Published in final edited form as:

Nat Cell Biol. 2010 January ; 12(1): 31–9. doi:10.1038/ncb2001.

Orphan nuclear receptor TLX activates Wnt/ β -catenin signalling to stimulate neural stem cell proliferation and self-renewal

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Abstract

The nuclear receptor TLX (also known as NR2E1) is essential for adult neural stem cell self-renewal; however, the molecular mechanisms involved remain elusive. Here we show that TLX activates the canonical Wnt/ β -catenin pathway in adult mouse neural stem cells. Furthermore, we demonstrate that Wnt/ β -catenin signalling is important in the proliferation and self-renewal of adult neural stem cells in the presence of epidermal growth factor and fibroblast growth factor. Wnt7a and active β -catenin promote neural stem cell self-renewal, whereas the deletion of Wnt7a or the lentiviral transduction of axin, a β -catenin inhibitor, led to decreased cell proliferation in adult neurogenic areas. Lentiviral transduction of active β -catenin led to increased numbers of type B neural stem cells in the subventricular zone of adult brains, whereas deletion of Wnt7a or TLX resulted in decreased numbers of neural stem cells retaining bromodeoxyuridine label in the adult brain. Both Wnt7a and active β -catenin significantly rescued a *TLX* (also known as *Nr2e1*) short interfering RNA-induced deficiency in neural stem cell proliferation. Lentiviral transduction of an active β -catenin increased cell proliferation in neurogenic areas of TLX-null adult brains markedly. These results strongly support the hypothesis that TLX acts through the Wnt/ β -catenin pathway to regulate neural stem cell proliferation and self-renewal. Moreover, this study suggests that neural stem cells can promote their own self-renewal by secreting signalling molecules that act in an autocrine/paracrine mode.

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Note: Supplementary Information is available on the Nature Cell Biology website.

AUTHOR CONTRIBUTIONS

Y.S. conceived and designed the study. Y.S., Q.Q., G.S., W.L., S.Y., P.Y. and C.Z. performed the experiments and analysed the data. Y.S., Q.Q., G.S., F.H.G. and R.M.E. interpreted the data. Y.S. wrote the paper with comments from Q.Q., G.S., R.T.Y., F.H.G. and R.M.E.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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The finding of neurogenesis in the adult brain led to the discovery of adult neural stem cells. Neural stem cells are defined as a subset of undifferentiated precursors that retain the ability to proliferate and self-renew and have the capacity to give rise to both neuronal and glial lineages^{1–4}. Under normal conditions, neurogenesis in the adult mammalian brain is restricted to two discrete germinal centres: the subgranular layer of the hippocampal dentate gyrus³ and the subventricular zones of the lateral ventricles^{5,6}. A complete understanding of adult neural stem cells requires the identification of molecules that determine the self-renewal and multipotent characteristics of these cells.

TLX is an orphan nuclear receptor that is expressed in vertebrate forebrains^{7,8}. We showed previously that TLX is an important regulator of neural stem cell maintenance and self-renewal in both embryonic and adult brains^{9,10}. TLX maintains neural stem cells in the undifferentiated and self-renewable state by complexing with histone deacetylases to repress downstream target genes¹¹ and forms a negative regulatory loop with the microRNA miR-9 (ref. ¹²). The feedback loop with miR-9 provides a strategy for controlling the balance between neural stem cell proliferation and differentiation¹². The TLX-positive neural stem cells in the dentate gyrus are important in spatial learning and memory¹³, whereas the TLX-positive cells in the subventricular zones were identified as the slowly dividing type B neural stem cells¹⁴.

Wnt proteins represent a growing family of secreted signalling molecules that perform multiple functions^{15–17}. In canonical Wnt signalling pathways, binding of Wnts to their receptors inhibits the phosphorylation of β -catenin by glycogen synthase kinase 3 β (GSK3 β). This inhibition prevents β -catenin degradation, resulting in its accumulation in the cytosol¹⁸. β -Catenin translocates to the nucleus, where it binds to the lymphoid enhancer binding factor (LEF)/T-cell transcription factor (TCF) family of transcription factors¹⁹ to induce expression of their target genes, such as *cyclin D1* (refs ^{20–22}). Wnts have been shown to regulate the self-renewal of multiple types of stem cell, including hematopoietic stem cells^{23,24} and epidermal/gut progenitors^{25,26}. The role of Wnt signalling in progenitor cell expansion in the developing nervous system has also been studied extensively^{27–32}. Recently, the role of Wnt signalling in neural stem cell proliferation in adult brains has begun to be identified³³.

We show here that TLX activates the Wnt/ β -catenin pathway in adult mouse neural stem cells to stimulate neural stem cell proliferation and self-renewal. Both Wnt7a and an active β -catenin rescued *TLX* short interfering RNA (siRNA)-induced deficiency in neural stem cell proliferation. Intracranial transduction of an active β -catenin increased cell proliferation in the neurogenic area of *TLX*^{−/−} adult brains significantly. These results strongly indicate that TLX regulates neural stem cell proliferation and self-renewal through activation of the Wnt/ β -catenin pathway in adult brains.

RESULTS

TLX activates Wnt/ β -catenin signalling

We have shown that TLX is essential for maintenance of the undifferentiated and self-renewable state of neural stem cells in the adult brain⁹. To identify downstream target genes of TLX, a gene profiling analysis was performed with RNAs isolated from adult brains of wild-type and TLX-null mice. Among genes with altered expression levels, a significant downregulation of Wnt7a expression was detected in adult TLX mutant brains (Fig. 1a and Supplementary Information, Fig. S1). Neural stem cells were isolated from *TLX*^{+/−} forebrains containing the hippocampal dentate gyrus and subventricular zones and cultured in N2-supplemented DMEM F12 medium containing epidermal growth factor (EGF) and fibroblast growth factor (FGF), as described⁹. *Wnt7a* was highly expressed in proliferating neural stem cells, and its expression was decreased markedly on differentiation, in a similar manner to the expression profile of *TLX* (Fig. 1a). Co-expression of TLX and Wnt7a in neural stem cells was

confirmed by immunofluorescence analyses, showing nuclear staining of TLX and cytoplasmic/membrane staining of *Wnt7a* (Fig. 1b). These results suggest that TLX could be an activator of *Wnt7a*.

Direct regulation of *Wnt7a* by TLX was suggested by a sequence analysis of the *Wnt7a* promoter, which revealed the presence of two consensus TLX binding sites (5'-AAGTCA-3') with a six-nucleotide spacer (5'-AACTCA-3') (-587 to -570). Gel shift and chromatin immunoprecipitation analyses revealed binding of TLX to the consensus sites in the *Wnt7a* promoter (Fig. 1c, d). To investigate whether TLX activates *Wnt7a* transcription, reporter assays were performed with a luciferase reporter gene linked to a 1.85-kilobase *Wnt7a* promoter (-1700 to +150) containing the consensus TLX binding sites. Transfection of TLX led to a 2.5-fold activation of the *Wnt7a* promoter, and mutation of the consensus TLX binding sites abolished the activation (Fig. 1e). These results demonstrate that TLX functions as a transcriptional activator of *Wnt7a* through direct binding to its promoter.

TLX represses transcription when bound to the *p21* promoter, which contains a monomeric TLX binding site, but activates transcription when bound to the *Wnt7a* promoter, which has two adjacent TLX binding sites (Supplementary Information, Fig. S2). Exchanging the monomeric TLX response element from the *p21* promoter with the sites contained in the *Wnt7a* promoter reversed the effect of TLX from a repressor to an activator of *p21*, whereas replacing the TLX response elements from the *Wnt7a* promoter with that from the *p21* promoter turned TLX from an activator to a repressor (Supplementary Information, Fig. S2). These results suggest that the TLX response elements may dictate TLX transcriptional activity.

In the canonical Wnt signalling pathway, Wnt stabilizes β -catenin and activates its downstream target genes. To determine whether TLX regulates Wnt/ β -catenin signalling, a β -catenin-responsive luciferase reporter Topflash was co-transfected with β -catenin or TLX, or both, into CV-1 cells. Co-transfection of TLX and β -catenin led to synergistic activation of the Topflash reporter (Fig. 2a), indicating that TLX potentiates β -catenin-responsive gene expression. To determine whether *Wnt7a* induction is necessary for TLX-mediated potentiation of the β -catenin reporter, we designed *Wnt7a*-specific siRNAs. Among the three siRNAs tested, *Wnt7a* siRNAs 1 and 3 had marked knockdown effect (Fig. 2b) and were used for subsequent studies. Treatment with *Wnt7a* siRNAs 1 and 3 led to a significant decrease in Topflash reporter activity (2.9-fold) in cells co-transfected with TLX and β -catenin (Fig. 2a). A decrease in reporter activity by *Wnt7a* siRNAs was also observed in β -catenin-transfected cells (1.7-fold), but this was not as marked as the decrease in activity in cells co-transfected with TLX and β -catenin. These results indicate that *Wnt7a* induction is important for the synergistic activation of Topflash by TLX and β -catenin.

Cyclin D1 is a well-characterized β -catenin downstream target gene that is activated by β -catenin-TCF-LEF complexes. To determine further whether TLX activates Wnt/ β -catenin signalling, we examined whether TLX regulates cyclin D1 expression. siRNA knockdown of TLX in neural stem cells led to significantly decreased expression of both *Wnt7a* and *cyclin D1* (Fig. 2c), whereas ectopic expression of TLX in 3T3 cells induced both *Wnt7a* and *cyclin D1* expression (Fig. 2d), in parallel with an increased accumulation of β -catenin protein levels in the cytosol (Fig. 2e). These results indicate that TLX activates cyclin D1 expression, presumably through activation of Wnt7a/ β -catenin.

Overexpression of TLX enhanced *Wnt7a* expression in neural stem cells and promoted neural stem cell proliferation (Supplementary Information, Fig. S3). Treatment with *Wnt7a* siRNAs knocked down *Wnt7a* expression and decreased cell proliferation in both control and TLX-overexpressing neural stem cells (Supplementary Information, Fig. S3), suggesting that *Wnt7a* is important for the effect of TLX on neural stem cell proliferation.

Wnt7a/ β -catenin stimulates neural stem cell proliferation and self-renewal

To further determine the role of Wnt7a in neural stem cell proliferation and self-renewal, we generated Wnt7a-overexpressing cells. Wnt7a was expressed as a fusion protein to human IgG in HEK 293 cells (Fig. 3a). Neural stem cells were co-cultured with the Wnt7a-transfected cells in a transwell system³⁴. Both cell proliferation, as revealed by cell numbers (Fig. 3b), and self-renewal, as determined by clonal rate (Fig. 3c), increased significantly in neural stem cells co-cultured with Wnt7a-expressing cells, compared with those co-cultured with control IgG-expressing cells. The clonal derivative of neural stem cells that were co-cultured with Wnt7a-expressing cells remained multipotent, with the ability to differentiate into neurons (Tuj1-positive), astrocytes (glial fibrillary acidic protein (GFAP)-positive), and oligodendrocytes (marker O4-positive) (Fig. 3d). These results suggest that Wnt7a stimulates neural stem cell proliferation and self-renewal in the presence of EGF and FGF.

Neural stem cells were also transduced with a constitutively active β -catenin harbouring a deletion of the first 90 amino acids (Δ N90) containing the GSK3 β phosphorylation site to mimic active Wnt signalling³⁵. Expression of the transduced Δ N90 β -catenin was shown by immunoblotting (Fig. 3e). Cell cycle analysis revealed that the active β -catenin promoted neural stem cell proliferation, resulting in an increase in cell population in the S/G2/M phases from 33.8% (control cells) to 49.4% (Fig. 3f). The β -catenin-transduced cells also self-renewed to a greater extent than control vector-transduced cells did, as revealed by an increased clonal rate and an enlarged clonal size (Fig. 3g, h). The clonal derivatives of the β -catenin-transduced cells were multipotent, with the ability to differentiate into both neurons and glia (Fig. 3i). These results suggest that the active β -catenin promotes neural stem cell proliferation and self-renewal.

To test whether Wnt signalling is required for neural stem cell growth, neural stem cells were treated with Frzb, a soluble Wnt antagonist that contains the cysteine-rich domain for Wnt interaction but lacks the transmembrane domain of the Frizzled receptors^{36,37}. Neural stem cells were cultured in Frzb-IgG or control IgG-containing conditioned medium with EGF and FGF. The protein levels of control IgG and Frzb-IgG in the conditioned medium were determined by western blot analysis (Fig. 4a). Treatment of Frzb led to decreased levels of cytosolic β -catenin protein (Fig. 4b) and significant inhibition of neural stem cell growth but had no effect on cell survival (Fig. 4c, d, and Supplementary Information, Fig. S4a, b).

In addition to Frzb, Wnt signalling was also inhibited by the expression of axin, an intracellular β -catenin inhibitor³⁸. Neural stem cells were transduced with control vector or axin-expressing retrovirus. The expression of viral axin was shown by northern blot analysis (Fig. 4e). Decreased cytosolic β -catenin protein levels were observed on treatment with axin (Fig. 4f). The axin-transduced cells showed a marked decrease in cell growth and self-renewal with no significant difference in cell survival (Fig. 4g, h, and Supplementary Information, Fig. S4c, d). These results further support the notion that Wnt/ β -catenin signalling is required for neural stem cell proliferation.

Wnt7a/ β -catenin rescues *TLX* siRNA-induced proliferation deficiency in neural stem cells

To determine whether Wnt/ β -catenin acts downstream of *TLX* to regulate neural stem cell proliferation and self-renewal, neural stem cells were treated with *TLX* siRNA-expressing lentivirus, which led to significant inhibition of neural stem cell proliferation. Introduction of the constitutively active β -catenin Δ N90 into *TLX* siRNA-transduced neural stem cells led to an up to 60% rescue of neural stem cell proliferation in the presence of EGF and FGF (Fig. 5a, b). These results strongly support the hypothesis that *TLX* acts in part through Wnt/ β -catenin to regulate neural stem cell proliferation.

The fact that TLX activates a secreted Wnt molecule suggests that TLX may stimulate neural stem cell proliferation through an autocrine/paracrine mode, in addition to the cell-autonomous regulation. To test this hypothesis, neural stem cells were infected with control vector or *TLX* siRNA-expressing lentivirus and seeded in the upper chambers of transwells. Non-transduced neural stem cells were plated at low cell densities at the base of transwells. Co-culture with *TLX* siRNA-transduced cells led to a significantly decreased proliferation of non-transduced neural stem cells, in comparison with co-culture with control vector-transduced cells (Fig. 5c). Addition of Wnt7a-expressing cells to the co-culture resulted in a significant recovery of cell proliferation (Fig. 5c), suggesting that TLX stimulates neural stem cell proliferation in part through activating a secreted Wnt7a molecule.

Regulation of neural stem cell proliferation by Wnt7a and β -catenin *in vivo*

The dentate gyrus of the hippocampus and the subventricular zone are two active adult neurogenic areas. Bromodeoxyuridine (BrdU) labelling analyses in *Wnt7a* knockout mice³⁹ revealed that loss of Wnt7a expression led to significant decrease in BrdU incorporation in the hippocampal dentate gyrus of adult brains (Fig. 6a, b), suggesting decreased cell proliferation. Decreased BrdU labelling was also observed in the subventricular zone of adult *Wnt7a*^{-/-} mice (Fig. 6c, d). These results suggest that Wnt7a is important for adult neural stem cell proliferation *in vivo*.

Furthermore, the β -catenin inhibitor axin was transduced into the sub-ventricular zone of adult wild-type brains by intracranial lentiviral delivery. Viral expression of axin was monitored by a co-expressed green fluorescent protein (GFP). Transduction of axin led to significantly decreased cell proliferation, as revealed by decreased numbers of GFP⁺BrdU⁺ cells compared with that in the control vector-transduced brains (Fig. 6e, f). These results further support the concept that Wnt/ β -catenin signalling is important for neural stem cell proliferation in adult brains.

To determine whether Wnt/ β -catenin acts downstream of TLX to regulate neural stem cell proliferation *in vivo*, the active β -catenin Δ N90 was introduced into the subventricular zone of *TLX*^{-/-} adult brains by stereotaxic lentiviral transduction. Viral expression of β -catenin was monitored by co-expressed GFP. Intracranial injection of β -catenin Δ N90 lentivirus led to considerable rescue of cell proliferation in the subventricular zone of *TLX*^{-/-} adult brains, as revealed by a marked increase in the percentage of GFP⁺BrdU⁺ cells out of total GFP⁺ cells, compared with that in control GFP-transduced *TLX*^{-/-} brains (Fig. 6g, h). These results provide strong *in vivo* evidence that TLX regulates Wnt/ β -catenin signalling to control neural stem cell proliferation in the neurogenic areas of adult brains.

GFAP⁺ type B cells have been proposed to be neural stem cells in the subventricular zone of adult brains⁴⁰. Next we examined the effect of Wnt signalling on GFAP⁺ type B cells by lentiviral transduction of the active β -catenin Δ N90 into the subventricular zone of wild-type adult brains. Viral expression of the active β -catenin was monitored by co-expressed GFP. Transduction of β -catenin Δ N90 lentivirus led to a significant increase in the percentage of GFAP⁺GFP⁺ cells out of total GFP⁺ cells in the subventricular zone, compared with that in control GFP-transduced brains (Fig. 7a, b). These results suggest that Wnt/ β -catenin signalling acts on type B neural stem cells in the subventricular zone of adult brains, in addition to its effect on type C cells³³.

It has been shown that the slowly dividing, BrdU-label-retaining cells correspond to neural stem cells in the adult brain⁴¹. To determine whether the TLX–Wnt signalling regulates neural stem cells *in vivo*, we treated 6-week-old Wnt7a-null mice and their wild-type littermates with BrdU for 7 days. The treated mice were allowed to survive for 3 weeks. The number of BrdU-label-retaining cells was markedly lower in the subventricular zone of Wnt7a-null mice than

in their wild-type littermates (Fig. 7c, d), suggesting that Wnt7a is an important regulator of neural stem cells in the adult brain. A substantial decrease in BrdU-label-retaining cells was also detected in the subventricular zone of adult *TLX*^{-/-} mice (Fig. 7e, f), which is consistent with a recent observation that TLX is expressed in type B neural stem cells in the subventricular zone of adult brains and is essential for the self-renewal of these cells¹⁴. The results from Wnt7a-null mice and TLX-null mice together provide strong evidence that the TLX–Wnt signalling cascade regulates neural stem cell population in the adult brain.

DISCUSSION

TLX has been shown to be essential in neural stem cell maintenance and self-renewal in adult brains⁹. To uncover the molecular mechanisms involved, we have shown here that TLX activates the canonical Wnt/β-catenin signalling in adult neural stem cells. Wnt/β-catenin in turn regulates neural stem cell proliferation and self-renewal in the presence of EGF and FGF. Our observations that both Wnt7a and a constitutively active β-catenin rescued *TLX* siRNA-induced proliferation deficiency in neural stem cells strongly support the notion that TLX acts in part by activating the Wnt/β-catenin pathway to regulate neural stem cell proliferation and self-renewal.

Wnt signalling has been shown to regulate the self-renewal of multiple types of stem cell. The role of Wnt signalling in the expansion of stem cells in developing brains is supported by studies in several mouse models^{27,29,30}. Our study demonstrated that the Wnt/β-catenin signalling is also important for adult neural stem cell proliferation and self-renewal. Wnt signalling has multiple functions in stem cells: in addition to its essential role in neural stem cell proliferation and self-renewal, Wnt signalling has also been shown to regulate neuronal differentiation of neural precursor cells^{42–44}. How stem cells respond to Wnts is likely to depend not only on cell-intrinsic properties but also on their specialized microenvironment⁴⁵. It is possible that Wnt/β-catenin signalling stimulates neural stem cell proliferation and self-renewal in the presence of mitogens and promotes neurogenesis on receiving differentiation cues. The presence of multiple Wnts also allows various outcomes of Wnt signalling. The Wnt pathways are therefore poised at a critical crossroads in balancing neural stem cell self-renewal versus differentiation.

The ability of TLX to repress transcription of the genes encoding intracellular molecules, such as GFAP⁹ and p21 (ref. ¹¹), indicates that TLX is a cell-autonomous regulator of neural stem cell self-renewal. Regulation of a secreted Wnt-encoding gene by TLX suggests that TLX may also function through an autocrine/paracrine mode in neural stem cells, in addition to its cell-autonomous actions. In this sense, our results support a novel concept that neural stem cells can stimulate their own proliferation by secreting signalling molecules, in addition to garnering support from other cell types, such as endothelial cells³⁴ and astrocytes⁴⁶, in the progenitor niche. The difficulty in culturing neural stem cells at a low cell density supports the concept that neural stem cell growth requires stimulation by autocrine effect or by cell–cell contact. The fact that CCg, a molecule secreted by neural stem cells, stimulates neural stem cell proliferation and self-renewal⁴⁷ lends additional support to this concept.

TLX has been shown to function as a transcription factor to repress the expression of its downstream target genes, such as p21. However, we show here that TLX activates Wnt7a expression. This activation could occur by direct binding to the *Wnt7a* promoter or by repression of a *Wnt7a* repressor. Binding of TLX to the consensus sites in the *Wnt7a* promoter has been observed in our study, although this does not exclude the possibility that TLX also activates *Wnt7a* by repressing a *Wnt7a* repressor. An explanation for the activation of *Wnt7a* by TLX binding could be the fact that DNA can act as an allosteric regulator to provide gene-specific regulation, the best-studied example of which is the glucocorticoid receptor⁴⁸. Our

TLX-binding-element swap experiment suggests that a specific configuration of the TLX response elements may dictate TLX to be a *Wnt7a* activator.

A substantial decrease in the number of neural stem cells retaining BrdU label was detected in the subventricular zone of both *Wnt7a*^{-/-} and *TLX*^{-/-} adult brains, providing strong evidence that TLX–Wnt signalling regulates the neural stem cell population in the adult brain. The decrease in the number of cells retaining BrdU label in the subventricular zone of adult *TLX*^{-/-} mice is more severe than that in *Wnt7a*^{-/-} mice. This discrepancy could be explained by the fact that TLX, as a transcription factor, regulates a spectrum of downstream target genes. Other functional targets, in addition to *Wnt7a*, may contribute to the substantial depletion of neural stem cell pool in *TLX*^{-/-} brains. Alternatively, the possible existence of a *Wnt7a*-independent stem cell population may also provide an explanation for the less severe decrease in neural stem cell numbers in *Wnt7a*^{-/-} brains.

Stem cell technology holds great promise for the treatment of many diseases that currently lack effective therapies. Identifying factors that control stem cell maintenance and self-renewal is an important step in moving stem cell technology from bench to bedside. Our finding that TLX activates *Wnt* to stimulate neural stem cell self-renewal suggests that purified Wnt proteins or molecules activating Wnt signalling can be developed as tools to facilitate the expansion of neural stem cells as a source for cell replacement therapies in the treatment of neurodegenerative diseases and brain injury.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturecellbiology/>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Jeremy Stark and John Zaia for a critical reading of the manuscript; Shengxiu Li and Richard Stewart for technical help; Mariko Lee and Brian Armstrong for microscopy support; Angela Barth (Stanford University) for the constitutively active β -catenin construct; Frank Costantini (Columbia University) for providing the axin cDNA; Andrew Lassar (Harvard Medical School) and Eldad Tzahor (Weizmann Institute) for providing the IgG and Frzb–IgG expression vectors and stably transfected cells; and Bert Vogelstein (Johns Hopkins University) for Topflash plasmid. Y.S. is a Kimmel Scholar. G.S. is a Herbert Horvitz Postdoctoral Fellow. F.H.G. is the Adler Professor of Age-Related Neurodegenerative Diseases and is supported by the National Institutes of Health (NIH) and the Picower Foundation. R.M.E. is an Investigator of the Howard Hughes Medical Institute at the Salk Institute and March of Dimes Chair in Molecular and Developmental Biology. This work was supported by the Whitehall Foundation, the Margaret E. Early Medical Trust and the NIH National Institute of Neurological Disorders and Stroke RO1 NS059546 (to Y.S.).

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Figure 1.

Expression of TLX and Wnt7a in adult TLX-null brains and adult neural stem cells. **(a)** Northern blot analysis of *Wnt7a* expression in wild-type (+/+), TLX-heterozygote (+/-), and TLX-null (-/-) adult brains, or in adult mouse neural stem cells (NSCs) cultured under proliferation (P) or differentiation (D) conditions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a loading control. **(b)** Immunofluorescence analysis of TLX (green) and Wnt7a (red) in adult mouse neural stem cells. Scale bar, 20 μm. **(c)** Binding of TLX to *Wnt7a* promoter in gel shift assays. ³²P-labelled DNA oligonucleotides containing the consensus TLX binding sites in the *Wnt7a* promoter were used as probe. TLX-DNA complexes and free probes are indicated. n.s., non-specific binding; C, control lysates. **(d)** Binding of TLX to *Wnt7a* promoter in chromatin immunoprecipitation analysis. DNA input was included in lane 1. Control IgG was in lane 2 and TLX antibody was used in lane 3. **(e)** TLX activates Wnt7a in reporter assays. Luciferase reporter gene (*wnt-luc*) was placed downstream of the wild-type (WT) or mutant (Mut) *Wnt7a* promoter. Luciferase activity in cells transfected with control vector (-TLX) or TLX-expressing vector (+TLX) was measured and normalized with β-galactosidase activity for transfection efficiency. Error bars show s.d.; assays were performed in triplicate. Uncropped images of blots in (a) and (d) are shown in Supplementary Information, Fig. S5.

Figure 2.

TLX activates Wnt/β-catenin/cyclin D1. **(a)** TLX potentiates a β-catenin-responsive reporter gene. The β-catenin-responsive reporter gene Topflash was transfected into CV-1 cells along with β-catenin or TLX, or both, in the presence of a control siRNA (C) or *Wnt7a* siRNAs (siWnt7a-1 and siWnt7a-3). Reporter luciferase activity was measured and normalized with β-galactosidase activity. Error bars show s.d.; assays were performed in triplicate. **(b)** Knockdown of *Wnt7a* expression with *Wnt7a*-specific siRNAs as revealed by luciferase assays. *Wnt7a*-specific siRNAs 1, 2 and 3 (si1, si2 and si3) were co-transfected with a luciferase reporter gene upstream of *Wnt7a* cDNA. The knockdown effect was determined by relative luciferase activity. *GFP* siRNA was included as a control (C). Error bars show s.d.; assays were performed in triplicate. Asterisk, $P < 0.005$ by Student's *t*-test. **(c)** Expression of *Wnt7a* and *cyclin D1* in TLX siRNA (siTLX)-treated adult mouse neural stem cells as revealed by RT-PCR analysis. Actin was included as a loading control. **(d)** Expression of *Wnt7a* and *cyclin D1* in TLX-expressing 3T3 (3T3-TLX) cells and parental 3T3 cells as revealed by northern blot analysis. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was included as a loading control. **(e)** Expression of TLX and cytosolic β-catenin in 3T3-TLX and parental 3T3 cells demonstrated by western blot analysis. Uncropped images of blots in (c)–(e) are shown in Supplementary Information, Fig. S5.

Figure 3.

Wnt7a and active β -catenin stimulate neural stem cell (NSC) proliferation and self-renewal. (a) Western blot analysis of control IgG and Wnt7a-IgG expression in stable HEK 293 cells with the use of human IgG antibody. (b) Proliferation of low-density adult mouse NSCs during co-culture with control IgG (C) and Wnt7a-IgG (Wnt)-expressing cells was monitored by cell numbers in each input well. Data are means $\pm$ s.d.; asterisk, $P < 0.01$ by Student's t -test, $n = 6$. (c) The self-renewal capacity of adult mouse NSCs co-cultured with cells expressing control IgG and Wnt7a-IgG was determined by secondary clonal rate. Data are means $\pm$ s.d.; asterisk, $P < 0.05$ by Student's t -test, $n = 5$. (d) Multipotency of the Wnt7a-treated NSCs. Cells were immunostained for a neuronal marker (Tuj1), an astrocyte marker (GFAP) and an oligodendrocyte marker (O4). Scale bar, 100 μ m. (e) Expression of β -catenin Δ N90 in

transduced adult rat NSCs analysed by western blot analysis with anti- β -catenin antibody. WT, wild-type β -catenin; Δ N90, β -catenin Δ N90. **(f)** Cell-cycle analysis of control and β -catenin Δ N90-transduced adult rat NSCs. The percentages of cells at G1 phase and at G2/S/M phases are indicated. **(g)** The self-renewal capacity of adult rat NSCs transduced with control vector (C) or β -catenin Δ N90-expressing virus (Δ N90) was determined by secondary clonal rate. Data are represented as means $\pm$ s.d.; asterisk, $P < 0.05$ by Student's t -test, $n = 5$. **(h)** Representative clonal derivatives of adult rat NSCs transduced with control vector or β -catenin Δ N90-expressing virus. d1, d10: days 1 and 10 of clonal analysis, respectively. Scale bar, 100 μ m. **(i)** Multipotency of β -catenin Δ N90-transduced NSCs. Cells were differentiated and immunostained for Tuj1, GFAP, and O4. 4,6-Diamidino-2-phenylindole (DAPI) staining is shown in blue. The right-hand panel is the merged image. Representative O4-positive cells are shown in the inset. Scale bar, 50 μ m. Uncropped images of blots in (a) and (e) are shown in Supplementary Information, Fig. S5.

Figure 4.

Inhibition of adult neural stem cell growth by Frzb and Axin. **(a)** Western blot analysis of control IgG and Frzb-IgG expression in stable HEK 293-cell-conditioned medium. **(b)** Expression of cytosolic β -catenin in adult mouse neural stem cells (NSCs) treated with control IgG (C) or Frzb-IgG (Frzb), demonstrated by western blot analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a loading control. **(c)** Proliferation of low-density adult mouse NSCs that were treated with control IgG (C) or Frzb-IgG (Frzb). **(d)** Self-renewal of adult mouse NSCs that were treated with control IgG (C) or Frzb-IgG (Frzb) is shown by secondary clonal rate. **(e)** Northern blot analysis of viral axin expression in axin-transduced adult rat NSCs. C, control cells; axin, axin-transduced cells. **(f)** Expression of cytosolic β -catenin in control vector (C) or axin-transduced adult rat NSCs demonstrated by western blot analysis. GAPDH was included as a loading control. **(g)** Proliferation of low-density adult rat NSCs that were transduced with control vector (C) or axin-expressing virus. **(h)** Self-renewal of control vector (C) or axin-transduced adult rat NSCs as revealed by secondary clonal rate. Data are represented as means $\pm$ s.d.; asterisk, $P < 0.01$ by Student's t -test for (c) and (g) ($n = 6$); two asterisks, $P < 0.05$ by Student's t -test for (d) and (h) ($n = 5$). Uncropped images of blots in (a) and (e) are shown in Supplementary Information, Fig. S5.

Figure 5.

Constitutively active β -catenin and Wnt7a rescued *TLX* siRNA-mediated proliferation deficiency in neural stem cells (NSCs). (a) Active β -catenin rescued *TLX* siRNA-induced NSC proliferation deficiency. Control vector (C), *TLX* siRNA (siTLX), active β -catenin, or siTLX and β -catenin double-transduced adult mouse NSCs were cultured in N2-supplemented medium containing EGF and FGF for 46 h and pulse-labelled with BrdU for 2 h. Cell proliferation was determined by BrdU labelling (red). Merged images of BrdU labelling and phase contrast images (grey) are shown in the lower panels. Scale bar, 100 μ m. (b) Quantification of the percentage of BrdU-positive (BrdU⁺) cells in total living cells of control vector (C), active β -catenin (β -cat.), *TLX* siRNA (siTLX), or both *TLX* siRNA and active β -catenin-transduced NSCs. Data are represented as means $\pm$ s.d.; asterisk, $P < 0.01$ by Student's t test, ($n = 3$). (c) Recovery of *TLX* siRNA-mediated NSC proliferation deficiency by Wnt7a. Untreated adult mouse NSCs were plated at low cell density at the base of transwells and co-cultured with control vector or *TLX* siRNA-transduced NSCs, or co-cultured with *TLX* siRNA-

transduced NSCs plus Wnt7a-IgG (Wnt7a)-expressing cells that were seeded in the upper chambers of transwells. Cells were cultured in N2-supplemented medium containing EGF and FGF. NSC proliferation was monitored by cell numbers in each input well at the base of transwells on day 5 of co-culture. Data are represented as means $\pm$ s.d.; asterisk, $P < 0.05$ by Student's t test, ($n = 3$).

Figure 6.

Regulation of cell proliferation by Wnt/β-catenin in adult brains. **(a)** Decreased BrdU staining in the dentate gyrus (DG) of *Wnt7a*^{-/-} adult brains. Hippocampal DG sections of adult wild-type (WT) and *Wnt7a*^{-/-} mice were immunostained for BrdU (green). Scale bar, 100 μm for all panels. **(b)** Quantification of BrdU-positive (BrdU⁺) cells in hippocampal DG of adult WT and *Wnt7a*^{-/-} mice. Data are represented as means ± s.d.; asterisk, $P < 0.05$ by Student's *t*-test, $n = 12$. **(c)** Decreased BrdU staining in the subventricular zone (SVZ) of *Wnt7a*^{-/-} adult brains. SVZ sections of adult WT and *Wnt7a*^{-/-} mice were immunostained for BrdU (red). V, ventral wall; L, lateral wall; D-DL, dorsal and dorsal-lateral region of the lateral ventricle. **(d)** Quantification of BrdU-positive (BrdU⁺) cells in SVZ of adult WT and *Wnt7a*^{-/-} mice. Data are represented as means ± s.d.; asterisk, $P < 0.05$ by Student's *t*-test, $n = 3$. **(e)** Decreased BrdU staining of axin-transduced cells in the SVZ of adult brains. SVZ sections from control vector (GFP) or axin (axin-GFP)-transduced and BrdU-treated mice were immunostained for BrdU (red). **(f)** Quantification of GFP-positive and BrdU-positive (GFP⁺BrdU⁺) cells from GFP⁺ cells in the SVZ of viral-infected mice. Data are represented as means ± s.d.; asterisk, $P < 0.05$ by Student's *t*-test, $n = 6$. **(g)** Rescue of cell proliferation in the SVZ of *TLX*^{-/-} adult brains with the use of active β-catenin ΔN90. SVZ sections from control vector (GFP) or β-catenin ΔN90 (β-catenin-GFP)-transduced and BrdU-treated *TLX*^{-/-} mice were

immunostained for BrdU (red). Rescue of cell proliferation was revealed by the increased percentage of GFP⁺BrdU⁺ cells out of total GFP⁺ cells. The insets are enlarged images of the cells indicated by arrows. **(h)** Quantification of GFP⁺BrdU⁺ cells in the SVZ of virus-transduced *TLX*^{-/-} brains. Data are represented as means $\pm$ s.d.; asterisk, $P < 0.05$ by Student's *t*-test, $n = 8$ for control GFP-transduced brains, $n = 5$ for β -catenin–GFP-transduced brains.

Figure 7.

TLX-Wnt/β-catenin signalling regulates neural stem cell population in the adult brain. **(a)** Lentiviral transduction of active β-catenin increases the numbers of GFAP⁺ B cells in the SVZ of wild-type (WT) brains. Brains were injected with control GFP lentivirus (GFP) or β-catenin ΔN90-GFP virus (β-catenin-GFP). Scale bar, 10 μm. **(b)** Quantification of the percentage of GFAP⁺GFP⁺ cells out of total GFP⁺ cells in control GFP (C) and β-catenin-GFP (β-cat.)-transduced SVZ. Data are represented as means ± s.d.; asterisk, $P < 0.05$ by Student's t -test, $n = 10$. **(c)** Decrease in number of BrdU-label-retaining cells in the SVZ of *Wnt7a*^{-/-} brains. BrdU-label-retaining cells were labelled in green. S100β staining (red) is included to show the structure of the SVZ. Scale bar, 20 μm. **(d)** Quantification of the BrdU-label-retaining (BrdU⁺) cells in the SVZ of WT and *Wnt7a*^{-/-} (*Wnt*^{-/-}) brains. The fold change in BrdU⁺ cells in the SVZ of WT mice is calculated by dividing the number of BrdU⁺ cells in the SVZ of WT mice by the number of BrdU⁺ cells in the SVZ of *Wnt7a*^{-/-} SVZ. The fold change in *Wnt7a*^{-/-} SVZ is expressed as 1. Data are represented as means ± s.d.; asterisk, $P < 0.001$ by Student's t -test, $n = 8$ for WT brains and $n = 12$ for *Wnt7a*^{-/-} brains. **(e)** Decrease in number of BrdU-label-retaining cells in the SVZ of *TLX*^{-/-} brains. BrdU labelling is shown in green and S100β in red. Scale bar, 20 μm. **(f)** Quantification of BrdU-label-retaining (BrdU⁺) cells in the SVZ of WT and *TLX*^{-/-} brains. The fold change in BrdU⁺ cells in the SVZ is calculated

in the same way as in (d). Data are represented as means $\pm$ s.d.; asterisk, $P < 0.001$ by Student's t -test, $n = 5$ for both wild-type and $TLX^{-/-}$ brains.