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Smarcd3b and Gata5 promote a cardiac progenitor fate in the zebrafish embryo

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SUMMARY

Development of the heart requires recruitment of cardiovascular progenitor cells (CPCs) to the future heart-forming region. CPCs are the building blocks of the heart, and have the potential to form all the major cardiac lineages. However, little is known regarding what regulates CPC fate and behavior. Activity of GATA4, SMARCD3 and TBX5 – the 'cardiac BAF' (cBAF) complex, can promote myocardial differentiation in embryonic mouse mesoderm. Here, we exploit the advantages of the zebrafish embryo to gain mechanistic understanding of cBAF activity. Overexpression of *smarcd3b* and *gata5* in zebrafish results in an enlarged heart, whereas combinatorial loss of cBAF components inhibits cardiac differentiation. In transplantation experiments, cBAF acts cell autonomously to promote cardiac fate. Remarkably, cells overexpressing cBAF migrate to the developing heart and differentiate as cardiomyocytes, endocardium and smooth muscle. This is observed even in host embryos that lack endoderm or cardiac mesoderm. Our results reveal an evolutionarily conserved role for cBAF activity in cardiac differentiation. Importantly, they demonstrate that Smarcd3b and Gata5 can induce a primitive, CPC-like state.

KEY WORDS: Cardiovascular progenitor, Cell fate, Zebrafish

INTRODUCTION

Cardiovascular progenitor cells (CPCs) represent the building blocks of the developing vertebrate heart. CPCs have the potential to form the major cardiovascular cell types (cardiomyocytes, endothelial and smooth muscle cells), and are therefore of great clinical interest for cell therapy-based cardiac repair approaches (Kattman et al., 2006; Moretti et al., 2006). Furthermore, defects in recruitment and differentiation of CPCs lead to congenital heart defects, which affect up to 1% of live births (Laugwitz et al., 2008; Pierpont et al., 2007). A much greater understanding of CPC biology is therefore required. Cells with CPC characteristics have been isolated from the developing mouse embryo, differentiating embryonic stem (ES) cells and adult hearts (Bu et al., 2009; Kattman et al., 2006; Moretti et al., 2006; Yang et al., 2008). Elegant fate-mapping studies in multiple vertebrate species have shown that progenitors of the cardiac lineage are among the first cells to undergo gastrulation movements, migrating to bilateral positions in the anterior lateral plate mesoderm (ALPM) (Schoenwolf and Garcia-Martinez, 1995; Stainier et al., 1993; Tam et al., 1997). At this stage, expression of the vertebrate *tinman* homologue *Nkx2.5* is initiated, which demarcates the cardiac mesoderm (Harvey, 1996). Expression of *Nkx2.5*, and subsequent cardiac differentiation, is regulated by the proper balance of signals (including BMP, FGF, secreted Wnt inhibitors and HH) found in this region of the ALPM (Alsan and Schultheiss, 2002; Marvin et al., 2001; Schneider and Mercola, 2001; Schultheiss et al., 1997; Thomas et al., 2008).

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An unanswered question is whether CPCs are 'primed' to differentiate upon arrival at the ALPM. Position in the pre-gastrulation vertebrate embryo predicts later cardiac fate. However, it is unclear if CPCs must receive additional signal(s) during gastrulation to be properly specified. Numerous explant studies using early pregastrula chick and mouse embryos have yielded conflicting results (e.g. Montgomery et al., 1994; Schoenwolf and Garcia-Martinez, 1995). However, recent work suggests that presumptive cardiac progenitors from the pregastrula embryo do have the potential to form cardiomyocytes when placed in ectopic (normally non-cardiogenic) embryonic locations (Lopez-Sanchez et al., 2009). Studies in mouse and sea squirt *Ciona intestinalis* have shown that Mesp family basic helix-loop-helix transcription factors are essential for cardiac mesoderm formation (Kitajima et al., 2000; Satou et al., 2004), and indeed Mesps can promote cardiac differentiation of ES cells in vitro (Bondue et al., 2008). However, as mutation of *Mesp1* and *Mesp2* in the mouse prevents migration of cells to the ALPM during gastrulation, the question of specification of CPCs cannot be directly addressed using this model.

A 'master regulator' of CPC fate, akin to *MyoD* in the skeletal muscle lineage, has not been identified. Work from many labs has shown that a number of transcription factors, notably Gata4/5/6, Srf, Mef2c, Nkx2.5 and Tbx5 are required to effectuate myocardial differentiation (Bruneau et al., 2001; Kuo et al., 1997; Lin et al., 1997; Lyons et al., 1995; Molkentin et al., 1997; Weinhold et al., 2000). These transcription factors interact to form complexes on many cardiac-specific promoter elements (for reviews, see Cripps and Olson, 2002; Evans et al., 2010). Of these, Gata4/5/6 factor activity is absolutely required for cardiogenesis, as loss of *Gata4/6* in mice, or the related *gata5/6* genes in zebrafish, results in an acardia (heartless) phenotype (Holtzinger and Evans, 2007; Zhao et al., 2008). A combination of Gata4, Mef2c and Tbx5 has been shown to direct fibroblasts to form cardiomyocytes in vitro at a low frequency (Ieda et al., 2010). As *Gata4/5/6* genes are broadly expressed in the mesendoderm, it is unclear how cardiac progenitor-specific GATA activity is effectuated.

Mounting evidence indicates that the initiation of cell differentiation is highly regulated at the epigenetic level. In vertebrates, the Swi/Snf-like multi-subunit BAF chromatin remodeling complex plays a key role in modifying DNA-histone contacts (Kwon et al., 1994). Work in ES cells and neurons has shown that the BAF complex can engage in a number of cell-specific events via differential incorporation of subunit variants, providing a 'combinatorial code' of activity (Ho et al., 2009; Yoo et al., 2009). *Smarcd3/Baf60c*, which encodes a variant Smarcd/Baf60 subunit, plays an essential role in early murine heart development (Lickert et al., 2004). Recently, lipofection of mouse embryos with *Smarcd3*/*Gata4*/*Tbx5* was shown to promote myocardial differentiation of non-cardiogenic mesoderm (Takeuchi and Bruneau, 2009). The authors demonstrated that these three proteins bind to each other and form part of a cardiac BAF (termed cBAF) complex that synergistically upregulate the expression of myocardial-specific genes. Smarcd3 was further shown to recruit Gata4 to target genes, possibly acting as a 'pioneer' factor to effect myocardial differentiation. Although these recent cardiac reprogramming experiments illustrate that myocardial 'reprogramming' of cells is feasible, they do not address the in vivo mechanisms through which the earliest events of cardiac differentiation are initiated.

Here, we exploit the advantages of the zebrafish embryo to study cBAF activity in vivo. We find that Gata5/Smarcd3b activity promotes myocardial development, and that combined loss of *gata5*, *smarcd3b* and *tbx5* results in pronounced defects in cardiogenesis. Via transplantation, we find that cBAF plays a cell autonomous role in promoting cardiac fate (cardiomyocytes, smooth muscle and endocardium), regardless of where cells are placed in host embryos. This is achieved by localization of cBAF overexpressing cells to the heart-forming region, and occurs even in the absence of host cardiac or endoderm tissue. The fate of cBAF cells can be modulated, as FGF signaling inhibition leads to decreased myocardial, but not endocardial, differentiation. Therefore, cBAF (Gata5/Smarcd3b) can promote formation of cells that home or localize to the heart-forming region. As these cells can form all the lineages of the developing heart, these results show that cBAF can drive, in vivo, a CPC-like state.

MATERIALS AND METHODS

Zebrafish lines, imaging and stages

Zebrafish embryos were maintained and staged using standard methods (Westerfield, 1993). *Tg(kdrl:EGFP)^{s483}*, *Tg(myl7:EGFP)^{twu34}*, *Tg(hsp70:dnfgfr1-EGFP)^{pd1}* and *Tg(myl7:dsRedExp-nuc)^{hsc4}* fish have been previously described (Beis et al., 2005; Huang et al., 2003; Lee et al., 2005; Takeuchi et al., 2011). *Tg(tcf21:GVEcR; UAS:EGFP)^{el133}* was generated using the Tol2 kit as previously described (Kwan et al., 2007). Approximately 4 kb of upstream sequence from the *tcf21* (*capsulin*) promoter was PCR amplified (primers 5′-GGG GAC AAC TTT GTA TAG AAA AGT TGC ATG TGA TGG CAT CGG TAA G-3′ and 5′-GGG GAC TGC TTT TTT GTA CAA ACT TGC AAG GGT GGA GTG GAG AAA A-3′), as were GVEcR F′ (Esengil et al., 2007) and UAS:EGFP, and combined to create *tcf21:GVEcR; UAS:EGFP* flanked by Tol2 sites. Transgenic zebrafish were created using a standard Tol2 transgenesis approach (Urasaki et al., 2006). Upon addition of 10 μM tebufenozide (Sigma) to activate GVEcR (Esengil et al., 2007), stable *Tg(tcf21:GVEcR; UAS:EGFP)^{el133}* showed pharyngeal arch mesoderm expression from 28 hpf onwards, similar to that observed for endogenous *tcf21* transcripts. Imaging was performed using a Leica DFC320 camera on a Leica M205FA stereomicroscope. Time-lapse imaging was processed with OpenLab software. All confocal imaging was carried out on a Zeiss LSM510 confocal microscope.

Microinjection, morpholinos, mRNA, RNA probes and RNA in situ hybridization

Morpholinos used to inhibit *smarcd3b* (5′-ttccctccgcttctcctgccttttg-3′), *gata5* (5′-tgttaagatttttacctatactgga-3′), *tbx5* (5′-gaaaggtgtcttcactgtccgccat-3′) and *sox32* (5′-cagggagcatccggtcgagatacat-3′) have been previously described (Ahn et al., 2002; David and Rosa, 2001; Takeuchi et al., 2007; Trinh et al., 2005), and were purchased from Gene Tools. RNA in situ hybridization using DIG-labeled antisense RNA probes was carried out as previously described (Thisse and Thisse, 2008). Probes for *nkx2.5*, *cmlc2/myl7*, *fkd2/foxa3*, *sox17*, *ntl* and *mespa/b* have been previously characterized (Field et al., 2003; Yelon et al., 1999), or were generated by PCR from cDNA template. For overexpression, full-length coding sequences of zebrafish *gata5*, *smarcd3b* and *tbx5* were subcloned into pCS2+. In vitro transcribed RNA (150-200 pg; made using a mMessage mMachine kit, Invitrogen) for each gene was injected per embryo.

Zebrafish embryonic cell culture

At shield [6 hours post-fertilization (hpf)] stage, embryos were dissociated as previously described (Westerfield, 1993). Dissociated cells were plated on laminin-coated glass coverslips and cultured at 28.5°C in L-15 media (Sigma) supplemented with 5% fetal bovine serum, 0.3 mg/ml Glutamine, 50 U/ml Penicillin, 0.05 mg/ml Streptomycin, 0.8 mM $CaCl_2$ and 10% zebrafish embryo extract.

Co-immunoprecipitation

N-terminal Myc, Flag and HA epitope tags were subcloned in-frame with Smarcd3b, Gata5 and Tbx5 full-length coding sequences in pCS2+. Cos2 cells were transfected using Lipofectamine 2000. Immunoprecipitation was performed as previously described (Chen et al., 2009) using protein-G agarose beads (Invitrogen), mouse anti-Myc and anti-HA antibodies (Invitrogen), and mouse anti-Flag antibody (Sigma).

Transplantation and heat-shock treatment

Transplantation experiments were carried out as previously described (Scott et al., 2007; Westerfield, 1993). Donor embryos were injected with 5% tetramethylrhodamine dextran or rhodamine green dextran (10,000 MW, Molecular Probes) as lineage tracer and RNA as indicated. A total of 40-50 cells were removed from 4 hpf donor embryos and transplanted to equivalently staged host embryos. For heat-shock, shield (6 hpf) stage transplantation embryos were incubated for 20 minutes in pre-warmed 37°C media, and then transferred to a 28.5°C incubator. *Tg(hsp70:dnfgr1-EGFP)* embryos were sorted based on EGFP fluorescence 90 minutes after heat-shock. EGFP-negative transplants were used as negative controls.

Chemical inhibitors

Transplantation embryos were treated with 40 μM SB-505124 (Sigma) or 10 μM dorsomorphin (Tocris) in egg water/0.1% DMSO from 4 or 6 hpf onwards as previously described (Hagos and Dougan, 2007; Yu et al., 2008). Control embryos were treated with 0.1% DMSO.

DAF-2DA staining

DAF-2DA (4,5-diaminofluorescein diacetate) staining was carried out as previously described (Grimes et al., 2006). Live embryos were transferred from embryo medium directly into a 10 μM solution of DAF-2DA (Calbiochem) in embryo medium adjusted to pH 7.0 and incubated for 12 hours in the dark at 28.5°C.

RESULTS

cBAF components synergistically regulate myocardial differentiation

To determine the endogenous cBAF function in heart development, morpholinos (MOs) targeting *smarcd3b*, *gata5* and *tbx5* were used. Zebrafish *gata5* has characteristics analogous to murine *Gata4* (Holtzinger and Evans, 2007; Peterkin et al., 2007), and is a key regulator of heart development (Reiter et al., 1999). Zebrafish contains two *smarcd3* genes (Ochi et al., 2008); however, MO targeting *smarcd3a* was found to have no effect on heart development (data not shown). Sub-optimal doses (1 ng) of *gata5*,

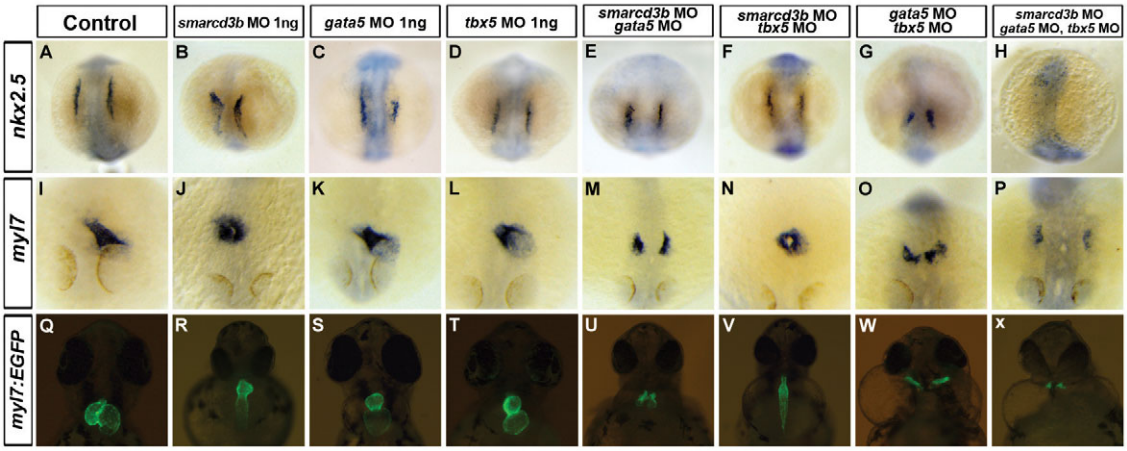

Fig. 1. Simultaneous inhibition of cBAF complex components leads to defects in myocardial specification. Suboptimal (1 ng of each MO in all cases) levels of morpholinos targeting *smarcd3b*, *gata5* and *tbx5* were injected as indicated. (**A-H**) RNA in situ hybridization for *nkx2.5* expression at the eight-somite stage (13 hpf). Dorsal views with anterior towards the top of images. (**I-P**) RNA in situ hybridization for *myl7* expression at 24 hpf. Dorsal views with anterior towards the bottom of images. (**Q-X**) Fluorescent images of 48 hpf hearts using the myocardial-specific *myl7:EGFP* transgene. Ventral views are shown with anterior towards the top of images.

smarcd3b and *tbx5* MOs were used that individually had minimal effects on early expression of *nkx2.5* (Fig. 1A-D), heart tube development (Fig. 1I-L) or chamber formation [Fig. 1Q-T, *smarcd3b* MO results in heart looping defects as previously reported (Takeuchi et al., 2007)]. Combined injection of *smarcd3b*, *gata5* and *tbx5* MOs led to progressively stronger effects on cardiac differentiation, with co-injection of all three MOs resulting in a marked absence of *nkx2.5* expression, cardia bifida and a greatly reduced number of *myl7* (*cmlc2*)-positive cardiomyocytes (Fig. 1E-H,M-P,U-X). To verify the specificity of cBAF function, the development of endoderm and noncardiac mesoderm was examined in triple *smarcd3b*/*gata5*/*tbx5* MO-injected embryos (morphants). Expression of endoderm markers *sox17* and *fkd2*/*foxa3* persisted in triple morphants at 6 and 24 hpf (see Fig. S1A-D in the supplementary material). Using *Tg(tcf21:GVEcR; UAS:EGFP)* embryos, at 48 hpf transgene expression was evident in the ventricle and cranial mesoderm of wild-type embryos (see Fig. S1E in the supplementary material). In triple morphants, whereas expression in the ventricle was absent, EGFP-positive cells remained evident in the (dismorphic) jaw (see Fig. S1F in the supplementary material). Vascular endothelial cell development was assayed using *kdrl:EGFP* embryos, which express EGFP in all endothelial/endocardial cells (see Fig. S1G in the supplementary material). Knockdown of *smarcd3b*/*gata5*/*tbx5* led the loss of endocardium; however, gross patterning of the vascular network remained intact (see Fig. S1H in the supplementary material). These experiments suggest a crucial and combinatorial role for the cBAF complex in zebrafish myocardial specification.

Our MO results are consistent with zebrafish Gata5/Smarcd3b/Tbx5 acting in a physical complex, as was previously described in the mouse (Lickert et al., 2004). To verify that these physical interactions are conserved, co-immunoprecipitation (co-IP) experiments were carried out in Cos2 cells using constructs encoding zebrafish Gata5, Smarcd3b and Tbx5 proteins. After co-transfecting constructs encoding Myc-tagged Smarcd3b and Flag-tagged Gata5, IP was performed with anti-Flag antibody. Myc-tagged Smarcd3b could be readily detected in the IP fraction (see Fig. S2A in the supplementary material). Similar co-IPs were carried out with Myc-tagged Smarcd3b and HA-tagged Tbx5, with a Tbx5/Smarcd3b interaction being apparent (see Fig. S2B in the supplementary material). These results suggest that physical interaction between zebrafish Smarcd3b, Gata5 and Tbx5 is conserved.

Overexpression of cBAF promotes myocardial differentiation

To further investigate the function of the cBAF complex, we globally overexpressed *smarcd3b*/*gata5*/*tbx5* in the zebrafish embryo via RNA injection at the one-cell stage. Following cBAF overexpression, defects in embryonic morphogenesis were evident at 24 hpf (Fig. 2G,I). Via RNA in situ hybridization, increased expression of *nkx2.5* and *myl7* was evident in these embryos at 13 and 24 hpf, respectively, with an enlarged heart apparent at 24 hpf (Fig. 2A,C,D,F,H,J). As expected (Reiter et al., 1999), overexpression of *gata5* alone led to upregulation of *nkx2.5* and *myl7* (Fig. 2B,E), although expansion of *myl7* expression at 24 hpf was not as robust as seen with cBAF (compare Fig. 2D with 2E). Overexpression of *smarcd3b* alone had no appreciable effects on embryonic or heart development (data not shown). Although enhanced myocardial gene expression was observed following *gata5* or *gata5/smarcd3b/tbx5* overexpression, ectopic regions of cardiac differentiation were not observed. Localization of *nkx2.5*, *myl7* and *a/vmhc* transcripts was consistently observed only in the heart-forming region of the embryo (Fig. 2; data not shown). Taken together with loss-of-function experiments, these results suggest that cBAF activity promotes cardiogenesis.

To examine the autonomy of cBAF pro-myocardial activity, we first cultured dissociated zebrafish embryos. Shield stage (6 hpf) *myl7:EGFP* embryos were dissociated, plated on laminin-coated coverslips and cultured for 24 hours. Using wild-type embryos, contractile cells expressing EGFP were observed, albeit representing a modest fraction of all cells (see Fig. S3G-I in the supplementary material). Following cBAF overexpression, an elevated proportion of cells were found to express EGFP (see Fig. S3A-C in the supplementary material). In many cases, clusters of EGFP-positive contractile cells formed following cBAF overexpression (see Fig. S3D-F in the supplementary material), suggesting an alteration in adhesive or proliferative properties.

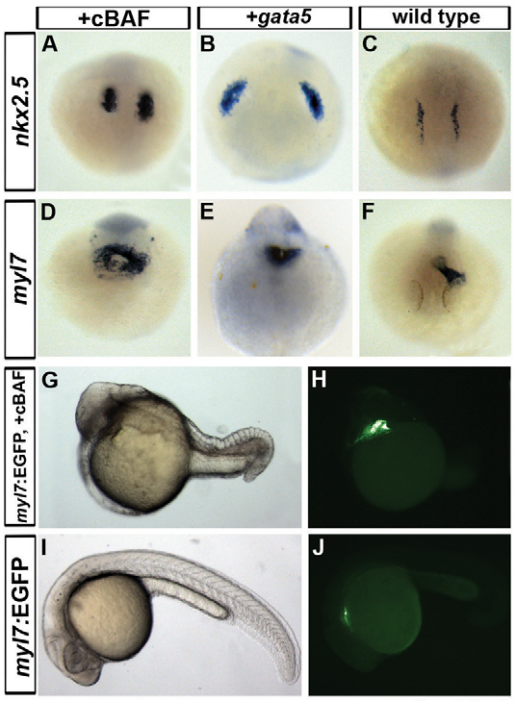

Fig. 2. Global overexpression of *gata5*/*smarcd3b*/*tbx5* in zebrafish results in expanded expression of myocardial-specific genes. (**A-C**) Expression of *nkx2.5* visualized by RNA in situ hybridization on 8-somite stage (13 hpf) wild-type (C), *gata5*-overexpressing (B) and cBAF-overexpressing (A) embryos. Dorsal views with anterior towards the top of images. (**D-F**) RNA in situ hybridization for *myl7* expression at 24 hpf in wild-type (F), *gata5*-overexpressing (E) and cBAF-overexpressing (D) embryos. Dorsal views of the heart region are shown. (**G-J**) Light and fluorescent images of 24 hpf *myl7:EGFP* transgenic wild-type (I,J) and cBAF overexpressing (G,H) embryos. Lateral views with anterior towards the left are shown.

These dissociated embryo culture results support the hypothesis that the cBAF complex promotes cardiomyocyte differentiation, perhaps in a cell-autonomous manner.

To examine more directly the autonomy of cBAF activity, transplantation experiments were performed. Injection of *tbx5* was found to be unnecessary for cBAF pro-myocardial activity, and was therefore excluded. Previous work in zebrafish has shown that at 6 hpf cardiomyocytes arise from the margin, displaced 60-140° bilaterally from the dorsal (shield) side of the embryo (Keegan et al., 2004). Transplantation of cells from a donor embryo (regardless of position) to this 'heart field' region of a host embryo can result in these cells adopting a cardiac fate (Lee et al., 1994). We first carried out transplant experiments in which transgenic *myl7:EGFP* donor embryos were injected at the one-cell stage with tetramethylrhodamine dextran (as lineage tracer) and either no (wild-type) or *gata5*/*smarcd3b* (cBAF) RNA. At sphere stage (4 hpf) cells were transplanted from either the margin or animal pole of donor embryos into the margin of wild-type host embryos (Fig. 3A). Myocardial differentiation of donor cells was scored at 48 hpf via the presence of EGFP expression. Overexpression of *gata5*/*smarcd3b* markedly increased (roughly threefold) the potency of donor cells to contribute to the myocardium. In 44% of transplants (n=203) using cBAF donor cells, we observed EGFP-positive cells in the heart (Fig. 3B-D,H-J), compared with 15% (n=110) of cases in control transplants (Fig. 3E-G,K-M). Strikingly, there was also a pronounced difference in the extent of myocardial contribution in individual EGFP-positive embryos. Although control transplants typically contained only a few EGFP-positive cells (less than 10 cells per heart), following cBAF overexpression much higher numbers of EGFP-positive donor cells were evident, forming large proportions of wild-type appearing hearts (compare Fig. 3B,H,E,K). In separate experiments, transplantation of *smarcd3b* morphant donor cells to the margin of host embryos resulted in greatly reduced myocardial differentiation (observed in only 2% of cases, n=92), further highlighting the importance of endogenous Smarcd3b/cBAF in early cardiac development.

cBAF promotes myocardial differentiation in a cell-autonomous, position-independent manner via directed migration to the heart-forming region

At 4 hpf, when transplants were performed, the shield of the host embryo was not apparent. As such, the high rate of cardiac contribution (44%) from cBAF donors suggested that cBAF may drive cells to a myocardial fate even if they are localized outside the heart field. To test this hypothesis directly, more stringent transplants were performed in which donor cells were placed in the animal pole of host embryos at 4 hpf (Fig. 3N). As previously reported (Lee et al., 1994), wild-type donor cell contribution to the heart is not observed when transplantation to the animal pole is performed (Fig. 3R-T,X-Z), with cells transplanted to this region typically giving rise to central nervous system and other ectodermal derivatives (as noted by rhodamine label in the head). Donor cells overexpressing *gata5*/*smarcd3b* transplanted to the animal pole contributed to the myocardial lineage (Fig. 3O-Q,U-W), a fate never observed when wild-type donors were used. Surprisingly, myocardial contribution occurred at a comparable efficiency (47%, n=121) to that seen in transplantation to the margin. Although injection of *smarcd3b* or *tbx5* mRNA alone could not confer this pro-myocardial activity, *gata5* alone was sufficient to do so, albeit at a lower efficiency (and with fewer myocardial cells formed per embryo, see below) than *gata5/smarcd3b* (Fig. 4A-C, 25% of transplants EGFP-positive for *gata5* alone, n=81). Endogenous *tbx5* and *smarcd3b* were required for full pro-myocardial activity, as injection of *tbx5* MO lowered cBAF efficiency to 14% of transplants (n=85), and *smarcd3b* MO lowered the efficiency of *gata5* mRNA to 15% (n=73) (Fig. 4D-F). These results indicate that cBAF can drive cells to a myocardial fate regardless of initial position in the pregastrula embryo. However, ectopic sites of *myl7:EGFP* expression were not observed in these experiments (similar to what was observed in embryos globally overexpressing *gata5*/*smarcd3b*, see Fig. 2). This suggests that cBAF may promote myocardial differentiation by regulating the localization/migration of cells to the future heart-forming region in the ALPM, subsequently allowing their proper differentiation.

To quantify cardiac potency directly, we employed *Tg(myl7:dsRedExp-nuc)* donor embryos. Following transplantation to the animal pole of *myl7:EGFP* host embryos, donor-derived cardiomyocytes could be readily counted at 48 hpf (Fig. 4G-I). Overexpression of cBAF in donor embryos resulted in a 2.3-fold higher number of cardiomyocytes per successful transplant (average 34 cardiomyocytes, n=8) when compared with *gata5* alone (average 15 cells, n=9), with combined knockdown of *smarcd3b* inhibiting the pro-myocardial activity of *gata5* (average 5.5 cells, n=7) (Fig. 4J).

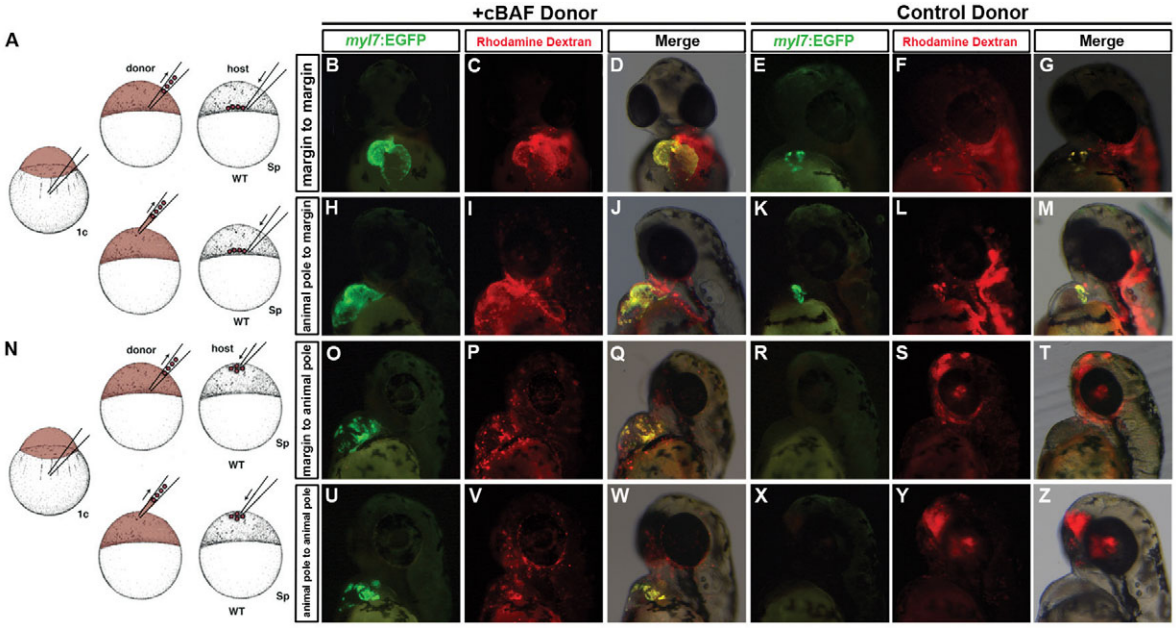

Fig. 3. Promotion of myocardial differentiation by *gata5*/*smarcd3b* in transplant experiments. (**A**,**N**) Schematics of experiments in which cells are transplanted to the host embryo margin (A) or animal pole (N) at sphere (Sp, 4 hpf) stage. (**B-M**) Transgenic *myl7:EGFP* donor cells from wild-type (E-G,K-M) or *gata5*/*smarcd3b*-overexpressing (B-D,H-J) donors were transplanted to the margin of wild-type host embryos. Rhodamine (red) indicates the lineage tracer in all donor-derived cells, EGFP indicates donor cells that have differentiated to form cardiomyocytes. (**O-Z**) Transplants were performed as above, with wild-type (R-T,X-Z) or *gata5*/*smarcd3b*-overexpressing (O-Q,U-W) donor cells placed in the animal pole of 4 hpf hosts. Embryos are 48 hpf, ventral (B-D) and lateral (E-Z) views with anterior towards the top.

The above experiments suggest an autonomous role for the cBAF complex in myocardial differentiation. Non-autonomous regulators of cardiac differentiation have been identified in many model systems (Foley and Mercola, 2005; Marvin et al., 2001; Schneider and Mercola, 2001). To investigate further the autonomy of cBAF activity, co-transplantation experiments were carried out. At sphere stage, donor cells from both: (1) *myl7:EGFP* transgenic embryos injected with *gata5*/*smarcd3b* RNA and tetramethylrhodamine dextran, and (2) wild-type embryos injected with rhodamine green were co-transplanted into the animal pole of 4 hpf wild-type hosts (Fig. 5A). Although co-transplanted cells were initially intermingled (Fig. 5A, inset), as gastrulation proceeded cells overexpressing *gata5*/*smarcd3b* (red) relocalized from the developing head to form two stripes of cells in the ALPM by 14 hpf (Fig. 5B,C, *myl7:EGFP* is inactive until after 14 hpf). By 24 hpf *gata5*/*smarcd3b*-expressing cells were clearly evident in the developing heart of the embryo (Fig. 5D,E) and, as evident by *myl7:EGFP* expression at 48 hpf, formed cardiomyocytes (Fig. 5F-H). In no cases (three experiments, n=50 for each) did co-transplantation of cBAF and wild-type donor cells result in wild-type cells localizing to the heart-forming region and/or differentiating as cardiomyocytes (Fig. 5A-F and data not shown, rhodamine green signal is much fainter than that of *myl7:EGFP*). The pro-myocardial activity of *gata5*/*smarcd3b* therefore appears strictly cell-autonomous in nature

To examine further how *gata5*/*smarcd3b*-expressing cells localize to the heart, time-lapse imaging was carried out from 6-10 hpf. Wild-type cells transplanted to the animal pole passively spread on the surface of the embryo, and were subsequently incorporated into the developing head (see Movie 1 in the supplementary material). By contrast, starting at roughly 8 hpf (70% epiboly), donor cells overexpressing *gata5*/*smarcd3b* started to form cellular aggregates and move posteriorly as gastrulation proceeded. This resulted in stripes of cells being found outside the head region, localized in the ALPM by 10 hpf (see Movie 2 in the supplementary material). These low-magnification time-lapse movies suggested migration of cBAF cells to the heart-forming region. To examine this in greater detail, time-lapse confocal imaging over a 30-minute period starting at 8 hpf was carried out. As expected, wild-type cells transplanted to the animal pole moved in register with each other, passively migrating with neighboring host cells. Prominent cellular protrusions or other signs of active directed migration were rarely seen in these cells (Fig. 6C,D). By contrast, *gata5*/*smarcd3b*-overexpressing cells formed aggregates and extended a large number of cellular protrusions/fillopodia-like structures (Fig. 6A,B). To examine molecular pathways through which cBAF exerts pro-myocardial activity, we next examined expression of *ntl* (mesodermal marker), *mespa* and *mespb* (CPC/mesoderm marker), and *nkx2.5* (a myocardial progenitor marker) in transplant embryos at shield (6 hpf) and tailbud (10 hpf) stages. As with wild-type donor transplantation controls and wild-type embryos, we did not observe ectopic expression of these genes at the animal pole or head following transplantation of cBAF overexpressing cells (Fig. 6E-P and data not shown). These results suggest that cBAF overexpression leads to cell-autonomous changes in cell adhesion and/or migratory properties, leading to their aggregation and exclusion from the animal pole region and subsequent relocalization to the ALPM.

cBAF promotes myocardial, endocardial and smooth muscle cell fates

In many transplants, we observed rhodamine-positive cBAF cells that were either adjacent to or in the heart, yet did not express the myocardial *myl7:EGFP* transgene (Fig. 5G, asterisks). Additional transplants were next carried out to examine other

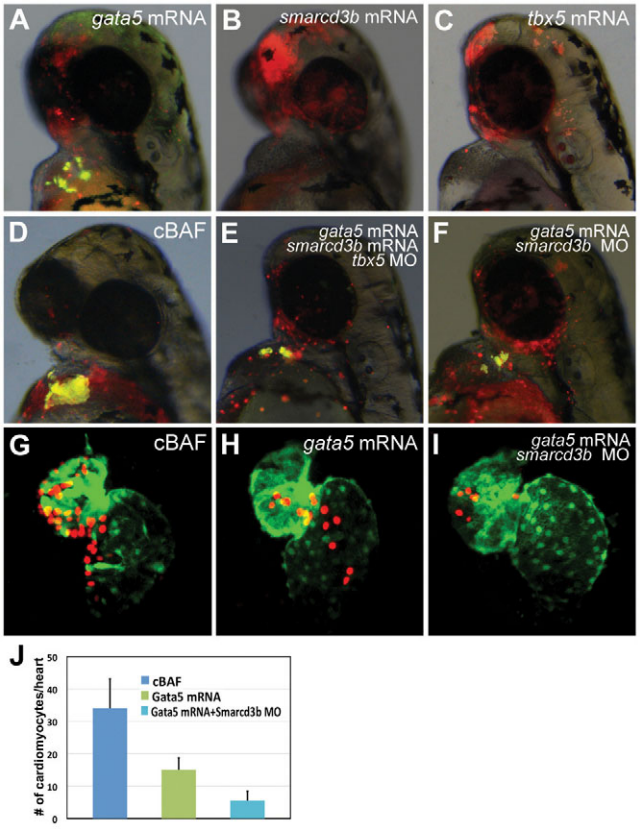

Fig. 4. Role of single components of cBAF in promotion of cardiomyocyte differentiation. (**A-F**) Transgenic *myl7:EGFP* donor embryos were injected with tetramethylrhodamine dextran (red) and *gata5* mRNA (A), *smarcd3b* mRNA (B), *tbx5* mRNA (C), *gata5*/*smarcd3b* mRNA (D), *gata5*/*smarcd3b* mRNA and *tbx5* morpholino (E), or *gata5* mRNA and *smarcd3b* morpholino (F). At 4 hpf, donor cells were transplanted to the animal pole of wild-type hosts. Donor cell cardiomyocyte differentiation was assessed at 48 hpf by EGFP fluorescence. All images are overlays of red channel, green channel and bright field, such that donor cell-derived cardiomyocytes appear yellow. (**G-J**) Transgenic *myl7:dsRedExp-nuc* donor embryos were injected with *gata5*/*smarcd3b* RNA, *gata5* RNA alone or *gata5* RNA in conjunction with *smarcd3b* morpholino. Transplantation was carried out as above. At 48 hpf, donor-derived cardiomyocytes were quantified via nuclear-localized dsRed signal. (J) Results shown as number of donor cardiomyocytes per positive transplant embryo (mean±s.e.m., $P<0.05$).

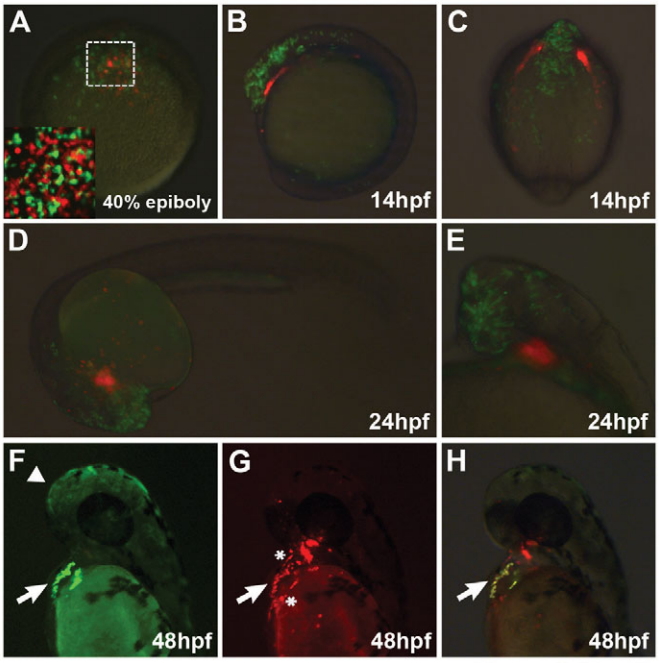

Fig. 5. Cell-autonomous migration of *gata5*/*smarcd3b*-expressing cells to the heart. (**A-E**) Donor cells overexpressing *gata5*/*smarcd3b* (red) and wild-type cells marked by rhodamine green were co-transplanted to the animal pole of a wild-type 4 hpf host embryo (A). Boxed area in A is shown at higher magnification in the inset, showing that wild-type and cBAF donor cells are uniformly dispersed in the animal cap following transplantation. (**F-H**) Co-transplants where *gata5*/*smarcd3b*-expressing donor cells carry the *myl7:EGFP* transgene. Faint rhodamine green signal is evident in the head (arrowhead), whereas stronger EGFP is seen in the heart (arrow). (A) Dorsal view; (B,F-H) lateral views with anterior towards the top; (C) dorsal view onto head; (D,E) lateral views with anterior towards left. Asterisks in G denotes rhodamine-positive cells inside and adjacent to the heart that do not express EGFP.

possible cardiovascular fates for these cells. Following transplantation of endothelial cell-specific *kdrl:EGFP* donors injected with *gata5*/*smarcd3b* to the animal pole of host embryos, EGFP was evident in the endocardium in 38% of transplants (Fig. 7A,B, $n=61$). Notably, EGFP signal was not evident in endothelial cells of the head or other extracardiac regions of the embryo, and was never observed when wild-type donors were used. Previous work has shown that the NO indicator DAF-2DA strongly stains smooth muscle of the outflow tract region from 48 hpf onwards (Grimes et al., 2006). Following transplantation of *gata5*/*smarcd3b*-expressing cells to the animal pole of host embryos, overlap of DAF-2DA and rhodamine (donor) signals in the outflow tract was observed in 32% transplants at 72 hpf (Fig. 7C,D, $n=43$). We further performed transplants using donor embryos bearing a *tcf21/capsulin* transgenic line that demarcates cranial mesoderm of the jaw. In 34% of animal cap transplants, EGFP signal was observed in the ventricle (potentially epicardium), with 3% of transplants showing EGFP signal in cranial mesoderm (Fig. 7E,F, $n=52$). DAF-2DA or *capsulin* expression was never observed from wild-type donor cells placed in the animal cap. To explore the potency of cBAF overexpressing cells in post-gastrulation embryos, heterochronic transplantation was employed. Sphere stage (4 hpf) *myl7:EGFP* donor cells were transplanted to the head and trunk regions of 14 somite (16 hpf) wild-type host embryos. Although some localized dispersal of cBAF-overexpressing donor cells was evident, no EGFP signal or localization to the heart was observed at 48 hpf ($n=26$ and $n=23$) (Fig. 7G,H). Taken together, these results show that cBAF can promote cells to localize to the heart-forming region of the early embryo and form myocardium, endocardium, smooth muscle, facial muscle and potentially epicardium with a high frequency.

cBAF acts downstream of many key cardiovascular signals

We next investigated the relationship between cBAF pro-myocardial activity and known cardiovascular development signaling pathways. In vertebrates, the adjacent endoderm

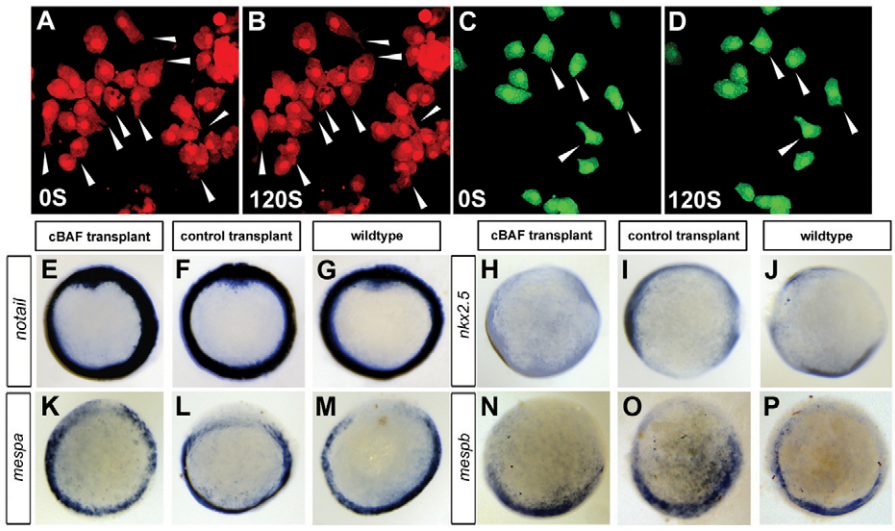

Fig. 6. Properties of cBAF-overexpressing cells. (**A-D**) Series of still images from Movies 1 and 2 in the supplementary material showing cBAF-overexpressing cells (A,B) demonstrating a large number of fillopodia-like structures (arrowheads) when compared with wild-type cells (C,D). (**E-P**) Expression of *ntl*, *nkx2.5*, *mespa* and *mespb* visualized by RNA in situ hybridization at shield (6 hpf, E-G, K-P) and bud (10 hpf, H-J) stages in cBAF donor transplants, control (wild-type donor) transplants and wild-type (no transplantation) embryos. All images are animal cap views.

promotes cardiac differentiation (Schultheiss et al., 1995). To assay whether host endoderm provides attractive or instructive cues for cBAF-expressing cells, host embryos were injected with MO targeting translation of *sox32*. Morphant and mutant *sox32* embryos lack all endoderm and display cardia bifida (two bilateral hearts) (Dickmeis et al., 2001; Kikuchi et al., 2001; Sakaguchi et al., 2001). Donor cells overexpressing *gata5*/*smarcd3b* formed bilateral clusters of myocardium in *sox32* morphant hosts, with *myl7:EGFP* evident in 31% of animal pole to animal pole transplants (n=44) and 33% of animal pole to margin transplants (n=41) (Fig. 8A-F). We next investigated the role of host cardiac tissue in recruiting cBAF-expressing cells. Embryos lacking *gata5* and *gata6* are devoid of myocardium (Holtzinger and Evans, 2007; Peterkin et al., 2007). Transplantation of cells from *myl7:EGFP* donor embryos injected with *gata5*/*smarcd3b* RNA into the animal pole of *gata5*/*gata6* MO-injected hosts resulted in clusters of donor-derived EGFP-positive contractile cells in 28% (n=37) of otherwise heartless embryos (Fig. 8G-I). Taken together, these results show that the promotion of migration to the ALPM and cardiac differentiation by the cBAF complex is not reliant on signals or chemotactic cues emitted from host endoderm or cardiac mesoderm.

In multiple vertebrate models, BMP signaling is essential for cardiogenesis (Schultheiss et al., 1997). More recently, BMPs have been shown to act as an attractive cue for cardiac progenitors in *Ciona intestinalis* (Christiaen et al., 2010). We therefore followed transplantation of *myl7:EGFP* cBAF donors to wild-type hosts with treatment by the BMP inhibitor dorsomorphin (Yu et al., 2008) from 6 hpf onwards. The concentration chosen for these experiments (10 μM) resulted in mild dorsalization phenotypes. At

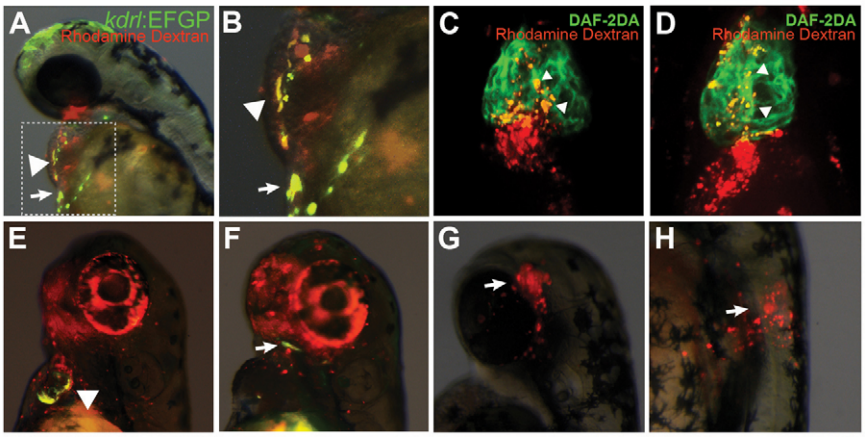

Fig. 7. Cells overexpressing *gata5*/*smarcd3b* form multiple cardiovascular lineages. (**A**,**B**) Donor *kdrl:EGFP* embryos were injected at the one-cell stage with *gata5*/*smarcd3b* RNA and tetramethylrhodamine dextran, and transplanted to the animal pole of wild-type host embryos at 4 hpf. Contribution to endocardium (arrowheads) and blood vessels neighboring the heart (arrows) was evident at 48 hpf. Lateral view of a 48 hpf embryo is shown, with the boxed region in A shown at higher magnification in B. (**C**,**D**) Donor embryos bearing no transgene were injected with *gata5*/*smarcd3b* RNA and tetramethylrhodamine dextran, and transplanted as in A,B. At 72 hpf, DAF-2DA staining was carried out. Arrowheads indicate the overlap of DAF-2DA staining (green) and tetramethylrhodamine dextran (red) in the outflow tract of the heart. Ventral view with anterior towards the top of images. (**E**,**F**) Donor *Tg*(*tcf21:GVEcR;UAS:EGFP*) embryos were injected with *gata5*/*smarcd3b* RNA and tetramethylrhodamine dextran and transplanted as in A,B. Contribution to ventricle (E, arrowhead) and head mesoderm/facial muscle (F, arrow) was evident at 48 hpf. (**G**,**H**) Heterochronic transplantation of 4 hpf donor cells from *myl7:EGFP* embryos injected with *gata5*/*smarcd3b* RNA and tetramethylrhodamine dextran to the head (G) and trunk (H) of 16 hpf host embryos. Combined bright-field/EGFP/rhodamine images are shown of transplant embryos at 48 hpf. Lateral views with anterior towards the top.

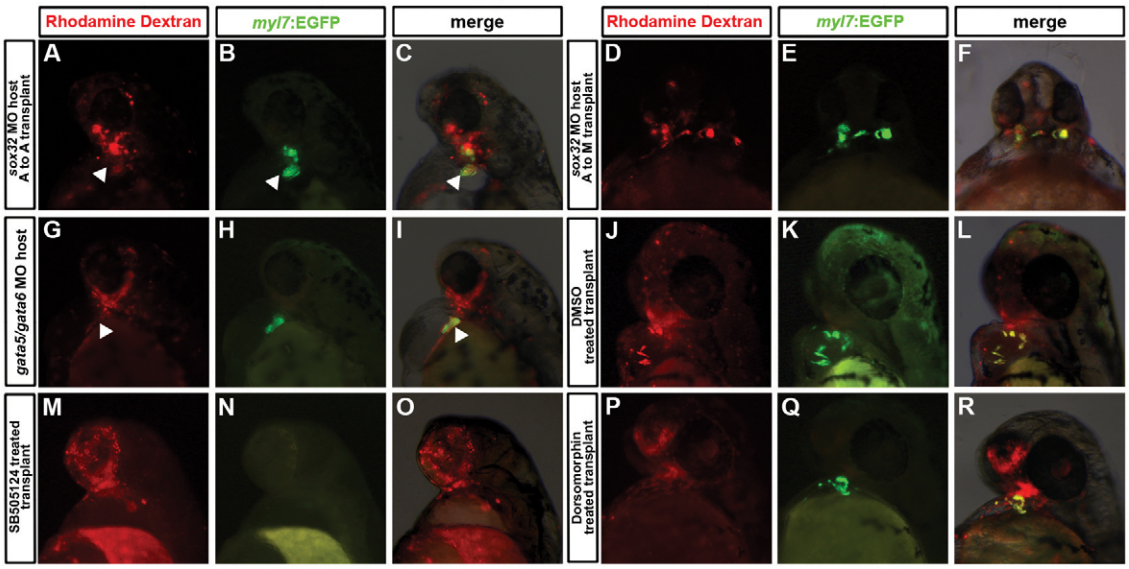

Fig. 8. Effects of host tissues and signaling pathways on the ability of cBAF-expressing cells to form myocardial cells. (**A-R**) Transgenic *myl7:EGFP* donor embryos were injected with tetramethylrhodamine dextran and *gata5/smarcd3b* RNA, and transplanted at 4 hpf into the animal pole of host embryos injected with morpholinos targeting *sox32* (A-F), and *gata5* and *gata6* (G-I). (J-R) Donor cells as per above were transplanted into the animal pole of wild-type host embryos, with transplants treated from sphere stage (4 hpf) onwards with DMSO [as drug treatment control (J-L)], 40 μM SB-505124 (M-O) or 10 μM dorsomorphin (P-R). In A-F, cells were transplanted from the animal pole (A) to the host margin (M) or animal pole (A) as indicated. Embryos are shown at 48 hpf. (A-C,G-R) Lateral views with anterior towards the top of images; (D-F) ventral views with anterior towards the top.

48 hpf, cBAF donor cells contributed to myocardium in 32% of dorsomorphin-treated transplants (*n*=40), a similar percentage seen in control (untreated) transplants (38%, *n*=36) (Fig. 8P-R). Although these experiments cannot definitively exclude a role for BMPs (as higher dorsomorphin concentrations were toxic), they suggest that cBAF activity is not exquisitely sensitive to BMP signaling. To examine whether cBAF activity can override the requirement for Nodal signaling in mesendoderm formation, we treated transplant embryos at 4 hpf with SB-505124, a Nodal signaling inhibitor shown to have potent activity in zebrafish embryos (Hagos and Dougan, 2007). In these transplants, *myl7:EGFP* donor cells (overexpressing cBAF) remained in the head of host embryos and failed to undergo myocardial differentiation (Fig. 8M-O, 0%, *n*=61). Finally, we evaluated the function of FGF signaling, a crucial regulator of myocardial differentiation (Alsan and Schultheiss, 2002), in cBAF activity. Inhibition of FGF signaling specifically in cBAF-expressing cells was achieved through use of *Tg(hsp70:dnfgfr1-EGFP)* zebrafish (Lee et al., 2005) that express a dominant-negative (truncated) form of the Fgfr1 upon heat-shock. Donor embryos were collected from intercrosses of homozygous *myl7:EGFP* and hemizygous *hsp70:dnfgfr1-EGFP* fish and injected with *gata5*/*smarcd3b* RNA and tetramethylrhodamine dextran. After transplantation to the animal pole of wild-type host embryos, embryos were given a 20-minute 37°C heat-shock at 6 hpf. Following a further 90 minutes at 28.5°C, embryos were sorted for presence of the *hsp70:dnfgfr1-EGFP* transgene based on expression of EGFP (*myl7* is not expressed at this stage). EGFP-negative transplants embryos were used as controls for heat-shock treatment. We found that when FGF signaling was inhibited, donor cells overexpressing *gata5*/*smarcd3b* formed *myl7:EGFP*-positive cardiomyocytes with a significantly lower efficiency (18% of embryos, *n*=53) compared with control donors (38% of embryos, *n*=39). Furthermore, following FGF inhibition the number of *myl7:EGFP*-positive cells formed per embryo was greatly reduced relative to heat-shock controls (compare Fig. 9A-C with 9D-F), with large clusters of cardiomyocytes evident in few transplants. Following FGF inhibition, many transplant embryos had large numbers of EGFP-negative/rhodamine-positive cells in the heart (Fig. 9C). We therefore repeated transplantation experiments using *kdrl:EGFP* donor embryos to trace endothelial/endocardial fate. We found that the percentage of *kdrl:EGFP*-positive transplants following FGF inhibition (31%, *n*=47) was similar to control sets (34%, *n*=59), with the number of EGFP-positive cells in individual embryos also being comparable (Fig. 9G-L). These results demonstrate that the fate of cBAF-induced cardiovascular cells can be modulated, with Nodal signaling being essential for cBAF activity and FGF signaling being required to optimize myocardial (but not endocardial) differentiation.

DISCUSSION

Here, we have shown that Gata5 and Smarcd3b can promote the development of CPC-like cells in vivo. This work both contrasts and expands on previous findings that *Gata4*/*Smarcd3*/*Tbx5* can promote myocardial differentiation in the mouse embryo (Takeuchi and Bruneau, 2009), and that *Gata4*/*Mef2c*/*Tbx5* can reprogram fibroblasts to cardiomyocytes in vitro (Ieda et al., 2010). In these cases, myocardial fate was promoted either in embryonic mesoderm or directly without an apparent intermediate progenitor state. In this current work, we believe our model reflects a closer representation of primitive embryonic CPC for several reasons: overexpression of *gata5*/*smarcd3b* drives cells to localize to the site of embryonic heart development (even from improper locations in the embryo); these cells are capable of forming multiple cardiovascular lineages (cardiomyocytes, smooth muscle and endocardium); and these cells differentiate to cardiovascular fates

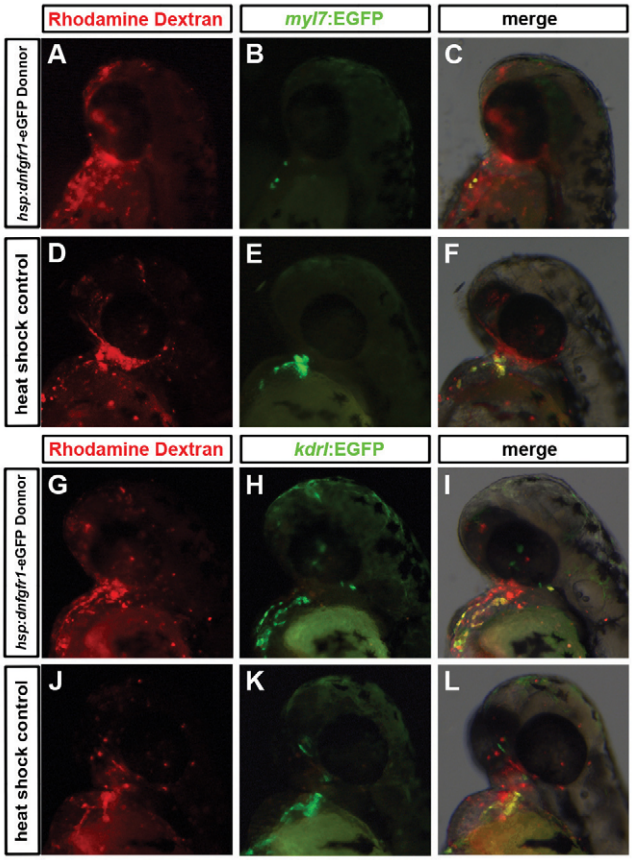

Fig. 9. FGF signaling is required for the promotion of myocardial, but not endocardial, fate by the cBAF complex. (**A-F**) Transgenic *myl7:EGFP* donor embryos injected with *gata5/smarcd3b* RNA and tetramethylrhodamine dextran bearing (A-C) and not bearing (D-F) a *hsp:dnfgfr1-EGFP* transgene were transplanted to the animal pole of wild-type host embryos at 4 hpf, with heat-shock subsequently applied at 6 hpf. (**G-L**) Experiments were performed as in A-F, with donors carrying a *kdrl:EGFP* transgene in place of *myl7:EGFP*. All images are lateral views of 48 hpf embryos with anterior towards the top.

only if localized to the heart-forming region proper. Therefore, although it remains to be seen whether individual cBAF cells are multipotent, the cBAF complex imbues embryonic zebrafish cells with several properties of CPCs.

It has been proposed that early cardiac differentiation and migratory behaviors are tightly linked (Christiaen et al., 2008); however, the pathways that regulate CPC specification and migration to the ALPM are poorly understood. In the zebrafish embryo, cBAF cells demonstrated hallmarks of active migration, suggesting that they are sensing a chemotactic cue (and overriding inhibitory cues). If this is the case, it remains unclear what attracts cBAF cells to the heart-forming region, as it is not signals emitted from the cardiac mesoderm or underlying endoderm. It is of course possible that overexpression of cBAF drives exclusion of cells from wild-type neighbors based on differential expression of cell-adhesion molecules. We currently favor a guided migration model for cBAF activity, as localization to the heart of cBAF-overexpressing cells appears largely independent of original position in the pre-gastrula embryo. Further research will be required to dissect the precise mechanism of cBAF-mediated localization to the heart-forming region. Induction of cardiac progenitor (*mespa/b*) and mesoderm (*ntl*) markers by cBAF was not evident. However cBAF activity was dependent on Nodal signaling, a key regulator of mesendoderm (and cardiac) differentiation (Gritsman et al., 1999; Xu et al., 1999). It is interesting to note that jaw musculature and potentially epicardial differentiation, as denoted by *tcf21* expression (Liu and Stainier, 2010), was observed for cBAF cells placed in the animal cap. In both mice and *Ciona*, the early *Mesp*-expressing lineage has been shown to form both cardiac and cranial mesoderm (Saga et al., 1999; Stolfi et al., 2010). The intermediate phases of cBAF reprogramming require further investigation.

Mechanistically, an intriguing question is how endogenous cBAF-like complexes are recruited to appropriate target genes to initiate CPC specification in vivo. Work in mouse suggests Smarcd3 is required to recruit Gata4 to appropriate target genes (Takeuchi and Bruneau, 2009). In skeletal muscle, Smarcd similarly enhances stable occupancy of myogenic promoters by Myod (de la Serna et al., 2005). In pluripotent cells, many differentiation-associated genes are 'poised' for activation (Zeitlinger et al., 2007). GATA factors have been shown to act as pioneer transcription factors in endoderm, providing the original impetus to activate key endoderm-specific genes (Cirillo et al., 2002). Although Gata4/5/6 have been known to be key regulators of myocardial differentiation (Grepin et al., 1995; Holtzinger and Evans, 2007; Peterkin et al., 2007; Zhao et al., 2008), potential transcriptional targets in CPCs remain unknown. As cBAF reprogramming of CPC-like cells is robust in zebrafish, future worked will be aimed at identifying early transcriptional targets of Gata5/Smarcd3. This will allow both identification of potentially novel regulators of early CPC development and, via analysis of the order of promoter/enhancer occupancy and chromatin status of target genes, start to delineate a transcriptional hierarchy of CPC differentiation. Future examination of Gata5/Smarcd3 target genes will provide an excellent starting point to uncover molecular pathways key for CPC specification, homing/migration behavior and terminal differentiation versus progenitor maintenance decisions.

Previous work has shown that *gata5* overexpression alone is sufficient to promote myocardial differentiation outside of the heart proper (Reiter et al., 1999). In the large number of cBAF overexpression and transplant experiments we have performed, this has not been observed. Examining how cBAF pro-myocardial activity is kept in check is therefore as important as how this activity is effectuated, as cell fate decisions involve both positive and inhibitory mechanisms. In our experiments, Gata5 alone has pro-myocardial activity, which is both bolstered by (and dependent on) endogenous Smarcd3b. In the mouse, *Smarcd2* could substitute for *Smarcd3* in the promotion of cardiac differentiation by *Gata4* (Takeuchi and Bruneau, 2009). It is therefore possible that cBAF activity is regulated both positively by the availability of Smarcd3 (and perhaps Smarcd2) and negatively by Smarcd1, analogous to what has been reported for BAF complex variant subunits in neural progenitor cells (Lessard et al., 2007; Wu et al., 2007). Expression of Smarcd subunits may therefore act as a 'rheostat' to adjust Gata4/5/6 activity.

Future work to determine how the early steps of CPC development are regulated has great potential implications for therapeutic approaches to heart disease. As CPCs home to the embryonic heart, it will be of interest to see whether cBAF can reprogram differentiated cell types to migrate to the heart and form

cardiac cells. Alternatively, cBAF activity may be useful for reprogramming of endogenous stem cells or cardiac fibroblasts to boost cardiac repair/regeneration. Given the amenability of the zebrafish embryo to chemical genetic approaches (Kaufman et al., 2009), future efforts at finding small molecules that can recapitulate cBAF activity may lead to greatly improved (CPC-based) cell therapy approaches for the damaged heart. Furthermore, as reprogramming of differentiated cells to other cell types (neurons, blood, etc.) has been achieved (Szabo et al., 2010; Vierbuchen et al., 2010), it will be of great interest to determine whether cBAF-like activity can also be generalized to other lineages.

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Competing interests statement
The authors declare no competing financial interests.

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