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Nemo-like kinase (NLK) acts downstream of Notch/Delta signalling to downregulate TCF during mesoderm induction in the sea urchin embryo

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Studies in *Caenorhabditis elegans* and vertebrates have established that the MAP kinase-related protein NLK counteracts Wnt signalling by downregulating the transcription factor TCF. Here, we present evidence that during early development of the sea urchin embryo, NLK is expressed in the mesodermal precursors in response to Notch signalling and directs their fate by downregulating TCF. The expression pattern of *nlk* is strikingly similar to that of Delta and the two genes regulate the expression of each other. *nlk* overexpression, like ectopic activation of Notch signalling, provoked massive formation of mesoderm and associated epithelial mesenchymal transition. NLK function was found to be redundant with that of the MAP kinase ERK during mesoderm formation and to require the activity of the activating kinase TAK1. In addition, the sea urchin NLK, like its vertebrate counterpart, antagonizes the activity of the transcription factor TCF. Finally, activating the expression of a TCF-VP16 construct at blastula stages strongly inhibits endoderm and mesoderm formation, indicating that while TCF activity is required early for launching the endomesoderm gene regulatory network, it has to be downregulated at blastula stage in the mesodermal lineage. Taken together, our results indicate that the evolutionarily conserved TAK/NLK regulatory pathway has been recruited downstream of the Notch/Delta pathway in the sea urchin to switch off TCF-β-catenin signalling in the mesodermal territory, allowing precursors of this germ layer to segregate from the endomesoderm.

KEY WORDS: NLK, Delta, TCF/Lef, ERK, MAP kinase, TAK1, Germ layers, Mesoderm, Endoderm, Sea urchin embryo

INTRODUCTION

The mesoderm of the sea urchin embryo originates from two populations of precursors, the primary mesenchyme cells (PMCs) and the secondary mesenchyme cells (SMCs), which are both generated at the vegetal pole of the embryo. The PMCs derive from the four micromeres that form during the asymmetric fourth cleavage. At blastula stage, the 32 cells that constitute this lineage detach from the epithelium and ingress into the blastocoele by a process of epithelial mesenchymal transition (EMT) that requires transient activation of the MAP kinase ERK (Fernandez-Serra et al., 2004; Rottinger et al., 2004). The SMCs derive from the sister cells of the micromeres called macromeres, which also give rise to most of the endoderm (Cameron et al., 1991; Ruffins and Ettensohn, 1993). This population of mesodermal precursors gives rise to four cell types: the pigment cells, the blastocoelar cells, the coelomic pouch precursors and the circumoesophageal muscles.

Fate mapping experiments and gene expression studies have shown that at blastula stage precursors of the SMCs are present in a ring of cells within the vegetal plate, the centre of which is occupied by the PMCs (Ruffins and Ettensohn, 1996). Indeed, embryological studies have revealed that during early development the SMCs require a signal from the micromeres to be specified. Removal of the micromeres result in embryos devoid of SMCs, while recombining micromeres with blastomeres derived from the animal pole is sufficient to induce formation of SMCs (McClay et al., 2000; Sweet et al., 1999). This signal has been identified as the Delta ligand that activates the Notch receptor (Sherwood and McClay, 1999; Sweet et al., 2002). Overactivation of the Notch pathway causes overproduction of SMCs, while inhibition of Notch signalling prevents specification of the SMCs (Sherwood and McClay, 1999; Sweet et al., 2002). Delta is first expressed in the micromere progeny starting at early blastula stage, then, after ingression of the PMCs, a second wave of Delta expression is initiated in the SMC precursors. Further, experiments using chimeric embryos have established that the first expression of Delta in the micromere progeny is essential for specification of the pigment cells and blastocoelar cells, while its later expression in the SMC territory at mesenchyme blastula stage is important for specification of the blastocoelar cells and muscle cells and for refining the position of the ectoderm-endoderm boundary (Sherwood and McClay, 2001).

Another signalling pathway crucial for specification of the mesoderm is the Wnt/β-catenin pathway (Emily-Fenouil et al., 1998; Logan et al., 1999; Wikramanayake et al., 1998). Starting at the 16-cell stage, β-catenin is translocated into the nuclei of micromeres and macromeres, where, associated with the transcription factor TCF, it activates endomesodermal genes (Huang et al., 2000; Vonica et al., 2000). Expression of Delta in the micromeres also requires nuclear translocation of β-catenin (McClay, 2000). Up to late blastula stage, a high level of β-catenin is detected in the nuclei of mesodermal precursors (PMCs and SMCs). However, after hatching, β-catenin is progressively downregulated in these cells and by early mesenchyme blastula stage it is no longer detected in the nuclei of SMCs (Logan et al., 1999).

One mechanism allowing the downregulation of TCF-β-catenin signalling has emerged from recent work in *Caenorhabditis elegans* and vertebrates. This mechanism relies on a crosstalk between the canonical Wnt pathway and a derived MAP kinase pathway involving the MAP kinase-related proteins Nemo Like Kinase (NLK)

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and TAK-1 (Behrens, 2000; Thorpe and Moon, 2004). In *C. elegans*, NLK regulates the segregation of the endodermal (E) and mesodermal (MS) precursors at the eight-cell stage through the asymmetric nuclear localization of POP-1, the *C. elegans* homologue of TCF (Bowerman and Shelton, 1999; Meneghini et al., 1999; Rocheleau et al., 1997; Rocheleau et al., 1999; Thorpe et al., 1997). Epistasis analysis and biochemical experiments have demonstrated that TAK-1 activates NLK, which in turn phosphorylates TCF and promotes its export from the nucleus (Lo et al., 2004; Rocheleau et al., 1999). The role of this MAP kinase pathway as a negative regulator of TCF activity seems to be conserved in vertebrates, as illustrated by its ability to suppress the axis-inducing activity of β-catenin in *Xenopus* (Ishitani et al., 1999). Biochemical studies have demonstrated that vertebrate NLK binds directly to the TCF/β-catenin complex and phosphorylates two conserved residues on TCF, inhibiting its DNA-binding activity (Ishitani et al., 2003b).

In this study, we analyse the role of NLK during sea urchin development. We found that *nlk* is expressed in a pattern strikingly similar to that of Delta, that expression of *nlk* in the mesodermal lineage is regulated by Notch/Delta signalling, and that *nlk* and Delta strongly synergize during mesoderm formation. Furthermore, we provide evidence that the Delta-induced expression of *nlk* serves as a mechanism to inhibit TCF in the mesodermal lineages and propose that downregulation of TCF in these cells is required for the segregation of the mesoderm from the endomesoderm.

MATERIALS AND METHODS

Animals, embryos and treatments

Adult sea urchins (*Paracentrotus lividus*) were collected in the bay of Villefranche. Embryos were cultured as described in Lepage and Gache (Lepage and Gache, 1989; Lepage and Gache, 1990). Treatments with U0126 were performed as described previously (Rottinger et al., 2004). Lithium was used at 30 mmol/l and dexamethasone at 10 μmol/l.

Northern blot

Total RNA was extracted from embryos at various stages by the method of Cathala (Cathala, 1983). RNA samples (10 μg per lane) were electrophoresed through a formaldehyde agarose gel, transferred to a Nylon membrane, and hybridized with probes labelled by random priming corresponding to the 3′UTR or to the coding sequence of the *nlk* transcript. Hybridization was carried out using standard methods (Sambrook et al., 1989). Both probes gave similar results.

Detection of phosphorylated ERK

For immunolocalization, embryos were fixed in methanol and following rehydration were incubated with a monoclonal antibody specific for the dually phosphorylated form of MAP kinase (ERK1 and ERK2) (Sigma M8159). An anti-mouse secondary antibody conjugated to alkaline phosphatase and the chromogenic substrates NBT/BCIP were used for detection.

Constructs, RNA and morpholino injections

The coding sequence of *nlk* was amplified by PCR using the Pfx DNA polymerase (Invitrogen) and inserted at the *Cla*I-*Xba*I sites of pCS2 + (Turner and Weintraub, 1994). RNA encoding a catalytically inactive version of NLK (NLK K78M) was obtained by replacing, within the ATP-binding site, a conserved lysine residue by a methionine using PCR. RNA encoding wild-type NLK or NLK K78M were injected at 900 μg/ml.

pCS2-DN-TCF encodes a dominant negative TCF and was made by deleting the β-catenin-binding domain of the *P. lividus* TCF (C.G., unpublished). The *pCS2-TCF-VP16-GR* was constructed by using as starting plasmid the *pCS2 ENR-Tbx2/3-GR* (Horb and Thomsen, 1999). The fragment encoding ENR-Tbx was removed by digestion and replaced by an N-terminally deleted TCF fused to VP16 (C.G., unpublished) (Triezenberg et al., 1988). *pCS2-TCF-VP16-GR* was used at 400 μg/ml. *PCS2Lv Delta* was constructed by amplifying the coding sequence of *Lytechinus variegatus* Delta (Sweet et al., 2002) and cloning it in pCS2. To construct the dominant negative Delta, we used the *P. lividus* Delta sequence. Using PCR, we introduced a stop codon at amino acid 626, resulting in a truncation of the region located after the transmembrane domain. RNAs encoding Delta or the dominant negative Delta were used at 400 μg/ml and 200 μg/ml, respectively.

In the experiments to test for a synergistic effect, *nlk* and Delta were co-injected at 450 μg/ml and 200 μg/ml, respectively. The following morpholino oligonucleotides were used at 0.9 mmol/l: 5′-CGAG-ATCCACAAACAGCCATATCAC-3′ (NLK ATG); 5′-TCGGAGGCA-GACCAGCAGCGAGAAA-3′ (NLK 5′UTR); 5′-GATTCAAGGCGA-GCCATTTTGGATG-3′ (TAK-1).

All the experiments were repeated two or three times, and for each experiment 100-150 embryos were observed. Only representative phenotypes (present in 80-90% of the injected embryos) are shown.

In situ hybridization

In situ hybridization was performed following a protocol adapted from Harland (Harland, 1991) with antisense RNA probes and staged embryos. Most of the probes used in this study were isolated in the course of an in situ hybridization screen (T.L., unpublished). The *bhmt* (betaine-homocysteine S-methyltransferase), *bpnt* (PAP-inositol 1-4 phosphatase), *papss* (3′-phosphoadenosine 5′-phosphosulfate synthase). *ets1*, *ske-T*, *alx1*, *Delta*, *goosecoid* and *brachyury* are the *P. lividus* homologues of these genes. The accession numbers of the mRNA sequences used in this study are as follows: *nlk*, AY442297; *TCF*, AM179826; *Delta*, DQ536193; *tak1*, DQ531771; *gcm*, DQ666827; *alx1*, DQ536192; *bhmt*, DQ531773; *bpnt*, DQ531772; *papss*, DQ531774.

TOP FLASH assays

Reporter gene assays were performed as described by Vonica et al. (Vonica et al., 2000). Briefly, the linearized TOP FLASH reporter plasmid was injected at 5 μg/ml together with carrier DNA at 50 μg/ml. Embryos injected with the TOP FLASH reporter plasmid alone, with *nlk* mRNA, or that were treated with lithium were collected after hatching and lysed in 70 μl of lysis buffer (Promega) for 30 minutes at room temperature. After centrifugation, the supernatant was stored at –20°C. To perform the luciferase assay, 60 μl of supernatant was mixed to 150 μl of assay buffer (Promega) and the luminescence was measured on a Glomax luminometer (Promega). About 200 embryos were used for each experiment and the experiments were repeated three times.

RESULTS

Identification of *nlk*

The sea urchin orthologue of NLK was identified in the course of an in situ hybridization screen as a gene displaying restricted expression during early development. The sea urchin *nlk* transcript encodes a protein of 438 amino acids homologous to the vertebrate NLK proteins (Brott et al., 1998; Hyodo-Miura et al., 2002; Ishitani et al., 1999; Thorpe and Moon, 2004), to *Drosophila* Nemo (Choi and Benzer, 1994) and to *C. elegans* LIT-1 (Meneghini et al., 1999; Rocheleau et al., 1999). An alignment of these NLK protein sequences shows that conservation is very high in the central kinase domain and the C-terminal region, while the N-terminal part is not conserved (see Fig. S1 in the supplementary material).

Northern blot analysis revealed the presence of a single 7.6 kb transcript expressed maternally and throughout cleavage. The level of this mRNA declines at the blastula stage then increases strongly during gastrulation, probably as a consequence of zygotic transcription (Fig. 1A). After gastrulation, expression of *nlk* decreases abruptly, a low level of mRNA is still detected at the prism stage, but expression is barely detectable at the pluteus stage.

nlk belongs to the Delta synexpression group

During development, *nlk* is expressed in a pattern that resembles that of Delta, the Notch ligand. This similarity is first apparent at the early blastula stage when, after an initial phase of ubiquitous

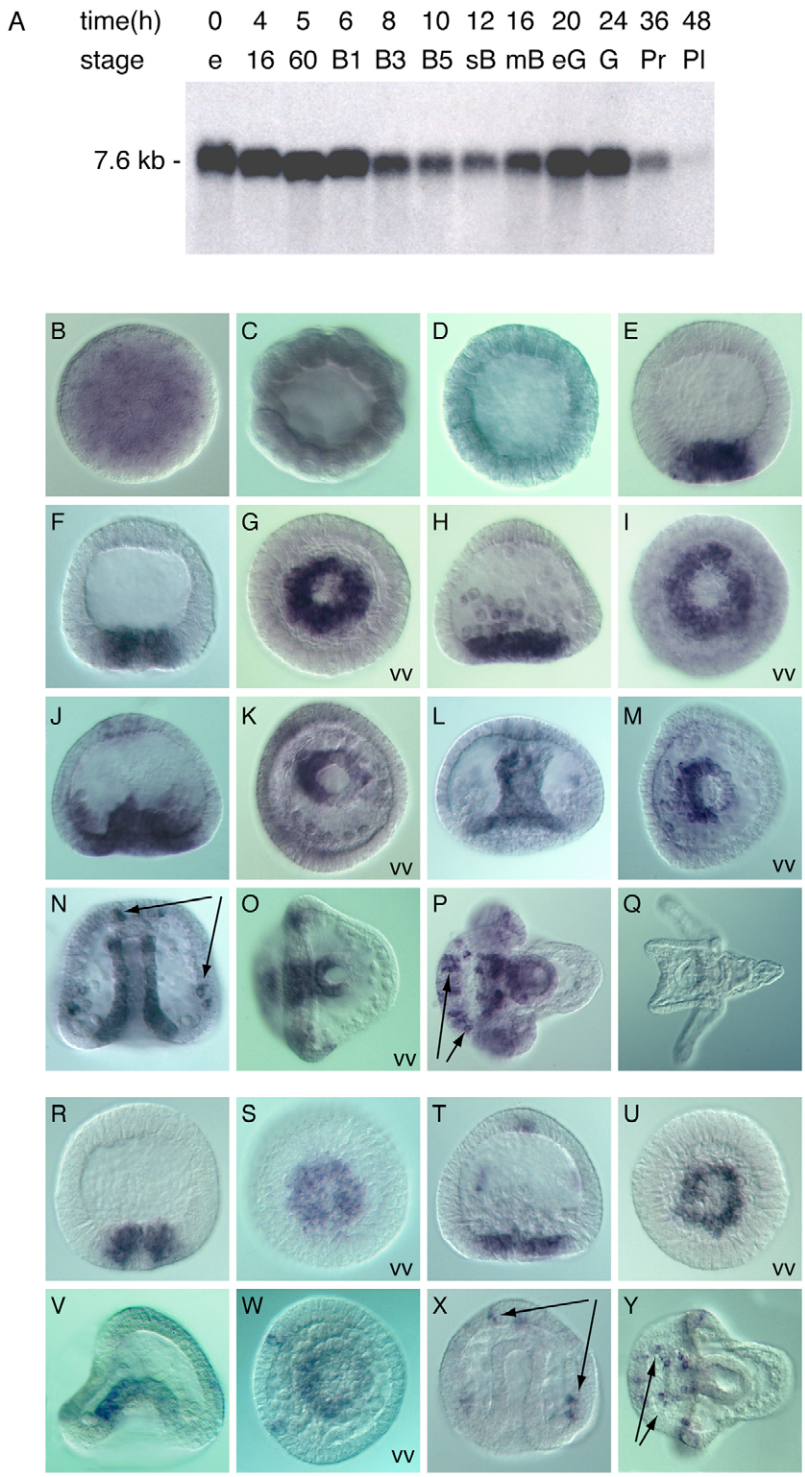

Fig. 1. Temporal and spatial expression of *nlk* during sea urchin development. (**A**) Northern blot (e) Egg, (16) 16-cell stage, (60) 60-cell stage, (B1) very early blastula, (B3) early blastula, (B5) hatching blastula, (sB) swimming blastula, (mB) mesenchyme blastula, (eG) early gastrula, (G) gastrula, (Pr) prism, (Pl) pluteus. (**B-Q**) Spatial distribution of *nlk* and (**R-Y**) *Delta* transcripts. Embryos at the indicated stage were hybridized with sense (not shown) and antisense probes for *nlk* or *Delta*. (B) Egg, (C) 60 cell stage, (D) early blastula, (E) hatching blastula, (F,G,R,S) swimming blastula, (H,I,T,U) mesenchyme blastula, (J,K,V,W) early gastrula, (L,M,X) late gastrula, (N,O) prism, (P,Y) early pluteus, (Q) pluteus. All pictures are lateral views with the animal pole at the top and the oral side on the left, except G,I,K,M,O,S,U,W, which are (vv) vegetal pole views. Note the similarities in the salt and pepper expression pattern of *nlk* and Delta gastrula and early pluteus stages (arrows in panels N,P and X,Y).

expression (Fig. 1B-D), *nlk* transcripts become restricted to a ring of about 30 cells around the vegetal pole that correspond to the precursors of the PMCs (Fig. 1E-G). Delta is also strongly expressed in the PMCs at this stage (Fig. 1R,S) (Sweet et al., 2002). As for Delta, *nlk* expression remains elevated in the PMCs until they start to ingress into the blastocoele but is no longer detected in these cells after ingression is completed. At mesenchyme blastula stage, *nlk* and Delta are both expressed in a domain located at the centre of the flattened vegetal plate that, according to the fate map, corresponds to the presumptive SMCs (Fig. 1H,I) (Ruffins and Ettensohn, 1993).

During gastrulation, *nlk* is expressed strongly in the archenteron (Fig. 1J-M) asymmetrically along the oral-aboral axis, with strongest expression on the oral side (Fig. 1K,M,O). Similarly, Delta is expressed in the archenteron, although at a low level and only early during gastrulation (Fig. 1V,W). Remarkable similarities in the expression patterns of NLK and Delta continue to be observed at the late gastrula, prism and pluteus stages. Novel domains of expression of *nlk* and Delta appear at the animal pole and on each side of the lateral ectoderm, and, intriguingly, they are often restricted to single cells (Fig. 1N,X). Finally, at the pluteus stage, both *nlk* and Delta are

expressed in cells scattered throughout the oral ectoderm that, based on their number and location, may correspond to neural cells (Fig. 1P,Y) (Nakajima et al., 2004).

Cross-regulatory interactions between *nlk* and Delta

To analyse the relationship between *nlk* and Delta, we first tested whether *nlk* is a transcriptional target of Notch/Delta signalling. Embryos were injected with mRNA encoding Delta or a dominant negative form of Delta and the expression of *nlk* was examined by whole-mount in situ hybridization. Injection of Delta mRNA caused *nlk* to be ectopically expressed throughout the vegetal plate (Fig. 2A-D). By contrast, in embryos overexpressing a truncated, dominant negative form of Delta (see Fig. S2 in the supplementary material), *nlk* expression in the PMC and SMC territories was abolished (Fig. 2E-J). These results indicate that *nlk* is positively regulated by the Notch/Delta pathway. Reciprocally, we tested whether expression of Delta was sensitive to manipulations of *nlk*. Injection of *nlk* mRNA caused large patches of ectodermal cells to express Delta ectopically, indicating that overexpression of *nlk* could in turn upregulate Delta (Fig. 2K,L). This analysis indicates that *nlk* is a transcriptional target of Delta/Notch signalling and that *nlk* and Delta regulate the expression of each other.

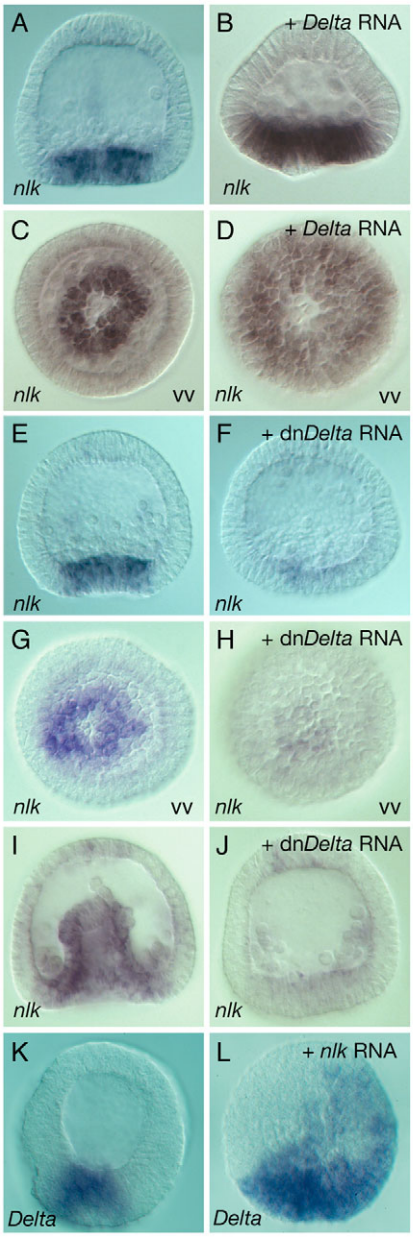

Fig. 2. *nlk* and Delta regulate each other. (**A-L**) Embryos were injected with mRNA encoding *Delta* (B,D), dnDelta (F,H,J) or *nlk* (L); (A,C,E,G,I,K) control uninjected embryos. In situ hybridization was performed with the indicated probes. (A-D) Mesenchyme blastula, (G,H,K,L) swimming blastula, (I,J) late gastrula.

Overexpression of *nlk* or Delta causes the same phenotypes

To gain insight into the role of NLK, we overexpressed it by mRNA microinjection into unfertilized eggs. Overexpression of *nlk* severely perturbed patterning of the embryo (Fig. 3). The first visible defects were observed at the gastrula stage. While in control embryos the gut had elongated and the PMCs had formed bilateral clusters on each side of the oral ectoderm, in the injected embryos, elongation of the archenteron was strongly delayed, the PMCs remained disorganized, and no visible sign of oral aboral polarity was apparent (Fig. 3A, part a). A second remarkable phenotype was observed following overexpression of *nlk* (although with high variability, from 10 to 90%, depending on the batch of embryos). This phenotype was characterized by massive EMT, which started at the vegetal pole and propagated to the rest of the embryo (Fig. 3A, part d). This wave of EMT resulted in the extrusion of an excess of mesenchymal cells at the vegetal pole. These mesenchymal cells later accumulated a red pigment, and therefore probably corresponded to pigment cells.

When the control embryos reached the pluteus stage, about half of the *nlk*-injected embryos had gastrulated but displayed a short and wide archenteron that remained straight (Fig. 3A, part f). Frequently, this archenteron was surrounded by multiple abnormal spicules, indicating that ectodermal patterning was perturbed and oral-aboral polarity was disrupted (Fig. 3A, part g). The other half of *nlk*-overexpressing embryos exogastrulated and also appeared radialized (Fig. 3A, part h). Their ectoderm consisted of a small hollow sphere containing abnormal spicules, and their digestive tract appeared enlarged. In addition, all the injected embryos were strongly pigmented, suggesting that overexpression of *nlk* had caused production of an excess of pigment cells.

This range of phenotypes, which comprises extrusion of pigment cells, radialization, exogastrulation and overdevelopment of the endomesoderm, is very reminiscent of the phenotypes caused by overexpression of an activated form of Notch (Sherwood and McClay, 1999) or of Delta (Sweet et al., 2002) (Fig. 3A, parts i-l). These observations provide further evidence that NLK function is linked to the Notch/Delta signalling pathway.

nlk overexpression expands the mesoderm territory

To characterize the NLK overexpression phenotype we examined the expression of several genes expressed in the main embryonic territories. We first analysed the expression of the hatching enzyme gene (*HE*), which is a marker of the presumptive ectoderm (Lepage et al., 1992), and of *brachyury*, which is expressed dynamically at the ectoderm-endoderm boundary (Croce et al., 2001; Gross and McClay, 2001). In control embryos, the territory expressing *HE* covers about two-thirds of the blastula (Fig. 3B, part a). Overexpression of NLK caused this territory to shrink to one-third of the embryo, indicating that the boundary between the ectoderm

and endomesoderm had been shifted toward the animal pole (Fig. 3B, part g). In agreement with this conclusion, the territory expressing *brachyury* was dramatically displaced toward the animal pole following overexpression of *nlk* (Fig. 3B, parts b,h). Conversely, the expression of *goosecoid* in the presumptive oral ectoderm was abolished in these embryos, consistent with the suppression of oral-aboral polarity and the absence of a stomodeum caused by ectopic *nlk* mRNA (Fig. 3B, parts c,i).

To examine the effects of NLK overexpression on mesoderm and endoderm, we analysed the expression of several novel markers that are specifically expressed in the these territories (see Materials and methods). *bhmt* is expressed in the presumptive midgut and hindgut, while *bpnt* and *papss* are expressed in the presumptive SMC territory, starting at mesenchyme blastula stage. In most of the *nlk*-injected embryos, the ring of cells expressing the endodermal marker *bhmt* was not particularly expanded but rather shifted toward the animal pole, suggesting that in these embryos, NLK had not caused formation of an excess of endoderm but had shifted the ectoderm-endoderm boundary (Fig. 3B, parts d,j). By contrast, expression of the mesodermal markers *bpnt* (Fig. 3B, parts e,f,k,l) and *papss* (data not shown) was dramatically expanded in *nlk*-injected embryos. Instead of a ring of cells surrounding the vegetal pole, the territory expressing these genes covered most of the vegetal plate and extended up to the equatorial region of the embryo. In addition, we confirmed that overexpression of Delta caused the same effects on mesodermal and endodermal markers as overexpression of *nlk* (data not shown; see Fig. 5).

On the basis of this molecular analysis, we conclude that overexpression of *nlk*, like overexpression of Delta, causes primarily an expansion of the mesoderm territory and a shift in the endoderm territory toward the animal pole, with a concomitant reduction of the ectodermal territory.

Strong activation of mesoderm formation following co-expression of *nlk* and Delta

The similarities in the expression patterns of *nlk* and Delta, together with the finding that they cross-regulate each other and that both genes produce similar phenotypes when overexpressed, suggested that *nlk* interacts with the Notch/Delta pathway. To test for a possible synergy, we co-injected both factors at concentrations that individually were not sufficient to produce the effects described previously. In this case a striking phenotype was observed. Starting at the mesenchyme blastula stage, all the injected embryos started to extrude mesenchymal cells from the vegetal pole, then a wave of EMT swept across the embryos, resulting in all embryos being transformed into mesenchymal cells (Fig. 4A,B,D,E). These mesenchymal cells accumulated a red pigment, suggesting that they had been transformed into pigment cells (Fig. 4C,F). Molecular

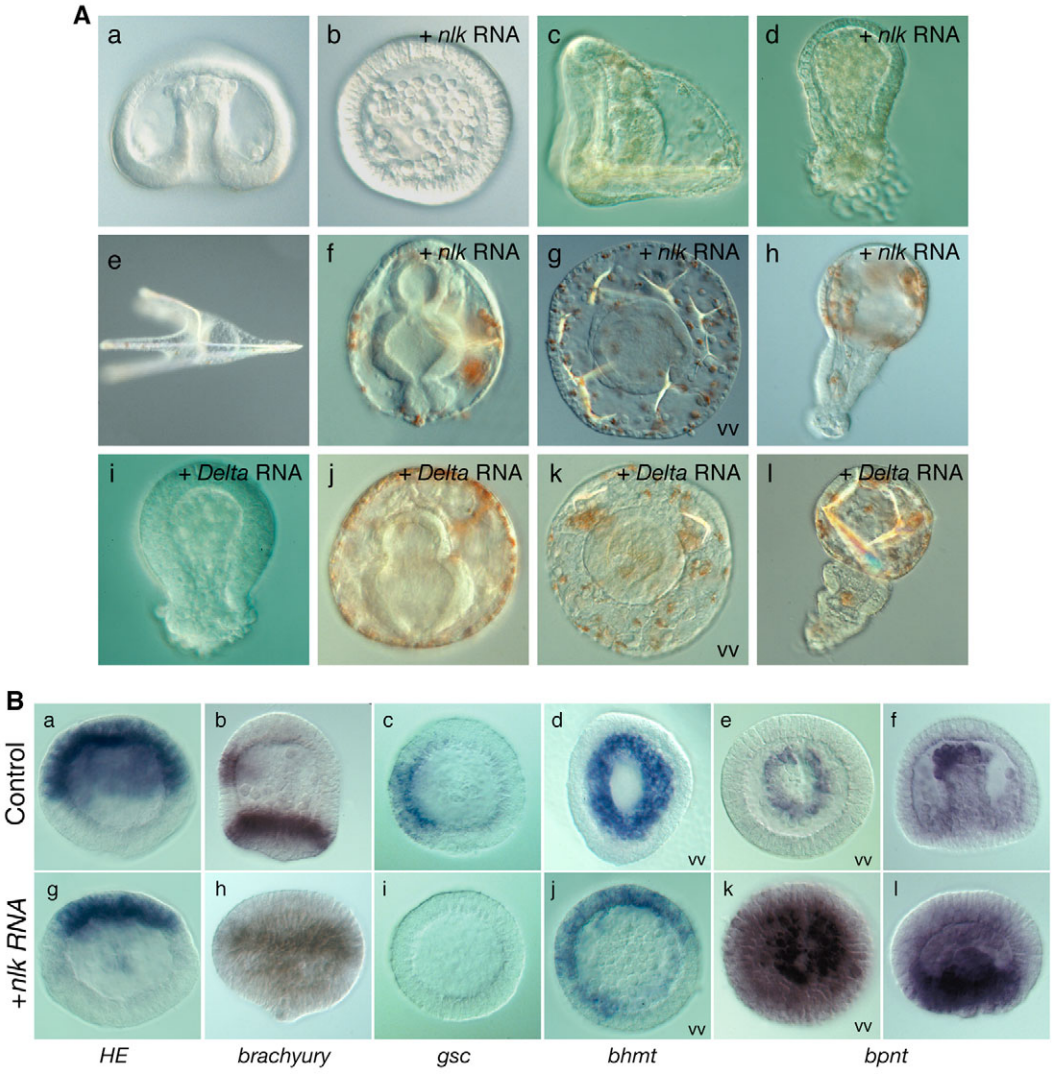

Fig. 3. Overexpression of *nlk* causes the same phenotypes as overexpression of *Delta*. (**A**) Morphology of embryos overexpressing *nlk* or *Delta*. (a,c,e) Control embryos. (b,d,f,g,h) Embryos injected with *nlk* mRNA. (i-l) Embryos injected with *Delta* mRNA. (a,b) Late gastrula, (c,d,i) prism stage, (e-h,j-l) pluteus stage. (vv) Vegetal pole view. (**B**) Effects of *nlk* misexpression on the expression of ectodermal, endodermal and mesodermal genes. In situ hybridization was carried out between blastula and gastrula stages. *HE* (a,g), *bhmt* (b,h), *brachyury* (c,i), *goosecoid* (d,j) and *bpnt* (e,f,k,l). (b-f) Control embryos. (g-l) Injected embryos.

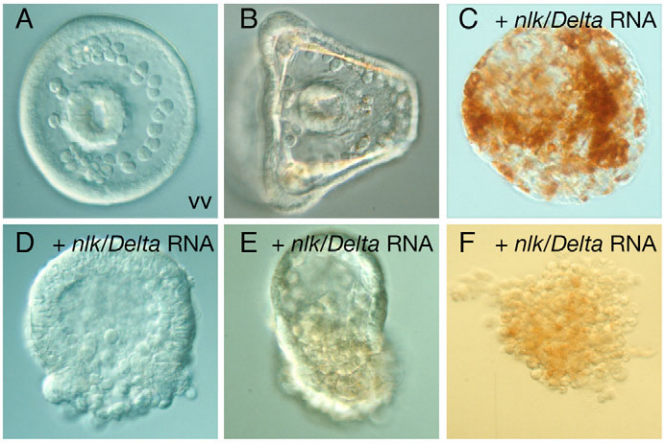

Fig. 4. Strong synergy between NLK and Delta in mesoderm formation. Embryos were injected with *nlk* mRNA together with *Delta*. (**A**,**B**) Control embryos. (**C-F**) Co-injected embryos. (D) Mesenchyme blastula, (A,E) late gastrula, (B,C,F) early pluteus.

characterization of this phenotype showed that co-expressing *nlk* and Delta abolished expression of the early PMC markers *skeT* (Fig. 5D) and *alx1* (Fig. 5H), while expression of the mesenchymal cell marker *ets1* (Fig. 5L) and of the pigment cell markers *gcm* and *papss* (Fig. 5P,T) was dramatically increased, extending to cells of the animal region. Therefore, co-expression of *nlk* and Delta had converted most cells of the embryo into SMCs. Importantly, the phenotypes obtained by co-injection of both reagents were much more severe than those caused by injection of either mRNA alone, indicating that NLK and Delta act synergistically during mesoderm formation.

The overproduction of mesenchymal cells caused by ectopic expression of *nlk* or Delta, or by the co-expression of both factors, is reminiscent of the phenotype caused by overexpression of an activated form of MEK (Fernandez-Serra et al., 2004). This observation raised the possibility that ectopic expression of *nlk* and/or Delta could activate ERK, which in turn would be responsible for part of the observed phenotypes. In control embryos at blastula stage, activation of ERK is restricted to the precursors of the PMCs (Fig. 5U) (Fernandez-Serra et al., 2004; Rottinger et al., 2004). However, following overexpression of *nlk* or Delta, ERK activation could be detected in an enlarged area of the vegetal pole that encompassed the region where the SMCs normally form (Fig. 5V,W). Most strikingly, in the embryos overexpressing *nlk* and Delta, a strong activation of ERK was detected in all cells, foreshadowing the massive conversion into mesenchymal cells observed at later stages (Fig. 5X).

Partial redundancy during mesoderm formation between the MEK/ERK and the TAK1/NLK pathways

To determine the function of NLK, we used three different strategies. First we constructed a kinase dead version of NLK (K78M), equivalent to the mouse NLK (K155M), which was shown to be unable to block TCF binding to DNA and to function as a dominant negative form (Ishitani et al., 1999). Second, we designed two antisense morpholino oligonucleotides directed against *nlk* transcripts, one directed against the translational start and the other further upstream in the 5′UTR. Finally, using a different morpholino oligonucleotide, we attempted to block the translation of the mRNA encoding TAK1, the kinase that is thought to be responsible for activating NLK. Embryos microinjected with the antisense morpholino oligonucleotides against *nlk* and/or *tak1* transcripts or with mRNA encoding the kinase dead mutant of NLK, always developed normally, even when very high concentrations (1.5 mmol/l oligonucleotide and 1.5 mg/ml RNA) of these reagents were used. A possible explanation for the absence of effect of these reagents is that the activity of another factor may compensate for the loss of NLK activity in these embryos. Because NLK is highly related to ERK, and because ectopic activation of the MEK/ERK pathway causes the same phenotypes as the overexpression of *nlk*, the kinase ERK itself appears a good candidate for this redundant factor. To test this idea, we injected *nlk* morpholinos (Mo-*nlk*) and treated half the embryos with the MEK inhibitor U0126 to block ERK activation (Fig. 6). Untreated Mo-*nlk*-injected embryos developed normally, and the control embryos treated with U0126 developed without PMCs and very few SMCs, as shown previously (Fernandez-Serra et al., 2004; Rottinger et al., 2004). A surprising phenotype was observed following the double knockdown of NLK and ERK. All these embryos failed to hatch and gastrulated within the fertilization envelope, and their archenteron was very smooth, suggesting that SMCs had failed to form (Fig. 6D). In Mo-*nlk*-injected embryos, the expression of *gcm*, which marks the pigment cell territory (Ransick et al., 2002), *papss* (Fig. 6I,O) and Delta (data not shown) was normal, while in the embryos treated with U0126, the expression of these genes was reduced but clearly detectable (Fig. 6H,N), consistent with previous studies (Fernandez-Serra et al., 2004). By contrast, in the U0126-treated Mo-*nlk*-injected embryos, no expression of *papss*, *gcm* or Delta could be detected, confirming that specification of the SMCs had failed (Fig. 6J,P and data not shown). The same effects were observed after simultaneous inhibition of *tak1* mRNA translation and MEK activity, including inhibition of hatching and complete loss of expression of *gcm*, *papss* and Delta, consistent with the position of TAK1 upstream of NLK in other systems. These results demonstrate that the TAK1/NLK pathway is partly redundant with the MEK/ERK pathway during mesoderm formation in the sea urchin embryo. They also show that while *nlk* overexpression results in formation of an excess of mesoderm, knocking down its function causes a loss of mesoderm, consistent with a positive requirement for NLK in mesoderm formation.

Overexpression of NLK downregulates TCF

Taken together, the results presented so far indicate that, in the sea urchin embryo, NLK is a target of Notch/Delta signalling and functions together with ERK to promote formation of the mesoderm. However, previous studies carried out on different organisms have shown that NLK, by phosphorylating TCF and suppressing its DNA-binding activity, acts primarily as an antagonist of the Wnt pathway. To examine whether overexpression of *nlk* influences the transcriptional activity of TCF in the sea urchin embryo, we performed a TCF reporter gene assay using the TOP FLASH construct that contains multiple copies of a TCF-binding site upstream of the luciferase coding sequence. This plasmid was injected into the eggs, either alone or together with *nlk* mRNA, and stimulation of the Wnt pathway was achieved by continuous treatment with lithium following fertilization. The embryos were collected at the blastula stage and luciferase activity was measured. When the embryos were injected with the reporter gene and treated with lithium, a dramatic increase in the activation of the reporter gene was observed compared with controls (Fig. 7B). When *nlk* was co-injected with the TOP FLASH plasmid, it completely prevented

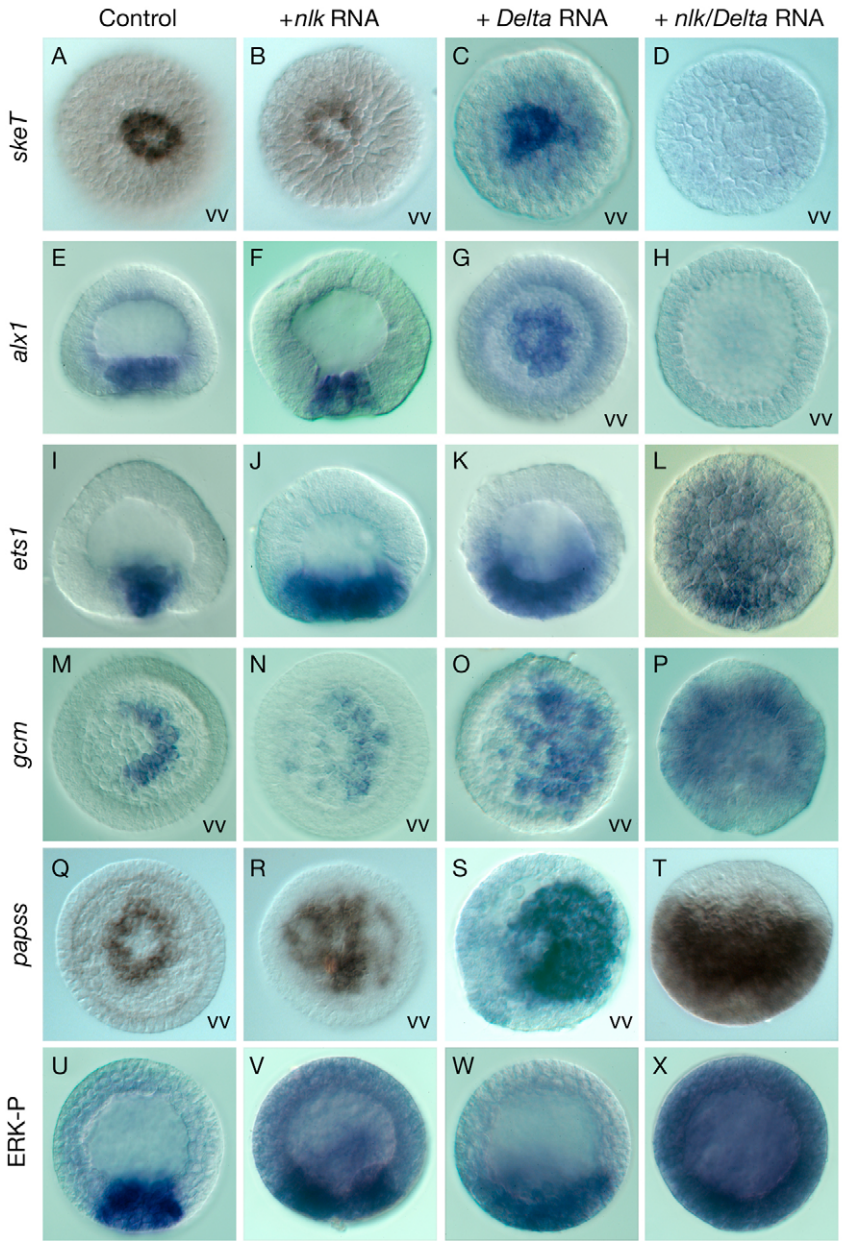

Fig. 5. Effects of *nlk/Delta* misexpression on mesoderm specification. (**A-X**) Following injections of the indicated mRNA, in situ hybridization was performed, with probes indicated on the left. vv, vegetal pole view.

the lithium-induced activation of the reporter gene. Consistent with these results, we found that the sea urchin TCF sequence contains a conserved SPGTP phosphorylation site within the central region of the protein, which has been shown to be phosphorylated by NLK (Fig. 7A) (Ishitani et al., 2003b), and that an NLK-GFP fusion protein localizes within the nucleus in the sea urchin embryo (see Fig. S2 in the supplementary material).

In the course of these experiments, we noted that when the *nlk*-injected lithium-treated embryos reached the mesenchyme blastula stage, they underwent a massive EMT similar to that observed following overactivation of the Notch pathway or co-expression of *nlk* and Delta (Fig. 8D). These mesenchymal cells were probably SMCs, as they became pigmented (Fig. 8H). While in the control embryos treated with lithium or injected with *nlk,* the endodermal marker *bhmt* was either overexpressed (Fig. 8J) or displaced towards the animal pole (Fig. 8K), in the *nlk*-injected lithium-treated embryos, the expression of this marker was abolished (Fig. 8L). Similarly, the expression of the PMC marker *skeT* was undetectable in these embryos (Fig. 8P). By contrast, expression of the SMC markers *papss* (Fig. 8T) and Delta (Fig. 8X) was dramatically increased and extended up to the animal pole. Thus, these results show that overexpression of NLK profoundly modifies the effects of lithium treatment on cell fate specification. While lithium treatment normally promotes predominantly endodermal cell fates, leaving the mesodermal territory largely unaffected, the presence of overexpressed NLK causes most cells of the embryo to express Delta and become mesoderm instead of endoderm.

Gastrulation and maintenance of the endomesoderm gene regulatory network requires downregulation of TCF after hatching

The spatial expression pattern of *nlk* together with the results from our functional analysis suggest that during normal development the role of this kinase is to downregulate TCF in the mesodermal precursors at blastula stages. If downregulating TCF before gastrulation is important for mesoderm formation, then

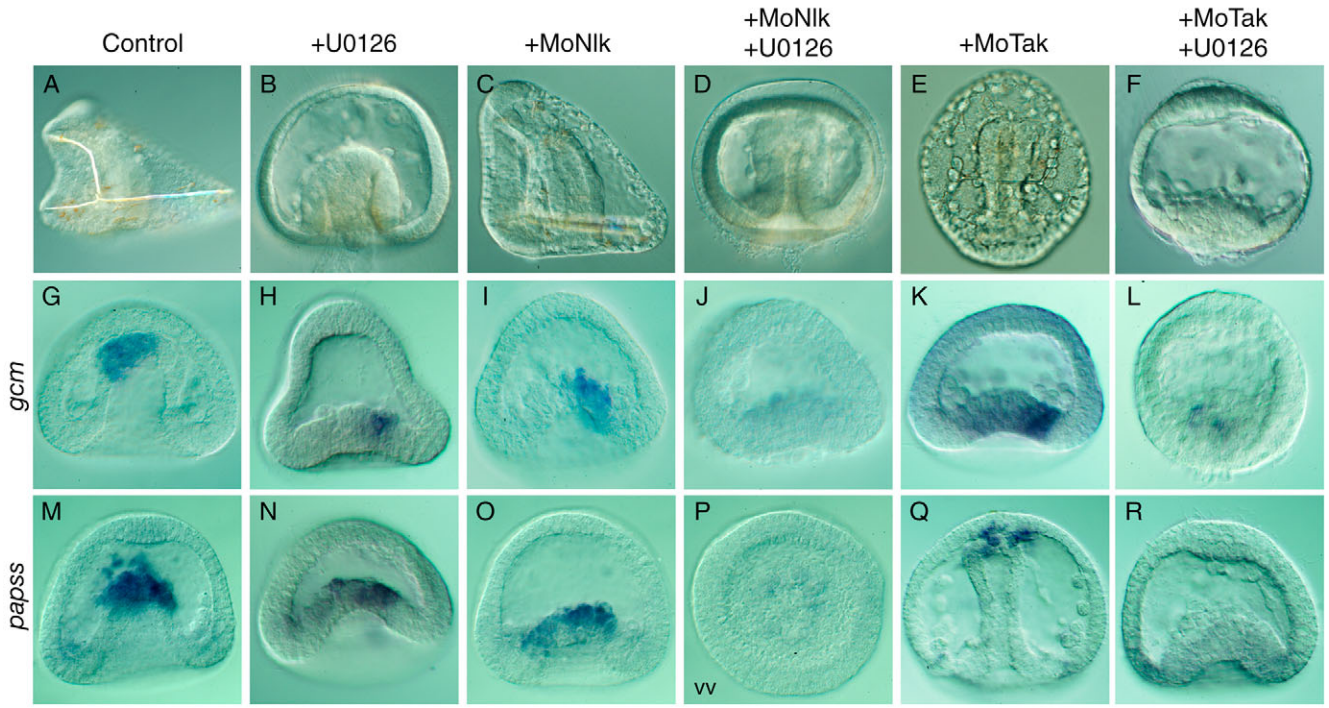

Fig. 6. Partial redundancy between the TAK1/NLK and the MEK/ERK signalling pathways. (**A-R**) Embryos were injected at the egg stage with the indicated reagents, treated or not with U0126 starting at the two-cell stage. In situ hybridization was performed for *gcm* (G-L) and *papss* (M-R). (A,G,M) Control embryos (B,D,F,H,J,L), U0126-treated embryos (C,D,I,J,O,P). Embryos injected with a morpholino oligonucleotide against *nlk* (Mo-*nlk*) or against *tak1* (Mo-TAK1) (E,F,K,L,Q,R). (A-F) Early pluteus, (G-R) gastrula. vv, vegetal pole view.

maintaining high activity of TCF would be predicted to interfere with differentiation of PMCs and SMCs. To test this prediction, we used a conditional, constitutively active version of TCF fused to the transcriptional activator domain of the viral VP16 protein and to the hormone-binding domain of the mouse glucocorticoid receptor (GR). Chimeric RNA encoding this construct was injected in the egg, and the nuclear uptake of the fusion protein was triggered at different stages by addition of dexamethasone (Fig. 9A). In the absence of dexamethasone, the embryos injected with this RNA developed normally. As expected, when dexamethasone was added immediately after fertilization, the injected embryos developed into extremely vegetalized larvae that expressed *bhmt* up to the animal pole (Fig. 9C,G). Treatments with dexamethasone started at the early blastula stage also caused a strong vegetalization, indicating that activation of the Wnt pathway to a sufficiently high level can still change the fates of the animal blastomeres towards endoderm after the cleavage period (data not shown). When dexamethasone was added around the time of hatching, a marked decrease in the degree of vegetalization was observed (Fig. 9D). The embryos formed primary and secondary

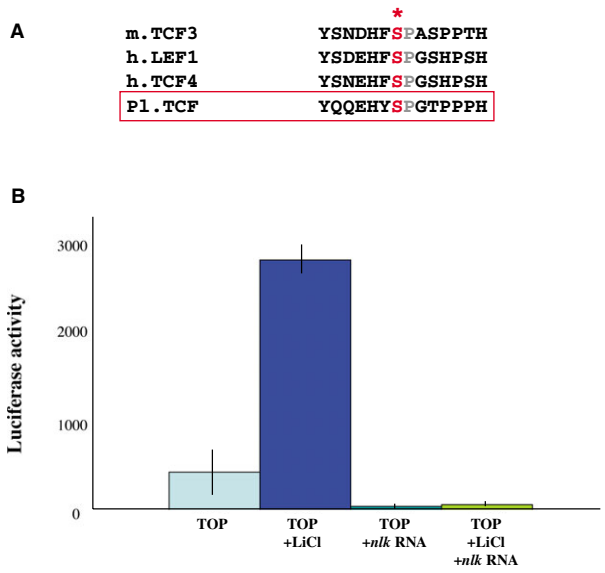

Fig. 7. NLK antagonizes the activity of TCF in TOP FLASH assays. (**A**) Partial sequence alignments between the sea urchin TCF (Pl.TCF) and vertebrate TCF proteins sequences, showing the conservation of the MAPK phosphorylation consensus site. (**B**) Ectopic expression of NLK downregulates TCF. Embryos were injected at the egg stage and treated with LiCl (30 mmol/l) from the two-cell stage on. Embryos injected with the TOP FLASH construct alone (grey column), plus lithium treatment (dark blue column). Embryos co-injected with the TOP FLASH construct and *nlk* mRNA (light blue column), plus lithium treatment (green column).

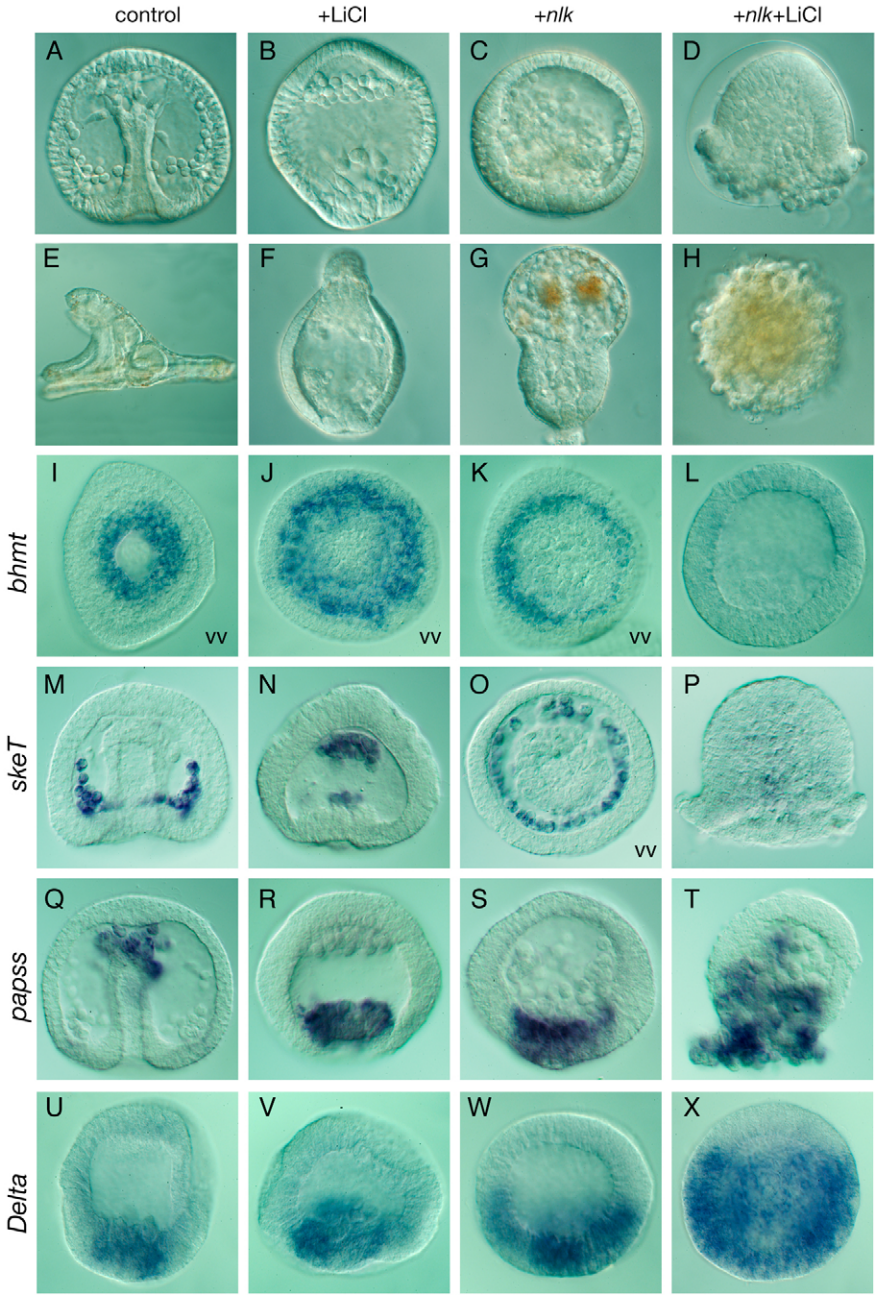

Fig. 8. Lithium treatment strongly potentiates the mesoderm-inducing activity of NLK. Embryos were injected at the egg stage and treated with LiCl at the two-cell stage and either observed for the phenotype (**A-H**) or fixed for in situ hybridization (**I-X**) performed with the probes indicated on the left. (M-P) Mesenchyme blastula (I-L), early gastrula (A-D,Q-T,U-X), late gastrula (E-H), pluteus. vv, vegetal pole view.

mesenchymal cells, gastrulated and formed pigment cells. In these embryos, the endodermal marker *bhmt* was overexpressed but the SMC marker *papss* was expressed at normal levels (Fig. 9H,Q). Finally, when dexamethasone was added at the early mesenchyme blastula stage, the stage at which zygotic expression of *nlk* peaks, a very different phenotype was observed (Fig. 9E). Activation of TCF at this stage instantaneously blocked migration of the PMCs and prevented invagination of the archenteron. These embryos later formed a rudimentary invagination and very few mesenchymal cells that remained unpigmented. Molecular analysis revealed that expression of the endodermal marker *bhmt*, of the PMC marker *skeT* and of the SMC marker *papss* was strongly inhibited 12 hours after activation of the fusion protein (Fig. 9I,N,R). These results show that a high level of TCF activity at the mesenchyme blastula stage severely interferes with development of the endomesoderm. These results are therefore consistent with the idea that NLK, expressed in response to Delta, functions as a negative feedback inhibitor of TCF/β-catenin signalling to allow segregation of the mesoderm (Fig. 10).

DISCUSSION

NLK and Notch/Delta signalling in the sea urchin embryo

Several lines of evidence strongly suggest that in the sea urchin embryo NLK cooperates with the Notch pathway during mesoderm development. First, the complex expression patterns of *nlk* and Delta are largely congruent. Second, *nlk* expression requires Delta signalling and NLK and Delta cross-regulate each other. Overexpression of either gene is sufficient to induce ectopic expression of the other. Furthermore, overexpression of either NLK or Delta causes strikingly similar phenotypes, including overdevelopment of the mesodermal derivatives, perturbation of the

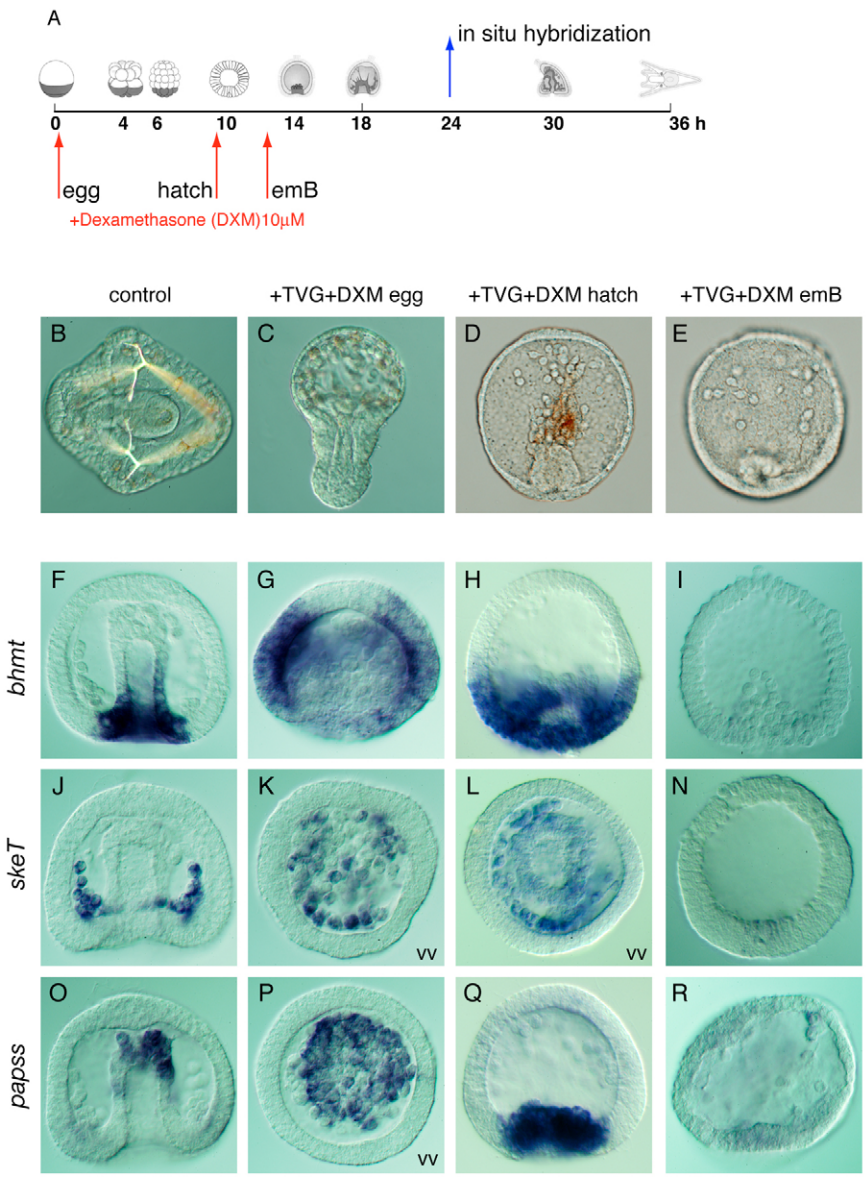

Fig. 9. Gastrulation and formation of the endomesoderm requires downregulation of TCF. (**A**) Scheme of the experiment. (**B-R**) Embryos were injected at the egg stage and treated continuously with Dexamethasone (DXM) starting either at the egg cell stage (C,G,K,P),or at the hatching Blastula (D,H,L,Q) or the early mesenchyme Blastula (vemB) stage (E,I,N,R). The morphology of the embryos is documented in B-E. (F-R) In situ hybridization with the probes indicated on the left. (B,F,J,O) Control embryos. vv, vegetal pole view.

oral-aboral polarity and massive epithelial mesenchymal transition. Finally, when co-expressed at doses that normally are not sufficient to induce mesoderm, *nlk* and Delta strongly synergize, resulting in nearly all cells of the embryo being converted into mesodermal, migratory cells.

While it is clear that the expression and function of NLK are linked to the Delta/Notch signalling pathway, the exact relationships between the activity of this kinase and the different phases of Delta signalling are not yet clarified. One role of NLK might be to promote the expression of Delta in the PMCs, allowing the emission of the first Delta signal responsible for specification of pigment cells. In line with this idea, *nlk* is expressed maternally while Delta is not, and downregulation of *nlk* and ERK prevents expression of Delta in the mesodermal precursors. Conversely, zygotic expression of Delta is initiated in the precursors of the skeletogenic mesenchyme at the seventh cleavage, i.e. before zygotic expression of *nlk* in this territory can be detected. Therefore, it is possible that the first Delta signal (micromere-derived) is responsible for activating the zygotic expression of *nlk* first in the PMCs, then in the presumptive SMC territory. This hypothesis is supported by the finding that early *nlk* expression is strongly dependent on Delta function. Finally, NLK may participate in the maintenance of Delta expression in the SMC territory, allowing the second Delta signal to occur and the other mesodermal derivatives to be specified. As several mesodermal derivatives, and notably pigment cells, are affected by misexpression of *nlk*, the activity of this kinase may well be involved in the two phases of Delta signalling. Additional experiments will be required to fully address these issues.

NLK and the maternal Wnt/β-catenin pathway

Intriguingly, NLK was first characterized in *Drosophila* as a gene required for planar polarity in the eye, a process that requires Delta/Notch signalling between photoreceptors R3 and R4 downstream of *frizzled* (Choi and Benzer, 1994). Mutant alleles of *Drosophila nlk* were also isolated in the course of a genetic screen for dominant modifiers of the activated Notch phenotype (Verheyen et al., 1996). However, a number of studies carried out in *C. elegans* and in vertebrates have subsequently shown that NLK primarily acts as a modulator of Wnt signalling (Ishitani et al., 2003a; Ishitani et al., 2003b; Ishitani et al., 1999; Meneghini et al., 1999; Thorpe

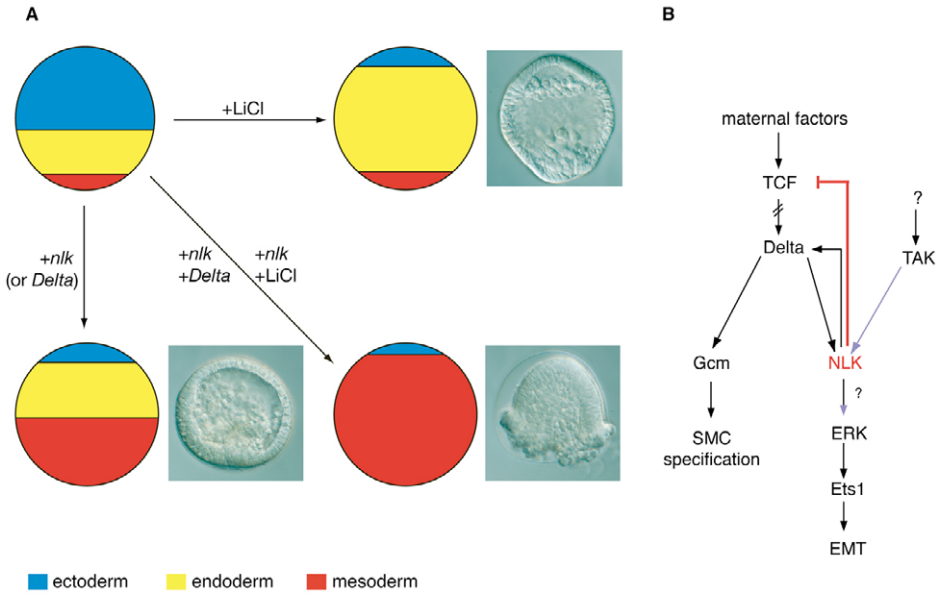

Fig. 10. Model of NLK function during germ layer specification and position of NLK in the endomesoderm gene regulatory network. (**A**) Model of NLK function during segregation of the mesoderm. Treatment with lithium increases the size of the endodermal territory and shifts the ectoderm/endoderm boundary towards the animal pole. Overerexpression of NLK or Delta causes the mesodermal territory to expand and the endodermal/ectodermal boundary to be displaced towards the animal pole. Co-injection of NLK and Delta converts most cells of the embryo into mesoderm. (**B**) Role of NLK in the gene regulatory network regulating mesoderm formation. During cleavage stages, the maternal Wnt pathway acting through TCF induces the endomesoderm. At the blastula stage, Notch/Delta signalling upregulates the expression of nlk in mesodermal precursors. NLK promotes segregation of the mesoderm from the endomesoderm by downregulating TCF in the presumptive SMCs, allowing the establishment of a novel regulatory domain that expresses mesodermal genes such as gcm. NLK and Delta maintain the expression of each other. The NLK and ERK pathways converge to maintain specification of SMCs, while NLK, possibly acting upstream of the MAP kinase pathway, promotes epithelial mesenchymal transition by stimulating phosphorylation of ERK and expression of *ets1*.

and Moon, 2004). In line with these data, sequence analysis confirmed that the sea urchin TCF contains a conserved putative MAP kinase phosphorylation site in a region that has been shown to be phosphorylated by NLK. Further, the TOP FLASH assays clearly showed that, like in vertebrates, NLK inhibits TCF. Taken together, these results strongly suggest that the role of NLK as a kinase that phosphorylates TCF and downregulates its activity is conserved in the sea urchin. However, while in *C. elegans* and in vertebrates NLK appears to act predominantly downstream or in parallel of the Wnt pathway, in the sea urchin, the function of this kinase appears to have been recruited downstream of the Notch/Delta signalling pathway.

The results obtained with the TOP FLASH reporter are, however, difficult to reconcile with the results of overexpression experiments. While experiments with the reporter gene did show that sea urchin NLK antagonizes TCF activity, gain-of-function experiments indicate that NLK overexpression does not interfere with the action of the TCF-β-catenin-mediated maternal Wnt pathway, which is believed to act during cleavage, as specification of the endomesoderm occurs normally in these NLK overexpressing embryos. Not only does overexpression of NLK not block the vegetalizing activity of Lithium, which activates the Wnt pathway, but lithium treatment strongly potentiates the action of NLK, resulting in formation of a large excess of mesoderm. Similarly, in zebrafish, overexpression of *nlk* does not interfere with transduction of the maternal Wnt/β-catenin pathway, which is required for specification of the dorsal ventral axis (Thorpe and Moon, 2004). So why does overexpression of *nlk* in zebrafish or sea urchin embryos not interfere with maternal Wnt/β-catenin signalling? It should be kept in mind that NLK is a MAP kinase that requires phosphorylation by an upstream MAP kinase cascade to be activated and therefore that it is the activity of the kinase that is important and not solely its presence. Thus, a possible explanation for the lack of effect of overexpressed NLK on the maternal TCF-β-catenin pathway is that the activity of NLK is regulated spatially or temporally. If this were the case, then overexpression of *nlk* would not necessarily be predicted to interfere with the early activity of TCF-β-catenin but may only affect late patterning of the embryo, which is what we observe.

Partial redundancy between NLK and ERK

We have shown previously that the strong activation of the MAP kinase ERK that occurs transiently before gastrulation is largely restricted to the presumptive PMC territory and that inhibition of this kinase suppresses formation of the skeletogenic mesenchyme but only partially affects the secondary mesenchymal cells (Rottinger et al., 2004). The finding that another MAP kinase is expressed specifically and at high levels in the SMC precursors raised the possibility that NLK in the SMCs was playing a role similar to that played by ERK in the PMCs. Surprisingly, inhibition of NLK function using dominant negative approaches or with morpholino oligonucleotides failed to demonstrate a requirement for this kinase in either the PMCs or SMCs. Based on the fact that NLK and ERK are both members of the MAP kinase family, which recognize and phosphorylate the same PXS/TP motifs, we then hypothesized that these two kinases may play redundant roles in the SMCs. Indeed, when the function of both kinases was inhibited, the SMCs failed to form, revealing the redundant roles of these kinases. Interestingly, these embryos also failed to hatch. This phenotype is reminiscent of the phenotype resulting from inhibition of the transcription factor Ets4, which regulates the hatching enzyme gene (Wei et al., 1999) and is a potential target of MAP kinase (Rottinger et al., 2004). This observation thus suggests that in addition to its role in the endomesoderm, NLK may cooperate with ERK to regulate the activity of ectodermal transcription factors such as Ets4.

The idea that the activities of ERK and NLK are partially redundant is further supported by the phenotypes observed following activation of ERK. Using an activated form of MEK, Fernandez-Serra et al. (Fernandez-Serra et al., 2004) have shown that activation of the MAP kinase pathway causes overproduction of SMCs, which

extrude from the vegetal pole, i.e. the *nlk* overexpression phenotype. Because, NLK probably acts by phosphorylating TCF, and because the specificities of ERK and NLK are largely overlapping, it would be tempting to hypothesize that the phenotypes caused by overactivation of ERK result from ERK phosphorylating TCF and downregulating its activity. However, preliminary experiments performed to test this hypothesis indicate that ERK is not able to downregulate TCF and therefore that ERK and NLK may promote mesoderm formation by different mechanisms (T.L., unpublished). Therefore, the relationships between NLK and ERK and between ERK and TCF are still enigmatic.

Downregulation of the transcriptional activity of TCF and segregation of the mesoderm from the endomesoderm

In the sea urchin embryo, the endodermal and mesodermal precursors originate from a common endomesodermal territory, which is initially specified by activation of a maternal TCF/β-catenin pathway at the vegetal pole. It is thought that activation of this pathway is sustained during cleavage by the expression of the *Wnt8* ligand in this area. However, starting at the hatching blastula stage, *Wnt8* expression is progressively downregulated in the endomesodermal territory and shifted towards the animal pole so that at the mesenchyme blastula stage, *wnt8* expression is restricted to the ectoderm (Wikramanayake et al., 2004) (E.R., J.C., G.L., L.B., C.G. and T.L., unpublished). Similarly, Logan et al. (Logan et al., 1999) have shown that up to hatching β-catenin is present in the nucleus in the precursors of the mesoderm and endoderm but that after hatching the level of nuclear β-catenin is downregulated in the precursors of the mesoderm (PMCs and SMCs). Therefore, while the role of the Wnt/β-catenin pathway is crucial during the early phase of development to specify the endomesoderm, the spatial expression pattern of *Wnt8* and the pattern of nuclear localization of β-catenin suggest that this pathway has to be downregulated in these cell types after hatching. The results presented in this study agree with this idea. NLK has been shown to inhibit the transcriptional activity of TCF and is expressed at the right place and at the right time to participate to that downregulation at the premise of gastrulation. Further, maintaining a high level of activity of TCF at the beginning of gastrulation strongly interfered with the programme of differentiation of the mesodermal cells.

Finally, the role of NLK in segregation of the mesoderm and endoderm in the sea urchin is highly reminiscent of the role of this kinase in *C. elegans*. In both species, segregation of the mesoderm and endoderm relies on the TAK-1/NLK pathway to mediate inductive interactions between blastomeres. However, while in *C. elegans* NLK appears to act downstream or in parallel of Wnt signalling to promote endoderm development, in the sea urchin NLK appears to act both downstream of Wnt signalling and in cooperation with Delta signalling to promote mesoderm specification. Our findings provide new insights to understand how the primary germ layers of the sea urchin embryo are established and how the mesodermal and endodermal precursors segregate from a bipotential endomesodermal territory, a process that occurs during development of many organisms (Rodaway and Patient, 2001).

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/21/4341/DC1

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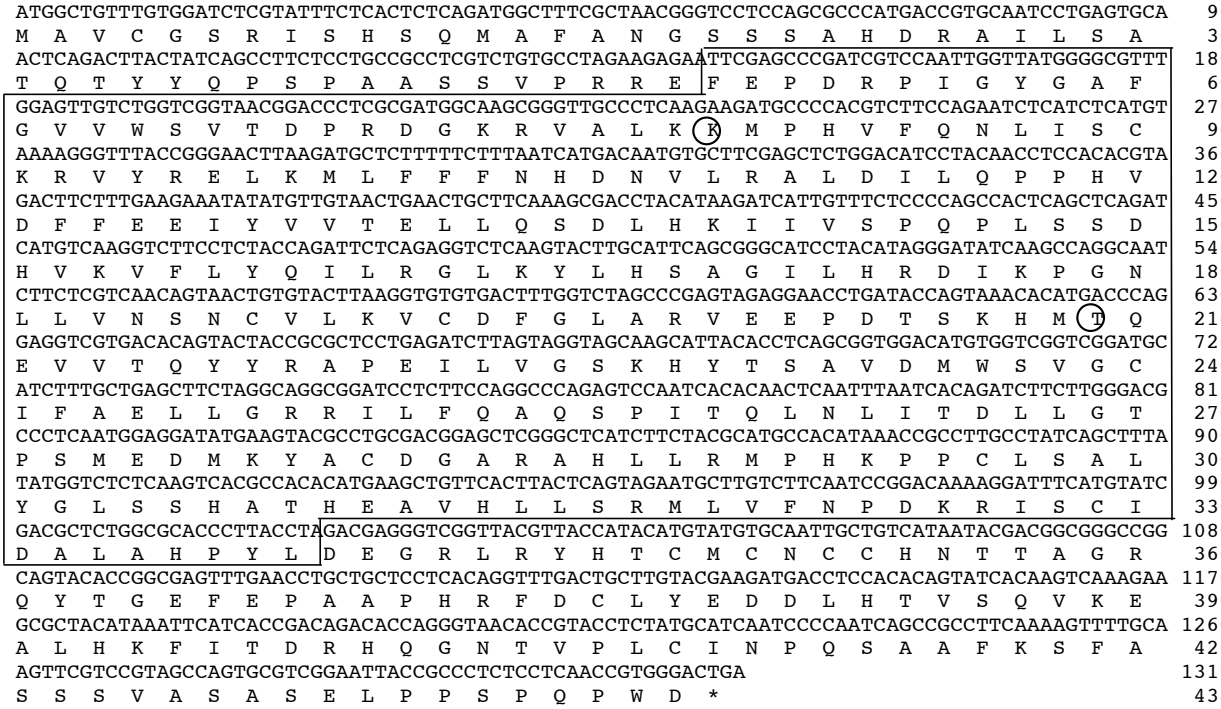
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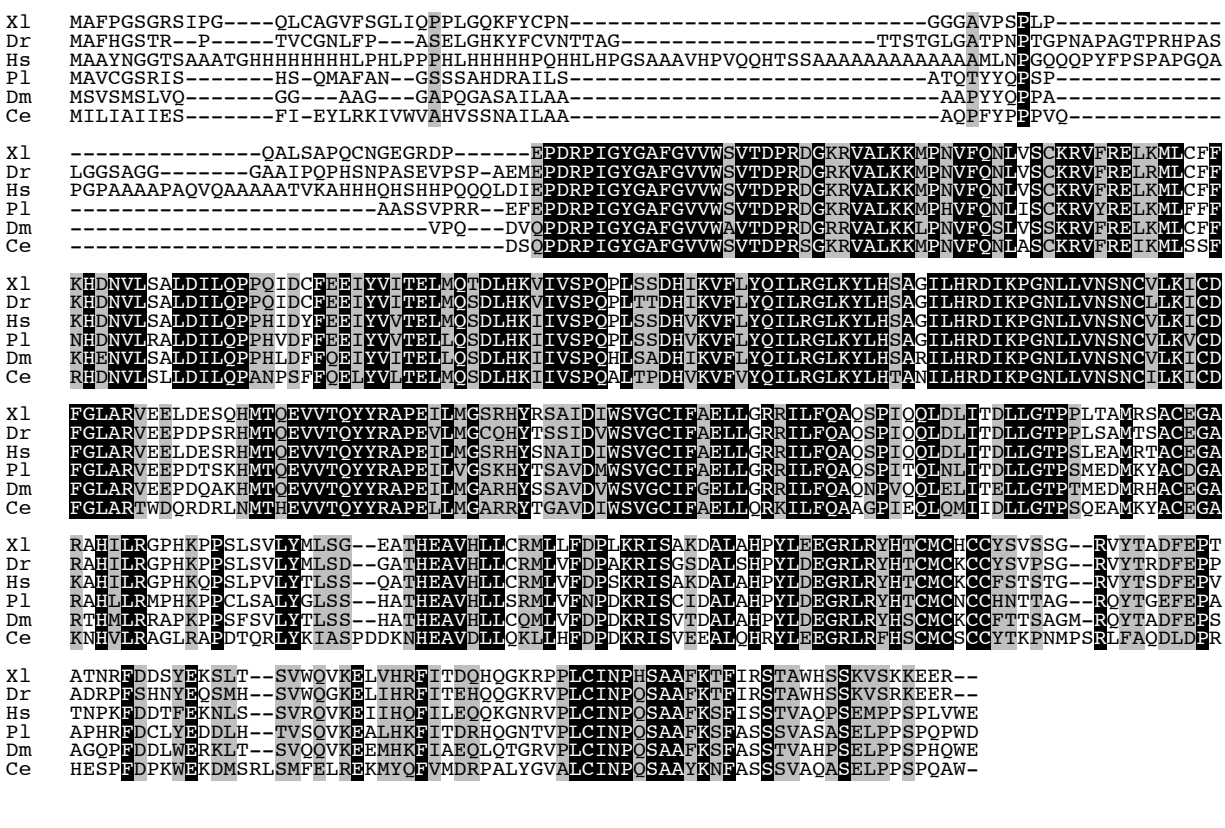
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