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NLK positively regulates Wnt/β-catenin signalling by phosphorylating LEF1 in neural progenitor cells

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Nemo-like kinase (NLK/Nlk) is an evolutionarily conserved protein kinase involved in Wnt/β-catenin signalling. However, the roles of NLK in Wnt/β-catenin signalling in vertebrates remain unclear. Here, we show that inhibition of Nlk2 function in zebrafish results in decreased Lymphoid enhancer factor-1 (Lef1)-mediated gene expression and cell proliferation in the presumptive midbrain, resulting in a reduction of midbrain tectum size. These defects are related to phosphorylation of Lef1 by Nlk2. Thus, Nlk2 is essential for the phosphorylation and activation of Lef1 transcriptional activity in neural progenitor cells (NPCs). In NPC-like mammalian cells, NLK is also required for the phosphorylation and activation of LEF1 transcriptional activity. Phosphorylation of LEF1 induces its dissociation from histone deacetylase, thereby allowing transcription activation. Furthermore, we demonstrate that NLK functions downstream of Dishevelled (Dvl) in the Wnt/β-catenin signalling pathway. Our findings reveal a novel role of NLK in the activation of the Wnt/β-catenin signalling pathway.

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Introduction

The T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors regulate Wnt/β-catenin signalling, which controls cell proliferation and fate decision during embryogenesis and adult tissue homeostasis (Logan and Nusse, 2004; Arce *et al*, 2006; Clevers, 2006; Hoppler and Kavanagh, 2007). TCF/LEF transcriptional activity is switched in a manner dependent on Wnt/β-catenin signalling (Logan and Nusse, 2004; Arce *et al*, 2006; Clevers, 2006; Hoppler and Kavanagh, 2007). In unstimulated cells, the levels of cytoplasmic β-catenin, a co-activator of TCF/LEF, are kept low by a degradation complex that includes Axin and glycogen synthase kinase 3β (GSK-3β). This kinase catalyses the phosphorylation of β-catenin, which promotes its ubiquitination and subsequent proteasomal degradation (Logan and Nusse, 2004; Clevers, 2006). In the absence of stimulation, TCF/LEF represses the expression of Wnt/β-catenin signalling-target genes by interacting with transcriptional co-repressors such as histone deacetylase 1 (HDAC1) and Groucho (Cavallo *et al*, 1998; Roose *et al*, 1998; Billin *et al*, 2000; Arce *et al*, 2009). The Wnt/β-catenin signalling pathway is induced when the secreted glycoprotein Wnt binds to the cell surface Frizzled (Fz) receptor and its co-receptor LRP5/6. This Wnt-bound receptor complex recruits the cytoplasmic protein Dishevelled (Dvl), which in turn brings the Axin–GSK-3β complex to the membrane and induces the phosphorylation of LRP6. Phosphorylated LRP6 promotes the dissociation of the β-catenin degradation complex (Davidson *et al*, 2005; Zeng *et al*, 2005, 2008). This series of events result in the accumulation of cytoplasmic β-catenin (Niehrs and Shen, 2010; MacDonald *et al*, 2011). The increased β-catenin concentration drives its migration into the nucleus where it forms complexes with TCF/LEF proteins, which then activate gene expression. However, the mechanism(s) by which TCF/LEF is converted from a repressor to an activator is poorly understood.

Nemo-like kinase (NLK) is an evolutionarily conserved MAP kinase-like kinase that regulates diverse signalling processes via phosphorylation of several transcription factors (Ishitani *et al*, 1999, 2010; Kanei-Ishii *et al*, 2004; Ohkawara *et al*, 2004; Kojima *et al*, 2005; Zeng *et al*, 2007). In *Caenorhabditis elegans*, the NLK homologue LIT-1 regulates POP-1, the *C. elegans* homologue of TCF/LEF (Meneghini *et al*, 1999; Rocheleau *et al*, 1999; Herman, 2001; Siegfried and Kimble, 2002; Siegfried *et al*, 2004). POP-1 represses the expression of genes required for endoderm induction. LIT-1 co-operates with the *C. elegans* β-catenin homologue WRM-1 to promote the phosphorylation and consequent nuclear export of POP-1, resulting in the transcriptional activation of POP-1-repressed genes (Meneghini *et al*, 1999; Rocheleau *et al*, 1999). LIT-1 also functions as a positive regulator of POP-1 in the fate specification of gonadal precursor cells (Herman, 2001; Siegfried and Kimble, 2002; Siegfried *et al*, 2004). However, the mechanism underlying this positive regulation is unclear. The regulation of POP-1 activity by LIT-1 is cell context dependent. The negative regulation of TCF/LEF by NLK has been also observed in human embryonic kidney 293 (HEK293) cells and the cervical epithelioid carcinoma cell line HeLa (Ishitani *et al*, 1999, 2003b). In these cell lines, overexpression of NLK inhibits β-catenin–TCF/LEF complex-mediated transcription via phosphorylation of TCF/LEF. On the other hand, positive regulation of TCF/LEF by NLK has not yet been observed in vertebrates.

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Mouse NLK is expressed in neural tissues, suggesting that mammalian NLK might play a role in nervous system development. Indeed, mice lacking NLK display various neurological abnormalities (Kortenjann *et al*, 2001). In the present studies, we demonstrate that NLK positively regulates the transcriptional activity of LEF1, a member of the TCF/LEF family, in zebrafish midbrain and mammalian neural progenitor cell (NPC)-like cell lines. We further show that Dvl activates NLK in the Wnt pathway and that NLK promotes the release of HDAC1 from LEF1 by phosphorylating LEF1. Our findings provide evidence that NLK mediates Wnt/β-catenin signalling, and consequently NPC proliferation through LEF1 phosphorylation.

Results

Nlk2 is essential for Wnt/β-catenin signalling in zebrafish midbrain

We used zebrafish as a model animal to investigate the roles of NLK in Wnt/β-catenin signalling *in vivo*. Zebrafish has two *nlk* genes, *nlk1* and *nlk2* (Supplementary Figure S1A and B). Nlk1 protein is more related to *Xenopus laevis* NLK1 (73% identical) than to human NLK (68% identical), while Nlk2 protein is most similar to human NLK (97% identical). Nlk2 and human NLK, but not Nlk1, contain histidine-rich (His-rich) and carboxyl terminal conserved regions (Supplementary Figure S1B). Vertebrate NLK proteins can be classified into two groups by phylogenetic analysis: type-I NLK, which includes *X. laevis* NLK1 and Nlk1, and type-II NLK, which includes mammalian NLK and Nlk2 (Supplementary Figure S1A). Recent studies show that Nlk1 regulates primary neurogenesis, ventrolateral mesoderm formation and brain anterior-posterior patterning in early embryogensis (Thorpe and Moon, 2004; Ishitani *et al*, 2010). We therefore investigated the physiological roles of Nlk2.

Expression of *nlk2* was observed in head tissues from the late somite stage (Figure 1A), suggesting that *nlk2* is involved in later brain development. To monitor activity of the zebrafish LEF1 homologue, Lef1, we used a transgenic zebrafish line carrying a Wnt/β-catenin signalling reporter construct (TOPdGFP), in which destabilized green fluorescent protein (dGFP) is driven by a promoter containing multiple TCF/LEF-binding sites, and thus indicates tissues where Lef1 is transcriptionally active (Dorsky *et al*, 2002). This transgenic zebrafish exhibited dGFP expression in the developing midbrain from the late somite stage (Figure 1B; Supplementary Figure S2A). As shown in Figure 1C, dGFP expression was observed in the dorsal and lateral marginal regions of the midbrain at 24 and 27 h.p.f. At 30 h.p.f., dGFP was expressed in the entire dorsal midbrain, while expression in lateral dorsal region was decreased. Consistent with this reporter activity, *lef1* and *nlk2* mRNAs were detected by *in-situ* hybridization in the entire dorsal midbrain of zebrafish embryos at 24 h.p.f. (Figure 1D). At 27 h.p.f., their expression levels in the lateral dorsal part of the midbrain were relatively low (Figure 1D) and their expression patterns were similar to that of TOPdGFP at 30 h.p.f. (Figure 1C). dGFP expression was attenuated by knockdown of zygotic Lef1 using a morpholino oligo (MO) that blocks *lef1* splicing (*lef1* spl MO) (Figure 1B; Supplementary Figure S2A and B; Supplementary Table SI), confirming that Lef1 indeed mediates the transactivation induced by Wnt/β-catenin signalling in the developing

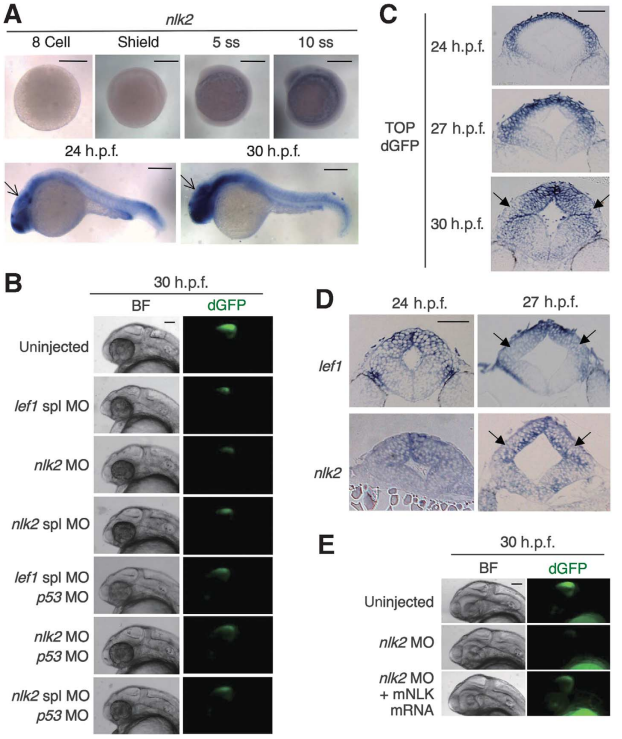

Figure 1 Nlk2 and Lef1 are required for the activation of Wnt/β-catenin signalling in zebrafish developing midbrain. (**A**) Whole mount *in-situ* hybridization staining for *nlk2* in zebrafish embryos at the indicated stage. Scale bar: 250 μm. The expression of *nlk2* in midbrain is indicated with arrows. (**B**, **E**) TOPdGFP-transgenic zebrafish embryos injected with *lef1* spl MO, *nlk2* MO, *nlk2* spl MO, or *p53* MO with or without mouse NLK (mNLK) mRNA, as indicated. Panels show the left side head views of 30 h.p.f. embryos with the anterior to the left. Cells expressing dGFP were visualized by fluorescence microscopy (right panels). Bright-field (BF) images are shown in left panels. Scale bar, 50 μm. Note that mNLK partially rescued *nlk2* MO-induced reduction of TOPdGFP activity ($n = 28$, 50%). (**C**, **D**) *In-situ* hybridization staining for TOPdGFP (**C**), *lef1* (**D**), and *nlk2* (**D**) in the transverse section at the level of midbrain in the indicated stage zebrafish embryos. Scale bar: 50 μm. The lateral dorsal region is indicated with arrows.

midbrain. We next examined the effect of *nlk2* inactivation on TOPdGFP reporter expression in the developing midbrain of zebrafish embryos using a translation-blocking MO against *nlk2* (*nlk2* MO) and an *nlk2* splice-blocking MO (*nlk2* spl MO) (Supplementary Figure S3). We found that, similarly to *lef1* spl MO-injected TOPdGFP fish embryos, the embryos injected with *nlk2* MO or *nlk2* spl MO showed lower TOPdGFP activity than the uninjected embryos at 24 h.p.f. (Supplementary Figure S2A and B; Supplementary Table SI) and 30 h.p.f. (Figure 1B; Supplementary Table SI). Co-injection of a validated MO for *p53* (Robu *et al*, 2007; Tsukada *et al*, 2010; Gerety and Wilkinson, 2011) together with an MO for *nlk2* or *lef1* had no effect on the phenotype induced by MO-mediated knockdown of *nlk2* or *lef1* (Figure 1B; Supplementary Table SI), eliminating the possibility that this phenotype was due to artificial MO-induced p53 activation (Robu *et al*, 2007). Furthermore, the *nlk2* MO-induced reduction of TOPdGFP activity in the midbrain was partially rescued by co-injection with mouse NLK mRNA (Figure 1E). We confirmed that injection of *lef1* spl MO or *nlk2* MO reduced dGFP expression levels in the midbrain but had no effect on the midbrain formation at 27 h.p.f. (Supplementary

Figure S2C). By observing the expression of brain maker genes, we also confirmed that neither injection of *nlk2* MO nor *lef1* spl MO affected the patterning of 24 h.p.f. zebrafish midbrain (Supplementary Figure S4A and B). These results suggest that Nlk2 positively regulates Wnt/β-catenin signalling in the developing zebrafish midbrain.

Nlk2 contribute to midbrain tectum development in zebrafish

To investigate the physiological roles of Nlk2 in the midbrain, we injected MOs for *nlk2* into a transgenic zebrafish line carrying the HuC:Kaede reporter, which expresses the fluorescent protein Kaede in neurons under the control of the neuron-specific *HuC/elavl3* promoter (Sato *et al*, 2006). Injection of either *nlk2* MO or *nlk2* spl MO reduced the size

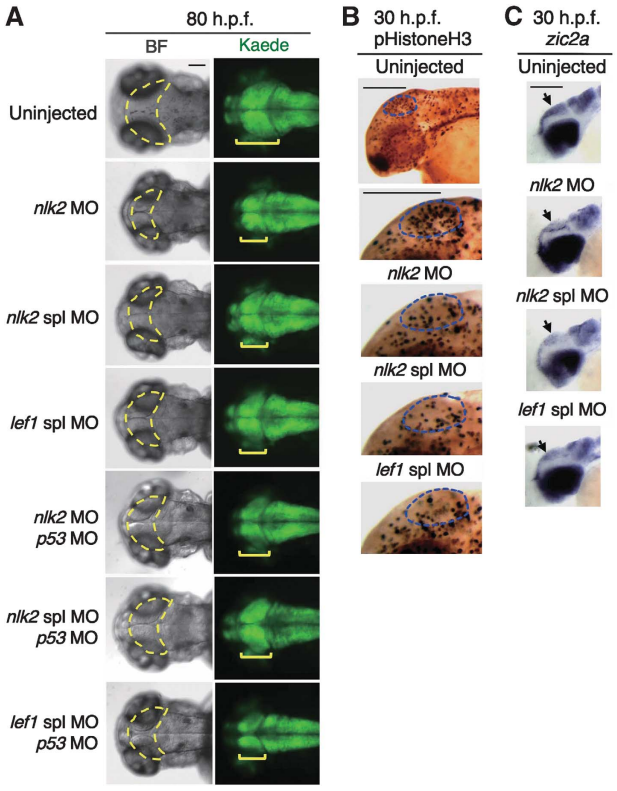

Figure 2 Nlk2 and Lef1 are essential for the midbrain tectum development in zebrafish. (**A**) HuC:kaede-transgenic zebrafish embryos injected with *nlk2* MO, *nlk2* spl MO, *lef1* spl MO, or *p53* MO as indicated. Panels show the dorsal head views of 80 h.p.f. embryos with the anterior to the left. Neurons expressing Kaede were visualized by fluorescence microscopy (right panels). Rectangles indicate the tectum. Bright-field (BF) images are shown in left panels. Broken lines indicate the tectum or presumptive tectal region. Scale bar, 50 μm. (**B**) Knockdown of either *nlk2* or *lef1* decreases the number of proliferating cells in midbrain. Anti-phospho-histone H3 immunostaining of 30 h.p.f. zebrafish embryos injected with *nlk2* MO, *nlk2* spl MO, or *lef1* spl MO, as indicated. Top panels show the left side head views of embryos with the anterior to the left. The other panels show the left side midbrain view of embryos with the anterior to the left. Broken lines indicate the presumptive tectal region. Scale bar, 250 μm. (**C**) Knockdown of either *nlk2* or *lef1* reduces expression of *zic2a* in midbrain. Panels show whole mount *in-situ* hybridization for *zic2a* in 30 h.p.f. embryos. Embryos were injected with *nlk2* MO, *nlk2* spl MO, or *lef1* spl MO as indicated. Panels show the left side head views of embryos with the anterior to the left. Expression of *zic2a* in midbrain is indicated with arrows. Scale bar, 250 μm.

of the midbrain tectum at 80 h.p.f. (Figure 2A; Supplementary Figure S5; Supplementary Table SI) but did not affect the development of the hindbrain (Supplementary Figure S5). Injection of *lef1* spl MO also resulted in a phenotype similar to that observed following injection of *nlk2* MOs. Co-injection of *p53* MO together with an MO for *nlk2* or *lef1* had no effect on the phenotype induced by MO-mediated knockdown of *nlk2* or *lef1* (Figure 2A; Supplementary Table SI). These results suggest that Nlk2 and Lef1 contribute to midbrain tectum development.

We next explored the mechanism by which Nlk2 and Lef1 contribute to tectum development. A previous report has shown that Lef1-mediated Wnt/β-catenin signalling promotes the proliferation of NPCs by activating the transcription of the *zic2a* and *zic5* genes in zebrafish developing midbrain (Nyholm *et al*, 2007). To examine whether Nlk2 contributes to the proliferation of midbrain NPCs, 30 h.p.f. zebrafish embryos were immunostained with anti-phospho-histone H3 antibody, which labels the nuclei of proliferating cells. Knockdown of *nlk2* or *lef1* decreased the numbers of phospho-histone H3-positive cells in the midbrain (Figure 2B; Supplementary Table SI), suggesting that Nlk2 and Lef1 are required for cell proliferation in the developing midbrain. We also found that expression of *zic2a* was decreased in the midbrain, but not in other regions of the brains of 30 h.p.f. embryos injected with *nlk2* MO, *nlk2* spl MO or *lef1* spl MO (Figure 2C; Supplementary Table SI). Using quantitative PCR (qPCR), we confirmed that injection of *nlk2* MO or *lef1* spl MO reduced the expression levels of *zic2a* in the midbrain (Supplementary Figure S2B). Our results suggest that Nlk2 and Lef1 promote the proliferation of NPCs through Wnt/β-catenin signalling in the developing midbrain.

Nlk2 phosphorylates Lef1 in zebrafish

We have previously reported that NLK phosphorylates human LEF1 at Thr-155 and Ser-166 *in vitro* (Ishitani *et al*, 2003b). The Thr residue is conserved between human and zebrafish (Figure 3A). To examine whether Nlk2 phosphorylates Lef1 at Thr-151, we generated an antibody that specifically recognizes phosphorylation of Lef1 at the conserved Thr residue (Figure 3A). This anti-phospho-Lef1 (anti-pLef1) antibody recognized Lef1 when it was co-expressed in mammalian neuro-2a cells with Nlk2, but not with kinase-negative Nlk2 (Figure 3B). These data suggest that Nlk2 phosphorylates Lef1 Thr-151. To verify antibody specificity, we generated a Lef1 mutation, Lef1(T151A), in which Thr-151 was changed to alanine. Lef1(T151A) was not detected by anti-pLef1 antibody when co-expressed with Nlk2 (Figure 3B).

We next investigated whether Nlk2 phosphorylates Lef1 in zebrafish embryos. We performed immunoblotting assays with anti-pLef1 and anti-Lef1 antibodies. Anti-Lef1 detected a protein of about 50 kDa in zebrafish embryo extracts (Figure 3C). In embryos injected with a translation-blocking MO against *lef1* (*lef1* MO) (Ishitani *et al*, 2005), levels of the protein detected by anti-Lef1 antibody decreased (Supplementary Figure S6), confirming that this protein corresponds to Lef1. Western blotting with anti-pLef1 antibody revealed that Lef1 phosphorylation could be detected in 24 h.p.f. embryo extracts (Figure 3C). Injection of *nlk2* MO reduced Lef1 phosphorylation, but had little effect on Lef1 protein levels. Thus, Nlk2 is able to phosphorylate Lef1 in 24 h.p.f. zebrafish embryos.

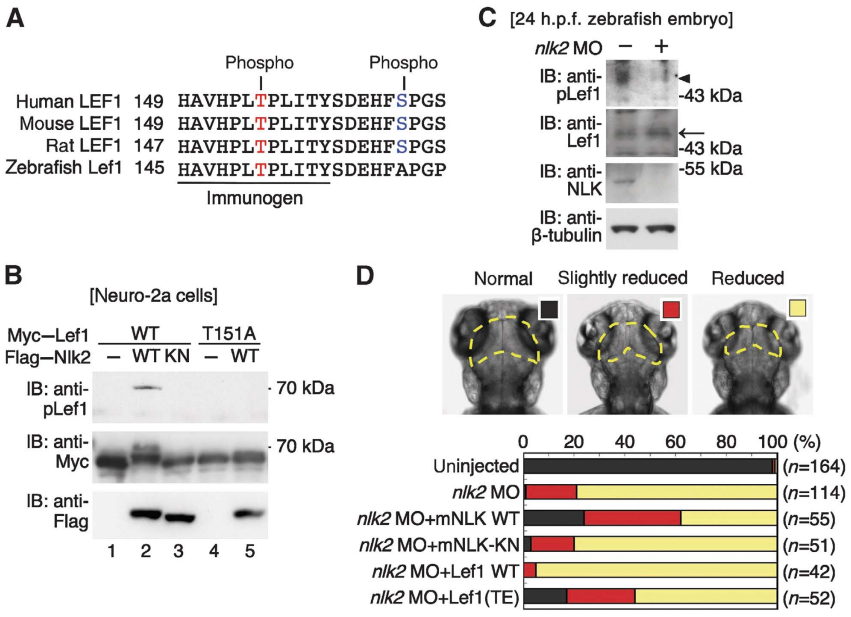

Figure 3 Nlk2 is involved in Lef1 phosphorylation in zebrafish. (**A**) Amino-acid sequence alignment of the NLK phosphorylation regions within vertebrate LEF1/Lef1 proteins. The Thr and Ser residues, which are phosphorylated by NLK, are indicated with red and blue letters, respectively. The bar under 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

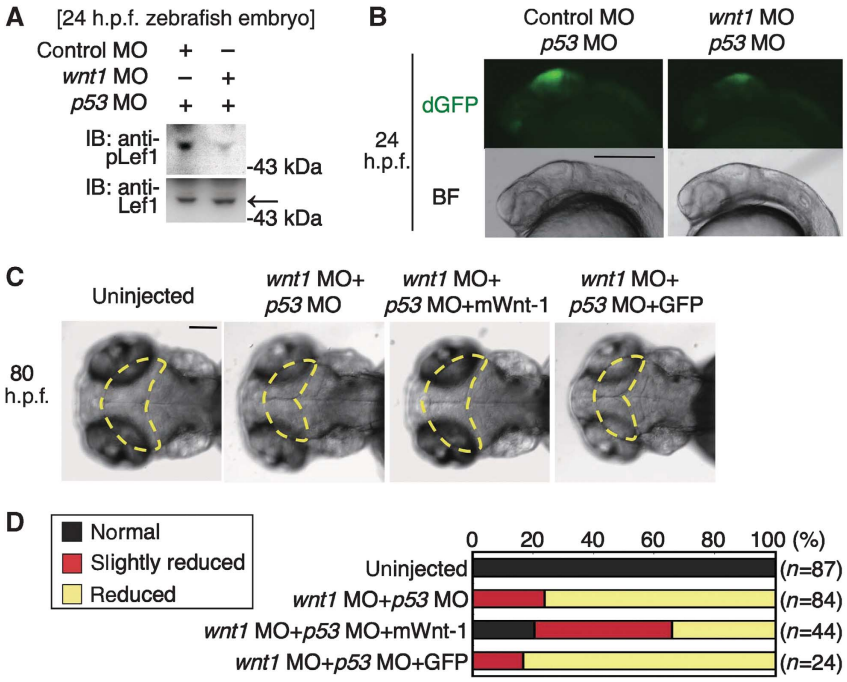

Figure 4 Wnt1 is involved in Lef1 phosphorylation in zebrafish. (**A**) Wnt1 is required for Lef1 phosphorylation at the conserved Thr residue in zebrafish. Zebrafish embryos were co-injected with *p53* MO and control MO or *wnt1* MO, as indicated. Extracts were harvested from the embryos at 24 h.p.f., 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

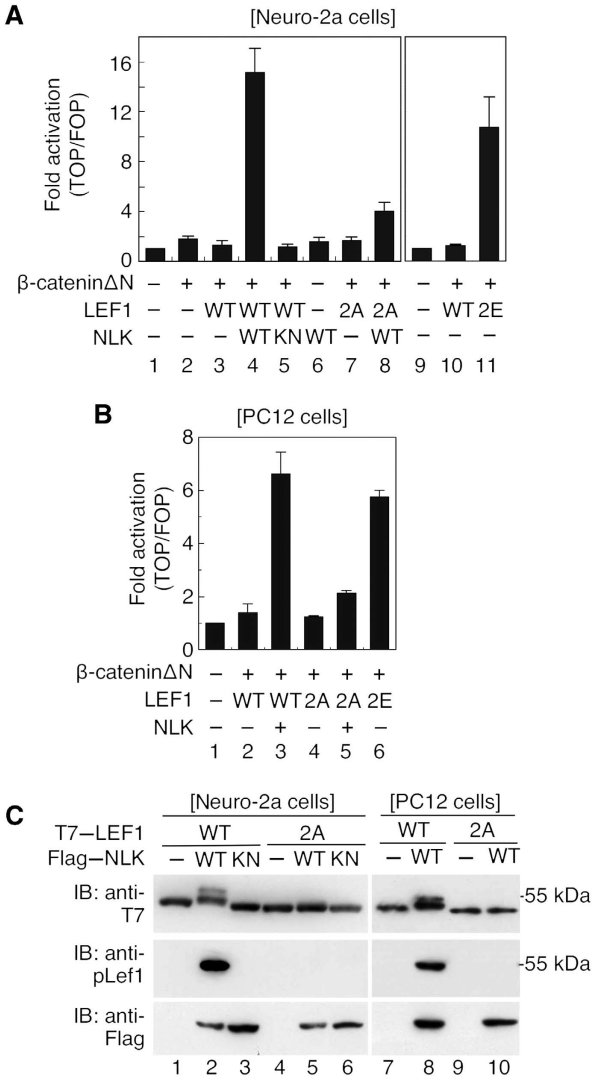

Figure 5 NLK promotes LEF1-mediated transcription in NPC-like mammalian cell lines. (**A**, **B**) The Wnt/β-catenin signalling reporter plasmids and expression plasmids encoding β-cateninΔN, human LEF1-WT, LEF1-2A, LEF1-2E, mouse NLK-WT, and NLK-KN were transfected as indicated and the luciferase activities 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.

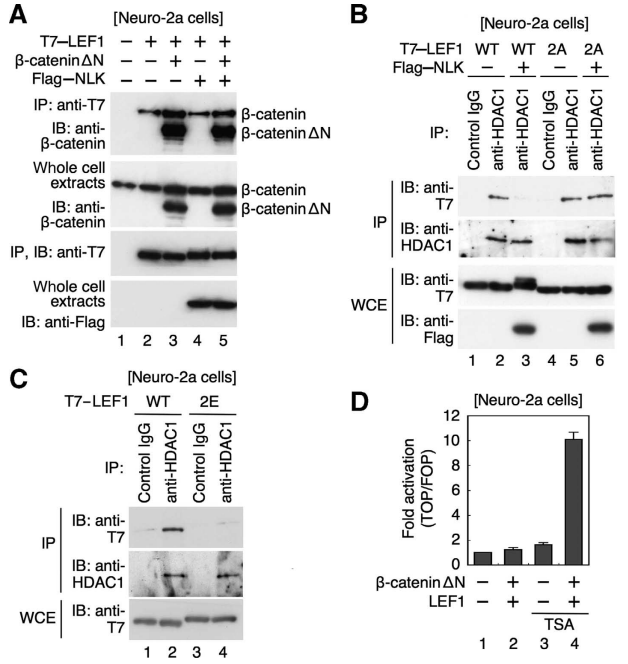

Figure 6 HDAC1 interacts with unphosphorylated LEF1. (**A**) NLK does not affect the interaction of LEF1 with β-catenin in neuro-2a cells. Neuro-2a cells were transfected with T7-tagged human LEF1, β-cateninΔN, and Flag-tagged mouse NLK as indicated. Cell extracts were subjected to immunoprecipitation 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.

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 (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

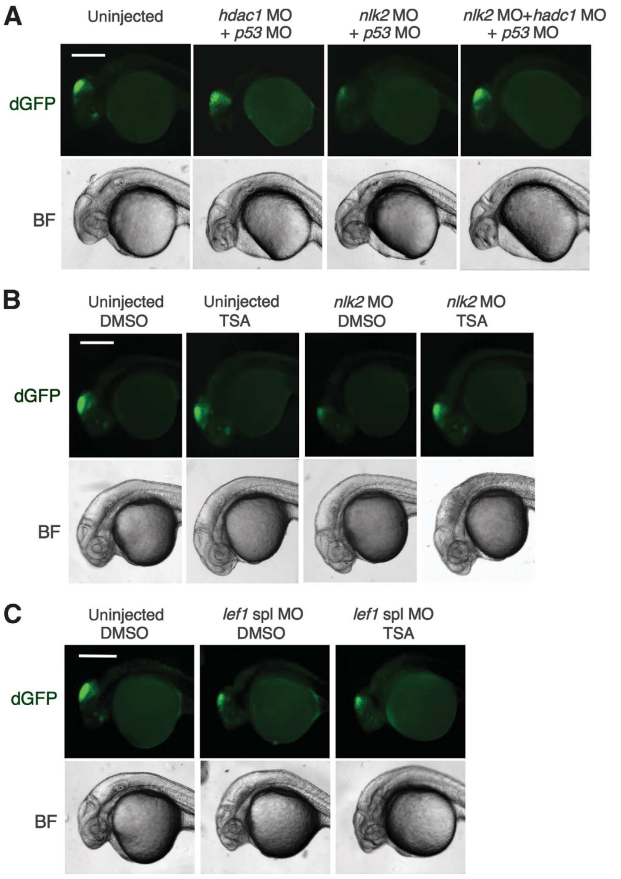

Figure 7 Nlk2 positively regulates Wnt/β-catenin signalling by blocking Hdac1-mediated inhibition in zebrafish midbrain. TOPdGFP-transgenic zebrafish embryos were uninjected or injected with *hdac1* MO, *nlk2* MO, *lef1* spl MO or *p53* MO at one-cell stage, as indicated. At 24 h.p.f., embryos were untreated (**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

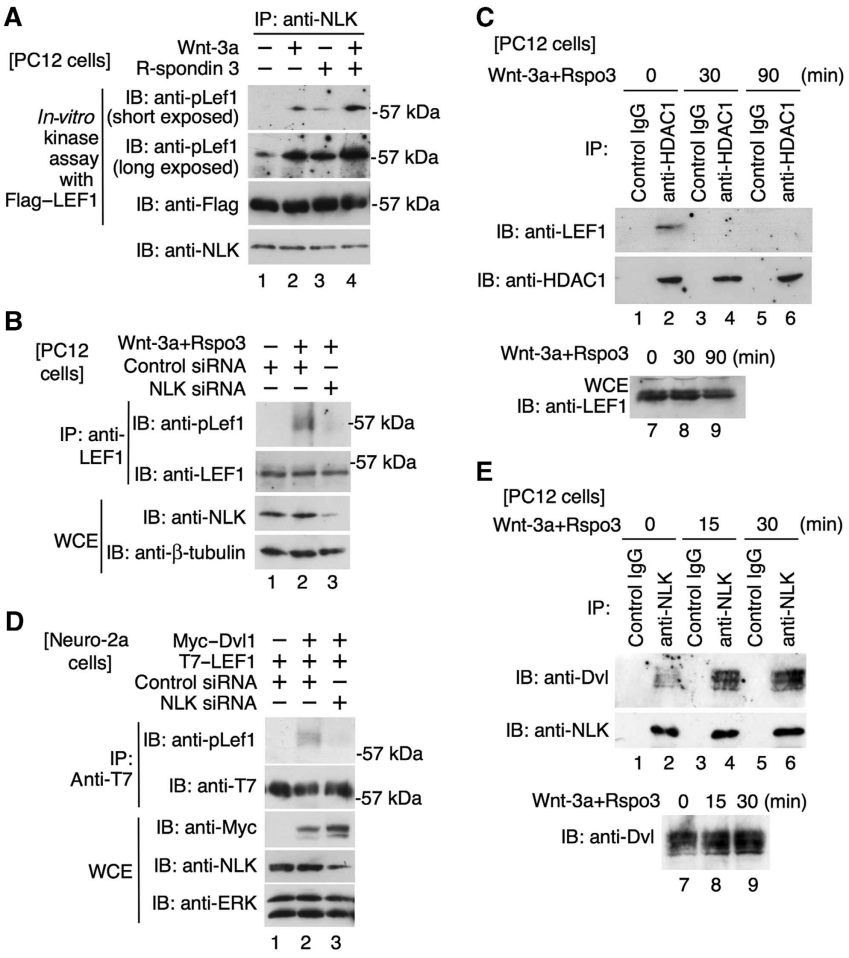

Figure 8 NLK functions downstream of Dvl in the Wnt/β-catenin signalling pathway. (**A**) Wnt-3a signalling activates the kinase activity of endogenous NLK. PC12 cells were untreated or treated with Wnt-3a and/or R-spondin 3 (Rspo3) for 30 min and endogenous NLK was immunoprecipitated (IP) with anti-NLK 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).

Acknowledgements

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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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