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Wnt5a and Wnt11 inhibit the canonical Wnt pathway and promote cardiac progenitor development via the Caspase-dependent degradation of AKT

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ABSTRACT

Wnt proteins regulate cell behavior via a canonical signaling pathway that induces β-catenin dependent transcription. It is now appreciated that Wnt/β-catenin signaling promotes the expansion of the second heart field (SHF) progenitor cells that ultimately give-rise to the majority of cardiomyocytes. However, activating β-catenin can also cause the loss of SHF progenitors, highlighting the necessity of precise control over β-catenin signaling during heart development. We recently reported that two non-canonical Wnt ligands, Wnt5a and Wnt11, act cooperatively to attenuate canonical Wnt signaling that would otherwise disrupt the SHF. While these data reveal the essential role of this anti-canonical Wnt5a/Wnt11 signaling in SHF development, the mechanisms by which these ligands inhibit the canonical Wnt pathway are unclear. Wnt11 was previously shown to inhibit β-catenin and promote cardiomyocyte maturation by activating a novel apoptosis-independent function of Caspases. Consistent with these data, we now show that Wnt5a and Wnt11 are capable of inducing Caspase activity in differentiating embryonic stem (ES) cells and that hearts from Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos have diminished Caspase 3 (Casp3) activity. Furthermore, SHF markers are reduced in Casp3 mutant ES cells while the treatment of wild type ES cells with Caspase inhibitors blocked the ability of Wnt5a and Wnt11 to promote SHF gene expression. This finding was in agreement with our *in vivo* studies in which injecting pregnant mice with Caspase inhibitors reduced SHF marker expression in their gestating embryos. Caspase inhibition also blocked other Wnt5a/Wnt11 induced effects, including the suppression of β-catenin protein expression and activity. Interestingly, Wnt5a/Wnt11 treatment of differentiating ES cells reduced both phosphorylated and total Akt through a Caspase-dependent mechanism and phosphorylated Akt levels were increased in the hearts Caspase inhibitor treated. Surprisingly, inhibition of either Akt or PI3K in ES cells was an equally effective means of increasing SHF markers compared to treatment with Wnt5a/Wnt11. Moreover, Akt inhibition restored SHF gene expression in Casp3 mutant ES cells. Taken together, these findings suggest that Wnt5a/Wnt11 inhibit β-catenin to promote SHF development through Caspase-dependent Akt degradation.

Introduction

Congenital heart disease (CHD) affects nearly 1% of live births and is the most common class of birth defect (Tennstedt et al., 1999). The intercellular signals that coordinate heart development and how defects in these cues cause CHD have therefore become areas of intense interest within developmental biology since a better understanding of these processes may aid the discovery of new methods to detect and treat CHD. Cardiac muscle is derived from two groups of progenitor cells initially located within a region of mesoderm underlying the head folds of early embryos called the cardiac crescent (Buckingham et al., 2005; Cai et al., 2003; Kelly et al., 2001; Kelly and Buckingham, 2002; Marguerie et al., 2006; Zaffran et al., 2004). First heart field (FHF) cardiac progenitor cells occupy the lateral region of the cardiac crescent, which will come into contact at the ventral midline as the body wall closes (Buckingham et al., 2005; Zaffran et al., 2004). Once at

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the midline, FHF progenitors form a tube and differentiate rapidly to form the linear heart of the early embryo, which will later give rise to the left ventricle (Buckingham et al., 2005; Zaffran et al., 2004). Second heart field (SHF) cardiac progenitors, which are identified by their expression of the lim/homeobox transcription factor Islet1 (Isl1), occupy more medial areas within cardiac crescent that become the mesothelium dorsal to the heart tube called the dorsal mesocardium (DM) (Cai et al., 2003; Verzi et al., 2005). In contrast to FHF progenitors, SHF progenitors remain undifferentiated and expand within the pharyngeal mesenchyme and DM before migrating to the poles of heart tube to become the majority of cardiomyocytes in the remaining chambers (Cai et al., 2003; Verzi et al., 2005).

In addition to the FHF and SHF, the cardiac neural crest (CNC) plays an essential role in cardiac development. CNC cells are initially located in the dorsal neural tube but delaminate and migrate ventrally into the heart, where they form the initial septum between the aorta and pulmonary trunk and contribute smooth muscle to the proximal outflow tract and ascending aorta (Snider et al., 2007). CNC cells also invade the semilunar cushion and are essential for outflow tract (OFT) valve development even though their descendants are not maintained in the adult structures (Snider et al., 2007). SHF progenitor and CNC cells lie in close proximity to one another and communication between the two cell types is essential for the proper development of both pools of progenitors (Rentschler et al., 2010; Rochais et al., 2009; Snarr et al., 2008; Snider et al., 2007; Vincent and Buckingham, 2010). Perturbations of the SHF and CNC therefore cause a similar spectrum of OFT defects, including persistent truncus arteriosis, double outlet right ventricle, tetralogy of Fallot and ventricular septal defects (Gittenberger-de Groot et al., 2013; Keyte and Hutson, 2012; Snider et al., 2007). Moreover, recent intersectional fate-mapping experiments have identified a lineage of cells that expresses both Wnt1 and Isl1 at some time in their history (Engleka et al., 2012). Since Wnt1 is only expressed in CNC cells within the neural tube and repressed shortly after these cells delaminate and begin their migration toward the heart (Danielian et al., 1997), these data have been interpreted to mean that a subset of CNC cells express Isl1, though it is also possible that there is transient Wnt1 expression in some SHF progenitors that has yet to be detected.

Wnt proteins are secreted ligands that signal through multiple pathways to regulate critical cellular behaviors, including the determination of cell fates, the rates of cellular proliferation, survival and differentiation as well as the levels cellular motility and adhesion (Cadigan and Nusse, 1997; Niehrs and Acebron, 2012; Teo and Kahn, 2010). Effects of Wnt proteins on gene expression are most often mediated by a canonical Wnt signaling pathway that inactivates two constitutively active kinases, Glycogen synthase kinase 3α and Glycogen synthase kinase 3β (Gsk3α/β), that act in a complex with other proteins to target the unbound form of β-catenin, a cell-cell adhesion protein, for degradation (Doble and Woodgett, 2003; Wu and Pan, 2010). Therefore, in the presence of Wnt signaling, β-catenin accumulates to high levels within the cytoplasm and nucleus, where it complexes with TCF/Lef1 family transcription factors to induce target gene transcription (Angers and Moon, 2009; Eastman and Grosschedl, 1999). In addition to signaling through β-catenin, Wnt proteins can activate non-canonical effectors such as Rho-family small GTPases, Mitogen activated protein kinases (MAPK) and Protein kinase C (PKC) (Kuhl, 2002; Strutt, 2003; Tada et al., 2002). This non-canonical Wnt signaling regulates the cytoskeleton to control cellular polarity, motility and adhesion. Additionally, non-canonical Wnt signaling frequently inhibits the canonical Wnt pathway and is believed to restrict the levels and duration of canonical Wnt signaling in several contexts (Mikels and Nusse, 2006; Topol et al., 2003; Westfall et al., 2003).

The balance between canonical and non-canonical Wnt signaling is essential for the growth and differentiation of SHF cardiac progenitor cells. We as well as others have found that loss of β-catenin in both the FHF and SHF reduces the numbers of SHF progenitor cells and disrupts SHF-derived structures without affecting the FHF, suggesting that the SHF is uniquely dependent on β-catenin dependent transcription (Ai et al., 2007; Cohen et al., 2008, 2007; Klaus et al., 2007; Kwon et al., 2007; Lin et al., 2007). In contrast, expressing a cre-inducible constitutively active form of β-catenin can either increase or decrease the numbers of SHF progenitor cells depending on the cre-line used to induce its expression (Cohen et al., 2007; Kwon et al., 2007, 2009; Qyang et al., 2007). Consistent with these later data, two non-canonical Wnt proteins expressed at the anterior pole to the heart tube during SHF migration, Wnt5a and Wnt11, promote cardiogenesis in the normally non-cardiogenic posterior mesoderm of early embryos as well as differentiating stem cells by inhibiting canonical Wnt signaling (Eisenberg and Eisenberg, 1999; Koyanagi et al., 2005, 2009; Pandur et al., 2002; Schneider and Mercola, 2001; Terami et al., 2004). Yet while these data suggest that Wnt5a and Wnt11 act in early cardiac progenitors, *Wnt5a* and *Wnt11* mutations cause mild heart defects due to problems in cell–cell adhesion and cytoskeleton organization in differentiating cardiomyocytes (Nagy et al., 2010; Schleiffarth et al., 2007; Zhou et al., 2007). However, we have more recently shown that mice lacking both Wnt5a and Wnt11 die early in embryogenesis with single chambered hearts resembling those caused by SHF ablation (Cohen et al., 2012). Further analysis revealed a loss of SHF progenitor cells and an associated increase in canonical Wnt signaling within the OFT and adjacent mesenchyme of $Wnt5a^{-/-}$; $Wnt11^{-/-}$ embryos relative to controls. Treating embryoid bodies (EBs) with Wnt5a and Wnt11 also caused a synergistic increase in cardiac progenitor gene expression, an effect that is blocked by forced β-catenin activation and mimicked by canonical Wnt pathway inhibition. Together, these data indicate that Wnt5a and Wnt11 act cooperatively to restrain canonical Wnt signaling in the SHF that would otherwise disrupt cardiac progenitor development.

The effects of Wnt5a and Wnt11 on SHF progenitors may be mediated by the Caspase-dependent inhibition of the canonical Wnt pathway. Wnt11 overexpression has been shown to promote later events of cardiomyocyte differentiation, such as the expression of cardiac Troponin T and the appearance of sarcomeres, in cultures of differentiating P19 embryonic carcinoma cells by inhibiting the canonical Wnt pathway (Abdul-Ghani et al., 2011). Interestingly, these effects of Wnt11 were associated with an increase in the activity of Caspases, a large family of cysteine proteases that play critical roles in programmed cell death (Elmore, 2007; McIlwain et al., 2013), and can be blocked by the addition of Caspase inhibitors to the culture media (Abdul-Ghani et al., 2011). Importantly, the increase in Caspase activity observed with Wnt11 was not associated with an increase in the numbers of cells marked by TUNEL staining (Abdul-Ghani et al., 2011), suggesting it is unrelated to apoptosis. Caspases have also been shown to have apoptotic-independent roles in the differentiation of skeletal muscle (Fernando et al., 2002; Murray et al., 2008). All in all, these data suggest that Wnt11 promotes cardiomyocyte maturation via the Caspase-dependent inhibition of the canonical Wnt pathway. While the aforementioned study focused on later cardiac development, these data raised the possibility that the cooperative effects of Wnt5a and Wnt11 on the early development of cardiac progenitor cells are similarly mediated by non-apoptotic Caspase signaling.

The data presented in this manuscript suggest that cooperative Wnt5a/Wnt11 signaling inhibits the canonical Wnt pathway and promotes SHF development via the Caspase-dependent inhibition of Akt. Treatment of mouse ES cells derived EBs with recombinant Wnt5a and Wnt11 increased the processing of Casp3, Caspase 6 (Casp6) and Caspase 7 (Casp7) into their active forms, which correlated with increased function as evidenced by the activation of a luciferase based Casp3/7 reporter. Conversely, staining for

cleaved-Casp3 was reduced in the OFT and surrounding mesenchyme of Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos relative to controls. Furthermore, SHF markers are reduced in Casp3 mutant ES cells while the treatment of wild type ES cells with Z-VAD-FMK, a broad spectrum Caspase inhibitor, blocked the ability of Wnt5a and Wnt11 to promote SHF gene expression. Consistent with these data, SHF marker expression was reduced in the embryos of pregnant mice injected with Z-VAD-FMK relative to controls. Caspase inhibition also prevented the reduction of β-catenin proteins and activity associated with Wnt5a/Wnt11 treatment. Interestingly, staining for phosphorylated Akt was increased in the hearts of Z-VAD-FMK treated embryos relative to wild type controls. Wnt5a/Wnt11 treatment also caused the Caspase-dependent loss of both phosphorylated and total Akt protein in differentiating ES cells while inhibiting either Akt or its upstream activator PI3K increased SHF marker expression to levels similar to those observed following Wnt5a/Wnt11 treatment. Finally, treating EBs made from Casp3$^{-/-}$ ES cells with an Akt inhibitor restored SHF gene expression to levels similar to those in control EBs. Taken together, these data suggest a model in which cooperative Wnt5a/Wnt11 signaling inhibits β-catenin and promotes SHF development by inducing the Caspase-dependent reduction of Akt activity.

Materials and methods

Generation of Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos and in utero inhibition of Caspase function

Mice carrying previously published alleles of Wnt5a and Wnt11 were interbred to produce Wnt5a$^{-/-}$; Wnt11$^{-/-}$ and wild type embryos as previously described (Cohen et al., 2012). Genotyping for Wnt5a and Wnt11 was performed as described (Majumdar et al., 2003; Yamaguchi et al., 1999). For the *in utero* inhibition of Caspase function, male and female CD1 mice were obtained from Charles River and mated. The presence of copulation plugs was used to identify pregnant females on day 0.5 of gestation (E0.5). Pregnant females were given intra-peritoneal injections of 5 μl/g body weight of either 1 mM Z-VAD-FMK (Calbiochem, 627610), a synthetic peptide that inhibits a broad range of Caspase family members (Garcia-Calvo et al., 1998; Slee et al., 1996), 1 mM Ac-DEVD-CHO (Calbiochem, 235420), a potent and more selective inhibitor of Casp3 (Garcia-Calvo et al., 1998; Nicholson et al., 1995), or their vehicle DMSO on E6.5, E7.5 and E8.5 of pregnancy. Injected females were then sacrificed on E9.5 and their gestating embryos harvested for subsequent analysis. The University Committee on Animal Resources (UCAR) at the University of Rochester approved all protocols and procedures.

Histology and immunological staining

Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos and their wild type siblings as well as embryos from pregnant females injected with Z-VAD-FMK and DMSO were collected at E9.5 and fixed in 4% PFA before being dehydrated with a series of washes in increasing ethanol concentration, embedded in paraffin and sectioned as described (Cohen et al., 2012, 2007; Nagy, 2003). Immunohistochemical and immunofluorescent staining with primary antibodies specific for Isl1 (Millipore, AB4326), PlexinA2 (Santa Cruz, sc-10144), Nkx2.5 (Santa Cruz, sc-8697), cleaved-Casp3 (Cell Signaling, 9664), phospho-ser473-Akt (Cell Signaling, 4060) and active-β-catenin (Millipore, 05-665) as well as biotinylated secondary antibodies recognizing mouse, rabbit and goat IgG (Vector Laboratories, BA-9200, BA-1000 and BA-9500, respectively), Alexa Fluor 488 conjugated anti-goat IgG (Jackson Immunoresearch, 705-545-147) or Dylight 594 conjugated anti-rabbit IgG (Vector Laboratories, DI-1094) was performed as described (Cohen et al., 2012, 2007; Nagy, 2003). Biotinylated secondary antibodies were detected using the Vectastain Elite ABC and DAB Peroxidase Substrate kits (Vector Laboratories, PK-1000 and SK-4100, respectively) according to the manufacturer's protocol. TUNEL staining was performed using the ApopTag® Red kit (Millipore, S7165) according to the manufacturer's protocol.

Generation and culture of EBs

The TL1 line mouse ES cells was generated by Dr. Patricia A Labosky and had previously been used to model the effects of Wnt5a and Wnt11 on cardiac progenitor development *in vitro* (Cohen et al., 2012). The Casp3$^{-/-}$, Casp3$^{-/+}$, rev-Casp3-CA and rev-Casp3-mut mouse ES cell lines were previously used to examine the roles of Casp3 in the switch from self-renewal to differentiation described (Fujita et al., 2008). All lines of mouse ES cells were grown on inactivated mouse embryonic fibroblasts (Millipore, PMEF-N) in high glucose DMEM (Life Technologies, 11965-092) supplemented with 15% FBS (Hyclone, SH309100HI), antibiotic-antimycotic (Life Technologies, 15240-062), Glutamax® (Life Technologies, 35050-061), non-essential amino acids (Life Technologies, 11140-050), sodium pyruvate (Life Technologies, 11360070), β-mercaptoethanol (Fisher Scientific, O3446I-100) and LIF (Millipore, ESG1107) as described (Cohen et al., 2012). To initiate differentiation, cells were dissociated, suspended at 5×10^4 cells/ml in differentiation media (same as growth media but with 20% FBS and without LIF) and used to make 20 μl drops on the underside of petri-dish lids (Cohen et al., 2012; Fuegemann et al., 2010). Cells were then cultured as hanging drops for 48 h to form EBs, which were then cultured for another 2 days in poly-HEMA (Sigma-Aldrich, P3932) coated dishes.

On day 4 of differentiation, EBs were transferred to gelatin (Sigma-Aldrich, G1393) coated dishes containing control media, media containing 200 ng/ml recombinant Wnt5a (RnD Systems, 645-WN-10), 200 ng/ml Wnt11 (RnD Systems, 6179-WN-10) or 100 ng/ml Wnt5a and 100 ng/ml Wnt11. When indicated, 10 μM Z-VAD-FMK, 1 μM Akt-i-VIII (Calbiochem, 124018), 1 μM PI-103 (Calbiochem, 528100), 50 nm Bio (Calbiochem, 361550), 50 nM meBio (Cabiochem, 361556), 100 nM XAV939 (Selleck Chemicals, S1180) or DMSO was added to the culture media. EBs were then either cultured for 48 h and lysed in Trizol (Life Technologies, 15596-026) for total RNA extraction or cultured for 24 h and lysed in RIPA buffer supplemented with a mix of protease and phosphatase inhibitors (Thermo Scientific, PI-89901 and PI-78420, respectively) for Western blotting. To determine if blocking Akt function was sufficient to restore Isl1 expression in Casp3$^{-/-}$ ES cells, EBs made from Casp3$^{-/-}$ and Casp3$^{-/+}$ ES cells were treated with either 1 μM Akt-Inhibitor VIII or DMSO between days 3 and 5 of differentiation before being lysed in Trizol RNA isolation. To determine if Caspase overexpression was sufficient to mimic the effects of Wnt5a and Wnt11 on Isl1 expression, EBs made from rev-Casp3-ca and rev-Casp3-mut ES cells were treated with 1 μg/ml doxycycline from days 4 to 5 of differentiation to induce Casp3 expression as described (Fujita et al., 2008).

Measuring Caspase and TCF activity in EBs

To measure the effects of Wnt5a and Wnt11 on the activities of Casp3 and Casp7, EBs were made from TL1 ES cells and transferred to gelatin-coated dishes containing either control media or media supplemented with recombinant Wnt5a and Wnt11 proteins on day 4 of differentiation. EBs were then cultured for 24 h before being lysed in a hypotonic extraction buffer as described (Botto et al., 2011). Protein concentrations were determined using the DC Protein Assay System (Bio-Rad, 500-0111) according to manufacturer's protocol,

allowing each sample to be adjusted to a final concentration of 1 mg/ml. 5 μL aliquots of lysate from each sample were then placed into the wells of an opaque white 96-well assay plate containing 50 ml of the Caspase-Glo 3/7 Assay reagent (Promega, G8090) and incubated for 30 min. The luminescence of each well was then determined using a Biotek Synergy HT Microplate Reader. Reported values are the mean ± the standard deviation for measurements taken from at least three independent replicates. $P < 0.05$ by two-tailed Student's *t*-test indicated statistical significance.

To measure the effects of Wnt5a and Wnt11 on β-catenin-dependent transcription, TL1 mouse ES cells were transiently transfected with Super 8x TOPFlash (Addgene, 12456), a reporter which uses 8 tandem repeats of the consensus TCF/LEF binding site to drive firefly luciferase expression in response to β-catenin signaling, as well as the constitutively active *Renilla* luciferase vector pRL-TK (Promega, E2241) using Effectene reagent (Qiagen, 301425) as described (Ko et al., 2009). Transfected ES cells were used to generate EBs, which were then treated with Wnt5a, Wnt11 or Wnt5a and Wnt11 on day 4 of differentiation as described. After 24 h, luciferase activity was assayed using the Dual Luciferase Assay System (Promega, E1910). *Renilla* luciferase values were used to normalize for transfection efficiency. Reported values are the mean ± standard deviation. $P < 0.05$ by two-tailed Student's *t*-test indicated statistical significance.

Quantitative PCR (Q-PCR) and Western blotting

To examine the effects of Wnt5a and Wnt11 on transcription, relative gene expression was calculated by the ΔC_T method using Gapdh as an endogenous control. For Q-PCR analysis of embryonic hearts, graphs represent the average results from three independent pools of RNA isolated from the heart-containing regions, defined as the heart and mesenchyme ventral to the foregut from the second pharyngeal arch to the septum transversum, of at least five embryos. For Q-PCR analysis of EBs, graphs represent the average values from four pools of RNA isolated from independent experiments. RNA was reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad, 170-8840) and Q-PCR performed on a Bio-Rad CFX386 thermocycler with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, 172-5270). The primer pairs used to detect Isl1, Nkx2.5, Tnni3 and Gapdh were previously described (Cohen et al., 2012). The primers used to detect Akt1, Casp3, Casp6, Casp7, PlexinA2 and p75 are listed in Supplementary Table 1. The ΔC_T values of all samples were normalized to the average ΔC_T of control samples, thus allowing the standard deviation to be calculated from the ΔC_T values of controls. Error bars represent s.d. Student's *t*-test with $P < 0.05$ indicated statistical significance.

To examine the effects of Wnt5a and Wnt11 on protein levels, EBs were treated with Wnt5a and Wnt11 for 24 h as described and lysed in RIPA buffer. Lysates were subjected to SDS-PAGE and transferred to PVDF membranes, which were then subject to Western blotting with antibodies recognizing cleaved forms of Casp3, Casp6 and Casp7 (Cell signaling, 9664, 9761 and 9491, respectively), total Casp3, Casp6 and Casp7 (Cell Signaling, 9662, 9762 and 9492, respectively), phospho-ser473-Akt, pan-Akt, Akt1 and Akt2 (Cell Signaling, 4060, 4691, 2938 and 3063, respectively), activated (hypo-phosphorylated) β-catenin (Millipore, 05–665), total β-catenin (BD Transduction Laboratories, 610154), β-Tubulin (Cell Signaling, 2128) and Gapdh (Thermo Scientific, PA1987) as well as HRP conjugated anti-mouse and anti-rabbit secondary antibodies (Vector Laboratories, PI-2000 and PI-1000, respectively). Signal was detected using Super Signal West Pico and Super Signal West Femto ECL substrates (Thermo Scientific, PI-34078 and PI-34094, respectively) and either Hyperfilm ECL (GE Healthcare Biosciences, 28-9068-39) or a Bio-Rad Chemidoc XRS imaging system.

Results

Cooperative Wnt5a/Wnt11 signaling induces executioner Caspase activity in vivo and in vitro

Wnt11 was shown to promote the maturation of P19 cell derived cardiomyocytes via Caspase-dependent β-catenin inhibition (Abdul-Ghani et al., 2011). We therefore sought to determine if the loss of SHF progenitors and increased canonical Wnt signaling observed in $Wnt5a^{-/-}$; $Wnt11^{-/-}$ embryos was associated with altered Caspase activity (Cohen et al., 2012). Caspases are separated into two classes based on their roles in apoptotic signaling (Elmore, 2007; McIlwain et al., 2013). Initiator Caspases are activated by extrinsic stimuli such as death receptor signaling or intrinsic stimuli such as the release of Cytochrome C from mitochondria. Once activated, initiator Caspases will cleave the zymogen forms of the downstream executioner Caspases, Casp3, Casp6 and Casp7, into their active forms, which then act upon numerous targets. Among executioner Caspases, Wnt11 was shown to specifically activate Casp3 in P19 cells (Abdul-Ghani et al., 2011). Sections of E9.5 $Wnt5a^{-/-}$; $Wnt11^{-/-}$ embryos and their wild type littermates were therefore stained with an antibody against Casp3 cleaved at asparagine 175 (cleaved-Casp3). In wild type embryos, cleaved-Casp3 was prominent in the walls of the OFT (arrows in Fig. 1A) and DM (arrowheads in Fig. 1A), regions containing SHF progenitors, as well as the roof of the OFT (dashed bracket in Fig. 1A) and foregut endoderm. On the other hand, cleaved-Casp3 was strongly reduced in these regions of $Wnt5a^{-/-}$; $Wnt11^{-/-}$ embryos (arrows, arrowheads and dashed brackets in Fig. 1C). There were slightly higher levels of residual cleaved-Casp3 staining in the OFT walls than in the roof of the OFT and DM in $Wnt5a^{-/-}$; $Wnt11^{-/-}$ embryos, suggesting that there may be redundant mechanisms regulating Casp3 activity in these areas. Importantly, TUNEL staining did not overlap with cleaved-Casp3 stained areas in the walls of the OFT or DM in either wild type or $Wnt5a^{-/-}$; $Wnt11^{-/-}$ embryos (arrows, arrowheads and dashed brackets in Fig. 1B and D). In contrast, large numbers of cells located within the otic placode (arrows in Fig. 1E and F) and neural tube (arrowheads in Fig. 1E and F) of $Wnt5a^{-/-}$; $Wnt11^{-/-}$ embryos stained strongly for both cleaved Casp3 and TUNEL. Moreover, while there were more TUNEL positive cells in the pharyngeal mesenchyme of $Wnt5a^{-/-}$; $Wnt11^{-/-}$ than of control embryos, the increase in apoptotic nuclei correlated with decreased levels of cleaved-Casp3 staining. Taken together, these data suggest that cleaved-Casp3 in the SHF is dependent on Wnt5a and Wnt11 signaling and distinct from the Casp3 activation associated with programmed cell death.

The loss of cleaved-Casp3 staining in the roof of the OFT was interesting since this area contains high numbers of CNC cells (Bradshaw et al., 2009), suggesting that the combined loss of Wnt5a and Wnt11 may also disrupt the CNC. To address this issue, sections of $Wnt5a^{-/-}$; $Wnt11^{-/-}$ and control embryos were fluorescently co-stained for Isl1 and PlexinA2, a Semaphorin receptor expressed in migrating neural crest (Brown et al., 2001). In wild type embryos, Is1 positive cells were present in the anterior (arrowhead in supplemental Fig. 1A and C) as well as posterior walls of the OFT and DM (large arrows in supplemental Fig. 1A and C). Cells expressing lower levels of Isl1 were also present in the roof of the OFT (small arrows in supplemental Fig. 1A and C), which is contiguous with the foregut endoderm, as well as in cells located within the lumen of the OFT (double-headed arrows in supplemental Fig. 1A and C). While the Isl1 positive cells in the anterior wall of the OFT did not express PlexinA2 (arrowhead in supplemental Fig. 1B and C), PlexinA2 was expressed by the Isl1 positive cells in the posterior wall of the OFT and DM (large arrows in supplemental Fig. 1B and C). Furthermore, some of the cells expressing low levels of Isl1 in the roof and lumen of the OFT (small arrows in supplemental Fig. 1B and C) and

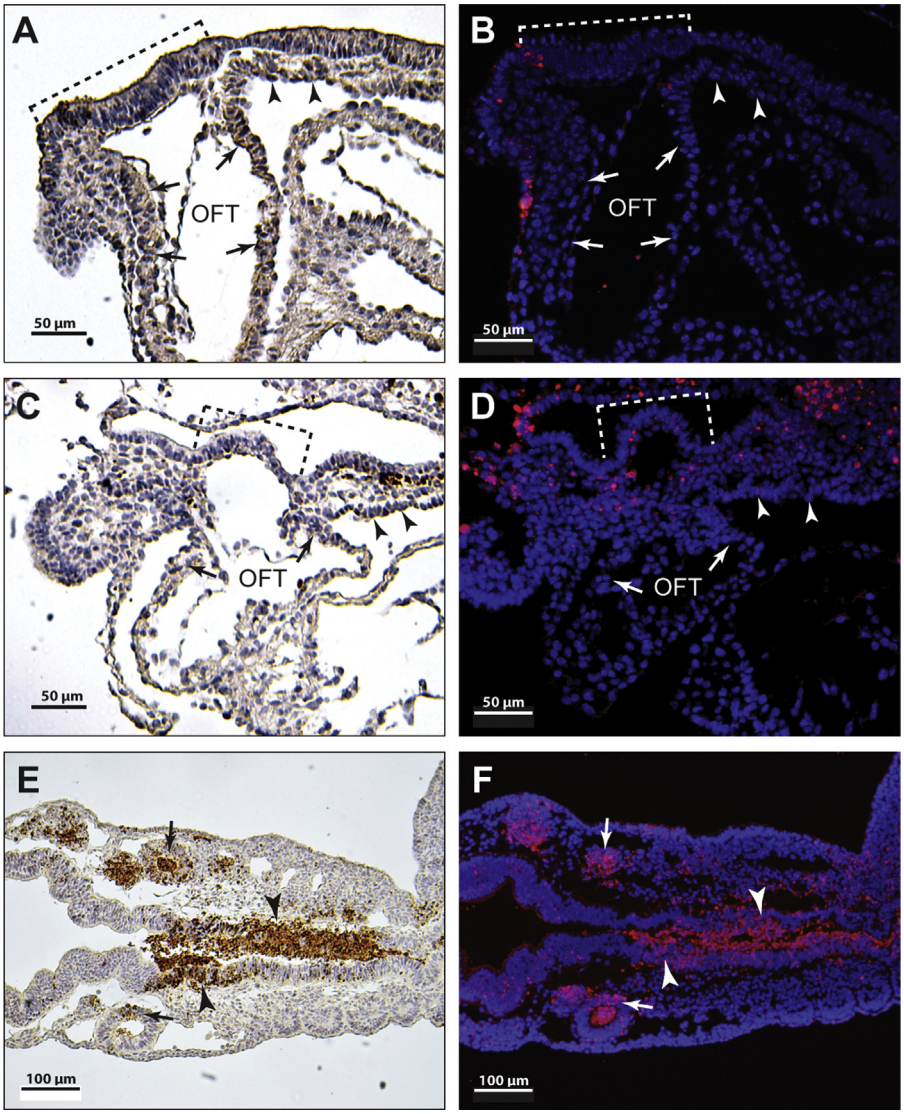


Fig. 1. Wnt5a and Wnt11 are co-required for non-apoptotic Caspase activity in the SHF. (A) and (C) Longitudinal sections of E9.5 wild type and Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos were stained for cleaved-Casp3 (brown staining). Cleaved-Casp3 levels were higher in the walls of the outflow tract (OFT), dorsal mesocardium (DM, arrowheads) and the roof of the OFT (dashed brackets) of wild type than of Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos. (B) and (D) Cells in the OFT (arrows), DM (arrowheads) and the roof of the OFT (dashed brackets) were not labeled by TUNEL staining in adjacent sections of wild type (B) or Wnt5a$^{-/-}$; Wnt11$^{-/-}$ (D) embryos. (E) and (F) In contrast, large numbers of cells within the otic placodes (arrows) and neural tubes (arrowheads) stained strongly for both cleaved-Casp3 and TUNEL in frontal sections of E9.5 Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos.

OFT lumen (double-headed arrows in supplemental Fig. 1B and C) also expressed PlexinA2. In contrast, the expression of both Isl1 and PlexinA2 were reduced or absent in the anterior (arrowheads in supplemental Fig. 1D–F) and posterior wall of the OFT and DM (large arrows in supplemental Fig. 1D–F) in the hearts of Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos relative to controls. While the lower level expression of Isl1 and PlexinA2 in the lumen of the OFT (double-headed arrows in supplemental Fig. 1D–F) was also absent in Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos, some cells in the roof of the OFT (small arrows in supplemental Fig. 1D and E) continued to express Isl1 but not PlexinA2. Together, these data suggest that both SHF progenitor and CNC cells, including those that express Isl1, are affected by the combined loss of Wnt5a and Wnt11 function.

Although Wnt11 was solely sufficient to inhibit canonical Wnt signaling and induce cardiac maturation in P19 cells (Abdul-Ghani et al., 2011), the adenoviral vector used in these studies may have produced Wnt11 at high enough concentrations to circumvent the need for Wnt5a. To determine if the cooperative effects of Wnt5a and Wnt11 on cardiac progenitor cells correlated with altered Caspase activity, EBs were generated from TL1 mouse ES cells as previously described (Cohen et al., 2012). On day four of differentiation, EBs were plated on gelatin-coated dishes in the presence of Wnt5a, Wnt11 or both. After 48 h, the levels of mRNA for Isl1, the pan-cardiac progenitor marker Nkx2.5 and the executioner Caspases, Casp3, Casp6 and Casp7, were analyzed by Q-PCR (Fig. 2A). Consistent with our previous findings, Isl1 and Nkx2.5 levels were modestly increased in Wnt5a treated EBs, moderately increased in Wnt11 treated EBs and strongly increased in EBs treated with both Wnt5a and Wnt11. In contrast, the levels of Casp3, Casp6 and Casp7 mRNA were unaffected by Wnt5a/Wnt11 treatment. To determine if the synergistic effects of Wnt5a and Wnt11 on Isl1 and Nkx2.5 expression were associated with increased Caspase activity, lysates from treated and control EBs were subject to Western blotting with antibodies recognizing cleaved-Casp3 as well as the activated forms of Casp6 and Casp7 generated by initiator Caspase cleavage at asparagine 162 and 198, respectively (Fig. 2B). Interestingly, the levels of cleaved-Casp3, cleaved-Casp6 and cleaved-Casp7 were moderately elevated in EBs treated with Wnt11 alone and strongly elevated in EBs treated with Wnt5a and Wnt11 in combination.

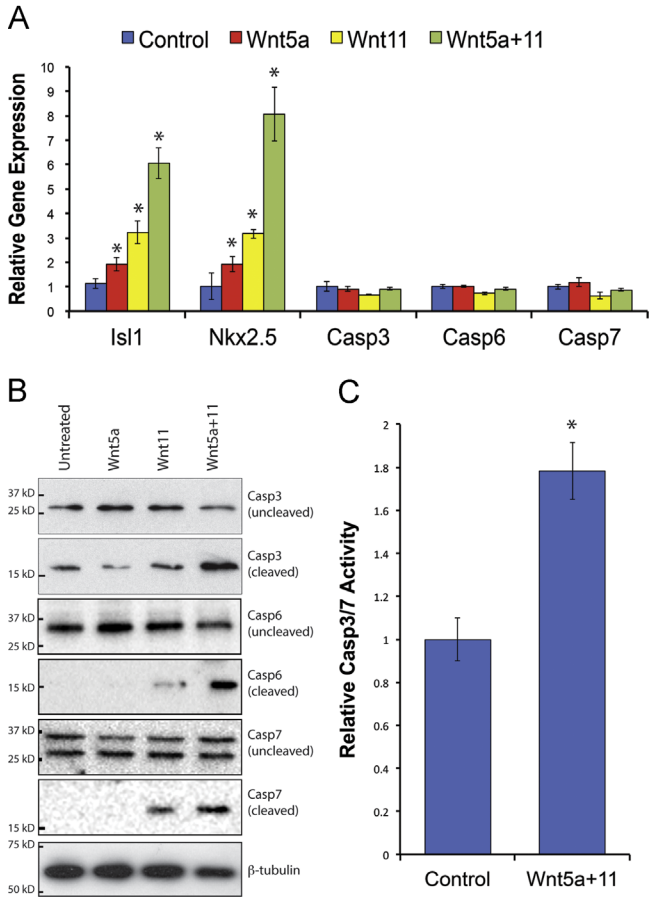


Fig. 2. Wnt5a and Wnt11 signal cooperatively to activate Caspases in differentiating ES cells. (A) Graph shows the results of Q-PCR for Isl1, Nkx2.5, Casp3, Casp6 and Casp7 performed on RNA from EBs treated with 200 ng/ml Wnt5a, 200 ng/ml Wnt11 or 100 ng/ml Wnt5a and 100 ng/ml Wnt11 on day 4 of differentiation and harvested after 48 h. (B) Western blots for both the cleaved and uncleaved forms of Casp3, Casp6 and Casp7 as well as β-tubulin were performed on lysates from EBs treated with Wnt5a, Wnt11 or Wnt5a and Wnt11 on day 4 of differentiation as described and harvested after 24 h. (C) Graph shows the results of CaspaseGlo-3/7 assays, a luciferase-based reporter of Casp3 and Casp7 activity, performed on EBs treated with control media or media containing both 100 ng/ml Wnt5a and 100 ng/ml Wnt11 on day 4 of differentiation and harvested 24 h later. Graphs represent the mean of 3 independent replicates ± the standard deviation. Astericks (*) indicate $P < 0.05$ vs. controls.

A commercially available luciferase-based reporter, CaspaseGlo 3/7 (Promega) was used to assess whether the elevated levels of cleaved-Casp3 and cleaved-Casp7 observed by Western blotting corresponded to an increase in activity (Fig. 2C). As expected, CaspaseGlo 3/7, revealed that Casp3/7 activity was higher in Wnt5a/Wnt11 treated EBs compared to untreated controls. Importantly, levels of the unprocessed forms of Casp3, Casp6 and Casp7 were not increased by Wnt5a/Wnt11 treatment, suggesting that the effects of these ligands on Caspase activity are achieved through increased post-translational processing.

Caspase activity is required for SHF progenitor cells and Wnt5a/Wnt11 signaling

While Wnt11 was shown to promote the maturation of P19 cell derived cardiomyocytes through the Caspase-dependent inhibition of the canonical Wnt pathway (Abdul-Ghani et al., 2011), these experiments focused on later events of cardiac differentiation, such as the expression of contractile proteins and formation of sarcomeres, and did not examine the effects of Wnt-induced Caspase activity on cardiac progenitor cells. To address this issue, EBs were generated from the TL1 strain of wild type mouse ES cells as well as ES cells that were either heterozygous or homozygous for a null allele of Casp3 (Cohen et al., 2012; Fujita et al., 2008). Q-PCR revealed that Casp3 mRNA was lower in Casp3$^{+/-}$ EBs than in WT EBs and below detection in Casp3$^{-/-}$ EBs on day 5 of differentiation while the expression of Casp6 and Casp7 were unaffected (Fig. 3A). The levels of Isl1 and Nkx2.5 mRNA were also moderately reduced in Casp3$^{+/-}$ EBs and strongly reduced in the Casp3$^{-/-}$ EBs relative to wild type control EBs at this time, indicating that Casp3 is required for cardiac progenitor development *in vitro*. To assess how the loss of Casp3 function affected SHF development over time, the levels of Isl1 mRNA were examined in Casp3$^{+/-}$ and Casp3$^{-/-}$ EBs between days 2 and 6 of differentiation (Fig. 3B). Although the levels of Isl1 mRNA increased sharply between days 3 and 4 of differentiation in Casp3$^{+/-}$ EBs, Isl1 expression was only slightly higher on day 4 of differentiation than it was on day 3 in Casp3$^{-/-}$ EBs. Furthermore, while the levels of Isl1 mRNA declined between days 4 and 6 of differentiation in Casp3$^{+/-}$ EBs, Isl1 levels increased in Casp3$^{-/-}$ EBs over this period and were nearly equivalent to those found in Casp3$^{+/-}$ EBs by day 6 of differentiation. These data suggest that Casp3 loss-of-function causes a delay rather than a loss of SHF development and may explain why the Casp3 mutant mice from which the Casp3$^{+/-}$ and Casp3$^{-/-}$ ES cell lines were generated were reported to be partially viable and have morphologically normal hearts (Fujita et al., 2008; Kuida et al., 1996).

While the low levels of Isl1 and Nkx2.5 mRNA in Casp3$^{-/-}$ EBs relative to WT and Casp3$^{+/-}$ EBs suggest that Casp3 plays a role in SHF development, it remains possible that these SHF markers are reduced as a secondary consequence of the poor health of Casp3$^{-/-}$ EBs. We therefore sought to determine if Casp3 overexpression was sufficient to induce Isl1 and Nkx2.5. EBs were made from ES cells that expressed either a constitutively active (rev-Casp3-CA) or an inactive mutant (rev-Casp3-mut) form of Casp3 in response to doxycycline (Fujita et al., 2008). On day 4 of differentiation, EBs were treated with doxycycline for 24 h before having their levels of Isl1 and Nkx2.5 mRNA examined by Q-PCR (Fig. 3C). In the presence of doxycycline, EBs made from rev-Casp3-CA cells expressed Isl1 and Nkx2.5 at higher levels than in they do in control media, suggesting that Casp3 activation is sufficient to promote cardiac progenitor development during ES cell differentiation. In contrast, doxycycline treatment does not affect Isl1 and Nkx2.5 expression in EBs made from rev-Casp3-mut ES cells.

Although these data indicate that Casp3 is necessary and sufficient for SHF progenitor gene expression, the effects of Casp3 on Isl1 and Nkx2.5 mRNA levels could still be distinct from those caused by Wnt5a and Wnt11. However, if Caspases act in a pathway that mediates Wnt5a/Wnt11 signaling, Caspase function will be required for Wnt5a and Wnt11 to induce SHF gene expression. EBs were therefore treated with recombinant Wnt5a and Wnt11 proteins as described but with the addition of either Z-VAD-FMK or its vehicle DMSO to the culture media at the time of Wnt treatment on day 4 of differentiation. On day 6 of differentiation, mRNA was isolated from EBs and examined for changes in Isl1 and Nkx2.5 expression (Fig. 3D and E, respectively). Consistent with our previous data, Isl1 and Nkx2.5 mRNA levels were increased by combined Wnt5a/Wnt11 treatment without the inhibitor. In contrast, Isl1 and Nkx2.5 were reduced in EBs treated with Wnt5a and Wnt11 in the presence of Z-VAD-FMK, suggesting that Caspase activity is required for Wnt5a and Wnt11 to induce cardiac progenitor gene expression in differentiating ES cells.

While the ability of z-VAD-FMK to inhibit multiple Caspases circumvents problems caused by functional redundancy, it prevents this inhibitor from being used to distinguish between roles played by individual Caspase family members. Moreover, since z-VAD-FMK can inhibit other families of cysteine proteases such as the Papains (Garcia-Calvo et al., 1998; Slee et al., 1996), Calpains (Rozman-Pungercar et al., 2003) and Cathepsins (Wolf et al., 1999), it is unclear if the effects of

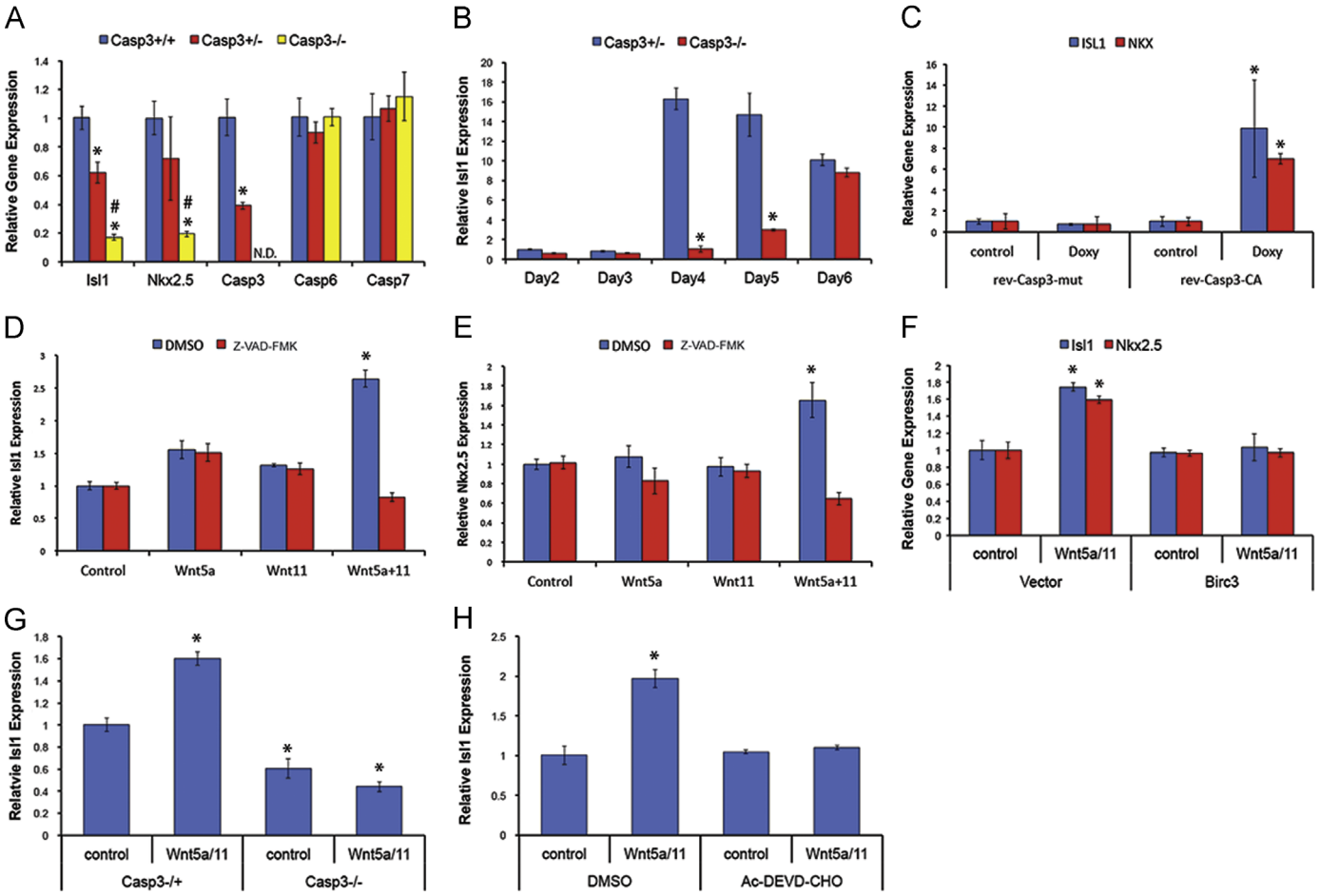


Fig. 3. Caspase activity is necessary and sufficient for the effects of Wnt5a and Wnt11 on cardiac progenitor cells *in vitro*. (A) Casp3$^{+/+}$, Casp3$^{+/-}$ and Casp3$^{-/-}$ EBs were harvested on day 5 of differentiation and analyzed for Isl1, Nkx2.5, Casp3, Casp6 and Casp7 expression. Graph shows the fold change in the expression of each gene in Casp3$^{+/-}$ and Casp3$^{-/-}$ EBs relative that found in Casp3$^{+/+}$ EBs, which are normalized to one. Casp3 mRNA was below the levels of detection in Casp3−/− EBs and therefore marked as not detected (N.D.). (B) Casp3$^{+/-}$ and Casp3$^{-/-}$ EBs were harvested once every 24 h between days 2 and 6 of differentiation and analyzed for Isl1 expression. The graph shows the levels of Isl1 in Casp3$^{+/-}$ and Casp3$^{-/-}$ EBs on each day expressed as a fold change relative to those in Casp3$^{+/-}$ EBs on day 2 of differentiation, which were normalized to one. (C) EBs made from rev-Casp3-CA and rev-Casp3-mut ES cells were treated with doxycycline between days 4 and 5 of differentiation and then anazyzed for Isl1 and Nkx2.5 expression. Graphs show the fold changes in Isl1 and Nkx2.5 expression relative to the levels found in rev-Casp3-mut EBs cultured in the absence of doxycycline. (D) and (E) EBs made from wild type mouse ES cells were treated with Wnt5a, Wnt11 or both Wnt5a and Wnt11 as described but with the addition of either 10 μM Z-VAD-FMK or DMSO to the culture media. Graph shows the fold changes in Isl1 (D) and Nkx2.5 (D) expression under each condition relative to the levels in untreated EBs in DMSO containing media, which were normalized to one. (F) EBs made from ES cells transfected with empty and Birc3 expressing vectors were cultured in control media and media with 100 ng/ml Wn5a and 100 ng/ml Wnt11 between days 4 and 5 of differentiation before being harvested and analyzed for Isl1 and Nkx2.5 expression. Graph shows the levels of Isl1 and Nkx2.5 for each condition expressed as a fold change relative to the levels in empty vector transfected EBs cultured in control media. (G) Casp3$^{+/-}$ and Casp3$^{-/-}$ EBs were cultured in the presence or absence of Wnt5a and Wnt11 as described between days 4 and 5 of differentiation before being harvested and analyzed for Isl1 expression. Graph shows the fold changes in Isl1 levels for each condition relative to those present in untreated Casp3$^{+/-}$ EBs. (H) EBs made from TL1 ES cells were treated with Wnt5a and Wnt11 as described in media containing either 1 μM Ac-DEVD-CHO or its vehicle DMSO from days 4–5 of differentiation before being harvested and analyzed for Isl1 expression. Graph shows the fold changes in Isl1 expression under each condition relative to that found in untreated EBs cultured in DMSO containing media, which were normalized to one. All graphs show the mean results of 3 independent replicates ± the standard deviation. Asterisks (*) indicates $P < 0.05$ vs. controls.

this inhibitor on Wnt5a/Wnt11 signaling stem from a loss of Caspase activity. To address this issue, wild type ES cells were transfected with either an empty expression vector or an expression vector encoding the cellular Inhibitor of apoptosis protein 2 (cIAP2), also known as Baculoviral IAP repeat 3 (Birc3), which binds and ubiquitinates Casp3 and Casp7 to inhibit their activity (Schotte et al., 1999). Birc3 expressing and control EBs were generated from the transfected cells and cultured in either control or Wnt5a/Wnt11 containing media between days 4 and 6 of differentiation before being harvested for the examination of gene expression. While Isl1 and Nkx2.5 levels were higher in vector transfected EBs that had been treated with Wnt5a and Wnt11 than in those that were cultured in control media (Fig. 3F), Wnt5a and Wnt11 did not affect the expression of these genes in Birc3 expressing EBs suggesting that Casp3 and Casp7 are required for the effects of Wnt5a and Wnt11 on SHF gene expression. To further determine if Casp3 was uniquely required for Wnt5a/Wnt11 signaling, Casp3$^{+/-}$ and Casp3$^{-/-}$ EBs as well as WT EBs treated with either DMSO or Ac-DEVD-CHO, a potent inhibitor and more specific inhibitor of Casp3 (Huang et al., 2000; Verhagen et al., 2001), were cultured in the presence or absence of Wnt5a and Wnt11 between days 4 and 5 of differentiation before being harvested for Q-PCR. In agreement with our previous data, the levels of Isl1 mRNA were higher in Casp3$^{+/-}$ and DMSO treated EBs that had been treated with Wnt5a and Wnt11 than in their untreated counterparts (Fig. 3G and E, respectively). In contrast, Wnt5a and Wnt11 did not increase Isl1 expression in Casp3$^{-/-}$ or Ac-DEVD-CHO treated EBs, consistent with Casp3 being uniquely required for the effects of Wnt5a and Wnt11 on cardiac progenitor gene expression during ES cell differentiation.

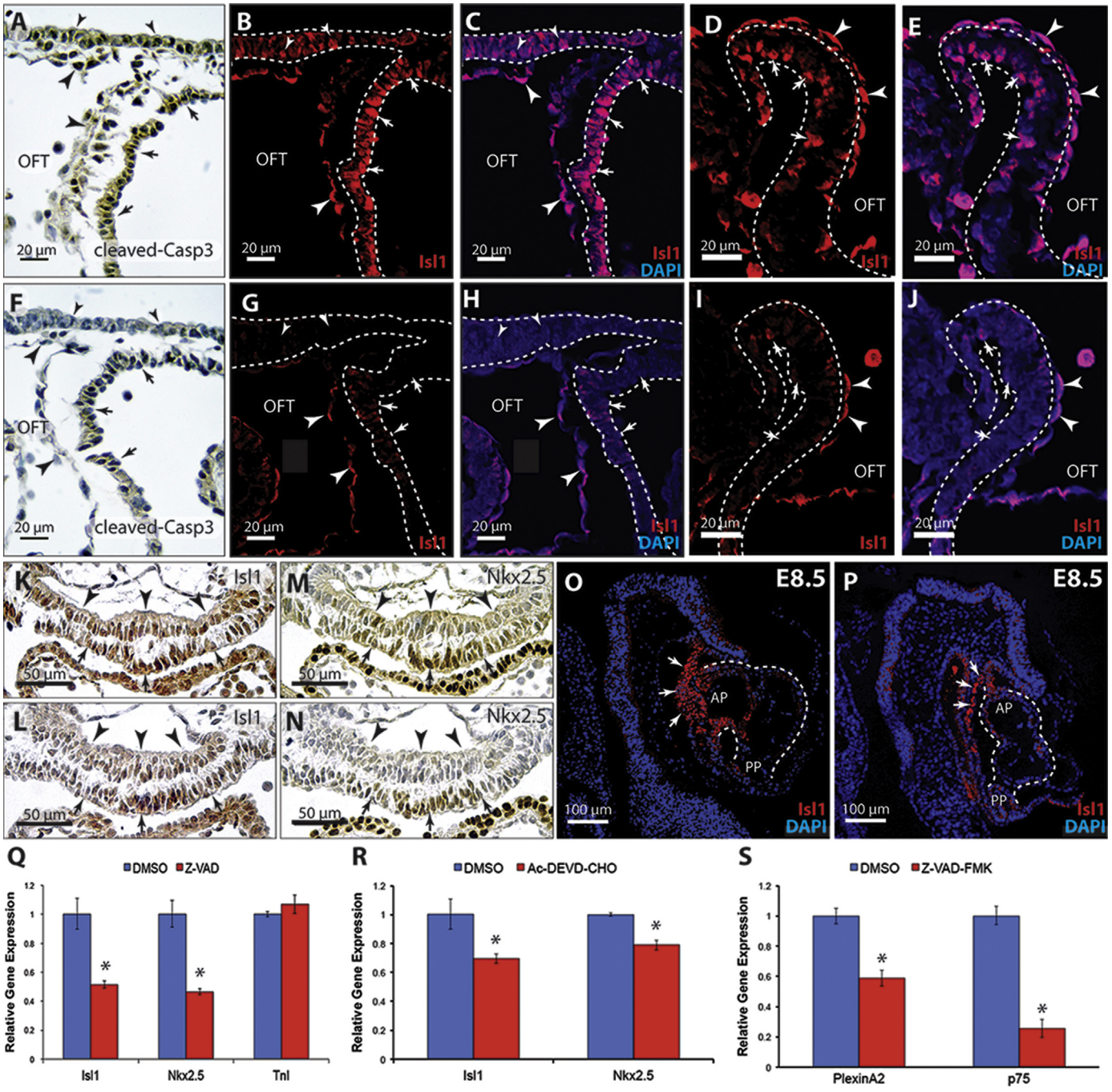


Fig. 4. ***In utero*** **pharmacological inhibition of Caspase activity reduces SHF gene expression in gestating embryos.** (A)–(K) Pregnant dams were injected with Z-VAD-FMK and DMSO as described in the Materials and methods section. (A)–(N) Embryos were harvested on E9.5 and processed for histological sectioning. (A)–(J) Longitudinal sections through the OFT of DMSO (A–E) and Z-VAD-FMK treated embryos (F–J). (A) and (F) Staining for cleaved-Casp3 (brown) is reduced in the OFT walls (arrows), OFT roof (small arrowheads) and endocardium (large arrowheads) of Z-VAD-FMK treated embryos (F) relative to those of DMSO treated controls (A). Counterstaining with hematoxylin (blue in A and F) is unaffected. (B), (C), (G) and (H) Staining for Isl1 (red) is reduced in the posterior wall (arrows) and roof (small arrowheads) of the OFT in Z-VAD-FMK treated embryos (G and H) relative to DMSO treated controls (B and C) while staining with DAPI (blue in C and H) is unaffected. Isl1 is not affected in the endocardial cells (large arrowheads) of Z-VAD-FMK treated embryos relative to control. (D), (E), (F) and (J) Isl1 (red) is similarly reduced in the anterior wall of the OFT (arrows) in Z-VAD-FMK treated embryos (I and J) relative to DMSO treated controls (D and E) but remains unaffected in endocardial cells (large arrowheads). DAPI staining (blue in E and J) is unaffected by Z-VAD-FMK treatment. (K)–(N) Cross sections of Z-VAD-FMK (L and N) and DMSO (K and M) treated embryos stained for Isl1 (brown in K and L) and Nkx2.5 (brown in M and N). Isl1 and Nkx2.5 are reduced in the both the DM (arrows) and foregut endoderm (arrowheads) in Z-VAD-FMK treated embryos relative to controls. (O) and (P) Longitudinal sections of Z-VAD-FMK (P) and DMSO (O) treated embryos collected at E8.25–E8.5 (prior to turning) stained for Isl1 (red) and DAPI (blue). The numbers of Isl1 positive SHF progenitors (arrows) entering the anterior pole (AP) of the heart tube (outlined by dashed lines) are decreased in Z-VAD-FMK treated embryos relative to DMSO treated controls. Very few Isl1 positive cells are present in the posterior pole (PP) of the heart tube in either condition. (Q) Graph shows Isl1, Nkx2.5 and Tnni3 mRNA levels in E9.5 Z-VAD-FMK treated embryos as fold changes relative to the levels of each gene in DMSO treated controls, which were normalized to one. (R) Graph shows Isl1 and Nkx2.5 mRNA levels in the hearts of E9.5 embryos from Ac-DEVD-CHO injected females (described in the Materials and methods section) as fold changes relative to the levels each gene present in controls. (S) Graph shows the levels of PlexinA2 and p75 mRNA in E9.5 Z-VAD-FMK treated embryos as fold changes relative to DMSO treated controls. Graphs in (Q)–(S) represent the mean for 3 pools of RNA for each condition ± standard deviation. Asterisks (*) indicate $P < 0.01$.

Caspase activity is required for SHF development in vivo

A pharmacological approach was used to determine if inhibiting the activities of multiple Caspases would disrupt SHF development *in vivo*. Pregnant female mice were given daily intraperitoneal injections of either Z-VAD-FMK or DMSO between E6.5 and E8.5 of gestation before being sacrificed on E9.5 to study the gestating embryos. Examination of H&E stained sections of Z-VAD-FMK and DMSO treated embryos revealed that their overall morphology was similar (data not shown). However, cleaved-Casp3 staining was reduced in the OFT and DM (arrows in Fig. 4A and B) as well as the roof of the OFT (small arrowheads in Fig. 4A and B) and endothelial cells (large arrowheads in Fig. 4A and B) of Z-VAD-FMK treated embryos relative to controls, confirming the efficacy of Caspase inhibition. To determine if Isl1 positive SHF progenitor and CNC cells were affected by Caspase inhibition, adjacent sections were fluorescently co-stained with an antibody recognizing Isl1 (red staining in Fig. 4B–E and G–J) and DAPI, which labels cell nuclei (blue staining in Fig. 4B–E and G–J). In DMSO treated embryos, strong Isl1 staining was present in the nuclei of cells lining the both the DM and posterior wall of the OFT (arrows in Fig. 4B and C) as well as the anterior wall of the OFT (arrows in Fig. 4D and E). Furthermore, endothelial cells (large arrowheads in Fig. 4B–E) as well as many of the cells within the roof of the OFT (small arrowheads in Fig. 4B–D) also expressed Isl1. In contrast to DMSO treated embryos, Isl1 staining was either present at reduced levels or absent in the DM and posterior wall of the OFT (arrows in Fig. 4G and H) as well as in the anterior wall of the OFT (arrows in Fig. 4I and J) of Z-VAD-FMK treated embryos. Isl1 expression in the roof of the OFT was also reduced or absent in Z-VAD-FMK treated embryos (small arrowheads in Fig. 4G and H), while Isl1 expression in endothelial cells was largely unaffected (large arrowheads in Fig. 4G–J). Staining cross sections through additional Z-VAD-FMK treated and control embryos for Isl1 (Fig. 4K and L) and Nkx2.5 (Fig. 4M and N) revealed reduced staining for both markers in the DM (arrows in Fig. 4K–N) and foregut endoderm (arrowheads in Fig. 4K–N). In agreement with these findings, Q-PCR revealed that the levels of Isl1 and Nkx2.5 mRNA were lower in the hearts and adjacent mesenchyme of Z-VAD-FMK treated embryos relative to controls (Fig. 4Q). In contrast cardiac Troponin I (TnI), a marker of differentiated cardiomyocytes, was expressed at similar levels in Z-VAD-FMK treated and control embryos, suggesting that the more differentiated descendants of FHF progenitors were unaffected by Caspase inhibition. To confirm that the loss of SHF markers caused by Z-VAD-FMK injection reflects its inhibition of Caspases and not off target effects on other cysteine proteases, pregnant female mice were injected with either DMSO or the Casp3 inhibitor Ac-DEVD-CHO before being sacrificed and having their embryos collected on E9.5. Performing Q-PCR on the hearts and surrounding mesenchyme of these embryos revealed that Isl1 and Nkx2.5 expression was similarly reduced in the hearts of Ac-DEVD-CHO injected embryos relative to controls (Fig. 4R). Taken together, these data strongly suggest that Caspases play an essential role in SHF growth and development *in vivo*.

The loss of Isl1 expression in the posterior walls and roof of the OFT suggested that Caspase activity was also required for the development of CNC cells. To address this possibility, the levels of PlexinA2 and another marker of migratory neural crest cells, the low-affinity nerve growth factor receptor (p75) (Andrade-Rozental et al., 1995; Rao and Anderson, 1997; Tropepe et al., 2009), were examined in the hearts and surrounding mesenchyme of E9.5 DMSO and z-Vad-Fmk treated embryos by Q-PCR (Fig. 4S). Interestingly, mRNA for both PlexinA2 and p75 were significantly reduced in the hearts of Z-VAD-FMK treated embryos relative to controls, suggesting that Wnt5a and Wnt11 were indeed required for CNC development. However, the effects of combined Wnt5a/Wnt11 loss-of-function on SHF progenitor cells are unlikely to be secondary to the loss of CNC since Isl1 expression is reduced in the hearts of z-Vad-Fmk treated embryos relative to controls by E8.5, prior to the CNC cells entering the heart (Fig. 4O and P).

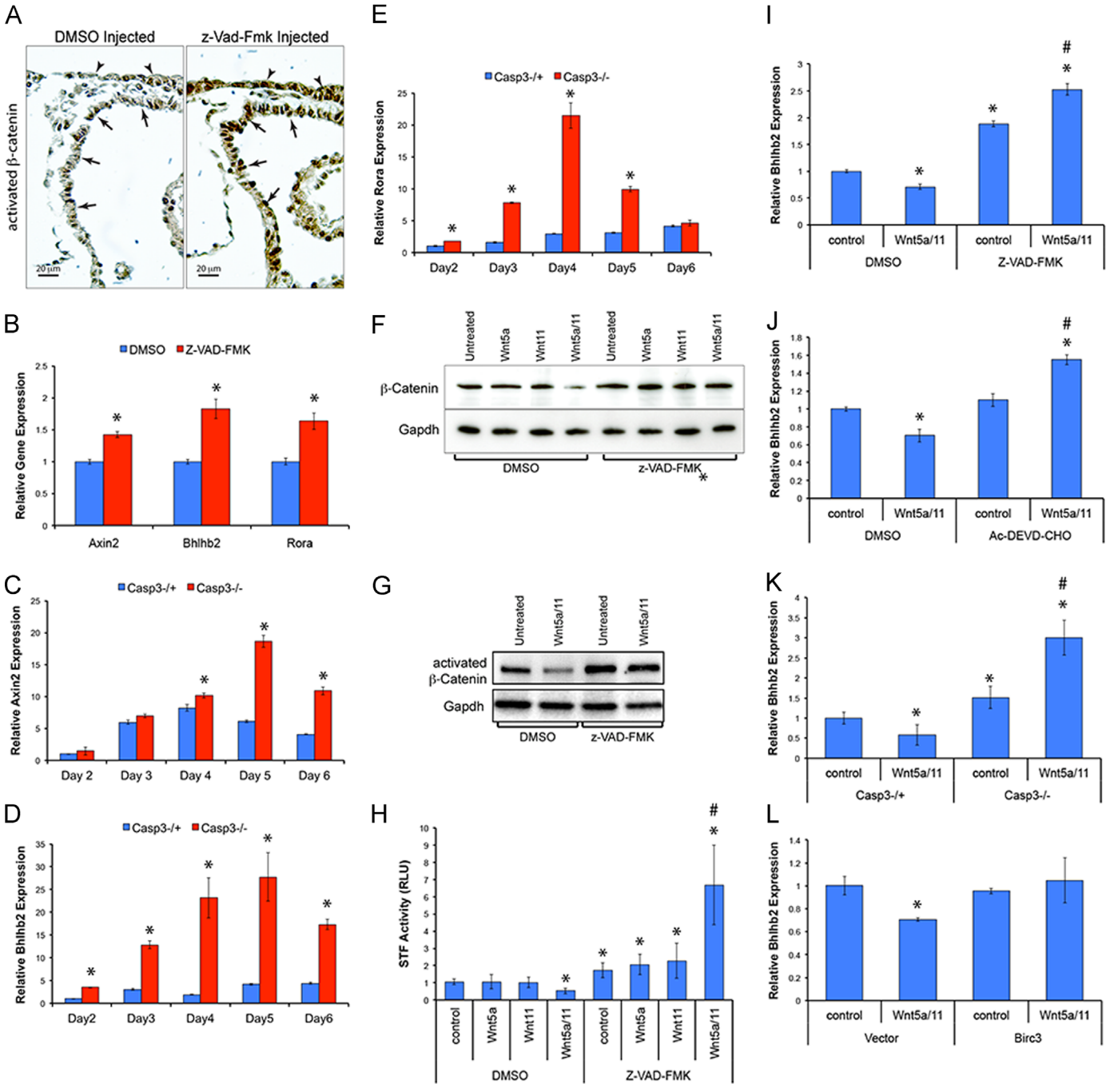
Caspase activity is required for Wnt5a/Wnt11 signaling to inhibit the canonical Wnt pathway

We previously showed that the loss of Isl1 positive SHF progenitor cells in Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos is associated with increased canonical Wnt signaling in the OFT and surrounding mesenchyme (Cohen et al., 2012). To determine if Caspase activity is similarly required to prevent the inappropriate β-catenin signaling in the SHF, longitudinal sections of E9.5 Z-VAD-FMK treated and control embryos were stained with an antibody specific to the activated, hypo-phosphorylated form of β-catenin. While low levels of activated β-catenin staining were observed in OFT walls of embryos from DMSO injected mothers, levels of activated β-catenin were noticeably increased in the OFT walls of embryos from Z-VAD-FMK injected females (arrows in Fig. 5A). Moreover, Q-PCR indicated that the canonical Wnt target gene Axin2 as well as two genes activated by β-catenin in Isl1 positive progenitor cells, Bhlhb2 and Rora, were expressed at higher levels in the hearts of Z-VAD-FMK treated embryos than in those of controls (Fig. 5B). These results are

Fig. 5. Caspase activity is required for Wnt5a and Wnt11 to inhibit the canonical Wnt pathway. (A) Pregnant dams were injected with DMSO or Z-VAD-FMK as described before being sacrificed at E9.5 and having their embryos harvested for histological sectioning. Longitudinal sections of DMSO (left) and Z-VAD-FMK (right) injected embryos were stained for activated β-catenin. Staining was broader and more intense in the DM and OFT walls (arrows) as well as the roof of the OFT (arrowheads) of Z-VAD-FMK treated embryos than in controls. (B) Graph shows the fold changes in Axin2, Bhlhb2 and Rora expression in the hearts of E9.5 Z-VAD-FMK injected embryos relative to the levels of each gene in DMSO treated controls. (C)–(E) Graphs show the fold changes in Axin2 (C), Bhlhb2 (D) and Rora (E) mRNA in Casp3$^{+/-}$ and Casp3$^{-/-}$ EBs collected every 24 h from days 2–6 of differentiation as relative changes from the levels of each gene in Casp3$^{+/-}$ EBs on day 2. (F) EBs were treated with Wnt5a, Wnt11, or Wnt5a and Wnt11 from days 4–5 of differentiation in the presence of DMSO or 10 μM Z-VAD-FMK. Western blotting reveals that β-catenin protein levels are reduced in EBs treated with Wnt5a and Wnt11 in the presence of DMSO but not presence of Z-VAD-FMK. (G) EBs were cultured in either plain media or media containing 100 ng/ml Wnt5a and 100 ng/ml Wnt11 from days 4–5 of differentiation in the presence of either DMSO or 10 μM Z-VAD-FMK. Western blotting reveals that the levels of activated β-catenin are reduced in EBs treated with Wnt5a and Wnt11 in the presence of DMSO but not in the presence of Z-VAD-FMK. (H) ES cells were co-transfected with the β-catenin reporter SuperTopflash (STF) and a plasmid that constitutively expresses *Renilla* luciferase, which was used to control for transfection efficiency. EBs were generated from these cells and treated with Wnt5a, Wnt11 or Wnt5a and Wnt11 from days 4–5 of differentiation before being lysed and having their luciferase activity levels determined using the Dual Luciferase Assay System (Promega). While STF activity was reduced in EBs treated with Wnt5a and Wnt11 in the presence of DMSO, Wnt5a and Wnt11 increased STF activity relative to controls when EBs were treated in media containing 10 μM Z-VAD-FMK. (I) and (J) EBs were cultured in either plain media or media containing Wnt5a and Wnt11 from days 4–6 of differentiation in the presence of 10 μM Z-VAD-FMK (I), 1 μM Ac-DEVD-CHO (J) or DMSO before being harvested and analyzed for Bhlhb2 expression. Bhlhb2 are lower in EBs treated with Wnt5a and Wnt11 in the presence of DMSO than in untreated EBs under equivalent conditions. In contrast, Bhlhb2 levels are increased in EBs treated with Wnt5a and Wnt11 in the presence of either 10 μM Z-VAD-FMK or 1 μM Ac-DEVD-CHO relative to untreated EBs cultured in the presence of inhibitor. (K) Graph shows the Bhlhb2 mRNA levels in Casp3$^{+/-}$ and Casp3$^{-/-}$ EBs cultured in either plain media or media containing Wnt5a and Wnt11 from days 4–5 of differentiation relative to those in untreated Casp3$^{+/-}$ EBs, which are normalized to one. (L) Graph shows Bhlhb2 mRNA levels in EBs made from ES cells transfected with either empty vector or Birc3 expression plasmid cultured in either plain media or media containing Wnt5a and Wnt11 from days 4–5 of differentiation relative to those in untreated EBs made from empty vector transfected cells, which are normalized to one. Graphs show the mean of 3 independent replicates ± the standard deviation. Asterisks (*) indicates $P < 0.05$ vs. controls. The hashtags (#) in (H) and (I) indicate $P < 0.05$ vs. untreated EBs in Z-VAD-FMK containing media. The hashtag (#) in (J) indicates $P < 0.05$ vs. untreated EBs in Ac-DEVD-CHO containing media. The hashtag (#) in (K) indicates $P < 0.05$ vs. untreated Casp3$^{-/-}$ EBs.

consistent with the reported increase in the activity of canonical Wnt reporters observed in the myocardium of E12.5 embryos treated with Caspase inhibitors in an *ex vivo* culture system (Abdul-Ghani et al., 2011) and suggest that Caspase activity is required to suppress excessive canonical Wnt signaling in the SHF during mouse embryogenesis.

To determine if the reduced expression of Isl1 and Nkx2.5 in Casp3$^{-/-}$ EBs was similarly associated with increased canonical Wnt signaling, the levels of Axin2, Bhlhb2 and Rora mRNA in Casp3$^{-/-}$ and Casp3$^{-/+}$ EBs were analyzed by Q-PCR at several times during their differentiation. Axin2 expression increased between days 2 and 4 in both Casp3$^{-/-}$ and Casp3$^{-/+}$ EBs with slightly higher levels of Axin2 mRNA in the Casp3 mutants (Fig. 5C). However while Axin2 expression declined in Casp3$^{+/-}$ EBs between days 4 and 6 of differentiation, Axin2 expression continued to increase until day 5 in Casp3$^{-/-}$ EBs and reached levels nearly three-fold higher than those present in Casp3$^{+/-}$ EBs before beginning to decline between days 5 and 6. Moreover, while the levels of Bhlhb2 mRNA increased between days 2 and 3 of differentiation and then remained relatively constant through day 6 in Casp3$^{+/-}$ EBs, Bhlhb2 expression increased dramatically in Casp3$^{-/-}$ EBs and reached levels over six-fold higher than those found in Casp3$^{-/+}$ EBs by day 5 before beginning to decline between days 5 and 6 (Fig. 5D). Rora expression also increased slowly throughout the differentiation of Casp3$^{+/-}$ EBs but rose sharply between days 2 and 4 in Casp3$^{-/-}$ EBs, reaching levels



that were over seven-fold higher than those found in Casp3$^{+/-}$ EBs on day 4 before declining (Fig. 5E). Taken together, these data indicate that Casp3 inhibits β-catenin dependent transcription in differentiating ES cells and suggest that the loss of Isl1 and Nkx2.5 in Casp3$^{-/-}$ EBs results from increased canonical Wnt pathway activation.

Our previous data indicated that canonical Wnt pathway inhibition was both necessary and sufficient for the effects of cooperative Wnt5a/Wnt11 signaling on Isl1 and Nkx2.5 expression in EBs (Cohen et al., 2012). The failure of Wnt5a and Wnt11 to increase Isl1 and Nkx2.5 mRNA levels in the presence of Z-VAD-FMK therefore suggests that Caspase activity may be required for the effects of these ligands on the canonical Wnt pathway. To test this hypothesis, EBs were treated with recombinant Wnt5a and Wnt11 proteins in the presence or absence of Z-VAD-FMK as described. EBs were then lysed and subjected to Western blotting for total β-catenin (Fig. 5F) or the activated form of β-catenin (Fig. 5G). Consistent with our previous findings, β-catenin levels were reduced in EBs treated with both Wnt5a and Wnt11 in the presence of DMSO relative to EBs treated with either Wnt on its own or untreated control EBs. In contrast, Wnt5a/Wnt11 treatment did not reduce β-catenin levels in the presence of Z-VAD-FMK, suggesting that the effects of cooperative Wnt5a/Wnt11 signaling on the canonical Wnt pathway are Caspase-dependent.

To more directly test whether Caspase activity is required for cooperative Wnt5a/Wnt11 signaling to inhibit β-catenin dependent transcription, ES cells were transiently transfected with super-Topflash (STF), a reporter that uses tandem repeats of the consensus TCF binding site to drive firefly luciferase expression in response to canonical Wnt signaling (Veeman et al., 2003), as well as a plasmid that constitutively expresses *Renilla* luciferase, which was used to normalize for transfection efficiency. EBs were made from these STF transfected ES cells and treated with Wnt5a and Wnt11 proteins in the presence or absence of Z-VAD-FMK as described. After being treated for 24 h, EBs were lysed and the levels of luciferase activity determined. Consistent with our previous data, the STF activity was reduced in EBs following combined Wnt5a/Wnt11 treatment in the presence of DMSO relative to untreated controls (Fig. 5H). Surprisingly, combined Wnt5a/Wnt11 treatment increased STF activity relative to controls in the presence of Z-VAD-FMK without increasing β-catenin protein. To determine if this change in the effects of Wnt5a and Wnt11 on STF activity correlated with changes in the expression of endogenous β-catenin target genes, the levels of Bhlhb2 mRNA were examined by Q-PCR (Fig. 5I). Consistent with our previous data, the levels of Bhlhb2 mRNA were significantly reduced in EBs treated with Wnt5a and Wnt11 in DMSO containing media relative to untreated control EBs. Moreover, the levels of Bhlhb2 mRNA were higher in EBs cultured in Z-VAD-FMK containing media than in DMSO treated controls and increased by Wnt5a/Wnt11 treatment. Similar increases in Bhlhb2 expression were observed in EBs treated with Wnt5a and Wnt11 in the presence of Ac-DEVD-CHO (Fig. 5J) and Wnt5a/Wnt11 treated Casp3$^{-/-}$ EBs (Fig. 5K). While the mechanisms behind this increase in β-catenin-dependent transcription remain unclear, these data are consistent with the decreased Isl1 and Nkx2.5 expression observed in EBs treated with Wnt5a and Wnt11 in the presence of Z-VAD-FMK. These data suggest that the changes in the effects of Wnt5a/Wnt11 signaling on β-catenin dependent transcription are specifically caused by the inhibition of Caspases and not the result of off target effects on other cysteine proteases. Interestingly, while Birc3 prevents the increase in Isl1 and decrease in Bhlhb2 expression caused by Wnt5a and Wnt11 (Fig. 3F and 5L, respectively), Wnt5a and Wnt11 do not increase Bhlhb2 expression in EBs made from Birc3 transfected cells suggesting that Birc3 may inhibit Wnt5a/Wnt11 signaling via mechanisms that extend beyond their inhibition of Caspase function.

Wnt5a/Wnt11 signaling promotes SHF development via the Caspase-dependent degradation of AKT

Wnt11 was shown to induce the maturation of P19 cell derived cardiomyocytes by activating the Caspase-dependent cleavage of β-catenin (Abdul-Ghani et al., 2011). However, we had previously found that inhibiting Gsk3α/β blocked the ability of Wnt5a and Wnt11 to inhibit the canonical Wnt pathway and promote cardiac progenitor gene expression in differentiating ES cells (Cohen et al., 2012). Since the direct proteolysis of β-catenin by Caspases would circumvent the need for Gsk3α/β to target β-catenin to the proteasome for degradation, these later data suggest that Wnt5a/Wnt11 induced Caspase activity inhibits the canonical Wnt pathway by targeting components that lie upstream of or in parallel to Gsk3α/β.

Phosphorylation by the serine/threonine kinase Akt has been shown to inhibit Gsk3α/β activity and increase β-catenin signaling in many cell types, including cardiac progenitor cells and cardiomyocytes (Cross et al., 1995; Doble and Woodgett, 2003). (Moreover, Caspases have been shown to cleave Akt and down-regulate its activity (Riesterer et al., 2004; Rokudai et al., 2000; Widmann et al., 1998). Taken together, these data raised the question of whether Wnt5a/Wnt11 signaling promoted cardiac progenitor development by reducing Akt activity. To investigate this hypothesis, sections of Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos were stained with an antibody recognizing the phosphorylation of Akt at serine 473 (p-Akt), which correlates with its activity (Jacinto et al., 2006; Sarbassov et al., 2005). Examination of the stained sections by light microscopy revealed that p-Akt levels were higher in the OFT and adjacent mesenchyme of Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos (arrows in Fig. 6A and B). p-Akt staining was similarly increased in the OFT and neighboring mesenchyme of Z-VAD-FMK treated embryos relative to controls (arrows in Fig. 6C and D). Taken together, these data suggest that Wnt5a and Wnt11 are co-required for the Caspase-mediated reduction of Akt signaling during SHF development.

Caspases have previously been shown to proteolyze Akt (Jahani-Asl et al., 2007; Widmann et al., 1998), suggesting that Wnt5a and Wnt11 may reduce Akt activity by inducing its degradation. To determine if Wnt5a and Wnt11 induced the Caspase-dependent loss of Akt activity during ES cell differentiation, EBs were treated with Wnt5a and Wnt11 between days 4 and 5 of differentiation in plain media as well as media containing either DMSO or Z-VAD-FMK and then analyzed for p-Akt levels. Interestingly, p-Akt levels were specifically reduced in EBs treated with both Wnt5a and Wnt11 in plain (Fig. 6E) and DMSO containing media (Fig. 6F) but not in media containing Z-VAD-FMK. Moreover, Western blotting with a pan-Akt antibody (Fig. 6E and F) as well as an antibody specific for Akt1 (Fig. 6F) revealed similar reductions in total Akt levels in EBs treated with Wnt5a and Wnt11 in plain and DMSO containing media but not in Z-VAD-FMK containing media. Since Akt1 mRNA was not affected in Wnt5a/Wnt11 treated EBs (Fig. 6G), these data suggest that Wnt5a and Wnt11 regulate Akt1 through a post-transcriptional mechanism such as Caspase-mediated proteolysis. In contrast to Akt1, Akt2 was only mildly reduced in EBs treated with either Wnt5a or Wnt11 relative to controls and not further decreased by combined Wnt5a/Wnt11 treatment, suggesting that the effects of Wnt5a and Wnt11 are isoform specific

To determine if blocking Akt function would mimic the effects of Wnt5a/Wnt11 signaling on cardiac progenitor cells, EBs were treated with recombinant Wnt5a and Wnt11 proteins as described in addition to either Akt Inhibitor VIII (Akt-i-VIII), an isozyme-selective inhibitor of AKT1 and 2 (Lindsley et al., 2005), PI-103, an inhibitor of PI3K (Knight et al., 2006) or DMSO (Fig. 6H). As expected, levels of Isl1 mRNA were significantly higher in EBs treated with Wnt5a and Wnt11 in DMSO containing media than in untreated EBs cultured in DMSO. However, treatment with Akt-i-VIII or PI-103 in EBs without

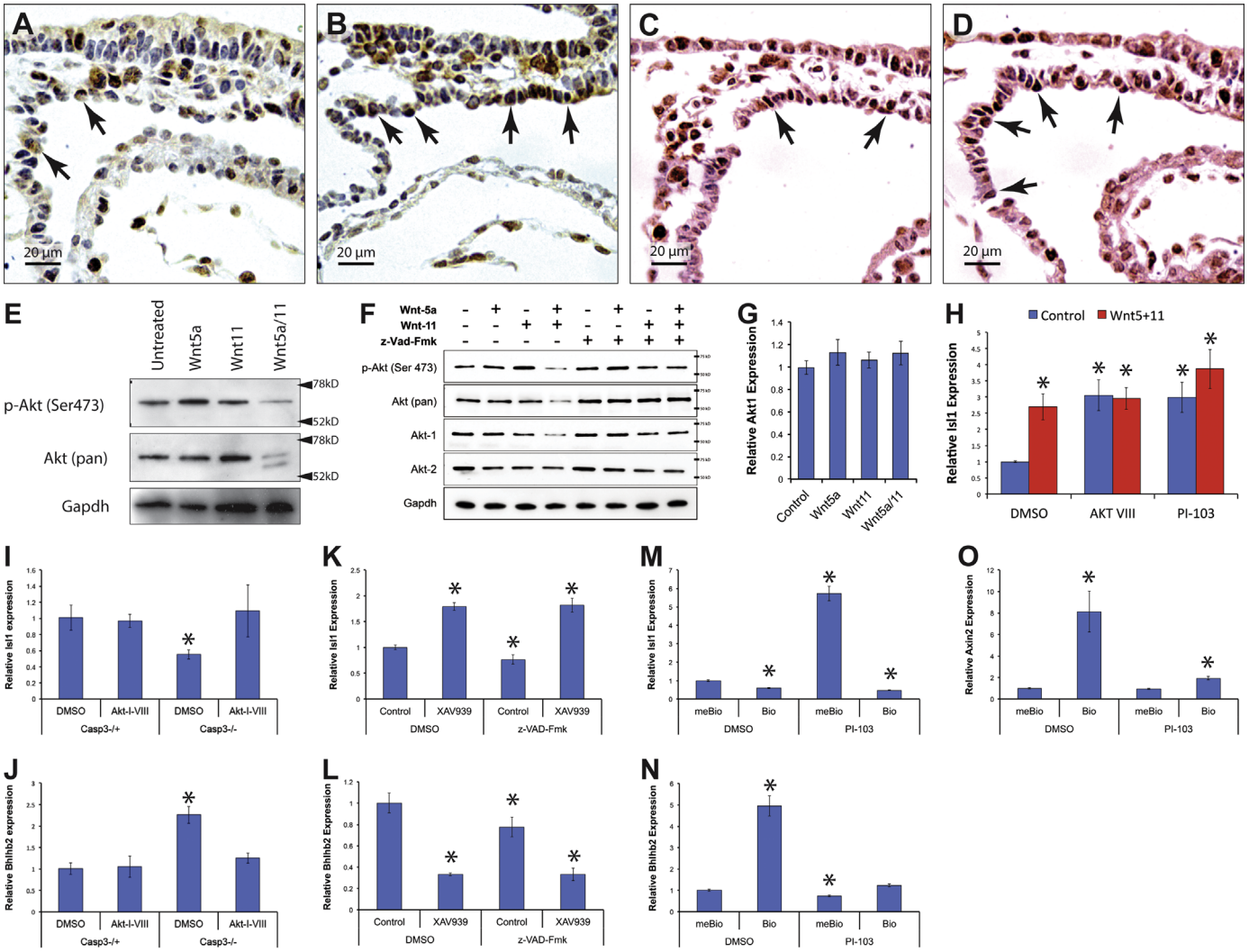


Fig. 6. Cooperative Wnt5a/Wnt11 signaling promotes SHF development by inducing the Caspase dependent loss of Akt protein. (A)–(D) Longitudinal sections of Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos (B) and wild type siblings (A) as well as embryos from Z-VAD-FMK (D) and DMSO (C) injected mothers were stained for Akt phosphorylated at serine-473. Phospho-Akt (p-Akt) levels are higher in the OFT and DM of Wnt5a$^{-/-}$; Wnt11$^{-/-}$ and Z-VAD-FMK treated embryos than in controls (arrows). (E) Wild type EBs were treated with Wnt5a, Wnt11 or both Wnt5a and Wnt11 from days 4–5 of differentiation and harvested to analyze the levels of p-Akt as well as the total levels of all Akt isoforms (pan-Akt) by Western blotting. Both p-Akt and pan-Akt levels were specifically reduced in Wnt5a/Wnt11 treated EBs relative to controls. (F) EBs were treated with Wnt5a, Wnt11 or both Wnt5a and Wnt11 from days 4–5 of differentiation and harvested. Western blotting revealed that the levels of p-AKT, pan-Akt and Akt1 were all specifically reduced in EBs treated with both Wnt5a and Wnt11 in the presence of DMSO but not Z-VAD-FMK. (G) EBs were treated with Wnt5a, Wnt11 or Wnt5a and Wnt11 from days 4–5 of differentiation and harvested to analyze the levels of Akt1 mRNA by Q-PCR. Graph shows the levels of Akt1 expression for each condition expressed as fold change from those in untreated controls, which were normalized to one. (H) EBs were treated with Wnt5a and Wnt11 from days 4–5 of differentiation in media containing either DMSO, 1 μM Akt-i-VIII or 1μM PI-103. Graph shows the fold changes in Isl1 expression in EBs cultured under each condition relative to those in untreated EBs cultured in DMSO containing media. (I) and (J) Graphs show the fold changes in the levels of Isl1 (I) and Bhlhb2 (J) mRNA in Casp3$^{+/-}$ and Casp3$^{-/-}$ EBs treated with either DMSO or 1μM Akt-i-VIII from days 4–5 of differentiation relative to the levels in DMSO treated Casp3$^{+/-}$ EBs, which are normalized to one. (K) and (L) Graphs show the fold changes in Isl1 (K) and Bhlhb2 (L) mRNA in EBs cultured from days 4–5 in media containing 100 nM XAV939 and 10 μM Z-VAD-FMK alone or in combination relative to the levels in EBs cultured in DMSO containing media. (M), (N), and (L) Graphs show the fold changes in Isl1 (M), Bhlhb2 (N) and Axin2 (L) mRNA levels in EBs treated with either 50 nM Bio or 50 nM meBio in media containing either 1 μM PI-103 or DMSO relative to those in untreated EBs cultured in the presence of DMSO. Graphs show the mean of 3 independent replicates ± the standard deviation. Astericks (*) indicate $P < 0.01$ vs. controls.

Wnt5a/Wnt11, elevated Isl1 expression to levels similar to those seen with Wnt5a/Wnt11 treatment alone. Moreover, Wnt5a/Wnt11 plus Akt-i-VIII or PI-103 treated EBs did not increase Isl1 expression beyond that observed in EBs treated with the inhibitors alone. Taken together, these data suggest that inhibiting Akt mimicks the effects of Wnt5a/Wnt11 on the SHF.

To determine if blocking Akt was sufficient to rescue the effects of Casp3 loss-of-function on SHF development, Casp3$^{+/-}$ and Casp3$^{-/-}$ EBs were cultured in media containing either DMSO or Akt Inhibitor VIII between days 4 and 5 of differentiation and then analyzed for Isl1 and Bhlhb2 expression. While the levels of Isl1 mRNA were lower in Casp3$^{-/-}$ EBs relative to Casp3$^{-/+}$ EBs in DMSO containing media, treating Casp3$^{-/-}$ EBs with Akt-i-VIII increased Isl1 expression to levels equivalent to those present in Casp3$^{+/-}$ EBs (Fig. 6I). Conversely, although Bhlhb2 expression was higher in Casp3$^{-/-}$ EBs than in Casp3$^{+/-}$ when cultured in the presence of DMSO, the levels of Bhlhb2 mRNA in Akt-i-VIII treated Casp3$^{-/-}$ EBs was equivalent to those found in Casp3$^{+/-}$ EBs. Together, these data suggest that Akt inhibition is sufficient to rescue the effects of Casp3 loss-of-function on both SHF development and the canonical Wnt pathway.

The restoration of Isl1 and suppression of Bhlhb2 expression in Akt-i-VIII treated Casp3$^{-/-}$ EBs suggests that Caspases lie upstream of Akt in the molecular pathway that mediates the effects of Wnt5a and

Wnt11 on the SHF. To further confirm the relationship between β-catenin and Caspase activity in this pathway, wild type EBs were cultured in either DMSO containing media or media containing the Tankyrase inhibitor XAV939, which stabilizes Axin and blocks canonical Wnt signaling (Huang et al., 2009), and Z-VAD-FMK, individually as well as in combination, between days 4 and 5 of differentiation before being analyzed for Isl1 and Bhlhb2 expression. Consistent with our previous data, Isl1 mRNA levels were increased in XAV939 treated EBs relative to DMSO treated EBs while Bhlhb2 expression was reduced. Furthermore, while the levels of Isl1 and Bhlhb2 expression were both mildly reduced in Z-VAD-FMK treated EBs relative to controls, the increased Isl1 and decreased Bhlhb2 expression caused by XAV939 treatment was unaffected by Z-VAD-FMK consistent with Caspases acting upstream of β-catenin in the regulation of SHF development.

To examine the relationship between PI3K/Akt signaling and β-catenin signaling, wild type EBs were treated with either the Gsk3α/β inhibitor Bio or (Meijer et al., 2003) or its inactive form meBio in media containing either DMSO or PI-103 between days 4 and 5 of differentiation and analyzed for Isl1 and Bhlhb2 expression. Consistent with our previous data, the levels of Isl1 mRNA in Bio treated EBs were reduced relative to those present in meBio treated EBs and increased in PI-103 treated EBs relative to DMSO treated EBs (Fig. 4M). Conversely, the levels of Bhlhb2 are higher in Bio treated EBs than in meBio treated EBs and reduced in PI-103 treated EBs relative to EBs cultured in the presence of DMSO (Fig. 4N). Consistent with Akt acting upstream of β-catenin in the SHF, levels of Isl1 in EBs treated with Bio and PI-103 together are not elevated but reduced to levels similar to those found in EBs treated with Bio alone. Surprisingly however, the levels of Bhlhb2 in the EBs treated with Bio in the presence of PI-103 were increased less than two fold relative to those found in EBs treated with meBio and PI-103 and not significantly increased relative to the levels found in EBs treated with meBio in the presence of DMSO. Similar results were observed for the expression of Axin2 (Fig. 6O). These data suggest inhibiting Gsk3α/β blocks the effects of PI-103 on Isl1 expression without activating β-catenin dependent transcription, although the mechanisms underlying this effect remain to be determined.

Discussion

Wnt5a and Wnt11 are necessary and sufficient for Caspase activity in the SHF

We previously demonstrated that Wnt5a and Wnt11 signal cooperatively to restrain canonical Wnt signaling in the SHF that would otherwise disrupt cardiac progenitor development (Cohen et al., 2012). However, while these data revealed an essential role Wnt5a and Wnt11 in the SHF, the mechanisms underlying the anti-canonical effects of these ligands were uncertain. A previous study had shown that Wnt11 promoted later aspects of cardiomyocyte differentiation, such as the expression of contractile proteins and subsequent appearance of sarcomeres, through the Caspase-dependent inhibition of β-catenin (Abdul-Ghani et al., 2011). Consistent with these data, the levels of cleaved Casp3 observed in the hearts of Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos were lower than those found in wild type siblings, providing *in vivo* genetic evidence for non-canonical Wnt ligands being required for Caspase activity in the SHF. Moreover, treating EBs with Wnt5a and Wnt11 caused a strong synergistic increase in the levels of cleaved Caspases, mirroring the effects of these ligands on Isl1 and Nkx2.5 expression. Together, these data suggest that the effects of Wnt5a and Wnt11 on cardiac progenitor cells may be Caspase mediated.

Recently, a lineage of cells that expresses both the CNC marker Wnt1 and Isl1 at some time in its history has been identified (Engleka et al., 2012). Since Wnt1 is only expressed in CNC cells in the dorsal neural tube and rapidly repressed after these cells delaminate and migrate toward the heart (Danielian et al., 1997), these results suggest that a subset of CNC cells express Isl1 in addition to SHF progenitor cells. Sections of Wnt5a$^{-/-}$; Wnt11$^{-/-}$ and wild type embryos were therefore co-stained for Isl1 and the CNC marker PlexinA2 to determine if these Isl1 expressing CNC cells are affected by Wnt5a/Wnt11 loss of function. Surprisingly, the majority of Isl1 positive cells in the posterior wall of the OFT and adjacent DM co-stained for both markers, suggesting that these cells were in fact Isl1 expressing CNC and not SHF progenitor cells. Moreover, the expression of both Isl1 and PlexinA2 were reduced or absent throughout the hearts of Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos, suggesting that these ligands are co-required for both SHF progenitors and CNC cells, including those that express Isl1. However, the effects of Wnt5a/Wnt11 and Caspase loss-of-function on SHF progenitors are unlikely to be secondary effects caused by the loss of CNC cells since the numbers of Isl1 positive cells were reduced in both Wnt5a$^{-/-}$; Wnt11$^{-/-}$ (Cohen et al., 2012) and Z-VAD-FMK treated embryos by E8.5, prior to the arrival of CNC cells to the heart. Moreover, it remains possible that some SHF progenitor cells may express PlexinA2 and it will be necessary to confirm these results by analyzing the effects of Wnt5a/Wnt11 and Caspase loss-of-function on Wnt1^{cre} fate mapping.

Caspase activity is necessary and sufficient for the effects of Wnt5a and Wnt11 on SHF progenitors

Although SHF progenitors have yet to be examined in Caspase mutant mice, heart phenotypes had been observed in Casp8 mutant and Casp3/Casp7 double mutants (Lakhani et al., 2006; Varfolomeev et al., 1998). However, subsequent studies showed that the heart phenotypes of Casp8$^{-/-}$ mice could be partially rescued by culturing embryos *ex vivo* and mimicked by conditionally deleting Casp8 in endothelial cells (Kang et al., 2004; Sakamaki et al., 2002), suggesting that these defects resulted from the disruption of the yolk-sac vasculature. Moreover, the heart defects found in Casp3$^{-/-}$; Casp7$^{-/-}$ mice are relatively mild and likely to result from later defects in myocardial maturation (Lakhani et al., 2006), suggesting that effects on the SHF may have been masked by the extensive overlap between the functions of Caspase-family members. To circumvent these issues, an *in utero* pharmacological approach was used to determine if Caspase function was required for SHF development. Using this approach, we found that Isl1 and Nkx2.5 were reduced as a result of Caspase inhibition. Since Isl1 and Nkx2.5 are both essential for the growth and differentiation of SHF progenitor cells (Cai et al., 2003; Prall et al., 2007), the above findings indicate that Caspase activity is indeed required for SHF development *in vivo*. Isl1 and Nkx2.5 are also reduced in Casp3$^{-/-}$ EBs and increased by the expression of a constitutively active form of Casp3. Moreover, Wnt5a and Wnt11 treatment does not increase the expression of cardiac progenitor markers or decrease the expression of β-catenin target genes in either Casp3$^{-/-}$ or Caspase inhibitor treated EBs relative to controls, suggesting that Caspase activation is necessary and sufficient for the effects of cooperative Wnt5a/Wnt11 signaling on the SHF. Interestingly, Wnt5a and Wnt11 increased the levels of STF activity in Casp3$^{-/-}$ and Caspase inhibitor treated EBs. While it is unclear why blocking Caspase function reversed the effects of Wnt5a and Wnt11 on β-catenin-dependent transcription, Wnt5a and Wnt11 were shown to synergistically activate canonical Wnt signaling during in early *Xenopus* embryos (Cha et al., 2008, 2009). These data raise the possibility that Caspases may function as a molecular switch that determines whether Wnt5a and Wnt11 promote or antagonize Wnt/β-catenin signaling.

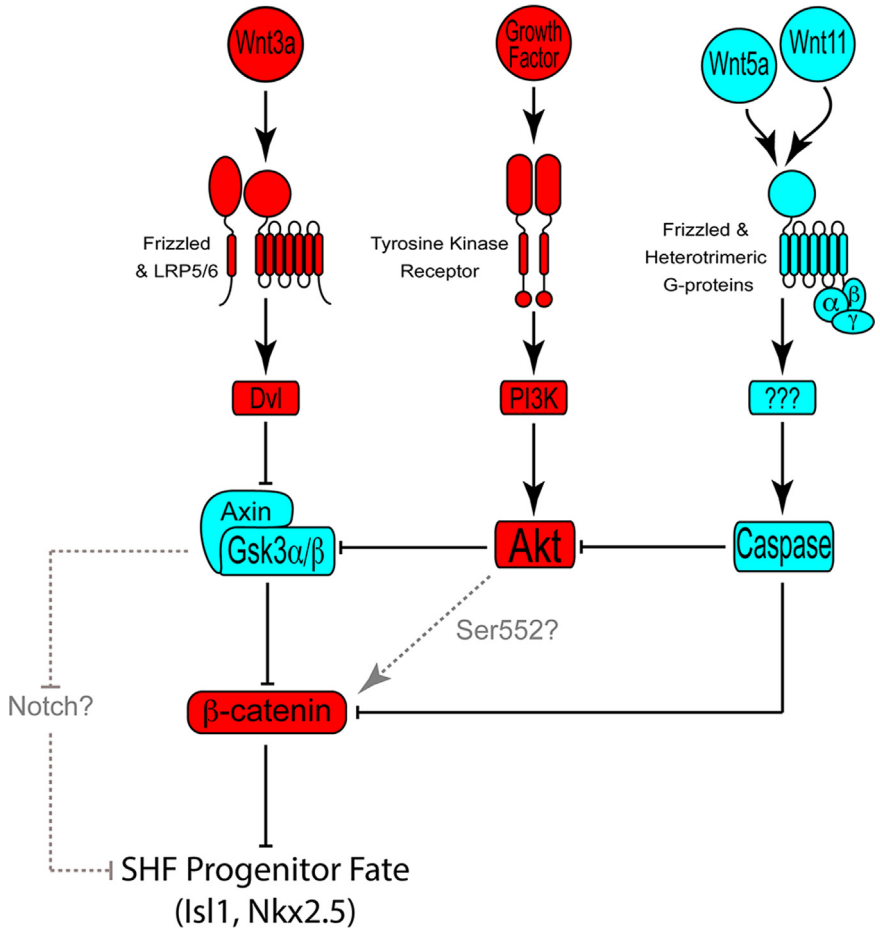


Fig. 7. Proposed model for how Wnt5a/Wnt11 signaling may reduce canonical Wnt signaling in the SHF by activating the Caspase-dependent degradation of Akt. Binding of canonical Wnt proteins such as Wnt3a to a complex consisting of Frizzled and LRP5/6 activates DVL, which recruits Axin and Gsk3α/β to the plasma membrane to prevent the phosphorylation of β-catenin. Binding of growth factors to tyrosine kinase receptors enhances canonical Wnt signaling by activating PI3K and Akt, which phosphorylates Gsk3α/β and inhibits its activity. Wnt5a and Wnt11 induce Caspase-dependent Akt degradation, which disrupts the effects of PI3K/Akt signaling on β-catenin-dependent transcription. A detailed explanation of the proposed pathway is provided in the discussion section. Factors that promote canonical Wnt signaling are shown in red while factors that inhibit Wnt/β-catenin signaling are shown in blue.

Wnt5a and Wnt11 may promote SHF development via the Caspase-dependent loss of Akt protein

While the inhibition of canonical Wnt signaling by Wnt11 in P19 cells has been attributed to the direct cleavage of β-catenin by Caspases (Abdul-Ghani et al., 2011), we found that inhibiting Gsk3α/β blocked the effect of Wnt5a and Wnt11 on both cardiac gene expression and canonical Wnt signaling in EBs (Cohen et al., 2012). Since inactivating Gsk3α/β would not affect canonical Wnt signaling if β-catenin was being degraded, these data suggested that Wnt5a and Wnt11 impinge on the canonical Wnt pathway upstream or in parallel to the β-catenin degradation complex (see Fig. 7). (Caspases have been shown to cleave a number of signaling proteins, including the serine/threonine kinase Akt (Riesterer et al., 2004; Rokudai et al., 2000; Widmann et al., 1998). It is well established that Akt phosphorylates serine-21 and serine-9 of Gsk3α and Gsk3β, respectively, to inhibit their activities (Cross et al., 1995; Doble and Woodgett, 2003). Though the phosphorylation of Gsk3α/β by Akt does not affect β-catenin dependent transcription in all cell types (Doble and Woodgett, 2003; Patel et al., 2004; Wu and Pan, 2010; Yuan et al., 1999), activating Akt signaling has been shown to promote canonical Wnt signaling during the cardiac differentiation of both P19 and ES cells (Lian et al., 2013; Naito et al., 2005). Surprisingly, we found that staining for the phosphorylation of Akt at serine-473 was increased in both $Wnt5a^{-/-}$; $Wnt11^{-/-}$ and Caspase inhibitor treated embryos relative to controls. Conversely, the levels of phosphorylated and total Akt protein were reduced in EBs treated with Wnt5a and Wnt11 proteins in media containing DMSO but not a Caspase inhibitor, suggesting that these ligands induce Caspase-dependent Akt degradation. Furthermore, treating EBs with inhibitors of Akt or its upstream activator PI3K increased Isl1 expression to levels comparable to those found in Wnt5a/Wnt11 treated EBs. Inhibiting Akt also restored Isl1 and reduced Bhlhb2 expression in EBs made from $Casp3^{-/-}$ ES cells. Together, these data suggest that Wnt5a and Wnt11 promote SHF progenitor growth and differentiation through the Caspase-dependent loss of Akt.

Intriguingly, while treating EBs with the Gsk3α/β inhibitor Bio blocked the increase in Isl1 expression caused PI-103, blocking PI3K/Akt signaling also blocks the increased expression of β-catenin target genes caused by Gsk3α/β inhibition. The ability of PI-103 to block canonical Wnt signaling in the presence of Bio may reflect the direct phosphorylation of β-catenin by Akt on Ser552, which has been shown to promote β-catenin dependent transcription (Fang et al., 2007). In contrast however, the ability of Bio to block the increase in Isl1 expression caused by PI-103 without increasing the transcription of β-catenin target genes is unexpected and suggest that Gsk3α/β may promote SHF development through an

additional as of yet unidentified mechanism. Interestingly, Gsk3α/β has been shown to phosphorylate the intracellular domain of Notch2 and attenuate the transactivation of its target genes (Espinosa et al., 2003). Since Notch has been shown to inhibit cardiogenesis in differentiating stem cells (Jang et al., 2008; Li et al., 2012), Gsk3α/β may play dual roles in restraining both canonical Wnt and Notch signaling in the SHF that would otherwise disrupt cardiac progenitor development, although this hypothesis remains to be tested.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.11.015.

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