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Alcam-a and Pdgfr- α are essential for the development of sclerotome derived stromal cells that support hematopoiesis in vivo.

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Title: Alcam-a and Pdgfr- α are essential for the development of sclerotome derived stromal cells that support hematopoiesis in vivo.

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1 **Abstract**

2 Mesenchymal stromal cells are essential components of hematopoietic stem
3 and progenitor cell (HSPC) niches, regulating HSPC proliferation and fate decisions.
4 Their developmental origins are largely unknown. In zebrafish, we previously found
5 that the stromal cells of the caudal hematopoietic tissue (CHT), a niche functionally
6 homologous to the fetal liver in mammals, arise from the ventral part of caudal somites.
7 We have now discovered that this ventral domain is actually the sclerotome, and that
8 two typical markers of mammalian mesenchymal stem/stromal cells, Alcam and Pdgfr-
9 α , are distinctively expressed there and instrumental for the emergence and migration
10 of stromal cell progenitors, which in turn conditions the proper assembly of the vascular
11 component of the CHT niche. Furthermore, we find that the trunk somites are similarly
12 dependent on Alcam and Pdgfr- α to produce mesenchymal stromal cells that foster
13 the initial emergence of HSPCs from the dorsal aorta. Thus the sclerotome contributes
14 essential stromal cells for each of the key steps of developmental hematopoiesis, and
15 likely is the embryological origin of most if not all mesenchymal stem/stromal cells
16 found in non-cephalic tissues.

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1 **Introduction**

2 Hematopoietic stem and progenitor cells (HSPCs) are multipotent precursors
3 that have self-renewal capacity and continuously replenish all mature blood cells
4 throughout the life span. In zebrafish as in mammalian development, they initially
5 emerge from the dorsal aorta of the embryo through an endothelial hematopoietic
6 transition (EHT)^{1,2,3,4}. Then they enter the bloodstream to reach their first niche, the
7 caudal hematopoietic tissue (CHT)⁵, where they expand and undergo their first multi-
8 lineage differentiation⁶. Thus the CHT in fish is the hematopoietic homolog of the fetal
9 liver in mammals. In our previous work, we showed that the CHT niche was mainly
10 composed of a transient venous plexus arisen from the primitive caudal vein, and
11 stromal reticular cells (SRCs) interconnecting as well as lining the branches of this
12 venous plexus^{5,7}.

13 We then discovered that the progenitors of these stromal cells initially arose
14 from the ventral part of the caudal somites through an epithelial mesenchymal
15 transition (EMT)⁷. These stromal cell progenitors (SCPs) then emigrated as strings of
16 2-3 cells further ventrally, in close interaction with the endothelial cells sprouting from
17 the primitive caudal vein that would make up the venous plexus⁷. This early interaction
18 of the precursors of the two cell types that would constitute the CHT niche was
19 presumably important for the very structure and functionality of that niche. In the
20 present study we have more closely characterized the process of SCP emergence and
21 emigration from the caudal somites and identified important molecular actors in this
22 process. Moreover, since the sclerotome compartment of the somites lies at their
23 ventro-medial side, and undergoes EMT and subsequent emigration to produce the
24 various sclerotome derivatives^{8,9}, we have addressed its relationship with the ventral
25 cell clusters of the caudal somites that give rise to the SCPs.

26 Activated leukocyte cell adhesion molecule (Alcam or CD166) is a large cell
27 surface glycoprotein of the immunoglobulin superfamily that mediates adhesion
28 through homophilic interactions in various tissues¹⁰, and heterophilic interactions with
29 CD6 at the interface of T cells and antigen-presenting cells¹¹, and with galectin-8 in the
30 extracellular matrix¹². While expressed in a wide variety of tissues, Alcam is often found
31 in cell subsets involved in dynamic growth and/or migration processes including neural
32 development¹³, pharyngeal pouch formation¹⁴, angiogenesis¹⁵, kidney development¹⁶
33 hematopoiesis¹⁷, and tumor progression¹⁸; it is also found in mesenchymal stem cells
34 (MSCs) / bone marrow and fetal liver stromal cells^{19,20,21}. The short cytoplasmic tail of

1 Alcam was shown to interact indirectly with the actin cytoskeleton via actin-binding
2 proteins such as ezrin and syntenin-1²². These interactions of the cytoplasmic domain
3 dynamically regulate the clustering of Alcam molecules at the cell surface and the
4 strength of the homophilic and heterophilic interactions^{23,24}.

5 Platelet-derived growth factor receptor α (PDGFR- α) is a highly conserved
6 receptor tyrosine kinase. Binding of PDGFs to PDGF receptors induces their
7 dimerization, which unlocks their tyrosine kinase activity and results in
8 autophosphorylation of specific tyrosine residues, which then act as binding sites for
9 intracellular Src homology 2 (SH2) domain-containing signaling molecules^{25,26}.
10 PDGFR- α mediated signaling regulates various processes of embryonic development
11 and organogenesis, notably the proliferation then migration and differentiation of
12 specialized mesenchymal cells in various organ anlagen²⁷. Like Alcam, PDGFR- α is
13 also a marker of bone marrow MSCs^{19,28}.

14 Here we identify Alcama and Pdgfr- α as two essential transmembrane proteins
15 in the process of SCP emergence from somite epithelial cells, their subsequent
16 migration to become stromal cells, and for the resulting structure and functionality of
17 the CHT niche. We further show that the somite ventral clusters giving rise to them
18 coincide with the caudal sclerotome, and that Alcama and Pdgfra are similarly
19 important for the emergence from the trunk somites of sub-aortic mesenchymal cells
20 that are essential for the induction of HSPCs from the aorta.

1 Results

2

3 **Alcama is required for stromal cell development from the somites**

4 We previously described that cell clusters containing SCPs appeared at the
5 ventral side of caudal somites by 21-22 hpf⁷. Somite formation occurs in rostro-caudal
6 direction and we found that the SCP clusters first become apparent by live VE-DIC
7 microscopy in the transition from somite maturation stage S4 to S5 (4th and 5th somite
8 from the tailbud)²⁹ (Fig. 1A). During cluster formation, the cells located on the ventral
9 side of the somites adopt a rosette-like structure with a small cavity in the center (Fig.
10 1A-a; Movie 1). To visualize the subsequent emigration of SCPs from these ventral
11 clusters (VCs), we previously used TCF-driven fluorescent reporters expressed in all
12 somite cells, which were merely inherited by the emigrating SCPs⁷. To analyze the fate
13 of these SCPs, we first used the *Tg(pax3a:eGFP)* and *Tg(ET37:eGFP)* lines³⁰.
14 *Tg(pax3a:eGFP)* labelled somite cells weakly, then the VCs more strongly, as well as
15 the migrating SCPs up to 2.5 dpf (Movie 2). The *Tg(ET37:eGFP)* line also highlights
16 somite cells weakly³⁰, but then starts to label SCPs more strongly from the onset of
17 their emigration from the somites (Fig. S1A). While these two lines proved useful for
18 our study, we also set out to create a new Tg line that would label the SCPs more
19 specifically and throughout their development. We firstly searched for genes that
20 seemed specifically expressed in the VCs in the ZFIN whole-mount in situ hybridization
21 (WISH) databank. Our subsequent confirmation by WISH of candidate genes between
22 23-48 hpf led us to target the chondroitin sulfate proteoglycan 4 (*cspg4*) gene (Fig.
23 S1B). We then generated a BAC-based Tg line expressing the GAL4 transcription
24 factor (TF) from the *cspg4* regulatory regions. This *TgBAC(cspg4:GAL4)* line crossed
25 with a *Tg(UAS:RFP)* reporter line faithfully recapitulates the *cspg4* gene expression
26 pattern spatiotemporally (Figs. 1B, S1C). With the exception of the notochord, RFP
27 expression highlights only the VCs and their derivatives in *Tg(cspg4:GAL4;UAS:RFP)*
28 embryos. By 29 hpf, the GFP signal from *Tg(ET37:eGFP)* was also clearly visible and
29 the migration of RFP⁺GFP⁺ cells was observed towards not only the ventral but also
30 the dorsal side (Fig. S1C). The latter cell population migrated along the medial face of
31 the somites towards the notochord (Fig. S1D), suggesting chondro-progenitor and/or
32 tenocyte fates^{8,9,31}. Ventral-wards migrating SCPs gave rise to stromal cells throughout
33 the CHT by 38 hpf, and some migrated even further ventrally to become fin

1 mesenchymal cells³⁰ (FMCs) in the caudal fin (Fig. 1B-b). These three fates of somite
2 VC derived cells are recapitulated in Fig. S1D.

3 To understand how somite epithelial cells compartmentalize to give rise to the
4 VCs and their derivatives, we also searched for cell adhesion molecules expressed
5 specifically in the VCs. We found that *alcama* (activated leukocyte cell adhesion
6 molecule a, aka CD166), was expressed in the VCs more strongly than in the bulk of
7 the somites at 23 hpf (Fig. 1C). Immunofluorescence allowed us to detect the Alcama
8 protein at the very core of the clusters as they first appeared morphologically, i.e. at
9 somite stage S5 (Fig. 1D-a,b). Upon somite maturation, Alcama protein signals then
10 gradually propagated to delineate cell boundaries within the clusters (Fig. 1D-a,b). We
11 then detected the Alcama protein at joints between cluster-derived cells migrating both
12 ventral- and dorsal-wards (Fig. 1D-c). We first examined the impact of *alcama* knock-
13 down by using an antisense morpholino oligonucleotide (MO), that completely blocked
14 translation of the *alcama* transcript in vivo (Fig. S1E,F). Injection in
15 *Tg(pax3a:eGFP;TCF:nls-mCherry)* embryos revealed that while in control embryos,
16 SCPs initially migrated as strings of 2-3 cells interconnected by a rather large contact
17 surface (Fig. 1E-a,c), which immunostaining showed to be enriched in Alcama protein
18 (Fig. 1D-c), in *alcama* morphants, adhesion between migrating SCPs was reduced to
19 a very small surface at the tip of an elongated cell protrusion (Fig. 1E-b,d). We next
20 crossed our *TgBAC(cspg4:GAL4;UAS:RFP)* line with *Tg(UAS:Lifeact-eGFP)*³², in
21 which Lifeact-eGFP allows to highlight F-actin in vivo, so as to visualize actin dynamics
22 and cellular projections during SCP emergence and migration. Lifeact-eGFP⁺ VC-
23 derived cells were clearly less numerous in *alcama* morphants by 26 hpf (Fig. 1F) - a
24 finding confirmed in the *Tg(ET37:eGFP)* background (Fig. 1G). This reduced number
25 was later mirrored by a similarly (2.7-fold) lower number of SCPs after migration into
26 the CHT by 30 hpf (Fig. 1G), and a clearly lower number of both stromal cells in the
27 CHT and FMCs by 46 hpf (Fig. 1H).

28

29 **Alcama regulates the migration behavior of SCPs by modulating F-actin**

30 Time-lapse imaging of migrating Lifeact-GFP⁺ SCPs in *alcama* morphants
31 revealed a characteristic cell morphology compared to SCPs in control embryos (Fig.
32 2A). Filopodial projections in migrating leader SCPs usually occur mainly at the leading
33 edge, whereas they were 50% more numerous (Fig. S2A-a) and more randomly
34 scattered around the migrating cell in *alcama* morphants (Fig. 2B). In addition, even

1 though they were slightly longer on average in *alcama* morphants (Fig. S2A-b), unlike
2 in control embryos their length did not correlate with their position relative to the
3 direction of migration (Fig. 2C). A further quantitative analysis of confocal live images
4 showed that i) a high Lifeact-GFP (F-actin) signal was present throughout migrating
5 SCPs in *alcama* morphants whereas the F-actin signal was observed predominantly
6 at the leading edge in controls (Fig. S2B), and (ii) the surface area of migrating SCPs
7 in *alcama* morphants was much larger than in controls (Fig. S2C). These changes in
8 morphology and F-actin distribution correlated with a strong defect in SCP migration,
9 as their linear distance of migration in *alcama* morphants was less than half that in the
10 control group (Fig. 2D). Later on, by 46 hpf, this migration defect had translated in a
11 much thinner distribution of ET37:GFP⁺ stromal cells in the CHT (Fig. 1H).

12 To gain more insight into the implication of Alcama in SCP migration, we took
13 advantage of *Tg(cspg4:Gal4)* driver line to express specifically in these cells three
14 different forms of Alcama fused to eGFP at its C-terminus – a WT full-length Alcama
15 (Alcama-FL-eGFP), and two mutant forms, i) Alcama- Δ N-eGFP, lacking both N-
16 terminal Ig-like V-type domains that are known to mediate homophilic cell adhesion³³,
17 and ii) Alcama- Δ PDZ-eGFP, in which we introduced in the short cytoplasmic domain
18 of Alcama three amino acid changes predicted to suppress the binding of PDZ domain
19 containing proteins (Fig. S2D), such as syntenin²². These different forms were inserted
20 downstream of a UAS promoter, and the constructs were injected in
21 *Tg(cspg4:Gal4;UAS:RFP)* embryos. Expression of Alcama-FL-eGFP in the VCs did
22 not perturb SCP emergence and migration (Fig. S2E, Movie 3). In contrast, SCPs
23 expressing Alcama- Δ N-eGFP appeared disconnected from their neighbors within the
24 VCs, and soon developed an intense filopodial dynamics while still within the somite
25 (Fig. 2Ea-c, Movie 4), but most often with no resulting ventral-wards migration towards
26 the CHT, whereas internal control neighboring RFP⁺GFP⁻ cells migrated normally (Fig.
27 2E-d). Alcama- Δ PDZ-eGFP⁺ VC cells and their SCP derivatives displayed much less
28 filopodia compared to WT or Alcama- Δ N-eGFP expressing cells; they still showed
29 some GFP enrichment at cell-cell contacts, but no clear polarity (Fig. 2Fa-c, Movie 5).
30 Like Alcama- Δ N-eGFP expressing cells, Alcama- Δ PDZ-eGFP⁺ SCPs barely migrated
31 to the ventral side, compared with (RFP⁺GFP⁻) SCPs in the same embryo (Fig. 2F-d).
32 However, the dorsal-wards migration was rather retained for GFP⁺ cells in both cases,
33 as well as for Alcama-FL-eGFP expressing cells (Figs. 2G, S2F,G). In vivo tracking of
34 GFP⁺ cells in both Alcama- Δ N-eGFP and - Δ PDZ-eGFP expressing embryos confirmed

1 that the cells that egressed ventrally from the clusters then wandered around and
2 migrated only shortly towards the ventral side, whereas the dorsal-wards migration
3 was similar to that of wild-type SCPs or Alcama-FL-eGFP expressing cells (Fig. 2H,I).
4 Statistical analysis of their fates confirmed that the phenotype of GFP⁺ mutant cells
5 was predominantly 'immobile' or 'dorsal migration', with occasional 'ventral migration'
6 limited to a short distance (Fig. 2J). Altogether, these data show that Alcama is cell-
7 autonomously involved in SCP emergence and migration.

8

9 **Alcama and PDGFR- α deficiency similarly affect SCP development**

10 A prominent feature of *alcama* morphant and dominant-negative mutant
11 phenotypes was the inhibition of SCP ventral migration. Therefore, we investigated the
12 gene expression of receptors for cytokines and growth factors that might be involved
13 in SCP migration. We found that *pdgfr- α* , a bone marrow stromal cell marker gene in
14 mammals²⁸, was specifically expressed in the somite VCs, starting by somite
15 maturation stage S6, and then in the migrating cells derived from them (Fig. 3F-a,c).
16 Therefore we hypothesized a possible cross-talk between Alcama and Pdgfr- α . It is
17 known that PDGF signaling leads to an increase in AKT and ERK phosphorylation^{34,35}.
18 We firstly investigated the impact of *alcama* knock-down on pERK signaling in the
19 caudal region at 26 hpf. The number of pERK⁺ cells was drastically decreased in the
20 CHT of *alcama* morphants, while it recovered to almost the control level in the rescued
21 group (Fig. 3A). Then, we analyzed the impact of Pdgfr- α deficiency, using a previously
22 validated MO against *pdgfra*³⁶. First, we analyzed the colocalization of pERK-positive
23 cells with SCPs marked by pax3a-GFP in *alcama* and *pdgfra* morphants. While in
24 control embryos we could observe pERK⁺GFP⁺ migrating SCPs, in *alcama* and *pdgfra*
25 morphants the overall number of pERK⁺ cells was reduced, and no pERK signal was
26 observed among migrating SCPs (Fig. S3A).

27 Time-lapse imaging of SCP emergence in *pdgfra* morphants by 23-24 hpf
28 revealed that the cohesion of the VCs was compromised, with groups of cells often
29 delaminating together from the somite (Fig. S3B), and then several SCPs per somite
30 cluster or cell group starting to emigrate simultaneously (Fig. 3B), yet with a clearly
31 reduced migration efficiency relative to WT. Quantification showed that the numbers
32 of CHT stromal cells and FMCs originating from SCPs were reduced 2.4-fold and 2.0-
33 fold, respectively, in *pdgfra* morphants by 40 hpf (Fig. 3C), similar to what we
34 previously found for *alcama* morphants (Fig. 1H). Live imaging also showed that like

1 in *alcama* morphants, the extent of ventral-wards migration of SCPs by that time was
2 much reduced in the *pdgfra* morphants (Movie 6), and this apparently directly impacted
3 on the co-migration of endothelial cells that made the venous plexus, resulting in a
4 much thinner venous plexus, often reduced to a single convoluted tube, in tight
5 apposition to the overlying caudal artery, which could locally show a reduced diameter
6 (Fig. 3D). We then attempted to identify PDGF ligands that would activate *Pdgfr- α*
7 signaling in the VCs and migrating SCPs. pERK immunostaining was performed on
8 embryos overexpressing either of three different PDGFs whose ventro-caudal specific
9 expression had been confirmed by WISH (Fig. S3C). All three ligands were found to
10 activate pERK signaling, hence likely *Pdgfr- α* signaling; however the effect of *Pdgfaa*
11 was statistically most significant (Fig. S3D).

12

13 **PDGFR- α drives SCP migration through PI3K signaling**

14 PI3K signaling is known to be activated downstream of various growth factor
15 receptors including PDGF receptors³⁷ following their activation. Pharmacological
16 inhibition of PI3K isoforms using AS605240 (inhibiting PI3K isoforms γ , α), LY294002
17 (inhibiting isoforms α , β , δ) or CAL101 (inhibiting isoform δ) revealed that the migration
18 of SCPs was compromised in embryos treated with LY294002, and more so with
19 AS605240, resulting in a 3.5-fold reduction in stromal cell numbers by 40 hpf (Fig. 3E),
20 whereas *pax3a-GFP^{high}* neural crest cells migrated with only a slight delay (Fig. S3E,
21 blue arrows). To monitor SCP migration over longer intervals, AS-treated embryos
22 were fixed and labeled with a *pdgfra* in situ probe at stages encompassing the entire
23 SCP migration process. In the control group, dorsal-wards migration of VCs derived
24 cells had already started by 23 hpf, whereas no migration was observed in the AS-
25 treated embryos (Fig. 3F-a,c). By 36 hpf, SCP migration had not started yet in the AS-
26 treated group, in which SCPs accumulated at the zone of their emergence, while it was
27 already complete in the control group (Fig. 3F-b,d).

28 Since the involvement of PI3K in SCP migration was revealed, we then mutated
29 two tyrosine residues in the intracellular domain of *Pdgfr- α* that are responsible for the
30 binding of class IA (α / β / δ) PI3K to *Pdgfr- α* following its activation and
31 autophosphorylation³⁴ (Fig. 4A), and we cloned the mutated or WT *pdgfra* ORF
32 followed by a tandem HA-tag downstream of a *hsp70* promoter³⁸ (*hsp70:pdgfra- Δ PI3K-
33 HA* or *hsp70:pdgfra^{WT}-HA*, respectively). Transient transgenics resulting from injection

1 of the hsp70:pdgfra- Δ PI3K-HA or hsp70:pdgfra^{WT}-HA construct in *Tg(pax3a:GFP)*
2 embryos were heat-shocked at 20 hpf, then imaged in vivo from 23 hpf, or fixed for
3 immunofluorescence at the same time point. The anti-HA antibody positive signals
4 sometimes showed a mosaic distribution, but were sufficiently present in the targeted
5 tissue of hsp70:pdgfra- Δ PI3K-HA and hsp70:pdgfra^{WT}-HA embryos at 24 hpf (Fig.
6 S4A). Heat-shocked hsp70:pdgfra- Δ PI3K-HA embryos typically showed a less clear
7 outline of the somite VCs than controls. In addition, 38% of them showed multiple SCPs
8 simultaneously initiating migration from one somite, reflecting the lack of leader cell,
9 and 20.5% had at least one somite showing an 'overflow' phenotype in which the VC
10 collectively delaminated into the ventro-caudal cavity, reminiscent of the phenotype
11 observed above in *pdgfra* morphants; these cell populations tended to disperse over
12 time, but did not go through the normal migration process (Figs. 4B, S4B). In embryos
13 overexpressing the *pdgfra*^{WT}-HA construct, more apoptotic bodies were detected than
14 in WT embryos, but no abnormalities were observed in the VCs and emigration of
15 SCPs (Fig. S4C). Interestingly, the morphology of migrating SCPs in *pdgfra- Δ PI3K*
16 expressing embryos was quite similar to that observed in *alcama* morphants, with long
17 and numerous filopodia and the cell's main axis often orthogonal to the ventral-wards
18 direction of migration (Fig. 4C, Movie 7), and with increased 3D cell surface area and
19 volume (Fig. S4D,E). Importantly, the number of VCs derived GFP⁺ cells was clearly
20 more decreased for those migrating ventral-wards (SCPs) than for those migrating
21 dorsal-wards (DMCs) (Fig. 4D), again as previously observed upon *Alcama* deficiency.
22 The total number of SCPs at 36 hpf in the CHT of *pdgfra- Δ PI3K* expressing embryos
23 was also strongly reduced (Fig. 4E), as in *Alcama*- or *Pdgfra*-deficient embryos.

24

25 **Alcama and Pdgfr- α expression are regulated by sclerotomal transcription** 26 **factors**

27 Since our live imaging revealed that the somite VCs giving rise to SCPs also
28 gave rise to cells migrating dorsal-wards towards the notochord, it suggested an
29 overlap or identity of SCP clusters with the sclerotome. Our WISH analysis revealed
30 that TFs considered as sclerotome markers, such as *Pax9*, *Snai2*, *Twist1a* and *Twist1b*
31 were indeed expressed in the VCs of caudal somites from the S4/S5 somite stage, and
32 also quite earlier - from the S1 stage - for *Snai2* (Fig. 5A). Therefore, we investigated
33 if these TFs regulate the expression of *alcama* and *pdgfra* during SCP development.

1 To this end, we first isolated promoter regions of the *alcama* (3.3 kb) and *pdgfra* (3.2
2 kb) genes from BACs, and cloned them into the pGL3 Basic vector to monitor their
3 promoter activity in vivo in various conditions through a Dual luciferase assay
4 performed on tail lysates at 23 hpf (Fig. 5B-a,E-a).

5 Co-injection of the pGL3-alcama-luc construct with either *snai2* MO or *pax9*
6 mRNA attenuated the *alcama* promoter activity, while co-injection with *snai2* mRNA or
7 *pax9* MO had no significant effect (Fig. 5B-b). Consistent with this, *alcama* mRNA
8 expression in the tail of *pax9* morphants was upregulated at 24 hpf (Fig. 5C), while it
9 was down-regulated in the tail of *snai2* morphants (Fig. 5G). We similarly analyzed the
10 effects of *twist1a/twist1b* and *snai2* on the *pdgfra* promoter by co-injection with pGL3-
11 *pdgfra*-luc. Double knockdown of *twist1a* and *1b* led to a drastic decrease in *pdgfra*
12 promoter activity in the tail samples, whereas co-injection of *twist1b* mRNA stimulated
13 it (Fig. 5E-b). In line with this, *pdgfra* mRNA expression in the tail was downregulated
14 in *twist1a/1b* morphants (Fig. 5F). *Snai2* MO also reduced the *pdgfra* promoter activity
15 (Fig. 5E-b), and consistently, *pdgfra* expression in the tail of *snai2* morphants was
16 significantly reduced (Fig. 5G). Finally, we found that these sclerotomal TFs regulated
17 each other in the tail, as *pax9* knockdown up-regulated *snai2* expression but down-
18 regulated *twist1b*, while *snai2* knockdown down-regulated *pax9* (Fig. 5D,G). The
19 network of genetic interactions deduced from all these data is depicted in Fig. 5H.

20

21 **Alcama and Pdgfr- α are also essential for the production of mesenchymal** 22 **derivatives of trunk somites, and of definitive HSPCs**

23 The expression of *Alcama*, *Pdgfra*, and the sclerotomal TFs mentioned above
24 in the ventral-most part of zebrafish somites is not restricted to the tail, but also extends
25 to the somites of the trunk (Fig. 6A). Moreover, as in the tail, *pax3a:eGFP⁺* or
26 *ET37:eGFP⁺* mesenchymal cells are present in the trunk by 36 hpf, notably just lateral
27 or ventral to the dorsal aorta, in close contact with it or with the dorsal wall of the
28 underlying axial vein (Fig. 6B,6C-a,6D-a, and Movie 8). As in the caudal region, we
29 found that *Alcama*, *Pdgfr-a* or *Pax9* deficiency led to a strong reduction of these
30 *pax3a:GFP⁺* or *ET37:eGFP⁺* stromal cells of the trunk (Fig. 6C,D). In addition, these
31 cells were found lateral or ventro-lateral to the aorta, but never just ventral to it (Fig.
32 6C,D). By 52 hpf, the total number of *GFP⁺* stromal cells in the trunk of all morphants
33 was even more reduced relative to control embryos than at 36 hpf (Fig. S5).

1 36 hpf is the developmental stage by which definitive HSPCs start to emerge by
2 EHT from the ventral wall of the aorta - a process that peaks by 48-52 hpf. We found
3 that *Alcama*, *Pdgfr- α* or *Pax9* deficiency all caused a dramatic reduction in *myb*⁺
4 HSPCs in the trunk by 36 hpf (Fig. 6E,F). At the same time-point, we could observe
5 the beginning of *myb*⁺ HSPC colonization of the CHT of control embryos, whereas
6 almost no *myb*⁺ cell was detected in the CHT of all morphants (Fig. 6E-a). Then, at 52
7 hpf, only few *myb*⁺ HSPCs were found in the trunk of all morphants, even less than at
8 36 hpf, while *myb*⁺ cells were numerous in control embryos, and very few *myb*⁺ HSPCs
9 were detected in the CHT of all morphants (Fig. 6E-b). Thus the severe impact of
10 *Alcama* or *Pdgfr- α* deficiency on the development of sclerotome-derived stromal cells
11 both in the trunk and in the tail led to a profoundly defective definitive hematopoiesis.
12

1 **Discussion**

2 Mouse studies have shown the presence of various stromal cell subsets in
3 hematopoietic stem cell niches³⁹. However, the developmental process and behavior
4 of these cells, and the molecules involved in cell-cell interactions during niche
5 formation, remain a mystery. In the present study, we have taken advantage of the
6 accessibility to in vivo observations of the zebrafish CHT, the hematopoietic homolog
7 of the fetal liver niche in mammals, to provide new insights into the genesis of stromal
8 cells. We previously found that the stromal cells of the CHT arose from ventral clusters
9 (VCs) within the caudal somites. We report here a new transgenic line,
10 *TgBAC(cspg4:GAL4)*, that specifically labels these VCs and their derivatives, notably
11 the SCPs throughout their development, and at least two other types of mesenchymal
12 cells - dorsal-wards migrating cells (DMCs) and fin mesenchymal cells (FMCs)³⁰.
13 Considering that the cell population originating from the caudal somites that
14 differentiates into osteoblasts in adult fish⁴⁰ may also be included, cells derived from
15 the VCs may have the potential of mesenchymal stem cells (MSCs). VC cells
16 specifically express sclerotome marker genes such as *twist1*, *snai2* or *pax9*, and their
17 anatomical location also supports their sclerotomal nature. The three transmembrane
18 molecules that we found here to be more specifically expressed in the VCs and their
19 derivatives, *Cspg4*, *Alcama* and *Pdgfr- α* , have all been used as markers of bone
20 marrow and/or fetal liver MSCs in mammals^{19,28, 39,41}. Our tracing of the SCPs back to
21 the VCs of the caudal somites allows us to connect the concept of MSCs, which arose
22 from studies of in vitro cell cultures from postnatal mammals, to the embryological
23 concept of sclerotome. The VC-derived DMCs would notably give rise to chondrocytes
24 and tenocytes^{8,9,31}. It will be important to study whether the 'commitment' process⁴²,
25 which corresponds to the first branching point in the differentiation of MSCs into various
26 cell lineages, may already take place within the VCs.

27 It is the outstanding optical clarity of the ventro-caudal region of zebrafish
28 embryos as the caudal somites develop that allowed us to identify for the first time the
29 earliest morphological individualization of the sclerotome, as a distinct ventral cell
30 cluster appearing at somite maturation stage S5, before these cells become
31 mesenchymal cells migrating both dorsal- and ventral-wards. Interestingly,
32 Naganathan et al.⁴³ recently found that up to stage S4, somites undergo a mechanical
33 adjustment of their A-P length that is facilitated by somite surface tension, which
34 requires the somite to be fully packed within an uninterrupted basal lamina. It therefore

1 makes sense that cell migrations out of the somite, which require to break the basal
2 lamina, are “allowed” to begin only once this mechanical rearrangement of the somite
3 has been completed, i.e. from somite stage S4/S5. Morphological individualization of
4 VCs by stage S5 appears to closely follow the onset of expression at the same place
5 of the sclerotomal TF genes as well as *alcama*. It may thus reflect the EMT triggered
6 by Twist1/Snai2/Pax9. At this stage, Alcama first appears in the center of the cluster
7 and then gradually spreads to all interfaces among cluster cells. Then as these cells
8 emigrate from the cluster, they do so as strings of cells still enriched in Alcama at their
9 interfaces. Suppression of Alcama function reduced the number of emigrating SCPs,
10 their contacts among each other, and their migratory capacity. The latter defect
11 correlated with a less efficient polarization of the cell and its F-actin dynamics relative
12 to the ventral-wards direction of migration. The augmented filopodial dynamics
13 observed in Alcama- Δ N expressing SCPs may be favored by the lack of Alcama-
14 mediated cell-cell contacts, while the still present short cytoplasmic domain of this
15 overexpressed Alcama- Δ N form may trigger more F-actin dynamics generating
16 filopodia. Conversely, VC cells overexpressing a mutant Alcama form in which the
17 cytoplasmic domain is no longer able to bind PDZ domain containing actin-binding
18 proteins such as syntenin-1^{22,23,24} showed a lack of filopodial dynamics, more extended
19 cell-cell contacts, and an even more complete lack of apparent cell polarity.

20 Suppression of Pdgfr- α function affected the emergence and ventral-wards
21 migration of sclerotome derivatives at least as strongly as Alcama deficiency. Our
22 finding that both Alcama and Pdgfr- α deficiencies led to a strong decrease in pERK
23 signaling in the ventro-caudal region suggests that the intracellular signaling pathways
24 downstream of Alcama and Pdgfr- α overlap within the stromal derivatives of the
25 sclerotome. In addition, we found PI3K activation to be an obligatory step downstream
26 of Pdgfr- α activation for the development of these cells. Interestingly, the dorsal-wards
27 migration of sclerotome cells was also affected in embryos treated with a PI3K α,γ
28 inhibitor, indicating that even though distinct pathways are likely involved in the dorsal
29 and ventral migrations of sclerotome derivatives, both use PI3K as a necessary
30 downstream effector (Fig. S6).

31 Clements & Traver⁴⁴ suggested that cells derived from the trunk sclerotome
32 were required for the emergence of HSPCs. Nguyen et al.⁴⁵ then found that somite-
33 derived pax3a:eGFP⁺ cells fostered HSPC emergence, but interpreted them as

1 dermomyotome-derived cells integrating into the DA endothelium. Here we have
2 confirmed their finding but clarified that these *pax3a:eGFP*⁺ cells are actually
3 sclerotome-derived, DA-associated mesenchymal cells. At least some of them, lateral
4 and ventral to the aorta, are most likely precursors of the mural cells later ensheathing
5 the DA that were shown to arise from trunk somites^{46,47} – more precisely the
6 sclerotome, as in mammals and birds^{48,49}, and to initially associate with the aorta floor.
7 Those seen in close association with the dorsal wall of the axial vein (PCV) underlying
8 the aorta are highly reminiscent of the stromal cells that we previously found by
9 electron microscopy bridging gaps between adjacent venous endothelial cells at this
10 very location⁶, which is where aorta-derived HSPCs enter circulation in zebrafish.
11 Altogether our present study leads to the unifying conclusion that the somitic VCs
12 identified as the sclerotome successively give rise to mesenchymal cells involved in
13 the first two main stages of HSPC development and function. Trunk somites first
14 contribute mesenchymal stromal cells that foster the emergence of HSPCs from the
15 neighboring DA floor, then the later formed caudal somites contribute mesenchymal
16 stromal cells that are key components of the first niche where the aorta-derived HSPCs
17 settle, expand and undergo multi-lineage differentiation.

18 Our study further revealed that *Alcama* and *Pdgfr- α* , known as mere markers of
19 MSCs in mammals, are actually instrumental for the very emergence and emigration
20 of mesenchymal stromal cells from the somites to build the hematopoietic niches both
21 in the trunk and tail. Our new *Tg(cspg4:Gal4)* line, which highlights mesenchymal
22 stromal cells from their sclerotomal origin onwards, will be a precious tool to discern,
23 notably through single-cell transcriptome analysis, whether cellular heterogeneities
24 prefiguring different fates are already present within the somitic VCs, and how different
25 the stromal cells fostering HSPC emergence from the aorta are from those that then
26 nurture HSPC expansion and multi-lineage differentiation in the CHT niche. Finally,
27 beyond stem/stromal cells found in hematopoietic organs, cells with MSC capacities
28 have been extracted from a variety of fetal and adult mammalian tissues, but their
29 precise origin within these tissues has remained elusive. Crisan et al.⁵⁰ presented data
30 suggesting that these various tissue MSCs were perivascular mesenchymal
31 cells/pericytes. Interestingly, *cspg4* expression, which we found here to mark the early
32 sclerotome and its derived cells, is a typical marker of pericytes^{51,52}. Therefore
33 altogether our study makes it reasonable to anticipate that developmentally, MSCs
34 from most if not all tissues posterior to the head will prove to arise from the sclerotome.

- 1 Elucidation of the molecular mechanisms of MSC development will contribute to the
- 2 field of regenerative medicine by facilitating the induction and manipulation of rare
- 3 MSC populations in adult tissues.

1 **Materials and Methods**

2

3 **Zebrafish**

4 Fish were maintained in our zebrafish facility at Institut Pasteur. Embryos were
5 obtained through natural crosses, raised at 28°C in embryo water [Volvic® water
6 containing 0.28 mg/ml Methylene Blue (M-4159; Sigma) and 0.03mg/ml 1-phenyl-2-
7 thiourea (P-7629; Sigma)], and staged according to Kimmel et al.⁵³ For this study, we
8 used the previously described transgenic lines *Tg(ET37:GFP)*⁵⁴, *Tg(pax3a:EGFP)*^{il150,}
9 ⁵⁵, *Tg(UAS:Lifeact-GFP)*^{mu271, 32}, *Tg(7xTCF-Xla.Siam:nlsmCherry)*⁵⁶, *Tg(kdrl:ras-*
10 *mCherry)*⁵⁷ and *Tg(lmo2:DsRed)*⁵⁸.

11

12 **BAC recombineering and transgenesis**

13 The tol2 and BAC recombineering vectors were kindly provided by K. Kawakami
14 (National Institute of Genetics, Mishima). A BAC DNA containing the *cspg4* gene
15 (DKEY-105I23) was purchased from Source BioScience. The *TgBAC(cspg4:GAL4)*
16 line was firstly generated by recombining a iTol2-amp cassette to the BAC vector⁵⁹,
17 then a second recombineering was performed targeting a cassette containing GAL4
18 (pGAL4FF-FRT-Kan-FRT) flanked by 50 bp arms homologous to the region around
19 the translation start of the *cspg4* gene. Primers used for the 1st and 2nd
20 recombineering steps are listed in the Supplementary information (Table 1).
21 Recombination was performed using RedET methodology (K001, GeneBridges) as
22 described in a previous report⁶⁰. BAC DNAs were prepared using NucleoBond BAC
23 100 (740579, Marchery-Nagel). *Tg(UAS:RFP)* embryos were injected with 1 nl of mix
24 solution of 100 ng/μl BAC DNA and 50 ng/μl Tol2 mRNA at 1-cell stage.

25

26 **In situ hybridization, immunostaining and TUNEL**

27 Whole-mount RNA *in situ* hybridization (WISH) was performed according to
28 Thisse [\(<https://wiki.zfin.org/display/prot/Thisse+Lab+-+In+Situ+Hybridization+Protocol+-+2010+update>\)](https://wiki.zfin.org/display/prot/Thisse+Lab+-+In+Situ+Hybridization+Protocol+-+2010+update). Riboprobes were synthesized from
29 PCR fragments amplified from cDNA extracted and synthesized from the tails of wild-
30 type embryos as a template, or by reverse transcription of linearized plasmids. The
31 probes used in this study are indicated in the Supplementary information (Table 2a,2b).

32 Whole-mount fluorescent immunostaining was carried out as described⁵. The
33 list of primary and secondary antibodies and concentrations used is shown in the
34

1 Supplementary material (Table 3). TUNEL staining was performed using ApopTag Red
2 In Situ Apoptosis Detection Kit (S7100, MerckMillipore) combined with peroxidase
3 (POD) coupled anti-DIG antibody (1/200, #11207733910, Roche) followed by
4 tyramide-based amplification⁶¹.

5

6 **Construction of mutant form of *alcama* and *pdgfra***

7 *UAS:alcama-ΔN-eGFP*, *UAS:alcama-ΔPDZ-eGFP* and *UAS:alcama-FL-eGFP*

8 Firstly, the *alcama* ORF was cloned to utilize as a template for the following
9 PCRs (details of cloning are indicated below). For *alcama-ΔN* construct, a DNA
10 fragment missing to encode amino acids 25-238 corresponding to the two amino-
11 terminal Ig-like domains of Alcama was amplified. For the *alcama-ΔPDZ* construct, 3
12 amino acid substitutions were introduced in a putative PDZ domain binding motif at
13 positions 533-539 (KTRQGSW->**MVRQGSG**) by site-directed mutagenesis. Primers
14 used for the mutagenesis are listed in Supplementary information (Table 4). Control
15 *alcama-FL* (full length) and mutated *alcama* fragments were cloned into the Nco I site
16 of UAS:eGFP vector in frame with eGFP using Gibson assembly (#E5510, NEB).

17

18 *hsp70:pdgfra-ΔPI3K-HA* and *hsp70:pdgfra-FL-HA*

19 A zebrafish *pdgfra-ΔPI3K* was designed based precisely on the human
20 dominant-negative *pdgfra*, with the substitutions of Y706F (Y731F in human) and
21 Y717F (Y742F)²⁵. Fragments containing amino acid substitutions were amplified by
22 PCR using the clone containing full-length wt *pdgfra* (kindly provided by J. Eberhart,
23 University of Texas at Austin) as a template. A 3 kb fragment covering the ORF was
24 amplified in three fragments and the point-mutations were introduced to the
25 appropriate fragments using primers listed in Supplementary information (Table 4). A
26 tandem HA-tag sequence was added in frame at a C-terminal of *pdgfra*. Fragments
27 encoding control or mutant *pdgfra* were cloned into *hsp70* vector using Gibson
28 assembly.

29

30 **Transient transgenesis**

31 Plasmid DNA for each construct was co-injected with capped mRNA coding for
32 the Tol2 transposase into 1-cell stage embryos at a concentration of 250 and 25 ng/μl,
33 respectively.

1

2 **RNA/cDNA synthesis, plasmid construction and injection**

3 Total RNA was extracted with the TRIzol reagent (15596026, Invitrogen) from
4 tails of anesthetized embryos at 23 hpf. Tail total RNA was reverse transcribed into
5 cDNA using a Superscript IV reverse transcriptase (18091050, Invitrogen). For *alcama*,
6 *pdgfaa*, *pdgfab*, *pdgfb* and *twist1b*, ORFs were amplified by PCR using tail cDNA as
7 a template for the subcloning into the TOPO vector and then reinserted into pExpress1
8 vector using Gibson assembly. ORFs of *pax9* and *snai2* were amplified by PCR from
9 tail cDNA then cloned directly into pExpress1 vector. Primers used for the cloning are
10 listed in Supplementary information (Table 5). mRNAs were synthesized using
11 mMessage mMachine transcription kit (AM1344, Ambion). Capped mRNAs were
12 injected into 1-cell-stage embryos at the amount of 100 pg.

13

14 **Morpholinos and qPCR**

15 Morpholino oligonucleotides (Gene Tools) were injected (0.5-1 nl) into 1-cell-
16 stage embryo at the amount specified; *alcama* MO¹⁴ (2 ng), *pdgfra* MO³⁶ (4 ng), *pax9*
17 MO^{62,63} (6 ng), *snai2* MO^{63,64} (8 ng) and *twist1a* and *twist1b* MOs⁶⁵ (2 ng each). MO
18 sequences are shown in the Supplementary information (Table 6). For quantitative
19 real-time PCR (qPCR), total RNA were extracted from 3 independent groups of 40 tails
20 dissected from embryos at 23 hpf to synthesize template cDNAs. The primers used for
21 qPCR are shown in the Supplementary information (Table 7). All qPCR experiments
22 were performed with measurements taken from 3 technical replicates. Fold changes
23 in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to *ef1a*.

24

25 **Heat-shock treatment**

26 Embryos were heat-shocked by placing them in pre-warmed Volvic water for 30
27 min at 39°C then transferred to 28°C until in vivo observation³⁸.

28

29 **Promoter cloning and Dual-Luciferase assay**

30 3.3 and 3.2 kb promoter regions of *alcama* and *pdgfra* genes were cloned from
31 BAC DNA (*alcama*: RP71-78P1, BACPAC Resources; *pdgfra*: DKEY-97C6, Source
32 BioScience) and cloned into the pGL3 basic vector (E1751, Promega) linearized with
33 Xho I and Hind III (pGL-*alcama*-luc and pGL-*pdgfra*-luc). Primers used for promoter
34 cloning are listed in Supplementary information (Table 8). To assess *alcama* and

1 *pdgfra* promoter activities in response to various TFs, WT embryos were injected at
2 the 1-cell stage with luciferase vectors (pGL-alcama-luc or pGL-pdgfra-luc), control
3 (Renilla) expression vector (pRL-TK; E2241, Promega), and specified amount of
4 Morpholino or appropriate mRNA encoding TF (co-injection condition is listed in
5 Supplementary information; Table 9). Tails were dissected from the injected embryos
6 at 23-24 hpf and lysed with passive lysis buffer (E1941, Promega) for subsequent
7 Dual-Luciferase assay following the manufacturer's instructions (E1910, Promega).

8

9 **Drug treatments**

10 Pharmacological inhibitors were all solubilized in DMSO (Sigma) and
11 appropriate DMSO controls (0.2 %) were used for all experiments. Embryos treated
12 with AS605240 (2 μ M; S1410, Selleckchem), LY294002 (20 μ M; S1105, Selleckchem)
13 and CAL101 (10 μ M; S2226, Selleckchem) from 19-20 hpf embryos then live-images
14 were taken with drugs in agarose and embryo water supplemented with tricaine. For
15 WISH, embryos were treated with drug from 19-20 hpf until the stage of interest, then
16 fixed in 4% methanol-free formaldehyde overnight.

17

18 **Live imaging, image processing and quantification**

19 VE-DIC (video-enhanced differential interference contrast) microscopy was
20 performed using a Polyvar2 microscope (Reichert) as described previously⁶⁶. Confocal
21 fluorescence microscopy was performed on Leica SPE and SP8 set-ups and Andor
22 spinning disk set-up as described previously^{4,7}.

23 The image processing was basically performed using Fiji⁶⁷. Measurements of
24 surface area and volume on migrating SCPs were performed using IMARIS software
25 (Bitplane). Cell tracking was performed using Manual tracking plugin (Fiji) and the
26 obtained data were 1) plotted with a Python script developed by S. Rigaud (Image
27 Analysis Hub (IAH) of Institut Pasteur) or 2) imported into the Chemotaxis and
28 Migration Tool plugin⁶⁸ (Fiji). For the analysis of filopodial dynamics, Filopodyan
29 plugin⁶⁹ was used on Fiji. First, we obtained time-lapse movies (6 min intervals, 23-38
30 hpf) of the migrating lifeact-GFP⁺ SCPs injected with control or alcama MO (7 leader
31 cells were analyzed for each condition). We extracted 7-10 timepoints from the time
32 that the leader cell left its followers and measured the orientation of the filopodia for
33 each timepoint. The cut-off threshold was set to 3 μ m.

1 The direction of SCP migration was obtained from the positional information at the first
2 and last timepoints. The angle of filopodia relative to the direction of SCP migration
3 was calculated with a Python script developed by D. Ershov (IAH, Institut Pasteur).

4 5 **Statistical analysis and replication**

6 Statistical analysis was performed using GraphPad Prism 9. For data that
7 followed a normal distribution, statistical significance was assessed by a two-tailed
8 Student's *t*-test, while Mann-Whitney test was introduced for two groups with unequal
9 variances. Significance levels were set at * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ and
10 **** $p < 0.0001$. Data are shown as mean or median \pm SD. All experiments are a
11 representation of at least three independent experiments unless stated otherwise. For
12 qPCR and luciferase assays, results were collected with three biological replicates.

13 14 15 **Acknowledgements**

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17 Institute of Genetics, Mishima) for providing us with the BAC recombineering plasmids,
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19 (University of Colorado) for the *cspg4:pJC53.2* plasmid, and A. Kudo (Tokyo Institute
20 of Technology, Nagatsuta) for the *pax9:pPICT3* plasmid. We also thank Stephane
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24 Luciferase assays. We are also thankful to N. Wolff and M. Genera (Institut Pasteur)
25 for their help with Western blotting.

26 27 **Author contributions**

28 E.M. conceived the project, designed, performed, and analyzed experiments, and
29 wrote the manuscript. C.V. conducted experiments and fish care. A.S. subcloned the
30 *hsp70* vector and assisted with helpful discussions. A-L.T. conducted experiments. P.H.
31 analyzed, discussed, and suggested experiments, and wrote the manuscript.

32 33 **Competing interests**

34 The authors declare no competing interests.

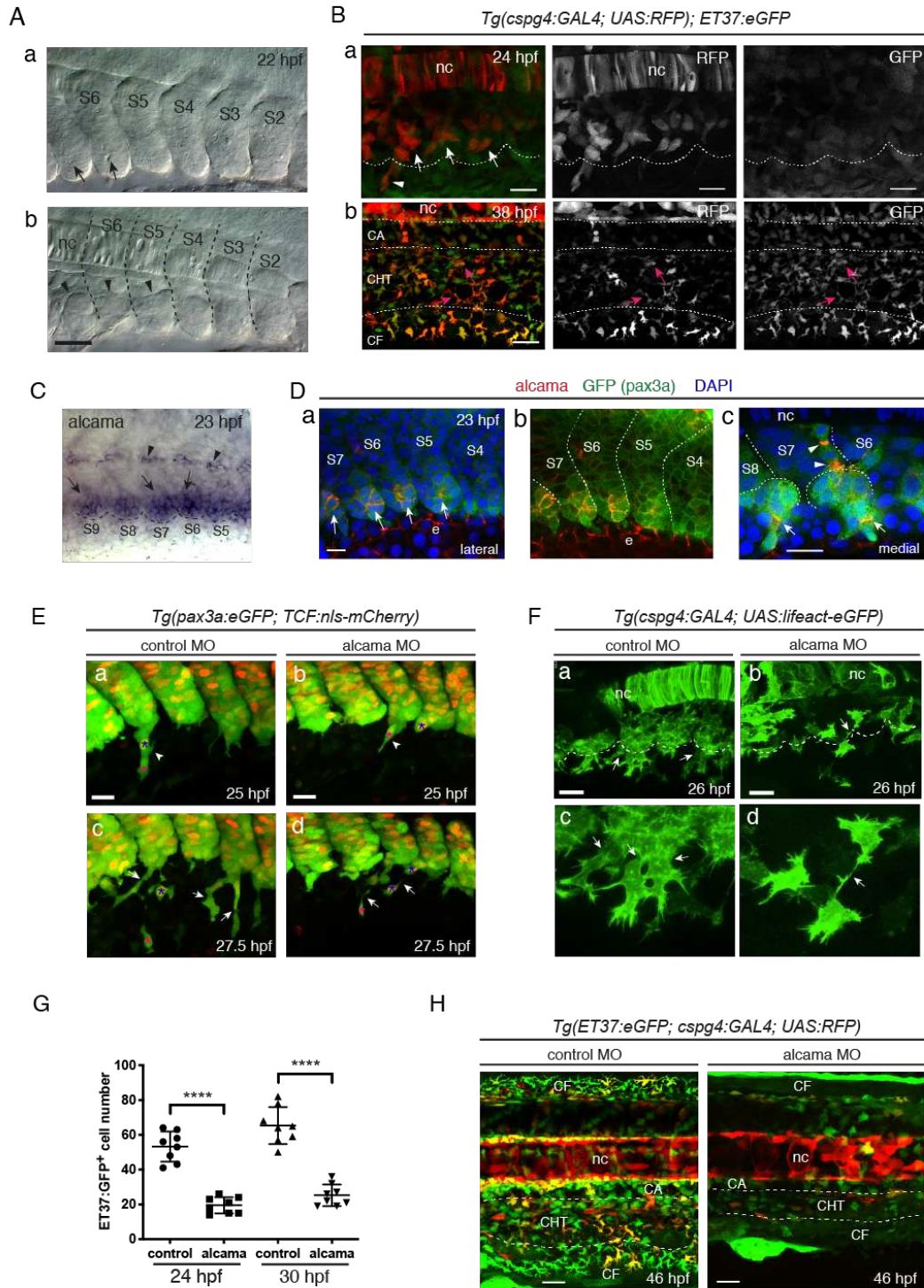
1

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7

Fig. 1



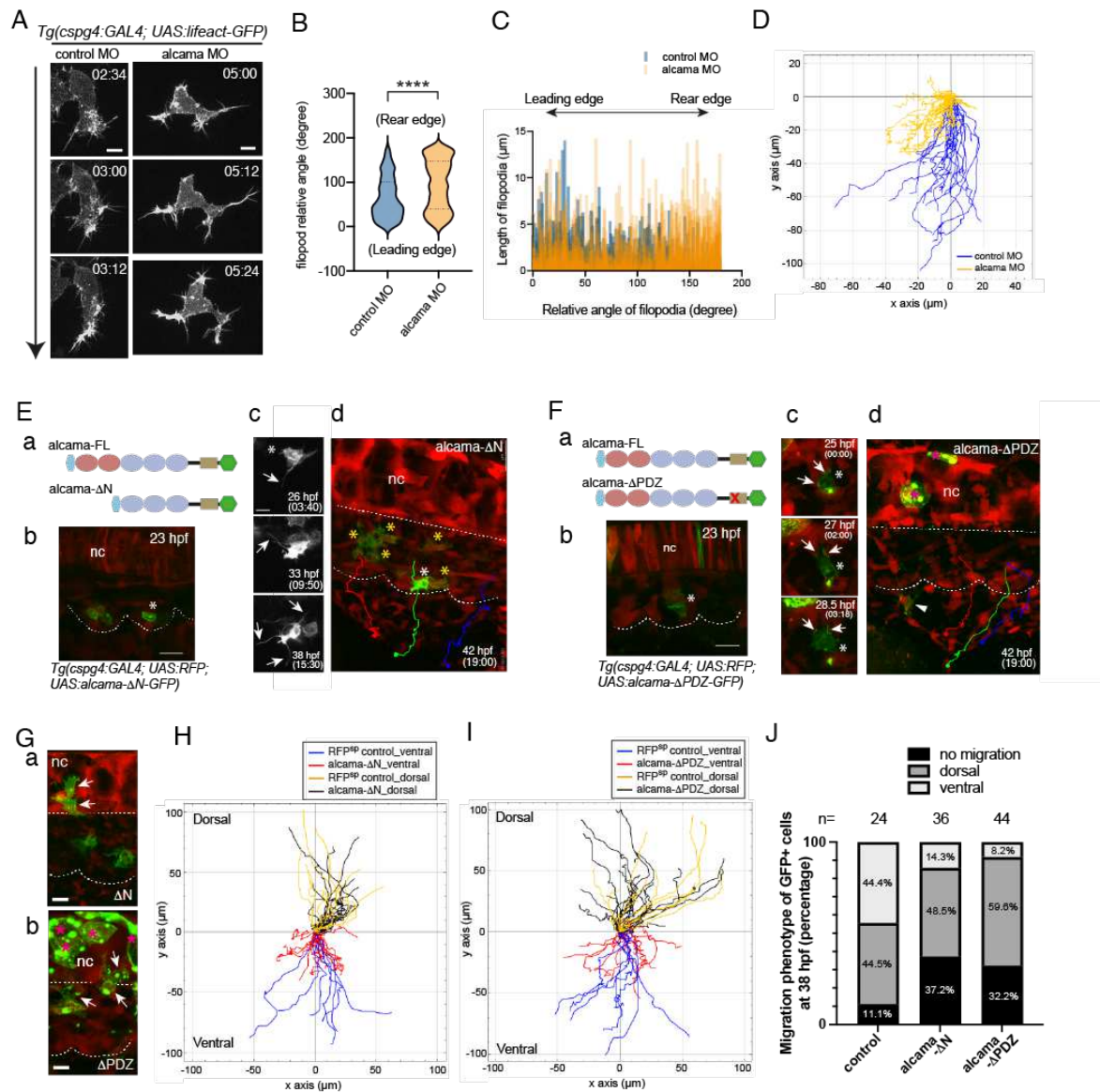
1

2 **Figure 1. SCP development and requirement for Alcama.**

3 **A**, VE-DIC microscopy of developing caudal somites at 22 hpf, rostral to the left, dorsal to the top.
 4 Somites are numbered S2-S6 according to their maturation stage counted from the tailbud. Lateral (a)
 5 and more medial (b) focal planes show the central lumen (a, arrows) formed within the ventral somite
 6 clusters, and the dorsal border of the clusters (b, arrowheads). Dashed lines indicate intersomitic
 7 boundaries. Scale bar, 40 μ m. **B**, Confocal projections of live *Tg(cspg4:GAL4; UAS:RFP; ET37:eGFP)*
 8 embryos at 24 (a) and 38 (b) hpf. a, Arrows and arrowhead point at VC cells and an emerging SCP,
 9 respectively. The dotted line outlines the ventral border of caudal somites. Magenta arrows in b point at

1 RFP/GFP double-positive cells in the CHT. Scale bars, 20 μ m. **C**, WISH for *alcama* at 23 hpf. Arrows
2 and arrowheads point at somite VCs and cells prefiguring the future horizontal myoseptum, respectively.
3 A dotted line outlines the somite ventral border. **D**, Immunostaining for *Alcama* (red), *pax3a:eGFP*
4 (green), together with DAPI (blue) at 23 hpf. **a**, Overlay of the red, blue and green signals; arrows point
5 at *Alcama* labeling in the somite VCs. **b**, Same image as **a** without DAPI staining. **e**, epidermis. **c**, At a
6 deeper focal plane, strong *Alcama* signals were detected at contacts between cells migrating semi-
7 collectively dorsal-wards (arrowheads) and ventral-wards (arrows). Dashed lines delineate the VCs and
8 somite borders. Scale bars, 20 μ m. **E**, Confocal maximum projections of control or *alcama* MO injected
9 *Tg(pax3a:eGFP; TCF:nls-mCherry)* embryos at 25 (**a,b**) and 27.5 (**c,d**) hpf. (**a,b**) Arrowheads show
10 SCPs in the early stages of migration; magenta and blue asterisks point to the same cells as in panels
11 **c** and **d**, respectively. (**c,d**) Arrows point at the intercellular junctions of migrating SCPs, which are much
12 thinner in **d**. Two blue asterisks in **d** indicate that the similarly labelled cell in **b** has undergone mitosis.
13 Scale bars, 25 μ m. **F**, Confocal projections of control or *alcama* MO injected *Tg(cspg4:GAL4;*
14 *UAS:Lifeact-eGFP)* at 26 hpf. Dashed lines indicate the somite ventral borders. Arrows point at the
15 connections between migrating SCPs. Magnified images are shown in **c** and **d**. Scale bars, 20 μ m. **G**,
16 Quantification of ET37:eGFP⁺ cells in the ventral tail of live control and *alcama* MO injected embryos,
17 at 24 and 30 hpf over a 5-somites width (n=8 embryos each; mean \pm SD; ****, P<0.0001. Student's *t*-
18 test). At 24 hpf, all GFP⁺ cells ventral to the notochord were counted; at 30 hpf, all GFP⁺ cells ventral to
19 the somites were counted. **H**, Confocal projections of control or *alcama* MO injected *Tg(ET37:eGFP;*
20 *cspg4:GAL4; UAS:RFP)* embryos at 46 hpf. Dashed lines delineate the CHT. nc, notochord; CA, caudal
21 artery; CHT, caudal hematopoietic tissue; CF, caudal fin.

Fig. 2



1

2 **Figure 2. Alcama regulates SCP migration.**

3 **A**, Time-lapse confocal images of a migrating SCP leader cell from control or alcama MO injected
4 *Tg(cspg4:Gal4; UAS:Lifeact-GFP)* embryos around 28 hpf. The time point from the initiation of SCP
5 migration is shown in the upper right corner of each panel. The time lag between control and alcama
6 morphant SCPs is due to the fact that the latter tend to take more time to fully egress from the somite.
7 Arrow indicates the direction of cell migration. Scale bars, 10 μm . **B**, Violin plot of filopodia angles relative
8 to the direction of migration for leader SCPs in control and alcama morphant embryos ($n=7$ SCPs for
9 each group, from 3 independent experiments; median \pm SD; ****, $P<0.0001$; Mann-Whitney test). **C**,
10 Filopodia length as a function of their relative angles for leader SCPs in control and alcama morphants.
11 **D**, Overlay of individual tracks of control and alcama-deficient SCPs during their migration towards the
12 CHT. In a trajectory plot, all (x, y) coordinates of the SCPs' starting points (in the clusters) are set to
13 (0,0) and negative or positive values on the Y axis indicate ventral-wards or dorsal-wards migration,
14 respectively. $n_{\text{Exp}}=4$, $n_{\text{embryos}}=6$, $n_{\text{tracked cells}}=19$ and 17 for control or alcama morphants, respectively.
15 **E**, **a**, Schematic representation of Alcama-FL-eGFP and Alcama- ΔN -eGFP mutant constructs. Pink and
16 blue ovals indicate the V-type and C2 type Ig-like domains, respectively. The beige rectangle represents

1 the short cytoplasmic domain. Light blue and green hexagons represent the signal peptide and GFP,
2 respectively. **b**, Appearance of Alcama- Δ N-eGFP⁺ SCPs at 23 hpf. White asterisk indicates the same
3 cell as shown in **c** and **d**. Dotted lines delineate somite and notochord borders. Scale bar, 20 μ m. **c**,
4 Morphological evolution over 15.5 hrs of the Alcama- Δ N-eGFP⁺ SCP shown in **b** (See also Movie 4).
5 Scale bar, 10 μ m. **d**, End point of the time-lapse imaging shown in (**b,c**) and Movie 4. GFP⁺ SCPs
6 marked by yellow asterisks did not migrate out of the somites, whereas RFP⁺/GFP⁻ WT SCPs showed
7 a normal emigration pattern exemplified by the three colored trajectories. A straight dotted line indicates
8 the notochord lower border. **F**, **a**, Schematic representation of Alcama-FL-eGFP and Alcama- Δ PDZ-
9 eGFP mutant constructs. The red X in the beige rectangle indicates the mutated PDZ binding motif in
10 the cytoplasmic domain. **b**, Appearance at 23 hpf of an Alcama- Δ PDZ-eGFP expressing VC cell
11 (asterisk) followed up over time in **c** (see also Movie 5). Scale bar, 20 μ m. **c**, Arrows point at large
12 contact areas between the GFP⁺ cell and neighboring RFP⁺ cells. **d**, End point of the time-lapse imaging
13 of the same embryo shown in **b**; the GFP⁺ SCP followed up in (**b,c**) had disappeared by 42 hpf, while
14 another such cell displayed short-distance migration (arrowhead). RFP⁺/GFP⁻ (WT) SCPs showed a
15 normal emigration pattern indicated by three colored trajectories. Straight and curvy dashes lines
16 delineate the ventral border of notochord and somites. **G**, Dorsal-wards migration of Alcama- Δ N-eGFP
17 and - Δ PDZ-eGFP expressing cells (arrows) by 27 hpf. Scale bars, 10 μ m. Magenta asterisks in **F-d** and
18 **G-b** label GFP⁺ notochord cells. **H**, Overlay of individual tracks of control (RFP⁺/GFP⁻ SCPs; orange and
19 blue lines) and Alcama- Δ N-eGFP expressing SCPs (black and red lines). $n_{Exp}=5$, $n_{embryos}=7$, $n_{tracked}$
20 $cells=29$ and 17 for Alcama- Δ N-eGFP⁺ and control RFP⁺/GFP⁻ SCPs, respectively. **I**, Overlay of individual
21 tracks of control (RFP⁺/GFP⁻ SCPs; orange and blue lines) and Alcama- Δ PDZ-eGFP⁺ SCPs (black and
22 red lines). $n_{Exp}=4$, $n_{embryos}=5$, $n_{tracked}$ $cells=32$ and 17 for Alcama- Δ PDZ-eGFP⁺ and control SCPs,
23 respectively. **J**, Frequency histogram of D/V migration patterns of Alcama- Δ N-eGFP⁺ and - Δ PDZ-eGFP⁺
24 cells compared with GFP⁺ cells of *Tg(cspg4:Gal4;UAS :GFP)* embryos at 38 hpf. The number of
25 embryos analyzed was 24 (WT), 36 (Alcama- Δ N) and 44 (Alcama- Δ PDZ) from three independent
26 experiments. nc, notochord.
27

Fig 3

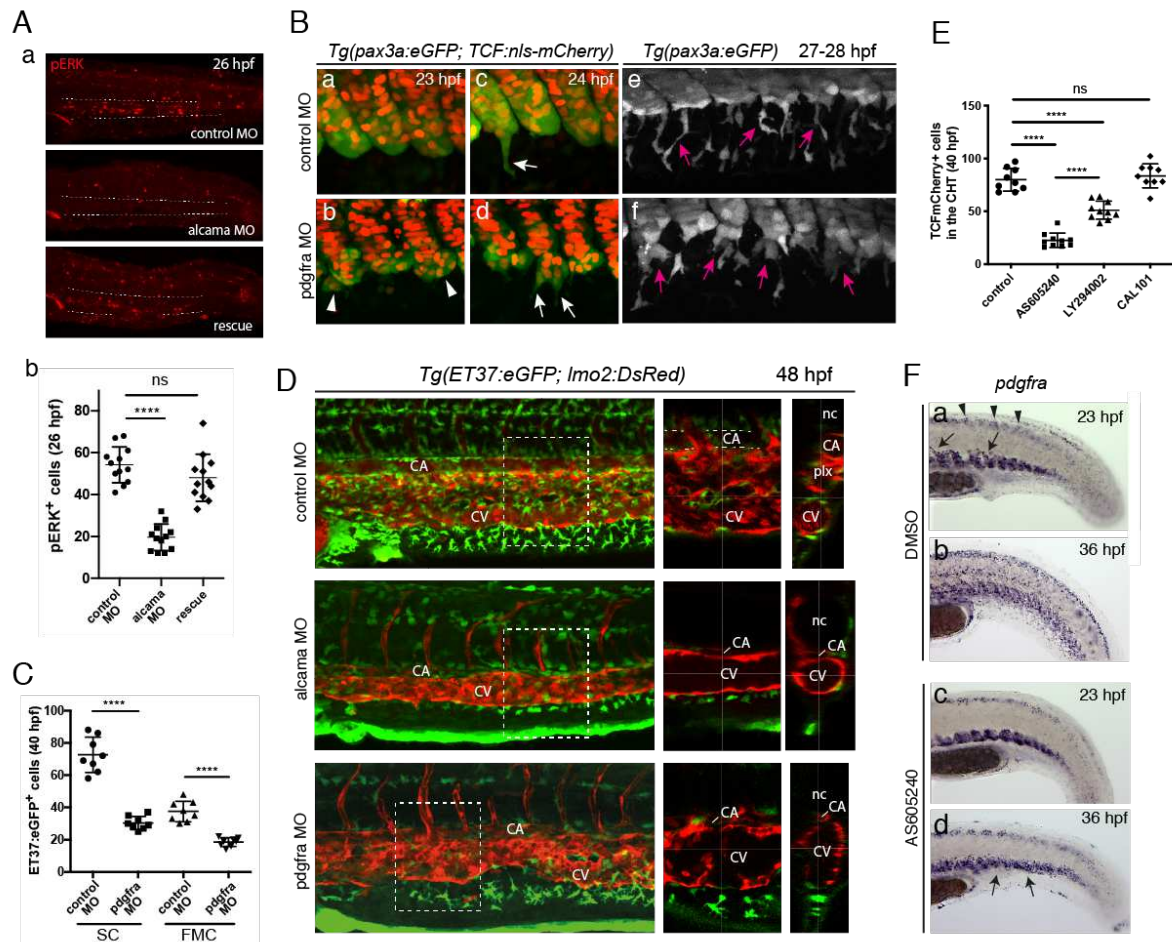
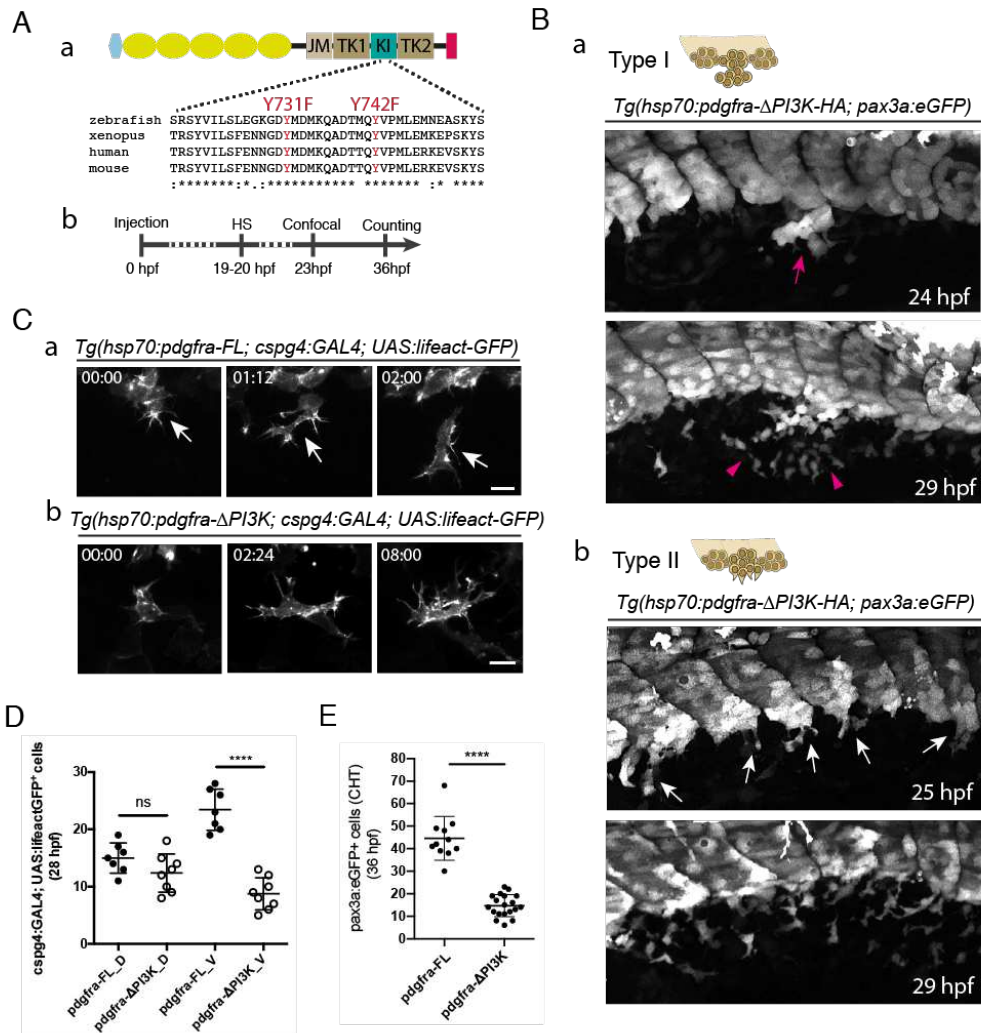


Figure 3. PDGFR α /PI3K signaling regulates SCP migration.

A, a, Immunofluorescence for pERK at 26 hpf in the tail region of embryos injected with control MO, alcama MO, or alcama MO + alcama mRNA (“rescue”). Representatives of 12 embryos from two independent experiments are shown. Dashed lines indicate the border of CHT. **b**, Quantification of **a** (n=12 embryos for each group; mean \pm SD; ****, $P < 0.0001$, Student's *t*-test). **B**, Confocal projections of live control (**a,c,e**) or *pdgfra* (**b,d,f**) MO injected *Tg(ET37:eGFP; TCF:nls-mCherry)* embryos from 23 to 28 hpf. Arrowheads point at cohesion defects observed in the VCs of *pdgfra* MO injected embryos. White and magenta arrows indicate emerging (**c,d**) and migrating (**e,f**) SCPs, respectively. **C**, Quantification of ET37:eGFP⁺ stromal cells and FMCs in control and *pdgfra* MO injected embryos at 40 hpf (n=8 for each group, from the same experiment; mean \pm SD; ****, $P < 0.0001$, Student's *t*-test). **D**, Confocal projections of control, alcama and *pdgfra* MO injected *Tg(ET37:eGFP; lmo2:DsRed)* embryos at 48 hpf. An enlarged view of the area enclosed by the dashed square is shown in the middle panel, and as an optical transverse section in the right panel. **E**, Quantification of TCF:nmCherry⁺ stromal cells in the CHT of control embryos (n=9), or embryos treated from 20 hpf with AS605240 (n=10), LY294002 (n=10), or CAL101 (n=9) at 40 hpf. Mean \pm SD; ****, $P < 0.0001$, Student's *t*-test. **F**, WISH for *pdgfra* at 23 and 36 hpf in 0.2 % DMSO (control) and AS605240-treated embryos. Arrows and arrowheads in **a** indicate VC cells migrating towards the notochord and dorsal mesenchymal cells in the control embryo at 23 hpf, respectively. Arrows in **d** show SCPs slightly dispersed around the VCs at 36 hpf. CA, caudal artery; CV, definitive caudal vein; Plx, venous plexus; nc, notochord.

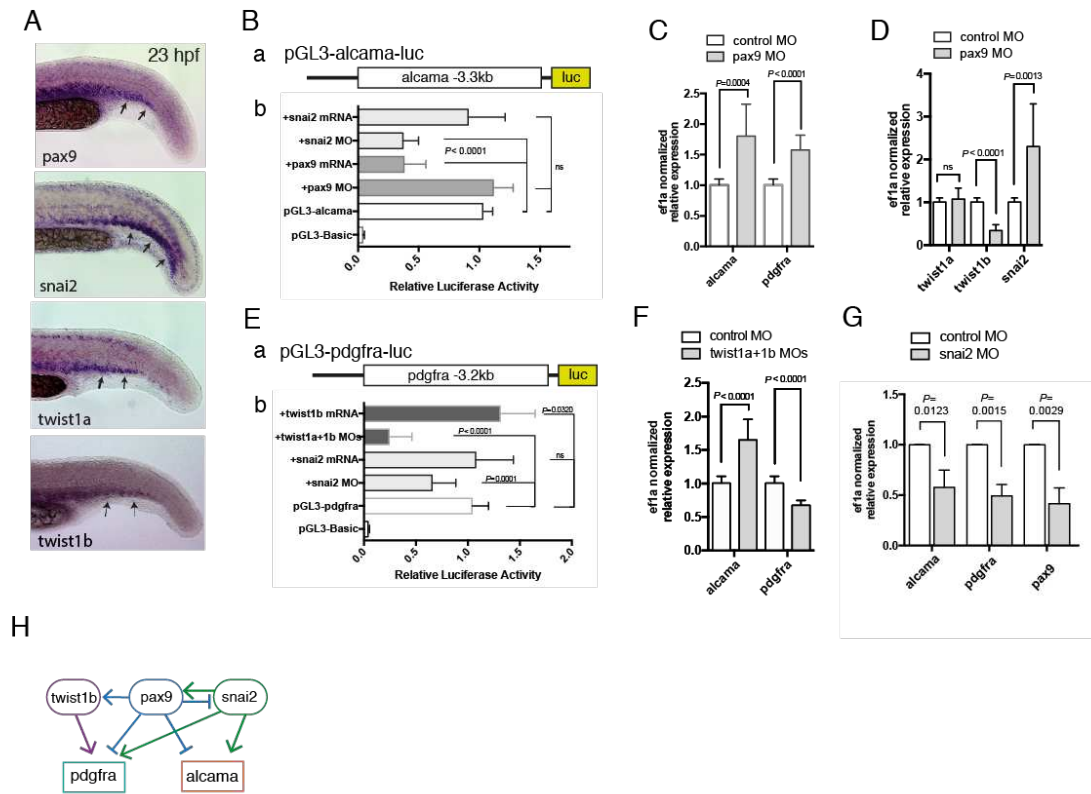
Fig. 4



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Figure 4. Pdgfra influences cluster cohesion and the following migration behaviour of SCPs through PI3K binding. **A. a**, Schematic representation of Pdgfra fused to tandem HA-tag (red) at its C-terminus, and amino acid alignment around the site of mutations in the KI (kinase insert) domain. The light blue hexagon and yellow ovals indicate the signal peptide and Ig-like extracellular domains, respectively; JM, juxtamembrane; TK1, tyrosine kinase domain 1; TK2, tyrosine kinase domain 2; tyrosine residues involved in PI3K binding when phosphorylated that we mutated into phenylalanines are shown in red. **b**, Time course of the experiment; HS, heat-shock. **B**. Confocal projections from live *Tg(hsp70:pdgfra-ΔPI3K; pax3a:eGFP)* embryos showing Type I (**a**) and Type II (**b**) cluster cohesion / emergence defects (see also Fig. S4B). Magenta arrow in **a** indicates SCPs overflow out of the cluster before the initiation of migration. This cell population was dispersed in situ after 5 hours (magenta arrowheads). **b**, Arrows indicate multiple SCPs emerging simultaneously from the cluster. **C**, Representative frames from a confocal time-lapse sequence of individual migrating SCP leader cell in *Tg(cspg4:GAL4; UAS:lifeact-eGFP)* embryos injected with the hsp70:pdgfra-FL (**a**) or hsp70:pdgfra-ΔPI3K (**b**) construct and heat-shocked at 20 hpf. Confocal imaging was performed from 23 hpf. Time is indicated in hrs and min. Scale bar, 25 μm. **D**. Quantification of *Tg(cspg4:GAL4; UAS:lifeact-eGFP⁺)* SCPs migrating dorsal-wards (D) or ventral-wards (V) in Pdgfra-FL (control) and Pdgfra-ΔPI3K expressing embryos at 28 hpf (mean±SD; n=7 embryos for control, n=8 for hsp70:pdgfra-ΔPI3K, from 3 independent experiments. ****, $P < 0.0001$, Student's *t*-test. **E**, Counting of *pax3a:eGFP⁺* SCPs in control and hsp70:pdgfra-ΔPI3K embryos in the CHT at 36 hpf, over a 5-somite width (mean±SD; n=11 for control, n=19 for hsp70:pdgfra-ΔPI3K, from 3 independent experiments. ****, $P < 0.0001$, Student's *t*-test.

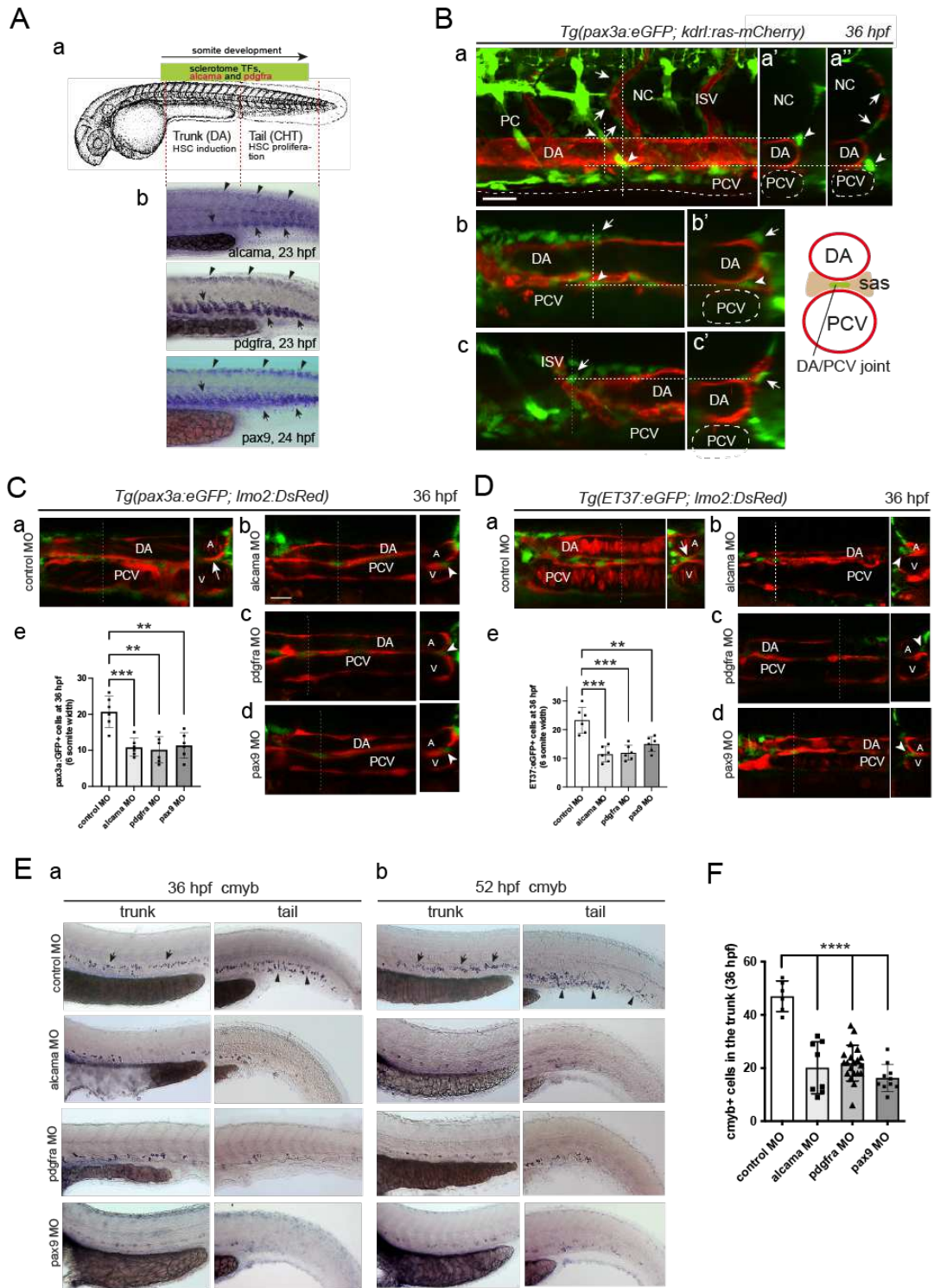
Fig. 5



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3 **Figure 5. *alcama* and *pdgfra* are regulated by sclerotome-associated TFs. A**, WISH for *pax9*, *snai2*,
 4 *twist1a* and *twist1b* at 23 hpf in the tail of WT embryos. Arrows point to expression of these genes in the
 5 somitic VCs. **B, a**, a DNA fragment encompassing the 3.3 kb region upstream of the of *alcama* gene
 6 transcription start site was inserted upstream of the luciferase (luc) coding region of the pGL3-Basic
 7 plasmid, to give pGL3-*alcama*-luc. **b**, in situ luciferase assays using pGL3-*alcama*-luc, which was
 8 injected at the 1-cell stage either alone or together with the indicated mRNA or MO; lysates obtained
 9 from 10-15 tails at 23 hpf for each condition were subjected to luciferase assay (n=9 for each, as
 10 biological triplicates from three independent experiments) or *pax9* (n=6 for each). The empty vector
 11 pGL3-Basic and pGL-*alcama*-luc (n=11 for each) were used as negative and positive controls,
 12 respectively. **C**, qPCR analysis for *alcama* and *pdgfra* in *pax9* morphants and control (n=9 for each, a
 13 set of biological triplicates from three independent experiments) at 23 hpf. **D**, qPCR analysis for *twist1a*,
 14 *twist1b* and *snai2* in *pax9* morphants and control (n=9 for each) at 23 hpf. **E, a** a DNA fragment covering
 15 the 3.2 kb region upstream of the *pdgfra* gene transcription start site was inserted upstream of the
 16 luciferase (luc) coding region of the pGL3-Basic plasmid, to give pGL3-*pdgfra*-luc. **b**, Same experimental
 17 scheme as in B-b, following injection of the indicated constructs and Mo or mRNA combinations. **F**,
 18 qPCR analysis for *alcama* and *pdgfra* (n=6 for each) in *twist1a*/*twist1b* double morphants and control
 19 (n=9) at 23 hpf. **G**, qPCR analysis for *alcama*, *pdgfra* and *pax9* in *snai2* morphants and control (n=3) at
 20 23 hpf (mean±SD). **H**, A proposed network for the regulation of TFs acting on *alcama* and *pdgfra* during
 21 SCP development. Line ending with arrow or bar represent activation or repression, respectively.
 22

Fig. 6



1

2 **Figure 6. *Alcama* and *Pdgfra* are also involved in stromal cell development in the trunk, which**
 3 **conditions the development of definitive HSPCs. **A**, a**, rostro-caudal extent of sclerotomal TFs and
 4 ***alcama* and *pdgfra* expression by 24 hpf. **b**, WISH for *alcama*, *pdgfra* and *pax9* at 23-24 hpf. Arrows**
 5 **point at their expression in the somite VCs/sclerotome in the trunk and tail. Arrowheads point at their**
 6 **expression in the smaller dorsal sclerotome³¹. **B**, Confocal maximum projection (**a**) and single confocal**

1 sections (**b,c**) of the trunk region of a *Tg(pax3a:eGFP; kdrl:ras-mCherry)* embryo at 36 hpf, with vertical
2 dashed lines showing the position of the optical transverse sections shown on the right (**a',a'',b',c'**).
3 Arrowheads point at GFP⁺ stromal cells located at the DA/notochord or DA/PCV junction, arrows point
4 at ISV-associated mesenchymal cells, known to originate from the dorsal-wards migration of ventral
5 sclerotome cells^{31,70}. Scale bar, 20 μ m. (**C,D**) Confocal sections of the trunk region of *Tg(pax3a:eGFP;*
6 *Imo2:DsRed)* (**C**) and *Tg(ET37:eGFP; Imo2:DsRed)* (**D**) embryos injected with control (**a**), alcama (**b**),
7 pdgfra (**c**) or pax9 (**d**) MOs. Dashed lines indicate the position of the corresponding transverse section
8 shown on the right. Arrows and arrowheads point at stromal cells located at the DA/PCV joint or
9 somewhat lateral to it, respectively. **e**, Quantification of pax3a:eGFP⁺ (**C**) or ET37:eGFP⁺ (**D**) cells in the
10 sub-aortic space of 36 hpf live embryos injected with control, alcama, pdgfra or pax9 MO (mean \pm SD;
11 n=6 embryos for each group. ***, $P \leq 0.001$; **, $P \leq 0.01$; Student's *t*-test). Counting was done over a 6-
12 somites width. **E**, WISH for *myb* at 36 (**a**) and 52 (**b**) hpf in embryos injected with control, alcama, pdgfra
13 or pax9 MO. Arrows and arrowheads point at myb⁺ signals in the trunk (arrows) and tail (arrowheads)
14 regions, respectively. **F**, Quantification of myb⁺ cells at 36 hpf as shown in **E-a** (mean \pm SD; n=6 embryos
15 for control, 8 for alcama, 21 for pdgfra and 10 for pax9 MO injected embryos. ****, $P \leq 0.0001$, Student's
16 *t*-test). DA or A, dorsal aorta; PCV or V, posterior cardinal vein; ISV, intersomitic vessel; sas, sub-aortic
17 space.

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- 6

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