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Multiple *Dlk1* splice variants are expressed during early mouse embryogenesis

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ABSTRACT Delta-like homologue 1 (*Dlk1*), an atypical Notch ligand, is known to have roles in growth and development, stem cell maintenance, and cancer. Evidence suggests that *Dlk1* expression patterns are more complex than previously appreciated, with multiple isoforms expressed in various tissues in both the embryo and adult. However, the early embryonic expression of *Dlk1* has not been well examined. Given that tissue specific *Dlk1* knockouts have to date failed to recapitulate phenotypes associated with the conventional *Dlk1* loss of function model, a better understanding of early *Dlk1* expression is important. To address this question, we have examined *Dlk1* expression during the early stages of mouse embryogenesis. *Dlk1* expression was first detected at Theiler Stage 14 (TS14), and its expression pattern persisted in specific tissues through TS20. Further, we found that all known *Dlk1* splice isoforms were expressed in early embryogenesis, with *Dlk1-A* and *Dlk1-C/C2* isoforms being expressed at the highest levels. The broad co-expression of multiple *Dlk1* isoforms corroborates recent work suggesting that *Dlk1*-mediated signaling may act through multiple DLK1 isoforms to balance differentiation.

KEY WORDS: *Dlk1*, *alternative splicing, embryonic expression*

Delta-like Homologue 1 (*Dlk1*) is a maternally imprinted gene that encodes a transmembrane protein with homology to the Delta/Jagged ligands of the mammalian Notch signaling pathway (Smas and Sul, 1993). The DLK1 protein comprises an extracellular domain with a signal peptide and epidermal growth factor (EGF)-like repeats, a juxtamembrane region with an isoform-specific TACE-mediated cleavage site, a transmembrane domain, and a small intracellular domain (Smas *et al*., 1994; Wang *et al*., 2006; Fig. 1A). *Dlk1* is alternatively spliced in humans and mice, with transcripts encoding soluble isoforms including TACE cleavage sites, and membrane-bound isoforms lacking the TACE cleavage sites (Smas *et al*., 1994; Wang *et al*., 2006; Fig. 1A).

During adipogenesis, soluble DLK1 has been shown to inhibit adipocyte differentiation, while the membrane-bound isoform is thought to promote differentiation (Garcés *et al*., 1999; Smas and Sul, 1993). In neural stem cells, membrane-bound DLK1 regulates stem cell number in the post-natal subventricular zone via a mechanism that requires soluble DLK1 (Ferrón *et al*., 2011). *Dlk1* has a known role in muscle satellite cell maintenance, and recent work has also suggested differential roles for DLK1 variants during embryonic muscle development (Andersen *et al*., 2013; Waddell *et al*., 2010). Taken together, the evidence supports the idea that *Dlk1* may play significant roles in balancing stem-like behavior with differentiation in a variety of cell types. This may further suggest that the embryonic functions of *Dlk1* require the regulated expression of multiple DLK1 isoforms, highlighting the importance of alternative mRNA splicing in *Dlk1*-mediated signaling.

Dlk1 mRNA distribution has not been studied in early embryogenesis. In late embryogenesis, *Dlk1* RNA expression has been reported in the liver, lung, muscle, vertebrae, pancreas, pituitary, and adrenal glands at embryonic day 12.5 (E12.5), and expression is found to be dramatically down-regulated at E16.5 (Yevtodiyenko and Schmidt, 2006). *Dlk1* null mice exhibit exhibit growth retardation, skeletal deformations, eyelid abnormalities, and postnatal hypertrophic adipocytes (Moon *et al*., 2002). In contrast, overexpression of *Dlk1* via transgene leads to an enhanced growth phenotype, defects in cartilage and bone, and perinatal lethality (Da Rocha *et al*., 2009). Together, these studies indicate roles for *Dlk1* in growth and development, with potential functions in tissues known to express *Dlk1* at E12.5. However, a recent study made conditional deletions of *Dlk1* in several tissues including pituitary somatotrophs, pancreatic beta cells and endothelial cells. None

Abbreviations used in this paper: Dlk1, Delta-like homologue 1; PSM, presomitic mesoderm; TS, Theiler Stage.

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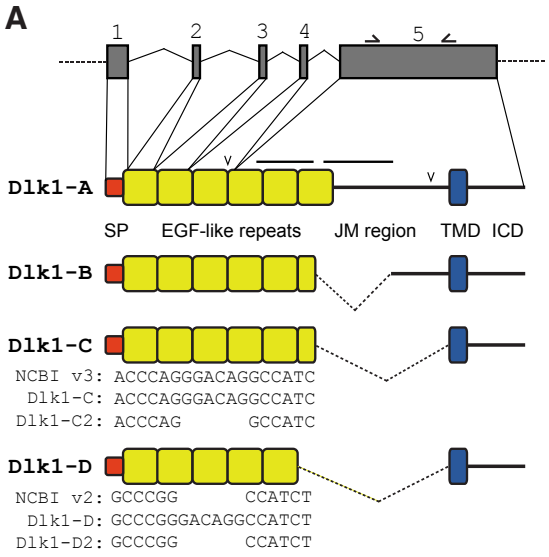

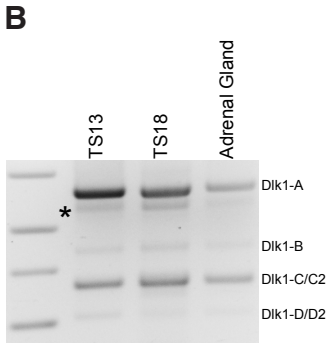

Fig. 1. All known *Dlk1* isoforms are expressed during early mouse embryogenesis. (A) *Schematic of previously described DLK1 protein isoforms. Some DLK1 variants contain potential TACE cleavage sites (arrow heads). All isoforms possess a signal peptide (SP), a varying number of epidermal growth factor (EGF)-like repeats, a transmembrane domain (TMD), and an intercellular domain (ICD). Full-length DLK1 (-A) and DLK1-B encode secreted forms of DLK1 while DLK1-C/C2 and DLK1-D/D2 encode membrane-bound isoforms. Lines above DLK1-A indicate the positions of* in situ *probes. The probe on the left detects all* Dlk1 *transcripts (pan-*Dlk1 *expression), while the right hand probe detects* Dlk1-A *expression. Both* Dlk1-*C and* Dlk1-D *encompass two variants that differ by 6 base pairs. The sequences of* Dlk-C *(NM_001190704), and* Dlk1-*D2 (NM_001190703) are shown along with the sequences of D and C2.* **(B)** *RT-PCR using primers flanking the alternatively spliced region of* Dlk1 *(arrows in A) were used to examine the expression of* Dlk1 *splice variants during early mouse development. All four major variants (*Dlk1-A, -B, -C, *and* -D*) and two sub-variants (*Dlk1-C2 *and* Dlk1-D2*) were detected when bands were cloned and sequenced. The unpredicted band of ~500bp (asterisk) was not clonable or sequenceable, and when purified and used as a template for further PCR, it gave rise to several* Dlk1 *isoforms. We conclude that this band represents a heteroduplex.*

of these tissue specific deletions recapitulate the phenotypes of *Dlk1* null animals, suggesting that the requirements for *Dlk1* during embryogenesis are complex, and that understanding the *Dlk1* expression pattern during early embryogenesis may give further hints about its functions (Appelbe *et al.*, 2013).

Given these facts, and the finding that DLK1 protein expression can be observed as early as E10 (Falix *et al.*, 2013), we performed a study of *Dlk1* expression during early mouse embryogenesis. Here, we demonstrate that *Dlk1* expression is detectable as early as Theiler Stage 13 (TS13, E8.5 on our background). Thus, *Dlk1* is expressed during the developmental periods in which terminal differentiation and organogenesis begin to occur in the mouse embryo. Expression patterns at these early stages are tissue specific, with *Dlk1* expression highest in regions where regulation of cell differentiation is important. Interestingly, we find that all known *Dlk1* isoforms are expressed during these early stages of embryogenesis, and we find that transcripts encoding the full-length and membrane-bound forms are co-expressed on the whole-tissue level. These results corroborate and extend previous *Dlk1* expression studies. Further, this suggests that the tissues utilizing *Dlk1*-mediated signaling do so through more than one isoform, possibly to mediate signaling between the stroma and parenchyma within a tissue in a paracrine fashion as has been previously suggested (Andersen *et al.*, 2013; Appelbe *et al.*, 2013).

Results

All known Dlk1 isoforms are expressed during early mouse embryogenesis

To determine which *Dlk1* isoforms are expressed during early embryogenesis, we examined mouse embryos at TS13 (~E8.5) and TS18 (~E10.5; Fig. 1). Using primers that flank the alternatively-spliced region of *Dlk1* exon 5 (Fig. 1A), we simultaneously amplified all *Dlk1* transcripts (Fig. 1B). PCR products were subcloned, and sequenced, and we find that all six previously identified *Dlk1* isoforms (A, B, C, C2, D, and D2) are expressed in mouse embryos (Smas *et al.*, 1994; Fig. 1A). An additional PCR product was subsequently determined to be a heteroduplex (Fig. 1B). The highest levels of expression detected at these stages were represented by the *Dlk1-A* and *Dlk1-C/C2* transcripts, which give rise to secreted and membrane-bound forms of DLK1, respectively (Smas *et al.*, 1997; Yevtodiyenko and Schmidt, 2006; Fig. 1B). These data indicate that all previously described *Dlk1* isoforms are expressed during

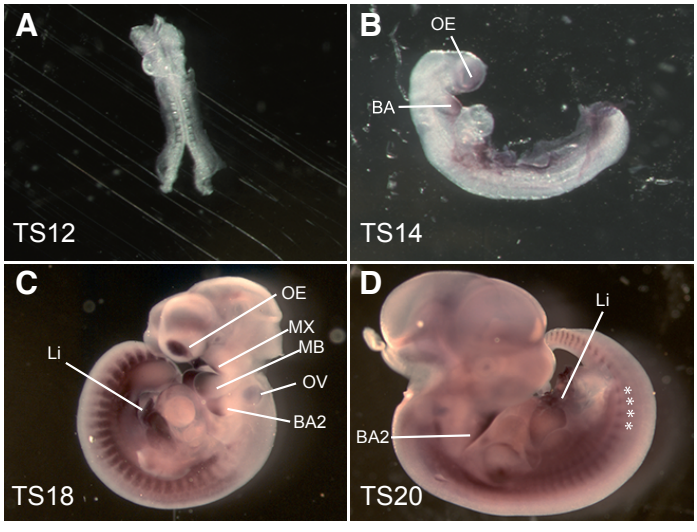

Fig. 2. Dlk1 expression is initiated at the onset of organogenesis, and is confined to a subset of embryonic tissues. *Whole mount* in situ *hybridization using an RNA probe designed to detect the expression of all* Dlk1 *isoforms simultaneously (pan-*Dlk1 *expression) was performed at several developmental stages.* **(A)** *No pan-*Dlk1 *expression is detected at Theiler Stage (TS) 12, which occurs at approximately E8.0 in the mouse.* **(B)** Dlk1 *expression is first detected in the branchial arches (BA) and olfactory epithelium (OE) at TS14.* **(C)** *At TS18 expression is detected in the mature somites (asterisks), in the liver (Li), the otic vesicle (OV), the maxillary (MX) and mandibular (MD) processes of the first BA, the second BA (BA2) and in the presomitic mesoderm (PSM; inset). Expression in the OE and BA is detected at higher levels than at previous stages.* **(D)** *At TS20, pan-*Dlk1 *expression is down-regulated in the somites (asterisks), the BA, and Li.*

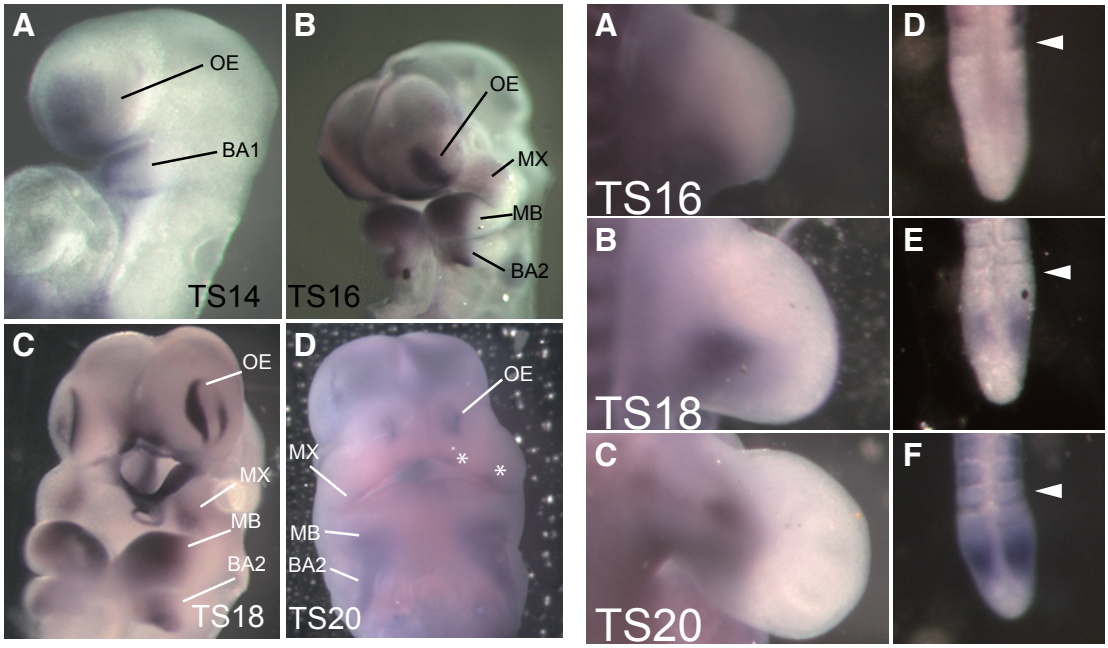

Fig. 3 (Left). *Dlk1* is expressed in a stage-dependent manner in the olfactory epithelium and branchial arches. *Whole mount* in situ *hybridization analysis of pan-*Dlk1 *expression in the developing olfactory epithelium and branchial arches was performed throughout the stages of detectable expression.* **(A)** *Pan-*Dlk1 *expression is first detected in the first branchial arch (BA1) and the olfactory epithelium (OE) of the olfactory placodes at TS14.* **(B)** *At TS16, as the OE has thickened,* Dlk1 *expression has intensified markedly. BA1 has bifurcated into the maxillary and mandibular prominences (MX and MB, respectively). Here, pan-*Dlk1 *expression has also intensified.* **(C)** *By TS18, pan-*Dlk1 *expression has become restricted to the periphery of the OE, with no detectable expression within the invaginating nasal pit. Expression has also become highly restricted in the MX, MB and BA2.* **(D)** *Expression in the OE has been reduced, but is still detectable at the periphery of the OE and in MX by TS20. In MB and BA2, expression has also been down-regulated.*

Fig. 4. (Right) *Dlk1* is expressed dynamically in the developing limb buds. *Pan-*Dlk1 in situ *hybridization was examined in limb buds and the PSM at various embryonic stages.* **(A)** *Pan-*Dlk1 *is expressed in a restricted pattern near the center of the developing limb bud at TS16, with expression also detectable at the midline.* **(B)** *At TS18, the pan-*Dlk1 *expression pattern closely resembles that observed at TS16, though expression has intensified.* **(C)** *By TS20, the pan-*Dlk1 *expression pattern is elongated along the ventro-medial axis of the limb bud, and* Dlk1 *expression appears to have been down-regulated in this tissue.* **(D-F)** *Pan-*Dlk1 *expression in the PSM is first detected after TS16, and appears as a broad stripe near the anterior boundary of the PSM. Expression here is not cyclic, and is detected throughout the developmental period during which somitogenesis occurs (data not shown).*

early mouse embryogenesis, and that it is the *Dlk1-A* and *Dlk1-C/C2* isoforms that predominate at these stages of development.

Dlk1 is expressed in a tissue-specific manner during embryogenesis

Having detected expression of *Dlk1* during early embryogenesis, we examined the spatial distribution of *Dlk1* transcripts during this period of development. *In situ* hybridization was performed to simultaneously examine the expression pattern of all *Dlk1* isoforms using a probe spanning EGF repeats 4-6, which are shared among all known isoforms (pan-*Dlk1* expression; Fig. 2). No expression was observed at TS12 (~E8.0; Fig. 2A). Robust *Dlk1* expression is first detected at TS14, in the first branchial arch (BA) and the olfactory epithelium (OE; Fig. 2B). At TS18, expression is also apparent in the liver, limb buds, otic vesicle, and mature somites (Figure 2C). At this stage, expression is strong in both the first and second branchial arch structures, as well as the OE. At TS20, expression has been refined in the OE and the BA and is maintained in the liver and somites (Fig. 2D). The expression patterns observed at these early developmental stages are consistent with the previously reported functions of *Dlk1*, though embryonic expression in the branchial arches and olfactory epithelium has not been previously reported.

Dlk1 expression in the branchial arches and olfactory epithelium is robust and specific

As the first specific site of pan-*Dlk1* expression is in the BA and OE at TS14, we examined these structures in more detail throughout early embryogenesis (Fig. 3). Pan-*Dlk1* expression is detected in the mandibular process of the first BA at TS14 (Fig. 3A). As craniofacial development proceeds, *Dlk1* expression is evident in the maxillary (MX) and mandibular processes (MD) as well as in the second branchial arch (Fig. 3B). *Dlk1* expression is refined as these tissues mature, becoming highly restricted in the MX, and more restricted in the MD and BA2 at TS18 (Fig. 3C). By TS20, *Dlk1* expression is downregulated in the craniofacial region, though it can still be observed in distinct puncta in the MX (Fig. 3D; asterisks).

The olfactory placodes are specialized epithelial thickenings that invaginate to form the nasal pit. Pan-*Dlk1* expression is evident in the olfactory epithelium (OE) of the placode at TS14 (Fig. 3A). Expression in the OE intensifies at TS16 as it thickens (Fig. 3B). Notably, as the placode invaginates at TS18, pan-*Dlk1* expression becomes restricted to the marginal rim of the OE (Fig. 3C). At TS20, pan-*Dlk1* expression is again largely extinguished, though the detectable expression remains at the periphery of the OE (Fig. 3D).

Dlk1 is expressed in the developing limb buds and presomitic mesoderm (PSM)

Given the previously suggested roles for *Dlk1* in muscle development (Andersen *et al.*, 2013), we further examined the pan-*Dlk1* expression pattern in the somites, limb buds and PSM. At TS16, *Dlk1* expression is robust at the posterior and midline of

the limb bud (Fig. 4A). At TS18, a condensation of *Dlk1* expression is detected near the central limb bud (Fig. 4B), and by TS20, pan-*Dlk1* expression is detected as a stripe at the midline of the limb bud (Fig. 4C). This observation corroborates studies that have found roles for *Dlk1* in embryonic muscle development and muscle precursor cells (Andersen *et al*., 2013; Moon *et al*., 2002; Waddell *et al*., 2010).

Dlk1 has been proposed to function in modulation of the Notch signaling pathway. In the presomitic mesoderm (PSM) oscillatory Notch1 signaling in the PSM is critical for the regulation of the axial skeleton by the segmentation clock. We therefore examined the expression of *Dlk1* in the PSM to determine whether its expression links it to the clock. Pan-*Dlk1* expression in the PSM is only weakly detectable prior to TS16 (Fig. 4D). Robust expression is observed at TS16, with a broad band of *Dlk1* expression in the posterior PSM (Fig. 4E), that is maintained at TS18 (Fig. 4F). Unlike the expression of some other Notch pathway members, however, expression of *Dlk1* in the PSM is stable, with no evidence of oscillatory expression (n=10). Thus it is unlikely that *Dlk1* plays a significant role in the segmentation clock, despite the occasional observation of rib fusions and other axial skeleton abnormalities in *Dlk1* mutant mice (Moon *et al*., 2002).

Distinct Dlk1 isoforms are co-expressed in developing tissues during early embryogenesis

An *in situ* probe which will hybridize specifically with the *Dlk1-A* transcript, while not detecting *Dlk1-B, -C* or *-D* (see Fig. 1), was used to compare the specific expression pattern of *Dlk1-A* to the pan-*Dlk1* expression patterns (Fig. 5). At TS18, we find that the overall expression pattern of *Dlk1-A* is strikingly similar to that of pan-*Dlk1* (Fig. 5). All tissues observed to express *Dlk1* were found to express *Dlk1-A*, although the intensity of staining varied somewhat from tissue to tissue. In the mature somites (Fig. 5A and E) and FLB (Fig. 5B and F), *Dlk1-A* expression levels seem similar to those detected using the pan-*Dlk1* probe. However, detection of *Dlk1-A* was weaker in the PSM (Fig. 5D and H) and during early limb bud development (Fig. 5C and G). However, given the non-quantitative nature of whole mount *in situ* analysis, it is difficult to ascertain whether these differences represent differential regulation of splicing in these tissues.

As an independent method to examine tissue specific splicing regulation, we examined *Dlk1* expression by RT-PCR using RNA extracted from whole embryos, PSMs, hind limb buds, and fore limb bud tissues at ~TS18 (Fig. 5I). In all tissues, all known *Dlk1* isoforms were amplified, with the *Dlk1-A* and *Dlk1-C/C2* isoforms exhibiting the highest levels of expression. As observed previously, *Dlk1-D/D2* is expressed at very low levels in all tissues examined. These data indicate that all *Dlk1* isoforms are expressed in a variety of tissues at this early stage of development.

Discussion

The expression patterns described here suggest that *Dlk1* may have specific functions during early stages of mouse development. *Dlk1* has a well-documented role in mesenchymal cell fate, and *Dlk1* function in the PSM may be related to a role in maintaining mesenchymal cells in an immature state prior to differentiation (Sul, 2009; Wang and Sul, 2009). The observed expression in the somites and limb buds could reflect the suggested role for *Dlk1* in regulating embryonic muscle development (Andersen *et al*., 2013; Waddell *et al*., 2010). *Dlk1* has previously been shown to be expressed in hepatoblasts and hepatic stellate precursor cells, thus the observed liver expression is likely indicative of these cell populations (Zhu *et al*., 2012).

We are the first to report strong expression of *Dlk1* in the branchial arches and their derivatives and in the olfactory epithelium. The early and robust expression in the branchial arch mesenchyme

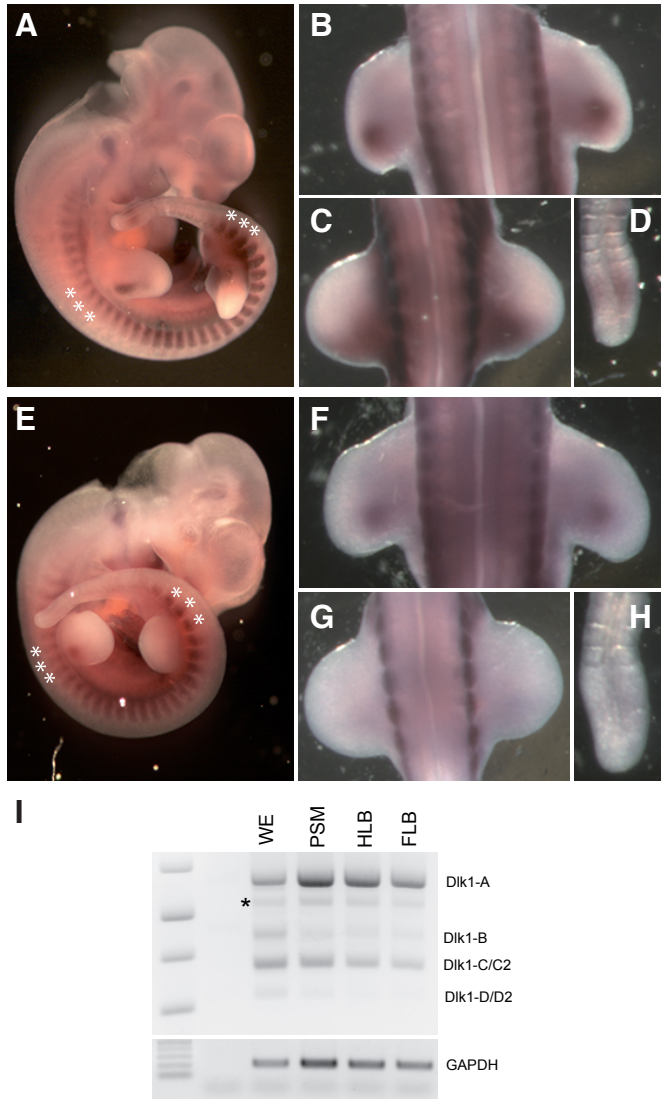

Fig. 5. Soluble and membrane-bound *Dlk1* isoforms are broadly co-expressed during early development. *Pan-*Dlk1 in situ *hybridization was compared to* in situ *hybridization using a* Dlk1-A *specific RNA probe (see figure 1).* Dlk1-A *expression is detected at a level similar to that of pan-*Dlk1 *expression in mature somites; see asterisks in* **(A,E)**, *and in the forelimb buds* **(B,F)**. *Differences in levels of expression are detectable in the hind limb buds* **(C,G)** *and in the PSM* **(D,H)**, *with lower levels of* Dlk1-A *detected relative to detection of pan-*Dlk1 *expression.* **(I)** *Expression levels of* Dlk1 *transcripts were analyed by RT-PCR using total reverse-transcribed cDNA extracted from whole embryo (WE), PSM, FLB and HLB tissues at ~TS18. Pan-*Dlk1 *expression analysis demonstrates similar expression patterns for all* Dlk1 *transcripts for each of the tissues examined. In all tissues examined, the* Dlk1-A *and* Dlk1-C/C2 *isoforms are detected at the highest levels.*

may reflect functional roles for *Dlk1* during craniofacial development, while expression of *Dlk1* at the periphery of the invaginating nasal pit, the domain of olfactory epithelial morphogenesis where olfactory neural stem and progenitor cells reside, is consistent with the described role of *Dlk1* in neural stem cell maintenance (Ferrón *et al*., 2011; Kawauchi *et al*., 2005; Schmidt *et al*., 2000). In support of this, asymmetric expression of *Dlk1* at the peripheral OE is reminiscent of Sox2 expression in this tissue (Chen *et al*., 2009).

A recent study used section IHC to detect DLK1 protein expression at similar stages to those reported here (Falix *et al*., 2013), and identified protein expression in many of the same structures during early embryogenesis. These results are complementary, as together these studies allow us to assess both protein and RNA expression in embryonic sections as well as in whole mounts. The data reported here identify additional sites of expression, and shed light on the control of transcript splicing, which is not possible using antibody analysis. Together, these findings allow us to assess the effects of transcriptional, post transcriptional and post-translational control of Dlk1 in the early embryo.

Our RT-PCR results demonstrate that *Dlk1* splice variants encoding both membrane bound and secreted versions of the DLK1 protein are co-expressed in an array of developmentally distinct tissues. The fact that in all tissues examined *Dlk1-A* (secreted) and *Dlk1-C/C2* (membrane bound) are expressed at the highest levels, indicates that both forms of the protein are functional during early embryonic development. Further examination will be needed to determine whether individual cells within the developing tissues may express specific *Dlk1* isoforms.

In summary, we have demonstrated for the first time that all known *Dlk1* isoforms are expressed during early embryogenesis. We find that *Dlk1* expression patterns can be correlated with previous *in vitro* and tissue-specific functional studies, and expression studies at later developmental stages. We have also described for the first time expression of *Dlk1* in the branchial arches and the olfactory epithelium. Finally, we have found that soluble and membrane-bound *Dlk1* isoforms are broadly co-expressed during early embryonic development, with both membrane bound and secreted isoforms being found in similar embryonic tissues. In light of previous work studying the functions of the DLK1 splice variants (Garcés *et al*., 1999), this suggests that both DLK1 variants have roles in organogenesis.

Materials and Methods

Timed pregnancy and embryo collection

Embryos were collected from timed pregnancies of FVB/NJ mice, with noon the day of plug identification designated as 0.5 d.p.c. Embryos were further staged according to Theiler. On this background, TS12 was ~ 8.0 d.p.c., TS14 was ~ 8.5 d.p.c., TS16 was ~ 9.5 d.p.c., TS18 was ~ 10.5 d.p.c., and TS20 was ~11.5 d.p.c. All mice were maintained under the care of the Ohio State University IACUC.

In situ *hybridization*

RNA is situ hybridization using digoxygenin-labeled probes was performed using standard protocols (Shifley and Cole, 2008). For simultaneous detection of all *Dlk1* transcripts (pan-*Dlk1* expression), a 382 bp fragment of the cDNA, was amplified by RT-PCR using primers SC-628 (5'-tgtcaatggagtctgcaagg-3') and SC-629: (5'-atcgttctcgcatgggttag-3'). For specific detection of *Dlk1-A*, a 185 bp fragment, corresponding to the intron of the *Dlk1*-B isoform was amplified by RT-PCR using the primers: SC-690 (5'-tgagcttcgagtgtctgtgc-3') and SC-691 (5'-tttcatggacaccttcagga-3').

RT-PCR

Total poly(A)$^+$-selected RNA was extracted from dissected embryos and first strand cDNA synthesis was completed (SuperScript III, Invitrogen). All *Dlk1* transcripts were amplified simultaneously using the primers SC-655 (5'-cacctgggttctctggaaag-3') and SC-656 (5'-acgcccaggatggtgaag-3'), which yields predicted bands corresponding to *Dlk1-A* at 595 bp, *Dlk1-B* at 442 bp, *Dlk1-C/C2* at 376 bp, and *Dlk1-D/D2* at 310 bp.

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