

# The G Protein-Coupled Receptor *Agtr1b* Regulates Early Development of Myocardial Progenitors

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DOI 10.1016/j.devcel.2007.01.012

## SUMMARY

While many factors that modulate the morphogenesis and patterning of the embryonic heart have been identified, relatively little is known about the molecular events that regulate the differentiation of progenitor cells fated to form the myocardium. Here, we show that zebrafish *grinch* (*grn*) mutants form a reduced number of myocardial progenitor cells, which results in a profound deficit in cardiomyocyte numbers in the most severe cases. We show that *grn* encodes the G protein-coupled receptor (GPCR) *Agtr1b*, a known regulator of adult cardiovascular physiology. Ectopic expression of *Apelin*, an *Agtr1b* ligand, results in the complete absence of cardiomyocytes. Data from transplantation and transgenic approaches indicate that *Agtr1* signaling plays a cell-autonomous role in myocardial specification, with activity being required coincident with the onset of gastrulation movements. These results support a model in which *agtr1b* regulates the migration of cells fated to form myocardial progenitors.

## INTRODUCTION

Organogenesis begins with the development of progenitor cells that later differentiate to form the specialized tissues required for organ function. In vertebrates, myocardial progenitors can first be distinguished by *nkx2.5* expression in two bilateral stripes of cells in the anterior lateral plate mesoderm (ALPM) (Harvey, 1996). Prior to the onset of *nkx2.5* expression, fate-mapping studies in several vertebrates have shown that myocardial progenitor cells ingress early during gastrulation (Stainier et al., 1993; Parameswaran and Tam, 1995; Schoenwolf and Garcia-Martinez, 1995; Keegan et al., 2004). Multiple signaling pathways, acting in both stimulatory and inhibitory fash-

ions, act to restrict cardiomyogenesis to a defined domain in the ALPM. These signals include TGF- $\beta$ s, Fgfs, Shh, and Wnts/Wnt inhibitors (Schultheiss et al., 1997; Reifers et al., 2000; Schneider and Mercola, 2001; Zhang et al., 2001).

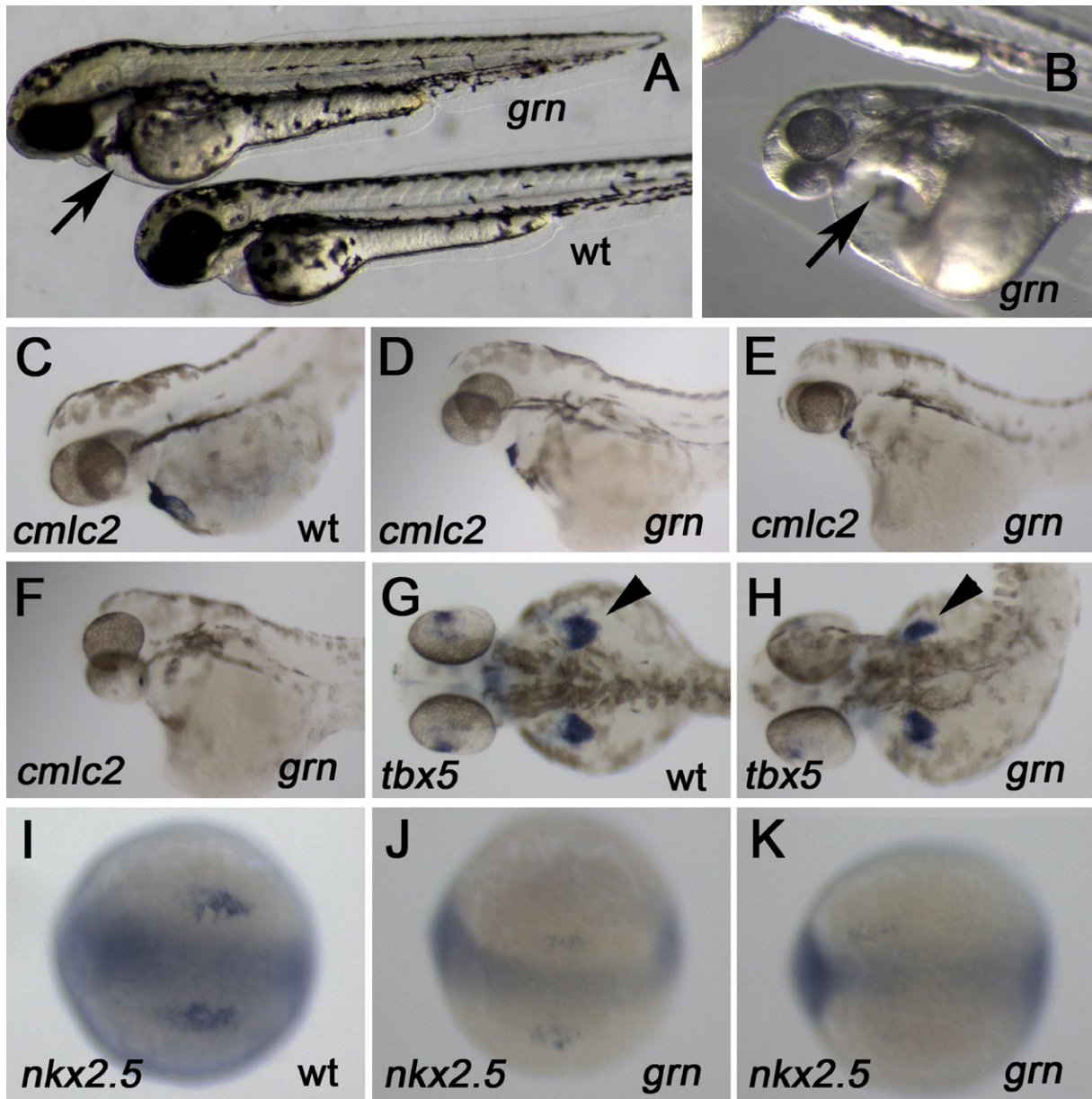
Relatively little is known regarding mechanisms that specifically affect myocardial progenitor specification and migration to the ALPM prior to the onset of *nkx2.5* expression. Explant studies in chick have suggested that signals are required for cardiomyogenesis prior to gastrulation (Antin et al., 1994). In gastrula-stage zebrafish embryos, retinoic acid (RA) signaling acts to restrict the number of myocardial progenitor cells formed (Keegan et al., 2005). Members of the *Mesp* transcription factor gene family are broadly expressed in the murine primitive streak, and they are required for the ingression of cardiovascular progenitors during gastrulation (Saga et al., 1999). Heart development is severely perturbed in *Mesp1/2* double mutants (Kitajima et al., 2000), as well as in ascidian embryos depleted of *mesp* function by injection of antisense oligonucleotides (Satou et al., 2004).

In this study, we used a forward genetic approach in zebrafish to identify possible novel regulators of early cardiac development. We describe a mutation, *grinch*, that greatly inhibits the development of the myocardium. Activity of the gene affected by this mutation, *agtr1b*, is shown to be required early in myocardial progenitors, coincident with the onset of gastrulation. To our knowledge, these results therefore identify a novel signaling pathway required for myocardial development.

## RESULTS

### *grinch* Mutants Have a Specific and Early Defect in Cardiomyogenesis

In order to identify additional regulators of cardiac development, we carried out a forward genetic screen and assayed heart morphology at 52 and 96 hours postfertilization (hpf) (Beis et al., 2005). A single allele of a novel recessive mutation, *grinch*<sup>s608</sup> (*grn*), was identified and caused a marked reduction in the size of the heart (Figures 1A and 1B). While the expressivity of the *grn* phenotype is



**Figure 1. The *grn*<sup>s608</sup> Mutation Affects Cardiomyocyte Specification**

(A and B) Lateral views of 48 hpf embryos from *grn*<sup>s608</sup> heterozygote incrosses. In severe cases, mutant embryos ([A], top; [B]) lack a distinguishable heart (arrow); however, this defect is variable within the same clutch.

(C–F) *cmlc2* ISH at 24 hpf on (C) wild-type (WT) and (D–F) *grn*<sup>s608</sup> mutant embryos.

(G and H) *tbx5* ISH on 48 hpf (G) WT and (H) *grn*<sup>s608</sup> mutant embryos. Arrowheads denote pectoral fin buds.

(I–K) *nkx2.5* in 14 hpf (I) WT and (J and K) *grn*<sup>s608</sup> mutant embryos; dorsal views, anterior is oriented toward the left.

variable, in the most extreme cases no or only a very small number of *cardiac myosin light chain 2* (*cmlc2*)-positive cardiomyocytes are present (Figures 1C–1F). The *grn* phenotype is partially affected by genetic background, as different pairs of mutant carriers reproducibly yield embryos with differing severities of phenotype (data not shown). A striking aspect of the *grn* phenotype is its specificity to the heart. Severely affected *grn* mutants are viable until 6–7 days postfertilization (dpf), with the only notably aber-

rant features being pericardial edema and the absence of a heart. Development of the endoderm, vasculature, posterior LPM, and somitic mesoderm appears unaffected (Figures S1A–S1E; see the Supplemental Data available with this article online). The pectoral fins, an ALPM derivative, form in *grn* mutants as in wild-type siblings (as shown by *tbx5* expression, Figures 1G and 1H). Therefore, the effects of the *grn* mutation on the LPM appear to be confined to the myocardial lineage.

To determine the nature of the *grn* myocardial defect, various stages of heart development were examined. At 12.5 hpf, *nkx2.5* expression is greatly reduced in a subset of *grn* mutants (Figures 1I–1K), indicating a defect in early myocardial specification. In contrast, later aspects of differentiation appear normal in the few cardiomyocytes formed in severe *grn* mutants. Expression of the chamber-specific genes *amhc* and *vmhc* (Yelon et al., 1999) is established (Figures S1F and S1G), and cells are contractile. Cardia bifida is not observed in *grn* mutants, indicating that the *nkx2.5*<sup>+</sup> cells formed can migrate to the midline. Finally, mild *grn* mutants with a wild-type-sized heart survive to adulthood and are fertile. Taken together, these data suggest that *grn* activity is required prior to the establishment of *nkx2.5*-positive myocardial progenitors in the ALPM, but appears dispensable for later aspects of embryonic heart development and function. The variable expressivity of the *grn* phenotype is not due to maternal contribution, as intercrosses of homozygous mutant *grn* carriers yield a phenotype indistinguishable from the zygotic one (data not shown). The observed variability could be due to the fact that *grn*<sup>s608</sup> is not a null mutation or that *grn* functions in parallel with other genetic pathways.

#### The *grinch* Phenotype Is Due to a Mutation in *agtrl1b*

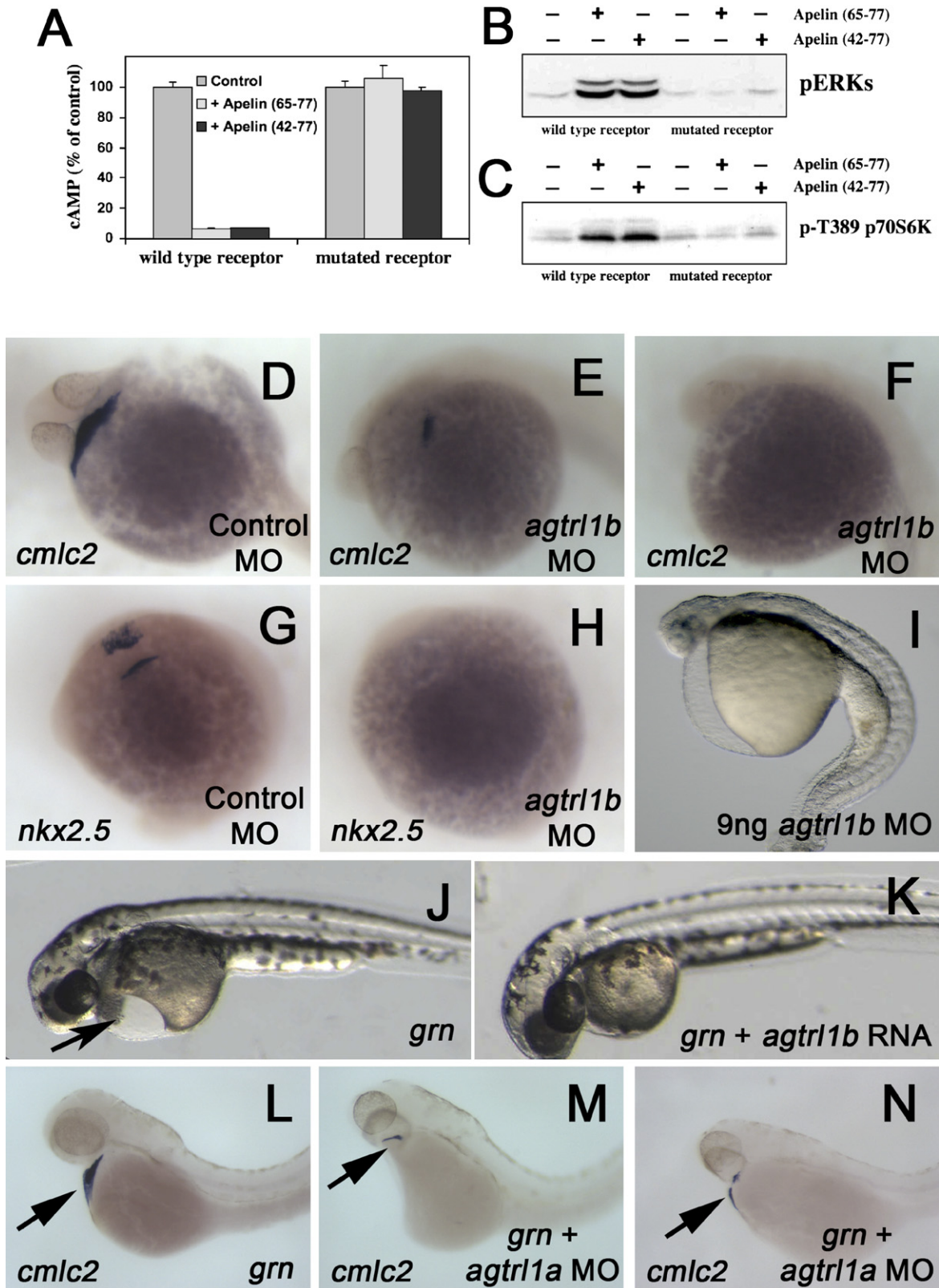
To gain further insight into the nature of *grn* activity, we sought to identify the gene affected by the *grn*<sup>s608</sup> mutation. Using single-sequence length polymorphisms (SSLP) and restriction fragment length polymorphism (RFLP) markers, the *grn* locus was shown to reside on linkage group 10, with tight linkage to the BAC zC102K8. Sequence analysis of zC102K8 revealed the presence of an ORF with homology to the GPCR gene *agtrl1* (a.k.a. *apj*, *X-msr*, and *angio1* [Audigier, 2006]) (Figure S2A). Sequencing of the *agtrl1b* cDNA showed that the s608 allele contains a G to T base pair change at position 269, creating a tryptophan to leucine amino acid change in the second transmembrane domain (Figures S2B and S2C). This tryptophan residue is fully conserved in all Agtr1 orthologs characterized to date, as well as in the related Angiotensin and CXC chemokine receptor families (data not shown). Therefore, it seems likely that the loss of this bulky hydrophobic amino acid in a transmembrane domain should have a significant impact on Agtr1b function.

To test whether a mutation in *agtrl1b* causes the *grn* phenotype, biochemical, loss-of-function, and rescue approaches were undertaken. Mammalian Agtr1 signals via binding of Apelin (Apln) peptide ligands (Tatemoto et al., 1998; Masri et al., 2002, 2004). Cells stably overexpressing zebrafish *agtrl1b* are responsive to both 13 and 36 amino acid human Apln peptides, resulting in adenylyl cyclase inhibition and phosphorylation of ERK and p70S6 kinase (Figures 2A–2C). These data validate the assignment of Agtr1b as a zebrafish ortholog of the Apln receptor. In contrast to the wild-type gene, the *grn*<sup>s608</sup> allele of *agtrl1b* is completely unresponsive to stimulation by Apln in these assays. Therefore, by biochemical criteria, the *grn*<sup>s608</sup> allele of *agtrl1b* appears to encode a functionally inactive

receptor. To further appraise the requirement for *agtrl1b* in heart development, morpholino (MO) antisense oligonucleotides were used. Injection of 2.0 ng of a MO targeting the 5'UTR of *agtrl1b* resulted in a strong reduction of cardiomyocyte differentiation, as assayed by *cmhc2* expression at 24 hpf (Figures 2D–2F), as well as decreased *nkx2.5* expression at the 10-somite stage (Figures 2G and 2H). In contrast, a 5 base pair mismatch control MO had no effect on heart development. Injection of higher concentrations of *agtrl1b* MO yielded a fully penetrant loss of heart formation. However, these morphants also exhibited stunted growth and brain necrosis, possibly due to toxic side effects of the MO (Figure 2I). In order to further assess whether *grn* encodes *agtrl1b*, rescue experiments were performed. Injection of *agtrl1b* mRNA into embryos derived from intercrosses of homozygous mutant *grn*<sup>s608</sup> carriers resulted in a substantial rescue of heart size and function (Figures 2J and 2K; Table S1). In many cases, circulation was restored in the injected embryos, which could, in turn, be raised to adulthood. Taken together, the preponderance of tight linkage, biochemical, MO loss-of-function, and RNA rescue data indicate that *agtrl1b* is indeed the gene affected by the *grn*<sup>s608</sup> mutation. Injection of RNA encoding Agtr1b<sup>s608</sup> at high concentrations had no effect on cardiac development in wild-type embryos (data not shown). Coupled with the absence of a heterozygous *grn* phenotype, these results strongly suggest that Agtr1b<sup>s608</sup> does not have dominant-negative effects.

#### *agtrl1b* Is Expressed Broadly in Mesendodermal Progenitors

To help define when *agtrl1b* acts to regulate myocardial differentiation, gene expression was examined by in situ hybridization. *agtrl1b* transcripts are detectable prior to the onset of gastrulation at sphere stage (4 hpf) in scattered cells in the animal pole of the embryo (Figure 3A). By 50% epiboly (5.3 hpf), *agtrl1b* expression is restricted to the ventral and lateral margins, the latter of which include the myocardial progenitors (Stainier et al., 1993; Keegan et al., 2004), but is excluded from the dorsal organizer region (Figure 3B). As gastrulation proceeds from 5 hpf onward, *agtrl1b* expression expands toward the animal pole, in concert with the movement of cells from the margin toward the axis of the embryo (Figure 3C). By bud stage (10 hpf), *agtrl1b* transcripts are localized posteriorly in the presomitic mesoderm and in adaxial cells adjacent to the midline, coincident with the first clearly detectable expression of *apln* in the embryonic midline proper (Figures 3D–3F). At somitogenesis stages, *agtrl1b* continues to be expressed in somitic mesoderm, with an additional domain of expression in the ALPM, medial to the *nkx2.5*<sup>+</sup> myocardial progenitors (Figures 3G–3J, compare arrows and arrowheads). At later stages, *agtrl1b* expression is prominent in the developing vasculature, including both major trunk vessels—the dorsal aorta and the posterior cardinal vein—as well as the branching inter-somitic vessels (Figures 3K and 3L). This later vascular expression of *agtrl1b* suggests that the medial ALPM



**Figure 2. *grn*<sup>5608</sup> Is a Mutation in *agtr1b***

(A–C) Analysis of *Agtr1b* signaling. (A) Inhibition of adenyllycylase activity, and (B and C) phosphorylation of ERK and p70S6 kinase by WT, but not mutant, *agtr1b* upon Apln addition.

expression at 11 hpf is likely in the nascent endocardial cells. As the endocardium appears to be dispensable for myocardial induction in zebrafish (Stainier et al., 1995), expression of *agtr1b* in the margin at blastula and gastrula stages, and exclusion of detectable *agtr1b* transcripts from the myocardium later, suggests an early role for Agtr1b function in the development of the myocardial progenitors. Further, as expression of *agtr1b* is highly similar to what has been described for the single *Xenopus agtr1* gene (Devic et al., 1996), this early function for *agtr1b* may be evolutionarily conserved.

The expression pattern observed for *agtr1b* at early stages of embryogenesis is similar to that described for *agtr1a*, a second *agtr1* homolog in zebrafish (Tucker et al., 2006). To investigate possible redundant functions of these two genes, an MO targeting *agtr1a* was injected into *grn* mutants. In crosses in which the *grn* phenotype was weak and heart development proceeded normally, further depletion of *agtr1a* resulted in a small heart phenotype (Figures 2L–2N). These data suggest that *agtr1a/b* act redundantly. However, additional genetic pathways appear to be required to regulate cardiogenesis (see Discussion).

#### Early Ectopic Activation of Agtr1b Signaling Blocks Myocardial Progenitor Formation

To gain additional insights into Agtr1b activity, gain-of-function experiments were conducted. Remarkably, injection of 25–100 pg zebrafish *apln* mRNA at the 1-cell stage resulted in the complete absence of myocardial differentiation. Expression of *cmlc2* at 24 hpf was severely inhibited by *apln* injection (Figures 4A and 4B). Similarly, early expression of *nkx2.5* was sharply reduced at 14 hpf (Figures 4C and 4D). These phenotypes were highly consistent, as opposed to the variable expressivity observed in the cases of *agtr1b* knockdowns via mutation or MO injection. Identical results were obtained from injection of murine *apln* mRNA (data not shown). In contrast to the severe effects on myocardial differentiation, addition of *apln* had no discernable effect on the development of other mesodermal populations, including the blood, skeletal muscle, and vasculature (Figures S3A–S3C). Despite the broad expression of *agtr1b* in the margin, the effects of Agtr1b signaling thus appear to be specific to the myocardial progenitors.

To determine the fate of myocardial progenitors in embryos injected with *apln*, we examined the morphology of the ALPM, where *nkx2.5* expression first arises. *gata5* and *hand2* expression analyses reveal the bilateral ALPM epithelial sheets in wild-type embryos at 12 hpf (Figure 4E, similar data not shown for *hand2*). In contrast,

embryos injected with *apln* RNA exhibited severe defects in ALPM morphogenesis (Figure 4F). Discontinuities in the ALPM were often evident (>80% of embryos, n = 200). In addition, streams of cells projecting laterally away from the ALPM were observed. A consistent decrease in the size of the ALPM was also characteristic of *apln*-injected embryos (Figure 4G). The effect of *apln* RNA on ALPM morphogenesis appeared to be specific, as coinjection of an *apln* 5'UTR MO abolished this phenotype (Figure 4H). These results suggest that cells fated to form the myocardium do not reach their correct position upon overexpression of *apln*. The defects in the integrity of the ALPM may therefore be secondary to an absence of these cells. In contrast to these results, the ALPM appears unaffected in *grinch* mutants (data not shown), suggesting that the phenotype observed after overexpression of *apln* is not simply due to a ligand-dependent downregulation of Agtr1b.

To define the developmental period over which Agtr1b signaling is required for myocardial differentiation, we used an inducible *apln* transgene driven by a zebrafish *hsp70* promoter fragment (Halloran et al., 2000). Embryos subjected to a 30 min heat shock at late blastula stages (3 hpf) developed an acardiac phenotype at 24–48 hpf (Figure 4I). Morphological defects were also apparent, with embryos displaying a shortened axis and clumping of cells in the trunk region. Heat shock was performed at various time points to examine the window over which ectopic signaling by Aplin blocked heart development. A sharp transition was observable at 5 hpf: prior to this time, heat shock of transgenic embryos resulted in no cardiomyocytes being formed. In contrast, heat shock at 6 hpf or later had subtle or no effects on myocardial formation (Figures 4J–4L). Genotyping of embryos confirmed that all transgenic carriers (and only those) were affected if shocked prior to 5 hpf. These experiments therefore define a narrow period of time (between 3 and 5 hpf) during which Aplin expression throughout the embryo blocked cardiogenesis. Interestingly, gastrulation is initiated in zebrafish at 5 hpf. Therefore, the time period during which cardiogenesis is sensitive to *hsp70:apln* activation immediately precedes the onset of gastrulation.

#### *agtr1b* Activity Is Required in Myocardial Progenitors

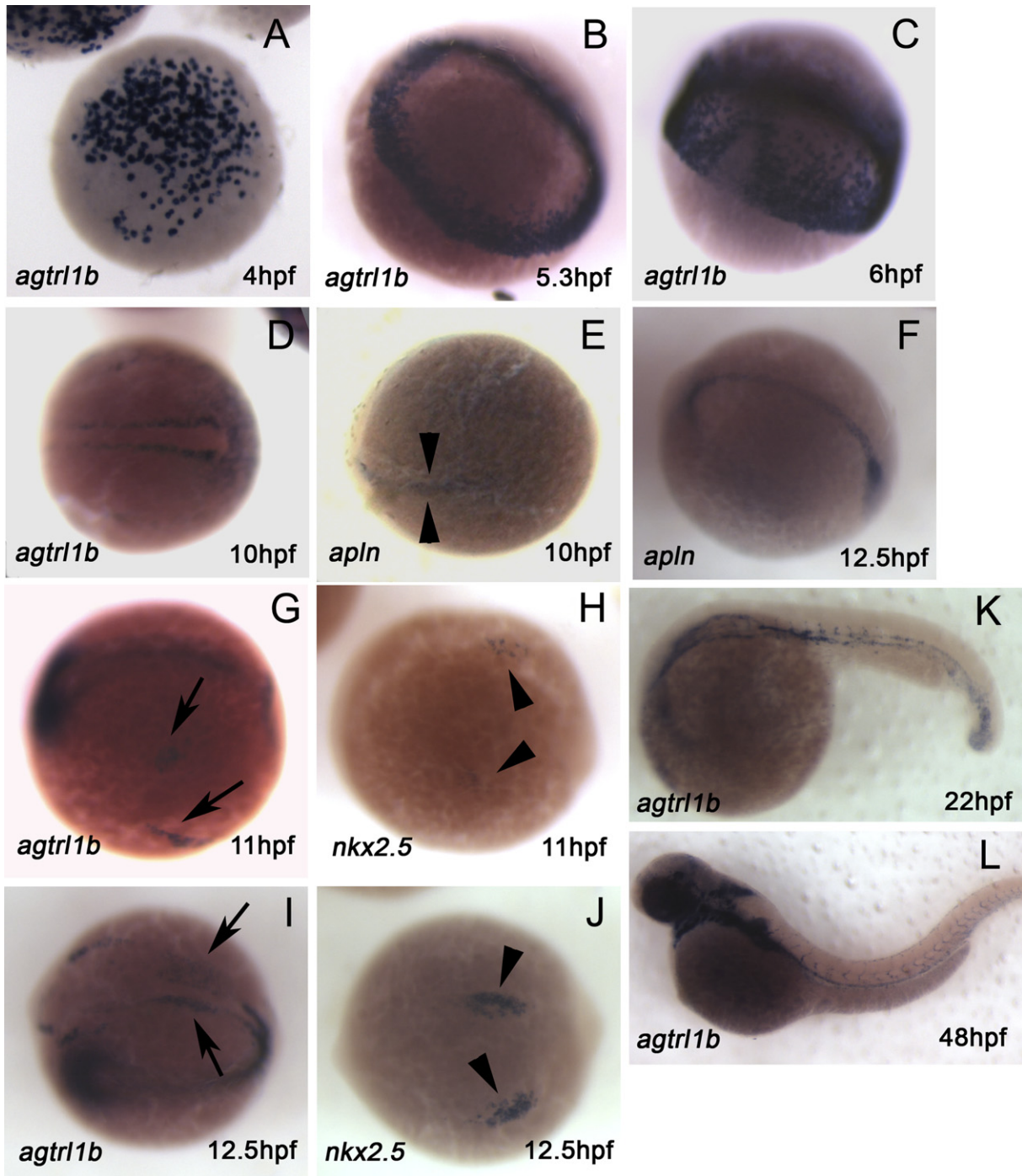
To determine whether Agtr1b signaling is required in myocardial progenitors or other cells, we performed transplantation experiments. Donor embryos carrying a *cmlc2:EGFP* transgene were used, allowing for the identification of donor cells that had taken a myocardial fate after transplantation. Transplantation of 3–4 hpf (late

(D–H) MO phenocopy of the *grn* phenotype. *cmlc2* ISH at 24 hpf on embryos injected with (D) mismatch control MO or a (E and F) 5'UTR *agtr1b* MO. *nkx2.5* ISH on 10-somite embryos injected with (G) mismatch control and (H) *agtr1b* MO.

(I) Abnormal morphogenesis and brain necrosis in a 24 hpf embryo injected with an increased concentration of *agtr1b* MO.

(J and K) Rescue of the *grn* phenotype by injection of *agtr1b* RNA. 48 hpf mutant embryos (J) uninjected or (K) injected with 100 pg *agtr1b* mRNA. The arrow denotes pericardial edema and lack of heart in uninjected controls.

(L–N) Redundancy of *agtr1a* and *agtr1b*. Relatively unaffected heart development in (L) mild *grn* mutants, as shown by *cmlc2* ISH (arrows) at 48 hpf, is worsened considerably by (M and N) coinjection of *agtr1a* MO.



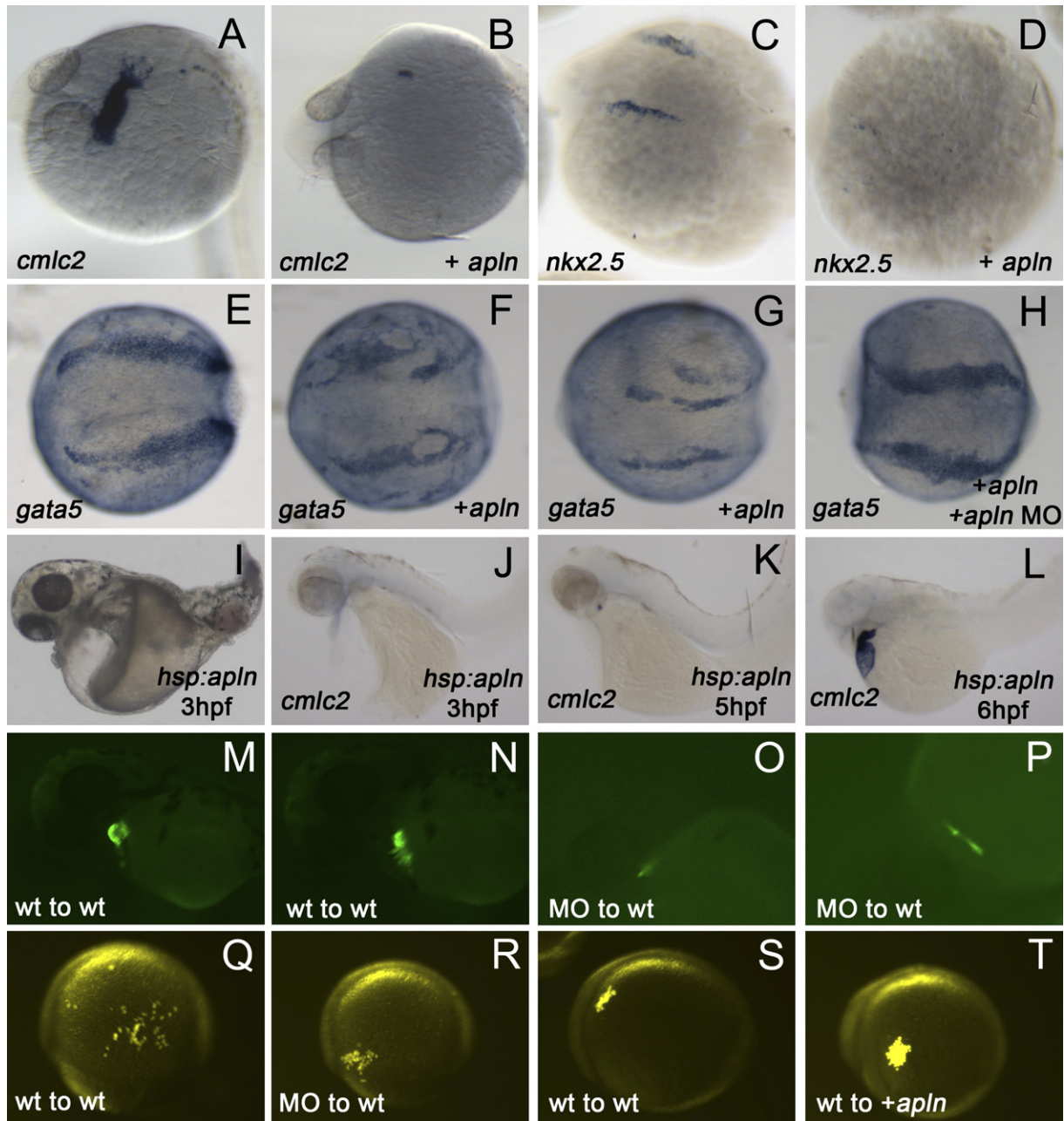
**Figure 3. Expression of *agtrl1b* Is Consistent with an Early Role in Myocardial Progenitor Development**

(A–D) Expression of *agtrl1b* at (A) 4 hpf (sphere), (B) 5.3 hpf (50% epiboly), (C) 6 hpf (shield), and (D) 10 hpf (bud) stages. *agtrl1b* is expressed broadly in the mesendoderm.

(E and F) Expression of *apl*n in the embryonic midline. *apl*n ISH at (E) 10 hpf and (F) 12.5 hpf (7-somite) stages. Arrowheads denote *apl*n expression in the midline.

(G–J) ALPM expression of *agtrl1b* in the endocardial progenitors; anterior is oriented toward the left. Expression of *agtrl1b* (arrows) at (G) 11 and (I) 12.5 hpf is medial to (H and J) *nkx2.5* expression (arrowheads) at equivalent stages.

(K and L) Vascular expression of *agtrl1b*. At (K) 22 and (L) 48 hpf *agtrl1b* transcripts localize to endothelial cells.



**Figure 4. Early Cell-Autonomous Role of Agtr1b Signaling in Cardiomyogenesis**

(A and B) 24 hpf embryos injected with 100 pg *apl*n mRNA. Fewer *cmlc2*-positive cardiomyocytes in (B) injected versus (A) uninjected embryos. (C and D) Injection of *apl*n affects myocardial progenitor specification. *nkx2.5* ISH on 14 hpf embryos (C) uninjected and (D) injected with 100 pg *apl*n mRNA.

(E–H) *gata5* ISH on 12 hpf embryos (E) uninjected, (F and G) injected with 100 pg *apl*n RNA, or (H) injected with 100 pg *apl*n RNA and 9.2 ng *apl*n 5'UTR MO; anterior is oriented toward the left.

(I–L) The *hsp:apl*n transgene can recapitulate *apl*n RNA effects on cardiac myogenesis. (I) Early heat shock at 3 hpf causes axial truncations. (J–L) Heat shock at 3 or 5 hpf, but not at 6 hpf, blocks cardiac myogenesis, as shown by *cmlc2* expression at 48 hpf.

(M–P) Transplantation of *cmlc2:EGFP* cells to WT host embryos. (M and N) WT donor cells can populate the myocardium extensively. (O and P) In contrast, donor cells injected with *agtr1b* MO form a small number of cardiomyocytes in WT host embryos (see Table 1).

(Q–T) Perturbation of Agtr1b signaling disrupts migration during gastrulation. Labeled donor cells were transplanted to the margin of host embryos at 4 hpf. (R) Cells injected with *agtr1b* MO remained at the margin area for a prolonged period of time compared to (Q) WT cells (shown at 8 hpf). (T) WT cells transplanted into embryos injected with *apl*n similarly displayed delayed migration when compared to (S) WT cells placed in uninjected hosts (shown at 10 hpf).

**Table 1. Transplantation Experiments Reveal a Cell-Autonomous Requirement for *Agtr1b* in Cardiomyocyte Formation**

Experiment	Donor; Host	GFP <sup>+</sup> Hearts/ Total	% Age GFP <sup>+</sup> Hearts
1	WT; WT	5/20	25%
1	MO; WT	0/23	0%
2	WT; WT	12/44	27%
2	MO; WT	6/102	5.9%
3	WT; WT	7/46	15%
3	MO; WT	16/138	11.6%
Total	WT; WT	24/110	21.8%
Total	MO; WT	22/263	8.4%

Cells from WT or *agtr1b* morphant (MO) embryos bearing a *cm1c2:EGFP* transgene were transplanted into WT hosts at 3–4 hpf. At 48 hpf, the percentage of host embryos containing GFP-positive cardiomyocytes was determined by fluorescence microscopy. In the third experiment, a higher percentage of host hearts contained MO-injected donor cells; however, 50% of donor embryos left to develop after transplantation contained beating tissue (data not shown). The elevated contribution of MO donor cells observed was therefore likely due to the incomplete penetrance of the MO phenotype.

blastula) wild-type cells to wild-type hosts resulted in 21.8% of host embryos with GFP<sup>+</sup> myocardial cells when observed at 48 hpf (Figures 4M and 4N; Table 1). In about half the cases in which GFP was observed in the heart, six or more myocardial cells were evident. In contrast, cells derived from embryos injected with *agtr1b* MO formed GFP<sup>+</sup> myocardial cells in 8.4% of the wild-type host embryos (Table 1). In addition, hearts contained only 1–3 GFP<sup>+</sup> cells, as opposed to the greater number frequently seen when wild-type donors were used (Figures 4O and 4P). Morphant donor cells contributed well to other lineages, including skeletal muscle, blood cells, and neurons, as evidenced by observing the rhodamine lineage tracer (Figures S3G and S3H; Movie S1). Given the incomplete expressivity of the *agtr1b* MO phenotype at the lower MO doses used in these experiments (to ensure health of the donor cells), these experiments indicate that placing *agtr1b* morphant cells in a wild-type environment did not appreciably rescue their ability to form myocardium, suggesting that *Agtr1b* signaling is required autonomously in myocardial progenitors.

To examine the effects of inactivating or hyperactivating *Agtr1b* signaling on cellular behavior, rhodamine-labeled donor cells were tracked over time. Cells injected with *agtr1b* MO and transplanted into wild-type embryos consistently migrated more slowly from the margin toward the axis of the embryo (Figures 4Q and 4R). In addition, wild-type cells transplanted into embryos injected with *apln* RNA similarly demonstrated reduced migratory behavior (Figures 4S and 4T). It should be noted that these effects were moderate, and that in both cases donor cells did reach the embryonic axis, albeit in a delayed fashion.

## DISCUSSION

We present evidence for a, to our knowledge, novel function for *Agtr1b* in the early development of myocardial progenitors. *Agtr1* has been attributed to a number of functions in adult physiology, including the regulation of blood pressure, heart contractility, and heart failure (Chen et al., 2003; Ishida et al., 2004; Ashley et al., 2005; Masri et al., 2005). Targeted mutagenesis of *Agtr1* in mouse has not been reported to affect early heart development (Ishida et al., 2004). However, a lower than expected frequency of postnatal homozygous mutant pups (19% versus 25%) was recovered. More recent work has found that mouse *Agtr1* mutants do indeed have severe cardiovascular defects, with the penetrance of the phenotype being influenced by genetic background (T. Quertermous, personal communication), indicating that *Agtr1* may regulate early cardiac development in mouse as well. It is interesting to note that our work describes an embryonic role for a signaling pathway that also regulates adult cardiac function. The question of whether these functions are ultimately achieved by differing or similar signaling mechanisms will be interesting to pursue, as will the effects, if any, of the *grinch* mutation on adult zebrafish heart function.

An angiogenic role for *Agtr1*, based on the use of MOs in *Xenopus* embryos, has also recently been reported (Cox et al., 2006; Inui et al., 2006). While *agtr1b* is expressed in the developing vasculature, primary angiogenesis and branching of the intersomitic vessels appear unaffected in *grn* mutants (Figure S1). However, as the absence of circulation impairs later aspects of angiogenesis (Lawson and Weinstein, 2002), the role of *agtr1b* in this process could not be directly evaluated. It is interesting to note that the two *Xenopus* publications reported differing results with respect to the phenotype observed after depletion of *Agtr1*. In one study (Cox et al., 2006), angiogenesis, including intersomitic vessel branching, was perturbed. In the second study (Inui et al., 2006), much more severe effects on cardiovascular development were observed, including a decreased formation of endothelial cells and severe cardiac abnormalities. The authors ascribed the cardiac phenotype as being secondary to the absence of signals from endothelial/endocardial cells. However, based on the results of our experiments (see below), we believe that, at least in zebrafish embryos, *Agtr1b* is required directly in myocardial progenitors. It is important to note that the myocardial phenotype observed in *grinch* mutants is caused by gene mutation, not by the use of MOs, and therefore is likely a more robust indicator of *agtr1b* function. A further examination of *agtr1a/b* double mutants will be required to fully assess the role of *Agtr1* signaling in zebrafish angiogenesis.

Tellingly, both the loss and ectopic activation of *Agtr1b* signaling result in a specific reduction of myocardial progenitors at the earliest stages that can be assayed by gene expression, a novel phenotype in vertebrates. These data suggest that *Agtr1b* signaling is not required for the induction of cardiomyogenesis. Further, coinjection of

a p53 MO, which inhibits apoptosis in zebrafish embryos (Langheinrich et al., 2002), did not alleviate the *agtr1b* MO phenotype (data not shown). Agtr1b signaling therefore does not appear to be required for the survival of myocardial progenitors. Instead, we find more likely a model in which Agtr1b signaling regulates the migration of myocardial progenitors to the correct position in the ALPM (Figure S4). With defective migration, these myocardial progenitors would not receive the inductive signals required to promote their differentiation. In contrast, ectopic expression of an Agtr1 ligand would disrupt an endogenous chemotactic gradient, similarly resulting in a failure to migrate. Transplantation data support this model, as both *agtr1b* morphant cells in a wild-type environment and wild-type cells in a ubiquitous *apln* milieu appear to migrate toward the midline more slowly. The defects observed in ALPM morphology upon ectopic *apln* expression may reflect the failure of the migrating cells to form a cohesive epithelial sheet. The partially penetrant nature of the *grn* phenotype may therefore indicate in part the sensitivity of myocardial progenitors, one of the first groups of cells to ingress during gastrulation, to subtle alterations in their migration.

Importantly, Agtr1 signaling has previously been shown to have promigratory activity in vitro (Hosoya et al., 2000; Hashimoto et al., 2005). The demonstration by use of *hsp70:apln* transgenic embryos that this sensitivity to ubiquitous Agtr1b ligand is confined to a period prior to about 5 hpf also supports this model, as gastrulation (and the resulting ingression of myocardial progenitors) occurs precisely over this period of time. In transplantation assays, *agtr1b* appears to be required autonomously in myocardial progenitors. We therefore propose that signaling via Agtr1b promotes migration of these cells, either by directly responding to a chemotactic gradient, or by acting permissively to bolster their migratory capacity. The *mesp* family of bHLH factors also regulates migration of myocardial progenitors during mouse and ascidian gastrulation (Davidson et al., 2005; Saga et al., 1999; Kitajima et al., 2000; Satou et al., 2004). However, no effects on *mesp* expression were observed in *grinch* mutants (data not shown).

Midline expression of the Agtr1b ligand Aplin suggests that it may provide a signal for migration of the myocardial progenitors. However, injection of three independent *apln* MOs (designed against the 5'UTR and the splice donor site in intron 1) caused severe defects in body axis elongation and morphogenesis (Figure S3D), but did not recapitulate the *grn* myocardial phenotype, as significant myocardium was formed in the morphants (Figure S3E). The MOs appear to be functional, as coinjection of the 5'UTR-targeted MO was sufficient to reverse the inhibition of cardiomyogenesis caused by *apln* RNA levels far exceeding those normally found in vivo (Figure S3F). While exogenous *apln* can therefore recapitulate the *grn* phenotype, and Aplin can act as a ligand to stimulate Agtr1b activity, an *apln* mutant will be required to definitively test whether Aplin is the in vivo signal for Agtr1b during myocardial progenitor development. While not the focus of

this study, the midline expression of *apln*, coupled with the convergence extension-type defects observed in *apln* morphants and embryos subjected to excess *apln*, suggests that this gene may play an important role in early embryonic morphogenesis.

The incomplete expressivity of the *grn* myocardial phenotype, despite the fact that the *grn*<sup>s608</sup> mutant allele appears to encode a biochemically inactive GPCR, suggests that Agtr1b acts redundantly or in parallel with other signaling pathway(s). Coinjection of *agtr1a* MO increased the penetrance of the *grn* phenotype to a certain degree, but it still did not result in a fully penetrant cardiac phenotype. The requirement for multiple pathways may explain why while *agtr1b* is broadly expressed in the mesoderm, its activity (even upon stimulation with exogenous Aplin) appears to be specific to myocardial progenitors. This proposed role for Agtr1b is consistent with the requirement for GPCR signaling in the migration of several cell types, including immune system components (Campbell et al., 2003), neurons (Knaut et al., 2005), and primordial germ cell progenitors (Doitsidou et al., 2002; Knaut et al., 2003; Kunwar et al., 2003; Molyneaux et al., 2003). Therefore, the numerous GPCRs encoded in metazoan genomes may regulate the migration of different cell populations.

## EXPERIMENTAL PROCEDURES

### Zebrafish Strains, Microinjections, and In Situ Hybridization

*grn* mutants were derived from a diploid genetic screen, as previously described (Beis et al., 2005). RNA in situ hybridization (ISH) was carried out as previously described (Yelon et al., 1999), by using standard probes. DNA fragments for *agtr1b*, *apln*, and *mespa* in situ probes were amplified by RT-PCR (sequences available upon request). For RNA overexpression, cDNAs encoding *apln* and the mutant and wild-type versions of *agtr1b* were amplified by RT-PCR and subcloned into pCS2+. *Tg(flk1:EGFP)*<sup>s843</sup> and *Tg(cmlc2:EGFP)* fish have been previously described (Huang et al., 2003; Jin et al., 2005). A PCR fragment of zebrafish *apelin* was subcloned into a vector with the 1.5 kb zebrafish *hsp70* promoter (Halloran et al., 2000) flanked by *I*SceI meganuclease restriction sites. Embryos injected with this *hsp70:apln* construct and *I*SceI were raised to adulthood, and then screened for germline transmission by mating with wild-type adults and PCR genotyping of the resulting embryos. A carrier, denoted *Tg(hsp70:apln)*<sup>hsc1</sup>, that transmits the transgene at a frequency of 10% was used for all experiments. Heat shocks were carried out for 30 min at 37°C. Induction of expression at all time points studied was confirmed by *apln* ISH.

### Sequences

Sequences for zebrafish *agtr1a* (NM\_001075105), *agtr1b* (NM\_001030197), and *apln* (EB962402) are available in the National Center for Biotechnology Information.

### Positional Cloning of *grn*

Diploid mutant embryos were obtained from *grn*<sup>s608</sup> carriers outcrossed to a WIK background. The region occupied by *grn* was repeat rich, and as such both the radiation hybrid panels and genome assembly were uninformative. Fortunately, tight linkage (0 recombinants out of 1324 diploid embryos) was found for the SSLP marker Z9473. PCR-based screening of a BAC pool library revealed that Z9473 is located on the 56 kb BAC zC102K8. After sequencing of the BAC zC102K8, RFLP markers were designed by comparison of sequences from 500 bp PCR products from homozygous mutant and wild-type

embryos. The *grn* region was found to include sequence between the markers 880/1 and 782/3. As *agtr1b* is a single-exon gene, sequencing in *grn* mutants was carried out on PCR products from both genomic and cDNA. The *grn*<sup>608</sup> allele of *agtr1b* eliminated a Sau96I restriction site, allowing for RFLP-based genotyping, which showed that it is very tightly linked to the *grn* locus (0 recombinants in 1324 diploid embryos). Unfortunately, zC102K8 did not encompass the entire *grn* critical region, and further attempts to extend the genomic contig beyond the end of zC102K8 have, to date, proven unsuccessful.

### Morpholinos

Morpholinos targeting the 5'UTR of *agtr1b* (5'-CAGAGAAGTTG TTTGTCATGTGTCT-3'), the 5'UTR of *apln* (5'-GATCTTCACATTCAT TTCTGCTCTC-3'), the first intron splice donor of *apln* (5'-CACGCT CCCGACTTACCAGCACTGG-3' and 5'-AACAGCCGTCACGCTCCC GACTTAC-3'), the 5'UTR of *agtr1a* (5'-TGTATTCCGACGTTGGCTC CATTG-3'), the 5'UTR of p53 (5'-GCGCCATTGCTTTGCAAGAA TTG-3'), and a mismatch control *agtr1b* MO (5'-CACAGAAC~~TT~~GT TT~~CT~~CAT~~CT~~CTCT-3'; underlined nucleotides denote changes made for the mismatch MO) were purchased from Gene Tools and Open Biosystems. A total of 2.0–8.0 ng of each MO was injected into 1-cell-stage embryos.

### Analysis of *Agtr1b* Signaling

The Flip-In system (Invitrogen) was used to generate isogenic, stable CHO cell lines expressing wild-type or mutant versions of *agtr1b*. For adenylcyclase assays, cells were treated with medium containing 20 mM forskolin and 0.5 mM 3-isobutyl-1-methylxanthine in the presence or absence of 1  $\mu$ M of the indicated APLN fragment. After incubation for 15 min, cells were lysed, and cAMP levels were determined by radioimmunoassay (Immunotech, Beckman Coulter). ERK and p70S6 kinase activities were determined in serum-starved cells stimulated at 37°C for 5 min in the absence or presence of 1  $\mu$ M APLN fragment. Western blotting was carried out by using anti-phospho-ERK and anti-phospho-Thr389 p70S6 kinase primary antibodies, followed by incubation with HRP-conjugated secondary antibody and chemiluminescent detection of HRP activity (Amersham Biosciences).

### Transplantation

Transplantation experiments were carried out as previously described (Parker and Stainier, 1999). Donor *cm1c2:EGFP* embryos were injected with 2 ng *agtr1b* ATG MO or mismatch control MO and were labeled by coinjection of 5% tetramethyl rhodamine dextran (MW 10,000, Molecular Probes). A total of 10–20 cells were removed from the margin of 3–4 hpf donor embryos and were transplanted to the margin of wild-type host embryos. In order to control for variation in contribution to the heart at different time periods, transplantation of wild-type and morphant embryos were staggered during the same experiment such that results could be compared directly.

### Supplemental Data

Supplemental Data include Figures S1–S4, Table S1, and Movie S1 and are available at <http://www.developmentalcell.com/cgi/content/full/12/3/403/DC1/>.

### ACKNOWLEDGMENTS

We thank members of the D.Y.R.S. and H.B. labs for their help during the screen. Steve Waldron, Ana Ayala, and Natasha Zvenigorodsky provided invaluable help with fish husbandry. Thanks to Nilo Kaviani and Monica Dixon for technical assistance. BAC sequencing was kindly performed by Jan-Fang Cheng, Matt Hamilton, Yi Peng, and Roya Hosseini, whose efforts were supported by a National Heart, Lung, and Blood Institute (NHLBI) Program for Genomic Applications grant to Jan-Fang Cheng and Eddy Rubin at the Lawrence Berkeley National Laboratory. Thanks to Neil Chi, Brent Derry, Benoit Bruneau, and Janet Rossant for discussions and critical reading of the manuscript. Special thanks to Lila Solnica-Krezel, Tom Quertermous, and

Paul Krieg for sharing unpublished information. I.C.S. was supported by a postdoctoral fellowship from the Canadian Institutes of Health Research. This work was supported in part by grants from NHLBI, the American Heart Association, and the Packard Foundation to D.Y.R.S.

Received: September 14, 2006

Revised: December 18, 2006

Accepted: January 17, 2007

Published: March 5, 2007

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