**RESEARCH ARTICLE**

**Greб1 is required for axial elongation and segmentation in vertebrate embryos**

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**ABSTRACT**

During vertebrate embryonic development, the formation of axial structures is driven by a population of stem-like cells that reside in a region of the tailbud called the chordoneural hinge (CNH). We have compared the mouse CNH transcriptome with those of surrounding tissues and shown that the CNH and tailbud mesoderm are transcriptionally similar, and distinct from the presomitic mesoderm. Amongst CNH-enriched genes are several that are required for axial elongation, including Wnt3a, Cdx2, Brachyury/T and Fgf8, and androgen/oestrogen receptor nuclear signalling components such as Greб1. We show that the pattern and duration of tailbud Greб1 expression is conserved in mouse, zebrafish and chicken embryos, and that Greб1 is required for axial elongation and somitogenesis in zebrafish embryos. The axial truncation phenotype of Greб1 morphant embryos can be explained by much reduced expression of No tail (Ntl/Brachyury), which is required for axial progenitor maintenance. Posterior segmentation defects in the morphants (including misexpression of genes such as mespb, myoD and papC) appear to result, in part, from lost expression of the segmentation clock gene, her7.

**KEY WORDS:** Tailbud, Neural tube, Axial stem cells, Somites, Transcriptome, Progenitors, Clock

**INTRODUCTION**

Vertebrate embryos develop in a highly organized fashion, progressively laying down axial tissues as they elongate along the anteroposterior embryonic axis (Brown and Storey, 2000; Catala et al., 1996; Wilson and Beddington, 1996; Wilson et al., 2009). Serial transplantation and other lineage tracing studies in mouse and chick have shown that a self-maintaining region in the tailbud called the chordoneural hinge (CNH) includes multipotent stem-cell-like progenitors for axial structures (Brown and Storey, 2000; Catala et al., 1996; Wilson and Beddington, 1996; Wilson et al., 2009). These include bipotent neuromesodermal progenitors (NMPs) that can generate both neural and mesodermal cells (Cambray and Wilson, 2002; Cambray and Wilson, 2007; McGrew et al., 2008; Selleck and Stern, 1991; Tam and Tan, 1992; Tzouanacou et al., 2009).

Adjacent to the CNH is the tailbud mesoderm (TBM) that contains the unsegmented precursors of the paraxial mesoderm; the presomitic mesoderm (PSM; Fig. 1A). During elongation, the PSM is displaced posteriorly while its anterior buds off a series of somites, epithelial balls that develop into segmental mesodermal structures such as the axial skeleton and musculature (reviewed in Pourquié, 2011).

Several studies have illuminated how axial progenitors are maintained during anteroposterior elongation. Briefly, a positive feedback loop between Brachyury/T and Wnt3a maintains axial progenitors in the tail bud (Martin and Kimelman, 2010; Wilson et al., 2009). In parallel, Fgf signalling protects axial progenitors from differentiation induced by retinoic acid (RA) that is secreted by differentiating and young somites and diffuses into the PSM (Diez del Corral et al., 2003; Olvera-Martinez et al., 2012; Ribes et al., 2009).

However, Fgf8, Wnt3 and T are all expressed in much larger domains than the CNH and so do not specifically distinguish axial progenitors from more specialised cells such as the TBM. Transcriptome analysis of dissected axial progenitor tissue during the period of axial elongation and of in vitro-derived NMPs has identified genes that are differentially expressed between progenitors and presomitic mesoderm cells (Gouti et al., 2017; Olivera-Martinez et al., 2014; Wymeersch et al., 2019). However, the functional significance of many of these genes has yet to be defined.

In this paper, we explore the transcriptional profiles of the CNH, TBM and PSM of E10.5 mouse embryos. We find that the CNH transcriptome is very similar to that of the TBM, and significantly different from that of the PSM. Several genes are expressed in both the CNH and TBM but not in the PSM, although none exclusively mark the CNH. Amongst the CNH-enriched transcripts is Greб1, which encodes a transcriptional co-activator for androgen/oestrogen hormone signalling. We show that Greб1 is expressed in the tailbud in mouse, chick and zebrafish embryos, and is required for axial progenitor maintenance and somite compartmentalisation in zebrafish. Our results indicate that Greб1 plays an evolutionarily-conserved role during vertebrate axial extension and segmentation.

**RESULTS AND DISCUSSION**

CNH transcriptome is distinct from PSM but not TBM

To identify potential markers for the CNH, we used microarray analysis on dissected tissue regions to identify genes whose expression in the E10.5 mouse CNH is elevated relative to that in the PSM and TBM (Fig. 1A). Hierarchical clustering of the replicate transcriptome patterns confirmed that the transcriptional profiles of the CNH, PSM and TBM are distinct (Fig. 1B; see Materials and Methods). Differential gene expression analyses identified 150
upregulated and 98 downregulated genes comparing the CNH to the PSM. The most significantly changed genes are listed briefly in Table 1, and the complete list can be found in Table S1. Only 12 upregulated and two downregulated transcripts distinguished the CNH and TBM, which is consistent with the latter population being directly derived from the former (Table 1; Table S1).

To confirm that many genes identified by microarray analysis are selectively expressed in progenitor regions of the extending embryo,
Tables 1 and 1 Continued

| ILMN_Gene     | CNH versus PSM fold change | CNH versus PSM FDR |
|---------------|---------------------------|--------------------|
| DEFCR-RS7     | 11.9                      | 1.96E-08           |
| T             | 10.2                      | 6.68E-05           |
| FGFR17        | 7.4                       | 2.10E-09           |
| LOC100044289  | 6.8                       | 1.24E-05           |
| WNT3A         | 6.0                       | 1.62E-05           |
| EVX1          | 6.0                       | 6.83E-07           |
| DEFCR6        | 5.1                       | 1.09E-06           |
| FGFR3         | 4.0                       | 1.49E-06           |
| GREB1         | 3.8                       | 5.77E-07           |
| CDX2          | 3.8                       | 3.03E-05           |
| SP5           | 3.6                       | 1.27E-06           |
| HOXC6         | 3.6                       | 4.54E-05           |
| AB20080H07R1K | 3.3                       | 3.03E-05           |
| ETS2          | 3.0                       | 3.63E-07           |
| CPN1          | 2.9                       | 3.52E-06           |
| HOXA7         | 2.9                       | 2.01E-06           |
| CDKN1A        | 2.7                       | 3.63E-07           |
| DEFCR-RS2     | 2.7                       | 5.49E-03           |
| TPDS          | 2.6                       | 2.37E-06           |
| CD40          | 2.6                       | 2.91E-05           |
| LOC212396     | 2.6                       | 7.02E-08           |
| HOPX          | 2.5                       | 1.07E-04           |
| GAD1          | 2.5                       | 3.25E-06           |
| GCNT2         | 2.5                       | 1.71E-05           |
| C23009801R1K  | 2.5                       | 3.29E-04           |
| GPRB3         | 2.4                       | 1.99E-07           |
| LMO2          | 2.4                       | 8.26E-04           |
| DUSP4         | 2.4                       | 2.03E-03           |
| ETV4          | 2.3                       | 1.15E-05           |
| DICKKOP1      | 2.3                       | 1.12E-05           |
| TMAPR1S2      | 2.3                       | 1.21E-07           |
| IRF1          | 2.3                       | 1.39E-06           |
| SUSD4         | 2.3                       | 1.12E-05           |
| SLC2A3        | 2.3                       | 3.31E-05           |
| SOMX          | 2.3                       | 6.66E-05           |
| CDKN1C        | 2.3                       | 1.03E-04           |
| UAP1L1        | 2.3                       | 1.67E-03           |
| SERPINE2      | 2.3                       | 4.93E-03           |
| CYPSA1        | 2.2                       | 2.32E-04           |
| GLDC          | 2.2                       | 1.20E-04           |
| PITR1M        | 2.2                       | 4.98E-04           |
| ACOT7         | 2.2                       | 3.00E-05           |
| HAP1          | 2.2                       | 5.77E-07           |
| WNT5B         | 2.2                       | 1.01E-05           |
| SCARA3        | 2.2                       | 1.43E-06           |
| PDIA5         | 2.2                       | 2.64E-05           |
| ENDO1         | 2.2                       | 5.38E-04           |
| NUP210        | 2.1                       | 1.45E-05           |
| DEFCR3        | 2.1                       | 1.23E-04           |
| OGFRL1        | 2.1                       | 3.55E-04           |
| HOXC9         | 2.0                       | 7.68E-06           |
| HOXA11S       | 2.0                       | 1.49E-06           |
| CRIP2         | 2.0                       | 9.04E-05           |
| 2610027C15R1K | 2.0                       | 4.67E-06           |

Genes downregulated in the CNH

| ILMN_Gene | CNH versus PSM fold change | CNH versus PSM FDR |
|-----------|---------------------------|--------------------|
| FOXC1     | -2.2                      | 5.53E-03           |
| SIX1      | -2.2                      | 1.52E-06           |
| ADD3      | -2.2                      | 8.09E-04           |
| SMOC1     | -2.3                      | 1.72E-06           |
| EMID2     | -2.5                      | 1.95E-06           |
| RAB15     | -2.5                      | 3.22E-08           |
| MAML2     | -2.6                      | 2.37E-05           |
| PPP1R1A   | -2.7                      | 2.57E-05           |
| 5430433G21R1K | -2.8             | 1.12E-06           |
| PPP2P2    | -2.8                      | 3.48E-05           |
| MEIS1     | -3.0                      | 3.25E-06           |
| MYL1      | -3.4                      | 5.77E-06           |
| 2210417D09R1K | -3.4             | 3.25E-06           |
| FGFR2     | -3.7                      | 5.07E-06           |
| MEIS2     | -4.0                      | 3.25E-06           |
| MEOX1     | -4.7                      | 5.31E-03           |
| PDZRN3    | -4.8                      | 3.63E-07           |
| CXCL12    | -5.5                      | 9.87E-05           |
| TCF15     | -5.8                      | 2.09E-05           |

Genes marked with * are differentially expressed in the CNH comparison to TBM and PSM (fold change >1.5 or <−1.5). Genes with FDR <0.01 and fold change >1.5 and <−1.5. The detailed data for all expression differences with FC >1.5, or <−1.5 are presented in Table S1.

Dunty et al., 2014; Maruoka et al., 1998; Takada et al., 1994).

...expressed in more specialised progeny cells, i.e. somites, unsegmented mesoderm or neural tube (Table 2; Table S2).

**Greb1 expression coincides with axial elongation in vertebrate embryos**

We also compared our list of CNH-enriched genes with those previously identified in previous studies of the CNH or NMPs (Table S3; Gouti et al., 2017; Olivera-Martinez et al., 2014; Wymeersch et al., 2019). Expression of seven of the ten most-enriched genes (Fig. 1C) was previously reported in the CNH and TBM, and to be functionally important for axial development (Cambray and Wilson, 2007; Dunty et al., 2014; Maruoka et al., 1998; Takada et al., 1994).

The expression and roles of the two most CNH-enriched genes from our study [Defcr-rs7, Defcr-rs6 (which encode small immune-defect peptides)] during axial elongation and segmentation remains to be studied. *Greb1*, which encodes a co-activator of the oestrogen and androgen receptors that is active in human oestrogen-receptor-positive primary breast and prostate cancer cells (Lee et al., 2019; Mohammed et al., 2013), is another top CNH enriched gene. Indeed, androgen receptor nuclear signalling is the most CNH-enriched pathway revealed by pathway enrichment analysis of our differentially expressed genes (Fig. 1D; Table S4; see Materials and Methods). Other androgen-responsive genes also enriched in the CNH include P21, cyclinD1 and MMP2 (Table S3). Indeed, the MMP2 matrix metalloproteinase is required for axial elongation – morpholino knockdown of MMP2 in zebrafish embryos – results in...
### Table 2. Annotated gene expression patterns (E7.5–13.5 mouse embryos) for genes that are differentially expressed in the CNH

| Gene       | Anatomical regions                                                                 |
|------------|-----------------------------------------------------------------------------------|
| Acot7      | Future spinal cord                                                                |
| Cdkn1a     | Neural tube, somite, dermomyotome, myotome, dermomyotome, somite, future spinal cord, somite |
| Cdkn1c     | Somite, dermomyotome, future spinal cord                                           |
| Cdx2       | Tail bud, future spinal cord neural plate, neural tube, tail unsegmented mesenchyme, neural tube, tail mesenchyme |
| Cpn1       | Neural tube                                                                       |
| Cyp26a1    | Future spinal cord neural plate, migrating neural crest, tail bud, mesenchyme, tail neural plate, tail neural tube, tail sclerotome |
| Dock6      | Future spinal cord, tail unsegmented mesenchyme                                     |
| Dock7      | Somite                                                                            |
| Dusp4      | Future spinal cord, tail unsegmented mesenchyme                                     |
| Ets2       | Primitive streak, neural tube, somite, trunk somite, future spinal cord, tail unsegmented mesenchyme |
| Etv4       | Future spinal cord, primitive streak, future spinal cord neural plate, tail bud, dermomyotome, sclerotome, neural tube floor plate, future spinal cord |
| Evx1       | Primitive streak, primitive streak, primitive streak, primitive streak, tail bud, mesenchyme, future spinal cord, future spinal cord neural plate, future spinal cord, tail neural tube, future spinal cord |
| Fgf8       | Future spinal cord, primitive streak, future spinal cord neural plate, somite, tail bud, trunk somite, tail mesenchyme, tail unsegmented mesenchyme, tail neural plate, trunk dermomyotome, myotome, tail somite, neural tube |
| fgf17      | Tail somite, somite, primitive streak, tail mesenchyme, tail unsegmented mesenchyme, neural tube |
| Gad1       | Neural tube, tail bud, tail mesenchyme, tail paraxial mesenchyme, tail future spinal cord, tail neural tube, future spinal cord |
| Gcnt2      | Future spinal cord                                                                |
| Gldc       | Neural lumen                                                                      |
| Greb1      | Future spinal cord                                                                |
| Hoxa7      | Neural tube ventricular layer, trunk somite, somite, neural tube, tail paraxial mesenchyme |
| Hoxc8      | Neural tube, somite, primitive streak, trunk somite                                |
| Hoxc9      | Tail paraxial mesenchyme, trunk tube, somite, tail somite                          |
| Lmo2       | Neural tube and head somites                                                        |
| Pitrm1     | Somite and neural tube                                                             |
| Serpine2   | Trunk dermomyotome and neural tube                                                 |
| Slc2a3     | Neural tube                                                                       |
| Sp5        | Future spinal cord, somite, primitive streak, head somite, trunk somite, future spinal cord neural plate, neural tube, tail unsegmented mesenchyme, dermomyotome, tail somite, neural tube lateral wall, tail mesenchyme |
| T          | Dermomyotome, primitive streak, node, future spinal cord neural plate, tail neural tube, tail bud, mesenchyme, tail unsegmented mesenchyme, tail neural plate, caudal neuropore, neural tube |
| Wnt3a      | Tail bud, primitive streak, future spinal cord, tail mesenchyme, tail unsegmented mesenchyme, neural tube, neural tube roof plate, neural tube lateral wall, sclerotome, neural tube floor plane |
| Wnt5b      | Neural tube floor plate, future spinal cord, neural tube                           |
| **Gene downregulated in CNH** | **Gene expression patterns** (E7.5–13.5 mouse embryos) for genes that are differentially expressed in the CNH |
| Tcf15      | Primitive streak, head somite, trunk somite, neural tube, neural tube lateral wall, myotome, sclerotome |
| Foxc1      | Primitive streak, node, future spinal cord, trunk paraxial mesenchyme, head somite, trunk somite, trunk unsegmented mesenchyme, neural tube, somite, tail unsegmented mesenchyme, trunk dermomyotome, trunk sclerotome, tail sclerotome |
| Gadd45g    | Trunk unsegmented mesenchyme, neural fold, tail unsegmented mesenchyme, future spinal cord neural plate, future spinal cord neural plate, tail unsegmented mesenchyme, neural tube, tail unsegmented mesenchyme, myotome, neural tube |
| Ism1       | Somite, trunk unsegmented mesenchyme, somite, tail unsegmented mesenchyme, neural tube, dermomyotome |
| Maml2      | Neural tube                                                                       |
| Meis1      | Trunk somite, future spinal cord, trunk paraxial mesenchyme, somite, neural plate, neural fold, neural groove, trunk unsegmented mesenchyme, neural tube, trunk myotome, trunk sclerotome, myotome, sclerotome, neural tube floor plate, neural tube mantle layer |
| Meis2      | Neural plate, neural fold, neural groove, trunk unsegmented mesenchyme, neural tube, somite, future spinal cord, trunk somite, trunk myotome, trunk sclerotome, myotome, sclerotome, neural tube floor plate, neural tube mantle layer |
| Mesp1      | Primitive streak, trunk paraxial mesenchyme, head somite, trunk somite, trunk unsegmented mesenchyme, somite, somite, trunk unsegmented mesenchyme, myotome, neural tube, tail unsegmented mesenchyme, trunk unsegmented mesenchyme, trunk unsegmented mesenchyme, myotome, neural tube |
| Msc        | Trunk and tail dermomyotome                                                         |
| Myl1       | Somite, myotome, trunk somite, tail paraxial mesenchyme                            |
| Pdzm3      | Tail somite, tail unsegmented mesenchyme                                           |
| Pipp3      | Neural tube ventricular layer, somite, myotome                                      |
| Ppp1r1a    | Neural tube, node, somite                                                          |
| Rab15      | Neural tube                                                                       |
| Six1       | Trunk somite, trunk unsegmented mesenchyme, somite, tail unsegmented mesenchyme, somite |
| Smoc1      | Node, somite, primitive streak                                                     |
| Tcf15      | Primitive streak, head somite, trunk paraxial mesenchyme, trunk unsegmented mesenchyme, somite, tail paraxial mesenchyme, tail unsegmented mesenchyme, dermatome, myotome, sclerotome, tail sclerotome, trunk dermomyotome, dermomyotome, trunk sclerotome |
| Tenn4      | Future brain neural fold, future spinal cord neural plate, future spinal cord neural fold, trunk somite, tail somite, tail somite |

Expression patterns were extracted from the Mouse Genome Informatics (MGI) database. Details of the data, including references, are reported in Table S2.
severe axial truncations (Zhang et al., 2003). Further studies will be required to test if androgen signalling operates in axial patterning.

Expression of chick Greb1 in the axial stem cell zone has been described previously (Olivera-Martinez et al., 2014). We expanded this finding by examining Greb1 expression during segmentation, using three different vertebrate systems. First, we visualised Greb1 transcription in elongating mouse embryos using in situ hybridisation (E10.5–E13.5; see Materials and Methods). In early (E8.5) embryos, Greb1 is expressed in a posterior domain that encompasses the caudal lateral epiblast, the region that includes the axial progenitors (Fig. 2A,A′). By E10.5, labelling is restricted to the CNH and dorsal TBM (Fig. 2B,B′). Expression in these regions is maintained during axial elongation, albeit more weakly by E12.5, and is lost at E13.5 when axial elongation ceases (Fig. 2C,D).

The above results show that, although not restricted to the CNH, axial Greb1 transcription in early mouse embryos coincides in time and place with the processes of axial extension and segmentation. To test if this correlation is evolutionarily conserved, we examined Greb1 expression in chick and zebrafish embryos. In both animals, Greb1 expression in the tailbud starts during elongation, and terminates when elongation and segmentation is complete. Greb1 is expressed in the HH13 chick caudal neural plate, whose cells contribute to the neural tube, somites and notochord, node and primitive streak (Fig. 2E,E′). Its axial transcription then becomes confined to the region of the tailbud which includes the chick CNH and TBM (HH17; Fig. 2F; McGrew et al., 2008), and has almost completely decayed when elongation is complete (HH26; Fig. 2G).

In zebrafish embryos, Greb1 transcription becomes confined to the region of the tailbud that contains axial progenitors (Fig. 2H–L). It persists during segmentation (11–16 hpf; Fig. 2I–K), and disappears when axial elongation comes to an end (24 hpf; Fig. 2L). This conserved spatial and temporal time course in early vertebrate embryos strengthens the link between Greb1 expression and axial extension.

**Knockdown of GREB1 disrupts axial elongation**

To test if Greb1 is functionally required during elongation and segmentation, we knocked down its expression by injecting antisense morpholinos into 1–2-cell zebrafish embryos (see Materials and Methods). We used two Greb1 splicing-blocking morpholinos (M1 and M2) that target the exon2-intron2 and exon16-intron16 boundaries, respectively (Fig. S1). These oligos should interfere with mRNA splicing to cause skipping of the adjacent exon and a shifted translational reading frame. The ensuing premature translational termination would completely truncate Greb1 protein (M1) or encode one that is only 40% full-length.

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**Fig. 2. The timing of axial Greb1 expression is coincident with axial elongation in vertebrate embryos.** (A–D) Mouse embryos and their tail regions at different embryonic stages. (A,A′) Dorsal view of E8.5 embryo showing expression in the caudal lateral epiblast (CLE). PS: primitive streak. (B,B′) Lateral view of E10.5 embryo, showing the Greb1-expressing tail region. (C) Lateral view of E12.5 tail region, showing reduced Greb1 expression. (D) Lateral view of E13.5 tail region, showing that expression is lost. (E–F) Greb1 expression in chick embryos at different stages: (E,E′) dorsal views of HH13 embryo and its tail region; (F) lateral and (F′) ventral view of a tail region at HH17; (G) lateral view of HH26 embryo, showing that Greb1 expression in the tailbud is almost gone. H–L are lateral views of zebrafish embryos of the indicated ages (hpf, hours post-fertilisation). Each pattern was analysed in two independent experiments using, for each stage, at least five mouse, or 10–15 chicken or zebrafish embryos. Tailbud regions are arrowed. Boxes in lower magnification images show the tail regions with magnified views in the adjacent panel.
Greb1 is needed to maintain Ntl expression in the tailbud

The axial truncations of Greb1 morphants resemble the phenotype of embryos mutant for No tail (Ntl), the zebrafish homologue of Brachyury/T, which is expressed in the tailbud, posterior PSM and notochord of wild-type embryos (Halpern et al., 1993; Schulte-Mmerker et al., 1994). Ntl in the tailbud helps maintain axial progenitors by protecting them from premature differentiation induced by RA secreted by the anterior PSM and somites (Dize del Corral et al., 2003; Martin and Kimelman, 2010; Olivera-Martinez et al., 2012; Ribes et al., 2009).

Tailbud Ntl expression in Greb1 morphants is indeed much lower than in wild-type or control embryos (M2: 20/34; MM: 0/11; Fig. 3Q′,R,R′). Thus, Greb1 is required for efficient Ntl expression, and reduced Ntl levels can explain the morphant embryos’ truncated axis.

Greb1 depletion affects somite polarity via the segmentation clock

During axial segmentation in zebrafish embryos, a linear array of chevron-shaped somites is progressively generated from the PSM between 10–24 hpf (Fig. 3A,A′). As mentioned above, Greb1 morphants lack morphologically discrete somites (Fig. 3A–C).

To assess if this morphological phenotype is accompanied by altered gene expression at somite boundaries, we examined Xirp2a/mRNA expression in the myoseptum between myotomes (Deniziat et al., 2007; Schroter and Oates, 2010). Strong distinct posterior stripes of Xirp2a/mRNA expression are frequently lost in Greb1 morphant embryos (M1: 20/25; M2: 14/18; MM: 0/15; Fig. 3A–C), corresponding to the regions with abnormal somite appearance.

In wild-type embryos, boundaries arise between posterior and anterior compartments of adjacent somites, raising the possibility that Greb1 is needed for somite compartmentalisation. To test this idea, we studied myoD transcripts, which are normally expressed in the posterior half of each somite (Weinberg et al., 1996). By contrast, expression of myoD extends into the anterior compartment in Greb1 morphants (M1: 7/15; M2: 13/18; MM: 0/18; Fig. 3D–F), suggesting that anterior morphant cells have adopted a posterior character. M1 and M2 morphants show similar effects on axial morphology and Xirp2a and myoD expression, we only analysed M2 morphants in subsequent experiments.

Analysing papC, which is expressed in the anterior compartments of newly formed somites (Rhee et al., 2003) provides additional support for the idea that Greb1 contributes to the establishment of anterior compartmentalisation. In morphant embryos, papC levels are reduced and lack clear borders (M2:14/22; MM:0/22; Fig. 3G,L).

Expression of myoD is normally suppressed in anterior somite compartments by mespb, which together with mespa, is expressed there in newly-formed somites (Sawada et al., 2000). We examined expression of both mesp genes in the morphant embryos and found that, although mespa expression is not altered (M2: 0/24; MM:0/21; Fig. 3H,M), mespb expression is greatly lowered (M2: 6/10; MM: 0/21; Fig. 3I,N). This reduction explains why myoD is derepressed in Greb1 morphants, and reinforces our view that Greb1 is needed for somite compartmentalisation.

What might cause mis-specification of somite compartments?

During vertebrate axial extension, the regular production of equal-sized segments results from the action of a molecular oscillator (‘segmentation clock’), which drives cyclic transcription of many PSM genes with a period corresponding to that of somite formation (Dequeant et al., 2006; Niwa et al., 2007; Palmeirim et al., 1997; Pourquié, 2011). Together, axial extension and cyclic gene expression establish reiterated expression of genes that define somite compartmentalisation and, hence, somite boundaries.

We examined two such cycling genes, her1 and her7, which encode transcriptional repressors whose periodic expression in the zebrafish PSM form and pattern the somites (Oates and Ho, 2002; Pourquié, 2011; Takke and Campos-Ortega, 1999). In particular, her7 is a regulator of mespb expression in forming somites (Choorapoikayil et al., 2012; Oates and Ho, 2002). Expression of her1 is normal in the PSMs of Greb1 morphant embryos (M2 0/16; MM 0/19; Fig. 3J,O), but that of her7 is lost, in both the tailbud and PSM (M2 5/5; MM 0/5; Fig. 3K,P). The latter’s loss explains the reduced mespb expression and abnormal somite compartmentalisation in Greb1 morphant embryos.

Together, our experiments support the following model for the Greb1 morphant phenotypes (Fig. 3S). Axial extension is truncated due to reduced expression of Ntl and, thereby, loss of axial progenitors (Fig. 3Q′,R,R′; Martin and Kimelman, 2010), and the segmentation phenotype is caused by loss of her7. This model is consistent with the misregulation of mespb and loss of more posterior somite boundaries in both her7 mutants and Greb1 morphants (Fig. 3A′– F, A′–F; Oates and Ho, 2002).

Although we cannot completely exclude the possibility that the morphant morphological and molecular phenotypes are due to off-target knockdowns, this explanation seems unlikely. Each of the splice-blocking morpholinos was independently derived, and so they would not be expected affect similar sets of off-target transcripts. The combination of morphant phenotypes we observe has not previously been described, and we have also shown that they are not due to non-specific morphant toxicity. Future experiments using...
CRISPR/Cas9 gene-editing will clarify this point and allow further studies of Greb1 action.

As Greb1, Ntl and Her7 are all transcription factors, some of the effects on gene transcription that we observe may be direct. Greb1 is required for clock output via her7, and may also act directly on mespb. However, the oscillator circuitry remains intact: morphants retain cyclic her1 expression and low level, metameric xirp2 expression (Fig. 3B,C,J,O). The latter idea would explain why mespb expression is abolished in the Greb1 morphants (Fig. 3I,N). Although further experiments will be required to distinguish between direct and indirect actions of Greb1 and its potential targets, the evolutionarily conserved pattern and time-course of Greb1 expression that we have shown in mouse, chick and zebrafish (Fig. 2) suggest that Greb1 is an important component in vertebrate axial patterning.

Fig. 3. Greb1 is required for axial elongation. (A–C,A′–C′) Lateral views of zebrafish embryos at 24 hpf, showing: (A) wild-type chevrons of xirp2a expression and the tail region (bracketed); (B,C) posterior loss in M1 and M2 morphants. The tail regions that are truncated and contain disrupted somites are bracketed. (D–F,D′–F′): expression of myoD in control (MM, M1 and M2 morphants). (G,L) papc, (H,M) mespa, (I,N) mespb, (J,O) her1, (K,P) her7 (Q,R) and (Q′,R′) Ntl expression in the tail region of 15 hpf control and morphant embryos. (S) A tentative model for gene interactions between Greb1 and patterning genes. Anterior expression of Her7 restricts mespb expression to the posterior somite compartment, which, in turn, restricts myoD and papC expression to the anterior compartment. Continuous arrows indicate interactions shown by others as likely to be direct. Dashed arrows could be direct or indirect.
MATERIALS AND METHODS

Maintenance and collection of embryos

E10.5 mouse embryos were collected from CD1 and C57Bl/6J pregnant females (Charles River Laboratories International Inc., UK) in M2 media (Sigma-Aldrich, M7167). Fertilized chicken eggs from Henry Stewart & Co (Louth, UK) were incubated at 37°C, and embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992). Adult wild-type zebrafish were maintained at 27°C on a regular 14 h light/10 h dark cycle, and embryos were collected and staged as described by Kimmel et al. (Kimmel et al., 1995). p53 heterozygous and homozygous mutant zebrafish embryos were obtained by crossing p53 homozygous female to p53 heterozygous males (Robu et al., 2007). Animals used in this study were handled by professionals meeting all the requirements of the Animals (Scientific Procedures) Act 1986.

Transcription profiling

Mouse CNH, PSM and TBM explants per experiment were dissected as previously described (Fig. 1A; Cambray and Wilson, 2002). Approximately 50 pieces of each region were pooled, and total RNA extracted using the RNasy Mini Kit (Qiagen, cat. no. 74104). Before processing the RNA samples for microarray analysis, their quality was tested using the Bioanalyzer RNA 6000 Pico kit (Agilent, cat. no. 5067-1513). Samples with RNA integrity number (RIN) 8–10 were processed for transcriptional profiling at the Genome Centre (Blizard Institute, Barts and the London School of Medicine and Dentistry) using Illumina ‘Ref6v2’ beads arrays. Two biological and one technical replicate were carried out for each region – CNH, TBM and PSM.

Microarray data analysis

Analysis was performed using software packages developed for Bioconductor version 2.4.0 and R version 2.9.0. The Illumina dataset was processed using the probe intensity transformation (VST) and normalization (RSN) methods from the limma package (Ihaka and Gentleman, 1996; Team, 2009). Hierarchical clustering was used to assay the reproducibility of the biological replicates. Differential gene expression was assessed between tissue-type replicate groups using an empirical Bayes t-test as implemented in the ‘limma’ package and taking account of replicate group and batch effects (K., 2005). Three comparisons were performed: CNH versus PSM, CNH versus TBM, CNH versus Combined PSM and TBM. The resulting P-values were adjusted to control the false discovery rate (FDR) using the Benjamini and Hochberg method. Two lists of differentially expressed genes were produced using different thresholds: (1) all genes that exhibited FDR<0.05 in all three comparisons, or a fold change >1.5 in the same direction in all three contrasts were classified as differentially expressed. (2) ‘Top50’: genes were selected on the basis of FDR<0.05 and an absolute fold change >1.5 from the CNH versus PSM comparison, ordered by fold change, and the top 50 most-changed genes were selected and clustered using hierarchical clustering algorithm. Genes from the two lists were combined and used to perform a pathway enrichment and network analysis with MetaCore software from Clarivate Analytics.

In situ hybridisation

We visualised spatiotemporal transcript expression in mouse, chick and zebrafish embryos by in situ hybridisation using digoxigenin-labelled antisense RNA probes (Hansisch et al., 2013; Rallis et al., 2010; Stauber et al., 2009). In general, templates for making antisense RNA probes for in situ detection of Greb1 transcripts were generated by RT-PCR of embryonic mRNA, cloning into pCR2.1-TOPO-TA vector (Invitrogen; Table S5), linearization using SpeI or NotI, and transcription by T3 or T7 RNA polymerase. cDNA templates for generating other antisense-RNA probes were obtained from the Julian Lewis lab. Expression patterns were replicated and scored independently by at least two people.

Morpholino injection

To knockdown zebrafish Greb1 expression, we injected 2 nl of the following splicing-blocking morpholinos into 1–2-cell embryos at 2–8 ng/µl in 0.4 mM MgSO4, 0.6 mM CaCl2, 0.7 mM KCl, 58 mM NaCl, 5 mM HEPES pH 7.6; 0.05% Phenol Red: (M1) 5′-GGAAAGACTGTAAGAAGCTACCCCTA-3′, (M2) 5′-AAATCTGAATCACCTGCTCTCC-3′ (Fig. S1; Gene Tools, Philomath, OR, USA). Control injections used a mutated M2 oligo (MM) with five nucleotide mismatches: 5′-AAATGTC- AACATCAGCTGTGCTCC-3′. To test for non-specific toxicity, 4 ng/µl of blocking or control morpholino was co-injected with 6 ng/µl p53 antisense morpholino (Robu et al., 2007). Efficacy and specificity were tested by sizing and sequencing RT-PCR products of total RNA from morpholino-injected embryos, SuperScript III One-Step RT-PCR mix (Invitrogen, #12574035).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: R.S.P., D.I.-H.; Methodology: R.S.P.; Validation: R.S.P.; Formal analysis: R.M.; Investigation: R.S.P., A.V.; Data curation: R.M.; Writing - original draft: D.I.-H.; Writing - review & editing: R.S.P., R.M., D.I.-H.; Visualization: R.S.P.; Supervision: D.I.-H.; Project administration: R.S.P.; Funding acquisition: D.I.-H.

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Data availability

The non-normalised and normalized expression sequencing data and gene tables are available from the Gene Expression Omnibus (GEO) with accession number GSE141519.

Supplementary information

Supplementary information available online at http://bio.biologists.org/lookup/doi/10.1242/bio.047290.supplemental

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