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 We also found that Nlk1 can hardly phosphorylate the conserved Thr residue on Lef1 (unpublished observation), suggesting that Nlk1 does not act redundantly with Nlk2 in the regulation of zebrafish midbrain development.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

We thank R Moon, ZIRC, and NBRP zebrafish for providing TOPdGFP- and HuC:Kaede-transgenic fish; K Kawakami, A Kikuchi, and S Ishii for providing the plasmids; the Laboratory for Technical Support, MIB, and the Research Support Center, Graduate School of Medical Sciences, Kyushu University; Y Sado, S Kai, and H Matsuo for his/her technical support. This research was supported by Brain Science Foundation, Astellas Foundation for Research on Metabolic Disorders, Health Labour Sciences Research Grant, Research Foundation for Opto-Science and Technology (TI), and the Grants-in-Aid for Scientific Research programs in Japan (TI., M I, and KM).

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.

References

Aberle H, Bauer A, Stappert J, Kispert A, Kemler R (1997) β-Catenin is a target for the ubiquitin-proteasome pathway. *EMBO J* **16:** 3797–3804

Amoyel M, Cheng YC, Jiang YJ, Wilkinson DG (2005) Wnt1 regulates neurogenesis and mediates lateral inhibition of boundary cell specification in the zebrafish hindbrain. *Development* **132:** 775–885

Arce L, Pate KT, Waterman ML (2009) Groucho binds two conserved regions of LEF-1 for HDAC-dependent repression. *BMC Cancer* **9:** 159–172

Arce L, Yokoyama NN, Waterman ML (2006) Diversity of LEF/TCF action in development and disease. *Oncogene* **25:** 7492–7504

Bafico A, Liu G, Yaniv A, Gazit A, Aaronson SA (2001) Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nat Cell Biol* **3:** 683–686

Billin AN, Thirlwell H, Ayer DE (2000) β-Catenin-histone deacetylase interactions regulate the transition of LEF1 from a transcriptional repressor to an activator. *Mol Cell Biol* **20:** 6882–6890

Binnerts ME, Kim KA, Bright JM, Patel SM, Tran K, Zhou M, Leung JM, Liu Y, Lomas III WE, Dixon M, Hazell SA, Wagle M, Nie WS, Tomasevic N, Williams J, Zhan X, Levy MD, Funk WD, Abo A (2007) R-Spondin1 regulates Wnt signaling by inhibiting internalization of LRP6. *Proc Natl Acad Sci USA* **104:** 14700–14705

Bonner J, Gribble SL, Veien ES, Nikolaus OB, Weidinger G, Dorsky RI (2008) Proliferation and patterning are mediated independently in the dorsal spinal cord downstream of canonical Wnt signaling. *Dev Biol* **313:** 398–407

Brennan K, Gonzalez-Sancho JM, Castelo-Soccio LA, Howe LR, Brown AM (2004) Truncated mutants of the putative Wnt receptor LRP6/Arrow can stabilize beta-catenin independently of Frizzled proteins. *Oncogene* **23:** 4873–4884

Carmon KS, Gong X, Lin Q, Thomas A, Liu Q (2011) R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/β-catenin signaling. *Proc Natl Acad Sci USA* **108:** 11452–11457

Cavallo RA, Cox RT, Moline MM, Roose J, Polevoy GA, Clevers H, Peifer M, Bejsovec A (1998) Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature* **395:** 604–608

Clevers H (2006) Wnt/β-catenin signaling in development and disease. *Cell* **127:** 469–480

Cunliffe VT (2004) Histone deacetylase 1 is required to repress Notch target gene expression during zebrafish neurogenesis and to maintain the production of motoneurones in response to hedgehog signalling. *Development* **131:** 2983–2995

Davidson G, Wu W, Shen J, Bilic J, Fenger U, Stannek P, Glinka A, Niehrs C (2005) Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction. *Nature* **438:** 867–872

de Lau W, Barker N, Low TY, Koo BK, Li VS, Teunissen H, Kujala P, Haegebarth A, Peters PJ, van de Wetering M, Stange DE, van Es JE, Guardavaccaro D, Schasfoort RB, Mohri Y, Nishimori K, Mohammed S, Heck AJ, Clevers H (2011) Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature* **476:** 293–297

Dorsky RI, Sheldahl LC, Moon RT (2002) A transgenic Lef1/β-catenin-dependent reporter is expressed in spatially restricted domains throughout zebrafish development. *Dev Biol* **241:** 229–237

Dorsky RI, Snyderb A, Cretekosd CJ, Grunwaldd DJ, Geislere R, Hafftere P, Moon RT, Raibleb DW (1999) Maternal and embryonic expression of zebrafish *lef1*. *Mech Dev* **86:** 147–150

Gerety SS, Wilkinson DG (2011) Morpholino artifacts provide pitfalls and reveal a novel role for pro-apoptotic genes in hindbrain boundary development. *Dev Biol* **15:** 279–289

Grinblat Y, Slive H (2001) *zic* gene expression marks anteroposterior pattern in the presumptive neurectoderm of the zebrafish gastrula. *Dev Dyn* **222:** 688–693

Herman M (2001) *C. elegans* POP-1/TCF functions in a canonical Wnt pathway that controls cell migration and in a noncanonical Wnt pathway that controls cell polarity. *Development* **128:** 581–590

Hikasa H, Sokol SY (2011) Phosphorylation of TCF proteins by homeodomain-interacting protein kinase 2. *J Biol Chem* **286:** 12093–12100

Hoppler S, Kavanagh CL (2007) Wnt signalling: variety at the core. *J Cell Sci* **120:** 385–393

Ishitani T, Hirao T, Suzuki M, Isoda M, Ishitani S, Harigaya K, Kitagawa M, Matsumoto K, Itoh M (2010) Nemo-like kinase suppresses Notch signalling by interfering with formation of the Notch active transcriptional complex. *Nat Cell Biol* **12:** 278–285

Ishitani T, Ishitani S, Matsumoto K, Itoh M (2009) Nemo-like kinase is involved in NGF-induced neurite outgrowth via phosphorylating MAP1B and paxillin. *J Neurochem* **111:** 1104–1118

Ishitani T, Kishida S, Hyodo-Miura J, Ueno N, Yasuda J, Waterman M, Shibuya H, Moon RT, Ninomiya-Tsuji J, Matsumoto K (2003a) The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca^{2+} pathway to antagonize Wnt/β-catenin signaling. *Mol Cell Biol* **23:** 131–139

Ishitani T, Matsumoto K, Chitnis AB, Itoh M (2005) Nrarp functions to modulate neural crest cell differentiation by regulating LEF1 protein stability. *Nat Cell Biol* **7:** 1106–1112

Ishitani T, Ninomiya-Tsuji J, Matsumoto K (2003b) Regulation of lymphoid enhancer factor 1/T-cell factor by mitogen-activated protein kinase-related Nemo-like kinase-dependent phosphorylation in Wnt/β-catenin signaling. *Mol Cell Biol* **23:** 1379–1389

Ishitani T, Ninomiya-Tsuji J, Nagai S, Nishita M, Meneghini M, Barker N, Waterman M, Bowerman B, Clevers H, Shibuya H, Matsumoto K (1999) The TAK1-NLK-MAPK-related pathway antagonizes signalling between β-catenin and transcription factor TCF. *Nature* **399:** 798–802

Jho EH, Zhang T, Domon C, Joo CK, Freund JN, Costantini F (2002) Wnt/β-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol Cell Biol* **22:** 1172–1183

Kanei-Ishii C, Ninomiya-Tsuji J, Tanikawa J, Nomura T, Ishitani T, Kishida S, Kokura K, Kurahashi T, Ichikawa-Iwata E, Kim Y, Mtasumoto K, Ishii S (2004) Wnt-1 signal induces phosphorylation and degradation of c-Myb protein via TAK1, HIPK2, and NLK. *Genes Dev* **18:** 816–829

Kojima H, Sasaki T, Ishitani T, Iemura S, Zhao H, Kaneko S, Kunimoto H, Natsume T, Matsumoto K, Nakajima K (2005) STAT3 regulates Nemo-like kinase by mediating its interaction with IL-6-stimulated TGFβ-activated kinase 1 for STAT3 Ser-727 phosphorylation. *Proc Natl Acad Sci USA* **102:** 4524–4529

Kortenjann M, Nehls M, Smith AJ, Carsetti R, Schuler J, Kohler G, Boehm T (2001) Abnormal bone marrow stroma in mice deficient for nemo-like kinase, Nlk. *Eur J Immunol* **31:** 3580–3587

Lekven AC, Buckles GR, Kostakis N, Moon RT (2003) Wnt1 and wnt10b function redundantly at the zebrafish midbrain-hindbrain boundary. *Dev Biol* **15:** 172–187

Logan CY, Nusse R (2004) The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* **20:** 781–810

Lustig B, Jerchow B, Sachs M, Weiler S, Pietsch T, Karsten U, van de Wetering M, Clevers H, Schlag PM, Birchmeier W, Behrens J (2002) Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. *Mol Cell Biol* **22:** 1184–1193

MacDonald BT, Semenov MV, Huang H, He X (2011) Dissecting molecular differences between Wnt coreceptors LRP5 and LRP6. *PLoS One* **6:** e23537

Mao B, Wu W, Li Y, Hoppe D, Stannek P, Glinka A, Niehrs C (2001) LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature* **411:** 321–325

Meier N, Krpic S, Rodriguez P, Strouboulis J, Monti M, Krijgsveld J, Gering M, Patient R, Hostert A, Grosveld F (2006) Novel binding partners of Ldb1 are required for haematopoietic development. *Development* **133:** 4913–4923

Meneghini MD, Ishitani T, Carter JC, Hisamoto N, Ninomiya-Tsuji J, Thorpe CJ, Hamill DR, Matsumoto K, Bowerman B (1999) MAP kinase and Wnt pathways converge to downregulate an HMG-domain repressor in *Caenorhabditis elegans*. *Nature* **399:** 793–797

Nam JS, Turcotte TJ, Smith PF, Choi S, Yoon JK (2006) Mouse cristin/R-spondin family proteins are novel ligands for the Frizzled 8 and LRP6 receptors and activate beta-catenin-dependent gene expression. *J Biol Chem* **281:** 13247–13257

Nambiar RM, Ignatius MS, Henion PD (2007) Zebrafish colgate/hdac1 functions in the non-canonical Wnt pathway during axial extension and in Wnt-independent branchiomotor neuron migration. *Mech Dev* **124:** 682–698

Niehrs C, Shen J (2010) Regulation of Lrp6 phosphorylation. *Cell Mol Life Sci* **67:** 2551–2562

Nyholm MK, Wu SF, Dorsky RI, Grinblat Y (2007) The zebrafish *zic2a-zic5* gene pair acts downstream of canonical Wnt signaling to control cell proliferation in the developing tectum. *Development* **134:** 735–746

Ohkawara B, Shirakabe K, Hyodo-Miura J, Matsuo R, Ueno N, Matsumoto K, Shibuya H (2004) Role of the TAK1-NLK-STAT3 pathway in TGF-β-mediated mesoderm induction. *Genes Dev* **18:** 381–386

Robu ME, Larson JD, Nasevicius A, Beiraghi S, Brenner C, Farber SA, Ekker SC (2007) p53 activation by knockdown technologies. *PLoS Genet* **3:** e78

Rocheleau CE, Yasuda J, Shin TH, Lin R, Sawa H, Okano H, Priess JR, Davis RJ, Mello CC (1999) WRM-1 activates the LIT-1 protein kinase to transduce anterior/posterior polarity signals in *C. elegans*. *Cell* **97:** 717–726

Roose J, Molenaar M, Peterson J, Hurenkamp J, Brantjes H, Moerer P, Wetering MVD, Destree O, Clevers H (1998) The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* **395:** 608–612

Semënov MV, Tamai K, Brott BK, Kühl M, Sokol S, He X (2001) Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. *Curr Biol* **11:** 951–961

Sato A, Yamamoto H, Sakane H, Koyama H, Kikuchi A (2010) Wnt5a regulates distinct signalling pathways by binding to Frizzled2. *EMBO J* **29:** 41–54

Sato T, Takahoko M, Okamoto H (2006) HuC:Kaede, a useful tool to label neural morphologies in networks *in vivo*. *Genesis* **44:** 136–142

Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A (1999) The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci USA* **96:** 5522–5527

Siegfried KR, Kidd III AR, Chesney MA, Kimble J (2004) The sys-1 and sys-3 genes cooperate with Wnt signaling to establish the proximal-distal axis of the Caenorhabditis elegans gonad. *Genetics* **166:** 171–186

Siegfried KR, Kimble J (2002) POP-1 controls axis formation during early gonadogenesis in *C. elegans*. *Development* **129:** 443–453

Takeuchi M, Kaneko H, Nishikawa K, Kawakami K, Yamamoto M, Kobayashi M (2010) Efficient transient rescue of hematopoietic mutant phenotypes in zebrafish using Tol2-mediated transgenesis. *Dev Growth Differ* **52:** 245–250

Tetsu O, McCormick F (1999) β-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398:** 422–426

Thorpe CJ, Moon RT (2004) nemo-like kinase is an essential co-activator of Wnt signaling during early zebrafish development. *Development* **131:** 2899–2909

Tsukada Y, Ishitani T, Nakayama KI (2010) KDM7 is a dual demethylase for histone H3 Lys 9 and Lys 27 and functions in brain development. *Genes Dev* **24:** 432–437

Urasaki A, Morvan G, Kawakami K (2006) Functional dissection of the Tol2 transposable element identified the minimal cis-sequence and a highly repetitive sequence in the subterminal region essential for transposition. *Genetics* **174:** 639–649

van de Wetering M, Cavallo R, Dooijes D, van Beest M, van Es J, Loureiro J, Ypma A, Hursh D, Jones T, Bejsovec A, Peifer M, Mortin M, Clevers H (1997) Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell* **21:** 789–799

Wei Q, Yokota C, Semenov MV, Doble B, Woodgett J, He X (2007) R-spondin1 is a high affinity ligand for LRP6 and induces LRP6 phosphorylation and beta-catenin signaling. *J Biol Chem* **282:** 15903–15911

Yamaguchi M, Tonou-Fujimori N, Komori A, Maeda R, Nojima Y, Li H, Okamoto H, Masai I (2005) Histone deacetylase 1 regulates retinal neurogenesis in zebrafish by suppressing Wnt and Notch signaling pathways. *Development* **32:** 3027–3043

Zeng YA, Rahnama M, Wang S, Sosu-Sedzorme W, Verheyen EM (2007) Drosophila Nemo antagonizes BMP signaling by phosphorylation of Mad and inhibition of its nuclear accumulation. *Development* **134:** 2061–2071

Zeng X, Huang H, Tamai K, Zhang X, Harada Y, Yokota C, Almeida K, Wang J, Doble B, Woodgett J, Wynshaw-Boris A, Hsieh JC, He X (2008) Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions. *Development* **135:** 367–375

Zeng X, Tamai K, Doble B, Li S, Huang H, Habas R, Okamura H, Woodgett J, He X (2005) A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* **438:** 873–877