



Figures and figure supplements

BMP and FGF signaling interact to pattern mesoderm by controlling basic helix-loop-helix transcription factor activity

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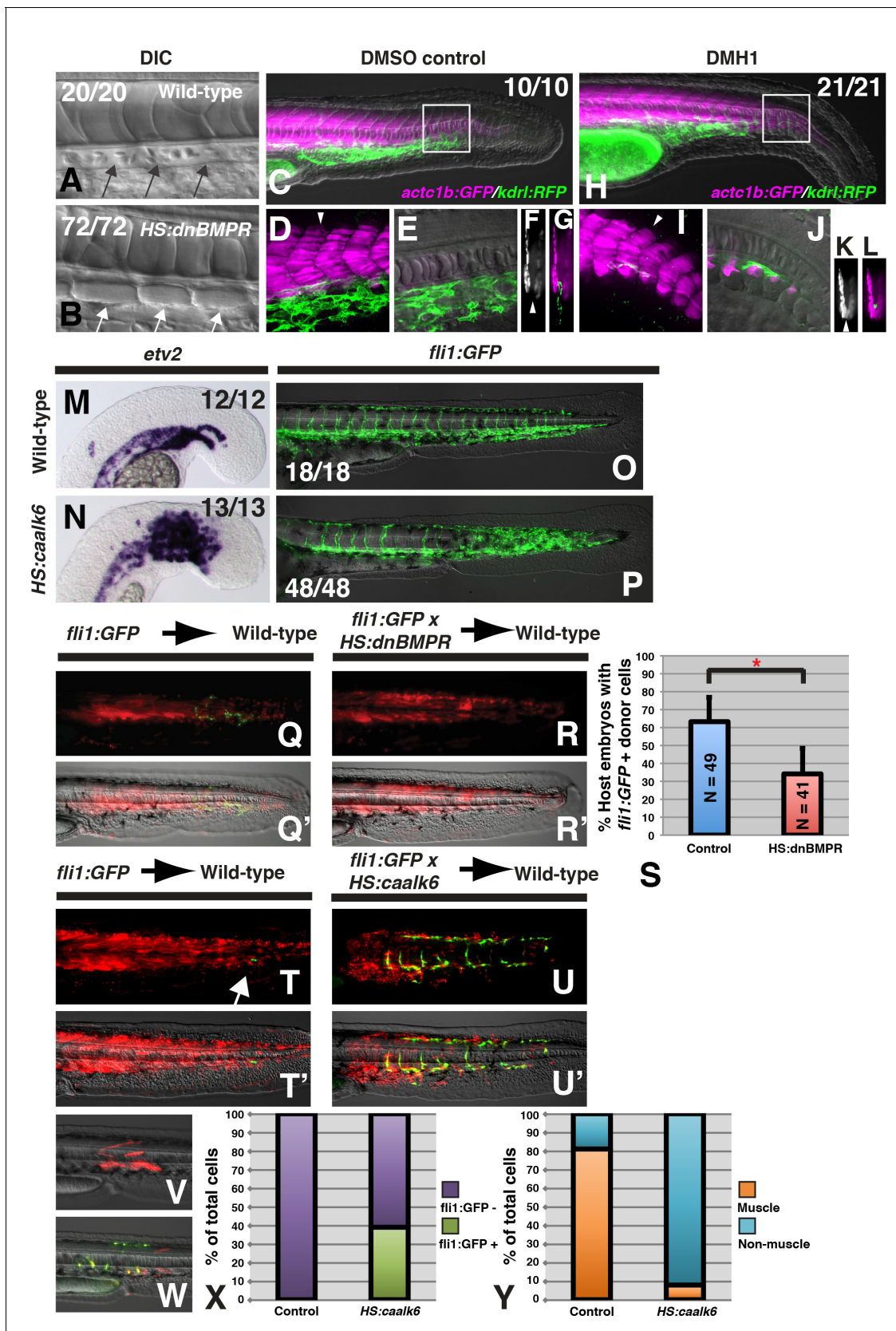


Figure 1. BMP signaling is necessary and sufficient for endothelial fate specification in tailbud-derived mesoderm. (A) Wild-type sibling embryos heat-shocked at the 12-somite stage exhibit normal formation of the dorsal aorta (black arrows, 20/20 normal). (B) *HS:dnbmpr* embryos heat-shocked at the Figure 1 continued on next page

Figure 1 continued

12-somite stage have ectopic segmented somite tissue where the dorsal aorta normally forms (white arrows, 72/72 with ectopic somite tissue). (C–L) Loss of BMP signaling using the small molecule DMH1 phenocopies *HS:dnbmpr* embryos. Embryos transgenic for both the *actc1b:GFP* (muscle, magenta) and *kdr:lrfp* (endothelium, green) transgenes were treated with DMSO (C–G) or DMH1 (H–L). A confocal Z-projection of the boxed region in C shows the presence of both muscle and endothelium in control DMSO treatment. A single z-slice at the midline shows the presence of endothelium and absence of muscle, which can also be observed in a digital cross section at the level of the white arrowhead in panel D. A confocal z-projection of the boxed region in H shows the presence of muscle and large reduction in endothelium (I). A single z-section at the midline shows the reduction of endothelium is accompanied by ectopic midline muscle formation, also observed in the digital cross-section at the level of the white arrowhead in panel I. (M, N) Transgenic *HS:caalk6* embryos heat-shocked at the 12-somite stage exhibit expansion of the endothelial marker *etv2* into the pre-somitic mesoderm 5 hr after the heat-shock (control N = 12, *HS:caalk6* N = 13). (O, P) At 36 hpf, *HS:caalk6* embryos heat-shocked at 12-somite stage have a dramatic expansion of *fli1:GFP* expression in posterior regions that would normally form somites, whereas there is no effect on anterior somites that formed before the heat-shock (Control N = 18, *HS:caalk6* N = 48). (Q–R') Rhodamine dextran (red) labeled *fli1:GFP* donor cells were transplanted into unlabeled wild-type host embryos to monitor for contribution of transplanted cells to endothelium. (Q, Q', S) Control cells contribute to endothelium in 63% of host embryos (N = 49). (R, R', S) Heat-shock induction of *dnbmpr* at the 12-somite stage significantly ($p=0.0107$) reduces the percentage of host embryos (34%) that have donor-derived endothelium (N = 41). (T–U') Induction of endothelium by BMP signaling is cell-autonomous, as exhibited in *HS:caalk6* x *fli1:GFP* cells transplanted wild-type host embryos. Host embryos were heat-shocked at the 12-somite stage and assayed for *fli1:GFP* expression at 36 hpf. (U, U') *HS:caalk6* transgenic cells do not contribute to somites and instead give rise to endothelium. One-cell transplants were done to quantify fate changes after BMP activation (W) compared to controls (V). 12-somite stage BMP activation resulted in 39% *fli:GFP* positive cells (four embryos, 49 cells), compared to 0% in control transplants (three embryos, 36 cells, $p<0.0001$) (X). The fate of control transplanted cells was 81% muscle, whereas only 8% of *HS:caalk6* cells adopted a muscle fate ($p<0.0001$) (Y). All embryos are pictured from a lateral view with the head to the left, except for F, G, K, and L which are digital transverse sections.

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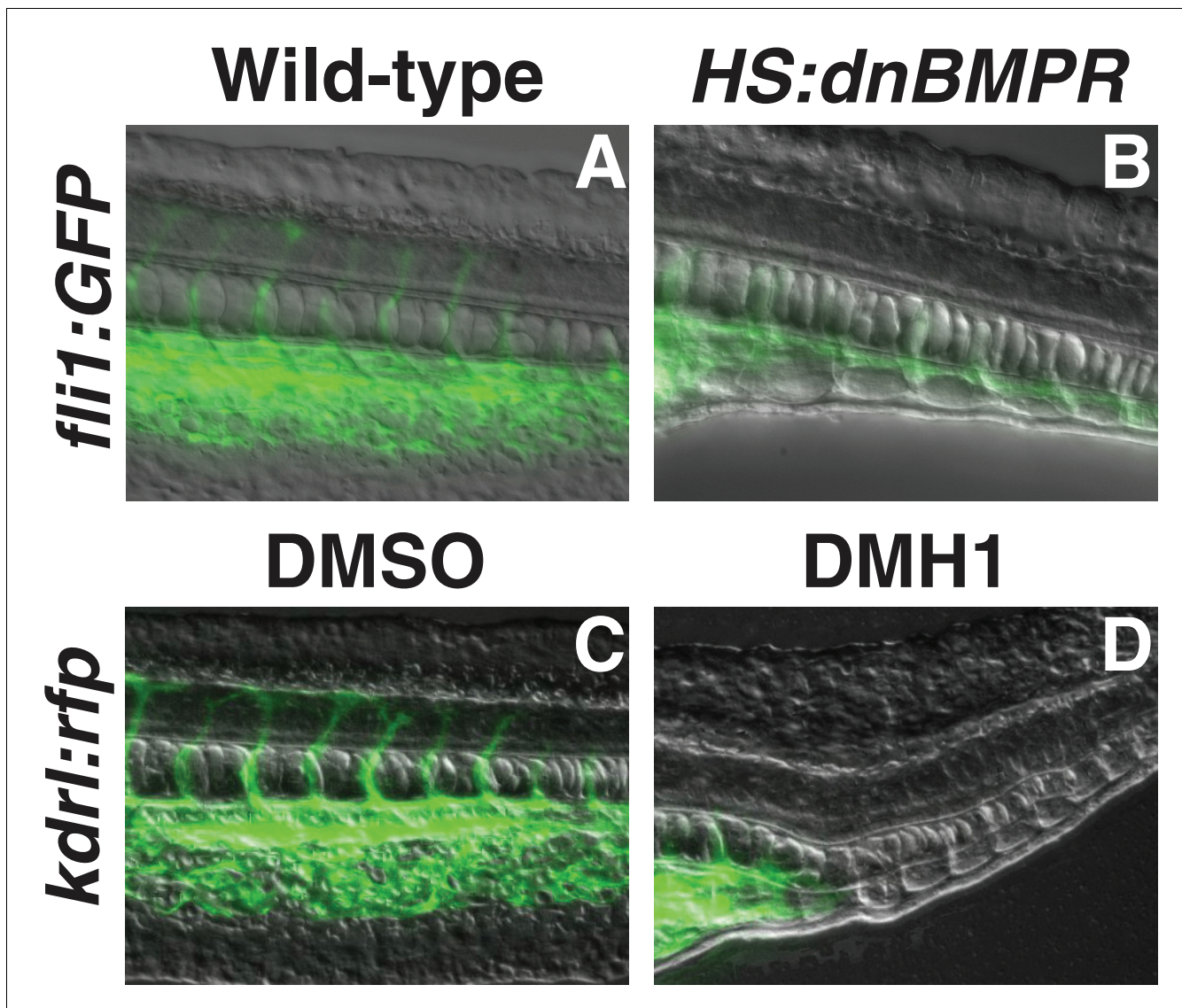


Figure 1—figure supplement 1. BMP signaling inhibition at bud stage medializes tail mesoderm. Transgenic *fli1:gfp* embryos were heat-shocked at the bud stage. Embryos that also had the *HS:dnbmpr* transgene exhibited a loss of endothelium and gain of somite tissue compared to control embryos (B compared to A). Similarly, *kdr1:GFP* embryos treated with the BMP inhibitor DMH1 showed a loss of endothelium and expansion of ectopic somite tissue compared to DMSO-treated control embryos (D compared to C).

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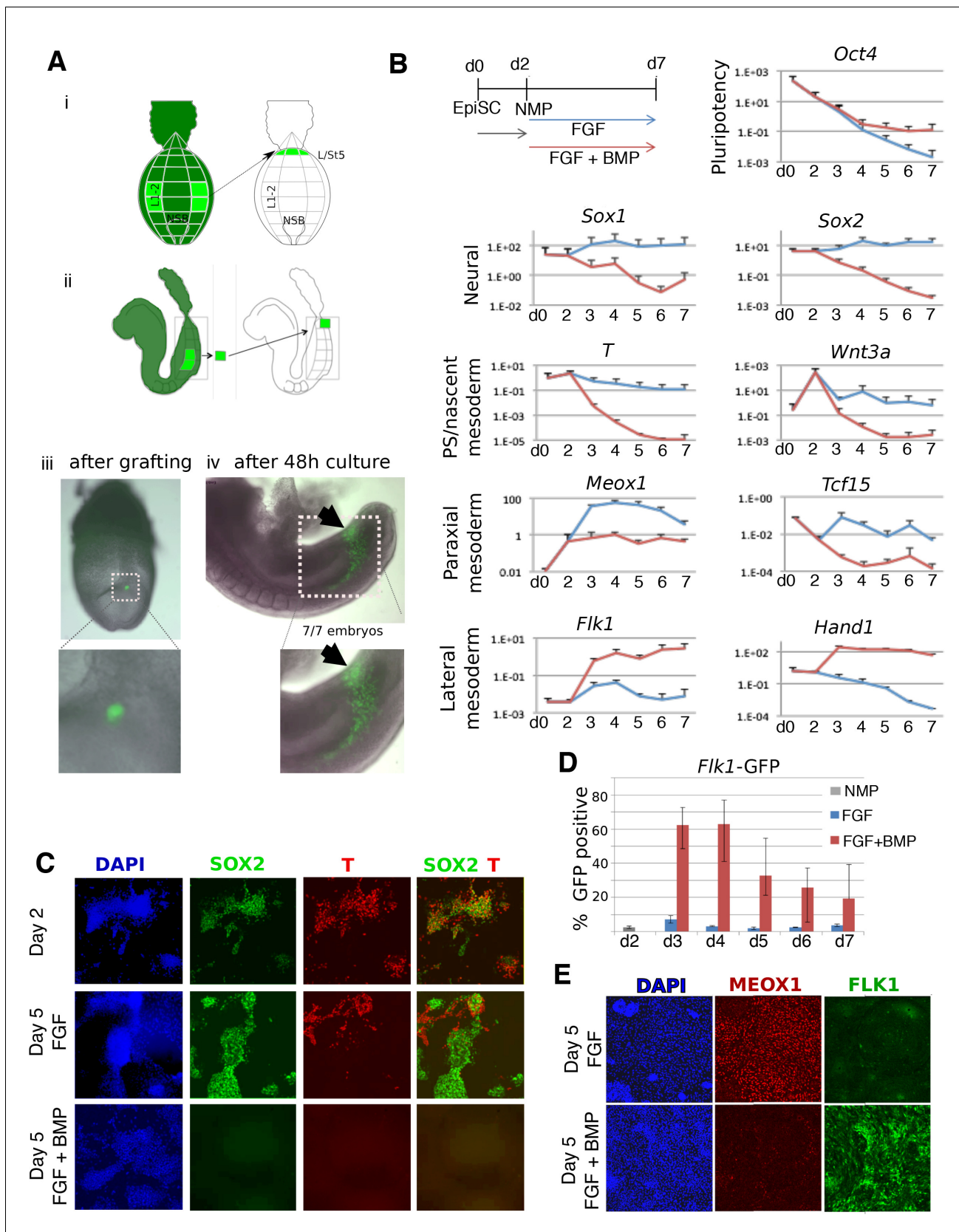


Figure 2. BMP redirects fate of mouse NMPs from paraxial to lateral mesoderm. (A) Heterotopic grafting from ubiquitous GFP embryos (Gilchrist et al., 2003) of NMP region fated for paraxial mesoderm at 2–5 somite stage (E8.0) into the posterior primitive streak region fated for lateral and ventral

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mesoderm, followed by 48 hr culture. (i) Posterior view (ii) Lateral view (iii) Representative embryo immediately after grafting showing position of GFP +grafted cells (iv) Representative embryo after 48 culture showing that descendants of grafted cells have adopted a lateral fate (arrowheads). (B) qPCR at indicated time points during the differentiation of EpiSCs into NMPs then treated with FGF2 or FGF2 +BMP4. Data shown relative to the housekeeping gene TBP. (C) Immunofluorescence detection of indicated markers in in vitro derived NMPs and their differentiating derivatives. (D) Flow cytometry of *Flk1-GFP* in the differentiating derivatives of in-vitro derived NMPs. (E) Immunofluorescence detection of indicated markers in the differentiating derivatives of in-vitro derived NMPs.

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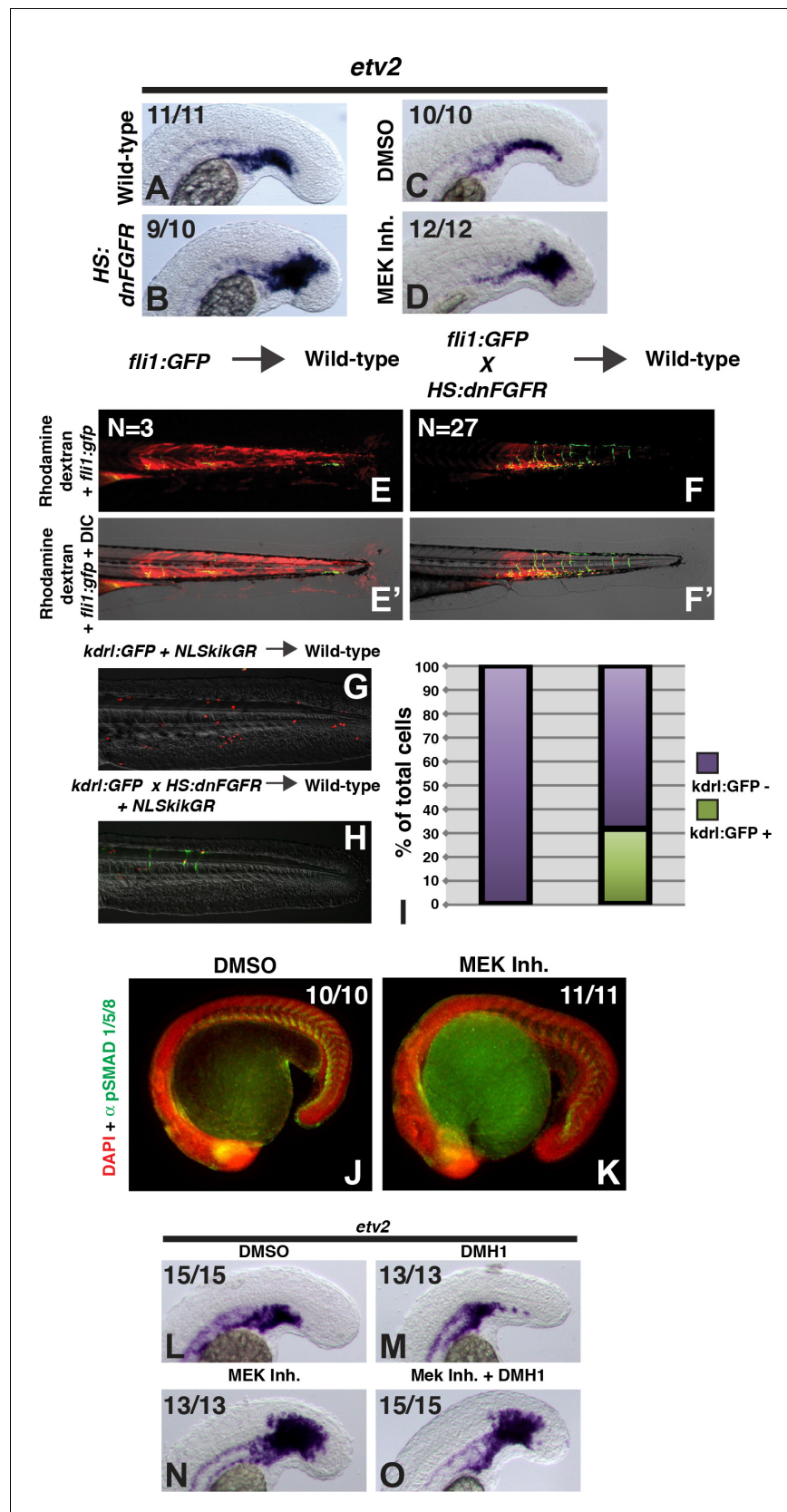


Figure 3. FGF signaling is necessary to maintain paraxial mesoderm fate and inhibit a default endothelial fate. Heat-shock induction of *dnfgfr* (B) or treatment with a MEK inhibitor (D) at the 12-somite stage causes an

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expansion of the endothelial marker *etv2* into the pre-somitic mesoderm 5 hr later compared to controls (A, C). (F, F') Transplanted *HS:dnfgr* x *fli1:GFP* show a cell-autonomous shift from somite to endothelial fate when heat-shocked at the 12-somite stage, whereas *fli1:GFP* transplants mostly contribute to muscle with minor endothelial contribution (E, E'). The same effect is seen with *HS:dnfgr* x *kdr1:GFP* transplanted cells when heat-shocked at the 12-somite stage (G, H). *NLS-kikume* was injected into donor embryos to quantify cell fate changes. 12-somite stage FGF inhibition resulted in 31% *kdr1:GFP*-positive cells (13 embryos, 308 cells), compared to 0% in control transplants (seven embryos, 587 cells, $p < 0.0001$) (I). Expansion of endothelium 5 hr after MEK inhibitor treatment is not due to an expansion of BMP signaling, as revealed by pSMAD 1/5/8 staining (K compared to J, green staining, red color is DAPI staining). (L–O) Similarly, treatment with the BMP inhibitor DMH1 does not prevent MEK-inhibitor-induced expansion of endothelium. Embryos were treated at the 12-somite stage and fixed 6 hr later. The expansion of the endothelial marker *etv2* into the PSM after MEK inhibitor treatment (N) is not inhibited by the addition of DMH1 (O).

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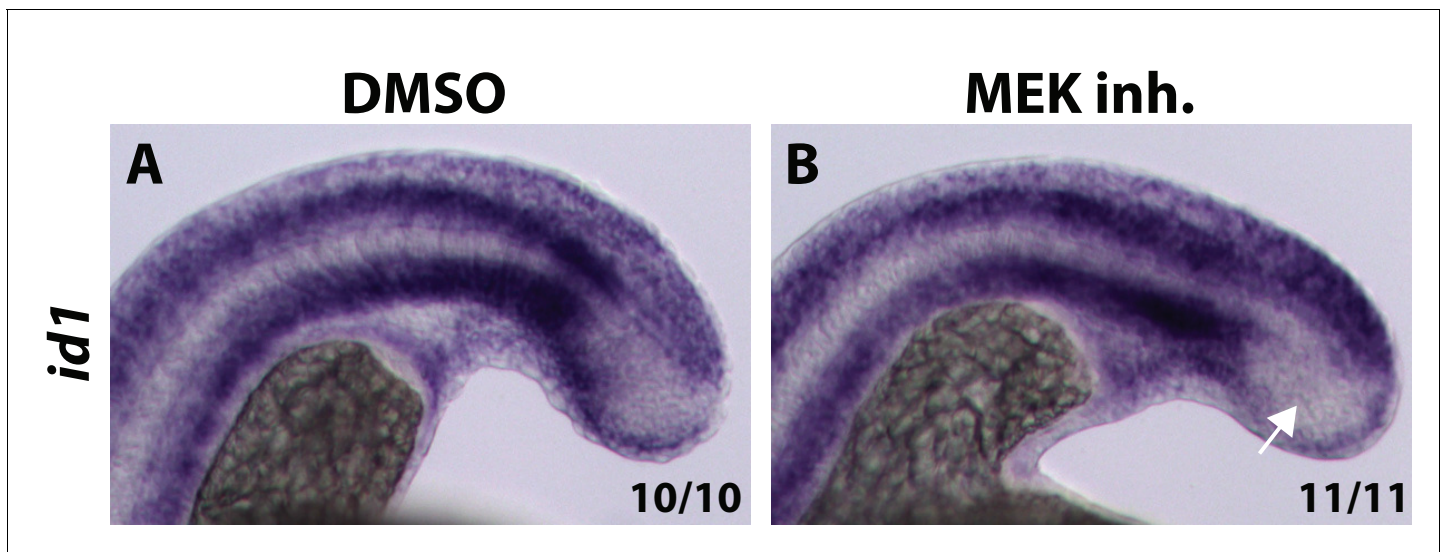


Figure 3—figure supplement 1. MEK inhibitor does not cause an expansion of *id1* expression into the PSM. Embryos were treated with a MEK inhibitor at the 12-somite stage and fixed and analyzed for *id1* expression six hours later. The expression of *id1*, which is a direct BMP target gene, does not expand into the PSM after MEK inhibition (B, arrow, compared to A).

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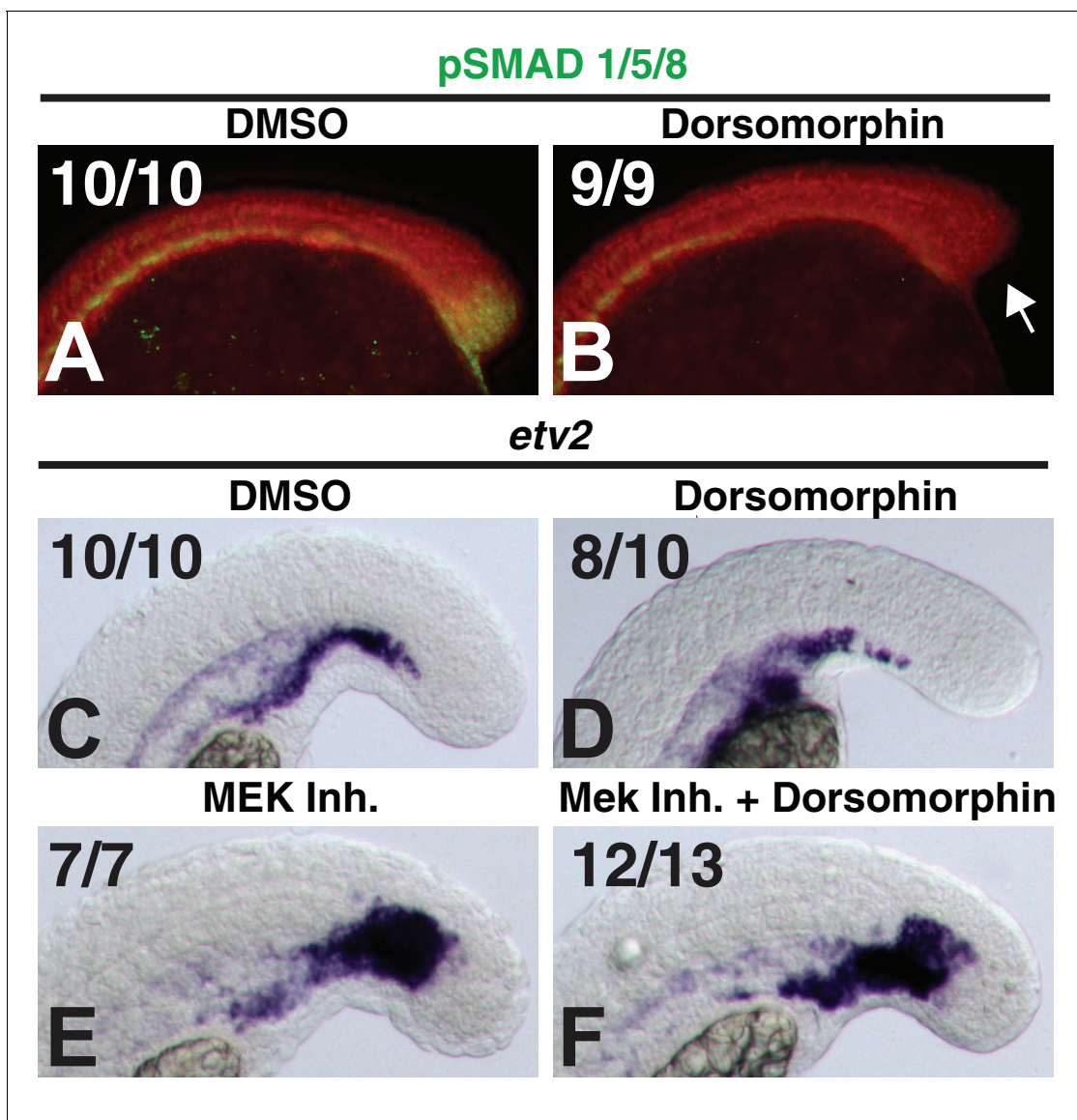


Figure 3—figure supplement 2. Dorsomorphin does not rescue MEK-inhibitor-induced endothelial expansion. Embryos were treated at bud stage with dorsomorphin and a subset of them were assayed at the 12-somite stage for the loss of pSMAD 1/5/8 staining in the tailbud (A, B, arrow indicates loss of pSMAD staining, red color is DAPI staining). (C–F) A subset of the remaining embryos were treated at the 12-somite stage with the MEK inhibitor, fixed 6 hr later and stained for mRNA expression of the endothelial marker *etv2*. Dorsomorphin did not rescue MEK-inhibitor-induced expansion of endothelium (F compared to E).

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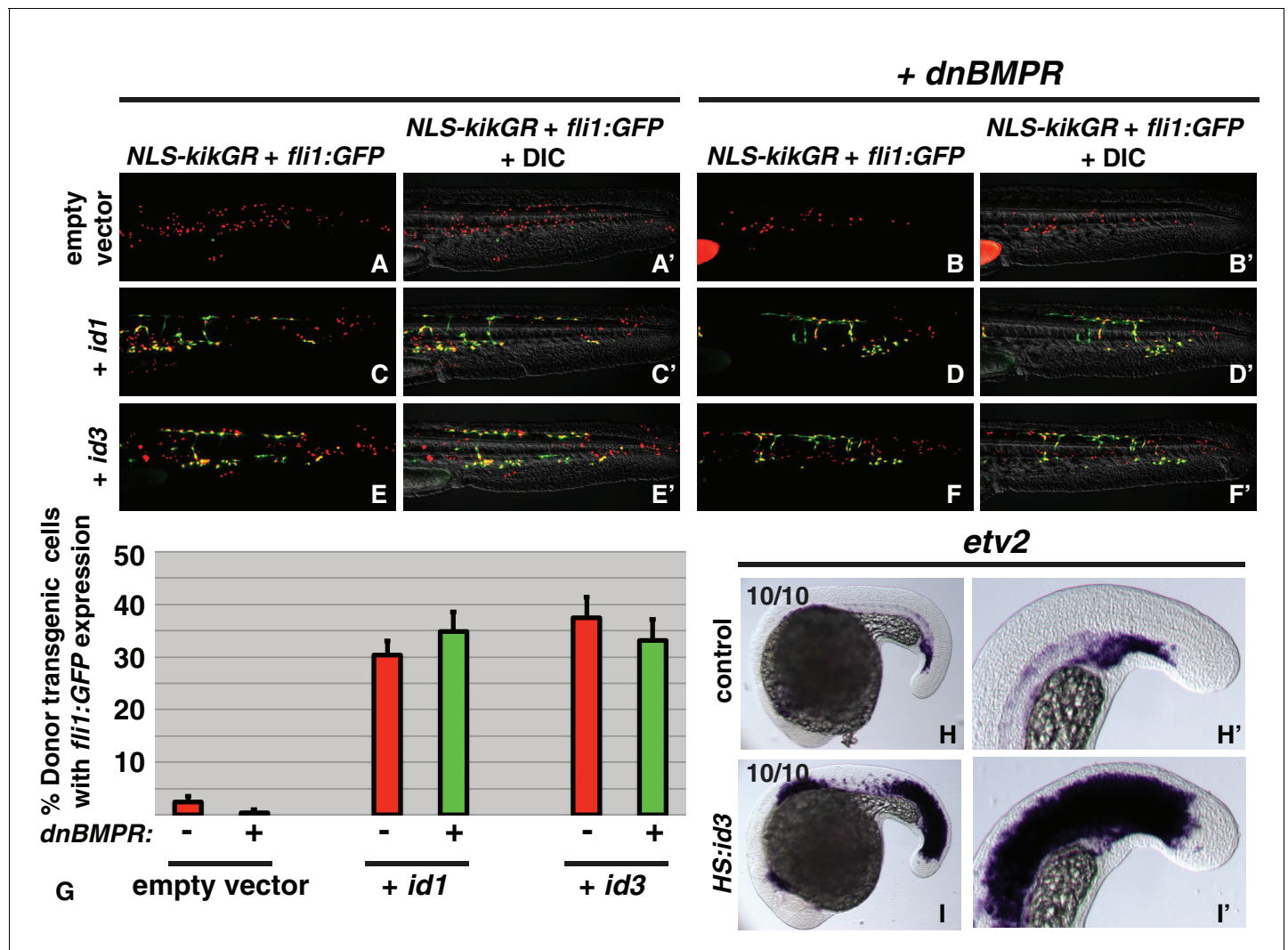
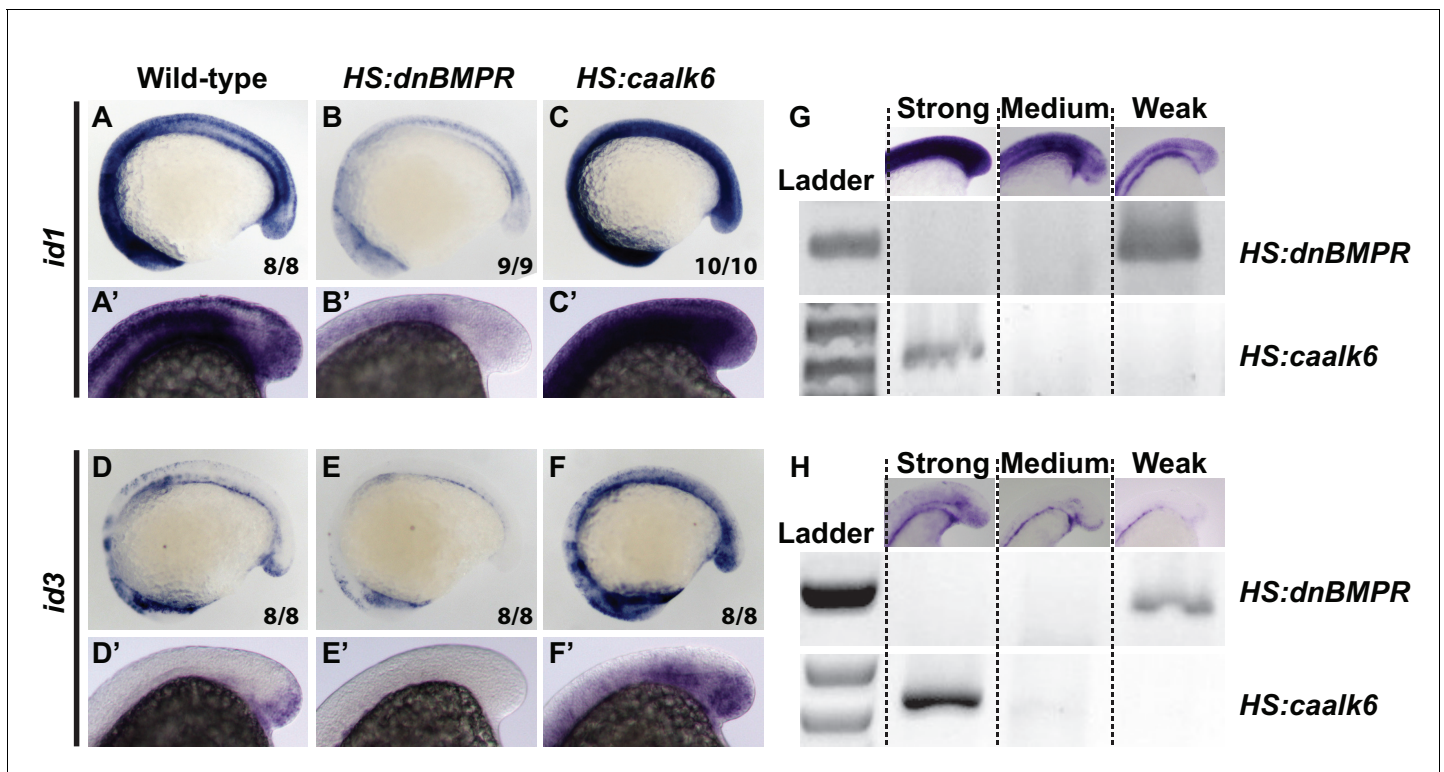


Figure 4. *id* genes are the essential BMP targets mediating endothelial induction. An assay was developed to quantify the percent of transplanted cells that adopt an endothelial fate (see text for details). Control cells transplanted to the ventral margin of host embryos and heat-shocked at the 12-somite stage exhibit a small percentage contribution to endothelium (green cells, A, A', G, empty vector $N^{\text{embryos}} = 19$, $N^{\text{cells}} = 500$), which is significantly reduced when BMP signaling is inhibited in transplanted cells (B, B', G, empty vector + *dnbmp* $N^{\text{embryos}} = 19$, $N^{\text{cells}} = 1022$, $p = 0.006$). Activation of *id1* or *id3* causes a significantly larger percentage of transplanted cells to adopt an endothelial fate (C, C', E, E', G, *id1* $N^{\text{embryos}} = 16$, $N^{\text{cells}} = 1159$, $p < 0.0001$, *id3* $N^{\text{embryos}} = 18$, $N^{\text{cells}} = 574$, $p < 0.0001$), and this effect is unchanged in cells that also lack BMP signaling (D, D', F, F', G, *id1* + *dnbmp* $N^{\text{embryos}} = 16$, $N^{\text{cells}} = 614$, $p < 0.0001$, *id3* + *dnbmp* $N^{\text{embryos}} = 12$, $N^{\text{cells}} = 531$, $p < 0.0001$). Cell fate quantification from these experiments is represented in panel G. A stable *HS:id3* transgenic line heat-shocked at the 12-somite stage and fixed 5 hr later exhibits a large expansion of the endothelial marker *etv2* (I, I') compared to heat-shocked wild-type embryos (H, H').

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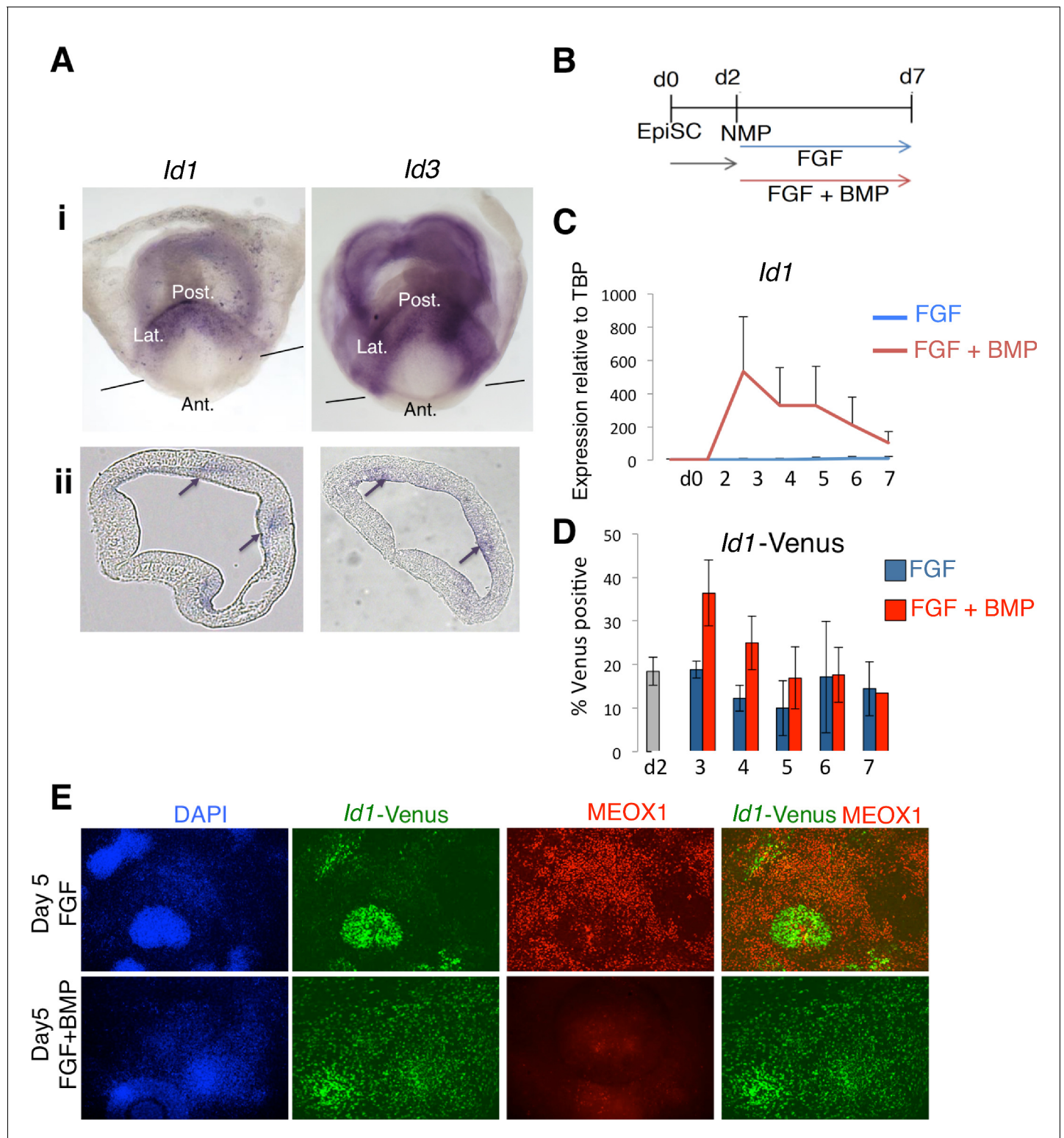


Figure 5. *Id1* and *Id3* are expressed in prospective lateral/ventral mesoderm at early somite stages in vivo, and are induced by BMP in NMPs in vitro. (A) In situ hybridisation for *Id1* and *Id3* in wholemount (i) and sections (ii) showing *Id1* and *Id3* expression restricted to the posterior (labelled 'Post.') and lateral (labelled 'Lat.') regions of the primitive streak. *Id1/3* are not detected in the anterior primitive streak (labelled 'Ant'). Lines in (i) indicate the plane of section. Arrows in (ii) indicate regions of expression in the posterior lateral regions of the primitive streak. (B) In vitro differentiation protocol. (C) *Id1* mRNA is expressed in response to BMP4 but not FGF2 during differentiation of NMP in culture (D) an *Id1*-Venus reporter is activated in response to

Figure 5 continued on next page

Figure 5 continued

BMP4 but not FGF2 during differentiation of NMP in culture (E) Immunofluorescence for indicated markers during differentiation of NMP in culture: expression of MEOX1 is mutually exclusive from expression of ID1-Venus, and is suppressed by addition of BMP4.

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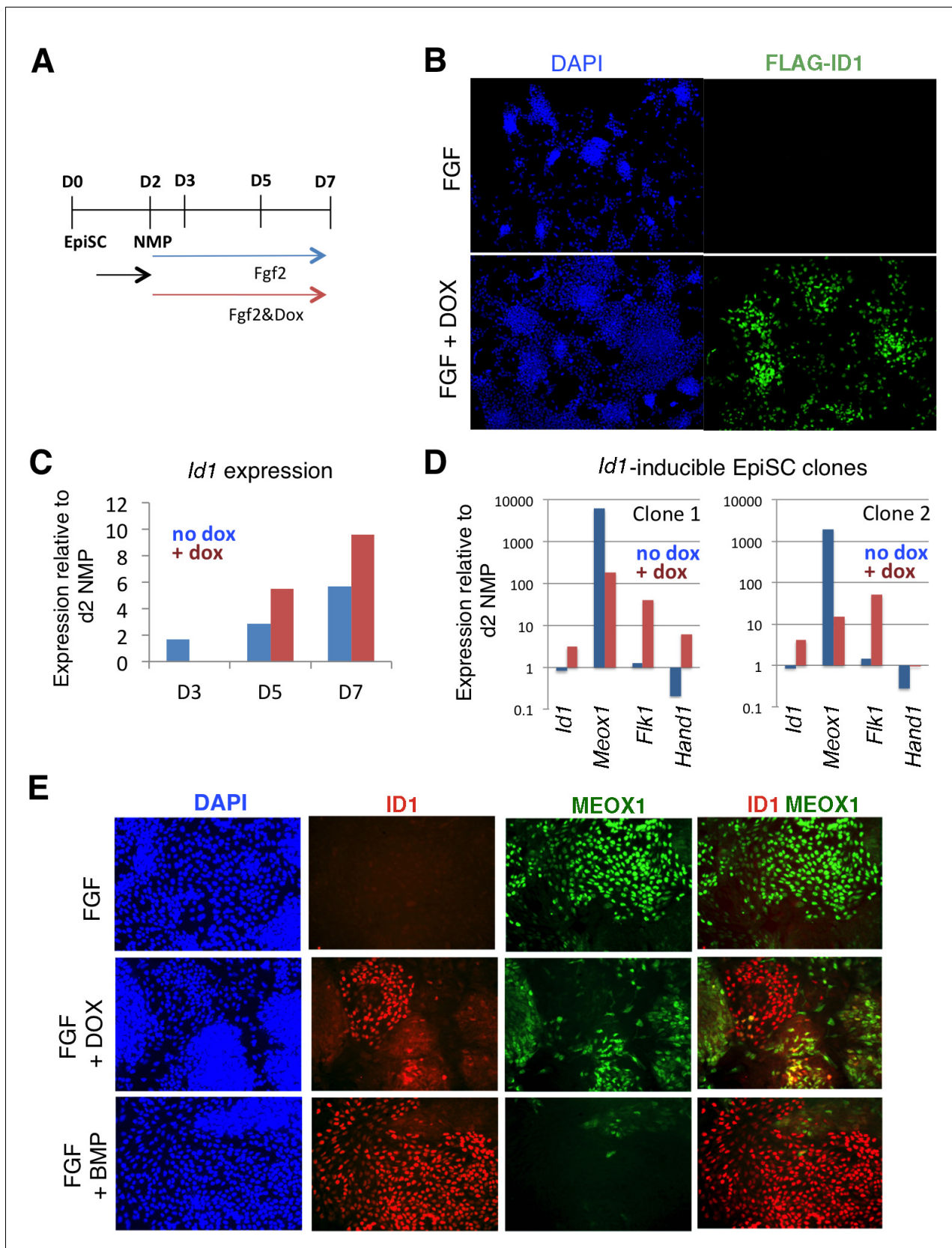


Figure 6. *Id1* GOF drives differentiation of lateral mesoderm at the expense of paraxial mesoderm. A: Differentiation protocol. B: Immunofluorescence detection for the Flag epitope in *Flag-Id1* inducible EpiSC indicates that addition of dox induces Flag-ID1 in a subset of cells. C: qPCR to detect *Id1*

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mRNA in the absence and presence of dox in *Flag-Id1* inducible EpiSC. D: qPCR to detect the indicated mesoderm markers in *Flag-Id1* inducible EpiSC the absence and presence of dox: data from two independent clonal lines is shown. D: Immunofluorescence detection of indicated markers: induction of *Id1* suppresses expression of MEOX1, recapitulating the effect of adding BMP4.

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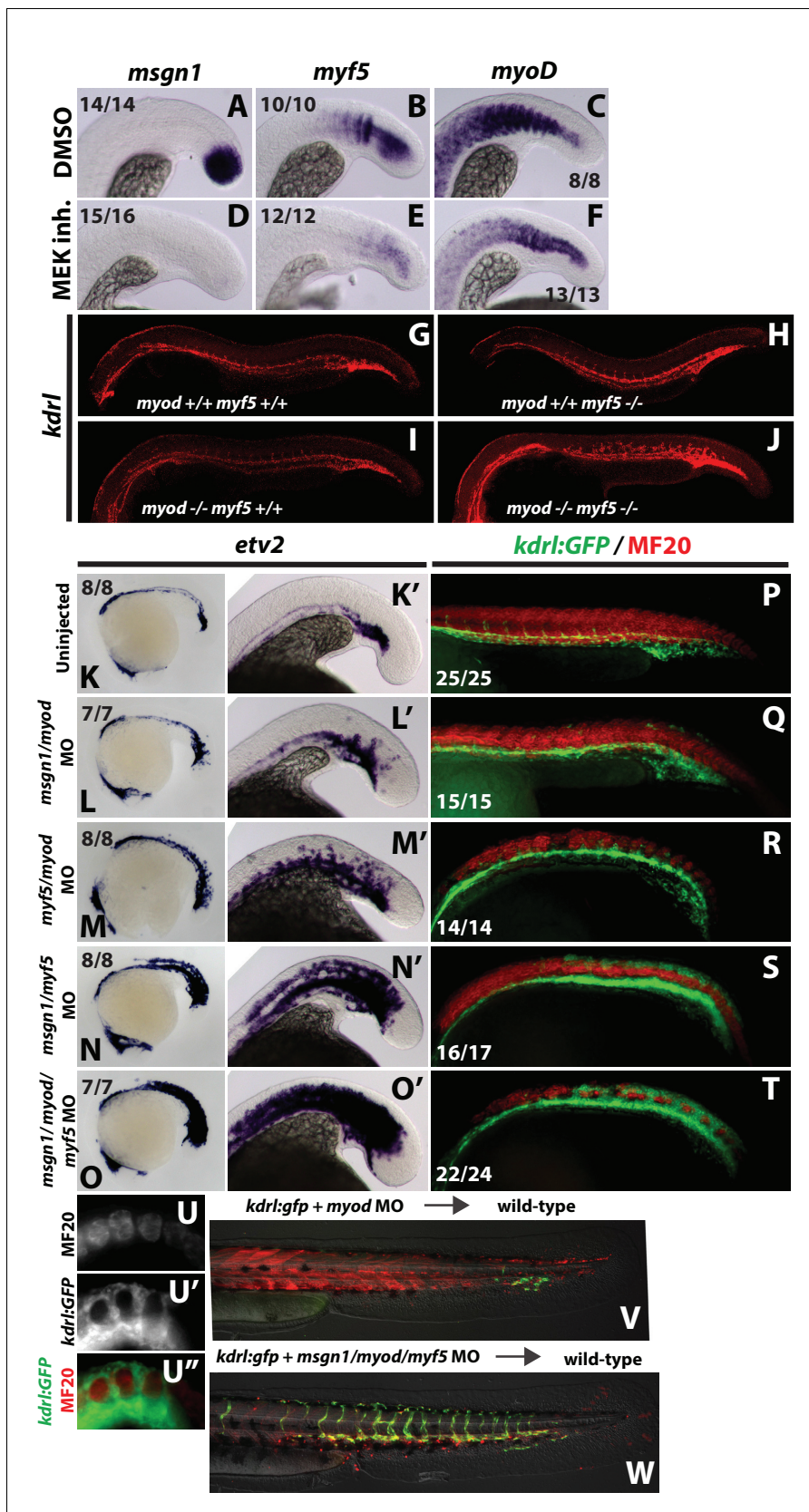


Figure 7. FGF signaling maintains paraxial mesoderm fate and inhibits endothelial fate through positive regulation of bHLH transcription factors. Wild-type embryos were treated with the MEK inhibitor or DMSO at the 12-somite stage and fixed five hours later. Expression of *msgn1* (D) and *myf5* (E) Figure 7 continued on next page

Figure 7 continued

were significantly downregulated compared to controls (**A**, **B**), whereas *myod* (**F**) exhibited only a minor reduction in expression compared to controls (**C**). Expression of *kdr1* (red) is expanded into somitic territories in *myod;myf5* double mutants compared to controls (**G**). $n = 44$ controls (pooled $+/+;+/+$, $+/+;+/-$; $+/-;+/-$, and $+/-;±$ genotyped embryos). 0/44 controls have expanded *kdr1*. $n = 4$ mutants ($-/-;-/-$ genotyped embryos). 4/4 show expanded *kdr1* (representative embryos shown). MO-mediated loss of function of *msgn1/myf5* (**L**, **L'**) or *myf5/myod* (**M**, **M'**) results in a moderate expansion of *etv2* expression at the 22 somite stage, whereas loss of *msgn1/myf5* causes a broad expansion of *etv2* (**N**, **N'**). Loss of function of all three genes further enhances *etv2* expansion (**O**, **O'**). MF20 (muscle, red) antibody staining in 30 hpf *kdr1:GFP* embryos demonstrates the gain of differentiated vasculature at the expense of differentiated muscle (**P–T**). U–U'' are high-magnification views of MF20 staining and *kdr1:GFP* expression in a *msgn1/myf5* loss of function embryo. Transplanted *kdr1:GFP* cells lacking *myod/myf5/msgn1* fail to join host somites and instead contribute predominantly to endothelium (**W**), whereas cells lacking *myod* behave normally, with most transplanted cells joining the somites and forming muscle (**V**).

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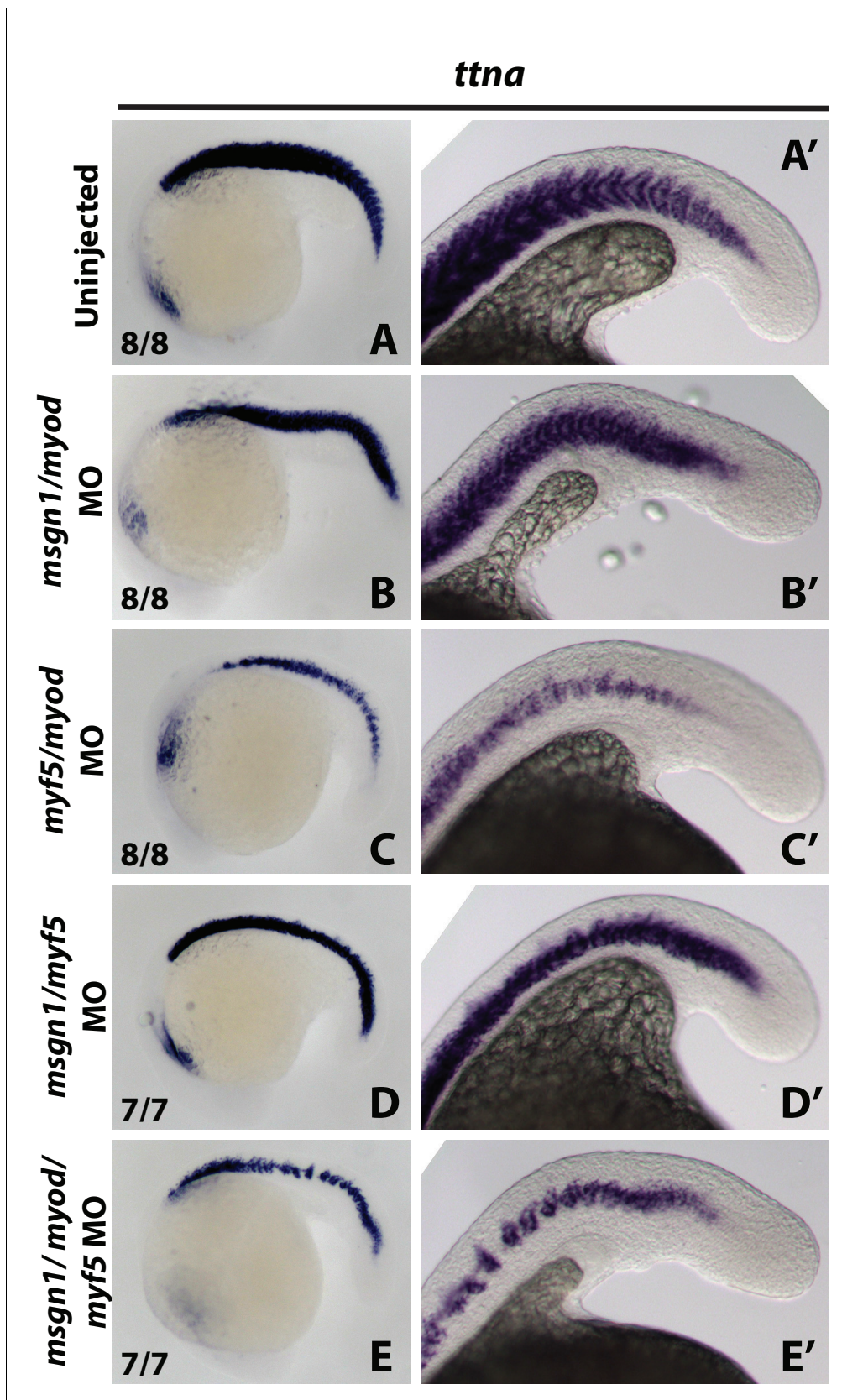


Figure 7—figure supplement 1. bHLH transcription factor knockdown inhibits skeletal muscle specification. A probe for *titin-a* (*ttna*) was used to label differentiating cardiac and skeletal muscle at the 22-somite stage. Loss of *msgn1* and *myod* function produced only a minor loss of skeletal muscle. Figure 7—figure supplement 1 continued on next page

Figure 7—figure supplement 1 continued

Loss of *myf5* and *myod*, *msgn1* and *myf5*, or *msgn1*, *myod*, and *myf5* caused a substantial loss of skeletal muscle, with the triple knockdown having the strongest effect.

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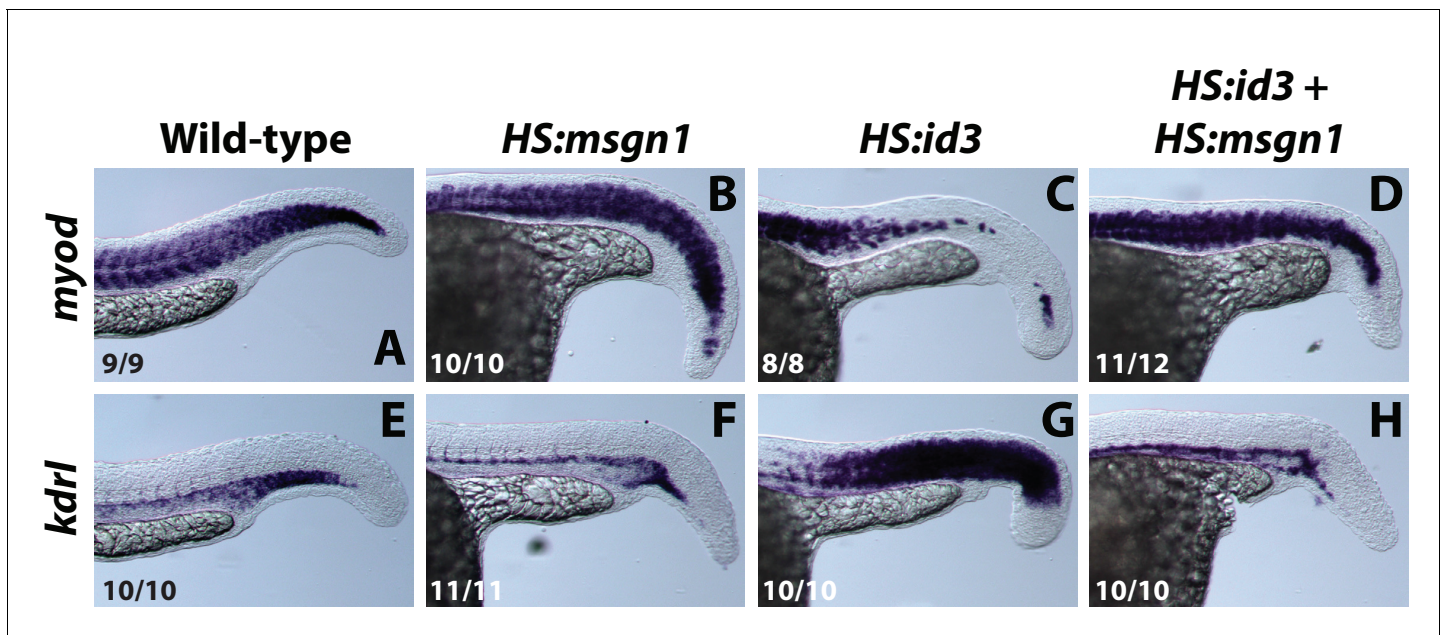


Figure 7—figure supplement 2. Over-expression of *msgn1* rescues *id3* over-expression. *HS:id3* and *HS:msgn1* lines were crossed to each other and heat-shocked at the 12-somite stage and fixed at 24 hpf for analysis of *myod* and *kdrl* expression. The activation of *msgn1* alone results in relatively normal *myod* expression and a posterior loss of *kdrl*. Activation of *id3* causes a strong loss of posterior *myod* expression and gain of *kdrl* expression throughout regions where somites normally form. Activation of *id3* and *msgn1* largely restores normal patterning of muscle and vasculature.

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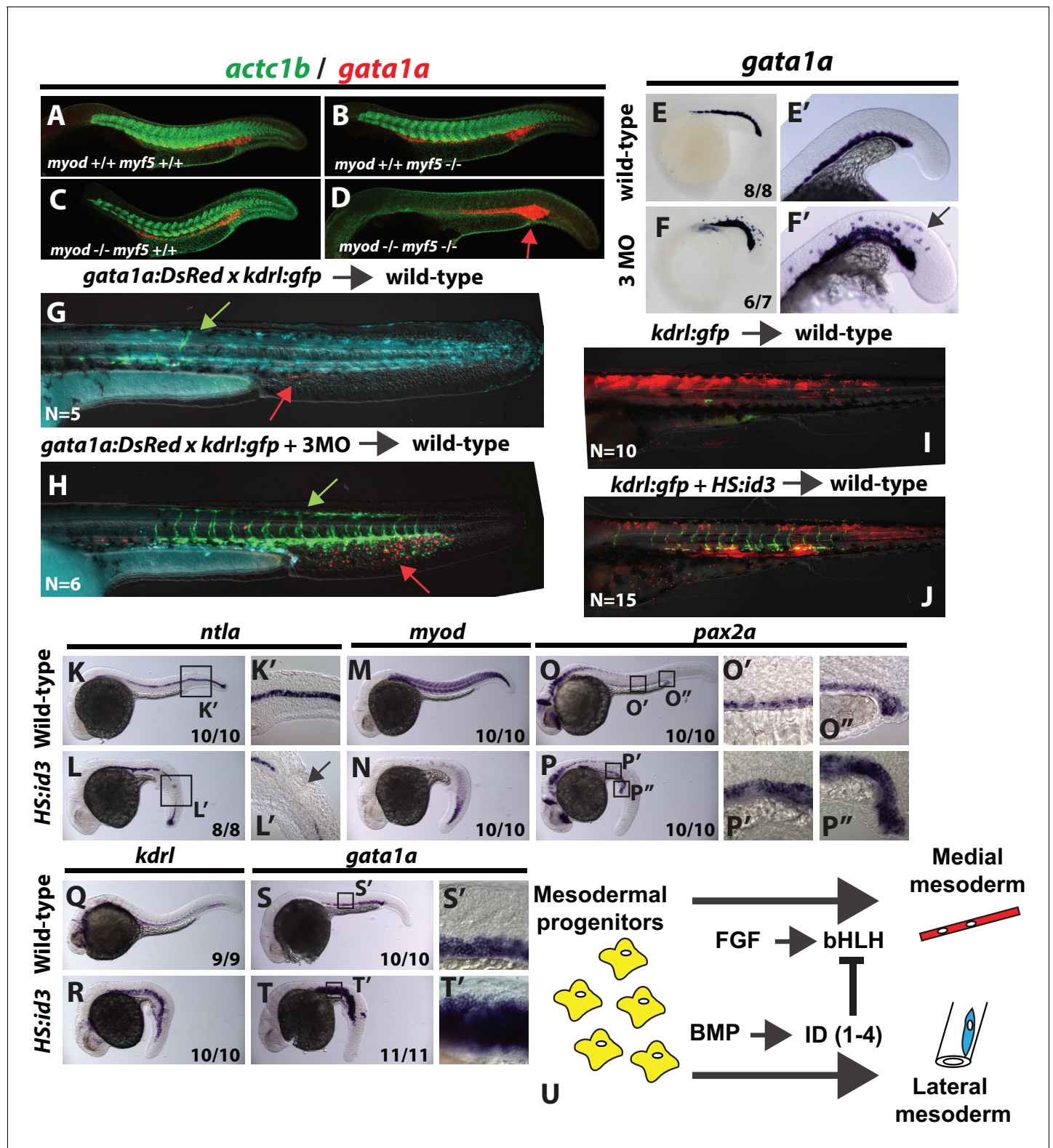


Figure 8. bHLH transcription factor activity provides mediolateral pattern to the entire mesodermal germ layer. Homozygous *myod;myf5* mutant embryos exhibit slightly expanded *gata1a* expression (D, red staining, arrow) and a complete loss of skeletal muscle marker *actc1b* (green staining). $n = 49$ controls (pooled $+/+;+/+$, $+/+;+/-$, $+/-;+/+$, and $+/-;+/-$ genotyped embryos). 49/49 controls show normal *actc1b*, 0/49 controls have expanded *gata1a*. $n = 9$ mutants ($-/-;-/-$ genotyped embryos). 9/9 show loss of *actc1b*, 7/9 show expanded *gata1a* (representative embryos shown). Loss of *msgn1/myod/myf5* function results in an expansion of *gata1a* expression into somitic domains at the 22 s stage (E–F'). Cells from transgenic *gata1a:dsRed* x *kdrl:gfp* embryos show normal expression patterns (G–J). Figure 8 continued on next page

Figure 8 continued

kdrl:GFP embryos injected with cascade blue dextran and *msgn1/myod/myf5* MOs transplanted into unlabeled host embryos are excluded from somites and contribute extensively to endothelium (H, green arrow) and red blood cell lineages (H, red arrow). Control cascade blue injected *gata1a:dsRed* x *kdrl:GFP* transplanted cells contribute primarily to somitic muscle with minor contributions to endothelium (G, green arrow) and red blood cells (G, red arrow). Heat-shock induction of *id3* at shield stage in mesodermally targeted transplanted cells that also contain the *kdrl:GFP* transgene causes a shift from predominantly somitic muscle fate to significant endothelial contribution in the trunk (J compared to I). Whole embryo induction of *id3* expression at shield stage and analyzed at 24 hpf indicates a loss of medial mesoderm (notochord and muscle, (K–N)), and an expansion of lateral mesoderm (pronephros, vasculature, and blood, (O–T')). Expression of *gata1a* in the trunk shows broad expansion into somite territories (S' compared to T'). (U) A model for how FGF and BMP signaling control mediolateral patterning of the mesoderm through modulation of bHLH transcription factor activity.

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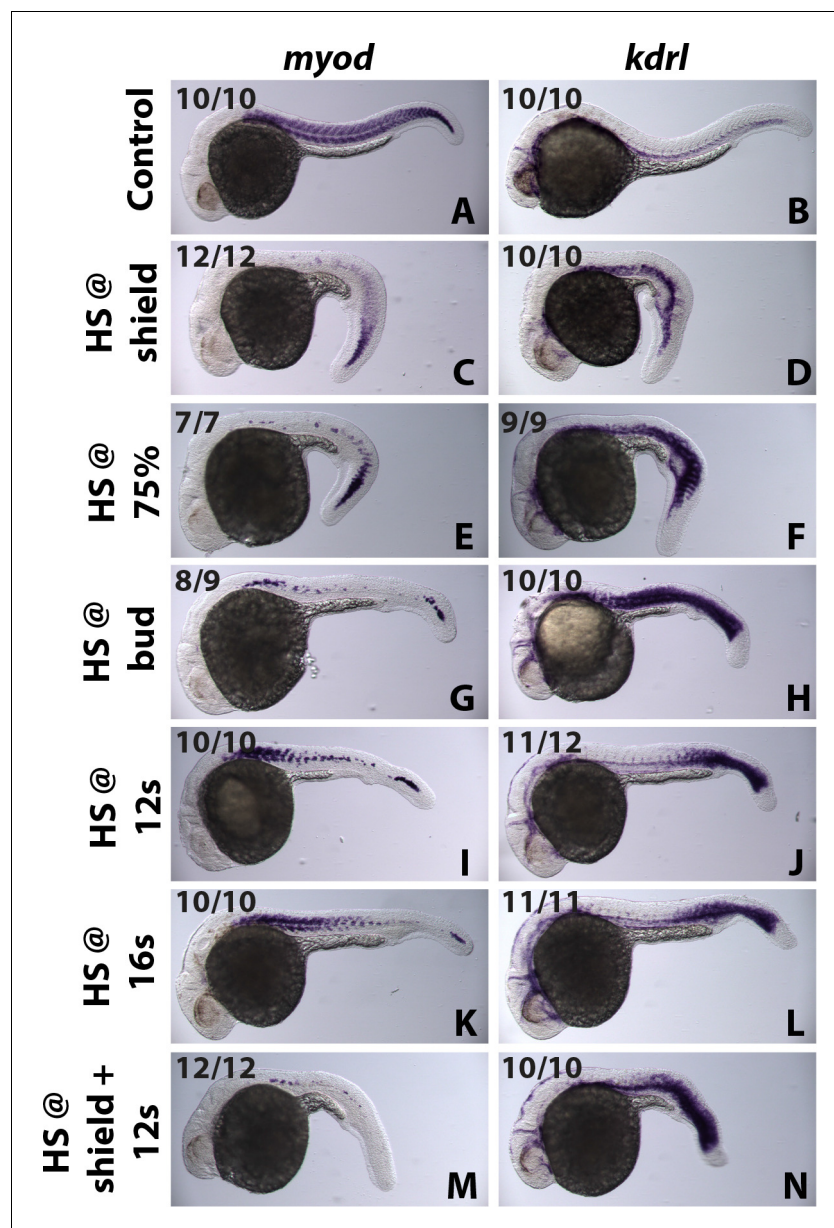


Figure 8—figure supplement 1. *id3* mediated patterning of the mediolateral mesodermal axis is coordinated with AP axis formation. A stage series of heat-shock inductions using the *HS:id3* transgenic line indicates that Id3 patterns mesoderm in coordination with anterior posterior axis formation. Early stage heat-shock inductions inhibit *myod* and expand *kdr1* expression mostly in anterior regions but posterior tissues are normal (C–F). An intermediate stage induction at the end of gastrulation inhibits *myoD* and expands *kdr1* everywhere except the extreme anterior and posterior regions (G, H). At later stages during somitogenesis, *id3* induction inhibits *myod* and expands *kdr1* expression in posterior but not anterior regions (I–L). Two heat-shock inductions at early and late stages indicates that recovery of posterior patterning in single early heat-shock inductions is due to turnover of the induced Id3 protein (M, N).

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