The Aplnr GPCR regulates myocardial progenitor development via a novel cell-non-autonomous, $G\alpha_{i/o}$ protein-independent pathway

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Summary

Myocardial progenitor development involves the migration of cells to the anterior lateral plate mesoderm (ALPM) where they are exposed to the necessary signals for heart development to proceed. Whether the arrival of cells to this location is sufficient, or whether earlier signaling events are required, for progenitor development is poorly understood. Here we demonstrate that in the absence of Aplnr signaling, cells fail to migrate to the heart-forming region of the ALPM. Our work uncovers a previously uncharacterized cell-non-autonomous function for Aplnr signaling in cardiac development. Furthermore, we show that both the single known Aplnr ligand, Apelin, and the canonical $G\alpha_{i/o}$ proteins that signal downstream of Aplnr are dispensable for Aplnr function in the context of myocardial progenitor development. This novel Aplnr signal can be substituted for by activation of Gata5/Smarcd3 in myocardial progenitors, suggesting a novel mechanism for Aplnr signaling in the establishment of a niche required for the proper migration/ development of myocardial progenitor cells.

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Introduction

A key step to organogenesis is the proper recruitment and differentiation of progenitors that form the mature cell types needed for organ development and function. Cardiovascular progenitor cells (CPCs) are the building blocks of the heart, and have the potential to form cardiomyocytes, endocardium and smooth muscle cells (Kattman et al., 2006; Moretti et al., 2006). CPCs are among the first to migrate during gastrulation, ultimately reaching bilateral regions of the anterior lateral plate mesoderm (ALPM) (Wu et al., 2008). Fate mapping studies in multiple vertebrate models have shown that CPCs arise from a fixed location in the pregastrulation embryo (reviewed in (Evans et al., 2010)). In zebrafish, cells fated to become cardiomyocytes arise from bilateral regions within the first few tiers of cells at the embryonic margin of the 4–6 hours post-fertilization (hpf) embryo, displaced 60–140° relative to the dorsal side of the embryo (Keegan et al., 2004; Stainier et al., 1993). Localization of cells to this “heart field” region of the pregastrula embryo can instruct later myocardial fate, as donor cells transplanted to this area can form cardiomyocytes *in vivo* (Lee et al., 1994). Upon arrival at the ALPM, multiple signals lead to initiation of cardiogenesis (reviewed in (Evans et al., 2010)), with expression of *Nkx2.5*, encoding an NK-class homeodomain transcription factor, being the earliest marker of myocardial progenitors in zebrafish and other vertebrates (Chen and Fishman, 1996; Komuro and Izumo, 1993; Lints et al., 1993; Tonissen et al., 1994).

It is apparent that myocardial progenitor cells arise from a specific location in the embryo and migrate during gastrulation to the ALPM, where they receive signals necessary for differentiation into cardiomyocytes. However, it remains to be determined whether cells fated to form the myocardium require additional signals during gastrulation to effectuate proper heart development, or if it is sufficient that cells reach the ALPM. Explant experiments, primarily done in the chick embryo, have yielded conflicting results on this question (reviewed in (Yutzey and Kirby, 2002)). Whereas some studies argue that cells exist in the early gastrula that are capable of forming cardiomyocytes regardless of later location in the embryo (Auda-Boucher et al., 2000; Lopez-Sanchez et al., 2009), others suggest that ultimate localization to the ALPM is sufficient for cardiac differentiation (Inagaki et al., 1993; Tam et al., 1997). A lack of mutants that specifically affect early myocardial progenitor development, and markers to isolate and characterize these cells, has hindered progress on this key question of early heart development. Members of the *Mesp* basic helix-loop-helix transcription factor family are essential for cardiogenesis in mouse and *Ciona intestinalis*, and can promote myocardial differentiation in embryonic stem cells (reviewed in (Bondue and Blanpain, 2010)). *Mesp* function in mice is required for proper migration of cardiac progenitors during gastrulation. Perturbations in BMP, FGF, Wnt (both canonical and non-canonical), and Nodal signaling prior to and during gastrulation lead to later deficits in development of *Nkx2.5*-expressing myocardial progenitors

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(Reifers et al., 2000; Reiter et al., 2001; Ueno et al., 2007). However, as embryos exhibit gross developmental defects when these pathways are manipulated, it is difficult to uncouple function in myocardial progenitor development from general patterning defects. Nevertheless, these results suggest that CPC potential and behaviour is influenced during gastrulation.

Our group has previously described a zebrafish mutant, *grinch (grn)*, in which there is a striking and specific deficit in myocardial progenitors (Scott et al., 2007). Characterization of the mutant showed that there was a decrease in, or in the most severe cases a loss of, *nkx2.5*-expressing cells. The *grn* mutation was mapped to the gene encoding Angiotensin II receptor-like 1b (*aplnrb*, *apj*, *msr1*, *agtrl1b*; referred to as *aplnrb* in this study), a G protein-coupled receptor (GPCR). Both loss of *aplnrb* and overexpression of *apelin* (which encodes the only known Aplnr ligand (Tatemoto et al., 1998)) during early gastrulation resulted in a heartless phenotype in zebrafish (Scott et al., 2007; Zeng et al., 2007). Aplnrb shares functional properties with chemokine receptors (Zou et al., 2000), and has been shown to promote angiogenesis in several contexts (Cox et al., 2006; Kasai et al., 2004; Sorli et al., 2007). The loss of heart following Apelin overexpression therefore suggested a chemotactic role for Aplnr/Apelin signaling to guide migration of myocardial progenitor cells to the ALPM during gastrulation. Both *apelin* overexpression and morpholino-mediated knockdown has been shown to affect migration of cells during gastrulation (Scott et al., 2007; Zeng et al., 2007). However, knockdown of *apelin* did not fully recapitulate the *grn*/*aplnrb* heartless phenotype. This suggested that Aplnrb may not simply act via Apelin signaling in cardiac progenitor development, and left as an open question the mechanism of the *aplnrb* heartless phenotype.

In this study we carried out a detailed analysis of the *aplnrb* loss-of-function phenotype to elucidate the mechanism through which Aplnr signaling regulates vertebrate heart development. We find defects in a specific region of the *aplnrb* mutant ALPM, coincident with the site of cardiac development. By tracking cells as they migrate from the heart field region of the early embryo, we find that these cells fail to reach the ALPM in the absence of *aplnrb* function due to a defect in the initiation of migration, resulting in the complete absence of cells in the heart-forming region. Unexpectedly, via transplantation analysis we find that Aplnrb function is required non-autonomously, in cells not destined to form cardiomyocytes, for proper myocardial progenitor development. This occurs independently of classic heterotrimeric G-protein signaling downstream of the GPCR Aplnrb. Finally, initial work suggests the activation of the cardiac chromatin remodeling complex, cBAF, in cardiac progenitors as a consequence of Aplnr signaling. This study therefore identifies a novel, non-autonomous function for Aplnr signaling to support proper myocardial progenitor development. Interestingly, this signal may provide a niche for proper CPC development and migration.

Results

Cells from the pregastrula heart field fail to reach the ALPM in *aplnra/b* morphant embryos

Our previous analysis suggested defects in ALPM formation in *aplnrb* morphants (morpholino-injected embryos) and mutants (Scott et al., 2007). To further characterize ALPM development, expression of additional ALPM markers was examined in *aplnrb* mutants. RNA *in situ* hybridization analysis demonstrated that, along with loss of *nkx2.5* expression, there is a decrease in expression of *spi1* and the more posterior domains of *fli1* and *tal1* in the ALPM, marking myeloid and presumed endocardial progenitors, respectively (supplementary material Fig. S1). This decrease in gene expression in *aplnrb* mutants may reflect a failure of cells to reach the APLM during development. Alternatively, cells may reach the ALPM but fail to differentiate into the proper cell types. To distinguish between these two possibilities, we performed lineage tracing to determine whether cells from the lateral embryonic margin migrate to the ALPM. For these experiments, the photoconvertible protein KikGR was employed. KikGR normally fluoresces with spectral characteristics similar to EGFP, however after exposure to UV light undergoes a permanent change in its fluorophore such that it fluoresces as a red fluorescent protein (Hatta et al., 2006). To obtain a more complete loss of Aplnr activity, we employed morpholinos (MOs) to inhibit *aplnrb* along with its paralog *aplnra* (Tucker et al., 2007). We have previously found that co-injection of these MOs results in a robust heartless phenotype and recapitulates loss of gene expression observed in the ALPM of *aplnrb* mutants (Scott et al., 2007; supplementary material Fig. S2). One-cell stage wildtype and *aplnra*/*aplnrb* MO-injected embryos were co-injected with mRNA encoding KikGR. At 6hpf, a cluster of about 50 cells at the lateral embryonic margin (displaced 90^0 from the shield) was photoconverted via exposure to UV light (Fig. 1B). Embryos were subsequently scored at 12–14hpf (6–10 somite stage) for the presence of photoconverted cells that had migrated to the heart-forming region of the ALPM (Fig. 1A). In 48% of wildtype embryos, cells from the lateral margin were found as a continuous stripe throughout the lateral plate mesoderm, beginning at the ALPM and extending posterior to the level of the anterior somites (Fig. 1D,D′). Photoconversion of lateral margin cells in *aplnra/b* morphant embryos similarly often resulted in a lateral stripe of cells being evident on one side of the embryo. However, we found a striking and significant difference (p=0.001) between wildtype and *aplnra/b* morphant embryos in the presence of photoconverted cells in the heart-forming region of the ALPM. In *aplnra/b* morphants, cells were rarely evident in this area (brackets in Fig. 1E′ compared to wildtype in Fig. 1D′). In some cases, cells were found both anterior and posterior to this area but were missing specifically form the heart-forming region (arrowhead in Fig. 1F′). In the few instances that cells were found in the ALPM, their organization into a stripe was highly disrupted (supplementary material Fig. S3). Contribution of photoconverted cells to the heart-forming region of the ALPM was thus markedly reduced in morphant embryos (27/57 in wildtype versus 3/104 in *aplnra/b* morphants, Fig. 1C). These data demonstrate that the deficit in myocardial progenitor development in *aplnra/b* morphants reflects the failure of cells from the lateral embryonic margin to reach the ALPM.

Portions of the ALPM are absent in *aplnra/b* morphants

To determine whether cells are present in the heart-forming region of the ALPM in *aplnra/b* morphants, we sectioned cardiomyocyte-specific *myl7:EGFP* (Huang et al., 2003) transgenic embryos at 20hpf and imaged cross-sections stained for filamentous Actin. Given the failure of lateral margin cells to reach the heart-forming region of the ALPM following loss of Aplnr signaling, it is possible that cells from other regions of the early embryo migrate to this area in their place. At 20hpf, the bilateral fields of cardiac mesoderm are folded epithelial sheets

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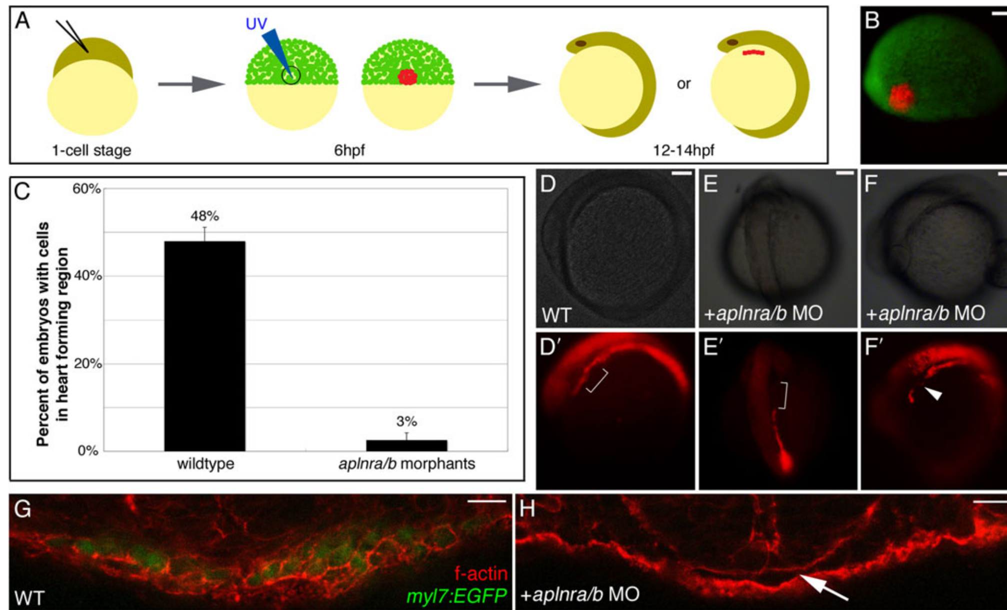

Fig. 1. Cells from the lateral embryonic margin fail to reach the heart-forming region in *aplnra/b* morphants. (A) Schematic of photoconversion method. mRNA encoding KikGR is injected at the 1-cell stage. Cells at the lateral embryonic margin (90˚ from dorsal, anatomically marked by the shield) are photoconverted by UV light at 6hpf. Embryos are scored at 12–14hpf for the presence of cells in the heart-forming region of the ALPM. **(B)** Merged fluorescent views of 6hpf embryo following photoconversion of lateral margin cells. **(C)** Graph of percentage of embryos with photoconverted cells that reach the heart-forming region. In wildtype embryo labeled cells are found in the lateral plate mesoderm **(D′)**, including the heart-forming region (bracket in D′). In *aplnra/b* morphants labeled cells are found in the lateral plate mesoderm but fail to extend anteriorly to the heart-forming region (bracket in **E′**) or are excluded from the heart-forming region (arrowhead in **F′**) while cells are found both and anterior and posterior to this region. **(D–F)** are bright field images of embryos in (D′-F′). (D,F) lateral views with anterior to the left, (E) dorsal view with anterior to the top. For (C) N=3, n=57 for WT; N=4, n=104 for *aplnra/b* morphants; p=0.001. **(G,H)** cross-sections through the ALPM of 20hpf wildtype (WT) and *aplnra/b* morphant *myl7:EGFP* embryos, respectively, stained with rhodamine-phalloidin to visualize cells. EGFP+ cardiac progenitors are present in wildtype embryos (G) while there is and absence of cells in the heart-forming region in *aplnra/b* morphants (arrow in H). See also supplementary material Fig. S3. Scale bars represent 100μm (B, D/D′, E/E′ and F/F′), 17μm (G) and 12μm (H).

that have begun their migration to the midline (Trinh and Stainier, 2004). Sections were selected for analysis based on the absence of head structures (such as brain ventricles) and the notochord to demarcate anterior and posterior ALPM boundaries, respectively. In wildtype embryos the expected folded epithelial sheet and cardiac-specific *myl7:EGFP* signal was observed (Fig. 1G). In contrast, there was a marked absence of cells in the ALPM of *aplnra/b* morphants (Fig. 1H). These data suggest that in *aplnra/b* morphants, and *aplnrb* mutants, there are no cells in the region of the ALPM that typically forms cardiac tissue.

Lateral margin cells exhibit early migration defects

To explore further the migration defect seen in the absence of Aplnr signaling, we performed time-course analysis of wildtype and *aplnra/b* morphant embryos. Cells were photoconverted as described above and embryos were imaged over time from shield stage (6hpf) to bud stage (10hpf), when gastrulation movements have been largely completed (Warga and Kimmel, 1990). Upon gross observation we saw the typical migration pattern in wildtype embryos with a streak of cells moving toward the animal pole (arrow in Fig. 2A,B). In *aplnra/b* morphant embryos, we found the leading cells of the migrating group to be absent (arrow in Fig. 2D,E). Importantly, cells in the leading streak are those that will form the ALPM (Sepich et al., 2005; Yin et al., 2009) and are found in the appropriate location in wildtype embryos (arrowhead in Fig. 2C) but not in *aplnra/b* morphants (arrowhead in Fig. 2F) at bud stage. We next examined this in more detail to determine when defects in migration are first apparent in *aplnra/b* morphants. By imaging embryos at higher magnification and at more frequent time intervals, we found that labeled cells exhibited a delay in the initiation of migration toward the animal pole in *aplnra/b* morphants. In contrast to wildtype embryos in which migration is well underway by 70% epiboly (Fig. 2G), cells in *aplnra/b* morphant embryos do not start migrating until roughly 85% epiboly (Fig. 2J; supplementary material Fig. S4), a lag of 1.0 to 1.5 hours. Our data therefore suggest that cells from the lateral embryonic margin in *aplnra/b* morphants fail to reach the ALPM due to a defect or delay in the initiation of migration towards the animal pole of the embryo.

Aplnr signaling functions cell-non-autonomously in the development of cardiomyocytes

We next performed transplantation analysis to determine whether Aplnr signaling functions cell-autonomously or cell-non-autonomously in the development of cardiomyocytes. It has been suggested that Aplnr signaling modulates a chemotactic cue that attracts cardiac progenitors to the heart-forming region of the ALPM (Scott et al., 2007; Zeng et al., 2007). If this were the case, a cell-autonomous role for *aplnrb* would be expected. While we have previously shown a decreased ability of *aplnrb* morphant cells to contribute to the heart (Scott et al., 2007), a possible non-autonomous role for this gene was not evaluated in these studies.

Cells from wildtype or *aplnra/b* MO-injected donor embryos harboring a *myl7:EGFP* transgene were transplanted into the

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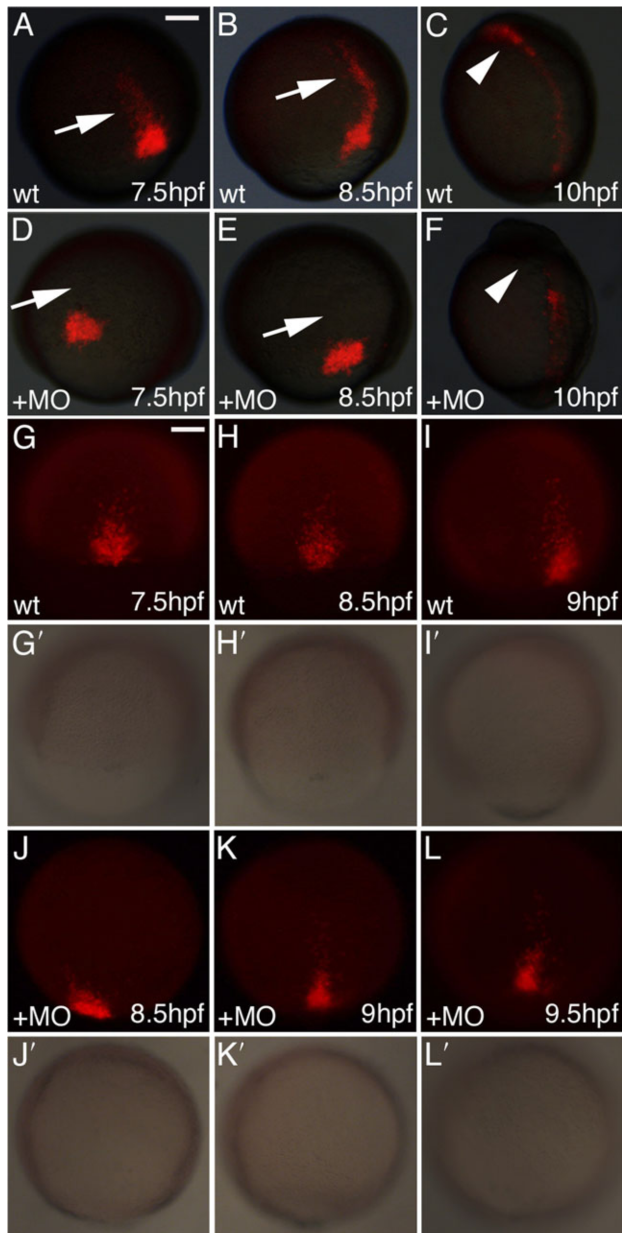

Fig. 2. Cells from the lateral embryonic margin display a defect in the initiation of migration. (A–F) Embryos injected with *KikGR* RNA were photoconverted at shield stage to mark cells in the lateral margin. Representative images of wildtype and *aplnra/b* morphant embryos at 70% epiboly (7.5hpf), 80% epiboly (8.5hpf), and bud (10hpf) stages. Arrows indicate leading streak of migrating cells in wildtype embryos and their absence in morphant embryos. Arrowheads indicate cells in the heart-forming region of wildtype embryos and absence of cells in morphants. **(G–L)** Time-lapse image of one wildtype and one *aplnra/b* morphant embryo during gastrulation demonstrating a delay in the initiation of migration of morphant cells. Corresponding images of the embryos are shown in **(G′-L′)** for each fluorescent image. Scale bar represents 100µm (A).

embryonic margin of wildtype or *aplnra/b* morphant host embryos. Transplant embryos were then scored at 48hpf for EGFP-positive cells, which would indicate donor cells that have formed cardiomyocytes. In control (wildtype donor to wildtype host) transplants, differentiated cardiomyocytes were evident in 19% of host embryos, in agreement with previously published work (Scott et al., 2007; Thomas et al., 2008; Fig. 3A,E). As expected, *aplnra/b* morphant cells had a greatly reduced capacity to differentiate into cardiomyocytes when placed in morphant host embryos (1% of tranplants were EGFP+, Fig. 3B,E), recapitulating the *aplnra/b* phenotype. Surprisingly, wildtype donor cells were rarely able to differentiate into cardiomyocytes in *aplnra/b* morphant hosts (2% of tranplants were EGFP+, n=190, Fig. 3C,E), whereas *aplnra/b* morphants cells had the capacity to differentiate into cardiomyocytes in wildtype hosts, albeit to a lesser extent than wildtype donor cells (13% of transplants were EGFP+, n=190; Fig. 3D,E). Our results demonstrate an unappreciated cell-non-autonomous role for Aplnr signaling in cardiomyocyte development.

We next wished to confirm that the non-autonomous function seen in *aplnra/b* morphants is specific to Aplnr activity and not secondary to a host embryo heartless phenotype. We utilized *gata5/6* morphants, in which myocardial differentiation is fully compromised (Holtzinger and Evans, 2007). When transplants were performed as described above we found that Gata5/6 activity is required cell-autonomously in cardiomyocyte development. Wildtype cells were able to contribute to heart in 18% of wildtype and 11% of *gata5/6* morphant hosts, whereas *gata5/6* morphant cells were unable to contribute to the heart in wildtype embryos (0/83 hosts) (data not shown). These data confirm that the non-autonomous function seen in *aplnra/b* morphants is specific to the loss of Aplnr activity, and not a general consequence of an absence of host cardiac progenitors.

Role of Apelin in Aplnr signaling during cardiac progenitor development

With our data revealing a non-autonomous role for Aplnr in cardiomyocyte development, we decided to revisit the role of the Apelin ligand in this process. While injection of *aplnra/b* MO leads to embryos lacking cardiac tissue, injection of *apelin* MO fails to recapitulate this heartless phenotype, with only a reduced heart size evident in the most severe cases ((Zeng et al., 2007), Fig. 4E). A trivial explanation for this discrepancy could be that the *apelin* MOs used do not fully inhibit Apelin function. To determine if this may be the case, we injected *apelin* MO into *hsp:apelin* transgenic embryos and compared the phenotype of the embryos with or without the induction of *apelin* over-expression. Heat-shock of *hsp:apelin* embryos prior to 6hpf greatly increased *apelin* expression compared to wildtype siblings (Fig. 4A). As we have previously found, *hsp:apelin* embryos heat-shocked at 4hpf resulted in a heartless phenotype (Scott et al., 2007; Fig. 4D). Injection of *apelin* MO resulted in a slight reduction in the size of the heart (Fig. 4E). Interestingly, when *hsp:apelin* transgenic embryos injected with *apelin* MO were heat-shocked, they re-capitulated the *apelin* morphant phenotype (small heart) rather than the *apelin* over-expression (heartless) phenotype (Fig. 4F). As the *hsp:apelin* transgene expresses, by RNA *in situ* hybridization, greatly elevated levels of *apelin*, this strongly suggested that the amount of *apelin* MO injected was sufficient to suppress the endogenous *apelin* transcript levels. Therefore, in wildtype embryos injection of *apelin* MO likely results in a complete knockdown of endogenous Apelin, and lends further support to the model that Apelin may not be the functional ligand for Aplnr signaling during myocardial progenitor development.

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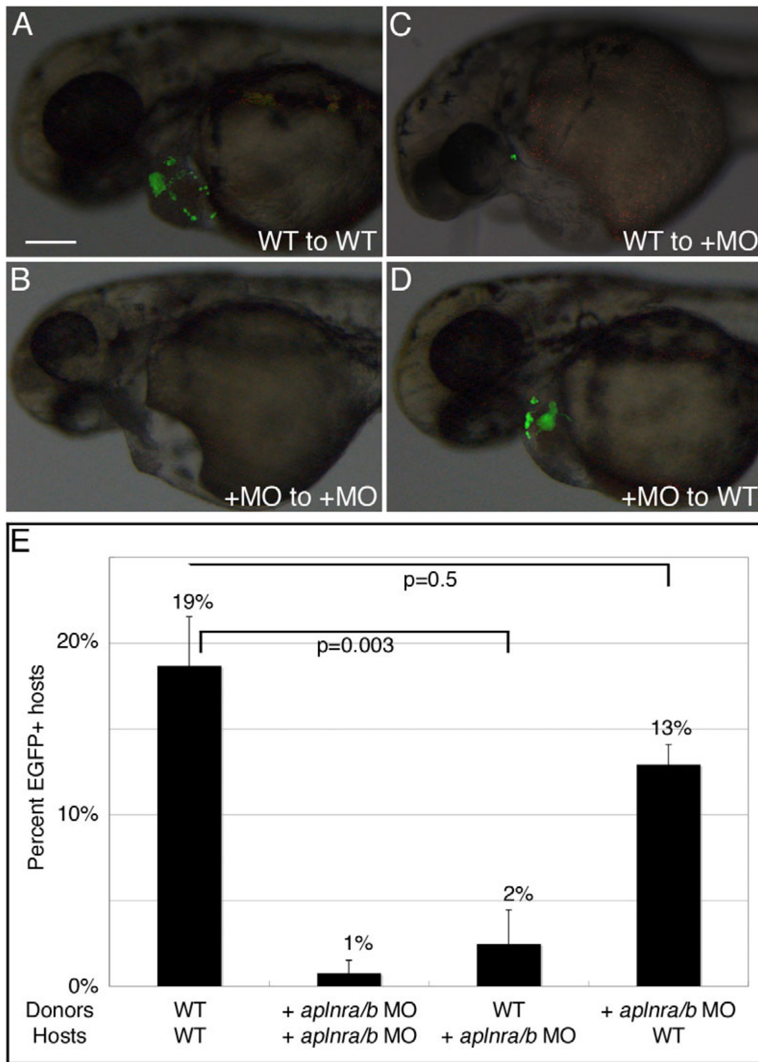

Fig. 3. Aplnr signaling functions cell-non-autonomously in the development of cardiomyocytes. Transplantation of wildtype (WT) or *aplnra/b* MO-injected (+MO) *myl7:EGFP* donor embryos to the margin WT or *aplnra/b* MO-injected host embryos was carried out at 4hpf. **(A–D)** Representative host embryos at 2dpf following transplantation; lateral view, anterior to left. (C) wildtype cells are unable to contribute to the heart in *aplnra/b* morphant host embryos, whereas *aplnra/b* morphant cells are able to contribute to the heart in wildtype hosts (D). **(E)** Graph of percentage of host embryos with contribution from *myl7:EGFP* donor cells. N=5, n=236 for wildtype donors and hosts; N=4, n=130 for *aplnra/b* morphant donors and hosts; N=4, n=190 for wildtype donors and *aplnra/b* morphant hosts; N=3, n=190 for *aplnra/b* morphant donors and wildtype hosts. Views in (A–D) are lateral views, with anterior to the left. Scale bar represents 200µm (A).

Aplnra/b signaling is $G\alpha_{i/o}$ protein-independent in cardiomyocyte development

The demonstration of a potential Apelin-independent role of Aplnrb in cardiac progenitor development next led us to examine the nature of Aplnrb signaling. *In vitro*, the human APLNR has been shown to signal in a classical heterotrimeric G-protein mediated fashion via the $G\alpha_{i/o}$ class of $G\alpha$ proteins (Masri et al., 2002). We sought to determine whether Aplnr signaling functions through $G\alpha_{i/o}$ proteins in the development of cardiomyocytes *in vivo*. Pertussis toxin (PTX) is a potent inhibitor of $G\alpha_{i/o}$ signaling

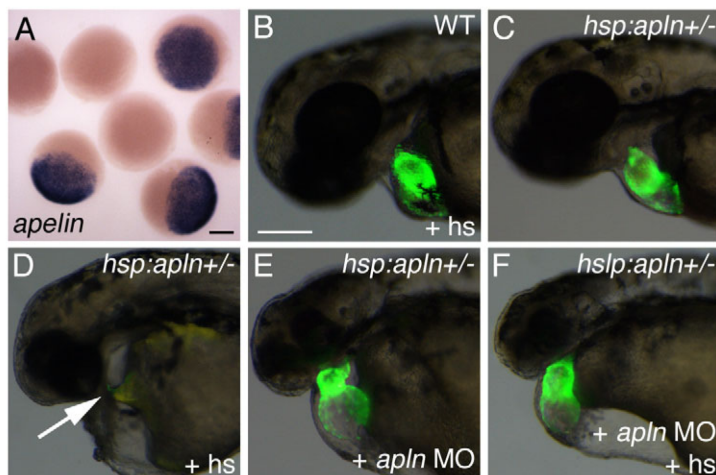

Fig. 4. Apelin is not necessary for the development of cardiomyocytes. **(A)** Embryos produced from crossing *hsp70:apln+/-* and wildtype fish showing robust up-regulation of *apelin* expression by RNA *in situ* hybridization following heat shock in embryos carrying the *hsp70:apln* transgene. **(B)** Wildtype fish are unaffected by heat-shock (+hs). **(C)** Unshocked *hsp70:apln+/-* embryos are phenotypically wildtype. **(D)** Overexpression of *apelin* leads to a loss in cardiac tissue (arrow shows empty cardiac region). **(E)** Injection of *apelin* MO leads to a heart that is present, but dismorphic. **(F)** Injection of *apelin* MO followed by heat shock of *hsp70:apln* embryos results in phenocopy of the *apelin* morphant heart phenotype. Scale bars represent 200µm (A and B, B scale bar is for images B–F).

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and acts by uncoupling $G\alpha_{i/o}$ proteins, and presumably their βγ subunits, from associated GPCRs (Codina et al., 1983). We first injected RNA encoding *PTX* at the 1-cell stage to globally inhibit $G\alpha_{i/o}$-mediated signaling. Global over-expression of *PTX* recapitulated the *aplnra/b* morphant embryo heartless phenotype (Fig. 5B,C). However, $G\alpha_{i/o}$ proteins both act downstream of multiple GPCRs and have been shown to regulate aspects of Hedgehog and Wnt signaling (Ogden et al., 2008; Slusarski et al., 1997). We could therefore not conclude from these results that the loss of cardiac tissue following *PTX* overexpression was specifically due to inhibition of $G\alpha_{i/o}$-mediated Aplnr signaling.

To further examine whether $G\alpha_{i/o}$ proteins are the major effectors of the Aplnr signaling pathway, we performed transplant analyses as described above using wildtype and *PTX* over-expressing embryos as both donors and hosts. In control experiments where *PTX* RNA-injected donor cells and host embryos were used, we observed no EGFP+ donor cells (n=80). Transplantation of *PTX* over-expressing cells to wildtype host embryos revealed a minor cell-autonomous role for $G\alpha_{i/o}$-mediated signaling in cardiac differentiation, with a reduction in transplant embryos with EGFP-positive cells being observed (EGFP+ cells in 13% of hosts, n=216, Fig. 5D,F). Surprisingly, and in contrast to *aplnra/b* morphant transplant experiments, we found that wildtype cells were able to differentiate into cardiomyocytes after being transplanted into *PTX* over-expressing host embryos (EGFP+ cells in 12% of hosts, n=158, Fig. 5E,F). These data suggest that the cell-non-autonomous effect of Aplnr signaling in cardiomyocyte development is independent of, or at least not fully dependent upon, signaling through $G\alpha_{i/o}$ proteins. In addition, we found that wildtype cells were able to contribute to heart in host embryos in which Gβγ subunit activity was inhibited by injection of RNA encoding the C-terminal fragment of GRK2 (Koch et al., 1994). In these experiments, EGFP+ cells were evident in 12% of hosts (n=75; data not shown), further supporting a $G\alpha_{i/o}$ protein-independent mechanism for Aplnr signaling.

Given the differences in the autonomy of Aplnr and $G\alpha_{i/o}$ function, we next sectioned *myl7:EGFP* embryos over-expressing *PTX* at 20hpf to examine the ALPM. Unlike what was observed in *aplnra/b* morphants, cells were present in the ALPM at the level of the heart-forming region in embryos injected with *PTX* RNA. However, these cells were unable to differentiate into cardiomyocytes, as noted by the absence of EGFP+ cells in *myl7:EGFP* transgenic embryos (arrow in Fig. 5H). Therefore, while global inhibition of $G\alpha_{i/o}$ protein signaling blocks differentiation of cells into cardiomyocytes, the autonomy and ultimate cause of this phenotype differs from that seen following *aplnra/b* knockdown. Taken together, these data suggest that Aplnr signaling acts via a mechanism independent of $G\alpha_{i/o}$ proteins in cardiac progenitor development (Fig. 6D).

Aplnr signaling functions upstream of the cardiac BAF complex

Recent work published by our lab demonstrated that overexpression of *gata5* and *smarcd3b*, two factors in a cardiac-specific chromatin remodeling complex (cBAF) effectively directed the migration of non-cardiogenic cells to the ALPM and their subsequent contribution to various

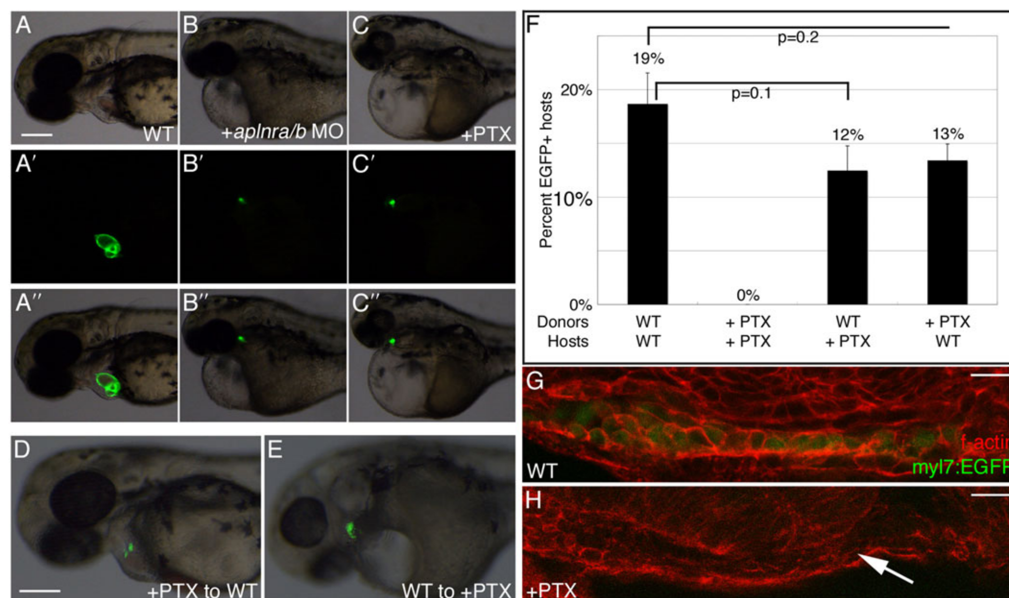

Fig. 5. Aplnr activity is independent of $G\alpha_{i/o}$ proteins in cardiomyocyte development. (A–C) Bright field images of wildtype (WT), *aplnra/b* morphant, and *PTX* overexpressing *myl7:EGFP* embryos at 2dpf. **(A′–C′)** Fluorescent images of embryos in (A–C), **(A″–C″)** are overlays of (A–C) and (A′–C′). Overexpression of *PTX* (C) recapitulates the *aplnra/b* morphant (B) cardiac phenotype. **(D–F)** Transplantation of *myl7:*EGFP donor cells from WT or *PTX* overexpressing cells to WT or *PTX* overexpressing host embryos at 4hpf was carried out. Hosts were subsequently scored at 2dpf for EGFP +′ve donor cells in the heart. (D–E) *PTX* overexpressing cells are able to contribute to the heart in wildtype embryos (D), and wildtype cells are able to contribute to the heart in *PTX* overexpressing host embryos (E), suggesting that $G\alpha_{i/o}$ proteins are not downstream of Aplnr signaling in cardiomyocyte development. (F) Graph showing percentage of host embryos with contribution from *myl7:EGFP* donor cells in transplants performed. N=5, n=236 for wildtype donors and hosts; N=2, n=80 for *PTX* O/E donors and hosts; N=3, n=158 for wildtype donors and *PTX* O/E hosts; N=3, n=216 for *PTX* O/E donors and wildtype hosts. O/E=overexpressing. **(G,H)** Cross sections through the ALPM of 20hpf *myl7:EGFP* wildtype and *PTX* overexpressing embryos. The ALPM is evident at the level of the heart-forming region following *PTX* addition (arrow in H) but cells fail to differentiate into cardiomyocytes as noted by the absence of EGFP +′ve cells. (A–E) are lateral views, with anterior to the left. Scale bars represent 200µm (A and D), 17µm (G) and 22µm (H).

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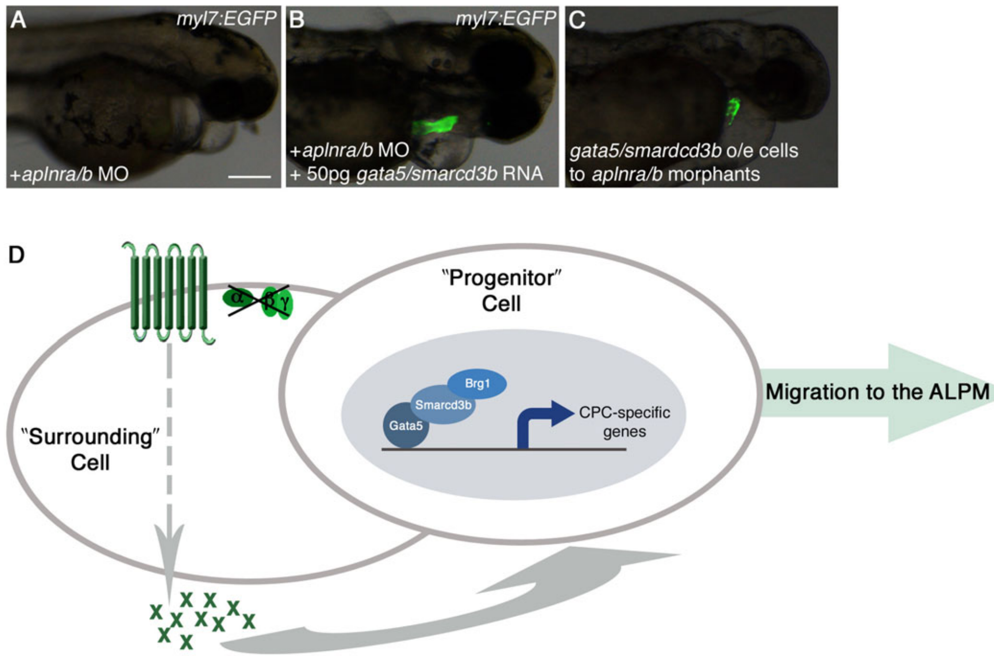

Fig. 6. Gata5/smarcd3b function downstream of Aplnr signaling to direct cells to the heart-forming region. (A,B) Overexpression of *gata5* and *smarcd3b* is able to rescue the *aplnra/b* morphant cardiac phenotype in *myl7:EGFP* embryos. **(C)** Cells from *myl7:EGF* embryos overexpressing *gata5* and *smarcd3b* can contribute to the heart when transplanted into *aplnra/b* morphant embryos. **(D)** Working model for Aplnr signaling in cardiac progenitor development. Aplnr activity functions independently of $G\alpha_{i/o}$ proteins in the production of extracellular factor(s)/cue(s). These factor(s)/cue(s) act cell-non-autonomously upon neighboring cells in the lateral embryonic margin, wherein *gata5/smarcd3b,* as part of the cBAF complex, functions cell-autonomously, facilitating their migration to the ALPM for development into cardiac progenitors. Aplnr activity is not required in progenitor cells. o/e: overexpressing. Scale bar represents 200μm (A).

cardiovascular lineages (Lou et al., 2011). As both Aplnr signaling and the cBAF complex influence the migration of cells to the heart-forming region of the ALPM, we investigated the relationship between these two activities. When *gata5* and *smarcd3b* were overexpressed in *aplnra/b* morphant embryos in a *myl7:EGFP* transgenic background, we were able to rescue the *aplnra/b* heartless phenotype as demonstrated through the presence of EGFP+ cardiomyocytes (Fig. 6B). Additionally, when cells from *myl7:EGFP* embryos overexpressing *gata5* and *smarcd3b* were transplanted into *aplnra/b* morphant host embryos, the cells were able to differentiate into cardiomyoctyes in a cell autonomous manner (Fig. 6C), with GFP+ cells in 18% (19/107) of host embryos. Taken together, these data suggest that the non-autonomous effects of Aplnr signaling may lead to the activation of the cBAF complex, which acts cell-autonomously ((Lou et al., 2011), this study) to guide the migration of cells to the heart-forming region of the zebrafish embryo (Fig. 6D).

Discussion

In previous studies of Aplnr signaling in zebrafish, a role for the migration of cells during gastrulation was inferred based on analysis of knockdown and global over-expression of Apelin (Scott et al., 2007; Zeng et al., 2007). Here, we have conducted a detailed analysis of ALPM development in the absence of Aplnr signaling. Our data demonstrate a role for Aplnrb signaling in the migration of cells from the lateral embryonic margin to the heart-forming region of the embryo, likely through the activity of cBAF (Fig. 6D). In the absence of Aplnrb signaling, a discrete domain of the ALPM, which overlaps with the site of *nkx2.5* expression, is devoid of cells. This effect on a confined region of the ALPM likely explains the cardiac specificity of the *aplnrb* mutant phenotype. Interestingly, we report here an unexpected cell-non-autonomous function for Aplnr signaling in heart development. Further, we show here that Aplnrb function in the context of cardiac progenitor development occurs via a $G\alpha_{i/o}$ protein-independent mechanism that is not dependent on the canonical Apelin ligand. The previous chemotactic model of Aplnrb function, in which it guides migrating cardiac progenitors to the ALPM via response to an Apelin gradient, therefore clearly must be revised.

It remains unclear why, despite the broad expression of *aplnrb* in gastrulating mesendoderm (Scott et al., 2007; Zeng et al., 2007), migration of cardiac progenitors (and presumably their neighbours in the ALPM) is specifically affected in *aplnrb* mutants. Cells contributing to cranial vasculature, circulating blood, pectoral fin mesenchyme and pharyngeal pouches have been shown to arise from overlapping regions of the ALPM (Keegan et al., 2004). However, the pectoral fin and cranial vasculature form normally in *aplnrb* mutants (this study and (Scott et al., 2007)). Thus Aplnr function appears to be necessary for specific cell populations that are found in a mixed progenitor pool at the lateral embryonic margin. Our photoconversion analysis demonstrated that cells from the lateral embryonic margin fail to migrate to the ALPM in the absence of Aplnr signaling. This may be due to a delay in the initiation of migration of "leading edge" mesoderm towards the animal pole ("anterior" of the embryo). While we did observe the eventual migration of cells toward the animal pole in *aplnra/b* morphants, the nature and fate of these cells remains unclear. They may represent myocardial progenitors that have initiated migration at a later time than in a wildtype embryo. Alternatively, they may be more posterior LPM progenitors that are initiating migration at the correct time. In this latter scenario, myocardial progenitors may have never been specified and/or failed to gastrulate, instead accumulating at the embryonic margin or moving passively with neighboring cells. From this perspective it is interesting to note that myocardial progenitors are among the first cell types to involute during vertebrate gastrulation (Garcia-Martinez and Schoenwolf, 1993; Parameswaran and Tam, 1995). The timing of this event may be essential for later heart development by allowing migration to the ALPM or rendering myocardial

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progenitors competent to initiate cardiogenesis. In this regard, the ability of Gata5/Smarcd3 to rescue myocardial differentiation in *aplnra/b* morphants is instructive (see below).

Many signals affecting cardiac progenitor migration to the midline, after the onset of *nkx2.5* expression, have been described, with perturbations resulting in cardia bifida (2 hearts, (Chen et al., 1996; Saga et al., 1999)). In the case of Aplnr signaling, mis-regulation of migration appears specific to a subset of gastrulating cells: those destined to reach the heart-forming region. This result is intriguing, as the molecular mechanisms regulating the migration of cardiac progenitors to the ALPM are poorly understood. Wnt3a/Wnt5a signaling has been shown to affect the path of cardiac progenitor migration in chick embryos, but not to affect cardiac progenitor specification (Sweetman et al., 2008; Yue et al., 2008). The non-canonical Wnt/planar cell polarity (PCP) signaling pathway regulates convergence and extension of the gastrulating vertebrate embryo (reviewed in (Roszko et al., 2009)). However, while perturbation of PCP leads to cardiac morphological defects (Phillips et al., 2007), even severe zebrafish PCP mutants, such as *MZtri* mutants (where maternal and zygotic *vangl2* are mutated) form a heart (data not shown). Accumulation of cells at the embryonic margin may also suggest failed regulation of cellular properties such as cell-shape changes or regulation of adhesion molecules, critical regulatory mechanisms for gastrulation (Speirs et al., 2010; Yin et al., 2009). Early patterning in the zebrafish embryo by a dorsoventral (DV) gradient of Bmp signaling has been shown regulate a gradient of Cadherin-based cell adhesion, in turn resulting in differential migratory behaviours for subsets of mesodermal populations along the DV axis (von der Hardt et al., 2007).

In the mouse, *Mesp1* and *Mesp2* are required for migration of cranio-cardiac mesoderm. In *Mesp1/2* mutants, presumptive cardiac progenitors fail to migrate from the primitive streak and as a consequence accumulate there, resulting in heartless embryos (Kitajima et al., 2000). However, we have not observed changes in *mespa/b* expression in *aplnrb* mutant embryos (data not shown).

The non-autonomous function of *aplnrb* in cardiac progenitor migration was unexpected, and provides further insight into the mechanism of Aplnrb function. In general, autonomous versus non-autonomous roles of Aplnr signaling have not been closely examined. In the context of angiogenesis, the receptor is expressed in endothelial cells, suggesting a cell-autonomous mechanism of action (Cox et al., 2006; Del Toro et al., 2010), however this has not been tested formally. The temporal requirement for Aplnr signaling in early heart development is not known, however use of a *hsp:apelin* transgene has shown that *apelin* overexpression prior to (but not after) 6hpf results in a heartless phenotype (Scott et al., 2007). We therefore favour at present a non-autonomous "niche" function for Aplnr signaling. The ultimate output of this signal is likely modulation of cell-cell contacts or ECM composition that results in an alteration of cardiac progenitor migration. It is interesting to note that a role for Aplnr/Apelin signaling in embryonic stem (ES) cell differentiation to cardiomyocytes was recently described (D′Aniello et al., 2009). In this study, Aplnr and Apelin were shown, via a PTX-sensitive mechanism, to act downstream of Nodal signaling to prevent neural differentiation at the expense of cardiomyocytes. It is unclear if this ES cell mechanism is fully conserved *in vivo*: in ES cells a $G\alpha_{i/o}$ signal is used, mediated by the Apelin ligand, which we do not observe in the context of zebrafish heart development. Further, the autonomy of Aplnr function in ES cells was not examined.

Numerous studies, both *in vitro* and *in vivo*, have described roles for Aplnr signaling in adult cardiovascular function (Ashley et al., 2005; Chandrasekaran et al., 2008; Charo et al., 2009; Szokodi et al., 2002). In these studies, a prerequisite role for Apelin, the only known Aplnr ligand (Tatemoto et al., 1998) in Aplnr function has been assumed. *In vitro*, PTX-sensitive signaling pathways downstream of Aplnr are activated by Apelin administration (Masri et al., 2002). Indeed, many *in vivo* studies of Aplnr function have relied on addition of Apelin ligand as a proxy for pathway activation. We were surprised to find that Aplnr signaling apparently acts independently of Apelin during early cardiac development in zebrafish. However, this result is consistent with *aplnrb* and *apelin* gene expression patterns, as *apelin* transcripts are not evident until 10hpf (Scott et al., 2007; Zeng et al., 2007), by which time cardiac progenitors have largely reached their target location in the ALPM. Obviously, our negative MO results for *apelin* with respect to heart formation cannot alone absolutely prove that Aplnrb is acting independently of Apelin. It is interesting to note, however, that analysis of *Apelin* and *Aplnr* mutant mice has in some cases noted discrepancies in what would be expected to be identical phenotypes (Charo et al., 2009; Ishida et al., 2004; Kuba et al., 2007). Notably, murine *Aplnr -/-* mutants exhibited cardiac developmental defects while *Apelin -/-* mutants did not. This led the authors to suggest that the Aplnr may act in an Apelin-independent manner in some contexts (Charo et al., 2009). Future evaluation of endogenous Aplnr function should therefore be careful to consider not only ectopic addition of Apelin, but also loss of Aplnr function.

Interestingly, our results strongly argue for a $G\alpha_{i/o}$-independent mechanism of Aplnr activity in the context of cardiac progenitor migration to the ALPM. While both loss of *aplnrb* and overexpression of *PTX* result in heartless embryos, this appears to be due to distinct mechanisms. *Aplnrb* was absolutely required in a non-autonomous fashion for heart development, whereas PTX did not show strict autonomous or non-autonomous functions. Further, embryos lacking Aplnr signaling did not contain cells in the heart-forming region of the ALPM, suggesting a failure of cells to reach this location. In contrast, inhibition of $G\alpha_{i/o}$ signaling via PTX did not affect the migration of cells to the ALPM. Instead, *PTX*-treated embryos had cells in the ALPM that could not initiate myocardial differentiation. An effect on cardiomyocyte development following global inhibition of $G\alpha_{i/o}$ proteins is not unexpected. $G\alpha_{i/o}$ proteins have been shown to be effectors of Hh signaling through their coupling to Smoothened (Riobo et al., 2006) and it was recently shown in zebrafish that Hh signaling functions cell-autonomously in the development of cardiomyocytes (Thomas et al., 2008). $G\alpha_{i/o}$ proteins also act downstream of Fzd1 in the Wnt/Ca2+ pathway, and are implicated in patterning of the zebrafish embryo, likely through a cell-non-autonomous mechanism (Slusarski et al., 1997). G-protein independent signaling by GPCRs has become appreciated as a critical component of many signaling events (Shenoy et al., 2006). In the absence of a G-protein mediated (and perhaps Apelin/Aplnr-mediated) signal, Aplnrb may be functioning in a number of ways. Signaling may be via recruitment of β-arrestin, and may occur following receptor internalization (Shenoy et al., 2006). Aplnrb may

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dimerize with other GPCRs, affecting their response to ligands, nature or strength of downstream signaling pathways (Han et al., 2009; Maurice et al., 2010; Monnier et al., 2011). Finally, signaling may occur via Aplnrb ligand(s) that remain to be discovered. Recent work suggests that the hypotensive effects of Apelin/Aplnr signaling in the vasculature may occur independently of $G\alpha_{i/o}$-mediated cAMP inhibition (Iturrioz et al., 2010). The mechanisms through which Apelin/Aplnr signaling regulates many described aspects of development, homeostasis and disease (Barnes et al., 2010; Carpene et al., 2007; Quazi et al., 2009) therefore requires further investigation.

Due to the specific involvement of both Aplnr signaling and cBAF activity in the migration of myocardial progenitor cells to the ALPM, and subsequent differentiation into cardiomyocytes, we investigated the relationship between the two. Interestingly, our results suggest that cBAF functions downstream of Aplnr signaling, in a cell-autonomous manner, to mediate the migration of cells to the heart-field region of the developing embryo. The extracellular factor(s) activated downstream of Aplnr is yet to be determined. However, in our hands activation of signaling pathways critical for early heart development (Fgf, Nodal, Bmp, canonical Wnt, and Shh) were not sufficient to rescue the *aplnra/b* morphant phenotype (results not shown). The relationship between Aplnr signaling and cBAF function requires future study. However, it is tempting to speculate that the Aplnr signaling may allow for formation of an active form of a Gata5 associated with Smarcd3-containing BAF complex. As neither the effectors of Aplnr signaling in cardiac progenitor migration nor the regulators of cBAF activity have been elucidated, the link between the two is an important connection that requires further investigation.

In summary, our work shows that Aplnr signaling regulates the migration of cardiac progenitor cells from the lateral embryonic margin to the ALPM (Fig. 6D), a step essential for cardiomyocyte development. The non-autonomous nature of Aplnr signaling in this context suggests that Aplnr supports a niche that cardiac progenitors require for their proper differentiation or migration. As a key early step in cardiac progenitor development has been postulated to involve changes in migratory behaviour (Christiaen et al., 2008; Tam et al., 1997), Aplnr signals may be instructive or permissive for this event. Inducers and effectors of this pathway remain to be elucidated, and their identification will be critical to understanding the earliest events of cardiac progenitor development. Further, this will likely aid future work in the differentiation and growth of cardiomyocyte populations from various stem cell populations for cell therapy, preclinical drug screening and disease mechanism study applications.

Materials and Methods

Zebrafish Strains and Embryo Maintenance

Zebrafish embryos were grown at 28℃ in embryo medium following standard procedures (Westerfield, 1993). *grn*^{s608} mutants, which harbour a mutation in *aplnrb*, as well as *tp53*^{zdf1}, *Tg(myl7:EGFP)*^{twu34} and *Tg(hsp:apelin)*^{hsc1} zebrafish lines have been previously described (Berghmans et al., 2005; Huang et al., 2003; Scott et al., 2007).

Microinjection

MOs used to target translation of *aplnra/agtrl1a* (5′ – cggtgta-ttccggcgttggctccat – 3′), *aplnrb/agtrl1b* (5′ – cagagaagttgtttg-tcatgtgctc – 3′), and *apelin* (5′ – gatcttcacattcatttctgctctc – 3′) have been previously described (Scott et al., 2007; Zeng et al., 2007) and were purchased from Gene Tools (Oregon, USA). For embryos injected with *aplnra/b* MO, 1 ng of *aplnra* and 0.5 ng of *aplnrb* MO was injected. 8 ng of *apelin* MO was injected. MOs targeting splicing of *gata5* (5′ – tgttaagatttttacctatactgga – 3′) and translation of *gata6* (5′ – agctgttatcacccaggtccatcca – 3′) were used as previously described (Peterkin et al., 2003; Trinh et al., 2005), with 2 ng of *gata5* MO and 0.5 ng of *gata6* MO co-injected into one-cell stage embryos. An 800 bp fragment of the *PTX* coding sequence was PCR amplified from pSP64T-Ptx (Hammerschmidt and McMahon, 1998) and subcloned into pCS2+. pCS2+ mypaCterm-GRK2, encoding the C-terminal portion of GRK2 fused to C-terminal palmitoylation and myristoylation sites, was constructed by Stephane Angers (University of Toronto). pCS2+-Gata5 and Smarcd3b constructs have been previously described (Lou et al., 2011). *PTX*, *mypaCterm-GRK2, gata5 and smarcd3b* mRNA was prepared from linearized plasmids using an mMessage mMachine kit (Applied Biosystems). 100 pg of *PTX* or 25 pg of *mypaCterm-GRK2* mRNA was injected into each one-cell stage embryo. For rescue experiments, 50 pg each of *gata5* and *smarcd3 b* mRNA was injected into each one-cell stage embryo.

RNA *In Situ* Hybridization and Histology

RNA *in situ* hybridization (ISH) was carried out as previously described (Thisse and Thisse, 2008) using riboprobes specific for *apelin*, *fli1*, *gata1*, *gata5*, *nkx2.5*, *myl7*, *spi1*, and *tal1*. DNA fragments for all probes were amplified by RT-PCR (sequences available upon request). ISH images were taken on a Leica MZ16 microscope at a magnification of 115X. Sectioning of embryos (embedded in 4% low-melt agarose) was performed on a Leica VT1200S Vibratome with a speed of 0.7 mm/s, amplitude of 3.0 mm, and section width of 150 µM. Images were taken on a Zeiss LSM510 confocal microscope.

Photoconversion

The KikGR coding sequence (purchased from MBL International, Japan) was amplified by PCR and subcloned into pCS2+. KikGR mRNA was made using the mMessage mMachine kit (Applied Biosciences) from a linearized pCS2+ KikGR template. 150 pg of *KikGR* mRNA was injected at the one-cell stage into wildtype embryos either with or without *aplnra/b* MO. Cells of the lateral embryonic margin (displaced 90˚ from the shield at 6hpf) were photoconverted on a Zeiss AxioImager M1by closing the fluorescence diaphragm such that only the cells of interest were visible. This region of the embryo was exposed to UV light (through the DAPI filter) for a period of one minute. Images of embryos were taken on a Zeiss AxioImager M1 microscope using either the 5X or 10X objective.

Transplantation

Transplantation was carried out as previously described (Scott et al., 2007). Donor and host embryos were injected with appropriate amounts of *aplnra/b* MO or *PTX* RNA as specified above. 10–20 cells from *myl7:EGFP* donor embryos were transplanted into the embryonic margin of unlabeled wildtype hosts. Hosts were scored at 48hpf for the presence of donor-derived EGFP+ cardiomyocytes in the heart. Images of embryos were taken on a Leica M205FA microscope at a magnification of 175X.

Statistics

For transplantation and photoconversion data, statistics were performed using an unpaired T-test with unequal variances.

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