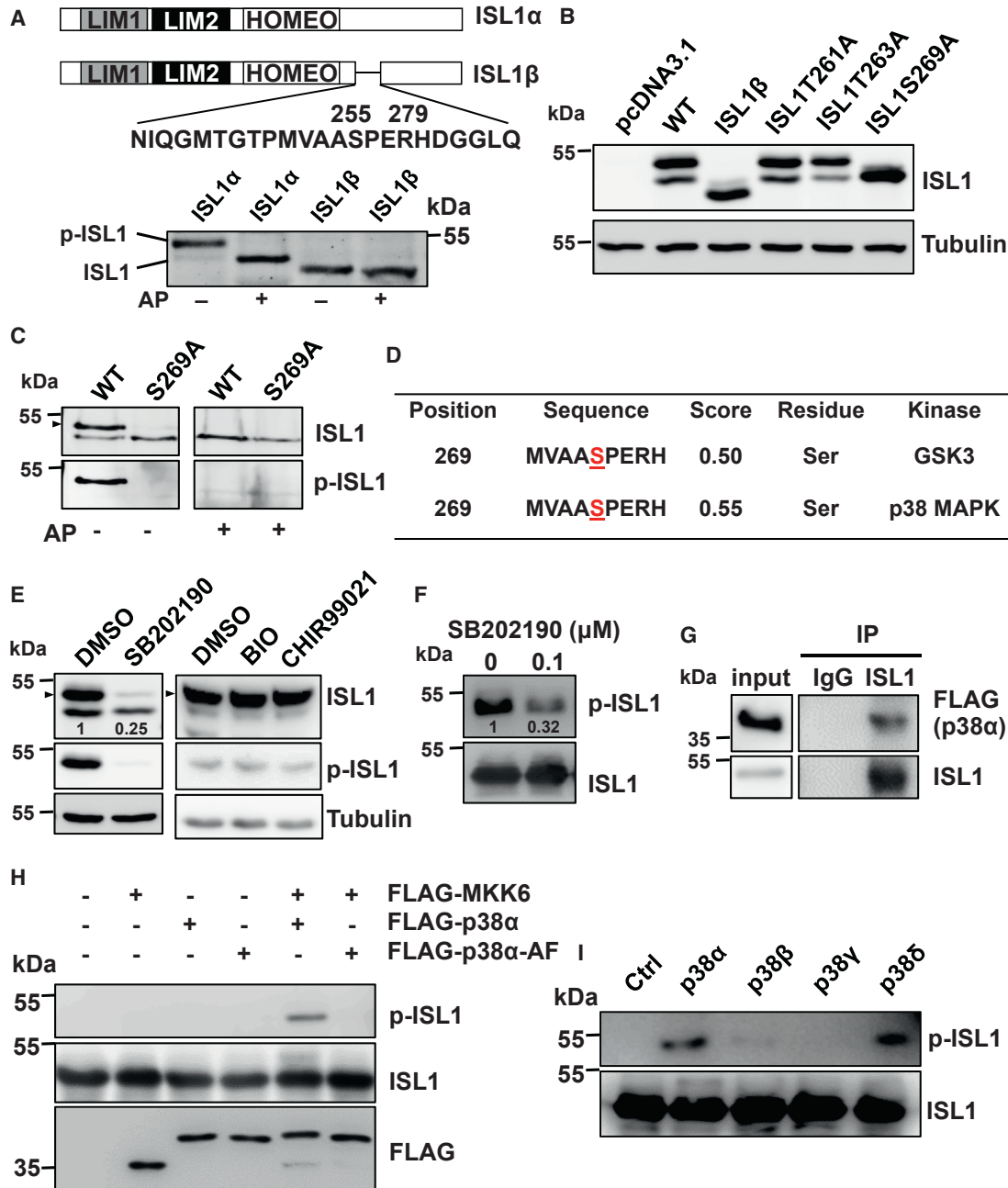


Figure 1. ISL1 is phosphorylated by p38 on serine 269

(A) Schematic representation of ISL1 α and ISL1 β (top panel). Alkaline phosphatase (AP) treatment of extracts from cells expressing either ISL1 α or ISL1 β , showing that the β form is not phosphorylated.

(B) Western blot (WB) analysis of extracts of cells expressing ISL1 α harboring different mutations in the serine or threonine residues within the 256–278 region. Tubulin served as loading control.

(C) AP treatment of extracts from cells expressing either WT ISL1 α or ISL1 α S269A, showing that ISL1 α S269A mutant is not phosphorylated. Antibody raised against synthetic ISL1 phospho-S269-peptide detected phosphorylated ISL1 only in extracts overexpressing ISL1 but not the S269A mutant. Arrowheads in this and the following figure panels indicate phosphorylated ISL1.

(D) Results of predictions of kinase-specific protein phosphorylation sites using NetPhosK1.0. The potentially modified residues are underlined.

(E) WB analysis of ISL1 overexpressing HEK293T cells treated either with DMSO, 50 μ M p38 inhibitor SB202190, or with the GSK3 inhibitors, 2.5 μ M BIO and 5 μ M CHIR-99021 for 16 h using ISL1 and phospho-ISL1 antibody.

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mobility identical to the lower unmodified band of ISL1 and to the wild-type (WT) ISL1 protein treated with alkaline phosphatase (Figures 1A–1C), suggesting that ISL1 is phosphorylated at S269. To further study ISL1 S269 phosphorylation, we generated an ISL1 phospho-specific antibody recognizing the phosphorylation consensus encompassing S269. We observed significant reactivity of the antibody with WT ISL1 protein and no reactivity with the ISL1 S269A mutant (Figure 1C). Next, we studied which kinase might induce ISL1 S269 phosphorylation. Computational prediction using NetPhosK1.0 revealed GSK3 and p38 MAPK as putative serine/threonine kinases responsible for ISL1 S269 phosphorylation (Figure 1D). Importantly, treatment of cells with the selective and potent p38 MAPK inhibitor SB202190 significantly inhibited ISL1 S269 phosphorylation, whereas GSK3 inhibition did not show an effect (Figure 1E). Similarly, an *in vitro* kinase assay using recombinant ISL1 protein and protein extracts treated with DMSO or SB202190 revealed significantly reduced ISL1 phosphorylation in the presence of the p38 inhibitor (Figure 1F). Consistent with a direct role of p38 on ISL1 phosphorylation, p38 α was immunoprecipitated together with ISL1 (Figure 1G) and an *in vitro* kinase assay using recombinant ISL1 protein and immunopurified kinases showed that p38 α but not its upstream kinase MKK6 or the kinase-deficient p38 α -AF could phosphorylate ISL1 (Figure 1H). Among the four different p38 MAPK isoforms that exist (Rose et al., 2010), mainly p38 α and p38 δ , were able to phosphorylate ISL1 (Figure 1I). Taken together, our data indicate that ISL1 α is phosphorylated by the p38 MAPK at S269.

Phosphorylation of ISL1 by p38 regulates its protein stability and activity during cardiogenesis downstream of BMP signaling

Phosphorylation of proteins often alters their stability, activity, and function. To test whether ISL1 phosphorylation might affect its transcriptional activity and stability, we transfected NIH3T3 cells with ISL1 together with a luciferase reporter construct containing the *Mef2c* cardiac-specific enhancer, which is bound and activated by ISL1 (Caputo et al., 2015). ISL1 is highly phosphorylated in NIH3T3 cells allowing us to study the effect of modulation of ISL1 phosphorylation on its transcriptional activity. Importantly, treatment with SB202190 led to dose-depen-

dent decrease of reporter activity (Figure 2A) and ISL1 levels (Figure 2B), suggesting that ISL1 phosphorylation by the p38 pathway regulates ISL1 transcriptional activity by affecting its protein stability. Consistent with these results, activation of p38 signaling by either adding serum to serum-starved NIH3T3 overexpressing *Isl1* or by treatment with the p38 agonist anisomycin led to an increase of ISL1 protein levels and transcriptional activity (Figures 2C–2F). We have previously reported that ISL1 protein levels are tightly regulated by the proteasome system (Caputo et al., 2015). To access whether p38 inhibition induces ISL1 ubiquitination and subsequent degradation, we co-expressed HA-tagged ubiquitin and FLAG-tagged ISL1 in HEK293T cells. As expected, cells treated with SB202190 showed massive increase in polyubiquitin chains conjugated to ISL1, suggesting that ISL1 phosphorylation prevents ISL1 polyubiquitination and proteasomal degradation (Figure 2G). In addition, inhibition of proteasome function with MG132 blocked the degradation of ISL1 induced by p38 inhibition (Figure 2H). Taken together, our data show that ISL1 phosphorylation by p38 protects it from proteasomal degradation and assures its transcriptional activity.

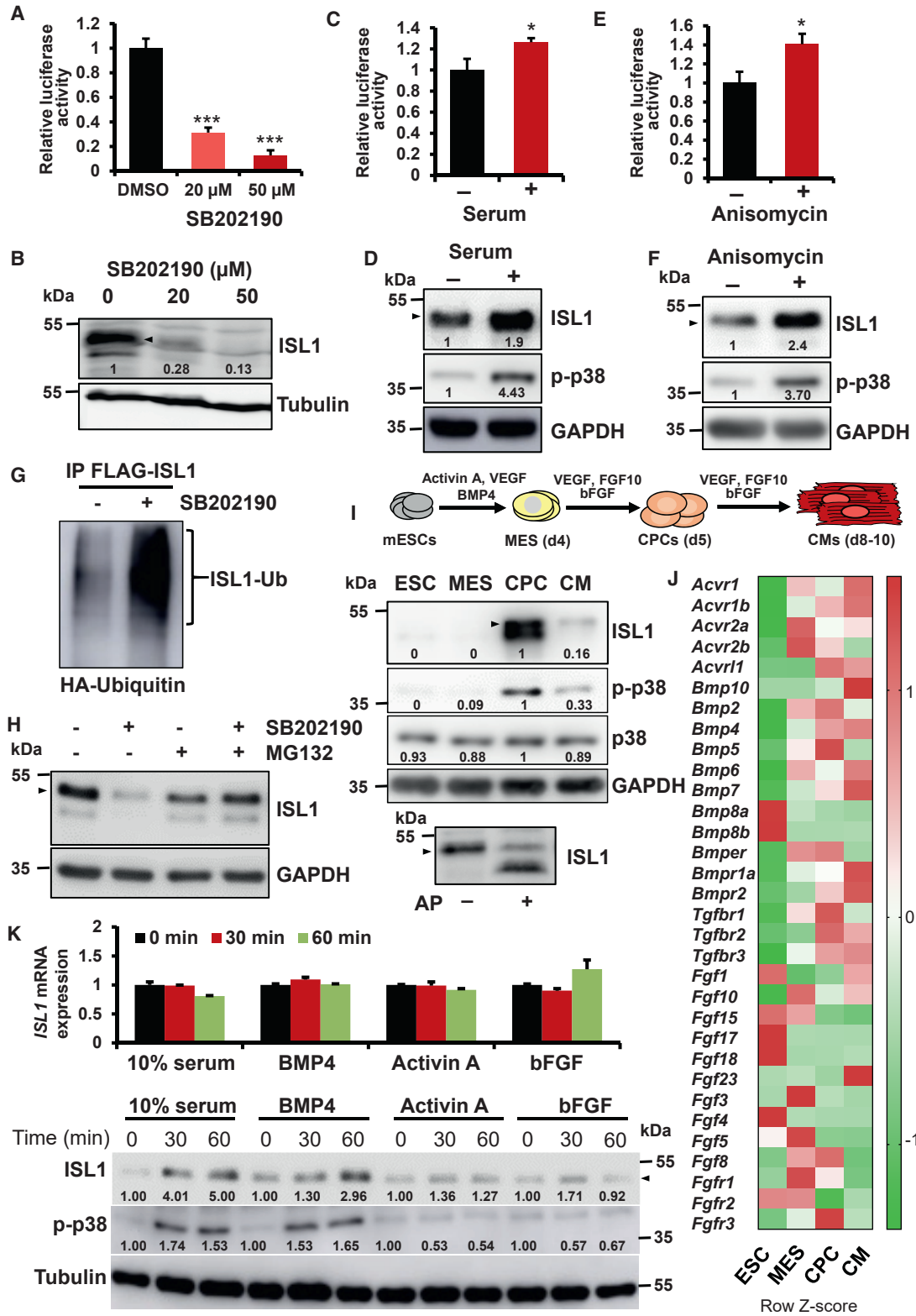
Interestingly, during the course of directed cardiac differentiation (Gao et al., 2019; Wamstad et al., 2012), although the total p38 levels remained constant, activated p38 levels were high only in cardiac progenitors, similar to ISL1, which was exclusively phosphorylated and expressed in 93% of cells at day 5 (d5) of differentiation, i.e., the CPC stage (Figures 2I, S1A, and S1B). To study the upstream signals leading to p38 activation and ISL1 phosphorylation during cardiogenesis, we treated *Isl1*^{-/-} mouse embryonic stem cells (mESCs) and lung epithelial BEAS-2B (B2B) cells overexpressing *Isl1* under a *CMV* promoter with various growth factors. The *CMV* promoter itself is not affected upon growth factor treatments and, therefore, does not interfere with the results (Figures 2K, top and S1C). Furthermore, B2B cells and mESCs have lower levels of activated phospho-p38, which allows us to study the timing of p38 and ISL1 phosphorylation. BMP4 treatment led to increase of activated phospho-p38 and ISL1, whereas treatment with activin A and basic FGF did not have a major effect (Figures 2K, bottom and S1D). BMP ligands and their transmembrane receptors are expressed after cardiac mesoderm commitment and are high in CPCs, consistent with the high level of activated p38 in CPCs (Figure 2J), suggesting

(F) *In vitro* kinase assay using recombinant ISL1 incubated with total protein extracts from cells treated with or without SB202190.

(G) Co-immunoprecipitation of protein extracts of HEK293T cells overexpressing ISL1 and FLAG-p38 α using ISL1 antibody followed by WB analysis with FLAG and ISL1 antibody.

(H) *In vitro* kinase assay with recombinant ISL1 incubated with combinations of immunopurified MKK6, p38 α , and kinase-deficient p38 α (p38 α -AF) proteins. FLAG-tagged constructs for these kinases were overexpressed alone or in combinations and immunoprecipitated using FLAG antibody.

(I) *In vitro* kinase assay with recombinant ISL1 incubated with immunopurified MKK6 together with different p38 MAPK isoforms.



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that the BMP-p38 MAPK signaling axis might regulate cardiogenesis by controlling ISL1 protein stability and activity.

To further study the role of p38 signaling in regulating ISL1 activity during cardiogenesis, we first differentiated mESCs using a directed cardiomyocyte (CM) differentiation protocol (Gao et al., 2019; Wamstad et al., 2012) and treated cardiac mesodermal (MES) precursors (d4) with either DMSO or SB202190. Treatment with the p38 inhibitor significantly decreased ISL1 protein levels in a dose-dependent manner (Figure 3A). In contrast, *Isl1* mRNA level was not decreased (Figure 3B), indicating that the decrease of ISL1 protein levels is not due to a decrease of *Isl1* gene expression but rather to posttranscriptional regulation. Next, we analyzed the expression of *Hand2* and *Mef2c*, known downstream targets of ISL1 (Caputo et al., 2015; Gao et al., 2019). Importantly, *Hand2* and *Mef2c* expression levels were strongly decreased upon p38 inhibition, whereas other progenitor marker genes, such as *Tbx1*, were not decreased (Figure 3C). Consistent with a direct role of ISL1 in the regulation of CM structural and contraction genes (Gao et al., 2019), p38 inhibition led to a dose-dependent decrease in the expression of the CM genes *Mlc2a*, *Myocd*, *Tnnt2*, and *Ttn* (Figure 3D). Similarly, silencing of p38 α , the only p38 MAPK isoform highly expressed during the course of cardiac differentiation (Figure S2A), led to a decrease of ISL1 protein levels without affecting *Isl1* mRNA levels (Figures 3E, 3F, and S2B) and downregulation of ISL1 target genes in CPCs and CMs (Figures 3G and 3H).

To gain a better understanding of the function of p38 in regulating ISL1 activity in cardiogenesis, we analyzed CPC and CM numbers. Fluorescence-activated cell sorting

(FACS) analysis for NKX2-5-GFP found no differences in CPC numbers upon p38 inhibition as well as p38 α silencing, similar to ablation of *Isl1* (Figure 3I). However, differentiation in CMs was severely impaired (Figure 3J), showing that, similar to *Isl1* depletion, p38 silencing or inhibition does not affect ISL1+ CPC specification, but rather their differentiation in CMs.

To further study the role of the BMP4-p38 MAPK signaling axis in cardiogenesis we treated cardiac MES precursors (d4) with Noggin, a potent BMP inhibitor. Treatment with Noggin led to significant decrease of *Isl1* mRNA as well as ISL1 protein levels (Figures S2C and S2D), consistent with the critical role of BMP signaling in cardiac specification (van Wijk et al., 2007). To dissect the role of BMP signaling in the regulation of ISL1 protein levels versus its role in controlling its expression, we utilized *Isl1*^{-/-} mESCs overexpressing CMV-driven *Isl1*. Treatment of cardiac MES precursors derived from *Isl1*^{-/-} mESCs overexpressing CMV-driven *Isl1* with Noggin led to a decrease in activated phospho-p38 and ISL1, although *Isl1* mRNA levels were even slightly elevated upon Noggin stimulation (Figure 3K). The expression of ISL1 downstream targets *Hand2* and *Mef2c* was also decreased in CPCs (Figure 3L) and we observed a dramatic decrease of *Mlc2a*, *Myocd*, *Tnnt2*, and *Ttn* expression in CMs upon Noggin treatment (Figure 3M), similar to *Isl1* ablation, consistent with a role of a BMP-p38-ISL1 signaling axis in regulating ISL1 protein stability and activity. Interestingly, *Tbx1* and *Isl1* levels were unchanged or increased in BMP, p38 MAPKs, or *Isl1* loss-of-function experiments (Figures 3C, 3G, and 3L). TBX1 and ISL1 are expressed in SHF CPCs and their expression is shut down upon differentiation (Liao et al., 2008); thus the elevated mRNA levels

Figure 2. ISL1 phosphorylation by the BMP4-p38 MAPK signaling axis affects its protein stability

(A and B) Luciferase assay of NIH3T3 cells expressing *Isl1* and a *Mef2c* anterior heart field (AHF) enhancer reporter construct (pGL4-*Mef2c*Promoter-Luc2-AHF), treated either with DMSO or the p38 inhibitor SB202190 ($n = 3$) (A) and WB analysis for ISL1 of protein extracts used in the reporter assays (B).

(C–F) Luciferase assay of NIH3T3 cells stably expressing *Isl1* transfected with the *Mef2c* reporter construct and treated with or without 10% fetal bovine serum for 1 h ($n = 3$) (C) or with or without 0.01 μ M anisomycin for 1 h ($n = 3$) (E), and WB analysis for ISL1 of protein extracts used in the reporter assays (D, F).

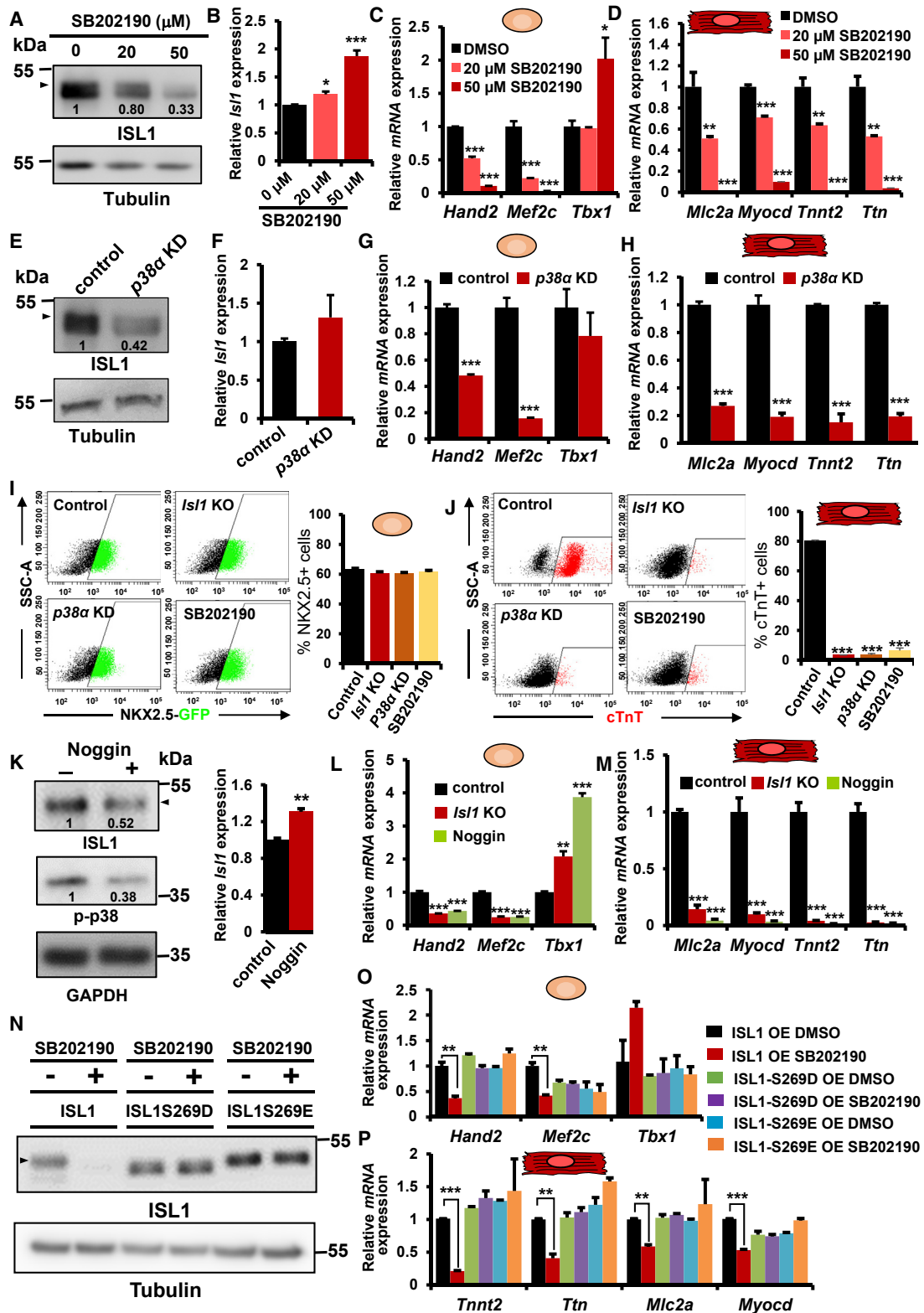
(G) HA-tagged ubiquitin and FLAG-tagged ISL1 were transiently expressed in HEK293T cells. Cells were either treated with DMSO or SB202190 together with MG132 for 6 h before harvesting. Equivalent amounts of total protein were immunoprecipitated with anti-FLAG (M2) antibody and detected with an anti-HA antibody.

(H) WB analysis for ISL1 of protein extracts of *Isl1* overexpressing NIH3T3 cells treated with DMSO or SB202190 with or without the proteasome inhibitor MG132 for 16 h.

(I) Scheme depicting the distinct stages of directed cardiac differentiation (top). MES, mesoderm; CPCs, cardiac progenitor cells; CMs, cardiomyocytes. WB analysis for ISL1 and activated phospho-p38 at different stages of directed differentiation of mESCs into CMs (middle). AP treatment of extracts from CPCs (bottom), showing that ISL1 is exclusively phosphorylated.

(J) Heatmap representation of expression data for genes involved in BMP, activin, and FGF signaling during the course of cardiac differentiation (Wamstad et al., 2012).

(K) *Isl1* mRNA expression in mESCs overexpressing CMV-driven *Isl1* treated with BMP4, basic FGF (bFGF), or activin for 30 or 60 min (top panel). WB analysis for ISL1 and activated phospho-p38 MAPK of protein extracts from *Isl1*^{-/-} mESCs overexpressing CMV-driven *Isl1* treated with BMP4, bFGF, or activin for 30 or 60 min (bottom panel).



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might be due to the differentiation defect, which we observe upon BMP, p38 MAPKs, or *Isl1* loss of function.

p38 MAPK signaling has been shown to regulate stem cell differentiation by targeting many regulatory factors that play crucial functions during cardiogenesis, such as GATA4, *Mef2a/c*, SRF, *Baf60c*, etc. (Yokota and Wang, 2016). To assess the specific effect of p38 signaling on ISL1-dependent transcription we overexpressed WT ISL1 and ISL1 protein carrying phosphomimetic mutations of serine 269 (ISL1S269D, ISL1S269E) under the *EF1 α* promoter in *Isl1* knockout mESCs (Figure S2E) and subjected them to directed cardiac differentiation. At the cardiac mesoderm stage, we treated the cells with either DMSO or SB202190. ISL1 protein levels strongly decreased upon SB202190 treatment, whereas ISL1S269D and ISL1S269E levels did not change (Figure 3N). Consistent with a crucial role of p38 signaling in regulating ISL1 activity during cardiogenesis, p38 inhibition led to a significant decrease of ISL1 primary downstream targets in CPCs and CMs overexpressing WT ISL1, whereas it had little effect on CPCs and CMs overexpressing ISL1S269D and ISL1S269E mutants (Figure 3O and 3P). Taken together, these data indicate a key role of p38 signaling in regulating ISL1 protein stability and activity during cardiac differentiation.

Inhibition of p38 *in vivo* results in ISL1 degradation and in defects in cardiac morphogenesis and function

To determine whether p38 MAPK signaling regulates ISL1 activity in cardiac development *in vivo*, we first treated

fertilized zebrafish embryos with SB202190 at the three somite stage, the time of ISL1 CPC specification (Witzel et al., 2012). The dose of SB202190 was titrated to avoid toxicity (Figures S3A and S3B). *In situ* hybridization at the ten somite stage, at which ISL1 target gene expression is significantly altered (Caputo et al., 2015; Witzel et al., 2012, 2017), revealed that, while *Isl1* and *Nkx2-5* mRNA levels were largely unaffected, the expression of ISL1 direct downstream targets *Mef2ca* and *Mef2cb* was significantly reduced (Figure 4A). Consistent with our cell culture-based studies, ISL1/2 protein levels were strongly reduced upon SB202190 treatment (Figure 4B). In zebrafish there are three islet family members (*Isl1*, *Isl2a*, and *Isl2b*) that show high overall homology and are recognized by the ISL1/2 antibody (Witzel et al., 2017). Importantly, the protein phosphorylation domain is conserved within the zebrafish islet family members, as well as in humans (Figures S3C and S3D), and computational prediction pointed to p38 MAPK as the only common putative kinase for all islet homologs (Figure S3D). Alkaline phosphatase treatment (Figure S3E) indicated that all zebrafish islet protein members are phosphorylated, suggesting that interfering with p38 signaling may affect the specific roles of all islet family members within the zebrafish heart (Witzel et al., 2017). *Isl2b* controls the development of the arterial pole and *isl2b* deficiency results in smaller ventricles and decreased expression of the zebrafish SHF marker *ltbp3* (Witzel et al., 2017; Zhou et al., 2011). In contrast, *Isl1* regulates the differentiation of CMs at the venous pole where contractions are initiated and *isl1*

Figure 3. p38 regulates ISL1 stability and function during cardiac differentiation

(A and B) WB analysis of protein extracts (A) and *Isl1* qPCR analysis (n = 4) (B) of mESC-derived CPCs treated at the MES stage (d4) with different concentrations of SB202190 for 24 h.

(C) Relative mRNA expression of *Tbx1* and ISL1 downstream targets (*Hand2* and *Mef2c*) in CPCs (d5) treated at the MES stage with different concentrations of SB202190 for 24 h (n = 4).

(D) Relative mRNA expression of CM marker genes in CMs (d8) differentiated from cells treated at the cardiac mesoderm stage with different concentrations of SB202190 for 96 h (n = 4).

(E and F) WB analysis of protein extracts (E) and *Isl1* qPCR analysis (n = 4) (F) of CPCs (d5) derived from mESCs stably overexpressing control small hairpin RNA (shRNA) or shRNA against *p38 α* .

(G and H) Relative mRNA expression of *Tbx1*, *Hand2*, and *Mef2c* in CPCs (d5, n = 4) (G) and of CM marker genes in CMs (d8, n = 4) (H), differentiated from control and *p38 α* knockdown mESCs.

(I) Representative FACS analyses of NKX2.5 + CPCs differentiated from *Isl1*^{-/-} and *p38 α* knockdown mESCs or mESCs treated with p38 inhibitor at the MES stage for 24 h (left). Percentage of NKX2.5+ CPCs at d5 determined by FACS analysis (n = 3) (right).

(J) Representative FACS analyses of cTnT+ CMs at d10 of ESC differentiation (left) and percentage of cTnT+ CMs at d10 determined by FACS analysis (n = 3) (right).

(K) WB analysis of protein extracts (left) and *Isl1* qPCR analysis (right) of *Isl1*^{-/-} CPCs overexpressing *CMV*-driven *Isl1* treated with Noggin at the MES stage for 24 h.

(L and M) Relative mRNA expression of *Tbx1*, *Hand2*, and *Mef2c* in CPCs (d5, n = 4) (L) and of CM marker genes in CMs (d8, n = 4) (M), differentiated from cells treated with Noggin from the MES stage.

(N) WB analysis of protein extracts from CPCs derived from *Isl1*^{-/-} mESCs overexpressing *EF1 α* -driven *Isl1* or *Isl1* carrying phosphomimetic mutations of serine 269 (ISL1S269D, ISL1S269E) treated either with DMSO or SB202190 at the MES stage for 24 h.

(O and P) Relative mRNA expression of *Tbx1*, *Hand2*, and *Mef2c* in CPCs (d5, n = 4) (O) and of CM marker genes in CMs (d8, n = 4) (P), differentiated from *Isl1*^{-/-} mESCs overexpressing ISL1, ISL1S269E, or ISL1S269D treated either with DMSO or SB202190 from the MES stage.

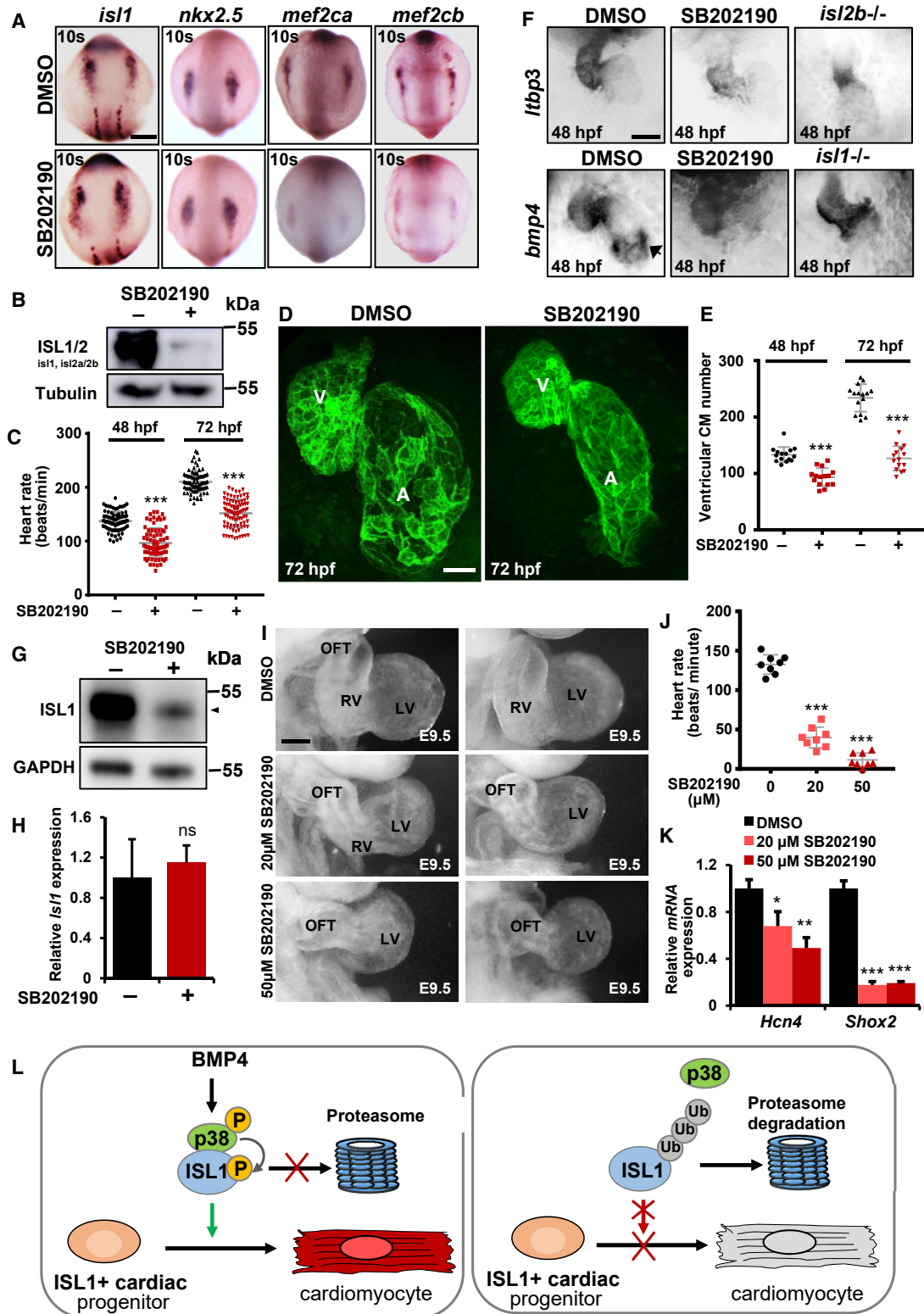


Figure 4. Inhibition of p38 results in ISL1 degradation *in vivo* and in defects in cardiac morphogenesis and function

(A) *In situ* hybridization of zebrafish embryos at the ten somite stage for *isl1*, *mef2ca*, *mef2cb*, and *nkx2.5* expression. The embryos were treated either with DMSO or SB202190 starting from the three somite stage. Scale bar, 0.2 mm.

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deficiency results in severe bradycardia and loss of ISL1+ *bmp4*-expressing cells at the sinus venosus (Arrenberg et al., 2010; de Pater et al., 2009). Importantly, similar to *isl1* mutant fish, zebrafish treated with SB202190 at 24 hpf (hours post-fertilisation) showed reduced heart rate and loss of *bmp4* expression at the venous pole (Figures 4C, 4E, and S3F). Furthermore, confocal imaging of zebrafish hearts of SB202190-treated embryos showed a smaller ventricle, significantly reduced number of ventricular CMs and pronounced cardiac looping defects (Figures 4D and 4E), similar to *isl2b*-deficient zebrafish embryos, while the overall embryo morphology was not affected (Figure S3G). *In situ* hybridization revealed that *ltbp3* was strongly decreased upon p38 inhibition similar to *isl2b*^{-/-} embryos (Figure 4F). Consistent with an important function of BMP4-p38 signaling axis in anterior and posterior SHF development, *p38α*, *Bmp4*, and *Isl1* are expressed in overlapping patterns in the developing heart (Figure S3H and (Witzel et al., 2017)).

Next, we studied the effect of p38 signaling on ISL1 activity using whole-embryo *ex vivo* culture of mouse embryos (Figures S4A–S4C). Inhibition of p38 signaling starting from embryonic day 8.5 (E8.5) (six somite stage) for 24 h led to a substantial decrease in ISL1 protein levels in embryos treated with SB202190, whereas *Isl1* mRNA levels were not significantly changed (Figures 4G and 4H). Consistent with an important role of p38 signaling in regulating ISL1 function, we observed dose-dependent defects in SHF development, including a dramatically smaller right ventricle (Figures 4I and S4D). The heart rate of mouse embryos treated with SB202190 was markedly lower compared with embryos treated with DMSO (Figure 4J) and the

expression of CM marker genes as well as the markers of the sinoatrial node (SAN) lineage *Hcn4* and *Shox2* was significantly decreased (Figure 4K and S4E), in line with the important role of ISL1 in CM differentiation and pacemaker development (Liang et al., 2015).

In conclusion, our study highlights a key function of ISL1 phosphorylation in regulating ISL1 protein stability and function during cardiogenesis, which is mediated by the BMP4-p38 signaling axis (Figure 4L).

DISCUSSION

Knowledge accumulated from developmental studies about the signaling pathways regulating the establishment of the distinct cardiac lineages has significantly improved the methods for *in vitro* differentiation of mESCs and induced pluripotent stem cells in the different cardiovascular lineages. However, the current limitations in the use of stem/progenitor cells in regenerative medicine calls for better understanding of the underlying mechanisms triggered by stage-specific addition of signaling molecules. Our study shows that activation of the p38 MAPK pathway downstream of BMP signaling results in ISL1 phosphorylation at S269, which prevents its degradation and ensures its transcriptional activity during cardiogenesis.

BMP ligands and receptors are expressed in specific patterns in the heart and play distinct as well as redundant functions during all stages of cardiogenesis (van Wijk et al., 2007). Ligand binding activates two major pathways: canonical signaling via SMADs and non-canonical SMAD-independent signaling via p38 MAPK, which are tightly intertwined through both positive and negative feedbacks

- (B) WB analysis of ISL1/2 in 72 hpf zebrafish embryos treated either with DMSO or p38 inhibitor at 24 hpf (at least 20 embryos per sample).
- (C) Heart beats per minute at 48 and 72 hpf of zebrafish embryos treated either with DMSO or p38 inhibitor at 24 hpf. Error bars represent SEM of four clutches. The numbers of embryos used per condition are indicated in Figure S3F.
- (D) Representative confocal images of control and p38 inhibitor-treated *Tg(my17:EGFP-HsHRAS)^{S883}* embryos at 72 hpf. Scale bar, 50 μm.
- (E) Quantification of ventricular CM numbers at 48 and 72 hpf of zebrafish embryos treated either with DMSO or p38 inhibitor at 24 hpf. DMSO, n = 15; 50 μM SB202190, n = 15, from 3 clutches.
- (F) Representative *in situ* hybridization for *ltbp3* (top) or *bmp4* (bottom) of zebrafish embryos at 48 hpf treated either with DMSO or p38 inhibitor at 24 hpf as well as of *isl2b*^{-/-} or *isl1*^{-/-} embryos. The arrow points to the *bmp4* expression at the venous pole lost upon p38 inhibitor treatment and in *isl1*^{-/-} embryos. Scale bar, 0.1 mm.
- (G) WB analysis for ISL1 of protein extracts from E8.5 mouse embryos treated either with DMSO or 50 μM SB202190 for 24 h in a whole-embryo *ex vivo* culture (at least five embryos per sample).
- (H) Relative *Isl1* mRNA expression in *ex-vivo*-cultured mouse embryos treated either with DMSO or 50 μM SB202190.
- (I) Representative right lateral views of the hearts of embryos treated either with DMSO (n = 27) or 20 μM (n = 9) and 50 μM SB202190 (n = 26) at E8.5 for 24 h. Scale bar, 0.2 mm.
- (J) Heart beats per minute of *ex-vivo*-cultured mouse embryos treated either with DMSO or SB202190 (n = 8).
- (K) Relative mRNA expression of SAN lineage markers *Hcn4* and *Shox2* in *ex-vivo*-cultured mouse embryos treated either with DMSO or 20 and 50 μM SB202190 starting at E8.5 for 24 h (n = 6).
- (L) Model of the role of BMP-p38 MAPK signaling axis in ISL1-driven cardiogenesis. In WT CPCs, BMP signaling activates p38 leading to ISL1 phosphorylation at S269 thereby preventing ISL1 degradation and ensuring its transcriptional activity during cardiogenesis. Interfering with the BMP-p38 MAPK signaling axis leads to degradation of ISL1 by the proteasome, resulting in defects in CM differentiation and impaired SHF development.



(Nohe et al., 2004). Myriad studies have shown the critical importance of canonical SMAD signaling in heart development; however, there is also evidence for an important role of non-canonical BMP signaling in cardiogenesis. For example, BMP activation of the TAK1-MAPK pathway induces CM differentiation by regulating the expression of key cardiac transcription factor genes (Monzen et al., 1999) and deletion of either TAK1 or TAB1, a protein necessary for TAK1 kinase activity, results in defects in cardiac development (Jadrich et al., 2006). We found that a BMP4-p38 MAPK signaling axis regulates ISL1+ CPC differentiation by controlling ISL1 protein stability. Loss of function of either BMP, p38 MAPKs or ISL1 did not affect CPC numbers but resulted in major defects in cardiac differentiation and in cardiac dysfunction. Consistent with the important role of p38 signaling in regulating ISL1 stability and activity, inhibition of p38 signaling after linear heart tube formation in mouse embryos resulted in dramatically decreased ISL1 protein levels, together with major defects in SHF development and heart function. Similarly, inhibition of p38 signaling in zebrafish embryos resulted in a profound decrease of ISL1 protein levels and defects in both arterial and venous pole development, which are controlled by *Isl2b* and *Isl1*, respectively (Witzel et al., 2017; Zhou et al., 2011). While these experiments support a key role of p38-mediated ISL1 phosphorylation in regulating ISL1 stability and SHF development *in vivo*, we cannot exclude that additional downstream effectors contribute to the observed phenotype upon p38 inhibition. Yet, experiments utilizing ISL1 phosphomimetic mutants of serine 269, which cannot be degraded by the proteasome upon p38 inhibition revealed the critical importance of p38-mediated ISL1 phosphorylation in ISL1+ CPC differentiation and ISL1 target gene expression. Nevertheless, several open questions remain, e.g., regarding the BMP receptors involved and the downstream signaling cascades converging on p38/ISL1. Interestingly, overexpression of TAK1 directs myocardial differentiation toward the SAN lineage (Brown et al., 2017). Similarly, ISL1 overexpression redirects the differentiation from working myocardial to a SAN cell type (Dorn et al., 2015). Since BMP inhibition (Sun et al., 2015), as well as p38 inhibition and *Isl1* depletion, resulted in decreased expression of the SAN lineage markers *Hcn4* and *Shox2* and decreased heart rate, and TAK1 activates p38 MAPK, it remains to be determined whether the BMP-TAK1-p38-ISL1 axis and ISL1 phosphorylation control the differentiation toward the SAN lineage. Interestingly, a recent study has shown that ISL1 is degraded through the ubiquitin-proteasome pathway selectively in the OFT but not in the SAN and the inflow tract region of postnatal hearts, raising the question whether the above-described mechanism is required for ISL1 protein stabilization in the SAN (Hatzistergos

et al., 2020). On the other hand, BMP-SMAD signaling directly suppresses *Isl1* expression through *miRNA17* and *miRNA20a* (Wang et al., 2010), ensuring proper differentiation of working myocardial cells. Thus, BMP signaling through different receptors and downstream pathways might regulate cardiac lineage diversification.

The protein phosphorylation domain is conserved within the zebrafish ISL1 family members, as well as in humans, suggesting that the proposed mechanism is likely an evolutionarily conserved way of regulating islet protein family function during cardiogenesis. Interestingly, S269 is located in the LIM homeobox 3 binding domain (LBD) of ISL1 (ISL1LBD), which binds in a similar manner to LIM domains as the adaptor molecule LDB1 (Bhati et al., 2008). Binding of LDB1 stabilizes ISL1 and promotes long-range promoter-enhancer interactions in cardiogenesis (Caputo et al., 2015); thus changes in ISL1 phosphorylation might affect its binding to LDB1 and result in alternate protein complexes formation that can target specific genes and thereby regulate different cell fates during embryonic development and cardiac lineage differentiation and diversification.

In conclusion, our study highlights a key role of BMP4-p38 MAPK signaling in controlling ISL1 protein stability and activity during cardiogenesis through p38-mediated ISL1 phosphorylation at S269 (Figure 4L).

EXPERIMENTAL PROCEDURES

A detailed description of the material and methods is provided in the [supplemental experimental procedures](#).

Cell lines, cell culture, and mESC differentiation

The generation of *Isl1* knockout and stable knockdown mESCs as well as mESC culture and differentiation were described (Gao et al., 2019). Details can be found in the [supplemental experimental procedures](#).

Zebrafish strains, *in situ* hybridization, and confocal microscopy

The following mutant and transgenic lines were used: *Tg(my-17:EGFP^HHRAS)^{s883}*, *isl1sa0029* (Sanger Institute, Zebrafish Mutation Resource) and *isl2b*^{-/-} (Witzel et al., 2017). All animal experiments were done according to the regulations issued by the Committee for Animal Rights Protection of the State of Hessen (Regierungspraesidium Darmstadt) and of the State of Baden-Württemberg (Regierungspraesidium Karlsruhe). Whole-mount staining, *in situ* hybridization, and imaging are described in detail in the [supplemental experimental procedures](#).

Ex vivo mouse embryo culture

E8.5 embryos were isolated retaining the connection between embryo and placenta and then cultured in DMEM (Life Technologies, 11960-044) supplemented with 50% house-made rat serum



overnight at 5% CO₂ and 20% O₂ atmosphere at 37°C (Gray and Ross, 2011). SB202190 was added at the indicated concentrations. Before harvesting proteins or RNAs, heart rate (beats per minute) was determined under a stereo microscope in embryos still in the yolk sac at a constant temperature of 37°C.

Antibodies, immunoblotting, immunoprecipitation, and kinase assay

Detailed materials and methods can be found in the [supplemental information](#).

Statistical analysis

All experiments were performed at least three independent times (unless otherwise noted) in at least triplicate and the respective data were used for statistical analyses. Results from quantitative analyses are presented as mean ± SEM. Statistical analyses were performed using Student's t test with two-tailed distribution. p values represent significance: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.stemcr.2021.06.017>.

AUTHOR CONTRIBUTIONS

Y.J., Y.R., and H.R.W. performed the experiments and analyzed the data. G.D. designed the experiments, analyzed the data, and wrote the manuscript. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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