Norrin mediates tumor-promoting and -suppressive effects in glioblastoma via Notch and WNT

Ahmed El-Sehemy, …, Peter Dirks, Valerie A. Wallace

*J Clin Invest*. 2020. [https://doi.org/10.1172/JCI128994](https://doi.org/10.1172/JCI128994).

**Research**  
**In-Press Preview**  
**Oncology**  
**Stem cells**

**Graphical abstract**

![Graphical abstract](image-url)
Norrin mediates tumor-promoting and -suppressive effects in glioblastoma via Notch and WNT.

Ahmed El-Sehemy\textsuperscript{1,2,3}, Hayden Selvadurai\textsuperscript{4,5}, Arturo Ortin-Martinez\textsuperscript{1,2,3}, Neno Pokrajac\textsuperscript{1,2,3}, Yasin Mamatjan\textsuperscript{7}, Nobuhiko Tachibana\textsuperscript{1,2,3}, Katherine Rowland\textsuperscript{4,5}, Lilian Lee\textsuperscript{4,5}, Nicole Park\textsuperscript{4,5}, Kenneth Aldape\textsuperscript{7,8}, Peter Dirks\textsuperscript{4,5,6}, Valerie A. Wallace\textsuperscript{1,2,3}

\textsuperscript{1}Department of Laboratory Medicine and Pathobiology, University of Toronto, Canada

\textsuperscript{2}Donald K. Johnson Eye Institute, Krembil Research Institute, University Health Network, Toronto. Canada

\textsuperscript{3}Department of Ophthalmology and Vision Sciences, University of Toronto

\textsuperscript{4}Developmental and Stem Cell Biology Program, and Arthur and Sonia Labatt Brain Tumour Research Centre, The Hospital for Sick Children, Toronto, Canada.

\textsuperscript{5}Department of Molecular Genetics, University of Toronto

\textsuperscript{6}Division of Neurosurgery, The Hospital for Sick Children.

\textsuperscript{7}MacFeeters Hamilton Centre for Neuro-Oncology Research, Princess Margaret Cancer Centre, Toronto, Canada

\textsuperscript{8}Laboratory of Pathology, National Cancer Institute, Bethesda, MD, USA

Address for correspondence (Lead contact)
Valerie A. Wallace, PhD
KRI, 60 Leonard St., Toronto, ON, M5T 0S8
Phone: 416-603-5800 x 7378
valerie.wallace@uhnresearch.ca

Declaration of Interests

The authors declare no potential conflicts of interest.
Abstract

Glioblastoma (GBM) contains a subpopulation of cells, GBM stem cells (GSCs), that maintain the bulk tumor and represent a key therapeutic target. Norrin is a Wnt ligand that binds the Frizzled4 (FZD4) receptor to activate canonical Wnt signaling. While Norrin, encoded by NDP, has a well-described role in vascular development, its function in human tumorigenesis is largely unexplored. Here, we show that NDP expression is enriched in neurological cancers, including GBM, and its levels positively correlated with survival in a GBM subtype defined by low expression of ASCL1, a proneural factor. We investigated the function of Norrin and FZD4 in GSCs and found that it mediated opposing tumor-promoting and -suppressive effects on ASCL1lo and ASCL1hi GSCs. Consistent with a potential tumor suppressive effect of Norrin suggested by the tumour outcome data, we found that Norrin signaling through FZD4 inhibited growth in ASCL1lo GSCs. In contrast, in ASCL1hi GSCs Norrin promoted Notch signaling, independently of WNT, to promote tumor progression. Forced ASCL1 expression reversed the tumor suppressive effects of Norrin in ASCL1lo GSCs. Our results identify Norrin as a modulator of human brain cancer progression and reveal an unanticipated Notch mediated function of Norrin in regulating cancer stem cell biology.

Statement of Significance

This study identifies an unanticipated role of Norrin in human brain cancer progression. In addition, we provide pre-clinical evidence suggesting Norrin and canonical Wnt signaling as potential therapeutic targets for glioblastoma subtype-restricted cancer stem cells.
Introduction

Glioblastoma (GBM) is the most common malignant brain cancer in adults [1, 2]. Consistent with its progressive nature, GBM remains significantly refractory to current therapeutic strategies [3, 4]. Temozolomide (TMZ) is the main chemotherapeutic agent for the management of GBM; however, it has been shown to be beneficial in a small subset of patients and usually only induces transient effects [5-7]. Despite the urgent need for new treatment approaches for GBM, developing such therapies is challenging due to the complex biology of GBM, the difficulty in delivering drugs across the blood brain barrier, and the remarkable heterogeneity of GBM tumors [8-10].

GBMs contain a subpopulation of cells with neural stem cell-like properties, GBM stem cells (GSCs) [11-14] that can propagate tumors and are thought to be the source of tumor recurrence and treatment resistance [15, 16]. Thus, targeting the GSC population presents a potentially effective therapeutic approach to overcome the problems facing traditional GBM therapies [17, 18]. To this end, a better understanding of GSC biology is required. The Wingless (Wnt) signaling pathway is a major regulator of stem cell growth in normal and tumorigenic contexts [19-21] and several Wnt pathway components are reported to regulate GBM progression through effects on GSCs [22]. Surprisingly, both inhibition and activation of Wnt signaling have been proposed as therapeutic strategies in GBM [22-26]. While this discrepancy can be attributed in part to different culture systems, models and experimental conditions used in the different studies, these variable results also highlight the complexity of the Wnt pathway and GBM biology.

Norrin, the protein product of Norrie disease protein (NDP) gene, is an atypical Wnt ligand that binds Frizzled receptor 4 (FZD4) and Low-density lipoprotein receptor-related protein 5 (LRP5) receptor complex in the presence of Tetraspanin 12 (TSPAN12) to activate canonical (B-catenin-dependent) Wnt signaling [27-29]. Activation of the Norrin/FZD4 signaling axis in endothelial
cells has a well-described role in regulating angiogenesis and blood brain barrier formation in the cortex, cerebellum, retina and inner ear [30-33]. Paracrine Norrin/FZD4 signaling extends to tumorigenesis, as activation of this pathway in the endothelium has recently been shown to inhibit medulloblastoma initiation in mice [34]. More recently, Norrin has been implicated in astroglial regulation of neuronal function in the cortex, pointing to a role for Norrin beyond the vasculature [35]. However, direct evidence for a functional role for Norrin/FZD4 in human brain tumor progression is lacking.

In this study, we show that NDP is widely expressed in a range of neurological and non-neurological cancers, and its expression level correlates with patient survival in neurological cancers. Our in vitro and in vivo analyses using human fetal neural stem cells and primary patient-derived GSCs reveals an interesting and endothelial cell-independent role for NDP in regulating GSC proliferation, cell cycle progression and tumorgenicity. Interestingly, our data show that NDP function and the growth modulatory effects of canonical Wnt signaling stratify based on GBM molecular subtype as defined by ASCL1 expression level, highlighting the importance of targeted therapy informed by molecular subtyping of tumor cells. In addition, we reveal a previously unexplored aspect of Norrin signaling, which is mediated through Notch, to maintain stemness of GSCs.

Results

**NDP expression is enriched in GBM and correlates with survival in neurological cancers**

To survey the distribution of NDP expression in human tissues, we queried the human protein atlas (HPA, www.proteinatlas.org) [36], and found that NDP expression, but not that of its receptor
**FZD4**, is enriched in several tissues, including the brain and cerebellum (Figure S1A). A similar survey of cancer cell lines (CCLE, https://portals.broadinstitute.org/ccle) [37] and primary tumors (the cancer genome atlas (TCGA)), shows that **NDP** is expressed in a variety of tumor types and is highly enriched in glioma cell lines (Figure S1B-boxed) and primary human gliomas, including low grade glioma (LGG) and GBM (Figure 1A-boxed). In addition, Gene Set Enrichment Analysis (GSEA) on GBM showed that **NDP** expression levels significantly correlate with classical GBM and aging brain gene sets (Figure 2A). **FZD4** is also expressed in different cancer types; however, its expression in GBM is comparable with other cancers (Figure S1A, S1B, 1B) and not as highly enriched as **NDP**. The discordance between **NDP** and **FZD4** expression in brain tumors could indicate that NDP, but not FZD4, levels are functionally limiting or that NDP has FZD4-independent. Consistent with the latter possibility, FZD4-independent and non-vascular functions of NDP have been reported in other contexts [38-41].

Next, we found that **NDP** expression correlates with survival in GBM, neuroblastoma, and brain astrocytoma (low grade glioma, LGG) (Figure 2B). Since transcriptome data is derived from whole tissue and tumor samples, it does not resolve the cell type specificity of gene expression, including expression within the tumor stem cell compartment. Therefore, we analyzed gene expression in human fetal neural stem cells (NSCs) and primary patient-derived GSCs, which were maintained in vitro using an established GSC culture protocol [42]. Quantitative real time qRT-PCR revealed that **NDP**, **FZD4**, **LRP5** and **TSPAN12** are expressed in hNSC and in the majority of the GSC lines we surveyed (Figure 2C). The enrichment of **NDP** expression in brain tumors, expression of Norrin/FZD4 signaling components in primary GSCs and the association between **NDP** expression level and survival in GBM suggest the possibility that **NDP** has a function in normal and transformed NSCs.
NDP function stratifies with ASCL1 expression levels

To investigate the role of NDP and FZD4 in growth and clonogenicity of non-transformed hNSC and GSCs we generated lentiviral constructs to express gene-specific short hairpin RNA oligonucleotides (2/gene) for knockdown, or full length cDNAs for overexpression. In these and subsequent experiments we confirmed the efficiency of knockdown and overexpression by qRT-PCR and western blotting, respectively (Figure S2). NDP and FZD4 knockdown in two hNSC lines significantly inhibited growth, whereas overexpression of these genes had the opposite effect, indicating that activation of the Norrin/FZD4 signaling pathway is growth promoting in hNSCs (Figure S2) and could play a direct physiological role in NSC growth that is independent of its function in endothelial cells.

The function of developmental signaling pathways, such as Notch and canonical Wnt, on GBM growth have been shown to differ based on ASCL1 expression levels [43, 44]. ASCL1 is a basic-helix-loop-helix (bHLH) transcription factor that is critically involved in regulating neuronal differentiation [45-47]. ASCL1\textsuperscript{hi} versus ASCL1\textsuperscript{lo} GBM exhibit different differentiation and invasion dynamics, and ASCL1\textsuperscript{hi} GBM requires Notch signaling for maintaining the GSC pool [44]. Based on these observations we compared the effects of NDP/FZD4 gain and loss of function on growth and sphere formation in two ASCL1\textsuperscript{lo} (G411, G564) and two ASCL1\textsuperscript{hi} GSC lines (G523, G472) [44] (Figure 3). ASCL1 expression status was confirmed in all GSC lines using qRT-PCR (Figure S3A) and the canonical Wnt-inducing capacity of NDP transgenes was confirmed using the TOP-Flash luciferase reporter assay in HEK293T cells (Figure S3B). The efficiencies of knockdown and overexpression in all lines were confirmed by overexpression by qRT-PCR and western blotting, respectively (Figure S3C). NDP and FZD4 knockdown in both ASCL1\textsuperscript{lo} GSC lines increased growth (Figure 3A) and sphere formation (Figure 3B), whereas overexpression had
the opposite effects (Figure 3C, D). These observations indicate that \textit{NDP/FZD4} expression suppresses growth and self-renewal in ASCL1\textsubscript{lo} GSCs.

In contrast, in both ASCL1\textsuperscript{hi} GSC lines, \textit{NDP} knockdown resulted in a striking inhibition of proliferation and sphere formation (Figure 3A, B). Notably, ASCL1\textsuperscript{hi} GSCs with \textit{NDP} knockdown appear to have a cell autonomous growth disadvantage relative to controls, as they are depleted in spheres (Figure S3H). To validate the effects of \textit{NDP} knockdown on the growth of ASCL1\textsuperscript{hi} GSCs, we designed a degenerate codon modified \textit{NDP} construct (\textit{MOD-NDP}) and confirmed that this version could rescue the growth inhibitory effect of short hairpin \textit{NDP} knockdown in ASCL1\textsuperscript{hi} GSCs (Figure S4). Interestingly, manipulating \textit{FZD4} in either ASCL1\textsuperscript{hi} GSC line failed to produce a significant phenotype. \textit{FZD4} knockdown with one of the two shFZD4 constructs (shFZD4-2) had a modest effect on proliferation, but not on sphere formation, in one of the ASCL1\textsuperscript{hi} GSC lines (G523) (Figure 3A, B), but this effect was not reproducible with the other shFZD4 construct (shFZD4-4) (Figure 3A, B) and was not observed in the other ASCL1\textsuperscript{hi} GSC line (Figure 3A, B). Moreover, \textit{FZD4} overexpression had no effect on proliferation or sphere formation in either ASCL1\textsuperscript{hi} GSC line (Figure 3C, D). In summary, these experiments in patient-derived GSCs (Summarized in Table S1), indicate that the function of Norrin stratifies with ASCL1 expression level. In ASCL1\textsubscript{lo} GSCs, Norrin/FZD4 is growth suppressing and in ASCL1\textsuperscript{hi} GSCs, Norrin is growth promoting and independent of FZD4.

Based on our in vitro observations we asked whether the correlation between \textit{NDP} expression and survival was associated with \textit{ASCL1} expression levels in GBM tumors. Therefore, we stratified GBM patient data on the basis of \textit{ASCL1} expression and then performed a new Kaplan-Meier survival analysis. Strikingly, we found that the survival advantage of \textit{NDP} expression stratified with tumors that had low levels of \textit{ASCL1} expression, while tumors with high \textit{ASCL1} levels did not show any correlation between \textit{NDP} expression level and survival outcomes (Figure 3E). Thus, the
survival advantage of ASCL1\textsuperscript{lo} GBM with higher \textit{NDP} expression is consistent with the growth inhibitory effect of \textit{NDP/FZD4} as we observed in ASCL1\textsuperscript{lo} GSCs. To further validate this conclusion, we stratified GBM patient samples based on both \textit{NDP} and \textit{ASCL1} expression levels simultaneously (Figure 3E). Interestingly, there was no survival advantage of patients with \textit{NDP/ASCL1}\textsuperscript{-}high versus \textit{NDP/ASCL1}\textsuperscript{-}low GBMs. This observation is consistent with our in vitro evidence (Figure 3A-D) that low \textit{NDP} expression in ASCL1\textsuperscript{lo} GSCs induces similar effects on tumor progression (growth promoting) to high \textit{NDP} expression in ASCL1\textsuperscript{hi} GSCs (Figure 3E). We also stratified patient samples based on combined \textit{NDP} and \textit{FZD4} expression then applied the same survival analysis (Figure 3E). Similarly, the addition of \textit{FZD4} expression resulted in compromising the survival advantage of \textit{NDP} expression alone (Figure 2B, 3E), supporting the uncoupled functions of \textit{NDP} and \textit{FZD4}, at least in a subset of the GSC cells.

\textit{NDP affects proliferation index and rate in GSCs}

To understand the cell biological basis for the effects of \textit{NDP} on GSC growth we examined the expression of proliferation, stemness and cell death markers in the cultures after \textit{NDP} knockdown. We first determined the proliferation index (e.g. the frequency of cycling progenitors) in the cultures by quantifying the proportion of Ki67\textsuperscript{+} cells. In parallel with our in vitro observations, we found that the proliferation index was increased in ASCL1\textsuperscript{lo} GSCs (Figure 4A, C), and reduced in ASCL1\textsuperscript{hi} GSCs (Figure 4B, D) after \textit{NDP} knockdown. Thus, in both GSC types \textit{NDP} affects growth by regulating the maintenance of cycling cells. To validate our results, we overexpressed \textit{NDP} in two ASCL1\textsuperscript{lo} GSC lines (G411 and G564), which resulted in significant reduction of the Ki67\textsuperscript{+} population (Figure S5). Consistent with a role for NDP in progenitor maintenance, we found that \textit{NDP} knockdown reduced the proportion of ASCL1\textsuperscript{hi} GSCs that express SOX2, a stemness marker (Figure 4B, D).
The proliferation index only provides information about the relative proliferative status of a mixed cell population but does not indicate how fast or slow the cells are cycling [48]. Therefore, to investigate the effect of NDP on proliferation rate we quantified the frequency of progenitors in S-phase. Control and NDP knockdown GSC cultures were exposed to a short pulse of EdU, a thymidine analogue that is incorporated into the DNA of cells in S-phase and the proliferation rate was determined by quantifying the proportion of EdU+/Ki67+ cells. Interestingly, the proliferation rate was significantly reduced in both ASCL1lo (Figure S6A) and ASCL1hi GSCs (Figure S6B) following NDP knockdown. Finally, we also assessed the effects of NDP knockdown on apoptosis by staining for cleaved Caspase-3 (Casp-3) (Figure S7). NDP knockdown resulted in a significant increase in the frequency of cleaved Casp-3+ cells in ASCL1hi (Figure S7B) but not ASCL1lo (Figure S7A) GSCs. Taken together, the growth and cell cycle analyses indicate that in ASCL1hi GSCs NDP expression stimulates proliferation by sustaining the progenitor pool and by promoting cell cycle progression and in the case of ASCL1hi GSCs, through effects on cell survival. In contrast, in ASCL1lo GSCs NDP affects growth via independent, but competing, mechanisms: it promotes cell cycle progression but reduces the cycling progenitor pool, with the later having the dominant effect on growth of the population.

**NDP regulates common and divergent transcriptional programs in ASCL1lo and ASCL1hi GSCs**

To identify the downstream transcription profiles that mediate the proliferative effects of NDP in GSCs we performed RNA-Seq analysis on one ASCL1lo (G411) and one ASCL1hi GSC line (G523), after NDP knockdown with two different shRNA constructs (Figure 5, S8). After filtering the data for gene expression changes common to both short hairpins (adjust FDR and p values < 0.05) we found that NDP knockdown resulted in overlapping and unique sets of altered transcripts
between ASCL1\textsuperscript{lo} and ASCL1\textsuperscript{hi} GSCs (Figure 5A, S8C-E, Table S2-S4), indicating that \textit{NDP} regulates common and unique downstream targets in both types of GSC subtypes. Interestingly, GSEA performed using the same strict criteria revealed a similar pattern of overlapping gene sets between both GSC subtypes, however, there were unique gene sets only in ASCL1\textsuperscript{lo} GSCs (Figure 5B, C, S8F, Table S5). The majority of the overlapping differentially expressed genes and enriched gene sets were related to cell cycle and cell division and included downregulation of cell cycle regulators including, Cyclins A2, G1, E2, B1, and B2 (Figure S8C, Table S5, S6). We validated the downregulation of cell cycle regulators following \textit{NDP} knockdown in both GSC subtypes by western blot analysis for CCNE2, CCNA2 (Figure 5D). Based on these data, we suggest that NDP regulates the expression of a cell cycle progression program common to ASCL1\textsuperscript{lo} and ASCL1\textsuperscript{hi} GSCs, which is consistent with the observation that \textit{NDP} knockdown in both types of GSCs has similar effects on cell cycle progression (Figure S6). GSEA results also support a model where \textit{NDP} regulates a second, independent gene expression program in ASCL1\textsuperscript{lo} GSCs. For example, while all of the significantly enriched gene sets in ASCL1\textsuperscript{hi} GSCs after \textit{NDP} knockdown overlapped entirely with the enriched genes sets in ASCL1\textsuperscript{lo} GSCs, there were additional enriched gene sets exclusive to ASCL1\textsuperscript{lo} GSCs after \textit{NDP} knockdown (Figure 5B, C, S8F).

Of the unique ASCL1\textsuperscript{lo} GSC RNA-Seq hits, many were related to migration, invasion, metastasis, EMT and extracellular matrix modulation (Figure S8D, Table S2). Similarly, unique ASCL1\textsuperscript{lo} GSC gene sets included cell movement and cytoskeletal organization (Figure 5B, S8F, Table S5). In contrast, the unique hits in ASCL1\textsuperscript{hi} GSCs were primarily related to cell cycle, proliferation, differentiation and DNA repair (Figure S8E, Table S3). One potential regulator of this divergent \textit{NDP}-regulated program in ASCL1\textsuperscript{lo} GSCs is the Wnt pathway, as NDP function in this subset was FZD-dependent. Consistent with this possibility we found that expression of selected Wnt targets was significantly in the RNA-seq library changed after \textit{NDP} knockdown in ASCL1\textsuperscript{lo} GSCs, and
unchanged in ASCL1\textsuperscript{hi} GSCs (Figure 5E). We propose that the regulation of genes associated with
tumor promoting processes and the Wnt pathway support the existence of a competing mechanism
mediating NDP function in ASCL1\textsuperscript{lo} GSCs and explains the dominant growth promoting effect of
NDP knockdown in ASCL1\textsuperscript{lo} GSCs.

**Growth inhibiting effects of canonical Wnt pathway activation segregate with ASCL1 status**

We show that in ASCL1\textsuperscript{lo} GSCs the phenotypic effect of NDP and FZD4 manipulation on growth
and self-renewal are the same whereas in ASCL1\textsuperscript{hi} GSCs manipulating NDP, but not FZD4
expression, affects growth and self-renewal. Moreover, NDP knockdown is associated with altered
expression of Wnt target genes in ASCL1\textsuperscript{lo} but not ASCL1\textsuperscript{hi} GSCs. Thus, we hypothesized that in
ASCL1\textsuperscript{lo} GSCs, Norrin/FZD4-mediated growth suppressive effects require canonical Wnt
pathway activation, whereas in ASCL1\textsuperscript{hi} GSCs, Norrin-mediated effects on growth are canonical
Wnt pathway-independent. To test this hypothesis, we overexpressed NDP in ASCL1\textsuperscript{lo} and
ASCL1\textsuperscript{hi} GSCs and treated them with Wnt inhibitors. In control assays we confirmed that treatment
with a function blocking anti-FZD4 antibody [49], tankyrase inhibitor (XAV939), which
stimulates B-Catenin degradation and blocks canonical Wnt signaling downstream of FZD
receptors, and IWP2, which inhibits Wnt secretion, all inhibited Wnt3- stimulated induction of a
TOP-FLASH luciferase reporter (Figure S9). Treatment with Wnt inhibitors increased growth in
NDP-expressing and control lentivirus infected ASCL1\textsuperscript{lo} GSC (Figure 6A). These observations
are consistent with endogenous and NDP-induced growth suppressive effect of FZD4 and Wnt/B-
catenin signaling in ASCL1\textsuperscript{lo} GSCs. In contrast, inhibiting canonical Wnt signaling had no effect
on proliferation of ASCL1\textsuperscript{hi} GSCs with or without NDP overexpression, indicating that NDP
function in these cells is independent of canonical Wnt signaling (Figure 6B). To confirm these
findings, we examined the effects of Wnt agonists, recombinant human rh-WNT3a and CHIR, a
GSK3 inhibitor, on the proliferation of ASCL1\textsuperscript{lo} and ASCL1\textsuperscript{hi} GSCs. In parallel with our observations in \textit{NDP}-overexpressing GSCs, activation of the canonical Wnt pathway inhibited proliferation of ASCL1\textsuperscript{lo} GSCs (Figure 6C) but had no effect on proliferation of ASCL1\textsuperscript{hi} GSCs (Figure 6D). Additionally, we found that modulating \textit{NDP} affected the levels of active B-catenin (Figure 6E, F) and the levels of Wnt targets CDK1 and phospho-NFkB-P65 [50-53] only in ASCL1\textsuperscript{lo} GSCs (Figure 6G). Taken together, we conclude that Norrin/FZD4 signaling stimulates the canonical Wnt pathway to suppress growth in ASCL1\textsuperscript{lo} but not in ASCL1\textsuperscript{hi} GSCs.

\textbf{Norrin promotes Notch signaling and inhibit differentiation in ASCL1\textsuperscript{hi} GSCs}

To address the mechanism underlying Wnt-independent effects of Norrin on promoting ASCL1\textsuperscript{hi} GSC progression, we started by asking whether Norrin function in this context is cell autonomous. Norrin is reported to function as a short-range paracrine signal, where Norrin secreted from one cell can activate FZD4/canonical Wnt signaling in a neighboring cell [27], which we confirmed in reporter assays in HEK293 cells (Figure S10D). However, the growth disadvantage of ASCL1\textsuperscript{hi} GSCs with \textit{NDP} knockdown in spheres (Figure S3G) suggested an autocrine or juxtacrine requirement for \textit{NDP} expression, because it does not appear to be rescued by Norrin provided by neighboring wildtype cells. To investigate this possibility, we first treated cells with recombinant Norrin protein (rNorrin) and observed effects on proliferation in vitro. Interestingly, rNorrin treated replicated the effects of \textit{NDP} overexpression only in ASCL1\textsuperscript{lo} but not ASCL1\textsuperscript{hi} GSCs (Figure 7A). Next, we performed a competition assay, where we mixed equivalent numbers of ASCL1\textsuperscript{hi} GSCs infected with Lenti- shNDP-GFP or Lenti-shScrambled-GFP with cells infected with Lenti-mCherry and measured the ratio of mCherry\textsuperscript{+}: GFP\textsuperscript{+} cells over time. If Norrin functions as an autocrine signal, the ratio of mCherry\textsuperscript{+}: Norrin-deficient GFP\textsuperscript{+} cells should increase owing to a cell-autonomous growth disadvantage of cells with \textit{NDP} knockdown. Equal seeding of the
cultures at day 1 was confirmed by flow cytometric analysis (Figure S10 A, B) and then reassessed after 6 days. Interestingly, the ratio of mCherry\(^+\): GFP\(^+\) cells remained almost equivalent in cultures expressing the scrambled shorthairpin control, whereas the ratio shifted dramatically towards the mCherry\(^+\) cohort in the NDP knockdown cultures (Figure S10 A-C). Given that the two populations were intermixed, this result strongly supports an autocrine or at least juxtaparacrine function for Norrin in ASCL1\(^{hi}\) GSCs.

Notch, which functions via juxtaparacrine signaling, is considered one of the master regulators of cancer stem cells [54]. Previously, Park et. al. reported that Notch inhibition leads to differentiation in ASCL1\(^{hi}\) but not ASCL1\(^{lo}\) GSCs [44] mediated, in part, through ASCL1-dependent chromatin remodeling of differentiation genes. Interestingly, the growth disadvantage of NDP knockdown in ASCL1\(^{hi}\) but not ASCL1\(^{lo}\) GSC lines suggests effects on differentiation as a possible underlying mechanism. Given the phenotypic similarity between NDP knockdown and Notch inhibition, we assessed the impact of NDP manipulation on Notch signaling in GSCs. We found that NDP knockdown resulted in a significant downregulation of Notch signaling in ASCL1\(^{hi}\) and ASCL1\(^{lo}\) GSCs, as shown by the reduction of cleaved (activated) Notch1 and Notch targets (Figure 7B), demonstrating that Norrin is required to maintain Notch signaling. Consistent with a role for Notch function downstream of Norrin in ASCL1\(^{hi}\) cells, we found that treatment with Notch inhibitor (GSI; Gamma Secretase Inhibitor) abrogated the effect of NDP overexpression in ASCL1\(^{hi}\) but had no effect on ASCL1\(^{lo}\) GSC (Figure 7C) and that NDP knockdown promoted expression of neuronal differentiation markers in ASCL1\(^{hi}\) GSCs (Figure 8).

ASCL1 functions as a pioneer factor to promote accessibility of differentiation inducing targets in response to Notch inhibition [44], which raises the possibility that ASCL1\(^{lo}\) cells were unaffected by loss of Notch signaling after Norrin depletion because of insufficient ASCL1 activity. To test this hypothesis, we stably transduced ASCL1\(^{lo}\) (G564) cells with human *ASCL1*, then knocked
down *NDP* to see if *ASCL1* overexpression is able to reverse the effects of *NDP* knockdown. Strikingly, *ASCL1* overexpression in ASCL1<sup>lo</sup> cells resulted in a complete reversal of the effects of *NDP* knockdown on proliferation (Figure 7D) and induced a phenotype similar to ASCL1<sup>hi</sup> GSC lines (Figure 3A). Collectively, these findings provide evidence for Norrin as a modulator of Notch signaling, and show that ASCL1 levels are the reason for the opposing effects of Norrin depletion in ASCL1<sup>hi</sup> and ASCL1<sup>lo</sup> GSCs.

**NDP modulation affects tumorigenicity in xenografted ASCL1<sup>lo</sup> and ASCL1<sup>hi</sup> GSCs**

To validate whether the growth inhibitory effects of *NDP* manipulation we observed in vitro translate to the complex environment of tumour growth in vivo, we orthotopically xenografted Nod-SCID Gamma (NSG) mice with ASCL1<sup>lo</sup> or ASCL1<sup>hi</sup> GSCs with *NDP* and *FZD4* (in case of ASCL1<sup>lo</sup> GSCs only) overexpression or knockdown (Figure 9, 10). Overexpression of *NDP* or *FZD4* in ASCL1<sup>lo</sup> GSCs, which inhibit growth in vitro, significantly prolonged survival in xenografted mice (Figure 9D). The tumors that did form were reduced in GFP<sup>+</sup> cells relative to tumors with control Lenti-GFP infection (despite being grafted with cells that were over 90% GFP<sup>+</sup>), suggesting that there was a selection bias against *NDP* or *FZD4*-overexpressing cells during tumor progression (Figure 10B). *NDP* or *FZD4* knockdown in ASCL1<sup>lo</sup> GSCs, which promotes growth in vitro, did not have a significant effect on survival, likely because the strikingly rapid kinetics of tumor formation (3-4 weeks) of this GSC line would make it difficult to detect faster tumor formation (Figure 9C, 10A). *NDP* knockdown in ASCL1<sup>hi</sup> GSCs, which inhibits proliferation in vitro, significantly prolonged survival in mice grafted with ASCL1<sup>hi</sup> GSCs (Figure 9E) and overexpressing *NDP* in this GSC line, which is growth promoting in vitro, significantly shortened survival (Figure 9F). Similar to our observations with ASCL1<sup>lo</sup> xenografts, human tumors derived from ASCL1<sup>hi</sup> GSCs with *NDP* knockdown were depleted of GFP<sup>+</sup> cells, despite the
grafts containing >90% GFP+ cells at the time of transplantation (Figure 10C). This observation suggests that *NDP* knockdown in ASCL1<sup>hi</sup>GSCs results in a selective growth disadvantage during tumor progression. Combined, these data demonstrate that *NDP* manipulations in ASCL1<sup>lo</sup> and ASCL1<sup>hi</sup> GSCs strongly affect the dynamics of overall tumour progression in a complex in vivo environment and confirm our in vitro observations.

Last, we used serum-induced differentiation to examine the effects of *NDP* knockdown on differentiated GSCs. Interestingly, serum differentiation abrogated the phenotypic divergence of *NDP* knockdown between ASCL1<sup>lo</sup> and ASCL1<sup>hi</sup> GSCs and resulted in a similar phenotype in both (Figure S11), supporting the correlation between Norrin functional divergence and tumor cell stem cell biology in this context.

**Discussion**

GBM remains one of the most lethal malignancies because it is largely refractory to the current standard treatment approaches. Due to the well-defined cellular hierarchy in GBM, targeting the GSC population has been suggested as a promising treatment strategy, however, there is a critical need for a better understanding of the biological pathways that control this population and how these pathways are affected in GBM subtypes. Canonical Wnt signaling is one of the most heavily studied pathways in the context of normal and cancer stem cells, however, there is a paucity of studies characterizing the role of the atypical Wnt ligand, Norrin, in primary tumors, likely because of the historical link of this gene to congenital eye disease [30, 31, 55]. Analysis of tumor databases revealed that *NDP* expression is widespread across several tumor types and is enriched in and associated with increased survival in several neurological cancers, including GBM. Our gain and loss of function studies in primary patient-derived GSCs revealed that *NDP* has opposite effects on
GSC growth, where it is growth inhibitory in ASCL1\(^{lo}\) GSCs and growth promoting in ASCL1\(^{hi}\) GSCs. Moreover, we show that the requirement for FZD4 and canonical Wnt signaling also segregates with ASCL1 status, such that Norrin acts through FZD4 and the canonical Wnt pathway in ASCL1\(^{lo}\) GSCs but is FZD4/canonical Wnt-independent in ASCL1\(^{hi}\) GSCs. To the best of our knowledge, this is the first demonstration that Norrin exhibits context specific canonical Wnt pathway activation in human tumor stem cells.

There is extensive evidence for tumor promoting mutations in Wnt pathway components in several cancers [20, 21, 56]. While Wnt pathway genes are rarely mutated in GBM, canonical Wnt signaling is frequently deregulated in GBM, typically through repression of secreted Wnt inhibitors [43, 57] or genetic alterations that promote B-catenin activity [19, 58, 59]. The functional observations from these and other studies have led to the general conclusion that canonical Wnt signaling is tumor promoting in GBM through effects on stemness, proliferation and invasion [60]. However, Wnt activation has been reported to attenuate growth and tumor progression in primary GSCs and GBM cell lines [23, 26]. While the basis for this discrepancy is not clear, it does highlight a possible context dependent function for canonical Wnt signaling in GBM, which is consistent with previously published data from studies on untransformed NSC and neural progenitors, where Wnt can promote opposing effects on growth, stemness and differentiation [61]. By targeting endogenously expressed Wnt pathway ligands and receptors we show that there are context specific functions of canonical Wnt signaling on the growth of GBM. Our results that canonical Wnt signaling is exclusively growth suppressive in ASCL1\(^{lo}\) GBM also have important clinical significance, as we show that XAV939 (tankyrase inhibitor), a compound that is currently being considered for treatment of CNS and non-CNS tumors [62, 63], might stimulate rather than inhibits the growth of ASCL1\(^{lo}\) GSCs. However, because of the heterogeneity of ASCL1\(^{lo}\) GSCs
in terms of growth properties, further validation of our observation in a larger cohort of ASCL1\textsuperscript{lo} GSCs would be beneficial. Notably, our data does not exclude the potential therapeutic advantage of Wnt inhibition in GBM but rather highlights the critical need for considering the molecular and cellular context in targeting responsive tumors. In parallel with our observations, it was recently reported that HIF1A/Wnt signaling strongly stimulates neuronal differentiation under hypoxic conditions, further supporting the significance of cellular and molecular contexts in determining responsiveness to Wnt [64].

We also show that NDP is a potent upstream regulator of Notch signaling. NDP knockdown results in a loss of Notch activation and the growth promoting effect of NDP overexpression in ASCL1\textsuperscript{hi} GSCs requires active Notch signaling. A role for Norrin in modulating Notch signaling, adds an exciting axis to the current knowledge about the non-canonical function ascribed to Norrin in several cellular contexts [38-41]. Notch inhibition is a potent promoter of neuronal, but not astrocyte differentiation, which has therapeutic relevance, since neuronal differentiation is terminal and leads to permanent cell cycle exit [44]. Therefore, NDP knockdown might present an exciting differentiation therapy approach to target ASCL1\textsuperscript{hi} GBM. Norrin function has also been implicated in the maintenance of blood brain barrier, pathological neovascularization, neuron survival [reviewed in 65] and cortical neuron dendritic morphology [35] in the adult brain. However, developing localized and FZD4-independent approaches to Norrin targeting could help circumvent potential brain toxicity of general Norrin/FZD4 disruption.

Despite differences in the phenotypes produced by manipulating NDP in both GSC subtypes, our ICC experiments uncovered a surprising overlap in its cell biological effects. In both GSC subtypes, NDP knockdown reduced the proliferation rate, indicating a slower cell cycle, and was associated with overlapping changes in cell cycle gene expression and gene set enrichment. This phenotype in ASCL1\textsuperscript{lo} GSCs is particularly notable because the net effect of NDP knockdown is
growth promoting, suggesting the existence of other Norrin-dependent growth inhibiting pathways in this GSC subtype. This interpretation is consistent with the observation that NDP knockdown in ASCL1\textsuperscript{lo} GSCs was associated with gene expression and gene set enrichment changes related to invasion, migration, metastasis and extracellular matrix modulation that were unique to this subtype. This competition between the Wnt–dependent and independent Norrin functions result in the overall phenotype after manipulating this gene in ASCL1\textsuperscript{lo} GSC lines. The underlying basis for the effects of NDP on growth in ASCL1\textsuperscript{lo} and ASCL1\textsuperscript{hi} GSCs is mediated, at least in part, by ASCL1, because re-introducing \textit{ASCL1} expression reverses the effect of NDP knockdown on the growth of ASCL1\textsuperscript{lo} GSCs.

In this study we focused our experiments on GSCs; however, further studies are required to assess the role of NDP in maintaining the pool of tumor differentiated cells and for interactions with the stroma in particular, as Norrin/FZD4 signaling is required for establishment of the BBB in several regions of the brain [66]. In addition to these observations in GBM, our analysis of publicly available tumor databases suggests that the role of NDP might extend to several other non-CNS cancers including breast, prostate and reproductive organ tumors, which could reflect physiological roles for Norrin in these tissues, particularly in the female reproductive system where Norrin is essential for embryo implantation [67]. While our primary focus in this study was GBM stem cells, we show that the Norrin/FZD4 signaling axis also significantly controls the proliferation of primary fetal hNSC cells in vitro. It would be interesting to follow up on this observation and investigate how Norrin functions in this context. Ultimately, these observations can serve as an initiative for investigating Norrin function in regenerative medicine applications.

In summary, we uncover a previously un-defined role of NDP in regulating the progression of GBM through effects on proliferation and self-renewal of GSCs, and a striking mechanism in
which Norrin harbors tumor suppressor functions by activating Wnt signaling in GBM with low levels of ASCL1, and oncogenic functions through Wnt-independent mechanisms in the GBM with high levels of ASCL1. Additionally, we provide evidence of Norrin function in modulating Notch signaling, which has significant impacts on several cellular contexts.

**Methods**

**Computational and in silico analysis**

The Cancer Genome Atlas Data (TCGA) data is publicly available from the Genomic Data Commons (GDC) data portal (https://gdc.nci.nih.gov/). The gene expression datasets were measured using the Illumina Hiseq _RNASeqV2 and log2 transformed by the UCSC Cancer Browser team. *NDP* gene expression (Boxplot) across cancers was queried using cBioPortal – a public online database. Correlation between gene expression and survival was produced by Kaplan-Meier method using Partek Genomics Suite software (Partek, St. Louis USA) and a log-rank test was performed to calculate p values (0.05 was considered as the threshold for significance). For the Gene-set enrichment analysis (GSEA), we compared *NDP* low vs. high expression (in IDH wild type GBM) after data was normalized and differential expression was used to perform pathway analysis. Statistical significance was determined based on the following criteria: FDR < 0.05, Fold change >1.7 or < -1.7. We also used the web-based servers of the Cancer Cell Line Encyclopaedia (CCLE) [37] and the human protein atlas (HTA) [36] to assess *NDP* expression levels in different contexts.

**Primary tumor cell lines and culture**

All GBM stem cell (GSC) and human fetal neural stem cell (hNSC) lines used in this study were obtained under MTA from the laboratory of Dr. Peter Dirks, at Sickkids Hospital, Toronto,
These GSC lines were derived from primary tumors, as previously described [42], in accordance with the Research Ethics Board at The Hospital for Sick Children (Toronto, Canada). Primary-derived GSC and hNSC lines were cultured in neural expansion conditions to promote and maintain the stem cell phenotype [42]. Briefly, cells were cultured on laminin coated surfaces in Neurocult media (StemCell Technologies), supplemented with BSA (Life Technologies) and 5 ml of 200mM L- Glutamine (Wisent) per 500 ml bottle of media. This base was then supplied with in-house equivalent to N2 hormone mix, 10 ng/ml recombinant human Epidermal Growth Factor (EGF) (Sigma Aldrich), 10 ng/ml recombinant human Basic Fibroblast Growth Factor (bFGF) (StemCell Technologies), 2 μg/ml Heparin (Sigma Aldrich) and 1X B27 Supplement (Life technologies) and cells were passaged for a maximum of 20 passages to maintain the stem cell phenotype, as described in [42]. To dissociate and split cells, we used brief Accutase (Sigma Aldrich) treatment (5 mins, 37 °C). In this study we used three ASCL1<sup>hi</sup> GSC (G523, G472, G440), two ASCL1<sup>lo</sup> GSC (G411, G564) and two hNSC (hNSC-1, hNSC-3) lines. GSC lines were tested periodically for the expression of a panel of stem cell and differentiation markers, including SOX2, O4, TU-J, MAP2, GFAP and Nestin, using immunocytochemistry to ensure the maintenance of the stem cell phenotype. For lentiviral production, dual-luciferase reporter system assay, and proof of concept experiments we used a Human Embryonic Kidney cell line (HEK-293T) obtained from (ATCC).

**Recombinant DNA, plasmids and cloning**

*NDP* and *FZD4* specific shRNAs were cloned into pGFP-C-shLenti plasmid by its provider (Origene). Gene knockdown and overexpression were confirmed by qRT-PCR and western blotting, respectively. Sequences of shRNA constructs:

- **ShNDP-A:** GCACCACATGTGGATTCTAT,
- **shNDP-C:** GCACCACATGTGGATTCTAT,
shFZD4-2:  CTCAAGTGTGGCTATGATGCTGGCTTATA,
shFZD4-4:  CATCACCTCAGGCATGTGGATTGGTCTCG.

Negative control of pGFP-C-shLenti containing scrambled non-effective shRNA was obtained from Origene (Cat. #TR30021). To rescue the effects of short hairpin NDP knockdown, a degenerate codon NDP transgene (Mod-NDP) was cloned into a pLV-mCherry plasmid (Addgene) by GeneArt services (Thermo Fisher Invitrogen). pLV-mCherry was a gift from Pantelis Tsoulfas (Addgene plasmid # 36084; http://n2t.net/addgene:36084; RRID: Addgene_36084). Briefly, we cloned a version of the NDP coding sequence consisting of degenerate codons (modified NDP, Mod-NDP), which is not complementary to the shNDP targeting sequences, into a lentiviral vector that expresses mCherry as a fluorescence marker. Next, we co-transduced G523 cells with Mod-NDP and either shScrambled, shNDP-A, or shNDP-C (with GFP marker). The expression and function Norrin from Mod-NDP was confirmed by western blotting and by TOP-FLASH reporter assay.

Sequence of Mod-NDP:

```
a tgagaaaca tgtagattc gcatcttttt ctatgtctc cctgttgttg ataatgggag atacagacag taaaacggac  
agctcattca taatggactc ggacccctga cgctgcatga gacatcattc cgggtgatat atgctcatca 
agatgggtgc tcttggccag gtcggagggg cactgcagcc aggcgtcacg ctccgagcct ttggtgtcct tcagcactgt 
cctcaagcagcccagctgtca ctctctgctca ctctgtccctg ccccaagctgcagcagc ccaatcgaa ggcacgcggg cgcagatgctt 
cagggggcat gcgacactct ggtacatctt ctcgtgctac tgcgaggaat gcaattcctg 
```

Sequence of wild-type human NDP:

```
a tgagaaaca tgtagattc gcatcttttt ctatgtctc cctgtgtgtg ataatgggag atacagacag taaaacggac  
agctcattca taatggactc ggacccctga cgctgcatga gacatcattc cgggtgatat atgctcatca 
agatgggtgc tcttggccag gtcggagggg cactgcagcc aggcgtcacg ctccgagcct ttggtgtcct tcacgatgctt 
cctcaagcagcccagctgtca ctctctgctca ctctgtccctg ccccaagctgcagcagc ccaatcgaa ggcacgcggg cgcagatgctt 
```
caggggcat gcgactcact gccacctacc ggtacatcct ctcctgtcac tgcgaggaat
gca attcctg

For ectopic NDP and FZD4 overexpression experiments, we used TrueORF cDNA clones from Origene. pLenti-C-mGFP was used for NDP while pLenti-C-MYC-DDK was used for FZD4.

To overexpress hASCL1, the cDNA was PCR amplified from pTight-hAscl1-N174 (Addgene #31876; a gift from Jerry Carbtree) then cloned into pLenti-CAG-P2A-GFP plasmid vector (modified from pLenti-CAG-IRES-GFP; Addgene #69047; a gift from William Kaelin).

**Preparation of lentiviral particles and infection**

We used a 3\textsuperscript{rd} generation lentiviral transduction system to knockdown or overexpress genes of interest. To produce virus particles, HEK293T cells were cultured in 15 cm dishes (BD Falcon) and allowed to adhere for 24 hours. The following day, cells were co-transfected with the lentiviral expression vector in combination with plasmids expressing virus coat and assembly proteins (REV, RRE, and VSVG) using Lipofectamin 3000 reagent (Life technologies). Conditioned media containing virus particles was collected 24 and 48 hours after transfection. Virus conditioned medium was passed through 0.45 μm low protein binding membranes (Sarstedt) and then concentrated by ultracentrifugation 22000 g for 2 hours. Virus pellets were then reconstituted in PBS. To assess virus titer, we infected a series of HEK293T culture vessels with 1 μL of a dilution series of the reconstituted virus pellet (1X, 1/10X, 1/10\textsuperscript{2}X, 1/10\textsuperscript{3}X etc.). 48 hours after the infection cells were analyzed using fluorescence microscopy and infectious particle titer was assessed by manual counting of cells expressing the fluorescent protein reporter. For lentiviral plasmids that do not express fluorescence proteins, we used qRT-PCR using LentiX qRT-PCR kit (Clontech, Takara).

**In vitro cell proliferation assay**

Cells were counted and seeded in coated 24 well plates at a density of 20,000 cells/well for slower
growing GSC lines, and 10,000 cells/well for faster growing GSC lines. Cells were quantified at two time points (3 and 6 days), or after 6 days for more complex experiments. To quantify absolute cell numbers, cells were incubated with Accutase for 5 minutes at 37 °C then live cells were counted using a hemocytometer slide after addition of Trypan Blue exclusion dye (Sigma Aldrich). Cell numbers were normalized to the seeding density of the first day in culture to assess percentage of cell proliferation. At least 3- 4 technical replicate wells were seeded for each sample.

**In vitro extreme limiting dilution assay (ELDA)**

To assess sphere formation ability, we used the in vitro extreme limiting dilution assay (ELDA), as previously described [68]. Briefly, cells were dissociated using Accutase and thorough pipetting to ensure the formation of single cell suspension. After counting, cells were seeded in suspension culture 96- well plate (Sarstedt) at a density of 4000 cells/well, with a minimum of 3-4 replicates per sample. Peripheral rows and columns of the plate were filled with PBS and not included in the experiment to reduce variability associated with plate position effects. Cells in the first column (4000 cells/well) were then serially diluted from one column to the next until cell density reached 4 cells/well in the last column. Plates were incubated for two weeks at 37 °C, and sphere forming wells were scored at these time points, as per the protocol instructions. Scoring results are then analyzed using ELDA software (http://bioinf.wehi.edu.au/software/elda/) from Walter+Eliza Hall Institute for Medical Research (WEHI) according to the developer instructions. Secondary sphere assays were performed as a proof of concept to confirm our results.

**Cell and tissue immunostaining and microscopy**

Cells were seeded at least 24 hours prior to the staining procedure on 8-well chamber slides (BD Falcon) coated with PLO and Laminin, as described above. Once the cells reached the appropriate
confluency and/or intended time point, the culture media was removed and cells were washed briefly with PBS. Cells were fixed by incubation with electron microscopy grade 4% Paraformaldehyde (PFA) (Bio-Rad) for 10 minutes, permeabilized in 0.1% Triton-X in PBS (PBST) for 5 minutes and blocked in 5% BSA in PBST all at room temperature. Cells were incubated overnight at 4 °C with primary antibodies diluted in blocking solution. After washing 3 times with PBS (5 minutes each), cells were incubated for 1 hour at room temperature with secondary antibodies and DAPI (nuclear stain) diluted in blocking solution, followed by 3 washes with PBS (5 minutes each), mounted in mounting medium (DAKO) and stored at 4 °C. Cells were imaged at 20X magnification on a Zeiss M2 epifluorescence microscope with apotome or by confocal microscopy unless otherwise indicated. Zeiss software was then used to analyze the images, which were then quantified manually. Cells were pulsed with 10 μM EdU for 3 hours prior to harvesting and signal developed with the Click-iT kit (Life Technologies), according to manufacturer’s instructions. For immunostaining in tissue sections, animals were perfused transcardially. Briefly, animals were injected with 10 ml PBS in the heart to clear the circulatory system, followed by 10 ml 4% PFA injection. Then, the brain was dissected and fixed in 4% PFA overnight at 4°C. Tissues were washed in PBS and cryoprotected in 30% sucrose/PBS overnight at 4°C. After cryoprotection, tissues were equilibrated in a 50:50 mixture of Optimal cutting temperature compound (OCT compound):30% sucrose, embedded into plastic molds and snap-frozen in liquid nitrogen. Brain sections were taken at 16 μm in the coronal plane using a Leica CM1850 cyrostat, air dried (1-2 hr) on Superfrost Plus positively charged slides (Fisher Scientific) and stored with desiccant at −20°C. Slides were washed in PBS and permeabilized in 0.1% Triton X-100 in PBS followed by blocking with 10% donkey serum (Sigma Aldrich) in PBS for 30 min at room temperature. Primary antibodies were diluted in 10% donkey serum and incubated with the slides overnight at 4°C. Next day, sections were incubated with secondary antibodies
(Molecular Probes) diluted at 1:1000 for 1 hour at room temperature, and nuclei were stained with Hoechst and a coverslip was attached with fluorescence mounting medium (Dako S3023). Fluorescent images of the tumors were captured using an LSM 780 confocal microscope (Zeiss) at 20X magnification.

**Cell competition assay**

Cells were divided into two groups; one was infected with a lentivirus expressing mCherry only (pLV-mCherry), while the other was infected with a lentivirus expressing GFP in addition to shRNA oligonucleotide. After both groups expressed the fluorescence dyes, mCherry\(^+\) and GFP\(^+\) cells were mixed in 1:1 ratio and a sample of the resulting cell mixture was directly analyzed by flow cytometry to determine the seeding density and ratio. Next, mixed cells were cultured on pre-coated 24 well plates (BD Falcon) for 6 days at 37 °C. After 6 days, cells were dissociated using Accutase and analyzed by flow cytometry to detect the ratio of mCherry\(^+\): GFP\(^+\) cells. Additionally, cell mixtures were cultured on pre-coated 8-well chamber slides (BD Falcon) and analyzed under the fluorescence microscope for visual inspection and imaging. The assay was repeated in two independent biological replicates.

**Cell lysis and western Blotting**

For preparation of cell lysates, cells were incubated with RIPA buffer (Cell Signaling Technology) supplemented with protease inhibitor complex (Roche) and phosphatase inhibitor complex (Cell Signaling Technology) for 5 minutes, followed by sonication and then centrifuged (13000 RPM, 15 minutes) to remove nucleic acids and cell debris. Bradford assay was used according to manufacturer instructions to assess protein concentration. Protein concentrations were measured using a benchtop spectrophotometer (Eppendorf). After addition of Laemmli loading buffer (Sigma Aldrich), lysates are incubated at 95 °C to ensure complete protein denaturation. Western
blotting was performed according to standard protocols (wet transfer, PVDF membranes), and images were developed using Odyssey fluorescence scanner system. BLUeye prestained protein ladder (GeneDireX) was used as a marker to identify the molecular weights of target proteins. One representative blot of the loading control GAPDH (housekeeping gene) is shown for each western blotting experiment.

**Small molecules, recombinant proteins and antibodies**

For cell treatments we used: Anti-FZD4 blocking antibody (Lexicon pharmaceuticals), Anti-KLH blocking antibody (Lexicon pharmaceuticals), IWP-2 (Sigma-Aldrich # I0536), XAV939(Sigma-Aldrich #X3004), rhNorrin (R&D systems # 3014-NR), rhWNT3a (R&D systems # 5036-WN), DKK1 (R&D systems # 5439-DK), DAPT (GSI; Sigma Aldrich #D5942), CHIR (CHIR 99021: 6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile; Axon Medchem #CT99021), BIO ((2’Z,3’E)-6-Bromoindirubin-3′-oxime; Sigma Aldrich #B1686). The following primary antibodies were used for western blotting and immunohistochemistry: anti-GAPDH (Millipore Sigma # CB1001, 1:5000), anti-Norrin (R&D systems #AF3014, 1:500), anti-MYC (Abcam #ab9106, 1:1000), anti-B-Catenin (CST #9562, 1:1000), anti-nonphospho-B-Catenin (CST #8814, 1:1000), anti-CCNA2 (Abcam #ab32286, 1:10000), anti-CCNE2 (Abcam #ab32103, 1:500), anti-CDK1 (Abcam #ab18, 1:1000), anti-NF-kB p65 (CST #8242, 1:1000), Anti-phospho- NF-kB p65 (CST #3033, 1:1000), anti-NICD1 (CST #4147, 1:1000), anti-Hes1 (Abcam #ab71559, 1:200), anti-HEYL (Abcam #ab26138, 1:1000), anti-Ki67 (BD Biosciences #550609, 1:100), anti-Sox2 (Abcam #ab97959, 1:1000), anti-HuAg (EMD Millipore #MAB1281, 1:1000), anti-Cleaved Caspase-3 for IHC (BD Pharmingen #559565), anti-Cleaved Caspase-3 for ICC (CST #9661, 1:400). Sections were counterstained with Haemotoxylin and Eosin (Sigma-Aldrich #MHS16), and nuclei (Hoechst 33342; Life Technologies #3570).
**Dual-Luciferase reporter assay system**

To assess Wnt activity, we used the Dual-Luciferase reporter assay system (Promega) to detect the signal produced by TOP-flash (with a B-Catenin activated promoter) reporter plasmid. Briefly, HEK293T cells were transiently transfected with a plasmid mixture containing *NDP, FZD4, LRP5, TSPAN12, TOP-FLASH*, and Renilla as a transfection control. These plasmids were a kind gift from Dr H. Junge. We used recombinant human Norrin (R&D Systems), recombinant human WNT3a (R&D systems), *WNT3a* overexpression, *NDP* overexpression, or small molecule GSK3 inhibitors (CHIR or BIO) as positive controls to activate canonical Wnt signaling in different experiments. Cells were incubated for 24 or 48 hours and cell lysates were prepared by passive lysis according to the manufacturer’s instruction. Luminescence signals were measured and normalized to the Renilla internal control using a bench top luminometer.

**Flow cytometric analysis**

Flow cytometry analysis was used to quantify the percentage of mCherry+ and GFP+ cells for the competition assay. Cells were lifted using Accutase, washed with PBS then fixed using 4% PFA. Wild type and single fluorescent marker cells were used as controls for each experiment. The flow cytometry run, analysis and quantification were performed at The SickKids-UHN Flow and Mass Cytometry Facility with the assistance of Ms. Emily Reddy, and results were visualized using FlowJo software.

**RNA extraction and qRT-PCR**

RNA was extracted using RNeasy mini-prep (Qiagen), according to manufacturer’s instructions. The concentration and purity of RNA were assessed using a bench top Nanodrop. First strand
complementary cDNA was reverse transcribed using QuantiTect (Qiagen) kit, according to manufacturer’s instructions. All samples included a no-reverse transcriptase (NRT) negative control to ensure total elimination of genomic DNA. The resulting cDNA was stored in -20 °C. For the qRT-PCR analyses, we used the iQ SYBR Green Supermix (Bio-Rad), as per manufacturer’s instructions. Results were statistically analyzed according to the standard protocols to generate double-delta CT values and comparative fold changes in gene expression relative to the controls. All qRT-PCR products were confirmed by gel electrophoresis, sequencing, as well as the existence of only one melting curve peak/gene product.

**PCR primers**

qRT-PCR primers were generated using NCBI primer blast tool with the standard parameters, and specifically designed to span an exon-exon junction to avoid genomic gDNA amplification. Primers were synthesized by ACGT company, Toronto, Canada.

NDP-Forward, TGCCTTCCCCCTAAGCTGTG; NDP-Reverse, ACCACGACGGAGAGCATAGA; FZD4-Forward, CTGACTGTAGCCGGGAAAG; FZD4-Reverse, TGACCCCATTTGAGTCTGC; TSPAN12-Forward, CTGCAAGAAACGAGGTTAGAGG; TSPAN12-Reverse, ACGCCACAAGCCAGTCTAC; LRP5-Forward, GTCCTCGGTGACAGGTTACA; LRP5-Reverse, AGCAAGCATTACGTCCCTCTG; B-Actin-Forward, GAGCACAGAGCCTCGCC; B-Actin-Reverse, TCATCATCCATGGTGAGCTGG; GAPDH-Forward, GAGCTCTTACGACCCGCTCA; GAPDH-Reverse, AGGAAAAGCATCACCAGTGAG; hPRT-Forward, CGGCAAGACGTTCAGATCC; hPRT-Reverse, AGTTGTGCATCACCCTGCTT; ASCL1-Forward, GGGCTCTTACGACCCGCTCA; ASCL1-Reverse, AGGTTGTGCATCACCCTGCTT.
RNA-Seq analysis

Cells were infected with shScrambled, shNDP-A, or shNDP-C-expressing lentiviruses and after 48h GFP reporter expression was confirmed and RNA was extracted using RNeasy mini kit (Qiagen). A portion of each sample was used to synthesize cDNA and confirm NDP knockdown efficiency using qRT-PCR, as described above. Samples were submitted to the Genome Quebec center, where RNA quality was confirmed using Bioanalyzer (Agilent), then RNA-Seq libraries were run. The screen consisted of two GSC lines; G523 and G411. Each GSC line had 9 samples; 3 biological replicates of shScrambled controls, 3 biological replicates of shNDP-A, and 3 biological replicates of shNDP-C transduced cells. Sample extracts were enriched for stranded poly(A)-mRNA and sequenced on Illumina HiSeq 4000 PE100. Bioinformatics analysis was carried out using the GenPipes RNA-seq pipeline [69]. Briefly: paired-ends sequencing reads were clipped for adapter sequence, trimmed for minimum quality (Q30) in 3' and filtered for minimum length of 32 bp using Trimmomatic [70]. Surviving read pairs were aligned to the Ensembl release 87 GRCh38 Homo sapiens genome assembly using the STAR [71] two-pass method. Exploratory analysis was conducted using various functions and packages from R and the Bioconductor project [72]. A gene-level count-based gene quantification against Ensembl annotations was performed using HT-seq count [73] in the intersection- nonempty mode. Differential expression was conducted using both edgeR [74] and DEseq [75]. Terms from the Gene Ontology were tested for enrichment with the GOseq [76] R package. Transcript-level assembly, quantification and differential expression analysis was performed using Cufflinks [77] and Cuffdiff [78]. Geneset enrichment analysis was carried out using fGSEA (doi: http://doi.org/10.1101/060012). The results of the enrichment analysis were used to create an enrichment map using the Enrichment Map [79] application for Cytoscape [80]. RNA-Seq data was deposited in the NCBI Gene Expression Omnibus (GEO) database (GEO identifier; GSE128255).
Animals

For in vivo transplantation experiments we used 5 to 8-week-old NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) female mice. All mice were purchased from Animal Research Center (ARC), UHN, Toronto, Canada. Experimental groups consisted of at least 5 mice/group. Randomization was not performed. Mice were housed in our facility located at the Krembil Discovery tower, Toronto Western Hospital.

Orthotopic xenografting

Lentiviral infected GSCs were dissociated using Accutase, then reconstituted to a concentration of 50000 cells/μl in PBS. Mice were anaesthetized with Ketamine/Metomidine and immobilized using a stereotaxic head frame. After shaving the head, an incision was made at the midline, then a bore-hole was drilled in the skull 1 mm lateral and 2 mm posterior to Bregma. Using a Hamilton syringe with a 27G round bottom needle, cells were uniformly injected with an automated nano-injector over the period of 3 minutes. After injection, the needle was left in place for 5 minutes to avoid cell reflux, then removed slowly. Finally, the skull was covered with bone wax, the incision was closed with sutures (size 5.0) followed by reversal of the anesthetic. Tramadol (Sigma Aldrich) was used for analgesia according to the ethics board protocols and recommendations for major surgeries. After surgery, animals were observed on a daily basis until they developed symptomatic tumors. Upon tumor formation, mice were sacrificed, perfused with PBS and 4% PFA, and brains were collected and fixed according to protocols for immunohistochemical analysis, as described above. Mice that developed complications due to surgery were removed from the study.

Quantification and statistical analysis

Unless otherwise is indicated, all experiments were repeated at least three independent times (3
biological replicates), each of them included at least three technical replicates. All groups in each experiment were matched in regard to number of biological and technical replicates. In addition, cells were matched in regard to passage number and culture conditions, as well as chemicals and reagents stocks. Quantification of immunohistochemical markers staining was performed manually with the assistance of Zeiss software by visually detecting and marking positive cells, then manually counting them. Quantification of qRT-PCR experiments was performed using the standard double-delta CT (ΔΔCT) method, comparing the expression levels of experimental samples to internal controls of housekeeping genes then experimental control of untreated or unmodified cells. Statistical significance was tested using unpaired two-tailed Student’s t-test when comparing two groups, and one-way analysis of variance (ANOVA) when performing multiple comparisons. Significance of in vivo transplantation and survival experiments was assessed using Log-rank test. In all figures, statistical significance (*); p < 0.05.

**Study Approval**

Primary-derived GNS and hNSC lines were obtained, derived and maintained under the Research Ethics Board at The Hospital for Sick Children (Toronto, Canada) and all animal experimental protocols were approved by the ethics and biosafety board of the Animal Research Center (ARC), UHN (Toronto, Canada).

**Acknowledgments**

We thank Drs. C. Schuurmans, H. Junge, M. Taylor and L. Megeney for helpful discussions and comments on the manuscript. We also thank Dr H. Junge for reagents and technical advice and Drs. François Lefebvre and Jose Hector Galvez at the Canadian Centre for Computational Genomics (C3G) for their help with the RNA-seq analysis. The C3G is a Node of the Canadian
Genomic Innovation Network and is supported by the Canadian Government through Genome Canada. This work was supported by operating grants to V.A.W. from the Cancer Research Society and the Canadian Institutes of Health Research.

**Author Contributions**

References:

48. Tanaka, R., et al., Accurate determination of S-phase fraction in proliferative cells by dual


Figures:
Figure 1

<table>
<thead>
<tr>
<th>TCGA</th>
<th>NDP Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
</tr>
</tbody>
</table>

RNA Seq V2 (log2)

B

FZD4 Expression

RNA Seq V2 (log2)

- Truncating (VUS)
- Missense (VUS)
- Not mutated
- No profiled for mutations
Figure 1. *NDP* is expressed a wide range of cancers, and is enriched in CNS and glioma tissues

(A, B) Analysis of *NDP* (upper) and *FZD4* (bottom) expression levels in primary human tumors from TCGA using cBioportal web server. *NDP* expression was significantly enriched in GBM and lower-grade glioma relative to the average of all cancer types (upper graph), while *FZD4* expression was comparable to other tumor types.
Figure 2

A  Gene Set Enrichment Analysis

GBM Classical

<table>
<thead>
<tr>
<th>NDP-High</th>
<th>NDP-Low</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Aging Brain

<table>
<thead>
<tr>
<th>NDP-High</th>
<th>NDP-Low</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B  Kaplan Meier survival analysis (NDP)

<table>
<thead>
<tr>
<th>Neuroblastoma</th>
<th>Brain Astrocytoma (LGG)</th>
<th>Glioblastoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival probability</td>
<td>Survival probability</td>
<td>Survival probability</td>
</tr>
<tr>
<td>n=47 Log-Rank p-Value 0.001</td>
<td>n=86 Log-Rank p-Value 0.003</td>
<td>n=143 Log-Rank p-Value 0.035</td>
</tr>
</tbody>
</table>

C  Expression of NDP/FZD4 pathway components in GBM and hNSC

Fold changes relative to mean

<table>
<thead>
<tr>
<th>NDP</th>
<th>FZD4</th>
<th>LPR5</th>
<th>TSPAN12</th>
</tr>
</thead>
<tbody>
<tr>
<td>G411</td>
<td>G523</td>
<td>G567</td>
<td>G729 G752r G789</td>
</tr>
<tr>
<td>G440</td>
<td>G472</td>
<td>G564</td>
<td></td>
</tr>
</tbody>
</table>

Fold changes relative to mean

<table>
<thead>
<tr>
<th>hNSC-1</th>
<th>hNSC-2</th>
<th>hNSC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. *NDP* is expressed in GSCs and correlates with survival in neurological tumors

(A) Gene set enrichment analysis reveals correlation between *NDP* expression and “Glioblastoma classical” and “Aging Brain” gene sets.

(B) Kaplan-Meier analysis correlating *NDP* expression with patient survival in neurological cancers.

(C) Expression of components of the *NDP/FZD4* signaling axis in a panel of 9 patient-derived GSCs (left graph) and 3 primary fetal hNSC lines (right graph). Blue boxes, ASCL1<sub>lo</sub> GSC lines; red, ASCL1<sub>hi</sub> GSC lines indicate the GSC lines selected for functional analysis.
Figure 3

<table>
<thead>
<tr>
<th>ASCL1&lt;sup&gt;lo&lt;/sup&gt; (NDP)</th>
<th>ASCL1&lt;sup&gt;hi&lt;/sup&gt; (NDP)</th>
<th>ASCL1&lt;sup&gt;lo&lt;/sup&gt; (IDHwt)</th>
<th>ASCL1&lt;sup&gt;hi&lt;/sup&gt; (IDHwt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G411</td>
<td>G564</td>
<td>G523</td>
<td>G472</td>
</tr>
</tbody>
</table>

### Proliferation

#### Loss of function

<table>
<thead>
<tr>
<th></th>
<th>shScrambled</th>
<th>shNDP-A</th>
<th>shNDP-C</th>
<th>shFZD4-2</th>
<th>shFZD4-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Day 2</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Day 3</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Day 4</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Day 5</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Day 6</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

#### Sphere formation

<table>
<thead>
<tr>
<th></th>
<th>shScrambled</th>
<th>shNDP-A</th>
<th>shNDP-C</th>
<th>shFZD4-2</th>
<th>shFZD4-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Day 2</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Day 3</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Day 4</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Day 5</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Day 6</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

### Gain of function

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>hNDP</th>
<th>hFZD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Day 2</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Day 3</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Day 4</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Day 5</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Day 6</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

### Sphere formation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>hNDP</th>
<th>hFZD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Day 2</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Day 3</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Day 4</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Day 5</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Day 6</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

### Kaplan Meier survival analysis in GBM patients

<table>
<thead>
<tr>
<th></th>
<th>ASCL1&lt;sup&gt;lo&lt;/sup&gt; (NDP)</th>
<th>ASCL1&lt;sup&gt;hi&lt;/sup&gt; (NDP)</th>
<th>ASCL1&lt;sup&gt;lo&lt;/sup&gt; (IDHwt)</th>
<th>ASCL1&lt;sup&gt;hi&lt;/sup&gt; (IDHwt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ND-P-High</td>
<td>ND-P-Low</td>
<td>ND-P-High</td>
<td>ND-P-Low</td>
</tr>
<tr>
<td></td>
<td>n=71</td>
<td>n=72</td>
<td>n=143</td>
<td>n=143</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>100</td>
<td>80</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>Survival (months)</td>
<td>0-10</td>
<td>10-20</td>
<td>0-100</td>
<td>10-20</td>
</tr>
</tbody>
</table>

Log-Rank p-Value: 0.54, 0.47, 0.93, 0.7
Figure 3. *ASCL1* subtype-dependent effects of *NDP* and *FZD4* on proliferation and sphere formation

(A, B) Effect of *NDP* or *FZD4* knockdown (two independent shRNAs/gene) on growth using the Trypan Blue proliferation assay after 3 and 6 days in culture (A) sphere formation using the ELDA assay after 2 weeks in suspension culture (B). n=3, error bars, mean ± SEM. Statistical significance (*) was tested using one-way ANOVA for the proliferation assay and Chi-square for ELDA; p < 0.05. Note that both shNDP constructs behaved similarly in G472 cells, so representative lines almost overlap on the graph.

(C, D) Effect of *NDP* and *FZD4* overexpression on GSC proliferation (C) and sphere formation (D). n=3, error bars represent mean ± SEM. n=3, error bars, mean ± SEM. Statistical significance (*) was tested using one-way ANOVA for the proliferation assay and Chi-square for ELDA; p < 0.05.

(E) Kaplan-Meier survival analysis correlating *NDP* expression with patient survival ASCL1\textsuperscript{lo}, ASCL1\textsuperscript{hi} GBM and in GBM stratified for NDP/ASCL1 and NDP/FZD4 expression.
Figure 4
A  \textbf{ASCL1}^{lo} (G564)

<table>
<thead>
<tr>
<th></th>
<th>shScrambled</th>
<th>shNDP-A</th>
<th>shNDP-C</th>
<th>shFZD4-2</th>
<th>shFZD4-4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ki67</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SOX2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B  \textbf{ASCL1}^{hi} (G472)

<table>
<thead>
<tr>
<th></th>
<th>shScrambled</th>
<th>shNDP-A</th>
<th>shNDP-C</th>
<th>shFZD4-2</th>
<th>shFZD4-4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ki67</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SOX2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C  \textbf{ASCL1}^{lo} (G564)

- Ki67* cells
- Sox2* cells

D  \textbf{ASCL1}^{hi} (G472)

- Ki67* cells
- Sox2* cells
Figure 4. Divergent effects of *NDP* and *FZD4* knockdown on the proliferation indices of GSC cultures

(A, B)  Representative images of ICC staining for Ki67 (red) and SOX2 (green) after *NDP* or *FZD4* knockdown in ASCL1^lo^ (G564) (A) and ASCL1^hi^ (G472) (B) GSCs. Scale bar, 50 μM.

(C, D)  Quantification of the frequency of Ki67^+^ and SOX2^+^ cells after *NDP* and *FZD4* knockdown in G564 (C) and G472 (D) GSCs. n=3 (with the exception of Ki67 in G564; we used n=9 because cells are very large and the number of cells per field or vessel is quite small). Error bars, mean ± SEM. Statistical significance (*) was tested using one-way ANOVA; p < 0.05.
Figure 5. RNA-Seq analysis reveals divergent and overlapping effects of *NDP* knockdown on gene expression in ASCL1<sup>lo</sup> and ASCL1<sup>hi</sup> GSCs

(A) Venn diagram representing the number of overlapping and common differentially expressed genes in each cell line. Roughly half of G523 hits and one third of G411 hits are overlapping. Notably, the number of hits in G411 is higher than in G523.

(B) Unbiased enrichment maps of the identified hits in both lines (the blue circles represent G411, red circles represent G523). The overlapping sets predominantly center on cell cycle, cell division and proliferation, while G411 unique sets are related to cytoskeletal re-arrangement and movement. Refer to Figure S6 for more detail.

(C) Venn diagram representing the number of unique and common enriched gene sets.

(D) Western blot validation of CCNE2 and CCNA2 expression in G411 and G523 cells with *NDP* knockdown.

(E) Log-Fc values for selected Wnt targets from the RNA-Seq library results. The selected genes show significant differential expression after *NDP* knockdown in G411 but not G523 cells. Represented bars show the average fold change of selected hits between for two shNDP (shNDP-A, shNDP-C) constructs in each cell line.
Figure 6

A  Wnt inhibition in ASCL1<sup>lo</sup> (G411)  

![Graph A: Wnt inhibition in ASCL1<sup>lo</sup> (G411)]

B  Wnt inhibition in ASCL1<sup>hi</sup> (G523)  

![Graph B: Wnt inhibition in ASCL1<sup>hi</sup> (G523)]

C  Wnt activation in ASCL1<sup>lo</sup> (G411)  

![Graph C: Wnt activation in ASCL1<sup>lo</sup> (G411)]

D  Wnt activation in ASCL1<sup>hi</sup> (G523)  

![Graph D: Wnt activation in ASCL1<sup>hi</sup> (G523)]

E  Non-Phospho (active) B-Cat levels after knocking down of NDP  

![Graph E: Non-Phospho (active) B-Cat levels after knocking down of NDP]

F  NDP over expression  

![Graph F: NDP over expression]

G  Wnt target validation  

![Graph G: Wnt target validation]
Figure 6. NDP function is Wnt-dependent in ASCL1\textsuperscript{lo} and Wnt-independent in ASCL1\textsuperscript{hi} cells

(A, B) Effects of canonical Wnt pathway inhibitors on proliferation of control and NDP-overexpressing G411 (ASCL1\textsuperscript{lo}) GSCs (A) and G523 (ASCL1\textsuperscript{hi}) GSCs (B) after 6 days. n=3, error bars, mean ± SEM. Statistical significance (†) within experimental groups and (*) between groups was tested using one-way ANOVA with multiple comparisons; p < 0.05.

(C, D) Effects of Wnt agonists WNT3a and CHIR on proliferation of G411 (C) and G523 GSCs (D) cells after 6 days. n=3, error bars, mean ± SEM. Statistical significance (*) was tested using one-way ANOVA with multiple controls; p < 0.05.

(E, F) Representative western blot analysis and quantitation of the levels of active (non-phosphorylated) and total B-catenin in NDP-knockdown G411 and G523 GSCs (E) and NDP-overexpressing G411 and G523 GSCs (F). Quantification was performed using Image Studio Lite software analysis package. n=3, error bars, mean ± SEM. Statistical significance (*) was tested using one-way ANOVA; p < 0.05.

(G) Representative western blot analysis (n=3) of selected Wnt targets following NDP-knockdown G411 and G523 GSCs.
Figure 7

A  Norrin treatment

ASCL1<sup>α</sup> (G411)  ASCL1<sup>β</sup> (G523)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>rNorrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation (%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B  Effects of NDP knockdown on Notch

<table>
<thead>
<tr>
<th></th>
<th>ASCL1&lt;sup&gt;α&lt;/sup&gt; (G411)</th>
<th>ASCL1&lt;sup&gt;β&lt;/sup&gt; (G523)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Notch1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HES1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeyL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C  NDP OE + GSI

ASCL1<sup>α</sup> (G411)  ASCL1<sup>β</sup> (G523)

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>GSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fold change in cell number</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D  ASCL1 OE + NDP KD in ASCL1<sup>α</sup> (G564)

<table>
<thead>
<tr>
<th></th>
<th>shScr</th>
<th>shNDP-A</th>
<th>shNDP-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fold change in cell number</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 7. *NDP* knockdown inhibits Notch signaling in ASCL1<sup>hi</sup> GSCs

(A) Effects of recombinant Norrin treatment on the proliferation of G411 and G523 GSCs after 6 days in culture. n=3, error bars, mean ± SEM. Statistical significance (*) was tested using Student’s t-test; p < 0.05.

(B) Representative western blot analysis for the indicated Notch pathway components in G411 and G523 GSCs after *NDP* knockdown.

(C) Effects of Notch inhibition on the growth promoting effects of *NDP* overexpression in G411 and or G523 GSCs after 6 days in culture. n=3, error bars, mean ± SEM. Statistical significance (*) was tested using one-way ANOVA; p < 0.05.

(D) Effect of *NDP* overexpression on the proliferation of G564 (ASCL1<sub>lo</sub>) GSCs after *ASCL1* overexpression. n=3, error bars, mean ± SEM. Statistical significance (*) was tested using one-way ANOVA; p < 0.05.
Figure 8

<table>
<thead>
<tr>
<th>A</th>
<th>ASCL1&lt;sup&gt;hi&lt;/sup&gt; (G523)</th>
</tr>
</thead>
<tbody>
<tr>
<td>shScrambled</td>
<td>shNDP-A</td>
</tr>
<tr>
<td>βIII-Tubb</td>
<td>Sox2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>Quantification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of cells</td>
<td></td>
</tr>
<tr>
<td>shScr.</td>
<td>shNDP-A</td>
</tr>
</tbody>
</table>

* *
Figure 8. *NDP* knockdown leads to differentiation in ASCL1 hi GSCs

(A) Representative images from ICC staining of BIII-Tubulin, SOX2, GFAP, MAP2 in G523 cells 3 weeks after *NDP* knockdown. Scale bar, 50 μM.

(B) Quantification of respective stains. n=3, error bars, mean ± SEM. Statistical significance (*) was tested using one-way ANOVA; p < 0.05.
Figure 9

<table>
<thead>
<tr>
<th>ASCL1&lt;sup&gt;lo&lt;/sup&gt;</th>
<th>ASCL1&lt;sup&gt;hi&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong> Xenograft in ASCL1&lt;sup&gt;lo&lt;/sup&gt; (G411)</td>
<td><strong>B</strong> Xenograft in ASCL1&lt;sup&gt;hi&lt;/sup&gt; (G523)</td>
</tr>
</tbody>
</table>

Kaplan Meier survival analysis in ASCL1<sup>lo</sup> (G411)

<table>
<thead>
<tr>
<th>Loss of function</th>
<th>Gain of function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C</strong> Loss of function</td>
<td><strong>D</strong> Gain of function</td>
</tr>
</tbody>
</table>

Kaplan Meier survival analysis in ASCL1<sup>hi</sup> (G523)

<table>
<thead>
<tr>
<th>Loss of function</th>
<th>Gain of function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E</strong> Loss of function</td>
<td><strong>F</strong> Gain of function</td>
</tr>
</tbody>
</table>

---

Log-Rank p-Value shScramble vs. shNDP=0.216
Log-Rank p-Value shScramble vs. shFZD4=0.554
n=8 per group

Log-Rank p-Value Control vs. shNDP=0.003
Log-Rank p-Value Control vs. shFZD4=0.029
n=8 per group

Log-Rank p-Value Control vs. shNDP=0.023
Log-Rank p-Value Control vs. shNDP-C=0.001
n=8 per group

Log-Rank p-Value Control vs. shNDP=0.045
n=5 per group
Figure 9. Effects of NDP on progression of GBM in vivo depend on ASCL1 expression status

(A, B) Representative H&E staining of orthotopically xenografted G411 (ASCL1lo) (A) and G523 (ASCL1hi) GSC (B) tumors (dotted line; tumor outline). Shown are tile scans taken at 2.5X magnification (left) and boxed regions are shown at higher magnification (20X) on the right. G411 GSCs form localized and G523 GSCs form diffuse tumors. Scale bars: 10 μM, 50 μM respectively.

(C, D) Kaplan-Meier survival analysis of mice orthotopically transplanted with G411 GSCs after NDP or FZD4 knockdown (C) and overexpression (D). Statistical significance (log-rank); p < 0.05.

(E, F) Kaplan-Meier survival analysis of mice orthotopically transplanted with G523 GSCs after NDP knockdown (E) and overexpression (F). Statistical significance (log-rank); p < 0.05.
<table>
<thead>
<tr>
<th></th>
<th>Loss of function in ASCL1(^{h}) (G411)</th>
<th>Loss of function in ASCL1(^{h}) (G523)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Hoechst / HuAg / GFP</td>
<td>Hoechst / HuAg / GFP</td>
</tr>
<tr>
<td></td>
<td>shScrambled</td>
<td>shScrambled</td>
</tr>
<tr>
<td></td>
<td>shF224</td>
<td>shF224</td>
</tr>
<tr>
<td></td>
<td>shNDP-C</td>
<td>shNDP-C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>Gain of function in ASCL1(^{h}) (G411)</th>
<th>Gain of function in ASCL1(^{h}) (G523)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hoechst / HuAg / GFP</td>
<td>Hoechst / HuAg / GFP</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>hNDP</td>
<td>hNDP</td>
</tr>
</tbody>
</table>
Figure 10. *NDP* modulation results in selective advantage or disadvantage in xenografted tumors depending on *ASCL1* expression status

(A, B) Representative images of IHC on *NDP* or *FZD4* knockdown (A) or overexpression (B) G411-derived tumors. Scale bar, 50 μM.

(C, D) Representative images of IHC on *NDP* knockdown (C) and overexpression (D) G523-derived tumors. Scale bar, 50 μM.
Graphical Abstract