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Report

A Mutation in Zebrafish *hmgcr1b* Reveals a Role for Isoprenoids in Vertebrate Heart-Tube Formation

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Summary

In vertebrates, the morphogenetic assembly of the primitive heart tube requires the medial migration and midline fusion of the bilateral myocardial epithelia [1, 2]. Several mutations that result in abnormal heart-tube formation have been studied; however, an understanding of the underlying molecular and cellular mechanisms of the migration and fusion of these epithelial sheets is far from complete [1–4]. In a forward genetic screen to identify genes regulating early zebrafish heart development, we identified a mutation in the *3-hydroxy-3-methylglutaryl-Coenzyme A reductase 1b* (*hmgcr1b*) gene that affects myocardial migration to the midline and subsequent heart-tube morphogenesis. The mutant phenotype can be rescued with injections of mevalonate, the direct product of HMGCR activity. Furthermore, treatment of embryos with pharmacological inhibitors of isoprenoid synthesis, which occurs downstream of mevalonate production, resulted in defective heart-tube formation. Interestingly, in *hmgcr1b* mutant embryos and embryos treated with HMGCR inhibitors, both RasCT20-eGFP and RhoaCT32-eGFP fusion proteins were mislocalized away from the plasma membrane in embryonic myocardial cells. We conclude that protein prenylation, acting downstream of Hmgcr1b and possibly through Ras and, or, Rho signaling, is required for the morphogenesis of the myocardial sheets for formation of the primitive heart tube.

Results and Discussion

A Novel Mutant with Early Heart Morphogenesis Defects

In order to more thoroughly understand the molecular, genetic, and cellular mechanisms of early heart formation, we carried out a forward genetic screen in zebrafish to identify additional genes regulating early heart development. The *s617* mutation was originally identified as causing delayed myocardial migration to the midline. By 24 hr postfertilization (hpf), all *s617* mutant embryos exhibit a thin, midline heart, a thick yolk extension, and pericardial edema (Figure 1). These defects become more pronounced by 36 and 48 hpf. Circulation is always absent in the mutant embryos.

In order to examine the heart phenotype more closely, we used a transgenic line, *Tg(cmlc2:egfp)* [5], in which the *cmlc2* promoter drives eGFP expression in the myocardial cells. We found that 21-somite-stage *s617* mutant embryos show delayed migration of the bilateral myocardial cell populations to the midline compared to their wild-type siblings (Figures 1F and 1G). By 24 hpf, the wild-type hearts have jogged to the left (Figure 1H), whereas *s617* mutant hearts remained at the midline (Figure 1I). The more severely affected mutant embryos also exhibited a delay in heart tilting and extension (Figure 1I). By 48 hpf, when wild-type hearts have undergone looping morphogenesis, *s617* mutant hearts were unlooped, thin midline structures.

Many mutations that cause myocardial migration defects have been shown to primarily affect endoderm development [6–11], suggesting that early heart morphogenesis requires an intact endoderm. In order to determine whether the *s617* mutation was affecting endoderm development, we used a transgenic line in which the *her5* promoter drives eGFP expression in the pharyngeal endoderm (*Tg(0.7her5:egfp)*^{ne2067}) [12]. No obvious differences in pharyngeal endoderm development were observed between mutant embryos and their wild-type siblings before 24 hpf (Figures 1J and 1K). These data suggest that endoderm differentiation and early morphogenesis are unaffected in *s617* mutant embryos.

The *s617* Gene Encodes 3-Hydroxy-3-methylglutaryl coenzyme A reductase 1b

To gain a better molecular understanding of the *s617* phenotype, we isolated the *s617* gene by positional cloning (Figure 2). By using bulk-segregant analysis, we mapped the *s617* locus to LG21. Fine mapping with 1301 diploid mutant embryos narrowed the *s617* genomic region to a 1.6 cM span between CA repeat markers z6295 and z7405. Further fine mapping revealed that the *s617* locus was located on the BAC CH211-149I8 (CR318660). One recombinant and zero recombinant markers were found in the 5′ and 3′ UTR regions of the *hmgcr1b* gene, respectively (Figure 2A). Because HMGCR had been implicated in regulating germ-cell migration in zebrafish and *Drosophila* [13, 14], we focused on *hmgcr1b* as a candidate for the *s617* locus. Sequencing *hmgcr1b* from *s617* mutant embryos revealed a G to A (GGT→GAT) transition at position 1575 (Figure 2B). This mutation results in an amino acid change from glycine (Gly) to aspartic acid (Asp) at codon 497. Interestingly, this glycine residue lies at a highly conserved position in a region required for homodimerization or tetramerization of HMGCR (Figure 2C) [15, 16]. We confirmed that this mutation occurs

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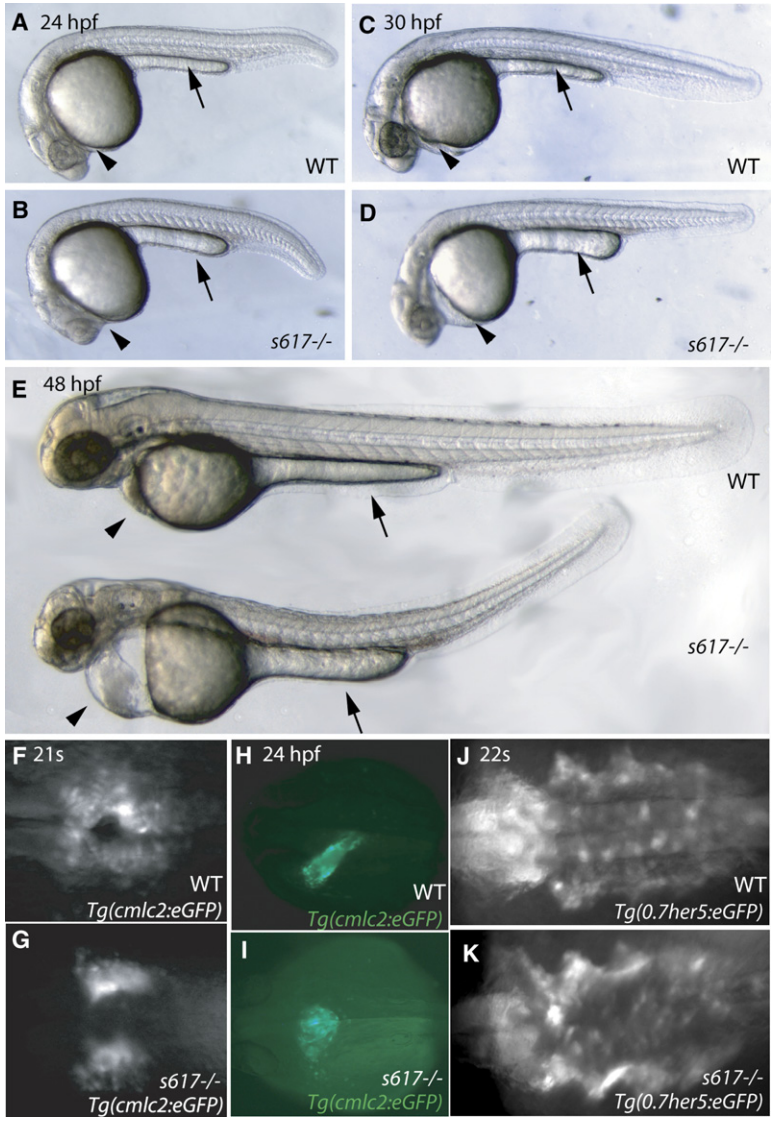

Figure 1. *s617* Mutant Embryos Exhibit an Early Heart Morphogenesis Defect

(A and B) By 24 hr postfertilization (hpf), mutant embryos (B) exhibit a slightly thicker yolk extension (indicated by the arrow) and pericardial edema (indicated by the arrowhead) compared to their wild-type siblings (A).

(C, D, and E) By 30 (C and D) and 48 (E) hpf, the thick yolk extension (indicated by the arrow) and edema (indicated by the arrowhead) have become more obvious in mutant embryos. Lateral views with anterior to the left are shown in (A)–(E).

(F and G) Compared to a fused midline heart in wild-type embryos (F) at the 21-somite stage (19.5 hpf), the myocardial epithelia in *s617* mutant embryos (G) exhibit delayed medial migration.

(H and I) At 24 hpf, wild-type hearts (H) extend toward the left side of the embryo. In contrast, *s617* mutant hearts (I) usually remain at the midline and are delayed in their formation.

(J and K) Anterior endoderm morphogenesis, as assayed by *Tg(0.7her5:egfp)*^{ne2067} expression in the wild-type (J) and mutant (K), appears unaffected at the 22-somite stage. Dorsal views with anterior to the left are shown in (F)–(K).

at the genomic level in *s617* mutant embryos by sequencing the relevant genomic region (data not shown).

To further test whether a defect in *hmgcr1b* is sufficient to cause a heart morphogenesis phenotype, we injected a morpholino antisense oligo designed against the *hmgcr1b* initiation ATG sequence into one-cell-stage embryos. Morpholino injections resulted in a phenocopy of the *s617* mutation, including pericardial edema, thick yolk extension, and heart morphogenesis defects. However, as expected based on the fact that *hmgcr1b* mRNA is present maternally [13], more severe defects were common when the MO was injected at high doses (10 ng/embryo), with axis defects and frequent embryonic death by 24 hpf. Lower doses (5 ng/embryo) caused slightly less severe phenotypes that more closely resembled the *s617* phenotype (Figure 2D).

In order to further establish whether the *s617* mutant heart defects are due to a mutation in *hmgcr1b*, we treated embryos with statins, specific inhibitors of HMGCRs, and were able to phenocopy the *s617* mutation (Figures 2E and 2F). Embryos were soaked in either 10 μM Atorvastatin or 1.2 μM Mevinolin starting at the end of gastrulation. Atorvastatin treatment resulted in embryos with slightly thicker yolk extensions and kinked notochord as previously described [13], as well as midline, unlooped hearts (Figure 2E). The heart phenotype looked very similar to that seen in *s617* mutant embryos. Soaking embryos in Mevinolin resulted in a much more severe heart and general somatic phenotype: Compressed and straight somites, a short tail, and a thick yolk extension were present as previously described [13]. Mevinolin treatments also blocked myocardial cell migration to the midline, resulting in cardia bifida (Figure 2F).

To further confirm that HMGCR activity is required for heart morphogenesis, we injected mevalonate, the direct downstream product of HMGCR, and were able to rescue the *s617* mutant phenotype (Figure 2G). Injection of 2.3 nl of a 0.5 M mevalonate solution into one-cell-stage embryos resulted in a rescue of the *s617* mutant phenotype through the first 72 hpf. Embryos were analyzed at 48 hpf and then genotyped with a restriction fragment length polymorphism (RFLP) generated by the *s617* mutation. Heart morphogenesis and function,