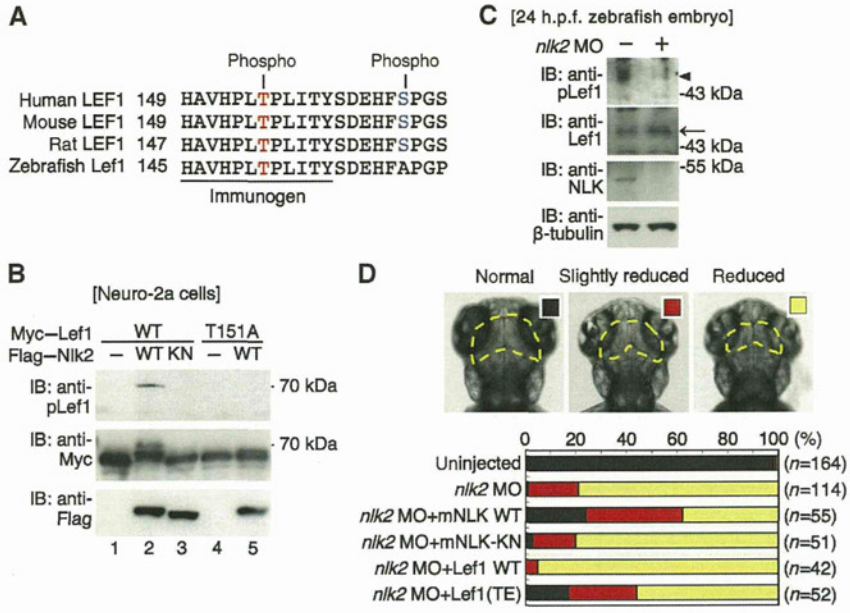

Figure 3 Nlk2 is involved in Lef1 phosphorylation in zebrafish. (**A**) Amino-acid sequence alignment of the NLK phosphorylation regions within vertebrate LEF1/Lef1 proteins. The Thr and Ser residues, which are phosphorylated by NLK, are indicated with red and blue letters, respectively. The bar under the sequence alignment indicates the immunogen for anti-pLef1 antibody. (**B**) Nlk2 phosphorylates Lef1 at the conserved Thr residue. Neuro-2a cells were transfected with Flag–Nlk2 wild-type (WT), Flag–Nlk2 kinase-negative mutant (KN), Myc–Lef1 (WT) and Myc–Lef1(T151A) as indicated. Cell lysates were immunoblotted with anti-pLef1, anti-Myc, and anti-Flag antibodies. (**C**) Nlk2 is required for phosphorylation of Lef1 at the conserved Thr residue in zebrafish. Zebrafish embryos were not injected or injected with *nlk2* MO, as indicated. Extracts were harvested from the embryos at 24 h.p.f., and immunoblotted with anti-pLef1, anti-Lef1, anti-NLK, and anti-β-tubulin antibodies. Arrow and arrowhead indicate Lef1 and phosphorylated Lef1 proteins, respectively. Anti-NLK antibody, which was generated in rabbit with a synthetic peptide corresponding to the carboxyl terminal conserved region of NLK, can recognize Nlk2, but not Nlk1. (**D**) The *nlk2* MO-induced tectum size reduction phenotype is rescued by expression of mouse NLK (mNLK) and zebrafish Lef1(T151E). Zebrafish embryos were injected with *nlk2* MO with or without transposase mRNA and Tol2-donor plasmid containing cDNA encoding mNLK-WT, mNLK-KN, Lef1-WT, or Lef1(TE), and then tectum size was determined. Embryos were classified into three groups based on the extent of tectum size reduction (normal, slightly reduced, and reduced). Upper panels show an example of each class. Broken lines indicate the tectum or presumptive tectal region. Lower graph shows the percentages of embryos exhibiting each class of tectum size reduction. The number shown in the right side of graph is the total number of embryos. Figure source data can be found in Supplementary data.

We examined whether Lef1 phosphorylation by Nlk2 is important for tectum development. Expression of mouse NLK, but not of kinase-negative NLK-KN, partially reversed the reduction in tectum size caused by *nlk2* MO in 80 h.p.f. *nlk2* morphants (Figure 3D). These results suggest that NLK determines tectum size in a manner dependent on its kinase activity. We then tested the effect of the Lef1(T151E) mutation, in which Thr-151 was replaced with glutamic acid to mimic phosphorylation at Thr-151. Expression of Lef1(T151E) partially reversed the reduction in tectum size caused by *nlk2* MO (Figure 3D). In contrast, wild-type Lef1 failed to suppress the *nlk2* MO-induced phenotype. These data suggest that Nlk2 regulates zebrafish tectum development via Lef1 phosphorylation.

Zebrafish has four *tcf* genes: *tcf7*, *tcf7l1a*, *tcf7l1b*, and *tcf7l2*, in addition to *lef1*. Among these *tcf* genes, *tcf7*, *tcf7l1a*, and *tcf7l2* were expressed in the Wnt/β-catenin signalling-active midbrain tissue (Supplementary Figure S7A). When co-expressed with Nlk2 in mammalian neuro-2a cells, Tcf7l1a and Tcf7l2, but not Tcf7 were recognized by the anti-pLef1 antibody (Supplementary Figure S7B). However, injection of a *tcf7l1a* splice-blocking MO (*tcf7l1a* spl MO) or a *tcf7l2* translation-blocking MO (*tcf7l2* MO) did not affect TOPdGFP activity in the midbrain of 24 h.p.f. embryos (Supplementary Figure S6C). These results suggest that Tcf7l1a and Tcf7l2 are not involved in Nlk2-mediated activation of Wnt/β-catenin signalling.

We next examined whether Wnt signalling regulates Lef1 phosphorylation and tectum development in zebrafish. The *wnt1* gene transcript was strongly expressed in 24 h.p.f. zebrafish dorsal midbrain (Supplementary Figure S4A and B), where the *nlk2* and the TOPdGFP reporter are also expressed. When *wnt1* was partially knocked down by MO in zebrafish embryos, Lef1 phosphorylation was reduced (Figure 4A). Furthermore, *wnt1* MO decreased TOPdGFP activity and *zic2a* expression at 24 h.p.f. (Figure 4B; Supplementary Figure S2B; Supplementary Table SII) and 27 h.p.f. (Supplementary Figure S2C), but had no effect on the expression of brain marker genes at 24 h.p.f. (Supplementary Figure S4C). Tectum size was also reduced in *wnt1* morphants at 80 h.p.f. (Figure 4C; Supplementary Figure S5; Supplementary Table SII). Expression of mouse Wnt-1 partially reversed the reduction in tectum size caused by *wnt1* MO (Figure 4C and D). Taken together, these results suggest that Wnt1 and Nlk2 regulate tectum development by inducing Lef1 phosphorylation in zebrafish midbrain.

NLK positively regulates Wnt/β-catenin signalling by phosphorylating LEF1 in NPC-like mammalian cell lines

The above results raised the possibility that NLK positively regulates Wnt/β-catenin signalling by phosphorylating Lef1 in zebrafish midbrain NPCs. We therefore examined the relationship between NLK and the Wnt/β-catenin pathway in the NPC-like mammalian cell lines, mouse neuroblastoma

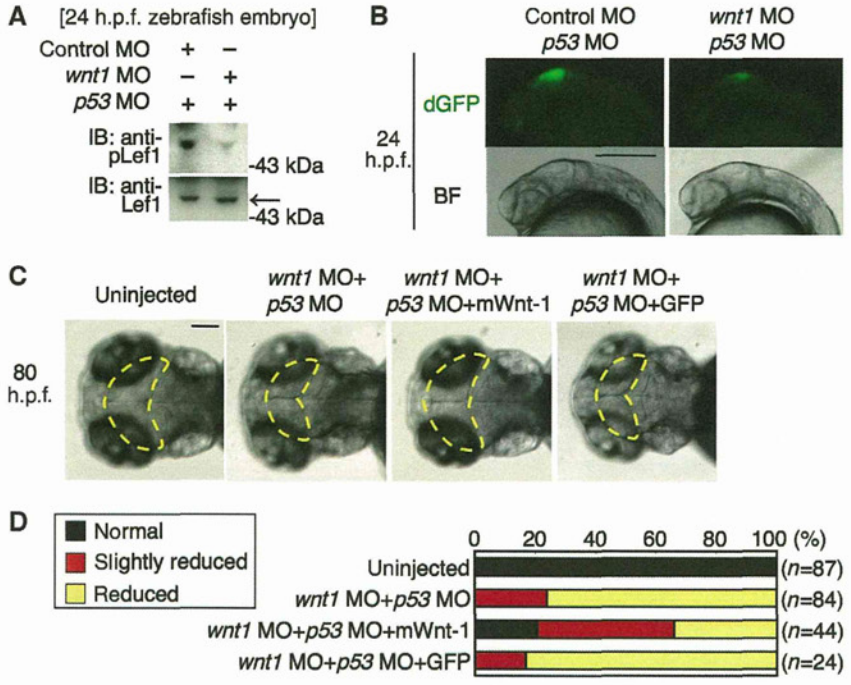

Figure 4 Wnt1 is involved in Lef1 phosphorylation in zebrafish. (**A**) Wnt1 is required for Lef1 phosphorylation at the conserved Thr residue in zebrafish. Zebrafish embryos were co-injected with *p53* MO and control MO or *wnt1* MO, as indicated. Extracts were harvested from the embryos at 24 h.p.f., and immunoblotted with anti-pLef1 and anti-Lef1 antibodies. (**B**) Wnt1 is required for activation of TOPdGFP in zebrafish midbrain. TOPdGFP-transgenic zebrafish embryos were co-injected with *p53* MO and control MO or *wnt1* MO, as indicated. Panels show the left side head views of 24 h.p.f. zebrafish embryos with the anterior to the left. Cells expressing dGFP were visualized by fluorescence microscopy (upper panels). Bright-field (BF) images are shown in lower panels. Scale bar, 250 μm. (**C**, **D**) Wnt1 is required for the formation of 80 h.p.f. zebrafish midbrain. Zebrafish embryos were injected with *p53* MO and *wnt1* MO with or without transposase mRNA and a Tol2-donor plasmid containing cDNA encoding mouse Wnt-1 (mWnt-1) or GFP, as indicated. Panels in (**C**) show a typical example. Broken lines indicate the tectum or presumptive tectal region. Embryos were classified into three groups based on the extent of tectum size reduction (normal, slightly reduced, and reduced). Graph in (**D**) shows the percentages of embryos exhibiting each class of tectum size reduction. The number shown in the right side of graph is the total number of embryos. Figure source data can be found in Supplementary data.

neuro-2a, and rat pheochromocytoma tumour PC12 cells. For this purpose, we used qPCR analysis and the reporter assay with a Wnt/β-catenin signalling-responsive reporter (TOPFLASH), which is driven by multiple TCF/LEF-binding sites (van de Wetering *et al*, 1997; Roose *et al*, 1998). TOPFLASH reporter activity was normalized against that of a reporter containing mutated TCF/LEF-binding sites (FOPFLASH). Deletion of the N-terminal region in β-catenin (β-cateninΔN) results in the accumulation of β-catenin, thus mimicking constitutive activation of Wnt/β-catenin signalling (Aberle *et al*, 1997). In HeLa and HEK293 cells, overexpression of β-cateninΔN alone induced relatively weak activation of the TOPFLASH reporter, while co-expression of β-cateninΔN with LEF1 strongly activated the reporter and enhanced the expression of *Axin2*, a direct target of Wnt/β-catenin signalling (Jho *et al*, 2002; Lustig *et al*, 2002; Supplementary Figure S8A–D). Consistent with a previous observation (Ishitani *et al*, 1999, 2003b), overexpression of NLK inhibited β-cateninΔN-LEF1-induced TOPFLASH reporter activity and *Axin2* expression in HEK293 and HeLa cells (Supplementary Figure S8A–D). In contrast, in neuro-2a and PC12 cells, β-cateninΔN overexpression or co-expression of β-cateninΔN with LEF1 did not activate the reporter (Figure 5A and B). However, we found that co-expression of NLK with β-cateninΔN and LEF1 efficiently activated TOPFLASH reporter activity in these cells and enhanced mRNA expression of Wnt/β-catenin signalling-target genes, such as *Axin2* and *cyclinD1* (Shtutman *et al*, 1999; Tetsu and McCormick, 1999), in a manner dependent on its kinase activity (Figure 5A and B; Supplementary Figure S8E and F). These results suggest that NLK kinase activity is required for promoting β-catenin–LEF1 complex-mediated activation of transcription in NPC-like cell lines.

We have previously reported that NLK inhibits β-catenin–LEF1 complex-mediated transcriptional activation by phosphorylating LEF1 at two conserved Thr and Ser residues (Figure 3A; Ishitani *et al*, 2003b). Consistent with this, NLK failed to inhibit β-cateninΔN–LEF1-induced TOPFLASH reporter activity when β-cateninΔN was co-expressed in HeLa cells with the LEF1-2A mutant, in which Thr-155 and Ser-166 were changed to alanines (Supplementary Figure S8A). We therefore tested the possibility that these sites are also involved in NLK-mediated Wnt/β-catenin signalling activation in neuro-2a and PC12 cells. We confirmed that NLK phosphorylates LEF1 at these residues in neuro-2a and PC12 cells. In SDS–PAGE gels, NLK induced a shift in the migration of wild-type LEF1, but not of the LEF1-2A mutant (Figure 5C). We next investigated the effect of the LEF1-2A mutation on NLK-dependent transcriptional activation. Compared with wild-type LEF1, co-expression of LEF1-2A with β-cateninΔN and NLK induced TOPFLASH reporter activity less effectively in neuro-2a and PC12 cells (Figure 5A and B). We next tested the LEF1-2E mutant, in which both Thr-155 and Ser-166 were changed to glutamic

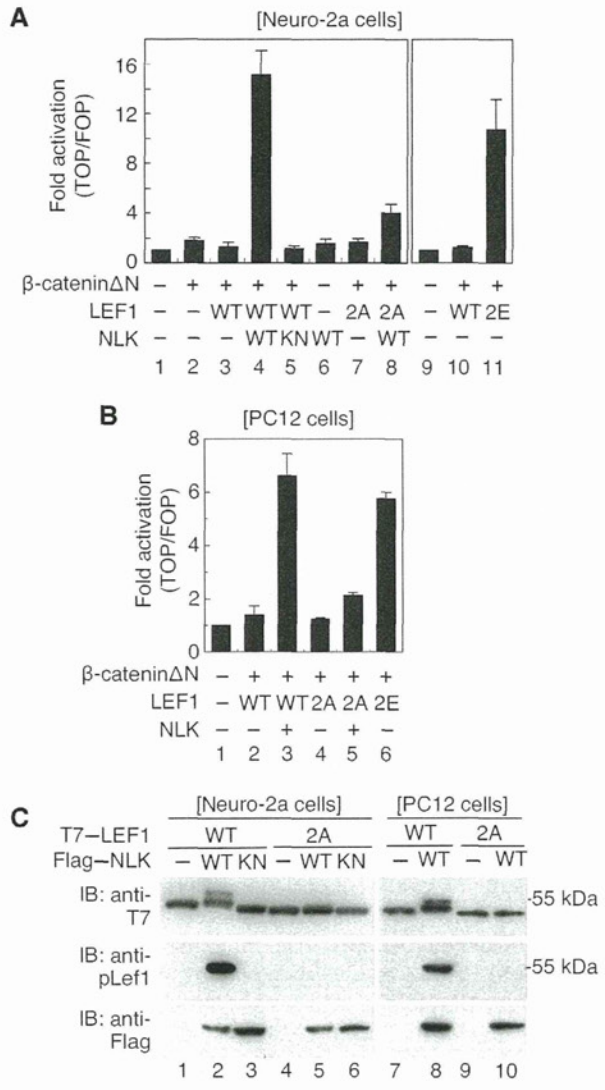

Figure 5 NLK promotes LEF1-mediated transcription in NPC-like mammalian cell lines. (**A**, **B**) The Wnt/β-catenin signalling reporter plasmids and expression plasmids encoding β-cateninΔN, human LEF1-WT, LEF1-2A, LEF1-2E, mouse NLK-WT, and NLK-KN were transfected as indicated and the luciferase activities were measured in neuro-2a (**A**) and PC12 (**B**) cells. (**C**) NLK phosphorylates LEF1 at Thr-155 and Ser-166 in neuro-2a and PC12 cells. Neuro-2a and PC12 cells were transfected with Flag-tagged mouse NLK (Flag–NLK-WT), Flag–NLK-KN, T7-tagged human LEF1 (T7–LEF1-WT) and T7–LEF1-2A mutant as indicated. Cell lysates were immunoblotted with anti-T7, anti-pLef1, and anti-Flag antibodies. Note that anti-pLef1 also recognized the phosphorylation of human LEF1. Figure source data can be found in Supplementary data.

acid to mimic phosphorylated threonine and serine. Co-expression of LEF1-2E with β-cateninΔN was able to activate the TOPFLASH reporter even in the absence of NLK overexpression (Figure 5A and B). These results suggest that LEF1 phosphorylation at Thr-155 and Ser-166 residues by NLK is important for its transcriptional activation of Wnt/β-catenin signalling in NPC-like mammalian cell lines.

NLK inhibits the interaction of LEF1 with HDAC1

We next examined how NLK promotes LEF1-mediated transcription in NPC-like mammalian cell lines. We have previously shown that NLK inhibits the DNA binding of TCF7L2

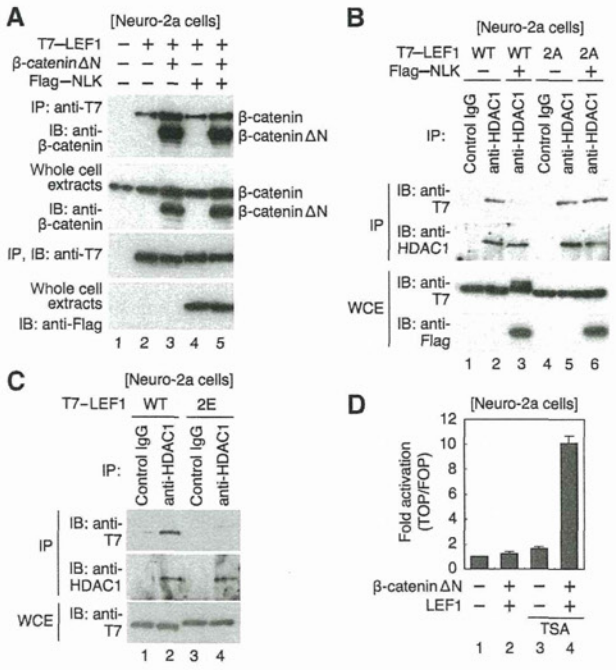

Figure 6 HDAC1 interacts with unphosphorylated LEF1. (**A**) NLK does not affect the interaction of LEF1 with β-catenin in neuro-2a cells. Neuro-2a cells were transfected with T7-tagged human LEF1, β-cateninΔN, and Flag-tagged mouse NLK as indicated. Cell extracts were subjected to immunoprecipitation with anti-T7 antibody. Immunoprecipitated complexes were immunoblotted with anti-β-catenin and anti-T7 antibodies. The amounts of β-catenin, β-cateninΔN, and Flag–NLK were confirmed by immunoblotting with anti-β-catenin and anti-Flag antibodies. (**B**, **C**) NLK-mediated LEF1 phosphorylation inhibits the interaction of HDAC1 with LEF1 in neuro-2a cells. Neuro-2a cells were transfected with plasmids encoding T7-tagged human LEF1-WT, T7–LEF1-2A, T7–LEF1-2E, and Flag-tagged mouse NLK as indicated. Cell extracts were immunoprecipitated with control IgG or anti-HDAC1 antibody. Immunoprecipitated complexes were immunoblotted with anti-T7 and anti-HDAC1 antibodies. The amounts of T7–LEF1 and Flag–NLK proteins were confirmed by immunoblotting with anti-T7 and anti-Flag antibodies, respectively. (**D**) Trichostatin A (TSA) treatment activates β-catenin–LEF1 complex-mediated transcription. Neuro-2a cells were transfected with Wnt/β-catenin reporter plasmids and plasmids encoding β-cateninΔN and LEF1. At 24 h after transfection, cells were left untreated or treated with 50 ng/ml TSA for 24 h and luciferase activity was measured. Figure source data can be found in Supplementary data.

(also known as TCF4), a member of the TCF/LEF family (Ishitani *et al*, 1999, 2003b). Indeed, a ChIP assay in HeLa cells showed that NLK inhibited the binding of LEF1 to the *Axin2* regulatory element (Supplementary Figure S9A). We therefore examined the effect of NLK on the binding of LEF1 to the target genes in neuro-2a cells. NLK overexpression had no effect on LEF1 binding to the *Axin2* and *cyclinD1* regulatory elements (Supplementary Figure S9B). These results suggest that NLK affects LEF-1-mediated transcription in neuro-2a cells through some mechanism other than inhibition of its DNA-binding ability.

The transcriptional activity of LEF1 is regulated positively by β-catenin and negatively by co-repressors such as HDAC1 (Cavallo *et al*, 1998; Roose *et al*, 1998; Billin *et al*, 2000; Logan and Nusse, 2004; Clevers, 2006; Arce *et al*, 2009). As shown in Figure 6A, NLK overexpression had no effect on the interaction between LEF1 and β-cateninΔN. We therefore considered the possibility that NLK might relieve negative

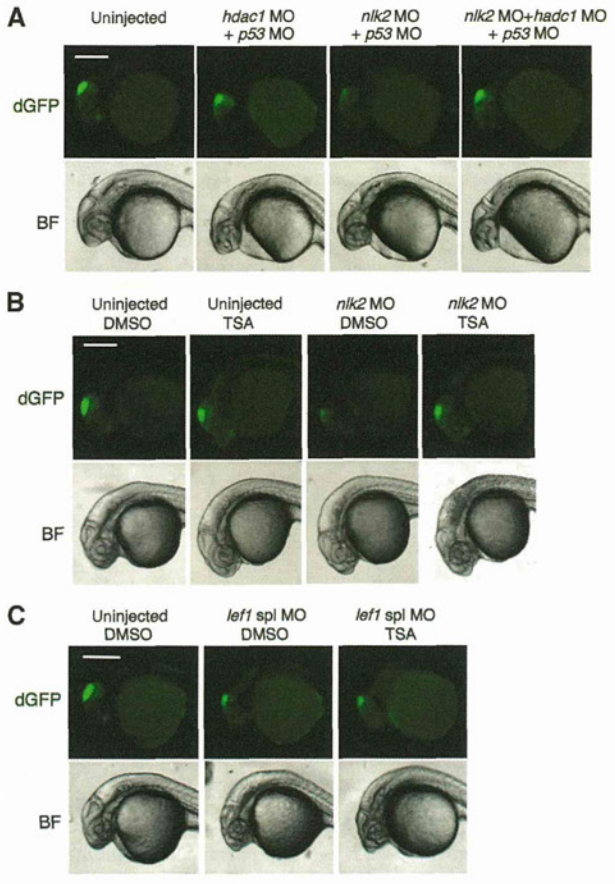

Figure 7 Nlk2 positively regulates Wnt/β-catenin signalling by blocking Hdac1-mediated inhibition in zebrafish midbrain. TOPdGFP-transgenic zebrafish embryos were uninjected or injected with *hdac1* MO, *nlk2* MO, *lef1* spl MO or *p53* MO at one-cell stage, as indicated. At 24 h.p.f., embryos were untreated (**A**) or left treated with DMSO or 1.2 μM TSA for 6 h (**B**, **C**). Panels show the left side head views of 30 h.p.f. zebrafish embryos with the anterior to the left. The cells expressing dGFP were visualized by fluorescence microscopy (upper panels). BF images are shown in lower panels. Scale bar, 250 μm.

regulation of LEF1. We found that LEF1 strongly interacted with endogenous HDAC1 in neuro-2a cells and that overexpression of NLK reduced this association (Figure 6B). We also found that the non-phosphorylated form of LEF1-2A stably interacted with HDAC1 regardless of NLK overexpression. In contrast, the LEF1-2E mutant that mimics constitutive phosphorylation failed to interact with HDAC1 (Figure 6C). Thus, HDAC1 binds preferentially to the unphosphorylated form of LEF1, and this interaction is disrupted by NLK-mediated phosphorylation. The above data raised the possibility that NLK promotes LEF1 activity by antagonizing HDAC1-mediated inhibition. To test this possibility, we examined the effect of trichostatin A (TSA), a specific inhibitor of HDAC1, on LEF1-mediated transcription in neuro-2a cells. Similarly to the effect of NLK overexpression (Figure 5A), TSA treatment strongly activated the TOPFLASH reporter in the presence of β-cateninΔN and LEF1 (Figure 6D).

It has been reported that the zebrafish HDAC1 homologue Hdac1 negatively regulates NPC proliferation mediated by Wnt/β-catenin signalling in zebrafish retina (Yamaguchi *et al*, 2005). In addition, *hdac1* transcripts are selectively expressed in the central nervous system (CNS), including the midbrain, at 24 h.p.f. (Cunliffe, 2004). We therefore investigated the possibility that Nlk2 positively regulates Wnt/β-catenin signalling by blocking Hdac1-mediated inhibition in zebrafish midbrain. Injection of *hdac1* MO or treatment with TSA reversed the reduction in Wnt/β-catenin signalling-induced TOPdGFP reporter activity observed in the midbrain of *nlk2* morphants (Figure 7A and B; Supplementary Tables SIII and SIV). In contrast, the *lef1* spl MO-induced phenotype was not suppressed by TSA treatment (Figure 7C; Supplementary Table SV). Thus, our results suggest that NLK/Nlk2 promotes LEF1/Lef1 activity by antagonizing HDAC1/Hdac1-mediated inhibition.

NLK functions downstream of Dvl in the Wnt signalling pathway in NPC-like mammalian cell lines

We examined whether Wnt-1 family proteins regulate NLK-mediated LEF1 activation in NPC-like mammalian cells. When PC12 cells were treated with Wnt-3a, a member of the Wnt-1 family of proteins, the TOPFLASH reporter was weakly activated (Supplementary Figure S10A). R-spondin 3, a member of the R-spondin family of secreted proteins, binds to a cell surface receptor of the Lgr family and facilitates Wnt-3a signalling by forming an Lgr/Fz/LRP complex (Carmon *et al*, 2011; de Lau *et al*, 2011). Treatment of PC12 cells with Wnt-3a and R-spondin 3 strongly activated the TOPFLASH reporter activity, and this activation was blocked by NLK siRNA treatment (Supplementary Figure S10A), suggesting that signalling by Wnt-3a and R-spondin 3 is transduced via NLK in PC12 cells. This combined treatment induced activation of NLK kinase activity (Figure 8A), phosphorylation of Lef1 (Figure 8B), and dissociation of HDAC1 from Lef1 (Figure 8C). The phosphorylation of Lef1 induced by Wnt-3a and R-spondin 3 was also inhibited by NLK siRNA (Figure 8B). These results suggest that Wnt-3a signalling activates the NLK kinase, which phosphorylates LEF1, resulting in its dissociation from HDAC1.

R-spondin promotes the Wnt/β-catenin pathway through LRP6 (Nam *et al*, 2006; Binnerts *et al*, 2007; Wei *et al*, 2007) and Dkk1 inhibits the Wnt/β-catenin signalling through an interaction with LRP6 (Bafico *et al*, 2001; Mao *et al*, 2001; Semënov *et al*, 2001). We found that expression of Dkk1 inhibited NLK activation induced by Wnt-3a and R-spondin 3 (Supplementary Figure S10B), suggesting that LRP6 is involved in the activation of NLK. Indeed, in PC12 cells, overexpression of constitutively active LRP6ΔN, which lacks the N-terminal extracellular domain (Brennan *et al*, 2004), activated the Wnt/β-catenin signalling reporter and this activation was reduced by NLK siRNA treatment (Supplementary Figure S11A).

Where in the Wnt signalling pathway does NLK function? Since it is known that Wnt signalling is transduced via Dvl (Logan and Nusse, 2004; Clevers, 2006), we investigated the relationship between Dvl and NLK in mediating Wnt signalling in PC12 and neuro-2a cells. In PC12 cells, overexpression of Dvl1 alone activated the Wnt/β-catenin signalling reporter and this activation was reduced by NLK siRNA treatment (Supplementary Figure S11A). In neuro-2a cells, Dvl1-induced reporter activation was relatively weak, but co-expression with LEF1 strongly enhanced reporter activity (Supplementary Figure S11B). This activation was blocked by NLK siRNA (Supplementary Figure S11B) or co-expression of the non-phosphorylated LEF1 mutant LEF1-2A (Supplementary Figure S11C). In addition, overexpression of Dvl1

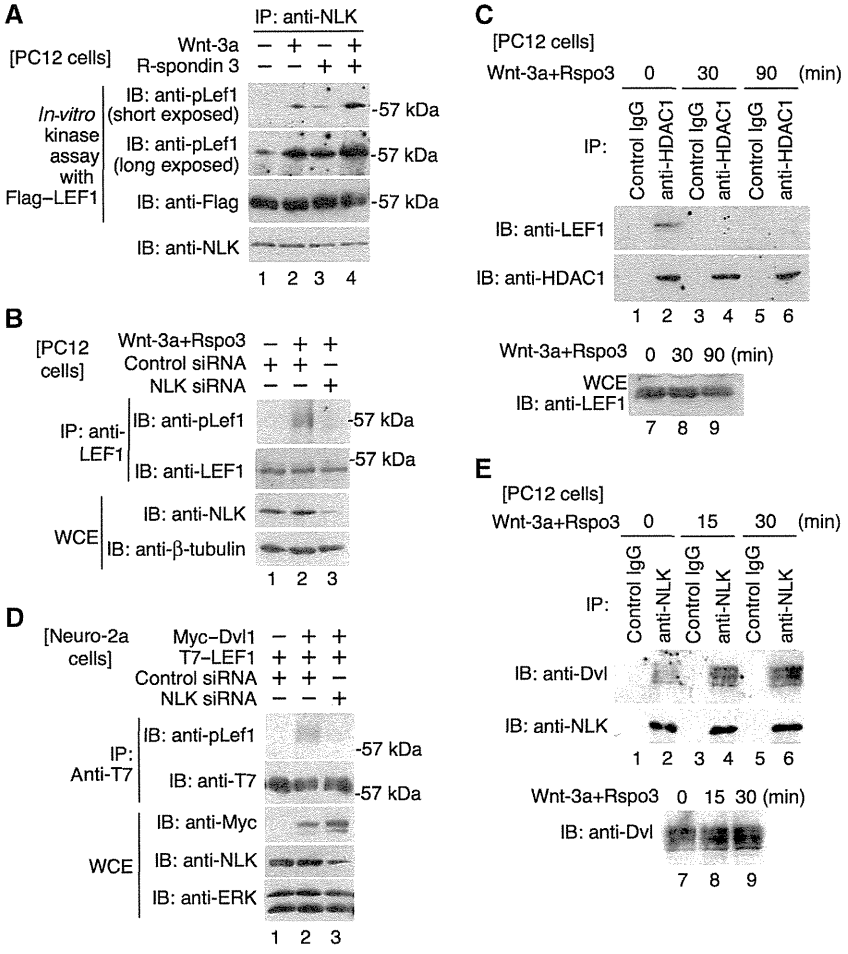

Figure 8 NLK functions downstream of Dvl in the Wnt/β-catenin signalling pathway. (**A**) Wnt-3a signalling activates the kinase activity of endogenous NLK. PC12 cells were untreated or treated with Wnt-3a and/or R-spondin 3 (Rspo3) for 30 min and endogenous NLK was immunoprecipitated (IP) with anti-NLK antibody. Aliquots of purified Flag–LEF1 and IP NLK proteins were subjected to a non-RI *in-vitro* kinase assay, and immunoblotted with anti-pLef1 antibody. Flag–LEF1 and NLK were confirmed by immunoblotting with anti-Flag and anti-NLK antibodies, respectively. (**B**) Wnt-3a signalling induces LEF1 phosphorylation. PC12 cells were treated with either control or NLK siRNA and then treated with or without Wnt-3a and Rspo3 for 30 min. Cell extracts were immunoprecipitated with anti-LEF1 antibody. IP complexes were immunoblotted with anti-pLef1 and anti-LEF1 antibodies. The amounts of endogenous NLK proteins were confirmed by immunoblotting with anti-NLK antibody. β-Tubulin was used as a loading control. (**C**) Wnt-3a signalling reduces the interaction of LEF1 with HDAC1. PC12 cells were treated with or without Wnt-3a and Rspo3 for the indicated time and then cell extracts were immunoprecipitated with control IgG or anti-HDAC1 antibody. IP complexes were immunoblotted with anti-LEF1 and anti-HDAC1 antibodies. The amounts of endogenous LEF1 were confirmed by immunoblotting with anti-LEF1 antibody. (**D**) Dvl1 induces LEF1 phosphorylation in a manner dependent on NLK. Neuro-2a cells transfected with Myc–Dvl1 and T7–Lef1 were treated with either control or NLK siRNA. Cell extracts were immunoprecipitated with anti-T7 antibody. IP complexes were immunoblotted with anti-pLef1 and anti-T7 antibodies. The amounts of Myc–Dvl1 and endogenous NLK were confirmed by immunoblotting with anti-Myc and anti-NLK antibodies, respectively. ERK was used as a loading control. (**E**) NLK binds to Dvl in a manner dependent on Wnt-3a signalling. PC12 cells were treated with or without Wnt-3a and Rspo3 for the indicated times and then cell extracts were immunoprecipitated with either control IgG or anti-NLK antibodies. IP complexes were immunoblotted with anti-Dvl and anti-NLK antibodies. The amounts of endogenous Dvl proteins were confirmed by immunoblotting with anti-Dvl antibody. Figure source data can be found in Supplementary data.

induced phosphorylation of exogenous LEF1 and this phosphorylation was blocked by NLK siRNA in neuro-2a cells (Figure 8D). Furthermore, we found that endogenous NLK associated with endogenous Dvl in PC12 cells and that Wnt-3a signalling enhanced this association (Figure 8E). The interaction of Dvl with NLK may activate its kinase activity. Taken together, these results suggest NLK functions downstream of Dvl in the Wnt signalling pathway in NPC-like mammalian cell lines.

Discussion

In the present study, we have shown that NLK positively regulates Wnt/β-catenin signalling in the developing midbrain of zebrafish and in NPC-like mammalian cell lines. In zebrafish, NLK-mediated signalling contributes to midbrain tectum development by promoting NPC proliferation. In NPC-like mammalian cells, Wnt-Dvl signalling activates NLK, which induces phosphorylation of LEF1, leading to its dissociation from HDAC1 and induction of LEF1 target gene expression.

The roles of zebrafish NLKs in Wnt/β-catenin signalling pathways

Zebrafish contains two NLK genes, *nlk1* and *nlk2*. A previous report has shown that Nlk1 is ubiquitously expressed from the early developmental stages and positively regulates

Wnt/β-catenin signalling through the regulation of Tcf7l1a and Tcf7l1b, members of the Tcf/Lef family of proteins that function as transcriptional repressors (Thorpe and Moon, 2004). Knockdown of *nlk1* reduces expression of the *lef1* gene in 60% epiboly-stage zebrafish embryos and this reduction is reversed by co-knockdown of *tcf7l1a* and *tcf7l1b*. Tcf7l1a and Tcf7l1b also repress *lef1* gene expression (Thorpe and Moon, 2004). Thus, Nlk1 functions as a positive regulator of Wnt/β-catenin signalling by negatively regulating Tcf7l1a and Tcf7l1b. However, the mechanism by which Nlk1 negatively regulates Tcf7l1a and Tcf7l1b remains unknown.

In this study, we show that Nlk2 is expressed in the CNS from the late somite stage and positively regulates Wnt/β-catenin signalling by phosphorylating Lef1 in zebrafish midbrain. When *nlk2* or *lef1* was knocked down in zebrafish embryos, the size of the midbrain tectum was reduced. Nlk2 knockdown in zebrafish embryos decreased the phosphorylation and transcriptional activity of Lef1. Furthermore, the phenotype caused by *nlk2* knockdown was suppressed by expression of an Lef1 mutant that mimics a constitutively phosphorylated state. These data suggest that Nlk2 is required for Wnt/β-catenin signalling through Lef1 phosphorylation in zebrafish midbrain. We also found that Nlk1 can hardly phosphorylate the conserved Thr residue on Lef1 (unpublished observation), suggesting that Nlk1 does not act redundantly with Nlk2 in the regulation of zebrafish midbrain development. Thus, it is likely that Nlk1 and Nlk2 have different substrate specificities and control different cellular events regulated by Wnt/β-catenin signalling in zebrafish.

NLK positively regulates Wnt/β-catenin signalling in NPC-like mammalian cell lines

In this study, we discovered that NLK also positively regulates Wnt/β-catenin signalling by phosphorylating LEF1 in NPC-like mammalian cell lines. Overexpression of mouse NLK induced phosphorylation of LEF1 and activated transcription mediated by the β-catenin–LEF1 complex in NPC-like neuro-2a and PC12 cells. In addition, we showed that LEF1 phosphorylation mediates the dissociation from HDAC1, resulting in the activation of LEF1-mediated transcription. The phosphorylation-dependent interaction between LEF1 and HDAC1 is consistent with a three-step mechanism of LEF1 activation by NLK. Upon NLK activation, HDAC1 dissociates from LEF1, resulting in de-repression of target gene transcription. Subsequently, β-catenin binds to LEF1 and further augments gene expression. It is worth noting that binding of β-catenin to LEF1 is not dependent on NLK-mediated phosphorylation.

Signalling in response to the Wnt-1 class of secreted proteins, such as Wnt-1 and Wnt-3a, leads to activation of Dvl, which then promotes the stabilization of β-catenin and induces β-catenin–TCF/LEF complex-mediated transcription (Logan and Nusse, 2004; Clevers, 2006). Here, we show that NLK functions downstream of Dvl in the Wnt-3a signalling pathway in NPC-like mammalian cells. Either Wnt-3a signalling or Dvl1 overexpression activated NLK kinase activity, which in turn induced phosphorylation and transcriptional activation of LEF1. Introduction of NLK RNAi blocked Wnt-3a- and Dvl1-induced phosphorylation and transcriptional activation of LEF1 in neuro-2a and PC12 cells. These data suggest that Dvl has two functions that serve to activate LEF1-mediated transcription in the Wnt signalling pathway. One is activation of NLK, resulting in phosphorylation of LEF1. The other is stabilization of β-catenin. Phosphorylated LEF1 dissociates from HDAC1 and activates transcription by forming a complex with β-catenin.

HDAC1 suppresses Wnt/β-catenin signalling and NLK blocks this HDAC1-mediated suppression in NPC-like mammalian cells. NLK-mediated Wnt/β-catenin signalling also contributes to NPC proliferation in zebrafish midbrain. Our results suggest that Lef1 phosphorylation by Nlk2 mediates its dissociation from Hdac1, resulting in the activation of Lef1 in zebrafish midbrain. Consistent with this possibility, treatment with the HDAC1 inhibitor TSA reversed the reduction in Wnt/β-catenin signalling-induced TOPdGFP reporter activity observed in the midbrain of *nlk2* morphants. Thus, HDAC1 inhibits Wnt/β-catenin signalling especially in the CNS. NLK is required for Wnt/β-catenin signalling in midbrain NPCs to relieve negative inhibition by HDAC1. Interestingly, a previous report has shown that acetylated Histone H4 can be strongly detected in the promoter region of the TOPFLASH reporter when Wnt/β-catenin signalling is active, but not when inactive (Billin *et al*, 2000). LEF1-bound HDAC1 might therefore inhibit transcription via deacetylation of histone and competition with histone acetyltransferases.

In contrast to the observations in NPC-like mammalian cells and zebrafish midbrain, overexpression of β-cateninΔN and LEF1 is sufficient for the activation of Wnt/β-catenin signalling in HEK293 and HeLa cells. We found that the HDAC1 inhibitor TSA failed to enhance the TOPFLASH reporter activation induced by β-cateninΔN and LEF1 in HEK293 (Supplementary Figure S8B) and HeLa cells (unpublished observation). Furthermore, LEF1 protein was not detected in the anti-HDAC1 immunoprecipitates from HEK293 cells, either transfected or not transfected with an LEF1 expression plasmid (unpublished observation). These observations suggest that HDAC1 has weak or no inhibition of β-catenin–LEF1 complex-mediated transcription in HEK293 and HeLa cells. This difference may explain why NLK-mediated inhibition of HDAC1 is not required for the activation of Wnt/β-catenin signalling in HeLa and HEK293 cells.

Effect of phosphorylation on LEF1 transcriptional activity is cell context dependent

We have previously reported that, in HEK293 and HeLa cells, overexpression of NLK inhibits transcriptional activity of the β-catenin–TCF/LEF complex by reducing its DNA-binding activity (Ishitani *et al*, 1999, 2003b). In addition, we showed that NLK was unable to inhibit TOPFLASH reporter activity when co-expressed with the LEF1-2A mutant (Supplementary Figure S8A), suggesting that NLK negatively regulates LEF1-mediated transcription via phosphorylation at Thr-155 and Ser-166 of LEF-1. However, co-expression of β-cateninΔ with the LEF1-2E mutant, which mimics constitutive phosphorylation, was still able to activate TOPFLASH reporter activity in HeLa cells (Supplementary Figure S12A). We found that NLK overexpression slightly induced phosphorylation of the LEF1-2A mutant in HeLa cells (Supplementary Figure S12B). The LEF1-5A mutant, in which Ser-132, Thr-155, Ser-166, Ser-200, and Thr-265 were changed to alanine residues, was not phosphorylated by NLK overexpression (Supplementary Figure S12B). Thus, NLK phosphorylates LEF1 at Ser-132, Ser-200, and Thr-265 residues in addition to Thr-155 and

Ser-166 residues in HeLa cells. Furthermore, co-expression of β-cateninΔN with the LEF1-5E mutant was unable to activate the TOPFLASH reporter (Supplementary Figure S12A). These results suggest that phosphorylation of LEF1 at Thr-155 and Ser-166 is essential but not sufficient for NLK-mediated inhibition of LEF1 transcriptional activity in HeLa cells. In contrast, in NPC-like mammalian cells, NLK promotes β-catenin–LEF1 complex-mediated transcription and has no effect on the DNA-binding activity of LEF1. In neuro-2a and PC12 cells, co-expression of LEF1-2E with β-cateninΔN was able to activate the TOPFLASH reporter even in the absence of NLK. Thus, phosphorylation of LEF1 at Thr-155 and Ser-166 is sufficient for its activation in neuro-2a and PC12 cells.

Recently, Hikasa and Sokol (2011) have shown that *Xenopus* HIPK2 phosphorylates LEF1 at Ser-132, Thr-155, and Ser-166 residues *in vitro* and that overexpression of HIPK2 reduces the binding of LEF1 to the *vent2* gene promoter in *Xenopus* embryo. We thus examined the effect of HIPK2 on LEF1 activity in neuro-2a cells. We found that co-expression of mouse HIPK2, but not the kinase-negative HIPK2, with β-cateninΔN and LEF1 efficiently activated TOPFLASH reporter activity (Supplementary Figure S12C). In addition, HIPK2 failed to enhance TOPFLASH reporter activity in the presence of β-cateninΔN and the LEF1-2A mutant in neuro-2a cells (Supplementary Figure S12C). These results suggest that, similarly to NLK, HIPK2 promotes Wnt/β-catenin signalling by phosphorylating LEF1 at Thr-155 and Ser-166 in neuro-2a cells. However, it is likely that HIPK2 has only a minor contribution to Wnt signalling-induced LEF1/Lef1 phosphorylation in NPC-like mammalian cells and zebrafish midbrain, because NLK/Nlk2 knockdown strongly inhibited this phosphorylation.

Materials and methods

Plasmids, reagents, and antibodies

Expression plasmids carrying Flag-tagged mouse NLK-WT and KN, and mouse HIPK2-WT and KN, β-cateninΔN; GFP-tagged LRP6ΔN; T7-tagged human LEF1; and HA-tagged human LEF1-WT and 2A have been described previously (Ishitani *et al*, 1999, 2003b, 2005; Kanei-Ishii *et al*, 2004; Sato *et al*, 2010). Flag-tagged human LEF1-WT; T7-tagged human LEF1-2A, 2E, and 5E; Myc-tagged zebrafish Lef1-WT, Lef1(T151A), and Lef1(T151E); Flag-tagged zebrafish Nlk2-WT and KN were generated by PCR. Anti-NLK antibody was previously described (Ishitani *et al*, 2003a). Anti-zebrafish Lef1 antibody was generated in rabbits using synthetic-peptide CIQEPASGTGQRMKTAYI as immunogens. Anti-pLef1 antibody was generated in rabbits using the synthetic phospho-peptide CHAVHPLT*PLITYS (T*: phospho-T) as immunogen. Details for the other antibodies are provided in Supplementary data. Mouse and rat NLK siRNA can reduce the expression of endogenous NLK proteins (Ishitani *et al*, 2009, 2010). Negative control siRNA oligonucleotides were obtained from Gene-net. MOs were obtained from Gene Tools. Translation-blocking MOs against *p53* (*p53* MO), *wnt1* (*wnt1* MO), *tcf7l2* (*tcf7l2* MO), *hdac1* (*hdac1* MO), and *lef1* (*lef1* MO) and splice-blocking MOs against *lef1* (*lef1* spl MO) and *tcf7l1a* (*tcf7l1a* spl MO) were previously described (Amoyel *et al*, 2005; Ishitani *et al*, 2005; Yamaguchi *et al*, 2005; Meier *et al*, 2006; Nyholm *et al*, 2007; Robu *et al*, 2007; Bonner *et al*, 2008). Sequences of siRNA oligos and MOs were given in Supplementary Table VI. TSA was obtained from Wako and dissolved in DMSO. The digoxigenin-labelled RNA antisense probes for *in-situ* hybridization were prepared from templates encoding *nlk2*, *lef1* (Dorsky *et al*, 1999), or *zic2a* (Grinblat and Slive, 2001), which were generated by PCR.

Cell culture, transfection, and treatment

Neuro-2a, HeLa, and HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). PC12 cells were grown in DMEM supplemented with 10% FBS and 5% horse serum. Neuro-2a, HeLa, and HEK293 cells were transfected with the expression plasmids using Polyethylenimine MW 25000 (Polysciences). PC12 cells were transfected with the expression plasmids using Lipofectamine LTX (Invitrogen). For RNAi in neuro-2a and PC12 cells, siRNA oligomers (final 20 nM) were transfected twice into cells using Lipofectamine RNAi MAX (Invitrogen) and Lipofectamine LTX (Invitrogen), respectively. PC12 cells were treated with 100 ng of recombinant Wnt-3a, 100 ng of recombinant R-spondin 3, and 250 ng of recombinant Dkk1 (R&D Systems).

Reporter gene assays

PC12, neuro-2a, and HeLa cells were transfected with the TOPFLASH or FOPFLASH reporter gene plasmids along with expression vectors as indicated. After 48 h, Firefly and *Renilla* luciferase activities were determined with the Promega Dual luciferase assay system. The pRL-EF vector, which expresses *Renilla* luciferase under the control of the EF-1α promoter, was used for normalizing transfection efficiency of the luciferase reporters. TOPFLASH activities were normalized against FOPFLASH activities. The mean of two (Supplementary Figures S10A and S11B), three (Figure 6D, Supplementary Figures S8A and B, S11A and C and S12A and C), or four (Figure 5A and B) independent experiments performed in duplicate is shown. The error bars indicate the standard deviations.

In-vitro kinase assay

Neuro-2a cells expressing Flag–LEF1 and PC12 cells were lysed and immunoprecipitated with anti-Flag M2 Agarose Affinity Gel (Sigma) and anti-NLK, respectively. Immunoprecipitated Flag–LEF1 proteins were released from Affinity Gel using Flag peptides (Sigma). Aliquots of immunoprecipitated endogenous NLK proteins were incubated with Flag–LEF1 with 1 mM ATP in 50 μl of kinase buffer at 30°C for 60 min.

Cloning of zebrafish nlk2

nlk2 gene sequences were amplified from a zebrafish RACE library. The RACE library was constructed using SMART PCR cDNA Synthesis Kit (TaKaRa). The sequence of *nlk2* was submitted to INSD (accession numbers AB372222).

MO and mRNA injections

For all injections, 1–5 ng control MO, 2–3 ng of *nlk2* MO, 2–5 ng of *lef1* spl MO, 5 ng of *lef1* MO, 0.5–1.5 ng of *wnt1* MO, 3 ng of *tcf7l2* MO, 4 ng of *tcf7l1a* spl MO, 2 ng of *hdac1* MO, or 5 ng of *p53* MO was injected at the one-cell stage. Note that embryos that were injected with a high dose (3–5 ng) of *wnt1* MO or *hdac1* MO did not form an intact midbrain (unpublished observation), perhaps because Wnt1 and Hdac1 are required for early embryogenesis, as was reported previously (Lekven *et al*, 2003; Cunliffe, 2004; Nambiar *et al*, 2007), and a high dose of *wnt1* MO or *hdac1* MO artificially activates p53 pathway. We therefore injected a lower dose of *wnt1* MO or *hdac1* MO with *p53* MO.

Rescue experiments by mRNA injection

Embryos injected with a high dose (10–20 pg) of mouse NLK mRNA did not form an intact midbrain (unpublished observation), perhaps because Wnt/β-catenin signalling is required for early brain anterior-posterior patterning. We therefore injected a lower dose (5 pg) of mouse NLK mRNA into fertilized eggs at the one-cell stage.

Rescue experiments by Tol2-mediated transgenesis

Injection of mRNA is the only effective rescuing phenotype at early embryonic stages due to mRNA instability. Therefore, we performed plasmid DNA rescue experiments (Figures 3D, 4C and D) using Tol2 transposon-mediated transgenesis (Takeuchi *et al*, 2010). A CMV promoter; cDNA for Flag-tagged mouse NLK-WT, NLK-KN; Myc-tagged zebrafish Lef1-WT and Lef1(T151E), mouse Wnt-1; and a polyA sequence are subcloned into a Tol2-donor plasmid, pT2AL200R150G (Urasaki *et al*, 2006). In all, 10 pg (Figure 3D) or 1 pg (Figure 4C and D) of Tol2 donor plasmids containing each cDNAs (Supplementary Figure S13A) was injected with 25 pg of transposase mRNA at the one-cell stage. As shown in Supplementary Figure S13B and C, we confirmed by immunostaining that 80 h.p.f. *nlk2* morphants injected with both a Tol2 donor plasmid containing Flag–NLK or Myc–Lef1 and transposase mRNA

expressed Flag–NLK or Myc–Lef1 proteins. We further confirmed that embryos injected with both a Tol2 donor plasmid containing GFP and transposase mRNA expressed GFP in the most of the cells in the head at 30 and 50 h.p.f. (Supplementary Figure S13D). These results support the effectiveness of our Tol2-mediated transgenesis system.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: TI mainly designed the experiments and wrote the manuscript; KM and MI also participated in designing the experiment and writing the manuscript; TI, SI, and SO performed most of the experiments; TI, SO, SI, and NS analysed the data.

Conflict of interest

The authors declare that they have no conflict of interest.

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Review

Nemo-like kinase, a multifaceted cell signaling regulator

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ABSTRACT

Nemo-like kinase (NLK) is an evolutionarily conserved MAP kinase-related kinase. Although NLK was originally identified as a *Drosophila* gene affecting cell movement during eye development, recent studies show that NLK also contributes to cell proliferation, differentiation, and morphological changes during early embryogenesis and nervous system development in vertebrates. In addition, NLK has been reported to be involved in the development of several human cancers. NLK is able to play a role in multiple processes due to its capacity to regulate a diverse array of signaling pathways, including the Wnt/β-catenin, Activin, IL-6, and Notch signaling pathways. Although the molecular mechanisms that regulate NLK activity remain unclear, our recent research has presented a new model for NLK activation. Here, we summarize the current understanding of the function and regulation of NLK and discuss the aspects of NLK regulation that remain to be resolved.

Contents

Abbreviations: NLK, Nemo-like kinase; IL-6, intereukin-6; MAPK, mitogen activated protein kinase; ERK, extracellular-signal regulated kinase; Pro, proline; Ser, serine; Thr, threonine; *C. elegans*, *Caenorhabditis elegans*; POP-1, Posterior pharynx defect protein 1; LEF1, Lymphoid enhancer factor; TCF, T-cell factor; NARF, NLK associated RING finger protein; NPC, neural progenitor cell; HDAC1, histone deacetylase 1; Foxo, forkhead box O; STAT3, signal tranducer and activator of transcription 3; CPEB, cytoplasmic polyadenylation element-binding protein; Notch1-ICD, Notch1 intracellular domain; CSL, CBF-1, suppressor of hairless, LAG-1; SETDB1, SET domain bifurcated 1; PPAR-γ, peroxisome proliferator activated receptor-γ; BMP, bone morphogenetic protein; Eya, Eyes absent; Even-skipped, Eve; MAP1B, microtubule-associated protein 1B; NGF, nerve growth factor; TGF-β, transforming growth factor-β; MAPKK, MAPK kinase; Tyr, tyrosine; MAP3K, MAPK kinase kinase; Glu, glutamic acid; Cys, cysteine; TAK1, TGF-β-activated kinase 1; HIPK2, homeodomain interacting protein kinase 2; miRNA, microRNA; HCC, Hepatocellular carcinoma; ZIPK, Zipper-interacting protein kinase; GSK-3β, Glycogen synthase kinase-3β; LiCl, lithium chloride; IMPase, inositol monophosphatase; IPPase, inositol polyphosphate 1-phosphatase.

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1. Introduction

Nemo-like kinase (NLK) was originally identified as *nemo*, a gene involved in cell motility (ommatidia rotation) during eye development in *Drosophila*. While wild-type flies form hexagonal-shaped ommatidia, *nemo* mutants form square-shaped ommatidia [1]. The name "nemo" derives from the Korean word for square. The vertebrate homolog of the *nemo* gene was discovered by Brott et al. in 1998, and was named "Nemo-like kinase" [2]. NLK is evolutionarily conserved from worms to humans (Fig. 1A). Invertebrate genomes possess only one NLK gene, while vertebrates have either one or two NLK genes. Vertebrate NLK proteins can be classified into two groups, type-I and type-II, by phylogenetic analysis (Fig. 1A) [3]. Amphibians and fish possess both type-I and type-II NLK, while mammals and chickens have only type-II NLK [3]. Type-II NLK, but not type-I NLK, contains histidine-rich amino-terminal and carboxyl-terminal conserved regions (Fig. 1B) [3]. Type-I NLK is known to be involved in early embryogenesis, in processes such as mesoderm induction [4,5]. However, type-II NLK knockout mice [6] and zebrafish *nlk2* knockdown embryos [3] display no early embryonic deficiencies. This suggests that the two NLKs have different functions in vertebrates. The differences in the biochemical properties of type-I NLK and type-II NLK remain unclear. In mice, the type-II NLK amino acid sequence is 54.5% similar and 41.7% identical to that of mouse mitogen activated protein kinase-1/extracellular-signal regulated kinase-2 (MAPK1/ERK2) [2]. Therefore, similar to MAPK1/ERK2, NLK is thought to function as a proline (Pro)-directed kinase, which phosphorylates proteins at a serine (Ser) or threonine (Thr) residue that is immediately preceding a Pro residue. In fact, NLK phosphorylates Lymphoid enhancer factor 1 (LEF1), a pivotal transcription factor in the Wnt/β-catenin signaling pathway, at the Thr and Ser residues of the Thr155-Pro156 and Ser166-Pro167 sequences [3,7]. However, the exact consensus target sequence of NLK has not been characterized. Over the past several years, evidence has emerged showing that NLK plays crucial roles in the regulation of diverse signaling pathways, including Wnt/β-catenin and Notch signaling pathways, and is involved in embryonic patterning, nervous system development, and cancer cell proliferation. Here, we discuss the function and regulation of NLK, with particular focus on vertebrate NLK.

2. NLK as a cellular signaling modifier

2.1. Discovery of the molecular function of NLK

In 1999, we and others found that the *C. elegans* mutant lacking endoderm, *lit-1*, possessed a mutation in a NLK homolog gene and that the gene product of *lit-1* inhibited nuclear localization of Posterior pharynx defect protein 1 (POP-1) by phosphorylating it during endoderm induction [8,9]. This was the first discovery of a molecular function of NLK. POP-1 is a homolog of the mammalian T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors that regulate Wnt/β-catenin signaling. Therefore, we hypothesized that mammalian NLK was involved in Wnt/β-catenin signaling. We found that mammalian NLK could phosphorylate vertebrate TCF/LEF transcription factors, including *Xenopus laevis* TCF7L1/TCF3 and human TCF7L2/TCF4 and LEF1. Moreover, the TCF7L2 proteins phosphorylated by NLK in the human embryonic kidney cell line HEK293 lacked DNA-binding activity in electron mobility shift assays [7,10] (Fig. 2A). In addition, NLK-mediated LEF1 phosphorylation inhibited binding of LEF1 to its target gene, the *Axin2* promoter, in HeLa cells [3]. Furthermore, we discovered that overexpression of NLK inhibited TCF/LEF-mediated transcription in both HeLa and HEK293 cells [3,7,10] (Fig. 2A). Yamada et al. also reported that NLK promotes NARF (NLK associated RING finger protein)-mediated ubiquitination and the subsequent proteasomal degradation of TCF7L2 and LEF1 [11]. Thus, NLK is considered a negative regulator of TCF/LEF in vertebrates.

2.2. Dual and opposite effects of NLK-mediated LEF1 phosphorylation in Wnt/β-catenin signaling

Interestingly, LIT-1 functions as a positive regulator of POP-1 during the fate specification of gonadal precursor cells in *C. elegans* [12–14]. Recently, we reported that vertebrate NLK also functions as a positive regulator of LEF1 in neural progenitor cells (NPCs) [3] (Fig. 2B). In the NPC-like mammalian cell lines, rat pheochromocytoma tumor PC12 cells and mouse neuroblastoma neuro-2a cells, NLK-mediated LEF1 phosphorylation at Thr-155 and Ser-166 induced

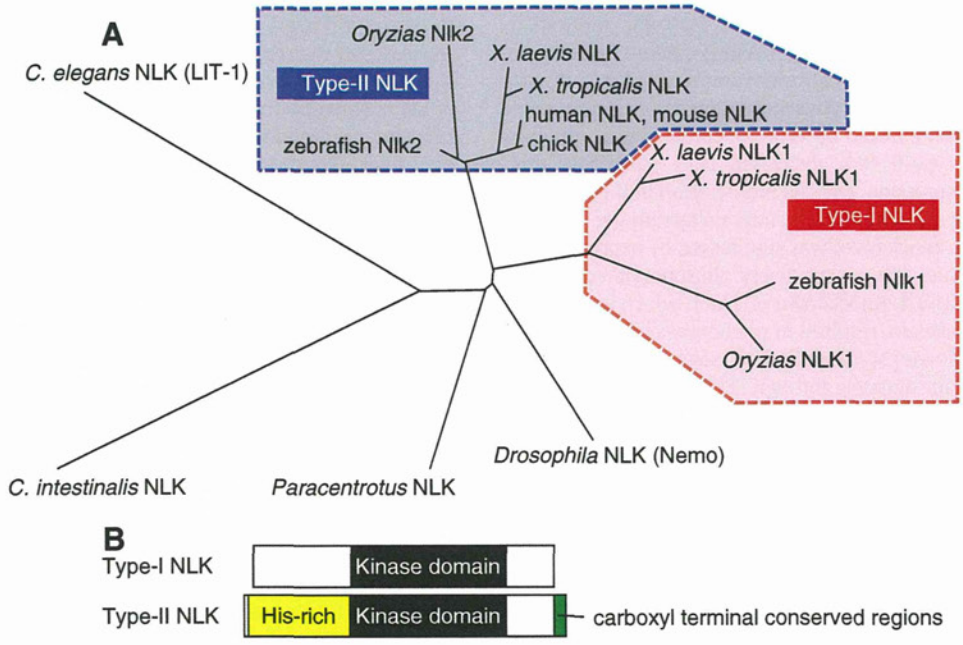

Fig. 1. NLK family proteins. (A) Phylogenetic analysis of NLK homologs by comparison of amino acid sequences. Vertebrate type-I and type-II NLKs are shown in blue and red, respectively. (B) Schematic diagrams of Type-I and Type-II NLKs. Note that type-II NLKs, but not type-I NLKs, have conserved histidine-rich (His-rich) and carboxyl-terminal regions, which are indicated by yellow and green boxes, respectively.

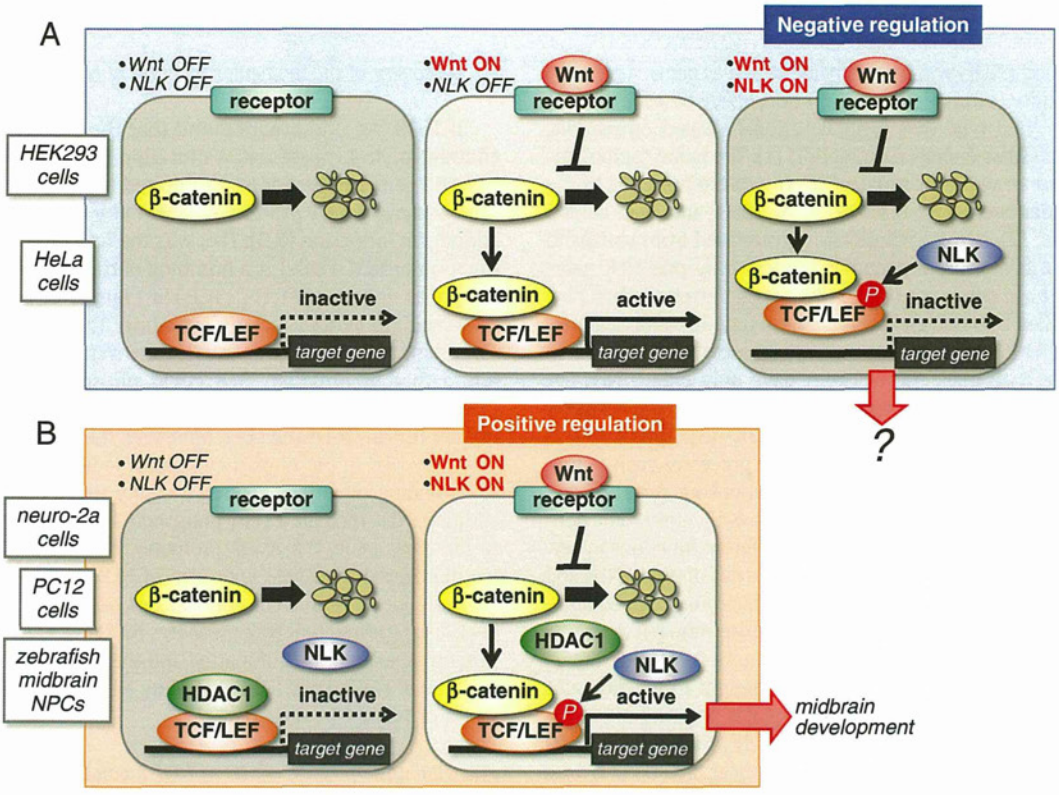

Fig. 2. Cell context-dependent positive and negative regulation of Wnt/β-catenin signaling by NLK. Under unstimulated conditions, β-catenin is destabilized and TCF/LEF represses target gene expression in all cell types (left panels in A and B). In neuro-2a and PC12 cells and zebrafish midbrain NPCs, but not in HEK293 or HeLa cells, HDAC1 strongly inhibits LEF1 transcriptional activity (left panels in A and B). The binding of Wnt ligand to the receptor induces the stabilization of β-catenin in all cell types (middle panel in A and right panel in B). In HEK293 and HeLa cells, stabilized β-catenin forms a complex with TCF/LEF, resulting in the activation of target gene expression (middle panel in A). Overexpression of NLK inhibits Wnt ligand-induced target gene expression by blocking its DNA-binding activity (right panel in A). In neuro-2a and PC12 cells and zebrafish midbrain NPCs, Wnt ligand-activated Dvl promotes Wnt/β-catenin signaling target gene expression via formation of the β-catenin-LEF1 complex and phosphorylation of LEF1 by NLK and the consequent dissociation of LEF1 from HDAC1 (right panel in B). NLK-mediated LEF1 positive regulation contributes to midbrain development (A), while the physiological roles of the negative regulation of LEF1 by NLK have not been determined (A).

dissociation of LEF1 from histone deacetylase HDAC1, thereby promoting transcription activation. The Lef1 phosphorylation did not affect the binding of LEF1 to its target gene promoter. Signaling induced by Wnt-3a, a member of the Wnt-1 family of Wnt/β-catenin ligands, promoted NLK kinase activation, LEF1 phosphorylation and HDAC1 dissociation from LEF1. In agreement with this, knockdown of NLK attenuated transcriptional activation induced by Wnt-3a. Furthermore, inhibition of Nlk2, a zebrafish type-II NLK, decreased LEF1 phosphorylation, LEF1-mediated gene expression, and cell proliferation in the presumptive midbrain, resulting in a reduction in midbrain tectum size. The phenotype caused by Nlk2 knockdown was suppressed by expression of a LEF1 mutant that mimics the constitutively phosphorylated state or by co-knockdown of HDAC1. Knockdown of Wnt1, which is expressed in the presumptive midbrain, resulted in phenotypes similar to those caused by Nlk2 knockdown [3]. These findings suggest that Nlk2 is required for Wnt/β-catenin signaling through LEF1 phosphorylation and for the abrogation of HDAC1-mediated LEF1 inhibition in zebrafish midbrain. Thus, NLK-mediated LEF1 phosphorylation has a dual and opposite effect in Wnt/β-catenin signaling. The first is the inhibition of LEF1 DNA-binding activity in HeLa and HEK293 cells, and the second is the release of HDAC1 from the LEF1 transcriptional complex in NPCs (Fig. 2 and Table 1).

It is unclear why NLK is required for activating TCF/LEF-mediated transcription in the zebrafish midbrain and NPC-like mammalian cell lines but not in HEK293 and HeLa cells. Interestingly, we found that the inhibitory effect of HDAC1 on LEF1-mediated transcription is relatively weak in HEK293 and HeLa cells [3]. Differences in the inhibitory effect of HDAC1 in different cell types may explain the seemingly contradictory effects of NLK mentioned above. The molecular mechanisms underlying these dual and opposite roles of NLK in Wnt/β-catenin signaling have yet to be characterized. The phosphorylation level of LEF1 in HeLa cells is much higher than that of LEF1 in neuro-2a and PC12 cells [3], suggesting that perhaps additional phosphorylation results in negative regulation of LEF1. Based on the results discussed above, comparison of the TCF/LEF-binding proteins and TCF/LEF phosphorylation sites in these cell lines may help to elucidate this mechanism.

2.3. Modulation of multiple transcriptional regulators by NLK

Over the past decade, a number of transcriptional regulators have been identified as substrates of vertebrate NLK. c-Myb is a transcription factor that regulates hematopoietic stem cell proliferation and differentiation [15,16]. In CV-1 cells, NLK phosphorylates c-Myb, resulting in its degradation [17] (Table 1). Another member of the Myb gene family, A-Myb, can be also phosphorylated by NLK. Phosphorylation of A-Myb inhibits its association with the transcriptional coactivator CBP, but does not induce A-Myb degradation [18] (Table 1). The Foxo family of transcription factors modulates the expression of target genes involved in apoptosis, the cell cycle, stress response, longevity, DNA repair, and glucose metabolism [19]. Of the members of the Foxo family, Foxo1, Foxo3a, and Foxo4 are phosphorylated by NLK. NLK-mediated Foxo1 phosphorylation has been shown to inhibit Foxo1-mediated transcription by promoting its nuclear export in the CV-1 cell derivative cell line, Cos-1 [20,21] (Table 1). However, the physiological roles of c-Myb and Foxo phosphorylation remain undefined.

Table 1
Targets of NLK family of protein kinases.

Targets	Direct effects	Effects on cells and/or tissues *in vivo*
C. elegans POP-1 (a homolog of TCF/LEF)	Inhibition of POP-1 nuclear localization in *C. elegans* embryos	Endoderm induction
C. elegans POP-1 (a homolog of TCF/LEF)	Promotion of POP-1 mediated gene expression in *C. elegans* gonadal precursor cells	Establishment of the proximal–distal axis of the gonad
Human TCF7L2	Inhibition of TCF7L2 DNA-binding *in vitro*	Unknown
Xenopus TCF7L1	Unknown	Unknown
Human LEF1	Inhibition of LEF1 DNA-binding in HeLa cells	Unknown
Human/mouse/ zebrafish LEF1	Promotion of LEF1-mediated gene expression in neuro-2a and PC12 cells and zebrafish midbrain.	Size expansion on midbrain tectum
Mouse c-Myb	Degradation of c-Myb and inhibition of c-Myb-mediated gene expression in CV-1 cells	Unknown
A-Myb	Dissociation of CBP from A-Myb and inhibition of A-Myb-mediated gene expression in CV-1 cells	Unknown
Foxo1	Export of Foxo1 from nucleus and inhibition of Foxo1-mediated gene expression	Unknown
Xenopus/mouse STAT3	Promotion of STAT3 transcriptional activity in *Xenopus* embryos and in HepG2 cells	Mesoderm induction in *Xenopus* embryod
Xenopus MEF2A	Promotion of MEF2Aactivity in *Xenopus* embryos	Anterior head formation
Xenopus CPEB	Degradation of CPEB in *Xenopus* embryos	Oocyte maturation in the absence of progesterone (in NLK overexpressed oocytes)
Human/mouse/ zebrafish Notch1	Inhibition of the active Notch1-ICD transcriptional complex formation in neuro-2a cells	Neurogenesis in zebrafish neural plate
SETDB1	Inhibition of PRAR-γ-mediated transactivation in ST2 cells	Unknown
Drosophila Mad	Inhibition of Mad nuclear localization and consequent suppression of BMP signaling in *Drosophila* wing disc	*Drosophila* wing development
Drosophila Period	Promotion of Period protein stability in *Drosophila*	Coordination of circadian rhythm
Drosophila Eya	Promotion of Eya mediated gene expression in wing disc	Unknown
Drosophila Eve	Enhancement of Eve-mediated gene repression	Patterning of *Drosophila* early embryos
Rat MAP1B	Unknown	Unknown
Rat Paxillin	Unknown	Unknown

During early embryogenesis in *X. laevis*, the type-I NLK, xNLK1, phosphorylates two transcription factors, STAT3 and MEF2A. Phosphorylation of STAT3 enhances its transcriptional activity and contributes to mesoderm induction [4] (Table 1), while MEF2A phosphorylation is involved in anterior head formation [22] (Table 1). Ota et al. also reported that overexpression of xNLK1 in *Xenopus* oocytes could induce the phosphorylation of key regulators of translational control, including Pumilio1, Pumilio2, and cytoplasmic polyadenylation element-binding protein (CPEB), as well as CPEB degradation and oocyte maturation in the absence of progesterone [23] (Table 1). However, they failed to demonstrate the physiological role of xNLK1 in oocyte maturation by xNLK1 knockdown.

We recently identified Notch1 intracellular domain (Notch1-ICD), a critical transcriptional regulator of Notch signaling, as an NLK substrate [24]. NLK phosphorylates Notch1-ICD at seven Ser residues on conserved Ser-Pro motifs *in vitro* and in neuro-2a cells. Overexpression of NLK inhibited Notch1-ICD-mediated gene expression via Notch1 phosphorylation in HEK293, HeLa, neuro-2a, and PC12 cells and in the colorectal cancer cell line SW480, and knockdown of NLK with RNAi enhanced Notch1-ICD-mediated gene expression in neuro-2a and SW480 cells. NLK-mediated Notch1-ICD phosphorylation prevented complex formation of Notch1-ICD with the DNA-binding factor CSL (CBF-1, suppressor of hairless, and LAG-1) and a transcriptional coactivator of the Mastermind family in neuro-2a cells and *in vitro* [24] (Table 1). These findings suggest that NLK is a negative regulator of Notch signaling. We also found that knockdown of zebrafish type-I NLK (Nlk1) enhanced Notch signaling and inhibited neurogenesis in the zebrafish neural plate. In addition, the effects of a phosphorylation-resistant Notch1-ICD mutant on Notch signaling activation and neurogenesis suppression in zebrafish neural plate were stronger than those of wild-type Notch1-ICD, while the effects of a Notch1-ICD mutant that mimics the phosphorylated state were weaker [24]. This suggests that NLK-mediated regulation of Notch signaling is essential for proper neurogenesis in the zebrafish neural plate [24]. More recently, it was reported that inhibition of NLK-mediated Notch signaling contributes to natural killer cell development *in vitro* [25].

NLK is also reported to participate in chromatin regulation. NLK regulates chromatin downstream of Wnt-5a signaling by phosphorylating SET domain bifurcated 1 (SETDB1), a methyltransferase of Histone-H3 [26] (Table 1). Wnt-5a signaling activates NLK-mediated SETDB1 phosphorylation, which suppresses peroxisome proliferator activated receptor-γ (PPAR-γ)-mediated transactivation in the mouse bone marrow-derived stromal cell line, ST2 [26]. Although it has been shown that Wnt-5a induces osteoblastogenesis through attenuating PPAR-γ-induced adipogenesis in ST2 cells [26], the relationship between SETDB1 phosphorylation and osteoblastogenesis and adipogenesis remains unclear.

Recent studies in *Drosophila* reveal novel nuclear functions of NLK. During early embryogenesis, Nemo phosphorylates Even-skipped (Eve) [27], a pair-rule gene that encodes a homeobox transcription repressor and plays essential roles in establishing metameric segmentation [28]. Nemo-mediated phosphorylation of Eve enhances Eve-mediated suppression of gene expression and contributes to embryonic patterning [27]. During wing development, Nemo blocks bone morphogenetic protein (BMP) signaling by phosphorylating Mad [29], the *Drosophila* homolog of the BMP signaling transcription factor, Smad [30], which inhibits its nuclear accumulation [29] (Table 1). During eye formation, Nemo phosphorylates Eyes absent (Eya), a transcriptional regulator essential for eye specification in a variety of organisms [31]. This phosphorylation of Eya enhances its transactivation function [32] (Table 1). Nemo also regulates circadian rhythms [33] through its role as a transcriptional regulator of Period, the circadian clock component that behaves as the primary phospho-timer [34]. Nemo-mediated phosphorylation promotes the stability of Period and coordinates circadian rhythm [33] (Table 1). All of these Nemo substrates and their molecular functions and regulation are highly conserved in vertebrates, suggesting that NLK may regulate these proteins in a similar manner.

2.4. NLK activity near the plasma membrane

In many mammalian cells, exogenous NLK proteins are localized primarily to the nucleus [2,35], suggesting that this is the primary site of NLK function. However, we discovered that in rat PC12 and mouse neuro-2a cells, endogenous NLK localizes to the perinuclear region, including the Golgi apparatus, and not to the nucleus. In addition, stimulation with nerve growth factor (NGF) promoted not only the translocation of endogenous NLK into the nucleus and leading edges of the cell, but also induced the enzymatic activation of NLK [36]. These findings suggest that NLK functions both in the nucleus and near the plasma membrane. In agreement with this, we recently found that following treatment with NGF, NLK phosphorylated the focal adhesion adaptor protein paxillin at Ser-126 and the microtubule-associated protein 1B (MAP1B) at the leading edge of PC12 cells [36]. NLK knockdown reduces the phosphorylation of paxillin Ser-126 and MAP1B, actin network formation in the leading edges, and neurite growth in NGF-treated PC12 cells [36]. Although the physiological roles of these phosphorylations remain unclear, these results suggest that NLK may contribute to the formation and extension of neurites in differentiating neurons *in vivo*. Invertebrate NLK can also localize to the plasma membrane. *C. elegans* LIT-1 localizes to the cell cortex during asymmetric cell division [37], and *Drosophila* Nemo localizes to the plasma membrane during ommatidial rotation [38]. These observations support the theory that NLK family proteins function both in the nucleus and near the plasma membrane. Further investigation of the functions of NLK at the plasma membrane will be important.

3. Regulation of NLK

3.1. Molecular mechanisms that regulate NLK kinase activity

A variety of extracellular signals can stimulate the activation of NLK kinase activity. Endogenous NLK can be activated in Wnt-3a-treated PC12 cells [3], NGF-treated PC12 cells [36], Activin A-treated HEK293 cells [4], and Wnt-5a-overexpressing HEK293 cells [39]. Treatment with IL-6 or transforming growth factor-β (TGF-β) can activate exogenous NLK in the human hepatocellular carcinoma cell line HepG2 [40]. However, the molecular mechanisms that regulate NLK kinase activity remain unclear. As described above, the amino acid sequence of NLK is similar to that of ERK2 MAPK [2]. The canonical MAPKs, including ERK2, possess a characteristic MAPK-activating phosphorylation sequence, Thr-Xxx-Tyr (TXY), in the activation loop just upstream of the conserved kinase domain VIII [41]. MAPKs are activated by the phosphorylation of Thr and Tyr residues in the TXY motif by a family of dual-specificity MAPK kinases (MAPKKs) [41–43]. MAPKK-mediated ERK2 phosphorylation not only stimulates the kinase activity of ERK2 but also induces dimerization (Fig. 3A) [44–46]. Thus, the activity of canonical MAPKs is tightly regulated by their upstream kinases. However, we and others showed previously that overexpression of NLK alone is sufficient for activation of its kinase activity in mammalian cells [2,35], suggesting that when overexpressed, NLK can become active without being phosphorylated by upstream kinases. Interestingly, NLK family proteins do not possess a TXY motif in their activation loop. Instead they possess the sequence Thr-Xxx-Glu (TQE) at an analogous site [2]. Glutamic acid, a negatively charged amino acid, can mimic the phosphorylated amino acid. Taken together, these observations suggest that phosphorylation of Thr-286 in the TQE sequence may be involved in NLK activation.

Recently, we discovered that NLK can become activated by autophosphorylating Thr-286 when overexpressed [35]. Size exclusion gel-filtration chromatography revealed that exogenous NLK expressed in HEK293 cells forms homo-dimers. Upon homo-dimer formation, NLK autophosphorylates Thr-286 in a *trans* manner within the dimer. Substitution of Thr-286 with a valine residue or phosphatase treatment strongly reduced NLK kinase activity. Mutation of NLK at Cys-425, which corresponds to the defect in the *C. elegans* mutant *lit-1*, prevented

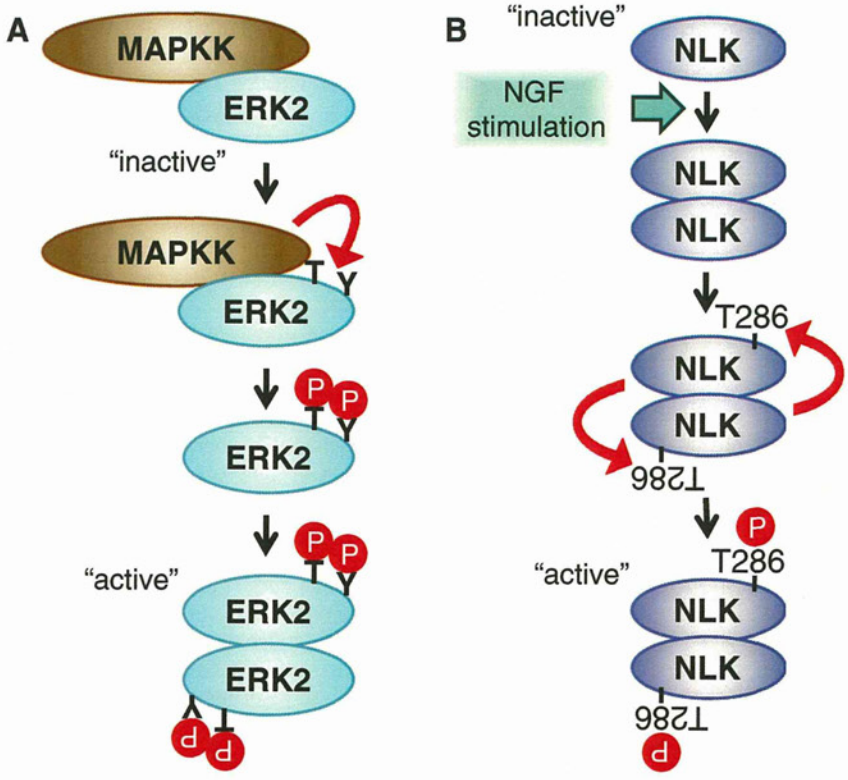

Fig. 3. Model comparing the known mechanism of ERK2 MAPK activation with NLK autophosphorylation/autoactivation. ERK2 MAPK is activated by phosphorylation of Thr and Tyr residues in the TXY motif by MAPKKs. This ERK2 phosphorylation not only activates the kinase activity of ERK2 but also induces its dimerization (A). NGF signaling induces NLK dimerization and consequent Thr-286 autophosphorylation of NLK (B).

NLK dimerization, rendering NLK defective in kinase activity. By contrast, the addition of exogenous NGF induced dimerization and autophosphorylation of Thr-286 in endogenous NLK proteins [35]. These findings suggest that NLK dimerization and the consequent NLK autophosphorylation of Thr-286 is essential for NLK activation (Fig. 3B).

Although the molecular mechanisms that regulate NLK dimerization remain unclear, some kinases that activate NLK have been identified. During endoderm induction in *C. elegans*, the MAPK kinase kinase (MAP3K)-related kinase MOM-4 activates LIT-1 [8,47]. Activation of the vertebrate MOM-4 homolog, TGF-β-activated kinase 1 (TAK1), can promote NLK autophosphorylation activity [10] and is essential for Wnt-5a-induced NLK activation in HEK293 cells [39]. Interestingly, an interaction between TAK1 and NLK was not detected by yeast two-hybrid (unpublished data), suggesting that TAK1 does not directly bind to NLK. Kanei-Ishii et al. reported that homeodomain-interacting protein kinase 2 (HIPK2) is directly phosphorylated by TAK1 and binds to and activates NLK [17]. Ohnishi et al. reported that p38, a well-known downstream MAPK of TAK1 MAP3K signaling, directly interacts with NLK and regulates its kinase activity [48]. However, neither HIPK2 nor p38 phosphorylate NLK at Thr-286 [17,48]. It will be interesting to determine whether phosphorylation by HIPK2 or p38 is involved in NLK dimerization.

3.2. Regulation of NLK by microRNA

Several recent reports have shown that NLK expression is regulated by microRNA (miRNA). During natural killer cell development, the levels of miRNA-181a and miRNA-181b transcripts increase [25]. miRNA-181a and miRNA-181b reduce the levels of NLK protein and abrogate NLK-mediated negative regulation of Notch signaling, thus promoting human natural killer cell development *in vitro* [25]. miRNA-181a, miRNA-181b, miRNA-181c, and miRNA-181d also negatively regulate the expression level of NLK protein in hepatocellular carcinoma (HCC) cell lines [49]. miRNA-181 family members may control NLK expression in a variety of tissues.

3.3. Mechanisms by which NLK selectively controls signaling pathways

As described above, NLK phosphorylates and regulates a variety of signaling molecules. It is noteworthy that, in several cases, the phenotypes of cell- or stage-specific NLK knockout/knockdown animals depend on the specific NLK target. For example, the midbrain tectum size reduction phenotype in zebrafish Nlk2-knockdown embryos was completely reversed by expression of a phosphorylation-state mimicking mutant of LEF1 [3]. Knockdown of Nlk1 failed to induce the increased-neuron phenotype in the neural plate of Notch-knockdown zebrafish embryos [24]. These observations suggest that NLK targets may be selected in a cell context and/or condition-dependent manner. However, little is known about NLK target selection, although some evidence exists suggesting that STAT3 and Zipper-interacting protein kinase (ZIPK) may contribute to this system. STAT3 forms a complex with both NLK and its activator TAK1 and specifically promotes IL-6 signaling-induced activation of the TAK1-NLK pathway and the consequent phosphorylation of STAT3 by NLK [40]. ZIPK interferes with the interaction of NLK with its substrate TCF7L2 in HEK293 cells [50], suggesting that ZIPK may selectively block NLK-mediated regulation of Wnt/β-catenin signaling. To fully understand the *in vivo* roles of NLK, the mechanism by which NLK targets are selected must be elucidated.

4. The roles of NLK in vertebrate development

4.1. The roles of NLK in nervous system development

Vertebrate NLK family genes are highly expressed in neural tissues [2,3,5,48] and play crucial roles in nervous system development. *X. laevis* type-I NLK, xNLK1, regulates the development of anterior neural structures by regulating the activity of MEF2A [22]. Zebrafish type-I NLK, Nlk1, controls brain anterior/posterior patterning, possibly in cooperation with Wnt/β-catenin signaling [5], and promotes primary neurogenesis in the neural plate by inhibiting Notch signaling [24]. Zebrafish type-II NLK, Nlk2, promotes neural progenitor cell proliferation in the presumptive midbrain by positively regulating Wnt/β-catenin signaling, and contributes to the size expansion of the midbrain tectum [3]. Mammalian type-II NLK also contributes to NGF-induced neurite outgrowth in PC12 cells. Thus, NLK is involved in a variety of processes during nervous system development. Interestingly, Kortenjann et al. observed that type-II NLK -deficient mice suffer from various neurological abnormalities, including cerebellar ataxia [6]; however, a detailed phenotype was not provided. Future analysis of the effects of knockdown of NLK on neuronal development may reveal whether the neuronal functions of type-II-NLK are conserved in vertebrates.

4.2. The roles of NLK in non-neuronal tissues

NLK has also been shown to regulate bone formation. Nifuji et al. reported that overexpression of NLK, but not a kinase-inactive mutant of NLK, suppressed the expression of bone markers in primary calvarial osteoblasts and the bone marrow stromal cell line ST2, while knockdown of NLK with siRNA enhanced bone marker expression [51]. Zanotti and Canalis showed that knockdown of NLK in ST2 cells promoted BMP and Wnt/β-catenin signaling [52], pathways that play a central role in osteoblastic differentiation [53]. These observations suggest that NLK negatively regulates osteoblastic differentiation by blocking BMP and Wnt/β-catenin signaling. However, the mechanism by which NLK regulates BMP and Wnt/β-catenin signaling and the molecular link between this regulation and osteoblastic differentiation remain unknown. Conversely, Kortenjann et al. reported that osteogenesis was unaffected in NLK knockout mice, although these mice were only maintained until 4 weeks after birth [6]. Further investigation of osteoblastgenesis and osteogenesis in NLK knockout mice at later stages will be important.

In some NLK-deficient mice at 3–4 weeks after birth, adipogenesis in bone marrow was enhanced, while the number of hematopoietic cells was reduced [6], suggesting that NLK is involved in adipogenesis and hematopoiesis. As described above, NLK suppresses PPAR-γ-mediated transactivation, a process that positively regulates adipogenesis [26]. This may explain the increased adipogenesis observed in NLK null mice, although the exact molecular mechanisms by which NLK regulates adipogenesis and hematopoiesis have not been clarified.

5. NLK expression and activity in cancer

During the past several years, a number of studies have reported a correlation between NLK expression and activity and cancer development. Jung et al. discovered that expression of NLK is up-regulated in HCCs and showed that NLK positively regulates the expression of cyclinD1, a core component of cell cycle regulation [54]. It is well-known that the development and progression of HCCs are associated with increased Wnt/β-catenin signaling [55]. Interestingly, NLK promotes cyclinD1 expression by positively regulating Wnt/β-catenin signaling in neuro-2a cells [3]. NLK might contribute to the growth of HCC by enhancing Wnt/β-catenin signaling activity. However, NLK appears to function as a tumor suppressor in prostate cancer and glioma. Emami et al. found that NLK expression is decreased during prostate cancer progression and demonstrated that NLK inhibits androgen receptor (AR) expression and subsequent AR-mediated transcription and promotes apoptosis in prostate cancer cell lines [56]. Cui et al. reported that NLK expression levels are higher in human glioma tissues from lower grade tumors and that the survival rate of patients with gliomas expressing low levels of NLK is significantly shorter than that of patients with gliomas expressing high levels of NLK [57]. It will be important to clarify the direct target (substrate) of NLK activity and the mechanisms that regulate NLK expression in these cancer cells.

6. Kinases that share molecular functions with NLK

Zebrafish Nlk1 and xNLK1 are involved in the processes of early embryogenesis, such as mesoderm induction [4,5]. Interestingly, type-II NLK knockout mice do not display early embryonic deficiencies [6], even though mammals do not possess type-I NLK [3]. This may be explained by the potential for other genes to compensate for the loss of type-I NLK activity and maintain early embryogenesis. Recently, HIPK2 was found to regulate Wnt/β-catenin signaling in a manner similar to NLK [58,59]. Hikasa et al. reported that *Xenopus* HIPK2 was able to phosphorylate LEF1, TCF7L1, and TCF7L2, but not TCF7, *in vitro*, and that overexpression of HIPK2 reduced the binding of LEF1 and TCF7L1 to the *vent2* gene promoter in *Xenopus* embryos [58,59]. These HIPK2 functions resemble those of NLK in HeLa and HEK293 cells. We have also reported that, similar to NLK, HIPK2 promoted Wnt/β-catenin signaling by phosphorylating LEF1 at Thr-155 and Ser-166 in neuro-2a cells [3]. Thus, NLK and HIPK2 may cooperate to regulate Wnt/β-catenin signaling activity in vertebrates. Glycogen synthase kinase-3β (GSK-3β) also shares molecular functions with NLK. GSK-3β can phosphorylate paxillin at Ser-126 and MAP1B and contributes to NGF-induced neurite outgrowth [60–63]. In addition, GSK-3β negatively regulates not only Wnt/β-catenin signaling [64,65] but also Notch signaling [66,67]. GSK-3β may function cooperatively and/or redundantly with NLK to regulate several signaling processes.

7. NLK is a lithium chloride (LiCl)-sensitive kinase

We recently found that NLK is sensitive to LiCl [36], which is well-known to inhibit GSK-3, but not specifically [68–70]. LiCl treatment in PC12 cells inhibited NGF-induced endogenous NLK activation. In addition, NLK immunoprecipitated from mammalian cells was unable to undergo autophosphorylation in the presence of LiCl [36]. These observations suggest that LiCl directly inhibits the kinase activity of NLK.

LiCl interferes with several signaling processes, including Wnt/β-catenin signaling- and Notch signaling-mediated gene expression and NGF-signaling-induced neurite outgrowth [66,67,71–73], which are also regulated by NLK [10,24,36]. In addition, LiCl is used to treat neurological diseases, such as bipolar disorder [74]. Previous studies have identified a number of LiCl target enzymes, including GSK-3β, inositol monophosphatase (IMPase), and inositol polyphosphate 1-phosphatase (IPPase) [75]. Despite the therapeutic benefits of lithium, its precise mechanism of action remains elusive. Berridge et al. proposed that depletion of inositol caused by lithium-mediated inhibition of IPPase and IMPase activities leads to mood stabilization [76]. Recently, Beaulieu et al. showed that lithium exerts its behavioral effects in mice by activating the Ser/Thr kinase Akt, which directly inhibits GSK-3β activity, by disrupting a signaling complex composed of Akt, β-arrestin 2, and protein phosphatase 2A [77,78]. Since NLK is highly expressed in the brain, the behavioral effects induced by lithium might also be mediated by changes in NLK activity. Reexamination of LiCl-sensitive events in light of the role for NLK might lead to novel insights not only into the therapeutic mechanism of lithium activity but also into the physiological roles of NLK.

8. Conclusion

NLK was first identified as a gene that contributes to cell movement in *Drosophila*. Conversely, NLK in *C. elegans* was isolated as a gene required for cell fate decisions. These differences in functionality predicted that NLK may play multiple roles at both the molecular and cellular levels. However, studies on NLK to date show that the roles of NLK are more diverse than had been expected. NLK controls gene expression and cytoskeletal architecture by phosphorylating and regulating a variety of cell signaling components and cytoskeleton-associated proteins. However, it is still not clear how the specific substrates are targeted and regulated by NLK under different conditions. It is also expected that NLK is able to regulate multiple targets simultaneously to coordinate the activities of multiple signaling pathways in response to specific stimuli. These issues are the focus of future studies. In both basic biology fields and medical research fields, such as cell fate control and cancer research, NLK is emerging as a remarkable and multifaceted gene. Although specific chemical inhibitors of NLK have not yet been developed, they are likely to be a useful tool in the treatment of multiple diseases.

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