

# TLX controls angiogenesis through interaction with the von Hippel-Lindau protein

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## Summary

TLX is known as the orphan nuclear receptor indispensable for maintaining neural stem cells in adult neurogenesis. We report here that neuroblastoma cell lines express high levels of TLX, which further increase in hypoxia to enhance the angiogenic capacity of these cells. The proangiogenic activity of TLX appears to be induced by its direct binding to the von Hippel-Lindau protein (pVHL), which stabilizes TLX. In turn, TLX competes with hydroxylated hypoxia-inducible factor (HIF- $\alpha$ ) for binding to pVHL, which contributes to the stabilization of HIF-2 $\alpha$  in neuroblastoma during normoxia. Upon hypoxia, TLX increases in the nucleus where it binds in close proximity of the HIF-response element on the VEGF-promoter chromatin, and, together with HIF-2 $\alpha$ , recruits RNA polymerase II to induce VEGF expression. Conversely, depletion of TLX by shRNA decreases the expression of HIF-2 $\alpha$  and VEGF as well as the growth-

promoting and colony-forming capacity of the neuroblastoma cell lines IMR-32 and SH-SY5Y. On the contrary, silencing HIF-2 $\alpha$  will slightly increase TLX, suggesting that TLX acts to maintain a hypoxic environment when HIF-2 $\alpha$  is decreasing. Our results demonstrate TLX to play a key role in controlling angiogenesis by regulating HIF-2 $\alpha$ . TLX and pVHL might counterbalance each other in important fate decisions such as self-renewal and differentiation, as well as angiogenesis and anti-angiogenesis.

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Key words: TLX, VHL, HIF-2 $\alpha$ , Angiogenesis, Neuroblastoma

## Introduction

The orphan nuclear receptor TLX (NR2E1) has been recognized as an important transcription factor for maintenance of neural stem cells in an undifferentiated state. TLX is expressed in neural stem cells in the hippocampal dentate gyrus and the subventricular zone where neurogenesis continues in adulthood (Shi et al., 2004). These stem cells reside in a so-called “niche”, which is localized in close proximity to blood vessels, supporting stem cells/progenitors and their expansion and migration (Shen et al., 2008). TLX-null mice exhibit progressive retinopathies due to the inability of retinal astrocytes to produce TLX, playing an important role in the vascular development (Uemura et al., 2006). Indeed, in TLX-null mutant mice, the retinal astrocyte network is disorganized along with poorly developed blood vessels (Miyawaki et al., 2004; Yu et al., 2000). It has been proposed that a hypoxic environment in the developing retina upregulates TLX in immature astrocytes to induce angiogenesis. When astrocytes mature by establishing a close contact with blood vessels, a normoxic environment prevails and TLX becomes downregulated in the astrocytes, suppressing angiogenesis. However, the regulation of TLX expression during hypoxia and the mechanism behind this angiogenic switching still remains unresolved.

The progression of tumors depends on the capacity to maintain its self-renewing cell population, i.e., tumor-initiating cells

(TICs). We therefore examined whether TLX might be involved in the angiogenesis important for maintaining the TICs of the nervous system tumors. Neuroblastoma is the most common extracranial solid childhood tumor of the sympathetic nervous system. It is thought to arise from the embryonic neural crest cells during and after their migration from the neural tube. Aggressive tumor cells typically lack differentiation markers of the sympathetic nervous system, similar to the neural crest progenitors (Pietras et al., 2008). The hallmark of undifferentiated neuroblastoma is its high degree of angiogenicity via a fine network of capillaries. TICs can be enriched from neuroblastoma, which constitutively express HIF-2 $\alpha$  to activate angiogenesis in and around the tumors (Holmquist-Mengelbier et al., 2006). In this regard, it is interesting to examine whether TLX may be involved in the development and progression of neuroblastoma, not only by stimulating self-renewal of TICs but also through its proangiogenic function. Furthermore, since HIF is a key regulator of the cellular response to hypoxia, we wanted to elucidate the relationship between TLX and HIF proteins.

HIF proteins are heterodimers, composed of one of the three  $\alpha$  subunits and the HIF-1 $\beta$ , a constitutive nuclear protein. In the presence of oxygen, HIF- $\alpha$  is prolyl hydroxylated, followed by interactions with von Hippel-Lindau protein (pVHL) that possesses E3 ubiquitin-ligase activity, and becomes degraded

by the proteasome (Kaelin, 2007b). The stabilized HIF heterodimer activates hypoxia-inducing genes through binding to their hypoxia-responsive elements (HREs), resulting in induction of angiogenic factors such as vascular endothelial factor (VEGF) and erythropoietin (Bunn et al., 1998; Wiesener et al., 2001). The inactivation by mutations of the VHL gene is associated with an increased risk of a variety of tumors, such as clear cell renal carcinoma, pheochromocytoma, and hemangioblastoma, in an allele-specific manner. However, HIF- $\alpha$  activation is not sufficient for the development of these tumors (Kaelin, 2007a). Herein, we report a novel interaction between TLX and VHL, facilitating the stabilization of HIF- $\alpha$ s in normoxia, and binding of TLX to the VEGF promoter in hypoxia. These results suggest a role of TLX in the switch mechanism of angiogenesis, in addition to the maintenance of neural stem cells. This finding might provide an integral view on the roles of VHL and TLX in oxygen control and cell proliferation.

## Materials and Methods

### Cell cultures and chemicals

The human neuroblastoma cell lines IMR-32, SH-SY5Y, and SK-N-BE2C, as well as the primate kidney epithelial Cos-1 cell line were cultured as described previously (Wetterskog et al., 2009). IMR-32 cells were cultured in a defined medium for neurosphere formation (Chavali et al., 2011). The VHL-deficient 786 cells, stably transfected with VHL or its control vector (786-VHL, 786-0), were cultured as described (Iliopoulos et al., 1995). Porcine aorta endothelial cells (PAEC) were cultured in the same manner as described for SH-SY5Y. For hypoxia studies, cells were grown in 1.7% O<sub>2</sub> unless indicated otherwise. For testing the stability of TLX, Cos-1 cells were treated with 25  $\mu$ g/ml cyclohexamide. For silencing TLX, shRNA vectors (20 nmol/L; Qiagen) were microporated into IMR-32 and SH-SY5Y using a microporator (Digital Bio) according to vender's instruction. When siRNA oligonucleotides (Qiagen) were used, FugeneHD (Roche) was employed for transfection. The concentrations of other chemicals used were 10  $\mu$ M for MG132 and 100  $\mu$ M for 2,2'-Bipyridyl (BP), both purchased from Sigma-Aldrich.

### qPCR

Cells were harvested and RNA was isolated using the TRIzol reagent (Invitrogen) and ethanol precipitation. cDNA was synthesized using oligo-dT primers and M-MuLV reverse transcriptase (Thermo Scientific). Quantitative real-time PCR analysis was performed using the StepOne Real-Time PCR system (Applied Biosystems). Relative mRNA expression was determined by normalizing to expression of the reference gene HPRT1 or GAPDH. Primer sequences used for qPCR are as follows: HPRT, Forward primer (FP), 5'-TTT GCT TTC CTT GGT CAG GC-3', Reverse primer (RP), 5'-GCT TGC GAC CTT GAC CAT CT-3'; GAPDH, 5'-AAA AGC GGG GAG AAA GTA GG-3', RP, 5'-CTA GCC TCC CGG GTT TCT CT-3'; TLX, FP, 5'-CTG GCT GTA TCT GGC ATG AA-3', RP, 5'-TCT AAA TCG AGC CAC CAC CT-3', VHL, FP, 5'-ATT AGC ATG GCG GCA CAC AT-3', RP, 5'-TGG AGT GCA GTG GCA TAC TCA T-3'.

### Immunoblotting

Cells were seeded in six-well plates, lysed, and proteins were immunoblotted as previously described (Wetterskog et al., 2009). The following primary antibodies were used. TLX (LifeSpan Biosciences, Santa Cruz Biotechnology, R&D Systems), VHL and VEGF-A (BD Biosciences), HA, Flag, and actin (AC-40) (Sigma-Aldrich), HIF-1 $\alpha$ , HIF-2 $\alpha$ ,  $\beta$ -actin,  $\beta$ -tubulin, Glucose transporter-1 (Glut1) (Santa Cruz Biotechnology), and GAPDH (Millipore). BAF57 was made as described in (Shiota et al., 2008). Separation of proteins into nuclear and cytoplasmic fractions (NF, CF) was done as described elsewhere (Kashiwagi et al., 2011).  $\beta$ -tubulin, BAF57, and  $\beta$ -actin were used as the cytoplasmic, nuclear, and total protein controls, respectively.

### Immunofluorescence staining

Cells were plated on chamber slides and used for immunostaining (Chavali et al., 2011) with antibodies described above and KI67 (Chemicon). As specificity controls, we used transfection of expression plasmid or control vector into cells not expressing the protein of interest as well as omission of primary antibody. Alexa 488 or 594-conjugated IgG (Invitrogen) was used as a secondary antibody, and stained cells were observed by epi-illumination fluorescence microscope (Olympus High Content microscopy). Images were acquired digitally by using the software CellR (Olympus).

### Protein binding assays (immunoprecipitation)

To generate TLX deletion constructs, cDNA of full-length mouse TLX (kind gift of Dr. Uemura) was used as template for PCR using primers as listed below, with flanking restriction sites for EcoRI and XhoI. The amplified cDNAs were cleaved and ligated into the EcoRI/XhoI sites of the pcDNA3 vector containing N-terminal Flag-tag. Primer sequences for TLX deletion constructs are as follows: TLX-FL, FP, 5'-GTT GAA TTC ATG AGC AAG CC GCC-3', RP, 5'-GCG CTC GAG TTA GAT GTC ACT GGA TTT G-3', TLX- $\Delta$ N, FP, 5'-TAT GAA TTC GAA TCA GCT GCC AGG CTT-3', TLX  $\Delta$ C, RP, 5'-TTT CTC GAG TTA ACA CAC GGA CTC AGT-3', TLX  $\Delta$ C341-385, RP, 5'-TTT CTC GAG TTA GAA TCG GCA GGG TTG G-3'.

HA-VHL<sub>30</sub> and its mutant VHL<sub>30</sub> $\Delta$ 95-123 in pcDNA3 were kind gifts of Dr. Krek (Hergovich et al., 2003). At 48 h after transfection using Fugene HD (Roche) with various expression vectors, cells were lysed and lysates were used for immunoprecipitation and for expression control of transfected plasmids by immunoblotting as previously described (Izumi et al., 2001). The different deletion constructs of TLX expressing recombinant glutathione S-transferase (GST) fusion protein and the full-length VHL<sub>30</sub> and VHL<sub>30</sub> $\Delta$ 95-123 expressing thioredoxin (Thio)-His fusion proteins were made from the plasmid (His-Patch ThioFusion System, Invitrogen) in *E. coli* and subsequently purified and used for in vitro binding assay, as described previously (Uramoto et al., 2002). For precipitation and blotting, antibodies against GST or Thio (Invitrogen) were used.

### Promoter reporter assay

Promoter assays were performed as described by using a VEGF-promoter in a pGL3 vector (Ryuto et al., 1996). Expression vectors used were HA-VHL<sub>30</sub> and Flag-TLX as described above. SH-SY5Y cells were incubated with 2,2' BP for the indicated time periods before being harvested. Standardization was done by normalization with co-transfected *renilla* activity.

### ELISA

Supernatants from IMR-32 cells was collected after 0, 4, and 24 h of hypoxia and VEGF concentrations were measured using the human VEGF colorimetric ELISA kit (ThermoFisher) according to the manufacturer's instruction.

### Colony formation assay in soft agar

Agar (Chemicon) was dissolved in culture medium to 0.8% and plated in culture plates (bottom layer). After the bottom layer had coagulated, cells were seeded in 0.4% agar and added on top of the bottom agar layer. Cells were covered with medium and cultured at 37°C in a 5% CO<sub>2</sub> incubator. Colonies were counted manually after three weeks.

### Chromatin immunoprecipitation (ChIP) assay

Protein and DNA were cross-linked by incubating cells with formaldehyde at a final concentration of 1% for 10 min at RT, and ChIP assay was done as previously described (Chavali et al., 2011). The fixed chromatin was incubated at 4°C with 2  $\mu$ g of antibodies against TLX, HIF-2 $\alpha$ , RNA Polymerase II (PolII), VHL and mouse or rabbit Ig (Santa Cruz) as a negative control. Actin promoter was used to rule out non-specific binding. The primers for VEGF promoter were 5'-TTT TCA GGC TGT GAA CCT TG-3' and 5'-GAT CCT CCC CGC TAC CAG-3' yielding a 233 bp product.

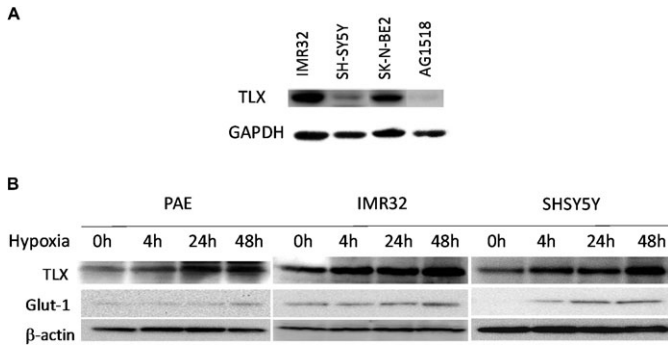
### Statistics

All quantitative analysis were repeated at least three times and values represent means  $\pm$  SD of one representative experiment, each done in triplicate. Overall significance was determined by submitting data to one-way analysis of variance. Significance of between-group differences was determined by various post-hoc comparisons as noted in Legends. Significance is labeled as \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.

## Results

### Expression and subcellular localization of TLX and VHL in neuroblastoma cell lines upon hypoxia

We first examined by immunoblotting whether neuroblastoma cell lines derived from the developing peripheral nervous system might express TLX. TLX expression differs among the IMR-32, SK-N-BE2C, and SH-SY5Y neuroblastoma cell lines (Fig. 1A). However, it is clearly higher compared with the human normal fibroblast AG1518 that expresses almost no TLX. It is well recognized that hypoxia up-regulates the expression of TLX in neuroprogenitors and retinal astrocytes during development (Chavali et al., 2011; Uemura et al., 2006). To test whether

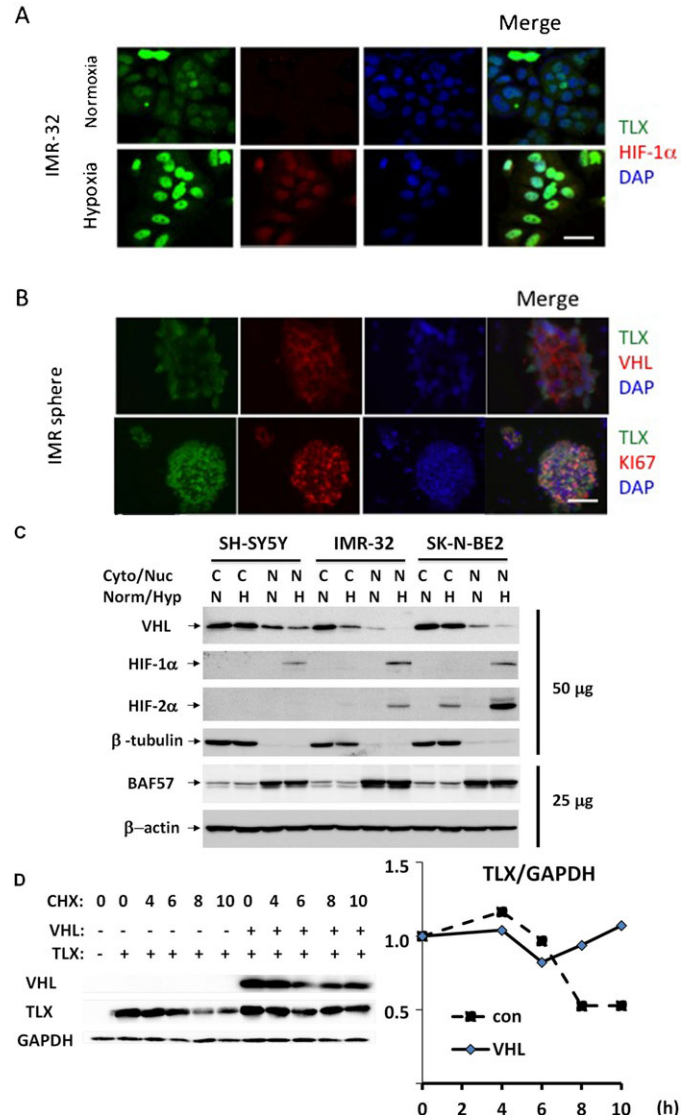


**Fig. 1. Hypoxia increases TLX expression in neuroblastoma.** (A) TLX expression in 3 neuroblastoma cell lines was evaluated by immunoblotting and compared with the normal fibroblast cell line AG1518 with GAPDH as a loading control. (B) Immunoblotting of TLX in PAEC, IMR-32, SH-SY5Y at indicated time points following hypoxia. Glut-1 is used as hypoxia control and  $\beta$ -actin as input control.

hypoxia induces TLX expression in neuroblastoma, IMR-32 and SH-SY5Y were cultured in 1.7% O<sub>2</sub>, which efficiently induces neuroprogenitor proliferation (Chavali et al., 2011), for 0, 4, 24, 48 h. The porcine aorta endothelial cell (PAEC) line was included because of its high hypoxia-sensitivity. The TLX expression increased in neuroblastoma cells and in PAEC following 4, 24, and 48 h of hypoxic treatment, along with glucose transporter-1 (Glut-1), which was used as a marker for hypoxia (Fig. 1B).

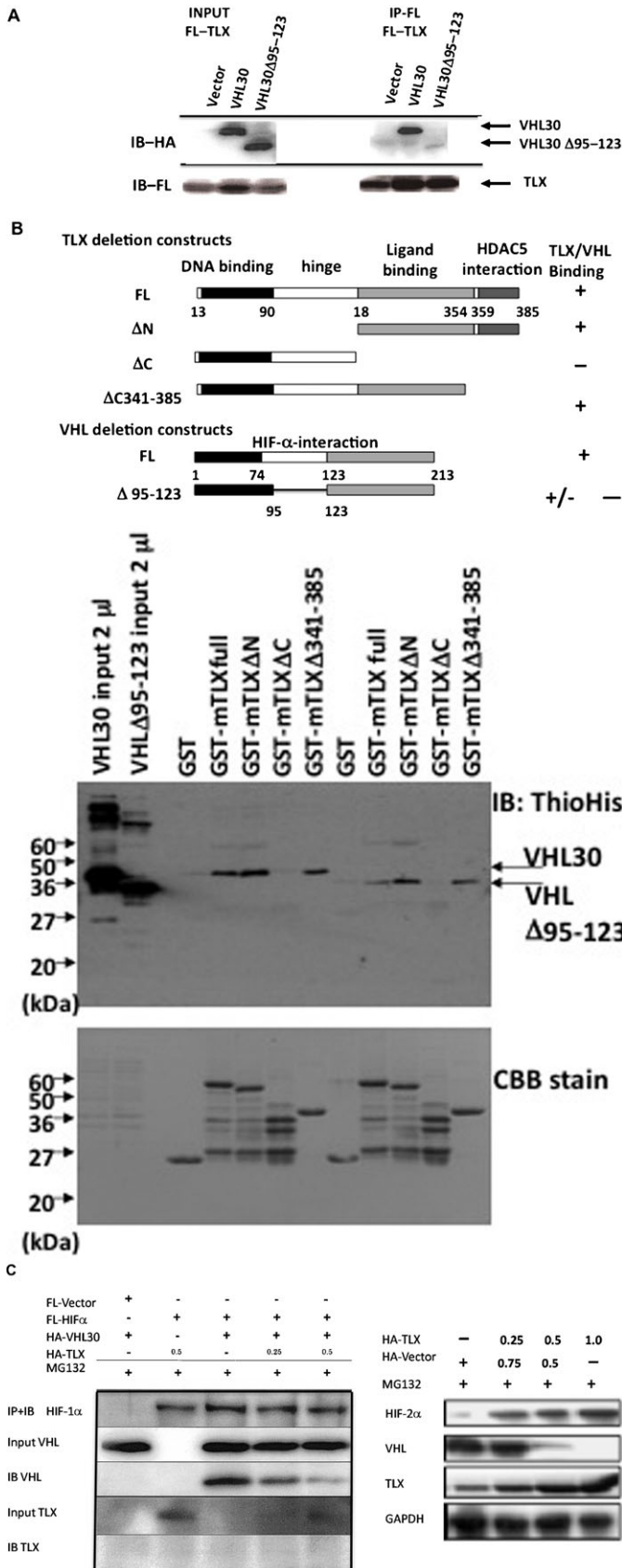
Since HIF-1 $\alpha$  is the most well-studied hypoxia-induced transcription factor with multiple target genes, we compared its expression pattern with that of TLX in IMR-32 following 6 h of hypoxia (Fig. 2A). Almost all cells stained positive for both TLX and HIF-1 $\alpha$ , whereas none of these cells expressed HIF-1 $\alpha$  in normoxia, even though a few cells expressed TLX quite strongly. In normal cells, HIF- $\alpha$ s are constantly degraded by pVHL. However, in neuroblastoma HIF-2 $\alpha$  is often constitutively expressed, which is further upregulated upon hypoxia, but the up-regulation occurs much later than with HIF-1 $\alpha$  (Holmquist-Mengelbier et al., 2006). In order to find out whether TLX has any relation with VHL we next stained IMR-32 cultured in spheres for TLX and VHL (Fig. 2B). TLX became up-regulated when neuroprogenitors were cultured in a defined medium stimulating neurosphere formation (Chavali et al., 2011). In this condition, TLX was actually expressed stronger in the cells located close to the surface of the spheres while cells in the center are stained more for pVHL that was mostly in the cytoplasm. In fact, TLX colocalized with KI67, a marker for dividing cells, indicating that TLX is associated with proliferating cells in the spheres.

In order to see the subcellular localization of pVHL and TLX in normoxia, proteins from IMR-32, SH-SY5Y, and SK-N-BE2C were fractionated into nuclear and cytoplasmic proteins and separately immunoblotted (supplementary material Fig. S1). In this condition, 3% (IMR-32), 14% (SK-N-BE2C), and 17% (SH-SY5Y) of the total VHL protein was detected in the nucleus. TLX expression was found in the nuclear fraction in both IMR-32 and SK-N-BE2C cells, whereas in SH-SY5Y a small fraction of TLX was found in the cytoplasm. We then examined whether VHL expression changes upon hypoxia with regard to its level and localization in relation to those of HIF-1 $\alpha$  and -2 $\alpha$ . Fractionated proteins from IMR-32, SH-SY5Y, and SK-N-BE2C were



**Fig. 2. Subcellular localization of TLX.** (A) Immunofluorescence staining of IMR-32 for TLX (green), HIF-1 $\alpha$  (red) and nuclei (DAPI; blue) in normoxia and after 6 h of hypoxia. The staining was standardized digitally to optimize the difference (upper panel). Bar, 50  $\mu$ m. (B) IMR-32 cells cultured in neurosphere media were stained for TLX (green) and VHL (red, upper panel) or KI67 (red, lower panel) and the images were taken as Z-stacks. (C) Nuclear (N) and cytoplasmic (C) expression of VHL, HIF-1 $\alpha$ , HIF-2 $\alpha$ , was compared by immunoblotting in normoxia (N) and after 12h hypoxia (H) in SH-SY5Y, IMR-32, and SK-N-BE2C cells.  $\beta$ -tubulin, BAF57, and  $\beta$ -actin were used as the cytoplasmic, nuclear, and total protein controls. (D) Effect of transfected pVHL<sub>30</sub> on the stability of TLX protein was examined with cyclohexamide pulse chase at 48 h after transfection of Flag-TLX and HA-VHL into Cos-1 cells, and cell lysates were analyzed at indicated time points. Changes of TLX expression during the time course was depicted in a graph. Values were standardized by GAPDH. Comparing means of 3 repeated experiments yielded a significant difference.

immunoblotted before and after 12 h hypoxia (Fig. 2C). pVHL was mostly expressed in the cytoplasm in agreement with the immunofluorescence (Fig. 2B). The pVHL in the nucleus decreased upon hypoxia, especially in IMR-32 and SK-N-BE2C. Adequate separation was confirmed by cytoplasmic  $\beta$ -tubulin and mostly nuclear BAF57 transcription factor. In hypoxia both HIF-1 $\alpha$  and HIF-2 $\alpha$  were accumulated only in the



nucleus in contrast to pVHL that decreased. SK-N-BE2C cells contained HIF-2 $\alpha$  in the nucleus and even slightly in the cytoplasm. These results suggest that in the nucleus the expression of VHL and TLX/HIF- $\alpha$  correlate negatively even though they may exist simultaneously in the nucleus. However, the mRNA levels of VHL and TLX as examined by qPCR remained unaltered in hypoxia (supplementary material Fig. S2). Thus, the protein expression of TLX seems to be stabilized upon hypoxia as in neuroprogenitors (Fig. 1B) (Chavali et al., 2011).

Since pVHL acts as a ubiquitin ligase, we wanted to confirm that TLX is not a degradation target of pVHL. We thus performed a cyclohexamide (CHX) pulse-chase experiment in Cos-1 cells over-expressing exogenous Flag-TLX together with HA-VHL or vector (Fig. 2D). Cells were harvested 48 h after transfection following treatment with CHX for various time periods. Coexpression of HA-VHL resulted in stabilization of TLX. This was also tested in VHL-deficient renal carcinoma 786-0 cells, where levels of pVHL and endogenous TLX were analyzed after transfection of VHL or its control plasmid in the presence of the proteasome inhibitor MG132 (supplementary material Fig. S3).

**TLX binds VHL and stabilizes HIF- $\alpha$ , enhancing the VEGF-promoter activity**

Next, we examined whether there is any interaction between TLX and pVHL, which might explain the stabilization of TLX and HIF- $\alpha$ . In order to determine TLX binding site to pVHL, Cos-1 cells were cotransfected with Flag-TLX and HA-full-length VHL<sub>30</sub>, or the deletion mutant VHL<sub>30</sub> $\Delta$ 95-123, which failed to bind microtubules (Hergovich et al., 2006). Twenty residues on the  $\beta$ -sheets of pVHL N67 to W117 are direct binding sites to HIF- $\alpha$ s (Min et al., 2002) and among these amino acids a mutation of Y98, Y111, or Y117 made it incapable to bind HIF- $\alpha$ s (Ohh et al., 2000). TLX was co-precipitated with the full-length pVHL<sub>30</sub> but hardly with pVHL<sub>30</sub> $\Delta$ 95-123 (Fig. 3A). This suggests that the region 95-123 is important for binding to TLX.

We also examined whether TLX and pVHL can bind each other directly without any involvement of other proteins, and if this is the case, we wish to determine which region of TLX would bind pVHL. To this end we used GST-pull down of different GST-fused TLX deletion mutants and ThioHis-tagged full-length VHL and its deletion mutant as shown in Fig. 3B. The result showed that deletion of the C-terminus ( $\Delta$ 187-385) of TLX abrogated VHL binding, while the N-terminal deletion construct ( $\Delta$ 1-187) was able to bind to pVHL. Even deletion of the C-terminal histone deacetylase (HDAC)5 interaction site ( $\Delta$ 341-385) could bind, indicating that the ligand-binding site is necessary for pVHL binding. However, pVHL<sub>30</sub> $\Delta$ 95-123 bound

**Fig. 3. Physical interaction of TLX and VHL.** (A) Cotransfection and coimmunoprecipitation of Flag-TLX with VHL<sub>30</sub>, VHL<sub>30</sub> $\Delta$ 95-123, or vector alone using Cos-1 cells. (B) Illustration of various TLX deletion constructs and the full-length and deletion mutant of VHL, and the result of binding (upper panel). In vitro coimmunoprecipitation of various deletion constructs of GST-TLX (FL,  $\Delta$ N,  $\Delta$ C,  $\Delta$ C341-385) and ThioHis-VHL<sub>30</sub> or ThioHis-VHL<sub>30</sub> $\Delta$ 95-123 by GST pull-down. Protein expression was detected by CBB stain (lower panel). (C) Competition assay between HIF- $\alpha$  and TLX for the binding to pVHL<sub>30</sub> in the presence of MG132. Same amounts of Flag-HIF-1 $\alpha$  or Flag-vector and HA-VHL or HA-vector were cotransfected into Cos-1 cells with increasing amounts of HA-TLX and lysates were precipitated by anti-Flag (left panel). IMR-32 was transfected with increasing amounts of TLX and protein lysate was coimmunoprecipitated with endogenous HIF-2 $\alpha$  by using a HIF-2 $\alpha$  antibody and immunoblotted with HIF-2 $\alpha$ , VHL, TLX, and GAPDH antibodies (right panel).

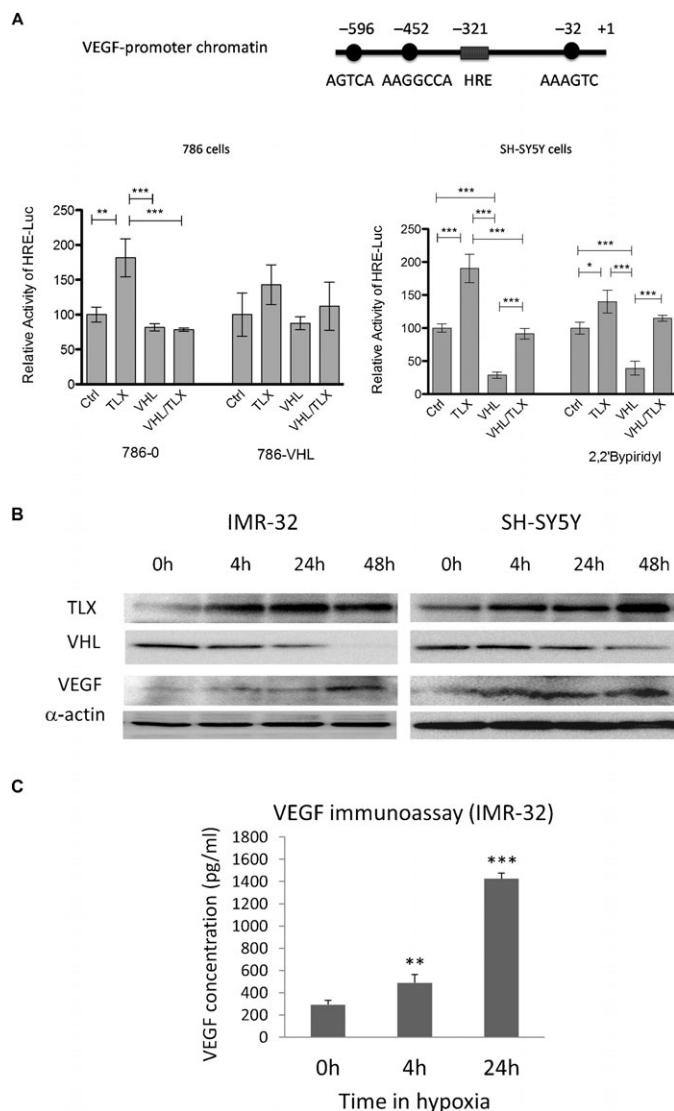
very weakly to the full-length TLX. The N-terminal deleted construct ( $\Delta$ 1-187) and to a lesser extent the far-most C-terminal  $\Delta$ 341-385 construct could bind pVHL<sub>30</sub> $\Delta$ 95-123, suggesting that exposing the ligand-binding region of TLX might increase its affinity to pVHL.

We further examined whether HIF- $\alpha$  and TLX could compete in binding to VHL involving the region containing residues 95-123. We thus treated cells with MG132 and performed coimmunoprecipitation with overexpressed Flag-HIF-1 $\alpha$  in normoxia (Fig. 3C, left panel), since the pVHL-binding sites of all HIF- $\alpha$ s are conserved. Cos-1 cells were transfected with Flag-HIF-1 $\alpha$  together with HA-VHL<sub>30</sub> and HA-TLX. When cells were transfected with increasing amounts of HA-TLX plasmid, the binding of pVHL to HIF-1 $\alpha$  decreased. Furthermore, when we overexpressed increasing amounts of TLX in IMR-32 cells and the lysate was immunoprecipitated by endogenous HIF-2 $\alpha$ , we saw that increased amounts of TLX diminished the amount of VHL to be coprecipitated with HIF-2 $\alpha$ . These results suggest that increased amounts of TLX could prevent pVHL from binding to prolyl-hydroxylated HIF- $\alpha$  (Fig. 3C, right panel).

Since TLX protein levels increased in the IMR-32 nucleus in hypoxia, we examined whether TLX could cooperate with HIF- $\alpha$  through binding to the TLX-binding motif (AAGTCA) close to the HRE element in the VEGF-promoter (Fig. 4A). In the 5' and 3' of the HRE-sequence, 3 TLX-consensus-like sequences are present in the VEGF-promoter. We used the VHL-deficient 786 cells stably transfected with VHL and its control vector (786-VHL, 786-0) for the promoter-reporter assays. We transfected those cell lines with TLX, VHL, or both TLX and VHL, together with the VEGF promoter-luciferase vector in these cell lines and measured their respective promoter activities (Fig. 4A). In 786-0, TLX activated the promoter-reporter 1.8-fold in normoxia in the presence of stabilized HIF- $\alpha$  caused by the loss of VHL. Thus, TLX activates the reporter not only by sequestering pVHL. As expected, VHL overexpression, which destabilizes HIF- $\alpha$ , decreased the activity. In 786-VHL, overexpression of TLX did not render any significant increase in the reporter activity. This may be due to depletion of HIF- $\alpha$  by abundant pVHL. We made a similar experiment in SH-SY5Y cells by using 2.2'BP that mimics a hypoxic condition. In normoxia, similarly to 786-0 cells, over-expression of TLX increased the promoter activity 2-fold, and excessive VHL decreased the activity to 30% of the control. In hypoxia, the activation due to TLX overexpression was marginal, although it counteracted the effect of VHL overexpression. These results suggest that TLX enhances the effect of HIF- $\alpha$ . Indeed, during hypoxia TLX protein levels increased, which was accompanied by a decrease of VHL protein levels and an apparent increase of VEGF protein levels after approximately 48 h. This supports the hypothesis that TLX protein might induce expression of VEGF. (Fig. 4B,C). The effect of TLX is larger in normoxia, since TLX stabilizes HIF- $\alpha$  by sequestering VHL in accordance with VHL overexpression, which will remove this effect.

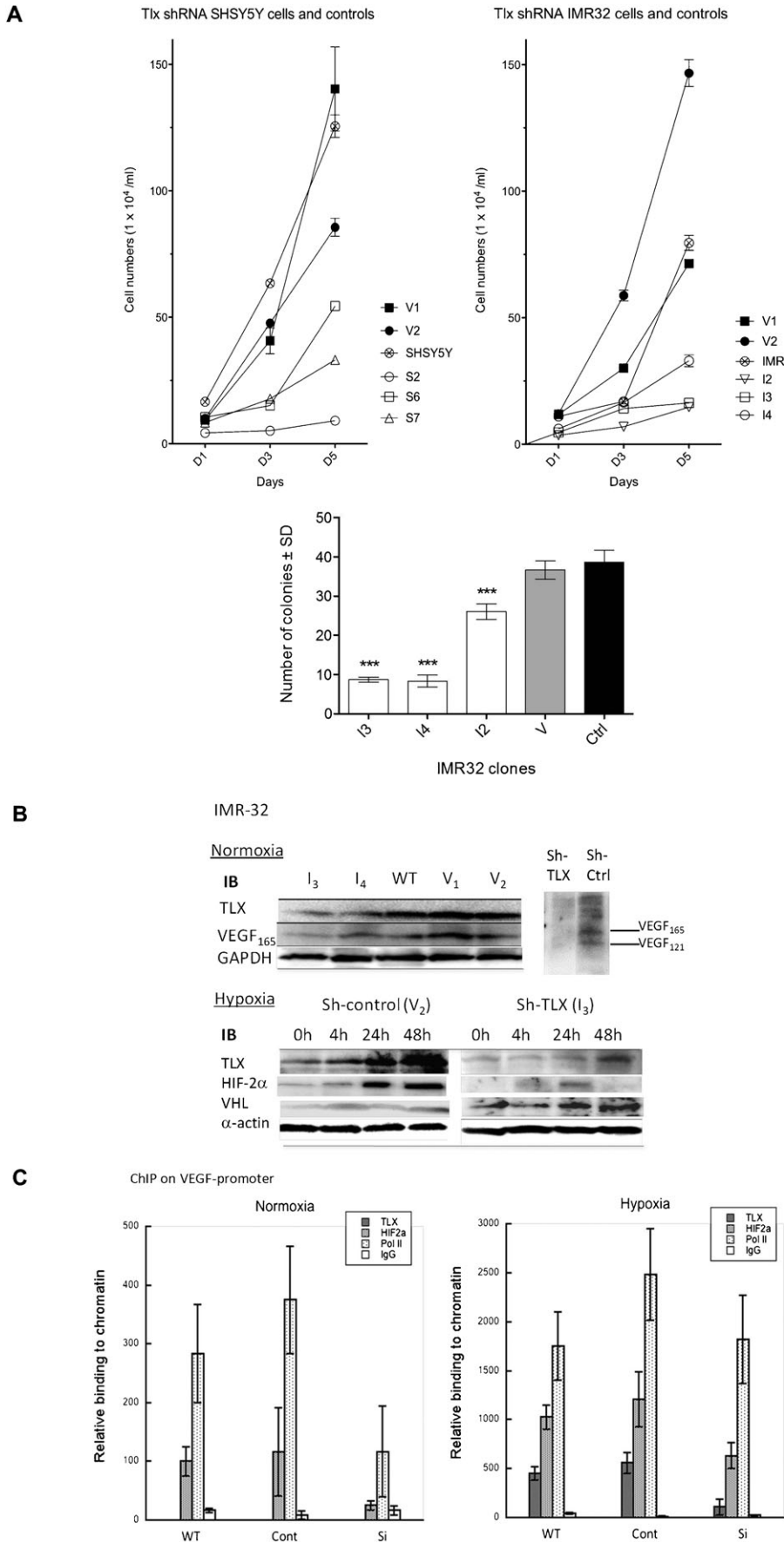
**TLX silencing suppresses cell growth, colony formation, and VEGF production of neuroblastoma cells**

In order to investigate the biological roles of endogenous TLX in neuroblastoma, we silenced TLX in IMR-32 and SH-SY5Y cells. Several stable cell lines carrying TLX shRNA were made from both cell lines (supplementary material Fig. S4A). In agreement with the role of TLX in self-renewal of neural stem cells, the



**Fig. 4. TLX activates but VHL represses the VEGF promoter.** (A) The activity of VEGF promoter-luciferase was measured after transfection of TLX, VHL<sub>30</sub>, TLX and VHL<sub>30</sub>, and control in 786-0 compared with 786-VHL (left panel) or in SH-SY5Y in normoxia compared to 24 h treatment with 200 mM of hypoxia mimetic 2,2'BP (right panel). The proximal VEGF promoter, indicating one HRE and three TLX-consensus-like sequences, is illustrated in the upper panel. The relative reporter activity is expressed with control set to 100  $\pm$  SD. For the 786 cells, overall ANOVA showed highly significant between-groups differences with overall F=7.55 (p=0.0004). For SH-SY5Y cells, overall F=59.22 (p<0.0001). Pairwise differences were analyzed with Tukey's post-hoc tests, as shown. (B) Protein expression of TLX, VHL<sub>30</sub>, VEGF, and  $\alpha$ -actin in IMR-32 and SYSH-5Y at indicated time points in hypoxia. (C) Concentrations of VEGF in supernatant of IMR-32 were analyzed by ELISA following hypoxia for 0, 4, and 24 h.

growth of silenced clones was clearly slower compared with the sh-controls (Fig. 5A). Concordant with its potent proliferation-promoting activity, decrease of TLX expression led to differentiation of both of these neuroblastoma cell lines (supplementary material Fig. S4B,C). Since IMR-32 has N-Myc amplification and a more immature phenotype than SH-SY5Y, we examined in more detail the IMR-32 shTLX clones and compared them with the wild type and vector controls. When these clones were spread on agar plates, and the numbers of formed colonies were counted after 21 days, a significant



**Fig. 5. Effects of TLX silencing on cell growth and angiogenic factors.** (A) Cell growth curves of wildtype, vector controls (V1, V2), and TLX-depleted SH-SY5Y (S2, S6, S7) and IMR-32 (I2, I3, I4) clones during 1, 3, 5 days after seeding. Bars indicate means  $\pm$  SD. Colony transformation assay of vector (V2) and wild type (Ctrl) controls, and shTLX (I2, I3, I4) clones of IMR-32 (right panel). Overall ANOVA showed highly significant differences with  $F=125.4$  ( $p < 0.0001$ ). Using Dunnett's post-hoc test, each clone was compared with the vector (V) as shown (\*). (B) Comparison of the expression of VEGF<sub>165</sub> in normoxia between shTLX clones (I3, I4), control vector clones (V1, V2), and wild type, with GAPDH as loading control (upper left panel). The expression of different isoforms of VEGF is shown in sh-TLX and sh-control (upper right panel). Expression of TLX, HIF-2 $\alpha$ , and VHL is compared by immunoblotting at indicated time points upon hypoxia. Expression of  $\alpha$ -actin is shown as a loading control (lower panel). (C) ChIP assay was performed to see proteins bound to the proximal VEGF-promoter with antibodies against TLX, HIF-2 $\alpha$ , RNA polymerase II (PolII), and non-specific IgG, using chromatin collected from IMR-32 wild type, control vector (Cont), and shTLX (I3) in normoxia (left panel) and at 24 h of hypoxia (right panel). Amounts of PCR products covering HRE and its upstream region are shown as relative values  $\pm$  SD with the binding of HIF2- $\alpha$  in normoxia as 100%.

reduction of colonies was found in shTLX clones as compared with the sh-control and wild-type cells (Fig. 5A).

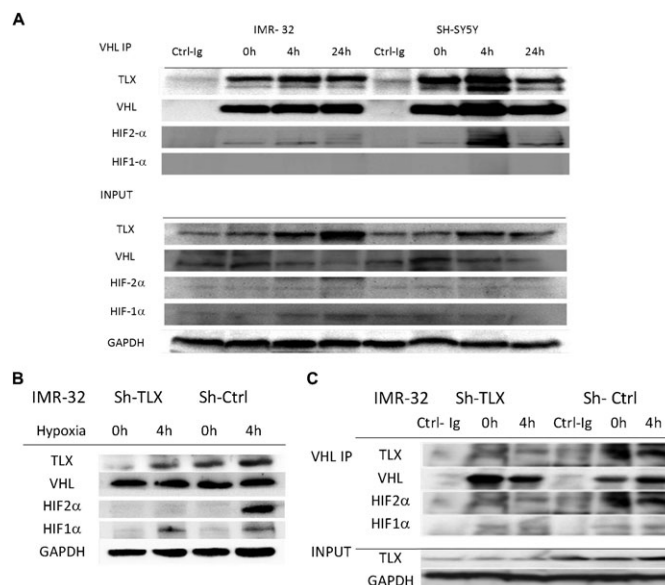
Next, we examined whether silencing of TLX affects the response of neuroblastoma to hypoxia. Silencing of TLX indeed decreased the expression of VEGF in normoxia (Fig. 5B). In hypoxia, TLX gradually increased in controls, whereas in shTLX cells it remained low until 48 h when the silencing effect was diminished. In this experiment, 3% O<sub>2</sub> was applied to prevent detachment of cells at later time points. The expression of HIF-2 $\alpha$  is markedly reduced in shTLX cells. We found that VEGFA<sub>121</sub> and VEGFA<sub>165</sub> isoforms (Koch et al., 2011) were expressed in shTLX control cells, and both isoforms were indeed diminished in shTLX. In addition, VHL was increased in shTLX cells, supporting the notion that expression of TLX correlates negatively with that of VHL but positively with HIF-2 $\alpha$ . The increase of VHL might contribute to the differentiation of TLX-silenced IMR-32 cells.

So far, we found that TLX overexpression activated the VEGF-promoter in normoxia but, in hypoxia, the effect was somewhat mild, which could be explained by the endogenous increase of TLX. In order to see whether TLX actually binds the VEGF-promoter chromatin, ChIP was performed in IMR-32-derived cell clones, wild type, sh-control, and shTLX, on the VEGF-promoter containing the HRE-consensus site (Fig. 5C). In normoxia, no TLX chromatin binding was detected in any of the cell lines, which agrees with the fact that TLX binds VHL and becomes sequestered. However, HIF-2 $\alpha$  and RNA polymerase II (PolII) bound in both wild type and sh-control, while binding was greatly diminished in the shTLX clone. This suggests that in normoxia, TLX will indirectly activate HRE-regulated promoters through stabilizing HIF-2 $\alpha$ . In hypoxia, both TLX and HIF-2 $\alpha$  bind chromatin with an apparent difference from shTLX cells. However, the decrease of PolII binding was relatively small in the shTLX cells. VHL was not detected on the promoter chromatin at any time (not shown).

#### TLX and VHL interaction increases in early hypoxia, stabilizing HIF- $\alpha$ to induce angiogenesis

Having seen the binding of both HIF-2 $\alpha$  and TLX in proximity to the HRE site of the promoter, we asked whether both TLX and HIF-2 $\alpha$  exist in endogenous complexes with pVHL. Thus, endogenous pVHL was immunoprecipitated in both IMR-32 and SH-SY5Y, and the bound proteins were evaluated at 0, 4, and 24 h in 1.7% hypoxia (Fig. 6A). In SH-SY5Y, the amount of precipitated pVHL increased to its maximum at 4 h in the hypoxic condition, whereas its expression in input protein decreased. In both cell lines, the same transient increase of TLX coimmunoprecipitated with pVHL at 4 h was seen, which might facilitate a faster stabilization of the remaining HIF- $\alpha$ . TLX expression in input protein continued to increase at 24 h, especially in IMR-32. Interestingly, HIF-2 $\alpha$ , but not HIF-1 $\alpha$ , was coimmunoprecipitated with pVHL, reaching maximum after 4 h in hypoxia and then to decrease at 24 h in SH-SY5Y. However, much lesser amount of HIF-2 $\alpha$  was precipitated in IMR-32, where only faint bands were detected for input of both HIF- $\alpha$ s. We also demonstrated that endogenous TLX could reciprocally coimmunoprecipitate VHL in wild type-, shTLX-, and the sh-control-IMR-32 cells (supplementary material Fig. S5).

We next examined the expression of TLX, VHL, HIF-1 $\alpha$ , HIF-2 $\alpha$  in shTLX, sh-control, and wild type cells in normoxia and after 4 h of hypoxia (Fig. 6B). As expected, shTLX cells



**Fig. 6. TLX and VHL complex in vivo.** (A) Proteins (160  $\mu$ g) coimmunoprecipitated with a VHL antibody from cell lysates of IMR-32 and SH-SY5Y taken at different time points in hypoxia (0, 4, 24 h) were blotted with the indicated antibodies. Lysates in normoxia precipitated with non-specific IgG were used as controls. Expression of each input protein is shown by immunoblotting, with GAPDH as an input control. (B) Cell lysates of IMR-32 (shTLX, sh-control) in normoxia and 4 h hypoxia were blotted with indicated antibodies. (C) Cell lysates as above were coimmunoprecipitated with VHL antibody and blotted with indicated antibodies in the same manner as above.

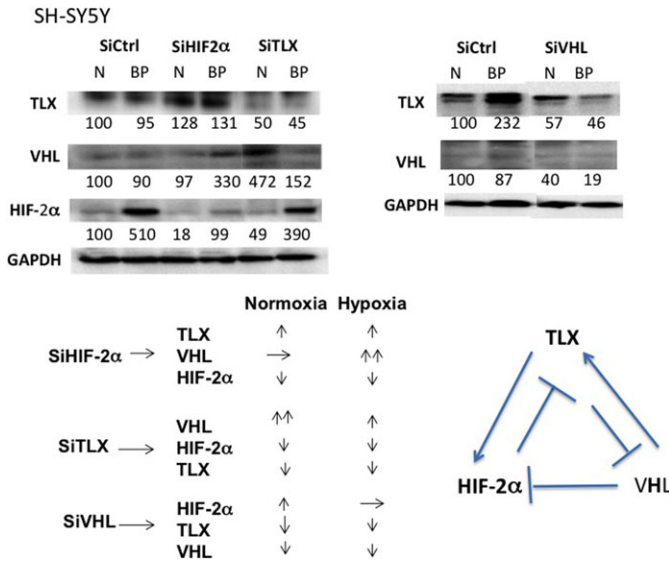
expressed hardly any HIF-2 $\alpha$  but HIF-1 $\alpha$  remained almost unaffected. We then asked whether the decreased expression of TLX affects the amounts of HIF- $\alpha$ s in the VHL complex in normoxic and hypoxic conditions (Fig. 6C). Binding of HIF-1 $\alpha$  was unaltered in IMR-32 cells in both conditions, but HIF-2 $\alpha$  was clearly decreased in shTLX cells in both normoxia and hypoxia, when compared with the sh-controls.

#### Silencing of HIF-2 $\alpha$ and VHL affect the expression of TLX, HIF-2 $\alpha$ , and VHL

In order to see whether decreased HIF-2 $\alpha$  or VHL expression will affect TLX expression, we used siRNA oligos for HIF-2 $\alpha$ , VHL, and TLX in normoxic and hypoxic conditions (Fig. 7). Since hypoxia affected the attachment of transiently siRNA-transfected IMR-32 cells, we used 2.2'BP to mimic hypoxic condition in SH-SY5Y. HIF-2 $\alpha$  depletion led to a slight up-regulation of TLX in both conditions and an increased VHL level only in hypoxia. Depletion of VHL resulted in decreased TLX levels, as expected. In concordance with Fig. 5B, TLX depletion increased VHL in normoxia and slightly so in hypoxia, but decreased HIF-2 $\alpha$  in both normoxic and hypoxic conditions.

#### Discussion

Hypoxia is a well-recognized mechanism contributing to tumor progression both through stimulation of blood vessels and by direct effects on tumor growth and invasion. We demonstrate herein that neuroblastoma cells express enhanced levels of TLX protein, which further increase in hypoxia to maintain cancer stem cells. In our search for a mechanism behind the angiogenic activity of TLX, we identified a physical



**Fig. 7. Effects of silencing HIF-2α and VHL.** Silencing HIF-2α, VHL and TLX was performed by siRNA oligonucleotides. Effects on the expression levels of TLX, HIF-2α, or VHL in normoxia and hypoxia were examined by immunoblotting. Hypoxia was created by addition of 2.2'BP for 8 h at 24 h after transfection, and immunoblotting data was normalized by GAPDH. Si-controls at normoxia were set to 100, and comparisons were done with values of si-controls, in normoxia and hypoxia, respectively (upper panel). Schematic illustration of the stimulatory and inhibitory relationships between TLX, HIF-2α, and VHL are depicted (lower panel).

interaction of TLX with pVHL, leading to stabilization of TLX and HIF-2α. In normoxia, TLX is able to stabilize HIF-α by sequestering pVHL, which might be a reason for the increased HIF-2α often seen in stem cells of advanced tumors (Qing and Simon, 2009), such as glioma (Li et al., 2009) and neuroblastoma (Holmquist-Mengelbier et al., 2006). HIF-2α has also been suggested to be more important than HIF-1α for the development of renal tumors (Covello et al., 2005; Kondo et al., 2002). Depletion of HIF-2α in VHL-null renal carcinoma was shown to efficiently suppress tumor-forming capacity in vivo. Furthermore, HIF-1α and HIF-2α possess different roles in hypoxic gene control (Qing and Simon, 2009). In concordance with this, the expression kinetics of TLX resembles more that of HIF-2α than HIF-1α. pVHL binds to a larger amount of TLX in early hypoxia than in normoxia, which may be a mechanism for a rapid stabilization of a partially hydroxylated pool of HIF-α. Although TLX and HIF-2α do not directly bind to each other, both are stabilized in the nucleus during hypoxia and bind the VEGF promoter, recruiting RNA polymerase II. Indeed, a similar mechanism has been proposed for coactivation between the orphan nuclear receptor HNF4 and HIF-αβ recruiting p300 on the erythropoietin gene (Bunn et al., 1998). TLX would thus employ somewhat different mechanisms in promoting angiogenesis, i.e., in normoxia by sequestering pVHL and in hypoxia by acting as a transcription factor binding the VEGF-promoter.

Several mechanisms have been proposed to explain how HIF-α becomes stabilized in normoxia or mild hypoxia in solid tumors, creating a hypoxic microenvironment. In aggressive neuroblastoma, rapid proliferation of TICs may render local hypoxia. Since dividing cells in the IMR-32 tumorspheres express TLX, HIF-2α can be stabilized. Furthermore, TLX was shown to induce Wnt7α (Qu et al., 2010) that inhibits GSK3, which may also

lead to HIF-α stabilization in early hypoxia (Mottet et al., 2003). In normoxia and early hypoxia, TLX occupies the HIF-binding sites of pVHL, resulting in stabilization of HIF-α, which concurs with the reduction of HIF-α by depletion of TLX. Moreover, the pVHL-bound TLX appears to be more stable, leading to further stabilization of HIF-2α. Interestingly, HIF-2α, but not HIF-1α, was coimmunoprecipitated with pVHL, which agrees with the finding that HIF-2α may sustain for a longer time than HIF-1α upon hypoxia in neuroblastoma (Holmquist-Mengelbier et al., 2006).

Recent reports demonstrate that pVHL can be SUMOylated at K171 and becomes stabilized to shuttles into nuclei, which might have otherwise been ubiquitinated and degraded (Cai and Robertson, 2010). Moreover, the SUMOylation of pVHL increases in early hypoxia (<6 h) and inactivates the ubiquitin ligase and other tumor suppressor functions of VHL (Cai et al., 2010). In fact, the binding of TLX to pVHL was slightly increased at 4 h in SH-SY5Y cells, indicating that pVHL might be stabilized upon hypoxia. The exact relationship between TLX and the modified pVHL during the course of hypoxia remains to be elucidated. Hypoxia might lead inactivation of pVHL suppressor functions, stabilizing TLX to stimulate proliferation of neuroblastoma TICs.

Silencing of HIF-2α or VHL by siRNA affects the expression levels of TLX, HIF-2α, and VHL in normoxic and hypoxic conditions. We thus confirmed that TLX regulates expression levels of HIF-2α, and pVHL regulates those of TLX. However, silencing of HIF-2α slightly upregulated TLX, suggesting that TLX could prolong a hypoxic environment when HIF-2α decreased. The exact mechanism behind the effect could not be completely identified in this study. However, the fact that the reduction of pVHL or HIF-2α rapidly affected the levels of other two proteins might suggest that there are intimate relationships between these three proteins. Furthermore, the observed differentiation of neuroblastoma by TLX depletion (supplementary material Fig. 4B,C) might also be due to an increase of pVHL, in agreement with a report where VHL overexpression induced neuronal differentiation of neuroblastoma cells (Murata et al., 2002).

Taken together, the relationship between TLX and pVHL changes according to the degree of oxygenation. In normoxia, their effects might rather be directed towards counterbalancing proliferation and differentiation. In hypoxia, TLX increases to stimulate angiogenesis, but when blood vessels are fully developed and the oxygen level increases, TLX becomes downregulated, which may suppress angiogenesis and differentiate neural stem cells. In this way, TLX may act as a switch molecule for angiogenesis, sustaining the homeostasis in the body.

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### Competing Interests

The authors declare that there are no competing interests.

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