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Characterization of a novel pharmacological Notch activator

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Eidesstattliche Erklärung

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I consent to the use of an anti-plagiarism software to check my thesis. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen 'Satzung der Justus-Liebig-Universität Giessen zur Sicherung guter wissenschaftlicher Praxis' in carrying out the investigations described in the dissertation.

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SUMMARY

The Notch signaling cascade is evolutionarily conserved across metazoan species. It exhibits pleiotropic actions during embryonic and post-natal development by governing a variety of cellular processes such as lineage commitment, maintenance of tissue stemness, proliferation, and apoptosis. Notch pathway activation takes place by the establishment of an extracellular interaction between the Notch transmembrane receptor and its cognate ligand on two adjacent cells followed by sequential proteolytic processing of the Notch receptor itself. This results in the release and translocation of the Notch intracellular domain (NICD) to the nucleus. Interaction of NICD with the DNA binding protein and transcription factor RBPJ forms the molecular assembly resulting in the transcription of Notch target genes. While a number of potent Notch inhibitors including γ-secretase inhibitors (GSIs) have been identified, Notch-'boosting' compounds remained elusive. Such compounds could be used in the context where Notch has been described to have tumor-suppressive functions such as in acute myeloid leukemia (AML).

The aim of my Ph.D. thesis was to characterize the Notch-activating small molecule Isoxazole 9 (ISX9). This compound showed significant stimulation of the Notch pathway in several cell lines. In the non-small cell lung cancer cell line, ISX9 resulted in strong induction of Notch target genes and activation of NOTCH1 and NOTCH3 receptors. Pharmacological blockade by γ-secretase inhibitors prevented ISX9-mediated NOTCH1 activation but not NOTCH3 activation. Using CRISPR/Cas9 technology, I was also able to demonstrate that the induction of Notch target genes by ISX9 was greatly reduced in NOTCH3-deficient cells (*NOTCH3* KO). Mechanistically, the Notch response elicited by ISX9 was found to be dependent on the transcription factor RBPJ. Moreover, ISX9 treatment leads to the expression of additional isoforms of NICD3 that are independent of S3 cleavage (γ-secretase-mediated). I was also able to show that splicing was necessary for the ISX9-activating effect suggesting that *de novo* transcription and/or splicing is required for ISX9 induction. Furthermore, I could show that ISX9 treatment causes an increase in intracellular calcium and that calcium is required for the induction of activated NICD3.

Considering the translational aspect, I investigated the effect of the Notch activating small molecule ISX9 in acute myeloid leukemia (AML). AML is characterized by differentiation blockade and rapid proliferation of immature myeloid cells where forced Notch activation promotes terminal differentiation. I was able to achieve a consistent effect in ISX9-treated AML cells. In addition to cell cycle arrest and upregulation of differentiation markers, induction of activated NICD3 and upregulation of Notch target genes were reported in ISX9-treated AML cells. In summary, terminal differentiation is induced by treatment with ISX9 and therefore ISX9 is a promising molecule for potential differentiation therapy in AML patients.

ZUSAMMENFASSUNG

Der Notch Signalweg ist eine evolutionär hoch-konservierte Signalkaskade. Dieser Signalweg reguliert sowohl embryonale als auch postnatale Entwicklungsprozesse indem sie sowohl Differenzierung als auch den Erhalt von Stammzellen, Proliferation und Apoptose steuert. Die Aktivierung von Notch findet extrazellulär durch Zell-Zell-Kontakte statt, wobei der Notch-Transmembranrezeptor auf der empfangenden Zelle durch den entsprechenden Notch-Liganden auf der benachbarten sendenden Zelle interagiert. Die Liganden-Rezeptor-Interaktion löst zwei aufeinanderfolgende proteolytische Spaltungen aus, die zur Freisetzung der intrazellulären Domäne von Notch (NICD) führen. Nach der Translokation in den Zellkern assoziierte NICD mit dem Transkriptionsfaktor RBPJ und induziert die Expression von Notch Zielgenen. Obwohl spezifische Notch Inhibitoren wie gamma-Sekretase Inhbitoren (GSI) bereits genutzt werden, sind Notch-stimulierende Substanzen noch weitgehend unbekannt. Solche Substanzen könnten zum einen therapeutisch genutzt werden im Zusammenhang von Krebserkrankungen, in denen Notch als Tumorsuppressor fungiert wie zum Beispiel der Akuten myeloischen Leukämie (AML) oder Lungenkrebs. Zum anderen können Notch-stimulierende Substanzen auch experimentell für ex-vivo Anwendungen eingesetzt werden.

Das Ziel meiner Doktorarbeit war es die Notch-aktivierende Substanz Isoxazole 9 (ISX9) zu charakterisieren. Dieser niedermolekulare Wirkstoff zeigte signifikant positive Effekte auf die Aktivierung von Notch Zielgene in mehreren Zelllinien. Eine starke Stimulation der Notch-Aktivität durch ISX9 habe ich in der nicht-kleinzelligen Lungen-Krebs Zelllinie festgestellt, mit starker Induktion von Notch Zielgenen und Induktion von aktivierten NICD1 und NICD3. Pharmakologische Blockade durch gamma-Sekretase Inhibitoren verhinderte die Aktivierung von NOTCH1 aber nicht die Aktivierung von NOTCH3. Mittels CRISPR/Cas9-Technologie konnte ich zeigen, dass bei Notch3-defiziente Zellen (*Notch3* KO) eine Induktion von Notch-Zielgene stark vermindert war. Mechanistisch ist der ISX9-aktivierende Effekt auch abhängig von Transkriptionsfaktor RBPJ. ISX9-Behandlung führt zur Expression von zusätzlichen Isoformen von NICD3, die unabhängig von der S3-Spaltung (gamma-Sekretase) ist. Ich konnte weiterhin zeigen, dass Splicing notwendig für den ISX9-aktivierenden Effekt war; also wird de novo Trankription und / oder Splicing für die ISX9 Induktion benötigt. Weiterhin konnte ich zeigen, dass ISX9-Behandlung einen Anstieg von intrazellulärem Calcium bewirkt und das Calcium benötigt wird für die Induktion von aktivierten NICD3.

Therapeutisch gesehen habe ich den Effekt von ISX9 in der akuten myeloischen Leukämie (AML) untersucht. Charakteristisch für AML ist die Blockade in der Differenzierung und Proliferation von unreifen myeloischen Zellen. Notch-Aktivierung führt zur Differenzierung und genau diesen Effekt konnte ich auch mittels ISX9 erzielen: Neben Zellzyklus-Arrest konnte man die Hochregulation von Differenzierungsmarkern nachweisen, sowie aktiviertes NICD3 und die Hochregulation von Notch Zielgenen. Zusammengefasst wird durch die Behandlung mit ISX9 die terminale Differenzierung induziert und deswegen ist ISX9 ein vielversprechendes Molekül für eine mögliche Differenzierungstherapie von AML Patienten.

1. INTRODUCTION

1.1 Notch signal transduction pathway

Cell-to-cell communication governs organismal development and tissue homeostasis. The generation of appropriate cellular responses relies on the transmission of an extracellular message to the nucleus. Among several mechanisms, the successful relay of biochemical cues from the cellular exterior to the interior is mainly attributed to a group of evolutionarily conserved signal transduction cascades. The Notch signaling pathway, being one of those, is pivotal for the intercellular transmission of biochemical signals. Cell-to-cell communication is established by the interaction between the transmembrane ligands on one cell and the Notch receptor on the surface of the neighboring cell (Kopan, 2012). By integrating the extracellular cues to the nucleus, the Notch pathway plays a key role in the establishment of a pattern of gene expression, thereby determining cell fate choices during embryonic development as well as maintaining adult tissue stemness (Fiúza & Arias, 2007).

1.1.1 Molecular architecture (pathway components)

Despite the comparatively simple molecular architecture with a limited number of core pathway components, the Notch signaling pathway determines a variety of cellular functions in several metazoans as well as in multiple cell types (Andersson et al., 2011).

The Notch gene was first described in association with the notched wing phenotype by John S. Dexter in 1914 (Dexter, 1914). Later, Thomas H. Morgan identified the corresponding allele responsible for the Notch phenotype (Morgan & Bridges, 1916). Molecular cloning of the *Drosophila* Notch gene revealed that Notch encodes a transmembrane receptor which interacts with membrane-bound ligands on the neighboring cells (Wharton et al., 1985; Fehon et al., 1990).

Notch ligands are Type-I transmembrane proteins. In *Drosophila*, there are two Notch ligands: one Delta and one Jagged homolog while several Delta/Serrate/Lag-2 (DSL) Notch ligand homologs are present in *C. elegans*. In mammals, there are five different Delta-Serrate-Lag (DSL) type ligands: Jagged1 (Jag1), Jagged2 (Jag2) and Delta-like 1 (Dll1), Delta-like 3 (Dll3) and Delta-like 4 (Dll4) (Greenwald, 1994; Maine et al., 1995; Lissemore & Starmer, 1999).

The fly genome encodes only one Notch receptor while *C. elegans* have two redundant Notch receptor proteins (Fitzgerald et al., 1993), and mammals have four Notch receptors: NOTCH1, NOTCH2, NOTCH3, and NOTCH4, with both redundant and exclusive functions (Krebs et al., 2003a; Cheng et al., 2007).

The effect of induced mutations has been studied in mice for each of the four Notch receptors (Notch1–Notch4) and four Notch ligand genes (Dll1, Dll4, Jag1, and Jag2). Mice with homozygous disruption of either Notch1 (Swiatek et al., 1994; Conlon et al., 1995) or Notch2 (Hamada et al., 1999) are embryonic lethal while Notch3-null (Krebs et al., 2003b) and Notch4-null mice (Krebs et al., 2000) survive without any apparent phenotypic abnormalities. However, Notch3 and Notch4 play important roles in embryonic vascular development (Krebs et al., 2000; Domenga et al., 2004). Targeted disruption of Dll1, Dll4, or Jag1 leads to embryonic lethality due to defects in somite boundary formation or in embryonic vascular development (Hrabě de Angelis et al., 1997; Gale et al., 2004; Xue et al., 1999) while Jag2 mutant homozygotes die at birth because of impaired craniofacial and T-cell development (Jiang et al., 1998). All these studies point to the non-redundant roles of the individual Notch genes and their ligands in mouse embryogenesis.

Notch receptors are single-pass transmembrane proteins with an extracellular domain that mediates ligand interaction and an intracellular domain engaged in the activation of target genes. The extracellular domain is composed of a conserved array of epidermal growth factor (EGF)-like repeats for ligand interaction followed by a negative regulatory region (NRR), which is composed of three cysteine-rich Lin12-Notch repeats (LNR) and a juxtamembrane heterodimerization domain (HD). The single transmembrane domain of the Notch receptor is followed by the intracellular components which include the RBPJ association module (RAM), seven ankyrin (ANK) repeats, and nuclear localization sequences located on both sides of the ANK domains. The end of the intracellular domain (C-terminus) contains conserved proline/ glutamic acid/ serine/ threonine-rich motifs (PEST domains) which regulate the stability of NICD, and the poorly defined transactivation domain (TAD) (Yochem et al., 1988; Lubman et al., 2004; Fleming, 1998).

1.1.2 Mechanism of activation

After translation, the NOTCH precursor protein is translocated from the endoplasmic reticulum to the Golgi apparatus. Before the Notch receptor gets integrated into the plasma membrane, it undergoes S1 cleavage in the Golgi apparatus by furin-like convertase forming the Notch heterodimer (Notch extracellular domain-Notch transmembrane and intracellular domain) (Logeat et al., 1998; Nichols et al., 2007) held together by calcium-dependent ionic bonds at the heterodimerization domain. Notch signaling activation is initiated by the interaction between the DSL (Delta–Serrate–LAG-2) domain at the amino terminus of the ligand and EGF repeats in the Notch receptors (Rebay et al, 1991; Shimizu et al., 1999).

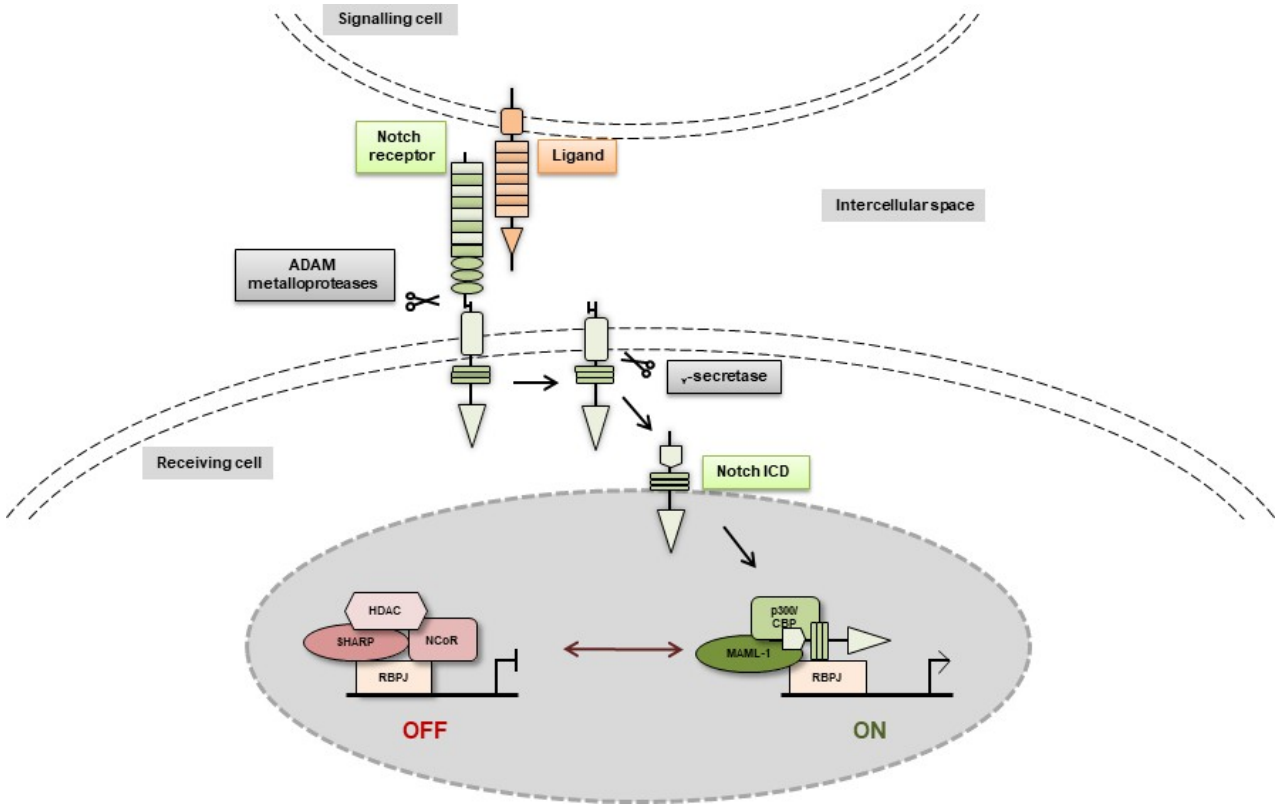


Fig.1 Mechanism of Notch pathway activation. Ligand stimulation triggers the sequential proteolytic processing of the Notch receptor releasing the Notch intracellular domain (NICD) which translocates to the nucleus and associates with the transcription factor RBPJ. This drives the transcriptional activation of Notch target genes. In the absence of stimuli, RBPJ recruits co-repressors to repress the expression of target genes.

Interaction of the Notch receptor with one of its ligands generates the pulling force to expose an extracellular metalloprotease site (S2 cleavage site) (Gordon et al., 2007 & 2015). S2 cleavage by transmembrane proteases of the ADAM/TACE (A disintegrin and metallopeptidase/ tumor necrosis factor α-converting enzyme) family results in a membrane-tethered intermediate called NEXT (NOTCH extracellular truncation) (Mumm et al., 2000). When the S2 cleavage step has occurred, the truncated receptor is subjected to S3/S4 cleavage by γ-secretase complex consisting of presenilin, as the enzymatic component, nicastrin, presenilin enhancer 2 (Pen2), and anterior pharynx-defective 1 (Aph1) (Siebel & Lendahl, 2017). In addition to Notch, this complex exhibits its catalytic activity on a number of other proteins such as APP, CD44, ErbB4, and the p75 NTR (Selkoe & Wolfe, 2007). It was traditionally assumed that the S3 cleavage of Notch follows the regulatory S2 processing more or less constitutively. However, recent pieces of evidence suggest that γ-secretase-mediated cleavage is also regulated with regard to cleavage efficacy and the position of the cleavage site within the receptor (Jorissen & De Strooper, 2010). Detailed analysis of the S3 cleavage product has identified several NICD variants with diverse N-

termini (NICD-V, NICD-L, NICD-S) amongst which NICD-V (starting at Val1744) exhibits the highest stability according to the N-end rule (Varshavsky, 1996) and represents the most of the Notch activating signal (Blat et al., 2002; Tagami et al., 2008).

After the final proteolytic processing, the intracellular domain of Notch (NICD) translocates into the nucleus where it acts as a co-activator of the transcription factor RBPJ (Su(H) in *Drosophila*, and Lag-1 in *C. elegans*). RBPJ is composed of three structural domains: Amino- and Carboxy-terminal domains (NTD and CTD respectively) flanking a central β-trefoil domain (BTD) (Kovall & Blacklow, 2010). RBPJ binds to the consensus CGTGGGAA motifs of the target genes via the NTD and BTD domains (Tamura et al., 1995). In the unstimulated state characterized by insufficient NICD, RBPJ represses transcription by assembling HDAC-containing co-repressors like SHARP (SMRT/ HDAC1-associated protein) (Oswald et al., 2002), SMRT (silencing mediator of retinoid and thyroid receptors) (Kao et al., 1998), N-CoR (nuclear receptor co-repressor) (Hörlein et al., 1995), KyoT2 (Taniguchi et al., 1998), and ETO (Salat et al., 2008) leading to transcriptional repression.

When Notch signaling is activated, binding of NICD to the BTD and CTD domain of RBPJ alters the RBPJ repressor complex displacing the co-repressors (Tamura et al., 1995). NICD-RBPJ complex is then stabilized by Mastermind (MAML) forming an NICD-RBPJ-MAML ternary complex. This ternary complex recruits cofactors, namely acetyltransferase p300 and Mastermind (Wallberg et al., 2002) to assemble an active transcription complex on downstream Notch target genes and trigger their transcriptional activation.

1.1.3 Signal downregulation

Prolonged Notch activation could be deleterious. Therefore, NICD half-life is tightly regulated at various levels, and anomalies in this regard lead to uncontrolled activation. Notch signaling activation is regulated by constitutive endocytosis and trafficking which, in turn, is modulated by several ubiquitin ligases such as NUMB (Guo et al., 2017; Luo et al., 2020), ASB (Diks et al., 2008), DTX1 (Hori et al., 2004), NEDD4 (Sakata et al., 2004), ITCH (Chastagner et al., 2008), CBL (Jehn et al., 2002), and FBXW7 (Oberg et al., 2001; Conner, 2016; Dutta et al., 2022). Endocytosed NOTCH receptors can either be activated or degraded in the cytoplasm while the rest are expressed on the cell membrane to transmit signals. This suggests that the availability of Notch receptor proteins for ligand binding is determined through the balance between degradation and receptor cycling (Le Borgne & Schweisguth, 2003).

NICD is phosphorylated by CDK8 within the TAD and PEST domains leading to its degradation by the E3 ubiquitin ligase FBXW7 (Tsunematsu et al., 2004; Fryer et al., 2004) until a new round of Notch activation commences. Upon degradation of NICD, the ternary complex disassembles and the signaling module is set for the next round of signaling upon stimulation.

1.1.4 Notch target genes

The best understood direct Notch target genes are Hairy and Enhancer-of-split [E(spl)] (HESR) genes in *Drosophila* and the related Hes and Hey genes in mammals that encode for the bHLH (basic helix-loop-helix) transcriptional repressors. These Notch pathway target genes negatively regulate the expression of downstream target genes such as tissue-specific transcription factors (Iso et al., 2003).

Notch target genes of the fly genome encode for class VI HLH proteins playing regulatory roles in fly development including segmentation, myogenesis, or neurogenesis (Klämbt et al., 1989; Rushlow et al., 1989). Typical characteristics of the Hairy and E(spl) proteins are a proline residue at a specific position in their basic domain and a highly conserved carboxyterminal tetrapeptide motif WRPW that recruits the co-repressor Groucho (Fisher & Caudy, 1998).

The mammalian counterpart of Hairy and E(spl) proteins are Hes transcription factors. The Hes proteins bind N- and E-box DNA sequences (CACNAG, CANNTG) and recruit TLE1-4 co-repressors (the orthologs of Groucho) through the WRPW motif (Iso et al., 2003). On the contrary, *Hey* gene family proteins bind to E-box sequences instead of N-box sequences and do not bind to TLE co-repressors because of the presence of YRPW (or YQPW) motif instead of the WRPW tetrapeptide (Iso et al., 2001). The function of Hes proteins is associated with the development of the nervous system (Nakamura et al., 2000) and sensory organs (Cau et al., 2000), pancreatic and endocrine cells (Jensen et al., 2000), and lymphocytes (Tomita et al., 1999) while Hey transcription factors play critical roles in the development of the cardiovascular system (Gessler et al., 2002).

Drosophila E(spl) proteins and mammalian Hes and Hey family members form homodimers and heterodimers that bind to the DNA target sequences via their HLH domains (Alifragis et al., 1997; Iso et al., 2001). Furthermore, they can interact with other HLH proteins and recruit transcriptional co-repressors. Hes proteins recruit TLE co-repressors that associate further co-repressors (Grbavec & Stifani, 1996) while Hey proteins interact with N-CoR and mSin3A which also subsequently attract other co-repressors (Iso et al., 2001). Interestingly, evidence based on promoter-reporter assays indicates distinct repression mechanisms associated with Hes and Hey proteins. Hes factors regulate transcription through both DNA-binding-dependent or -independent mechanisms whereas Hey proteins lead to transcriptional repression mainly via protein-protein interactions (Fischer & Gessler, 2007).

However, in parallel with the HESR genes, the Notch-activated transcriptome also includes several other signatures. Consistent with the role of Notch signaling in the commitment of lymphoid progenitor cells to the T-cell lineage, genes associated with T-cell development such as *pTα* (pre-T-cell receptor alpha chain) are Notch target genes (Reizis & Leder, 2002). Also, Gata3 is a direct transcriptional Notch target with implications in T helper (Th) cell differentiation (Fang et al., 2007). Two other Notch target genes *Deltex1* and *Nrap* serve as negative modulators of Notch activity resulting in B-cell development at the expense of T-

cell development and an increase in neuronal precursors, respectively (Izon et al., 2002; Lamar et al., 2001). Other Notch target genes include cyclin D1 (Ronchini & Capobianco, 2001), cyclin D3 (Joshi et al., 2009), p21 (Rangarajan et al., 2001), c-Myc (Palomero et al., 2006b), NF-κB2 (Oswald et al., 1998) and Notch3 (Palomero et al., 2006a; Tottone et al., 2019).

The diversity of the Notch-activated transcriptome is generated either at the level of promoter regulation or through crosstalk with the synergizing pathways. In general, RBPJ preferentially binds to the CGTGGGAA motifs of target gene promoters suppressing transcription in the absence of Notch ligands and switching to the co-activator complex upon ligand binding. Tissue-specific expression patterns of different NOTCH receptor homologs and the associated Notch-driven functional output could be explained by the induction of a distinct set of downstream genes by different NICDs. For example, activation of Notch2 promotes tumor growth of medulloblastoma cells whereas Notch1 activation has an antagonistic effect on the same tumor type (Fan et al., 2004). Activation of Notch3 leads to accelerated differentiation of pancreatic endocrine cells while a constitutively active form of the Notch1 receptor in the developing pancreas prevents differentiation of pancreatic progenitors towards both endocrine and exocrine fate (Hald et al., 2003). Target gene selectivity of different Notch receptors might be influenced by the DNA-binding preferences of different NICDs. For example, the Notch1-responsive promoters mainly consist of paired RBPJ-binding sites while a Notch3-responsive promoter contains a single RBPJ binding site with proximal binding sites for a zinc finger transcription factor (Ong et al., 2006).

Another contributing factor to the generation of a broader repertoire of Notch output in response to the extracellular cues is the specificity of the receptor-ligand pairing. Discrimination of cognate ligands by Notch receptors is mediated by the EGF repeats of the receptors in certain contexts. The *Drosophila* Notch with a point mutation at the EGF repeat 8 or the mouse *Notch2* with the corresponding mutation can only be activated by Delta but not by Serrate, or by Dll1 but not by Jag1, respectively (Yamamoto et al., 2012; VanHook, 2012).

Also, the two Delta ligand paralogs Dll1 and Dll4 perform redundant functions, for example, the maintenance of crypt progenitors in the adult small intestine of mice where their expression pattern overlaps significantly (Benedito & Duarte, 2005). However, context-dependent functional divergence was observed for these ligands in the aspect of T-cell development. Dll4-mediated Notch signaling activation is indispensable for T-cell fate determination of hematopoietic progenitors (Hozumi et al., 2008) while T-cell development is unaffected in Dll1-null mice (Hozumi et al., 2004). Further, patterning defects in mice lacking Dll1 are largely rescued by mesodermal expression of exogenous Dll1 but not Dll4 suggesting the inability of Dll4 to replace Dll1. Mechanistically this is supported by the *in vitro* findings that Dll4 but not Dll1 is a potential cis-inhibitor of Notch signaling resulting in an overall reduction in net Notch activity (Preuße et al., 2015). Recently, the contrasting roles for Notch ligands have been conceptually explained by the findings that Dll1 and Dll4 can activate the same receptor Notch1 with distinct signaling dynamics. This has been

demonstrated through the dynamic encoding model where Dll1 generates a clustering-dependent NICD pulse and Dll4 can activate a sustained signaling independent of the clustering process resulting in distinct transcriptional responses (Nandagopal et al., 2018).

There is compelling evidence that the crosstalk between the Notch pathway and other signaling cascades might also influence the diversity of signaling response. Crosstalk between Notch and Wnt signaling has been implicated in several developmental processes. Dishevelled, the cytoplasmic phosphoprotein of the Wnt pathway, relays the Wnt signal from receptors to downstream effectors. In fly, Dishevelled was demonstrated to antagonize Notch signaling activity by blocking Notch and its ligand Delta with a direct impact on bristle development (Axelrod et al., 1996). Glycogen synthase kinase-3beta (GSK3β) has a positive role in Notch signaling through phosphorylation of the intracellular domain of Notch, thereby protecting it from proteasomal degradation (Foltz et al., 2002). Furthermore, Notch and β-catenin positively stabilize the endogenous or exogenous levels of each other, and the NICD-dependent transcriptional activity is enhanced by β-catenin (Jin et al., 2009). In hair follicle pre-cortex, the Wnt β-catenin pathway regulates Notch activation through induction of its downstream target Jag1 and plays a key role in hair lineage commitment (Estrach et al., 2006). Wnt signaling also modulates vascular development and endothelial differentiation in mice through upregulation of Dll4 (Corada et al., 2010). In colon cancer cells, overactivation of β-catenin increases *NOTCH2* promoter activity suggesting a direct crosstalk of Wnt signaling with the Notch pathway leading to target gene activation (Ungerbäck et al., 2011).

Concerted action of Notch and TGFβ/BMP signaling have also been reported in many aspects of cellular functions. During smooth muscle cell differentiation, TGF-β1 promotes the expression of smooth muscle-specific genes through a significant inhibition of Notch3 and a reciprocal increase in Hes1 transcription (Kennard et al., 2008). In C2C12 myoblast cells and in mouse embryonic endothelial cells, Notch and BMP signaling synergistically activate the transcription of Notch target genes while BMP had little effect by itself (Itoh et al., 2004). This synergistic interplay between Notch and BMP signaling is also reflected by the effect of Smad downregulation on the reduced expression of stalk-enriched transcripts including *Hes1*, *Hey1*, and *Jag1* during angiogenic sprouting (Moya et al., 2012) and on the impairment of NICD-induced chondrocyte differentiation (Shang et al., 2016).

There are several indications supporting the fact that Notch signaling is linked to the hypoxia pathway. Hypoxia directly affects the Notch signaling by upregulating the expression of the Notch ligand Dll4 with a functional impact on tumor angiogenesis (Patel et al., 2005), arterial cell fate decision (Diez et al., 2007), and choroidal neovascularization (Dong et al., 2011). Also, HIF-1α-dependent transcriptional activation of Jag2 mediates hypoxia-regulated crosstalk between breast tumor cells and endothelial cells promoting vascular branching of the latter (Pietras et al., 2011). Exposure of cultured human neuroblastoma to hypoxic conditions leads to an increase in Notch1 protein which is thought to contribute to the development of a non-neuronal phenotype through the downregulation of SNS (sympathetic nervous system)-marker genes (Jögi et al., 2002). In fact, HIF, a global regulator of oxygen

homeostasis, is recruited at Notch-responsive promoters and it directly activates the Notch downstream targets which all together maintain an undifferentiated state in cortical neural stem cells, myogenic satellite cells, and C2C12 cells (Gustafsson et al., 2005). In addition to stem cell maintenance, hypoxia promotes epithelial-to-mesenchymal transition (EMT) phenotype and metastatic potential through Notch activity in breast (Chen et al., 2010), cervical, colon, glioma, ovarian (Sahlgren et al., 2008), and oral cancer (Ishida et al., 2013). However, in progenitor T-cells, hypoxia causes a reduction in FIH-mediated hydroxylation of NICD and its reduced interaction with the deubiquitinase USP10. This, in turn, leads to the destabilization of NICD and subsequent downregulation of Notch target genes (Ferrante et al., 2022).

It is well established that cell fate determination of neural progenitor cells relies on the morphogen signaling of Sonic Hedgehog (Shh). Notch activity regulates the localization of the Shh receptor Patched1 and mediates the trafficking of Smoothened, the key effector of Hedgehog signaling (Kong et al., 2015). Also, in NIH-3T3 fibroblasts, Notch signaling activation leads to accumulation of Smoothened and an increased level of full-length Gli3, the transcription factor of the Hedgehog pathway, priming the progenitor cells for their response to Shh (Stasiulewicz et al., 2015). Enhanced Gli1 activity increases the expression of Notch pathway components including receptors, ligands, and Notch target genes in the neuronal background (Takanaga et al., 2009; Stecca & Ruiz I Altaba, 2009). Also, the Hedgehog signaling directly regulates Hes1 as confirmed by the binding of Gli2 to the *Hes1* locus (Wall et al., 2009). Conversely, Gli2 and Gli3 have been described as direct downstream targets of NICD/RBPJ transcriptional complex in neural stem cells (Li et al., 2012). In sum, the integrative role of Notch and Hedgehog pathways is critical for the assignment of neural cell fate decisions (Borggrefe et al., 2016).

1.2 Non-canonical Notch signaling

Several biological processes are regulated through the canonical Notch signaling pathway. However, protease-independent or transcription-independent functions of Notch have also been described. Non-canonical Notch signaling has been investigated mostly in fruit flies. Although the association of non-canonical Notch signaling events in vertebrates is not well understood, the emerging relevance of non-canonical Notch signaling in association with mammalian pathologies is opening avenues for the intervention of Notch-dependent diseases including cancer and immune modulation (Alfred & Vaccari, 2018).

1.2.1 RBPJ-independent

One of the earliest pieces of evidence supporting the existence of an RBPJ-independent pathway was that activated Notch receptor blocks muscle cell differentiation by non-classical Notch signaling where mutant forms of NOTCH1 lacking sequences for RBPJ-NICD interactions effectively repress myogenesis. Furthermore, overexpression of HES1, the transcriptional repressor of MyoD, does not prevent muscle-specific gene expression in myoblast confirming the fact that NOTCH1-induced inhibition of muscle cell differentiation

does not involve transactivation by RBPJ (Shawber et al., 1996). RBPJ-independent activities of Notch have also been demonstrated to play a key role in keratinocyte differentiation (Rangarajan et al., 2001). Notch1, being a direct transcriptional target of KLF4 (Krüppel-like factor 4), mediates KLF4-mediated transformation of an adenovirus E1A-immortalized rat kidney cell line, RK3E. However, suppression of canonical Notch signaling by double negative mutants of MAML1 or RBPJ did not interfere with malignant transformation by KLF4, indicating that KLF4 could contribute to tumor progression by triggering Notch1 synthesis which signals through a non-canonical Notch1 pathway (Liu et al., 2009). In vascular endothelial cells, activated NOTCH4 plays a protective role in maintaining vascular stability in inflammatory situations. As one of the potential mechanisms, NICD4 generates an anti-apoptotic response through the inhibition of JNK activation via RBPJ-dependent signals. Additionally, the full cytoprotective activity of NOTCH4 is accomplished by upregulating Bcl-2 expression via an RBPJ-independent pathway indicating that Notch4 provides endothelial protection through both canonical and non-canonical signaling (MacKenzie et al., 2004).

1.2.2 Protease- and ligand-independent

Several reports suggest that perturbation of γ-secretase activity does not inhibit all Notch-related responses in transformed tumor cells indicating a non-canonical role of Notch signaling (Ayaz & Osborne, 2014). In T-cell acute lymphoblastic leukemia, which accounts for 10% to 15% of pediatric and 25% of adult acute lymphoblastic leukemia cases, NOTCH1 gain-of-function due to either chromosomal translocation of the *NOTCH1* gene (Ellisen et al., 1991; Pear et al., 1996) or point mutations at the heterodimerization domain (Malecki et al., 2006; Sulis et al., 2008) results in ligand or γ-secretase-independent spontaneous activation of the receptor driving T- cell oncogenesis. Additionally, two kinds of Notch gene fusion or rearrangements have been reported in breast cancer cell lines resulting in either membrane-tethered Notch1 proteins devoid of S2-cleavage site and hence are only regulated by γ-secretase or a rearranged and truncated cytoplasmic NICD2 which mediates transcriptional activation independent of γ-secretase cleavage (Robinson et al., 2011).

1.3 NOTCH3

The human neurogenic locus notch homolog protein 3 (NOTCH3) is the third identified mammalian member of the Notch receptor family. In addition to the central nervous system, where NOTCH3 was first identified (Lardelli et al., 1994), tissue distribution of NOTCH3 is mainly restricted to vascular smooth muscle cells (Joutel et al., 2000), and T_{Reg} cells (Anastasi et al., 2003).

Despite similar domain organization, NOTCH3 differs from NOTCH1 and NOTCH2 in certain features (Fig. 2). NOTCH3 consists of relatively smaller extracellular domains with a lower number of EGF repeats (NOTCH3 has 34 EGF repeats whereas NOTCH1 and NOTCH2 have 36 EGF repeats) and lacks the transactivation domain which is implicated in its weaker

transactivation activity (Aburjania et al., 2018; Hosseini-Alghaderi & Baron, 2020). NOTCH1 contains the longest PEST domain while the PEST domain of NOTCH3 is the shortest, and the lengths of the PEST domain of NOTCH2 and NOTCH4 are somewhere in between (Yuan et al., 2020).

Additionally, a comparison of intracellular subdomains of NOTCH1 and NOTCH3 reveals that the percentage amino acid identity is highly similar in the ankyrin repeat region (72%) while that for the RAM domain, the RE/AC region (the region flanked by ankyrin repeats and the C-terminal region that encompasses 118 amino acid residues in NICD1, and 125 amino acid residues in NICD3), and the C-terminal region are 41%, 50%, and 21%, respectively (Beatus et al., 2001). Although NOTCH3 engages the canonical pathway activation mechanism through binding to membrane-tethered type-I classical DSL domain-containing ligands, recent findings indicate a considerably high background of ligand-independent Notch3 signaling in comparison to Notch1 and Notch2 (Xu et al., 2015).

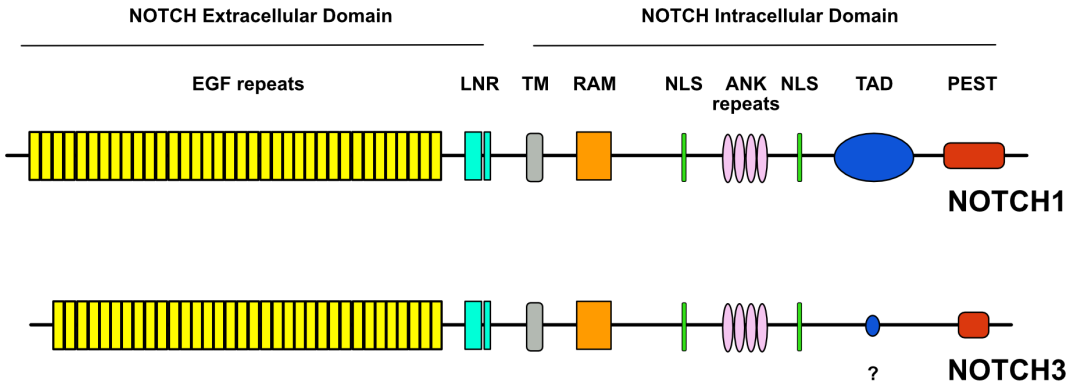


Fig. 2 Schematic view comparing the structural organization of human NOTCH3 and NOTCH1. Yellow: EGF (epidermal growth factor) repeats, Cyan1: Lin12-Notch repeats (LNR), Cyan2: HD (heterodimerization domain), Gray: TM (transmembrane domain), Orange: RAM (RBPJ-interacting domain), Green: NLS (nuclear localization signal), Pink: ANK (ankyrin repeats), Blue: TAD (transactivation domain), Red: PEST (proline, glutamate, serine, and threonine-rich domain)

The basal activity of Notch3 is mainly attributed to the difference in the first two LNR modules of the NRR domain which masks the metalloprotease cleavage site in the absence of a ligand. X-ray crystallographic analysis revealed that the interface between the first two LNR domains is stabilized by an aromatic conserved cluster of three tryptophan residues (W1412, W1425, and W1434). A histidine residue (H1471 in Notch1 and H1446 in Notch2) in the first LNR domain engages the third tryptophan in an aromatic π-stacking interaction which is replaced by a proline residue in Notch3, resulting in a less intrinsically stabilized conformation of autoinhibited Notch3. Additionally, other NRR structures of Notch3 are more disordered in comparison to the analogous structures of Notch1 and Notch2 (Xu et al., 2015). Furthermore, non-canonical Notch3 signaling might also involve non-canonical ligand- or endocytosis-mediated activation (Rauen et al., 2009; Gera & Dighe, 2018; Hu et al., 2014).

Although *Notch3*-knockout mice do not show any phenotypic defects, Notch3 is the predominant Notch receptor in vascular smooth muscle cells and plays a key role in differentiation towards the vascular lineage. In adult organisms, *Notch3* deficiency leads to vascular defects including reduced vessel integrity, hemorrhage, loss of blood–brain barrier function, and compromised adaptability in hypertensive conditions (Belin de Chantemèle et al., 2008). This is consistent with the fact that dominant mutations in the *NOTCH3* gene are associated with the most common hereditary cause of stroke and dementia in adults known as CADASIL (Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) with 100% penetrance (Hack et al., 2000). In CADASIL brains, mutations within the extracellular EGF-like repeats of the NOTCH3 receptor impair the clearance of the NOTCH3 ectodomain leading to the accumulation of NOTCH3 aggregates in the vicinity of vascular smooth muscle cells (Joutel et al., 2000). The developmental role of NOTCH3 has also been reported in neuronal differentiation (Kawai et al., 2017), and esophageal squamous cell differentiation (Ohashi et al., 2010).

Similar to other Notch receptors, constitutive activation of the Notch3 receptor (mainly in the form of gain-of-function mutations) is associated with several pathological conditions. Notch3 triggers a similar oncogenic program to drive T-cell leukemogenesis as in activated Notch1-driven T-ALL via upregulation of *Myc* (Choi et al., 2017). Notch3 mutations have been demonstrated in the NRR domain in TALL-1 and the endometrial cancer cell line Ishikawa, and in the PEST domain in breast and lung cancer cell lines leading to increased ligand-independent NOTCH3 activation (Bernasconi-Elias et al., 2016,). *Notch3* gene amplification correlating with its protein level promotes tumor progression in ovarian serous carcinoma (Park et al., 2006). Further, aberrant NOTCH3 expression is associated with distant metastases in a breast cancer model (Leontovich et al., 2018). Constitutively activated Notch3 in the developing lung of a transgenic mouse model prevents terminal epithelial differentiation (Dang et al., 2003). Also, activated Notch3 is involved in NF-κB and pTα-driven T-cell tumorigenesis (Bellavia et al., 2000 & 2002).

Expression of Notch3 is observed in $CD4^-CD8^-$ double negative (DN) thymocytes and the expression is lost before the transition to the double positive (DP) stage. Activated Notch3 in thymocytes leads to the development of aggressive T-cell lymphomas due to sustained CD25 expression and constitutive activation of NF-κB (Bellavia et al., 2000).

1.4 Pharmacological manipulation of the Notch signaling pathway

The implication of the Notch signaling cascade in a plethora of developmental decisions speaks in favor of its association with human pathologies and diseases (Siebel & Lendahl, 2017). Therefore, extensive research has taken place to investigate the signaling nodes that are amenable to therapeutic targeting.

Notch plays a key role in cellular differentiation and stem cell maintenance. Numerous oncogenic functions such as promoting cell survival, inhibition of apoptosis, development of treatment resistance, and crosstalk with other critical oncogenes have been ascribed to the Notch signaling pathway. Given the undifferentiated state of the cancer stem cells and the stem-cell-promoting properties of the Notch pathway, Notch signaling inhibition as a cancer therapy has been extensively investigated.

The attractive approaches for pharmacological intervention of the Notch signaling cascade are listed below: -

1.4.1 Antibody-mediated inhibition of Notch ligand-receptor interaction

The DSL (Delta-Serrate-LAG2) domain of the ligands and the EGF repeats of the Notch receptor are essential for ligand-receptor interaction which could be targeted by antibodies.

Jag1-overexpressing breast tumors often develop bone metastasis (Sethi et al., 2011). Fully human monoclonal antibodies against Jag1 reduce bone metastasis and show a synergistic effect with paclitaxel in reducing metastatic burden (Zheng et al., 2017). DLL3 is often upregulated and aberrantly expressed in neuro-endocrine tumors such as SCLC (small cell lung cancer) (Sabari et al., 2017). A DLL3-targeted antibody-drug conjugate (ADC) consisting of humanized DLL3-specific IgG1 monoclonal antibody shows effective targeting of DLL3-expressing tumor-initiating cells with durable tumor regression in patient-derived xenograft (PDX) tumors (Saunders et al., 2015). Inhibition of Notch signaling by a fully human IgG1 monoclonal antibody that binds human DLL4 exerts anti-tumor activity in the ovarian tumor xenograft model by promoting non-functional angiogenic sprouting (Kuhnert et al., 2015).

To circumvent the cytotoxicities associated with pan-Notch inhibition, monoclonal antibodies have been developed against individual Notch receptor paralogs. Oncogenic activation or overexpression of Notch1 has been reported in hematological tumors such as T-ALL (Ellisen et al., 1991), colorectal cancer (Liao et al., 2018), glioma (Purow et al., 2005), and in several chemotherapy-refractory tumors (Ferrarotto et al., 2017). A Notch1-blocking monoclonal antibody, Brontictuzumab, shows clinical benefits in adenoid cystic carcinoma patients with high NICD1 levels (Ferrarotto et al., 2018). The cross-reactive antibody OMP-59R5, inhibiting NOTCH2 and NOTCH3, promotes apoptotic cell death in NOTCH3-expressing pancreatic and other solid tumors (Yen et al., 2015). Another novel anti-Notch3 antibody–drug conjugate (ADC) PF-06650808 also shows early signs of antitumor potency in advanced breast cancer patients, and the drug response is correlated with *NOTCH3* expression (Rosen et al., 2020).

1.4.2 Modulation of proteolytic processing

The full-length Notch receptor undergoes S1 cleavage catalyzed by Furin proteases in the Golgi apparatus before being integrated into the plasma membrane. Inhibition of this processing, therefore, should hypothetically block the signaling response. Sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) has been demonstrated to be essential for Notch S1 cleavage and receptor trafficking to the cell surface, as revealed by a genetic complementation study in *Drosophila* (Periz & Fortini, 1999). Treatment with Thapsigargin, a small molecule inhibitor of SERCA, leads to reduced levels of the Furin-processed transmembrane NOTCH1 in human T-ALL cell lines with activating Notch1 mutations. The inhibitory effect of Thapsigargin on Notch maturation is also translated to suppression of leukemia growth *in vitro* and in T-ALL xenografts, and perturbation of *Drosophila* intestinal stem cell differentiation (Roti et al., 2013).

S2 cleavage of the Notch receptor is immediately prompted by ligand-receptor interaction and serves as the rate-limiting step of the Notch signaling pathway. Inhibition of ADAM metalloproteases, which catalyze the S2 cleavage reaction, might potentially dampen the amplitude of Notch signaling. In colorectal tumor xenografts, targeted inhibition of ADAM10 results in reduced NICD1 levels with a substantial decline in tumor growth and disease relapse, indicating an effective targeting of chemo-resistant cancer stem cells (CSCs) where Notch activity is thought to contribute to stemness and chemoresistance (Atapattu et al., 2016). Similarly, ADAM10 inhibitor GI254023X induces apoptosis in Jurkat cells through inhibition of Notch1 activation and downregulation of *HES1* transcripts (Ma et al., 2015). Because ADAMs regulate the processing of several other proteins, this approach is associated with off-target effects.

S2 cleavage could also be targeted by inhibitory antibodies specific for the NRR region of the Notch receptor. This strategy prevents ligand-directed unfolding of the NRR and subsequent processing of the Notch receptor by ADAMs.
Cleavage of the Notch receptor at the S2 site is followed by constitutive proteolysis at the S3 site mediated by the γ-secretase enzyme complex. Notch signaling can therefore be targeted by pharmacological blocking of the γ-secretase via GSIs (γ-secretase inhibitors). γ-secretase inhibitors suppress Notch signaling and decrease neurosphere formation in malignant gliomas (Gilbert et al., 2010). Inhibition of Notch pathway signaling by PF-03084014, a GSI, improves the anti-tumor activity of docetaxel in prostate cancer cells (Cui et al., 2015). Also, the downregulation of Notch signaling by GSI sensitizes hepatocellular carcinoma cells towards IL-24-induced cell apoptosis (Han et al., 2015). Another GSI NMK-T-057 leads to significant induction of apoptotic cell death and inhibition of EMT in breast cancer cells (Das et al., 2019).

However, the inhibition of Notch signaling by GSIs affects all the Notch receptors and this approach cannot be followed for specific targeting. Furthermore, the multi-protein γ-secretase complex is also involved in the processing of the amyloid precursor protein and

GSIs were originally developed to block the same. Since, γ-secretase complex is key for activation of the Notch pathway and Notch prevents the differentiation of gastrointestinal precursor towards the secretory cell fate, GSI treatment for Notch inhibition invariably results in the accumulation of goblet cells (van Es et al., 2005; Purow, 2012). This on-target toxicity along with the risk of other symptoms including infections, and non-melanoma skin cancers limit the therapeutic application of GSIs for Alzheimer's disease (Doody et al., 2013). However, pharmacological inhibition of presenilin-1 (PSEN1) prevents oncogenic NOTCH signaling with subsequent cell cycle arrest and improves overall survival in T-ALL without any associated gastrointestinal tract toxicity (Habets et al., 2019).

1.4.3 Targeting NICD-RBPJ interaction

Proteolytic processing of the Notch receptor liberates the Notch intracellular domain (NICD) which enters the nucleus and binds RBPJ and MAML. The NICD-RBPJ-MAML ternary complex brings about the activation of Notch target gene expression. RBPJ serves as the core regulator of this pathway playing the role of a molecular switch. Consistent with its function as a repressor, its depletion by genetic approaches or by RNA interference results in the derepression of Notch target genes (demonstrated by Morel & Schweisguth, 2000; Yuan et al., 2019, and in the current study). Alternatively, overexpression of the dominant negative version of MAML, which interacts with NICD and RBPJ but does not recruit the transcriptional machinery, blocks the Notch signaling (Fryer et al., 2002; Weng et al., 2003). A small molecule RBPJ Inhibitor-1 (RIN1), that perturbs the functional association of RBPJ with SHARP and NICD, suppresses the proliferation of NOTCH-dependent hematologic tumor cell lines (Hurtado et al., 2019). Also, disruption of the activating transcriptional ternary complex assembly by IMR-1 (Inhibitor of Mastermind Recruitment-1) leads to downregulation of Notch target genes with an inhibitory effect on xenograft tumor model and Notch-dependent somite development in zebrafish embryo (Astudillo et al., 2016). Another Notch inhibitor CB-103, interfering with the assembly of the Notch transcription complex, elicits growth arrest in Notch-addicted tumor cells such as DND-41 T-ALL cells and triple-negative breast cancer cells and, re-sensitizes chemo-resistant colorectal cancer cells (Lehal et al., 2020).

1.4.4 Benefits of Notch pathway activation

Consistent with the role of Notch signaling in normal organismal development, it can both promote or suppress disease progression. So far, several Notch signaling pathway blockers have been described with many under pre-clinical evaluation (Andersson & Lendahl, 2014). Consistent with the wealth of evidence on the oncogenic role of Notch, Notch-targeted therapies have mostly followed Notch inhibition approaches. However, cytoprotective and pro-differentiating effects conferred by activated Notch in a variety of cellular backgrounds point to the application of Notch-'boosting' agents in clinical or pharmaceutical contexts. These include differentiation therapy for AML patients (Lobry et al., 2013), regenerative therapy (to promote osteogenesis in osteoporosis patients or for bone fracture repair) (Xu

et al., 2022), or *ex vivo* applications such as the expansion of precursor T-cells (Varnum-Finney et al., 1998), and neural induction of embryonic stem cells (Lowell et al., 2006). However, there are very few reports on Notch- 'boosters' that could potentially enhance the Notch response.

Consistent with the notion that Notch signaling involves interaction between membrane-bound ligands on the signaling cell and the membrane-bound-receptor on the surface of the responding cell, cell-to-cell contact is a pre-requisite for activation of Notch signaling. In addition to membrane-tethered ligands, soluble ligands have been demonstrated in the past to bind to Notch and display biological relevance mainly through antagonistic effects on downstream signaling (Varnum-Finney et al., 2000). However, secreted forms of DSL ligands with a soluble extracellular domain lead to Notch1 activation upon antibody-mediated clustering or oligomerization in cultured cells suggesting that Notch signal transduction following ligand binding requires ligand multimerization (Hicks et al., 2002). In fact, a soluble form of human Delta-like-1 (hDLL1) promotes the expansion of murine hematopoietic progenitor cells indicating suppression of hematopoietic differentiation by ligand-mediated Notch activation (Han et al., 2000). Interestingly, in a co-cultured experiment, liver parenchyma endothelial cells were demonstrated to secrete a soluble form of Jag1 and promote cancer stem cell phenotypes such as tumorigenicity, metastasis, and chemoresistance of human colorectal cancer cell line by activating Notch signaling in a paracrine/angiocrine manner (Lu et al., 2013). This presents the prospect of testing genetically engineered soluble ligands for gene therapy applications such as tissue engineering and for artificial induction of cell growth, immune function, and expansion of T cells (Varnum-Finney et al., 1998).

Notch signaling has both oncogenic and tumor-suppressive roles in several malignancies. In neuroblastoma cell lines that lack detectable Notch activation, exposure to immobilized Notch ligand Jag1 inhibits cell proliferation by triggering G_0/G_1 arrest that resembled the effect in cells overexpressing Notch receptor intracellular domains, demonstrating a tumor-suppressive role of Notch signaling in neuroblastoma (Zage et al., 2012). In anaplastic thyroid carcinoma (ATC) which also shows a significantly low level of Notch1 expression, naturally occurring compounds Chrysin and Hesperetin suppress tumor growth by inducing apoptosis through functional activation of the Notch pathway (Yu et al., 2013; Patel et al., 2014). A potent NOTCH3-activating antibody that binds to an epitope within the negative regulatory region stimulates ligand-independent processing and activation of NOTCH3. This agonist antibody mimics the ligand-induced functional effects in cultured cells including increased proliferation and migration, and decreased cell death (Li et al., 2008) warranting further investigation for therapeutic application in Notch-hypomorphic diseases, for example, CADASIL, Alagille syndrome, and spondylocostal dysostosis characterized by mutations in the *NOTCH3*, *JAG1*, and *DLL3* gene, respectively (Siebel & Lendahl, 2017). An engineered DLL4 variant ($Delta^{MAX}$) with improved receptor-binding affinity, expression, and thermostability activates both endogenous and overexpressed Notch receptors more potently than wildtype DLL4. In functional assays, $Delta^{MAX}$ enhances the percentage of proliferating $CD8^+$ T-cells with increased expression of T-cell effectors IFNγ (interferon-γ)

and Granzyme B indicating highly effective Notch pathway modulation which could be used in T-cell-related applications (Gonzalez-Perez et al., 2023).

Genomic analysis identified several missense, nonsense, or splice-site loss-of-function mutations of the Notch receptor in a variety of squamous cell carcinomas correlating with reduced Notch transcript or protein level, and reduced Notch activation (Lefort et al., 2007; Taleb et al., 2014; Zhang et al., 2016). Transgenic mice with conditional epidermal expression of dominant MAML develop cutaneous squamous cell carcinoma suggesting a protective role of Notch signaling against malignant keratinocyte lesions (Proweller et al., 2006). Further, constitutive activation of the Notch1 receptor leads to G_0/G_1 cell cycle arrest and anti-proliferative effects in a human tongue cancer cell line (Duan et al., 2006). Furthermore, pharmacological activation of Notch signaling represses the Notch target ASCL1, a basic helix-loop-helix transcription factor, and neuroendocrine markers, eventually leading to a significant reduction of carcinoid tumor growth *in vivo* suggesting an inhibitory role of the Notch pathway against neuroendocrine neoplasms (Pinchot et al., 2011).

Inactivating Notch pathway mutations have been identified in chronic myelomonocytic leukemia (CMML) which has a propensity for progression to secondary acute myeloid leukemia (AML) (Patnaik et al., 2014), and ectopic Notch expression could suppress the aberrant expansion of granulocyte/monocyte progenitors and CMML-like disease pathology (Klinakis et al., 2011). Similarly, Notch activation signatures are suppressed in human and mouse acute myeloid leukemia, and conditional activation of Notch transgene or exogenous stimulation through Notch ligand-agonists enforces differentiation of leukemia-initiating cells towards macrophage and dendritic cell lineage and induces apoptotic death suggesting that Notch pathway reactivation could therapeutically target AML cells for disease regression (Lobry et al., 2013). Collectively, Notch signaling could negatively regulate tumorigenesis depending on the cellular context and therapeutic activation of Notch signaling could be an attractive strategy for augmenting Notch-mediated tumor suppression.

1.5 Role of Notch signaling in cellular differentiation

The Notch signaling pathway is indispensable for several stages of the cellular differentiation process. In certain situations, Notch signaling blocks differentiation programs to reserve the stem or progenitor cell pool while in some contexts, Notch activation promotes a differentiated cell fate.

The process for the development of somites (somitogenesis) giving rise to skeletal muscle, cartilage, tendons, endothelium, and dermis is regulated by the oscillatory gene expression pattern of the Notch pathway components such as Dll1, Lunatic Fringe, and the Notch downstream target gene Hes7 (Wahi et al., 2016). During myogenesis, Pax3-expressing progenitor cells in the myotome compartment of the somites rely on Notch1 activation for differentiation towards endothelial and smooth muscle cell fates (Mayeuf-Louchart et al., 2014). Repair of injured adult muscle is controlled by the stem cells called satellite cells through Notch signaling (Tran et al., 2013), and arterial differentiation is also supported by

Notch signaling at the expense of venous lineage. Heterozygous deletion of Dll4 (Gale et al., 2004) or conditional knockout of Hes1 or Hes5 (Kitagawa et al., 2013) or double deletion of Hey1 and Hey2 (Fischer et al., 2004) perturbs arterial cell fate decision, suggesting that Notch signaling is critical for arterial fate specification. Further, angiogenic sprouting is tightly regulated by Notch-signaling-mediated crosstalk between the tip and stalk cells. Tip-stalk cell identity is determined by the reciprocal interplay between VEGF and Notch signaling (Kangsamaksin et al., 2014). Activation of VEGF signaling induces Dll4 expression in the tip cell. Dll4 ligand leads to the activation of Notch signaling in the neighboring stalk cell where it suppresses VEGF signaling and promotes stalk cell signatures (Blanco & Gerhardt, 2013). In addition to the endothelial cells, Notch signaling plays a key role in the development of VSMCs through its antagonistic effect on the transcription factors Pax1, Scx, and Sox9 (Briot et al., 2014). Specific expression of Notch3 has been detected in VSMCs (Prakash et al., 2002) and pericytes (Joutel et al., 2000) where Notch3 signaling is required for arterial differentiation and maturation of VSMCs (Domenga et al., 2004) and for pericyte to caSMC (coronary artery smooth muscle cell) differentiation (Volz et al., 2015). The association of dysregulated Notch signaling and heart diseases further indicates the role of the Notch pathway in cardiac development. Hey1 and Hey2 are two Notch-responsive genes that affect endocardial differentiation (Kokubo et al., 2005). Also, Notch activation contributes to myocardial remodeling after cardiac injury or infarction (Ferrari & Rizzo, 2014).

Studies of Notch-driven cancers reveal that Notch signaling contributes to the development of innate and acquired immune systems and also to the homeostasis and immune response of mature immune cells. Notch signaling is also essential for the development of the correct number and type of hematopoietic cells of both lymphoid and myeloid lineages at the correct time point. It is pivotal for thymocyte development where it controls binary cell-fate choices at decisive checkpoints including T-cell- versus B-cell-specific gene expression, $CD4^+$ versus $CD8^+$ lineage specification, and αβ versus γδ T-cell-receptor expression (Osborne & Minter, 2007).

Notch signaling is continuously required throughout the early stages of intrathymic T-cell development from the generation of the earliest intrathymic T-cell precursors to the various stages of thymocyte maturation. Upon entry of the lymphoid progenitors into the thymus, localized expression of Notch ligands on cortical epithelium triggers activation of the Notch1 receptor initiating the process of thymopoiesis. The generation of early thymic progenitors and their progression through the initial stages of intrathymic maturation (double negative or DN1 to DN3) are dependent on Notch activation. The expression of a functional TCRβ chain allows survival and transition of the DN3 subpopulation to DN4, a process known as β-selection. The resulting pre-TCR signaling allows differentiation into $CD4^+CD8^+$ double positive (DP) thymocytes which will undergo positive and negative selection based on the interaction between the αβ TCR and the self-antigens presented by the MHC of the thymic epithelial cells (reviewed by Li & von Boehmer, 2011; Brandstadter & Maillard, 2019). Commitment to the $CD4^+CD8^+$ αβ lineage is supported by Notch activation over the $CD4^-CD8^-$ γδ lineage at the β-selection checkpoint and is impaired by conditional ablation

of RBPJ (Tanigaki et al., 2004) or inhibition of Notch signaling by dominant-negative Mastermind-like 1 (DNMAML) (Maillard et al., 2006). Studies in later stages of T-cell development suggest a controversial role of the Notch pathway in generating the $CD4^+CD8^-$ or $CD4^-CD8^+$ thymocytes (Laky & Fowlkes, 2008).

Fully differentiated effector T-cells can be of two types: Th1 or Th2 cells, depending on their characteristic cytokine profiles. Expression of Dll1 on APCs (antigen-presenting cells) favors the development of IFNγ-secreting Th1 cells whereas those T-cells exposed to Jag1-expressing APCs are directed towards Th2-cell fate to produce IL-4 and IL-5 (Amsen et al., 2004). Expression of Notch3 in T-cells improves protection against experimentally induced autoimmune diabetes through the expansion of T_{Reg} cells suggesting an important role for Notch signaling in their expansion (Anastasi et al., 2003). Upon antigen presentation by MHC class I-expressing cells, naive $CD8^+$ T-cells differentiate into cytotoxic T lymphocytes (CTLs). Notch2 signaling has been reported to promote CTL differentiation and effector functions (Maekawa et al., 2008; Sugimoto et al., 2010).

At the B-cell lineage of lymphoid development, Notch signaling regulates the development of marginal zone B-cells but not follicular B cells. The Notch ligand Dll1 is essential for marginal zone B-cell specification (Hozumi et al., 2004). Conditionally targeted deletion of Notch2 results in a diminished number of marginal zone B-cells (Saito et al., 2003), a phenotype also observed in mice lacking MAML1 (Wu et al., 2007). On the contrary, genetic ablation of SHARP, the negative regulator of Notch signaling, leads to an increase in marginal zone B-cells and a reduction in the follicular B-cell pool (Yabe et al., 2007).

Genetic studies in *Drosophila* indicated a key role of Notch signaling in the development of the central and peripheral nervous system. Notch-null mutant flies are associated with neuronal differentiation at the expense of epidermal structures leading to the developmental defect known as neurogenic phenotype (Lehmann et al., 1981). Notch signaling also plays a role in maintaining the neuronal progenitors and in determining two lineages of the vertebrate CNS (central nervous system): neurons, and glia. Ectopic activation of the Notch receptor in the retina of *Xenopus laevis* (Coffman et al., 1993) or in the developing eye of *Drosophila* (Fortini et al., 1993) shows inhibition of neuronal differentiation, while conditional ablation of Rbpj leads to accelerated neuronal differentiation in embryonic neurospheres (Gao et al., 2009). Further, mimicking Notch activation by expression of Notch downstream targets Hes1 or Hes5 was shown to inhibit neuronal differentiation at an early embryonic stage (Ohtsuka et al., 2001). In contrast to the inhibitory role of Notch activation on neuronal differentiation, in later stages of CNS differentiation, Notch signaling promotes gliogenesis enforcing differentiation of many glial subtypes except oligodendrocytes (Dorsky et al., 1995; Bao & Cepko, 1997; Scheer et al., 2001). In rodents, the ectopic expression of Notch downstream targets Hes1 or Hes5 promotes gliogenesis whereas ablation of these targets inhibits glial cell fate specification (Hojo et al., 2000; Furukawa et al., 2000). Activation of Notch signaling also contributes to the maintenance of neural stem/progenitor cells which is abrogated in conditional knockout mice lacking Rbpj (Imayoshi et al., 2010; Ehm et al., 2010).

Multiple pieces of evidence speak in favor of the pivotal role of Notch signaling in pancreatic specification and differentiation by regulating the fate of the Pdx1-positive progenitor cells towards endocrine or exocrine cell lineage. Inhibition of Notch activation in mice lacking Dll1 shows premature differentiation of the progenitor cells into endocrine cells. This is mainly attributed to the inhibitory effect of Notch signaling on the pro-endocrine transcription factor Neurogenin3 (Apelqvist et al., 1999). In this line of reasoning, sustained expression of a constitutively active form of the Notch1 receptor in the developing pancreas confirms the repressive activity of Notch1 on the differentiation of common pancreatic precursor cells (Hald et al., 2003). In addition to determining fate choice in the developing pancreas, Notch signaling also controls the plasticity of terminally differentiated pancreatic cells in adults (Hosokawa et al., 2015).

1.6 Regulation of Notch signaling by calcium influx

Calcium signaling influences several aspects of the Notch pathway and this regulatory interplay is implicated in various biological processes.

Notch activation depends on extracellular calcium concentration as the interaction between the Notch receptor with the ligands is calcium-dependent and mediated by the extracellular domains of the receptor and the ligand (Fehon et al., 1990). Spontaneous shedding of the Notch extracellular domain and consequent intracellular Notch activation is observed upon calcium chelation, and the same functional consequences are observed upon deletions or point mutations in Lin12-Notch repeats (LNR). This suggests that receptor activation is regulated by the calcium-dependent interaction of the Notch extracellular domain and the Notch transmembrane domain (Rand et al., 2000). Furthermore, many of the EGF repeats of the Notch1 receptor are known to possess calcium-binding motifs that are involved in ligand binding. Mutations that disrupt the consensus calcium-binding sequences affect the proteolytic susceptibility of the Notch receptor suggesting that calcium-dependent structural features influence the affinity of the Notch receptor for its ligand and subsequent activation (Cordle et al., 2008).

Notch signaling plays an integral role in the left–right asymmetry determination process during gastrulation of chick embryos which is, in turn, determined by the local and asymmetric accumulation of extracellular Ca^{2+}, as predicted by a mathematical model (Raya et al., 2004). Primary murine keratinocytes, when exposed to high-calcium media or transduced with retroviral vectors encoding NICD, express spinous differentiation markers suggesting that calcium induces spinous differentiation through canonical Notch signaling (Blanpain et al., 2006).

Calcium ion has been demonstrated to stabilize and enhance γ-secretase cleavage of Alzheimer Amyloid Precursor Substrate suggesting that calcium levels can influence the efficiency and kinetics of Notch receptor cleavage (Ho et al., 2010).

In prostate cancer cells, a significant reduction in NICD1 protein level and Hes1 mRNA expression was observed upon treatment with either KN-93, an inhibitor of CaMKII, or GSI, an inhibitor of the γ-secretase complex. Further, upregulation of Hes1 was observed in cells transfected with CaMKII-α expressing plasmid suggesting that CaMKII and intracellular calcium regulate Notch signaling (Mamaeva et al., 2009). In hepatocellular carcinoma, mitochondrial calcium uniporter regulator 1 (MCUR1)-mediated mitochondrial Ca^{2+} uptake promotes EMT and metastasis through Nrf2-mediated Notch1 activation suggesting that Ca^{2+} homeostasis is attributed to the regulation of the Notch signaling pathway (Jin et al., 2019). A subpopulation of NSCLC (Non-small cell lung cancer) cells that exhibit stem cell-like behavior is marked by surface expression of the voltage-gated calcium channel α2δ1 subunit. $\alpha2\delta1^{+}$ cells have Notch signaling-dependent self-renewing potential both *in vitro* and *in vivo* which is suppressed upon exposure to cyclosporin A, a calcineurin inhibitor. In this study, a reporter assay indicated the existence of NFATc2-binding at the *NOTCH3* promoter suggesting that calcium influx in NSCLC cells promotes tumor-initiating properties via Ca^{2+}-NFAT mediated activation of the Notch pathway (Ma et al., 2021).

1.7 Calcium-induced cellular differentiation

Calcium is a flexible and ubiquitous second messenger molecule. It plays a key regulatory role in various intracellular activities and gene expression patterns by transmitting extracellular signals to the cell's interior. Calcium is predominantly stored in the ER, lysosomes, and mitochondria where calcium concentrations are 100-fold higher than the steady-state calcium concentration in the cytoplasm. Cytoplasmic calcium buffering in resting cells is achieved by the collaborative action of more than 200 calcium-binding proteins (Gilabert, 2020). Intracellular calcium dynamics is an orchestrated outcome of the complex feedback mechanisms between cytoplasmic calcium, intracellular calcium stores, and influx and efflux mechanisms across the plasma membrane (Dupont & Combettes, 2016). Modulation in calcium dynamics influences several calcium-regulated mechanisms including contractility of muscle cells, memory formation in neurons, insulin secretion from pancreatic β-cells, release of hormones and neurotransmitters, and transcription factor activity (Li et al., 1998; Berridge et al., 2000).

Calcium-associated stimuli such as hormones, growth factors, and neurotransmitters activate the GPCRs (G protein-coupled receptors) or the protein tyrosine kinase-linked receptors leading to the activation of the phospholipase C family members linked to the receptors and subsequent generation of IP3 (inositol 1,4,5-trisphosphate). IP3 is produced by the catalytic hydrolysis of the precursor PIP2 (phosphatidylinositol 4,5-bisphosphate) to DAG (diacylglycerol) and IP3 itself. The binding of the second messenger IP3 to the IP3 receptors (IP3Rs) in the ER membrane leads to the release of calcium from the ER to the cytoplasm (Fedorenko et al., 2014). Depletion of ER calcium stores is sensed by the STIM1 proteins which associate with the CRAC channels in the plasma membrane resulting in a subsequent influx of calcium from the extracellular space. Calcium could also be released from other organelles such as mitochondria and lysosomes through other mechanisms.

Mechanical, electrical, or hormonal stimulations trigger calcium release from intracellular stores while neurotransmitters lead to the opening of the plasma membrane channel proteins for the entry of external calcium (Bootman et al., 2001).

An increase in cytoplasmic calcium signal could be sustained, transient, or oscillatory with variable outcomes. Regulation of gene expression and protein functions depends on the frequency, duration, and amplitude of calcium spikes (Dolmetsch et al., 1998). Response to calcium signals includes the function of numerous calcium-regulated proteins such as kinases and phosphatases, calcium-associated transcription factors, and Ca^{2+} channel proteins or enzymes. These effector proteins contain Ca^{2+}-binding motifs and relay the signal to downstream effectors that regulate cellular activities based on their localization and post-translational modifications coupled with changes in calcium concentration (Bagur & Hajnóczky, 2017). Calcium stimulation is reversed by sarcoendoplasmic reticulum Ca^{2+}-ATPases (SERCA) that return it from the cytosol to intracellular stores or to the external milieu (Bootman, 2012; Clapham, 2007).

Calcium-mediated signal transduction is initiated by the binding of cytosolic Ca^{2+} to a number of signaling molecules, one of which is the ubiquitous calcium-binding protein calmodulin (CaM). Calcium-bound CaM activates Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) which phosphorylate and activate the transcription factor CREB (cAMP response element-binding protein). CREB, in turn, binds to cAMP response elements (CREs) and drives the transcriptional activation of downstream genes (Barbado et al., 2009). For example, CREB supports the differentiation, and morphological and functional maturation of neural progenitor cells (Lepski et al., 2013).

Further, Ca^{2+}-bound calmodulin binds and activates the phosphatase calcineurin which dephosphorylates the cytosolic nuclear factor of activated T-cells (NFAT). This unmasks the nuclear localization signal of NFAT causing it to translocate to the nucleus where it acts as a transcription factor for a wide variety of genes (Hogan et al., 2003, Park et al., 2020). Upon pharmacological inhibition of calcineurin, NFAT is re-phosphorylated. Thereafter, the resulting conformational change of NFAT masks the nuclear localization sequence preventing its nuclear entry, and target genes are repressed (Oh-hora & Rao, 2009; Sharma et al., 2011). NFATc1 and NFATc2 are essential for differentiation and effector functions such as cytotoxicity, functional receptor expression, and cytokine production of T-cells, and also for maintenance of homeostasis in B-cells (Peng et al., 2001). NFAT-directed gene transcription also plays a crucial role in several aspects of neurogenesis such as synaptic plasticity (Graef et al., 1999), axonal growth (Nguyen & Di Giovanni, 2008), and neuronal survival (Vashishta et al., 2009).

Also, calcium-activated CaMKII phosphorylates HDAC4, a negative regulator of the transcription factor myocyte enhancer factor 2C (MEF2C), and promotes its exit from the nucleus thereby allowing MEF2C to induce muscle differentiation (Lu et al., 2000). Also, in hippocampal neurons, the chromatin modifier MeCP2 controls several processes of

neuronal development which is, in turn, regulated through its phosphorylation by calcium-induced CaMKII (Buchthal et al., 2012).

Elevation of calcium concentration in collaboration with receptor-mediated DAG synthesis results in membrane recruitment of conventional protein kinase Cs (Cullen, 2003) which, in turn, phosphorylates and modulates the activity of several downstream effectors including transcription factors and chromatin remodeling proteins, thereby regulating gene expression programs that drive cellular differentiation. For example, PKC activation is known to modulate signaling networks in oligodendroglial (Damato et al., 2021) and neuroblastoma (Leli et al., 1992) cell lines.

In neuronal development, calcium signaling contributes to the specification of neuronal identity. Calcium stimulation inhibits the transcriptional repressor DREAM (Downstream Regulatory Element [DRE] Antagonist Modulator) and thereby regulates the expression of genes involved in neural induction like c-fos and voltage-gated Ca^{2+} channels (Carrión et al., 1999; Naranjo & Mellström, 2012). NeuroD, a transcription factor regulating the survival and differentiation of many neuronal tissues, is activated by CaMKII-mediated phosphorylation and represents another example of calcium-mediated regulation in neurogenic processes (Gaudillière et al., 2004; Deisseroth et al., 2004).

In immune cells, calcium plays an essential role in the regulation of differentiation programs. For example, an increase in extracellular calcium is a danger signal in the synovial fluid of rheumatoid arthritis patients. Calcium and calciprotein particles (CPPs) induce the differentiation of monocytes into pro-inflammatory macrophage-like cells called calcium-macrophages through the activation of NLRP3 inflammasome (Murthy et al., 2022). Also, suppression of differentiation of immature $CD4^+CD8^+$ thymocytes into mature $CD4^+CD8^-$ and $CD4^-CD8^+$ cells for export to the peripheral lymphoid organs by Cyclosporin A, an inhibitor of calcium-dependent gene activation, indicates that calcium signaling is important for T-cell differentiation (Gao et al., 1988).

1.8 Small molecule ISX9 as an inducer of differentiation

In the seminal work by Schneider et al. (2008), Isoxazole 9 or ISX9 (N-cyclopropyl-5-(thiophen-2-yl)-isoxazole-3-carboxamide), belonging to a family of 3,5-disubstituted isoxazoles, was first described as a synthetic inducer of neuronal differentiation. This pro-neural effect of the small molecule ISX9 on cultured adult hippocampal neural progenitor cells involves the induction of Ca^{2+} signal and subsequent activation of CaMKII which, in turn, phosphorylates HDAC5 and relieves the repression of MEF2C inducing neurogenic response (Schneider et al., 2008). Similarly, ISX9 enhances Mef2-dependent adult hippocampal neurogenesis *in vivo* to increase dendritic complexity and improve spatial memory (Petrik et al., 2012). Also, mouse fibroblasts could be chemically reprogrammed to functional neurons using a cocktail of small molecules where ISX9, as a constituent, is essential for the cell-lineage switch characterized by the enrichment of neuronal-specific genes and downregulation of fibroblast hallmark genes (Li et al., 2015). Interestingly, ISX9

shows distinct effects on different types of neural progenitor populations. While ISX9 enhances differentiation in neural progenitor/stem cells, it induces cytotoxicity in oligodendrocyte precursor cells (OPCs), and suppresses angiogenic tube formation in mouse endothelial progenitor cells (EPCs) (Koh et al., 2015).

ISX9 stimulates the expression of the neuronal transcription factor NeuroD1 (neurogenic differentiation 1), which plays a key role in the development of the neuroendocrine cells originating from the lung, intestine, and pancreas. In fact, NeuroD1 is represented as one of the glucose-sensitive pancreatic β-cell-specific transcription factors that efficiently regulates the expression of the insulin gene (Aramata et al., 2005). ISX9 improves the expression of NeuroD1 and enhances glucose-stimulated insulin secretion in long-term *ex vivo* culture of human islets and in isolated pancreatic β-cells by ERK 1/2-dependent phosphorylation and regulation of p300 activity (Dioum et al., 2011). Consistent with this, ISX9 has been demonstrated to rescue β-cell dysfunction via CN/NFAT (calcineurin/ cytoplasmic nuclear factor of the activated T-cells)-mediated activation of calbindin D28K, a Ca^{2+}-binding protein that shows Ca^{2+} buffering and anti-apoptotic activity in neurons and pancreatic β-cells (Rabinovitch et al., 2001; Phillips et al., 1999; Kook et al., 2014). ISX9 shows its protective role against apoptotic death upon chronic serum withdrawal and inflammation in cultured pancreatic β-cells via recruitment of the transcriptional complex consisting of NFATc1/NFATc2, p300, and Creb1 to promote transcriptional upregulation of D28K. This was also reflected by the glycemic improvement of the transplanted human islets in streptozotocin (STZ)-induced diabetic mice, as indicated by lowering of blood glucose and increase in human C-peptide level in the plasma, upon daily administration of ISX9 (Pujol et al., 2018) suggesting the therapeutic potential of ISX9 to promote islet function in diabetic patients (Eizirik & Darville, 2001).

ISX9 induces the expression of several cardiac transcription factors such as Nkx2.5, GATA4, ISL-1, and Mef2c in hiPSCs indicating successful differentiation towards cardiac progenitor cells (CPCs). Transcriptomic analysis also revealed ISX9-mediated induction of several cardiac differentiation signaling pathways which is dependent on Wnt and TGFβ pathways, and upregulation of anti-apoptotic signaling and cardiac hypertrophy-associated miRNAs. Successful engraftment and differentiation of ISX9-induced CPCs into three different cardiac lineages leading to reduced fibrosis in ischemic mice further confirm the efficacy of ISX9 as a pharmacological tool for functional improvement of infarcted myocardium (Xuan et al., 2018).

In addition to pancreatic and neuronal differentiation, the specification of enteroendocrine (EEC) fate is mediated by NeuroD1 (Mutoh et al., 1997). Exposure of mouse small intestine-derived organoids and human terminal ileal organoids to ISX9 increases the expression of EEC lineage-specific transcription factors, leading to the enrichment of differentiated cells of the endocrine branch at the expense of non-endocrine lineages which is dependent on calcium signaling (Tsakmaki et al., 2020).

The two molecular subtypes of neuroblastoma: adrenergic (ADRN) and mesenchymal (MES) are determined by the super enhancer (SE)-driven activity of the associated transcription factors. Interestingly, ISX9 treatment enforces the expression of MES-like signatures comprising of gene sets of benign well-differentiated ganglioneuroma tumors with concomitant downregulation of ADRN genes. ISX9 increases global transcription except for the ADRN genes by modulating oncogenic SE circuits and establishing a chromatin accessibility landscape associated with low-risk neuroblastoma (Koeniger et al., 2023).

Furthermore, in neuroblastoma cells, ISX9 activates GLI1, the transcription factor of Hedgehog signaling, without the need for upstream Hh pathway elements, resulting in significant growth-inhibitory effects. Therefore, the application of ISX9 as a promising drug candidate for anti-neuroblastoma therapy has been proposed (Koeniger et al., 2021). Since Hedgehog signaling is known to exhibit regulatory crosstalk with the Notch signaling pathway (Borggrefe et al., 2016), it could be likely that ISX9 promotes the Notch signaling cascade. Also, the tumor-suppressive function of ISX9 in neuroblastoma cells is attributed to the capacity of ISX9 to induce differentiation terms including NOTCH3, a mesenchymal subtype marker (Koeniger et al., 2023). Similar to neuroblastoma, acute myeloid leukemia (AML) is characterized by impaired cellular differentiation, and therefore, reactivation of the Notch pathway has previously been reported to have pro-differentiation and tumor-suppressive effects in AML (Lobry et al., 2013). Following this concept, ISX9, if proven to be a Notch-inducing agent, would provide therapeutic benefits in certain clinical conditions where pharmacological activation of Notch alleviates disease symptoms.

2. AIM OF THE STUDY

The ancient and highly conserved Notch signaling is implicated in several aspects of metazoan development and adult tissue repair. Aberrant functioning of Notch signaling components leads to a spectrum of human disorders ranging from developmental syndromes such as CADASIL (cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy), Alagille syndrome, etc. to adult-onset diseases like cancers. Therefore, Notch signaling has been proven to be an attractive target for pharmaceutical intervention, and interest in its therapeutic targeting is rapidly expanding.

From a drug development standpoint, extensive research on the role of Notch signaling in tumorigenesis has resulted in the development of a vast majority of small molecule Notch antagonists while the identification of compounds that enable Notch activation has not drawn much attention. However, recent reports suggest that activation of Notch signaling might be promising in several clinical and pharmaceutical circumstances.

1. Since no pharmacological modifier to ‘boost’ the Notch signaling axis is commercially available, my first goal was to identify a small molecule that positively supports the Notch response and to delineate its mechanism of action.

2. My next aim was to provide insights into the therapeutic application of the Notch ‘booster’ for enforcing Notch reactivation in appropriate clinical contexts.

3. MATERIALS & METHODS

3.1 Materials

3.1.1 Antibodies

Anti-mouse IgG HRP	Cell Signaling (7076)
Anti-rabbit IgG HRP	Cell Signaling (7074)
Anti-rat IgG HRP	Jackson Immuno Research (112-035-072)
Cleaved Notch1 (Val1744) (D3B8), Rabbit polyclonal	Cell Signaling (4147)
NOTCH2 (D76A6) XP®, Rabbit monoclonal	Cell signaling (5732)
NOTCH3, Rabbit monoclonal	Cell Signaling (5276)
NOTCH4 (L5C5), Mouse monoclonal	Cell Signaling (2423)
GAPDH, Mouse monoclonal	Abcam (ab8245)
H3, Rabbit polyclonal	Abcam (ab1791)
H3K27ac, Rabbit polyclonal	Diagenode (pAb-174-050)
H3K27ac, Rabbit monoclonal	Cell Signaling (8173)
RBPJ, Rabbit monoclonal	Cell Signaling (5313)
ERK 1/2, Rabbit polyclonal	Cell Signaling (9102)
Phospho ERK 1/2, Rabbit monoclonal	Cell Signaling (4370)

3.1.2 Cell culture reagents, and materials

Ampicillin	Roth
BMS-906024	MedChem Express
Cell culture dishes	TPP
Cell culture flasks	Corning
Cell culture plates	Corning
DAPT	Enzo Life Science
DMSO	Amresco
PBS (DPBS)	Gibco
FCS (Lot No. P150702)	PAN Biotech GmbH
Hinokiflavone	Extrasynthese
Histamine dihydrochloride	Sigma
IMDM	Gibco
Isoginkgetin	PhytoLab
ISX9	Cayman
Lipofectamine 2000	Invitrogen
ML-792	Medkoo
Penicillin/Streptomycin	Gibco
Polyethylenimine (PEI)	Polysciences
Puromycin	Serva
RPMI 1640	Gibco

Trypan blue	Gibco
Trypsin	Gibco

3.1.3 Cell lines and media

Beko (Mouse pre-T lymphocyte cell line)	IMDM, 2% FCS, 2.3x SF-, 2 µL β-mercaptoethanol
H1299 (Human non-small cell lung cancer cell line)	RPMI-1640, 10% FCS
H69 (Human small cell lung cancer cell line)	RPMI-1640, 10% FCS, 1.25 mg/ml Amphotericin B
MT cell (Mouse mature T cell line)	IMDM, 10% FCS
THP1 (Human acute monocytic leukemia cell line)	RPMI1640, 10% FCS
Composition of freezing media	50% of FCS, 40% of the corresponding complete media, 10% of DMSO

3.1.4 Chemicals

1-butanol	Sigma-Aldrich
2-propanol	Sigma-Aldrich
4x Laemmli Sample Buffer	Biorad
Absolute QPCR ROX (500 nm) Mix	Thermo Scientific
Acrylamide solution 29:1	VWR
Agarose	VWR
Ammonium persulfate (APS) 98+%	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
Bovine serum albumin (BSA) 10 mg/mL	NEB
Bradford protein assay solution	Sigma-Aldrich
β-mercaptoethanol	Sigma-Aldrich
Chloroform	Millipore
Dithiothreitol (DTT)	Amresco
DNA Loading Dye	Thermo Scientific
dNTP Set (High concentration)	Thermo Scientific
ECL Select Western Blotting Detection Reagent	GE Healthcare
EDTA disodium salt dihydrate	Applichem
Ethanol	Sigma-Aldrich
Ethidium bromide solution	Roth
Glycerol	Applichem
Glycine	Applichem
HEPES	Sigma-Aldrich
Hydrochloric acid 1N	Millipore
Igepal CA-630 (NP-40)	Sigma-Aldrich
Methanol	Sigma-Aldrich

Nonfat dry milk powder	Roth
Phenylmethansulfonylfluoride (PMSF) >99%	Amresco
Potassium chloride	Millipore
Protease inhibitor cocktail mix	Roche
Sodium acetate	Roth
Sodium azide	Merck
Sodium chloride	Sigma-Aldrich
Sodium dodecyl sulfate	Roth or Biorad
Sodium fluoride	Sigma-Aldrich
Sodium hydroxide	Millipore
Sodium orthovanadate	Sigma-Aldrich
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Tris base (Trizma)	Roth
Triton X-100	Amresco
Trizol	Ambion
Tween 20	Amresco

3.1.5 Common buffers and solutions

LB agar plates	1% bacto-trypton (w/v), 0.5% bacto-yeast extract (w/v), 0.5% NaCl (w/v), 1.5% LB agar
LB medium	1% bacto-trypton (w/v), 0.5% bacto-yeast extract (w/v), 0.5% NaCl (w/v)
SF^- (100X)	40X MEM NEAA, 40X penicillin/streptomycin, 12.5 mg/mL Peptone, 0.2 mg/mL Insulin
TAE (50X)	2 M Tris-HCl pH 8.0, 57.1 mL/L acetic acid, 0.05 M EDTA
TBS (10X)	0.25 M Tris-HCl pH 7.42, 1.37 M NaCl, 27 mM KCl
TE	10 mM Tris-HCl pH 8.0, 1 mM EDTA
0Ca-Tyrode's solution	140 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 12.2 mM Glucose, 1 M $MgCl_2$, pH 7.4
1Ca-Tyrode's solution	140 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 12.2 mM Glucose, 1 M $MgCl_2$, 1.25 mM $CaCl_2$, pH 7.4

<u>Alkaline lysis method for plasmid isolation</u>

GTE	25 mM Tris-HCl pH 8.0, 50 mM Glucose, 10 mM EDTA
NaOH/ SDS	0.2 mM NaOH, 1% SDS (w/v)
KAc buffer	3 M KAc, pH 5.5

<u>Extraction method for mammalian genomic DNA isolation</u>

DNA Extraction buffer	10 mM Tris-HCl pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5 % Sarcosyl, added freshly: 1mg/ml Proteinase K
Precipitation buffer	75 mM NaCl in 100 % EtOH

Whole cell extraction

Lysis buffer	20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40 (v/v), 10% glycerol (v/v), 0.5 mM Na_3VO_4, 10 mM NaF, 1 mM PMSF, 1x protease inhibitor cocktail mix

Nuclear Extraction

Sucrose buffer	320 mM sucrose, 3 mM $CaCl_2$, 2 mM MgAc, 0.1 mM EDTA, 10 mM Tris-HCl pH 8.0, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, 0.5% NP-40, 1x Protease inhibitor
High salt buffer	20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 800 mM KCl, 0.2 mM EDTA, 1% NP-40, 0.5 mM PMSF, 1x Protease inhibitor mix, 0.5 mM DTT

SDS-PAGE and Western blotting

Stacking buffer	0.5 M Tris-HCl pH 6.8, 0.4% SDS
Resolving buffer	1.5 M Tris-HCl pH 8.8, 0.4% SDS
Running buffer	25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% SDS (w/v)
Transfer buffer	25 mM Tris-HCl pH 8.0, 190 mM glycine, 20% methanol (v/v)
Washing buffer	1X TBS, 10% Tween 20
Stripping buffer	10% SDS, 1M Tris-HCl, pH 6.0, vacuum-filtered

3.1.6 Enzymes and standards

6x DNA loading dye	Thermo Fisher Scientific
BSA	NEB
DNA MW marker GeneRuler 1 kb Plus #SM0313	Thermo Fisher Scientific
DNA MW marker GeneRuler 100 bp Plus #SM0323	Thermo Fisher Scientific
DNase I (For RNA-Seq)	Qiagen
DNase I (For RNA isolation)	Ambion
Dream Taq	Thermo Fisher Scientific
M-MuLV reverse transcriptase	NEB
PageRulerTM Plus protein ladder 10-180 kDa #26616	Thermo Fisher Scientific
PageRulerTM Plus protein ladder 10-250 kDa #26619	Thermo Fisher Scientific
Phosphatase	NEB
Proteinase K	Roche
Restriction enzymes	NEB
RNase A	Roche
Q5	NEB
T4 DNA Ligase	NEB
Trypsin	Gibco

3.1.7 Strains and plasmids

E. coli Top10 (Invitrogen) F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ *lac*X74 *rec*A1 *ara*D139Δ(*araleu*)7697 *gal*U *gal*K *rps* L (StrR) *end*A1 *nup*G

Eukaryotic expression vectors

Table 1.

Plasmid	Source
pcDNA3.1-Flag2	From Francesca Ferrante
pcDNA3.1(+) mNICD3	From Mark Chiang

Genome targeting vectors

Table 2.

Plasmid	Source
pSpCas9(BB)-2A-Puro (PX459) V2.0	**Addgene**
px459 V2.0 pSpCas9(BB)-2A-Puro_hRBP2N guide1E20_CRISPR #9 (gRNA #1)	From D. Giaimo
px459 V2.0 pSpCas9(BB)-2A-Puro_hRBP2N guide2E20_CRISPR #15 (gRNA #2)	From D. Giaimo
px459 V2.0 pSpCas9(BB)-2A-Puro-hNOTCH3 guide #1_E1 (gRNA #1)	This study
px459 V2.0 pSpCas9(BB)-2A-Puro-hNOTCH3 guide #2_E33 (gRNA #2)	This study

3.1.8 Primers, probes, and oligos

3.1.8.1 Primers

3.1.8.1.1 Primers for qPCR

Table 3.

Gene name	Probe	Sequence
Human		
GAPDH	#45	F: ACACCCACTCCTCCACCTTT R: TGACAAAGTGGTCGTTGAGG
HPRT	#73	F: TGACCTTGATTTATTTTGCATACC R: CATCTCGAGCAAGACGTTCA
HEY1	#17	F: CAGGGAGCCAGCATGAAG R: GAGCCGAACTCAAGTTTCCA
HEY2	#60	F: CCAGCAGTGCATCAGTATGTC R: CAGGCACTTACGAAACACGA
HEYL	#78	F: TCCCCACTGCCTTTGAGA R: TTTCAAGTGATCCACCGTCA
HES4	#78	F: GCTCAGCTCAAAACCCTCAT

		R: CTCACGGTCATCTCCAGGAT
NOTCH1	#27	F: GTGTGCACTGCGAGGTCA R: CACAGATGCCCAGTGAAGC
NOTCH2	#51	F: GACATTGATGACTGCCTTGC R: GGCACTTATCCCCAGTGAAA
NOTCH3 (EXON 9-10)	#32	F: AGCCCGATGGCTACGAGT R: ATGAGAAGCTGGCGATGC
NOTCH3 (EXON 24-25)	#67	F: CCTAGTCCTGGCTCCGAAC R: CATCGGGGAAGCAGTGAT
NOTCH3 (EXON 27)	#10	F: CCAAGGGTGAGAGCCTGAT R: CCATGCCTGGCTCCTCTA
NOTCH4	#27	F: GTCTCTGAGTGGTGGCTGTG R: CTGCTGACATCAGGGGTGT
RBPJ	#21	F: CAGCAAGCGGATAAAAGTCA R: AACTGTCTGGGATCGTAGTCG
Mouse		
GusB	#25	F: TGTGGGCATTGTGCTACCT R: ATTTTTGTCCCGGCGAAC
β-Actin	#63	F: GGATGCAGAAGGAGATTACTG C R: CCACCGATCCACACAGAGTA
Tbp	#97	F: GGGGAGCTGTGATGTGAAGT R: CCAGGAAATAATTCTGGCTCAT
Gapdh	#45	F: CCAAAACATCATCCCATCGT R: AACTGACACGTTTGGGGTTG
Hes1	#20	F: TGCCAGCTGATATAATGGAGAA R: CCATGATAGGCTTTGATGACTTT
Gm266	#81	F: CAAGGCCGACCTAGATGC R: GTCGTGATTTCCAGGAACG
Hey1	#17	F: CATGAAGAGAGCTCACCCAGA R: CGCCGAACTCAAGTTTCC
PreTCR	#45	F: CAGCTCTCCTTGCCTTCTGA R: CCTGGCTGTCGAAGATTCC
Dtx1	#49	F: GCCACATGTATCACCTGCTC R: ATGGCTTTGCAGGTTGGA
Cd25	#89	F: CAATGGAGTATAAGGTAGCAGTGG R: CATCTGTGTTGCCAGGTGAG
Lgmn	#85	F: GAATTCCCACGGTTCTGC R: AGCACCAGGCTGAGAAGC

3.1.8.1.2 Primers for sequencing

Table 4.

Plasmid	Primer name	Sequence
px459 V2.0 pSpCas9(BB)-2A-Puro-hNOTCH3 guide #1_E1	U6-seq	GAGGGCCTATTTCCCATGATTCC
px459 V2.0 pSpCas9(BB)-2A-Puro-hNOTCH3 guide #2_E33	U6-seq	GAGGGCCTATTTCCCATGATTCC

3.1.8.1.3 Primers for PCR validation

Table 5.

Purpose	Primer name	Sequence
hRBPJ knockout screening	hRBPJ screen fw	ATCATCTGTACTGTCTTGG
	hRBPJ screen rev	AGATGAATAAAAAAGGCTCC
hNOTCH3 knockout screening	hNOTCH3 screen fw	CAGGGCTAACTTGAGCTCCC
	hNOTCH3 screen rev	CCAGGAGCACTCCAACTGAC

3.1.8.2 Probes

LNA® Locked Nucleic Acids residues are marked with capital letters.
All the probes contain Fluorescein/Rhodamine dyes: 5'-FAM-TAMRA-3'

Table 6.

Probe	Sequence
#2	TTCTCCTG
#10	CCACCTCC
#17	AGGAGCTG
#21	CAGAGCCA
#27	CAGGCAGC
#32	CTGCTCCC
#45	CTGGGGCT
#49	TGGTGGCC
#51	GGCAGGAG
#60	CTTCCCCA
#73	TCCTCAGC
#78	AGCTGGAG
#103	CTCTTCCC
#110	CAGAGGCT

3.1.8.3 Oligos for cloning guide RNAs

Table 7.

Guide RNAs	Oligos for cloning
hNOTCH3 gRNA #1	F: CACCGGTGGCGGCGACATCGGGCGA R: AAACTCGCCCGATGTCGCCGCCACC
hNOTCH3 gRNA #2	F: CACCGTATCAGTGGCCAATTCGAGG R: AAACCCTCGAATTGGCCACTGATAC

3.1.9 Database and software

CRISPR Design Tool: https://crispor.tefor.net
Image and Illustration Tool: https://affinity.serif.com/en-us/
Ensembl- Geome browser for verterbrate genomes: www.ensembl.org/
NCBI- National Center for Biotechnology Information: www.ncbi.nlm.nih.gov/
Adobe Acrobat 9.0 Adobe System
Microsoft Office 2016: Excel, Word, PowerPoint
Prism 5.0- GraphPad
QuantStudioTM Design & Analysis v1.5.1
SnapGene®
Vilber Fusion-Capt for Fusion Fx7: EvolutionCaptv18-02

3.1.10 Technical equipment

Agarose Gel Chamber	Peqlab
Centrifuges	Beckman, Eppendorf 5417R, Thermo Scientific Heraesus Megafuge 16, Hettich Mikro 22R
Developer (Western-blot)	Vilber Fusion FX7
Gel documentation system	Intas
Incubators	Nuare 5810E
Incubator shaker	Edmund Buhler Gmbh SM30 Control
Light microscope	Olympus CK2
PCR-Thermoblock	Biometra T3 Thermocycler
pH meter	Mettler Toledo Seven Compact
Photometers	NanoDrop® ND2000c Spectrophotometer
Real-Time PCR device	Applied Biosystems™ QuantStudio
SDS-PAGE	Biorad Protean 2/3
Tissue culture hood	Heraeus Instruments
Western blotting	Biorad Mini Trans-Blot Cell

3.2 Methods

3.2.1 Bacterial cell culture methods

3.2.1.1 *E. coli* culture methods

E. coli. Top10 strain was utilized for plasmid amplification. Cells were grown in LB media supplemented with 50 mg/mL Ampicillin by continuous shaking at 200 rpm for 10h at 37°C. Bacterial culture and glycerol were mixed in a 1:1 ratio in a total volume of 1 mL and frozen as glycerol stock at -80°C.

3.2.1.2 Competent cell transformation

2 µg of DNA was added to 50 µL of competent cells and incubated on ice for 20 min, followed by heat shock at 42°C for 45 sec and then recovery for 2 min on ice. 250 µL of pre-warmed LB media was added to the cells followed by incubation at 37°C for 90 min.100 µL of this suspension was plated on LB agar plates supplemented with 50 µg/mL Ampicillin.

3.2.2 Eukaryotic cell culture methods

3.2.2.1 Cell culture conditions

All eukaryotic cell lines were maintained at 37°C in the incubator supplied with 5% CO_2 for optimal growth. Cells were passaged once 80% confluence was achieved. For passaging, cells were either collected after trypsinization for adherent cells (eg. H1299) or directly for suspension cells (eg. THP1), followed by centrifugation at 1200 rpm for 5 min. The cell pellet was further dissolved in fresh media and split into cell culture plates.

The concentration of the compounds applied in all experiments in the thesis are listed below:

Table 8.

Compounds	**Concentration**
ISX9	20 µM
DAPT	23.1 µM
BMS-906024	200 nM
Isoginkgetin	30 µM
Hinokiflavone	30 µM
ML-792	0.25 µM
Histamine	100 µM

An equal amount of DMSO was used for the control treatments in each experiment except for histamine treatment where H_2O was used as the solvent control.

3.2.2.2 ISX9 treatment in H1299 cells

2 X 10^6 H1299 cells were seeded in a 15 cm cell culture dish. After attachment, the media is aspirated and replaced with 20 mL of media with either DMSO or 20 µM ISX9. After 12h or 24h incubation, the media was removed and the cell monolayer was collected in 1 mL fresh media by scraping. Trypsin-mediated cell dissociation of cell monolayer leads to transient activation of NOTCH1 and upregulation of Notch response genes. Hence, trypsin was used for routine sub-culturing of cells but not for the collection of treated cells for accurate interpretation of results (Liu et al., 2014).

3.2.2.3 Transfection of H1299 cells using polyethylenimine

For overexpression, 1 X 10^6 H1299 cells were seeded in 10 cm cell culture dish with complete RPMI media. After attachment of the cells, 325 µL of sterile 1X PBS was mixed with 10 µg of plasmid DNA in tube I, and 309 µL of 1X PBS was combined with 20 µL of PEI in tube II. The solution from tube II was added to tube I, the contents were mixed together by pipetting and incubated for 30 min at room temperature. In the meantime, the medium of the seeded cells was replaced with serum-free medium and the incubated solution was added dropwise to the cell monolayer. After 6h, the medium was aspirated out and replaced with fresh complete medium.

3.2.2.4 Generation of CRISPR/Cas9-depleted H1299 cells

Genomic depletion of RBPJ and NOTCH3 was achieved by CRISPR/Cas9-based technique. The guide RNAs for genetic depletion of NOTCH3 were designed using the online tool available at http://crispor.tefor.net/. The desired 5' overhangs were added and the oligos were phosphorylated, ligated, and introduced into the dephosphorylated vector backbone (BB)-2A-Puro-(PX459)-V2.0 digested with BbsI. The *RBPJ*-knockout clones were generated using the combination of hRBPJ guide RNAs gRNA #1 and gRNA #2. The *NOTCH3*-knockout clones were generated using the combination of hNOTCH3 guide RNAs gRNA #1 and gRNA #2 and the guide sequences are as follows: -

Table 9.

Gene name	Guide name	Guide sequence
RBPJ	hRBPJ gRNA #1	TCATGCCAGTTCACAGCAGT*GGG*
RBPJ	hRBPJ gRNA #2	TCCTTCTACATGCAAGTATC*TGG*

Table 10.

Gene name	Guide name	Guide sequence
NOTCH3	hNOTCH3 gRNA #1	GTGGCGGCGACATCGGGCGA*CGG*
NOTCH3	hNOTCH3 gRNA #2	TATCAGTGGCCAATTCGAGG*TGG*

The underlined letters in the guide sequences indicate the PAM sequences. The sequence of the guide RNA oligos are mentioned in Section 3.1.8.3.

0.5 X 10^6 H1299 cells were seeded and after attachment, cells were transfected with the appropriate targeting plasmid(s) (10 µg each of two plasmids each encoding for one guide RNA or 20 µg of the empty vector) using Lipofectamine 2000 Transfection reagent according to the manufacturer's instructions. After 6h of incubation, the medium was replaced with the fresh complete medium. After 48h of transfection, cells were subjected to antibiotic selection by the addition of puromycin (for the first day with 2 µg/ mL and then subsequently with 1 µg/ ml for 2 days) to the culture medium. The following day, the medium was replaced with an antibiotic-free medium for recovery and waited till the monolayer became confluent. Thereafter, the monolayer was trypsinized, cells were counted and serial dilution was performed to establish single-cell clones. The diluted cell suspension was aliquoted in 96-well plates and each well was filled up to 200 µL with complete medium. Single-cell clones were further expanded, and divided into four parts for freezing, subsequent expansion, gDNA extraction, and preparation of whole cell extract for characterization and knockout validation. Based on the feature of the PCR product for all examined clones compared to the empty vector control, the potential knockout clone was anticipated. The effect of genomic depletion on both transcript and protein levels was analyzed by RT-qPCR and Western blotting respectively.

3.2.3 Molecular biology methods

3.2.3.1 Purification of plasmid DNA from bacteria

Competent cells were transformed with genetically manipulated plasmids and plated on antibiotic-containing LA plates. Selected bacterial colonies were inoculated in 3 mL of LB broth supplemented with the appropriate antibiotic for selection and incubated for 12-18h at 37°C with continuous shaking. From the overnight grown bacterial culture, 2 mL was reserved as glycerol stock for subsequent expansion, and the rest of the culture was utilized for plasmid DNA extraction by alkaline lysis method. For miniprep purification of plasmid DNA, the bacterial suspension was centrifuged at 5000 rpm for 3 min. The cell pellet was resuspended in 150 µL lysis buffer. Thereafter, 300 µL of NaOH/SDS solution was added and mixed by inverting. This was followed by neutralization with 150 µL 3M KAc buffer, mixed by inverting, and incubated on ice for 10 min. The lysate was then centrifuged at full speed for 10 min and then the supernatant was transferred to new tubes and mixed with 500 µL isopropanol followed by vortexing and incubation on ice for 10 min. The DNA was pelleted down and washed twice with 70% EtOH and then finally resuspended in TE buffer. The purified plasmid DNA was validated by sequencing and used for transfection.

3.2.3.2 Genomic DNA extraction from mammalian cells

Genomic DNA was isolated for PCR-based screening for positive cell clones bearing the desired CRISRP/Cas9-mediated genomic deletion. The expanded single-cell clones were washed with PBS and the cell pellet was dissolved in 200 µL of DNA extraction buffer and incubated for 2 days at 37°C. Subsequently, lysates were transferred into fresh tubes and mixed with precipitation buffer (a mixture of ice-cold 100% EtOH and 50 mM NaCl). The

content of the tubes was mixed properly by inverting several times until the DNA spool was visible which was then transferred to 70 % EtOH and DNA was precipitated by centrifuging at 10000 rpm for 5 min at 4°C. Afterward, the supernatant was removed, and the genomic DNA pellet was dried at room temperature and dissolved in 50 µL of TE for 12-24h at 37°C and was further used for PCR screening.

3.2.3.3 Quantification of nucleic acids

DNA and RNA concentrations were quantified spectrophotometrically by measuring OD_{260} with the Nanodrop2000c.

3.2.3.4 Restriction digestion

For restriction digestion, DNA templates were incubated with the New England Biolabs (NEB) enzymes and respective buffers according to the manufacturer's protocol. All enzymatic reactions were incubated at 37°C for 2h. For the purpose of cloning, the digested plasmid DNA was dephosphorylated to prevent re-circularization. Digested DNA products were analyzed by agarose gel electrophoresis and the desired product with the appropriate size was purified using GelElute Extraction Kit (Eurogentec).

3.2.3.5 Polymerase Chain Reaction (PCR)

The specific DNA products were amplified by Polymerase Chain Reaction using commercially synthesized primers. The PCR was performed in automated, temperature-dependent cycles of denaturation, annealing, and elongation in a thermal cycler. The annealing temperature was set according to the GC content of the primers and the extension time was determined on the basis of the amplicon length (60 sec/1000bp).

Below are the two separate PCR conditions for the detection of gene editing in *RBPJ*- and *NOTCH3*-knockouts.

For detection of successful gene editing at the h*RBPJ* locus, the composition of the PCR reaction mixture was set as below: -

Table 11.

Components	**Volume (µL)**
DNA templates	200 ng
Dream Taq Buffer	2
dNTPs (10 µM for each)	1
Forward primer (100 pmol/µL)	0.2
Reverse primer (100 pmol/µL)	0.2
Dream Taq DNA polymerase	0.2
H_2O	Up to 20 µL

The PCR conditions were as follows: -

Initial denaturation 95°C, 5 min
Denaturation 95°C, 30 sec
Annealing 56°C, 30 sec
Elongation 72°C, 30 sec
Repeat from 2 for 32 cycles
Final extension 72°C, 10 min

For detection of successful gene editing at the h*NOTCH3* locus, the composition of the PCR reaction mixture was set as below: -

Table 12.

Components	**Volume (µL)**
DNA templates	200 ng
Dream Taq Buffer	3
dNTPs (10 µM for each)	2
Forward primer (100 pmol/µL)	0.1
Reverse primer (100 pmol/µL)	0.1
DMSO	1.5
Dream Taq DNA polymerase	0.2
H_2O	Up to 20 µL

The PCR conditions were as follows: -
Initial denaturation 95°C, 5 min
Denaturation 95°C, 30 sec
Annealing 60°C, 30 sec
Elongation 72°C, 32 sec
Repeat from 2 for 38 cycles
Final extension 72°C, 10 min

PCR amplicons were analyzed by agarose gel electrophoresis. The concentration of agarose gel was decided based on the sizes of the DNA fragments to be separated. DNA ladders were used for reference.

3.2.3.6 Ligation

The ligation reaction was carried out with digested vector and insert DNA at a molar ratio of 1:3. The reaction was set up as below: -

Table 13.

Components	**Volume (µL)**
Digested vector	50 ng
Digested insert	1
10X T4 DNA ligase Buffer	1

T4 ligase	1
H_2O	Up to 10 µL

The ligation reaction mix was incubated overnight at 16°C and then transformed into competent cells and plated on agar plates containing appropriate antibiotics for selection.

3.2.3.7 Total RNA isolation and reverse transcription

The cell pellet was resolved in 700 µL of cold TRIzol Reagent and incubated for 5 min at 4°C to ensure complete lysis of the cells. This was followed by the addition of 140 µL of chloroform, mixing of the contents by vigorous shaking, and incubation on ice for 5 min. The suspension was then centrifuged at 12000 rpm for 15 min. The homogenate formed three separate layers: a clear upper aqueous phase containing RNA, an interphase, and a pink-colored organic layer containing DNA and proteins. The aqueous layer was carefully transferred to newly labeled tubes, ensuring that the interphase was not disturbed. An equal volume of 2-propanol was added to this suspension, mixed, and incubated on ice for 10 min. RNA from the resulting suspension was then precipitated by centrifugation at 12000 rpm for 10 min. The obtained RNA pellet was washed twice with 1 mL 70% EtOH and then resuspended in RNase-free TE buffer pH 8.0.

To remove genomic DNA contamination, the extracted RNA sample was mixed with 10X Ambion RNAse-free DNase buffer and DNase I and incubated at 37°C for 30 min. The DNase I enzyme was heat-inactivated by incubating the RNA suspension at 65°C for 10 min.

1 µg of total RNA was mixed with 2 µL of random hexamer and the volume was made up to 12 µL with water. This was first incubated at 72°C for 5 min in the thermocycler to denature the secondary structure of RNA. After incubation, a cocktail of 2 µL 10X M-MuL V Buffer, 0.4 µL of 2.5 mM dNTPs, 1 µL 0.1 M DTT, 0.25 µL of 200 U/µL M-MuL V Reverse Transcriptase, and 4.35 µL of H_2O was added to the tube and incubated for 50 min at 37°C. The cDNA synthesis was stopped by incubating the reaction mixture at 75°C for 10 min. The synthesized cDNA sample was half-diluted and gene expression was analyzed by RT-qPCR.

3.2.3.8 Preparation of total RNA and library for RNA-Seq

Total RNA was purified by Rneasy Mini Kit (Qiagen), the QIAshredder (Qiagen), and the DNase I (Qiagen) digestion was performed according to the manufacturer's instructions.

3.2.3.9 Real-Time PCR

The quantitative real-time PCR was carried out in A QuantStudioPlus Real-Time PCR System. Levels of specific mRNAs were detected by double-dye tagged hydrolysis probes. The Taqman reaction contains a probe sequence that is designed to hybridize specifically to a target region in the cDNA flanked by a primer pair. The detection probes are fluorogenic

oligonucleotides covalently attached to a reporter dye (6-FAM) at the 5'-end and a quencher (TAMARA) at the 3'-end. When the probe is intact, due to the proximity of the fluorophore and the quencher, reporter fluorescence is suppressed due to Fluorescence Energy Transfer (FRET) as the emission of the emission spectrum of the reporter overlaps with the excitation of the quencher molecule. However, during extension, the 5'-exonuclease activity of the Taq DNA polymerase will degrade the 5'-end of the probe releasing the reporter dye. Release of the reporter and the resulting fluorescence signal increase in each PCR cycle depending on the amount of target sequences in a given sample indicating the physical separation of the reporter from the quencher.
The reaction was run using the following program: -

Hold stage 1: 50°C	2 min
Hold stage 2: 95°C	15 min
Denaturation Step 3: 95°C	15 sec
Annealing & extension Step 4: 60°C	1 min

Steps 3 and 4 were programmed to repeat for 45 cycles and the analysis was performed using QuantStudio.

3.2.3.10 DNA analysis by agarose gel electrophoresis

PCR amplicons and restriction digests were analyzed by agarose gel electrophoresis. 6X gel loading dye was added to the samples to a final 1X concentration and resolved at 120 V.

3.2.4 Biochemical methods

3.2.4.1 Preparation of whole cell extract from eukaryotic cells

Cells were pelleted down by centrifuging at 1200 rpm at 5 min and the pellet was washed twice with ice-cold 1X PBS. Then the pellet was resuspended in Whole cell extraction buffer and incubated on ice for 15 min. The suspension was centrifuged at 13200 rpm for 15 min at 4°C. The clear supernatant was collected in a fresh tube and the amount of protein was quantified by Bradford Assay and resolved by SDS-PAGE.

3.2.4.2 Preparation of nuclear extract from eukaryotic cells

Cells were centrifuged at 1200 rpm for 5 min and the pellet was washed with 10 ml PBS. The pellet was resuspended in 100 µl of sucrose buffer and incubated for 20 min on ice and vortexed in 20 sec intervals. Cell lysis was ensured by staining with Trypan Blue followed by microscopic examination (lysed cells are blue and 95% of cell lysis should be achieved). The lysate was centrifuged at 4000 rpm at 4°C for 5 min. The cell pellet was washed with pre-cooled PBS and then resuspended in 100 µl of high salt buffer. The lysate was incubated for 20 min on ice in the cold room followed by centrifugation for 15 min at 14000 rpm at 4°C. The supernatant representing the nuclear extract was then transferred to fresh tubes and subjected to protein quantification.

3.2.4.3 Protein estimation by Bradford assay

The protein amount was quantified by the Bradford assay using BSA (Bovine Serum Albumin) as a standard. The assay is based on the principle that binding of proteins to an acidic solution of Coomassie brilliant blue G-250 shifts the absorbance from 465 nm to 595 nm. From each experimental sample, 1 µl was taken and mixed with 1 ml of H_2O. The BSA standard samples with different dilutions were made in a total volume of 1 mL with H_2O. 200 µL of Bradford reagent was added to both standard and test samples, and absorbance was measured at 595 nm. The protein concentration in test samples was determined by referring to the absorbance of the standard samples. For any given experiment, all the protein samples were mixed with 4X Laemmli loading buffer to obtain equal concentration across all the samples, boiled, and subsequently analyzed by Western blotting.

3.2.4.4 SDS-PAGE

The proteins in whole cell extracts were analyzed by discontinuous SDS-PAGE. Gels were made according to the size of the protein of interest. This system consists of two parts: a resolving gel in which proteins are resolved on the basis of their molecular weights, and a stacking gel in which all the proteins are concentrated into a compact horizontal zone prior to entering the resolving gel.

Composition of stacking gel
Table 14.

Components	**Volume**
40% Acrylamide	650 µL
H_2O	3.05 mL
Stacking buffer	1.25 mL
APS	31 µL
TEMED	6.5 µL

Composition of resolving gel
Table 15.

Components	**Volume for 10%**	**Volume 12%**
40% Acrylamide	1.875 mL	2.25 mL
H_2O	3.75 mL	3.375 mL
Resolving buffer	1.875 mL	1.875 mL
APS	31 µL	31 µL
TEMED	6.5 µL	6.5 µL

The assembled polyacrylamide gel was placed into a Biorad chamber containing SDS-PAGE running buffer. The samples were first run at 80 V until properly stacked and then the voltage was increased to 120 V and the samples were resolved until the protein ladder of

the intended molecular size was still retained in the gel. The gel was disassembled and used for Western blotting.
For some purpose, Mini-Protain Precast gels 4-20% from Bio-Rad were used.

3.2.4.5 Western blotting

After the samples were resolved by electrophoresis, proteins were transferred to a PVDF membrane by wet transfer for 1h 15 min at 0.38 A in transfer buffer in the cold room using the Biorad Mini Trans-Blot system. Following this, the membranes were blocked with 5% non-fat milk or 5% BSA in 1X TBST for 1 hour and incubated overnight with primary antibody at 4°C. The next day, the membranes were washed 5 times each with 1X TBST for 5 min on a rocker. Then the membrane was incubated with appropriate HRP-conjugated secondary antibody for 1h at room temperature. The excess unbound secondary antibody was then washed off 5 times each with 1X TBST for 5 min. Thereafter, membranes were finally incubated with an enhanced chemiluminescent substrate (ECL), and the emitted chemiluminescence signal corresponding to the protein of interest was detected by a Vilber Fusion FX7 system.

3.2.4.6 Membrane stripping for reblotting

After developing the chemiluminescence signal by probing the membrane with the first antibody, the membrane was washed twice with the relevant washing buffer for 10 min and then the membrane was inserted in a falcon tube containing 50 mL of membrane stripping buffer freshly supplemented with 350 mL of β-mercaptoethanol. The membrane was then incubated in a water bath at 70°C for 30 min and then the membrane was washed twice again for 10 min with the relevant washing buffer. The membrane was then blocked with the appropriate blocking buffer and then incubated with a primary antibody followed by the necessary steps of developing the signal for the next protein of interest.

3.2.4.7 Chromatin Immunoprecipitation assay

RBPJ-ChIP was performed by Dr. Benedetto Daniele Giaimo (Institute of Biochemistry, Justus Liebig University, Giessen). Briefly, H1299 cells were washed twice with PBS and fixed for 1h at room temperature in 10 mM dimethyladipimate (DMA, Thermo Scientific 20660) dissolved in PBS. Thereafter, cells were washed once in PBS followed by crosslinked with 1% FMA for 30 min at room temperature. The FMA reaction was blocked by adding 1/8th volume of 1 M glycine pH 7.5 and incubating for 5 min at room temperature. Chromatin immunoprecipitation (ChIP) was performed as previously described by Giaimo et al., 2017 with only one modification that chromatin from *Drosophila* Schneider cells was used for spike-in purposes (each of 25 µg of mouse chromatin and 25 ng of *Drosophila* chromatin were used in ChIP against RBPJ transcription factor) in presence of 2 µg of anti-His2Av (Active Motif 61686) for each immunoprecipitation. For the immunoprecipitation of RBPJ, an anti-RBPJ antibody (Cell Signaling Technology, 5313S) was used. Library preparation was carried out using the Diagenode MicroPlex Library Preparation kit v3

(Diagenode C05010001) following the manufacturer's instructions with a few modifications. Libraries were purified with Agencourt AMPure XP Beads (Beckman Coulter, #A63881), quantified, analyzed on an Agilent Bioanalyzer, and pooled. Finally, the samples were sequenced on a NovaSeq device at Novogene UK.

3.2.4.8 Calcium imaging

Ca^{2+} imaging experiments were performed in cooperation with AG Diener (Institute for Veterinary Physiology and Biochemistry, Justus Liebig University, Giessen) as described by Ballout et al., 2022. The experiments were carried out at room temperature with the Ca^{2+}-sensitive fluorescent dye Fura-2 to measure changes in the cytosolic Ca^{2+} concentration. For this purpose, an inverted microscope (IX-50; Olympus, Hamburg, Germany) endowed with an epifluorescence setup was used, which was connected to a charged coupled device (CCD) camera and a computer equipped with an image analysis system (Till Photonics, Martinsried, Germany). The H1299 cells were seeded on glass coverslips (diameter 13 mm, 1000 cells / well) in a 4-well chamber the day before. The next day, the H1299 monolayer was loaded with 3 µM of the membrane-permeable form of Fura-2, [Fura-2 acetoxymethyl ester (Fura-2/AM, Thermo Fisher Scientific)] combined with an equal volume of the non-ionic detergent pluronic acid (20% [w/v] stock solution in DMSO; Thermo Fisher Scientific) for 90 min. Thereafter, cells were washed with Tyrode's solution, and the coverslips were mounted in the imaging chamber and covered with 1 ml Tyrode's solution. The cells were excited alternately with 340 nm or 380 nm, the emission was measured at wavelength >440 nm, and the ratio was calculated (340 nm/380 nm) in different regions of interest (ROI), each representing one individual cell. After a stabilization period over several minutes, either ISX9 (20 µM, dissolved in DMSO) or 0.1 % DMSO (v/v) was added to the imaging chamber. A response to the respective drug was accepted when a) the amplitude of the change exceeded the 4-fold standard deviation of the scattering in the Fura-2 ratio during the stabilization period and b) the amplitude of the change in the Fura-2 ratio exceeded an absolute value of 0.1. At the end of each experiment, 50 µM cyclopiazonic acid (CPA), a blocker of sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA), was administered as a viability control. Cells were accepted as viable if CPA induced an increase in the Fura-2 ratio.

3.2.5 Bioinformatic analysis

Bioinformatic analysis was previously described by Friedrich et al., 2022. In short, raw sequencing reads were adaptor and quality trimmed using TrimGalore (https://github.com/FelixKrueger/TrimGalore) and subsequently aligned to the hg19 reference genome using HISAT2 (Kim et al., 2019). Differentially expressed genes were detected by DESeq2 (Love et al., 2014) based on read counts generated by the summarizeOverlaps (Lawrence et al., 2013) function and the hg19 gene transfer format file. Enrichment analyses were performed based on the clusterProfiler package (Wu et al., 2021). PCR duplicates were removed from ChIP-seq files using Picard tools (https://broadinstitute.github.io/picard/) and subsequent peak calling was performed using

MACS2 (Zhang et al., 2008). RBPJ peaks had to be conserved between two replicates and the association of RBPJ sites to corresponding genes was performed using ChIPseeker (Yu et al., 2015). Motif analysis was performed using MEME suite (Bailey et al., 2015).

3.2.6 Statistical analysis

Representation of graphical data and the corresponding statistical analyses were performed using GraphPad Prism 5.0 software. The statistical significance of the differences between experimental groups was tested by Student's t-test. All data were represented by mean ± SD (standard deviation) of the indicated number of independent experiments. The level of statistical significance was presented by asterisks as P (p-value) ≤ 0.05 = (*), P ≤ 0.01 = (**), P ≤ 0.001 = (***), and P > 0.05 represented non-significant (ns).

4. RESULTS

4.1 ISX9 is a small molecule activator of the Notch signaling pathway

The small molecule ISX9, previously known as a pro-neural compound (Schneider et al., 2008), has been demonstrated to induce Hedgehog/Gli1 activity in neuroblastoma cells as well as in several other human carcinoma cell lines by Koeniger et al. in 2021. Another subsequent publication from the same group revealed that ISX9 stimulates the activation of NOTCH signaling in order to induce the differentiation of neuroblastoma cells towards the mesenchymal subtype (Koeniger et al., 2023). Based on these previous findings, I investigated the role of ISX9 as a Notch 'booster' in more detail.

To gain insights into the molecular mechanism of a potential Notch activator, I needed an experimental model with a low basal expression of Notch compartments. Therefore, I used several 'Notch-OFF' cell lines to determine the likelihood of Notch-activation by ISX9. ISX9 drove marked Notch induction in the non-small cell lung cancer (NSCLC) cell line H1299, where I could detect a significant upregulation of canonical Notch signatures *HEY1, HEY2, HEYL,* and *HES4* upon treatment with ISX9 (Fig. 3). The effect of ISX9 treatment on gene expression was comparable between 12h and 24h incubation. Hence, I considered 12h as the standard time point for carrying out further experiments to eliminate any secondary effects.

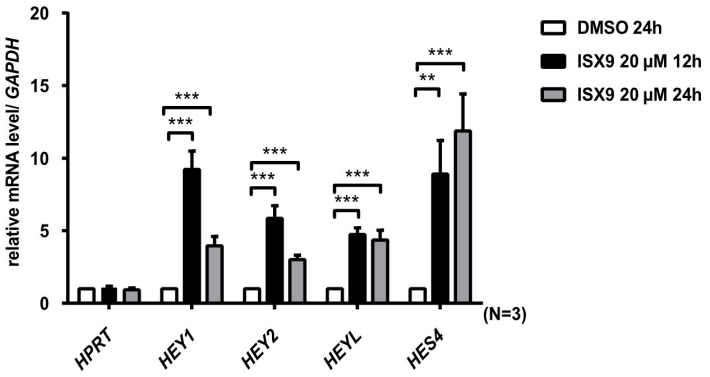


Fig. 3 Small-molecule ISX9 is an activator of Notch target gene expression. ISX9 treatment leads to the upregulation of Notch target genes. H1299 cells were treated with 20 µM ISX9 or DMSO as control for 12h and 24h. Total RNA was isolated and subjected to reverse transcription. cDNAs were analyzed by RT-qPCR using gene-specific primers and *GAPDH* was used as the housekeeping control for normalization. Mean ± SD of three independent experiments was represented [P (p-value) > 0.05 = (ns or not significant), P ≤ 0.05 = (*), P ≤ 0.01 = (**), P ≤ 0.001 = (***), unpaired Student's *t*-test].

H1299 represents an ideal system to evaluate the Notch-'boosting' potential of the compound of interest for certain features. I took advantage of the information on genome-wide analysis of human proteins available on The Human Protein Atlas

(https://www.proteinatlas.org). In H1299 cell line, the expression of the Notch receptors and the ligands is significantly low: *NOTCH1* =2.2 nTPM, NOTCH2 =21.8 nTPM, *NOTCH3* =2.6 nTPM, *DLL1* =0.6 nTPM, *DLL3* =6.8 nTPM, *DLL4* =0 nTPM (nTPM =normalized transcript per million) designating that the metastatic NSCLC cell line H1299 is a 'NOTCH-low' cell type as the expression of the major Notch pathway components are low or minimal. This baseline 'poor Notch' status complements capturing the Notch-inducing potential of ISX9. Moreover, overexpression of the constitutively activated form of the Notch receptor resulted in marked upregulation of Notch target genes in H1299 cells (Fig. 4 A). Further, the same Notch receptor target genes were found to be upregulated in H1299 cells upon removal of the transcriptional repressor of the Notch signaling pathway, RBPJ, indicating derepression at the target gene promoters (Fig. 4 B). These, all together, imply that Notch signaling activity in H1299 cells is low but inducible representing an ideal system to delineate the Notch-activating capacity of ISX9.

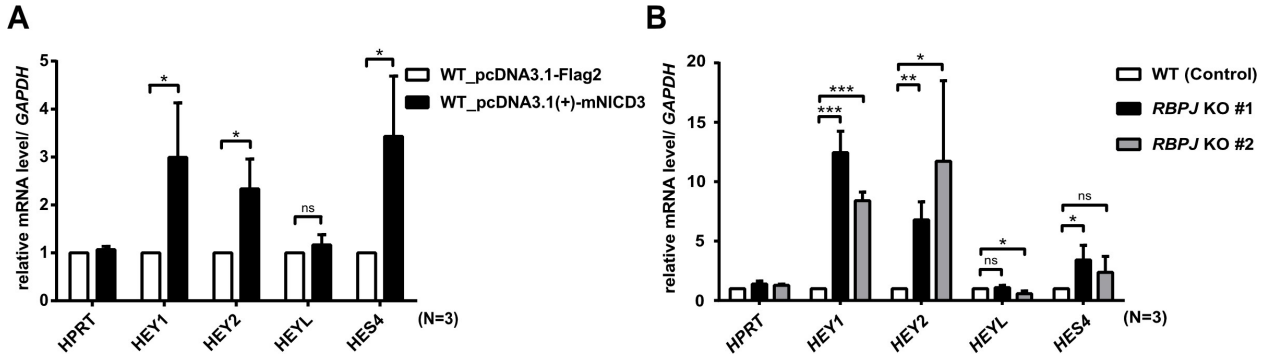


Fig. 4 Notch pathway is inducible in H1299 cells. (A) Forced activation of cleaved NOTCH3 in H1299 cells activates Notch target gene expression. Wildtype H1299 cells were transfected with mouse NICD3-overexpressing vector pcDNA3.1(+)-mNICD3 and empty vector control. After 48h of transfection, total RNA was isolated and reverse transcribed. cDNAs were analyzed by RT-qPCR using gene-specific primers and *GAPDH* was used as the housekeeping control for normalization. (B) The Notch pathway is derepressed upon depletion of RBPJ in H1299 cells. Total RNA from wildtype (control) or RBPJ-depleted H1299 cells was reverse transcribed into cDNA and expression of Notch target genes *HEY1, HEY2, HEYL,* and *HES4* was analyzed by RT-qPCR using gene-specific primers. Data were normalized to the housekeeping gene *GAPDH*. Mean ± SD of three independent experiments was represented [P (p-value) > 0.05 = (ns or not significant), P ≤ 0.05 = (*), P ≤ 0.01 = (**), P ≤ 0.001 = (***), unpaired Student's *t*-test].

Using two different cell lines with high and low background Notch activity, next, I tested whether ISX9 treatment results in a contrasting effect on the stimulation of Notch target gene expression. While in the murine pre-T cell line (called Beko), where the Notch signal transduction is constitutively active, no significant Notch induction was observed upon ISX9 treatment (Fig. 5 A), in the mouse hybridoma mature-T cell line (MT cells), known to be devoid of Notch activity (Xu et al., 2017; Yuan et al., 2019; Friedrich et al., 2022), ISX9 showed robust activation of Notch signatures (Fig. 5 B).

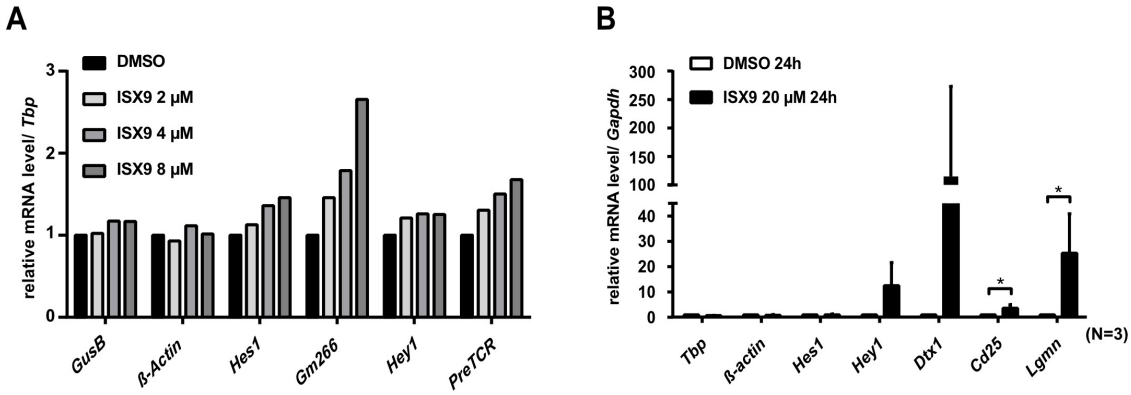


Fig. 5 Basal Notch activity determines ISX9-triggered Notch target gene expression. (A) ISX9 treatment causes moderately low induction of the Notch signatures in the Notch-active background. Beko cells were treated with 2 μM, 4 μM, and 8 μM ISX9 or DMSO as control for 24h. Total RNA was isolated and subjected to reverse transcription. cDNAs were analyzed by RT-qPCR using gene-specific primers and *Tbp* was used as the housekeeping control for normalization. A single experiment was represented and no significance test was performed. (B) ISX9 treatment leads to the upregulation of Notch target genes in the 'Notch-OFF' background. MT cells were treated with 20 μM ISX9 or DMSO as control for 24h. Total RNA was isolated and subjected to reverse transcription. cDNAs were analyzed by RT-qPCR using gene-specific primers and *Gapdh* was used as the housekeeping control for normalization. Mean ± SD of three independent experiments was represented [P (p-value) > 0.05 = (ns or not significant), P ≤ 0.05 = (*), P ≤ 0.01 = (**), P ≤ 0.001 = (***), unpaired Student's *t*-test].

In order to analyze the effect of ISX9 on a genome-wide level, I performed deep sequencing (RNA-Seq analysis) using the RNA isolated from DMSO or ISX9-treated H1299 cells. RNA-Seq analysis was performed in duplicates which revealed upregulation of 3849 genes and downregulation of 139 genes, and upregulation of 3894 genes and downregulation of 130 genes upon 12h and 24h of ISX9 treatment, respectively (Fig 6 A). The gene expression pattern of Notch target gene induction in the replicates of RNA-Seq experiments was reproducible and could be validated by RT-qPCR. Furthermore, different Notch signaling-related functional terms (GO terms and KEGG pathways) were found to be enriched in genes upregulated upon ISX9 treatment (Fig 6 B). These genome-wide results are in line with the previous results that ISX9 activates Notch target genes.

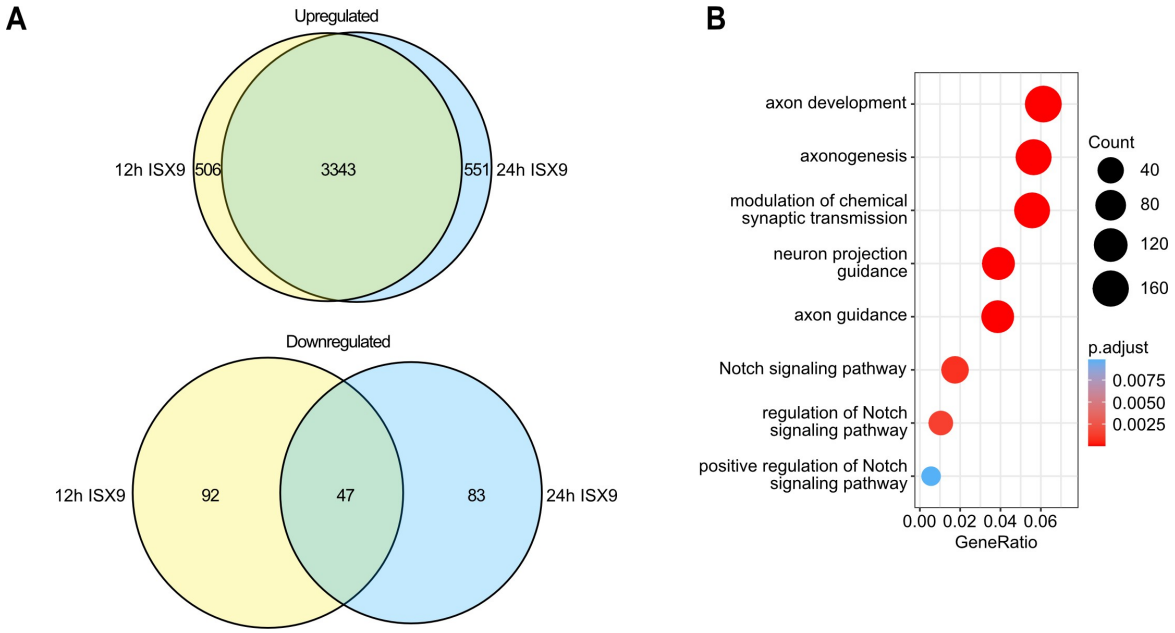


Fig. 6 Genome-wide analysis indicates Notch-specific induction by ISX9. (A) ISX9 treatment for 12h and 24h shows a significant overlap of differentially expressed genes. Venn diagram representation of the overlap between significantly upregulated and downregulated genes in H1299 cells upon ISX9 treatment for 12h and 24h. (B) Genes upregulated upon ISX9 treatment are enriched for multiple Notch-related terms. Bubble plot showing the results of the gene ontology (GO) 'Biological Process' analysis.
Deep sequencing was performed at Novogene (UK) Company Limited and data analysis was performed in collaboration with Tobias Friedrich and Dr. Benedetto Daniele Giaimo.

4.2 Notch activation by ISX9 depends on the transcription factor RBPJ

The expression of Notch target genes is a consequence of active Notch signaling, if not triggered by any other converging signaling events. Based on that, the next question was whether the components of the Notch pathway *per se* are induced by ISX9 treatment. I observed a marked increase in *NOTCH1* mRNA (Fig. 7 A) as well as in cleaved or activated NOTCH1 receptor protein (NICD1) (Fig. 7 B) upon ISX9 treatment compared to DMSO.

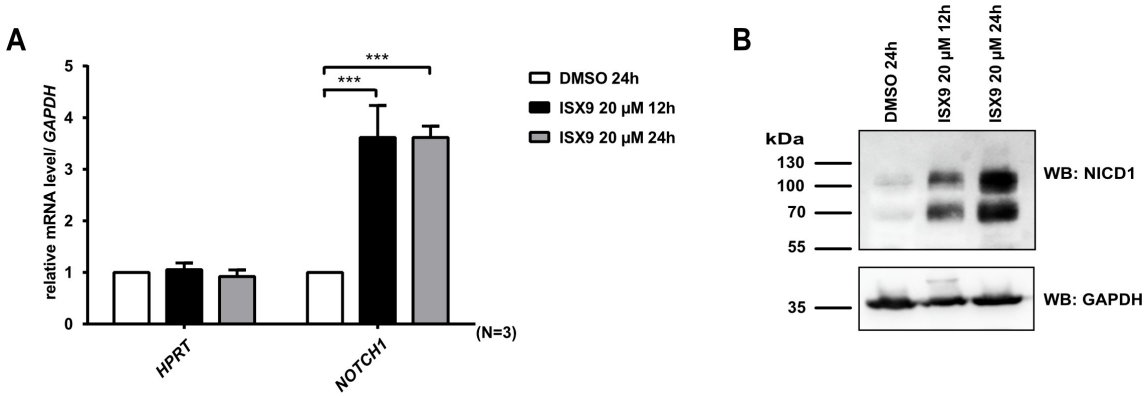


Fig. 7 ISX9 triggers Notch activation. (A) ISX9 stimulates *NOTCH1* expression. H1299 cells were treated with 20 µM ISX9 for 12h and 24h or DMSO as control. Upon RNA extraction and reverse transcription, cDNAs were analyzed by RT-qPCR. *GAPDH* was used as the housekeeping control for normalization. Mean ± SD of three independent experiments was represented [P (p-value) > 0.05 = (ns or not significant), P ≤ 0.05 = (*), P

≤ 0.01 = (**), P ≤ 0.001 = (***), unpaired Student's *t*-test]. (B) ISX9 treatment leads to increased NICD1 protein level. H1299 cells were treated for 12h and 24h with 20 µM ISX9 or DMSO as control and the whole cell extract was analyzed by Western blotting for detection of the endogenous cleaved NICD1 protein or GAPDH as the loading control. The experiment was repeated three times independently.

Since RBPJ is a core downstream regulator of the Notch signaling, potential Notch target genes are depicted as genes bound by RBPJ and deregulated upon depletion of RBPJ. To evaluate whether ISX9-driven gene expression depends on transcription factor RBPJ, I wanted to establish an RBPJ loss-of-function system in H1299 and perform a comparative analysis of Notch target gene expression in the parental and the RBPJ-depleted background. At the same time, this also served as an ideal control to define direct Notch target genes.

In order to deplete RBPJ in H1299 cells, I used the CRISPR/Cas9 technology. Genomic *RBPJ* exon (ENSG00000168214/ GRCh38) at genomic loci Chr 4: 26,424,335-26,424,485 encoding the DNA binding domain of RBPJ was chosen to design the guide RNAs using the Zhang Lab CRISPR Design Tool (Cong et al., 2013) (sequence of the guides in Table 9) to disrupt the DNA binding function of all RBPJ isoforms. Potential knockouts were obtained by simultaneous targeting with two guide RNAs as shown in Fig. 8 A. A PCR assay amplifying the genomic locus that encompasses the CRISPR/Cas9 targeted fragment was designed for the identification of potential knockout clones. The absence of 289 bp PCR product in the clones *RBPJ* KO #1 and KO #2 in comparison to empty vector-transfected clones indicated successful genomic disruption (Fig. 8 B) and these clones were further subjected to biochemical characterization as *RBPJ*-knockouts. Complete depletion of RBPJ was validated at the protein level by Western blotting (Fig. 8 C) and at the RNA level by RT-qPCR analysis (Fig. 8 D)

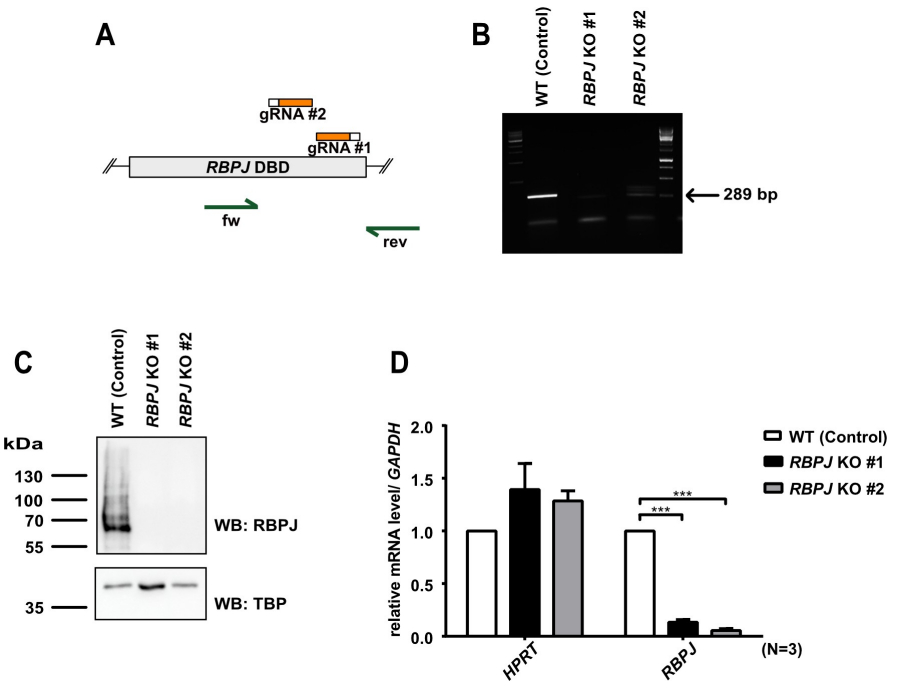


Fig. 8 RBPJ was efficiently depleted by CRISPR/Cas9 in H1299 cells. (A) Schematic representation of genomic targeting of *RBPJ* by CRISPR/Cas9. The targeted genomic locus, guide-RNAs (gRNA #1 and gRNA #2), and PAM sequences are depicted in gray, saffron, and white rectangles, respectively. Green Arrows (forward and backward) indicate forward and reverse primers (fw and rev) used for PCR screening for detecting genomic deletion. (B) Validation of genomic depletion of *RBPJ*. Cut-site-specific PCR amplification of genomic DNA was carried out using fw and rev primers at the targeted *RBPJ* locus. The PCR product (289 bp) consisting of the genomic locus simultaneously targeted by two guides: gRNA #1 and gRNA #2 was analyzed by gel electrophoresis. PCR amplicon detected in H1299 wildtype (control) cells was absent in H1299 *RBPJ* KO #1 and *RBPJ* KO #2 clones indicating potential genomic disruption. (C) Validation of CRISPR/Cas9-mediated depletion of RBPJ at the protein level. Nuclear extract was prepared from wildtype (control) and RBPJ-depleted H1299 cells (clones KO #1 and KO #2) and protein level of RBPJ and nucleus-specific loading control TBP were analyzed by Western blotting. (D) RBPJ expression in *RBPJ*-knockout H1299 cells. Total RNA was extracted from wildtype (control) or RBPJ-depleted H1299 cells, reverse transcribed into cDNA, and analyzed by RT-qPCR using primers specific for *RBPJ*. Data were normalized to the housekeeping gene *GAPDH* and represent the mean values and standard deviation of three independent experiments [P (p-value) > 0.05 = (ns or not significant), P ≤ 0.05 = (*), P ≤ 0.01 = (**), P ≤ 0.001 = (***), unpaired Student's t-test]

By combining the RNA-Seq and ChIP-Seq data in RBPJ-depleted background, it is possible to successfully define a Notch signature in any given cell type (Friedrich et al., 2022). In order to understand the implication of the transcriptional function of RBPJ in the ISX9-stimulated overall transcriptional landscape, RNA-Seq was performed to detect significant gene expression changes in RBPJ-depleted H1299 cells in comparison to the wildtype (control) cells. Upon RBPJ depletion, 24 and 58 genes were upregulated and downregulated, respectively, in *RBPJ* KO #1; and 30 and 60 genes were upregulated and downregulated, respectively, in *RBPJ* KO #2. Consistent with the function of RBPJ as a transcriptional repressor (Raafat et al., 2009; Liefke et al., 2010; Xu et al., 2017; Yuan et al., 2019), Notch target genes such as *HEY1*, and *HEY2* were commonly derepressed in two H1299 *RBPJ* KO clones *RBPJ* KO #1 and #2 and as a proof of principle, *RBPJ* itself was downregulated in these clones (Fig. 9 A). The derepression of Notch target genes in RBPJ-depleted cells was also validated by RT-qPCR (Fig. 4 B). To further characterize the RBPJ-responsive sites, anti-RBPJ ChIP-Seq was performed in *RBPJ* KO #1. 1980 RBPJ binding sites were identified in wildtype (control) cells which were undetectable in *RBPJ*-knockout cells (*RBPJ* KO #1). RBPJ binding motif was also identified among the most significantly enriched motifs by MEME-ChIP (Fig. 9 B).

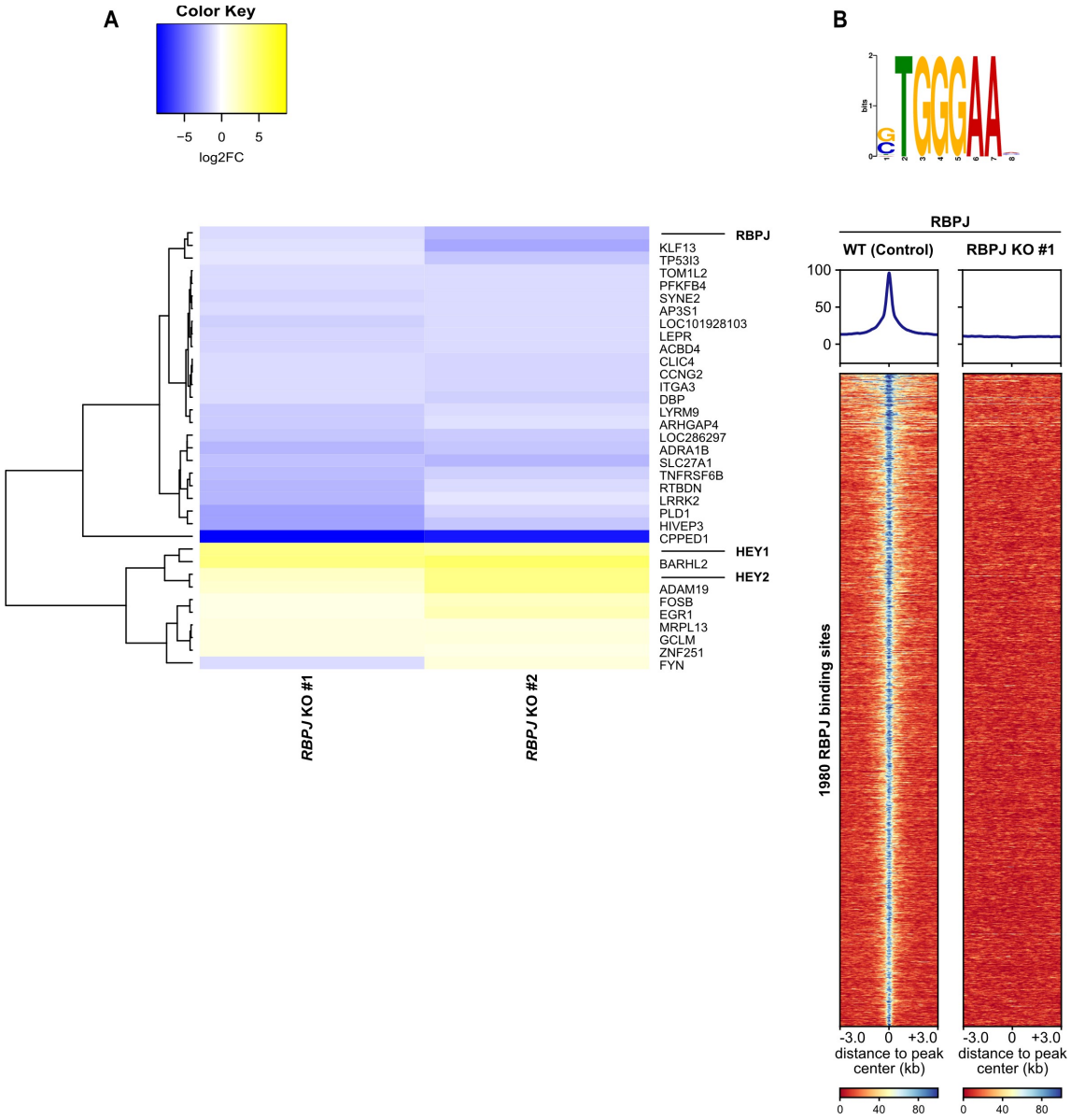


Fig. 9 RBPJ mediates repression of Notch target genes in H1299 cells. (A) Notch target genes are derepressed upon removal of RBPJ. Heat map showing deregulation of gene expression as $\log_2$ Fold Change in RBPJ-depleted H1299 cells compared to wildtype (control) cells. (B) Genome-wide occupancy of RBPJ is undetectable in RBPJ-depleted H1299 cells. H1299 wildtype (control) and *RBPJ*-knockout cells were subjected to ChIP analysis using antibodies against RBPJ or IgG as control. The heat map was sorted based on the mean RBPJ binding per region over all samples.
Library preparation and deep sequencing were performed at Novogene (UK) Company Limited and data analysis was performed in collaboration with Tobias Friedrich and Dr. Benedetto Daniele Giaimo.

Then, the ISX9-regulated genes were analyzed in-depth to assess their Notch pathway-specificity by following the same combination approach of RNA-Seq and ChIP-Seq data in ISX9-treated cells. This allowed me to define the bona fide Notch target genes based on RBPJ binding and transcriptional upregulation upon depletion of RBPJ. A selection of 17 genes that met these strict criteria (Fig 10 A, in yellow) were also upregulated upon ISX9 treatment. These genes include the well-known Notch targets *HEY1*, *HEY2*, *HES4*, and *NRAP*. As a confirmation, H1299 *RBPJ*-knockout cells were treated with ISX9, and target

gene induction was quantified by RT-qPCR. ISX9 treatment was unable to induce *HEY1*, *HEY2,* and *HES4* in RBPJ-depleted H1299 cells compared to the wildtype (control) cells (Fig. 10 B).

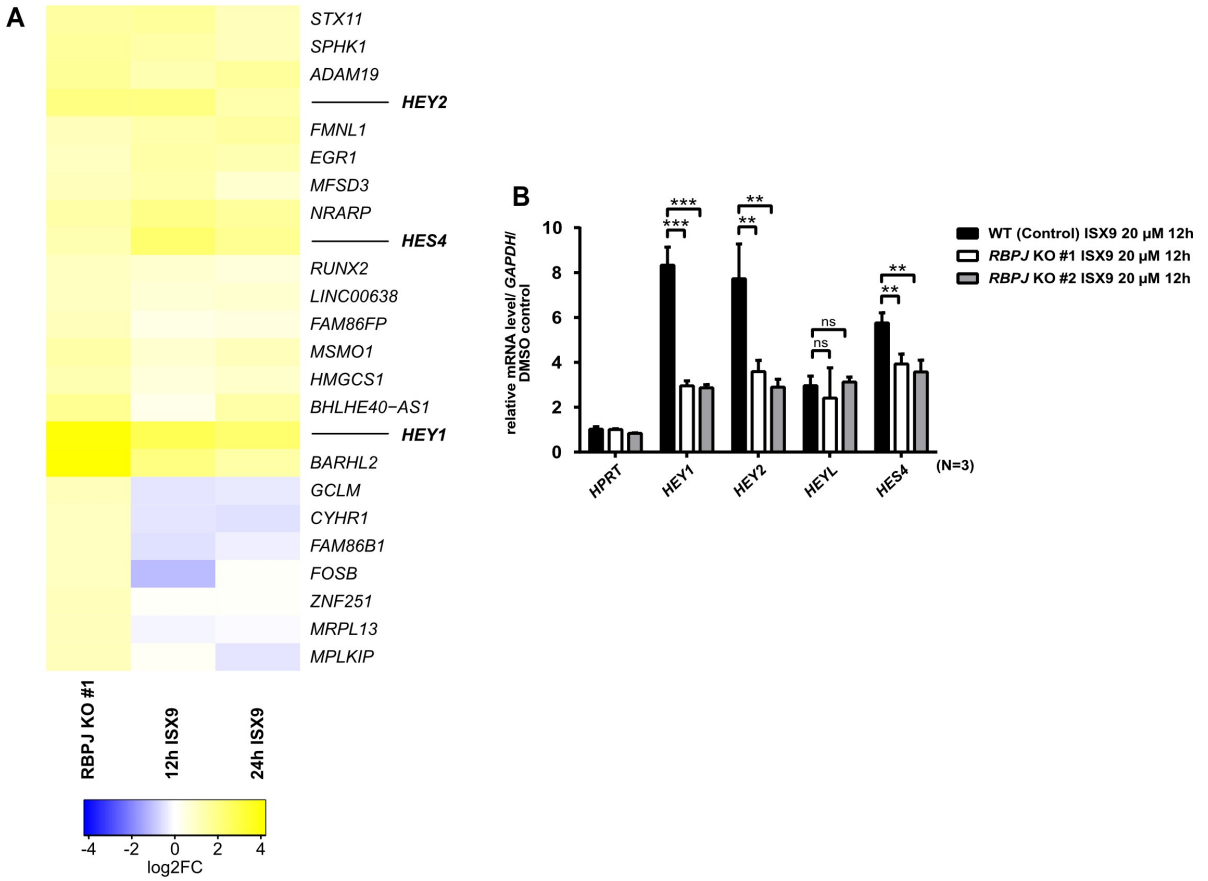


Fig. 10 ISX9-triggered Notch response is dependent on RBPJ. (A) Notch target genes are upregulated by ISX9. Heat map showing significant expression changes of RBPJ-bound genes upon ISX9 treatment. The gene signatures that undergo derepression in the *RBPJ*-knockout H1299 cells compared to wildtype (control) cells, representing *'bona fide*' Notch targets, were deregulated upon ISX9 treatment for 12h and 24h. The upregulated genes include *HEY1*, *HEY2*, and *HES4*. (B) ISX9-mediated Notch response is mediated by RBPJ. H1299 *RBPJ*-knockout cells and wildtype (control) cells were treated with 20 µM ISX9 for 12h and expression of Notch target genes was analyzed by RT-qPCR analysis. Relative fold changes in ISX9 treated wildtype (control), *RBPJ* KO #1, and *RBPJ* KO #2 clones were determined in comparison to DMSO control for each Notch target gene. Data were normalized to the housekeeping gene *GAPDH* and the data represented the mean ± SD of three independent experiments [P (p-value) > 0.05 = (ns or not significant), P ≤ 0.05 = (*), P ≤ 0.01 = (**), P ≤ 0.001 = (***), unpaired Student's t-test].
Library preparation and deep sequencing were performed at Novogene (UK) Company Limited and data analysis was performed in collaboration with Tobias Friedrich and Dr. Benedetto Daniele Giaimo.

4.3 ISX9 shows a differential impact on NOTCH3 over NOTCH1

Canonical activation of Notch signaling is established by the ligand-triggered enzymatic cleavage of the membrane-bound Notch receptor by γ-secretase to generate the active Notch intracellular domain (NICD). Based on that, I tested the hypothesis of whether ISX9-

mediated Notch signaling response is perturbed upon inhibition of the final Notch receptor cleavage. Along with the ISX9 treatment, S3 cleavage, the final processing of the Notch receptor, was pharmacologically blocked by the γ-secretase inhibitor (GSI) DAPT [N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester]. Samples from the same experiment were processed for whole cell extraction and RNA isolation to correlate between the observed changes in protein level and gene expression. The prepared whole cell extract was analyzed by Western blotting. In comparison to DMSO control, the marked increase in NICD1 protein in cells treated with ISX9 only was abrogated in cells that received ISX9 and GSI in combination (Fig. 11 A, compare lane 2 with lane 3). The same samples were also assessed for gene expression analysis. Surprisingly, comparable induction of Notch target genes was observed upon ISX9 exposure with or without inhibition of the S3 cleavage indicating that perturbation of the Notch receptor processing by GSI does not abrogate the Notch signaling response elicited by ISX9 (Fig. 11 B, compare black solid bar with yellow solid bar).

So far, all the experiments were performed considering NOTCH1 as the most relevant representative of the Notch signaling pathway since NOTCH1 is an indispensable mediator of Notch signaling and the most extensively studied member of the Notch receptor family. However, the experimental results described in Fig. 11 A and B suggest that Notch pathway activation by ISX9 is not fully mediated by the NOTCH1 receptor. This led to the next step to assess the effect of ISX9 treatment on other members of the NOTCH receptor family. In addition to *NOTCH1*, *NOTCH2* and *NOTCH3* transcripts were also found to be significantly upregulated upon ISX9 treatment in RT-qPCR analysis (Fig. 11 C). As an aside, the *NOTCH4* mRNA level could not be detected by RT-qPCR using the available TaqMan probes. ISX9 treatment showed significant upregulation of *NOTCH1*, *NOTCH2*, and *NOTCH3*, however, induction of *NOTCH3* transcript was several folds higher in comparison to other NOTCH receptor transcripts. More importantly, out of all four NOTCH receptors, only the marked increase in cleaved NOTCH3 (NICD3) remained unaltered upon GSI treatment (Fig. 11 D, compare lane 2 with lane 3) suggesting that ISX9-exerted GSI-resistance is exclusive for NOTCH3 in comparison to all the other NOTCH receptor proteins (Fig. 11 E). This potentially explains the discordance in unperturbed induction of Notch response upon blocking of the most essential molecular event to generate NICDs by GSI treatment.

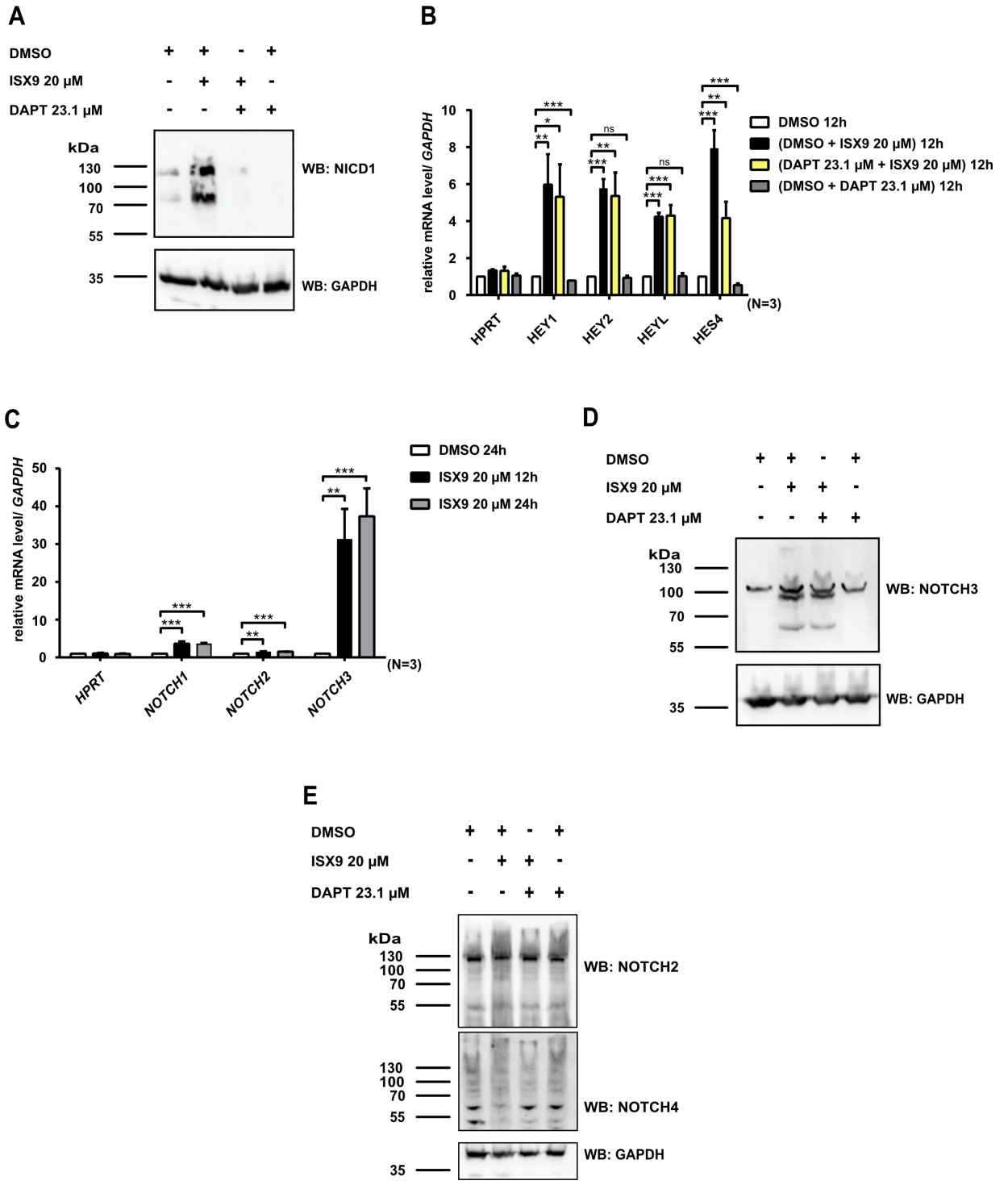


Fig. 11 ISX9 treatment enhances Notch signaling independent of NOTCH processing (A) GSI reduces ISX9-induced NICD1 protein. H1299 cells were treated with 20 µM ISX9 or DMSO for 12h while NOTCH receptor processing was blocked simultaneously with 23.1 µM DAPT or DMSO as control. The level of cleaved NOTCH1 or NICD1 and GAPDH (loading control) was detected by Western blotting using the whole cell extract of the treated H1299 cells. (B) Disruption of NOTCH receptor processing does not abrogate Notch target gene induction by ISX9. Total RNA was extracted from the same experiment in A, reverse transcribed and Notch target gene expression was analyzed by RT-qPCR using gene-specific primers. The housekeeping gene *GAPDH* was used for normalization and the mean ± SD of three independent experiments was represented. (C) ISX9 induces the expression of NOTCH receptors. H1299 cells were treated with 20 µM ISX9 or DMSO

as control for 12h and 24h. Total RNA was isolated and subjected to reverse transcription. The expression of different Notch receptors was analyzed by RT-qPCR considering the housekeeping gene *GAPDH* as the normalization control. Mean ± SD of three independent experiments was represented. (D) ISX9-induced NICD3 is not susceptible to perturbation of S3 cleavage. The whole cell lysate from A was used to detect endogenous NOTCH3 and GAPDH protein levels by Western blotting. (E) ISX9 triggers GSI-resistant activation of NOTCH3 but not any other NOTCH protein. The whole cell extract in A was used for the detection of NOTCH2 and NOTCH4 receptor proteins, and the loading control GAPDH by Western blotting [P (p-value) > 0.05 = (ns or not significant), P ≤ 0.05 = (*), P ≤ 0.01 = (**), P ≤ 0.001 = (***), unpaired Student's t-test].

To test whether the differential effect of ISX9 on NOTCH3 in comparison to NOTCH1 is a general phenomenon, the effect of ISX9 on the protein level of Notch receptors was monitored in the constitutively active Notch background of Beko cells. While ISX9 failed to enhance the level of activated Notch proteins, GSI treatment led to the complete removal of basal NICD1 (Fig. 12 A, compare lane 3 with lane 4) but not NICD3 (Fig. 12 B, compare lane 3 with lane 4) protein in the presence of ISX9 (Fig. 12) reinforcing the fact that ISX9 confers a differential effect on NOTCH3 compared to NOTCH1.

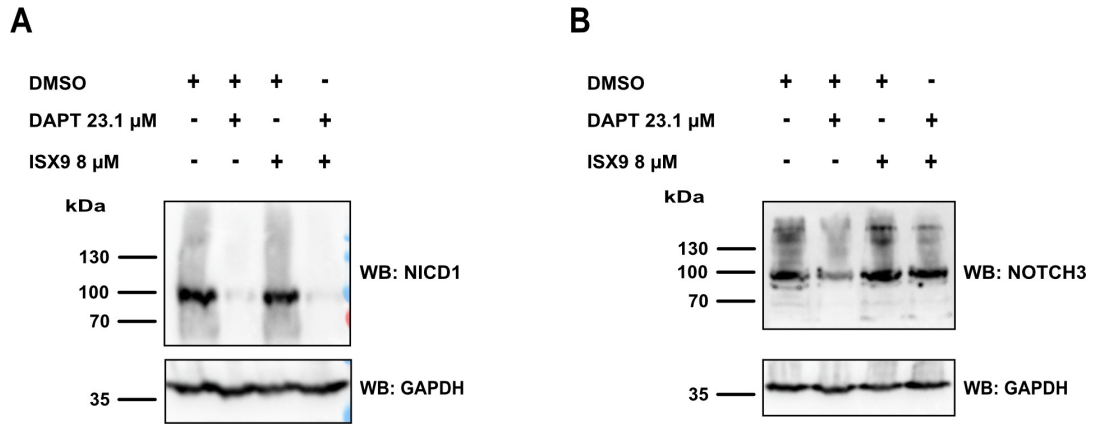


Fig. 12 ISX9 exerts a differential impact on the basal level of NOTCH3 compared to NOTCH1. (A) Inhibition of Notch processing by GSI results in the complete removal of basal-level NICD1 protein. Beko cells were treated with 8 μM ISX9 or DMSO for 24h while NOTCH receptor processing was blocked simultaneously with 23.1 μM GSI or DMSO as control. The level of cleaved NICD1 and GAPDH (loading control) was detected by Western blotting using the whole cell lysate extracted from the treated cells. (B) Inhibition of γ-secretase does not alter the basal level NICD3. The whole cell lysate from A was used to detect endogenous NOTCH3 and GAPDH protein levels by Western blotting.

Pieces of evidence demonstrated that various GSIs show differential profiles of inhibition on the processing of various NOTCH substrates. In order to eliminate the impact of substrate-specific bias (if any) on different endogenous NOTCH substrates by DAPT, a GSI used in previous experiments, cells were exposed to ISX9 in combination with BMS-906024, the only known GSI to inhibit S3 processing of all NOTCH substrates nearly equivalently (Ran et al., 2017). In the presence of ISX9, BMS-906024 treatment also resulted in efficient depletion of processed NOTCH1 (Fig. 13 A, compare lane 2 with lane 3) but the NICD3 isoform remained induced and unchanged (Fig. 13 B, compare lane 2 with lane 3). Finally, at the transcript level, ISX9 treatment significantly upregulated the Notch response which was not inhibited by BMS-906024 (Fig. 13 C, compare black solid bar with yellow solid bar).

Altogether, it was observed that ISX9 treatment enhanced the GSI-resistant active NOTCH3 protein level while ISX9-induced NOTCH1 receptor activation was still sensitive to the blockade of its proteolytic processing. This suggests that exposure of H1299 cells to ISX9 leads to a significant increase in the active NOTCH3 receptor protein by bypassing the canonical Notch pathway events rendering it non-responsive to the GSI treatment.

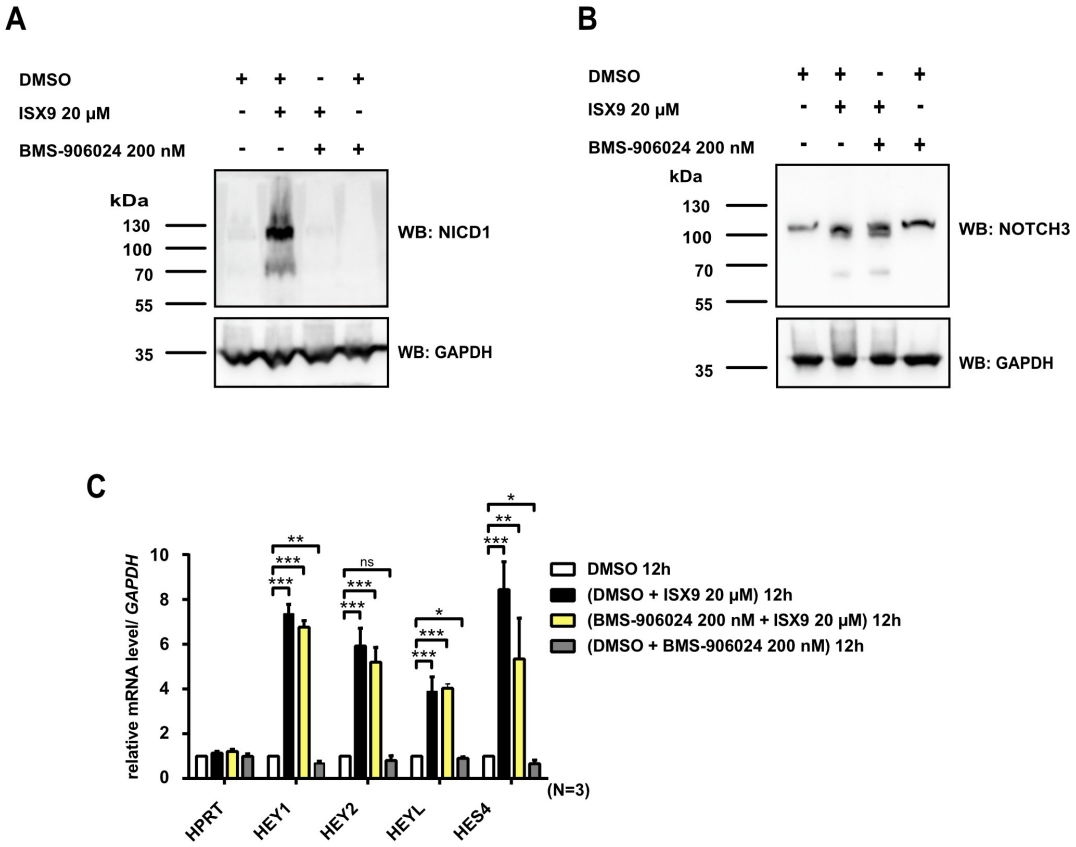


Fig. 13 ISX9-mediated induction of processed NOTCH1 but not NOTCH3 is subjected to blockage by the 'superior' GSI, BMS-906024 (A) ISX9-stimulated increase in active NOTCH1 is prevented by the GSI BMS-906024. H1299 cells were treated with 20 µM ISX9 for 12h with or without 200 nM BMS-906024 to ensure maximum and equivalent inhibition of all Notch receptor processing. The level of cleaved NOTCH1 or NICD1 was detected in the whole cell extract of the treated H1299 cells by Western blotting and GAPDH was used as a control to ensure equal sample loading. (B) NICD3 induction by ISX9 is not susceptible to inhibition of S3 processing. The same whole cell lysate from A was used to detect NOTCH3 protein and GAPDH as the loading control. (C) Inhibition of Notch receptor cleavage does not perturb ISX9-triggered upregulation of Notch target gene expression. Total RNA was extracted from the same experiment in A, subjected to reverse transcription, and expression of Notch target genes was quantified by RT-qPCR using gene-specific primers. The data was normalized considering the housekeeping gene *GAPDH* and the mean ± SD of three independent experiments was represented [P (p-value) > 0.05 = (ns or not significant), P ≤ 0.05 = (*), P ≤ 0.01 = (**), P ≤ 0.001 = (***), unpaired Student's t-test].

4.4 Induction of Notch signaling by ISX9 is NOTCH3-dependent

Collectively, the results from Section 4.3 precisely indicated a potential role of NOTCH3 in mediating the effects of ISX9. Therefore, I asked whether ISX9-triggered transactivation of Notch target genes is inhibited upon abolition of endogenous NOTCH3. The classical means

of ‘Notch inhibition’ is pharmacological blockage of the S3 processing of the Notch receptor by GSI. This approach, however, would not be suitable to test my hypothesis as GSI treatment would deplete all the NOTCH receptor derivatives or NICDs. To assess whether NOTCH3 is pivotal for the Notch-inducing response by ISX9, I generated NOTCH3-depleted H1299 cells by using the CRISPR/Cas9 technology. To maximize the likelihood of *NOTCH3*-specific genomic loss, one guide RNA (gRNA #1) targeting the first exon: chr19:15200788-15200995 and the other (gRNA #2) targeting the last exon: chr19:15159038-15159645 of human *NOTCH3* (ENSG00000074181/ GRCh38) were used in combination to transfect the parental H1299 cells (sequence of the guides in Table 10) (Fig. 14 A). Individual clones were expanded and screened for detection of NOTCH3 protein by Western blotting (Fig. 14 B). I could isolate three different clones showing significant depletion of NOTCH3. Next, a PCR assay was designed to characterize the genomic disruption at the target locus of the potential *NOTCH3* KO clones. The amplification of the PCR product spanning the Exon 1 target site failed, most likely due to the high GC content (82%). Therefore, potential genomic disruption was detected based on the PCR amplification of the Cas9-targeted site at the Exon 33 of *NOTCH3*. Loss of cut-site-specific PCR amplicon in *NOTCH3* KO clones #2 and #3 indicated potential genomic perturbation (Fig. 14 C). Further, the final validation was done by evaluating the effect of genomic depletion on the transcript level of *NOTCH3* by RT-qPCR analysis (Fig. 14 D).

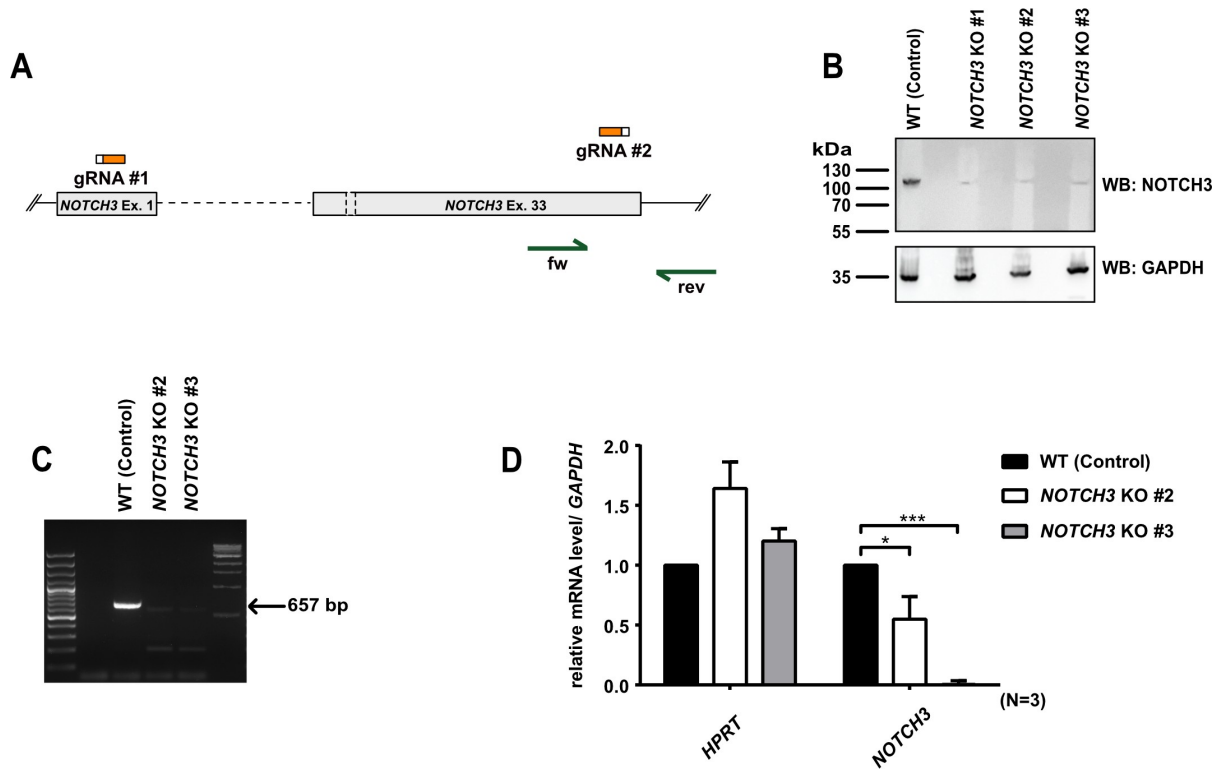


Fig. 14 Significant depletion of NOTCH3 was achieved in H1299 cells. (A) Schematic representation of genomic targeting of *NOTCH3* by CRISPR/Cas9. To ensure the complete depletion of functional NOTCH3 protein from H1299 cells, the whole genomic locus encoding human NOTCH3 protein was targeted by the combination of one guide RNA targeting the Exon 1 and the other one targeting the last exon of the *NOTCH3* gene: Exon 33. The targeted genomic loci are depicted in gray rectangles and guide RNAs (gRNA #1 and

gRNA #2) are shown in saffron with PAM sequences shown in white rectangles. The green forward and backward arrows indicate forward and reverse primers (fw and rev) respectively, that were used for PCR screening for the detection of successful genomic deletion. (B) Quantification of NOTCH3 depletion at the protein level. The expression level of NOTCH3 protein was detected in the whole cell extract of wildtype control (Empty vector-transfected single clone) and NOTCH3-depleted (gRNA-transfected single clones) H1299 cells by Western blotting. Three different clones (clones KO #1, KO #2, and KO #3) showed significant depletion of NOTCH3 in comparison to wildtype (control) H1299 cells. (C) Validation of genomic depletion of targeted *NOTCH3* locus in single-cell clones. Genomic DNA was isolated from potential H1299 *NOTCH3*-knockouts KO #2 and KO #3 and the genomic depletion at the targeted *NOTCH3* locus of Exon 33 was detected by PCR amplification using fw and rev primers as depicted in A. Given the limitations to amplifying the targeted locus of Exon 1, it was considered that genomic DNA of knockout clones with deletion of full locus, including Exon 1 and Exon 33, would also be devoid of the target site at Exon 33. PCR amplification of the 657 bp genomic region encompassing the Cas9 cut-site of gRNA #2 was detected in H1299 wildtype (control) cells and was absent in H1299 *NOTCH3* KO #2 and *NOTCH3* KO #3 clones as depicted by agarose gel electrophoresis indicating potential genomic disruption. (D) Analysis of *NOTCH3* transcripts in *NOTCH3*-knockout H1299 cells. Total RNA was extracted from wildtype (control) or NOTCH3-depleted H1299 cells, reverse transcribed into cDNA, and analyzed by RT-qPCR using primers specific for *NOTCH3*. Data were normalized to the housekeeping gene *GAPDH* and the mean ± SD of three independent experiments was represented [P (p-value) > 0.05 = (ns or not significant), P ≤ 0.05 = (*), P ≤ 0.01 = (**), P ≤ 0.001 = (***), unpaired Student's t-test].

Subsequently, to define the role of NOTCH3 in the ISX9-driven induction of Notch signaling, NOTCH3-depleted clones were subjected to ISX9 treatment, and differential expression of Notch target genes was investigated. The induction of well-known canonical Notch signatures *HEY1* and *HEY2* were significantly compromised in two different *NOTCH3* KO clones compared to an empty vector clone indicating that ISX9 activates the Notch pathway indeed via NOTCH3 (Fig. 15 A).

To mechanistically demonstrate that stimulation of the Notch response by ISX9 is indeed NOTCH pathway-dependent, I ectopically overexpressed mouse NICD3 in *RBPJ* KO cells. The experiment was replicated until comparable overexpression of NICD3, as detected by Western blotting for NOTCH3, was achieved in wildtype (control) and *RBPJ* KO clones at least in three independent experiments (Fig. 15 B). Overexpression of NICD3 resulted in Notch target gene activation, similar to ISX9, in the wildtype (control) but not in the *RBPJ* KO clones (Fig. 15 C). Altogether, these data suggest that ISX9-mediated induction of Notch target genes depends on NOTCH3.

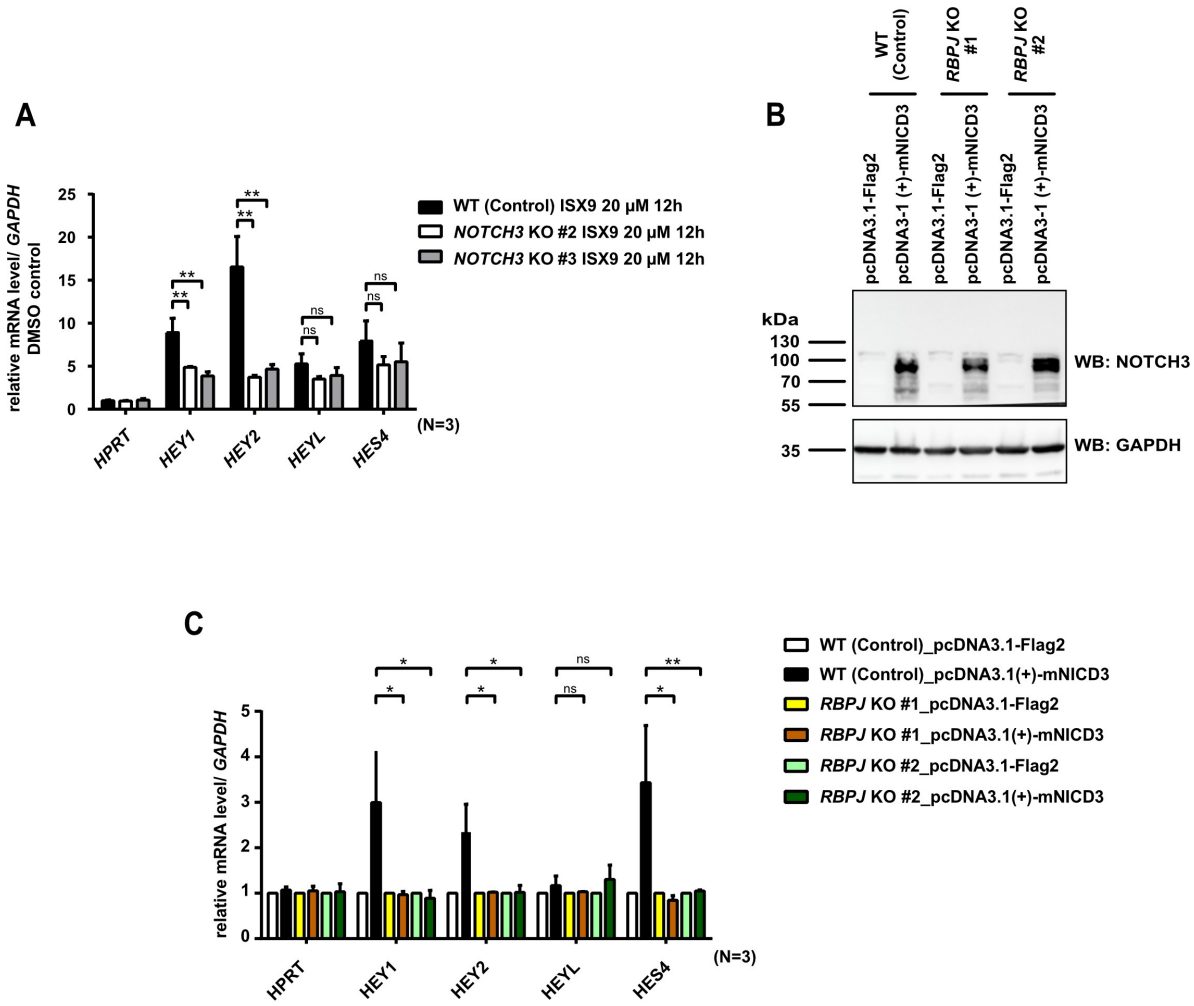


Fig. 15 Pivotal role of Notch3 in ISX9-mediated induction. (A) Depletion of NOTCH3 reduces Notch target gene activation by ISX9. Two H1299 *NOTCH3*-knockout clones KO #2 and KO #3 and wildtype (control) cells were treated with 20 µM ISX9 for 12h. Total RNA was extracted; reverse transcribed and RT-qPCR analysis was performed to quantify the expression of Notch-responsive genes. Relative fold change in ISX9 treated wildtype (control), *NOTCH3* KO #2 and KO #3 was determined in comparison to DMSO control for each Notch target gene. *GAPDH* served as the housekeeping control. The mean values and standard deviation of three independent experiments were represented. (B) Overexpression of mice NICD3 was successfully achieved in H1299 wildtype (control) and *RBPJ* KO cells. Wildtype (control) and RBPJ-depleted H1299 cells (clones KO #1 and KO #2) were transfected with a plasmid expressing mNICD3 and cells expressing pcDNA3.1-Flag2 were used as control. Cells were collected 48 hours post-transfection; whole cell lysate was prepared and the levels of NOTCH3 and GAPDH were detected by Western blotting. Experiments were repeated until overexpression of NOTCH3 was comparable in at least three independent experiments. (C) Overexpression of NICD3 mimics the effect of ISX9-mediated Notch induction in wildtype cells but not in cells lacking RBPJ. Total RNA was extracted from H1299 wildtype (control) or *RBPJ* KO cells overexpressing mouse NICD3, reverse transcribed and RT-qPCR analysis was performed to quantify the expression of Notch-target genes. Relative fold change in mNICD3-transfected wildtype (control), *RBPJ* KO #1 and #2 was determined in comparison to vector-transfected counterparts and was normalized to the housekeeping gene *GAPDH*. The mean values and standard deviation of three independent experiments were represented [P (p-value) > 0.05 = (ns or not significant), P ≤ 0.05 = (*), P ≤ 0.01 = (**), P ≤ 0.001 = (***), unpaired Student's t-test].

4.5 ISX9 induces spurious transcription of NOTCH3

ISX9 treatment induces the expression of NOTCH3 at both transcript and protein levels. Western blot analysis of ISX9-treated H1299 whole cell protein extract displayed two band signals (Fig 11 D, lane 2) for induced cleaved NOTCH3 protein that slightly differed in their apparent molecular weights. The ISX9-induced additional NICD3 isoform might potentially be a variant of active NOTCH3 resulting from the translation of truncated transcripts or from the alternative proteolytic processing of the receptor. I already demonstrated that the induction of the NICD3 fragment by ISX9 is insensitive towards inhibition of S3 site cleavage (Section 4.3) referring to the association of a transcriptional remodeling event rather than regulated proteolysis. Synthesis of truncated *NOTCH3* transcripts might be a consequence of transcription from a cryptic initiation site or alternative splicing. Therefore, I hypothesized that ISX9 leads to the synthesis of a spurious *NOTCH3* transcript which is shorter than the canonical full-length mRNA and is likely to be initiated from a cryptic promoter situated downstream to the γ-secretase S3 cleavage site. This transcript would translate into a shorter form of NICD3 that would be active and independent of γ-secretase cleavage. To test this hypothesis, the relative level of *NOTCH3* transcripts corresponding to the different parts of the *NOTCH3* gene was analyzed using TaqMan probe #32 to amplify Exon 9-10 (which encodes a part of the extracellular domain), TaqMan probe #67 (which encodes C-terminal part of the extracellular domain) and TaqMan probe #10 (which encodes N-terminal part of NICD3) in ISX9-treated H1299 cells normalized to DMSO-treated cells. Induction of *NOTCH3* transcript variants corresponding to different locations from 5' to 3'-ends was comparable in the RT-qPCR assay indicating no significant preference for 3'-direcetd transcription of NOTCH3 (Fig. 16 A).

Next, I asked whether the NOTCH3 truncated protein results from ISX9-triggered specific or alternative splicing events. To address this question, the ISX9 response was determined upon perturbation of the splicing machinery by treating the H1299 cells in combination with a general inhibitor of splicing, Isoginkgetin (O'Brien et al., 2008). ISX9-treated H1299 cells could no more show an increase in NICD1 or the unique NICD3 isoform in the presence of Isoginkgetin (Fig. 16 B, compare lane 2 with 3). Furthermore, another plant biflavone Hinokiflavone with a stronger effect on pre-mRNA splicing showed comparable results (Fig. 16 C, compare lane 2 with lane 6) re-enforcing the fact that splicing of pre-synthesized transcripts is essential for Notch stimulation of ISX9. Notably, Hinokiflavone is known to modulate splicing activity rather than just inhibiting it by promoting exon retention or exon skipping depending on the cell lines and the dosage. Mechanistically, Hinokiflavone inhibits SENP1 (Sentrin-specific proteases 1)-mediated de-SUMOylation of spliceosome proteins leading to altered interactions between the splicing factors and significant changes in the splicing pattern, suggesting a potential link between splicing and protein SUMOylation (Pawellek et al., 2017). Since SUMOylation of protein is a reversible process and depends on the balance between SUMO conjugation and deconjugation reactions, the effect of ML-792, a small-molecule inhibitor of SAE (SUMO activating enzyme) (He et al., 2017), on ISX9-mediated NOTCH3 activation was evaluated. In comparison to SENP1 (SUMO deconjugation enzyme) inhibition by Hinokiflavone, inhibition of SUMO conjugation by ML-

792 showed no interference with the NICD3 induction by ISX9 (Fig. 16, compare lane 2 with lane 5).

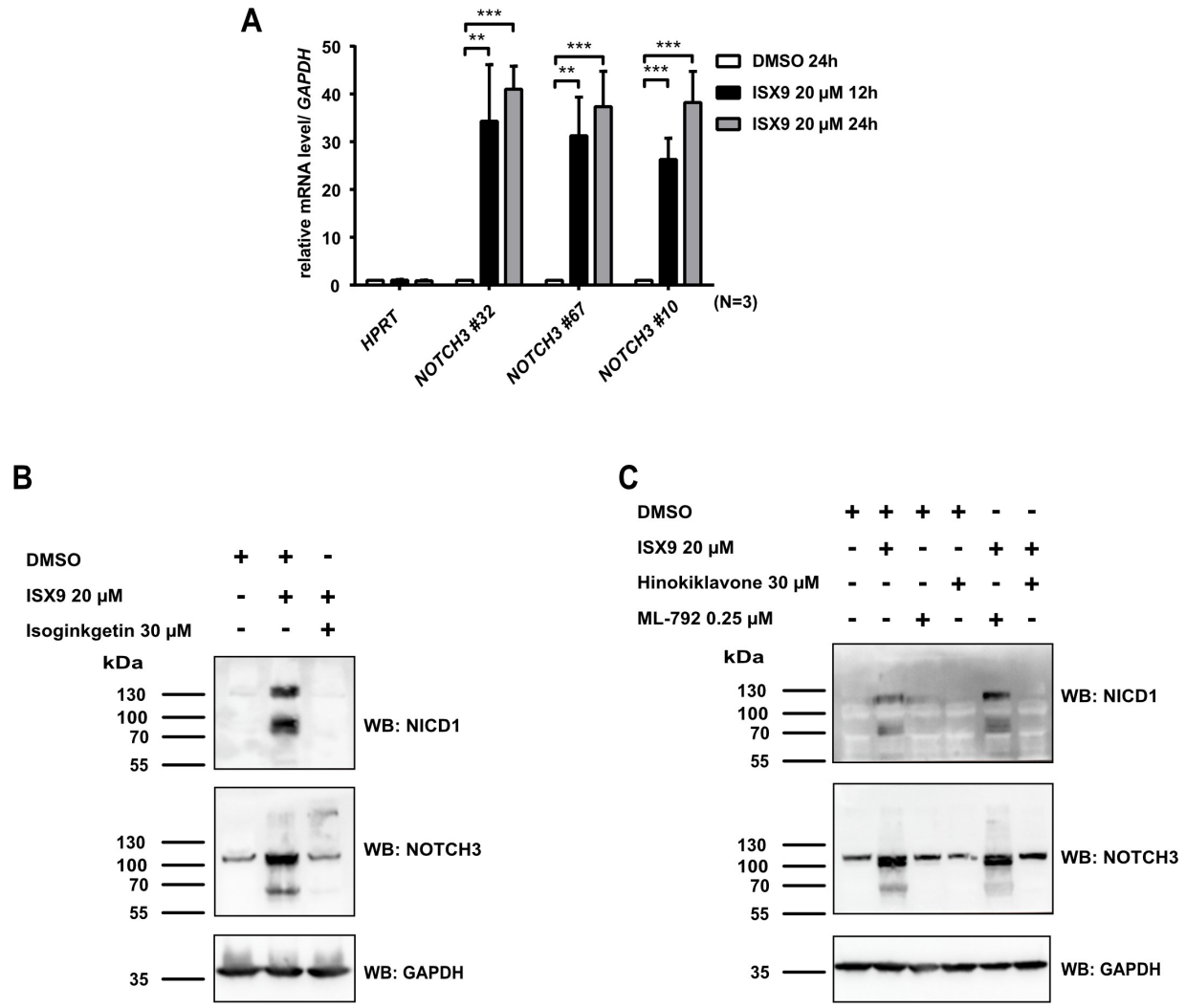


Fig. 16 Notch activation by ISX9 is dependent on splicing machinery. (A) ISX9-mediated transcriptional induction is uniform across the *NOTCH3* transcript. H1299 cells were treated with 20 μM ISX9 for 12h and 24h or DMSO as control. Upon RNA extraction and reverse transcription, cDNAs were analyzed by RT-qPCR using TaqMan probes #32, #67, and #10 to amplify the NOTCH3 extracellular domain, the C-terminal part of the NOTCH3 extracellular domain, and the N-terminal part of NICD3, respectively. *GAPDH* was used as the housekeeping control for normalization. Mean ± SD of three independent experiments was represented. (B) Isoginkgetin, a general splicing inhibitor, suppresses the ISX9-stimulated Notch response. H1299 cells were treated with 20 μM ISX9 for 12h with or without 30 μM Isoginkgetin in combination. The amount of activated NOTCH1, NOTCH3, and GAPDH (loading control) was analyzed by Western blotting. (C) Hinokiflavone with a stronger inhibitory effect on pre-mRNA splicing than Isoginkgetin prevents ISX9-mediated activation of NOTCH1 and NOTCH3. H1299 cells were treated with 20 μM ISX9 or 30 μM of Hinokiflavone separately or in combination. DMSO was used as vehicle control. Cells were subjected to whole cell lysate preparation and activated NOTCH1 and NOTCH3 protein level was analysed by Western blotting and GAPDH served as the loading control. Since Hinokiflavone leads to significant changes in alternative splicing patterns by increasing protein SUMOylation of splicing factors, the effect of inhibition of SUMO-conjugation on ISX9-mediated Notch activation was determined. H1299 cells were treated with 0.25 μM ML-792 alone (Inhibitor of SUMO activating enzyme), or with 20 μM ISX9 in combination with ML-792. The whole cell extract was subjected to Western blotting for cleaved NOTCH1 and NOTCH3, and GAPDH as the loading control [P (p-value) > 0.05 = (ns or not significant), P ≤ 0.05 = (*), P ≤ 0.01 = (**), P ≤ 0.001 = (***), unpaired Student's t-test].

4.6 ISX9 activates calcium influx

In undifferentiated HCN cells (hippocampal neural stem cells), ISX9 has been demonstrated to induce neuronal response. Pharmacological inhibition of Ca^{2+} flux (with Ca^{2+} influx inhibitors, Ca^{2+} channel blockers, and CaM kinases inhibitors) impairs this pro-neurogenic effect indicating a calcium agonistic property of ISX9 (Schneider et al., 2008). Such a calcium-inducing response imposed by ISX9 has also been reported in non-neural backgrounds with a significant impact on diverse cellular functions. In addition to promoting β-cell differentiation in human and mouse pancreatic islets, the pharmacological application of ISX9 protects pancreatic beta cells from glycemic stress and improves islet function by regulating calcium homeostasis through activation of CN/NFAT signaling pathway (Pujol et al., 2018). Further, calcium influx by ISX9 repairs the age-related decay of functional CaMKII, a major regulator of circadian rhythm, suggesting its pharmacological potential for rewiring healthy aging (Li et al., 2022a).

Consistent with these reports, the next question was whether exposure to ISX9 facilitates an increase in cytoplasmic Ca^{2+} in H1299 cells. In fact, the measurement of intracellular calcium levels in the ISX9-treated H1299 cells by the Ca^{2+}-sensitive fluorescent dye Fura-2 unequivocally showed that ISX9 is able to trigger calcium influx rapidly (Fig. 17 A II & III) compared to the DMSO control (Fig. 17 A I).

Since ISX9 activates Notch-specific response via NOTCH3, it was intriguing to explore whether there is an interplay between calcium flux and ISX9-triggered activation of NOTCH3. To accomplish this, I investigated how the removal of exogenous calcium affects the Notch signaling response in H1299 cells subjected to ISX9 treatment. In this context, a few aspects, that might confound the interpretation of the experimental results to reveal the implication of calcium signaling in the mechanism of action of ISX9, were taken into consideration: a) The concentration of calcium in RPMI-1640 media used for H1299 culture is 0.42 mM [Conrad R. (sigma technical documents); Fujisaki et al., 2018] and b) commercially available FCS, used as a supplement for maintaining H1299 cells, contains calcium oxalate crystals either in monohydrate or dihydrate forms (Pedraza et al., 2008). Thus, the effect of ISX9 exposure was determined in a calcium-poor environment and not in calcium-free RPMI media in order to eliminate the effect of calcium salts already present in the normal culture media. H1299 cells were treated with ISX9 in the presence of calcium-free Tyrode's solution and the level of Notch-specific stimulation was compared with that of the ISX9-induced cells in the presence of regular serum-supplemented RPMI-1640 media. Of note, the constituents of calcium-depleted Tyrode's solution are minimal yet sufficient to maintain normal physiological functions of H1299 cells and the cell viability was sufficient for RNA and protein extraction for further analysis. Interestingly, ISX9 failed to stimulate the unique NICD3 isoform in a calcium-deficient condition (Fig. 17 B, compare lane 2 with lane 4) indicating that calcium signaling is essential for NOTCH3 activation by ISX9. Further, the ISX9-triggered NICD3 fragment reappeared upon supplementation of the Tyrode's solution with external calcium (Fig. 17 B, compare lane 4 with lane 6). Also, upregulation of *NOTCH3* (Fig. 17 C) and the Notch target gene *HES4* (Fig. 17 D) by ISX9 was found to be significantly

low in calcium-free Tyrode's solution compared to that in complete media (white dotted bar vs. yellow dotted bar) and was restored upon reintroduction of calcium (yellow dotted bar vs. gray dotted bar) reconfirming the direct association of calcium in ISX9-directed molecular consequences.

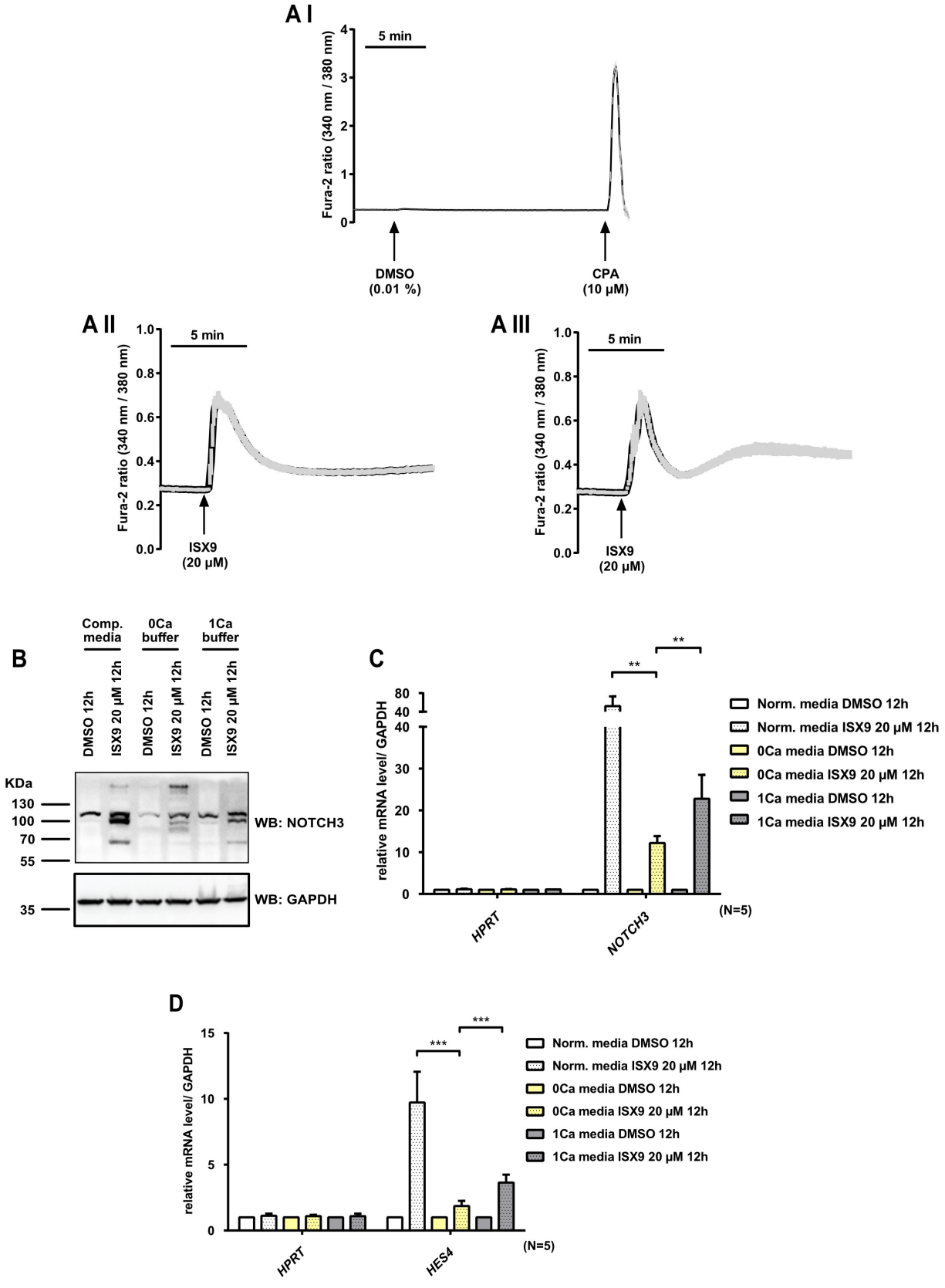


Fig. 17 ISX9-mediated NOTCH3 activation depends on extracellular calcium. (A) ISX9 increases cytosolic calcium in H1299 cells. Cytosolic Ca^{2+} level was measured as a quantification of Fura-2 (340/380) ratios from one respective experiment shown as mean (black line) ± SEM (gray area) after stimulation with DMSO control and CPA (n = 17 cells) (A I), or with ISX9 (dissolved in DMSO) (A II & A III). In most of the responding cells, ISX9 induced either a transient peak in the Fura-2 signal which declined to reach a stable plateau (A II, n = 7 cells), or was followed by a secondary long-lasting increase (A III, n = 9 cells). The maximal DMSO concentration was 0.1 % (1 µl DMSO in 1 ml Tyrode's solution). (B) The induction of activated NOTCH3 by ISX9 depends on exogenous calcium. H1299 cells were treated with ISX9 20 µM or DMSO as control for 12h in complete media or in Tyrode's solution without (0Ca) or with (1Ca) external calcium (1.25 mM). Cells were collected and whole cell lysate was prepared followed by Western blotting for detection of NOTCH3 and GAPDH as the loading control. (C & D) ISX9-driven upregulation of *NOTCH3* and *HES4* is impaired by calcium depletion and is restored when calcium is replenished. Total RNA was extracted from B and reverse transcribed. cDNAs were used for gene expression analysis by RT-qPCR. *GAPDH* was used as the housekeeping gene for normalization. Mean ± SD of three independent experiments was represented [P (p-value) > 0.05 = (ns or not significant), P ≤ 0.05 = (*), P ≤ 0.01 = (**), P ≤ 0.001 = (***), unpaired Student's t-test].
Ca^{2+} imaging experiments were performed in cooperation with Prof. Dr. Diener (Institute for Veterinary Physiology and Biochemistry, Justus Liebig University, Giessen).

The above findings revealed a key role of cellular calcium in the Notch signaling response of ISX9. Intracellular calcium is one of the conserved cellular tools linking biochemical events at the cell surface to gene expression changes in the nucleus. For example, in excitable and non-excitable cells, Ca^{2+} microdomains in the vicinity of open Ca^{2+} channels recruit Ca^{2+}-dependent transcription factors such as NFATs and c-fos and regulate downstream signaling pathways (Yeh & Parekh; 2018). Therefore, I asked whether ISX9-driven activation of the Notch signaling is solely a consequence of ISX9-triggered calcium influx. To address this question, cytosolic calcium concentration was artificially triggered, and subsequent changes in Notch target gene expression were examined. Previous studies have shown that histamine, a biogenic amine mainly synthesized and released by mast cells, induces Ca^{2+} influx in human umbilical vein endothelial cells and pulmonary artery endothelial cells (Mauban et al., 2006). Similarly, a significant increase in cytoplasmic Ca^{2+} concentration was elicited upon application of histamine to H1299 cells (Fig. 18 A). However, histamine-induced calcium response did not correlate with Notch target gene expression as there was no induction of Notch signatures upon stimulation of H1299 cells with histamine (Fig. 18 B). Consolidating all these results, I concluded that a cytosolic calcium spike is required but not sufficient for ISX9-mediated Notch activation. This also hints at the influence of an additional key factor regulating Notch induction by ISX9. As Notch signaling is initiated at the cell surface through ligand-receptor interaction, I asked a fundamental question of whether ISX9 influences the expression level of the Notch ligands. Expression of the canonical Notch ligand *JAG2* was significantly elevated by ISX9 (Fig. 18 C) indicating that ligand expression could be a key for Notch-specific transcriptional response elicited by ISX9.

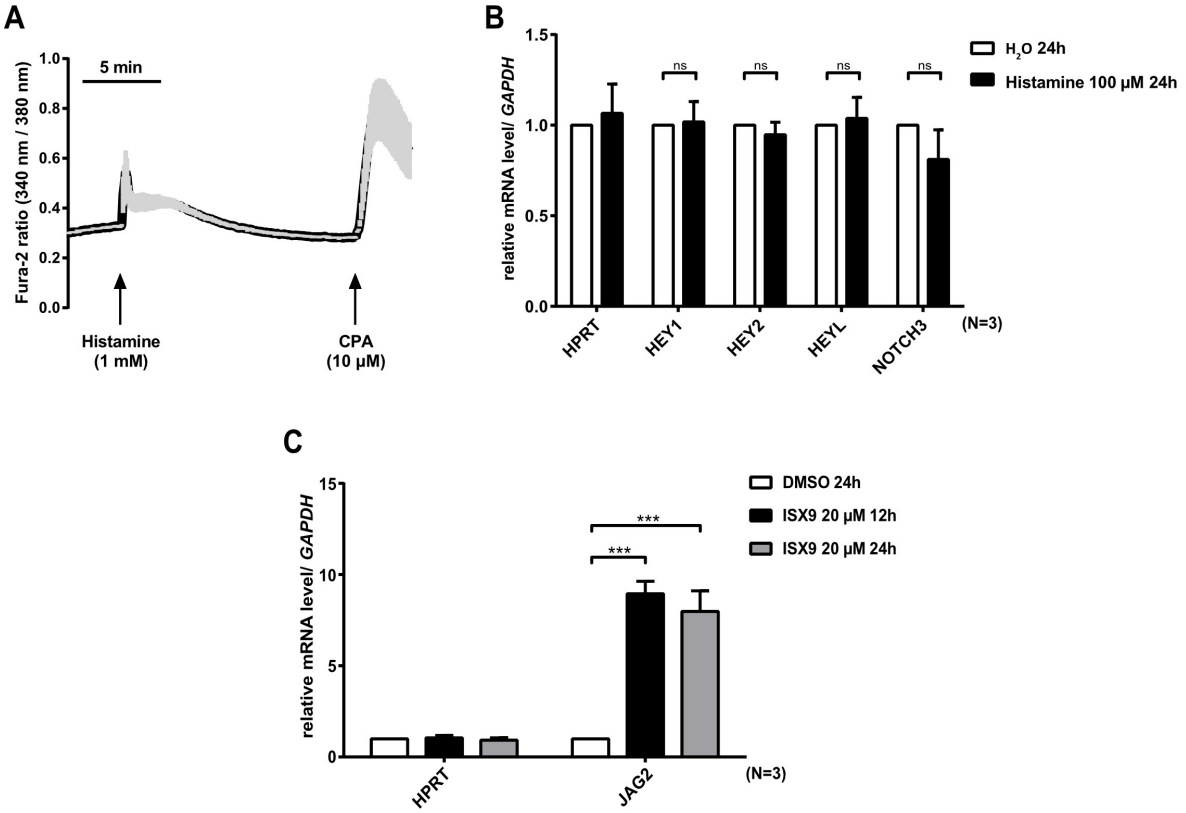


Fig. 18 Ectopic Ca^{2+} spike fails to mimic ISX9-mediated Notch response. (A) Histamine stimulation causes an increase in free cytosolic calcium. H1299 cells were treated with 1 mM histamine and cytosolic calcium concentration was measured using Fura-2-based Ca^{2+} imaging microscopy. Data was presented as mean ± SE of three independent measurements. (B) Histamine has no effect on Notch target gene expression. H1299 cells were stimulated with 100 µM histamine for 24h and total RNA was isolated followed by cDNA synthesis. RT-qPCR was performed to analyze gene expression using gene-specific primers and *GAPDH* was considered as the housekeeping gene. (C) ISX9 treatment leads to upregulation of Notch ligand *JAG2* in H1299 cells. H1299 cells were treated with 20 µM ISX9 or DMSO as control for 12h and 24h. Total RNA was isolated and subjected to reverse transcription. cDNAs were analyzed by RT-qPCR using gene-specific primers and *GAPDH* was used as the housekeeping control for normalization. Mean ± SD of three independent experiments was represented [P (p-value) > 0.05 = (ns or not significant), P ≤ 0.05 = (*), P ≤ 0.01 = (**), P ≤ 0.001 = (***), unpaired Student's t-test].
Ca^{2+} imaging experiments were performed in cooperation with Prof. Dr. Diener (Institute for Veterinary Physiology and Biochemistry, Justus Liebig University, Giessen).

4.7 ISX9-triggered Ca^{2+} dynamics and NOTCH3 induction show temporal correlation

Amongst the wide range of Ca^{2+}-regulated cellular processes, kinetically late cellular events such as nuclear transcriptional activation followed by protein synthesis require Ca^{2+} signaling to operate over a sustained period of time. Based on this, I assumed that two consecutive Ca^{2+} waves elicited by ISX9 (Fig. 17 A III) might have a mechanistic relevance with the kinetics of NOTCH3 induction. To test this, I analyzed the expression NOTCH3 at both transcript and protein levels at early and late time points of ISX9 treatment in H1299 cells. In fact, the ISX9-driven increase in the *NOTCH3* transcript level was detected much

earlier than the increase in NICD3 protein. In conclusion, I envisage that ISX9 elicits an initial calcium trigger followed by an additional delayed Ca^{2+} response which is essential for setting the stage for optimal Notch response.

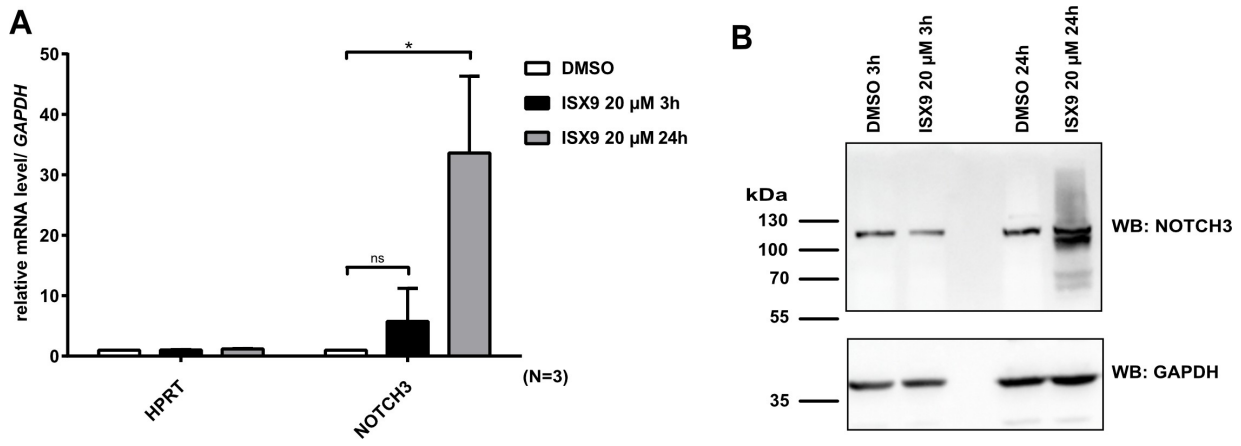


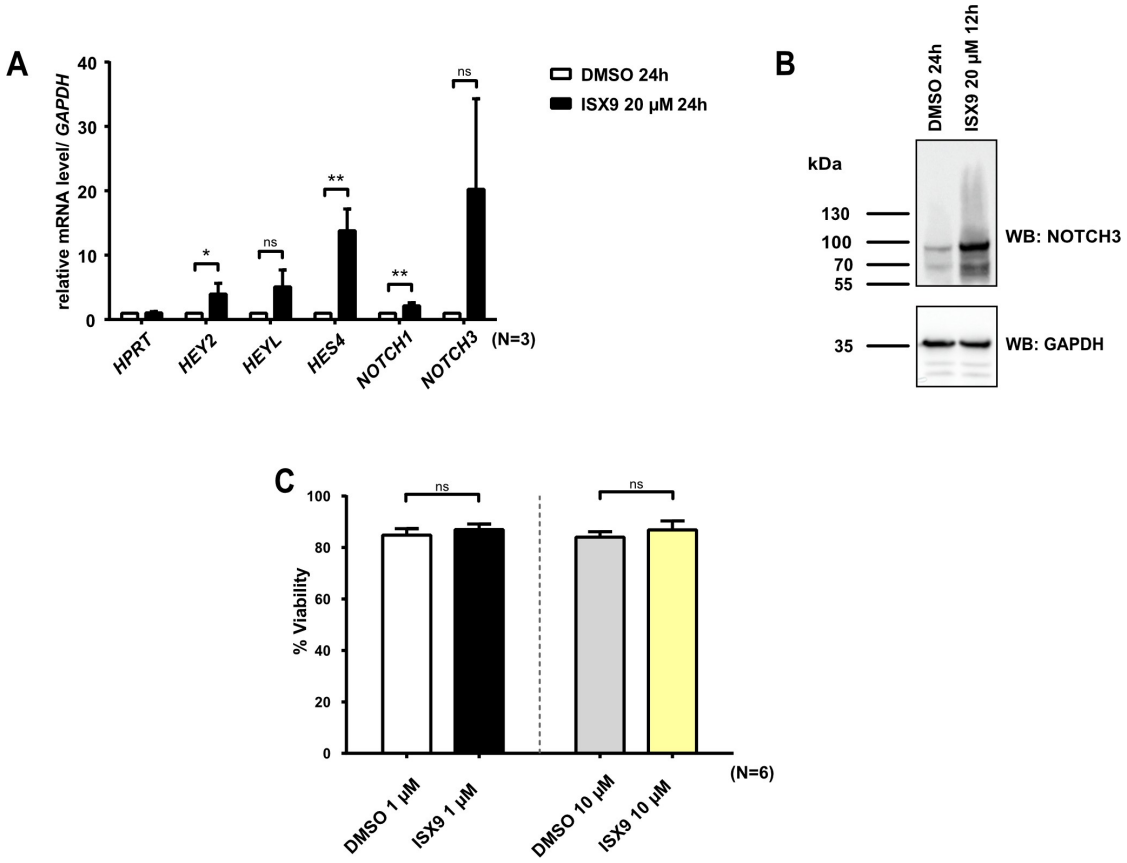
Fig. 19 Induction of NOTCH3 transcript and protein by ISX9 follows distinct temporal dynamics. (A) ISX9 stimulates *NOTCH3* expression. H1299 cells were treated with 20 μM ISX9 or DMSO as control for 3h and 24h. Upon RNA extraction and reverse transcription, cDNAs were analyzed by RT-qPCR. *GAPDH* was used as the housekeeping control for normalization. Fold change was calculated compared to the DMSO control of the respective time points. Mean ± SD of three independent experiments was represented [P (p-value) > 0.05 = (ns or not significant), P ≤ 0.05 = (*), P ≤ 0.01 = (**), P ≤ 0.001 = (***), unpaired Student's t-test]. (B) ISX9 treatment leads to increased NICD3 protein level. H1299 cells were treated for 3h and 24h with 20 μM ISX9 or DMSO as control and the whole cell extract was analyzed by Western blotting for detection of NOTCH3 protein or GAPDH as the loading control. The experiment was repeated three times independently.

4.8 ISX9 induces Notch signaling and differentiation in AML cells

Acute myeloid leukemia (AML), the most common acute leukemia diagnosed in adults, is characterized by blockage of myeloid differentiation and accumulation of progenitor cells (Olsson et al., 1996). Previous findings manifest a tumor-suppressive role of Notch signaling in AML, and therapeutic activation of Notch signaling has been demonstrated to inhibit oncogenic program in AML cells (Kannan et al., 2013). Reasoning that, my next goal was to investigate whether the small molecule ISX9, with its prominent Notch-'boosting' effect, exerts anti-tumor activity in AML cells. So far in this study, ISX9-mediated Notch activation has been elucidated in the non-small cell lung cancer cell line H1299. To assess whether the Notch-inducing potential of ISX9 is also relevant in the myeloid context, I assessed the Notch signaling response to ISX9 in the human acute myeloid leukemia cell line THP1. ISX9 treatment led to significant upregulation of the Notch target genes *HEY2* and *HES4* and the *NOTCH1* receptor gene (Fig. 20 A). Also, a marked increase in *NOTCH3* transcript (Fig. 20 A) and activated NOTCH3 protein (Fig. 20 B) was observed in the ISX9-treated THP1 cells suggesting that ISX9 is capable of inducing Notch signaling in AML cells where baseline Notch activity is remarkably low (Kannan et al., 2013). Next, I performed functional assays to test whether Notch pathway reactivation in AML cells leads to ectopic myeloid differentiation. To accomplish this, the impact of ISX9 on the AML cell line THP1 was

characterized through several functional assays including evaluation of overall viability, cell-cycle distribution (G1, S, and G2/M), and expression of several differentiation markers. A slight but non-significant increase in the viability of THP1 cells was observed upon treatment with two dosages of ISX9 (1 µM and 10 µM) compared to DMSO control (Fig. 20 C). In FACS-based assays, no significant change in the population of cells at different phases of the cell cycle was observed after treating THP1 cells with 1 µM ISX9. However, a significant increase (80.1%) in the proportion of THP1 cells in the G1 phase was observed upon treatment with 10 µM ISX9 compared to DMSO-treated cells (58.1%) with a concomitant decrease in the proportion of cells in the S and G2/M phase from 15.1% to 7.2% and from 12.1% to 3.6%, respectively (Fig. 20 D).

Further, immuno-phenotypical characterization was performed to evaluate the induction of differentiation in THP1 cells upon exposure to ISX9. A significant increase in the proportion of CD11b-expressing cells (8.5% to 27.5%) was observed when treated with 1 µM ISX9 while other populations remained unchanged (CD14: 7% vs. 7.4%; CD86: 10.6% vs. 12.2%). A significant increase in the expression of surface markers: CD33 (87.1% to 97.1%), CD11b (5.3% to 92.3%), and CD86 (8.6% to 81.2%) was observed after treatment with 10 µM ISX9 compared to DMSO control while the proportion of CD14-positive cells remained unchanged (6.3% to 8.4%) (Fig. 20 E). These observations collectively demonstrated that the potential Notch-'booster' ISX9 provokes proliferative arrest and terminal differentiation in AML cells.



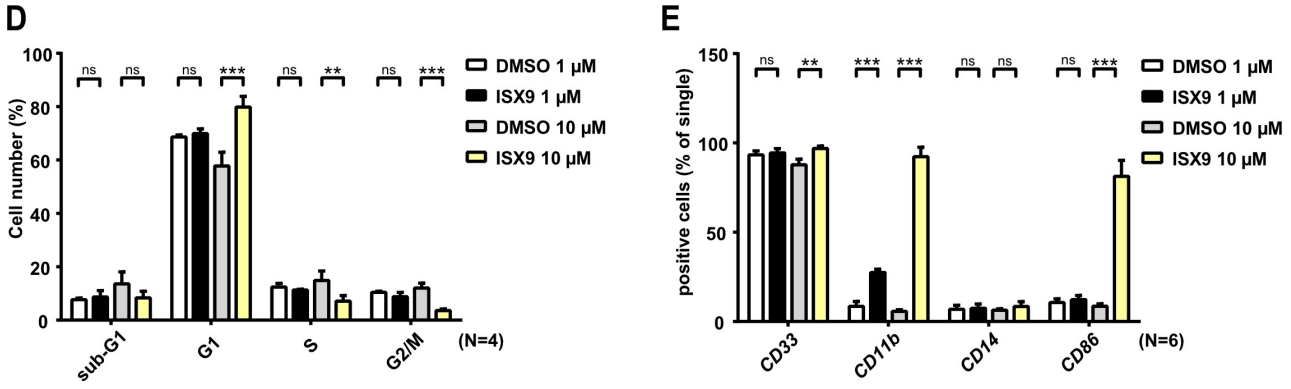


Fig. 20 ISX9 treatment induces Notch signaling and promotes cell cycle arrest and differentiation in AML cells. (A) ISX9 leads to a marked increase in Notch target gene expression in THP1 cells. The human AML cell line THP1 was treated with 20 µM ISX9 for 24h. Total RNA was extracted; reverse transcribed, and RT-qPCR analysis was performed to quantify the expression of Notch-target genes. *GAPDH* served as the housekeeping gene. Mean ± SD of three independent experiments was represented. (B) ISX9 treatment results in a significant increase in activated NOTCH3 protein. THP1 cells were treated with 20 µM ISX9 or DMSO control for 24h and the whole cell extract was analyzed by a gradient gel followed by Western blotting for detection of endogenous NOTCH3 protein or GAPDH as the loading control. The experiment was repeated three times independently. (C) THP1 cell viability is not grossly affected by ISX9. THP1 cells were treated with 1 µM (black bar) and 10 µM (yellow bar) ISX9 or DMSO (white and gray bars) as the vehicle control for 48h. The incubated THP1 cells were stained with DAPI and acridine orange, and the viability was determined by NucleoCounter NC 3000 based on the staining. DAPI-positive cells indicated dead cells which were distinguished from acridine orange-positive living cells. (D) Cell cycle progression of THP1 cells is arrested by ISX9. THP1 cells were treated with 1 µM and 10 µM ISX9 or DMSO as control for 48h. The cells were fixed and then incubated with DAPI. Cell cycle dynamics of the ISX9 and DMSO-treated THP1 cells were determined by the DNA content detected by DAPI staining. (E) ISX9 strongly promotes the surface expression of differentiation markers in THP1 cells. THP1 cells were exposed to 1 µM or 10 µM ISX9 or DMSO control and the level of surface antigens CD33, CD11b, CD14, and CD86 was analyzed by flow cytometry. Mean ± SD of 5-6 independent experiments was represented in C, D, and E [P (p-value) > 0.05 = (ns or not significant), P ≤ 0.05 = (*), P ≤ 0.01 = (**), P ≤ 0.001 = (***), unpaired Student's t-test].
Cell viability, cell cycle distribution, and flow cytometry assays were performed at the laboratory of Prof. Dr. Oswald (Center for Internal Medicine, University of Ulm, Ulm).

4.9 ISX9 stimulates the Notch pathway in SCLC cells

The Notch agonistic effect of ISX9 could also be beneficial in other clinical contexts, one of which is to drive tumor immunity in small-cell lung cancer (SCLC) cells. A study by Roper et al. showed that *in vitro* activation of Notch signaling induces a low neuro-endocrine phenotype in SCLC cells which is also consistent with the fact that expression of Notch pathway genes is associated with improved response to immune checkpoint blockade in SCLC patients (Roper et al., 2021). I performed preliminary experiments in the SCLC cell line H69, where ISX9 treatment led to the upregulation of several canonical Notch target genes such as *HEY2*, *HEYL,* and *NOTCH3* indicating that the pharmacological modulation of the Notch pathway by ISX9 might be promising for the development of immunotherapeutic strategies for SCLC.

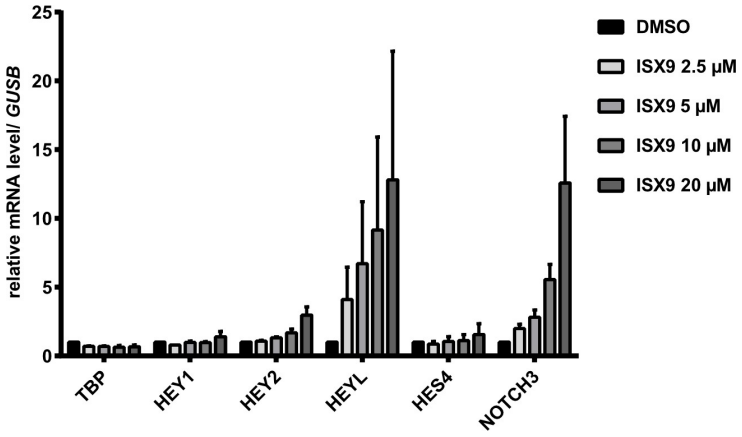


Fig. 21 ISX9 triggers Notch target gene expression in small cell lung cancer cell line H69. H69 cells were treated with different dosages of ISX9 or DMSO as the control for 24h. Upon RNA extraction and reverse transcription, cDNAs were analyzed by RT-qPCR. *TBP* was used as the housekeeping control for normalization. Fold change was calculated in comparison to the DMSO control. Mean ± SD of two independent experiments was represented and no significance test was performed.

4.10 The MAPK/ERK pathway is activated by ISX9

Next, I went ahead to shed more light on the molecular mechanism of ISX9. Considerable diversity in Notch-induced transcriptome is often generated through the direct interaction between core Notch pathway components with other signaling cascades. There are pieces of evidence that activation of the MAPK/ERK pathway regulates the transcriptional activity of NOTCH (Sadek et al., 2008; Tremblay et al., 2013; Li et al., 2022b). More importantly, ISX9 is known to improve the anti-diabetic activity of pancreatic β-cells by enhancing the phosphorylation of ERK 1/2 (Dioum et al., 2011). I, therefore, examined the effect of ISX9 on the activation of ERK 1/2 by the detection of phosphorylated ERK 1/2 (p-ERK 1/2). Induction of p-ERK 1/2 was observed after 10 min of ISX9 exposure which persisted till 30 min and then declined over time while the total ERK 1/2 level remained unaltered. This result indicates the potential involvement of the MAPK/ERK pathway in the stimulation of Notch response by ISX9 and certainly deserves further attention.

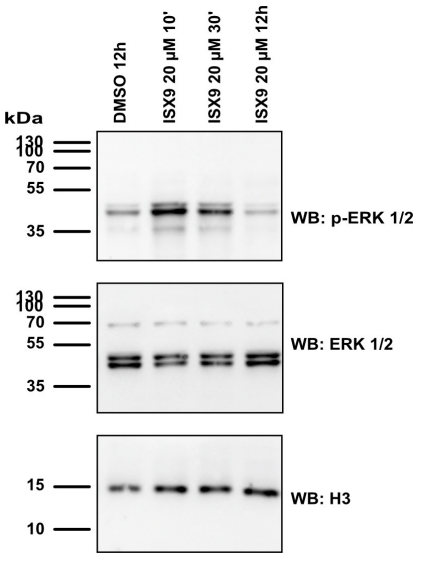


Fig. 22 ISX9 enhances ERK 1/2 phosphorylation. Time course of ISX9-induced activation of ERK 1/2. H1299 cells were treated with 20 μM ISX9 for 10 min, 30 min, and 12h, and with DMSO for 12h as control. The whole cell extracts were analyzed by Western blotting to detect the level of phospho-ERK 1/2, total ERK 1/2, and H3 as the loading control. The experiment was repeated three times independently.

My findings identified ISX9 as a novel tool for pharmacological reinforcement of Notch signaling and also as a synthetic inducer of cellular differentiation. Although the mechanism of action of ISX9 has been partially understood, the prominent Notch-driving feature of ISX9 makes it an attractive drug candidate for application in relevant pathophysiological conditions. AML cell cycle arrest in response to Notch signaling activation has been described previously (Kannan et al., 2013; Ye et al., 2016) which is consistent with my results in Section 4.8. In conclusion, I reported that ISX9 is a potent Notch inducer and it shows anti-proliferative effects in AML cells holding great promises for future development as a novel agent beneficial to patients with AML.

5. DISCUSSION

The objective of my thesis was to identify a pharmacological Notch agonist and to unravel its molecular mechanism(s). Surprisingly, the mechanism is specific to one particular Notch receptor, Notch3, and also involves the canonical transcription factor RBPJ. The results deciphered that ISX9 is a potent small-molecule compound capable of activating the Notch response and holds great promise for Notch pathway reactivation.

5.1 Does the Notch-'boosting' effect of ISX9 depend on the choice of the experimental system?

In the present study, the Notch activating potential of the small molecule ISX9 was evaluated with an approach to monitor its effect on Notch signaling events in terms of gene expression.

Cellular response to pathway-manipulating drugs could be presumed to be dependent on the baseline activity status. In this line, the induction of Notch signal transduction by ISX9 was found to be a reciprocation of the background Notch activity status in the experimental model system. For example, the murine T-cell lymphoma cell line Beko, derived from TCRβ-deficient mice, is characterized by constitutively active Notch signaling, and treatment with γ-secretase inhibitor DAPT leads to significant downregulation of several Notch target genes such as *Deltex-1*, *pre-Tα*, *Hes1*, *Hey1*, and *CD25* (Liefke et al., 2010). In this 'NOTCH-ON' state, ISX9 treatment leads to a minimal dose-dependent induction of Notch target genes *Hes1*, *Gm266,* and *preTCR,* and almost no effect was observed for *Hey1* (Fig. 5 A). On the contrary, in the murine hybridoma mature T-cell line (MT) with significantly low Notch activity (Oswald et al., 2016), ISX9 showed a significant activation of Notch signatures (Fig. 5 B) indicating that the baseline level of transcription determines the extent of amenability of the Notch signaling cascade.

Amongst several human carcinoma cell lines, the strongest and most consistent Notch-inducing activity of ISX9 was observed in the non-small cell lung cancer (NSCLC) cell line H1299. This metastatic cell line is associated with poor expression of Notch pathway components representing a 'NOTCH-low' mode. Also, I demonstrated that ectopic activation of the Notch pathway (by overexpression of NICD3) in H1299 cells leads to upregulation of Notch target genes (Fig. 4 A) and also that depletion of RBPJ leads to derepression of Notch target genes (Fig. 4 B) confirming that the Notch pathway is basal and inducible. This strongly suggests that H1299 represents an ideal system for capturing the Notch-'boosting' potential of the compound of interest.

5.2 Distinct response of Notch1 and Notch3 receptors to ISX9

The Notch signaling response is regulated at the level of proteolytic processing of the membrane-bound form of the Notch receptor to liberate the active NOTCH intracellular fragment which subsequently results in transcriptional activation. Therefore, the Notch

signaling pathway could be modulated in several steps such as ligand-receptor interaction, receptor processing, or transcriptional activation. Mechanistically, I could experimentally prove that ISX9 activates the *NOTCH3* gene locus and that in so doing, generates a novel γ-secretase-independent NICD3 isoform that activates the downstream Notch signatures. Moreover, a number of Notch target gene signatures activated by ectopic expression of the cleaved NOTCH3 receptor (mouse NICD3) (Fig. 4 A) are also upregulated by ISX9 (Fig. 3 A) and this provides a comprehensive evidence of activation of the NOTCH pathway by the small molecule ISX9 in a NOTCH3-dependent manner. It is intriguing to extend this comparative study on a genome-wide scale to finally establish that ISX9 promotes activation of the Notch pathway through the upregulation of NOTCH3-specific signatures.

My observations elucidated that ISX9 exerts a differential effect on NOTCH3 compared to NOTCH1 as a) it results in a relatively stronger induction (upregulation) of *NOTCH3* transcript compared to *NOTCH1* (Fig. 11 C) and b) it promotes the synthesis of a shorter functionally active NOTCH3 protein (Fig. 11 D) which is speculated to be devoid of the S3 cleavage site and therefore GSI-resistant. Given that *NOTCH3* is a canonical downstream transcriptional target of NOTCH1, the distinct effect of ISX9 on protein turnover of two different members of the Notch receptor family: NOTCH1 and NOTCH3, as reported in this study, refers to a unique pharmacologically induced scenario. This unveils that the machinery regulating NOTCH3 can operate autonomously without requiring NOTCH1 activation under the influence of ISX9.

Of note, this inference is based on the NOTCH3 Western blot presented in Fig. 11 D. Unlike the rabbit monoclonal antibody (Cell Signaling #4147), which specifically detects endogenous levels of cleaved or activated Notch1 (corresponding to Val1754 or Val1744 S3 cleavage site in human or mouse Notch1 respectively) without recognizing the full length or any other cleaved form of the Notch1 protein, the rabbit monoclonal antibody for NOTCH3 (Cell Signaling #5276) detects endogenous levels of total NOTCH3 protein including both full-length as well as the truncated versions.

Although I received the recombinant monoclonal rabbit anti-human NICD3 antibody directed at the human NOTCH3 Val1662 S3 cleavage site as a kind gift from Dr. Christian W. Siebel (Choy et al., 2017), the NOTCH3 Western blot using this antibody yielded a weak signal. Therefore, I decided to use the anti-NOTCH3 antibody (Cell Signaling #5276) and detected activated or cleaved NOTCH3 based on the molecular mass for evaluating the effect of ISX9 treatment on NOTCH3 activation. However, the truncated form of the ISX9-induced NICD3 needs to be determined by mass spectrometry.

Furthermore, to investigate whether NOTCH3 is the key limiting factor of Notch pathway induction by ISX9, I performed CRISPR/Cas9-mediated depletion of NOTCH3 in H1299 cells and assessed the response of NOTCH3-depleted H1299 cells to ISX9 (Fig. 15 A). Two independent *NOTCH3*-knockout clones were analyzed to exclude clonal artifacts. Compared to the wildtype control cells, ISX9-driven induction of Notch targets such as *HEY1* and *HEY2* was significantly low but not completely abolished in the NOTCH3-depleted background hinting at residual NOTCH3-independent Notch pathway activity. One plausible

explanation is that ISX9-activated NOTCH1 could still generate signals (by synthesis of NICD1) for upregulating the Notch pathway in the absence of NOTCH3. Therefore, it would be interesting to investigate the effect of ISX9 on the expression of Notch target genes in GSI-treated NOTCH3-depleted cells.

Although induction of Notch response upon either overexpression of mouse NICD3 or ISX9 treatment was abolished upon RBPJ-depletion, the pattern of the perturbations did not completely overlap. The constitutively activated form of NOTCH3 did not induce the Notch target genes at all in the absence of RBPJ (Fig. 15 C), while this abrogation was only partially observed for *HEY1*, *HEY2,* and *HES4* and not for HEYL in ISX9-treated RBPJ-depleted cells (Fig. 10 B). This clearly indicates the association of an additional factor which is likely to be a non-canonical axis triggered by ISX9 and warrants further investigation.

Collectively, I consolidate my observations through a working model summarizing the mechanism of action of ISX9 (Fig. 23). The potential NOTCH 'booster' ISX9 enhances the Notch signaling response significantly. ISX9-facilitated Notch pathway modulation is not dependent on proteolytic cleavage, the key regulatory step of Notch receptor activation rather, ISX9 promotes the synthesis of biochemically active NICD3 fragments leading to the transactivation of Notch target genes. Furthermore, NOTCH3-induction by ISX9 partially relies on cytosolic calcium.

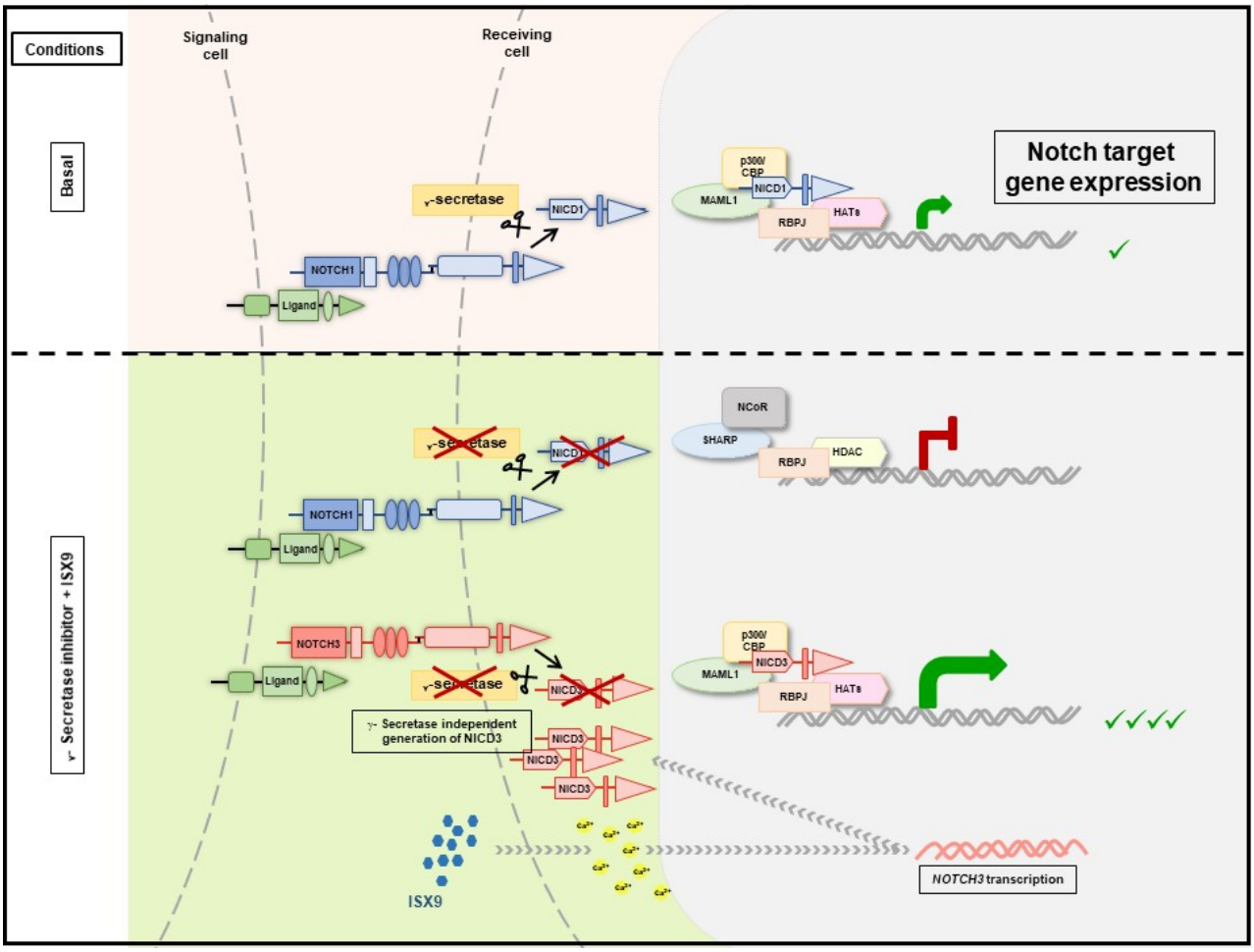


Fig. 23 Model: Notch target gene activation is mediated via small-molecule ISX9 through a Notch3-dependent mechanism. In an uninduced state, basal level NOTCH1 protein maintains a baseline Notch signaling activity in H1299 cells and expression of the NOTCH3 receptor is minimal. The Notch 'booster' ISX9 leads to a significant upregulation of *NOTCH1* and *NOTCH3* transcripts. Subsequently, γ-secretase-mediated activation of NOTCH1 takes place while induction of active NOTCH3 protein is not processing-dependent and is mediated by ISX9-driven rise in cytosolic Ca^{2+} concentration.

GPCR (G-protein coupled receptor) signal transduction is one of the crucial regulators of cytoplasmic calcium oscillations. GPCR integrates extracellular signals and potentiates phospholipase C activation to generate the secondary messenger IP3 (inositol 1,4,5-triphosphate), which binds to IP3R (inositol triphosphate receptor) in ER (endoplasmic reticulum) and releases calcium into the cytoplasm (Dhyani et al., 2020). Given the role of GPCRs in intracellular calcium mobilization and emerging evidence of ISX9-activated Ca^{2+} influxes, I speculate that the ISX9-GPCR mechanistic pathway might be the key upstream trigger resulting in downstream transcriptional activation of Notch target genes. Contextually, a functional target screen identified the extracellular proton-sensing GPCR OGR1 as a target protein of ISX9. In this article, ISX9 has been proposed to promote cardiomyogenic differentiation and pro-survival functions in infarcted myocardial cells, which is essentially mediated by OGR1-triggered Ca^{2+} fluxes (Russell et al., 2012). In this direction, it would be intriguing to carry out target-based analysis to identify the upstream protein or receptor targets of ISX9 which was beyond the scope of the present study.

Also, from the mechanistic point of view, I do not exclude the possibility of ISX9's function at the level of transcriptional regulation. Diverse chromatin modifying functions of ISX9 have been previously reported which include induction of nuclear export of HDAC5 (Schneider et al., 2008), stimulation of HAT activity (Dioum et al., 2011), or inhibition of class I HDACs (Koeniger et al., 2021). These strongly suggest that remodeling of the chromatin landscape by ISX9 might also contribute to its Notch-inducing effects in addition to other significant mechanisms of ISX9 action described in this study.

5.3 Mechanistic relevance of ISX9-triggered Ca^{2+} dynamics

The results in Fig. 17 unveil the significant mechanistic implication of calcium signaling in the Notch-agonistic effect of ISX9. The observation that induction of Notch signaling by ISX9 is abrogated upon withdrawal of exogenous calcium depicts that calcium is necessary for the stimulation of Notch response by ISX9. Interestingly, ISX9 evoked two different patterns of Ca^{2+} signals in H1299 cells: either a rapid initial phase of intracellular Ca^{2+} that declined to a stable plateau of basal Ca^{2+} levels (Fig. 17 A II) or an initial transient rise of cytosolic Ca^{2+} followed by a sustained phase of Ca^{2+} influx (Fig. 17 A III). This variation in the influx phase of Ca^{2+} signals could be attributed to pre-existing cell-to-cell variability in terms of the activity and the expression of IP3R channels (Yao et al., 2016) or voltage-gated ion channels (Vereb et al., 2005; Vetter & Lewis, 2010) in the H1299 cells. Therefore, manipulation of all major sources of Ca^{2+} influx in H1299 cells would provide further insights into the variable Ca^{2+} dynamics that were observed upon ISX9 treatment.

The level of intracellular calcium is determined by the balance between the release of calcium from internal stores or entry from an external medium and the removal of Ca^{2+} from the cytoplasm. This enables the maintenance of the resting level of Ca^{2+} ions at approximately 100 nM. Therefore, in a given cell type, the expression of a unique set of Ca^{2+}-signaling components (IP3R channels, SERCA, etc. [Lemon et al., 2003]) creates distinct spatial and temporal properties of its Ca^{2+} signaling system (Berridge et al., 2003) which, in turn, determines the calcium spike upon exposure to calcium agonists. Therefore, the combination of ISX9 with calcium channel blockers might further validate the association of calcium signaling with ISX9-driven Notch activation.

A feedback loop between Ca^{2+} signaling and the cell cycle has been described in regenerative contexts (Deng et al., 2015; Moore et al., 2023). Therefore, the diverse calcium response elicited by ISX9 (Fig. 17 A II & III) could also be a result of the heterogeneous mixture of asynchronous H1299 cells at different cell cycle stages. As the experimental cells were not synchronized, they consisted of both proliferating and quiescent cells with significant differences in their intracellular signaling modules coupled to Ca^{2+} mobilization. Therefore, calcium manipulation with synchronized cells might clarify whether the diverse Ca^{2+} signaling dynamics in ISX9-treated cells were due to cell cycle-specific transcription of Ca^{2+} signaling pathway genes.

Depletion of exogenous calcium reduced ISX9-mediated induction of *NOTCH3* and the Notch target gene *HES4* while their upregulation was restored upon replenishment of calcium (Fig. 17 C & D). However, other Notch target genes (*HEY1*, *HEY2*, and *HES4*) were not regulated in this manner (data not shown), or rather their expression did not correlate with the expression of the *NOTCH3* gene which refers to their distinct responses to the manipulation of external calcium and provides another evidence of NOTCH3-independent Notch pathway activation by ISX9. This might include an association of upstream Notch pathway components such as Notch ligands (discussed below) or other non-canonical signaling axis that positively supports Notch target gene expression.

It is of note that, as a positive control, stimulation of H1299 cells with histamine led to an increase of cytosolic calcium which, however, failed to induce Notch target gene expression. This, in turn, confirms that calcium is required but not sufficient for ISX9-mediated activation of Notch signaling suggesting that other aspects are additionally required to achieve full induction. Contextually, significant upregulation of the Notch ligand *JAG2* in ISX9-treated H1299 cells (Fig. 18 C) presents an open question of whether Notch ligands are the key determinants of Notch signal transduction in ISX9-treated cells other than NOTCH3 activation and cytosolic calcium rise. Future work should be aimed at illustrating the effect of ligand inhibition, for example, with blocking antibodies or by knockdown of specific ligands, on ISX9's Notch response.

Dynamics of NOTCH3 activation by ISX9 showed a distinct temporal pattern associated with the induction of *NOTCH3* mRNA and its protein product (Fig. 19). I speculate that this plausibly reflects the nature of ISX9-stimulated intracellular Ca^{2+} waves. It is presumed that a short-lived rapid Ca^{2+} spike is not enough to drive the Notch response completely and

therefore a subsequent cytosolic calcium rise is required for full induction of the Notch signaling axis. As a monophasic cytoplasmic calcium flux was also triggered in a significant proportion of H1299 cells upon ISX9 exposure, it is also possible that the second trigger for sustained Notch activity might not necessarily be a second Ca^{2+} spike. Considering the heterogeneity of Ca^{2+} response in ISX9-treated H1299 cells (Fig. 17 A II & III) and the results in Fig. 18 C, I surmise that ISX9-stimulated transient Ca^{2+}-flux evokes a second wave of Notch ligand activity (for example JAG2, a NOTCH1 ligand) which subsequently triggers the NOTCH1 receptor to sustain Notch signaling response. This needs to be elucidated in detail and my observation is a starting point for further investigation.

5.4 Future directions

Previous reports on the small-molecule ISX9 have highlighted its versatile applications, for example, in inducing neurogenesis and improving hippocampal memory (Petrik et al., 2012), protecting pancreatic β-cell against apoptotic death (Pujol et al., 2018), potentiating circadian amplitude in aging cells (Li et al., 2022a), and promoting cardiogenic regeneration (Sadek et al., 2008; Xuan et al., 2018). Despite the volume of work on ISX9-mediated diverse effects on cellular reprogramming, the Notch pathway-specific therapeutic targeting potential of ISX9 has not been previously reported.

Given the potential Notch-'boosting' effect of ISX9, it would be highly desirable to employ ISX9 for reactivation of the Notch pathway in acute myeloid leukemia, where the tumor-suppressive role of NOTCH has been manifested. Consistent with the fact that the expression of Notch signaling components and downstream targets is significantly low in AML patients and forced Notch activation by expression of constitutively active Notch receptors or exposure to Notch ligand peptide mimetics inhibits AML proliferation (Kannan et al., 2013), I could successfully demonstrate that ISX9 treatment not only enforces Notch pathway activation but also provokes proliferative arrest in the AML cell line THP1.

Since, differentiation blockade is one of the major pathological features of AML, manipulation of myeloid differentiation and subsequent apoptosis of terminally differentiated cells has been a successful strategy for the treatments of myeloid malignancies, referred to as differentiation therapy (Olsson et al., 1996; Stubbins & Karsan, 2021). Therefore, utilizing the pro-differentiating properties of ISX9 (Fig. 20 E), restoration of the cellular differentiation program should allow for an effective therapeutic response in AML and other cancers, when used in combination with conventional chemotherapy.

This study has provided compelling evidence to demonstrate the Notch-agonistic capacity of ISX9. I could identify the Notch-inducing effect of ISX9 in a variety of cell types indicating a broad and very fundamental mechanistic functionality of the small molecule ISX9. Therefore, ISX9 might offer therapeutic benefits in several clinical contexts such as for the treatment of cancers where Notch plays a tumor-suppressive role, for example, acute myeloid leukemia (Lobry et al., 2013), head and neck squamous cell carcinoma (Stransky et al., 2011) and skin cancer (Demehri et al., 2009) or in other pathological conditions

resulting from reduced Notch activity. I have discussed such applications in the context of anti-leukemic therapy in AML (Fig. 20) and immune activation therapy in SCLC (Fig. 21). Furthermore, given that Notch activity in bone endothelial cells regulates the differentiation of osteoprogenitor cells and promotes sprouting of bone vasculature (Ramasamy et al., 2014), ISX9 might serve as a promising drug candidate for the design of anti-osteoporosis drugs or for regeneration therapy with implication in bone fracture repair.

Pharmacological NOTCH activation shows therapeutic benefits in several clinical contexts. However, Notch signaling activation by viral vectors and peptide agonists faces limitations in application contexts due to issues like random viral insertion and low permeability or bioavailability, respectively (Craik et al., 2013). So far, the only known small molecule Notch agonist is a plant-derived compound NHMC (N-methylhemeanthidine chloride), which promotes proteolytic cleavage and activation of NOTCH1 and shows anti-leukemic activity *in vitro* and *in vivo* (Ye et al., 2016). This compound has been isolated from the whole plants of *Zephyranthes candida* (Rain lily) and has not been made commercially available. Therefore, ISX9 is one of the relatively few small molecules that hold great promise for the future development of mechanism-based interventions for Notch hypomorphic diseases without genetic manipulation.

Molecular exploration in this study has revealed a unique regulatory axis of the Notch signaling pathway in a pharmacologically induced condition which is coherent with several other studies where small molecules have been utilized as experimental probes to explore complex signaling networks. Although my work presents ISX9 as a new Notch response-promoting toolkit and a synthetic inducer of AML cell differentiation, it remains to be addressed whether ISX9-mediated proliferative arrest of AML cells is indeed a consequence of NOTCH activation. To accomplish that, it is necessary to test whether ISX9-mediated anti-AML effects could be reversed by γ-secretase inhibitor pretreatment.

Finally, as ISX9 has a NOTCH-inducing effect on several cell types, additional work should clarify if and how ISX9 can be deployed for specific clinical purposes. In the study by Petrik et al., where ISX9 was shown to improve memory in adult mice, it was well tolerated without any adverse health effects (Petrik et al., 2012). This suggests that ISX9 is safe for *in vivo* applications at least in animal models. It also remains for future studies to address the challenges associated with the specificity and efficacy of ISX9 administration in patients depending on the therapeutic goal.

Although ISX9 is known to be a cell-permeable synthetic compound, no direct evidence has shed light on its actual direct target and site of action. Therefore, future studies might employ systems pharmacology to predict the transport and distribution of ISX9 to elucidate further on the regulatory molecular alterations facilitated by ISX9. It would be also interesting to assess the biological effect of Isoxazole variants to see whether small structural alterations could impact biological activity.

LIST OF ABBREVIATIONS

ADAM	a disintegrin and metalloprotease
ADC	antibody-drug conjugate
ADRN	adrenergic
AML	acute myeloid leukemia
ANK	Ankyrin repeats
APC	antigen-presenting cells
Aph1	anterior pharynx-defective 1
APP	amyloid precursor protein
APS	Ammonium persulfate
ASB	ankyrin repeat and SOCS box containing 11
ASCL1	achaete-scute family bHLH transcription factor 1
ATC	anaplastic thyroid carcinoma
Bcl-2	B-cell lymphoma 2
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
bp	base pairs
BSA	bovine serum albumin
BTD	β-trefoil domain
C	Celsius
C. elegans	*Caenorhabditis elegans*
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
CADASIL	Cerebral arteriopathy with subcortical infractions and leukoencephalopathy
CaM	calmodulin
CaM	Calcium/calmodulin-dependent protein kinase II
CaMKs	Ca^{2+}/calmodulin-dependent protein kinases
Cas9	CRISPR associated protein 9
caSMC	coronary artery smooth muscle
CBL	Casitas B-lineage lymphoma proto-oncogene
CD4/8/11b/33/86	cluster of Differentiation 4/8/11b/33/86
CD44	CD44 molecule (Indian blood group)
CDK8	cyclin-dependent kinase-8
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
ChIP-Seq	ChIP-Sequencing
Chr	chromosome
CMML	chronic myelomonocytic leukaemia
CN	calcineurin
CN/NFAT	calcineurin/cytoplasmic nuclear factor of the activated T-cells
CNS	central nervous system
CO_2	carbon dioxide
CPA	cyclopiazonic acid
CPCs	cardiac progenitor cells
CPPs	calcium and calciprotein particles
CRAC	calcium release-activated channels
CREB	cAMP response element-binding protein
CREs	cAMP response elements
CRISPR	clusters of regularly interspaced short palindromic repeats
CSCs	cancer stem cells
CTD	C-terminal domain
C-terminal	carboxy-terminal
CTLs	cytotoxic T lymphocytes
D28K	calbindin D28K
DAG	diacylglycerol
DAPT	N-[N-(3,5-difluorophenylacetyl-L-alanyl)]-S-phenylglicine t-butyl ester
DLL1/3/4	delta-like1/3/4

DMA	dimethyladipimate
DMSO	dimethyloxaloylglycine
DN1/3/4	double negative 1/3/4
DNA	deoxyribonucleic acid
DNMAML	dominant-negative Mastermind-like
dNTP	deoxynucleotide
DP	double positive
DPBS	Dulbecco's Phosphate Buffered Saline
DREAM	Downstream Regulatory Element [DRE] Antagonist Modulator
DSL	Delta, Serrate, Lag-2
DTT	dithiothreitol
DTX1	deltex E3 ubiquitin ligase 1
E	Glutamic acid/Glu
E(spl)	Enhancer of split complex
E1A	Adenovirus early region 1A
E3	E3 ubiquitin ligase
ECL	enhanced chemiluminescent substrate
EDTA	Ethylenediaminetetraacetic acid
EEC	enteroendocrine cells
EGF	epidermal growth factor
EMT	epithelial-to-mesenchymal transition
EPCs	endothelial progenitor cells
ER	endoplasmic reticulum
ErbB4	erb-b2 receptor tyrosine kinase 4
ERK 1/2	Extracellular Signal-Regulated Kinase 1/2
ETO	Eight-Twenty One
EtOH	Ethanol
F/fw	forward primer
FAM	Fluorescein amidites
FBXW7	F-Box and WD repeat domain containing 7
FCS	fetal calf serum
Fig.	Figure
FIH	Factor-inhibiting hypoxia-inducible factor
FMA	formaldehyde
FRET	Fluorescence Energy Transfer
Fura-2	Fura-2-acetoxymethyl ester
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GATA3/4	GATA binding protein 3/4
gDNA	genomic DNA
Gli1/2/3	Gliotactin 1/2/3
GO	gene ontology
GPCRs	G protein-coupled receptors
gRNA	guide RNA
GSI	γ-secretase inhibitor
GSK3β	Glycogen synthase kinase-3 beta
GTE	Glucose Tris EDTA
GusB	glucuronidase, beta
h	hour
h	human
H/His	Histidine
H3	Histone 3
HCN cells	hippocampal neural stem cells
HD	heterodimerisation domain
HDAC 1/4/5	histone deacetylase 1/4/5
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HES1/4/5/7	Hairy/enhancer of split 1/4/5/7
HESR	hairy and enhancer of split-related
HEY1/2/L	Hairy/enhancer of split-like with YRPW motif 1/2/L
HIF-1α	hypoxia inducible factor 1α
hiPSCs	human induced pluripotent stem cells

HLH	helix-loop-helix
HPRT	Hypoxanthine guanine phosphoribosyl transferase
HRP	horseradish peroxidase
HuD	RNA-binding protein belonging to the human antigen (Hu) family
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL-4/5/24	Interleukin-4/5/24
IMDM	Iscove's Modified Dulbecco's Medium
IMR-1	Inhibitor of Mastermind Recruitment-1
IP3	inositol 1,4,5-trisphosphate
IP3R	inositol 1,4,5-trisphosphate receptor
ISX9	N-cyclopropyl-5-(thiophen-2-yl)-isoxazole-3-carboxamide
ITCH	itchy E3 ubiquitin protein ligase
JAG1/2	Jagged1/2
JNK	c-Jun N-terminal kinases
K	Lysine/Lys
KAc	Potassium Acetate
KCl	Potassium chloride
kDa	kilo Dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
KLF4	Krüppel-like factor 4
KO	knock out
L	Leucine/Leu or Liter
LA	Luria agar
LB	Luria broth
Lgmn	Legumain
LNA	locked nucleic acids
LNR	Lin12-Notch repeats
log	logarithm
m	milli
M	molar
m	mouse
mA	milliampere
MACS	Model-based analysis of ChIP-Seq
MAML1	mastermind-like1
MAPK	Mitogen-Activated Protein Kinase
MCUR1	mitochondrial calcium uniporter regulator 1
MeCP2	methyl CpG binding protein 2
Mef2	myocyte enhancer factor
MEM NEAA	MEM Non-essential amino acids
MES	mesenchymal
mg	milligram
MgAc	magnesium acetate
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
Min	minute
miRNAs	microRNAs
mL	milliliter
mRNA	messenger RNA
mSin3A	transcriptional regulator
MT	Mature hybridoma T cells
MW	molecular weight
MyoD	myogenic differentiation
n	nano
N_2	Nitrogen
Na_3VO_4	sodium orthovanadate
NACl	sodium chloride
NaF	sodium fluoride
NaOH	sodium hydroxide
N-CoR	nuclear receptor co-repressor

NEDD4	neural precursor cell expressed, developmentally down-regulated 4
N-end rule	N-terminal end rule
NeuroD	neuronal differentiation
NEXT	Notch extracellular truncation
NFATc1/2	Nuclear factor of activated T-cells, cytoplasmic 1/2
NF-κB	nuclear factor κB
NHMC	N-methylhemeanthidine chloride
NICD	Notch intracellular domain
NICD-V/L/S	NICD variants ending with Valine, Leucine, and Serine at N-termini
NIH-3T3	Cell line of mouse embryonic fibroblasts
Nkx2.5	NK2 homeobox 5
NLRP3	NLR Family Pyrin Domain Containing 3
NLS	nuclear localisation signal
Nm	nanometers
NP-40	Nonidet-P40
Nrf2	Nuclear factor E2-related factor 2
NRR	negative regulatory region
ns	not significant
NSCLC	Non-small cell lung cancer
NTD	N-terminal domain
N-terminal	amino-terminal (NH2-terminal)
NUMB	NUMB endocytic adaptor protein
OMP-59R5	Tarextumab
OPCs	oligodendrocyte precursor cells
P	Proline/Pro
p300	histone acetyltransferase protein 300
p75 NTR	the neurotrophin receptor p75
PAGE	polyacrylamide-gel electrophoresis
PAM	protospacer adjacent motif
Pax3	paired box 3
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDX	patient-derived xenograft
Pdx1	pancreatic and duodenal homeobox 1
PEI	Polyethylenimine
Pen2	Presinilin enhancer-2
PEST	proline-glutamine-serine and threonine-rich domain
PIP2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PMSF	phenylmethanesulfonylfluoride
PSEN1	presenilin-1
pTα	pre-T-cell receptor alpha chain
PVDF	Polyvinylidene fluoride or polyvinylidene difluoride
Q	Glutamine/Gln
R (amino acid)	Arginine /Arg
R/rev	reverse
RAM	RBPJ associated domain
RBPJ	Recombination Signal Binding Protein J
RE/AC	repression/activation domain
RIN1	RBPJ Inhibitor-1
RNA	ribonucleic acid
RNA-Seq	RNA-Sequencing
ROI	regions of interest
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute Media
RT-qPCR	quantitative reverse transcription polymerase chain reaction
S	Serine/Ser
S1/2/3	site 1/2/3 of cleavage of Notch receptor
SAE	SUMO activating enzyme
SCLC	small cell lung cancer

Scx	scleraxis bHLH transcription factor
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	super enhancer
sec	second
SEM	standard error of the mean
SENP1	SUMO deconjugation enzyme
SERCA	Sarcoendoplasmic reticulum Ca^{2+}-ATPase
sgRNA	single guide RNA
SHARP	SMRT-and HDAC1-asocciated repressor protein
Shh	sonic hedgehog
Smad	mothers against decapentaplegic family transcription factors
SMRT	silencing mediator of retinoid and thyroid hormone receptors
SNS	sympathetic nervous system
Sox9	SRY (sex determining region Y)-box 9
STIM1	Stromal interaction molecule 1
STZ	streptozotocin
Su(H)	Suppressor of Hairless
SUMO	Small Ubiquitin-related MOdifier protein
T	Threonine/Thr
TACE	tumor necrosis factor α-converting enzyme
TAD	transactivation domain
TAE	Tris-acetate-EDTA
T-ALL	T cell acute lymphoblastic leukemia
TAMRA	tetramethyl-6-carboxyrhodamine
TBP	TATA binding protein
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
TCR	T-cell receptor
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethylendiamin
TGFβ	transforming growth factor β
Th1/2	T helper cells 1/2
TLE	transducin-like enhancer of split
TM	transmembrane
T_{Reg}	Regulatory T cells
Tris	2-Amino-2-(Hydroxymethyl)-1,3-propandiol
Triton X-100	t-Octylphenoxypolyethoxyethanol, Polyethylene glycol tert-octylphenyl ether
Tween 20	Polyethylene glycol sorbitan monolaurate, Polyoxyethylenesorbitan monolaurate
USP10	Ubiquitin Specific Peptidase 10
v/v	volume per volume
Val1744	Valine 1744 residue
VEGF	vascular endothelial growth factor
VSMCs	Vascular smooth muscle cells
W	Tryptophan/Trp
Wnt	Wingless and Int-1
WT	wildtype
x g	g-force
Y	Tyrosine/Tyr
β-Actin	Actin beta isoform
µ	micro

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Ph.D. PORTFOLIO

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Topic of Ph.D. thesis work: Characterization of a novel pharmacological Notch activator

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Prof. Dr. Sandra B. Hake
Prof. Dr. Elena Evguenieva-Hackenberg
Prof. Dr. M. Lienhard Schmitz

Meetings of the thesis committee

Date	**Activity**
10.08.2020	Progress report Borggrefe/Hake/Schmitz Joint Meeting
05.02.2021	IMPRS TAC meeting
08.04.2021	TRR81 internal seminar
06.09.2021	Progress report Borggrefe/Hake/Braun/Schmitz Joint Meeting
26.01.2022	IMPRS TAC meeting
29.01.2021	MPI progress report meeting
28.01.2022	MPI progress report meeting
07.04.2022	TRR81 internal seminar
24.06.2022	MPI progress report meeting
11.07.2022	Progress report Borggrefe/Schmitz Joint Meeting
03.04.2023	Progress report Borggrefe/Schmitz Joint Meeting

Ph.D. Training

Date	General Courses
21.10.2019	Lung development
25.10.2019	Anatomy
25.10.2019	Gas exchange physiology
08.11.2019	Pulmonary circulation: Physiology and pathophysiology
08.11.2019	Lung regeneration & repair
13.11.2019	Heart disease and adaptations
29.11.2019	Anatomy & Physiology – the cardiovascular system
29.11.2019	Heart: Regeneration & repair
09.12.2019	Exam
20.03.2020	Physiological basis of immune responses
20.03.2020	Autoimmune diseases, Infectious diseases, Transplantation
27.03.2020	Tumor immunology and therapy
27.03.2020	Pancreatic hormones (Diabetes mellitus)
03.04.2020	Hypothalamus-pituitary- (gonad, adrenal) system
03.04.2020	Function and dysfunction of the digestive tract
17.04.2020	Lipid metabolism and its disorders
17.04.2020	Adipocyte biology: from lean to obese
24.04.2020	Development, function
24.04.2020	Diseases, pharmacotherapy
04.05.2020	Exam
23.10.2020	Epigenetics
23.10.2020	DNA replication & repair
06.11.2020	Transcription
06.11.2020	Signal transduction
13.11.2020	Translation
13.11.2020	RNA interference
27.11.2020	Regulation of cell death
27.11.2020	Cell cycle
04.12.2020	Autophagocytosis & protein degradation
04.12.2020	Protein sorting & processing
11.12.2020	Exam
12.03.2021	Stem cells and Development
12.03.2021	Vascular function I (endothelial system)
19.03.2021	Immunology, Infectious diseases- principles of pathogenicity
19.03.2021	Cancer, tumor biology
09.04.2021	Development (vasculogenesis / angiogenesis)
09.04.2021	Contractility
23.04.2021	Disease (atherosclerosis, hypertension etc.)
23.04.2021	Vascular function II (vascular smooth muscle)
14.05.2021	Blood components and functions
14.05.2021	Hemostasis and Thrombosis
17.05.2021	Exam

Date	Methods Seminar Series
13.05.2020	Proteomics and Mass Spectrometry
15.06.2020	Genome Engineering
28.09.2020	Flow Cytometry

09.05.2022 RNA and DNA sequencing
02-16.11. 2020 FELASA THEORY General Core Package & basic -Module
18.11.2020 FELASA course Hands-on training Basic Mouse Module

Presentation: Journal Club

Date	**Activity**
20.04.2020	Alteration of CTCF-associated chromatin neighborhood inhibits TAL1-driven oncogenic transcription program and leukemogenesis Li et al., Nucleic Acids Res. 2020
26.04.2021	Metabolic control of DNA methylation in naive pluripotent cells Betto et al. Nat Genet. 2021
30.08.2021	A bacterial protease depletes c-MYC and increases survival in mouse models of bladder and colon cancer Butler et al. Nat Biotechnol. 2021
01.08.2022	Mitochondrial RNA modifications shape metabolic plasticity in metastasis Delaunay et al. Nature 2022
26.06.2023	Genome-wide RNA polymerase stalling shapes the transcriptome during aging Gyenis et al. 2023 Nat Genet. 2023

Retreats

Date	**Activity**
09-11.10.2019	IMPRS Annual Retreat, Hohenroda, Germany
16-21.02.2020	TRR81 Annual Retreat, Kleinwalsertal, Austria
22-24.09.2021	IMPRS Annual Retreat (online)
10.11.2021	1st iCANx Retreat (online)
12-14.09.2022	IMPRS Annual Retreat, Weilburg, Germany

International Conferences

Date	**Activity**
05.03.2020	Chromatin Club, Institute of Biochemistry, University of Giessen
29.04.2020	PhD Club, Institute of Biochemistry, University of Giessen
02.07.2020	Chromatin Club Marburg Mini Conference (online)
14.01.2021	13th TRR81 PhD Minisymposium (online)
24.06.2021	14th TRR81 PhD Mini-Symposium (online)
01.11.2021	1st TRR81 & CRACKs Minisymposium
02.02.2022	15th TRR81 PhD Minisymposium
08.04.2022	PhD Club, Institute of Biochemistry, University of Giessen
27-29.09.2022	6th TRR81 symposium “Chromatin Changes in Differentiation and Malignancies, Egmond aan Zee, The Netherlands
26.01.2023	PhD Club, Institute of Biochemistry, University of Giessen

Seminars and meetings attended

Date	Activity	
05.03.2020	Chromatin Club	
	CTCF Clustering in Light of Senescence Entry	Spiros Palikyras
	How DNA methylation is read in the brain	Adrian Bird
	CHD7 cell-autonomously regulates cardiac neural crest cell development through ATP-dependent and – independent activities	Karim Bouazoune
	PWWP2A: A multivalent H2A.Z chromatin binder and gene regulator	Tim M. Wunderlich
29.04.2020	Institute of Biochmeistry, PhD Club	
25.06.2020	TRR81 Internal Seminar	AG Hake & Bartkuhn/ Goesmann
	Functional analysis of the PRTH complex: A novel member of the PWWP2A-H2A.Z chromatin axis?	Andreas Herchenröther
	Bioinformatics tools for combined epigenome and transcriptome analysis	Tobias Zimmermann
02.07.2020	Chromatin Club Marburg Mini Conference	
	From annotation to function of enhancer elements in cardiac myocytes	Ralf Gilsbach
	Gene regulation by long non-coding RNAs in human macrophage Immunity	Leon Schulte
	High Throughput Sample Preparation and Data Normalization for Epigenomics Studies	Sarantis Chlamydas
	HDAC3 functions as a positive regulator in Notch signal transduction	Benedetto Daniele Giaimo
	Histones: How much variation do we Need?	Sandra Hake
2.11.2020	TRR81 Seminar	
	Transcription at the centromere: Opportunity and Danger	Patrick Heun
12.11.2020	TRR81 internal Seminar	Team Bauer
	News about the PRMT6 / H3R2me2a recruitment mechanisms to promoter and enhancer sites	Caroline Bouchard
30.11.2020	TRR81 seminar	
	STAG2 regulates interferon signaling in melanoma via enhancer loop reprogramming	Lei Gu
17.12.2020	TRR81 internal Seminar	AG Kracht/ Papantonis
	Establishment of CAPTURE for inflammatory gene loci Proximity labeling of p65 NF-kB interactomes	Lisa Leib Jana Juli
	CTCF clustering: links to phase separation in senescence	Spyros Palikyras
14.12.2020	TRR81 internal Seminar	
	Put the periphery into the focus: spatial control of silent chromatin	Sigurd Braun
14.01.2021	TRR81 PhD Minisymposium	

	Molecular mechanism of gene regulation	
	Transcriptional control of macrophage Function	Francesco Gualdrini
	Mechanisms of transcriptional regulation during human cell transdifferentiation	Kseniia Lysakovskaia
	Mapping regulatory elements across ultra-long single-molecules of DNA	Sofia Luciana Battaglia
	Live visualization of genomic loci in mouse embryonic stem cells	Jente van Staalduinen
	Role of Pax5 in controlling loop extrusion and VH gene recombination at the Igh locus	Louisa Hill
	Understanding the molecular mechanism of X-linked dystonia- parkinsonism: from alternative splicing to epigenetics	Simona Capponi
21.01.2021	TRR81 internal Seminar	AG Liefke/ Schmitz
	Investigating the role of the CpG-island- binding protein SAMD1 in cancer	Clara Simon
	Functional analysis of H2B S6 Phosphorylation	Max Pfisterer
08.04.2021	TRR81 internal Seminar	AG Borggrefe & Stiewe
	Genome-wide characterization of the dynamic RBPJ-DNA binding	Tobias Friedrich
	Genetic and epigenetic p73 alterations in small cell lung cancer	Nastasja Merle
12.04.2021	TRR81 Seminar	
	Genome regulation by the BAF chromatin remodeler	Sandra Schick
20.05.2021	TRR81 internal Seminar	AG Hake/Bartkuhn/Goesmann
	Functional analysis of HMG20A and its role in H2A.Z biology	Andreas Herchenröther
	Integrative analysis of epigenomic and transcriptomic data with PETRA	Maximilian Dörrbecker
14.06.2021	TRR81 Seminar	
	Go with the flow or how metabolism impacts epigenetics and ageing	Peter Tessarz
24.06.2021	TRR81 PhD Mini-Symposium	
	Phase separation mediated transcriptional reprogramming upon proteotoxic stress	Prashant Rawat
	Centromere Regulation by RNA binding proteins in the germline of Drosophila melanogaster	Janina Luitz
	Neocentromere formation remodels chromatin fibre structure	Catherine Naughton
	Regulation of transposable elements by the inner nuclear membrane protein Lem2	Thomas van Emden
	Mechanisms underlying promoter interaction with gene regulatory elements	Angelika Feldmann
	TALENs outperform Cas9 in editing the heterochromatin region of the genome: A single-molecule study	Saurabh Shukla
01.07.2021	TRR81 internal Seminar	Team Braun/Yuan & Dobreva
	Suv4-20h1 protects muscle stem cell from transcription-replication collisions and rhabdomyosarcoma formation	Ting Zhang

	Enhanced H3K4me3 demethylation by inhibition of fatty acid oxidation enables heart regeneration	Xiang Li
	Interplay between epigenetics and metabolism in cancer cells	Arthur Mathes
	Interplay between epigenetics and metabolism in cardiac laminopathies	Yinuo Wang
26.08.2021	TRR81 internal Seminar	AG Brehm/ Vázquez
	Zinc-finger protein hangover interacts with chromatin regulating complexes in Drosophila melanogaster	Laura Schmelzer
	Peptides as Modulators of Epigenetic Protein Complexes	Van Tuan Trinh
07.10.2021	TRR81 internal Seminar	AG Bauer/Diederich/Kolb
	Ligand-based optimisation of the PRMT Modulator K002FT017- A Medicinal Chemistry driven approach	Sepideh Salehipour-Bavarsard & Christian Iking
25.10.2021	TRR81 Seminar	
	An emerging role for histone variants in memory	Iva Zovkic
01.11.2021	TRR81 & CRACKs Minisymposium	
	mRNA structures as hubs in post- transcriptional gene regulation	Julia Weigand
	The right substrate for mettl4: DNA or RNA?	Lei Gu
	Haploid expression of LncRNAs in the cardiac system	Phillip Grote
11.11.2021	TRR81 internal Seminar	AG Papantonis & Kracht
	RNA polymerase II is required for spatial chromatin reorganization following exit from mitosis	Nadine Übelmesser
	Chromatin Regulation at IL-1α-NF-κB driven genes	Lisa Leib & Jana Juli
02.02.2022	TRR81 PhD Minisymposium	
	Explore Chromatin-RNA interplay and beyond	
	Mechanisms and regulation of aberrant alternative splicing in cancers and the mechanisms and functions of arginine methylation on RNA splicing factors	Huan-Huan Wei
	Nuclear Proteomics to Study Gene Expression Regulation in Cancer	Marianna Maniaci
	Chromatin and Splicing	Reini Fernandez De Luco,
	Long non coding RNA in X chromosome inactivation	Joost Gribnau
	Large-scale approaches to the pre- clinical translational study of cancer therapeutics	William R. Sellers
	TRACTION - Translational Research to AdvanCe Therapeutics and Innovation in ONcology	Virginia Giuliani
20.01.2022	TRR81 internal Seminar	AG Liefke/ Schmitz
	Characterization of Elongin B/C as a possible drug target and the role of IRF2BP2 in AML	Sabrina Fischer
	Investigations on the CpG island-binding protein SAMD1	Robert Liefke
	Functional Analysis of H2B S6 Phosphorylation	Maximilian Pfisterer

27.01.2022	QMUL Epigenetics Webinars	
	The feedback from RNAs to chromatin	Richard Jenner
24.02.2022	TRR81 internal Seminar	AG Hake/ Bartkuhn
	The H2A.Z/PWWP2A/NuRD-associated protein HMG20A controls early head and heart development	Andreas Herchenröther
	How zinc fingers meet a histone variant: Lessons from ZNF512B - An unusual protein with pretty usual domains	Tim Marius Wunderlich
	New methods available through the Bioinformatics Service Project	Marek Bartkuhn
07.04.2022	TRR81 Internal Seminar	AG Borggrefe/ Stiewe
	Notch-dependent and -independent functions of transcription factor RBPJ	Dino Giaimo
	Deciphering the TP53 mutome by saturating CRISPR mutagenesis of the DNA-binding domain	Julia Funk
	Studying the role of gene alterations in neuroendocrine small cell lung cancer using CRISPR mouse models	Katharina Kochan
24.06.2022	Research Seminar	
	Mechanisms of activation and regulation of G protein-coupled receptors	Daniel Hilger
30.06.2022	TRR81 internal Seminar	AG Braun/Yuan & Dobreva
	Crosstalk of metabolism and epigenetics in heart development and disease	Andreea Bostean
	Rnf20: More than a Histone H2B ubiquitin ligase?	Arthur Mathes
01.07.2022	TRR81 Seminar	
	Understanding and exploiting chromatin regulation in cancer	Marcus Buschbeck
25.08.2022	TRR81 internal Seminar	AG Brehm & Vázquez
	Ambivalent hangovers: A transcription factor that interacts with activating and repressive chromatin modifiers	Jonathan Lenz
	Characterisation of the novel histone methyltransferase complex dG9a/CG9932 in D. melanogaster	Stephan Awe
	Peptides as Synthetic Tools for Modulation of Gene-regulating Protein -Protein Interactions (PPI)	Van Tuan Trinh
02.09.2022	TRR81 Seminar	
	Uncovering new mechanisms that regulate genome organisation and function: the fundamental roles of histone variants	David Tremethick
10.11.2022	TRR81 internal Seminar	AG Bauer, Diederich & Kolb
	Ligand-based optimisation of PRMT Modulators	Sepideh Salehipour- Bavarsad, Christian Iking & Jonas Kammertöns
01.12.2022	Institute Seminar	
	Stress-mediated translation control in physiology and disease	Jan Medenbach’
12.12.2022	TRR81 Seminar	
	Probing chromatin dynamics and its regulators using a novel histone timer	Felix Jonas

15.12.2022	TRR81 internal Seminar	AG Papantonis & Kracht
	Speckles and CTCF organise the senescent genome	Spyros Palikyras
	Functional follow-up analyses of the NF-κB p65 proximity-labelled interactome to reveal coregulators of the IL-1α-NF-κB system	Lisa Leib & Jana Juli
16.05.2023	RTG 2355 Seminar	
	RNA stability controlled by m6A methylation mediates X-to-autosome dosage compensation in mammals	Julian König
20.06.2023	RTG 2355 Seminar	
	The role of subcellular architecture in the regulation of gene expression during stress	Michaela Müller-McNicoll
20.07.2023	RTG 2355 Seminar	
	RNA localization controls RNA function	Gabrijela Dumbović

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