The infantile myofibromatosis NOTCH3 L1519P mutation leads to hyperactivated ligand-independent Notch signaling and increased PDGFRB expression

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Summary statement

Hyperactivated, ligand-independent Notch signaling acts epistatically over PDGF signaling in infantile myofibromatosis.

Abstract

Infantile myofibromatosis (IMF) is a benign tumor form characterized by the development of nonmetastatic tumors in skin, bone, muscle and sometimes viscera. Autosomal dominant forms of IMF are caused by mutations in the PDGFRB gene, but a family carrying a L1519P mutation in the NOTCH3 gene has also recently been identified. In this report, we address the molecular consequences of the NOTCH3\textsuperscript{L1519P} mutation and the relationship between the NOTCH and PDGFRB signaling in IMF. The NOTCH3\textsuperscript{L1519P} receptor generates enhanced downstream signaling in a ligand-independent manner. Despite the enhanced signaling, the NOTCH3\textsuperscript{L1519P} receptor is absent from the cell surface and instead accumulates in the endoplasmic reticulum. Furthermore, the localization of the NOTCH3\textsuperscript{L1519P} receptor in the bipartite, heterodimeric state is altered, combined with avid secretion of the mutated extracellular domain from the cell. Chloroquine treatment strongly reduces the amount of secreted NOTCH3\textsuperscript{L1519P} extracellular domain and decreases signaling. Finally, NOTCH3\textsuperscript{L1519P} upregulates PDGFRB expression in fibroblasts, supporting a functional link between Notch and PDGF dysregulation in IMF. Collectively, our data define a NOTCH3-PDGFRB axis in IMF, where an IMF-mutated NOTCH3 receptor elevates PDGFRB expression. The functional characterization of a ligand-independent gain-of-function NOTCH3 mutation is important for Notch therapy considerations for IMF, including strategies aimed at altering lysosome function.

Introduction

Infantile myofibromatosis (IMF, MIM 228550) patients suffer from nonmetastatic tumors that develop in skin, bone, muscle and viscera (Chung and Enzinger, 1981). IMF has an estimated incidence of 1:150,000-400,000 live births and was first described as a distinct disease entity in 1954 (Stout, 1954). The term infantile myofibromatosis was coined in 1981 (Chung and Enzinger, 1981). IMF tumors, which most often occur in children, are generally benign and sometime
regress, although visceral tumors can be lethal (Schurr and Moulsdale, 2008). Histological findings comprise fascicles of spindle-shaped cells separated by collagen fibers surrounding a central vascular area with features of hemangiopericytoma (Levine et al., 2012).

IMF can occur in both autosomal recessive and dominant forms, suggesting complex underlying genetics. For the autosomal dominant forms, two mutations in the PDGFRB gene (MIM 173410) have been identified (Cheung et al., 2013; Martignetti et al., 2013). One report identified PDGFRB c.1681C>T, p.Arg561Cys (R561C) and c.1978C>A, p.Pro660Thr (P660T) mutations in eight families (Martignetti et al., 2013), while another study identified 11 affected patients carrying the R561C mutation (Cheung et al., 2013). The PDGFRB mutations lead to ligand-independent receptor activation (Arts et al., 2017) and are likely gain-of-function mutations, as they are sensitive to kinase inhibitors (Mudry et al., 2017). Elevated expression of PDGF ligands and receptors has also been observed in pediatric fibromatoses and myofibromatosis (Gibson et al., 2007), further supporting the notion that elevated PDGF signaling can cause IMF. PDGF signaling is initiated by PDGF ligands, which cause PDGF receptor dimerization, leading to receptor autophosphorylation and phosphorylation of downstream target proteins, for example in the MAPK, PI3K and JAK/STAT pathways (Heldin, 2013). PDGFRB, and its main ligand, PDGF-B, are highly evolutionarily conserved (Hoch and Soriano, 2003). During development, PDGF signaling is paramount for the recruitment of pericytes during blood vessel formation and complete knockout of either the Pdgfb or Pdgfrb genes in mice leads to perinatal death (Leveen et al., 1994; Soriano, 1994).

Recently, mutations have also been identified in the NOTCH3 gene in IMF patients. Nine affected individuals in an IMF family carried a heterozygous c.4556 C>T mutation in NOTCH3, whereas seven unaffected family members did not harbor the mutation (Martignetti et al., 2013); for review see (Lee, 2013). The c.4556 C>T mutation results in a leucine to proline transition at position 1519 in the NOTCH3 receptor (L1519P), a site located in the so-called heterodimerization domain of the receptor (Fig. 1A). Notch receptors (NOTCH1-4) are large transmembrane receptors present at the cell surface as dipartite (heterodimeric) proteins after proteolytic cleavage in the Golgi compartment. This first cleavage, called Site 1 or S1 cleavage, is executed by furin-like convertase, generating the extracellular domain (ECD) and the transmembrane intracellular domain (TMIC)
The ECD and TMIC moieties are held together via the two halves of the heterodimerization domain, which is split up by the S1 cleavage (Fig. 1A). At the cell surface, the receptor interacts with Notch ligands (of the Dll and Jagged type) presented at a juxtaposed cell, and the ligand-receptor interaction results in a second proteolytic cleavage (S2 cleavage) conducted by ADAM metalloproteinases (Fig. 1A). The S2 cleavage occurs when a “hinge” region in the so called negative regulatory region (NRR; which encompasses the heterodimerization domain and the three LNR repeats, Fig. 1A) opens up in response to a pulling force from the ligand, exposing the S2 cleavage site. S2 cleavage yields the NEXT form of the receptor from the TMIC, and NEXT is directly processed by the γ-secretase complex (S3 cleavage) to produce the intracellular domain (ICD). The S3 cleavage is intramembranous and occurs at the cell surface or in endosomes (Fig. 1A) (Siebel and Lendahl, 2017). The S3 cleavage releases the Notch intracellular domain (Notch ICD), which acts as a transactivating factor after binding to the DNA-binding CSL (RBP-j) protein. In the absence of Notch activation, CSL acts as a transcriptional repressor but converts to become an activator upon binding of Notch ICD and a third protein, MAML, into a trimeric Notch ICD/MAML/CSL complex. Notch signaling is, like PDGF signaling, used in many organs and important for organ development as well as tissue homeostasis (Siebel and Lendahl, 2017). In addition to being mutated in IMF, mutations in NOTCH3 cause the stroke and dementia syndrome CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy; IMOM No 125310), which is characterized by white matter lesions, lacunar ischemic infarcts and degeneration of vascular smooth muscle cells in the brain vasculature (Coupland et al., 2018).

In this report, we analyze the molecular consequences of the L1519P IMF mutation in the NOTCH3 protein and the relationship between Notch and PDGF signaling in IMF. We find that NOTCH3<sup>L1519P</sup> generates hyperactivated Notch signaling, despite the fact that it does not appear at the cell surface. The dimeric form of NOTCH3<sup>L1519P</sup> is relocalized to the perinuclear area, and the ECD is avidly exported from the cell to the surrounding medium, indicating aberrant intracellular routing. We also show that NOTCH3<sup>L1519P</sup> exacerbates PDGFRB expression, supporting the notion of a NOTCH3-PDGFRB axis, where Notch is epistatic over PDGF signaling. The notion of a ligand-independent hyperactivated Notch receptor in IMF has important implications for Notch therapy development.
**Results**

**NOTCH3**\textsuperscript{L1519P} exhibits an imbalance between the full length and TMIC forms

To explore the molecular consequences of the NOTCH\textsuperscript{3L1519P} mutation, we engineered cell lines where the wildtype or L1519P forms of NOTCH3 were introduced into HEK 293T cells from which we had first ablated the NOTCH1, 2 and 3 genes by CRISPR/Cas9 (HEK 293T ΔN1-3 cell line), to rid the cells from endogenous Notch signaling and thus eliminate the potential risk of antibody cross-reactivity in the cellular localization assays described below (Fig. S1A). The wildtype or L1519P forms of NOTCH3 were stably integrated into the AAVS1 locus in the HEK 293T ΔN1-3 cell line, which is tetracycline regulated and thus allowing the levels of NOTCH3 receptor expression to be regulated by doxycycline stimulation (Fig. S1B). HEK 293T ΔN1-3 cell lines expressing similar amounts of NOTCH3 wildtype or NOTCH3\textsuperscript{L1519P} in the presence of doxycycline were selected (Fig. S1C). In the “Notch off” situation, i.e. without ligand stimulation, the wildtype NOTCH3 receptor as expected appeared as a combination of a band representing the full-length (unprocessed) form (250 kDa) and the S1-processed TMIC form (90 kDa) (Fig. 1B). In contrast, the NOTCH3\textsuperscript{L1519P} receptor appeared predominantly as the unprocessed full-length form, with only minor amounts of the TMIC and NEXT/ICD forms (NEXT and ICD migrate very closely together and cannot be separated under the western blot conditions used in this experiment) (Fig. 1B). In all, this suggests an imbalance between the full length and processed forms (TMIC and NEXT/ICD) of NOTCH3\textsuperscript{L1519P}.

**Aberrant intracellular routing of NOTCH3**\textsuperscript{L1519P}

The imbalance between the full-length and processed forms may indicate a problem with intracellular routing, in S1 processing, which occurs in the Golgi compartment (Siebel and Lendahl, 2017), or in the stability of the heterodimer formed by TMIC and ECD (Fig. 1A). To gain insights into the routing of the receptor from the ER to the cell surface, we assessed whether NOTCH3\textsuperscript{L1519P} routed to the cell surface. Immunostaining for the NOTCH3 ECD, on intact, non-permeabilized cells revealed the presence of wildtype NOTCH3 at the cell surface, while in contrast, NOTCH3\textsuperscript{L1519P} was undetectable at the cell surface, which was visualized by Na,K-ATPase staining (Fig. 2A). As a control for the amounts of wildtype and L1519P NOTCH3...
protein, immunostaining for NOTCH3 ICD after permeabilization of the cells revealed ample amounts of NOTCH3 protein in both wildtype and mutant cells (Fig. 2B). To corroborate these data, a combined image streaming and microscopy analysis for NOTCH3 cell surface immunostained cells confirmed that no or very little NOTCH3\textsuperscript{L1519P} was present at the cell surface (Fig. S2).

To gain further insights into the intracellular routing of NOTCH3\textsuperscript{L1519P}, we explored its localization in the endoplasmic reticulum (ER) and Golgi compartments. Co-immunostaining with an antibody to the extracellular domain and calnexin, a marker for the ER, revealed a more extensive colocalization with ER for NOTCH3\textsuperscript{L1519P} as compared to wildtype NOTCH3 in permeabilized cells (Fig. 2C). Quantification of the colocalization using Pearson’s correlation coefficient confirmed the increased accumulation of NOTCH3\textsuperscript{L1519P} in the ER (Fig. 2C). Co-immunostaining for the NOTCH3 extracellular domain and the Golgi compartment, using giantin as a Golgi marker, showed no statistical difference between the wildtype and NOTCH3\textsuperscript{L1519P} protein (Fig. 2D). In sum, these experiments suggest that NOTCH3\textsuperscript{L1519P} is absent from the cell surface but shows increased retention in the ER.

**NOTCH3\textsuperscript{L1519P} undergoes enhanced and ligand-independent S2 cleavage**

To further investigate the basis for the aberrant receptor routing and altered TMIC levels, we explored whether S2 processing was affected in NOTCH3\textsuperscript{L1519P}. In the “Notch off” situation, i.e. when cells were not ligand-stimulated, there was as expected a build-up of TMIC from the wildtype NOTCH3 receptor, but with very little accumulation of NOTCH3 NEXT or ICD (Fig. 3A). In contrast, there was less TMIC in NOTCH3\textsuperscript{L1519P}-expressing cells in the “Notch off” situation, but instead a band corresponding to NOTCH3 NEXT/ICD (Fig. 3A, see also Fig. 1B). This suggests that S2 processing occurs in NOTCH3\textsuperscript{L1519P} also in the absence of ligand activation. In keeping with this notion, blockade of S2 cleavage by treatment of the cells with the ADAM 10 secretase inhibitor GI254023X reduced the accumulation of the NEXT/ICD band in the NOTCH3\textsuperscript{L1519P}-expressing cells (Fig. 3A). As the NEXT moiety is rapidly processed to the ICD form by the \(\gamma\)-secretase-mediated S3 cleavage (see Fig. 1A), and ICD has short half-life because of rapid proteasome-mediated degradation (Oberg et al., 2001; Zhang et al., 2017), we explored the effect of treating the cells with the \(\gamma\)-secretase inhibitor DAPT to block the transition of the
NEXT into the ICD form. DAPT treatment resulted in a stronger NEXT/ICD band in NOTCH3\textsuperscript{L1519P}-expressing cells (Fig. 3A), indicating that blockage of S3 cleavage leads to accumulation of NEXT, while the TMIC level was unaffected in the NOTCH3 wildtype-expressing cells. The low levels of TMIC from NOTCH3\textsuperscript{L1519P} may thus be caused by enhanced S2 cleavage in the absence of ligand activation. In support of this notion, cycloheximide chase experiments demonstrated that the TMIC from NOTCH3\textsuperscript{L1519P} in the “Notch-off” situation was turned over more rapidly than the TMIC from the NOTCH3 wildtype receptor (Fig. 3B). In all, these experiments reveal enhanced and ligand-independent S2 processing of NOTCH3\textsuperscript{L1519P}.

**Relocalization of NOTCH3\textsuperscript{L1519P} in the dipartite ECD-TMIC state coupled with exacerbated export of the extracellular domain from the cell**

We next explored the possibility that the enhanced ligand-independent S2 cleavage and lack of NOTCH3\textsuperscript{L1519P} at the cell surface might be due to destabilization of the dipartite state of the receptor, i.e. the ECD-TMIC heterodimer, since the mutation is located in the conserved heterodimerization region (Fig. 1A) and may thus affect stability of the ECD and TMIC interaction. To address this, we established a proximity ligation assay (PLA) that records the proximity of the ECD and TMIC moieties, using antibodies to the ECD and TMIC domains, respectively (Fig. 4A). The ECD-TMIC PLA assay revealed a more widespread ECD-TMIC interaction for wildtype NOTCH3 as compared to NOTCH3\textsuperscript{L1519P}, for which much of the PLA signal localized to aggregates in a cytoplasmic area bordering the cell nucleus (Fig. 4B). 3D rendering analysis confirmed that the ECD-TMIC wildtype heterodimer was localized at both the plasma membrane and in the cytoplasm, while the ECD-TMIC heterodimer was predominantly observed in the cytoplasm near the cell nucleus (Fig. 4C). Together, these results indicate that the L1519P mutation leads to relocalization and possible destabilization of the ECD-TMIC heterodimer early during the transport to the cell surface and the absence of a dipartite NOTCH3\textsuperscript{L1519P} receptor at the cell surface.

The fact that the L1519P mutation shifted the localization of the ECD-TMIC heterodimer combined with absence of the mutated receptor at the cell surface led us to explore the fate of the NOTCH3\textsuperscript{L1519P} ECD. To test whether it was exported from the cell, we analyzed cell culture medium from cells expressing NOTCH3\textsuperscript{L1519P} or wildtype NOTCH3. Medium from the
NOTCH3\textsuperscript{L1519P}-expressing cells not activated by ligand contained considerably more ECD (Chapman et al., 2006), as compared to medium from wildtype NOTCH3 cells, which did not contain measurable amounts of ECD (Fig. 4D).

While export of NOTCH3\textsuperscript{L1519P} ECD to the cell medium was independent of ligand activation, we next asked whether it was dependent on S2 cleavage, endocytosis or autophagosome-lysosome function. To test this, cells expressing NOTCH3\textsuperscript{L1519P} were treated with GM6001 (a broad-spectrum matrix metalloproteinase inhibitor blocking S2 cleavage), MitMAB (a dynamin I/II inhibitor blocking endocytosis), GI254023X (to block S2 cleavage) and chloroquine (which inhibits fusion of the autophagosome). Treatment with GM6001, GI254023X, DAPT or MitMAB did not affect export of the ECD from NOTCH3\textsuperscript{L1519P} to the cell medium, indicating that S2 cleavage and endocytosis were not required for ECD export (Fig. 4E). In contrast, chloroquine strongly reduced the amount of NOTCH3\textsuperscript{L1519P} ECD in the medium (Fig. 4E). Chloroquine treatment also resulted in an accumulation of a NEXT/ICD band in whole-cell extracts from the NOTCH3\textsuperscript{L1519P}-expressing cells (Fig. 4E).

The effect of chloroquine treatment on export of the ECD from NOTCH3\textsuperscript{L1519P} prompted us to analyze the localization of wildtype and NOTCH3\textsuperscript{L1519P} protein to endosomes and lysosomes. NOTCH3\textsuperscript{L1519P} showed reduced localization to early endosomes, as compared with wildtype NOTCH3, using EEA1 as an early endosome marker and analyzed using Pearson’s correlation coefficient (Fig. 4F). In contrast, when colocalization with lysosomes was analyzed using LAMP1 as a lysosomal marker, NOTCH3\textsuperscript{L1519P} colocalized more extensively with LAMP1 as compared to wildtype NOTCH3 (Fig. 4G). Treatment with chloroquine decreased colocalization between NOTCH3\textsuperscript{L1519P} and lysosomal LAMP1 (Fig. 4H). In sum, these data indicate that the heterodimeric ECD-TMIC from NOTCH3\textsuperscript{L1519P} is relocalized in the cell, which may lead to the exacerbated export of its ECD from the cell in an autophagosome-lysosome fusion-dependent mode.
NOTCH3\textsuperscript{L1519P} produces NOTCH3 ICD in a ligand-independent manner leading to enhanced Notch downstream signaling output

To learn how ligand-mediated activation affected \textit{NOTCH3}\textsuperscript{L1519P} processing and downstream signaling, we first subjected cells expressing wildtype or \textit{NOTCH3}\textsuperscript{L1519P} receptor to ligand activation by culturing the cells on immobilized Jagged2 ligand. Ligand activation of the wildtype NOTCH3 receptor as expected led to the production of NEXT and a small amount of ICD when MG132 was supplemented to block proteasome-mediated degradation of NOTCH3 ICD (Oberg et al., 2001) (Fig. 5A), indicating that NEXT and ICD were rapidly turned over when proteasome activity was not inhibited. Blockade of S3 processing using DAPT under ligand-activating conditions abolished the ICD band while sustaining a small amount of NEXT (Fig. 5A), arguing that NEXT is rapidly degraded by the proteasome. For cells expressing \textit{NOTCH3}\textsuperscript{L1519P}, there was less TMIC (Fig. 5A), in keeping with the results presented in Fig. 1B. Treatment with MG132 resulted in a strong accumulation of both NEXT and in particular of ICD, and this accumulation was interestingly observed both in the “Notch-on” and “Notch-off” situations, i.e. both with and without ligand stimulation (Fig. 5A). Treatment with DAPT resulted in an accumulation of NEXT, also both in the “Notch-on” and “Notch-off” states. Collectively, these data suggest an enhanced S2 cleavage of NOTCH3\textsuperscript{L1519P}, leading to production of a NEXT moiety, which accumulates when S3 cleavage is blocked, but which otherwise rapidly turns over into the ICD form in a ligand-independent manner.

To learn whether the enhanced S2 processing and emergence of a NOTCH3 ICD even under non-ligand activation conditions resulted in enhanced Notch downstream signaling, NIH3T3 cells were transfected with a Notch reporter system, 12X-CSL-luc, to record Notch activation immediately downstream of the Notch receptor (Chapman et al., 2006). In control NIH3T3 cells not transfected with wildtype or L1519P NOTCH3, there was no induction of 12X-CSL-luc activity irrespective of ligand activation or not, while cells expressing wildtype NOTCH3 receptor showed low activity in the “Notch off” state and robust reporter activation upon ligand activation, which was abrogated by DAPT treatment (Fig. 5B). In the \textit{NOTCH3}\textsuperscript{L1519P} receptor-expressing cells, in contrast, the Notch reporter was activated also in the “Notch-off” state, almost to the same extent as under
ligand-activating conditions, while DAPT in both cases abrogated the activation (Fig. 5B). These data show that the NOTCH3\textsuperscript{L1519P} constitutively activates Notch downstream signaling.

**NOTCH3\textsuperscript{L1519P} upregulates PDGFRB expression**

As dysregulated PDGF signaling has been linked to IMF (Arts et al., 2017; Cheung et al., 2013; Martignetti et al., 2013; Mudry et al., 2017), we next sought to explore a possible link between Notch and PDGF signaling in fibroblasts, the presumed cell type of origin for IMF. To this end, we conducted the experiments in a fibroblast cell line derived from HMF tumor stromal fibroblasts, in which we had previously removed the NOTCH\textsuperscript{2} gene by CRISPR/Cas9 cells to generate a fibroblast cell line with very low endogenous Notch signaling due to the removal of NOTCH\textsuperscript{2} (HMF\textDelta\textsuperscript{N2}) (Strell et al., 2019), but with the endogenous NOTCH\textsuperscript{3} gene retained, thus more closely mimicking the human IMF heterozygous situation. Wildtype or mutant NOTCH\textsuperscript{3}\textsuperscript{L1519P} genes were then stably introduced into the HMF\textDelta\textsuperscript{N2} cells via insertion into the AAVS1 locus, as described above. Notch signaling has previously been reported to activate expression of PDGFRB in vascular smooth muscle cells (Jin et al., 2008), and we therefore analyzed how levels of PDGFRB were regulated by wildtype and NOTCH\textsuperscript{3}\textsuperscript{L1519P} expression. HMF\textDelta\textsuperscript{N2} cells expressed a low level of PDGFRB protein, and a similar level was observed in HMF\textDelta\textsuperscript{N2} cells expressing wildtype NOTCH\textsuperscript{3}, while the PDGFRB level was higher in NOTCH\textsuperscript{3}\textsuperscript{L1519P}-expressing HMF\textDelta\textsuperscript{N2} cells (Fig. 6A). To assess whether the observed upregulation was a result of transcriptional activation, we assessed the mRNA levels of PDGFRB, as well as of four well-established Notch downstream genes (Hes\textsubscript{1}, Hey\textsubscript{1}, Notch\textsubscript{3} and NRARP). Hes\textsubscript{1}, Hey\textsubscript{1}, Notch\textsubscript{3} and NRARP expression was upregulated by wildtype NOTCH\textsubscript{3} expression and further elevated in cells expressing NOTCH\textsubscript{3}\textsuperscript{L1519P} (Fig. 6B). For PDGFRB, there was no upregulation of expression in the NOTCH\textsubscript{3} wildtype cells, while there was increased expression in the NOTCH\textsubscript{3}\textsuperscript{L1519P}-expressing cells (Fig. 6B), in line with the protein data in Fig. 6A.

To assess possible effects of the NOTCH\textsubscript{3} L1519P not only for PDGFRB expression but also further downstream in the PDGFRB signaling cascade, we monitored the levels of PDGFRB autophosphorylation and phosphorylation of the downstream effectors AKT and MAPK (p42 and p44 MAPK). Upon treatment of cells expressing wildtype or L1519P NOTCH\textsubscript{3} with PDGF-BB ligand, we observed an increased level of PDGFRB autophosphorylation in the NOTCH\textsubscript{3}\textsuperscript{L1519P}-
expressing cells as well as increased AKT phosphorylation (Fig. 6C). p42/44 MAPK phosphorylation was likewise augmented in the NOTCH3\(^{L1519P}\)-expressing cells as compared to control cells, while this was not the case for NOTCH3\(^{L1519P}\)-expressing cells (Fig. 6C). Collectively, this suggests that NOTCH3 L1519P elicits elevated PDGFRB downstream signaling, and in line with this notion, there was also an increase in cell proliferation (as judged by increased Ki67 expression) in the PDGF-BB-stimulated NOTCH3\(^{L1519P}\)-expressing cells while this was not the case for NOTCH3 wildtype-expressing cells (Fig. S3).

If upregulation of PDGFRB is a critical part of the pathogenic function of NOTCH3\(^{L1519P}\), it may be assumed that the dominant PDGFRB mutations associated with IMF are gain-of-function mutations. To test this, we assessed the nature of the PDGFRB R561C and P660T IMF mutations. Transfection of the PDGFRB\(^{R561C}\) and PDGFRB\(^{P660T}\) into HEK 293T cells revealed that they upon PDGF-BB ligand activation exhibited a higher level of receptor phosphorylation as compared to control PDGFRB, while a kinase-dead version of PDGFRB (carrying the L658P mutation) did not elicit receptor phosphorylation (Fig. 7A). PDGFRB dimerizes and becomes auto-phosphorylated upon ligand binding and autophosphorylation can occur on as many as 13 cytoplasmic tyrosine residues (Andrae et al., 2008; Tallquist and Kazlauskas, 2004). We next analyzed phosphorylation of tyrosine residues 751, 771, 1009 and 1021, and in all cases, phosphorylation was enhanced in the PDGFRB\(^{R561C}\) and PDGFRB\(^{P660T}\) mutants compared to wildtype PDGFRB (Fig. 7B). Finally, activation of downstream signaling events was analyzed for the various PDGFRB mutants. Increased phosphorylation of 42/44 MAPK and AKT was observed in cells transfected with the PDGFRB\(^{R561C}\) and PDGFRB\(^{P660T}\) mutants (Fig. 7C). These data are in agreement with a previous report indicating that the PDGFRB mutations found in IMF patients are gain-of-function mutations (Mudry et al., 2017). Together, these data indicate that NOTCH3\(^{L1519P}\) upregulates expression of PDGFRB and that PDGFRB IMF mutations are gain-of-function mutations.

**Discussion**

Infantile myofibromatosis has been linked to mutations in PDGFRB (Arts et al., 2017; Cheung et al., 2013; Martignetti et al., 2013; Mudry et al., 2017), but patients with mutations in the NOTCH3 receptor gene (NOTCH3\(^{L1519P}\)) have recently also been identified (Arts et al., 2017; Martignetti et al., 2013). In this report, we decode the molecular consequences of the NOTCH3\(^{L1519P}\) mutation.
and demonstrate that it produces a ligand-independent hyperactivated form of the NOTCH3 receptor. The hyperactive nature of NOTCH3\(^{L1519P}\) is supported by an elevated downstream signaling output, both with regard to Notch reporter activity and expression of the Notch downstream genes *Hes1, Hey1, Notch3* and *NRARP*. In line with an increased signaling output, we also observed enhanced production of the ICD from the NOTCH3\(^{L1519P}\) protein. Interestingly, hyperactive signaling was shown to occur in a ligand-independent manner, as evidenced by that the NOTCH3\(^{L1519P}\) receptor generated the NEXT and ICD forms also in the “Notch-off” state, i.e. without ligand stimulation, and that the mutated receptor was never observed at the cell surface, thus precluding it from normal ligand-dependent activation.

How can the lack of cell surface presence combined with hyperactive signaling be reconciled? While we do not have a complete understanding of this, the data support a scenario where both the proteolytic processing and the intracellular routing of the NOTCH3\(^{L1519P}\) receptor are altered. Notch receptors undergo a series of proteolytic processing steps (Fig. 1A) and there are several lines of evidence pointing to an enhanced and ligand-independent S2 processing of NOTCH3\(^{L1519P}\). Firstly, there is less of the S1-processed form, TMIC, of the NOTCH3\(^{L1519P}\) protein and the mutated TMIC has a shorter half-life, indicating that it may be more rapidly processed. Secondly, the amount of the S2-cleaved form, NEXT, is increased and NEXT is generated also in the absence of ligand, suggesting ligand-independent S2 processing. This spontaneous ligand-independent cleavage is likely still conducted by ADAM proteins, as the generation of NEXT was blocked by the ADAM inhibitor GI254023X. Thirdly, in line with the spontaneous production of NEXT, NOTCH3 ICD, which is constitutively generated from NEXT by the \(\gamma\)-secretase complex, was also produced under “Notch-off” conditions when proteasome-mediated degradation was blocked by MG132. The hypothesis that S2 processing is affected is corroborated by the notion that the L1519P mutation is located in the heterodimerization domain (Fig. 1A) and may thus lower the affinity between the ECD and TMIC moieties, facilitating S2 cleavage. In line with such an effect, the L1519P mutation is likely to destabilize the receptor (-1.8Kcal/mol according to the DUET software) (Pires et al., 2014). Collectively, these observations demonstrate that NOTCH3\(^{L1519P}\) is constantly “on”, generating NEXT and ICD. The observed increase in ligand-independent signaling from NOTCH3\(^{L1519P}\) is also in keeping with a previous study (Xu et al., 2015).
Disturbed intracellular routing of $\text{NOTCH}^3_{\text{L1519P}}$ is manifested by increased retention of $\text{NOTCH}^3_{\text{L1519P}}$ in the ER as well as in the lysosome, combined with reduced amounts in early endosomes. The PLA assay reporting on NOTCH3 receptors in an uncleaved or dipartite, heterodimeric state with the ECD and TMIC in close proximity, also revealed an altered intracellular distribution for $\text{NOTCH}^3_{\text{L1519P}}$, with aggregates localized close to the cell nucleus. Together, these findings may argue for an initial problem in ER to Golgi routing, which results in aberrant shunting of the mutated receptors from the ER to the lysosomes. There are indeed emerging evidence for transport systems directly between ER/Golgi and lysosomes, where cells under certain conditions promote the formation of ER-lysosome contacts, facilitating the transfer of ER-associated proteins to the lysosomal surface (Lie and Nixon, 2019). It is also increasingly realized that lysosomes, in addition to their role in protein degradation and autophagy, can act as a signaling hub and thus have both degrading and signaling functions (Lie and Nixon, 2019). It may thus be hypothesized that the aberrant and ligand-independent S2 processing occurs in the lysosome, a notion supported by that the exacerbated export of the mutated NOTCH3 ECD from the cell was abrogated by chloroquine, which blocks autophagosome-lysosome fusion. Aberrant S2 processing may also be facilitated by autocatalytic cleavage, which should be favored by the low pH in the lysosome (Fairchild et al., 2001; Lidell et al., 2003; Thuveson and Fries, 2000). It is however of note that receptor processing occurs via the S2 and S3 sites, as it was sensitive to ADAM and $\gamma$-secretase inhibitors. In a broader $\text{NOTCH}^3$ mutation and disease context, it is interesting to observe that a similar leucine-to-proline mutation in amino acid 1515, i.e. located only four amino acid residues away from the IMF mutation at position 1519, produces a distinct disease outcome. A patient with the $\text{NOTCH}^3_{\text{L1515P}}$ mutation, which also generates a receptor with enhanced signaling and secretion of ECD, instead developed cerebral small vessel disease (Fouillade et al., 2008). The reason why two so closely related NOTCH3 mutations give different disease outcomes is not understood, and it will be interesting to learn whether IMF patients carrying the $\text{NOTCH}^3_{\text{L1519P}}$ mutation develop vascular problems later in life, and conversely, whether IMF would be more frequently occurring in patients with NOTCH3 mutations linked to vascular disease.
As IMF mutations are found in both the NOTCH3 and PDGFRB genes, it is of interest to learn whether Notch and PDGF signaling are linked. Our data show that in vitro expression of NOTCH3\textsuperscript{L1519P}, but not wildtype NOTCH3, causes an upregulation of PDGFRB expression, both at the mRNA and protein levels, in fibroblasts, i.e. the cell type that is the likely origin for IMF tumors. This reveals that Notch acts epistatically over PDGFRB expression, corroborating a previously observed Notch-PDGFRB axis in vascular smooth muscle cells (Jin et al., 2008). Together with our confirmation that the IMF mutations PDGFRB\textsuperscript{R561C} and PDGFRB\textsuperscript{P660T} are gain-of-function mutations (Cheung et al., 2013; Martignetti et al., 2013), the data argue for a Notch-PDGF axis in IMF, where a hyperactive NOTCH3 mutation causes elevated PDGFRB expression, or alternatively elevated PDGF signaling is achieved by gain-of-function mutations in PDGFRB. In line with this hypothesis, it has previously been observed that elevated levels of PDGFRB have been noted in pediatric fibromatosis and myofibromatosis (Gibson et al., 2007).

The ligand-independent hyperactive signaling from the NOTCH3\textsuperscript{L1519P} receptor has implications for Notch therapy considerations for IMF (Andersson and Lendahl, 2014). One consequence of the ligand-independent signaling is that Notch activation may occur in places and at time-points where a wildtype ligand-dependent NOTCH3 receptor would not be normally activated, including cell contexts where no Notch ligands are expressed. Secondly, the notion that NOTCH3 is itself a target gene of the augmented activation by NOTCH3\textsuperscript{L1519P} suggests that the aberrant activation via NOTCH3\textsuperscript{L1519P} could result in a loop, where an initial expression of the mutant receptor leads to further elevated receptor levels. Finally, the observation that NOTCH3\textsuperscript{L1519P} acts ligand-independently and never reaches the cell surface suggests that antibody-based therapies that block NOTCH3 function via clamping the NRR (Wu et al., 2010) or affect ligand-receptor interaction (Chung et al., 2017; Lafkas et al., 2015; Tran et al., 2013) may not be effective on this type of Notch mutations. Instead, approaches based on small molecules interfering with Notch function further down in the signaling cascade (Andersson and Lendahl, 2014), may be more appropriate candidates for future therapy for IMF patients with NOTCH3\textsuperscript{L1519P} mutations.
In conclusion, our study identifies a NOTCH3-PDGFRB axis in IMF, where Notch signaling is epistatic over PDGFRB and where activating mutations can occur in both the NOTCH3 and PDGFRB genes. This information is of relevance for therapy considerations for IMF and for understanding how Notch signaling can get derailed by missense mutations in a Notch receptor.

**Materials and Methods**

**Establishment of CRISPR knock-out and knock-in cell lines.**

To generate the HEK293T cell line in which NOTCH1, NOTCH2 and NOTCH3 were ablated (HEK293T ΔN1-3), single guide RNA (sgRNA) targeting Notch1, Notch2 and Notch3 (Table S1) was cloned into the guide RNA Cas9 vector (Addgene pX459). HEK293T cells were transfected with the gRNA vector, and puromycin dihydrochloride (Sigma-Aldrich) at 1µg/ml was used for selection. Single cell colonies were isolated and subjected to Western blot as previously described (Strell et al., 2019). To integrate the V5-Notch3 and V5-NOTCH3<sup>L1519P</sup> sequence into the AAVS1 locus in HEK293T ΔN1-3 cells, the AAVS1-T2 gRNA vector (Addgene #41818) were co-transfected with the AAVS1.V5-Notch3 or AAVS1.V5-NOTCH3<sup>L1519P</sup> donor vector. The transfected cells were cultured for 5 days, thereafter 1µg/ml puromycin was used to screen single cell clones.

**DNA constructs**

The detailed description of the cloning work is published as Supplementary information.

**Cell culture and treatments**

HEK293T, HEK293T ΔN1-3 and its derived NOTCH3- and PDGFRβ-expressing cell lines, NIH3T3 and HMFΔN2 cells were maintained in DMEM, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies) at 37°C in a humidified 5% CO₂ atmosphere. All transfections were conducted using Lipofectamine® 2000 or Lipofectamine® Plus (Life Technologies) according to the manufacturer’s instructions. Detailed description of PDGF-BB treatment is described in Supplementary information. All reagents used are listed in the Table S2.
**Image streaming**

Cells were immunostained with an antibody to the NOTCH3 extracellular domain (1E4) and sorted by an image streamer, in which cells are simultaneously imaged and sorted (image flow cytometry). The detailed description is published as Supplementary information and antibodies are listed in the Table S3.

**Luciferase assay**

NIH3T3 cells were transfected with 12xCSL-luc and CMV-β-galactosidase together with NOTCH3 wildtype, L1519P plasmid or pcDNA3 vector as a control. For more detail, see the Supplementary information.

**PLA assay**

The PLA assay was performed by using the Duolink In Situ Detection Reagents Red kit (DUO92008, Sigma-Aldrich), according to the manufacturer’s protocol. Briefly, HEK 293T ΔN1-3 V5-NOTCH3 and HEK 293T ΔN1-3 V5-NOTCH3L1519P cells were cultured for 24 h on coverslips and fixed with 4% paraformaldehyde in PBS for 10 min, followed by blocking with 5% BSA in 0.1% Triton X-100 for 1h at 37°C. Primary antibodies, 1E4 (mouse, Merck) and anti-Notch3 (rabbit, Abcam), were incubated overnight at 4°C. After washes with PBS, the slides were incubated with the PLA probes anti-mouse MINUS (DUO92004, Sigma-Aldrich) and anti-rabbit PLUS (DUO92002, Sigma-Aldrich), according to the manufacturer’s protocol. The plasma membrane was stained using the anti-Na/K ATPase-Plasma Membrane Marker (Alexa Fluor® 488) (Abcam). Subsequently, the cover slips were washed with PBS and mounted with Duolink in situ mounting medium containing DAPI.

**Activation of Notch signaling by immobilized ligand**

Cell culture plates were coated with recombinant human Jagged-2 Fc chimeric protein (#P78504, R&D Systems) at 0.5 µg/ml, with IgG Fc fragment as control. Duration of treatments is described in the supplemental information.
Western blot analysis
The detailed description is published as Supplementary information and antibodies are listed in the Table S3.

Immunoprecipitation
Cells were incubated in DMEM containing 20 ng/ml Doxycycline for two days. Conditioned medium was collected and cleared by centrifugation at 2000 RPM for 5 minutes and incubated with anti-V5 agarose (Novus Biologicals) overnight, followed by washing of the beads with lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, and protease inhibitor (Roche). Bound proteins were detected with immunoblotting using anti-V5 antibody (Life technologies).

RNA extraction and Real-time PCR
RNA extraction and cDNA synthesis were accomplished as previously described (Chapman et al., 2006). Real-time PCR analysis was carried out on a 7500 Fast Real-Time PCR system with Fast SYBR Green Master Mix (Applied Biosystems) according to the manufacturer’s protocol. Primers for qPCR are listed in Table S2.

Cell proliferation assay
The detailed description is published as Supplementary information and antibodies are listed in Table S3.

Immunocytochemistry and confocal microscopy
Cells were plated on glass coverslips for 24 h, then fixed for 10 min at room temperature with 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 in PBS followed by blocking with 0.1% Triton X-100 and 2% BSA in PBS for 1 h. Primary antibodies were incubated at +4 °C overnight, and after washes in PBS, samples were incubated with secondary antibodies for 1 hour at RT and mounted with Vectashield medium with DAPI (Vector Laboratories). Images were captured by confocal microscopy (LSM 880 META (Carl Zeiss AG)). Image analysis was carried out using Image J (NIH) and Adobe Photoshop software. Antibodies are listed in the Table S3.
Statistical analysis

GraphPad Prism 8.4.3 was used to generate the bar graphs, and results were analyzed by student’s T-test to assess the statistical differences between experimental groups, where $p \leq 0.05$ was considered statistically significant. Error bars represent standard deviation of the mean. Pearson’s correlation coefficient was carried out as previously described (Adler and Parmryd, 2010).
Acknowledgements

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Conflict of interest

UL holds research grants from AstraZeneca and Merck AG, no personal remuneration. No disclosures for any other of the authors.
References


Fig. 1. Aberrant processing of NOTCH3\textsuperscript{L1519P}. A Schematic overview of Notch proteolytic processing. B Expression of the full length (FL), TMIC and NEXT/NICD forms was analyzed by Western blotting of cell extracts from 293T ΔN1-3 cells (Control) or 293T ΔN1-3 cells expressing wildtype (WT) and NOTCH3 \textsuperscript{L1519P} (L1519). β-actin levels were used as loading control. Relative levels of TMIC/FL were quantified by Image J from three experiments and analyzed by student’s T-test, ** p < 0.01.
Fig. 2. Lack of cell surface expression and increased retention of NOTCH3<sup>L1519P</sup> in the endoplasmic reticulum (ER). A-B Confocal images of wildtype and NOTCH3<sup>L1519P</sup>-expressing cells without (A) and with (B) permeabilization. Na, K-ATPase was used as a membrane marker. C Immunocytochemistry for NOTCH3 ECD (green) and the ER marker calnexin (red) from permeabilized cells; the plot shows the Pearson’s correlation coefficients for the colocalization analysis. D Immunocytochemistry for NOTCH3 ECD (red) and Golgi marker Giantin (green); the graph shows the Pearson’s colocalization coefficients for the co-localization analysis. *** p < 0.001. Scale bar, 20µm
Fig. 3. NOTCH3L1519P undergoes enhanced and ligand-independent S2 cleavage. A Western blot analysis of TMIC and NEXT/ICD fragments in the presence of the S2-inhibitor GI254023X or the γ-secretase inhibitor DAPT. B Treatment of the wildtype and NOTCH3L1519P-expressing cells with cycloheximide at different time points, as indicated. The graph shows densitometric quantification of TMIC using Image J. Data are shown from three independent experiments.
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Fig. 4. Relocalization of the ECD-TMIC heterodimer and exacerbated export of NOTCH3<sup>L1519P</sup> ECD into the cell medium. A Schematic representation of the PLA assay identifying the ECD-TMIC heterodimer. B PLA (red) was performed on wildtype or NOTCH3<sup>L1519P</sup>-expressing cells, using mouse anti-ECD 1E4 and rabbit anti-ICD Notch3 antibodies. The cell membrane (green) was directly labelled with anti-Na/K ATPase-Plasma Membrane Marker (Alexa Fluor® 488). C 3D surface rendering of the PLA and cell membrane staining. D Immunoprecipitation of supernatants from cells expressing wildtype or NOTCH3<sup>L1519P</sup> mutation using V5-agarose beads followed by Western blot using anti-V5 antibody. β-actin levels were used as loading control. E Western blot analysis of the conditioned media from cells expressing NOTCH3<sup>L1519P</sup> treated with DMSO, GM6001, GI254023X, Chloroquine, DAPT and MitMAB, as indicated. β-actin levels were used as loading control for the whole-cell extracts. F Immunocytochemistry for NOTCH3 ECD (green) and the endosomal marker EEA1 (red). G Immunocytochemistry for NOTCH3 ECD (green) and LAMP1 (red). H Immunocytochemistry for NOTCH3 ECD (green) and LAMP1 in cells treated with chloroquine (CQ). The plots show the Pearson’s correlation coefficient for the colocalization analysis. **<i>p < 10^{-2}</i>, ***<i>p < 10^{-3}</i>. Scale bar, 10 µm.
Fig. 5. NOTCH3<sup>L1519P</sup> produces ICD in a ligand-independent manner. A Western blot analysis of full length (FL), TMIC and NEXT and ICD fragments from cell extracts using an anti-Notch3 ICD antibody, following activation of the cells by immobilized Jagged2 (Jag2-Fc) and treated with the γ-secretase inhibitor DAPT or MG132, as indicated. B NIH3T3 cells were transfected with wildtype, NOTCH3<sup>L1519P</sup> or control plasmid together with 12XCSL-luc reporter and β-gal plasmids and cultured on immobilized Jagged2 (Jag2) in combination with treatment by DMSO or DAPT, as indicated. ***p < 10<sup>-3</sup>, ****p < 10<sup>-4</sup>. 

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***p < 10<sup>-3</sup>, ****p < 10<sup>-4</sup>. 

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Fig. 6. NOTCH3L1519P hyperactivates expression of Notch downstream genes. A Western blot analysis of PDGFRβ expression in HMF ΔN2 cells expressing wildtype or NOTCH3L1519P as compared to the parental HMF ΔN2 cells (Ctrl). The graph shows PDGFRβ expression normalized to β-actin using Image J. B Quantitative RT-PCR analysis of the Notch downstream targets genes Notch3, Hes1, Hey1, NRARP and PDGFRβ from wildtype or NOTCH3L1519P-expressing HMF ΔN2 cells. C Western blot analysis of PDGFRB, AKT and p42/44 MAPK phosphorylation upon PDGF-BB stimulation as indicated. The blot with p42/44 MAPK was stripped and reprobed with the β-actin antibody for loading control. Relative levels were normalized to β-actin using Image J.
Fig. 7. PDGFRB IMF mutations are gain-of-function mutations. A Analysis of phosphorylation levels from different PDGRFB mutants after stimulation with PDGF-BB, as indicated. B Quantification of phosphorylation of specific tyrosine residues (Y751, Y771, Y1009, Y1021) in the various PDGFRB mutants, as indicated (N=3). C Analysis of phosphorylation levels of PDGFRB, SHP2, Akt, p42/44 MAPK and eIF4E (as control) at different time points after PDGF-BB stimulation (0, 5, 15 and 60 min), as indicated. *** p < 0.001 as compared to the stimulated WT control (black bar) and # p <0.05 and ### p<0.001 as compared to unstimulated WT control.
Supplementary information

Material and Methods

DNA constructs

The NOTCH3<sup>L1519P</sup> mutation (c.4556 C>T) was generated by using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent) with the primer pairs in Supplementary Table 1, according to the manufacturer’s instructions. The resulting mutation was verified by DNA sequencing. The V5-Notch3 and V5-NOTCH3<sup>L1519P</sup> plasmids were generated by using oligonucleotides encoding the V5 epitope sequences and fused in frame in the coding region of wild type NOTCH3 gene or NOTCH3<sup>L1519P</sup> mutation at the N-terminus by PCR using the KOD Xtreme<sup>™</sup> Hot Start DNA Polymerase (Merck) To introduce the AAVS1 sites in the Notch3 knock-in expression vector, the sequences of V5-Notch3 and V5-NOTCH3<sup>L1519P</sup> were amplified by PCR using the Taq polymerase as described above and cloned into the HincII site in the AAVS1-TRE3G-EGFP donor vector (Addgene, #52343) to obtain AAVS1.V5-Notch3 and AAVS1.V5-NOTCH3<sup>L1519P</sup>. Constructs of human wild-type (WT) and kinase-dead (KD) PDGFRβ subcloned into the pcDNA 3 vector (Life Technologies) were kindly provided by C.H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). The KD construct carries the K634A mutation in which the nucleotide-binding lysine of the protein-tyrosine domain has been changed to alanine<sup>1</sup>. To generate the PDGFR mutants, R561C and P660T, a QuikChange II Site-Directed Mutagenesis Kit was used according to the manufacturer’s instructions, with the primers in Supplementary Table 1. The sequence of the full insert was determined for each construct.

Image streaming

Cells were dissociated with TryplE and blocked with 2% FBS in PBS on ice for 15 minutes, followed by filtering through a 70-μm cell strainer (BD Biosciences) to get single-cell suspensions. Cells were incubated with primary antibody 1E4 for 30 min on ice and after washing with blocking buffer, the cell suspensions were stained with goat anti-mouse Alexa 488 secondary antibody for 30 minutes at r.t in the dark. Cell nuclei were counterstained with DRAQ5 (red) before the acquisition. Samples were analyzed using a BD LSR Fortessa X-20 cytometer.

PDGF-BB treatment
Recombinant human PDGF-BB was purchased from R&D Systems. For PDGFRβ autophosphorylation assays, cells were starved in 0% FBS overnight and cooled on ice for 15 min before addition of 40 ng/ml PDGF-BB, conditioned medium or DMEM-vehicle. Cells were treated on ice for 1 hour, then harvested. For time-course analysis, PDGF-BB was directly added to the normal growth medium at 37°C to reach a concentration of 40 ng/ml. Cells were rinsed in ice-cold PBS and snap-frozen at different time-points.

Luciferase assay

NIH3T3 cells were transfected with 12×CSL-luc and CMV-β-galactosidase together with Notch3 wildtype, L1519P plasmid or pcDNA3 vector as a control. DAPT was added at 6 hour post transfection, as indicated. After incubation for 24 hours, the cells were lysed in Cell culture lysis reagent (Promega) and luciferase activity was measured in triplicate by the GloMax® Multi Detection System apparatus (Promega) using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer’s protocol. In all assays, relative luciferase activity was calculated as the ratio of Luciferase values normalized to β-gal levels.

Western blot analysis

Cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail (Complete, Roche). Protein concentrations were determined using the BCA method (Thermo Scientific, Pierce). The lysates were separated in 4–12% Mini-PROTEAN TGX gels (Bio-Rad) or 10% SDS-PAGE, and transferred to nitrocellulose membranes or PVDF membrane using Trans-Blot Turbo transfer system (Bio-Rad). The membranes were after blocking with 5% nonfat milk in PBS, incubated with primary antibody overnight at +4 °C followed by probed with HRP-linked secondary antibody (GE Healthcare). Antibodies are listed in the Supplementary Table 3. When needed, membranes were stripped once using the Antibody stripping buffer (Interchim) and reprobed with different antibodies. Complete removal of the primary and secondary antibodies were checked by incubation of the stripped membrane with the HRP-coupled secondary antibody followed by chemiluminescent substrate (Clarity Western ECL substrate, BioRad or SuperSignal kit, Pierce). A high-resolution CCD camera (ChemiDoc MP, BioRad) was used for signal detection and optimal exposure was determined by the software. For the statistical analysis of band intensity, results from three experiments were included.

Cell proliferation assay

Cells were cultured on glass coverslips in 12-well plates to 30% confluency using the medium described in “Cell culture and treatments”, followed by 18 hours in serum-reduced medium containing 5% FBS with 50ng/ml of PDGF-BB. After three washes with PBS, cells were fixed
with 4% PFA and permeabilized with 0.2% Triton X-100 in PBS. Samples were stained with primary rabbit anti-Ki-67 antibody over night at 4°C, followed by incubation with secondary antibody for 1h and counterstained with DAPI. Images were taken at 40x magnification. The numbers of Ki-67 positive cells were normalized to DAPI staining.

References:

Table S1

Primers used for mutagenesis, CRISPR knock-out cell line and qPCR

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Table S2

List of reagents

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### Table S3

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Figure S1. **A** Western blot of whole cell lysates from HEK 293T ΔN1-3 cells using antibodies for Notch1, 2, 3 and β-actin (as loading control). **B** Schematic figure depicting the AAVS1/293T-tet system. **C** Expression of similar levels of wildtype and L1519P NOTCH3 in the HEK293T ΔN1-3 cells in the presence of doxycycline (50ng/ml).
Figure S2. Image stream flow cytometry analysis of NOTCH3 cell surface expression using an antibody to the NOTCH3 extracellular domain (1E4) for wildtype and Notch3<sup>L1519P</sup> expressing cells.
Figure S3. Cell proliferation illustrated as percentage of Ki-67-positive cells normalized to DAPI. Data were obtained from three independent experiments.