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NOTCH AND HEDGEHOG ON THE SCALES

Analysis and evaluation of the coactivator protein Mastermind-like 1 and its pivotal role in inter-pathway signalling

Doctoral Thesis

Presented by

Dipl. Ing. (FH) Dieter Matthias Lauer

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Professor Dr. A. Gulino
Director of PhD Programme

under supervision of

Professor Dr. I. Screpanti

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Una lingua con molte consonanti
è come un campo di patate.
Una lingua con molte vocali
è come un campo di fiori.

Enrico Caruso

Warum die Rose besingen, Aristokrat!
Besing die demokratische Kartoffel,
die das Volk nährt!

Heinrich Heine

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I Introduction

I.I A pathway through evolution

Cellular communication implies transduction of extracellular signals into cells and subsequently induction of intracellular response.[1] Signal transduction from one cell to another is achieved by transmitter molecules or ligands that can either be sequestered into the extracellular medium, mostly for intermediate and long range signalling, or execute their role in direct cell-cell contact as in the case of membrane bound proteins. Which cell is targeted depends on the expression of specific intra- or intermembrane receptor molecules, providing the cell with ligand specific binding sites on its surface. Non-covalent binding leads to conformational changes in the receptor that in turn is activating a second intracellular messenger. Because extracellular activation is step by step triggering secondary signal transduction events, involving a defined set of proteins, molecules and posttranslational modifications, one is talking about a signalling *pathway*.[2] The effect and final goal of pathway activation is a cellular response, either by direct alteration of the metabolism or indirectly by induction of transcriptional events leading to changed genetic expression levels inside the signal receiving cell.[3] Signalling pathways are absolutely essential for developmental processes in multi-cell organisms, providing the basic requirements for intercellular communication in order to control proliferation, differentiation and apoptosis. Many of indispensible biological characteristics, including specific ligand and receptor expression, intermediate messenger proteins and posttranslational modification events as well as mechanisms for target gene repression and activation have been highly conserved through evolution. RAS/MAPK, WNT, TGFß, JAK/STAT, NOTCH or HEDGEHOG are key genes for signalling pathways, processing conserved developmental cues in invertebrates like in vertebrates.[4,5] While controlled pathway activation can make the cell change from a spatio-temporal defined steady state into a responding cell, genetic or epigenetic deregulation of such a pathway can trigger inappropriate response mechanisms. In case of the involvement of key signalling pathways, such an uncontrolled change of genetic expression can disturb the fragile equilibrium between proliferation and apoptosis and cause genetic diseases and cancer.[6-18] Understanding of complex signalling pathway coordination in cells still poses a complex task for modern science and it will be necessary to combine parallel events of signal processing in order to compile whole cellular response and to understand how development and live is orchestrated in detail.[19-30]

I.II Notch

I.II.I NOTCH gene

Morgan and Bridges discovered in 1916 the NOTCH gene by X-linked dominant *Drosophila melanogaster* genetic mutants, exhibiting irregular *notches* of missing tissue at the tips of the insect's wing blades, explaining similar observations done by John S. Dexter two years before. In 1940 Poulson at al. found out that complete loss of NOTCH activity caused lethal hyperplasia of the embryonic nerve system.[31] In the following decades NOTCH was shown to have a unique role in binary cell fate determination.[32] Its ability of regulating symmetric versus asymmetric division in fetal and postnatal development[33] underlined the involvement of the NOTCH gene in organogenesis and tissue formation at multiple developmental steps in probably all cells of the animal and human body.[34-39]

Organ/tissue	Processes regulated	References
Brain	Controls the balance between gliogenesis and neurogenesis; stem cell maintenance; apicobasal polarity of neuroepithelial cells	(Ohata et al., 2011) (reviewed by Tanigaki and Honjo, 2010)
Breast	During pregnancy: alveolar development, maintenance of luminal cell fate, prevention of uncontrolled basal cell proliferation	(Buono et al., 2006)
Craniofacial structures	Palate morphogenesis: loss of Notch signaling results in cleft palate, fusion of the tongue with the palatal shelves and other craniofacial defects; Alagille syndrome includes craniofacial defects; also involved in tooth development	Jag2 (Jiang et al., 1998), Jag2/Notch1 (Casey et al., 2006), Dll3/Notch1 (Loomes et al., 2007), Jag1 (Li et al., 1997), tooth development (Mitsiadis et al., 2005)
Ear	Defines the presumptive sensory epithelium, determines hair cell and supporting cell fates	CSL (Yamamoto et al., 2011), Jag1 (Kiernan et al., 2006) (reviewed by Cotanche and Kaiser, 2010)
Esophagus	Regulates esophageal epithelial homeostasis	(Ohashi et al., 2010)
Eye	Fiber cell differentiation in the lens/lens development	CSL/Notch1 (Rowan et al., 2008; Jia et al., 2007), Jag1 (Le et al., 2009)
Heart	Cardiac patterning, cardiomyocyte differentiation, valve development, ventricular trabeculation, outflow tract development	(Reviewed by MacGrogan et al., 2010)
Hematopoietic system (including immune and lymphatic systems)	Required for the second wave of hematopoiesis in development; controls the balance of B-cell versus T-cell development; maintenance of hematopoietic stem cells; maintenance of myeloid homeostasis	(Reviewed by Bigas et al., 2010)
Intestine	Controls proliferation and differentiation (including absorptive fate versus secretory fate choices)	(Reviewed by Heath, 2010)
Kidney	Notch2 defines cell fate of podocytes and proximal tubules	(Cheng et al., 2007)
Limbs	Apical ectodermal ridge (AER) formation and digit morphogenesis, especially regulation of apoptosis	Notch1/Notch2 (Pan et al., 2005), Notch1/Jag2 (Francis et al., 2005), Jag1 (McGlinn et al., 2005), Jag2 (Jiang et al., 1998), Hairy (Notch target gene) (Vasiliauskas et al., 2003)
Liver	Regulates ductal plate formation and intrahepatic bile duct morphogenesis in mice	Notch2 (Geisler et al., 2008; Zong et al., 2009), Notch2/Jag1 (Lozier et al., 2008), Jag1 (Li et al., 1997)
Lungs	Lateral inhibition between tracheal cells prevents extra cells from assuming the lead position during tracheal branching morphogenesis	(Ghabrial and Krasnow, 2006)
Muscle	Promotes transition of activated satellite cells to highly proliferative myogenic precursor cells and myoblasts; prevents myoblast differentiation into myotubes after injury	(Reviewed by Tsivitse, 2010)
Neural crest	Controls patterning of neural crest precursors for the outflow tract region of the heart; regulates the transition from Schwann cell precursor to Schwann cell, controls Schwann cell proliferation and inhibits myelination; controls melanocyte stem cell maintenance	(Reviewed by Jain et al., 2010; Mirsky et al., 2008; Schouwey and Beermann, 2008)

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Organ/tissue	Processes regulated	References
Pancreas	Specifies endocrine cell differentiation through lateral inhibition: endocrine lineage cells inhibit endocrine differentiation of their neighboring cells; maintains pancreatic endocrine precursor cells, inhibits terminal acinar cell differentiation; controls pancreatic epithelium branching and bud size	(Reviewed by Kim et al., 2010)
Pituitary	Regulates pituitary growth/proliferation, melanotrope specification and gonadotrope differentiation	Hes1 (Monahan et al., 2009; Raetzman et al., 2007), Notch2 (Raetzman et al., 2006) (reviewed by Davis et al., 2010)
Placenta	Controls fetal angiogenesis, maternal circulatory system development, spongiotrophoblast development	(Reviewed by Gasperowicz and Otto, 2008)
Prostate	Required for epithelial differentiation and growth; expressed by progenitors that are required for branching morphogenesis (Notch1); stromal survival [Notch2 and Delta-like 1 homolog (Dlk1)]	(Wang, X. D. et al., 2006; Wang et al., 2004; Orr et al., 2009)
Sex organs and germ cells	Maintenance of Leydig progenitor cells in testis; regulation of spermatogenesis; controls oocyte growth via actomyosin-dependent cytoplasmic streaming and oocyte cellularization	(Tang, H. et al., 2008; Hayashi et al., 2001; Nadarajan et al., 2009) (reviewed by Barsoum and Yao, 2010)
Skin	Regulates cell adhesion, control of proliferation, hair follicle or feather papillae differentiation and homeostasis	(Reviewed by Hayashi et al., 2001)
Spine/spinal cord/somites	Somite segmentation through oscillation of genes	(Reviewed by Dunwoodie, 2009; Kageyama et al., 2010)
Spleen	Regulates generation of T lineage-restricted progenitors and marginal zone (MZ) B-cell development; controls homeostasis of $CD8^-$ dendritic cells in the spleen	(Reviewed by Yuan et al., 2010)
Stomach	Acts as a switch in choice between luminal and glandular cell fates	(Matsuda et al., 2005)
Thymus	Thymic morphogenesis, differentiation of gamma delta lineage T-cells	(Jiang et al., 1998)
Thyroid	Regulates the numbers of thyrocyte and C-cell progenitors and regulates differentiation and endocrine function of thyrocytes and C-cells	Hes1 (Carre et al., 2011)
Vasculature	Regulates arteriovenous specification and differentiation in endothelial cells and vascular smooth muscle cells; regulates blood vessel sprouting and branching	(Reviewed by Gridley, 2010)

fig. 1: Notch signalling in development, taken from Andersson et al., *Dev.*, 2011.[5]

Since in the 1980s the NOTCH gene was sequenced by Spyros Artavanis-Tsakonas and Michael W. Young[40-44], the US National Library of Medicine counts more than 8.000 publications dealing with 'Notch signal(l)ing', with an exponentially increasing number and nearly 3.000 entries only in the last three years from 2010 up to date.

A century of research on NOTCH brought us to a detailed understanding of the core signalling pathway, including the *Notch* receptor as well as many other auxiliary proteins and mechanisms that orchestrate signal transduction. However, its tangled involvement in a vast number of developmental processes, its increased complexity in higher species, pronounced by overlapping as well as distinct roles of multiple paralogues and interacting proteins, as well as mechanisms responsible for pathway regulation and fine tuning, seem to snowball and at the same time redefine our entire knowledge of cellular biology. NOTCH is so tremendously essential for live, that aberrant Notch signalling is the reason for many different genetic caused diseases.[9,45-54] Understanding the Notch signalling pathway opens up new possibilities for cancer therapy. Clinical trials are already on the way to hopefully produce effective pharmaceutics against genetically caused aberrant signalling diagnosed in a multitude of cancers.[55-59]

I.II.II The canonical Notch pathway

The core pathway of canonical Notch signalling is activated by ligand-receptor binding between two adjacent cells, inducing a cleavage process of the Notch receptor, a type I single-pass transmembrane protein heterodimer, transcriped from the NOTCH gene, posttranslationally modified and expressed on the surface of a signal receiving cell. The processed intracellular domain of the Notch receptor (NICD) is than translocating into the nucleus and induces, by derepression and binding to a CBF1/Suppressor of Hairless/LAG-1 (CSL) family DNA-binding protein, transcription of specific Notch related target genes.[31]

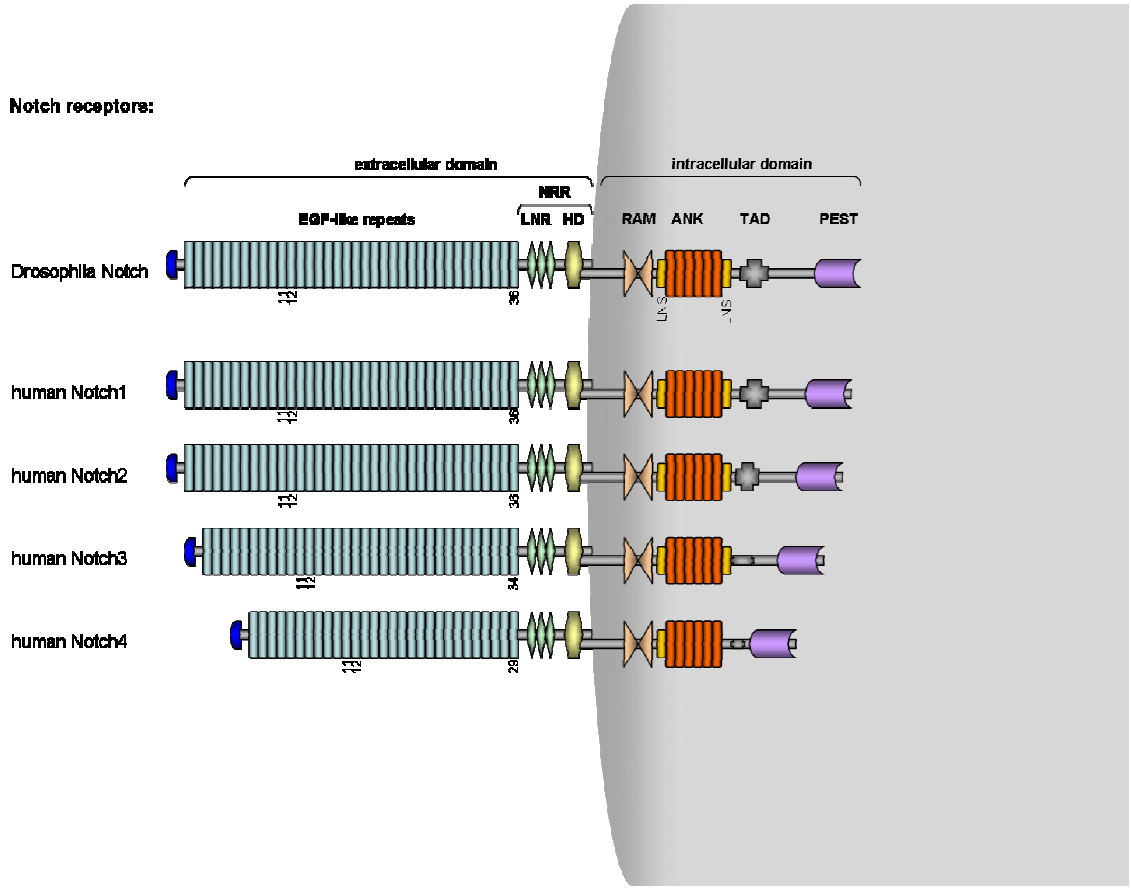

figure 2: Notch receptors. Notch receptors are expressed on the cell surface as heterodimers composed of a large extracellular domain non-covalently linked to the intracellular domain. Epidermal growth-factor (EGF)-like repeats and a negative regulatory region (NRR), comprising three LIN Notch (LNR) repeats and a heterodimerization domain (HD), define the extracellular part, while the intracellular domain contains the RAM domain and seven Ankyrin repeats (ANK), important for cofactor binding at the chromatin level. Notch receptors 1-3 contain two nuclear localization signals (NLS) compared to one NLS in Notch4. The transcriptional activation domain (TAD) is important for downstream target activation events and shows structural differences between the mammalian Notch family members. All four Notch receptors contain a C-terminal Pro Glu Ser Thr (PEST) sequence for degradation.

Initially the NOTCH gene is translated as a 300 kDa precursor protein which is immediately processed by furin-like convertase proteolysis at cleavage site S1 in the Golgi apparatus and reassembled as a non-covalently linked heterodimer[60] before being extensively N- and O- glycosylated and transported to the cell's membrane. Fucosylation by Pofut1 (in mammals, O-fut1 in Drosophila) and Fringe is essential for receptor ligand interaction and can modulate binding strength and partner preferences[61], while the chaperone activity of glycolysating enzymes in the endoplasmatic reticulum is essential for export of processed Notch receptors to the cell surface[62,63].

Compared to a single NOTCH gene present in *Drosophila melanogaster*, in mammals four different NOTCH genes have evolved, coding for four Notch receptor paralogues (Notch1-4).[31,64] The extracellular region of Notch receptors (NECD) is defined by tandem Epidermal Growth Factor (EGF)-like repeats, providing 36 repeats in Notch1 and Notch2, 34 repeats in Notch3 and 29 in Notch4 followed by three LIN-12 Notch repeats adjacent to the cell membrane. A single pass transmembrane region is connecting the NECD with the intracellular domain (NICD) of a Notch receptor heterodimer.[31] This EGF-like structure is shared by all receptors as well as by the Notch pathway related Delta and Serrate ligands (DSLs) in *Drosophila* and the five corresponding mammalian DSL-like homologues Delta-like1, 2, 4 and Jagged1 and 2. It enables receptor-ligand interactions through intra-domain disulfide bridge motifs, defined by calcium binding capacity of six specific cysteine residues embedded in single EGF-like repeats.[65-68] Ligand binding to the extracellular part of a Notch receptor is inducing trans-endocytosis of the ligand-NECD complex into the signal sending cell. The cell-cell contact is believed to induce a pulling force[69] that provokes a slight conformational lifting mechanism of the extracellular Notch receptor domain, exposing the cleavage site S2, buried inside the negative regulatory region (NRR)[70] C-terminal to the EGF-repeats of the extracellular domain. Here is where the LIN-12 Notch repeats and the heterodomain (HD) reside. In case of no ligand binding the S2 cleavage site remains inaccessible and shielded by the LIN-12 Notch domain, in order to prevent inappropriate Notch activation.[71] Only after ligand binding induced conformational changes in the NRR a metalloprotease of the ADAM10/TACE (in mammals; Sup-17 in *C. elegans*; Kuz in *Drosophila*) family can induce proteolysis, facilitating removal of the extracellular domain of the Notch receptor.[72] The fact that cleaved Notch ectodomains are transendocytosed together with the ligands into the signal sending cell is believed to play a role in feedback signalling inside the signal sending cell[73-79] but remains still to be fully understood.

The resulting Notch extracellular truncation (NEXT) in a signal receiving cell is subsequently endocytosed and further cleaved by the intramembrane aspartyl protease gamma-secretase complex, releasing the Notch intracellular domain (NICD).[80-85] Monoubiquitination of a juxtamembrane lysine residue inside the NEXT is required for this last cleavage step.[86] It has been proposed that specific ubiquitination at or immediately after endocytosis would be an ulterior controlling mechanism in the Notch signal cascade having inpact on endosomal sorting and thereby regulating further signal transduction, receptor recycling or degradation.[87-90] Only an activated and correctly processed intracellular domain of the Notch receptor proceeds signalling into the nucleus.

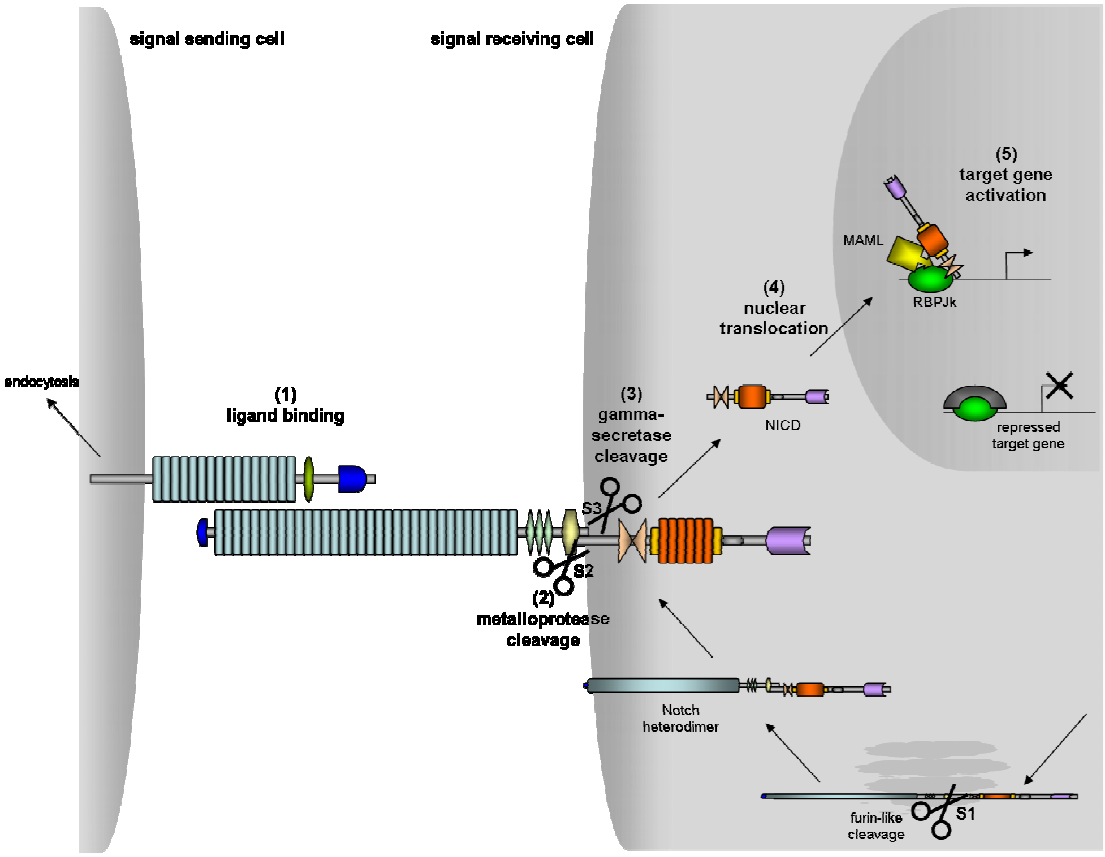

figure 3: Notch signalling. Notch proteins are synthesized as a single 300 kDa polypeptide. After fucosylation, full length Notch is cleaved at the trans-Golgi by a furin-like protease at the S1-cleavage site to generate the non-covalently linked Notch heterodimer. The Notch receptor is further glycosylated by Fringe glycosyltransferases before shuttled and associates to the plasma membrane. Interaction with a DSL ligand (1) and trans-endocytosis of the ligand bound extracellular portion induces proteolytic cleavage of the Notch receptor by the ADAM (a disintegrin and metalloproteinase) protease TACE (tumour-necrosis-factor-α-converting enzyme) (2). A final S3-cleavage by the γ-secretase complex is triggering the release of an intracellular Notch fragment which translocates into the nucleus (4), where it associates with and derepresses the transcriptional repressor RBPj (also called CBF1, suppressor of hairless or Lag1), inducing specific target gene activation(5).

Cleaved NICD is the distal transactivating component in the Notch signalling cascade. In correlation with its function, NICD contains a region called RAM (RBPj associated molecule) for RBPj (CBF1, suppressor of hairless, Lag1; CSL) binding, seven ankyrin repeats (ANK) and two nuclear localization sequences, one before and one after the ANK region. Due to the essential roles of RAM and ANK domains in ternary activator complex formation together with the DNA-binding protein RBPj and the coactivator Mastermind-like (MAML), a key mechanism of canonical Notch signalling, the respective protein sequences are highly conserved between different members of the Notch family[91]. The transactivation domain (TAD), C-terminal to the ANK repeats, shows more variations[92], indicating possible differences in transactivation strength or protein-protein interaction capacity with paralogue specific cofactors. At the very C-terminal end of the NotchICD is located a conserved region rich in proline (P) glutamic acid (E), serine (S) and threonine (T), known as the PEST domain and believed to be important for protein degradation.[93-97]

In the past decade, Notch specific transactivation of target genes has been intensively studied and subjected to powerful bioanalystic tools like x-ray crystallography and nuclear magnetic resonance (NMR) assays, providing us very detailed insights into molecular interactions during activator complex formation on the DNA. In the 'off' state of Notch signalling, respective target gene promoters are repressed by RBPj and additional corepressors, forming a complex that hinders inapropriate transcription initiation.[98] Presence of cleaved NotchICD inside the nucleus is triggering derepression of the promoter region by displacing corepressors and direct binding to RBPj.[99-101]

While the DNA-recognition and binding domain of RBPj is recruiting NICD onto the specific CSL promoter region in Notch target genes[101,102], a third partner in the ternary activator complex, is absolutely essential for full transactivation: Mastermind-like.[103-105]

In higher vertebrates like mouse and human there are three known members of the Mastermind-like protein family (MAML1-3) which can in part substitute for each other.[106] MAML1 is the best studied paralogue and, together with NICD and RBPj sufficient to induce full Notch transactivation. MAML1 has been shown to bind the NICD and RBPj on CSL sites only in a complexed dimer where a highly conserved N-terminal alpha-helical structure of the first 80 amino acids of MAML1 is fitting into a molecular groove formed by some parts of the C-terminal Notch ANK repeats and specific residues of the RBPj protein. The role of MAML is mainly considered to recruit additional cofactors like p300 or CDk8 which are inducing posttranslational modifications like acetylation, phosphorylation and ubiquitination, regulating the binding affinity and longevity of NICD engagement in transactivation.[107-115]

I.II.III Similarities and discrepancies between mammalian Notch paralogues

Since Notch family members can only in part substitute for each other[116], it is still a task to unravel specific properties and the evolutionary need for four slightly different receptors in more complex vertebrates, compared to a single NOTCH gene sufficient for signalling in *Drosophila melanogaster*. When simple target gene transactivation strength has been compared *in vitro*, using luciferase reporter constructs driven by the Hes1 promoter, a differential activity pattern was established in which the intracellular domain of Notch1 resulted as the prevalent transcription factor with the strongest response, followed by the other paralogues 2, 3 and 4.[116] Notch1 is the most studied Notch family member and often used in overexpression experiments aimed to simulate general Notch pathway activation. However, the presence of other Notch proteins and the differential coexpression in developing tissues are indicating that in higher organisms developmental control is encoded in simultaneously acting Notch paralogues, with specific roles, differing in cofactor binding capacities, ligand-receptor activation specificies or posttranslational properties deciding about proteic half life, quantitative availability at the transactivation site and strength as promoter specific transcription factors.[116-121] Further evidences for independent and specific roles of the four mammalian Notch receptors are given since mutations in respective genes were associated with different developmental defects and human diseases. Mutations in the NOTCH1 gene can cause T-cell acute lymphoblastic leukaemia (T-ALL)[122] and aortic valve disease[123], while NOTCH2 defects are leading to Alagille syndrome[124] or osteoporosis like in the case of Hajdu-Cheney syndrome[125]. Cerebral autosomal dominant arteriopathy with subcortial infarcts and leukoencephalopathy (CADASIL) is a stroke disorder caused by mutations in the NOTCH3 gene[126-129] and NOTCH4 may be involved in schizophrenia[130-136]. Shimizu et al. showed that expression levels of RBPj could differentially alter Notch induces transcriptional activity. On a TP1-luciferase promoter N1IC was the strongest activator compared to N2IC and N3IC, while coexpression of RBPj was reducing the overall NICD induced transactivation. Interestingly the NICD induced activation level of a Hes5 luciferase promoter showed an increased signal transduction capacity of N3IC when coexpressed RBPj while the ICD constructs of Notch1 and Notch2 copied the behaviour of negative RBPj relation, seen in the TP1-luc assay.[116] A deeper investigation of vertebrate Notch target selectivity was done by the group of Kopan. The authors of a comparative study on the transactivation capacity of different Notch family members, published in 2005, stated that “relative activation strength is dependent on protein module and promoter context”[101]. The RAM region of NotchICDs interprets CSL binding site proximity and

orientation while the transactivation domain of Notch is important for recruitment of cofactors into the activator complex formation. A difference in target site recognition was established in which Notch1ICD prefers paired, head to head orientated, CSL sites and Notch3ICD, providing a TAD region with only 40 % conservation of amino acid sequence with respect to the transactivation domain of Notch1, was more active on promoters like Hes5, with single so called *cryptic* CSL sites in conjunction to a proximal, yet unknown cis-element of the zinc finger character. Chimeric constructs demonstrated the Notch3 TAD to be the most potent transactivator on Hes5, when fused to the Notch1 RAM/ANK domain in order to get maximal CSL binding and MAML recruitment to the promoter.[101] In line with these observations x-ray crystallography studies from the group of Blacklow propose dimerization of two NotchICDs on paired CSL binding sites, including the cryptic Hes5 promoter.[137] Whether different Notch isoforms can positively cooperate on target gene activation or whether there are additional DNA-binding cofactors involved to explain target selectivity remains still a problem to be solved. J. W. Cave is deducing that "incorporation of different NICD paralogues into Notch transcription complexes increases the combinatory complexity with local activators"[138]. A part from mutations that directly affect the respective genes of Notch receptor paralogues, ligands[139-148] or pathway members like RBPj and Mastermind-like[149-151], the increasing number of identified NICD binding proteins and their specific roles in posttranslational modifications of Notch, are manifolding possible weak spots in the Notch pathway whose genetic defects in outer corepathway components may lead to additional modulation of Notch signalling: Deltex (Dtx1-4) controls Notch ubiquitination, processing and internalization at the membrane level[152-157] while the RBPj interacting and tubulin associated protein (RITA/C12ORF52) shuttles cleaved intracellular domain of Notch between cytoplasm and nucleus[158]. The Ski-interacting protein/nuclear receptor coactivator (SKIP/NCoA-62/SNW1) forms multidimers with NICD and MAML enableing association to RBPj and transcriptional activation.[159,160] Beta-catenin (Ctnnb1) synergizes with NICD and RBPj on target genes[161-163] while the Notch-regulated ankyrin repeat protein (Nrap) as well as tumor protein p73 α (p73α) bind and inhibit the NotchICD/CSL interface[164-166]. Also Smad family members (SMADs) enhance and fine-tune Notch signalling.[167-171] NICD was demonstrated to interacte with nuclear factor NF-κB[172-175] while the subunit alpha of hypoxia inducible factor 1 (HIF1α) can stabilize NICD and synergize in transcription of Notch target genes.[176-178] Cyclin dependent kinase 8 (CDK8) together with Cyclin C (CycC) phosphorylate the intracellular domain of Notch to target the NICD for ubiquitination and degradation.[115] While numb was shown to recruit the E3 ubiquitin ligase Itch to N1IC[121], which due to the membrane theathered domain of itch is

believed to act outside the nucleus[179,180], another E3 ubiquitin ligase, F-box/WD repeat protein 7 (Fbxw7/Cdc4), colocalizes to and ubiquitinates NICDs inside the nucleus.[96,115,181-183] Selective Notch target gene activation can also be primed to become transcriptionally active by the presence of epigenetic modifications regulating the chromatin microenvironment. Methylation of histone H3 lysine residue 3 (H3K4) and acetylation of H3K9 residues are known to trigger cofactor accessibility and gene activation while demethylation and deacetylation are associated with repressive effects on transactivation.[184-186] The protein p300 is known to trigger important posttranslational modifications on targets through its acetylation capacity. In a specific cellular context of mouse retina, Notch1 was proved to be more stable when being acetylated by p300. The general idea is that acetylation takes place at the same lysine residues that can also be targeted by E3 ubiquitin ligases in order to transfer ubiquitin chains to the protein and determine its proteasomal degradation.[187] The fact that MAML1 is able to bind p300 and recruit it into the NICD-CSL complex, highlights the finely tuned complex formation process in which interaction of Notch with various cofactors is orchestrated in a temporal and spatial manner.[114,188] The presence of cleaved NICD inside the nucleus, binding to RBPj and complex formation with MAML is inducing a series of events, leading step by step to complex stabilization, transcription initiation as well as sequential complex destabilization and NICD degradation, in order to render the cell as sensitive as possible to continued Notch signalling.[189-191] A further distinction between Notch paralogue's transactivation strength and protein stability became evident when phosphorylation studies on Notch have been done. Phosphorylation of specific serine or threonine residues, creating a required E3 ubiquitin ligase binding motif, were shown to antagonize protein acetylation in a competitive manner.[114] The fact that Notch3, in a leukaemia cell background, was less stable when HDAC inhibitors were used to increase Notch3 acetylation, by inhibition of deacetylating processes, indicates the highly complex and cell context dependent situation of posttranslational modifications of different Notch paralogues.[192] In order to demonstrate that phosphorylation, induced by the specific Nemo-like kinase (NLK), was leading to increased ubiquitination and sequential degradation of Notch1, transcriptional activity were tested in luciferase assays. While Notch1 was indicating a dose dependent decrease of activity, corresponding to protein phosphorylation and degradation, Notch3 was showing the exact opposite and exceeded by far the Notch1 induced signal strength.[193] Regarding differences in acetylation and stability of the intracellular domains of Notch members, it is important to mention that further ubiquitination and turnover processes of NICDs are just about to be understood.

I.II.IV Notch signalling in T-cell development

In haematopoiesis, including the lymphatic as well as the immune system, Notch is believed to be essential for maintenance of hematopoietic stem cells, myeloid homeostasis as well as the control of balance between B-cell versus T-cell development.[194]

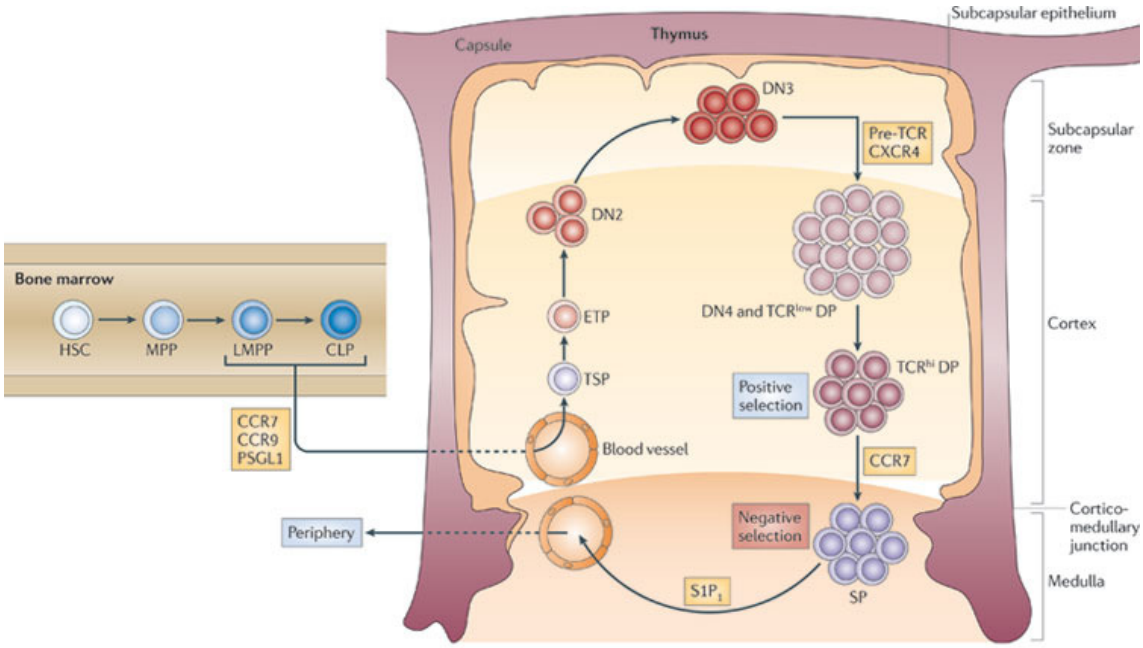

figure 4: T-cell development, taken from Love et al., *Nat. Rev. Immun.*, 2011.[195] Haematopoietic stem cells (HSCs) differentiate into multipotent progenitors (MPPs) within the bone marrow. Recombination activating gene 1 (RAG1) and RAG2 positive lymphoid-primed multipotent progenitors (LMPPs, CLPs) subsequently upregulate CC-chemokine receptor (CCR) 7 and 9. These thymus-settling progenitors (TSPs) enter the thymus near the cortico-medullary junction to generate early T cell progenitors (ETPs; also known as KIT$^+$ double negative 1 (DN1) thymocytes). ETPs in turn differentiate into DN2 and DN3 cells that migrate to the subcapsular zone. Expression of the pre-T cell receptor (preTCR) on DN3 thymocytes induces cell proliferation and differentiation to the DN4 and subsequently to the double positive (DP) stage. DP thymocytes that form appropriate interactions with self peptide–MHC complexes on cortical thymic epithelial cells (positive selection) upregulate expression of CCR7 and mature into single positive (SP) mature T cells. Negative selection in the medulla is followed by emigration into the periphery.

Notch1 has been shown to be involved in the generation of hematopoietic stem cells (HSCs) derived from endothelial cells but is dispensable for later embryonic haematopoiesis.[196,197] Jagged1 expression in osteoblasts may regulate the HSC homeostasis through Notch signalling, as presence of gamma-secretase inhibitors blocked a Jagged1-Notch induced increase in total numbers of HSCs but in how far Notch signalling is involved in stem cell niches is yet an open question.[198,199]

Haematopoietic stem cells differentiate into RAG1/2 (recombination activating gene 1 and 2) positive multipotent progenitors within the bone marrow. After upregulation of CC-chemokine receptor (CCR) 7 and 9 they enter the thymus near the cortico-medullary junction to generate early T cell progenitors (ETPs).[195] Notch1 other than Notch2 or 3 is through Delta-like4-Notch1-CSL signalling in a non redundant way necessary and sufficient for bone marrow derived lymphoid progenitor cells in becoming T-cells and no B-cells.[200,201] The block of B-cell lineage is in part mediated by Hes, a direct Notch target.[202] Lineage commitment is thought to happen by the entry of immature lymphoid precursors into the thymus as Notch ligands (mostly Jagged1 and Delta 4) are only expressed on the thymic epithelium.[203] Immature $CD4^-$ and $CD8^-$ double negative thymocytes (DN1: LIN^{low}, $SCA1^+$, KIT^+) differentiate into DN2 (LIN^{low}, $CD25^+$, KIT^{hi}) and DN3 (LIN^{low}, $CD25^+$, KIT^{low}) cells, migrating to the subcapsular zone.[195] Expression of the preT-cell receptor (preTCR) on late DN3 thymocytes induces cell proliferation and differentiation to the DN4 and subsequently to the double positive (DP) stage. Notch3 may coordinate growth and differentiation of late DN T-cells as Lck-driven N3IC overexpression *in vivo* did maintain abberant expression of the invariant chain of the preT-cell receptor and can regulate the function of T-cell differentiating factor SCL/Tal1.[204,205] The importance of Notch1 and Notch3 in thymocyte differentiation is further sustained by their protein expression patterns, peaking at the DN3 stage of early T-cells[206], and non-overlapping functions of both Notch paralogues.[120,207] Whether or not canonical Notch signalling is dispensable for post preTCR checkpoint lineages and differentiation into $CD4^+$ or $CD8^+$ single positive T-cells, remains still to be clarified. N1IC overexpression did affect SP cells but conditional inactivation of Notch1 or RBPj was not skewing nor versus one or the other lineage fate.[208,209] DP thymocytes that form appropriate interactions with self peptide MHC complexes on cortical thymic epithelial cells (positive selection) upregulate expression of CCR7 and mature into single positive (SP) mature T cells. Negative selection in the medulla is followed by emigration into the periphery.[195]

I.II.V Notch signalling in leukaemogenesis

Involvement of NOTCH in leukaemogenesis was evident since it has been shown that a specific chromosomal translocation, t(7;9)(q34;q34.3), was leading to the aberrant expression of a truncated form of Notch1IC, causing T-cell acute lymphoblastic leukaemia (T-ALL).[210,211] While this specific mutation was found in about 1 % of all human T-ALL disease cases other mutations in T-ALL patients have been identified,

leading all to hyperactivity of the Notch1 protein and increasing the incidence of Notch1 gain of function mutations in T-ALL up to 50 %.[122] Transgenic Lck-driven intra-thymic Notch3ICD overexpression in mice induced a developmental block at the double negative stage (DN) of T-cell development. Notch3ICD was able to bypass the preTCR checkpoint in pTα/preTCR deficient immature thymocytes through constitutive activation of a non-canonical NF-κB pathway, resulting in a more differentiated T-cell phenotype.[212,213] The observation that Notch3 does have an important role in T-cell development was confirmed by more recent publications.[175,204,213]

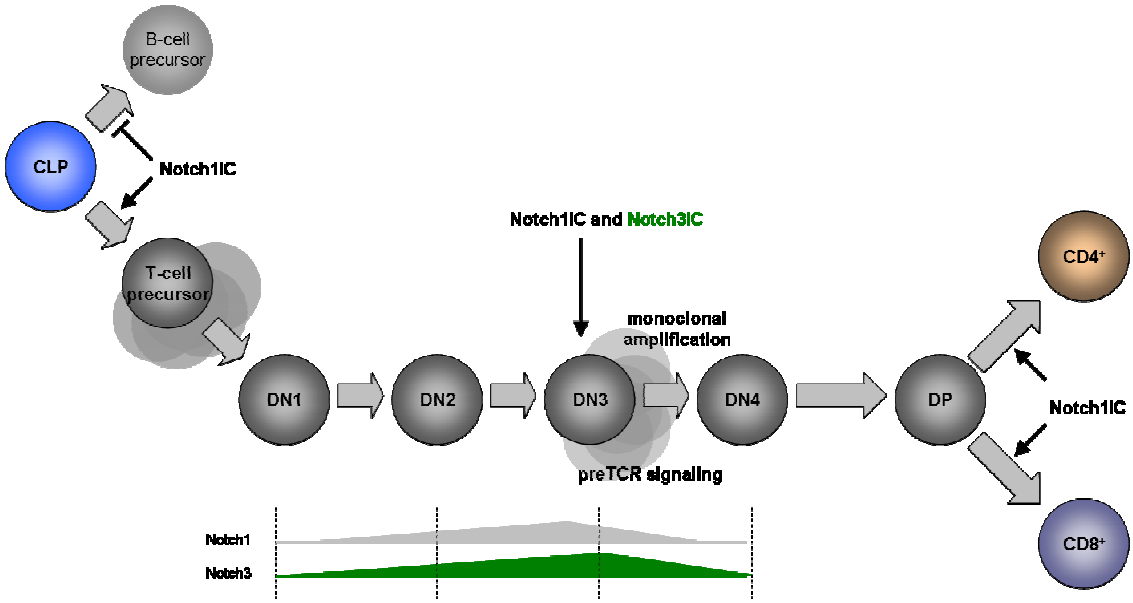

figure 5: Notch activity in T-cell development. Notch/RBPj signalling is involved in multiple steps of thymocyte differentiation as indicated. During the intrathymic T-cell development from double negative DN ($CD4^-CD8^-$) thymocytes into double positive DP ($CD4^+CD8^+$) and single positive SP ($CD4^+$ or $CD8^+$) thymocytes, Notch1 and Notch3 receptor expression are peaking at the DN3 stage, where preTCR signalling and monoclonal amplification happens, creating a T-cell pool subsequently subjected to positive selection during the DN4 stage.

Koyanagi et al. have done an exhausting Notch receptor and ligand expression profile during the different stages of T-cell development and underline the upregulation of Notch1 and Notch3 at the double negative stages DN2 to DN4. More precisely Notch1 receptor expression was detected until beta-selection, defined by the surface marker CD27, and immediately downregulated before gene rearrangement of the T-cell receptor beta-chain (TcR β^{low} and TcR β^{high}) while Notch3 receptor was downregulated in TcR β^{high} cells, after Notch1. Even in sorted double positive (DP) T-cells there were evidences of Notch3 receptor as well as Jagged1 ligand expression which could indicate a specific non-redundant role for Notch3, just at the transition from double negative to double positive cells during thymocyte differentiation.[206]

These observations are in line with our FACS analysis date from Notch3IC transgenic mice exhibiting lymphomas with a developmental block at the DP stage. Shi et al. have described non-overlapping functions for Notch1 and Notch3 during lymphopoiesis in the thymus demonstrating evidences for dramatic changes in expression of Notch3, in RNA and protein levels, from DN1/DN2 to DP T-cells peaking at the DN3 stage. Important to mention is that Notch3 levels have been normal despite simultaneous Notch1 deletion, excluding Notch3 upregulation to be caused by Notch1 gene transactivation.[120] A comparative approach aimed to decipher the role of variations between different Notch family members in leukaemogenesis was demonstrating that, while overexpression of all intracellular domains of Notch 1 to 4 support T cell development in thymic organ culture as well as in mice, N4ICD overexpression failed to induce T-ALL in xenotransplants. Failure of transactivation of important target genes like Hes1, which was shown to potentiate T-cell lymphomagenesis, and the inability to rescue Notch1 dependent T-ALL, were in the case of Notch4 at least in part caused by a structural divergence in the ANK domain.[214] Important to know is that NICD must cooperate with a second T-cell specific signalling event, mediated by the preT-cell receptor, to exert its oncogenic potential. When N1IC expressing bone marrow progenitors, derived from mice lacking functional preTCR signalling ($Rag2^{-/-}$, $Lcp2^{-/-}$ or SLP76 deficient), were transplanted into lethally irradiated mice, no T-cell leukaemia was detected until the T-cell receptor beta transgene was reintroduced into $Rag2^{-/-}$ mice.[215] The fact that aberrant upregulation of the intracellular domain of Notch (NICD1-3), the distal transcription factor in Notch signalling, is causing T-ALL was sustained by two further experimental setups. On the one hand constitutive expression of the Notch ligand Delta4 in bone marrow progenitors was able to activate the Notch signalling pathway inducing T cell leukaemia. On the other hand ineffective degradation of NotchICD, caused by Fbw7 mutations and consequently reduced NICD degradation, was detected in human T-ALL patients. With a 30% loss of function mutation coincidence in T-ALL, affecting three arginine residues in its target binding domain, FB(X)W7 represents the second most mutated gene in T-ALL patients, directly after mutations in the NOTCH1 gene, and should be considered as a major regulator of intracellular Notch signalling.[216] Interestingly Bellavia et al. reported that in virtually 100 % of human T-ALL cases, Notch3 is overexpressed without having specific genetic mutations like demonstrated for the NOTCH1 gene.[217]

I.III Hedgehog

I.III.I Hedgehog pathway

Like Notch also the Hedgehog pathway is one of the highly conserved representatives of key signalling pathways, controlling proliferation and differentiation in embryo- and organogenesis as well as pattern formation in pre- and postnatal vertebrate and invertebrates.[218-220] Binding of the mammalian, lipid-modified and secreted Sonic (Shh), Indian (Ihh) or Desert (Dhh) Hedgehog ligands to the 12 transmembrane receptor Patched is triggering pathway activation in the signal receiving cell. The non-covalent ligand receptor interaction is inducing derepression of Smoothened, a second transmembrane protein, which is then able to subsequently activate the intracellular transcription factor Gli that translocates into the nucleus and initiates target gene transcription.[221,222]

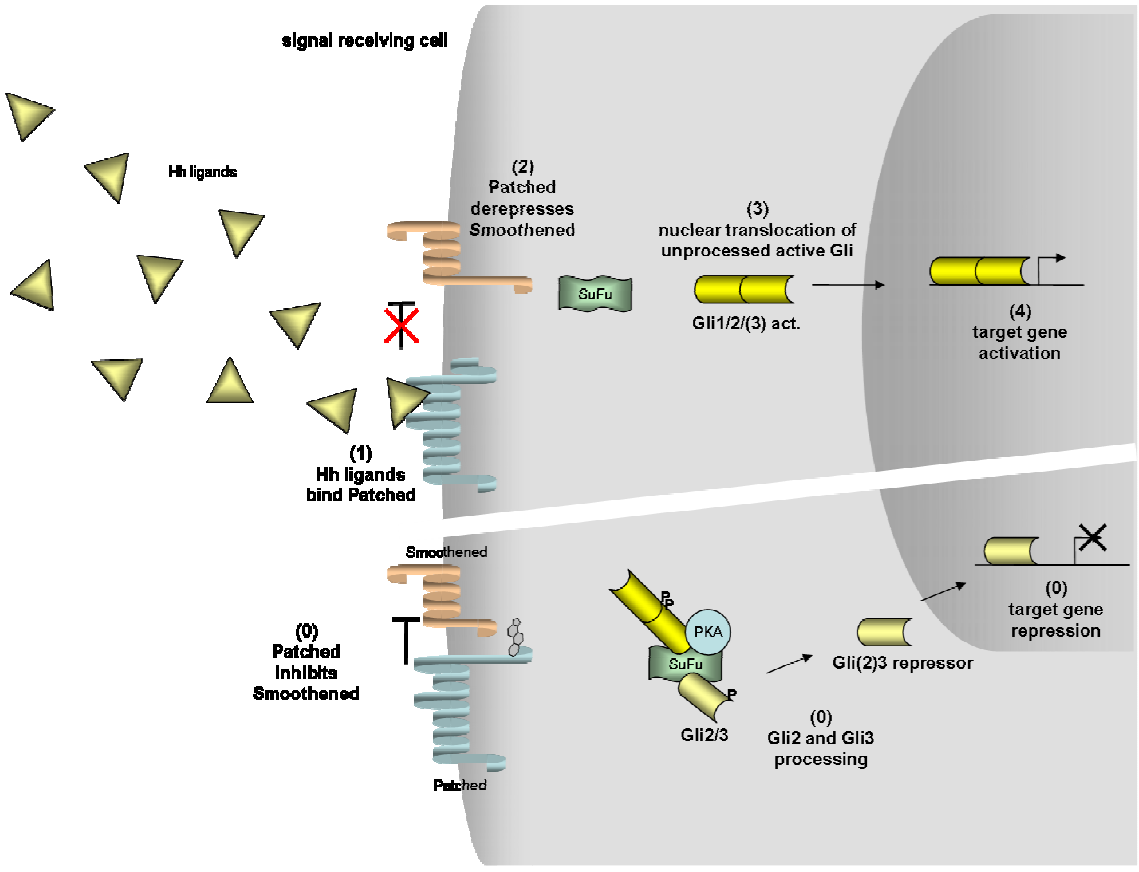

figure 6: Hedgehog signalling. In the absence of Hh ligands (0), the transmembrane receptor Patched is repressing Smoothened. Under these circumstances, kinases like PKA, GSK3β or CK1 phosphorylate Gli2/3 and initiated their processing into repressor forms which translocate into the nucleus in order to inhibit target gene activation. Upon binding of sequestered Hh ligand (1), Patched derepresses Smoothened (2) that now engages the signalling machinery, culminating in the appearance of activator forms of Gli that translocate into the nucleus (3) and regulate expression of Hh target genes (4).

The presence of Hedgehog ligands is in most cases graduated with the highest concentration at the signal sending source and low quantities in extracellular matrix distant from it. As cellular response is depending on concentration as well as duration of the signal, a gradient of sequestered ligands is important for proper developmental patterning processes, providing a position dependent intercellular communication and affecting cells juxtaposed to the ligand source differently than more distant cells.[223-225]
The receptor protein Patched works as a repressor of the 7 transmembrane protein Smoothened (Smo), inhibiting its function in the signalling *off* state.[226] In this case the transcription factors Gli2 and Gli3 are bound to a multiprotein complex, including Suppressor of Fused (SUFU) and the mammalian homologue of Costal2 (KIF7).[227,228] Three different and often sequentially acting kinases, protein kinase A (PKA), glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1) have been shown to phosphorylate Gli2 and Gli3 proteins at specific serine and threonine residues.[229,230] Once phospho-modified, Gli proteins undergo proteolytic processing, initiated by an enzyme called Slmb, producing shorter Gli2 and Gli3 repressor forms that translocate into the nucleus and repress, together with SUFU, SAP18 and SIN3, specific Hedgehog pathway target genes.[231-233] When activating the Hedgehog signalling pathway by extracellular Hh-ligand binding to Patched, Smoothened becomes derepressed and phosphorylated in its C-terminal intracellular tail.[234,235] In Drosophila, this modification is believed to induce recruitment of Cos2, disassembling the multiprotein complex formed around Cubitus interruptus (Ci), the analogue of mammalian Gli proteins.[236] In vertebrates Kif7 has been shown to have similar enzymatic functions regarding Gli1 processing and complex formation.[237,238] Non-phosphorylated or processed Gli proteins have the full potential to transactivate target genes by translocation into the nucleus and recruitment of enhancer proteins like Dyrk1 and CBP/p300.[239] Beside various target genes also the proper pathway members PATCHED1 as well as GLI1 are induced by active Hedgehog signalling, building up a complex feedback loop capable to reinforce ligand induced signalling inside the signal receiving cell as well as responding to the extracellular activation by Patched receptor production.[240,241]

I.III.II Hedgehog in T-cell development

Hedgehog signalling is involved in intrathymic T-cell development. While the ligand Shh is provided and sequestered by thymic stroma, the receptor Patched as well as the pathway activator Smoothened are expressed in the membrane of developing, double

negative (DN; $CD4^-$ and $CD8^-$) thymocytes. By the use of monoclonal antibodies blocking Shh, Crompton and colleagues have demonstrated that Hedgehog pathway inhibition was increasing the number of cells differentiated from DN into DP T-cells. In contrast, supply of Shh proteins could provoke a differential arrest at the $CD25^+$ DN3 stage.[242] Further it has been shown that in activated $CD4^+$ T-cells Shh plays a role in clonal expansion by promoting cell cycle transition from S to G2 phase.[243] Positive and negative selection of immature T-cells in the thymus ensure functional but not self-reactive TCR signal strength in order to give rise to mature thymocytes without autoimmunity effects.[244] Rowbotham et al. studied the effect of Hedgehog pathway activity during T-cell development and demonstrated that, while transgenic introduction of the T-cell receptor into wild type mice did result in more effective positive and negative selection, clonal deletion was ineffective when the TCR was overexpressed in mice with Gli2ΔN_2 driven, constitutive active Hedgehog signalling. Further evidence that Hedgehog signalling is counteracting the TCR was provided from $Shh^{-/-}$ mice in which TCR signalling is hyperactive leading to an abnormal high CD4:CD8 ratio.[245,246] How Hedgehog signalling is terminated in order to give rise to further development into DP and mature functional T-cells is not well understood and requires deeper investigation. Gli3 is upregulated in T-cells after preTCR signalling and considered to be easily processed into its repressor form which would add an additional repressing factor in intracellular Hedgehog signal regulation.[247] It has been proposed that the movement of developing thymocytes inside a Shh gradient, set up by the thymic architecture, is exposing T-cells to changing pathway activating conditions (fig. 7).[248] At the same time Smoothened has been shown to be downregulated by preTCR signalling.[242] In both cases intracellular Hedgehog signal transduction was downmodulated by the consequences of impaired Smoothened activity and consequent inactivation of Gli transcritption factors. Especially during transition from the DN3 stage before and after Notch induced preTCR signalling, till mature single positive $CD4^+$ T-killer or $CD8^+$ T-helper cells, the pool of differentiating thymocytes underlies exclusive checkpoints. For a successful passage through the beta-selection step, one of the important T-cell specifying checkpoints, thymocytes must have rearranged the beta-chain of the T-cell receptor (TCRβ) in order to signal through the preTCR. The importance of the Hedgehog signalling cascade in developing T-cells was further sustained by El Andaloussi et al. demonstrating the essential role of Smoothened derepression and Gli1/2 activation in promoting survival and proliferation at the stage preceding preTCR signalling.[249]

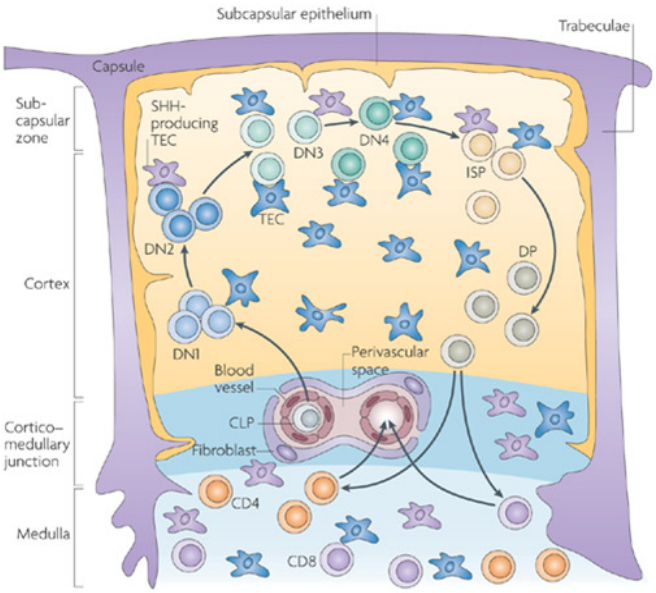

figure 7: T-cell development, taken from Crompton et al., *Nat. Rev. Immun.*, 2007.[248] Immature DN1 stage thymocytes are situated close to the site of thymic entry at the cortico-medullary junction. The DN2 population moves across the cortex and into the subcapsular zone. DN3 T-cells accumulate in the subcapsula where transition to the DN4 stage happens. During their passage through the thymus, thymocytes are exposed to a gradient of SHH produced by thymic epithelial cells (TECs) scattered in the subcapsular region, medulla and cortico-medullary junction.

While Hedgehog signalling is absolutely essential for many early developmental processes, Gli1 has been shown to be not essential for mouse development. Gli1, beeing a transcriptional target of its own, is autoamplifying Hedgehog induced signals. Important to mention is that Gli2 seems to able to substitute for Gli1 which might explain the lack of phenotype in Gli1-mutant mice.[250,251] Gli1 differs mainly from the mammalian GLI family members Gli2 and Gli3 by the lack of repressor function in the 'off' state of Hh-signalling and is considered as an unambiguous positive Hedgehog pathway transcription factor.[252] As Hedgehog signalling is transcriptionally controlling many target genes whose activation is decisive for proliferation, cell cycle regulation as well as initiation of differentiation, repression of those genes at defined developmental steps in lineage patterning is probably as important as their activation. Gli1, not providing a PPD site for being processed into a target gene repressor, is therefore proposed to have an outstanding role in mammalian Hedgehog signalling.[253,254] In fact Gli1 has a non-redundant role in intrathymic T-cell development. It positively affects T-cell differentiation before and negatively after preTCR signalling. Gli1 is in contrast to Gli2 and Gli3 not essential at the DN1 and DN2 stages of developing thymocytes but Gli1 knockout does affect the DN3 and DN4 populations. Gli1 expression is peaking in DN3 T-cells (fig. 8) but does not seem to be involved in the process of TCRβ chain rearrangement.[255]

I.IV Cross talk

I.IV.I Possible Notch and Hedgehog cross regulation

The case of Gli1 regulation has been subjected to multiple analyses in various organs, tissues and cell lines. An integrative genomic approach, done by Katoh and colleagues, is proposing that Gli1, beside other regulatory mechanisms, is under negative control of canonical Notch signalling. Gli1 was transcriptionally downregulated after Notch activation, possibly by the repressive effect of basic helix-loop-helix (bHLH) type Notch target genes of the HES and HEY family, binding to conserved double N-boxes in the first intron of the GLI1 gene.[256] Nguyen et al. came to the conclusion that there would be a multistep link between bHLH/Notch and Gli activities. They refer to previous work of Chitnis and Krintner who postulated that lateral inhibition by Delta-Notch signalling is essential for neurogenesis and demonstrate data in which a morfolino-drug against Gli3 could reverse the neurogenic effect caused by Delta-mutant Notch inhibition.[257,258]

A delicate balance between Notch and Hedgehog signalling is further sustained by the work of Kim et al. who stated that “endodermal Hedgehog signals modulate Notch pathway activity in the developing digestive tract mesenchyme”[259]. While complete absence of Notch signalling in conditional RBPJ knockout mice caused loss of subepithelial fibroblasts and abbreviated gut length, also Notch overactivity caused loss of mesenchymes and impaired organogenesis. Interestingly, the overexpression of Notch phenocopied Hh-deficient embryos. On the contrary, fetal gut mesenchymes in culture could be rescued by Shh-induced signalling counteracting the programmed cell death caused by Notch overactivity. Kim et al. assumed that Hedgehog signalling was restraining Notch pathway activity in order to give rise to proper organogenesis of the developing embryonic intestine. Double-null embryos for Shh and Ihh were exhibiting increased Notch signalling. An important observation is that increased Hes1 and Hes5 expression in embryos lacking Hedgehog pathway activity was not accompanied with increased expression of Notch receptors or ligands. That is why the authors suggest Hedgehog signalling to modulate the Notch pathway’s activity intracellulary and distal to receptor activation.[259]

Shivdasani’s group demonstrated a Hedgehog pathway antagonizing effect of Notch1ICD overexpression in cultured cells. It should be mentioned that quantitative PCR, western blotting as well as in-situ hybridization indicated Notch3 to play a major role in stomach endoderm and mesenchyme *in vivo*. Nevertheless, in this experimental

setting Notch3ICD efficacy in antagonizing the Hedgehog pathway was not tested, the localization of a possible cross talk between Notch and Hedgehog signalling, distal to the NotchICD release and upstream of Notch target gene activation, suggests the involvement of the intracellular domain of Notch or components of the Notch activator complex.

I.IV.II Notch and Hedgehog in complementing T-cell development

The work of Siggins et al. could add an interesting point to the role of Hedgehog signalling in haematopoiesis: Conditional Patched1 knockout mice had no effect on hematopoietic stem cell (HSC) activity and did not show Hh signal activation when hematopoiesis-specific Patched deletion was applied. Still a hematopoietic defect could be detected, pronounced in death of bone marrow (BM) derived preB-cells as well as a significant loss of double positive (DP) T-cells. Double negative (DN) stages of T-cell development were unaffected. While the authors explained the phenotype to be caused by epithelial cell extrinsic mechanisms, induced through Hh signal activation in non-hematopoietic tissue, the observed properties of B-cell deletion, T-cell defects at the DP stage and splenomegaly are very similar to hematopoietic defects in mice with hyperactive Notch signalling. Notch activity in early thymocyte progenitors is skewing cells to become T-cells instead of B-cells, while Notch3IC transgenic mice exhibited splenomegaly and developmental defects at the DN-DP transition of developing thymocytes.[204,260] Interestingly the exposure to cyclopamine, a small molecule inhibitor of Smoothened, did negatively affect early thymocyte progenitors (ETPs) and thymocytes of the DN2 stage of differentiation but did not alter the survival rate of $CD25^+$ preT cell lines, evidencing the importance of functional and timely regulated Hedgehog signalling before but not after preTCR signalling.[249] Deeper analysis of Gli1 deficient mice with healthy appearance showed a differential blockage at the DN3 stage of developing thymocytes right before preTCR signalling.[255]

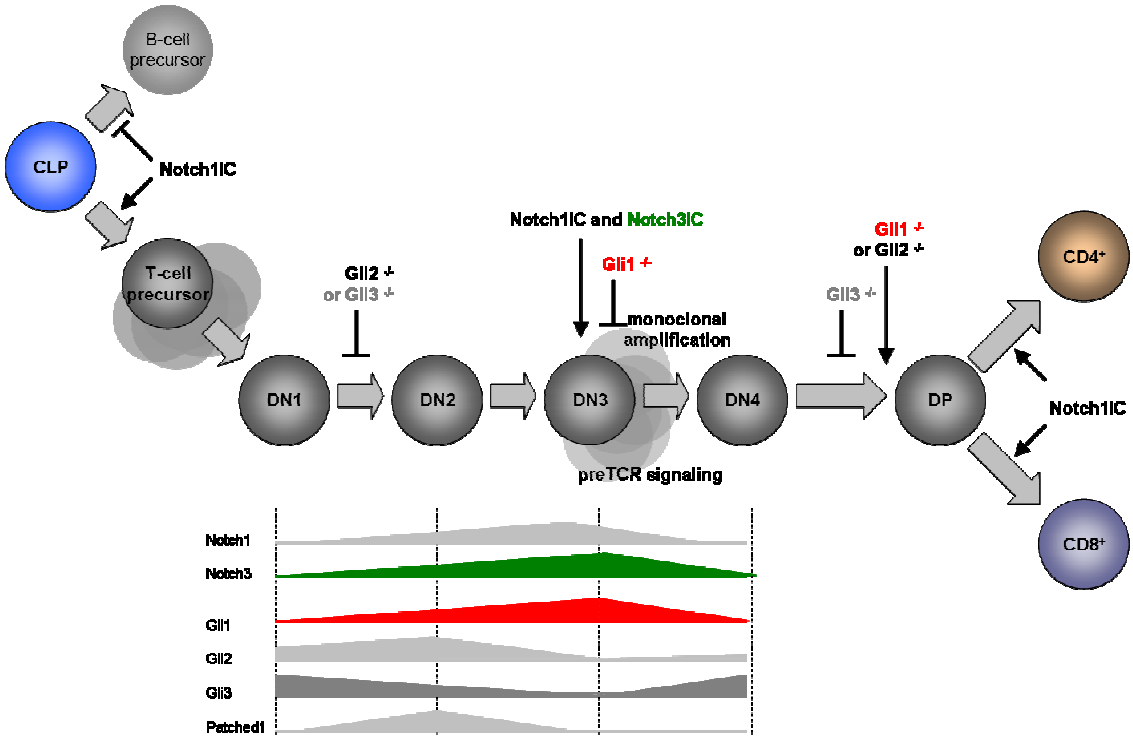

figure 8: Notch and Hedgehog activation during T-cell development: While Gli2 protein expression is positively correlated with Patched expression, peaking at the DN2 stage of developing thymocytes, expression of Gli3, believed to be a potent Hedgehog pathway repressor, is negatively correlated to Gli1 expression and most prominent at the DN1 as well as the DN4 stage. Gli1 expression is essential before preTCR signalling occurs and Notch3 and Gli1 have overlapping expression patterns peaking at the DN3 stage of DN3 thymocytes.

Gli1 expression is peaking at the double negative DN3 stage of immature thymocyte differentiation (fig. 8) indicating high levels of Hedgehog pathway activities.[249,255] Overlapping expression patterns of Gli1 and Notch3(IC), with their maximal levels at the exact same spatio-temporal window of T-cell development as well as their essential and distinct roles in orchestrating differentiation and proliferation before preTCR signal initiation, might indicate either a higher yet undefined molecular network regulating simultaneously both pathways or more probably a diret inter-pathway cross talk important to maintain intracellular balance and to coordinate signal response mechanisms.

I.V Aim of thesis

Both the Notch and the Hedgehog pathway, highly conserved from vertebrates to invertebrates, contribute indispensably to correct development and coordinate stemness maintenance, proliferation, differentiation and apoptosis. Aberrant signalling, generally caused by mutations in one or more genes of the many members or auxiliary proteins involved in the signalling cascades can provoke changes in the tightly regulated balance of gene expression and lead to abnormal and oncogenic characteristics of multiple cell types at various developmental stages. Combined targeting of both pathways has been proposed to be tested in patients. Regrowth and recurrence of tumours is believed to be initiated by a small pluripotent fraction of self renewing cancer stem cells and poses a major problem for clinical chemotherapy of cancer where slow proliferating cells often show a higher resistance against unspecific drugs.[261,262] Self renewing capacity and monoclonal cell pool amplification rely on control of the cell-cycle, proliferation and differentiation, believed to be managed by simultaneous Notch and Hedgehog signalling. Deciphering the molecular mechanism behind merging pathway coordination will be of essential importance for developmental biology as well as for the improvement of current cancer therapies. Due to their complementing roles in lineage patterning, the goal of this thesis was to verify a regulative cross talk between Notch and Hedgehog signalling during immature thymocyte development. The Hedgehog-Gli signal transduction cascade has been shown to be essential for intrathymic T-cell development until preTCR signalling occurs while in parallel the Notch pathway is playing an important role in initiation of preTCR signalling at the $CD4^-$ and $CD8^-$ double negative DN3 stage of developing thymocytes. In order to investigate the possibility of a cooperative role of Notch and Hedgehog signalling in T-cells, protein expression patterns of respective pathway transcription factors should be established. Initial experiments were aimed to elucidate Gli1 and Notch3 transcriptional activity in DN1 and DN3 -like thymocyte cell lines. Once ruled out a possible convergence point, the molecular background of interpathway signalling should be investigated.

II Materials and Methods

II.I Chemicals and Solutions

II.I.I Salts and Powders

The following salts, powders and reagents were purchased from Aurogene, Fluka, Merck and Sigma:

Agar, agarose, boric acid, bromphenol blue, EDTA, EGTA, glycin, KCl, LB, Na_3VO_4, NaCl, NaF, Na-pirophosphate, non fat dry milk, PMSF, SDS, Tris-HCl, Trizma base.

II.I.II Buffers and Solutions

The following salts, powders and reagents were purchased from Carlo Erba Reagents, Euroclone, Fluka, Gibco, Merck, Roche and Sigma:

acetone, acrilamid mix (30 %), ammonium persulfate (APS), bradford dye, Brij58, chloroform, complete protease inhibitor tablets, DMSO, DTT, ECL, ethanol, FBS, formaldehyde, glycerol, glycogen, HEPES, iso-propanol, L-glutamine, luciferase assay reagent and substrate, methanol, Na-deoxycholate, NP-40, PBS, penicillin-streptomycin, phenol, physiological solution, Stop&Glow, TEMED, trichlor acid (TCA), Triton-X, Trizol, TWEEN, β-mercaptoethanol.

TBE buffer (10x):	TRIS base (108 g/L), boric acid (54 g/L), EDTA 0.5 M, pH 8 (7.4 g/L)
WB running buffer (10x):	30.25 g/L Trizma base, 144 g/L glycine, 10 g/L SDS
WB transfer buffer (10x):	60 g/L Trizma base, 95 g/L glycine

RIPA lysis buffer:	20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA pH 8.0, 30 mM NaF, 2 mM Na-piro-Phosphate, 1 mM Na-orthovanadate (Na_3VO_4), Protease inhibitor cocktail tablets.
NP-40 lysis buffer:	50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 0.1 % NP-40, proteinase inhibitor cocktail tablets.
triton lysis buffer:	20 mM TrisHCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, Triton-X (1x), 30 mM NaF, 1 mM Na3VO4, 0.25 mM PMSF, proteinase inhibitor cocktail tablets.
buffer A:	10 mM HEPES, 10 mM KCl, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF
buffer C:	20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, proteinase inhibitor cocktail tablets.

II.I.III Others

Blue X-Ray film (Aurogene), hyperladder (bioline), immobilon transfer membrane (millipore), nitrocellulose membrane (PROTRAN, Whatman), metafectene pro (biontex), lipofectamine 2000 (invitrogen);

Reagents for standard PCR and reverse transcriptase PCR (buffer, $MgCl_2$, dNTPs, oligo dT primers, RNase inhibitor, Taq-polymerase) were purchased from Applied Biosystems, Bioline and Promega;

Restriction enzymes and buffers purchased from New England BioLabs.

Bi or tri- distilled water was used;

II.II Biological Material

II.II.I Primary antibodies used for immunoblotting

Anti-β-Actin (A5441)	(Sigma)	MAML1 (#4608)	(Cell Signaling)
ANTI-FLAG (F7425)	(Sigma)	Notch3 (#2889)	(Cell signaling)
ANTI-FLAG (M2)	(Sigma)	Notch3 (8G5)	(Cell signaling)
Anti-MAML1 (5975)	(Millipore)	Notch3 M134 (5593)	(Santacruz)
Anti-RBP-Jk	(Millipore)	Notch3 M20 (7424)	(Santacruz)
anti-Tubulin (8035)	(Santacruz)	NOTCH3 (23426)	(abcam)
GLI-1 H-300 (20687)	(Santacruz)	patched G19 (6149)	(Santacruz)
GLI-1 N-16 (6153)	(Santacruz)	RBPJK (25949)	(abcam)
HA-probe F-7 (7392)	(Santacruz)	RBPJk D20 (8213)	(Santacruz)
HA-probe Y-11	(Sigma)	RBPSUH (#5442)	(Cell Signaling)
Lck 3A5 (433)	(Santacruz)	Smo N-19 (6366)	(Santacruz)
Mam1 N-20 (18506)	(Santacruz)		

II.II.II Plasmids

N3IC-HA and N3IC-flag: Notch3-IC (aa 1664 - 2318) with C-terminal HA-tag was cloned into T7 CMVp expression plasmid, provided by Dr. U. Lendahl and described by Lardelli et al. 1996.[263] -HA to -flag exchange was done in Screpanti's lab. by standard cloning techniques. MAML1-flag: cDNA of human Mastermind-like 1 full-length was cloned in pFLAG-CMV2, described by Wu et al., 2000.[107] RBPjk: cDNA of murine RBPj was cloned into CDM8 vector, descrive by Chung CN et al., 1994.[264] Gli1-HA was provided by Dr. AE Oro and described by Kinzler et al., 1988.[265]

pTα luciferase promoter: Putative pTα promoter region (pubMed sequence U27268) was subcloned in TA-cloning vector and fused into luciferase pGL3-basic vector.[266] Patched wt and mut luciferase promoter were provided by R. Toftgard (KI, Sweden) and described by Agren et al., 2004.[267]; Gli 12x luciferase responsive element was provided by R. Toftgard and described in Kogerman et al., 1999.[268]

Plasmid amplification and purification was done following the QIAGEN protocol, using QIAGEN Plasmid Mini or Maxi kit.

II.II.III Cell lines

Cell lines were cultured at 37°C, 5% CO_2 in humidified atmosphere and split each second or third day.

Hek 293T cell line: Human embryonic kidney cells with SV40 Large T-antigen. Cultured in DMEM, 10% FBS, 100 U/ml penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine;

M31T cell line: Immortalized $CD4^-$ $CD8^-$ $CD3^+$ T cell line with α, β but no γ TCR, after secondary V_β rearrangement, described by Primi D. et al., 1988.[269] Cultured in DMEM, 10% FBS, 100 U/ml penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, β-mercaptoethanol;

preT 2017 cell line: Transformed Maloney virus-derived $CD4^-$ $CD8^-$ T cell line with $TCR\gamma^{high}$, described by Spolski R. et al., 1988.[270] Cultured in RPMI, 10% FBS, 100 U/ml penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, β-mercaptoethanol;

N3-232T-cell line: Immortalized immature thymocyte cell line derived from Notch3-IC transgenic mice. Cultured in RPMI, 10% FBS, 100 U/ml penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, β-mercaptoethanol.

II.III Methods

II.III.I Bradford assay

1 - 5 µL of cell lysate or volumes of BSA-solution (0 - 40 µg) were diluted in 800 mL H_2O and 200 mL Bradford-dye (Biorad protein assay). Spectrometrical absorption was determined at 595 nm and protein concentrations were calculated from BSA-trendline.

II.III.II Chromatin immunoprecipitation assay

Protein complexes were cross linked to DNA in living nuclei by adding formaldehyde directly to thymocytes to a final concentration of 1%. Crosslinking was allowed to proceed for 10 min at 37°C and then was stopped by the addition of glycine to a final concentration of 0.125M. Cells were washed twice with phosphate-buffered saline

containing 1mM PMSF. Nuclei were extracted with a 20 mM Tris pH 8.0, 3 mM $MgCl_2$, 20 mM KCl buffer containing protease inhibitors, pelleted and lysed by incubation in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Trischloride pH 8.1), containing protease inhibitors. Chromatin solution was sonicated for 15 pulses of 15 s to generate 300 – 600 bp DNA fragments. After microcentrifugation the supernatant was diluted 1:10 (dilution buffer: 0.01 % SDS, 1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Trischloride pH 8.1, 167 mM NaCl, containing protease inhibitors), precleared with Salmon Sperm DNA/Protein A agarose (#157, Upstate Biotechnology) and divided into aliquots. 5 µg antibodies were added per aliquot for incubation (in rotation over night, 4°C) and Antibody-protein-DNA complexes were isolated by immunoprecipitation with Salmon Sperm DNA/Protein A agarose. Following extensive washing, DNA bound fragments were eluted and analyzed by subsequent PCR using primers specific for Gli-binding sites on the mPtch-promoter. Primers used: mPtch1A fwd ACACACTGGCG CACTATCCA, mPtch1A rev ACACACTCACACGTACAGGA, mPtch1B fwd TAAGAAA GAAAGGAGGGGGG, mPtch1B rev GGAGGGCAGAAATTACTCAG.

II.III.III Immunoblotting

Nitrocellulose membranes were carefully washed (PBS, 0,05 % Tween), blocked with non fat dry milk dissolved in PBS and incubated with primary antibodies in 2 % or 5 % milk for 2 hours or over night. Membranes were washed three times with PBS-Tween for 10 min. each, reincubated with horseradish peroxidase-labeled goat-antirabbit, goat-antimouse or rabbit-antigoat secondary antibodies (Santa Cruz Biotechnology Inc.) and developed with the ECL detection system (Amersham).

II.III.IV Immunofluorescence microscopy

Cells were fixed in 4 % paraformaldehyde for 20 min. at RT, incubated in 0.2 % Triton X-100 to permeabilize cell membranes and incubated in blocking buffer (PBS with 3 % BSA). Primary antibodies were diluted in PBS and incubated 1,5 h. Samples were washed three times and then incubated with secondary antibodies for 30 min at room temperature in blocking solution. Nuclei were counterstained with Hoechst reagent.

II.III.V Immunoprecipitation assay

Cells were lysed, protein concentration was determined and 200 µg - 1000 µg of whole cell lysate were immunoprecipitated using specific antibodies and Protein A-Agarose Immunoprecipitation Reagent (sc-2001) or Protein G PLUS-Agarose Immunoprecipitation Reagent (sc-2002). HA peptide I2149 against aa98-106 of human influenza virus HA (Sigma-Aldrich) or normal mouse, rabbit or goat IgG (santa cruz) was used as negative controls. Precipitates were further resolved by SDS-PAGE and subjected to western blot analysis.

II.III.VI Luciferase assay

Luciferase activity was assayed with a Dual luciferase assay system 24 or 48 hours after transfection of plasmid DNA and Renilla-expressing vector pRL-TK, (Promega). Specific luciferase activity was determined (TD-20/30 luminometer, Turner Designs) in triplicates and normalized to renilla luciferase activity in a dual luciferase assay system (Promega, Madison, WI, USA), following manufacture's protocol.

II.III.VII Nuclear and cytosolic compartments

Pellet of 10 x 10^6 cells was washed in PBS, centrifuged (7 min., 4°C, 1.200 rpm), resuspended in 100 µL freshly prepared buffer A and incubated 15 min. on ice. After addition of 0.6 % NP-40 the lysate was vortexed for 10 sec. and centrifuged (30 sec., 4°C, 12.000 rpm) in order to subsequently separate the cytosolic compartment in the supernatant from pelleted nuclei. The supernatant was further centrifuged (20 min., 4°C, 12.000 rpm) in order to eliminate membrane residues. The nuclear pellet was washed carefully 3 times in buffer A + NP-40 (each time 30 sec., 4°C, 12.000 rpm) before being resuspendet in buffer C, vortexed for 15 sec. and incubated 10 min. on ice. Nuclear proteins were found in the supernatant of a final centrifugation (20 min., 4°C, 12.000 rpm).

II.III.VIII RNA quantification

Total RNA was extracted from resting cells, using Trizol (Gibco) following manufacture's protocol and purified by RNeasy Mini kit (Qiagen). mRNA expression was analysed by cDNA transcripts using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) employing TaqMan gene expression assay according to the manufacturer's instructions (Applied Biosystems). Each amplification reaction was performed in triplicate, and the average of the three threshold cycles was used to calculate the amount of transcripts in the sample (SDS software, ABI). All values were normalized to two endogenous controls, GAPDH and HPRT.

II.III.IX Transfection

Transient transfection experiments were performed by Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instructions.

II.III.X Western blotting

6 % or 8 % poly-acrylamide gels were freshly prepared. Samples containing Laemli-buffer and β-mercaptoethanol were boiled 5 min. and separated by SDS-PAGE, (running buffer, 100 mV) before blotted onto nitrocellulose membrane (chilled transfer buffer, 100 mV). Prestained Protein Marker HyperPAGE (BIOLINE) or ProSieve Quadcolor Protein Markers (Lonza Rockland) were used for band size determination. Protein blotting on the membrane was generally confirmed with Ponceau solution.

II.III.XI Whole cell lysates

Pellet of 10×10^6 cells was washed in PBS, centrifuged (7 min., 4°C, 1.200 rpm), resuspended in 100µL (NP-40 or Triton-x) lysis buffer, incubated 20 min. on ice and centrifuged (20 min., 4°C, 13.000 rpm). Protein concentration of supernatant was determined by Bradford assay.

III Results

III.I Antagonistic effect in target gene activation

III.I.I Gli1 interferes with Notch target gene transactivation

In order to investigate the paradigm of a Notch-Hedgehog cross talk in thymocytes, we decided to start with a straight forward approach, testing whether or not active Hedgehog pathway could directly affect Notch induced transcriptional activity on a Notch specific target gene. The alpha chain of the pre T-cell receptor (preTCRα or pTα) is a known Notch target and our lab could demonstrate previously that Notch3 is important for pTα expression at the DN3 stage of developing thymocytes.[213,271]

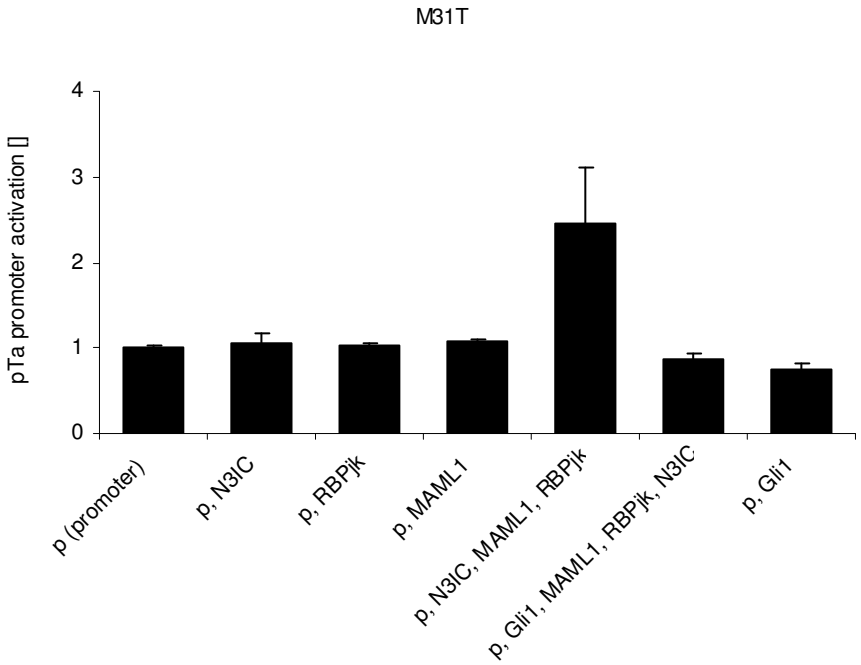

figure 9: pTα promoter activation by N3IC and cofactors. pTα driven luciferase signal strength in M31T cells, 48h post transfection. Cotransfection of N3IC together with MAML1 and RBPjκ results in maximal signal induction. Addition of Gli1 shows antagonistic effect on N3IC induced target gene transactivation.

To exclude multiple processing and activation steps of known members of the Hedgehog pathway, like Smoothened or the Suppressor of Fused (SuFu), which would render the final interpretation much more complex, we decided to cotransfect and overexpress the distal transcription factor Gli1 together with the required intracellular domain of Notch3 (N3IC) and its essential cofactors RBPjκ and MAML1 into the M31T (fig. 9) or preT 2017 (fig. 10) cell line, measuring luciferase expression 48h after transfection. In this experiment, coexpression of Gli1 and N3IC should simulate

intracellular T-cell conditions in which both pathway's distal transcription factors were active, in order to analyse changes in the final transcriptional outcome. Corresponding to previous work in our laboratory, N3IC required both cofactors, RBPjκ and MAML1, to gain full activation of the pTα promoter driven luciferase signal in the M31 T-cell background. Column five in figure 9 and 10 show maximal signal strength of Notch3ICD and coactivators. Interestingly the coexpression of Gli1, in a ratio 1:1 to all transfected protein expression plasmids, decreased Notch3IC induced promoter transactivation from 50 % (fig. 10, column 6) to 100 % (fig. 9, column 6). This antagonistic effect of Gli1 coexpression in Notch3IC driven pTα promoter transactivation assays did also work on other known Notch target genes like Hes1 and Hes5 (data not shown). As for maximal transactivation of Notch target genes it was necessary to transfect, in addition to NICD, also the cofactors RBPjκ and MAML1, interpretation of such an antagonistic effect of Gli1 resulted complex. To reduce the number of expressed proteins involved in a possible Notch-Gli cross reaction, we decided to switch the experimental setting to a Gli1 induced transactivation assay, trying to show N3IC overexpression in disturbing Gli1 driven luciferase expression in a similar antagonistic way. Gli1 is able to directly bind its target genes by at least the last three of its five zinc finger structures[272] and does therefore not require a DNA binding protein like RBPjκ in the canonical Notch pathway.

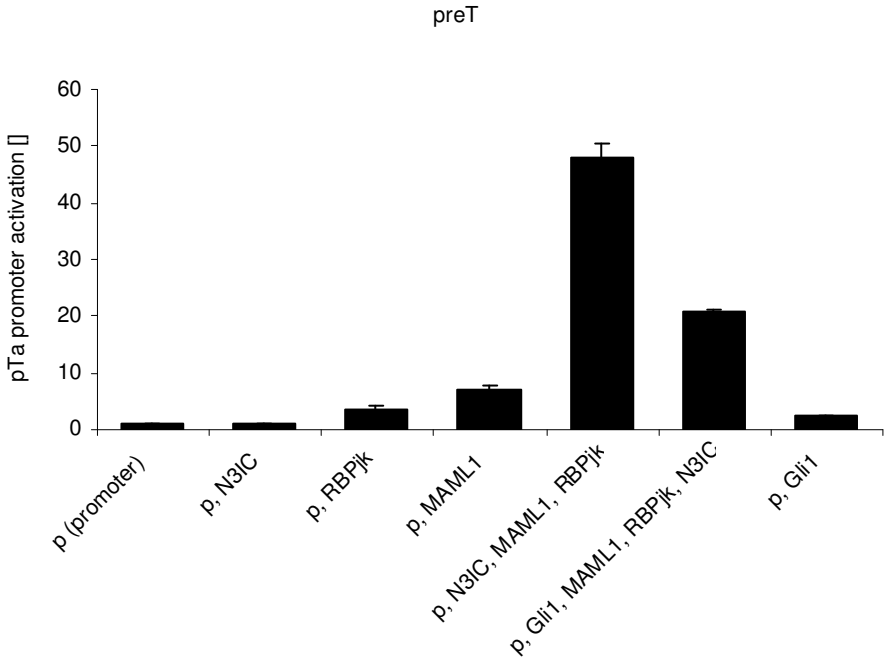

figure 10: pTα promoter activation by N3IC and cofactors. pTα driven luciferase signal strength in preT cells, 48h post transfection. Cotransfection of N3IC together with MAML1 and RBPjκ results in maximal induction. Addition of Gli1 shows antagonistic effect on N3IC induced target gene transactivation.

III.I.II Cell context dependent effect of N3IC on Gli1 induced transactivation

Using a luciferase reporter element, driven by a sequence of twelve times repeated Gli consensus binding sites (Gli12x-promoter) we were able to transfect our leukaemia T-cell lines M31T and preT and deduce from only promoter (p) transfections (p = 1 fold induction) versus promoter and cotransfected Gli1- expression vector the Gli1 induced transcriptional activity. As to be seen in figure 11, Gli1 was able to activate the luciferase-reporter while coexpression of N3IC failed to unspecifically activate Gli1 induced luciferase expression. The M31T cell line was used to represent the cellular context of developing thymocytes in an early double negative (DN1-like) stage of differentiation.[269,273] At this time point neither Notch3 nor Notch1 are activated, proved by the complete absence of the NICD in western blot assays (fig. 22 and data not shown). As overexpression of the intracellular domain of Notch3 did not affect Gli1 induced target gene transactivation in M31T cells (fig. 11a), we made two possible cases. Either there would be no direct cross talk of the pathway's distal transcription factors Gli1 and N3IC or for an indirect effect the given molecular context of M31T cells would not provide the proteic requirement for pathway interactions. To rule out a direct Gli1-Notch protein-protein interaction we performed immunoprecipitation assays, using whole cell lysate of transfected Hek293T cells, but failed to copurify Gli1-HA together witch N3IC-flag precipitation (data not shown).

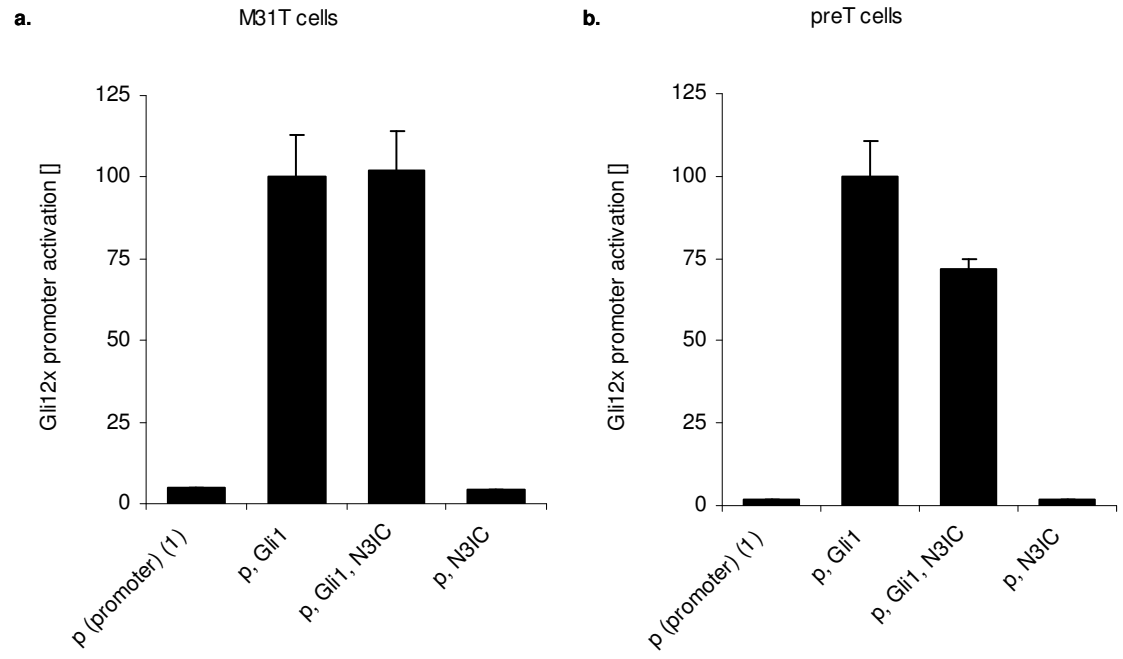

figure 11: Gli12x responsive element activation by Gli1. 12 Gli consensus binding site driven luciferase signal strength in M31T (**a.**) and preT (**b.**) cells, 48h post transfection. Gli1 transfection results in maximal induction. Addition of N3ICD shows a 28 % reduction of Gli1 induced target gene transactivation in preT but not in M31T cells.

Following our second hypothesis, that the cellular context would make the difference in indirect N3IC-Gli1 cross talk, we applied the same experimental approach using the preT 2017 cell line, representing a late DN3 stage of differentiation. Under normal *in vivo* conditions Notch3 as well as Gli1 expression are peaking short before the preTCR signalling initiation (Crompton et al.[248], Koyanagi et al.[206], Shi et al.[120], our data), like depicted in figure 8. The preT 2017 cell line derived from Maloney virus transformed $CD4^-$ $CD8^-$ double negative thymocytes, expressing $TCR\gamma^{high}$ is providing a DN3-like molecular background.[270] Interestingly, and different from our observations using M31T cells, in the preT cell line we could see a significant reduction of Gli1 induced luciferase expression when cotransfecting the constitutive active form of Notch3 (fig. 11b). In a ratio 1:1 of transfected plasmids the signal decrease was 28 %. Such an effect on Gli1 induced transactivation could have had several explanations. We excluded the possibility that N3IC would repress actively the Gli12x responsive element as luciferase expression was under the control of twelve Gli1 consensus binding sites without any CSL element needed for NICD binding. Still the overexpression of N3IC could inhibit Gli1 induced transcription by yet unknown Gli1 protein inactivating or degrading mechanisms. A third possibility of lowering transcriptional activity on target genes is the limitation of endogenous cofactors.[274] If a possible cross talk between the Notch and Hedgehog pathways would involve not or not only the distal transcription factors Gli1 and NICD, we were wondering whether or not known Notch binding partners could affect Gli1 activity while being differentially expressed in the two thymocyte cell lines used. We performed total (fig. 22), cytosolic (data not shown) and nuclear (fig. 12) protein extraction of the respective cell lines and compared specific cofactor expression levels by western blot analysis. While focusing on RBPjκ and MAML1, known Notch pathway members that have been shown to bind the intracellular domain of Notch and could have been affected or sequestered by N3IC in our experiments, we detected differences in nuclear protein levels. Figure 12 illustrates the significant higher level of MAML1 protein in the nuclear compartment of preT cells compared to M31T cells. RBPjκ did also show some slighter expression differences, with higher protein levels in M31T nuclei.

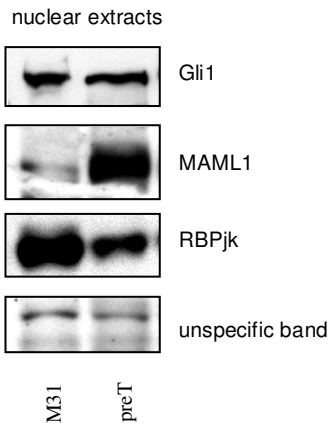

figure 12: Western blot of nuclear extracts of M31T and preT cells. Lysates were dissolved by SDS-PAGE. Nitrocellulose blots were cut at specific protein band sizes and incubated with the indicated antibodies.

III.I.III Mastermind-like1 coactivates Gli1-induced transcriptional activity

Different nuclear expression of the Notch coactivator Mastermind-like 1 (MAML1) in M31T and preT cells (fig. 12), its importance in Notch activator complex formation[189,275-277] and recruitment of additional factors[114,188] as well as its upcoming Notch-independent roles in other signalling pathways[278-281], led us to the following experimental setting: We cotransfected a plasmid coding for MAML1 into the M31T cell line while performing the same Gli1 target gene activation study using a Gli12x responsive element, in order to supply the DN1-like cell line with this potent cofactor, like expressed in DN3-like preT cells. By this approach, we were able to detect in our model an unexpected, more than 200 % increase of Gli1 induced luciferase expression (fig. 13a, compare columns 3 and 4). As Mastermind-like1 did not affect the Gli consensus site driven luciferase expression in the absence of overexpressed Gli1 protein (column 2), we assumed MAML1 to have an agonistic role in Gli1 induced gene transactivation. The effect of MAML1 was dose dependent, tested by increased MAML1:Gli1 ratios (data not shown). In preT 2017 cells the addition of MAML1 did reinforce Gli1-induced transcriptional activity up to 400 %, further sustaining the importance of Mastermind-like 1 protein expression levels at the DN3 stage of developing thymocytes.

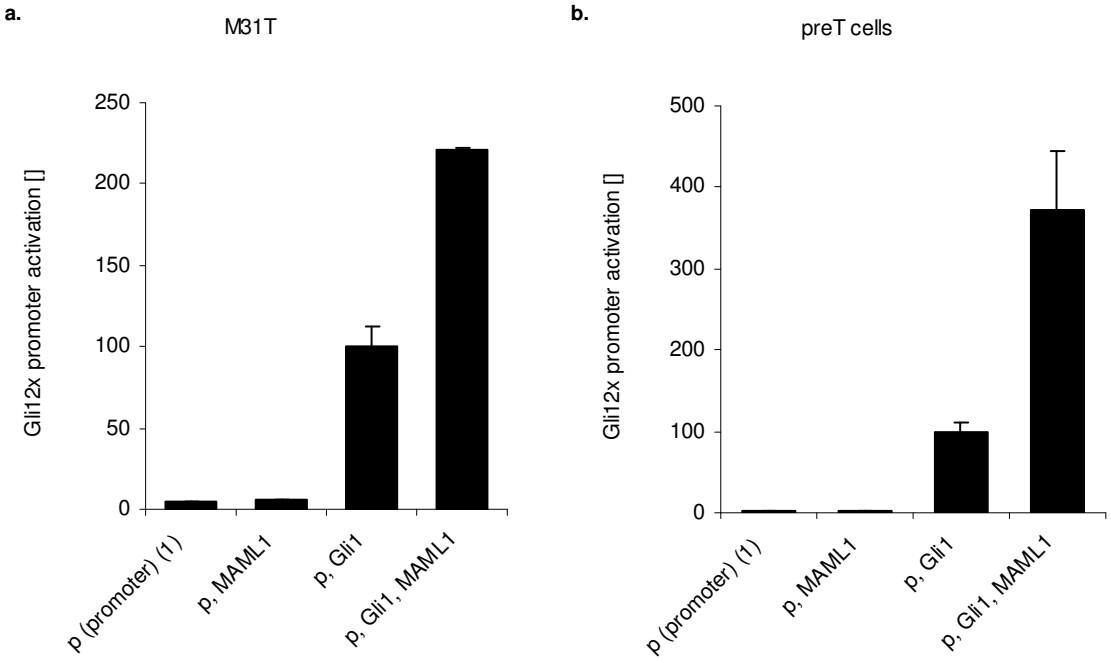

figure 13: Gli12x responsive element activation by Gli1. 12 Gli consensus binding site driven luciferase signal strengh in M31T (**a.**) and preT (**b.**) cells, 48h post transfection. Gli1 transfection results in about 100 fold promoter induction. Addition of MAML1 shows in Gli1 induced target gene transactivation a significant signal increment of more than 200% in M31T and 400 % in preT cells.

III.I.IV N3IC reverses Gli1 transactivation potentiated by MAML1

Once demonstrated that MAML1 can act as a cofactor in the Hedgehog pathway, able to increase significantly Gli1 induced transcriptional activity, we were wondering what would happen when we express N3IC in this Notch inactive background.

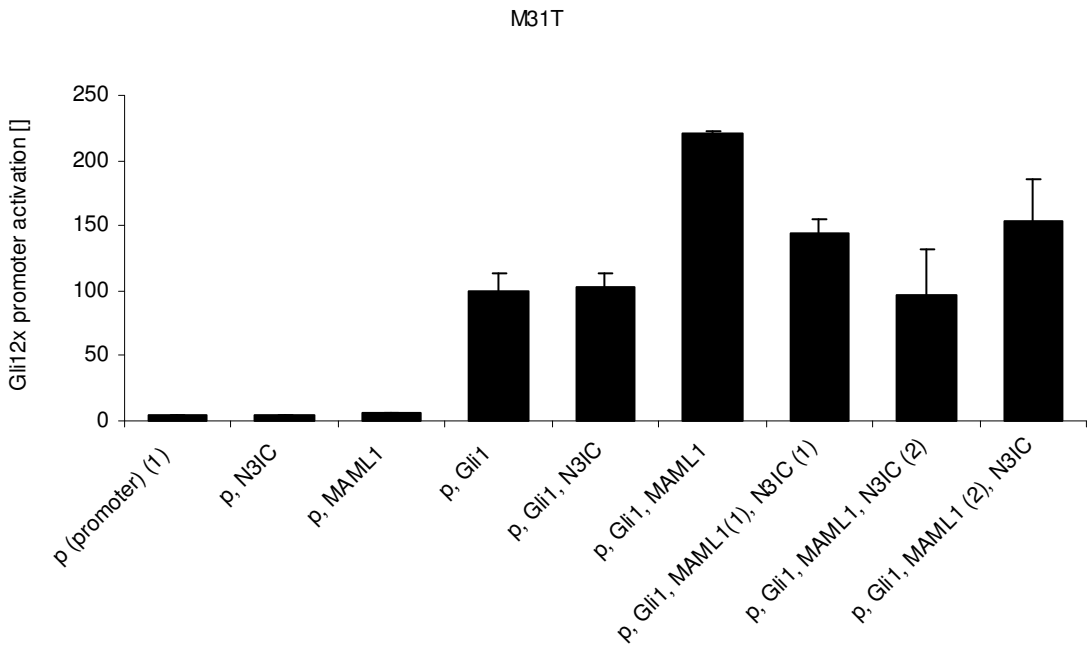

figure 14: N3IC induced antagonism in Gli1 and MAML1 induced transactivation. Gli12x driven luciferase signal strength in M31T cells, 48h post transfection. Addition of N3IC antagonizes the positive effect of MAML1 on Gli1 induced transactivation in a dose dependent manner.

In order to show a direct effect of Notch pathway activation on Gli1 induced signalling, we decided to cotransfect N3IC into the M31T or the preT cell line, analysing Gli1 induced and MAML1 potentiated signal strength in the presence of active Notch. Addition of increasing amount of N3IC resulted in a dose dependent antagonistic effect on Gli1 induced and MAML1 superactivated transcriptional activity. By the use of plasmids coding for Gli1, MAML1 and N3IC in a ratio 1:1:1 (fig. 14, column 7) the signal strength of promoter activation lay exactly in between the values of normal Gli1-induced activity (column 4) and the, by addition of MAML1 potentiated signal (column 6). Increasing the intracellular N3IC protein level, transfecting Gli1, MAML1 and N3IC in a ratio 1:1:2, was completely antagonising the coactivator effect of MAML1 on Gli1-induced transactivation (column 8). Supplying more MAML1 (ratio 1:2:1) was again reverting the N3IC caused antagonism (last column), indicating a stoichiometric competition of N3IC and Gli1 for the coactivator MAML1.

We repeated the same experiment to proof a dose dependent antagonism of Notch3 on Gli1 and MAML1 induced transactivation in preT cells and gained comparative results regarding the effect of MAML1 and N3IC on the transcription factor activity of Gli1 (compare fig. 14 and 15). MAML1 cotransfected together with Gli1 into preT cells showed a significant, 4-fold increase in luciferase signal strength. Most importantly and like in M31T cells seen before, this MAML1- induced boost of Gli1 transcriptional activity could again be antagonized by coexpression of N3IC in a dose dependent manner, as to be seen in figure 15. By increasing the intracellular overexpressed N3IC protein level, the coactivator effect of MAML1 on Gli1 induced transactivation was strongly antagonized. The supply of more MAML1 (Gli1:MAML1:N3IC in a ratio 1:2:1, last column) was again reverting the N3IC caused antagonism.

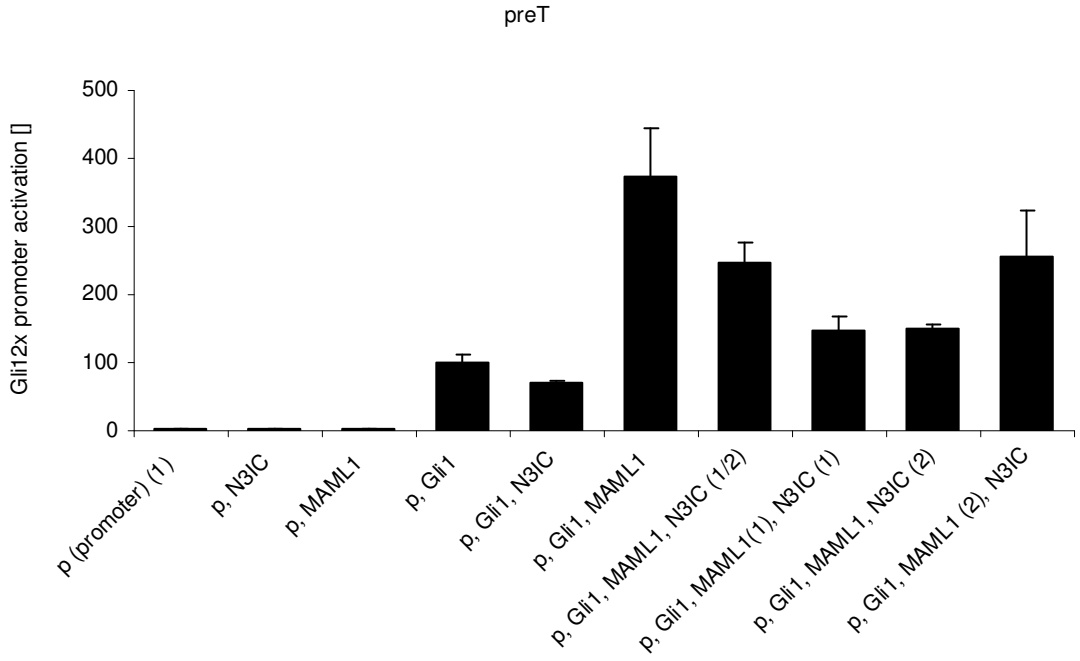

figure 15: Gli12x responsive element activation by Gli1. 12 Gli consensus binding site driven luciferase signal strength in preT cells, 48h post transfection. Addition of N3IC can antagonize the positive effect of MAML1 on Gli1 induced transactivation in a dose dependent manner. Increased supply of transfected MAML1 (last column) can counteract the antagonistic effect of N3IC.

III.I.V MAML1 and RBPjκ in Patched1 transactivation

As the used Gli12x responsive element is an artificial promoter, we decided to verify obtained results on the promoter of the target gene PATCHED (PTCH1), known to be direct transcriptional target of Gli1.[282] The *PtchWT* plasmid expresses luciferase under the control of 1000 bp of the Patched (Ptch) wild type (WT) promoter, upstream the transcription start side (TSS). The Patched-promoter sequence is providing two putative binding sites for Gli1 of which the one closer to the TSS has been published to be mandatory for target gene activation.[267] Following our hypothesis, and to be in line with results presented in the previous chapter, N3IC should quench the MAML1 protein and by doing so reduce its availability to be used as a cofactor in Gli1 induced Patched transactivation. We know from previous studies as well as from literature that MAML1 does only bind the intracellular domain of cleaved Notch after forming a ternary complex with RBPj. The RBPj-Notch dimer is forming a molecular groove on its surface that can provide sufficient binding energy for non-covalent binding of the helical N-terminal region of Mastermind-like 1.[110] With this molecular concept in mind, we decided to evaluate also the effect of RBPj in the following luciferase assays.

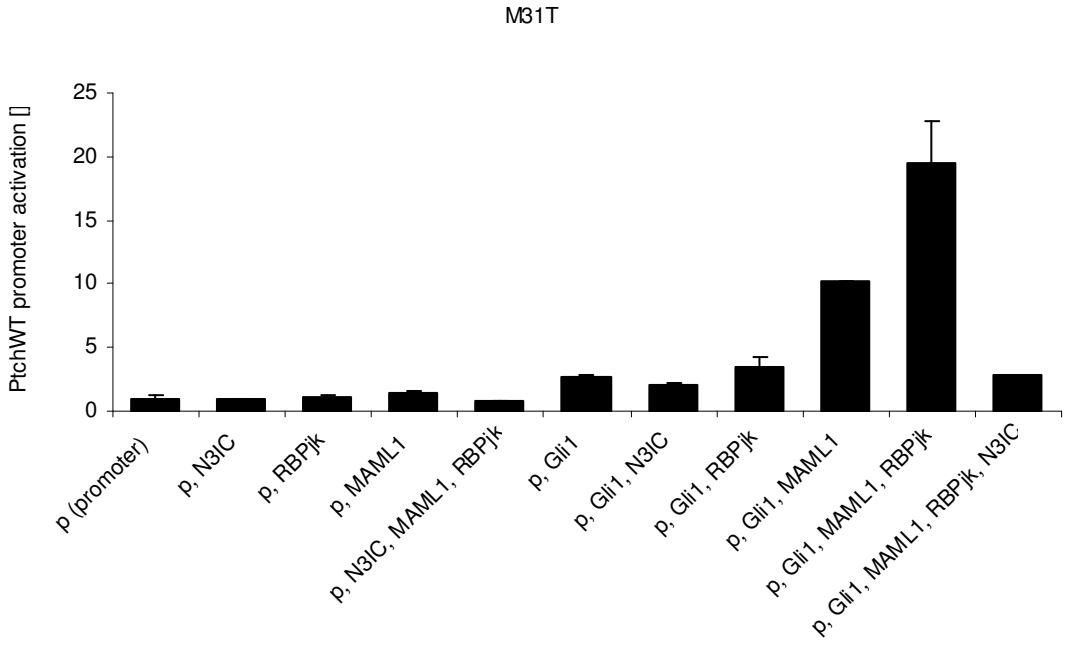

figure 16a: Ptch1 wt promoter activation. Patched1 promoter driven luciferase signal strength in M31T cells, 48h post transfection. Gli1 transfection results in 2.7 fold promoter induction. Addition of MAML1 shows a significant signal increment on Gli1 induced target gene transactivation up to 10.2. Cotransfection of both factors MAML1 and RBPjκ results in the maximal transactivation strength of 20 fold promoter induction, about 7 times Gli1 only activity. Addition of N3IC, in a ratio 1:1 to all transfected plasmids, can antagonize the positive effect of MAML1 and RBPjκ on Gli1 (last column).

In the M31T cell line, combined coexpression of RBPjκ and MAML1 increased Gli1 induced transactivation more than seven times of normal Gli1 activity and exceeded all single or double combinations tested (fig. 16a, column 6 - 10). The N3IC induced antagonistic effect on luciferase expression still worked even on the Patched promoter and restored a value similar to the one measured when transfected only Gli1, without MAML1 or RBPjκ (fig. 16a, compare column 6 with last column). To control Gli1 dependency and that the agonistic effects of MAML1 and RBPjκ, seen when cotransfected together with Gli1, was not caused by unspecific promoter binding, we used a mutated form of the described Patched promoter (PtchMUT), in which two point mutations in the mandatory Gli1 consensus binding site prevent Gli1 binding and transactivation.[267]

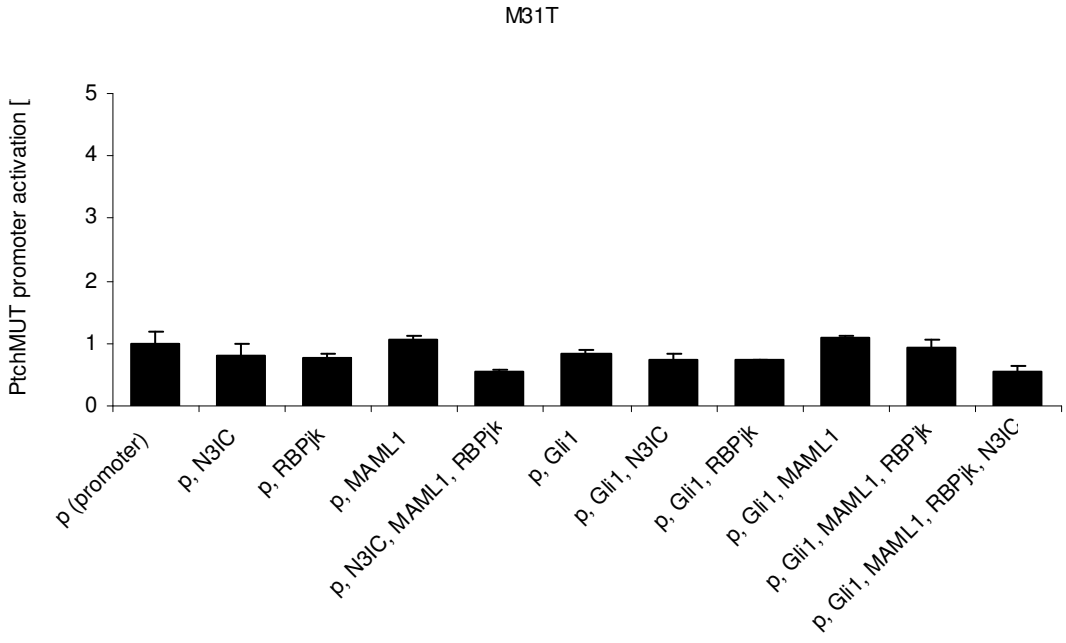

figure 16b: Ptch1 mut promoter activation. Mutated Patched1 promoter driven luciferase signal strength in M31T cells, 48h post transfection. No significant transactivation was observes throughout all transfected samples.

Use of the PtchMUT promoter did effectively inhibit Gli1 induced luciferase activation. The fact that also no signal was detected when MAML1 and RBPjκ were coexpressed underlined their Gli1 reinforcing but not Gli1-target gene autoactivating properties. Again we were able to demonstrate a dose dependency for the Notch3IC induced antagonism (fig. 17). Increased levels of transfected N3IC-plasmids did restore normal Gli1-induced Patched activation. From this effective antagonism seen in our *in vitro* luciferase assay series, we deduced that the exact level of MAML1 and intracellular Notch fragments would be of major importance in interfering with the Hedgehog pathway.

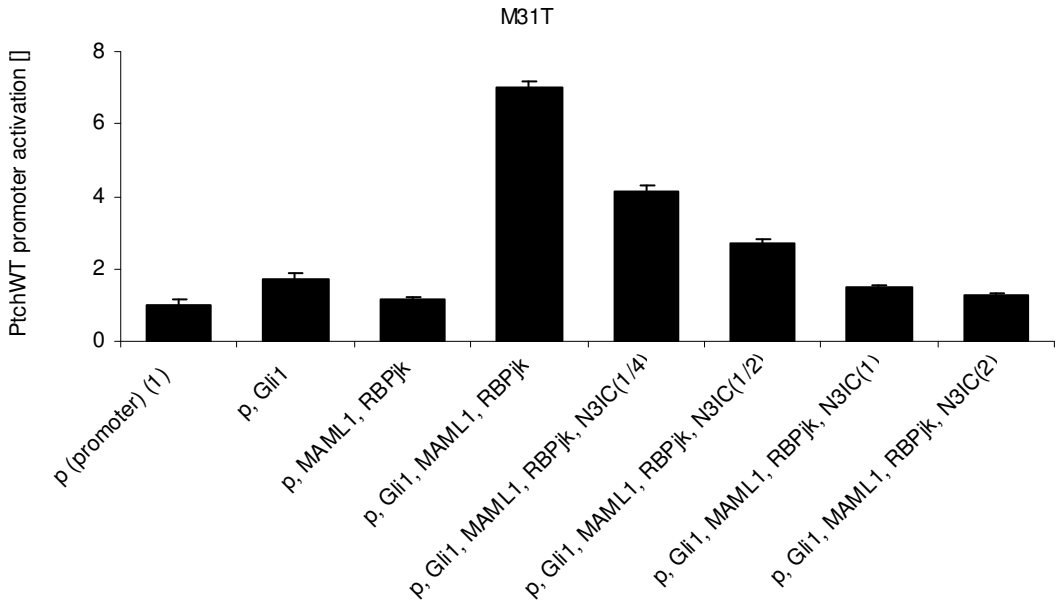

figure 17: Ptch1 wt promoter activation. Patched1 promoter driven luciferase signal strength in M31T cells, 48h post transfection. Cotransfection of both factors MAML1 and RBPjκ results in the maximal transactivation strength of about 4 times Gli1 only activity. Addition of N3IC can antagonize the positive effect of MAML1 and RBPjκ on Gli1 induced transactivation in a dose dependent manner.

The more N3IC was supplied, the more pronounced was its antagonistic effect on Gli1. Still MAML1 must play a decisive role in these pathway converging transactivation experiments as its absence uncouples N3IC from Gli1 in matter of target gene transactivation.

In order to evaluate the effect of RBPjκ in Gli1 induced transactivation and the possible role in N3IC induced antagonism, we did transfection studies with RBPjκ but without exogenous MAML1. The effect of RBPjκ coexpression in Gli1 induced transactivation of a Gli12x responsive element was in M31T cell negligible and in preT cells statistically not significant nor dose-dependent (fig. 18), most probably due to the lack of RBPjκ binding sites in the artificial and Gli specific luciferase promoter. Overall Gli1 induced promoter transactivation was more efficient in preT cells (fig. 18, column 4, grey vs. black), indicating that the cellular background of the preT 2017 cell line, representative for DN3-like thymocytes, may favour Gli1 target gene activation, while the differences in endogenous expression of MAML1 between M31T and preT cells (fig. 12) would correlate with its role as a potent Gli1 coactivator.

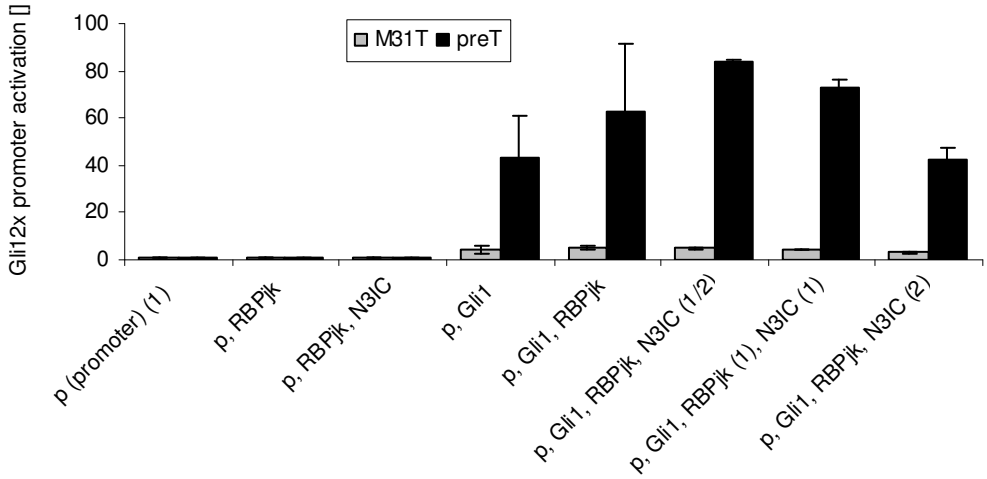

figure 18: Gli12x responsive element activation by Gli1. 12 Gli consensus binding site driven luciferase signal strengh in M31T (black) and preT (grey) cells, 48h post transfection. Gli1 induced transcriptional activity was only insignificantly altered by RBPjk and without a clear dose-dependency for additional N3IC.

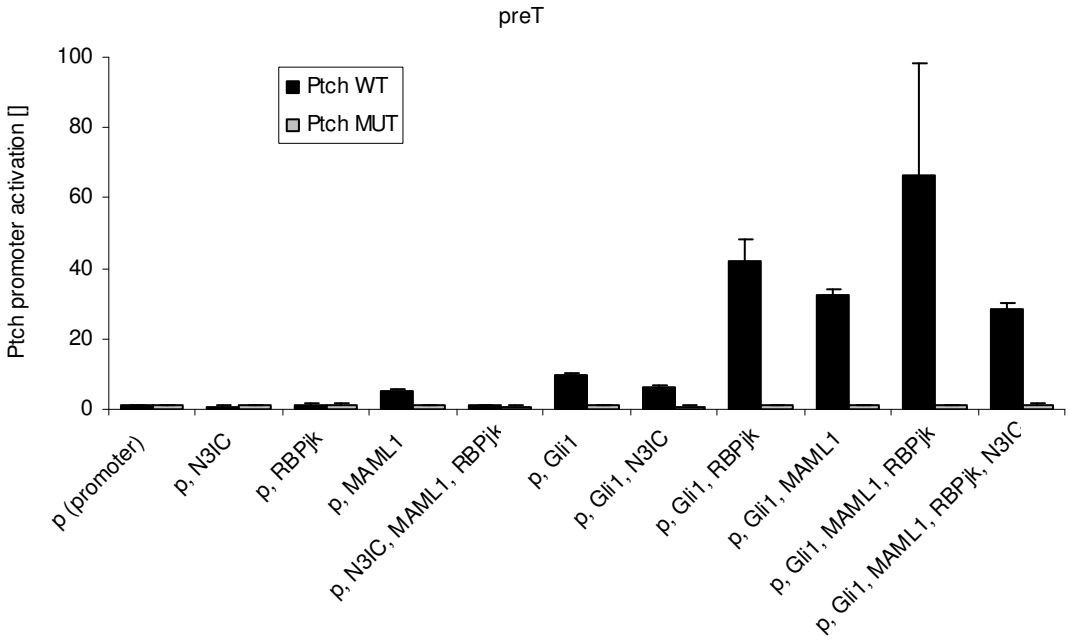

figure 19: Ptch1 promoter activation. Patched1 wt promoter driven luciferase signal strength (black columns) in preT cells, 48h post transfection. Gli1 transfection results in 10 fold promoter induction. Addition of MAML1 shows a significant signal increment on Gli1 induced target gene transactivation up to 32. While addition of RBPjκ shows an increment up to 42, cotransfection of both factors MAML1 and RBPjκ together results in the maximal transactivation strength of 66 fold promoter induction. Addition of N3IC, in a ratio 1:1 to all transfected plasmids, can antagonize the positive effect of MAML1 and RBPjκ on Gli1 (last column). Mutated Patched1 promoter driven luciferase signal strength (grey columns) in preT cells, 48h post transfection. No significant transactivation was observes throughout all transfected samples.

Interestingly, when using the Patched WT promoter in the preT cell line, we did see an agonistic effect of RBPjκ on Gli1-induced target gene activation (fig.19, black

column 7). The fact that RBPjκ-only transfection, without Gli1, did not activate the Patched wild type promoter in an unspecific way (fig. 19, black column 3) was indicating a coactivator role of RBPjκ in Gli1 induced Patched promoter but not through Gli-consensus element activation. Using the mutated form of the Patched promoter, transactivation was abolished completely (fig. 19, grey columns).

III.I.VI Enhanced Gli1 recruitment for Patched1 transactivation

Using the genomatix server for transcription factor binding site prediction, we identified a putative RBPjκ binding site upstream to the essential Gli-binding site of the Patched promoter, juxtaposed to a second non-consensus Gli-binding site, reported to be a potent enhancer element.[267]

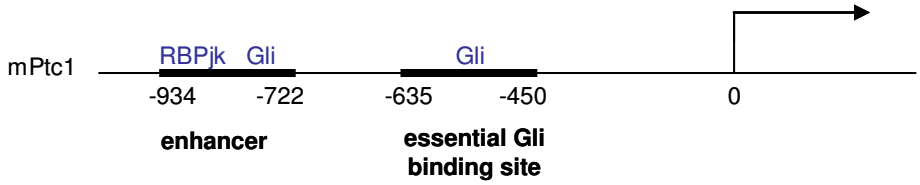

figure 20: mPtc1 promoter. Mandatory and sufficient Gli consensus binding site (635-450 bp upstream) and Gli enhancer element juxtaposed to RBPjk/Ikaros binding site upstream the transcription start site (934-722 bp upstream) of murine Patched1 gene.

Performing chromatin immunoprecipitation (ChIP) of M31T cells, we could detect endogenous Gli1, bound to the upstream enhancer element of the murine Patched1 promoter region, when we tranfected the cells with RBPjκ or MAML1 (fig. 21a). The fact that MAML1 and RBPjκ overexpression is followed by Gli1 binding to the Gli enhancer site in the Patched1 promoter region may reflect their agonistic effect seen before in luciferase activity assay (compare fig. 16a and 17). Interestingly, when performing chromatin immunoprecipitation assay with antibodies against RBPjκ, DNA fragments including the putative RBPjκ binding site coprecipitated only in the case of MAML1 transfection (fig. 21b). We did also re-ChIP assays to detect MAML1 bound to Gli1 on the Patched promoter but failed to gain reproductive results. Whether or not MAML1 is directly binding Gli1 on the chromatin remains to be demonstrated. As the effect of MAML1 and its involvement in Gli1 transactivation was more pronounced than that of RBPjκ we decided to focus in further experiments on the role of MAML1 in Gli1 signalling.

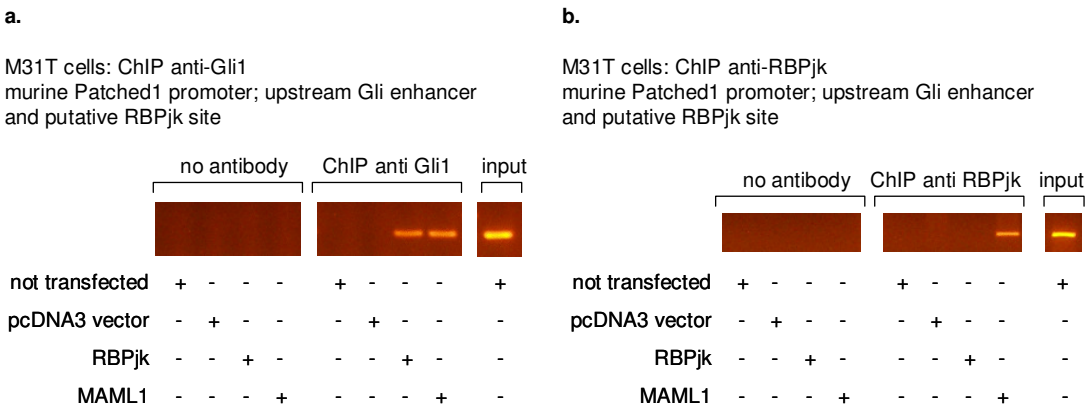

figure 21: ChIP of Gli enhancer element in Patched1 promoter: Chromatin Immunoprecipitation of normal or transfected M31T cells. **a:** ChIP against Gli1 reveals DNA fragment binding in the case of RBPjκ or MAML1 transfected M31T cells. **b:** ChIP against RBPjk reveals DNA fragment binding in the case of MAML1 transfected M31T cells.

Taken together, we were able to demonstrate a Notch independent cofactor role for MAML1 in Gli1 induced transcriptional activity when overexpressed *in vitro* in two different T-cell lines (fig. 14a and 15a). However, addition of the intracellular domain of Notch3 did abolish the coactivator effect of MAML1 on Gli1-induced transactivation in a dose dependent manner (fig. 14b and 15b). Also Notch transactivation, together with the essential cofactors RBPJκ and MAML1 was antagonized by Gli1 expression (fig. 10) indicating a co-usage of the MAML1 protein. Its differential nuclear expression in M31T and preT cells (fig. 13) as well as dose dependent antagonism seen in transactivation of Notch and Hedgehog target genes may argue for stoichiometric competition of both transcription factors NICD and Gli1 for MAML1 and the need for increased expression levels at the DN3-like stage of developing thymocytes (compare fig. 8), where under normal conditions both pathways are active and essential for preTCR signal induction.[206,249,255] Chromatin immunoprecipitation may further sustain a role of MAML1 in Gli1 induced transactivation as overexpression of MAML1 in preT cells led to Gli1 protein binding to the enhancer element of the endogenous target gene Patched1. Presence of an RBPjκ binding site in front of the Patched enhancer element could indicate a more complex regulatory mechanism. Our working model is giving MAML1 a central role in between these two highly conserved signalling pathways and we hypothesize an intrinsic molecular competition, where expression and activation levels of the transcription factors Gli1 and Notch3IC are decisive in single or shared usage of the interpathway cofactor MAML1.

III.II Protein-protein interaction

III.II.I Mastermind-like 1 interacts with Gli1

The results presented in chapter III.I evidence a specific coactivator role for MAML1 in Gli1 induced transcriptional activity. Proteins acting as transcriptional cofactors do in general directly or indirectly bind the respective transcription factor and thereby potentiate transactivation strength through posttranslational modifications or by the recruitment of additional cofactors.[283-285] Initially, we performed a comparative series of qPCR (fig. S1) and western blot (fig. 22 and data not shown) analysis of various thymocyte cell lines in order to screen for simultaneous expression of Notch and Hedgehog pathway members in RNA as well as in protein levels.

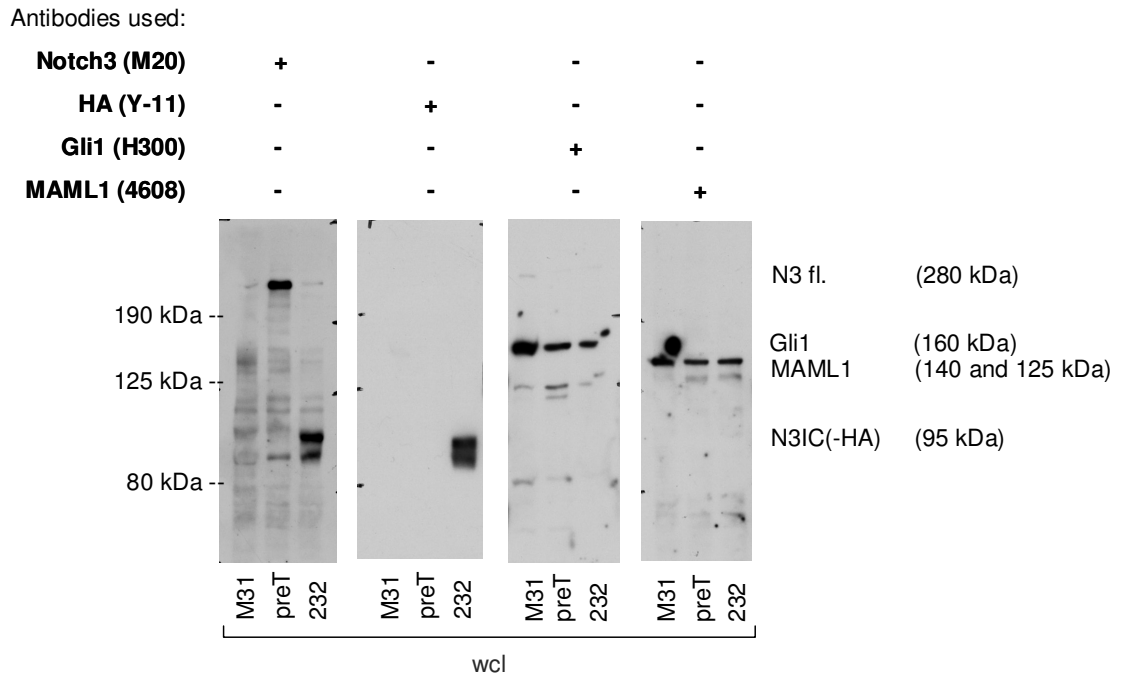

figure 22: WB of endogenouse proteins: Western blot analysis of three different cell lines: M31T (DN1-like thymocytes), preT 2017 (late DN3-like thymocytes) and 232T (murine N3IC^{+} T-cells). The 160 kDa Gli1 band corresponds to transfected human Gli1 protein. The lower and slighter 125 kDa MAML1 band corresponds to nuclear MAML1.

M31T and preT 2017 cells were used in our luciferase experiments as they represent DN1-like and DN3-like stages of developing double negative T-cells. The 232T cell line was established from immortalized *ex vivo* thymocytes of an Lck-driven N3IC-HA overexpressing transgenic mouse and was initially used as a control for endogenous expression of the intracellular domain of Notch3. 232T cells did express high levels of N3IC and Gli1 as well as their respective target genes Hes1 and Patched1, typical for

deregulated and simultaneously active Notch and Hedgehog pathways, like seen in other mouse and human leukaemia cell lines (unpublished data). Hybridization with specific antibodies against MAML1 did reveal two bands in western blots of preT and 232T cells, one at the size of 140 kDa and the other at 125 kDa. The lower one was less pronounced when whole cell lysates were analyzed but gained prevalent protein detection strength when SDS-PAGE and immunoblotting was performed with nuclear extracts (fig. 12).

It is known that MAML1 localizes to nuclear spots (compare fig. S3), where it can interact with the intracellular domain of Notch paralogues, RBPj or GSK3β.[109,112,188] To test which of the two bands seen in the western blot analysis of whole cell lysate was the specific NotchICD interacting one, we performed co-immunoprecipitation assay of Notch and MAML1. As 232T cells do constitutively express HA-tagged N3IC we precipitated Notch with anti-HA antibodies, resolved proteins by SDS-PAGE and transferred them onto a nitrocellulose membrane before hybridization with anti-MAML1 antibodies. While in nuclear extracts (fig. 23, left lanes) two MAML1 bands were visible, the co-immunoprecipitate (IP) of N3IC did only reveal the lower 125 kDa protein band.

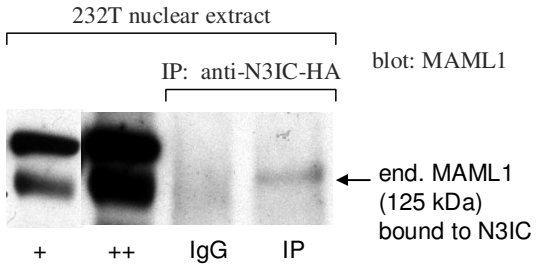

figure 23: MAML1 125 kDa binds N3ICD: Coimmuno-precipitation assay with anti-HA Agarose; precipitates of endogenous N3ICD-HA of nuclear extracts of 232T cells reveal interaction with the lower MAML1 band of the size 125 kDa but not with the upper and stronger pronounced 140 kDA band.

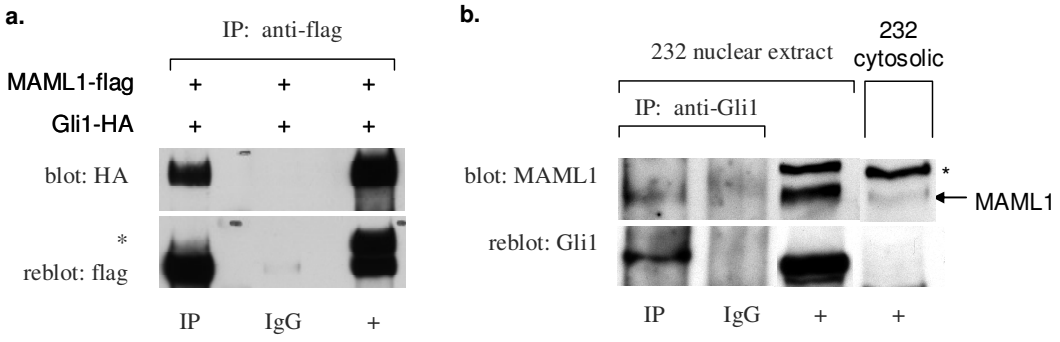

figure 24: CoIP of MAML1 and Gli1: a. Gli1-HA and MAML1-flag proteins were transfected into Hek293T cells and wcl were used to precipitate MAML1 with a flag-Agarose antibody. After western blotting onto nitrocellulose the membrane was first incubated with anti-HA antibodies in order to reveal coimmunoprecipitation of Gli1-HA. Reblotting with specific anti-flag antibodies did show the total protein pulldown. **b.** Endogenous immunoprecipitation of Gli1 reveals a slight coprecipitation of the lower MAML1 band of the size 125 kDa in nuclear extracts of 232T cells.

To understand the cofactor role of MAML1 in Gli1 induced target gene activation, we applied similar immunoprecipitation assays to detected direct protein-protein interactions. Due to the lack of good commercially available antibodies against murine MAML1 proteins, usable for endogenous co-immunoprecipitation, we decided to change the experimental setup from low efficient endogenous (fig. 24b) to exogenous protein interaction studies (fig. 24a). We took advantage of the flag-tag of MAML1, transfected into Hek293T cells, and incubated immunoprecipitates (IP), an IgG negative control as well as whole cell lysate (+) against the HA-tag of cotransfected Gli1-HA. Gli1 co-immunoprecipitated with MAML1, when overexpressed in Hek293T cells (fig. 24a).

III.II.II N3IC can affect Gli1-MAML1 interaction

For the purpose of understanding the molecular background of the antagonistic behaviour of Gli1 and Notch3ICD, seen in transactivation experiments with specific luciferase promoters, we initially tried to coprecipitate Gli1 with N3IC but failed to see a direct protein-protein interaction (data not shown). We therefore started to evaluate the interaction strength of Gli1 and MAML1 in the absence or presence of cotransfected N3IC.

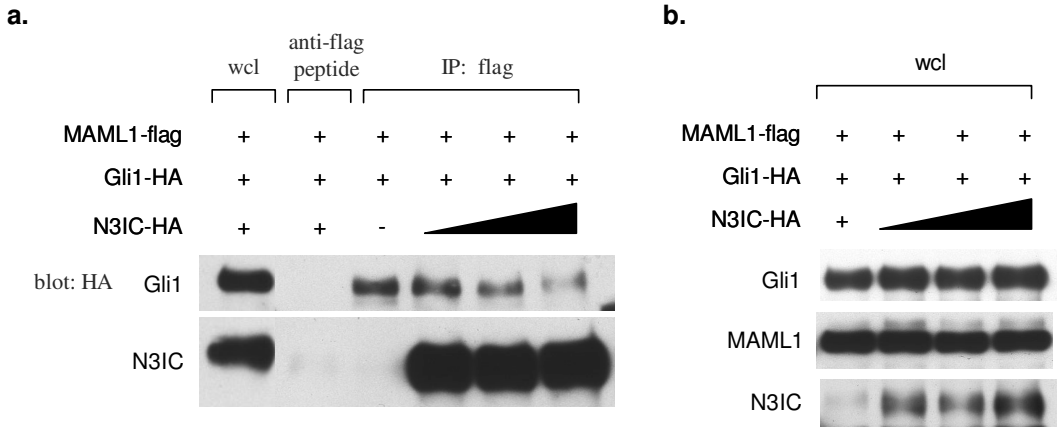

figure 25: Increasing amount of N3IC can negatively affect Gli1 binding to MAML1: a. Plasmids coding for the proteins Gli1-HA, MAML1-flag and N3IC-HA were transfected into Hek293T cells and wcl were used to precipitate MAML1 with a flag-Agarose antibody. After western blotting onto nitrocellulose the membrane was incubated with anti-HA antibodies in order to reveal coimmunoprecipitation of Gli1-HA and N3IC-HA at the same time. Increasing amount of transfected N3IC led to decreased Gli1 coimmunoprecipitating with MAML1. **b.** protein expression in whole cell lysates used for immunoprecipitation assay in a.

In the experiment shown in figure 25a, we cotransfected MAML1-flag as well as Gli1-HA and increasing amount of N3IC-HA expression plasmids into Hek293T cells and performed co-immunoprecipitation against MAML1-flag proteins. While western blotting of whole cell lysate (25b) confirmed constant expression levels of Gli1 and MAML1 and a dose-dependent increase of the N3IC protein, the amount of with MAML1 co-immunoprecipitated Gli1 was negatively correlated to N3IC coexpression and binding to MAML1. Increasing amount of N3IC, with respect to constantly coexpressed MAML1 and Gli1 protein levels, was assumed to induce a quencher effect, as the intracellular domain of Notch is reported to recruit MAML1 into a ternary complex with RBPJκ, exhibiting non-covalent protein interactions with high affinity. Interestingly, the expression of N3IC in whole cell lysates was less effective than in comparable transfection settings without coexpression of MAML1 (see also fig. 28). However N3IC binding to MAML1 was strong and seemed to displace Gli1 co-precipitation.

We know from literature that MAML1 needs the NICD-RBPj interface to efficiently bind to the intracellular domain of Notch, in order to form a strong ternary activator complex on target genes.[275] We decided to overexpress also RBPjκ to equilibrate protein expression ratios in transfected cells used for MAML1 co-immunoprecipitation. Densitometrical quantification was normalized against precipitated MAML1 and values of Gli1-only (fig. 26, first left red column) as well as N3IC-only coimmunoprecipitates (lane 6, green column) were set to 1. By co-immunoprecipitation of proteins bound to MAML1, in a set of transfected 293T cells, we were able to show a 20 %, 46 % or even 69 % reduction of MAML1-bound Gli1 protein (red columns, from left to right) when coexpressing N3IC, RBPjκ or both respectively. The diminished amount of Gli1 binding to MAML1 when overexpressed Gli1, MAML1 as well as N3IC in an expression plasmid ratio 1:1:1 is reassembling and confirming the data from previous experiments (i.e. fig. 25a, lanes 3 and 5). Hybridization with antibodies against RBPjκ revealed the presence of endogenous human RBPjκ in the N3IC-MAML1 precipitate of transfected Hek293T cells (fig. 26, lanes 3 and 6). Overexpression of a constant amount of mouse RBPjκ, the murine homologue of human RBPj in which binding properties to MAML1 are highly conserved, led to detection of a slightly higher murine RBPjκ band in MAML1 co-precipitates (lanes 4, 5, 7 and 8). Cotransfection of mRBPjκ did positively affect N3IC-MAML1 binding. While already in the reference sample (lane 6), in which only N3IC was coexpressed with MAML1 (= 1.0 x), endogenous hRBPjκ was recruited, supply of mRPBjk in an equivalent ratio to NICD and MAML1 did show a 2.7 fold increase of N3IC-MAML1 coprecipitation. These data sustain the need for RBPjκ in complex formation of the intracellular domain of Notch3 and its coactivator MAML1.

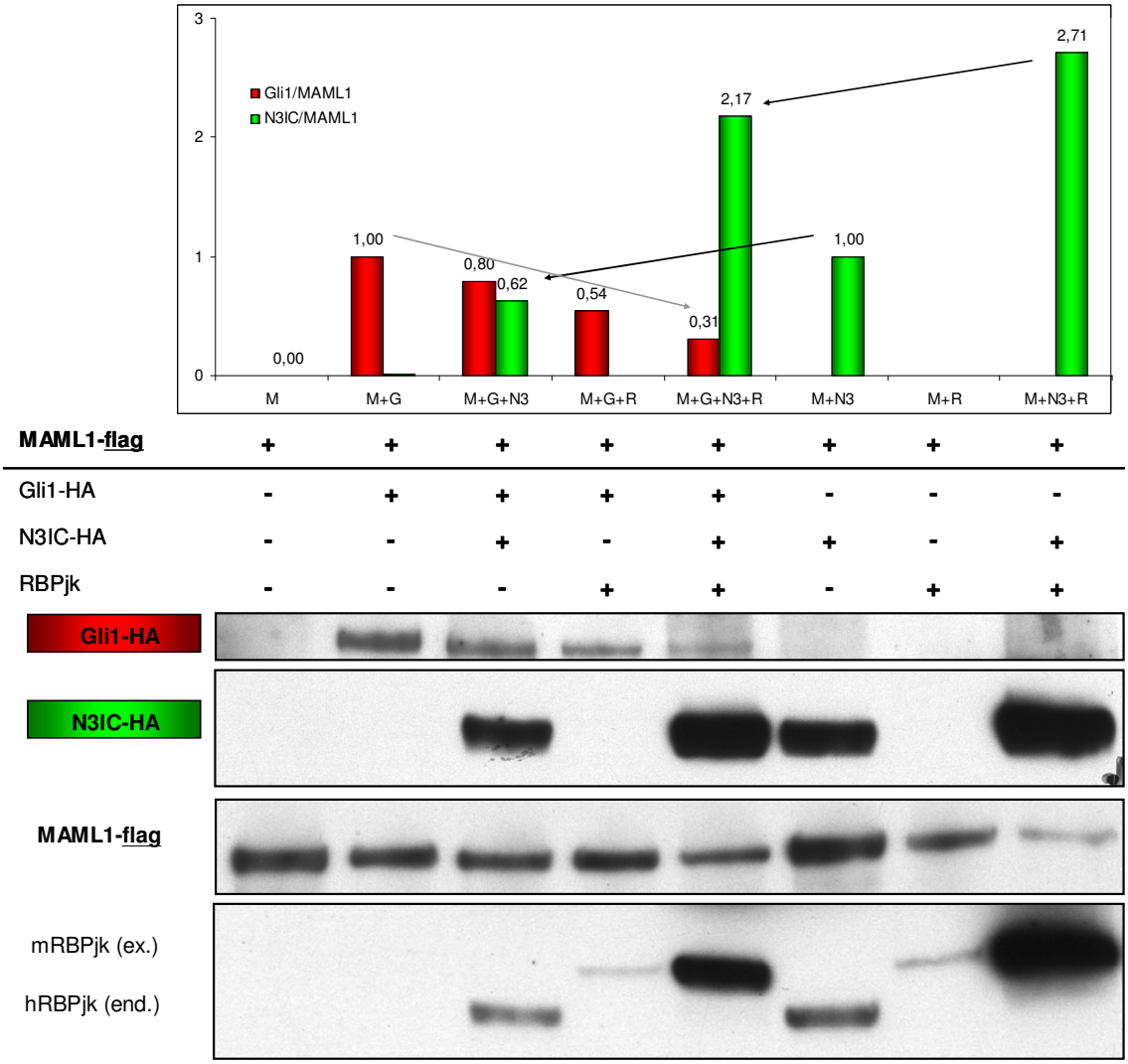

figure 26: N3IC and RBPjκ sequester MAML1 away from Gli1 into ternary complex: Plasmids coding for the proteins Gli1-HA, MAML1-flag, RBPjκ and N3IC-HA were transfected in different combinations into Hek293T cells and wcl were used to precipitate MAML1 with a flag-Agarose antibody. After western blotting onto nitrocellulose the membrane was incubated with specific antibodies in order to reveal coimmunoprecipitation of Gli1-HA, N3IC-HA, RBPjκ (slight band size differences between endogenous human RBPjκ/CSL and transfected mouse RBPjκ) and reblotted for total MAML1-flag precipitation. Densitometrical analysis was normalized to precipitated MAML1 amount. Gli1-only coimmunoprecipitates (first left red column) and N3IC-only coimmunoprecipitates (lane 6, green column) were set to 1.

On the one side, coexpression of Gli1 did reduce N3IC coprecipitation with MAML1 about 20 % (fig. 26, lane 8: 2.7, lane 5: 2.2), while on the other side the effect of N3IC coexpression, together with RBPj, reduced simultaneous Gli1 binding to MAML1 about 70 % (fig. 26, compare lanes 2 and 5). The fact that also binding of N3IC to MAML1 and RBPj is affected by coexpression of Gli1 might indicate a bilateral antagonism, affecting both Notch and Gli binding to a central MAML1 and is in line with results from our luciferase-activation experiments using the Patched or the pTα promoter.

III.III MAML1 induces N3IC turnover

III.III.I Gli1 collaborates with MAML1 in N3IC degradation

In order to link the antagonistic effect of Gli1 coexpression in Notch driven gene activation with competitive protein binding characteristics of MAML1 to N3IC and Gli1, we analyzed the amount of co-immunoprecipitated N3IC in a MAML1-flag IP while increasing the dose of Gli1 protein expression.

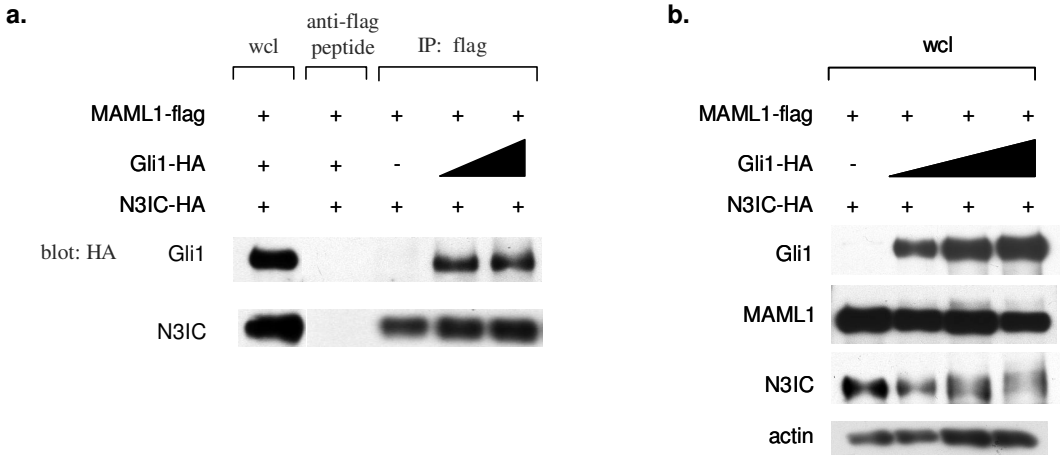

figure 27: Increasing amount of Gli1 can negatively affect N3IC protein expression: Plasmids coding for the proteins Gli1-HA, MAML1-flag and N3IC-HA were transfected into Hek293T cells and wcl were used to precipitate MAML1 with a flag-Agarose antibody. After western blotting onto nitrocellulose the membrane was incubated with anti-HA antibodies in order to reveal coimmunoprecipitation of Gli1-HA and N3IC-HA at the same time. **a.** Co-immunoprecipitation of increased Gli1 and constant N3IC expression. **b.** Increasing amount of transfected Gli1 leads to decreased N3IC expression when MAML1 is coexpressed in a constant and equal amount to N3IC.

While a decreased binding affinity of N3IC to MAML1, with increasing amount of Gli1 coexpression (27a), was not evident like in the equivalent experiment analysing the effect of a N3IC dose on Gli1 described above (fig. 25a), the overall protein expression control of whole cell lysates disclosed a strong reduction of N3IC protein expression (27b). The low N3IC protein expression was evident whenever we were coexpressing Gli1 and MAML1 together (see also fig. 25b and fig. 28a and c). To test whether the negative effect on Notch3IC expression, seen in figure 27b, was based on the combined overexpression of both proteins, MAML1 and Gli1, or caused by a single coexpression of one of the two proteins, we transfected independently from each other MAML1- or Gli1- doses, analysing the effect on expression of constantly coexpressed N3IC in whole cell lysates.

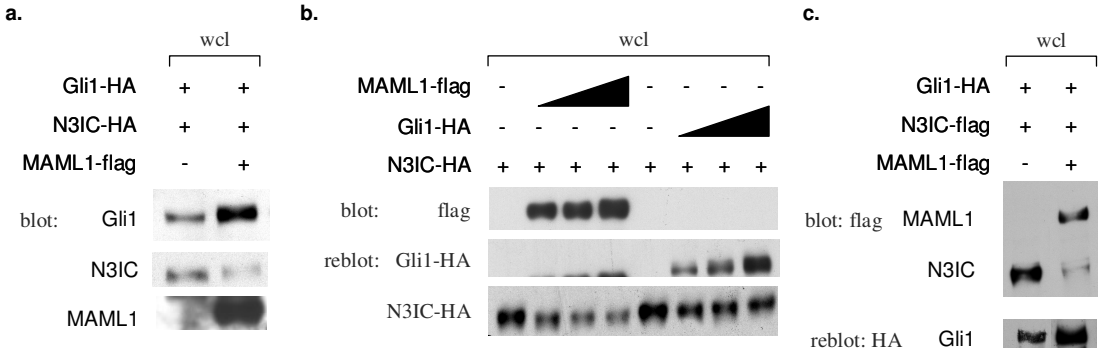

figure 28: N3IC protein expression is disturbed by MAML1: Whole cell lysate of Hek293T cells transfected with the same amount of Gli1 and N3IC (-HA in **a.** and N3IC-flag in **c.**) expression plasmids, show significant impairment of N3IC protein expression when simultaneously overexpressed MAML1 (right lane). Gli1 expression however is more pronounced in the presence of MAML1. **b.** Effect of increasing MAML1 or Gli1 doses on constant N3IC expression.

In the case of increased MAML1 coexpression we could detect a dose dependent reduction of N3IC protein expression (fig. 28b, lane 1 - 4) while increased amount of Gli1 did only show a modest and dose-independent effect (fig. 28b, lane 5 - 8). However Gli1 overexpression did have a dose dependent effect on N3IC expression when MAML1 was coexpressed in an equivalent amount to NotchICD protein (fig. 27b).

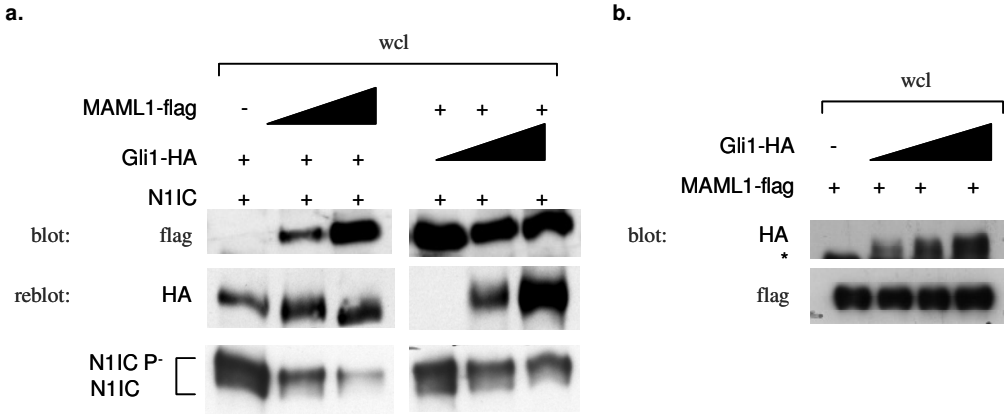

figure 29: N1IC protein expression is disturbed by MAML1: Whole cell lysate of Hek293T cells transfected with the same amount of Gli1 and N1IC expression plasmids, show significant impairment of N1IC protein expression when simultaneously overexpressed MAML1 in a dose-dependent manner (**a.** lane 1 - 3). **b.** Increasing Gli1 protein expression however did not alter constant MAML1 expression. Increasing amount of transfected Gli1 led to only minimal deceased N1IC expression when MAML1 was coexpressed in a constant and equal amount to N1IC (a. lane 4 - 6).

We decided to repeat the same set of experiments using the intracellular domain of Notch1 (N1IC) instead of N3IC to see whether a similar Notch paralogue would be affected by MAML1 and Gli1 in the same way or differ from data gained with Notch3.

By this we aimed to deduce protein structure related key functions important for a NICD paralogue specific turnover. The dose of MAML1 did strongly reduce N1IC expression in the presence (fig. 29a, lane 1 - 3) or absence (fig. 30b, lane 1 - 4) of Gli1. These data go hand in hand with published work on MAML1 induced Notch1ICD turnover.[286]

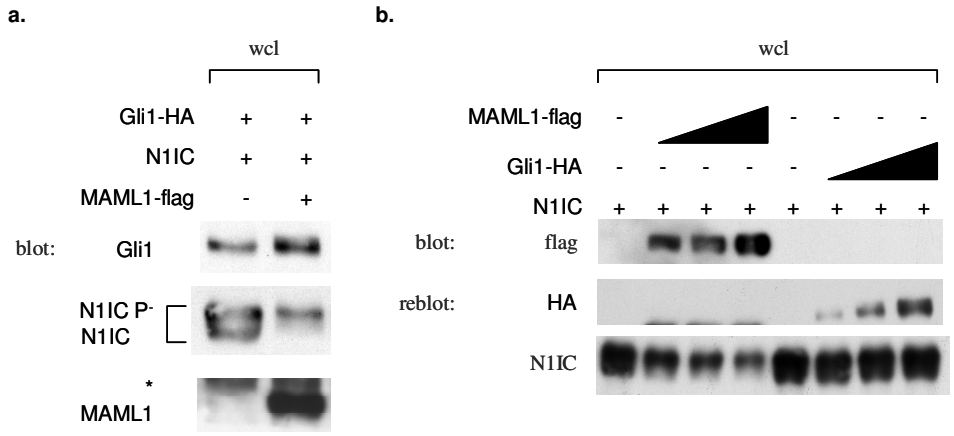

figure 30: N1IC protein expression is disturbed by MAML1: a. Whole cell lysate of Hek293T cells transfected with the same amount of Gli1 and N3IC expression plasmids, show specific loss of the lower N1IC protein band when simultaneously overexpressed MAML1 (right lane). Gli1 expression however is more pronounced in the presence of MAML1. **b.** Effect of increasing MAML1 or Gli1 doses on constant N1IC expression.

Loss of the lower band of N1IC (fig. 30a), visible when MAML1 was coexpressed and lysates were resolved by high resolution 6 % SDS polyacrylamid gel electrophoresis, could have been an indication for Notch1ICD phosphorylation, demonstrated to be important prior to ubiquitination and sequential degradation.[179,287] Interestingly Gli1 expression was increased by coexpression of MAML1 (fig. 28a,c, 29a and 30a). Preliminary experiments, in which the use of proteasomal inhibitors (MG132) during coexpression studies led to increased half-life of Gli are proposing MAML1 to actively induce Gli1 protein stabilization (unpublished). The underlying effect and involved posttranslational modifications including phosphorylation and acetylation through MAML1 recruitment, however remain to be deeper investigated. While the negative effect of MAML1 on N3IC protein expression could indicate a similar role in degradation initiation like demonstrated for Notch1ICD, the dose-dependent effect of Gli1 on N3IC was dependent on the presence of equal amount of MAML1. Assuming that both, MAML1 and Gli1 affect N3IC stability, we were wondering whether or not Gli1 would have a role in triggering ubiquitin mediated N3IC proteasomal degradation.

III.III.II Gli1 and MAML1 can alter ubiquitination of N3IC

For *in vitro* ubiquitination of N3IC we coexpressed HA-tagged ubiquitin together with N3IC and different combinations with or without Gli1 and MAML1. Presence of MAML1 without and with coexpression of Gli1 did again decrease N3IC expression levels in whole cell lysates (fig. 31 compare wcl in lanes 2, 3 and 4). Precipitating N3IC with a specific Notch antibody (or IgG as negative control in the IP) and hybridization of the western blot nitrocellulose membrane with anti-HA antibodies revealed a N3IC multi-ubiquitination pattern with at least two specific bands at 120 kDa and 160 kDa (fig. 31).

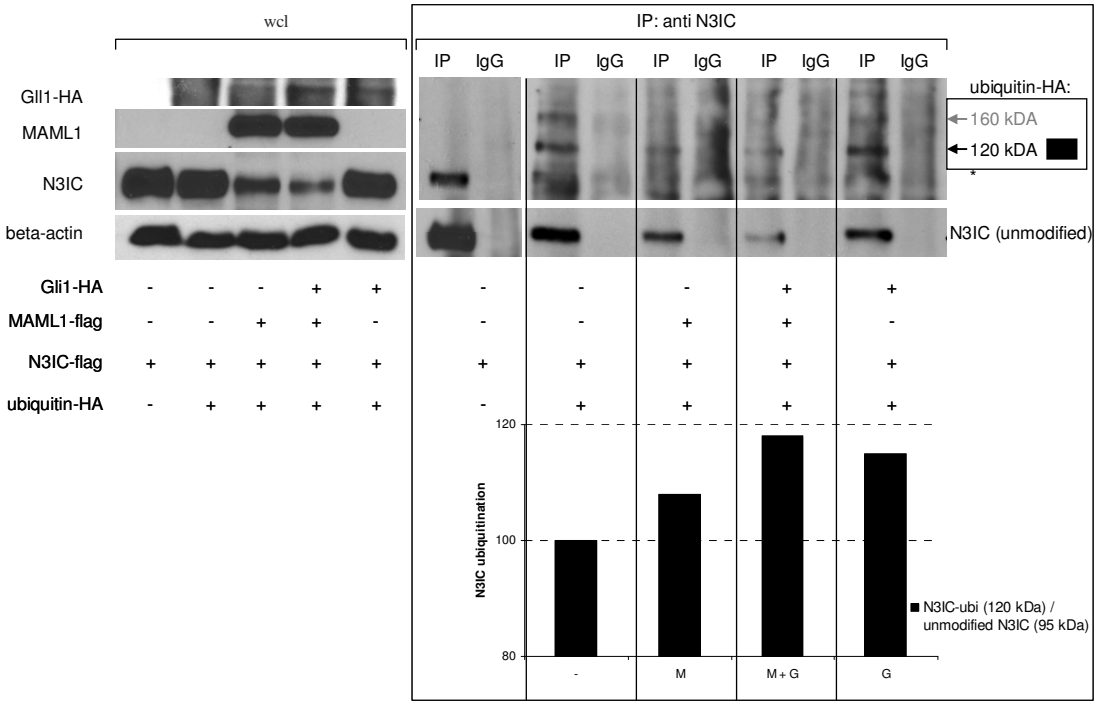

figure 31: Effect of MAML1 and Gli1 on ubiquitination of N3IC: Hek293T cells were transfected (24h) with equal amounts of indicated protein expression plasmids and whole cell lysate were subjected to western blotting of total proteins (left side) or immunoprecipitation against N3IC in order to detect ubiquitination.

Densitometrical comparison was done after normalization of detected amounts of ubiquitin modified N3IC 120 kDa isoforms with unmodified Notch3IC (~90 kDa). Interestingly, coexpression of MAML1 (fig. 31, diagram, columns “M”) was increasing the relative amount of ubiquitinated N3IC. Further addition of Gli1 (columns “M+G”) did reinforce this effect. Gli1 coexpression without MAML1 (diagram, columns “G”) did also increase the relative amount of the ubiquitin-modified 120 kDa N3IC isoform while N3IC protein expression in whole cell lysates (wcl) was unchanged (fig. 31, wcl lane 5).

IV Discussion

IV.I Cross talk

IV.I.I Point of convergence

Our general hypothesis is a cooperative role for Notch and Hedgehog signalling in monoclonal amplification, highly controlled by essential extracellular developmental cues. In this picture, stem or progenitor-like cells as well as developmental processes of the haematopoietic system require Hedgehog pathway activity for cellular proliferation and cell cycle control[288-290] as well as Notch signalling, in order to inhibit differentiation and sustain monoclonal amplification[291-293]. Hence the extracellular tissue and the presence, absence or the exact amount of Notch and Hedgehog ligands are shaping the microenvironmental niche and affect single cell fate decisions. As a monoclonal amplification needs to be under strict control of the organic compartment in which the cell resides, the specific niche must provide, in addition to ligands, other selective mechanisms to counteract the expanding cell pool that under deregulated conditions would most probably lead to tumour outgrowth. We decided to study the complex behaviour of Notch and Hedgehog signalling in T-cells where the combination of both pathways, at the DN3 stage of developing thymocytes, gives rise to monoclonal amplification in order to provide a sufficient pool of T-cells that can be subsequently subjected to beta-selection induced survival through preTCR signalling as well as MHC-driven positive and negative selection.

To proof a direct cross talk of active Notch and Hedgehog signalling, we decided to start measuring transactivation strength on Notch or Gli target genes by the use of luciferase expression plasmids under the control of specific promoter elements or consensus binding sites. 48 hours post transfection of transcription factors and luciferase responders into cultured T-cells, luciferase expression was deduced from luminometric signal strength in the various combinations of transcription and cofactors. N3IC, RBPjκ and MAML1 cotransfection resulted in maximal signal strength of Notch sensitive luciferase responders. Interestingly, additional of the Gli1 expression plasmid downmodulated transactivation of N3IC on its target pTα (as well as Hes1 and Hes5, unpublished). In parallel, Gli1 induced transactivation of a luciferase expression vector, driven by Gli consensus binding sites, was compared with double transfected cells where in addition to Gli1 also the intracellular domain of Notch3 had been overexpressed. N3IC led to a significant signal reduction in Gli1 induced target gene transactivation.

The presented experiments were indicating that distal components of both pathways, the Gli1 transcription factor and the cleaved intracellular domain of Notch3 might be responsible for interpathway cross talks, affecting and downmodulating opposite pathway's target gene transactivation strength.

Investigating the developing embryonic digestive tract, another model for studying the role of parallel Notch and Hedgehog signalling in developing cells, Shivdasani et al. demonstrated that Notch overexpression could, by the loss of mesenchymes and impaired organogenesis, phenocopy Hh-deficient embryos. Double-null embryos for Shh and Ihh were leading to increased Notch signalling. On the contrary, fetal gut mesenchymes in culture could be rescued from cell death, caused by Notch overactivity, through extracellular supply of Shh-ligands activating the Hedgehog signalling pathway. The authors assumed that Hedgehog signalling was restraining Notch pathway activity in order to give rise to proper organogenesis of the developing embryonic intestine.[259] This antagonistic pathway behaviour, proved *in vivo* in Notch and Hedgehog dependent developing cells of the intestine, was not further investigated on the molecular level but may sustain our *in vitro* data from developing Notch and Hedgehog depending thymocytes, pointing towards a more general interpathway cross talk. The observation that increased Hes1 and Hes5 expression in the digestive tract of embryos lacking Hedgehog activity was not accompanied with increased expression of Notch receptors or ligands would suggest that Hedgehog signalling is probably modulating the Notch pathway's activity distal to the step of receptor activation.[259] This is in line with the Gli1-N3IC transactivation antagonism in our experiments, as we were overexpressing the intracellular constitutively active domain of Notch3 that doesn't require any extracellular activation or cleavage steps.
Kim et al. demonstrated an antagonistic effect on the Hedgehog pathway by the use of Notch1ICD overexpression in cultured cells, while quantitative PCR, western blotting as well as in-situ hybridization indicated Notch3 to play a major role in their research on stomach endoderm and mesenchyme *in vivo*. Nevertheless Notch3ICD efficacy was not tested in this experimental setting, the localization of a possible cross talk between Notch and Hedgehog distal to the NotchICD release and upstream of Notch target gene activation could indicate the direct involvement of the intracellular domain or components of the Notch activator complex.[259] To this regard our data further sustain the possible convergence point of Notch and Hedgehog, responsible for parallel pathway's antagonism on target gene transactivation, to be intracellular, short before or at the level of target gene transactivation.

IV.I.II Deregulated coordination of Notch and Hedgehog signalling

Hedgehog is known to regulate stem and progenitor cell proliferation and cell cycle progression[288-290] while canonical Notch/CSL signalling can control symmetric versus asymmetric division[294,295] and is believed to have a fundamental role in preventing premature differentiation of progenitor cells[296-298]. In order to investigate a regulative network between Notch and Hedgehog signalling in neurogenic differentiation of neocortical progenitors, the group of Wainwright decided to inactivate both pathways. Conditional knockout of Patched did activate the Hedgehog pathway in progenitor cells and increased the amount of the radial glial progenitor cell compartment by sustained symmetric division. This dramatic expansion of stem and progenitor cells in the neocortex, could be reversed when simultaneously inactivating RBPj and attenuating Notch signalling. The expression of stem cell marker Sox2 in the ventricular zone of the neocortex was lost when RBPj was inactivated. Because the balance of symmetric versus asymmetric dividing progenitors was restored when both RBPj and Patched were lost, one could draw conclusion that excessive Hedgehog signalling is affecting Notch in controlling cell division symmetry.[299] This is in line with our model of cooperating pathway activities for monoclonal division, in which like in stem cells the combination of both pathways and their respective target genes lead to undifferentiated cell pool amplification.

While the authors show Hes1 to be upregulated in the case of hyperactive Hedgehog signalling and hypothesize that Hedgehog would cooperate with the Notch pathway through the Notch-RBPj signalling cascade[299], others have shown that Hedgehog and Gli2 signalling can bind and activate Hes1 in a Notch independent way, providing a plausible second explanation for Hes1 upregulation, independent of canonical Notch signalling[300]. Hes5 was not upregulated in the described experiment while in a different work by Hallahan et al., where transgenic Smoothened expression was used to activate Hedgehog signalling, resulting in medulloblastoma outgrowth, Hes5 and Notch2 were upregulated.[299,301] In the year 2004 Wainwright's group stated that "Notch2 and the Notch target gene, HES5, were also significantly elevated in Smoothened-induced tumors showing that Shh pathway activation is sufficient to induce Notch pathway signaling"[301]. In independent work Notch2, beside the presence of Notch1 and Notch3, was shown to be a general prevalent active Notch member in medulloblastoma[302] which could also explain the upregulation of typical Notch target genes.

In 2008 the group of Wainwright published data of a deeper analysis of the paradigm of a Notch and Hedgehog regulatory system in medulloblastoma, proofing that in fact at least the *canonical* Notch pathway could be excluded for Hedgehog-Smoothened induced upregulation of Hes1. “While Smoothened function was found necessary for upregulation of Hes1 in response to Sonic Hedgehog, the mechanism does not require gamma-secretase mediated cleavage of Notch receptors, and appears to involve transcription factors other than RBP-Jkappa.”[303] They describe their findings as “a novel mechanism for Hes1 regulation in stem-like cells that is independent of canonical Notch signalling.”[303] Very interesting, apart from the Hes1 regulatory mechanisms, was the fact that whether Notch cleavage by gamma-secretases nor RBPj was needed for Hedgehog induced medulloblastoma initiation. These data could be confirmed by the same group two years later demonstrating evidences that “Hedgehog-dependent medulloblastoma were not blocked by loss of RBP-J, indicating that canonical Notch signaling is not required for tumor initiation and growth in this model.”[304]

So far the presence of Notch in medulloblastoma, a model of deregulated neurogenesis, is beyond question but its role in tumour initiation and progression and its interaction with the Hedgehog signalling pathway is far from being fully understood. The upper described experiments may point to a non-canonical role of the intracellular domain of one or more Notch paralogues, downstream to extracellular Notch activation and upstream the canonical RBPjκ signal transduction.

Ajeawung et al. report in the October’s edition of 2012 in *Clinical and Investigative Medicine* that “thus far, two drugs which target the NOTCH and HEDGEHOG signalling have completed Phase I clinical trials [for medulloblastoma treatment], but with evidence of low efficacies”.[305] The work of Hatton et al. underlines the complexity of the Notch-Hedgehog paradigm in medulloblastoma: “In contrast to prior *in vitro* studies, pharmacologic inhibition of notch pathways did not reduce the efficacy of medulloblastoma xenotransplantation nor did systemic therapy impact tumor size, proliferation, or apoptosis in genetically engineered mouse medulloblastoma models. The incidence and pathology of medulloblastomas driven by the SmoA1 transgene was unchanged by the bi-allelic absence of Notch1, Notch2, or Hes5 genes.”[306] Hatton as well as Julian et al. were following the hypothesis that the observed upregulation of Notch signalling in medulloblastoma would in cooperation with Hedgehog-Gli deregulation cause tumour growth. A possible alternative we would like to propose is that the presence of Notch proteins may not be the cause but the result of Hedgehog induced medulloblastoma, in order to counteract the deregulated pathway’s activities. If

this would be truth, the use of gamma-secretase inhibitors (GSI) and even the loss of RBPj would probably not alter the pathology of Hedgehog induced medulloblastoma. If Notch upregulation is the result of an intrinsic molecular mechanism aimed to antagonize deregulated Hedgehog-Gli signalling, it would be interesting to analyse the opposite, activating Notch in order to antagonize hyperactive Gli1 signalling in medulloblastoma. On the one side it has been shown that Notch is upregulated in medulloblastoma, without playing a role in tumour initiation[307,308], while on the other side there are still evidences for overactivity of the Notch pathway in such developmental deregulated pathologies[309-313].

Growing numbers of identified Notch and Hedgehog/Gli coexpression patterns in various types of cancers may indicate a link between pathway coordination and tumour transformation. Notch deregulation can cause beside many other diseases gastrointestinal neuroendocrine carcinomas in which Gli1 was shown to be significantely upregulated and important for tumour growth.[314] We did find upregulated Gli1 protein expression in various human T-cell acute lymphoblastic leukaemia cell lines (unpublished) as well as in our murine 232T cell line that constitutively overexpress the active intracellular domain of Notch3. Although abnormal pathway activities could have been caused or selected during the process of cell line establishment, the *in vivo* data from mentioned pathologies argue for a close relationship in stem- or progenitor cells as well as cancer. As there is no proof for direct Hedgehog-Notch pathway transactivation most probably other cell intrinsic mechanisms, controlling Gli1 and NICD protein expression, longevity and turnover may be involved. The highly complex situation of cooperative Notch and Hedgehog signalling in developmental processes and cancer awaits further investigation. “Recent findings have shown that Notch signaling is dysregulated and contributes to the malignant potential of […] tumors. Growing evidence point towards an important role for cancer stem cells in the initiation and maintenance of glioma and medulloblastoma.”[315] In agreement with this a recent publication of Dave et al. concedes that “…the undoubted interaction observed at the developmental and stem cell level between Hedgehog and Notch signalling is reflected in part by an apparent RBPj dependent regulation by Hedgehog signalling of some, but not all, Notch effectors in the VZ.”[316] The involvement of RBPj in Notch Hedgehog cross talk still remains controversial and is probably depending on the cells specific developmental stage. The fact that loss of RBPj dependent Notch signalling is decreasing the pool of symmetric dividing stem-like progenitors of the neocortex[316], is in line with many other data, highlighting the importance of Notch in progenitor maintenance and inhibition of immature differentiation[291-293].

Because Hedgehog is known to regulate proliferation through cell cycle control, expansion of the radial glial cell compartment[316] could be caused by simultaneous activity of both pathways. Our observations indicate a role for RBPjκ in Patched1 expression in a cellular context were MAML1 is present but Notch is not cleaved. It needs to be confirmed that antagonism on transcription outcome induced by Notch activation also involves Patched promoter bound cofactors. MAML1 could stabilize RBPjκ binding to the upstream RBPjκ-Gli1 enhancer element of murine Patched1 promoter while exogenous supply of MAML1 and RBPj did lead to Gli1 binding to the enhancer element as well as increased transactivation strength in luciferase assays. Interpreting our *in vitro* data, knock-out experiments for RBPj might therefore not only have inhibited canonical Notch signalling but could have affected Hedgehog target gene activation through the lack of direct promoter binding effects like seen for Patched1, as well as indirectly deregulating the delicate balance of MAML1 in between cleaved Notch and activated Gli1.

IV.II Molecular concept of a Notch-Hedgehog antagonism

IV.II.I New roles for the coactivator MAML1

Mastermind-like 1 is known to be a transcriptional coactivator in the Notch pathway, however, several reports are disclosing novel roles for MAML1, independent of canonical Notch signalling. MAML1 was shown to coprecipitate together with the tumour suppressor p53 on chromatin of p53 targets, positively affecting transcriptional activity in a Notch independent manner.[317] Similar agonistic effect of MAML1 on Notch independent transactivation was demonstrated for the runt-related transcription factor 2 (Runx2), essential for osteoblastic differentiation as well as chondrocyte proliferation and maturation.[318] The ability of MAML1 to bind and recruit histone acetyl transferase p300 as well as cyclin dependent kinase CDK8 into transcription complexes, renders Mastermind-like1 a potent coactivator, regulating posttranslational modification events like acetylation and phosphorylation of juxtaposed binding partners.[111,114,115,188,319]
Data from various published work in independent biological contexts, as well as the research done in Screpanti's laboratory are merging together, highlighting an overall concept of coordinated and cross talking Notch and Hedgehog signalling. Patched deletion in embryonic mesenchymes of the developing intestine can mimic a Notch hyperactive phenotype[259], while Notch activation can antagonize Hh signalling by expression of Hes1 blocking Gli1[320]. Interestingly, one month ago, Kang et al. have published data from a MAML1 knockdown in human melanoma cells indicating a role for MAML1 in cellular senescence of cancer cells. Making the case that "targeting MAML1 [might] regulate the Notch, Wnt, and Hedgehog signalling pathways simultaneously", the authors transplanted lentiviral shMaml1 infected cells into syngeneic mice in order to demonstrate decreased tumour growth compared to control mice. They propose this approach as "a novel anticancer strategy by inhibiting cell proliferation and promoting differentiation and irreversible senescence". [321] Also Saint Just Ribeiro and Wallberg suggest that due to the "function of MAML1 as a coactivator for diverse activators, and MAML1 interaction with broadly used coactivators, […] MAML1 might be a key molecule that connects various signaling pathways to regulate cellular processes in normal cells and in human disease."[322]

To our knowledge we report here for the first time a direct coactivator role of MAML1 in Gli1-induced target gene transactivation. The described effect of MAML1 must differ from its role in canonical Notch signalling, as the used M31T and preT cell lines did not show evidence for active Notch signalling in western blot analysis of cleaved NICD and

qPCR of target gene transcripts. In fact does the agonistic role of MAML1 on Gli1 transactivation only come to full potential in the absence of active Notch. As soon as the intracellular domain of Notch3 was coexpressed, even to low doses, the effect of MAML1 in the Hedgehog pathway was antagonized, rendering the case of activated Notch signalling not to be independent but essential to be absent.

During the elaboration of this work, an independent research group discovered a similar cofactor role for MAML1 in Hedgehog signalling, strongly sustaining our results and highlighting the undoubted importance of limited amount of Mastermind in orchestrating developmental primed intracellular equilibrium between Notch and Hedgehog pathway activities. In a seminal experimental setting Kalderon and Vied declared Mastermind to be a crucial stem cell (SC) factor, specifically enhancing Hedgehog signalling in Drosophila ovary SCs. They identified Mastermind (Mam) as a dose-dependent modifier in a genetic screening for dominant suppressors of the Hedgehog-induced overproliferation of follicle stem cells (FSCs). Increased Hedgehog signalling, induced by Patched mutation, was leading to duplication and enhanced longevity of FSCs while loss of Mam entirely prevented the progressive, cell autonomous duplication. Reduced levels of Mam like in heterozygotes of Drosophila FSC $mam^{+/-}$ mutants failed to sustain Hh-induced FSC duplication, which highlights the importance of regulated Mastermind expression levels. In this cellular context Mastermind was shown to be essential for FSC maintenance and Hedgehog driven duplication while Notch-signalling was not required at that developmental time window, proved by Notch knockout mutants. Interestingly overactivated Notch signalling was impairing FSC maintenance that reflects the antagonistic behaviour in which Mastermind/MAML1 can serve as a coactivator in both pathways.[323] In fact the presence of overexpressed N3IC in 232T cells could have antagonized endogenous Gli1-MAML1 interaction, explaining poor yields in co-immunoprecipitation in our experiments (fig. 24b). We propose Mastermind to be responsible for intracellular balance between the Notch and the Hedgehog pathway, in order to orchestrate simultaneous activities in stem-like, non differentiating amplification.

IV.II.II MAML1 is quenched by the NotchICD-CSL interface

Final transcriptional outcome of the Notch-Hedgehog antagonism is most probably depending on many different factors, evolutionary balanced and timely and spatially coordinated during development. Pathway activation, nuclear translocation and quantitative availability of Gli1 or NICD proteins, as well as the expression level of MAML1 and the presence of additional cofactors and posttranslational modifications will decide about strength of complex stabilization and the respective binding affinity of Notch or Gli1 to Mastermind-like 1. Notch3ICD overexpression does not alter the expression of Gli1 protein (fig. 25b) but reduces its binding affinity to MAML1 (fig. 25a and 26). This quenching effect could explain decreased transcriptional activity of Gli1 like seen in our transactivation studies in a Notch3IC dose dependent manner (fig.14 and 15). While MAML1 cotransfection exceeds an agonistic effect on Gli1 induced transactivation we have demonstrated that supply of the constitutively active intracellular domain of Notch3 could counteract this hyperactivation.

The function of RBPj to bind DNA is essential to recruit NICD onto specific promoter sites in Notch dependent target genes. RBPj is non-covalently juxtaposing MAML1 to NICD in the inert ternary Notch activator complex.[189] Therefore also the availability of and binding affinity to RBPj are most probably playing a role in regulating a common multi-pathway usage of MAML1. Further investigation on this aspect of antagonistic and stochiometric molecular behaviour is needed. As the overexpression of Notch did counteract the essential cofactor role of MAML1 in Hedgehog induced stem cell maintenance, like observed by Kalderon and Vied[324], it would be interesting to know more about the molecular mechanism underlying this antagonistic effect. We have tried to extend our research by the use of immunofluorescence microscopy but failed to identify different subcellular localization of Gli1 when overexpressing MAML1 (compare S2 a and b). The nuclear import of Gli1 must depend on other mechanisms like phosphorylation events and interaction with Fused (Fu), the suppressor of Fused (SuFu) or additional cofactors like i.e. the protein Zic1.[325,326] The first two zinc fingers of Gli1 are suggested to act as a protein-protein interaction site.[257] Whether MAML1 is provoking posttranslational modifications on Gli1, for example by the recruitment of p300 and CDK8, or might be involved in reinforcing chromatin binding of Gli1 to the specific zinc-finger binding sites, has still to be shown.

IV.II.III Gli1 and MAML1 induced degradation of N3IC

In contrast to the antagonistic effect of N3IC on Gli1-MAML1 interaction, Gli1 overexpression does not significantly alter the with MAML1 coprecipitated amount of intracellular Notch3 protein. However, Gli1 does show similar antagonistic effects on N3IC target gene transactivation. Dose dependent decreased expression of N3IC with increased Gli1 in the presence of MAML1, detected in whole cell lysates, and finally increased N3IC ubiquitination were an indication that Gli1 might sustain MAML1-induced N3IC degradation. We could exclude that differences in protein expression would have resulted from altered transcription of protein expression plasmids, as their CMV2-promoters were constitutively active and RNA levels of N3IC did not change significantly after coexpression of MAML1 or Gli1 (data not shown). MAML1 was published to be important for turnover of the intracellular domain of Notch1. We gained similar results and dose dependent decreased protein levels of Notch3IC when increasing the amount of coexpressed MAML1 *in vitro*. In various studies on different gene expression mechanisms as well as on protein stability, including the intracellular domain of Notch, a phosphorylation-ubiquitination cascade was shown to initiate degradation. Genetic expression is believed to be terminated by proteasomal degradation of respective transcription factors, involving a series of important posttranslational modifications that culminate in protein disassembly. Kinases phosphorylate specific amino acid side chains and by doing so create recognition sites for further binding of ubiquitin ligases that attach ubiquitin chains to lysine residues. Transcriptional activator complex disassembly is initiated and ubiquitin modified factors are targeted for proteasomal degradation. Controlled turnover of transcription factors is necessary to render genetic transactivation sensitive to quantitative and time dependent signal transduction. MAML is absolutely essential for Notch induced transactivation[276] but many recent publications are indicating a step by step process in which Notch pathway activation leads to nuclear import of NICD, derepression of RBPj bound target gene promoters[327,328], ternary activator complex formation[110,329] and stabilization through recruitment of p300 and additional cofactors[330], as well as sequential phosphorylation[115,331], complex destabilization[332,333] and final NICD ubiquitination and degradation[96]. Before being targeted to the proteasome, the intracellular domain of Notch needs to be phosphorylated at specific serines or threonines. Multistep phosphorylation was shown to be initiated when MAML1 is recruited into the transcriptional activator complex of N1IC and RBPj. MAML1 recruits specific kinases like CK2, NLK or CDK8 in order to phosphorylate NICD, creating a phosphomotif important for target recognition and binding by E3 ubiquitin ligases.[115,331-

[333] Different classes of phosphorylating kinases and ubiquitin transferases with specific recognition sites have been identified, rendering a generalization of protein turnover initiating processes complex. Two E3 ligases have been proposed to be important for Notch receptor degradation: Deltex/Itch and the F-box and seven WD40 domain containing protein Fb(x)w7 (SEL-10, Cdc4 or Ago). Itch can interact with the membrane anchored form of Notch and posseses itself a C2 phospholipid binding domain, believed to be important in targeting respective protein to the plasma membrane. Fbw7 instead is found inside the nucleus and colocalizes with the intracellular domain of Notch.[182] Yet we don't know how Gli1 may affect phosphorylation and ubiquitination of the intracellular domain of Notch3 in detail. N3IC does not provide a binding site for Gli1 and we failed to see direct protein-protein interaction (data not shown). As the presence of MAML1 seems to be essential in this regulatory mechanism, Gli1 may reinforce MAML1 induced phosphorylation or ubiquitination processes on NICD by modifying directly the MAML1 protein or its interaction with recruited cofactors.

In order to start analysing a possible degradative relationship between Gli1 and N3IC, we were focusing on the E3 ubiquitin ligase Fbw7 that was shown to follow Cdk8 and GSK3β induced phosphorylation, colocalize to NICD inside nuclear spots, physically interact and trigger NICD ubiquitination and proteasomal degradation.[181,182,334-340] Fbw7 does specifically fine tune Notch signalling during T-cell development and functions as a tumour suppressor counteracting Notch induced leukaemia.[341-343] Taken together the published knowledge of Fbw7 induced degradation, yet only partially confirmed for Notch turnover, the overall picture can be summarized as followed: Starting at Notch induced transactivation, formation of the ternary NICD-RBPjκ-MAML complex is at the same time essential for target gene transactivation as well as the first step of programmed NICD turnover. MAML1 has been shown to recruit CDK8 into the complex where specific CDK8 induced phosphorylation is thought to initiate a second phosphorylation step on threonine or serine residues in close vicinity. Initial phosphorylation at a +4 position in the amino acid sequence of Fbw7 binding motifs of targeted proteins is followed by GSK3β induced phosphorylation at position 0. Comparison of amino acid sequences of experimentally proved targets of Fbw7 let us define a recognition motif that we used to screen for localization of conserved putative Fbw7 binding sites inside the intracellular domain of Notch paralogues. A part from the known Fbw7 degron in the very C-terminal PEST domain of all Notch family members, our motif search with the ExPASy server Prosite was evidencing a second putative binding site (fig. 32, blue marks) in mammalian NotchICDs 1-3.

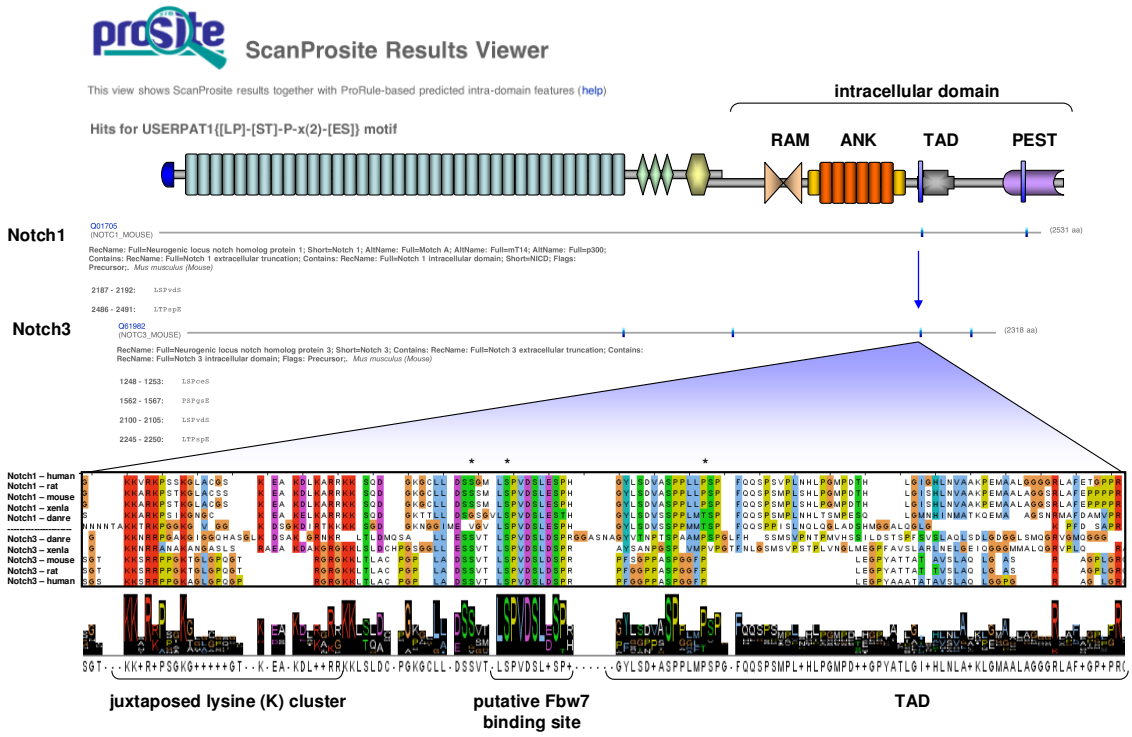

figure 32: NICD structure analysis: Using the ExPASy PROSITE server two conserved putative Fbw7 binding sites with the motif [LP]-[ST]-P-x(2)-[EST] were identified in the intracellular domains of Notch1 and Notch3. JalView order from top to bottom: Notch1 human, rat, mouse, zebrafish, frog; Notch3 frog, zebrafish, mouse, rat, human. The C-terminal motif is localized inside the PEST domain. A second upstream motif can be found infront of the TAD domain, juxtaposed to a specific partially conserved lysine cluster. N1IC stabilizing serine-arginine mutations[115] are indicated (*).

C-terminal to the ankyrin repeats (ANK), through which MAML1 interacts with NICD during the ternary activator complex, there is an about hundred amino acid long region with multiple regulative features that, together with the transactivation domain (TAD), initiate the most diverse part of proteins of the Notch family. The EP-domain, binding site for p300, has a positive role in Notch1 induced transactivation while it represses Notch3 activity.[344] The histone acetyl transferase protein p300 did lead to acetylation of specific lysine residues in Notch1, possibly antagonizing ubiquitination and increasing the overall Notch1ICD protein stability while our lab could demonstrate that Notch3ICD resulted more stable when deacetylated by the histone deacetylase HDAC1. Juxtaposed lysine residues are in part but not completely conserved between Notch1 and Notch3 which may provide the different family members with distinct residues for acetylation or ubiquitination. The serine/threonine rich region (STR) as well as the identified putative Fbw7 motif in front of the transactivation domain are most probably targeted by phosphorylation events. Specific serine to alanine point mutations near and inside the putative Fbw7 binding site (fig. 32, *) did result in decreased NICD degradation which could argue for a E3 ubiquitin ligase to bind in this region.[115] The

Nemo-like kinase (NLK) was shown to suppress “Notch signalling by interfering with formation of the Notch active transcriptional complex”[345]. Ishitani et al. describe the importance of phosphorylated serine-proline sites (SP) in the STR and TAD region, for Notch1ICD degradation. While analysing decreased transactivation strength of Notch1 in the presence of NLK, they show a significant increase of transcriptional activity for Notch3ICD but don’t go into further detail regarding the third paralogue of the Notch family.[345] MAML1 is together with Gli1 able to alter N3IC ubiquitination in a specific manner. While the intracellular domain of Notch1 was shown to be poly-ubiquitinated, our results of *in vitro* ubiquitination are proposing multi-ubiquitination for N3IC, sustaining results of similar investigations and the hypothesis of independent non-redundant regulatory mechanisms of the NICD.[121] Together with the decreased protein expression, the 120 kDa isoform of N3IC could indicate a specific ubiquitin-modification important for further proteasomal degradation. The identified putative Fbw7 binding site depicted in figure 32 is highly conserved through various species of the animal kingdom and its specific localization inside this important and at the same time non-redundant part of the intracellular domain of Notch may indicate the involvement of different regulatory mechanisms between the Notch family members, responsible for termination of transcriptional activity, Fbw7 binding and final protein degradation. Timely dependent upregulation of Notch3 and Gli1 during monoclonal amplification, could indicate a Notch3 specific cross talk through MAML1 and Gli1, possibly uncoupling other Notch family members for independent cellular regulatory processes.

IV.III Outlook - finding the right balance

Our hypothesis of a role for MAML1 in between the Notch and Hedgehog pathway during T-cell development, is in line with the observations of pathway antagonisms in different cell types at similar non-differentiating amplification events, short before developmental lineage decisions. We propose Mastermind to play a decisive role in putting Notch and Hedgehog pathway signalling on the scales in order to establish an intracellular equilibrium in which increased Notch or Hedgehog signalling would through co-usage of MAML1 lower the opposite's pathway activity. A major problem remains the choice of an adequate system to demonstrate MAML1 protein switching from Gli1 to NICD. Different cell lines with active or inactive pathways would involve also different cellular backgrounds that could have influence on protein expression levels or posttranslational modifications rendering comparative protein-protein interactions difficult. We tried to activate or inhibit *in vitro* Hedgehog signalling by specific drugs in order to detect altered Notch signalling. Unfortunately the effect of the Smoothened agonist *SAG* as well as the natural occurring chemical compound *cyclopamine* did show low efficacies in Hedgehog pathway activation or repression in our thymocyte and leukemia cell lines. T-cells do not have a primary cilium, the membrane structure which functions as a sensory organelle in many eukaryotic cells, and which is believed to be important for Smoothened, localization and activity after canonical activation of the Hedgehog signalling cascade[346-353]. It has been shown that intraflagellar transport (IFT) associated proteins, essential for ciliary assembly localize to the microtubule organizing centre (MTOC) and the Golgi and that they are important for T-cell receptor (TCR)/CD3 signalling. The lack of a primary cilium and its important membrane structure could have been in part substituted by the presence of the immune synapse in thymocytes.[354,355] However it still has to be shown whether or not Smoothened is localized to the immune synapse after derepression by Patched. Differences in cellular organizing structures and signalling processes in T-cell lines could explain low efficacies of pharmaceutical drugs raised against Smoothened activity of other cell lines. Interestingly the important role of Hedgehog signalling in immature thymocytes seems to disappear after preTCR signalling.[249] Simultaneous T-cell receptor constitution at the immune synapse and Smoothened downregulation at the DN3 stage of developing thymocytes is indicating an important switch in pathway reactivity and awaits to be linked to shared spatial features in the T-cell membrane.[356] Also interference of Smoothened by siRNA turned out to be difficult in our T-cell model. PreT or 232T cells, like many other thymocyte cell lines, demonstrated low transfection efficiencies, even by the use of electroporation methods. In addition, high levels of Gli1

in 232T or other leukemic cell lines do not necessarily result from canonical Hedgehog pathway activation. As we presume an intracellular balance between Notch and Hegdehog, the constitutively overexpressed N3IC in 232T cells could have caused intrinsic upregulation of Gli1 during cell line establishment, in order to antagonize the hyperactive Notch pathway. So far, main research in this field was done *in vivo*, using genetic knock-in or knock-out methods for the sake of a general physiological understanding of the roles of NOTCH and HEDGEHOG. Our results and proposed molecular mechanisms can hopefully not only complement *in vivo* data but also highlight developing thymocytes to be a good model for studying the underlying molecular crosstalk, due to simultaneous requirement of both pathways at a defined developmental stage. Our further investigations will focus on quantitative analysis of MAML1 expression that, following our model, defines the threshold in converging pathway's activities. The Mastermind (-like) protein is expressed early in development but a transcriptional activator has not yet been identified.[109,357] As both signalling cascades rely on extracellular cues in order to activate intracellular mechanisms and transcriptional events, research has started and will focus more and more on in depth analysis of complex regulatory mechanisms in various cell types and tissues, highly defined in spatial and temporal coordinates. The presence of multiple Notch receptors and ligands, the Gli paralogues as well as different members of the Mastermind-like families in mammals increase complexity and will have to be included in follow up experiments on cross talking pathways.

V Summary

As representatives of highly conserved key signalling pathways, the NOTCH and HEDGEHOG genes are involved in regulation of proliferation and cell cycle progression as well as differentiation and apoptosis in a multitude of organs and tissues. Both molecular mechanisms rely on extracellular ligand-receptor interaction, provoking further intracellular messenger processing and signal transduction, and culminate in transcription factor induced target gene transactivation. Their essential and specific roles in temporal and spatial defined developmental progression become evident when inter- and intracellular communication is deregulated. Genetic and epigenetic caused signalling defects have been identified in various types of cancers. In order to unravel coordination of molecular mechanism it is essential to understand the underlying signalling processes in detail. The Hedgehog pathway is getting activated by ligand binding to the transmembrane receptor Patched that derepresses Smoothened. Sequential induction of phosphorylation dependent processing and activation of Gli transcription factors enables their nuclear translocation and activation of specific Hedgehog pathway related target genes. Canonical Notch signalling is in contrast activated by Jagged or Delta-like ligands that provoke shedding of the Notch intracellular domain (NICD), its translocation into the nucleus and transcription activator complex formation together with essential cofactors like the DNA binding protein RBPj and Mastermind-like (MAML). The ability to recruit potent kinases and histone acetyl transferases or deacetylases into transcriptional complexes does not only assign Mastermind-like 1 an essential role in canonical Notch signalling but also implements MAML1 as a potent cofactor in Notch independent developmental processes, including amongst others signalling of the tumour suppressor p53.

Interestingly, evidences from independent research have emerged, pointing towards a direct regulatory relationship between the two signalling pathways, so far mostly considered to work parallel and independent from each other. Simultaneous protein expression patterns and transcription factor activation of both the Notch and the Hedgehog pathway have been described in embryonic stem cells as well as in lineage specifying processes like i.a. in the developing brain or during maturation of the haematopeietic system. In fact deregulated Hedgehog signalling in the cerebellum can cause medulloblastoma and Notch activity has been shown to be affected through yet unidentified mechanisms. On the contrary, preliminary data of our laboratory are indicating the Hedgehog pathway to be deregulated in Notch-induced T-cell leukaemia. The Hedgehog-Gli signal transduction cascade is essential for intrathymic T-cell

development until the checkpoint of preTCR signalling while Notch3 is playing an important role in initiation of preTCR signalling at the $CD4^-$ and $CD8^-$ double negative DN3 stage of developing thymocytes. Overlapping expression patterns of Gli1 and Notch3 proteins, as well as their rising importance in proliferation and maintenance of undifferentiated stem or progenitor cells led us focus especially on cross regulation of respective transcription factors. We were able to demonstrate a Notch independent cofactor role for MAML1 in Gli1 induced transcriptional activity when overexpressed *in vitro* in two different T-cell lines. However, addition of the intracellular domain of Notch3 (N3IC) did abolish the coactivator effect of MAML1 on Gli1 induced transactivation in a dose dependent manner. Also Notch transactivation, together with the essential cofactors RBPJ and MAML1 was antagonized by Gli1 expression, indicating a co-usage of the MAML1 protein. Its differential nuclear expression in M31T and preT cells as well as dose dependent antagonism seen in transactivation of Notch and Hedgehog target genes may argue for stoichiometric competition of both transcription factors N3IC and Gli1 for MAML1 and the need for increased expression levels at the DN3-like stage of developing thymocytes, where under normal conditions both pathways are active and essential for preTCR signal induction. Chromatin immunoprecipitation may further sustain a role of MAML1 in Gli1 induced transactivation as overexpression of MAML1 in preT cells led to Gli1 protein binding to an enhancer element of the endogenous target gene Patched1. We can draw conclusion that complex stability and availability of the pivotal MAML1 protein can regulate interpathway gene expression *in vitro* and may have influence on the developmental outcome *in vivo*. Microenvironmental primed pathway activation and the sequential co-usage of MAML1, would define a model of intrinsic equilibrium between Notch and Hedgehog with decisive importance on cellular response in terms of differentiation, proliferation and apoptosis. Our finding should be especially considered for Notch and Hedgehog dependent developmental processes and could be important for upcoming cancer treatments that combine pharmaceutical drugs against the Notch and the Hedgehog pathway.

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VI Appendix

VI.I Supplementary data

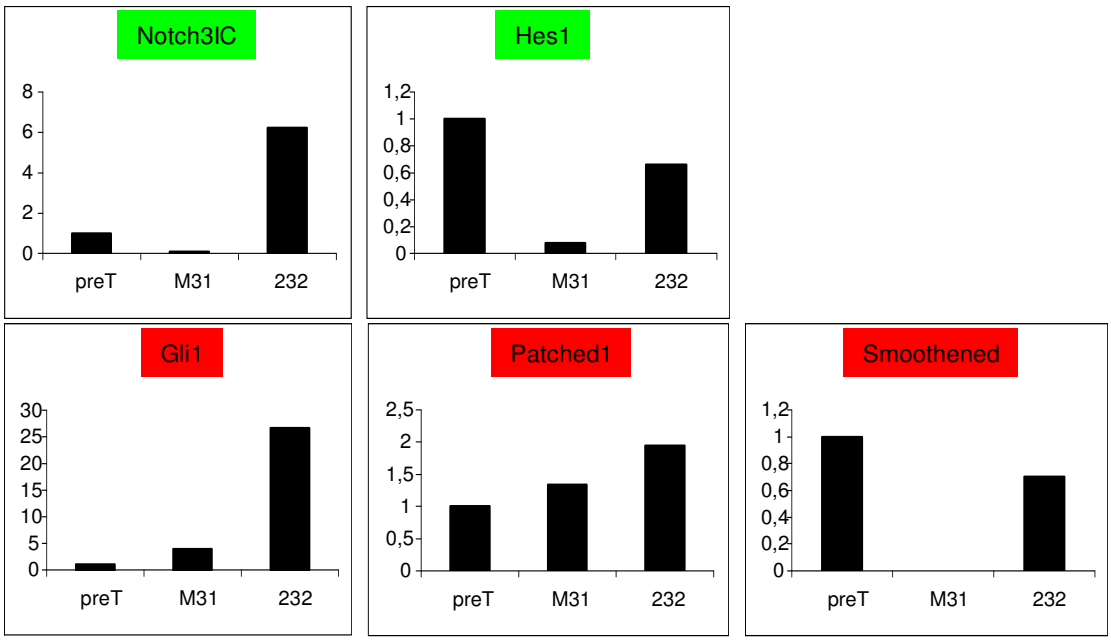


figure S1: RNA expression in T-cell lines: RNA extractions of M31T, preT and 232T cells were subjected to RT-PCR and sequential comparative qPCR in order to determine Notch and Hedgehog pathway activity inside the respective T-cell lines.

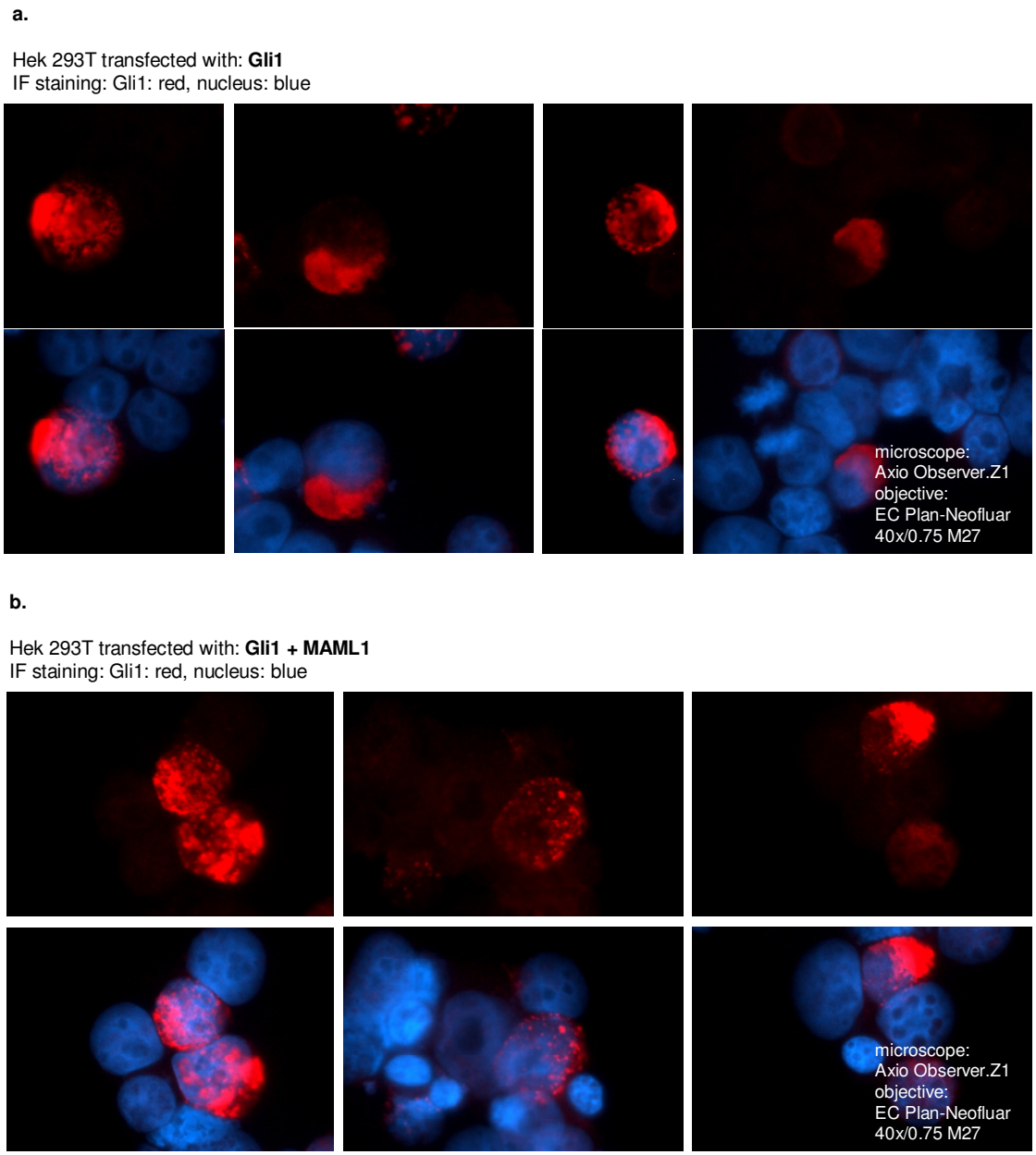
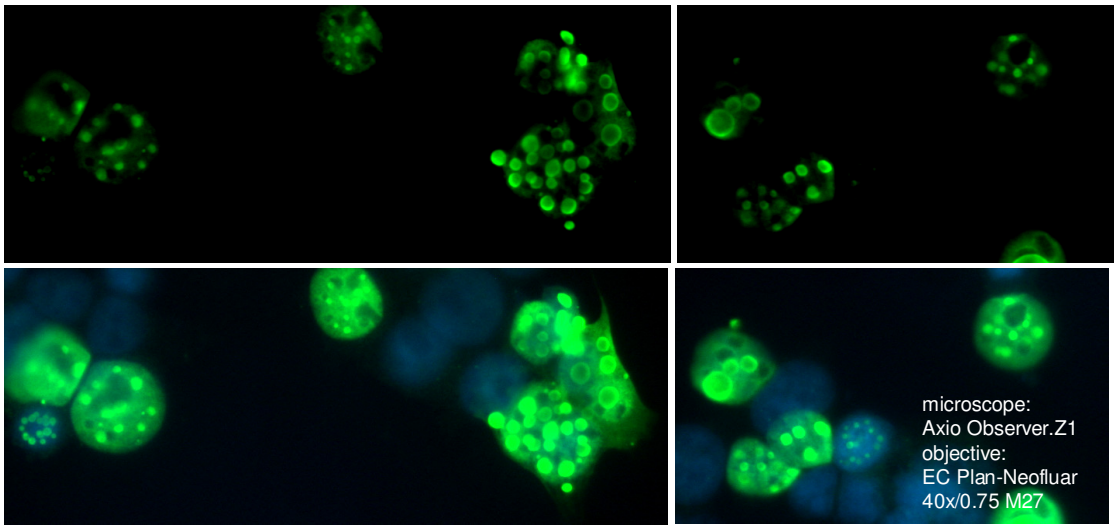


figure S2: Immunofluorescence microscopy: Gli1-proteins (red) overexpressed in Hek293T cells without (**a.**) or with MAML1 cotransfected (**b.**). In order to analyse the agonistic effect of MAML1 coexpression on Gli1 induced transcriptional activity, immunofluorescence microscopy was used to detect altered intracellular localization of Gli1. After coexpression of MAML1, Gli1 remained mostly perinuclear.

a.

Hek 293T transfected with: **MAML1**
IF staining: MAML1: green, nucleus: blue



b.

Hek 293T transfected with: **MAML1 + Gli1**
IF staining: MAML1: green, nucleus: blue

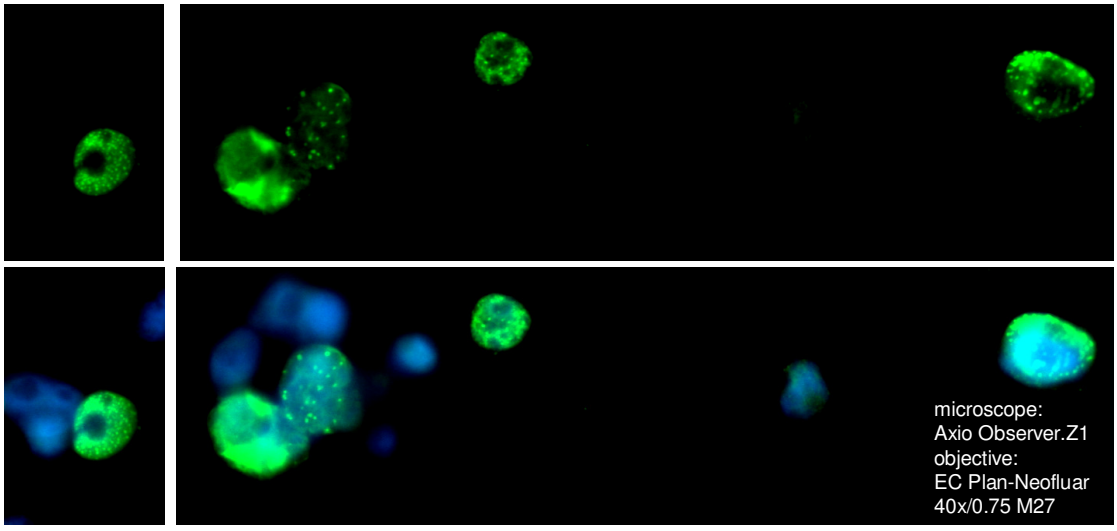


figure S3: Immunofluorescence microscopy: MAML1-proteins (green) overexpressed in Hek293T cells without (**a.**) or with Gli1 cotransfected (**b.**). As localization of MAML1 to nuclear spots is believed to be essential for its cofactor role, implying transcriptional activity and turnover of the intracellular domain of Notch, immunofluorescence microscopy was used to detect possible altered localization of MAML1 in the presence of Gli1. After coexpression of Gli1, speckled MAML1 localization appears less pronounced but remains nuclear.

VI.II List of abbreviations

ANK	Ankyrin repeats
Bp	Base pair
CBF-1	C-promoter binding factor-1
CD4	T-helper cells (cluster of differentiation 4)
CD8	T-killer cells (cluster of differentiation 8)
ChIP	Chromatin immunoprecipitation
CLP	Common lymphoid progenitor
CSCs	Cancer stem cells
CSL	CBF-1, Su(H), Lag-1
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
DN	Double negative T-cells ($CD4^-$ and $CD8^-$)
DP	Double positive T-cells ($CD4^+$ and $CD8^+$)
EGF-like	Epidermal growth factor like
ETP	Early T-cell progenitor
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FSCs	Follical stem cells
Fig.	Figure
Gli12x	Luciferase responsive element driven by 12 Gli consensus binding sites
GSI	Gamma secretase imhibitor
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
Hek	Human embryonic kidney cells
Hh	Hedgehog
HSC	Haematopoietic stem cell
IF	Immunofluorescence
IgG	Immunoglobulin G
IP	(Co-) immunoprecipitation
JAK	Janus kinase
LMPP	Lymphoid primed multipotent progenitor
Luc	luciferase
MAML1	Mastermind-like 1 protein
MAPK	Mitogen-activated protein kinase
MPP	Multipotent progenitor
MUT	Mutated

NICD	Notch intracellular domain
NLS	Nulcear localization sequence
PEST	Proline (P), Glutamic acid (E), Serine (S) and Threonine (T) rich region
PKA	Protein kinase A
PTα	pre T-cell receptor alpha
Ptch	Patched1
RAG	Recombination activation gene
RAM	RBP associated molecule
RAS	‘Rat sarcoma’ small GTPase protein superfamily
RBPJ(κ)	Recombining binding protein suppressor of hairless
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SCs	Stem cells
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Smo	Smoothened
SP	Single positive T-cells ($CD4^+$ or $CD8^+$)
Su(H)	Suppressor of Hairless
TAD	Transactivation domain
TGF	Transforming growth factor
TSP	Thymus-settling progenitor
STAT	Signal transducer and activator of transcription
WCL	Whole cell lysate
WNT	Gene / Signalling pathway
WT	Wild type

VI.III Literature

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Dipl. Ing. (FH)

Dieter Matthias Lauer

Josef-Rahier-Str. 12
52428 Jülich, Germany

E-mail: dieter.m.lauer@gmail.com

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Personal Information

Date of birth	October 22, 1983
Nationality	German
Gender	male

Education

Since 2009 — **Ph.D. student in Molecular Medicine**
Sapienza University of Rome, Italy

Ph.D. thesis: Analysis and evaluation of the coactivator protein Mastermind-like 1 and its pivotal role as a putative convergence molecule in inter-pathway signalling

2003 – 2009 — **Dipl. Ing. (FH) in Bioengineering**
University of Applied Sciences Aachen, Germany
4.5-year degree course (Oct. 2003 – Jan. 2009 including one year of biochemical studies abroad plus a studies-integrated internship for half a year)

Diploma thesis: Synthesis and Validation of modified L-lysine polycations as efficient non-viral gene delivery in somatic gene therapy.

Work Experience

Since June 2009 **Dept. of Molecular Medicine, University of Rome – La Sapienza**, Italy

Early Stage Researcher (ESR) in NotchIT project, *Marie Curie* scholarship during the FP7 of the European Commission, **www.notchit.eu**

in vitro and *ex vivo* experimentation (T-ALL mouse model and leukemia cell lines), lymphocyte purification, cloning, cell culture drug treatments, siRNA, luciferase assay, RT/q-PCR, WB, IP, ChIP, cloning DNA/RNA and protein extraction, IF-microscopy, bioinformatics

May 2008 – Oct 2008 **Faculty of Chemistry and Pharmacy, Ludwig Maximilians University**; Munich, Germany

Synthesis of PEGylated, pH- sensitive and membrane reactive poly-L-lysine constructs, HPLC purification, *in vitro* testing and validation of transfection efficacy vs. cell viability using luciferase-, MTT, EtBr excision, erythrocyte leakage assays and physicochemical particle analysis

Oct. 2007 – Apr. 2008 **Merck KGaA**; Darmstadt, Germany
Internship, research in proteomics and peptide modifications of membrane proteins for 2D gel electrophoresis, HPLC purification, WB

Jan. 2004 – Dec. 2005 **Agroisolab GmbH**; Jülich, Germany
Student assistant, analytics based on measurement of stable isotope

2003 – 2008 further practical instructions in the biochemical laboratories of the

- **University of Applied Sciences – Aachen**, Germany
  advanced courses in immobilized biocatalysts, genetics, molecular and applied enzyme technology
- **University Miguel Hernández – Elche**, Spain
  DNA sequencing, cloning, immunology
- **Research Centre Juelich,** Germany
  directed mutagenesis, electroporation, genetics

Research Skills and Qualifications

Extensive knowledge in Notch signalling and developmental biology as well as skilled training in advanced biochemical and biotechnological methods:

Jun. 28 – 30, 2010	**theoretical and practical training** in Notch signaling in developmental biology and in heart development; tissue regeneration in chicken and zebrafish; CNIC Madrid, Spain
Oct. 11 – 15, 2010	**theoretical and practical training** in signaling cascades cross talking to Notch in development and pathology; high throughput screening and structural biology, X-ray crystallography; Karolinska Institute (KI); Stockholm, Sweden
Oct. 19 – 20, 2010	**Illumina Sequencing Workflow,** 2-day theoretical course including library-preparation, cluster generation, sequencing and data analysis; Sapienza University of Rome, Italy
Oct. 24 – 26, 2010	**Notch Signaling and Haematopoiesis;** University of Edinburgh, Scotland
Nov. 15 – 19, 2010	**2 day course on ES cell differentiation;** **2 day course on Gene delivery;** Karolinska Institute (KI); Stockholm, Sweden
Nov. 21 – 23, 2010	**Notch IT workshop: Animal experimentation** – technical and ethical aspects; Sapienza University of Rome, Italy
Feb. 03 – 04, 2011	**Notch IT workshop on Basic Stem Cell Technology;** **7th annual SSCN meeting;** École polytechnique fédérale de Lausanne (EPFL), Switzerland
Feb 22 – 23, 2011	**Genomatix workshop:** Introduction of the Genomatix Mining Station (GMS) and training with the Genomatix Genome Analyzer (GGA) for Chip- and RNA-Sequencing; Sapienza University of Rome, Italy

Apr. 27 – 30, 2011	**Notch IT workshop: Computational Biology;** École polytechnique fédérale de Lausanne (EPFL), Switzerland
Sep. 19 – 20, 2012	**Workshop: Notch in vascular development and pathology;** Faculty of Medicine, Paris, France
Nov. 29 – Dec. 1, 2012	**Workshop: Proteomics of Notch signalling,** mass spectroscopy; Sapienza Universität - Rom, Italien

Presentations at International Meetings

- Lauer, Dieter (2010, Italy).	talk: *Notch signaling crosstalks in thymocyte development*
- Lauer, Dieter (2011, Switzerland)	poster on the SSCN conference: *Notch signaling and interpathway cross talking in thymocyte development,*
- Lauer, Dieter (2011, Italy)	talk: *Notch and Hedgehog*
- Lauer, Dieter (2011, Greece)	poster on the 5th Notch meeting: *Notch signaling*

Hobbies and Interests

Languages	German (mother tongue), fluent in English and Italian, advanced Spanish and basic French knowledge
Computing	MS Office, bioinformatics, multimedia and graphic design
Sports	swimming, climbing, running

Feb. 2013, Rome Dieter M. Lauer