Biochemical non-equivalence of the DSL proteins DLL1 and DLL3

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Abstract

The evolutionary conserved Notch signaling pathway mediates direct communication between adjacent cells and plays a pivotal role in somite formation and patterning during embryogenesis. The Notch ligands *Dll1* and *Dll3* are both essential for somitogenesis in mammals. However, despite their largely overlapping expression domains in the presomitic mesoderm of mouse embryos, *Dll1* and *Dll3* null mutant mice display strikingly different somite defects. Additionally, the DLL1 and DLL3 proteins differ with respect to various domains suggesting that both proteins are biochemically not equivalent and exert non-redundant functions during somitogenesis.

In this study, it was demonstrated that DLL3 does not induce Notch signaling in transactivation assays. Providing a 'trivial' explanation, the DLL3 protein does not localize to the cell surface but accumulates inside the cell. Subcellular localization studies in the presomitic mesoderm of mouse embryos revealed that endogenous DLL3 predominantly localizes to the Golgi apparatus whereas endogenous DLL1 is expressed at the cell membrane. *In vitro* analyses of cell surface presentation and subcellular localization of DLL1-DLL3 chimeric ligands demonstrated that the transmembrane domain and juxtamembrane sequences of DLL3 harbor recognition sequences that are responsible for Golgi retention of the protein. Furthermore, the DSL domain of DLL1 appears to be necessary in order to direct cell surface presentation. In combination with EGF-like repeats 1 and 2 and the transmembrane and intracellular domain, the DSL domain of DLL1 seems sufficient to activate Notch signaling as determined by transactivation assays. In addition, two conserved amino acid motifs in the DSL domain of DLL1 that are not present in the divergent DSL domain of DLL3, were shown to be necessary for efficient cell surface presentation and for DLL1 function.

The analysis of presomitic mesoderm of *Dll3* mutant pudgy embryos showed that the loss of Dll3 has only a low impact on Notch activation suggesting that DLL3 does not exert antagonistic but rather modulatory influence on Notch signaling.

As part of this study the *Dll3* coding sequence was inserted into the *Dll1* locus by targeted recombination, thus exchanging the endogenous expression of *Dll1* for that of *Dll3*. This presented a pivotal prerequisite for the analysis of the functional non-equivalence of *Dll1* and *Dll3 in vivo*.

Zusammenfassung

Der konservierte Notch-Signalweg vermittelt die Kommunikation zwischen benachbarten Zellen und spielt eine Schlüsselrolle in der Somiten- und Musterbildung während der Embryonalentwicklung. Die Notch-Liganden *Dll1* und *Dll3* sind beide unentbehrlich für eine normale Somitenbildung in Säugetieren. Trotz der weitgehend überlappenden Expressionsmuster im präsomitischen Mesoderm von Mausembryonen, zeigen Mäuse mit Nullallelen von *Dll1* und *Dll3* unterschiedliche Somitendefekte. Zusätzlich legen Unterschiede in der Proteinstruktur von DLL1 und DLL3 die Vermutung nahe, dass beide Faktoren biochemisch nicht äquivalent sind und unterschiedliche Funktionen während der Somitenbildung übernehmen.
In dieser Studie wurde gezeigt, dass DLL3 kein echter Notch-Ligand ist, da es keine Notch-Aktivierung auslöst. Dies ist darauf zurückzuführen, dass DLL3 nicht auf der Zelloberfläche präsent ist, sondern intrazellulär akkumuliert. Die Analyse der subzellulären Lokalisierung von DLL3 im präsomitischen Mesoderm von Mausembryonen zeigte, dass endogenes DLL3 im Gegensatz zu DLL1 überwiegend im Golgi-Netzwerk und nicht auf der Zelloberfläche lokalisiert ist. Die Untersuchung von Dll1-Dll3-chimären Liganden im Hinblick auf Zelloberflächenpräsentation und subzelluläre Lokalisierung der Proteine zeigte, dass die Transmembrandomäne von DLL3 zusammen mit benachbarten Regionen Signalsequenzen aufweist, die für das Zurückhalten des Proteins im Golgi-Apparat verantwortlich sind. Für eine effiziente Oberflächenlokalisierung der chimären Liganden war die DSL-Domäne von DLL1 zwingend erforderlich. Für das Transaktivierungspotential der chimären Liganden ist die Präsenz des N-Terminus einschließlich der DSL-Domäne und der ersten beiden EGF-ähnlichen Domänen zusammen mit der Transmembran- und intrazellulären Domäne von DLL1 ausreichend. Weiterhin wurde gezeigt, dass zwei konservierte Aminosäuremotive in der DSL-Domäne von DLL1, die in der DSL-Domäne von DLL3 fehlen, unerlässlich für die korrekte Lokalisierung und Aktivatorfunktion von DLL1 sind. Die Analyse von präsomitischem Mesoderm aus Dll3-mutanten Mausembryonen zeigte, dass der Verlust von DLL3 kaum Auswirkung auf das Ausmaß der Notch-Aktivierung hat. Diese Beobachtung legt nahe, dass die Funktion von Dll3 einen eher modulatorischen als antagonistischen Einfluß auf die Notch-Aktivierung während der Somitogenese ausübt. Als weiterer Teil dieser Studie wurde die kodierende Sequenz von *Dll3* in den *Dll1* Locus der Maus eingebracht, um die endogene Expression von *Dll1* durch *Dll3* zu ersetzen und so die Voraussetzung für die Analyse einer möglichen funktionellen Redundanz von *Dll1* und *Dll3 in vivo* zu schaffen.

Key words

Dll1, Dll3, Notch signaling

Schlagworte

Dll1, Dll3, Notch Signalweg

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Abbreviations

%	percent
α	anti
A	adenine (DNA) or alanine (protein)
aa	amino acid
Fig.	figure
ATP	adenosintriphosphate
AP	antero-posterior
BMP	bone morphogenetic protein
bp	base pairs
BSA	bovine serum albumin
°C	degree Celsius
C	cytosine
cDNA	complementary DNA
Ci	Curie
cm	centimeter
CMV	cytomegalovirus
cpm	counts per minute
dCTP	desoxycytosintriphosphate
dd	double destilled
DMEM	Dulbeccos modified eagle medium
DNA	desoxyribonucleic acid
dNTP	desoxynucleotidtriphosphate
DMSO	dimethyl sulfoxide
dpc	days post coitum
ECD	extracellular domain
EDTA	ethylendiamintetraacetate
EGF	epidermal growth factor
ES cells	embryonic stem cells
F	phenylalanine
FCS	fetal calf serum
FGF	fibroblast growth factor
g	gram oder gravity
G	guanine (DNA) or glycine (protein)
GFP	green fluorescent protein
h	hour
HA	hemagglutinin
ICD	intracellular domain
IRES	internal ribosomal entry site
JAK/STAT	Janus kinase/signaltransducer and activator of transcription
kb	kilo bases
l	liter
LacZ	beta-galactoside
LB	Luria Broth
m	milli
M	molar
mA	milli Ampere

MAGUK ..... membrane-associated guanylate kinase
mM ..... milli molar
µ ..... micro
µM ..... micro molar
min ..... minute
mRNA ..... messenger ribonucleic acid
ORF ..... open reading frame
PAGE ..... polyacrylamide gel electrophoresis
PBS ..... phosphate-buffered saline
PCR ..... polymerase chain reaction
PFA ..... paraformaldehyde
PGK ..... phosphoglycerine kinase
pH ..... power of the hydrogen
POD ..... peroxidase
PSM ..... presomitic mesoderm
PVDF ..... polyvinylidene difluoride
RNA ..... ribonucleic acid
rpm ..... rotations per minute
RT ..... room temperature
SDS ..... sodiumdodecylsulfate
sec ..... seconds
T ..... thymine
TAE ..... Tris acetate EDTA
TGF-beta ..... transforming growth factor beta
TM ..... transmembrane
Tris ..... Tris(hydroxymethyl)aminomethane
U ..... units
Univ. ..... university
UTR ..... untranslated region
o/n ..... overnight
V ..... valine or Volt
W ..... tryptophane
w/o ..... without
wt ..... wildtype
Y ..... tyrosine

1 Introduction

A limited set of signaling pathways is active during embryogenesis and crucial for the development of the embryo into a healthy organism. The major signaling pathways acting during vertebrate development are the Wnt, JAK/STAT, Hedgehog, receptor tyrosine kinase (e.g. FGF signaling), TGF-β (e.g. BMP signaling) and the Notch signaling pathway. These pathways are interconnected and together control the gene regulatory program required for proper embryonic development by inhibitory and/or activating crosstalk (Axelrod *et al.*, 1996; Shaye and Greenwald, 2002; Wahl *et al.*, 2007; Yoo *et al.*, 2004).

1.1 Somitogenesis in mice

The developing mouse embryo is a well established model to analyze the molecular genetics and function of signaling cascades involved in the regulation of growth and patterning. One example is the tightly regulated process of somite formation (somitogenesis) that is important for organizing the segmental pattern of the body during early embryonic development (Gossler and Tam, 2002).

During gastrulation the unsegmented paraxial mesoderm emerges as a mesodermal subpopulation from the primitive streak and locates bilaterally to the midline of the embryo. In this so-called presomitic mesoderm (PSM) morphologically distinct spherical units of mesenchymal cells (somitomeres) become compacted, epithelialize and eventually bud off the rostral end of the paraxial mesoderm to form a somite (Fig. 1.1). Throughout somite formation continuous proliferation of a pool of progenitor cells in the primitive streak and later in the tail bud ensures the supply of cells in the presomitic mesoderm.

Somites are transient metameric structures. Shortly after their formation the epithelial somites differentiate into sclerotome and dermomyotome by undergoing localized epithelial-mesenchymal transition. Later the sclerotome gives rise to the vertebrae, the intervertebral discs and the ribs. The dermomyotome forms the dermis of the dorsal skin, the skeletal muscle of the back, the body wall and the limbs.

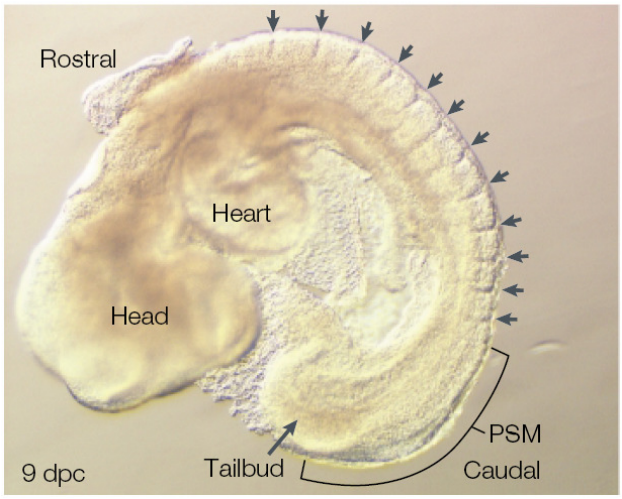

Fig. 1.1: Somitogenesis in the mouse embryo (Saga and Takeda, 2001). Epithelial somites bud off sequentially from the rostral end of the presomitic mesoderm (PSM), while more PSM cells are supplied from the paraxial mesoderm in the caudal region of the tailbud. Arrows show previously formed somite segment borders.

The formation of somites occurs at regular intervals in a coordinated manner at both sides of the neural tube. The periodicity is mediated by a 'segmentation clock' that intrinsically oscillates within the PSM in a rostro-caudal fashion. Through cyclic gene expression in the posterior presomitic mesoderm the formation of somites is spatially and temporally controlled (Aulehla and Herrmann, 2004; Pourquie, 2003). The period of wavelike gene expression correlates with the creation of a somitic boundary within 120 min in mice. For instance, dynamic expression of Lunatic fringe (Lfng), a modulator of Notch signaling, and its confined localization to the presumptive somite border region mediates the formation of morphological boundaries (Aulehla and Johnson, 1999; Sato *et al.*, 2002). Notch signaling plays an important role in the clock mechanism. However, somites still form when Notch signaling is impaired or abolished, suggesting that additional factors must be involved (Oka *et al.*, 1995). The origin of the autonomous oscillation of gene expression is still a subject of controversial discussions and several models were proposed to explain the mechanisms underlying somitogenesis (Baker *et al.*, 2006). The number of somites and the cycling period of somite formation are specific for each organism, and have been extensively studied in zebrafish, mouse and chicken embryos (Rida *et al.*, 2004; Tam, 1981).

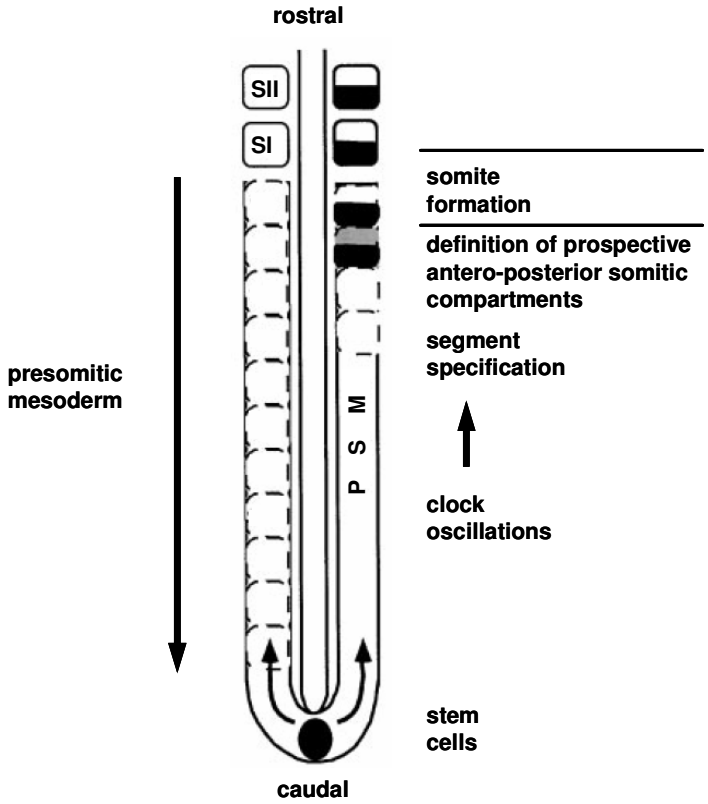

Fig. 1.2: Major events taking place in presomitic mesoderm (Pourquie and Kusumi, 2001). Proliferation of stem cells in the tail bud provides the supply of mesodermal cells in the presomitic mesoderm (PSM). Oscillations of the 'segmentation clock' lead to segment specification. In the anterior (rostral) PSM prospective antero-posterior somite polarity is defined prior to somite formation. SI and SII indicate already formed somites.

The presomitic mesoderm gains positional identity along the anterior-posterior (AP) body axis specified by the expression of particular Hox genes (Cordes *et al.*, 2004; Kessel and Gruss, 1991; Krumlauf, 1994; Nowicki and Burke, 2000). This positional specification of future mesoderm derivatives is coupled to Notch signaling and the segmentation clock (Cordes *et al.*, 2004). Similarly, the somitomeres themselves establish an antero-posterior (AP) polarity leading to a subdivision into an anterior and a posterior compartment of the somite that show differential cell properties and expression of marker genes (Fig. 1.2; Keynes and Stern, 1984; Keynes and Stern, 1988). Compartmentalization of the somites was demonstrated by rotating newly formed somites along their AP axis in transplantation experiments which results in the development of vertebrae with the corresponding inverse polarity (Aoyama and Asamoto, 1988). The antero-posterior pre-patterning originates on the basis of a molecular patterning in the anterior end of the unsegmented PSM. Several factors intrinsic to the somitic mesoderm of the vertebrate embryo are known to control AP polarity within somites prior to formation of epithelial somites. By interaction of the Notch signaling pathway with the basic helix–loop–helix transcription factor Mesp2 (mesoderm posterior 2), somite compartmentalization is established (Saga *et al.*, 1997). Complex feedback loops of Mesp2 function eventually lead to differential expression of the Notch ligand Dll1 in the somite compartments and to polarized expression of the transcription factors Tbx18 (anterior)

and Uncx4.1 (posterior) (Haenig and Kispert, 2004; Mansouri *et al.*, 1997; Takahashi *et al.*, 2000; Takahashi *et al.*, 2003). Concerted action of Tbx18 and Uncx4.1 is required to maintain the separation of anterior and posterior somite compartments presumably by preventing migration of anterior or posterior cells into the neighboring compartment (Bussen *et al.*, 2004). A link between segment polarity and somite morphogenesis is provided by expression of Eph/ephrin signaling components in polarized stripes in the PSM mediating cell contact repulsion and differential permissiveness of the somite compartments to migrating neural crest cell, motor neurons and intersegmental blood vessels (Adams *et al.*, 1999; Krull, 2001).
While somitogenesis itself proceeds without the requirement for continuous interactions with surrounding tissues, the further differentiation of the somites relies on inductive or inhibitory paracrine signals from proximal tissues such as the surface ectoderm, the neural tube and the notochord (Kieny *et al.*, 1972). The dorsal part of the somite retains its epithelial organization and becomes precursor tissue of the dermis and the myotome (Ikeya and Takada, 1998; Marcelle *et al.*, 1997; Pourquie *et al.*, 1996). The ventro-medial part of the somite differentiates into sclerotome which is subdivided into a cranial (anterior) and a caudal (posterior) half (Ebner, 1888; Fan and Tessier-Lavigne, 1994; Johnson *et al.*, 1994). In a resegmentation process, the caudal sclerotome of one somite fuses with cells from the cranial part of the adjacent somite to form one vertebra. While the vertebral body is composed of both somite halves, the development of proximal ribs and pedicles with transverse processes solely arises from the posterior somite compartment expressing Uncx4.1 (Leitges *et al.*, 2000). Cells of the center of the somite give rise to the intervertebral disc separating the vertebral bodies (Bagnall *et al.*, 1988).
An involvement of the Notch pathway in somitogenesis was first indicated by somite morphology defects observed in mice bearing targeted mutations in either the receptor Notch1 (Conlon *et al.*, 1995; Swiatek *et al.*, 1994) or the major intracellular effector, RBPJκ (Oka *et al.*, 1995). Notch signaling plays a fundamental role in the establishment of the antero-posterior somite polarity and in the maintenance of somite borders during somitogenesis. Thus, mutations affecting Notch signaling components give rise to aberrant vertebral formation in mice and humans.

1.2 The Notch signaling pathway

The phylogenetically highly conserved Notch signaling pathway is an intercellular signaling system that has been described in a variety of metazoan organisms (Greenwald, 1998;

Gridley, 2003; Sherwood and McClay, 1997; Shi and Stanley, 2006; Weinmaster, 1997). It serves as a central regulator of fundamental developmental processes such as lateral inhibition, lineage decision and boundary formation, as well as in adult tissue homeostasis and regeneration (Conboy *et al.*, 2003; Conboy and Rando, 2002; Ehebauer *et al.*, 2006; Kohler *et al.*, 2004; Nakamura and Chiba, 2007; Wilson and Radtke, 2006).

Unlike most other paracrine cell signaling pathways Notch signaling represents juxtacrine signaling with receptors and ligands both being transmembrane proteins mediating communication of adjacent cells. A direct cell-cell contact allowing the binding of the receptor to its ligand is required for *trans*-signaling events (Fehon *et al.*, 1990). Upon ligand binding, signal transduction is initiated and Notch receptors undergo complex proteolytic processes that eventually lead to the release of the intracellular domain of the receptor (Brou *et al.*, 2000; Mumm *et al.*, 2000; Schroeter *et al.*, 1998). The intracellular transduction of Notch signals is remarkably simple and involves no secondary messengers. The liberated intracellular domain of Notch directly translocates to the nucleus to activate transcription of a wide array of downstream target genes (Kopan *et al.*, 1996; Struhl and Adachi, 1998).

During embryonic development Notch signaling plays a pivotal role in cell fate specifications (Artavanis-Tsakonas *et al.*, 1999). The best understood effect of Notch signaling is the diversification of cell fates within a group of equivalent cells. A single cell expresses high levels of Notch ligand thereby activating Notch in the surrounding cells and inhibiting them from adopting the same fate. This Notch-mediated mechanism (termed *lateral inhibition*) governs, for instance, early neurogenesis in vertebrates and sensory hair cell formation in the vertebrate inner ear (Cabrera, 1990; Chitnis, 1995; de la Pompa *et al.*, 1997; Muskavitch, 1994). In other processes such as wing margin boundary formation in flies and somite segmentation in vertebrates, Notch-mediated *lateral induction* generates embryonic fields, domains of cells with the same fate (Lewis, 1998; Panin *et al.*, 1997). Negative and positive feedback loops triggering down- and upregulation of Notch ligand expression in the signal-receiving cells lead to these contrasting effects during organogenesis.

1.3 Biochemistry of the canonical Notch signaling pathway

Notch receptors (in mammals Notch1-4) are initially synthesized as ~300 kDa precursor proteins. Prior to their presentation on the cell surface, they are processed in the secretory pathway. The immature single-pass transmembrane precursor protein is subject to a first step of proteolytic processing by a furin-like convertase in the trans-Golgi network (Blaumueller *et*

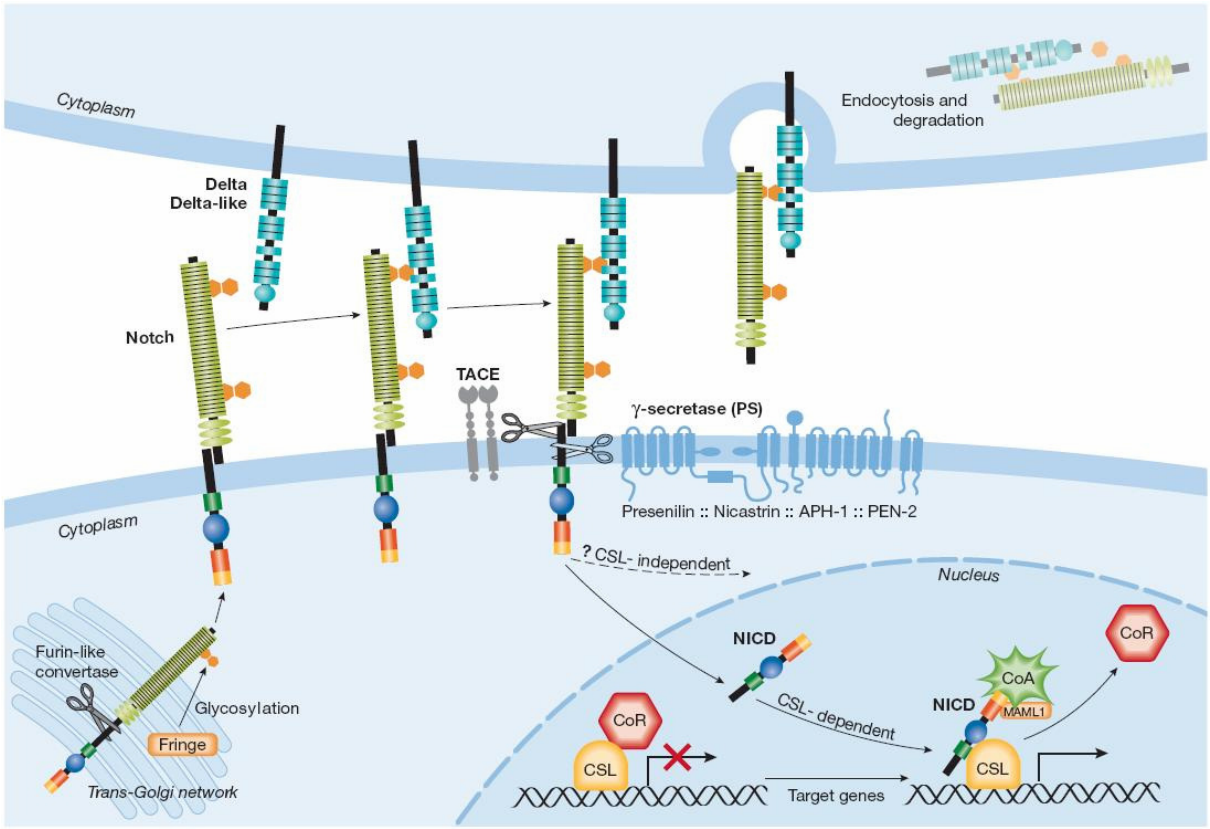

Fig. 1.3: Notch signaling (Radtke *et al.*, 2005). During their transport to the cell surface Notch receptors are cleaved by a Furin-like convertase and modified by Fringe glycolsyltransferases. Upon ligand interaction with the heterodimeric Notch receptor, signal transduction is initiated involving two sequential proteolytic cleavages. The first - within the Notch extracellular domain - is mediated by the metalloprotease TACE. The endocytosis of the extracellular subunit of the receptor by the neighbouring ligand-expressing cell facilitates this event. The second cleavage occurs within the Notch transmembrane domain and is mediated by the γ-secretase activity of a multi-protein complex of presenilins (PS). The released intracellular domain of Notch (NICD) translocates to the nucleus and binds to the transcription factor CSL. This interaction leads to transcriptional activation by displacement of corepressors (CoR) and simultaneous recruitment of coactivators (CoA), including mastermind-like proteins (MAML1).

al., 1997; Logeat *et al.*, 1998). This first cleavage results in two fragments: a large extracellular segment (NECD) comprising a series of tandemly arranged EGF-like repeats and a smaller subunit that consists of a short ectodomain, the transmembrane domain and an intracellular domain (Fig. 1.3). The two subunits form a mature heterodimer by calcium-dependent, non-covalent interactions within their extracellular regions preventing constitutive receptor activation in the absence of the ligand (Malecki *et al.*, 2006).

Multiple ligands, collectively known as DSL (Delta/Serrate/Lag-2) proteins, are known to elicit a signal through binding to the Notch receptor (Fehon *et al.*, 1990; Henderson *et al.*,

1994; Thomas *et al.*, 1991; Vassin and Campos-Ortega, 1987). In mammals, five DSL proteins have been described that are classified in two distinct families: homologues of the *Drosophila* Delta protein (*Dll1* (Delta-like1), *Dll3* and *Dll4*) and homologues of the *Drosophila* Serrate protein (Jagged1 and Jagged2) (Bettenhausen *et al.*, 1995; Dunwoodie *et al.*, 1997; Lindsell *et al.*, 1995; Shawber *et al.*, 1996a; Shutter *et al.*, 2000).

In response to ligand binding, E3 ubiquitin-ligases trigger endocytosis of the ligand-NECD complex by the ligand-presenting cell resulting in a physical dissociation of the Notch heterodimer (Itoh *et al.*, 2003; Lamar *et al.*, 2001; Nichols *et al.*, 2007; Parks *et al.*, 2000; Pavlopoulos *et al.*, 2001). The removal of the extracellular subunit of Notch leads to a conformational change in the membrane-tethered Notch derivative NEXT (Notch extracellular truncation). In consequence, a second cleavage site is exposed within the extracellular juxtamembrane region. It is recognized by the metalloprotease TACE (Tumor necrosis factor-alpha converting enzyme), belonging to the group of ADAM (a desintegrin and metalloprotease) enzymes that catalyzes shedding of the ectodomain (Brou *et al.*, 2000; Mumm *et al.*, 2000).

Subsequently, a third cleavage termed regulated intramembrane proteolysis (RIP) occurs within the transmembrane domain due to the γ-secretase activity of a proteolytic multi-protein complex consisting of the core components presenilin, nicastrin, APH1 (anterior pharynx defective1) and PEN2 (Presenilin enhancer 2) (Brown *et al.*, 2000; Schroeter *et al.*, 1998; Wolfe, 2006). It was suggested that the ubiquitination of Notch and its targeting to an endocytic vesicle is a prerequisite for the γ-secretase cleavage of Notch (Gupta-Rossi *et al.*, 2004). Interestingly, the presenilin/γ-secretase complex also mediates the proteolytic cleavage of amyloid precursor protein (APP) that is involved in Alzheimer disease (Borchelt *et al.*, 1996; Duff *et al.*, 1996; Price *et al.*, 1998).

The intracellular domain of Notch (NICD) contains a nuclear localization sequence and – after release from the cell membrane - undergoes translocation into the nucleus where it forms a short-lived complex with the major Notch effector, the DNA-binding transcriptional repressor CSL (CBF1 in human, Suppressor of hairless in *D. melanogaster*, LAG-1 in C. elegans and RBPJκ in mice) (Jarriault *et al.*, 1995; Shawber *et al.*, 1996b). In the absence of NICD, CSL forms a multiprotein transcriptional repressor complex together with corepressors such as SMRT (silencing mediator of retinoic and thyroid receptors), N-CoR (nuclear receptor corepressor) and CIR (CBF1 interacting corepressor) (Lai, 2002).

Upon CSL-binding NICD acts as a transcriptional coactivator by displacing the CSL-dependent corepressor complex including the histone deacetylase HDAC-1 which converts

local chromatin into a transcriptionally silent form (Kao *et al.*, 1998). Additionally, NICD promotes the recruitment of transcriptional coactivators (Jeffries *et al.*, 2002) such as the p300 histone acetyltransferase (HAT) complex (Oswald *et al.*, 2001; Rand *et al.*, 2000) and the Mastermind-like protein/LAG-3/SEL-8 (Nam *et al.*, 2006; Wilson and Radtke, 2006), a scaffold protein for the formation of a large multiprotein transcriptional activation complex that facilitates the activation of lineage-specific programs of gene expression. Known direct target genes of Notch include members of the hairy/enhancer-of split (HES), HES-related (HERP) and Mesoderm posterior (MESP) families of basic helix-loop-helix transcription factors as well as Lfng (Cole *et al.*, 2002; Jarriault *et al.*, 1995; Jouve *et al.*, 2000; Kageyama *et al.*, 2007; Maier and Gessler, 2000; Morales *et al.*, 2002; Morimoto *et al.*, 2005; Takahashi *et al.*, 2000). Other targets include CyclinD1, Ephrin B2, Nodal, Myc and smooth muscle alpha actin (Klinakis *et al.*, 2006; Krebs *et al.*, 2003; McDaniell *et al.*, 2006; Noseda *et al.*, 2004; Ronchini and Capobianco, 2001).

In the majority of developmental settings, signals are induced via the conserved canonical Notch signaling pathway described above. However, although poorly understood, there is evidence for alternative actions, for instance DSL-ligand independent Notch signaling, CSL-independent signaling or Notch-independent CSL auto-activation (Barolo *et al.*, 2000; Matsuno *et al.*, 1997; Rusconi and Corbin, 1999; Shawber *et al.*, 1996b).

1.4 Modulation of Notch signaling

Although the core of the Notch pathway is remarkably simple employing only three components, a ligand, a receptor and a transcription factor, signal transduction is embedded in a complex network of modulatory processes (Bray, 2006). Moreover, tissue-specific combinations of Notch modulators may contribute to different modes of regulation. Therefore, the effects on Notch signaling always depend on the cellular context and the available protein network. Regulation takes place at several levels of the Notch pathway: on the level of availability and affinity of ligands and receptors previous to signal transduction and after initiation of the signal by endocytosis and removal of bound protein. A third level of modulation includes the stability of the Notch intracellular domain (ICD) and its enhancer complex and the expression of modulators that act downstream of the Notch signal.

1.4.1 Regulation of Notch ICD turnover and negative feedback loops of Notch targets

Notch-mediated transcription is an extremely dynamic process. Rapid proteolytic turnover of Notch ICD and a short half life of the enhancer complex ensure a high sensitivity of the Notch signaling pathway.

The recruitment of negative regulators by the scaffold protein MAML facilitates the hyperphosphorylation of Notch ICD eventually leading to its proteasomal degradation (Foltz *et al.*, 2002; Fryer *et al.*, 2002; Qiu *et al.*, 2000).

The cytoplasmic protein Deltex acts as a transcriptional regulator of Notch signaling by interacting with Notch ICD independent of CSL proteins (Matsuno *et al.*, 1998; Matsuno *et al.*, 2002; Yamamoto *et al.*, 2001). The modulatory effect of Deltex on Notch signaling depends on the cellular context (Izon *et al.*, 2002). It involves targeting Notch ICD to the late endosomes where it accumulates, although the exact mechanism of this process is still unclear (Hori *et al.*, 2004). Deltex proteins are not expressed in the PSM and the somites but are thought to be involved in cytodifferentiation of neuronal tissues and in cell proliferation events in the eye, in vascular structures and during hematopoiesis (Mitsiadis *et al.*, 2001)

Transcripts of Nrarp (Notch-regulated ankyrin repeat protein), a transcriptional target of Notch signaling, are detected in the paraxial mesoderm. Nrarp functions as a feedback regulator of Notch signaling that attenuates ICD-mediated transcription by direct interaction with Notch and the CSL protein CBF-1 (Krebs *et al.*, 2001; Lamar *et al.*, 2001; Yun and Bevan, 2003).

Similarly, the Notch target gene Mesp2, encoding a transcription factor, establishes a feedback loop by suppressing Notch activity through induction of the Lunatic fringe gene (Morimoto *et al.*, 2005). Mesp2 is involved in the specification of somite polarity. It is expressed in the rostral presomitic mesoderm and becomes immediately down-regulated after the formation of the segmented somites (Saga *et al.*, 1997).

Other modulators of Notch (such as numb and numb-like) have important roles in somite maturation and in neural development (Holowacz *et al.*, 2006). These proteins exert their function by promoting Notch degradation and recruitment into endocytic vesicles (McGill and McGlade, 2003).

1.4.2 Processes modulating Notch receptors and ligands

Glycosylation of the extracellular domain of Notch modulates the sensitivity of Notch receptors for their ligands. In addition, fine-tuning of the signaling intensity of Notch can be

achieved by inhibitory associations of the Notch receptor with coexpressed ligands and effects on endocytosis rates. Moreover, the observed formation of homomultimers of Notch receptors and ligands might contribute to the modulation of Notch activity (Sakamoto *et al.*, 2005).

Glycosylation of Notch receptors and ligands

The affinity of Notch receptors for their ligands is regulated by the glycosyltransferases Pofut (protein o-fucosyltransferase) and Fringe that participate in the synthesis of O-fucose glycans attached to EGF repeats in the Notch receptor (Haines and Irvine, 2003; Haltiwanger and Lowe, 2004; Moloney *et al.*, 2000a).

O-fucosylation occurs in the endoplasmatic reticulum (ER) at specific serine or threonine residues in a consensus sequence of certain EGF repeats and is catalyzed by Pofut1 (Okajima *et al.*, 2003; Panin *et al.*, 2002). In the Golgi network, the elongation of O-fucose to a tetrasaccharide requires the β-1,3N-acetylglucosaminyl-transferase activity of proteins encoded by Fringe genes (in mammals: Lunatic fringe, Maniac fringe and Radical fringe). Glycosylation of EGF repeat 12 in the Notch receptor differentially alters Notch affinity for its ligands (Lei *et al.*, 2003; Rampal *et al.*, 2005; Shao *et al.*, 2003). For example, glycosylation by Lfng exerts a positive impact on Delta-Notch signaling whereas it negatively affects the association of the Notch ligand Serrate and Notch (Bruckner *et al.*, 2000; Fleming *et al.*, 1997; Hicks *et al.*, 2000; Klein and Arias, 1998; Moloney *et al.*, 2000b; Panin *et al.*, 1997). The weakened ligand-receptor interaction no longer effectively promotes Notch proteolysis which is required for activation of downstream signaling events (Yang *et al.*, 2005). Therefore Serrate-Notch signaling intensity is diminished by the action of Fringe.

Similar to the Notch receptor, consensus sites for O-fucosylation exist in the EGF-like repeats of Notch ligands. Studies in *Drosophila* have identified a hypomorphic allele of Delta (*sup5*) which exhibits a mutation in the O-fucosylation site in EGF3 (Lieber *et al.*, 1992). Additionally, a missense mutation resulting in the human disorder Alagille syndrome maps to a predicted O-fucose site in EGF-like repeat 5 of Jagged1 (Heritage *et al.*, 2000). These observations provide evidence that O-fucosylation is essential for normal ligand function. Although the exact functional significance of these modifications of Notch ligands is still not known, it was suggested that O-fucosylation might facilitate ligand multimerization (Panin *et al.*, 2002).

Loss of Pofut activity in mice leads to embryonic lethal phenotypes that resemble the complete absence of Notch signaling indicating that O-fucosylation is absolutely required for signaling through all Notch receptors (Shi and Stanley, 2003). In contrast to the essential role of Pofut in all contexts, Fringe activity has a rather modulatory effect on Notch signaling in

only a subset of Notch functions (Okajima *et al.*, 2003). Loss of Lunatic fringe (Lfng) activity causes a less severe phenotype compared to that of Pofut mutant mice. Lfng mutant mice show irregular shaped somites with disturbed antero-posterior polarity and truncation of the antero-posterior axis both in the trunk and tail (Zhang and Gridley, 1998).

Ubiquitination, endocytosis and multimerization of Notch ligands

Several reports highlight the importance of internalization of Notch ligands and receptors for the regulation of the Notch signal (reviewed in Le *et al.*, 2005). *Drosophila shibire* (dynamin) mutants that are endocytosis-deficient were used to demonstrate that dynamin-dependent endocytosis is required for efficient Notch signaling (Seugnet *et al.*, 1997). Internalization of the ligand-receptor complex leads to a dissociation of the Notch heterodimer and subsequent removal of the Notch extracellular domain (NECD) which is a prerequisite for Notch ectodomain shedding and Notch activation (Nichols *et al.*, 2007). Additionally, the endocytic process might be important for clearing bound ligands from the surface and re-sensitizing the cell for new Notch signals. It was suggested that ligand-NECD complexes dissociate in an endocytic vesicle and unbound Delta protein returns to the surface via recycling vesicles while NECD is retained internally and eventually destroyed (Chitnis, 2006). Wang and Struhl proposed that Notch ligands need to be targeted by mono-ubiquitination in order to enter specific endocytic recycling compartments as a prerequisite for their conversion into active ligands (Wang and Struhl, 2004; Wang and Struhl, 2005). Moreover, it was suggested that endocytosis and recycling may promote clustering of the Notch ligands in special microdomains at the cell surface thus enhancing Notch signaling (Chitnis, 2006).

1.5 Pathology of aberrant Notch signaling

In humans aberrant Notch signaling has been linked to numerous developmental abnormalities and pathologies. For instance, mutations in the Notch1 receptor can cause aortic valve disease (Garg *et al.*, 2005). A small subset of patients with Alagille syndrome (a congenital syndrome associated with liver, cardiovascular, and skeletal defects), normally associated with mutations of the Notch ligand Jagged1, shows alterations in the *Notch2* gene (Li *et al.*, 1997; McDaniell *et al.*, 2006; Oda *et al.*, 1997). The congenital vascular disorder CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leucoencephalopathy) is caused by missense mutations in the *Notch3* gene and associated with stroke and dementia (Joutel *et al.*, 1996; Joutel *et al.*, 1997). Patients suffering from

spondylocostal dysostosis display multiple vertebral segmentation defects and rib anomalies caused by mutations of the Notch ligand *Dll3*.

Notch signaling can affect tumorigenesis, e.g. by acting as an oncogene in T-cell acute lymphoblastic leukemia (T-ALL) which arises from a mutation in the *Notch1* gene that renders the receptor more susceptible to activation (Ellisen *et al.*, 1991; Malecki *et al.*, 2006; Radtke and Raj, 2003; Weng and Lau, 2005). Aberrant Notch4 signaling in mammary epithelial cells promotes the development of tumors in the mammary gland (Jhappan *et al.*, 1992; Politi *et al.*, 2004). On the other hand, Notch signaling is reduced in several cancers pointing to a potential function as a tumor suppressor dependent on the cellular context (Miele *et al.*, 2006; reviewed in Radtke and Raj, 2003). For instance, deletion of Notch1 in the epidermis results in the development of skin tumors (Nicolas *et al.*, 2003).

1.6 Components of the Notch signaling pathway

While receptors and ligands participating in Notch signaling were found as several homologues in mammals, signal transduction through all Notch receptors seems to use the same basic signaling pathway via one major downstream effector. In mice, this function is exerted by the highly conserved and ubiquitously expressed CSL transcription factor, RBPJκ. Mice carrying RBPJκ null alleles show severe phenotypes due to a complete loss of Notch signaling (Oka *et al.*, 1995).

1.6.1 Notch receptors

Ninety years ago, in 1917, Thomas Hunt Morgan described a mutant strain of the fruit fly *Drosophila melanogaster* that exhibited notches at the margins of the wing blades (Morgan, 1917). This trait was attributed to a partial loss of function (haploinsufficiency) of a gene from the neurogenic "notch" locus (Mohr, 1919) encoding a membrane-spanning receptor with EGF-like repeats that was cloned in the mid-1980's (Kidd *et al.*, 1986; Wharton *et al.*, 1985).

Four Notch genes (Notch1-4) have been identified in mammals. Among these homologues, Notch1 is probably the best studied member of the receptor family because of its involvement in a great variety of developmental processes during embryogenesis (reviewed in Bolos *et al.*, 2007; Chiba, 2006; Weinmaster and Kintner, 2003). The *Notch1* gene is expressed in derivatives of all three germ layers during early mouse development, including the primitive streak during gastrulation, the presomitic mesoderm during the process of somitogenesis,

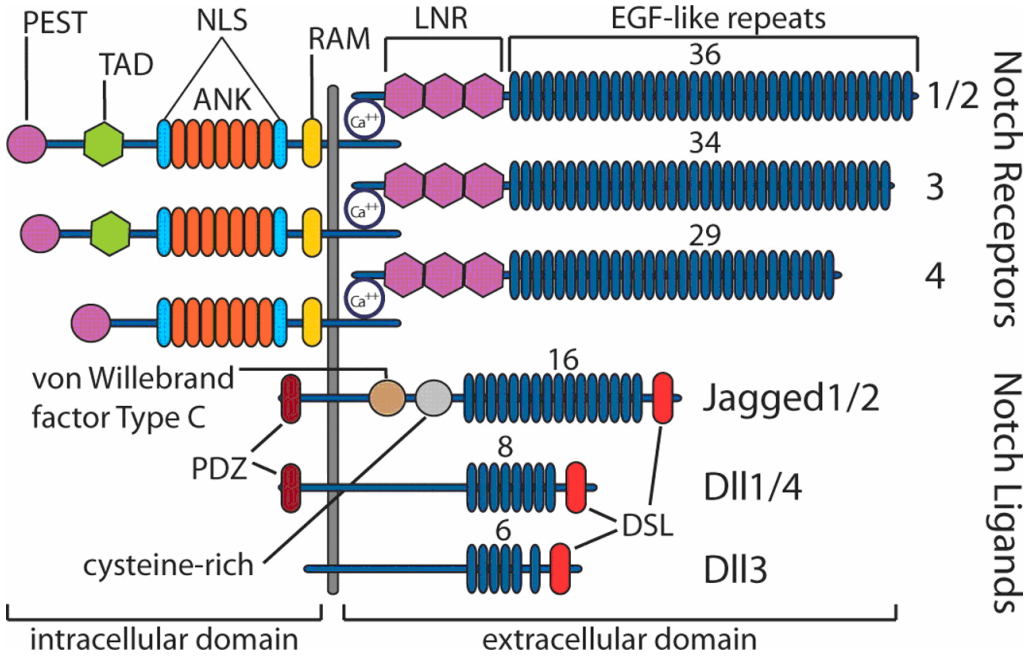

Fig. 1.4: Notch receptors and ligands (adapted from Niessen and Karsan, 2007). Mammals have four Notch receptors (Notch1–4) and five ligands [Jagged1/2, Delta-like (Dll)-1/3/4]. Notch receptors form heterodimers. In their extracellular domain they contain several epidermal growth factor (EGF)-like repeats, three Lin-12/Notch (LNR) repeats, and a heterodimerization domain that stabilizes Notch heterodimer formation through calcium-dependent interactions. The intracellular domain comprises an RBPJκ-associated molecule (RAM) domain, seven ankyrin (ANK) repeats, two nuclear localization signals (NLS), a transactivation domain (TAD), and a PEST domain. Notch ligands are also single-pass transmembrane proteins. The extracellular domains consist of a Delta/Serrate/Lag2 (DSL) domain unique to Notch ligands and multiple EGF-like repeats. Jagged proteins contain an additional cysteine-rich domain and a von Willebrand factor type C domain. The intracellular domains of Jagged1, Dll1 and Dll4 have been shown to contain PDZ binding motifs.

differentiating endothelial cells and the developing nervous system (Lieber *et al.*, 1992; Reaume *et al.*, 1992).

Notch1 plays a vital role in postimplantation development as Notch1 mutant mice die during embryogenesis around embryonic day 9.5 with vascular and somite defects (Conlon *et al.*, 1995; Huppert *et al.*, 2000; Krebs *et al.*, 2000; Swiatek *et al.*, 1994). Notch1 and Notch2 are both required for embryo viability. They are expressed in an overlapping pattern in the presomitic mesoderm and null alleles lead to severe defects in somite patterning emphasizing the importance of Notch signaling during somitogenesis (Krebs *et al.*, 2000; Shimizu *et al.*, 1999; Swiatek *et al.*, 1994). Ablation of Notch3 or Notch4 does not lead to aberrant embryonic phenotypes (Krebs *et al.*, 2000; Krebs *et al.*, 2003).

Regarding the protein structure of Notch receptors (Fig. 1.4), the extracellular domains comprise multiple tandem-arrayed epidermal growth factor (EGF)-like repeats, three

Lin12/Notch (LNR) motifs and a heterodimerization domain. The EGF-like repeats govern calcium-dependent ligand-binding and promote homodimerization of the receptor. EGF-like repeats 11 and 12 of *Drosophila* Notch alone are necessary and sufficient to mediate interactions with Notch ligands (Rebay *et al.*, 1991). Some EGF-like repeats are glycosylated at O-fucosylation sites by Pofut and Fringe resulting in differential affinity for Notch ligands (see chapter 1.4.2; Hicks *et al.*, 2000). The LNR motifs are responsible for heterodimerization and prevent receptor activation in the absence of receptor-ligand engagement (Fehon *et al.*, 1990; Greenwald and Seydoux, 1990; Rand *et al.*, 2000; Sakamoto *et al.*, 2005; Sanchez-Irizarry *et al.*, 2004).

The intracellular domain of Notch that mediates Notch signaling carries the RBPJκ associated molecule (RAM) domain close to the single-pass transmembrane domain and seven ankyrin repeats flanked by two nuclear localization signals. The RAM domain and the ankyrin repeats interact with the CSL transcription factor in the nucleus (Beatus *et al.*, 2001; Tamura *et al.*, 1995). In addition, Notch ICD contains a PEST (proline-glutamate-serine-threonine rich) sequence involved in regulating protein half-life and, except for Notch4, a transactivation domain (TAD) (Beatus *et al.*, 2001; Fryer *et al.*, 2004). The TAD domain recruits transcriptional activators such as Mastermind-like and the histone acetyltransferase (HAT) complex (Kurooka *et al.*, 1998; Tani *et al.*, 2001).

1.6.2 Notch ligands

Three of the five Notch ligands described in mammals are expressed in the presomitic mesoderm, namely Dll1, Dll3 and Jagged1. Dll1 and Dll3 are both essential for proper somite formation, indicated by severe somite defects in mutant mice, whereas Jagged1 mutant mice show no somitic phenotype (Dunwoodie *et al.*, 2002; Hrabe De *et al.*, 1997; Xue *et al.*, 1999). Jagged1 and Dll4 play vital roles in vascular development and remodeling, whereas Jagged2 participates mainly in limb, craniofacial, and thymic development (Jiang *et al.*, 1998; Krebs *et al.*, 2004; Valsecchi *et al.*, 1997; Xue *et al.*, 1999).

For all ligands, except for *Dll3*, a clear Notch activating function was demonstrated (Hicks *et al.*, 2000; Lindsell *et al.*, 1995; Shawber *et al.*, 1996a; Shimizu *et al.*, 2000a; Shutter *et al.*, 2000). Apart from their positive function, Notch ligands were shown to act in a dominant-negative manner and impair signal transduction when expressed at high levels in the same cell as Notch (cis-inhibition; de Celis and Bray, 2000; Dunwoodie *et al.*, 1997; Jacobsen *et al.*, 1998; Sakamoto *et al.*, 2002). The associated mechanism is not known but it appears to be

required, for instance, during wing development of flies by limiting Notch activation to a defined domain at the wing margin (Micchelli *et al.*, 1997).

Notch ligands are type I transmembrane proteins that share several structural features (Fig. 1.4). All Notch ligands have a DSL domain at the N terminus, a variable number of multiple epidermal growth factor (EGF)-like repeats with distinctive spacing of the six conserved cysteine residue in the extracellular domain and a relatively short cytoplasmic tail (Fleming *et al.*, 1990; Nye and Kopan, 1995). The DSL domain unique to Notch ligands shows similarities to the EGF-like repeats and is indispensable for Notch activation through binding to EGF-like repeats 11 and 12 of the Notch receptor (Shimizu *et al.*, 1999; Shimizu *et al.*, 2000a; Tax *et al.*, 1994). The EGF-like repeats contribute to stable ligand-receptor interaction and homodimerization and are substrates for glycosylation (Panin *et al.*, 2002; Rebay *et al.*, 1991; Sakamoto *et al.*, 2005).

Jagged proteins are characterized by an additional cysteine-rich domain in the extracellular domain and a von Willebrand factor type C domain. The cysteine-rich domain is thought to control Notch receptor specificity while the latter seems to be involved in ligand dimerization (Fleming, 1998).

The intracellular region of the Notch ligands is assumed to have a rather disordered nature without any known structural domains. Different ligand types show distinct cytoplasmic tails while within the same ligand type the ICD sequence is evolutionary well conserved (Pintar *et al.*, 2007). Recent reports suggest that, in addition to allowing regulated endocytosis (see chapter 1.4.2), the intracellular domain of some Notch ligands seem to function as transcriptional regulators. Similar to Notch receptors, some Notch ligands are prone to successive ADAM protease and γ-secretase cleavages that release the intracellular domain (LaVoie and Selkoe, 2003; Six *et al.*, 2003). Putative nuclear localization sequences were found in all Notch ligands except for DLL3 and DLL4. The intracellular moiety of DLL1 was detected in the cell nucleus and interaction with nuclear factors was demonstrated (Hiratochi *et al.*, 2007; Ikeuchi and Sisodia, 2003; Six *et al.*, 2003). These results suggest that DLL1 ICD contributes to the activation of transcriptional events indicating that Notch signaling might not be exclusively unidirectional.

The importance and properties of the cleaved extracellular domain of DLL1 is still not known. In *D. melanogaster* the Delta protein exists in transmembrane and soluble, truncated forms generated by the cleavage by an ADAM metalloprotease (Klueg *et al.*, 1998; Qi *et al.*, 1999). In *C. elegans* there is evidence that secreted DSL proteins act as natural ligands and can substitute for membrane-tethered ligands (Chen *et al.*, 2004).

Additionally, the intracellular domains of DLL1, DLL4 and Jagged1 carry a PDZ (**P**ost-synaptic density-95/**D**iscs large/**Z**onula occludens-1) ligand binding motif at the C-terminal end (Sheng and Sala, 2001). Recently, it has been shown that several scaffold proteins of the MAGUK (Membrane-Associated Guanylate Kinase) family interact with the C-terminus of DLL1 and DLL4 and that a deletion of the class I PDZ binding motif ATEV in DLL1 abolishes interaction with MAGUK protein family members (Pfister *et al.*, 2003; Six *et al.*, 2004). A zebrafish DeltaD variant that fails to bind PDZ- containing proteins functions normally as a Notch ligand (Wright *et al.*, 2004). Thus, the initiation of the ligand-induced Notch signal seems to be independent of this novel PDZ-dependent signaling mechanism inside the ligand expressing cell suggesting a cell-autonomous function of the Notch ligands DLL1 and Jagged1 (Ascano *et al.*, 2003; Six *et al.*, 2004).

Whereas Jagged1 exhibits a class II PDZ binding motif which was shown to interact with the ras-binding protein Afadin (AF6) (Ascano *et al.*, 2003; Hock *et al.*, 1998) the C-termini of DLL3 and Jagged2 do not contain any PDZ binding motif emphasizing the differences of the Notch ligands with respect to their interactions with other proteins.

Delta-like 1 (DLL1)

Mutations in Delta were initially described by Dexter (1914) on the basis of wing venation defects observed in a *Drosophila melanogaster* strain heterozygous for a loss-of-function mutation in the Delta gene. The Delta homologue, Delta-like 1 (*Dll1*), is probably the best studied Notch ligand in vertebrates. The DLL1 protein of vertebrates contains a DSL domain and eight EGF-like repeats in its extracellular domain. The DLL1 intracellular domain is lysine-rich and carries a PDZ ligand binding motif at its C-terminal end.

Dll1 is expressed during gastrulation and early organogenesis in a spatiotemporal-restricted manner in the presomitic and somitic mesoderm, in the nervous system and the spinal nerves (Bettenhausen *et al.*, 1995; Jouve *et al.*, 2000). *Dll1* mRNA expression in the paraxial mesoderm starts with the onset of gastrulation (E7) and continues until day 12.5 of murine development correlating with the period of somitogenesis (Beckers *et al.*, 1999). During this period strong mRNA expression is detected in the whole presomitic mesoderm. In the formed somites *Dll1* expression is restricted to the caudal halves. Additionally, *Dll1* transcripts were detected at later stages in epithelial ducts of several organs, in skeletal and smooth muscles, the central nervous system, some sensory epithelia as well as in endothelial cells of blood vessels (Beckers *et al.*, 1999).

During somitogenesis *Dll1* is required for the epithelialization of the somites and for the maintenance of somite borders (Hrabe De *et al.*, 1997). In the nervous system *Dll1*

participates in cell fate decisions and maintains cells in an undifferentiated state by inducing Notch signals (Chitnis, 1995; Lewis J, 1998).

Mice heterozygous for the *Dll1* null allele display subtle malformation of the vertebrae indicating a haploinsufficiency of *Dll1* (Cordes *et al.*, 2004). Homozygous *Dll1* mutant embryos exhibit severe patterning defects in the paraxial mesoderm and a hyperplastic central nervous system. Albeit an initial metameric unit is formed, somites are not fully epithelialized and their borders are not maintained leading to a perturbed arrangement of myotomes and sclerotomes. Lfng expression in the PSM is severely downregulated and in consequence the segments lack any detectable antero-posterior polarity as indicated by contiguous *Tbx18* expression and the loss of *Uncx4.1* expression, both representing somite polarity markers (Bussen *et al.*, 2004; Hrabe De *et al.*, 1997; Morales *et al.*, 2002). *Dll1*-deficient mice die around E12 from severe hemorrhagic bleedings as a secondary effect due to vascular defects.

Delta-like 3 (DLL3), a divergent DSL protein

The Delta-like 3 (*Dll3*) gene was isolated by a subtracted library screen as a gene expressed in the mesoderm and the primitive streak during gastrulation (Dunwoodie *et al.*, 1997). It was the third member of the Delta familiy identified in vertebrates. So far, the second homologue *Delta2* found in vertebrates has only been described in *Xenopus laevis* (Mansouri *et al.*, 1997) whereas *Dll3* has only been identified in mammals and is the most divergent ligand among the Delta homologues. DLL3 protein exhibits 6 EGF-like repeats and a highly modified DSL domain. The intracellular domain of DLL3 bears no homology to other DSL ligands, is about half their size and contains neither nuclear localization signals nor a PDZ binding motif at its C-terminus. As DLL3 ICD lacks lysine residues, it is unlikely to be ubiquitinylated and internalized via endocytosis.

Dll3 shows a diverse and dynamic pattern of mRNA expression during gastrulation and early organogenesis. *Dll3* transcripts localize to the primitive streak and later, at early somite stages, persist in the tail bud. The highest level of *Dll3* transcripts is found in the paraxial mesoderm. Expression is also detected along the length of the presomitic mesoderm and in the nascent somites but ceases as somites mature. *Dll3* is expressed in a broad band in the forming somite and in the anterior half of the nascent somites (Dunwoodie *et al.*, 1997). Additionally, lower levels of *Dll3* mRNA were described in the neuroectoderm and in the pituitary (Dunwoodie *et al.*, 1997; Raetzman *et al.*, 2004).

Analysis of *Dll3* null mutant embryos generated by gene targeting (Dunwoodie *et al.*, 2002) and of the radiation induced *Dll3* pudgy mouse mutant (Kusumi *et al.*, 1998) revealed an essential role of this gene in somite formation and skeletogenesis. In pudgy mice, a frame-

shift caused by a four-nucleotide deletion leads to an early truncation of the expected Dll3 product prior to its DSL domain (Grüneberg, 1961; Kusumi *et al.*, 1998). The loss-of-function mutations in the *Dll3* gene result in a highly disorganized vertebrocostal skeleton (Grüneberg, 1961). The developmental origin of these defects is a delayed and irregular somite formation. This results in the perturbation of antero-posterior somite polarity revealed by a salt and pepper expression pattern of the polarity marker *Uncx4.1* and fuzzy expression of the anterior compartment marker *Tbx18* (Bussen *et al.*, 2004; Takahashi *et al.*, 2003). The expression of Notch target genes as Lfng, Hes1, Hes5 and Hey1 is disrupted in the presomitic mesoderm of these mice (Dunwoodie *et al.*, 2002). Additionally, histological analyses of *Dll3* mutant embryos revealed a neural phenotype with incomplete penetrance. Whereas the neural tube of the trunk showed no defects, *Dll3* mutant mice appear to have an enlarged roof to the fourth ventricle of the brain with a reduction or absence of the neuroepithelium and malformations in the lateral ventricles (Kusumi *et al.*, 1998; Sparrow *et al.*, 2002).

Dll3 is essential for proper somitogenesis but dispensable for embryo vitality. Homozygous pudgy mice are viable although loss of homozygous pudgy progeny has been observed before E9.5 (Dunwoodie *et al.*, 2002; Kusumi *et al.*, 1998).

Numerous mutations of DLL3 protein have been reported in SCD (spondylocostal dysostosis) patients, all appearing to be pure loss-of function mutations either leading to premature translational termination with subsequent loss of important protein domains such as EGF-like repeats and/or the transmembrane domain or to cause missense or insertion mutations (Turnpenny *et al.*, 2003).

Contradicting evidence on *Dll3* function exists in the literature. Dunwoodie *et al.* (1997) showed that *Dll3* can inhibit primary neurogenesis when ectopically expressed in *Xenopus leavis*, whereas Ladi *et al.* (2005) demonstrated the opposite effect in a similar experiment. Thus, different potential functions have been suggested: Dll3 might act as a bona fide Notch ligand activating Notch signaling as demonstrated for the other Notch ligands. Alternatively, it might have a rather antagonistic function or act as a modulator of Notch signaling (Dunwoodie *et al.*, 1997; Ladi *et al.*, 2005; Weinmaster and Kintner, 2003).

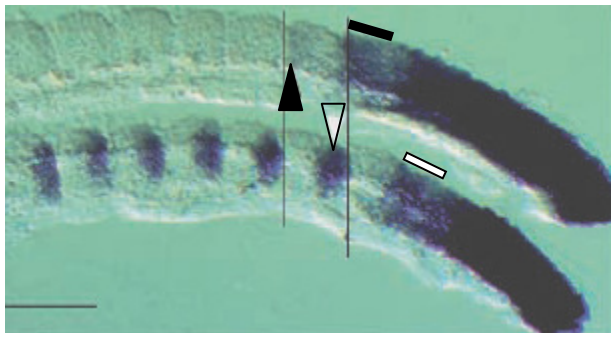

Fig. 1.5: mRNA expression of Dll3 and Dll1 in mouse presomitic mesoderm (Dunwoodie *et al.*, 1997). *In situ* hybridization of tail halves: *Dll3* (top) is expressed in a broad band at the anterior of the forming somite (short thick black line); at the anterior part of the nascent somite this expression is refined to a faint narrow band (black arrowhead). *Dll1* (bottom) is expressed in a broad band at the posterior end of the forming somite (white line); in the formed somites *Dll1* expression is restricted to the posterior half (white arrowhead).

Divergence of Dll3 from Dll1 and other homologues

In the mouse embryo *Dll1* and *Dll3* expression patterns overlap in the posterior PSM and are distinct in the anterior PSM and the somites (Fig. 1.5). In the forming somites, Dll1 expression coincides with the posterior half, while Dll3 is expressed in the anterior half. Additionally, Dll1 is expressed in the posterior halves of the already formed somites.

Dll1 and *Dll3* are both essential for normal somite formation and for correct specification of the antero-posterior segment polarity within the presomitic mesoderm (Dunwoodie *et al.*, 2002; Hrabe De *et al.*, 1997). However, null mutant mice display clearly distinct phenotypes. Loss-of-function mutations of the two DSL proteins differently influence segment polarity and activation of downstream targets. In *Dll1* mutant embryos expression of the posterior somite marker *Uncx4.1* is totally lost whereas in *Dll3* pudgy embryos *Uncx4.1* shows a random expression pattern. Expression of the Notch target genes *Lfng* and *Hes1* is severely downregulated or absent in *Dll1* null mutants while in *Dll3* null embryos the caudal expression domain of these genes is completely lost but the rostral stripe is retained (Barrantes *et al.*, 1999; Jouve *et al.*, 2000).

In addition to the distinct phenotypes of their null mutants, the DSL proteins DLL1 and DLL3 differ significantly in their amino acid sequences. DLL1 and DLL3 proteins share only 36% overall amino acid identity. The highest homology of DLL1 and DLL3 exists between EGF-like repeat 4 of DLL3 and repeat 6 of DLL1 (63%). Compared to DLL1 and other Delta homologues, the DLL3 extracellular domain contains an almost unrecognizable DSL domain and only six EGF-like repeats with altered spacing between some of them. Based on DLL1-sequence homology, DLL3 lacks EGF-like repeat 2 (and 3), which is a perfectly conserved EGF-like repeat among DSL proteins in vertebrates and *D. melanogaster* (Lissemore and

Starmer, 1999). The DSL domain of DLL3 displays only 18% amino acid identity with the DLL1 DSL domain compared to 51% identity between the DSL domains of DLL4 and DLL1. Additionally, conserved motifs present in the DSL domains of all other Notch ligands are absent in DLL3. Furthermore, the intracellular domains of DLL1 and DLL3 are highly dissimilar. While the DLL1 ICD comprises lysines and a C-terminal PDZ domain, DLL3 lacks both features.

DLL1 was unambiguously proven to be a bona fide Notch ligand whereas there are few reports on DLL3 function, some of them with contradictory results (Dunwoodie, 2002; Ladi, 2005). Based on these observations a hypothesis was put forward suggesting that *Dll1* and *Dll3* have non-redundant functions and that their functional non-equivalence is due to differences in protein structure and sequence.

Redundancy of Notch ligands

Some Notch ligands might have redundant functions. Due to the distinct expression patterns of the ligands in the mouse embryo a biochemical redundancy might not be revealed in null mutant mice. Experiments in *C. elegans*, *D. melanogaster* and mice have shown that Notch ligands may be interchangeable and exert partially redundant functions depending on the cellular context. Although in *C. elegans* the Notch ligands *Lag-2* and *APX-1* have a relatively low level of homology and their mutants show different phenotypes, *APX-1* expressed under the *Lag-2* promotor can fully substitute for the loss of *Lag-2* in the nematode (Fitzgerald and Greenwald, 1995; Gao and Kimble, 1995). Similarly, Serrate expression can functionally replace Delta activity during neuroblast segregation in the *Drosophila* embryo (Gu *et al.*, 1995). In cell fate specification in the *Drosophila* sensory organ lineage Delta and Serrate have redundant functions required for asymmetric cell divisions (Zeng *et al.*, 1998). Nobta *et al.* (2005) showed that during osteoblastic differentiation in mice *Dll1* and Jagged1 are functional redundant as they induce an identical cellular response. For *Dll1* and *Dll3*, functional redundancy was proposed in some cellular contexts such as the spinal cord and the melanotrope lineage in the pituitary gland where *Dll1* and *Dll3* are coexpressed as loss of *Dll3* did not lead to any phenotype in these tissues (Raetzman *et al.*, 2004).

Nevertheless, some developmental processes employ different ligands for distinct signaling events. In the inner ear of the mouse DLL1 and Jagged1 were found to have rather contrasting functions (Brooker *et al.*, 2006). While DLL1 mediates the function of Notch in lateral inhibition, Jagged1 is responsible for activating the prosensory function of Notch.

2 Aims of this study

The DSL proteins DLL1 and DLL3 are both essential for somitogenesis and were originally thought to constitute activating Notch ligands. However, despite the largely overlapping expression pattern of *Dll1* and *Dll3*, null alleles of both genes result in different phenotypes and both proteins differ with respect to various protein domains. The distinct phenotypes of mutant embryos and the structural divergence of the proteins suggested that DLL1 and DLL3 are functionally non-equivalent Notch ligands. Experiments in *D. melanogaster* and *C. elegans* revealed functional reduncancy of Notch ligands in certain contexts. The objectives of this study were to evaluate the biochemical equivalence and to elucidate the biochemical differences of the mouse homologues DLL1 and DLL3.

Towards these aims, an *in vitro* comparison of Dll1 and Dll3 and chimeric proteins with respect to transactivation potential, cell surface presentation and subcellular localization was devised to reveal potential differences in the biochemical properties of the protein domains. In order to analyze the biochemical equivalence of the two DSL proteins *in vivo* it was planned to replace *Dll1* with *Dll3* coding sequence by homologous recombination.

Additionally, since the DSL domain of Notch ligands was previously shown to be essential for Notch binding (Shimizu *et al.*, 1999), the significance of conserved sequences in the DSL domain of DLL1 not present in DLL3, was addressed by mutating these sequences in the DLL1 protein and analyzing the effect of these mutations with regard to Notch transactivation and protein localization.

Is summary, these experiments should help to clarify the significance of the divergent protein structures of DLL1 and DLL3 for their biochemical properties and their functionality in the developing embryo.

3 Material and Methods

3.1 Material

3.1.1 Primers

Primers were synthesized by MWG Biotech AG or Operon Biotechnologies GmbH.

Primers used for cloning of targeting and expression constructs:

Primer	**Sequence 5' to 3'**
Dll3BamHIfor	AGG CAG GCG GAT CCA CAG CGC
SacIDll3HArev	GAG CTC CTA TTA TCA AGC GTA GTC
SacI3xstopDll3rev	GAG CTC CTA TTA TCA GGC CTC TCG TGC ATA AAT GGA AG
ScaITDPIDll3EGF1for	AGT ACT GCA CTG ACC CAA TCT GTC GAC CAG GCT GCA GCC C
Dll3EGF3rev	ATC TTC ACC GCC AAC ACA CAA GCC
NdeDll1-Dll3for	CAT ATG GAG AGC CAG GGC GGG CCC TTC CCC TGG CTG CCT CCC GCC TTG GGG CTG CTG
Dll3Flag3xstopNotIrev	GCG GCC GCC TAT TAT CAT TTA TCG TCA TCG TCT TTG TAG TCT GCG GCC TCT CGT GCA TAA ATG GAA GGG
NotIfor3'Dll1	GCG GCC GCA CAG ACC TCC
EcoRI-Dll1kozakATG	GAA TTC GTC CAG CGG TAC CAT GG
BspEIDll3EGF2rev	TCC GGA CAC CTC ACA TCG AAG CCC GTA
BspEIDll1EGF3for	TCC GGA GTC ACG TGT ACT CAC CAT AAG CCG TGC
Dll1EGF5rev	CGT CCT CCA TTG AAG CAA GGG CC
NotIfor3'Dll1	GCG GCC GCA CAG ACC TCC
SalIDll1EGF2rev	GTC GAC AGT AGT TCA GGT CTT GGT TGC A
Dll3ECD-DLL1TMICDfor	GCG GAT CCA CAG CGC TTT CTT GTG GCC GTG TGT GCC GGG GTG
Flag3xSTOPNotIrev	GCG GCC GCC TAT TAT CAT TTA TCG TCA TCG TCT TTG
YY-AV-for	GTT TGT GTG TGA CGA GCA CGC CGT CGG AGA AGG TTG CTC TG
YY-AV-rev	CAG AGC AAC CTT CTC CGA CGG CGT GCT CGT CAC ACA CAA AC
GWKG-AAAA for	GAA GAT GTG CGA CCC TGC CGC GGC AGC CCA GTA CTG CAC TGA CC
GWKG-AAAA rev	GGT CAG TGC AGT ACT GGG CTG CCG CGG CAG GGT CGC ACA TCT TC

YY-AY for	GTT TGT GTG TGA CGA GCA CGC CTA CGG AGA AGG TTG CTC TGT GTT CTG C
YY-AY rev	GCA GAA CAC AGA GCA ACC TTC TCC GTA GGC GTG CTC GTC ACA CAC AAA C
YY-FY for	GTT TGT GTG TGA CGA GCA CTT CTA CGG AGA AGG TTG CTC TGT GTT CTG C
YY-FY rev	GCA GAA CAC AGA GCA ACC TTC TCC GTA GAA GTG CTC GTC ACA CAC AAA C
YY-YF for	GTT TGT GTG TGA CGA GCA CTA CTT CGG AGA AGG TTG CTC TG
YY-YF rev	CAG AGC AAC CTT CTC CGA AGT AGT GCT CGT CAC ACA CAA AC
YY-FF for	GTT TGT GTG TGA CGA GCA CTT CTT CGG AGA AGG TTG CTC TG
YY-FF rev	CAG AGC AAC CTT CTC CGA AGA AGT GCT CGT CAC ACA CAA AC

Primers used for genotyping of mice and embryos:

Name	**Sequence 5' to 3'**
EGF3'#1	TGT CAC GTC CTG CAC GAC G
EGF3'#2	GGT ATC GGA TGC ACT CAT CGC
EGFΔneo FOR	ATG GAC AGC ATT TCC TCC TGC CTC
EGFΔneo REV	GCC AGT CAG TTC CCA GTA AGA AGT C
Dll1 F2	CTC CTG CGC GGT GGA GGG AGG
Dll1 R1	GGA GTC GAC ACC CAG CAC TGG CG
Melta38	ATC CCT GGG TCT TTG AAG AAG
LacZ1/Dll1 Ko	CAA ATT CAG ACG GCA AAC
Dll3 pu1	ACG AGC GTC CCG GTC TAT AC
Dll3 pu2	AGG TGG AGG TTG GAC TCA CC

3.1.2 Synthetic DNA, Vectors and cDNAs

The gene fragment used for chimeric ligands J and M was synthesized by Entelechon GmbH (delivered doublestranded and subcloned).

Gene fragment:

Gene fragment	**Sequence 5' to 3'**
NDLL1-DSLDLL3-182	CAG TAG CGG CCG CAC AGA CCT CCG GTA CTC TTA CCG GTT TGT GTG CGA GCC GCC CGC CGT CGG GGC CGC CTG CGC GCG CCT GTG CCG CTC ACG CAG TGC CCC CTC GCG GTG TGG CCC GGG ACT GCG ACC CTG CAC GCC ATT CCC AGA CGA GTG CGA AGC CCC GTC TGT GTG TCG ACC AGG CT

Vectors and cDNA:

Vector / cDNA	**Origin**
pTracerCMV	Clontech
pTracerCMV-Dll1Flag	(Shimizu *et al.*, 2000b) S. Chiba, Univ. of Tokyo, Tokyo, Japan
pGEMzf	Promega
RBP-luc	(Minoguchi *et al.*, 1997) A. Israel, Inst. Pasteur, Paris, France
Hes1-luc	(Logeat *et al.*, 1998) A. Israel, Inst. Pasteur, Paris, France
pSL1180	Amersham
pBluescript II	Stratagene
pNEB193	New England Biolabs
pLitmus29	New England Biolabs
Dll3 cDNA	(Dunwoodie *et al.*, 1997) S. Dunwoodie, Victor Chang Cardiac Research Institute, Darlinghurst, Australia

3.1.3 Media

Bacterial culture

LB-medium: 10 g bacto-tryptone; 5 g yeast extract; 5 g NaCl ad 1000 ml
Ampicillin was added after autoclaving if required
(100 µg/ml ampicillin)

LB-agar plates: LB-medium (see above) with additional 1.5% bacto-agar
Additives were added shortly before pouring the plates if required
(100 µg/ml ampicillin; 1 mM IPTG; 50 µg/ml X-Gal)

Cell culture

CHO-medium: DMEM/F12 1:1, 10% FCS, 2 mM Glutamax, 100 U/ml penicillin, 100 µg/ml streptomycin

HeLa-medium:
(also OP9 and L cells) DMEM, 10% FCS, 2 mM Glutamax, 100 U/ml penicillin, 100 µg/ml streptomycin

HEK 293 medium: DMEM, 10% FCS, 2mM Glutamax, 1 mM sodiumpyruvate, 100 µM non-essential amino acids, 100 µM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin

Ingredients for cell culture media were obtained from GibcoBRL. Prior to use, fetal bovine serum (FCS) was heat inactivated by incubation at 56°C in a water bath for 30 min to destroy

complement proteins. Freezing media were prepared by adding 10% DMSO and 10% FCS to the normal medium.

3.1.4 Cells

Bacteria

The *Escherichia coli* variants XL1-blue (Stratagene) and TOP10 (Invitrogen) were used for amplification of vector DNA.

Eukaryotes

The type and source of cell lines used for transfection, generation of stable cell lines, transactivation assays, cell surface biotinylation, metabolic labeling and immunofluorescence stainings are listed below. All cell lines grow as an adherent monolayer in cell culture dishes.

Cell lines:

Cell line	**Origin**	**Source**
CHO	chinese hamster ovary	Gossler Lab
CHO-PSGL-1	chinese hamster ovary	D. Vestweber, Univ. of Münster, Münster, Germany
HeLa	human cervical cancer	Gossler Lab
HeLa-N1	human cervical cancer	A. Israel, Institute Pasteur, Paris, France
HEK293	human embryonic kidney	Gossler Lab
L cell-Dll1HA	mouse fibroblast	G. Weinmaster, UCLA, Los Angeles, USA
OP9	mouse stromal cells derived from bone marrow	A. Israel, Institute Pasteur, Paris, France
OP9MIG	mouse stromal cells derived from bone marrow	A. Israel, Institute Pasteur, Paris, France
OP9-Dll1	mouse stromal cells derived from bone marrow	A. Israel, Institute Pasteur, Paris, France

3.1.5 Antibodies

Primary and secondary antibodies used for Western blot analysis (WB), immunofluorescence (IF) of cells, whole mount immunofluorescence (WM-IF) of presomitic mesoderm and immunoprecipitation (IP) are listed below.

Primary antibodies:

Antibody	**directed against**	**Host species**	**Source**	**Application, dilution**
anti-Calreticulin		rabbit, polyclonal	Abcam	IF, 1:50
anti-cleaved Notch1 (Val1744)	Val1744 of the cleaved Notch ICD	rabbit, polyclonal	Cell Signaling	WB, 1:1000
anti-Dll1 (H-265)	C-terminus	rabbit, polyclonal	Santa Cruz	WM-IF, 1:100
anti-Dll1 1F9	extracellular peptide	rat, monoclonal	E. Kremmer, GSF Munich, Germany	IF, 1:10-1:50 IP, 1:50
anti-Dll1 2A5	intracellular peptide	rat, monoclonal	E. Kremmer, GSF Munich, Germany	IF, 1:10-1:50
anti-Dll1 2B3	extracellular peptide	rat, monoclonal	E. Kremmer, GSF Munich, Germany	IF, 1:10-1:50
anti-Dll3 C2	C-terminus (intracellular)	guinea pig, polyclonal	S. Dunwoodie, Victor Chang Cardiac Research Institute, Darlinghurst, Australia	IF, 1:50 WM-IF, 1:50
anti-Dll3 N2	N-terminus (extracellular)	guinea-pig, polyclonal	S. Dunwoodie, Victor Chang Cardiac Research Institute, Darlinghurst, Australia	IF, 1:50 WM-IF, 1:50
anti-Dll3-ICD (1D1)	intracellular domain	rat, monoclonal	E. Kremmer, GSF Munich, Germany	IF, 1:10
anti-Flag (M2)	peptide DYKDDDDK	mouse, monoclonal	Sigma	WB, 1:10000 IF, 1:5000
anti-GM130 (35)	C-terminus	mouse, monoclonal	BD Biosciences	IF, 1:250 WM-IF, 1:250
anti-HA (3F10)	peptide YPYDVPDYA	rat, monoclonal	Roche	IF, 1:100
anti-pan Cadherin (CH-19)	C-terminus	mouse, monoclonal	Sigma	WM-IF, 1:250
anti-PSGL-1 (4RA10)		rat, monoclonal	D. Vestweber, Univ. of Münster, Germany	WB,1:100 IP, 1:100

Secondary antibodies

antibody	**host species**	**Source**	**application, dilution**
anti-guinea pig Texas Red	donkey	Jackson ImmunoResearch Laboratories, Inc.	IF, 1:200 WM-IF, 1:200
anti-mouse Alexa Fluor®488	donkey	Invitrogen	IF, 1:500 WM-IF, 1:500
anti-mouse FITC	donkey	Jackson ImmunoResearch Laboratories, Inc.	IF, 1:1000
anti-mouse POD	sheep	Amersham	WB, 1:10000
anti-mouse Texas Red	donkey	Jackson ImmunoResearch Laboratories, Inc.	IF: 1:2000 WM-IF, 1:200
anti-rabbit Alexa Fluor®488	donkey	Invitrogen	WM-IF, 1:500
anti-rabbit FITC	donkey	Jackson ImmunoResearch Laboratories, Inc.	IF, 1:1000
anti-rat Alexa Fluor®488	donkey	Invitrogen	IF, 1:500 WM-IF, 1:500
anti-rat FITC	donkey	Jackson ImmunoResearch Laboratories, Inc.	IF, 1:1000
anti-rat POD	goat	Amersham	WB, 1:10000
anti-rat Texas Red	donkey	Jackson ImmunoResearch Laboratories, Inc.	IF, 1:2000 WM-IF, 1:200

3.1.6 Data bases

In the course of this project the following data bases were used:

Ensembl genome browser	(http://www.ensembl.org)
Expert Protein Analysis System (ExPASy)	(http://www.expasy.org)
Mouse Genome Imformatics (MGI)	(http://www.informatics.jax.org)
National Center for Biotechnology Information (NCBI)	(http://www.ncbi.nlm.nih.gov)

3.1.7 Computer programs

The following computer programs were used in the course of this project:

Text processing:	Word 2003 (Microsoft); Reader 6.0 (Adobe)
Image acquisition:	Fujifilm Photograb-300Z (Fuji Photo Film Co.); LSM 510 Confocal Software (Carl Zeiss MicroImaging, Inc) Fujifilm Image reader Version 1.8E (Fuji Photo Film Co) FotoLookSA3.03 (Agfa-Gevaert AG) BAS-100 MacBASV2.2 (Fuji Photo Film Co.)
Image processing:	Photoshop 7.0 und CS (Adobe), Illustrator CS (Adobe)
Sequence processing:	MacVectorTM 7.2 (Accelrys Inc.)
Online search:	Netscape Communicator 7.1 (Netscape Communications Corp.) Safari Version 2.0.4 (Apple Computer, Inc.)
Literature management:	Reference manager 11 (Thomson ResearchSoft)

3.2 Methods of molecular biology

3.2.1 Standard conditions and methods of molecular biology

Common methods of molecular biology such as precipitation of nucleic acids, restriction digests of plasmid DNA, ligation of DNA fragments, transformation of electrocompetent *E. coli* cells, analytic preparation of plasmid DNA by alkaline lysis, photometric quantification of nucleic acids and agarose gel electrophoresis to separate DNA fragments were essentially performed as described by Sambrook *et al.* (1989) and Ausubel *et al.* (1998). Restriction digests were performed with enzymes from New England Biolabs, MBI Fermentas, Stratagene and Roche with the supplied buffers as recommended by the manufacturer. Agarose gel electrophoresis was performed using TAE buffer, gel elution of DNA fragments was performed using the "NucleoSpin® Extract II" Kit (Macherey-Nagel). For preparation of "clean" plasmid DNA the "GenElute™ Plasmid Miniprep Kit" (Sigma) was used according to the manufacturer's protocol. For subcloning of PCR products the "pGEM®-T Easy Vector System" (Promega) was used. DNA sequencing was performed by Agowa GmbH or in house using the "BigDye™ Terminator Cycle Sequencing Ready Reaction kit" (PE Applied Biosystems).

3.2.2 Generation of expression constructs

For cloning of eukaryotic expression vectors the pTracerCMV plasmid was used carrying an ampicillin-resistance cassette for selection in bacteria, a zeocin-resistance cassette for selection in cell culture and a CMV-promotor upstream of the multiple cloning site. The pTracer-Dll1Flag plasmid was modified by inserting an IRES-neo cassette after the Dll1Flag ORF and served as a shuttle vector (pTracer-Dll1flagIRESneo) for expression of flag-tagged Dll1, Dll3 as well as mutant and chimeric ligands. In addition, HA-tagged versions of *Dll1* and *Dll3* were cloned into pTracer. The Dll3HA construct was partially cloned by Claudia Brockmeyer and the Dll3flag and Dll1HA contructs were cloned by Stephan Hegge under my supervision during their diploma thesis. The integrity of all constructs was verified by sequencing.

Chimeric ligands

Chimeric ligands were generated by conventional cloning methods. Junctions between the *Dll1* and *Dll3* sequences were created by PCR mutagenesis using primers with an overhang containing restriction sites without changing the amino acid sequence. In the case of chimeric ligands D and E, two gene fragments containing a deletion or an insertion between EGF1 and

2 were synthesized (GenScript). For cloning of the constructs J and M a gene fragment containing the N-terminus of DLL1 and the link to the DLL3 DSL domain was synthesized by Entelechon GmbH. The junctions of *Dll1* and *Dll3* sequences in chimeric ligands are shown in the table below.

Construct A (internal designation swap1/3):
By PCR the EGF-like repeats 1 to 3 of the *Dll3* ORF were amplified using a forward primer (ScaITDPIDll3EGF1for) derived from the start of EGF1 additionally containing a *Sca*I site and a *Dll1* sequence linker in the overhang and the reverse primer Dll3EGF3rev. The subcloned PCR product was digested with *Sca*I and *Xma*I and the resulting 262 bp fragment was isolated. Together with the ~690 bp *EcoR*I/*Sca*I *Dll1* fragment from pTracer-Dll1Flag it was subcloned into the pBSII vector. The resulting vector was digested with *EcoR*I/ *Xma*I and the ~950 bp chimeric fragment was then cloned into the pTracer-Dll3flagIRESneo together with the ~540 bp *Xma*I/*Sac*II fragment of the *Dll3* ORF.

Construct B (internal designation Dll1-Dll3):
By PCR the transmembrane and intracellular domain (TM-ICD) of the *Dll3* ORF including the flag tag and stop codons was amplified using a forward primer (NdeDll1-Dll3for) derived from the start of the DLL3 transmembrane domain additionally containing a *Dll1* sequence linker with a *Nde*I site in the overhang and the reverse primer Dll3Flag3xstopNotIrev. The subcloned PCR product was digested with *Nde*I and *Not*I and the ~350 bp fragment was isolated. Together with the ~1.6 kb *EcoR*I/*Nde*I *Dll1* fragment from pTracer-Dll1flagIRESneo it was subcloned into the pTracer-Dll1flagIRESneo vector.

Construct D (internal designation swap Bsp):
By PCR the N-terminus, DSL domain and EGF1 and 2 up to the start of EGF3 of chimeric ligand A were amplified using the forward primer EcoRI-Dll1kozakATG and a reverse primer (BspEIDll3EGF2rev) derived from the end of EGF2 and the following spacer sequence additionally containing a *Dll1* sequence linker with a *BspE*I site in the overhang. By a second PCR EGF2 to 4 of *Dll1* were amplified using a forward primer (BspEIDll1EGF3for) derived from the start of EGF2 including a *BspE*I site in the overhang and a reverse primer (Dll1EGF5rev) derived from the spacer sequence between EGF4 and 5 containing a *Blp*I site. The subcloned product of PCR1 was digested with *Age*I and *BspE*I and the ~430 bp fragment was isolated. Together with the ~200 bp *BspE*I/*Blp*I fragment from PCR2 it was subcloned into the pTracer-Dll1flagIRESneo vector. The *BspE*I site was generated by conservative mutations of the *Dll1* sequence without altering the amino acid sequence.

Construct G (internal designation swap Sal131):
Together with the ~1.7 kb *EcoR*I/*BamH*I fragment from construct L the ~600 bp *BamH*I/*Not*I fragment from construct K was cloned into the pTracer-Dll1flagIRESneo vector.

Construct I (internal designation swap 131):
Together with the ~1.5 kb *EcoR*I/*BamH*I fragment from construct A the ~600 bp *BamH*I/*Not*I fragment from construct K was cloned into the pTracer-Dll1flagIRESneo vector.

Construct J (internal designation N131):
The gene fragment NDLL1-DSLDLL3-182 containing a short sequence of the N-terminus of *Dll1* ORF and the transition to the *Dll3* DSL domain was digested with *Not*I and *Sal*I. The ~160 bp fragment was isolated and cloned together with the ~1.4 kb *Sal*I/*Not*I fragment from construct K into the pTracer-Dll1flagIRESneo vector.

Construct K (internal designation Dll3-Dll1):
By PCR the transmembrane and intracellular domain (TM-ICD) of *Dll1* ORF including the flag tag, stop codons and a *Not*I site were amplified using a forward primer (Dll3ECD-DLL1TMICDfor) derived from the start the *Dll1* transmembrane domain additionally containing a *Dll3* sequence linker with a *BamH*I site in the overhang and the reverse primer Flag3xSTOPNotIrev. The subcloned PCR product was digested with *BamH*I and *Not*I and the ~650 bp fragment was isolated. Together with the ~1.5 kb *EcoR*I/*BamH*I *Dll3* fragment from pTracer-Dll3flagIRESneo it was subcloned into the pTracer-Dll1flagIRESneo vector.

Construct L (internal designation swap Sal):
By PCR the DSL domain and EGF-like repeats 1 and 2 of the *Dll1* ORF were amplified using a forward primer (NotIfor3'Dll1) derived from the *Dll1* sequence upstream of the DSL domain containing a *Not*I site and a reverse primer (SalIDll1EGF2rev) derived from the end of EGF2 and the following spacer sequence additionally containing a *Dll3* sequence linker with a *Sal*I site in the overhang. The subcloned PCR product was digested with *Not*I and *Sal*I and the ~350 bp fragment was isolated. Together with the ~1.1 kb *Sal*I/*Not*I Dll3Flag fragment from pTracer-Dll3flagIRESneo it was cloned into the pTracer-Dll1flagIRESneo vector.

Construct M (internal designation N13):
The gene fragment NDLL1-DSLDLL3-182 containing a short sequence of the N-terminus of *Dll1* ORF and the transition to the *Dll3* DSL domain was digested with *Not*I and *Sal*I. The ~160 bp fragment was isolated and cloned together with the ~1.1 kb *Sal*I/*Not*I fragment from pTracer-Dll3flagIRESneo into the pTracer-Dll1flagIRESneo vector.

Junctions of Dll1 and Dll3 sequences in the chimeric ligands:

Construct	**Sequence junction**
A	...TGC ACT GAC CCA ATC tgt cga cca ggc tgc... **C T D P I** *c r p g c*
B	...GGG CCC TTC CCC TGG ctg cct ccc gcc ttg... **G P F P W** *l p p a l*
C	...AGC GCC ATG ACC tgc gca gat gga ccc... **S A M T** *c a d g p* ...cca cag cgc ttt ctt GTG GCC GTG TGT... *p q r f l* **V A V C**
D	...ACT GAC CCA ATC tgt cga cca ggc tgc... **T D P I** *c r p g c* ...gtg tcc gga gtc acg TGT ACT CAC CAT... *v s g v t* **C T H H**
E	...GGC CGC TAC TGC acg gtc cct gtc tcc acc agt agc **G R Y C** *t v p v s t s s* tgc ctg aac tcc agg gtt cct ggt cct gcc agc act *c l n s r v p g p a s t* gga tgc ctt tta cct ggg cct gga cct TGC ATC CGA *g c l l p g p g p* **C I R** TAC CCA... **Y P**
F	...ACT GAC CCA ATC tgt cga cca ggc tgc... **T D P I** *c r p g c* ...act gga ccc ctc tgc GAT GAG tgt gat ggg aac cca... *t g p l c* **D E** *c d g n p* ...gtg tcc gga gtc acg TGT ACT CAC CAT AAG... *v s g v t* **C T H H K**
G	...GAC CTG AAC TAC tgt cga cca ggc tgc... **D L N Y** *c r p g c* ...cca cag cgc ttt ctt GTG GCC GTG TGT... *p q r f l* **V A V C**
I	...TGC ACT GAC CCA ATC tgt cga cca ggc tgc... **C T D P I** *c r p g c* ...cca cag cgc ttt ctt GTG GCC GTG TGT... *p q r f l* **V A V C**
J	...TCT TAC CGG TTT GTG tgc gag ccg ccc gcc... **S Y R F V** *c e p p a* ...cca cag cgc ttt ctt GTG GCC GTG TGT... *p q r f l* **V A V C**
K	...cca cag cgc ttt ctt GTG GCC GTG TGT GCC... *p q r f l* **V A V C A**
L	...GAC CTG AAC TAC tgt cga cca ggc tgc... **D L N Y** *c r p g c*
M	...TCT TAC CGG TTT GTG tgc gag ccg ccc gcc... **S Y R F V** *c e p p a*

Constructs C, E and F were cloned by Dr. Katrin Serth and Patricia Delany-Heiken. Construct H was cloned by Claudia Brockmeyer under my supervision during her diploma thesis.

Dll1 sequences: UPPER case (NUCLEOTIDES)/**BOLD (AMINO ACIDS)**

Dll3 sequences: lower case (nucleotides)/*italics (amino acids)*

Dll1 DSL mutant ligands:
Plasmids for eukaryotic expression of the DLL1 DSL mutant proteins were generated by mutating the subcloned *Not*I/*Nde*I fragment of *Dll1* (Ago#951) using the "QuikChange Site-directed Mutagenesis Kit" (Stratagene) with the following primers.

Construct	Mutation	Forward primer	Reverse primer
DLL1 DSL-ΔY	YY→AV	YY-AV-for	YY-AV-rev
DLL1 DSL-ΔG	GWKG→AAAA	GWKG-AAAA for	GWKG-AAAA rev
DLL1 DSL-AY	YY→AY	YY-AY for	YY-AY rev
DLL1 DSL-FY	YY→FY	YY-FY for	YY-FY rev
DLL1 DSL-FF	YY→FF	YY-FF for	YY-FF rev
DLL1 DSL-YF	YY→YF	YY-YF for	YY-YF rev

The mutated *Not*I/*Nde*I fragment of *Dll1* was cloned into the pTracer-Dll1FlagIRESneo vector. The DLL1 DSL-AY and the DLL1 DSL-FY constructs were cloned by Claudia Brockmeyer under my supervision during her diploma thesis.

3.2.3 Generation of the targeting constructs

The strategy of the targeting constructs was adopted from a strategy developed by Dr. Ralf Cordes. For the generation of the $Dll1^{Dll3ki}$ and $Dll1^{Dll1DSLmut}$ constructs, homologous regions of the *Dll1* locus and transitions of different components were cloned from the $Dll1^{Dll1ki}$ construct (Ago#474). The *Dll3* ORF (with or without a C-terminal HA tag) was fused to a genomic *Dll1 Sac*I/*EcoR*I fragment containing part of exons 9, 10, and 11. A PGK neomycin expression cassette flanked by loxP sites was introduced 3′ to the Dll3-Dll1 fusion. A 4.6 kb *BamH*I/*Kpn*I fragment of *Dll1* genomic DNA upstream of the ATG fused in frame to *Dll3*, and ~3 kb of *Dll1* genomic DNA downstream of the *Sal*I site in exon 2 were included as regions of 5′ and 3′ homology, respectively. A diphtheria toxin A expression cassette was cloned both upstream and downstream of the homology arms.

3.2.4 Generation of *knock-in* mice targeting the *Dll1* locus

Linearized vector DNA was electroporated into 129Sv/ImJ embryonic stem cells and selected as described previously (Abdelkhalek *et al.*, 2004). Correctly targeted clones were identified by PCR using primers derived from the neo sequence and from genomic sequences downstream of the targeting vector. PCR-positive ES cell clones were verified by Southern blot analysis using external probes located 3′ and 5′ to the regions of homology in the vector. PCR-positive ES cell clones were used for blastocyst injection. Blastocysts were transferred into pseudo-pregnant females to generate chimeric mice. After germline transmission was

obtained the knock-in mice were passed through the germline of ZP3::Cre females (de Vries *et al.*, 2000) to remove the floxed neo cassette that was included in the targeting vectors.
Electroporation of the ES cells with the targeting constructs, ES cell culture as well as blastocyst injection of verified ES cell clones, blastocyst transfer in pseudo-pregnant females and subsequent crossing of chimeric mice were kindly performed by Dr. Karin Schuster-Gossler with technical assistance by Hannelore Burkhardt in the cell culture and Anatoli Heiser in the mouse work.

3.2.5 Southern blot analysis

PCR-positive ES cell clones and offspring of chimeric founder mice were tested for the correct homologous recombination event by Southern Blot Analysis (Chomczynski and Qasba, 1984; Southern, 1975).

Alkaline transfer

Denaturation buffer:	0.5 M NaOH, 1.5 M NaCl
Neutralization buffer:	50 mM NaP_i (prepared from 1 M NaP_i stock pH 6.7)

Genomic DNA was digested with *BamH*I (Roche) overnight at 37°C. DNA fragments were separated by agarose gel electrophoresis, stained with ethidiumbromide and documented with a size standard. To improve the transfer of large DNA fragments to the membrane, the DNA was depurinated by incubating the agarose gel in 0.15 M HCl for 15 min. Afterwards the agarose gels were rinsed in deionized water and soaked with denaturation buffer twice for 20 min with gentle rocking. To immobilize the DNA on a membrane the DNA fragments were transferred from the agarose gel onto a nylon membrane (Hybond-N, Amersham) by traditional semi-dry capillary blotting overnight. The gel/membrane sandwich was set up as follows (from bottom to top): two layers of Whatman 3MM-filter paper soaked with denaturing buffer, agarose gel, membrane, two layers of Whatman 3MM-filter paper soaked with denaturing buffer. A stack of dry tissue paper on top provided the soaking force. After the transfer the nylon membranes were neutralized in 50 mM NaP_i buffer for 20 min and crosslinked with 1200 J in a UV-Crosslinker (Stratagene).

Synthesis of radioactive labeled DNA probes

Radioactive labeled DNA probes were generated according to the method described by (Feinberg and Vogelstein, 1983). Using random primers, [α–^{32}P]-dCTP was incorporated into the newly synthesized DNA strand by the Klenow fragment of DNA polymerase I.
For the 3'probe 5 µg of the EGF3'probe plasmid (Ago#1667) were digested with *Eco*RI and the 500 bp fragment was isolated and used for labeling. For the 5' probe 5 µg of the

EGF5'probe plasmid (Ago#1668) were digested with *BamH*I and *Ava*II. The 320 bp fragment was separated from the 200 bp fragment on a 1.5% agarose gel, isolated and used for labeling. For the synthesis of radioactive labeled DNA probes the „Prime-It®II Random Primer Labeling Kit" (Stratagene) was used.

25 ng of the DNA fragment was denatured together with 10 µl random hexamer primer solution filled with ddH_2O up to a total volume of 33 µl at 96°C for 5 min. 10 µl labelling buffer, 5 µl [α–^{32}P]-dCTP (10 µCi/µl; 3000 Ci/mmol) and 1 µl exo-Klenow emzyme (5 units/µl) were added and the reaction mix was incubated for at least one hour (or overnight) at 37°C. Free nucleotides were removed by precipitation of the labeled DNA fragments. The DNA pellet was resolved in 100 µl ddH_20. 1 µl was measured in a scintillation-counter (LS 6000SE; Beckman).

Hybridization of the Southern blot

Wash buffer: 1% SDS, 40 mM NaP_i pH 6.7

Church buffer: 7% SDS, 300 mM NaP_i pH 6.7, 5 mM EDTA

The membrane filters with immobilized DNA fragments were placed in hybridization glass tubes and shortly incubated with prewarmed wash buffer at 65°C. Subsequently, the filters were saturated by three hour incubation with 20 ml prewarmed prehybridization buffer (Church) at 65°C with continuous rotation in the hybridization oven. 2 x 10^6 cpm/ml radioactive labelled DNA probe were added to the hybridization solution (Church) and boiled for 10 min. The prehybridization buffer was discarded and replaced with the hybridization solution. The membranes were incubated overnight at 65°C under continuous rotation in an hybridization oven. After washing three times for 20 min with wash buffer at 65°C the membranes were wrapped with transparent foil and exposed to X-ray films (Hyperfilm™ MP Amersham) with intensifying screen for several days at -80°C or to a phosphor imaging plate at room temperature to detect bound probe.

3.2.6 Genotyping of mice and embryos

DNA isolation from tail biopsies and yolk sacs

TE buffer:	10 mM Tris-Cl, pH 8.0, 1 mM EDTA
Proteinase K buffer:	0.1 M Tris/HCl pH 8.0, 5 mM EDTA, 0.5% SDS, 200 µg/ml proteinase K

To isolate DNA from tail biopsies the tail clippings of 2 to 5 mm length were incubated overnight in 500 µl proteinase K buffer at 56°C. The samples were centrifuged for 10 min at 16000 g to remove unsoluble debris. Genomic DNA in the supernatant was precipitated by addition of 450 µl isopropanol. After washing with 70% ethanol 100-200 µl TE buffer were added to the DNA pellet. Incubation of the DNA for 30 min at 65°C inactivated DNases and completely dissolved the DNA. Genomic DNA solution was stored at 4°C.

Genotyping by PCR

10x PCR reaction buffer: 500 mM KCl, 100 mM Tris/HCl pH 8.8, 0.1% Triton

To genotype mice or embryos, genomic DNA was extracted from tail clippings, yolk sacs or embryonic tissues and genotyped by PCR. All reactions were performed in thin-walled PCR tubes and were run in PCR cyclers Primus 96plus (MWG Biotech AG).

EGFΔneo- PCR

PCR program:

1x	94°C	5 min
30x	94°C	30 sec
	57°C	1 min
	72°C	30 sec

PCR reaction set up:

32.5 µl	ddH_2O
5 µl	10 x PCR reaction buffer
5 µl	15 mM $MgCl_2$
2.5 µl	DMSO
1 µl	EGFΔneo FOR (see 3.1.1)
1 µl	EGFΔneo REV (see 3.1.1)
1 µl	dNTPs (10 mM)
1 µl	Taq polymerase
1 µl	genomic DNA

To detect the $Dll1^{Dll3HA}$, $Dll1^{Dll3}$, $Dll1^{Dll1DSL\text{-}\Delta Y}$ or $Dll1^{Dll1DSL\text{-}\Delta G}$ alleles after Cre recombinase-mediated excision of the floxed PGKneo cassette a forward primer aligning to the 3'UTR of the 11th exon of *Dll1* and a reverse primer aligning to the 2nd intron were used to amplify a 280 bp PCR product.

Dll1LacZ-PCR

PCR program:

1x	94°C	5 min
40x	94°C	30 sec
	53°C	30 min
	72°C	30 sec

To detect the *Dll1* null allele $Dll1^{LacZ}$ the PCR reaction mix described for the EGFΔ neo- PCR was set up with the primers Melta38 and LacZ1/Dll1 Ko (see 3.1.1). After amplification with the program above a 580 bp PCR product indicated the $Dll1^{LacZ}$ allele.

Dll1 wt-PCR

PCR program:

1x	94°C	3 min
40x	94°C	30 sec
	63°C	30 min
	72°C	45 sec

To detect the *Dll1* wildtype allele the PCR reaction mix described for the EGFΔ neo- PCR was set up with the primers Dll1F2 and Dll1R1 (see 3.1.1). After amplification with the following program a 425 bp PCR product indicated the *Dll1* wildtype allele.

Dll3 pudgy-PCR

PCR program:

1x	94°C	3 min
40x	94°C	30 sec
	61°C	30 min
	72°C	45 sec

PCR reaction set up:

65 µl	ddH2O
10 µl	10 x PCR reaction buffer
10 µl	15 mM $MgCl_2$
5 µl	DMSO
2 µl	primer Dll3 pu1 (see 3.1.1)
2 µl	primer Dll3 pu2 (see 3.1.1)
2 µl	dNTPs (10 mM)
2 µl	Taq polymerase
2 µl	Genomic DNA

To detect the *Dll3* null allele (pudgy) the PCR reaction was set up and run as described above. After amplification, PCR products were precipitated, cleaved with *Hae*III and separated on

3% agarose gels. A 100 bp fragment indicated the *Dll3*^{pu} allele, a 65 bp fragment the *Dll3* wildtype allele.

3.2.7 PCR-screening of ES cell clones for correct targeting events

Mastermix 1:

1 µl	primer EGF3'#1 (see 3.1.1)
1 µl	primer EGF3'#2 (see 3.1.1)
1 µl	dNTPs
20 µl	ddH_2O

Mastermix 2:

5 µl	10x PCR buffer (Roche)
19 µl	ddH_2O
0.4 µl	High Fidelity Taq polymerase
1 µl	Taq polymerase (conc.)

PCR program:

1x	94°C	2min
10x	94°C	30 sec
	58°C	30 sec
	68°C	4 min
20x	94°C	30 sec
	58°C	30 sec
	68°C	4 min + 5 sec each cycle
	68°C	7 min

To genotype the G418-resistant ES cells and the offspring of the founder mice the "Expand High Fidelity PCR System" (Roche) was used. Two mastermixes were prepared. After filling 23 µl of Mastermix 1 in each tube, 2 µl ES cell DNA and 25 µl of Mastermix 2 were added PCR reactions were run on a 0.7% agarose gel. For correctly targeted alleles a 4.2 kb PCR product was expected.

3.3 Methods of protein biochemistry

3.3.1 SDS-PAGE

Stacking gel buffer:	0.5 M Tris/HCl pH 6.8, 0.4% SDS
Separating gel buffer:	1.5 M Tris/HCl pH 8.8, 0.4% SDS
2x sample buffer:	125 mM Tris/HCl pH 6.8, 20% glycerine, 4% SDS, 2% β-mercaptoethanol, ~10 µg/ml bromphenolblue
Electrode buffer:	25 mM Tris, 192 mM glycine, pH 8.3, 0.1% SDS

10% SDS-polyacrylamide gels were prepared using 30% acrylamide/0.8% bisacrylamide, stacking gel buffer, seperating gel buffer, SDS, ammoniumpersulfate and TEMED according to standard protocols (Ausubel *et al.*, 2007).

Samples were boiled in sample buffer for 5 min, spun down and loaded on the gel together with PageRuler^{TM} prestained protein ladder Plus (Fermentas). Gels were run in a electrophoresis chamber of the "Mini Protean II System" (Bio-Rad) at 200 V for ~45 min in electrode buffer. At this point the gels were either transferred to a membrane (see below) or stained with Coomassie.

3.3.2 Western blot

Transfer buffer:	25 mM Tris, 192 mM glycine, pH 8.3 (w/o adjusting the pH)
PBS:	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3
PBST:	PBS, 0.5% Tween20

The SDS-polyacrylamide gel was transferred to a PVDF membrane by wet tank blotting using the "Mini Trans-Blot Electrophoretic Transfer Cell" (Bio-Rad). For this purpose the gel was equilibrated and the membrane, filter paper and fiber pads were soaked in transfer buffer for 5-10 min. A transfer sandwich was prepared according to the instruction manual. A high intensity field transfer was performed at 100 V and limited to 350 mA for 1 h using the Bio-Ice cooling unit. After the transfer, the membrane was blocked with 5% milk in PBST for at least 30 min at RT. Subsequently, the membrane was incubated with the primary antibody diluted in 5% milk in PBST o/n at 4°C. The next day it was washed three times with PBST for 20 min and incubated with the secondary antibody coupled to peroxidase and diluted in 5% milk in PBST for 1-2 h at RT. Afterwards the membrane was washed again three times with PBST for 20 min and once with PBS. For POD detection the ECL Western blotting detection

reagents (Amersham) were used and the membrane was exposed to a Hyperfilm™ ECL film (Amersham) for the required time.

3.3.3 Cell surface biotinylation

PBS c/m	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$
Quench buffer:	50 mM glycine in DMEM
RIPA:	50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, supplemented with 2.8 µg/ml aprotinin, 0.15 mM benzamidine, 2.5 µg/ml leupeptin and 2.5 µg/ml pepstatin A

For the biotin-streptavidin pull down cells were plated on 60 mm ∅ cell culture dishes and grown to confluence. Plates were washed three times with cold PBS c/m and placed on ice with 500 µl PBS. 10 µl of Sulfo-NHS-LC-Biotin solution (5 mg/ml in 0.1 M sodium phosphate buffer, pH 7; Pierce Chemical Co.) were added three times in 10-min intervals. After 30 min, the biotin solution was aspirated, and the plates were washed once with 50 mM glycine in DMEM and incubated for 30 min to quench the biotinylation reaction. Cells were washed twice with PBS c/m and lysed with 400 µl RIPA. Lysates were incubated for 30 min on ice and centrifuged for 10 min at 12000 g to remove cellular debris. The biotinylated proteins were precipitated with streptavidin agarose (Sigma) overnight at 4°C. The streptavidin agarose beads were washed three times with RIPA before resuspension in 2× sample buffer. Equivalent amounts of lysates and precipitates were subjected to SDS-PAGE and analyzed by Western blotting as described.

3.3.4 Metabolic labeling with [^{35}S]-sodiumsulfate and subsequent immunoprecipitation

PBS:	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3
Sulfate-free Joklik MEM:	MEM Joklik (Sigma) supplemented with 1.8 mM $CaCl_2$, 100 µM non-essential amino acids, 10% dialyzed FCS
Lysis buffer:	PBS, 1%NP40, 1% TritonX-100, 2 µg/ml leupeptin, 1 mM PMSF
Coomassie Brilliant Blue:	methanol, 0.1% aqueous coomassie brilliant blue, glacial acetic acid, (2:2:1)
Destaining solution:	5% methanol, 7.5% glacial acetic acid
2x sample buffer:	125 mM Tris/HCl pH 6.8, 20% glycerine, 4% SDS, 2% β-mercaptoethanol, ~10 µg/ml bromphenolblue

CHO cells were grown to 80% confluence on 100 mm ∅ cell culture dishes. Cells were washed once with sulfate-free Joklik MEM and incubated with this medium for 24 h to empty

the endogenous sulfate storage. Afterwards the medium was exchanged for sulfate-free Joklik MEM medium with 100 µCi/ml (3.7 MBq/ml) [^{35}S]-Na_2SO_4 (PerkinElmer LAS GmbH). Cells were labeled for 24h at 37°C and 5% CO_2.

The next day the medium was discarded and cells were washed twice with PBS. Cells were lysed by addition of 700 µl lysis buffer and incubation on ice for 10 min. Cells were scraped off the dishes. Cell lysates were collected in separate tubes. After 30 min incubation on ice, lysates were centrifuged to remove unsoluble cell debris. Cleared lysates were incubated with antibodies against DLL1 or PSGL-1, respectively. After 2 h incubation at 4°C by continuous rotation on a wheel, ProteinG sepharose (GE Healthcare) was added and lysates were further incubated overnight.

Sepharose beads were washed three times with lysis buffer and precipitated by centrifugation for 30 sec at 1000 g. Beads were boiled in 2x protein sample buffer and spun down prior to loading on a SDS polyacrylamide gel. After gel electrophoresis, gels were fixed by staining with Coomassie-brilliant Blue and subsequently destained with destaining solution. Afterwards gels were incubated with NAMP100 Amplify™ fluorographic reagent (Amersham), vacuum-dried and wrapped in transparent film. Hyperfilm™ MP (Amersham) films were exposed to the gels for 2-3 weeks and subsequently developed. CHO cells were used as a negative control. Stable CHO cells expressing PSGL-1 were used as a positive control for monitoring sulfation efficiency.

3.3.5 Immunofluorescence staining

For immunocytochemistry cells were grown on gelatine-coated coverslips. Cells were rinsed twice with PBS and fixed with methanol for 10 min at 4°C. After three washes with PBS, the cells were blocked with 5% donkey serum in PBS for 30 min at room temperature. Cells were incubated with the primary antibody for 1-2 h at room temperature and, after three washes with PBS, with the fluorochrome-conjugated secondary antibody for 1 h. After washing, the coverslips were mounted in Gel/Mount (Biomeda) or ProLong Gold antifade (Invitrogen). Texas Red-, FITC- and/or Alexa Fluor®488-labeled cells were analyzed at room temperature by confocal laserscanning microscopy using the LSM 510 Meta (Carl Zeiss MicroImaging, Inc.) connected to the inverted microscope Axiovert 200M with a Plan Apochromat 63×/1.4 oil differential interference contrast objective. For image acquisition the LSM 510 software (Carl Zeiss MicroImaging, Inc.) was used. Pictures were processed and assembled using Photoshop and Illustrator CS.

3.4 Methods of cell biology

3.4.1 Cell culture

Cell lines were grown at 37°C and 5% CO_2 in cell culture dishes.

Cells were passaged with standard trypsinization. To store cells for a longer period, cells were frozen as cell suspension with freezing medium in a cryovial. For short-term storage cells could also be frozen as adherent monolayer in 24-well plates with added freezing medium. Rapid thawing of the cell cryostocks was achieved with prewarmed cell culture medium.

3.4.2 Transfection of cells

One day before transfection cells were seeded in cell culture dishes such that 50-70% confluence was achieved the next day. On the day of transfection medium was changed at least two hours before transfection. Cells were transfected using JetPEI transfection reagent (Biomol) according to the manufacturer's protocol.

3.4.3 Generation and subcloning of stable cell lines

For the generation of CHO cell lines stably overexpressing a specific protein, cells were transfected with the corresponding construct using the JetPEI transfection reagent (Biomol), splitted 24 h later 1:50-1:100 on several cell culture dishes and grown under selection (500 µg/ml G418; 250 µg/ml zeocin) for 7-12 days. CHO cell clones were picked and expanded on a 96-well plate under selection. Clones were further expanded on a 24-well plate and duplicated. Confluent cells from one plate were lysed with 2x sample buffer, sonified and protein expression levels were determined by Western blot analysis. Freezing medium was added to the cells on the other 24-well plate and the plate was frozen at -80°C. CHO cell clones with efficient protein expression were thawn and expanded. Clones that showed a heterogenous cell staining were subcloned by a limited dilution. CHO cells were seeded on 96-well plates in a dilution of 0.5 cells per well to get monoclonal cell populations. Cells from wells with single cell clones were expanded and analyzed as described above.

3.4.4 Notch transactivation assay

5x Extraction buffer:	125 mM Tris pH 7.8 (adjust pH with phosphoric acid), 10 mM EDTA, 50% glycerine, 5% TritonX-100 dilute 1:5 and add 10 mM DTT before use
Luciferase buffer:	25 mM diglycine, 15 mM $MgSO_4$, autoclave, store at 4°C add 5 mM ATP before use
Z-buffer:	60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, autoclave, store at 4°C add 35 µl β-mercaptoethanol per 10 ml buffer before use
Luciferin stock:	25 mM luciferin in 25 mM NaOH, store at-20°C dilute 1:100 before use
ONPG stock:	4 mg/ml o-Nitrophenyl-β-galactopyranoside in Z-buffer, store at -20°C
Stop solution:	1 M Na_2CO_3

HeLa cells stably expressing Notch1 (HeLa-N1) were transiently transfected with a luciferase reporter construct (RBP-luc; (Minoguchi *et al.*, 1997) or Hes1-luc; (Logeat *et al.*, 1998)) using JetPEI (Biomol), following the manufacturer's instructions. After ~16 h, 10^6 transfected HeLa-N1 cells were cocultivated on 6-well plates for 24 h with 10^6 CHO cells expressing different ligands. Each CHO cell line was cocultivated twice per experiment. Luciferase activity was measured using the "Dual-Luciferase Reporter Assay System" (Promega) following the manufacturer's instructions or as decribed below.

After cocultivation cells were washed once with PBS and lyzed by adding 200 µl extraction buffer. After 10 min, cells were scraped off the dishes and incubated for 5 min on ice. After centrifugation cleared lysates were used for analysis of luciferase and β-galactosidase activities. Just before measuring at the luminometer (Lumat LB9501, Berthold) 40 µl cell lysate were added to 300 µl luciferase buffer in the test tubes. 100 µl of luciferin solution were added by the luminometer to each sample. Samples were measured in duplicates. To quantify β-galactosidase activity 400 µl Z-buffer were added to 40 µl cell lysate. The reaction was started by addition of 100 µl ONPG-solution, incubated at 37°C and stopped by addition of 250 µl 1 M Na_2CO_3 when intense yellow staining was observed. Samples were measured in triplicates using a photometer at 405 nm. Firefly luciferase activity was normalized to cotransfected *Renilla* luciferase or LacZ activity (pRL-TK, Promega or pEQ176, Schleiss *et al.*, 1991). Expression of chimeric ligands was verified by Western blot analysis.

For the analysis of cis-inhibition, HeLa-N1 cells were cotransfected with the corresponding ligand pTracerCMV expression vector in addition to the luciferase constructs RBP-luc and pRL-TK. Expression levels of transiently expressed ligands were analyzed by Western blot analysis.

3.5 Methods of embryology

3.5.1 Mouse keeping, embryo preparation and PSM dissection

Mice were kept and bred in the central animal facility of the Medical School Hannover. Timed matings were set up and females were examined daily for a vaginal plug to obtain mouse embryos of a known gestation stage. Noon on the day of appearance of the vaginal plug was designated as day 0.5 post coitum (E0.5). Embryos were dissected from the uterus and the Reichert's membrane was removed under the stereomicroscope by standard techniques (Beddington, 1987).

Embryos for skeletal preparations were dissected in water.

Embryos for immunofluorescence stainings were dissected and rinsed in phosphate-buffered saline (PBS) and fixed overnight with 4% paraformaldehyde (PFA) in PBS.

For PSM protein lysates, the PSM of an E9.5 embryo was dissected in PBS and cut after the first somite with an insect needle. Each PSM was stored separately in 20 µl 2x sample buffer at -80°C. For genotyping, the rest of the embryo was digested with proteinase K, genomic DNA was isolated and genotyped by PCR. Prior to western blot analysis the PSMs were lysed in a sonifier water bath for ~25 min.

3.5.2 Whole mount immunofluorescence staining of embryos

PBS:	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3
PBS-TR:	0.1% Triton X-100 in PBS
Blocking solution:	1% BSA in PBS-TR
Antigen retrieval solution:	10 mM sodium citrate buffer pH 6

Embryos were dissected at E9.5, fixed in 4% paraformaldehyde/PBS overnight at 4°C, washed three times with PBS, dehydrated through a standard methanol/PBS series and stored in methanol at −20°C. Rehydrated embryos were washed three times in antigen retrieval solution, heated to 100°C for 10 min and allowed to cool down to room temperature. Embryos were washed in water and cracked for 8 min in 100% acetone prechilled to −20°C and then rehydrated in water. Embryos were blocked overnight in blocking solution at 4°C. Embryos were incubated with primary antibodies diluted in blocking solution at 4°C for 2–3 days with gentle agitation. Embryos were washed six times in PBS-TR for 30 min each and then reblocked for 1–2 h at room temperature. Fluorochrome-conjugated secondary antibodies

were diluted in blocking solution and incubated with embryos at 4°C overnight with gentle agitation. The embryos were washed six times in PBS-TR for 30 min each, cleared by successive 10-min washes in 25% glycerol, 50% glycerol, and 70% glycerol. The posterior third of the embryos was dissected and flat-mounted sagittally in ProLong Gold antifade (Invitrogen). Texas Red- and/or Alexa Fluor®488-labeled cells were analyzed by confocal laser-scanning microscopy using the LSM 510 Meta (Carl Zeiss MicroImaging, Inc.) connected to the inverted microscope (Axiovert 200M; Carl Zeiss MicroImaging, Inc.) with a Plan Apochromat 63×/1.4 oil differential interference contrast objective. For image acquisition, LSM 510 software was used. Pictures were processed and assembled using Photoshop and Illustrator CS (Adobe).

3.5.3 Skeletal preparation of E18.5 embryos

Alcian Blue cartilage staining sol.:	150 mg/l Alcian Blue 8GX in 80% ethanol, 20% acetic acid
Alizarin Red bone staining sol.:	50 mg/l Alizarin Red in 0.1% KOH

E18.5 embryos were dissected and macerated in water for 2 h. Embryos were incubated in 65°C warm water for 1-2 min and quenched with cold water. Afterwards embryos were skinned and eviscerated (inner organs were used for genotyping). Embryos were fixed in 100% ethanol for 1-2 days. Skeletons were stained as follows: Cartilage staining was performed by incubating embryos in Alcian Blue cartilage staining solution for 2-3 days. Subsequently, embryos were washed in 100% ethanol for 2 days and tissue was digested by incubation for 1-5 h in 0.5% KOH. Afterwards staining of the bones by incubation in Alizarin Red bone staining solution for 5-16 h was performed. Embryos were digested further in 0.05% KOH until the tissue was transparent. Skeletons were stored in 30% glycerine/0.01% KOH and photographed using a microscope (M420; Leica) with Apozoom 1:6 and Photo grab-300Z version 2.0 software (Fuji Photo Film Co.).

4 Results

At the beginning of this project little was known about the properties and functions of the divergent DSL protein DLL3. Initial studies in *Xenopus laevis* indicated that it acts as a Notch activator because ectopic *Dll3* expression inhibited primary neurogenesis (Dunwoodie *et al.*, 1997).

Given the distinct loss-of-function phenotypes of *Dll3* and *Dll1* null alleles, the divergent DSL domain and reduced number of EGF-like repeats in DLL3 the question was raised whether *Dll3* can still functionally substitute *Dll1* by its biochemical properties. To address this question *in vitro*, *Dll1* and *Dll3* as well as a number of mutant and chimeric ligands were overexpressed in cell culture, studied with respect to their subcellular localization and subsequently used in transactivation assays together with cells harboring a Notch-responsive reporter. Furthermore, in order to analyze a potential functional redundancy *in vivo*, a mouse model was generated by a targeted knock-in in which the *Dll3* cDNA was expressed under the regulatory control of *Dll1*.

4.1 DLL3 does not activate Notch signaling in vitro

To express wildtype DLL1 and DLL3 in cell culture, the full length cDNAs were cloned into the eukaryotic expression vector pTracer that contains a CMV promoter driving the expression of the inserted coding sequence and a zeocin resistance cassette for selection. A flag or HA tag was added C-terminally to allow for monitoring of protein expression levels. Previously, it was shown that the addition of a tag at the C-terminus does not interfere with binding of the ligand to the Notch receptor (Lindsell *et al.*, 1995; Oda *et al.*, 1997; Shimizu *et al.*, 2000b).

An internal ribosomal entry site (IRES) and a neomycin resistance cassette were added downstream of the inserted cDNA to allow for G418-selection. Thus, in the course of generating stable cell lines, treatment of transfected cells with G418 should select for cell clones expressing the inserted cDNA and therefore facilitate the search for cell clones with high expression levels. However, only 5 to 50% of the G418-resistent CHO cell clones showed efficient expression of the inserted coding sequence as determined by Western blot analysis.

To analyze the activation of Notch by DLL1, DLL3 and their variants a transactivation assay (Logeat *et al.*, 1998) was established in the laboratory and optimized. This assay was based on a luciferase reporter gene that was expressed under the control of a promoter containing RBPJκ binding sites. Activation of Notch in cells carrying such a reporter resulted in Notch-dependent expression of luciferase. Notch activation was achieved by cocultivation of ligand-expressing cells with Notch-expressing cells transfected with the Notch-responsive luciferase reporter construct. Subsequently, the cells were lysed and assayed for luciferase activity.

A HeLa cell line stably expressing Notch1 (HeLa-N1, a kind gift of A. Israel, Institute Pasteur, Paris) was used for the transactivation assay. Notch1 was shown to interact with DLL1, Jagged1 and Jagged2 (Shimizu *et al.*, 2000b) and is indispensable for somitogenesis. Accordingly, ligand interaction with Notch1 should reflect the situation in the presomitic mesoderm.

Since the cell type and the design of the reporter construct as well as the ratio of Notch1- and ligand-expressing cells influence the overall readout of the coculture experiment, various assay conditions were tested to improve the sensitivity of the transactivation assay. Initially, two different Notch-responsive luciferase reporter constructs were used in cocultures of transfected HeLa-N1 cells with OP9 cells stably expressing Dll1 (kindly provided by Dr. A. Israel, Institute Pasteur, Paris). The Hes1 luciferase construct (Hes1-luc) consisted of the endogenous Hes1 minimal promoter, comprising two RBPJκ binding sites, upstream of the firefly luciferase coding sequence (Jarriault *et al.*, 1995). The other reporter construct (RBP-luc) consisted of twelve copies of the RBPJκ binding site from the EBV TP1 promotor linked to the beta-globin promoter upstream of the firefly luciferase coding sequence (Minoguchi *et al.*, 1997). In the transactivation assay the RBP-luc vector showed more than two-fold stronger activation capacity than the Hes1-luc vector (Fig. 4.1 A). Therefore the RBP-luc construct was used as reporter in the subsequent experiments.

With OP9 cells it proved difficult to obtain single cell suspension to precisely count cell numbers hence cocultivation for transactivation was tested with transfected CHO cells (Chinese hamster ovary cells). Since transient expression of *Dll1* gave highly variable results in this transactivation assay due to the variance in expression levels, stable CHO cell lines were generated. After transfection with the respective pTracer expression construct, CHO cells were grown under zeocin-selection for integration and G418-selection for expression of the DLL1 protein. Resistant cell clones were expanded and screened by Western blot analysis for high expression levels of the overexpressed flag- or HA-tagged protein. After generation of stable CHO cell lines expressing Dll1 they were tested in the transactivation assay for their

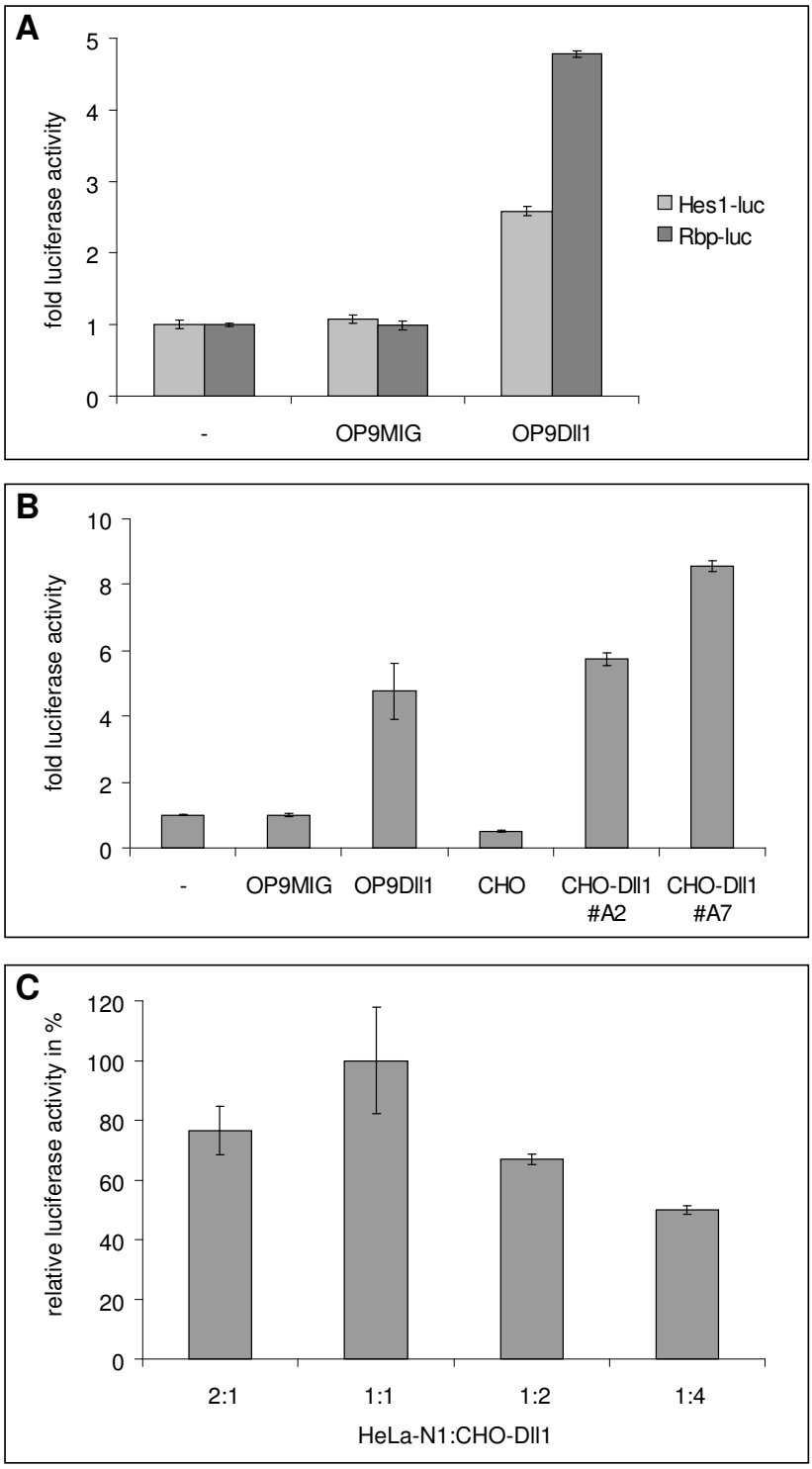

Fig. 4.1: Establishment and optimization of a transactivation assay. (A) After cocultivation with OP9 cells expressing Dll1, the transfected RBP-luc reporter construct induced Notch signaling in HeLa-N1 cells two fold stronger than the Hes1-luc plasmid. Transfected HeLa-N1 cells without cocultivation were set as reference. The vector control expressing GFP (OP9MIG) showed no transactivation. (B) CHO-Dll1 cell lines transactivated HeLaN1 transfected with RBP-luc reporter construct stronger than the OP9Dll1 cell line. C) A 1:1 ratio of HeLa-N1 and CHO-Dll1 cells showed the strongest induction of Notch1 signaling via the RBP-luc reporter plasmid. Transfection efficiency was normalized to the activity of β-galactosidase expressed by a cotransfected CMV-LacZ construct. Two cocultivations were performed for each experiment, bars represent standard deviations.

potential to induce Notch activation. The CHO-Dll1 cell line #A7 was able to induce Notch1 activation to a considerably larger extent than the corresponding OP9 cell line (Fig. 4.1 B) and therefore used for further experiments. With regard to the ratio of cell numbers, a cocultivation with equal cell numbers of the HeLa-N1 and CHO-Dll1 cell lines showed the best transactivation result (Fig. 4.1 C). These settings were used in all following transactivation assays.

To compare the transactivation ability of DLL3 to that of DLL1, stable cell lines were generated (for information concerning the CHO cell lines used for the experiments described in the figures of this thesis see appendix, Tab. 1). In transactivation assays DLL1 expressing CHO cells activated the Notch1 receptor more strongly compared to untransfected CHO cells. In contrast, CHO cells stably expressing DLL3 did not induce Notch1 signaling (Fig. 4.2).

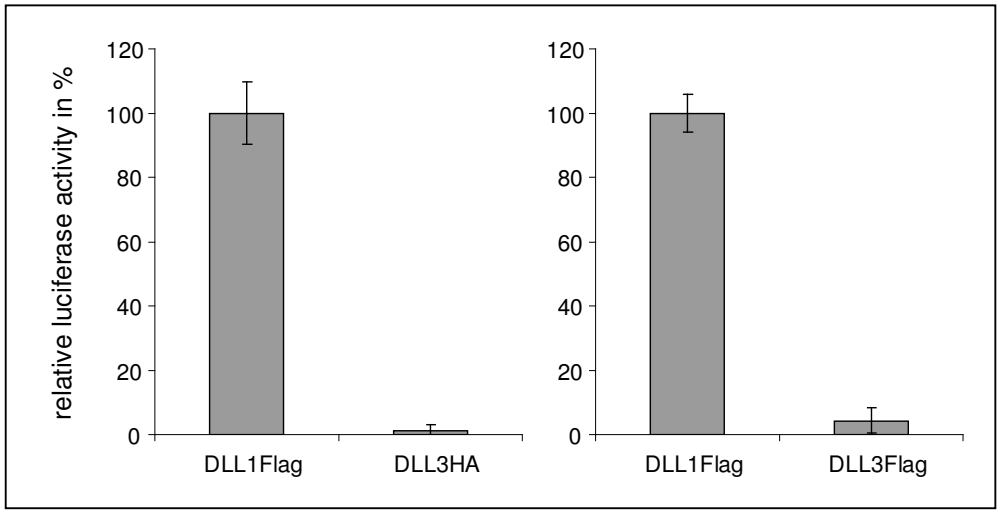

Fig. 4.2: Notch activation by DLL1 and DLL3. CHO cells stably expressing DLL1Flag strongly activated Notch1 signaling in with RBP-luc reporter transfected HelaN1 cells (set as 100%), while CHO cell lines stably expressing DLL3 did not induce Notch1 activation (DLL3HA ~1,3% ± 1,7%SD; DLL3Flag ~4,3% ± 4,1%SD). Untransfected CHO cells were set as 0%. Transfection efficiency was normalized to the activity of β-galactosidase or Renilla luciferase expressed by cotransfected CMV-LacZ or pRL-TK (Promega) construct, respectively. Four cocultivations were performed with DLL3HA CHO cells analyzed in two independent experiments each, including negative and positive controls. Six cocultivations were performed with DLL3Flag CHO cells analyzed in three independent experiments each, including negative and positive controls. Bars indicate the standard deviation. For information concerning CHO cell lines see appendix, Tab. 1.

4.2 *DLL3 is predominantly located inside the cell*

Based on the presence of a signal sequence and a transmembrane domain, all Notch ligands are assumed to be transmembrane proteins present on the surface of the signal-sending cells. Given the inability of Dll3 to activate Notch *in vitro* it was important to verify surface expression of this protein. To analyze cell surface presentation, proteins exposed on the cell surface of CHO cell lines were labeled with biotin. After cell lysis biotinylated proteins were precipitated by streptavidin and analyzed for the presence of DLL1 and DLL3, respectively, by western blot analysis.

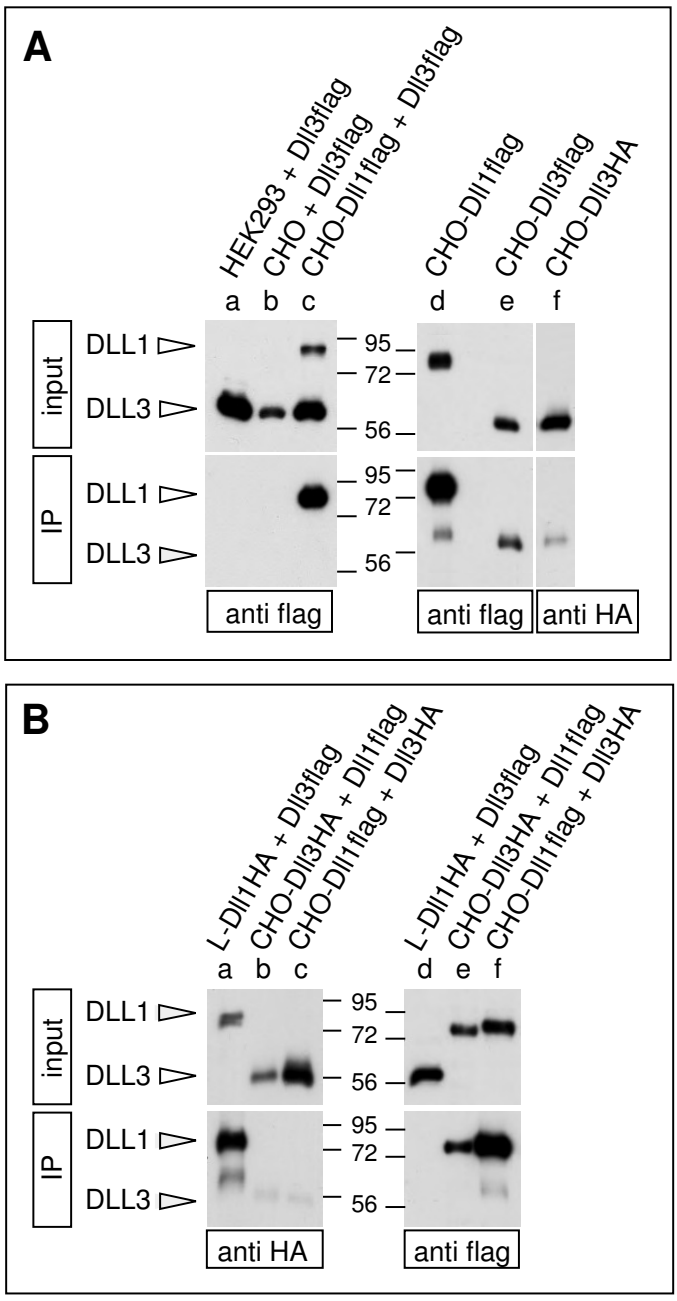

Fig. 4.3: Localization of DLL3 in various cell lines. Western blot analysis of cell lysates (input) and streptavidin-immunoprecipitated protein after surface biotinylation (IP). (A) Transiently expressed DLL3flag protein (a-c) was not detected on the cell surface. On the surface of CHO cells, stably expressed DLL1flag is readily detected (IP, c) although expressed at lower levels than transiently expressed DLL3 (input, c). In CHO cell lines stably expressing DLL3flag (e) or DLL3HA (f) only low amounts of DLL3 were present on the cell surface although total protein levels were similar to those of cells stably expressing DLL1 (d). (B) L cells coexpressing DLL3flag at significantly higher levels than DLL1HA present DLL1 efficiently on the surface but not DLL3 (a, d). CHO cells coexpressing DLL3HA and DLL1flag (b, c, e and f) present DLL1 efficiently on the surface but DLL3 only in trace amounts.

Surprisingly, the analysis of the surface expression in the stable CHO cell lines revealed that DLL3 presentation on the cell surface was highly reduced compared to DLL1.

In CHO and HEK293 cells with high transient DLL3 expression levels no DLL3 protein was present on the cell surface although DLL3 protein was readily detected in the whole cell lysates (Fig. 4.3 A lanes a, b). In the stable CHO-Dll1 cell line, total expression levels of DLL1 in the whole cell lysates were lower than those of transiently coexpressed DLL3. However, in contrast to DLL1, no DLL3 protein was present on the cell surface (Fig. 4.3 A, lane c).

Similarly, among the CHO cell lines stably expressing DLL3 several clones exhibited no detectable or only minor amounts of protein on the surface. The cell line shown in Fig. 4.3 A lane e showed the highest presentation of DLL3 on the surface among all lines examined.

Distinct surface expression of DLL1 and DLL3 was confirmed in CHO cells coexpressing Dll1 and Dll3 with different tags. *Dll3* was transiently expressed in CHO cells stably expressing Dll1 and *vice versa*. In both cases, DLL1 was easily detected on the cell surface, while DLL3 was not, or only in trace amounts (Fig. 4.3 B b, c and e, f). The same was true for DLL3 surface presentation in L cells stably expressing DLL1 and transiently expressing DLL3 (Fig. 4.3 B a, d; L-Dll1HA cells were a kind gift of G. Weinmaster, University of California, Los Angeles). Even when DLL3 was detected on the surface, the relative amounts of DLL3 were always considerably lower than those of DLL1 (compare Fig. 4.3 B lane a with lanes b and c).

To verify these differences in protein localization a second independent approach was pursued. Immunofluorescence stainings of CHO cells expressing either DLL1 or DLL3 or both were established with different antibodies to analyze the subcellular localization of the two DSL proteins *in situ*. In transiently as well as in stably expressing cells DLL1 was readily detected on the cell surface (Fig. 4.4 A, a-d). In some cases depending on the expression level, DLL1 protein was also detected in vesicular structures in the cytoplasm, but the majority of DLL1 protein was associated to the membrane. Occasionally, the contacting surfaces of the cells showed an accumulation of DLL1 protein (Fig, 4.4 A c).

In contrast, DLL3 staining with anti-flag or anti-DLL3ICD antibodies was mainly perinuclear (Fig. 4.4 A e-h) and not detectable at the membrane which is consistent with the surface biotinylation data. Given that the flag tag recognizes the intracellular domain of DLL3 as it is C-terminally linked to it, stainings with antibodies against the intracellular and extracellular domains of DLL1 and DLL3 were compared to exclude the possibility that cleavage of the intracellular domain leads to protein translocation into the cell. Confocal images of these

stainings showed no obvious differences between the detection of the intra- and the extracellular protein domain (Fig. 4.4 B).

When coexpressed, DLL1 and DLL3 colocalized in perinuclear structures but essentially not at the cell membrane (Fig. 4.5 A). To further investigate the subcellular localization of DLL3 protein, costainings with organelle markers were performed. In the perinuclear region, DLL3 partially colocalized with GM130, a marker for the cis-Golgi network. However, DLL3 showed no colocalization with Calreticulin, a marker for the endoplasmic reticulum (Fig. 4.5 B).

Taken together, these data suggest that in cells expressing DLL1 and/or DLL3, DLL1 is largely present on the cell surface while DLL3 localizes to perinuclear regions, including the Golgi apparatus. Since Notch signaling relies on the contact of proteins on the surface of opposing cells, the intracellular localization of DLL3 might provide a 'trivial' explanation for its failure to transactivate Notch.

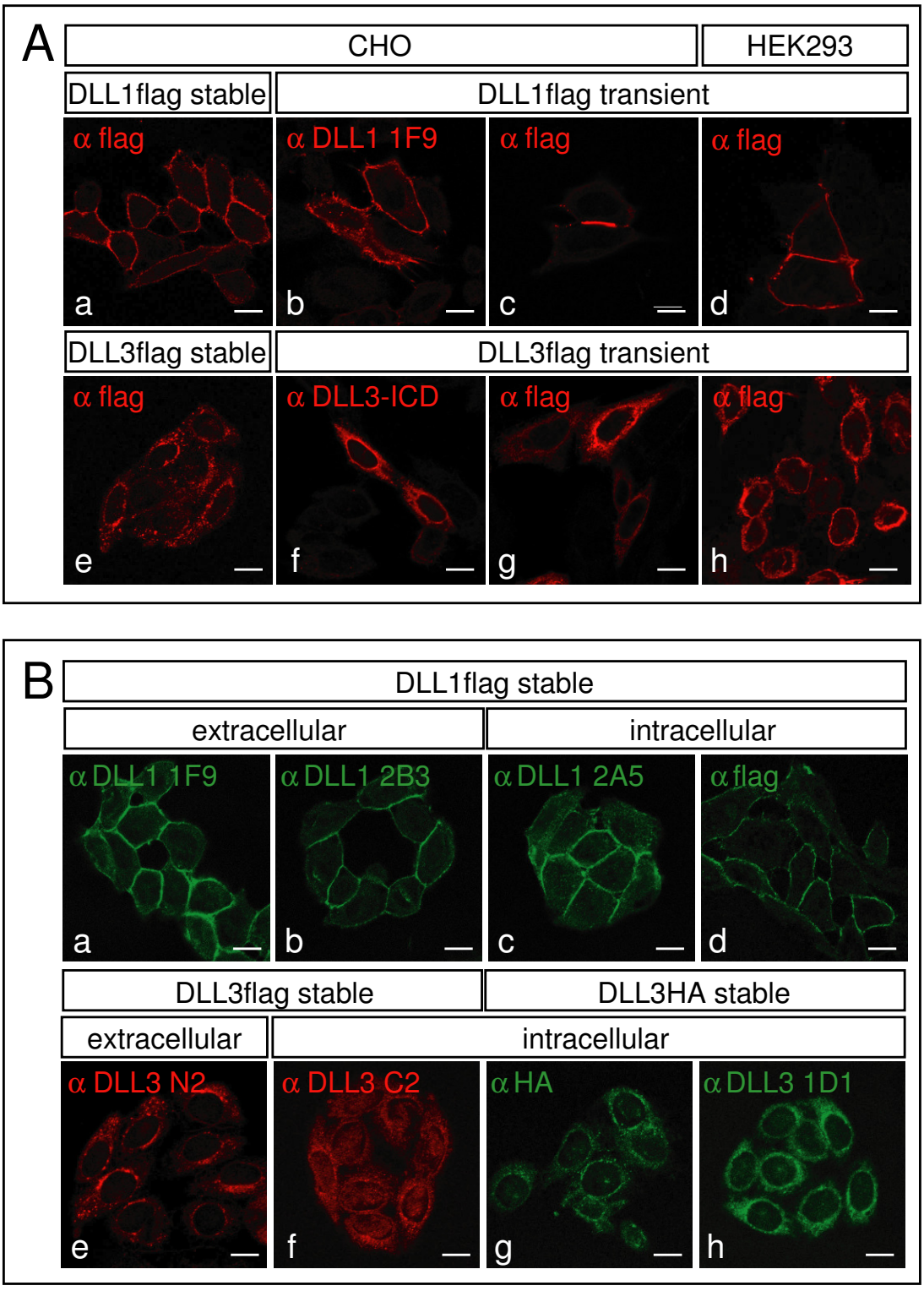

Fig. 4.4: Detection of DLL1 and DLL3 proteins by immunofluorescence in various cell lines. (A) In stably DLL1 expressing CHO cell lines (a) as well as in transiently expressing CHO (b, c) and HEK293 cells (d) DLL1 is present on the cell surface. Dll3 is detected in perinuclear structures in stably DLL3 expressing CHO cells (e) and in transient expressing cells (f-h) (B) Antibodies against the extracellular domain of DLL1 (a,b) or the intracellular domain (c, d) show the same membrane staining. Stainings with antibodies against the extracellular domain (e) or the intracellular domain (f, g) as well as the intracellular tag (HA, h) all reveal DLL3 localization inside the cells. Scale bars, 10 µm.

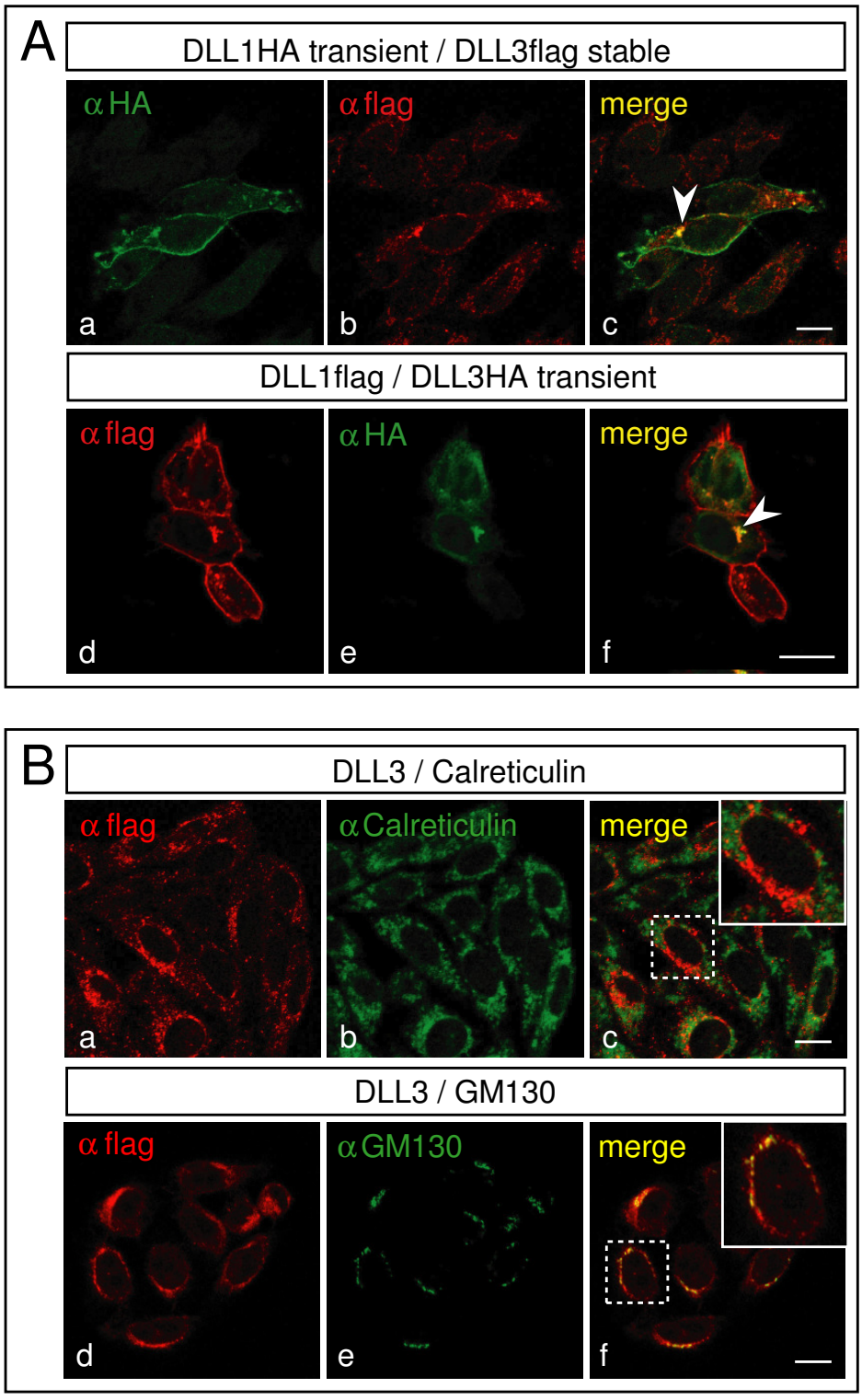

Fig. 4.5: Colocalization studies of DLL1 and DLL3. (A) Colocalization of DLL1 and DLL3 in overexpressing cells. CHO cells expressing DLL1 (a and d) show a clear cell surface staining, whereas DLL3 (b and e) is detected almost exclusively inside the cell. DLL1 and DLL3 colocalize only in some vesicular structures (c, f arrowheads) but not significantly at the membrane. (B) Colocalization of DLL3 with organelle markers. DLL3 colocalizes with the Golgi marker GM130 (f), but not with the marker for endoplasmatic reticulum, Calreticulin (c). Scale bars, 10 µm.

4.3 Endogenous DLL3 colocalizes with the Golgi-Marker GM130

Up to now, subcellular localization of the DSL proteins Dll1 and Dll3 was not studied *in vivo*. To exclude that the observations *in vitro* could be due to high protein levels caused by unphysiological overexpresssion in cell culture, the localization of the endogenous proteins under physiological conditions was addressed by whole mount immunohistochemistry of wildtype mouse embryos using a technique adapted from Gavin Chapman (Victor Chang Cardiac Research Institute, Darlinghurst, Australia). Data were obtained by confocal laser scanning microscopy. Previously, it was shown by whole mount *in situ* hybridization that *Dll1* and *Dll3* mRNAs are coexpressed in the presomitic mesoderm of E9.5 mouse embryos. In addition, mRNA levels were relatively high in the PSM, qualifying this embryonic tissue to study the localization of DLL1 and DLL3 protein in the same cells *in vivo*.

Similar to the observations obtained from cell culture experiments, endogenous DLL1 was predominantly present on the surface of presomitic mesoderm cells and colocalized with plasma membrane proteins detected by an anti-pan-Cadherin antibody (Fig. 4.6 a-c). In addition some intracellular DLL1 was detected that colocalized mainly with the Golgi matrix protein GM130 (Fig. 4.6 f).

Importantly, endogenous DLL3 was not detected at the membrane of PSM cells (Fig. 4.6 l). Two different polyclonal antibodies (C2 against the C terminus/intracellular domain or N2 against the N terminus/extracellular domain of DLL3) showed the same protein localization (data not shown) excluding that cleavage of the protein leads to different localization of extra- and intracellular domain of DLL3.

DLL3 was detected in intracellular regions largely overlapping with the cis-Golgi marker GM130 (Fig. 4.6 m-o), indicating that the localization of DLL3 in the Golgi network observed in overexpressing cells occurs also *in vivo*. Both DSL proteins colocalized in some intracellular areas but were otherwise essentially not overlapping (Fig. 4.6 g-i). Given that Notch ligands need to be present at the cell surface for Notch activation, together with the results from the transactivation assay the findings suggested that DLL3 does not serve as an activating Notch ligand as it is accumulated in the Golgi network under physiological conditions and only minor amounts of DLL3, if any, are present on the surface of presomitic mesoderm cells.

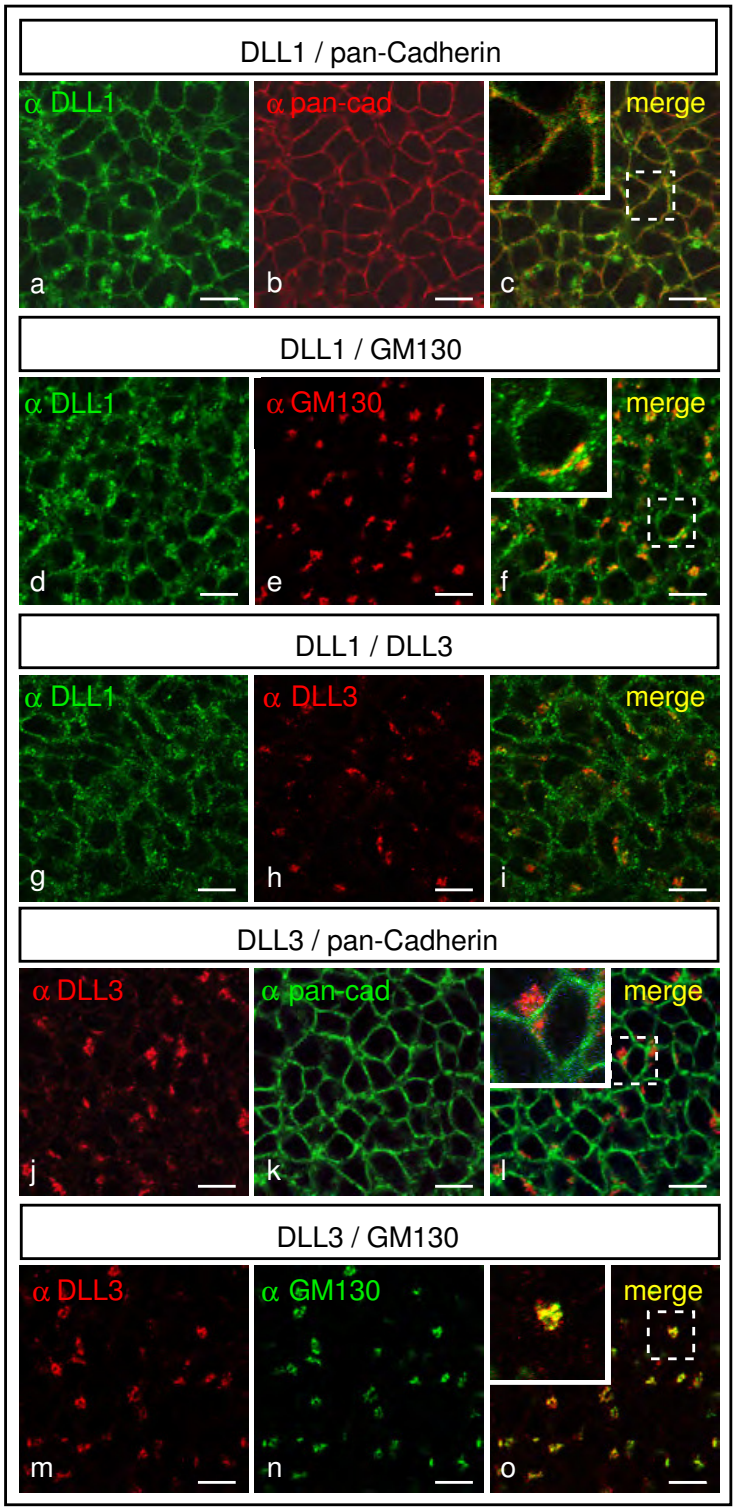

Fig. 4.6: Localization of endogenous DLL1 and DLL3 proteins. Immunofluorescent detection of DLL1 and DLL3 in PSM cells of E9.5 embryos. Endogenous DLL1 is present at the surface (a) and colocalizes with the membrane (c) and in vesicular structures with the cis-Golgi marker GM130 (f). DLL3 does not colocalize with anti-pan-Cadherin staining of the membrane (l) but is detected in vesicular structures in the cytoplasm (m), mostly overlapping with GM130 (o). DLL1 and DLL3 colocalize inside the cell but not at the membrane (i). Scale bars, 10 µm.

4.4 Generation of Dll3 knock-in alleles

In parallel to the *in vitro* experiments, a replacement of *Dll1* by tagged and untagged *Dll3* was approached *in vivo*. The *Dll1* locus was targeted by homologous recombination in ES cells in order to generate mutant mice. The rational behind this strategy was to analyze whether *Dll3* can rescue *Dll1* function under physiological conditions.

A *knock-in* of a "minigene" version of *Dll1* into the *Dll1* locus was previously generated (Schuster-Gossler *et al.*, 2007). Mice carrying this allele were viable and showed no obvious phenotype indicating that the alteration of the *Dll1* locus had no adverse effects on expression of the *Dll1* minigene.

The strategy for targeting the *Dll1* locus by a *Dll3* knock-in ($Dll1^{Dll3ki}$) was adopted from a strategy developed by Dr. Ralf Cordes (Schuster-Gossler *et al.*, 2007).The *Dll1* locus consists of eleven exons with the open reading frame starting at the end of the first exon. A chimeric *Dll3* "minigene" was fused in frame to the start-of-translation codon (ATG) of the endogenous *Dll1* gene, analogous to the $Dll1^{lacZ}$-null allele generated previously by Hrabe de Angelis *et al.* (1997). Thereby, the *Dll3* coding region was expressed under the control of the regulatory elements of the *Dll1* gene and *Dll1* function was simultaneously eliminated. In the *Dll3* minigene, the *Dll3* coding sequence was linked at the 3'end to genomic sequences of the *Dll1* gene containing exons 9–11 (Fig. 4.7 A) and including the 3'UTR that provides the polyadenylation signal. After processing of the primary transcript, the *Dll3* coding sequence was thus flanked by the 5' and 3' UTRs of *Dll1*, which should generate *Dll3* transcripts with stability and processing properties similar to those of the genuine *Dll1* mRNA. The *Dll3* cDNA is expected to be expressed in the same spatially and temporally regulated pattern as *Dll1* since in heterozygous $Dll1^{lacZ}$ mice LacZ expression reflects *Dll1* expression in all tissues analyzed (Beckers *et al.*, 1999; Beckers *et al.*, 2000).

A hemagglutinin (HA) epitope tag followed by three stop codons was included at the carboxyterminal end of *Dll3* coding sequence to monitor expression of the exogenous DLL3 protein. Lindsell *et al.* showed 1995 that the addition of a C-terminal epitope tag to the Jagged protein does not interfere with its function *in vivo*. As a control an untagged version of the *Dll3 knock-in* construct was generated to exclude that phenotypes in the $Dll1^{Dll3HAki}$ mice might attribute to the included HA tag.

A phosphoglycerine kinase (PGK) promoter driven neomycin resistance cassette flanked by loxP sites was introduced 3' to the Dll3-Dll1 fusion as a positive selection marker. Following

expression of Cre recombinase this selection cassette was excised. The removal of the PGK-neo cassette is essential for achieving physiological expression levels of the exogenous DLL3 since it was shown that the *Dll1*^{Dll1ki} allele still carrying the PGK-neo cassette represents a hypomorphic allele of *Dll1* (Schuster-Gossler *et al.*, 2007).

Diphtheria toxin A expression cassettes were included upstream and downstream of the homologous regions as negative selection markers to enrich for homologous recombination events by ablation of ES cell clones with an incomplete recombination.

Linearized vector DNA was electroporated into 129Sv/ImJ embryonic stem cells and G418-

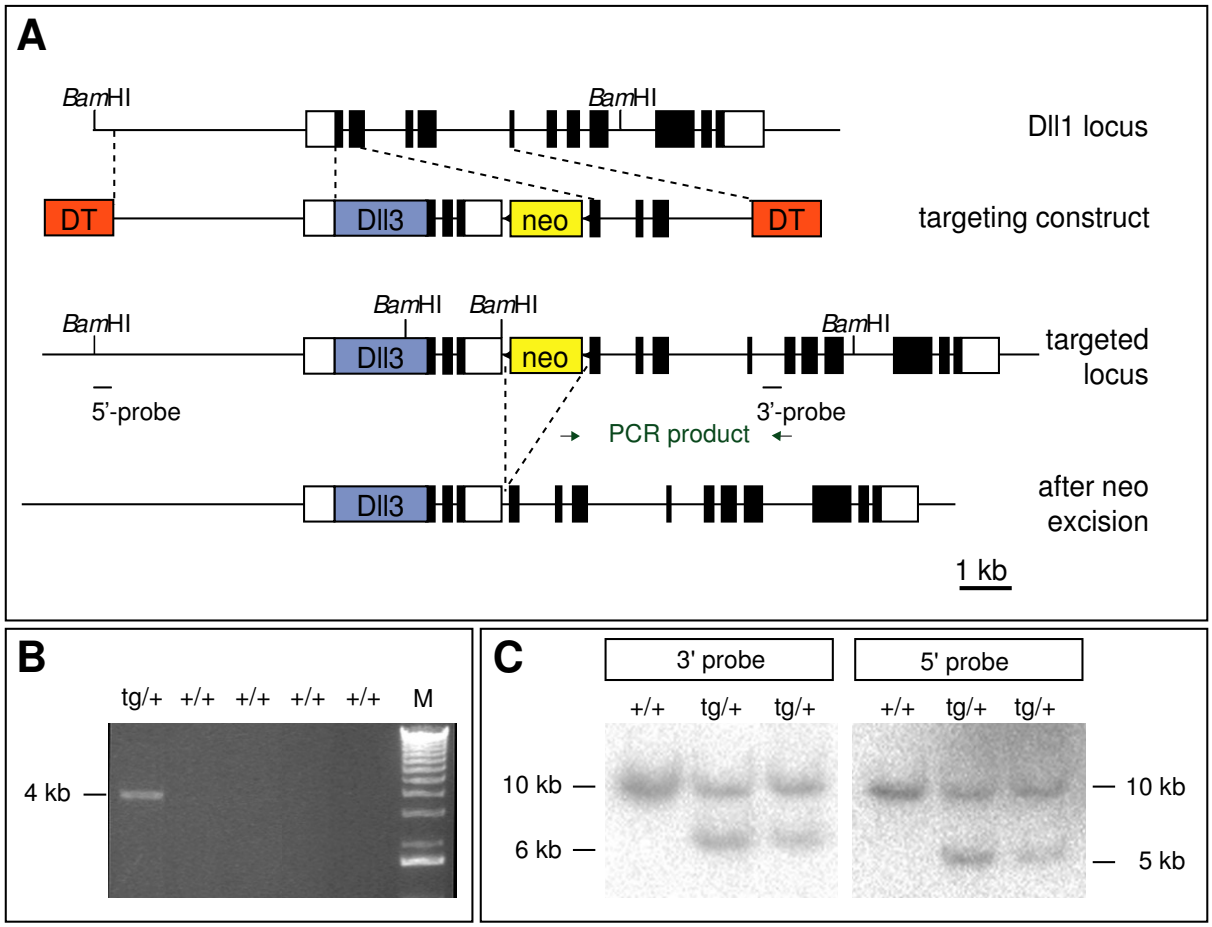

Fig. 4.7: Generation of Dll1^{Dll3HAki} and Dll1^{Dll3ki} mice. (A) Targeting strategy for introduction of Dll3 into the Dll1 locus. White and black boxes indicate noncoding and coding regions, respectively. The blue box indicates Dll3 or Dll3HA cDNA, respectively. PCR primers align to the neo cassette and to the fifth intron, DNA probes for Southern blot analysis lie outside the homologous regions. Neo, PGK-neomycin resistance cassette. DT, diphtheria toxin A chain. (B) PCR-screening of ES cell clones for homologous recombination. Example for a PCR-positive ES cell clone (tg/+) showing a 4kb PCR product. (C) Screening for correctly targeted ES cell clones by Southern blot analysis. The targeted allele shows a 6.5kb band (3'probe) or a 5.5 kb band (5'probe) whereas the wildtype allele displays a 10 kb band (both probes).

resistant ES cell clones were selected by Dr. Karin-Schuster-Gossler and Hannelore Burkhardt. Targeted clones (10 out of 480 analyzed $DLL3^{HAki}$) were identified by PCR using primers derived from the neomycin sequence and genomic sequences downstream of the targeting vector (Fig. 4.7 B). PCR-positive clones were further verified by Southern blot analysis. For this purpose genomic DNA isolated from the ES cells was digested with the restriction enzyme *BamH*I, separated by gel electrophoresis and blotted on nylon membranes. Two external probes located 3' and 5' to the regions of homology in the vector (Fig. 4.7 C) were used to examine the integrity of the mutated *Dll1* locus in these ES cell clones. The diagnostic bands were at 5.5 kb (5' probe) and 6.5 kb (3' probe) for the targeted allele and at 10 kb (both probes) for the wildtype allele. Correctly targeted ES cell clones were used for blastocyst injection (performed by Dr. Karin Schuster-Gossler) to generate chimeric mice and germline transmission of these alleles was obtained. After the excision of the neo cassette, analysis of the *knock-in* mice (performed by Dr. Katrin Serth) demonstrated that similar to the *in vitro* findings, Dll3 did not activate Notch *in vivo* (Geffers *et al.*, 2007).

4.5 Analysis of Notch activation in the presomitic mesoderm of Dll3 mutant mice

Based on the idea that the loss of Dll3 function should lead to an increase in Notch activation if Dll3 served as a direct antagonist of Notch signaling (Ladi *et al.*, 2005), protein levels of

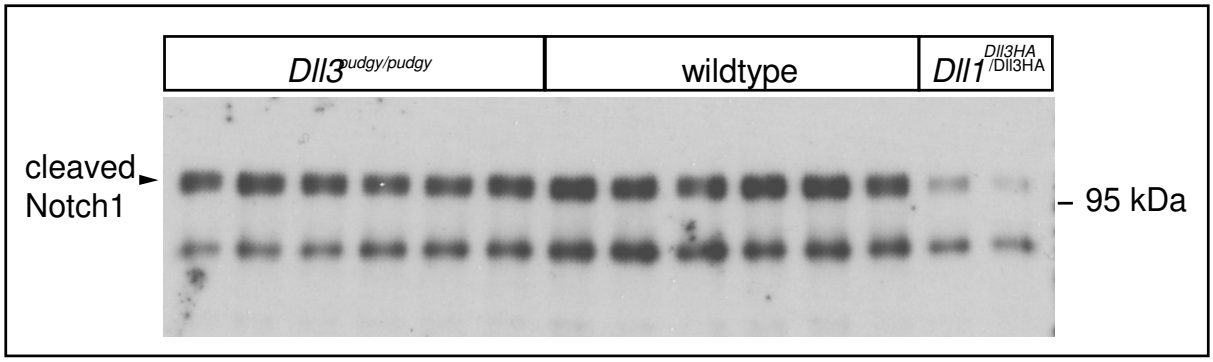

Fig. 4.8: Protein levels of activated NotchICD in the presomitic mesoderm. Cell lysates of a single presomitic mesoderm (PSM) of E9.5 wildtype, pudgy and $Dll1^{Dll3HA}$ embryos were loaded. Western Blot analysis was performed with an anti-cleaved Notch1 antibody (Val1744). In homozygous $Dll3^{pudgy}$ PSMs the levels of activated NotchICD were slightly lower than in wildtype PSMs. In PSMs of homozygous $Dll1^{Dll3HA}$ embryos very weak bands of cleaved Notch were detected. Arrowhead indicates the expected 110 kDa band of NotchICD. The second band is presumably unspecific detection by the antibody.

activated Notch were assessed by Western blot analysis of the presomitic mesoderm (PSM) of E9.5 pudgy, wildtype and *Dll1*$^{Dll3HAki/Dll3HAki}$ embryos (provided by Dr. Katrin Serth). PSMs were dissected, lysed in protein sample buffer and analyzed with antibodies against cleaved Notch1. The protein levels of activated Notch intracellular domain in the presomitic mesoderm of pudgy embryos were even slightly lower than in wildtype mice refuting a direct antagonistic function of DLL3 (Fig. 4.8). In the presomitic mesoderm of *Dll1*$^{Dll3HAki/Dll3HAki}$ embryos only very little cleaved Notch1 was detected.

4.6 DLL1 domains required for Notch activation

In vitro experiments revealed the intracellular localization of DLL3 and its inability to activate Notch indicating that DLL3 is not a bona fide Notch ligand. The DLL1 and DLL3 proteins show several differences in their amino acid sequences that might contribute to the functional non-equivalence of the two DSL proteins. Compared with DLL1, the DLL3 protein has a deviant DSL domain, fewer EGF-like repeats, and altered spacing between some EGF-like repeats. It also lacks lysine residues and a PDZ ligand binding domain in its intracellular domain.

To analyze which of these structural differences might contribute to the functional divergence, various chimeric ligands were generated by combination of different protein domains of DLL1 and DLL3 using PCR and site-directed mutagenesis without altering, deleting or inserting amino acids. All these C-terminally tagged chimeric *Dll1-Dll3* cDNAs (Fig. 4.9 A) were cloned into the pTracer vector for cell culture expression and establishment of stable CHO cell lines. Cell lines were analyzed for protein expression levels by Western blot analysis to identify clones expressing similar levels of different protein variants for further analyses. (Some constructs and the cell lines used for Notch activation assays were generated and analyzed by Dr. Katrin Serth as specified in chapter 3.2.2, page 29 and appendix Tab. 1)

Cell surface localization of chimeric proteins was assessed by surface biotinylation, followed by immunoprecipitation and Western blotting. Chimeric ligands A–H were readily detected on the cell surface though to different extents (Fig. 4.9 B). Comparing total protein levels (Fig. 4.9 B, input) with amounts on the surface (Fig. 4.9 B, IP), chimeric ligands B, D, E, F and H showed surface protein levels similar to DLL1, whereas chimeras A, C and G exhibited a diminished cell surface presentation.

However, only constructs C and G let to significant activation of Notch indicating that EGF–like repeats 3-6 of DLL3 are functionally equivalent to EGF-like repeats 5-8 of DLL1 as

assumed by their high sequence homology. Furthermore, the N-terminus including the DSL domain and EGF-like repeats 1 and 2 in combination with the intracellular domain of DLL1 are essential and sufficient to activate Notch1 (Fig 4.9 A; final transactivation assays performed by Dr. Katrin Serth are published in Geffers *et al.*, 2007).

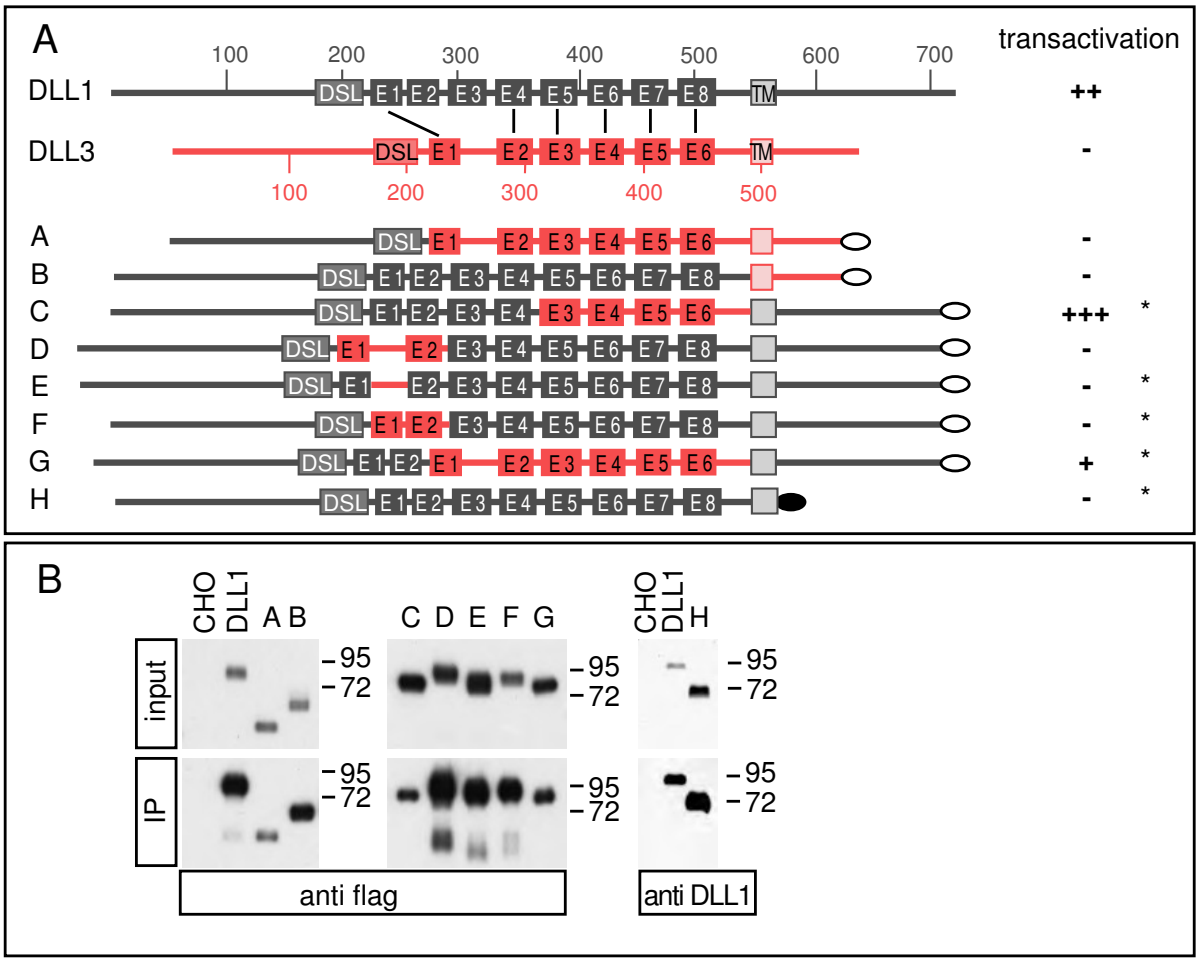

Fig. 4.9: Analysis of DLL1-DLL3 chimeric ligands. (A) Schematic overview of wildtype DLL1 and DLL3 and chimeric constructs used to generate stably expressing CHO cell lines. DLL1 protein is shown in black and DLL3 in red. Numbers indicate the amino acid residue numbers. DSL, DSL domain; E1–E8, EGF-like repeats; the flag tag is indicated by white ovals and the HA tag in construct H by a black oval. Corresponding EGF-like repeats of DLL1 and DLL3 are connected by black lines. The levels of Notch transactivation induced by the chimeras are indicated as follows: +++: very strong; ++: strong; +: weak; -: none. Transactivation results obtained by Dr. Katrin Serth are marked with *. (B) Western blot analysis of cell lysates (input) and streptavidin immunoprecipitated protein after surface biotinylation (IP). CHO cells stably expressing chimeric ligands show similar (input A and B) or even stronger (input C–H) expression compared with DLL1 expressing cells. All chimeric ligands are present on the cell surface (IP), chimeric ligands A, C, and G at lower levels and chimeric ligands B, D–F, and H at similar or even higher levels compared to DLL1.

4.7 Protein domains affecting subcellular localization

The surface biotinylation results suggested that chimeric ligands differ with respect to their propensity to localize to the surface. To analyze in more detail how different protein domains of DLL1 and DLL3 affect the distribution of the stably overexpressed chimeric ligands in the cell, their localization on the cellular level was studied by indirect immunofluorescence (Fig. 4.10). Additional chimeras were cloned and cell lines thereof generated to narrow down the region required for cell surface presentation of DLL1 and intracellular localization of DLL3 (Fig. 4.10 A, chapter 3.2.2 and appendix, Tab. 1).

Chimeric ligands containing the transmembrane and intracellular domain (TM-ICD) of DLL1 and at least the DLL1 N-terminus including the DSL domain fused to extracellular DLL3 sequences were detected on the cell surface in addition to variable intracellular expression (Fig. 4.10 B c -e).

Though haboring the DLL1 signal peptide, chimeric ligands containing only the N-terminus of DLL1 linked to DSL domain and EGF repeats of DLL3 (chimeras J and M) were exclusively detected in intracellular regions (Fig. 4.10 B f, k). The same was true for a chimera consisting of the DLL3 extracellular domain juxtaposed to the DLL1 TM-ICD (Fig. 4.10 B g). As the intracellularly truncated version of DLL1 (ligand H) was predominantly detected on the surface, the intracellular domain of DLL1 appeared to be dispensable for surface expression (Fig. 4.10 B b). Taken together, these findings demonstrate that the DSL domain of DLL1 is necessary to direct cell surface presentation of chimeric proteins.

In contrast to cells expressing DLL1 or construct H or B, that consistently showed strong expression on the surface, surface presentation of most chimeric ligands containing a combination of DLL1 and DLL3 domains in their extracellular part was variable and not detectable in all expressing cells. Especially chimeric ligands C and G exhibited variable surface staining. Furthermore, chimeric ligands containing the transmembrane domain and the juxtaposed sequences of DLL3 were predominantly found in intracellular regions (Fig. 4.10 B i-k) as a result of a strongly diminished cell surface presentation compared to chimeras with the corresponding *Dll1* sequences.

Collectively, it appears that while the DLL1 DSL domain is required for cell surface presentation, the DLL3 transmembrane domain together with adjacent sequences is involved in retention of chimeric ligands in intracellular compartments and localization of DLL3 to the Golgi network.

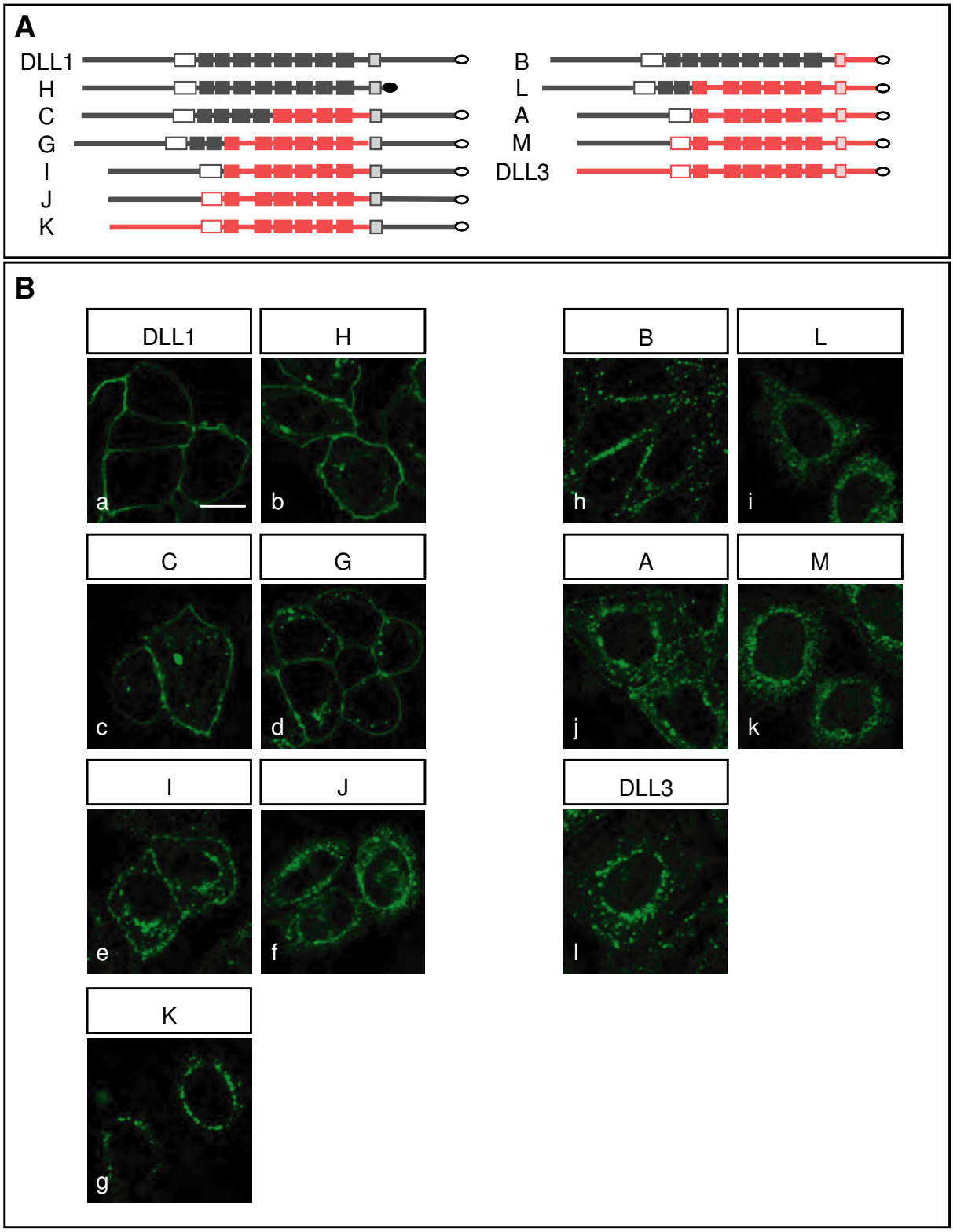

Fig. 4.10: Localization of chimeric DLL1 and DLL3 proteins. (A) Schematic representation of chimeric proteins containing the ICD of DLL1 (left) or DLL3 (right) arranged according to the extent of extracellular DLL1 sequences (top to bottom). Gray parts and red parts indicate DLL1 and DLL3 sequences, respectively. White filling indicates DSL domains, light gray or red shading indicates TMs, and filled boxes indicate EGF-like repeats. (B) Confocal images of CHO cells expressing chimeric ligands and stained by indirect immunofluorescence. Similar to DLL1 (a) and DLL1 lacking the ICD (b), chimeric ligands that contained the

TM-ICD of DLL1 and the DLL1 N-terminal portion including the DSL domain were detected on the cell surface (c–e), in addition to some variable intracellular expression. Presence of the DLL1 N-terminus alone was not sufficient to direct detectable surface expression (f), similar to the extracellular domain of DLL3 fused to DLL1 TM-ICD (g). Surface presentation of a chimera containing the DLL1 extracellular domain juxtaposed to the DLL3 TM-ICD (h). Chimeras that contain the DLL1 N-terminal portion including the DSL domain, and the DLL3 TM in the context of juxtaposed DLL3 sequences were retained intracellularly (i and j). Intracellular localization of DLL3 with the N-terminus replaced by the corresponding DLL1 sequence, and of DLL3, respectively (k and l). A–C, G, and H refer to chimeras shown in Fig. 4.9 A and I–M to additional ones. Chimera H (b) was detected with anti-DLL1 and all other chimeras with anti-flag antibodies. Scale bar, 10 μm.

4.8 Mutations in the DSL domain of DLL1 abolish its transactivation potential

In addition to the analysis of larger protein domains, differences in the DSL domain of DLL1 and DLL3 were analyzed in more detail. The conserved DSL domain of DLL1 is essential for Notch binding (Shimizu *et al.*, 1999) and appears to be necessary for surface presentation. The DSL domain of DLL3 is highly dissimilar to DSL domains of other Delta and Serrate homologues (Fig. 4.11). It lacks two conserved amino acid motifs, a YY motif (aa 182-183 in mDLL1) and a GWxG motif (aa 214-217 in mDLL1) and shows a different spacing between the conserved cysteine residues that contribute to the secondary structure of the domain by forming disulfide bonds.

These two conserved motifs were mutated in DLL1 to analyze the relevance of these features for subcellular localization of the protein and Notch transactivation (Fig. 4.11). The double tyrosine motif (Y182, Y183) was mutated in DLL1 by site-directed mutagenesis into alanine and valine (DLL1 DSL-ΔY) to mimic the corresponding sequence of the DLL3 DSL domain. In a different construct the GWxG motif of DLL1 (aa 214-217) was mutated into four alanines (DLL1 DSL-ΔG). These mutant DLL1 coding sequences were coupled to a flag tag and cloned into the expression vector pTracer and stable CHO cell lines were generated overexpressing the mutant protein.

Cell surface biotinylation experiments were performed in order to verify cell surface presentation of the mutant proteins. While the DLL1 DSL-ΔG mutant protein was readily detected on the cell surface, only trace amounts of the DLL1 variant with the deleted double tyrosine motif (DLL1 DSL-ΔY) was present on the cell surface (Fig. 4.12).

The first tyrosine residue (Y182 in DLL1) seems to be required for Notch signaling as it is perfectly conserved even in Notch ligands of *C.elegans* exhibiting a histidine or phenylalanine

```
dros  DL          TCDLNYYGSGCAKFCRPRDDSFGHSTCSETGEIICLTGWQGDYCH
dros  Serrate     QCAVTYYNTTCTTFCRPRDDQFGHYACGSEGQKLCLNGWQGVNCE
Apx-1             LCSSNYHGKRCNRYCIAN--AKLHWECSTHGVRRCSAGWSGEDCS
Lag-2             TCARNYFGNRCENFCDAHLAKAARKRCDAMGRLRCDIGWMGPHCG
xen  Dll1         SCDEHYYGDSCSDYCRPRDDNFGHYTCDEQGNRLCMSGWKGEYCA
xen  Dll2         SCDEHYYGDSCSDYCRPRDDNFGHYTCDEQGNRLCMSGWKGEYCA
xen  Serrate-1    ICDEHYYGFGCNKFCRPRDDFFGHYTCDLNGNKTCLEGWMGPECS
chick Dll1        VCDEHYYGEGCSVFCRPRDDRFGHFTCGERGEKVCNPGWKGQYCT
hum Dll1          VCDEHYYGEGCSVFCRPRDDAFGHFTCGERGEKVCNPGWKGPYCT
hum Jagged2       RCDENYYSATCNKFCRPRNDFFGHYTCDQYGNKACMDGWMGKECK
rat Dll1          VCDEHYYGEGCSVFCRPRDDAFGHFTCGERGEKMCDPGWKGQYCT
mJagged1          TCDDHYYGFGCNKFCRPRDDFFGHYACDQNGNKTCMEGWMGPDCN
mJagged2          RCDENYYSATCNKFCRPRNDFFGHYTCDQYGNKACMDGWMGKECK
mDll1             VCDEHYYGEGCSVFCRPRDDAFGHFTCGDRGEKMCDPGWKGQYCT
mDll4             ICSDNYYGESCSRLCKKRDDHFGHYECQPDGSLSCLPGWTGKYCD
consensus          C   YY   C   C           C   G   C  GW G  C
mDll3             RCEPPAVGAACARLCRSR---SAPSRCGP-GLRPCTP--FPDECE

Dll1 DSL mutants:   ΔY:    YY→AV
                    AY:    YY→AY
                    FF:    YY→FF
                    FY:    YY→FY
                    YF:    YY→YF
                    ΔG:    GWKG→AAAA
```

Fig. 4.11: Alignment of the DSL domains of various Delta homologues. DLL3 shows a divergent DSL domain with an altered spacing of the conserved cysteine residues (bold, black) and lacks two conserved amino acid motifs (bold, red). Designations of the generated DLL1 DSL mutants with altered amino acid sequence are shown below.

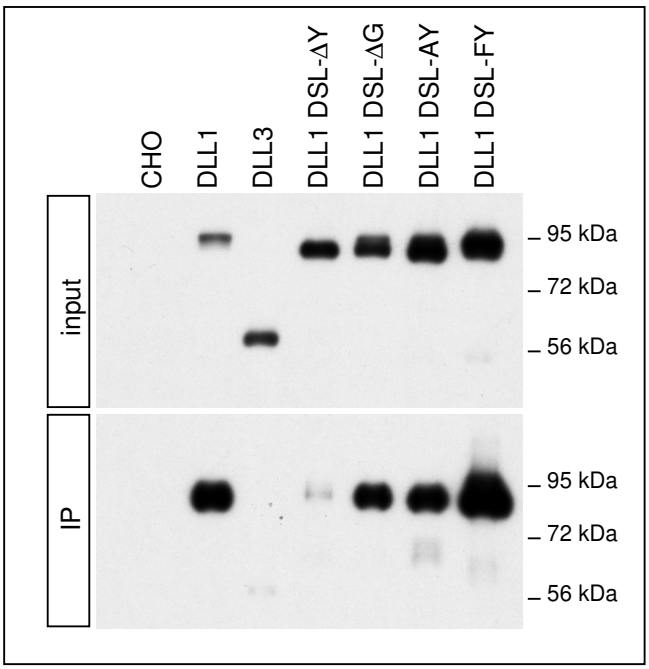

Fig. 4.12: Cell surface presentation of DLL1 DSL mutant proteins. Western blot analysis of cell lysates (input) and streptavidin immunoprecipitated protein after surface biotinylation (IP). DLL1 DSL mutant proteins ΔG, AY and FY were readily detected on the cell surface, whereas only very low amounts of DLL3 and DLL1 DSL-ΔY were detected in the streptavidin-precipitated fraction.

substituting for the second tyrosine (Y183). Mutations of the first tyrosine leaving the second tyrosine unaffected (DLL1 DSL-AY and DLL1 DSL-FY, generated by Claudia Brockmeyer during her diploma thesis) resulted in apparently normal surface localization of the proteins (Fig. 4.12). To confirm these findings immunofluorescence stainings were performed to analyze the subcellular distribution of the proteins (Fig. 4.13). As expected, DLL1 DSL-ΔY

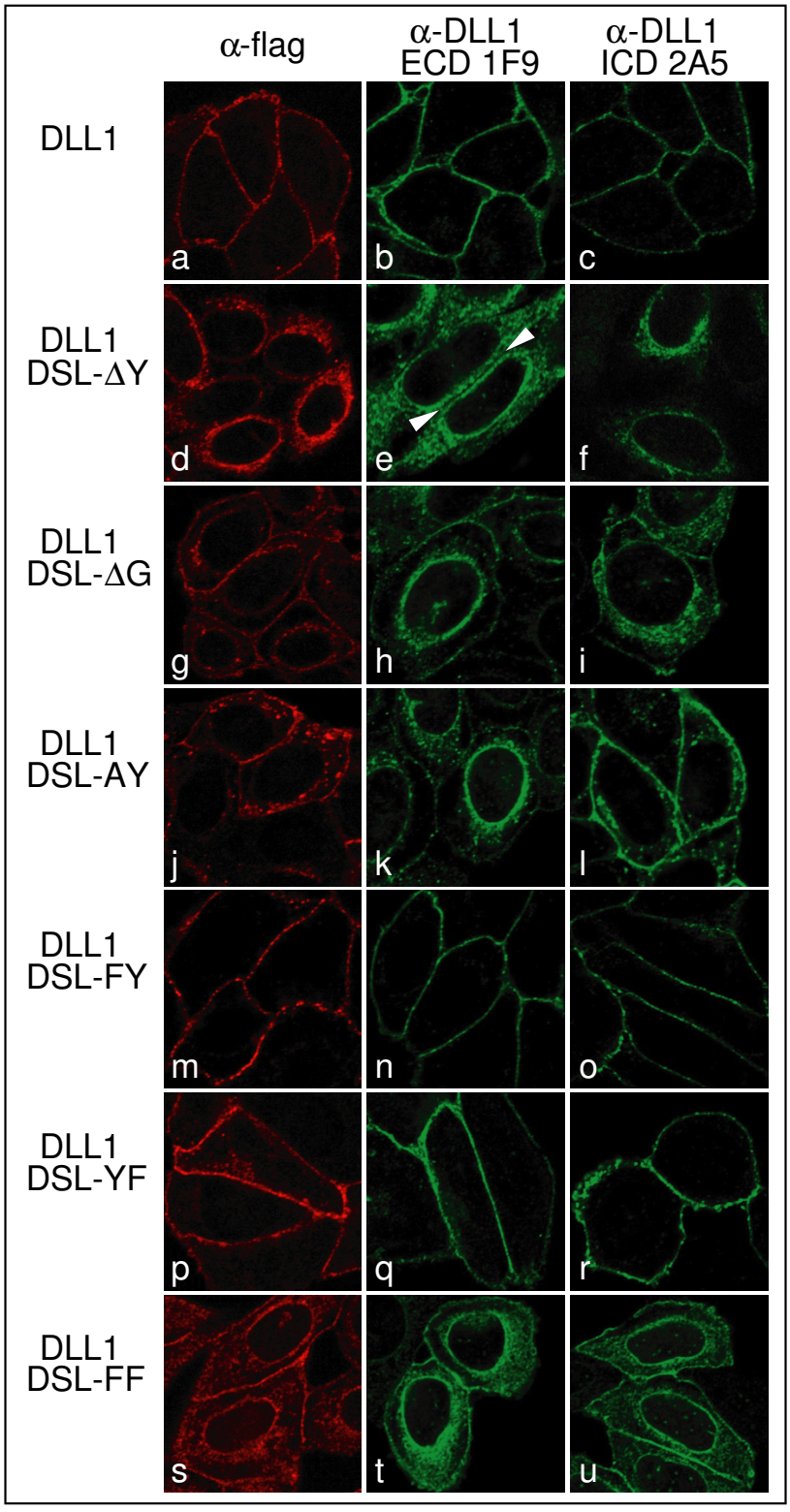

Fig. 4.13: Subcellular localization of DLL1 DSL mutant proteins with different antibodies. Mutations of the conserved tyrosine motif in the DSL domain of DLL1 leads to altered protein localization. Conservative mutations to hydrophobic amino acids retain normal cell surface presentation ability (m-o, p-r) while surface presentation is completely lost when tyrosines were mutated to alanine and valine (d-f) Arrowheads in (e) indicate the membrane which shows no staining. Semi-conservative mutations of only one tyrosine to alanine or of both tyrosines to hydrophobic phenylalanines diminish protein amounts present on the cell surface (j-l, s-u). A similar effect has the mutation of the GWxG motif into alanines (g-i).

mutant protein was only detected in some perinuclear structures and did not reach the cell surface (Fig. 4.13 d-e). In some cases DLL1 DSL-ΔY mutant protein was detected even in the whole cell except for the membrane which could be recognized as a black non-fluorescent line (Fig. 4.13 e, arrowheads).

DLL1 DSL-ΔG and DLL1 DSL-AY mutant proteins reached the cell surface although overexpressed protein was also detected to a large extent in the perinuclear region (Fig. 4.13 g-i and j-l). The conservative mutation DLL1 DSL-FY had no influence on protein localization as demonstrated by a surface staining similar to that of DLL1 (Fig. 4.13 m-o).

Two additional mutations DLL1 DSL-YF and DLL1 DSL-FF were cloned to in more detail investigate if the presence of an aromatic amino acid residue is sufficient for protein localization at the cell surface. Both proteins were present on the cell surface but DLL1 DSL-FF protein was additionally detected inside the cell (Fig. 4.13 p-r and s-u).

In colocalization studies with organelle markers DLL1 DSL-ΔY staining partially overlaped with staining for the Golgi marker GM130 but hardly with calreticulin, a marker for the endoplasmic reticulum (Fig. 4.14) indicating that the subcellular localization of DLL1 DSL-ΔY protein seems to be similar to that of DLL3.

To further explore the significance of the DSL mutations for Notch activation, CHO cell lines stably expressing the DLL1 DSL mutant proteins were analyzed in a transactivation assay for their potential to activate Notch. For two DLL1 variants (DLL1 DSL-FF and DLL1 DSL-YF) no clonal CHO cell lines existed which is a prerequisite for reproducible protein expression levels, thus transactivation assays were not performed. Because of the intracellular

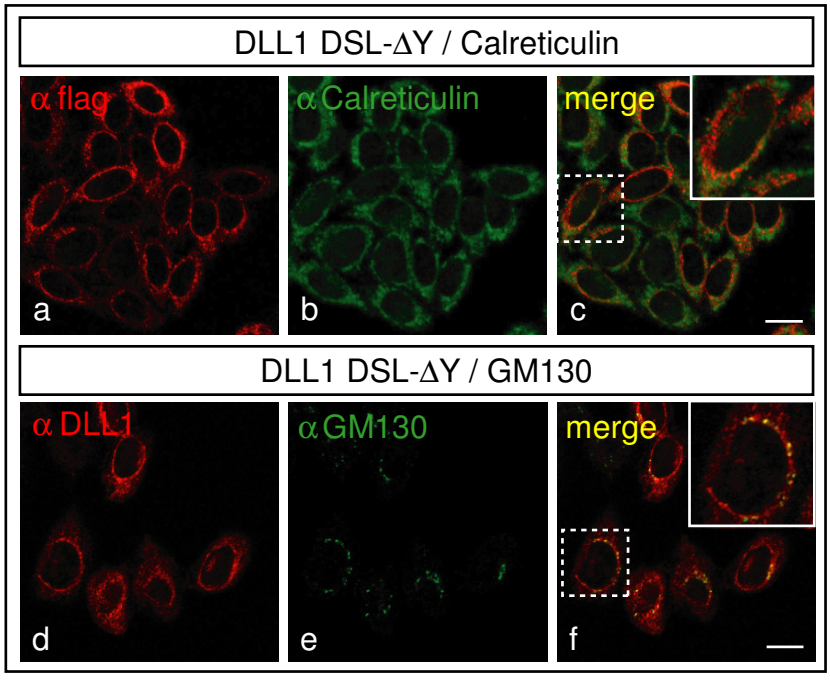

Fig. 4.14: Colocalization of DLL1 DSL-ΔY protein with organelle markers. DLL1 DSL-ΔY does hardly colocalize with calreticulin, an endoplasmic reticulum marker, (c), but localizes partially to the Golgi apparatus stained by the cis-Golgi marker GM130 (f).

localization of DLL1 DSL-ΔY mutant protein, the failure of this DLL1 variant to transactivate Notch did not allow one to draw conclusions concerning the relevance of the double tyrosine motif for Notch activation. However, the DLL1 DSL-ΔG mutant protein showed no transactivation potential although the protein was efficiently presented at the cell surface as determined by surface biotinylation (Fig. 4.15). Similarly, the mutations of the first tyrosine created DLL1 v ersions that exhibited a considerably diminished transactivation potential. Collectively, the mutation of the tyrosine182 or the GWxG motif in the DSL domain of DLL1 led to an abolishment of DLL1 function. Additionally, the presence of two hydrophobic amino acids with at least one tyrosine is necessary in the DLL1 DSL domain to result in normal cell surface presentation.

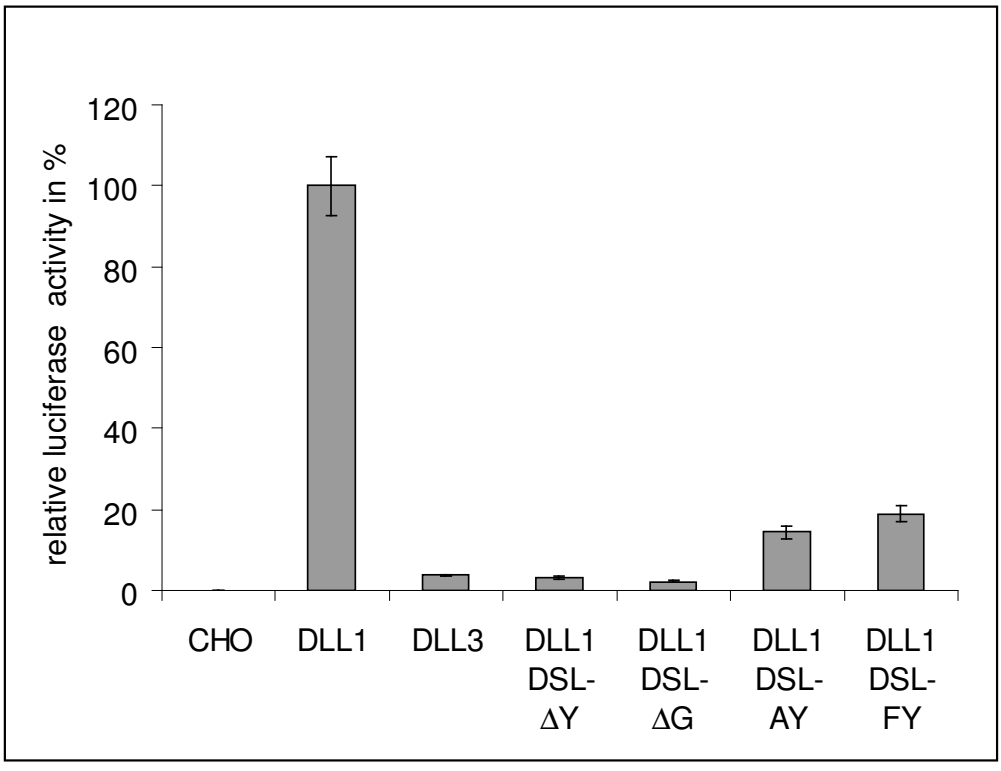

Fig. 4.15: Activation of Notch by DLL1 DSL mutant proteins. Transactivation assay by cocultivation of HeLa-N1 cells transfected with the RBP-luc reporter construct and CHO cells stably expressing DLL1, DLL3 or DLL1 DSL mutant protein. All examined DLL1 DSL mutant protein exhibit no or a strongly diminished Notch activation potential. CHO cells were set to 0%, luciferase activity induced by DLL1 was set to 100%. Transfection efficiency was normalized to the activity of Renilla luciferase expressed by a cotransfected pRL-TK construct (Promega). Four cocultivations were performed in two independent experiments each, including negative and positive controls. Bars indicate the standard deviation. For information concerning CHO cell lines see appendix, Tab. 1.

4.9 Cis-inhibition of Notch by DLL1 variants carrying a mutated DSL domain

Interactions of Notch receptors with coexpressed Notch ligands in the same cell were previously shown to result in an impairment of Notch signaling (de Celis and Bray, 2000; Jacobsen *et al.*, 1998). In vertebrates (chick), this cis-inhibition has been demonstrated for Serrate and Delta homologues (Sakamoto *et al.*, 2002). Recently, coculture luciferase assays revealed a cis-inhibitory function for DLL3 although it cannot activate Notch (Ladi *et al.*, 2005).

As the DLL1 DSL mutant proteins have lost their transactivation capacity and some of them showed diminished cell surface presentation, the question was raised whether these effects also influence their ability to cell-autonomously inhibit Notch transactivation by cis-interactions.

When coexpressed in Notch1-expressing cells, the DLL1 mutant proteins showed cis-inhibitory effects on Dll1-induced Notch signaling to different extents (Fig. 4.16 A). Coexpression of high levels of DLL1 wildtype protein showed the strongest Notch1 inhibition (Fig. 4.16 B). Although the DLL1 DSL-ΔG expression level was similar to that of DLL1, this DLL1 version exhibited less effective inhibition of Notch signaling. Compared to the DLL1 DSL-ΔG variant, the DLL1 DSL-ΔY mutant protein showed a similar inhibitory effect but was expressed at a significantly lower level indicating that its potential to inhibit Notch activation was actually stronger than that of the DLL1 DSL-ΔG mutant protein. Taken together these results suggest that transactivation of Notch by ligand binding and cis-inhibition of Notch by its interaction with coexpressed ligands in the same cell occur by two different modes and presumably require different regions of the ligands as interaction sites. Thus, dysfunction of Notch activation does not necessarily abolish the ability of DSL proteins to interfere with Notch in cis.

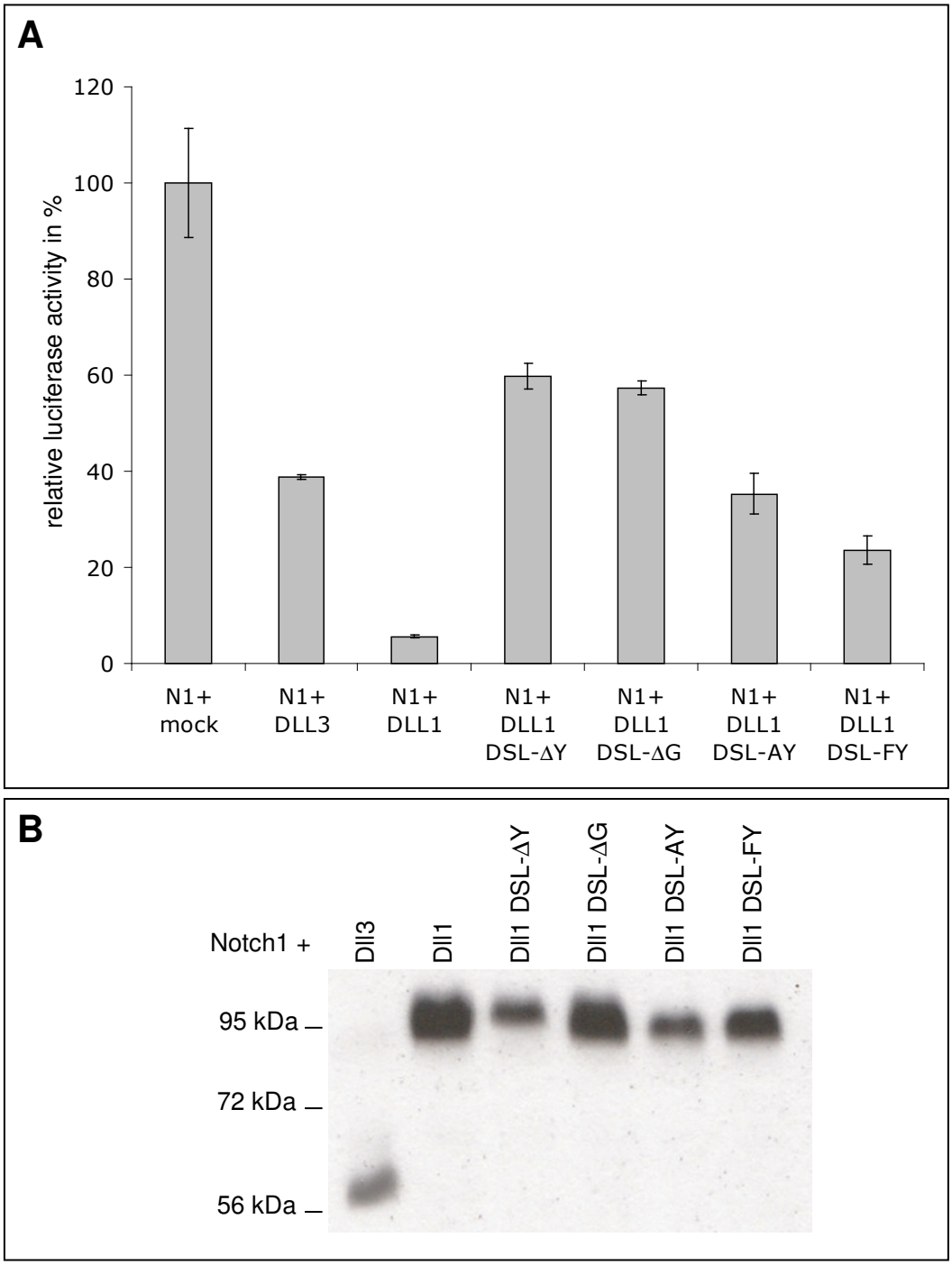

Fig. 4.16: Cis-inhibition of DLL1 DSL mutant proteins. (A) HeLa-N1 cells were cotransfected with RBP-luc plasmid and a ligand expression vector or empty vector (mock). Each transfected HeLa-N1 sample was cocultured with CHO-DLL1 cells and CHO wildtype cells, respectively. Within each set-up, luciferase activity was measured against negative control (CHO wild-type cells) set to 0%, HeLa-N1 without coexpressed ligands (N1+mock) was set to 100%. Expression levels of the transiently expressed proteins are shown in (B). All DSL protein variants showed a cis-inhibitory effect on Notch activation. DLL1 showed the strongest inhibition, DLL1 DSL-ΔY and DLL1 DSL-ΔG exhibited the weakest effect. (B) Western blot analysis of transfected HeLaN1 cells used for transactivation assay in (A). Protein expression levels of the cotransfected ligands were analyzed by Western blot of whole cell lysates with anti-flag antibodies.

4.10 Tyrosine O-sulfation of DLL1 is not detectable

The double tyrosine motif in the DSL domain is highly conserved in Delta homologues from *Drosophila melanogaster* to humans. Organisms that emerged earlier during evolution - like the nematode *C. elegans* or the green sea urchin - contain only the first tyrosine and another aromatic amino acid at the corresponding position in the DSL domain of Notch ligands (Fig. 4.11, *C.elegans* Notch ligands Apx-1 and Lag-2). The presence of this tyrosine motif might have potential importance for different processes. Given that the tyrosines are in the extracellular part of the DLL1 protein modifications such as tyrosine phosphorylation are unlikely to occur as they were described only for cytoplasmic parts of proteins.

In a search for modification sites in DLL1 the double tyrosine motif in the DSL domain of DLL1 was predicted to serve as a sulfation site. Tyrosine O-sulfation is a post-translational modification of proteins synthesized along the secretory pathway (Moore, 2003). In the Golgi apparatus, the tyrosylprotein sulfotransferases catalyze the transfer of sulfate from the universal sulfate donor adenosine 3'-phosphate 5'-phosphosulfate (PAPS) to the hydroxyl group of a peptidyltyrosine residue (Lee and Huttner, 1983). The general effect of this modification seems to be enhancement of protein-protein interaction (Wilkins *et al.*, 1995). Additionally, sulfation may have an impact on protein trafficking (Friederich *et al.*, 1988). Although common features for tyrosine O-sulfation have been described, there is no consensus sequence *per se* (Moore, 2003).

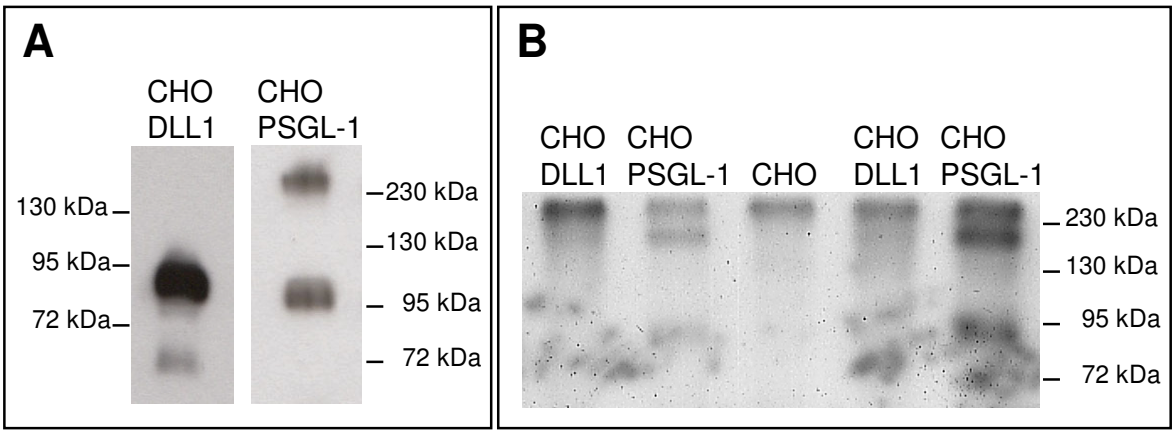

Fig. 4.17: Tyrosine O-sulfation. (A) Western Blot analysis after immunoprecipitation of DLL1 and PSGL-1 with anti-DLL1 (1F9) and anti-PSGL-1 antibodies, respectively. (B) Radiograph of [^{35}S]-labeled protein of CHO cells expressing DLL1 or PSGL-1 and of CHO control cells after immunoprecipitation of the respective protein (see A). No [^{35}S]-labeled DLL1 band was detetected.

To investigate this potential modification of DLL1 protein, metabolic labeling of CHO cells expressing *Dll1* was performed with [^{35}S]-sulfate. As a positive control P-selectin glycoprotein-1 (PSGL-1) which can be sulfated on a cluster of three tyrosine residues was labeled in CHO cells (Liu *et al.*, 1998). Afterwards the cells were lysed and protein lysates were subjected to immunoprecipitation by addition of protein G sepharose and antibodies against DLL1 and PSGL-1, respectively.

After separation of the precipitated protein fractions by SDS-PAGE the gels were exposed for three weeks. The positive control (PSGL-1) showed bands of the expected size indicating that the metabolic labeling procedure was successful. Although DLL1 was readily obtained by immunoprecipitation of protein lysates (Fig. 4.17 A) no [^{35}S]-labeled protein band was detected (Fig. 4.17 B). So far, there is no evidence of tyrosine O-sulfation of *Dll1*.

4.11 Generation and analysis of Dll1 DSL mutant mice

Parallel to the *in vitro* experiments, two of the generated DLL1 DSL mutations were analyzed *in vivo* by generation of Dll1 DSL mutant mice. Pursuing the same targeting strategy as for the *Dll3 knock-in* mice endogenous *Dll1* sequence was substituted by a *Dll1* minigene containing mutated DSL domain sequence (Fig. 4.18 A). Linearized vector DNA was electroporated into 129Sv/ImJ embryonic stem cells and G418-resistant ES cell clones were selected by Dr. Karin-Schuster-Gossler and Hannelore Burkhardt. Targeted clones were identified by PCR using primers derived from the neomycin sequence and genomic sequences downstream of the targeting vector. PCR-positive clones were further verified by Southern blot analysis. Two external probes located 3' and 5' to the regions of homology in the vector were used to examine the integrity of the mutated *Dll1* locus in these ES cell clones. The diagnostic bands were at 5.5 kb (5' probe) and 6.5 kb (3' probe) for the targeted allele and at 10 kb (both probes) for the wildtype allele (Fig. 4.18 B). Correctly targeted ES cell clones were used for blastocyst injection (performed by Dr. Karin-Schuster-Gossler) to generate chimeric mice and germline transmission was obtained. After excision of the neo cassette verified mice were used for further analyses. Heterozygous $Dll1^{Dll1DSLmut}$ mice showed no apparent phenotype demonstrating that the DLL1 DSL mutant proteins do not exert an obvious dominant negative function *in vivo*.

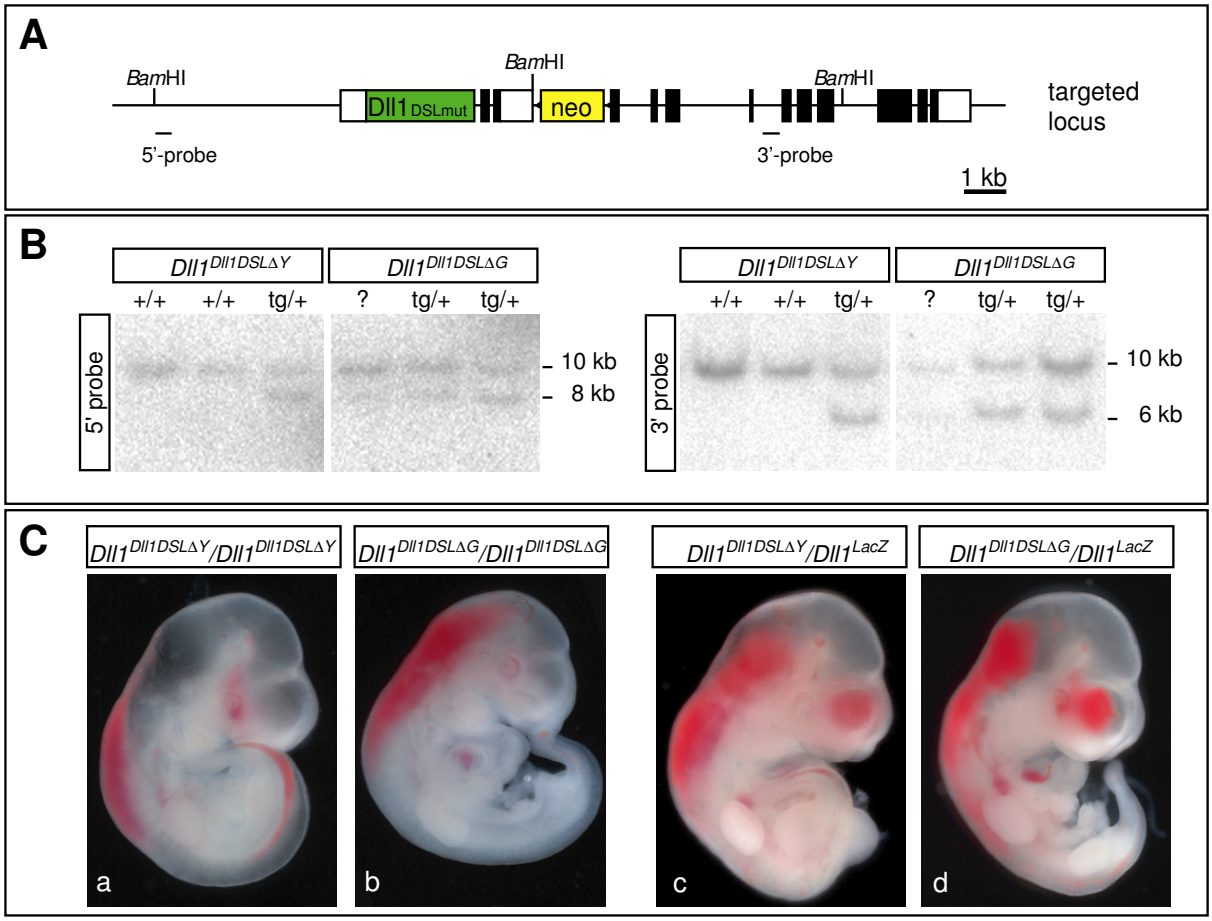

Fig. 4.18: Screening for targeted ES cell clones and phenotype of E10.5 *Dll1*^{Dll1DSLmut} embryos. (A) Targeted locus for the *Dll1*^{Dll1DSLmut} *knock-in*. The green box indicates the Dll1 DSL-ΔY and Dll1 DSL-ΔG cDNA, respectively. (B) Correctly targeted ES cell clones showed a shift from 10 kb to 6,5 kb with the 3' probe and a shift from 10 kb to 8 kb with the 5' probe on a Southern blot. (C) Homozygous E10.5 *Dll1*$^{Dll1\ DSL\text{-}\Delta Y}$(a) and *Dll1*$^{Dll1\ DSL\text{-}\Delta G}$ (b) mutant embryos showed hemorrhagic bleeding and lack somite borders. The same phenotype was detected in *Dll1*$^{Dll1\ DSL\text{-}\Delta Y}$/*Dll1*^{LacZ} (c) and in *Dll1*$^{Dll1\ DSL\text{-}\Delta G}$/*Dll1*^{LacZ} (d) embryos.

Homozygous *Dll1*^{Dll1DSLmut} embryos showed similar somite formation defects as *Dll1* null embryos and died around E11.5 due to severe hemorrhagic bleedings indicating that the mutations Dll1DSL-ΔY and Dll1DSL-ΔG both create functional *Dll1* null alleles (Fig. 4.18 C). The failure of the *Dll1*^{Dll1DSLmut} alleles to rescue the DLL1 function confirmed the results obtained *in vitro*.

The Dll1DSL-ΔY mutation resembles the DLL3 DSL domain which also lacks the double tyrosine motif. Additionally, DLL1 DSL-ΔY and DLL3 are both hardly detectable on the cell surface but colocalize partially with the Golgi marker GM130. These similarities raised the question if retention of DLL1 DSL-ΔY protein inside the cell results in a functional substitution of *Dll3* in *Dll3* mutant mice. To address this question mice double heterozygous

for the *Dll1*$^{Dll1DSL\Delta Y}$ and the *Dll3*^{pudgy} allele were intercrossed with heterozygous *Dll3*^{pudgy} mice. If the mutated DLL1 protein could (partially) rescue the loss of DLL3 in the presomitic mesoderm a milder phenotype than the pudgy phenotype would be expected in mice with *Dll1*$^{Dll1DSL\Delta Y/+}$; *Dll3*$^{pudgy/pudgy}$ genotype. Skeletal preparations of E18.5 embryos with this genotype showed a severely perturbed vertebral column similar to the *Dll3*^{pudgy} phenotype (Fig. 4.19). This finding indicated that retained DLL1 DSL-ΔY protein inside the cell cannot substitute for Dll3 function. Thus, forced retention of a DSL protein within the cells is not sufficient to adopt *Dll3* function *in vivo.*

Genotype	Phenotype	Embryos at E18.5 (Σ 41)
Dll1$^{+/+}$; Dll3$^{+/+}$	wildtype	5
Dll1$^{+/+}$; Dll3$^{pudgy/+}$	wildtype	13
Dll1$^{Dll1DSL\Delta Y/+}$; Dll3$^{+/+}$	wildtype	6
Dll1$^{Dll1DSL\Delta Y/+}$; Dll3$^{pudgy/+}$	wildtype	12
Dll1$^{+/+}$; Dll3$^{pudgy/pudgy}$	pudgy	4
Dll1$^{Dll1DSL\Delta Y/+}$; Dll3$^{pudgy/pudgy}$	similar to pudgy	1

Fig. 4.19: Skeletal preparations of E18.5 embryos. Alcian blue staining of the cartilage and alizarin red staining of the bones. Homozygous pudgy embryos display a severely perturbed vertebral column with rib malformations. *Dll1*$^{Dll1DSL\Delta Y/+}$; *Dll3*$^{pudgy/pudgy}$ embryos exhibit similar defects of the skeleton.

5 Discussion

The DSL proteins *Dll1* and *Dll3* are coexpressed in the presomitic mesoderm but their null alleles display distinct phenotypes. As the two proteins additionally differ on the amino acid level it could be presumed that these two DSL proteins exert different functions and cannot compensate for the loss of each other. This study addresses the biochemical and functional properties of the Notch ligands DLL1 and DLL3.

5.1 Unanticipated subcellular localization of DLL3

Based on the fact that DLL3 carries a signal peptide and a transmembrane domain and on the findings that ectopic expression of *Dll3* in *Xenopus laevis* led to Notch activation (Dunwoodie *et al.*, 1997), it was assumed that DLL3 is a bona fide Notch ligand. During the course of this study, it was demonstrated that DLL3 did not activate Notch1 in transactivation assays (Ladi *et al.*, 2005). Consistent with the inability of DLL3 to induce Notch signals DLL3 expressing cells did not suppress myogenic differentiation in C2C12 cells. Additionally, the authors demonstrated that in cells coexpressing *Dll1* and *Dll3*, the presence of DLL3 did not perturb DLL1-induced Notch signaling. Ladi *et al.* (2005) suggested that *Dll3* acts in an antagonistic fashion as coexpression of *Dll3* with Notch inhibits Notch activation. Furthermore, they showed that DLL3 cannot bind to Notch in trans although DLL3 protein was verified to be present on the cell surface. They concluded that the inability of DLL3 to bind Notch in trans gives rise to its failure to activate Notch.

In this project it was confirmed that DLL3 does not activate Notch in trans but that an unanticipated intracellular localization of DLL3 presumably accounts for this deficiency. These observations were contradictory to results published by Ladi *et al.* (2005) that by surface biotinylation demonstrated that DLL3 and DLL1 are both present to the same extent on the cell surface of L cells (mouse fibroblasts). This discrepancy was corroborated by independent lines of evidence in this study. By cell surface labeling of overexpressing CHO cells only trace amounts of DLL3 were found on the cell surface. Even a close recapitulation of the cell surface labeling experiments done by Ladi *et al.* (2005) could not reproduce the published findings. As Ladi *et al.* (2005) did not provide any immunofluorescence data to show the protein distribution in the cell, stainings with antibodies against different epitopes were performed to verify the distinct protein localization of DLL1 and DLL3. Confocal images revealed that DLL3 invariably localized to perinuclear structures inside the cell.

Moreover, the use of antibodies detecting either the intra- or the extracellular domain of DLL3 excluded that this intracellular staining is caused by protein cleavage.

Finally, the intracellular localization of DLL3 was confirmed *in vivo* by immunofluorescence staining of the presomitic mesoderm of wildtype E9.5 mouse embryos where endogenous *Dll1* and *Dll3* are coexpressed at high levels. While DLL1 was readily detected on the cell surface, DLL3 predominantly resided within the presomitic mesoderm cells and colocalized with DLL1 only in perinuclear regions. Verifiying the differential protein localization of DLL1 and DLL3, subcellular colocalization studies with organelle markers demonstrated an accumulation of DLL3 in the Golgi network *in vivo*. DLL3 colocalized with the cis-Golgi marker GM130 but not with the membrane marker pan-Cadherin whereas DLL1 expression clearly overlapped with membrane staining. Since in the presomitic mesoderm DLL1 and DLL3 were expressed at physiological levels this *in vivo* evidence was most important to exclude that the observed intracellular localization of DLL3 is due to artifacts by overexpressed protein. In addition, the absence of DLL3 from the cell surface is supported by localization studies in flies by Dr. Robert Jaekel and Dr. Thomas Klein (University of Cologne, Germany). DLL3 expressed in *D. melanogaster* wing disc cells does not localize to the apical membrane (Geffers *et al.*, 2007). Although not yet experimentally proven, it cannot be excluded that extensive recycling of the protein leads to lower steady state levels of the DLL3 protein on the cell surface. Even though the majority of DLL3 probably never reaches the cell surface - as observed in fly cells, in overexpressing CHO cells and in the murine presomitic mesoderm - it cannot be fully excluded that minor amounts of DLL3 might be sufficient for its biological function on the cell surface.

5.2 The transmembrane domain and surrounding sequences of DLL3 account for protein retention

Since DLL3 contains a predicted signal peptide at its N-terminus but appears to be absent from the cell surface, some retention signal seems to be present in its protein sequence. Interactions of specific sequences of DLL3 with other proteins located in the Golgi network might account for its confinement to intracellular compartments.

Chimeric ligands A-G composed of domains of DLL1 and DLL3 (Fig. 4.9) differed in their propensity to localize to the cell surface as determined by cell surface biotinylation although the N-terminus of DLL1 carrying a signal peptide responsible for the post-translational transport to the secretory pathway was present in all these chimeras. Comparing the total

protein level with the protein amount on the cell surface, chimeric ligands A, C and G (Fig. 4. 9) displayed a diminished cell surface presentation. Chimeric ligand A containing the N-terminus and DSL domain of DLL1 linked to the EGFs and intracellular domain of DLL3 was detected at the cell surface, albeit at much lower levels than the wildtype DLL1 protein. Chimeric ligands C and G contain the transmembrane (and intracellular) domain of DLL1 but the juxtaposed extracellular sequence of DLL3. Both chimeras exhibited a reduced propensity to localize at the cell surface. This decreased surface presentation was confirmed in immunofluorescence stainings. Although protein was detected on the cell surface of both cell lines, not all cells showed surface presentation of the ligands to a comparable extent. All three chimeric proteins contained the transmembrane domain of DLL3 and/or the juxtaposed extracellular DLL3 sequence indicating that these sequences might compose a Golgi retention signal. So far, there is no consensus sequence for Golgi retention described in the literature (Moore, 2003). However, the requirement of the transmembrane region of DLL3 for Golgi retention was consistent with findings in other Golgi-retained proteins, such as glycosyltransferases, though these enzymes are type II transmembrane proteins (Colley, 1997). In contrast, chimeric ligand B that also comprised the DLL3 transmembrane sequence showed nearly normal levels of protein on the cell surface. This ligand contains only DLL1 sequence in its extracellular domain indicating that the juxtaposed extracellular DLL3 region is also required for Golgi retention of the DLL3 protein. Consistently, a chimera consisting of the extracellular domain of DLL3 and the TM-ICD of DLL1 exhibits no surface expression. Collectively, the transmembrane domain of DLL3 together with adjacent extracellular sequences appears to contain some localization signal responsible for protein retention inside the cell and localization in the Golgi apparatus.

In further subcellular localization analyses, the DLL1 DSL domain appeared to be necessary to direct cell surface expression. Chimeric ligands containing only the N-terminus of DLL1 fused to DLL3 DSL domain and EGF repeats (chimeras J and M in Fig. 4. 10) exhibited no detectable membrane localization.

The presence of both confined regions, the DSL domain of DLL1 and the transmembrane region of DLL3 together with juxtamembrane sequences, led to ambiguous protein localization on the cell surface and within the cell.

5.3 Mutations of conserved motifs of the DLL1 DSL domain influence surface presentation

With regard to protein localization, the DSL domain of DLL3 could not substitute for the DSL domain of DLL1 as chimeras differing only in their DSL domain displayed different protein localization. On the amino acid level, the DSL domain of DLL3 greatly diverges from that of other Delta homologues. In addition to an altered spacing of conserved cysteine residues, two highly conserved amino acid motifs (YY and GWxG) are absent in the DLL3 DSL domain. In this study it was experimentally demonstrated that non-conservative alteration of either motif had an impact on the propensity of the generated DLL1 variants to localize to the cell surface. Conservative mutations of the double tyrosine motif (like the exchange of one of the tyrosines to a similar aromatic amino acid as phenylalanine in DLL1 variants DLL1 DSL-FY and DLL1 DSL-YF) did not change cell surface presentation. However, the loss of either both hydroxyl groups or of one of the aromatic rings influenced the localization of the mutant DLL1 protein. Semi-conservative mutations of either both tyrosines into phenylalanines (DLL1 DSL-FF) or of only one tyrosine into an alanine (DLL1 DSL-AY) reduced protein presentation on the cell membrane since a large fraction of the protein was detected inside the cell. DLL1 variants that lack both aromatic residues in the DSL domain (DLL1 DSL-ΔY) resided within the cell. The replacement of the GWxG motif in the DSL domain by four alanine residues (DLL1 DSL-ΔG) also partially impeded cell surface presentation of this DLL1 version.

According to modification predictions the tyrosines Y182 and Y183 in the DLL1 DSL domain might represent a sulfation site. Friederich *et al.* (1988) demonstrated that inhibition of tyrosine O-sulfation blocks protein secretion. Therefore mutation of these tyrosines might abolish sulfation and subsequent transport to the membrane. Additionally, it is conceivable that this potential modification might influence the affinity of DLL1 to Notch, as tyrosine O-sulfation was also implicated in protein-protein interaction (Wilkins *et al.*, 1995). However, it is unlikely that this modification occurs in DLL1, since no radioactive signal could be detected after metabolic labeling with [^{35}S]-sulfate of DLL1 expressing CHO cells and subsequent immunoprecipitation.

Recently, the DSL domain of Jagged1 was crystallized and the structure solved (Penny Handford, Univ. of Oxford, UK, personal communication). A comparison of the sequence of the DLL1 DSL domain with the three-dimensional model of the Jagged1 DSL domain allows a prediction of the relative position of these two conserved amino acid motifs within this

domain. The alignment reveals that both conserved motifs, the YY and the GWxG motif, presumably lie in the hydrophobic core preserving the intramolecular structure of the DSL domain. The YY motif seems to be involved in the connection of the N-terminus of the DSL domain with the part that resembles an EGF domain via hydrophobic interactions (Penny Handford, Univ. of Oxford, UK, personal communication). The perturbation of efficient folding by mutations of the conserved motifs in the DLL1 DSL domain is likely to account for the retention of the misfolded protein during its passage through the secretory pathway. Possibly, the perturbation of the DSL domain also leads to a rapid degradation of the protein in consequence of ER stress induced by misfolded protein.

5.4 Forced retention of a DSL protein cannot rescue DLL3 function

Concerning cell surface presentation and subcellular localization, DLL3 and the DLL1 variant, DLL1 DSL-ΔY, displayed similar properties. Both proteins were virtually absent from the cell surface and colocalized partially with the cis-Golgi marker GM130. Mice that were heteroallelic for the $Dll1^{Dll1DSL\Delta Y}$ allele and homozygous mutant for $Dll3^{pudgy}$ showed a severely perturbed vertebral column similar to the pudgy phenotype indicating that mutant DLL1 present in the Golgi apparatus can not adopt DLL3 function. The $Dll1^{Dll1DSL\Delta Y}$ allele even seemed to enhance the pudgy phenotype but due to low numbers of skeletons analyzed thus far one cannot draw clear conclusions from this data. The failure of DLL1 DSL-ΔY to rescue the loss of functional DLL3 is unlikely to be due to differences in their somite expression pattern since the DLL3 protein expressed from the *Dll1* locus in the *Dll3 knock-in* approach appeared to fully rescue the loss of wildtype endogenous *Dll3* (data by Dr. Katrin Serth; Geffers et al., 2007). Apart from its differences in protein localization, DLL3 function might rely on specific properties of the EGF-like repeats and the DSL domain that might simultaneously abolish its activating function as a Notch ligand. Previously characterized missense mutations in EGF2, 4 and 5 of *Dll3* each display a loss-of-function phenotype in mice indicating that the EGF-like repeats are essential for the correct function of DLL3 during somitogenesis (Shinkai *et al.*, 2004; Turnpenny *et al.*, 2003).

5.5 Conserved motifs in DLL1 DSL domain are essential for transactivation but not for cis-inhibition of Notch

Proper three-dimensional folding of the DSL domain of Jagged1 is essential to present the Notch binding site on the surface of the conserved DSL domain (Penny Handford, Univ. of Oxford, UK, personal communication). Thus, loss of aromatic tyrosine residues or the GWxG motif destabilizing the overall DSL domain folding in Notch ligands would affect the shape of the Notch binding site and accordingly the potential to activate Notch.

Consistent with these predictions, mutations of the DLL1 DSL domain preserving one tyrosine residue (DLL1 DSL-FY and DLL1 DSL-AY) induced Notch in transactivation assays, though to a strongly diminished extent. In contrast, the loss of the GWxG motif completely abolished Notch activation. Loss of both tyrosine residues led to an impaired cell surface presentation not allowing conclusions about the potential of the DLL1 variant to activate Notch.

The generated DLL1 DSL mutant proteins did not efficiently transactivate Notch, presumably because they contain a more or less perturbed DSL domain. However, all of them were able to cell-autonomously inhibit Notch1 when coexpressed with the receptor. These observations suggest that the interaction of Notch1 with coexpressed ligands does not require a functional DSL domain. Dlk1, a protein belonging to the epidermal growth factor (EGF)-like homeotic family, negatively regulates Notch signaling through interactions with specific EGF-like repeats (Baladron *et al.*, 2005; Nueda *et al.*, 2007). The protein structure of Dlk1 shows similarities to DLL3 but does not contain any DSL domain indicating that the interplay of EGF-like repeats themselves might be sufficient to induce an impeding effect on Notch signaling. Itoh *et al.* (2003) showed that the lack of the ICD of *Xenopus* Delta1 does not disrupt the cell-autonomous association and inhibition of Notch suggesting that the ICD and ICD-related processes such as endocytosis are dispensable for the cis-inhibition of Notch signaling. These findings rather point to an “unspecific” association of EGF-containing proteins *in vitro* and do not necessarily prove that all these proteins can interact with Notch *in vivo*.

Additionally, DLL3 and DLL1 DSL-ΔY exerted cell-autonomous inhibitory functions on Notch signaling although they resided within the cell. These findings are consistent with literature reporting that in vertebrates cis-interaction of Notch with its ligand occurs inside the cell, probably in the ER or the Golgi apparatus (Sakamoto *et al.*, 2002). In contrast, in *Drosophila* inhibition of Notch function by coexpressed Serrate appeared to originate from cis-interactions at the cell surface (Glittenberg *et al.*, 2006).

5.6 The DSL domain, EGF-like repeat 1 and 2 and the intracellular domain of DLL1 are required for Notch activation

DLL3 lacks sequences in its DSL domain that are otherwise conserved among Delta homologues. Mutations of these sequences in the DSL domain of DLL1 abolished its function. According to this, it is questionable whether DLL3 could activate Notch signaling even if it would be present on the cell surface. Transactivation analyses of chimeric ligands present on the cell surface and comprised of different protein domains of DLL1 and DLL3 (performed as detailed in Fig. 9 A) allow one to draw conclusions regarding the regions necessary for the function of DLL1 as an activating ligand. Additionally, the question of functional equivalence of the EGF-like repeats of DLL1 and DLL3 was addressed by this approach.

Although the DSL domain was shown to be responsible for Notch binding (Shimizu *et al.*, 1999), transferring the N-terminus including the DSL domain of DLL1 to the EGF-like repeats of DLL3 resulted in a non-activating ligand indicating that the DSL domain of DLL1 is not sufficient to confer transactivation potential on DLL3.

Additionally, the intracellular domain (ICD) of DLL1 is required for its function as a Notch ligand since the lack of the ICD or the replacement of the TM-ICD by the corresponding DLL3 sequence led to a complete abolishment of Notch transactivation. The intracellular domain of DLL3 is highly divergent from DLL1 and lacks several features that might be important for Notch activation. For instance, the C-terminus of DLL1 constitutes a PDZ ligand binding motif which was recently shown to mediate interactions with members of the MAGUK (membrane-associated guanylate kinase) protein family (Pfister *et al.*, 2003; Six *et al.*, 2004; Wright *et al.*, 2004). As the binding of PDZ domain-containing proteins depends on the presence of a C-terminal valine in DLL1 a C-terminal tag is likely to interfere with this interaction. However, DLL1 full length protein carrying a C-terminal tag was able to transactivate Notch1 in coculture experiments suggesting that processes associated with PDZ binding are not required for Notch signaling *per se*. This is further supported by the fact that a variant of the Notch ligand DeltaD that cannot interact with PDZ domains exhibits no Notch phenotype in zebrafish (Wright *et al.*, 2004).

Furthermore, the ICD of DLL1 is involved in processes such as ubiquitin-mediated endocytosis and multimerization. Trafficking of Notch ligands by endocytosis was shown to be initiated by the RING E3 ubiquitin ligases, Mindbomb and Neuralized (Itoh *et al.*, 2003; Lai and Rubin, 2001; Lamar *et al.*, 2001; Nam *et al.*, 2006; Pavlopoulos *et al.*, 2001). Both

enzymes interact with short amino acid motifs in the ICD of the Notch ligand Serrate to ubiquitinate specific lysines thereby targeting the protein for internalization by the endocytotic machinery (Overstreet *et al.*, 2004). Simultaneous absence of Neuralized and Mindbomb completely abolished Notch signaling in *Drosophila* indicating that ubiquitination is a prerequisite for Delta signaling (Lai *et al.*, 2005; Pitsouli and Delidakis, 2005). Deletion of the interacting motif or substitution of the lysine residues by alanine severely compromised ligand internalisation and Notch activation (Glittenberg *et al.*, 2006). Consistent with the requirement of ubiquitin-mediated endocytosis, substitution of the Delta ICD by an endocytotic motif of an unrelated transmembrane protein or ubiquitin itself resulted in an active ligand (Itoh *et al.*, 2003; Wang and Struhl, 2004; Wang and Struhl, 2005).

Moreover, endocytosis is involved in dissociation of the Notch heterodimer after ligand binding and removal of the Notch extracellular domain (NECD) which is a prerequisite for the Notch ectodomain shedding by the metalloprotease TACE (see chapter 1.3). For instance, the loss of E3 ligase in Mindbomb1 mutant mice resulted in a disruption of Notch signaling as these embryos failed to generate activated Notch ICD (Barsi *et al.*, 2005; Koo *et al.*, 2005).

Findings that the signal-transducing activity of soluble DLL1 was enhanced by antibody-mediated cross-linking indicates that multimerization of Notch ligands mediated by the intracellular domain also affects activation of Notch (Hicks *et al.*, 2002; Shimizu *et al.*, 2002). *Drosophila* Delta and Serrate proteins lacking their ICD acted to antagonize the function of the full-length ligands (Fleming *et al.*, 1997; Sun and Artavanis-Tsakonas, 1996). Additionally, the soluble ligand form impaired the transactivation activity of full length Delta when coexpressed in the same cell (Shimizu *et al.*, 2002). These observations imply a link between endocytosis, clustering and Notch signaling.

As the DLL3 ICD lacks lysine residues required for ubiquitin-conjugation, one can assume that the chimeric ligand comprised of the extracellular part of DLL1 and the TM-ICD of DLL3 is incapable to activate Notch due to a loss of ubiquitin-dependent processes or other modifications. Emphasizing the importance of ICD-mediated processes for Notch activation, a truncated DLL1 ligand lacking the ICD did not transactivate although efficiently presented on the cell surface.

A chimera composed of the N-terminal part of DLL1 including DSL and EGF domain 1 and 2, the six EGF repeats of DLL3 and the TM-ICD of DLL1 exhibited activating properties albeit weaker than the wildtype indicating that the two distal EGF repeats and the DSL domain of DLL1 are sufficient for Notch activation. Consistent with this finding is the

requirement for the DSL domain and EGF repeats 1 and 2 of Jagged1 for Notch binding *in vitro* (Shimizu *et al.*, 1999).

Substitution of the EGF-like repeats 1 and 2 in DLL1 by those of DLL3 with or without correct spacing led to a complete derogation of Notch signaling although both proteins localized to the cell membrane. These observations indicate that DLL1 EGF-like repeats 1 and 2 which are highly conserved among Delta homologues have specific properties that are not present in repeats 1 and 2 of DLL3. Interestingly, the disruption of the domain structure of EGF 1 or 2 of DLL1 by mutating conserved cysteines was sufficient to impair DLL1 function *in vitro* and *in vivo* (Geffers, Müller and Gossler, unpublished observations; Cordes, Schuster-Gossler and Gossler, unpublished observations). While mutation of EGF-like repeat 1 abolished cell surface presentation, DLL1 protein with a disrupted EGF-like repeat 2 localized to the cell membrane but showed no transactivation potential.

In addition to the requirement of EGF-like repeat 1 and 2 of DLL1, the correct spacing of these EGF repeats in DLL1 is essential for Notch activation as alteration of the length of the spacer sequence resulted in a loss of DLL1 function. EGF 1 and 2 might support the binding of the DLL1 DSL domain to Notch by additional interaction with EGF repeats of Notch. Concerning the functional equivalence of the EGF-like repeats of DLL1 and DLL3, EGFs 4 to 8 of DLL1 resemble EGFs 2 to 6 of DLL3 by sequence. These EGF repeats were exchanged in DLL1 without diminishing its Notch activating capacity. On the contrary, in transactivation assays this chimeric ligand induced even higher Notch activity in the receiving cells than the DLL1 wildtype. This effect might also be due to different expression levels as it was not possible to obtain CHO cell lines with closely matching levels of protein expression.

Analyzing the exchangeability of the EGF-like repeats of DLL1 and DLL3, not only the protein sequence of these domains but also modification sites has to be considered. Similar to the Notch receptors, Notch ligands might be targeted by glycosyltransferases Pofut1 and Fringe that participate in the synthesis of O-linked fucose glycans attached to EGF repeats (Haines and Irvine, 2003; Haltiwanger and Lowe, 2004; Moloney *et al.*, 2000a; Panin *et al.*, 2002).

In DLL1, five of the eight present EGF-like repeats carry potential consensus sequences for O-fucosylation: EGF 2, 3 and 8 have a broad consensus site, EGF 4 and 7 display a narrow O-fucose consensus site. Similarly, the narrow O-fucosylation site is present in EGF2 and 5 of DLL3 which are homologuous to EGF 4 and 7 of DLL1 (Panin *et al.*, 2002). There is evidence that Dll1 and Jagged1 incorporate *O*-fucose that is in turn elongated by Fringe *in vitro* (Panin *et al.*, 2002). Metabolic labeling demonstrated that DLL3 also served as a

substrate for O-fucosylation by Pofut1 (Müller and Gossler, unpublished observations). However, which of the potential fucosylation sites are modified in DLL1 and DLL3 *in vivo* still needs to be shown. The highly conserved DLL1 EGF repeat 2 together with EGF repeat 3 - both containing potential fucosylation sites - are missing in the DLL3 protein. Concerning the involvement of EGF1 and 2 of DLL1 in Notch activation the potential O-fucosylation in EGF-like repeat 2 of DLL1 may accessorily play a role in this context. Although the exact functional significance to these modifications is yet unknown it was suggested that O-fucosylation might facilitate ligand multimerization (Chitnis, 2006).

5.7 DLL3 is not an antagonist of DLL1 in vivo

On the basis of a detailed analysis of genetic interactions, Takahashi *et al.* (2003) suggested that DLL1 and DLL3 may have non-redundant, even counteracting functions. However, their conclusions were based on the assumption that DLL3 is able to activate Notch signal transduction.

Dunwoodie *et al.* (2002) discussed the possibility that the obvious differences in expression of Notch target genes in the PSM of *Dll1* and *Dll3* mutants do not reflect distinct functions but are rather due to the same function that affects target gene expression to different extents. The authors proposed that this issue could best be addressed by a cDNA *knock-in* approach that replaces *Dll1* by *Dll3* or *vice versa*.

In this study two mouse lines were generated expressing the Dll3 and Dll3HA cDNA, respectively, under the regulatory control of *Dll1*. Analyses of these mutant mice (performed by Dr. Katrin Serth; Geffers *et al.*, 2007) revealed that the phenotype of homozygous $Dll1^{Dll3HAki}$ embryos was virtually indistinguishable from the *Dll1* null phenotype as antero-posterior somite patterning (indicated by Uncx4.1 expression) and cyclic gene expression of the Notch modulator Lfng were similarly affected in both cases. These observations demonstrate that, consistent with *in vitro* findings, *Dll3* cannot substitute for *Dll1 in vivo*. However, DLL3 protein generated from the *knock-in* allele was functional as the *Dll3* null phenotype was rescued by *Dll3* expression from the $Dll1^{Dll3HAki}$ allele. Mice that were homozygous mutant for the null allele $Dll3^{pu}$ but heteroallelic for the *Dll1* wildtype and the $Dll1^{Dll3}$ *knock-in* alleles showed normal axial skeleton development as well as normal cyclic Lfng expression and stripy expression of Uncx4.1 in embryos of this genotype.

Given that the disrupted antero-posterior somite patterning of pudgy mice is restored in $Dll1^{Dll3HAki/+}$; $Dll3^{pu/pu}$ embryos, the *knock-in* into the *Dll1* locus might serve as an *in vivo*

assay to define features or protein domains of DLL3 responsible for its function. Additionally this *knock-in* approach shows that loss of distinct expression of *Dll3* in the anterior half of the somites does not influence the normal development of the vertebral column. Moreover, ectopic expression of *Dll3* in the posterior halves of the formed somites does not interfere with DLL1 function.

To investigate how DLL3 affects Notch activation *in vivo* the protein levels of activated Notch intracellular domain (NICD) in mouse PSM were analyzed by Western blot with anti-cleaved Notch antibodies. If DLL3 would act as an antagonist of Notch signaling during somitogenesis the loss of DLL3 should lead to an enhancement of Notch signaling and higher levels of activated NICD. However, the PSMs of pudgy embryos displayed slightly decreased activated NICD levels when compared to the wildtype PSM, thus – if anything - supporting a Notch *activating* potential of DLL3. As a control, PSMs of homozygous $Dll1^{Dll3HAki}$ mice were analyzed. Since the Notch ligand *Dll1* is eliminated and replaced by *Dll3* which proved not to activate Notch *in vitro*, signal transduction via these ligands is assumed to be completely disrupted in this tissue. Consistently, only very low levels of activated NICD were detected in these samples, probably originating from signal induction via another Notch ligand. Jagged1 might account for this Notch activation as it is strongly expressed in the posterior half of the forming somite, the tailbud and at low levels in the PSM (Shi and Stanley, 2003). However, Jagged1-induced signal transduction in the PSM seems to be dispensable for somitogenesis as Jagged1 null mutant embryos lack any detectable somite phenotype (Xue *et al.*, 1999). Consistently, altering the ratio of *Dll3* to *Dll1 in vivo* by combination of different *Dll3* and *Dll1* mutant alleles revealed no genetic evidence for antagonism of DLL3 and DLL1 during somitogenesis (data by Dr. Katrin Serth; Geffers et al., 2007).

Taken together our observations suggest that DLL3 does not obviously inhibit Notch signaling *in vivo* though it was shown that coexpression of DLL3 and Notch1 leads to cell-autonomous inhibition of Notch signaling *in vitro* (Ladi *et al.*, 2005). DLL3 seems to have a different function - possibly even outside the Notch pathway - as it is not acting as a simple Notch activator or antagonist.

5.8 Potential DLL3 functions

DLL3 does not exert pivotal functions outside somitogenesis as *Dl3* mutant mice survive though with an extremely perturbed vertebral column (Grüneberg, 1961; Kusumi *et al.*, 1998).

The normal function of DLL3 requires the protein to be anchored into the membrane. Mutations in the transmembrane domain of DLL3 resulting in a soluble extracellular DLL3 domain - due to protein truncation - produced a *Dll3* null allele (Turnpenny *et al.*, 2003).

Though harboring a signal peptide at its N-terminus, DLL3 appears to be absent from the cell surface. Therefore a direct contact between proteins on the neighboring cell surface and DLL3 appears unlikely. Analysis of the subcellular localization of stable CHO cell lines expressing chimeric ligands by either cell surface labeling or immunofluorescence and transactivation assays of these ligands revealed that DLL3 has acquired sequences that promote Golgi retention and several structural changes that are sufficient to abrogate Notch activating function. Furthermore, DLL3 involvement in processes such as ubiquitin-mediated endocytosis or those associated with PDZ-containing proteins seems improbable, as its intracellular domain lacks lysine residues required for ubiquitin-conjugation and the C-terminal PDZ binding motif present in other Delta homologues. Additionally, the absence of nuclear localization signals in the DLL3 intracellular domain eliminates the possibility to directly translocate to the nucleus as described for DLL1 ICD but it does not exclude that DLL3 ICD might act as a transcriptional regulator when co-transported together with other proteins into the nucleus (Hiratochi *et al.*, 2007; Ikeuchi and Sisodia, 2003; Six *et al.*, 2003). Collectively, DLL3 differs from DLL1 with respect to protein localization and its potential to interact with proteins such as E3 ligases and PDZ-containing proteins.

Intra- rather than intercellular associations of DLL3 with other proteins might be important for DLL3 function. Interacting intracellular partners could be Notch receptors and/or Notch ligands on their way through the secretory pathway to the cell surface. As the majority of DLL3 protein localizes to the Golgi apparatus, this cell compartment could present the site of association with potential interaction partners. DLL3 colocalization with DLL1 in intracellular structures of presomitic mesoderm cells, as reported in this study, raises the possibility that these proteins interact.

Most strikingly, a Notch modulator, the glycosyltransferase lunatic fringe (Lfng), which catalyzes the elongation of O-fucose linked to EGF repeats of Notch receptors and ligands, also resides within the Golgi network (Hicks *et al.*, 2000).

Dll3 and *Lfng* are largely coexpressed in the PSM, the somites and the neural tube. Despite subtle defects in the central nervous system of pudgy mice, *Dll3* null alleles cause skeletal defects by a disruption of the antero-posterior patterning of the somites. This phenotype is remarkably similar to that of mice carrying either *Lfng* loss-of-function or gain-of-function mutations (Serth *et al.*, 2003; Zhang *et al.*, 2002).

Ladi *et al.* (2005) demonstrated that DLL3 interferes with DLL1-induced Notch signaling by cis-inhibition and that expression of *Lfng* can override the inhibitory effect of DLL3. Additionally, as DLL3 was shown to be O-fucosylated *in vitro* (Müller and Gossler, unpublished observations), EGF-like repeats 2 and 5 of DLL3 that carry narrow O-fucosylation sites might be substrates of Lfng. These findings point to a potential interaction of DLL3 and Lfng which has to be investigated in more detail. As cis-inhibition seems not to interfere with cell surface presentation of Notch it remains an open question how this mechanism regulates Notch activity (Sakamoto *et al.*, 2002). Though coexpression of *Lfng in vitro* diminishes ligand-dependent cell-autonomous inhibition of Notch signaling (Ladi *et al.*, 2005; Sakamoto *et al.*, 2002), intracellular ligand-receptor interactions might still influence the interaction of Notch with POFUT and/or Lfng under physiological conditions. As a potential consequence, this might prevent crucial modifications of the Notch receptor. Thus, a modulatory effect on Notch signaling might emenate from DLL3 interactions with Lfng.

In *Drosophila* a Notch chaperone activity was demonstrated for the glycosyltransferase OFUT1 which was independent of its fucosyltransferase activity. Interaction of Notch with OFUT1 promoted normal folding of Notch in the endoplasmic reticulum and trafficking of Notch to the cell membrane (Okajima *et al.*, 2005). Additionally, OFUT1 served as an extracellular component that was essential for the constitutive endocytic trafficking of Notch in Drosophila (Sasamura *et al.*, 2007). Similarly, it is conceivable that intracellular DLL3 acts as a scaffold protein involved in trafficking or that its function comprises chaperone activity for other components of the Notch signaling pathway. Thus, Notch signaling might be directly or indirectly modulated by intracellular interactions of DLL3.

During evolution similar mechanisms emerge by the usage of different components. For instance, in fish and frog, oscillating expression of Delta-like ligands during somitogenesis evoke rhythmic changes in Notch signaling required for somite formation (Jiang *et al.*, 2000). In contrast, in mouse and chick, oscillations in Notch signaling in the presomitic mesoderm are generated by the wavelike expression of the Notch modifier Lfng while ligand expression in the caudal PSM is relatively uniform (Aulehla and Johnson, 1999; Forsberg *et al.*, 1998).

As Dll3 is only described for mammals, it might represent a novel intracellular component of Notch signaling, a modulator that has evolved in mammals and fulfils a function that is accomplished by other proteins in Dll3-lacking species like fish and frog.

Data obtained in this study unambiguously show that DLL3 does not transactivate Notch signaling and localizes to intracellular compartments including the cis-Golgi network. This provides first evidence that DLL3 might functions as an intracellular component of the Notch

signaling pathway. Although DLL3 interacts with Notch and the *Dll3* null phenotype is similar to that of Lfng (Ladi *et al.*, 2005), at this point it cannot completely be ruled out that in addition to this Dll3 exerts functions independent from Notch.

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Publications

Papers

Schröder N, Sekhar A, Geffers I, Müller J, Dittrich-Breiholz O, Kracht M, Wedemeyer J, Gossler A. **Identification of mouse genes with highly specific expression patterns in differentiated intestinal epithelium**. *Gastroenterology. 2006 Mar;130(3):902-7.*

Geffers I[*], Serth K[*], Chapman G, Jaekel R, Schuster-Gossler K, Cordes R, Sparrow DB, Kremmer E, Dunwoodie SL, Klein T, Gossler A. **Divergent functions and distinct localization of the Notch ligands DLL1 and DLL3 in vivo**. *J Cell Biol. 2007 Jul 30;178(3):465-76.*

Poster presentations

Insa Geffers, Karin Schuster-Gossler, Ralf Cordes, Hannelore Burkhardt, Anatoli Heiser and Achim Gossler **Functional non-equivalence of the Notch ligands Dll1 and Dll3**. *16. Wissenschaftliche Tagung der Gesellschaft für Entwicklungsbiologie in Münster. 06.-09. April 2005*

Insa Geffers[*], Katrin Serth[*], Karin Schuster-Gossler, Ralf Cordes, Hannelore Burkhardt, Anatoli Heiser and Achim Gossler **Functional non-equivalence of the Notch ligands Dll1 and Dll3**. *6. GfE-School in Günzburg. 28.-30. September 2006*

Insa Geffers, Claudia Brockmeyer, Karin Schuster-Gossler, Ralf Cordes, Elisabeth Kremmer and Achim Gossler **DLL3 might be an intracellular component of the Notch pathway**. *17. Wissenschaftliche Tagung der Gesellschaft für Entwicklungsbiologie in Marburg. 21.-24. März 2007*

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Appendix

Table 1: Cell clones used for this thesis and their origins

Figure	CHO cell line	Cell clone	Generated by	Subcloned by
4.2 left panel	Dll1Flag	#A7	I.G.	
	Dll3HA	#A5	I.G.	
4.2 right panel	Dll1Flag	#A7/A1	I.G.	I.G.
	Dll3Flag	#C3/38	I.G.	K.S./P.D.-H.
4.3	Dll1Flag	#A7/A1	I.G.	I.G.
	Dll3Flag	#C3/38	I.G.	K.S./P.D.-H.
	Dll3HA	#A5	I.G.	
4.4	Dll1Flag	#A7/A1	I.G.	I.G.
	Dll3Flag	#C3/38	I.G.	K.S./P.D.-H.
	Dll3HA	#A5	I.G.	
4.5	Dll3Flag	#C3/38	I.G.	K.S./P.D.-H.
4.9	Dll1Flag	#A7/A1	I.G.	I.G.
	A	#A5/8	K.S./P.D.-H.	K.S./P.D.-H.
	B	#A3	K.S./P.D.-H.	-
	C	#18/10	K.S./P.D.-H.	K.S./P.D.-H.
	D	#14	I.G.	-
	E	#B2	K.S./P.D.-H.	-
	F	#B11	K.S./P.D.-H.	-
	G	#A2/C5	K.S./P.D.-H.	K.S./P.D.-H.
	H	#13	K.S./P.D.-H.	-
4.10	Dll1Flag	#A7/A1	I.G.	I.G.
	A	#A5/8	K.S./P.D.-H.	K.S./P.D.-H.
	B	#A3	K.S./P.D.-H.	-
	C	#18/10	K.S./P.D.-H.	K.S./P.D.-H.
	G	#A2/C5	K.S./P.D.-H.	K.S./P.D.-H.
	H	#13	K.S./P.D.-H.	-
	I	#B3	I.G.	-
	J	#6	I.G.	-
	K	#B11/2C5	K.S./P.D.-H.	K.S./P.D.-H.
	L	#E1	K.S./P.D.-H.	-
	M	#7	I.G.	-
4.12	Dll1Flag	#A7/A1	I.G.	I.G.
	Dll3Flag	#C3/38	I.G.	K.S./P.D.-H.
	DLL1 DSL-ΔY	#16/6	I.G.	I.G.
	DLL1 DSL-ΔG	#16/8	I.G.	I.G.
	DLL1 DSL-AY	#13/11	C.B.	I.G.
	DLL1 DSL-FY	#3/5	C.B.	I.G.
4.13	Dll1Flag	#A7/A1	I.G.	I.G.
	DLL1 DSL-ΔY	#16/6	I.G.	I.G.
	DLL1 DSL-ΔG	#16/8	I.G.	I.G.
	DLL1 DSL-AY	#13/11	C.B.	I.G.
	DLL1 DSL-FY	#3/5	C.B.	I.G.
	DLL1 DSL-YF	#1	I.G.	
	DLL1 DSL-FF	#22	I.G.	
4.14	DLL1 DSL-ΔY	#16/6	I.G.	I.G.
4.15	Dll1Flag	#A7/A1	I.G.	I.G.
	Dll3Flag	#C3/38	I.G.	K.S./P.D.-H.
	DLL1 DSL-ΔY	#16/6	I.G.	I.G.
	DLL1 DSL-ΔG	#16/8	I.G.	I.G.
	DLL1 DSL-AY	#13/11	C.B.	I.G.
	DLL1 DSL-FY	#3/5	C.B.	I.G.

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