The mediator complex subunit Med10 regulates heart valve formation in zebrafish by controlling Tbx2b-mediated Has2 expression and cardiac jelly formation

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ABSTRACT

In search for novel key regulators of cardiac valve formation, we isolated the zebrafish cardiac valve mutant ping pong (png). We find that an insertional promoter mutation within the zebrafish mediator complex subunit 10 (med10) gene is leading to impaired heart valve formation. Expression of the T-box transcription factor 2b (Tbx2b), known to be essential in cardiac valve development, is severely reduced in png mutant hearts. We demonstrate here that transient reconstitution of Tbx2b expression rescues AV canal development in png mutant zebrafish. By contrast, overexpression of Forkhead box N4 (Foxn4), a known upstream regulator of Tbx2b, is not capable to reconstitute tbx2b expression and heart valve formation in Med10-deficient png mutant hearts. Interestingly, hyaluronan synthase 2 (has2), a known downstream target of Tbx2 and producer of hyaluronan (HA) - a major ECM component of the cardiac jelly and critical for proper heart valve development - is completely absent in ping pong mutant hearts. We propose here a rather unique role of Med10 in orchestrating cardiac valve formation by mediating Foxn4 dependent tbx2b transcription, expression of Has2 and subsequently proper development of the cardiac jelly.

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1. Introduction

The cardiac atrioventricular canal (AVC) ensures highly specialized functions, including cardiac chamber demarcation, AV conduction and the formation of endocardial cushions. Endocardial cushions are cells of the AVC that undergo an epithelial-to-mesenchymal transition (EMT) and are remodelled into functional cardiac valves during late cardiogenesis [1]. The molecular underpinnings that orchestrate vertebrate heart valve formation are only poorly deciphered yet.

Temporo-spatial control of transcription is of central importance during organogenesis and is mainly orchestrated by transcriptional activators and repressors. To convey the regulatory information from these transcriptional activators or repressors to the basal transcription machinery, the multiprotein Mediator complex (Med) serves as a connecting bridge between initiating and accomplishing elements. In this context, the Mediator complex physically links transcription factors bound to DNA to the RNA polymerase II and the basal transcription machinery. In vertebrates, the Med complex consists of more than 30 protein subunits, subdivided into a head, middle, tail and regulatory domain [2]. Whereas several Med subunits such as Trap80 or Med6 are indispensable for general gene transcription, other subunits including Med10, Med12 or Med13 are dispensable for general transcription but are involved in the regulation of specific gene programs, also in the heart [3–5]. Nevertheless, the role of the Mediator complex during cardiac development and particularly heart valve formation is only poorly understood.

In search for key-regulators of cardiac valve formation, we isolated the zebrafish heart valve mutant ping pong (png) [6]. We demonstrate that defective valve formation in png is caused by a...
promoter mutation within the mediator complex subunit 10 (med10) gene leading to reduction of med10 transcription. We find that tbx2b expression in the AVC of png mutants is absent and that re-introduction of Tbx2b rescues endocardial cushion development. Additionally, ectopic expression of foxn4, a known transcriptional regulator of Tbx2b, is not capable to reconstitute tbx2b expression in the AVC of png mutants, implying that Med10 might act as a mediator of Foxn4 signals to guarantee proper tbx2b expression in the AV myocardium. As a consequence, hyaluronan synthase 2, a known downstream target of Tbx2, is absent in png hearts leading to loss of hyaluronic acid (HA) production in the cardiac jelly and thereby the defective endocardial cushion development.

2. Methods

2.1. Zebrafish Strains, Histology, Immunostaining/-Blotting and in situ hybridization

Care and breeding of zebrafish Danio rerio was as described [7]. The study was performed after appropriate institutional approvals, which conform to EU Directive 2010/63/EU. Pictures and movies were recorded 24, 48 and 72 hpf. Embryos were fixed in 4% PFA and embedded in JB-4 (Polysciences, Inc.) and stained with H&E. Immunostaining of Dent’s fixed embryos was performed using distinct myosin heavy chains antibodies (MF20 and S46). In situ hybridization, hyaluronic acid (HA) staining and Western Blotting were performed as described [7–9].

2.2. Injection procedures, LiCl treatment and quantitative real-time PCR

Morpholinos were directed against the translational start site (MO1-med10 = 5'–CGAGGTATCGAACTTCTCCGCT−3'), the splice donor site of exon 1 (MO2-med10 = 5'–GATTAACTCA-CAGTTTCTGGTG−3') of the zmed10 gene and against the donor site of exon 2 (MO-foxn4 = 5'–TCAAAGGAATTCACCTCCCCTATCT−3') of the zfoxn4 gene. A standard control oligonucleotide (MO-control) (GENETOOLS, LLC) was injected at the same concentration as a negative control. Sense-capped RNA was synthesized using the mMESSAGE mMACHINE system (Ambion) from pCS2zmmed10, pCS2zfoxn4, pCS2zttbx2b, and pCS2zndr2 [7]. To induce Wnt signaling, the established heat-shock inducible transgenic line Tg(hsp70:wnlt8a-GFP) was used [10]. Embryos were treated with LiCl (0.1 M and 0.15 M) from 36 hpf to 48 hpf. qRT-PCR was performed according to standard protocols with the SYBR-Green method (Thermo Scientific) using an ABI 7000. cDNA was generated from 2 μg RNA of 48 hpf png mutants and wild-types using oligo (dT) primer and SuperScript II reverse transcriptase (Invitrogen).

2.3. Statistical analyses

Unpaired two tailed students’ t-test was performed to assess statistical significance of biological replicates of the quantitative data. For the qRT-PCR data t-test was performed to the delta Ct values. For each replicate, pooled samples (50 embryos) were analyzed.

3. Results

3.1. Zebrafish ping pong mutants display impaired AVC development

In search for key-regulators of AVC development, we isolated in a large-scale ENU-mutagenesis screen the recessive, embryonic lethal zebrafish mutant ping pong (pngm683) (Fig. 1A–D) [6]. During the first 48 h post fertilization (hpf), the png heart jogs leftwards, loops, myocardial and endocardial cell layers develop properly, and atrial and ventricular cardiomyocytes express chamber-specific myosin heavy chains (Fig. 1E–H; Movies 1 and 2, Fig. S1A–H). Usually, at 72 hpf, cardiac chambers in wild-types are demarcated by the AV ring and the endocardial cushions, specialized cells that give rise to the AV valves (Fig. 1F). By contrast, in png mutants heart chambers are not separated by an AV ring and no endocardial cushion cells are visible (Fig. 1H; Fig. S1I, J; Movies 3 and 4).

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.06.088.

To define the cardiac defects in png mutants, we first examined the expression of the myocardial chamber marker Atrial Natriuretic Factor (Anf). Usually, anf is expressed in both cardiac chambers but is absent from AVC in wild-types. We find anf distributed in both heart chambers and the AVC of png mutants (Fig. 1I, J), suggesting that AV myocardium is not properly specified. Upon Bmp4 stimuli endocardial cells usually accumulate at the AVC, increasingly express Notch1b, and then migrate into the AV jelly to undergo EMT [11]. Thus, to evaluate if altered Bmp4 expression in png mutant hearts affects endocardial notch1b expression, we assayed bmp4 and notch1b distribution and find them misexpressed in myocardial and endocardial cells of the atrium and the ventricle, respectively (Fig. S2A–D). Therefore, to analyze if loss of regular Notch1b expression leads to impaired initiation of EMT in the png AVC, we assessed the expression of secreted phosphoprotein 1 (spp1), a well-established marker for activated AV endocardial cells that migrate into the extracellular matrix and then undergo EMT [12,13]. As shown in Fig. 1K, L, spp1 RNA is completely absent from the AVC of png mutant hearts at 72 hpf, implicating impaired initiation of EMT of endocardial AVC cells to be responsible for the AVC defect.

3.2. Ping pong (pngm683) encodes the zebrafish mediator complex subunit 10 (Med10)

To identify the ENU-induced png mutation, we performed a positional walk. By a genome-wide study of segregation of microsatellite markers using bulked segregant analysis, we mapped the png mutation between the microsatellite markers Z9059 and Z11872 on chromosome 19. Genetic fine mapping and genotyping of 1497 png mutant embryos revealed two open reading frames within the critical mutation interval encoding for the zebrafish Mediator complex subunit 10 (Med10; AAW56465) and UBE2Q1L (ENSARG00000079276) (Fig. 1M). By sequencing of wild-type and png mutant cDNA, no alterations of the coding sequences of these genes were identified. To assess if altered Med10 or UBE2Q1L transcription is the molecular cause of the png mutant phenotype, we next assayed transcript levels of both genes by quantitative real-time PCR in wild-types and png mutants. We find significantly reduced zmed10 transcript levels in png mutants compared to wild-type littermates, whereas UBE2Q1L mRNA levels are unaltered in png mutants (Fig. 1N). Sequencing of the zmed10 promoter region reveals an 1193 bp insertion located 110 bp upstream of the transcriptional start site of the med10 gene (Fig. 1M). Interestingly, the identified ping pong promoter mutation is identical to the one isolated in tennis match (ten) zebrafish mutants [5], which also display defective AV canal formation.

To substantiate our finding that impaired AVC development in png mutants is due to altered Med10 function, we performed Morpholino-mediated med10 knockdown studies as well as gene rescue experiments and find that impaired Med10 transcription indeed accounts for the png mutant phenotype (Suppl. Fig. 3).
3.3. Defective Wnt and Nodal signaling does not account for impaired AV valve formation in png

Lin et al. demonstrated that Morpholino-mediated knock-down of med10 leads to defective tail bud formation due to enhanced Wnt and diminished Nodal signaling [5]. Interestingly, neither tennis match nor png zebrafish mutants show defective tail bud formation, implying both lines to be hypomorphic. Nevertheless, whether altered Wnt and Nodal signaling also accounts for the AVC canal defect in tennis match or png mutants is not known yet [5]. Hence, to analyze if enhanced Wnt signaling accounts for defective AV valve development in png mutants, we overexpressed Wnt8, known to effectively activate canonical Wnt signaling, using the transgenic zebrafish line Tg(hsp70l:wnt8a-GFP) that expresses wnt8a-GFP under control of the heat-shock inducible hsp70l promoter in wild-type embryos. However, activation of Wnt signaling using established heat-shock protocols [10] does not interfere with the development of the AVC as observed in png mutants (Fig. 2A–B'; Fig. S4A, B). Similarly, induction of Wnt signaling by LiCl does also not induce AVC defects in sensitized heterozygous...
*embryos (Fig. S4C–F), implicating that enhanced Wnt signaling does not account for the AVC defects in *png* mutants. Vice versa, increasing Nodal activity in homozygous *png* mutants by transient overexpression of *nodal related protein 2* (*cyclops, ndr2*) mRNA does not suppress the *png* mutant AVC phenotype (Fig. 2C–E). These findings indicate that the *png* mutant heart valve phenotype seems...
neither mediated by enhanced Wnt nor reduced Nodal signaling.

3.4. Foxn4-Med10-Tbx2b signaling is disrupted in png mutant hearts

The T-box transcriptional regulator Tbx2 was found to suppress expression in the myocardium of the AVC [14] to guarantee proper AVC development [15]. Thus, to evaluate whether altered Tbx2b signaling accounts for the misexpression of anf and the structural AVC defects in png mutants, we assayed expression of tbx2b and find that in png mutant hearts tbx2b mRNA is absent in the AVC (Fig. 2F I). Next, to assess if ectopic expression of Med10 can reconstitute the expression of tbx2b thereby suppressing the development of AVC defects in png mutants, we injected 800 pg of med10-mRNA into 1-cell stage png mutant embryos. As shown in Fig. 2J M, ectopic expression of med10-mRNA reconstitutes both, regular tbx2b and anf expression in AV myocardial cells of png mutants, suggesting a specific role for Med10 in the regulation of Tbx2b expression in the AV myocardium. Hence, to further examine whether loss of Tbx2b expression in the AVC is indeed the molecular cause for defective AVC development in png mutants, we injected 320 pg tbx2b-mRNA into png mutants and find that 19.70 ± 2.32% (P < 0.001) of homozygous png mutants show properly developed AVC accompanied by the formation of endocardial cushions (Fig. 3A E), suggesting that Med10 regulates AVC development mainly via the control of Tbx2b expression.

In slip jig mutant zebrafish, forkhead box transcription factor 4 (Foxn4) was found to regulate tbx2b transcription in AVC [15]. Hence, to assess if similar to the situation in slip jig mutants, ectopic expression of foxn4 can reconstitute tbx2b expression and thereby endocardial cushion formation in png mutants, we injected up to 320 pg of foxn4-mRNA into png mutants. However, we find that foxn4-mRNA injection does not lead to re-expression of tbx2b in the AVC and reconstitution of AV valve formation in png mutants.

![Fig. 3. Ectopic expression of Tbx2b rescues the valve defect in png mutants.](image-url)
implicating that Med10 either orchestrates Tbx2b expression in the AVC in a rather Foxn4-independent manner, or that Foxn4-mediated transcription of tlb2b strongly depends on intact Med10 function. Thus, we evaluated whether wild-type med10-mRNA is able to rescue the AVC defect in Foxn4 morphants. We find that ectopic expression of Med10 is unable to reconstitute AVC development in Foxn4-deficient embryos (Fig. 3I, J), implying that Med10 and Foxn4 signaling depend on each other. To ultimately prove whether Med10 and Foxn4 act in the same molecular pathway and depend on each other during heart valve development, we performed sensitizing experiments in a heterozygous png (png⁺⁻/⁻) background by reducing Foxn4 activity. To do so, we injected low doses of MO-foxn4 into heterozygous png embryos and analyzed the resulting cardiac phenotype. After injection of 2.4 ng MO-foxn4, 56.79% ± 31.36% of injected sensitized heterozygous png embryos display a heart phenotype similar to the png phenotype accompanied by the absence of AV valves and severe regurgitation of blood between ventricle and atrium (Fig. 3K, L). By contrast, only 31.31% ± 28.61% of injected homozygous wildtypes develop heart valve defects (Fig. 3K, L).

Tbx2 is known to regulate the expression of hyaluronan synthase 2 (has2) responsible for the production of hyaluronan (HA), a major ECM component of the cardiac jelly [16]. Interestingly, loss of Has2 leads to severe cardiovascular abnormalities including the failure of transformation of cardiac endothelial into mesenchymal cells (EMT) [16]. To evaluate whether defective Tbx2b signaling in png mutant hearts leads to the loss of has2 expression, we assayed has2 mRNA level and distribution and find that in png mutants has2 is completely absent in the AVC (Fig. 4A–D). Hence, to investigate if loss of Has2 in png hearts leads to reduced HA levels in the cardiac jelly, we performed HA staining of png mutant hearts and find the amount of HA in the cardiac jelly markedly reduced (Fig. 4E, F).

Next, to determine whether reduced HA levels in png mutant hearts interfere with the development of the cardiac jelly, we co-stained png x Tg(fli1a:EGFP) transgenic zebrafish with Phalloidin and Dm-grasp (Alcam) and find by confocal sectioning that the area between AV endocardium and myocardium is severely reduced in png mutant hearts (Fig. S5A, B), suggesting that Med10-deficiency interferes with the regular establishment of the cardiac jelly and that this leads to the inhibition of AV endocardial cell differentiation as shown by missing Dm-grasp-positive AV endocardial cells in png mutant hearts (Fig. S5A, B).

4. Discussion

In search for signaling cascades that guide cardiac valve development in vertebrates, we deciphered the molecular fundamentals of the zebrafish AVC mutant ping pong and find that the transcriptional Mediator complex subunit 10 (Med10) regulates the development of the AV valves by mediating Foxn4 signals and thereby controlling the expression of Tbx2b in AV myocardial cells and the formation of the cardiac jelly.

The Mediator complex (Med) integrates and transduces regulatory information from DNA-binding transcription factors to the

![Fig. 4. Defective Has2 signaling in png mutant hearts. (A-D) has2 staining is absent in AVC and OFT endocardium of png mutants at 48 (B) and 72 hpf (D). (E, F) Histological sections of wt (E) and png mutants (F) at 72 hpf stained for hyaluronic acid (HA) (brown) and counterstained with H&E. The amount of HA in the cardiac jelly of png mutants is severely reduced.](image-url)
basal RNA polymerase II (Pol II) machinery thereby serving as a connecting bridge between regulatory and accomplishing transcriptional elements without direct DNA binding [17]. Not much is known about the functions of distinct Mediator subunits in vertebrates. Med10 was shown to be dispensable for global transcriptional processes but found to be involved in formation of tail buds by regulating Wnt and Nodal signaling in zebrafish. Although a valve pathology was described in tennis match mutants, the role of enhanced Wnt and impaired Nodal signals on AV valve development in tennis match embryos was not evaluated in this study [5].

We find that neither transgenic overexpression of Wnt8 in wild-types nor increasing Wnt signaling in sensitized heterozygous pgp embryos result in defective AVC formation, indicating that the AVC defect in pgp mutants is not due to over-activated Wnt signaling as observed in APC mutant zebrafish [16]. Furthermore, overexpression of Nodal related protein 2, known to activate Nodal signaling [19], is not capable to reconstitute valve formation in homozygous pgp mutants, indicating that impaired valve formation in Med10-deficient embryos is neither mediated by Wnt nor Nodal signaling.

We find anexpressed in pgp AVC myocardial cells, cells that are usually devoid of anf mRNA [20]. Tbx2b was shown to act as a transcriptional repressor in AV myocardium, inhibiting Anf transcription by forming a repressive ternary complex on the Anf promoter together with Nkx-2.5 [21]. We find that the expression of tbxb2 is absent in AV myocardial cells in pgp, explaining the misexpression of Anf in pgp AV myocardium. Chi et al. reported that targeted ablation of tbxb2 leads to malformations of the AVC accompanied by missing endocardial cushions [15]. These findings are consistent with our findings that pgp mutants also show abrogated AVC development as a result of diminished tbxb2 expression. Overexpression of Tbx2b in pgp mutants is capable to reconstitute AVC morphogenesis, indicating that loss of Tbx2b in the AVC downstream of Med10 is causative for the cardiac phenotypes in pgp mutants. Analyses of the zebrafish mutant slip 1g revealed that Foxn4 plays a key-role in tbxb2 expression at the AVC. Loss of Foxn4 in zebrafish results in loss of tbxb2 expression specifically at the AVC and thereby a structurally and functionally defective AVC [15]. We find that ectopic expression of Foxn4 cannot reconstitute tbxb2 expression and AVC morphogenesis in pgp mutants. Vice versa, injection of med10-mRNA into Foxn4 morphants is also not capable to rescue AVC development, implicating that Med10 function might transduce Foxn4 signals to regulate the expression of tbxb2 at the AVC. In fact, we were able to show that injection of foxn4 Morpholinos into heterozygous pgp embryos leads to defective heart valve formation in these sensitized embryos, indicating that both proteins can act in the same pathway.

Shirai et al. were able to show that activated Tbx2b leads to enhanced EMT of endocardial cells [22]. In pgp, we find a complete loss of secreted phosphoprotein 1, a factor that was shown to be strongly expressed in activated AV endocardial cells that migrate into the cardiac jelly and undergo EMT [12]. The cardiac jelly is mainly composed of the glycosaminoglycan hyaluronic acid (HA), which is produced by hyaluronan synthase (Has2), a known downstream target of Tbx2b [22]. Indeed, we find Has2 and subsequently HA lost in pgp hearts, implying that impaired development of the cardiac jelly accounts for missing spp1 expression and the lack of EMT of AV endocardial cells. In fact, Has2-deficient mice that fail to form cardiac jelly resulting in defective EMT and endocardial cushion formation [16]. By contrast, Has2 upregulation leads to increased extracellular HA production, the expansion of the cardiac jelly and thereby excessive endocardial cushion cell differentiation [8].

In summary, we demonstrate that Foxn4-Med10-Tbx2b signaling plays a crucial role in cardiac jelly formation by regulating Has2 expression and HA production and thereby in vertebrate AV valve formation.

Disclosures

None.

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Appendix A. Supplementary data

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Transparency document

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