

Notch Receptor-Ligand Interactions During T Cell Development, a Ligand Endocytosis-Driven Mechanism

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Abstract Notch signaling plays an important role during the development of different cell types and tissues. The role of Notch signaling in lymphocyte development, in particular in the development and commitment to the T cell lineage, has been the focus of research for many years. Notch signaling is absolutely required during the commitment and early stages of T cell development. Activation of the Notch signaling pathway is initiated by ligand-receptor interactions and appears to require active endocytosis of Notch ligands. Studies addressing the mechanism underlying endocytosis of Notch ligands have helped to identify the main players important and necessary for this process. Here, we review the Notch ligands, and the proposed models of Notch activation by Notch ligand endocytosis, highlighting key molecules involved. In particular, we discuss recent studies on Notch ligands involved in T cell development, current studies aimed at elucidating the relevance of Notch ligand endocytosis during T cell development and the identification of key players necessary for ligand endocytosis in the thymus and during T cell development.

Abbreviations

ADAM	A disintegrin and metalloprotease
ANK	Ankyrin
BM	Bone marrow
CD	Clusters of differentiation
CDK	Cyclin dependent kinase

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CSL	CBF-1 in humans, suppressor of hairless in <i>Drosophila</i> , lag in <i>C. elegans</i>
Dll	Delta-like
DN	Double negative
DOS	Delta and OSM-11-like proteins
DP	Double positive
DSL	Delta/Serrate/Lag2
EGF	Epidermal growth factor
ETP	Early thymic progenitor
FL	Fetal liver
FoxN1	Forkhead box protein N1
GPI	Glycosylphosphatidylinositol
GSL	Glycosphingolipid
HSC	Hematopoietic stem cell
ICN	Intracellular Notch
ISP	Immature single positive
Jag	Jagged
LDL	Low density lipoprotein
Lfng	Lunatic fringe
Lqf	Liquid facets
LNR	LIN-12 Notch repeats
MamL	Mastermind-Like
Mib	Mindbomb
MINT	Msx interacting nuclear target protein
mRNA	Messenger ribonuclease
MMTV	Mouse mammary tumor virus
NECD	Notch extracellular domain
Neur	Neuralized
NHR	Neuralized homology repeats
NLS	Nuclear localization signal
NRR	Negative regulatory region
PEST	Polypeptide enriched in proline (P), glutamic acid (E), serine (S), and threonine (T)
PDZL	PSD-95/Dlg/ZO-1-ligand
PIPs	Phosphoinositol phosphates
RAG	Recombination activating gene
RAM	RBPJ association module
RBPJ	Recombination binding protein joining-kappa
RING	Really interesting new gene
siRNA	Small interference ribonuclease
SMRT	Silencing mediator of retinoid and thyroid receptor
SP	Single positive
TAD	Transactivation domain
TCR	T cell receptor

TEC	Thymic epithelial cells
TF	Transcription factor
TSPs	Thymic seeding progenitors
WT	Wildtype
Ub	Ubiquitin

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1 T Cell Development

The thymus is the site of development of the majority of T cells, and provides an essential stromal microenvironment necessary for the generation of a functional and diverse T cell repertoire, which is self-restricted and self-tolerant (Petrie and Zuniga-Pflucker 2007).

Cells emigrating to the thymus from either the fetal liver or the adult bone marrow, undergo a series of well-characterized checkpoints, and become increasingly more restricted to the T cell lineage, while eliminating the possibility of other cell fates. These emigrating thymic seeding progenitor cells (TSPs) have been the focus of many studies and is reviewed in Bhandoola et al. (2007).

Different stages of T cell development have been classically defined on the basis of CD4 and CD8 expression. The starting progenitors within the thymus lack the expression of CD4 and CD8, and are termed double negative (DN) cells. The DN subset can be further subdivided by the expression of the cell surface markers CD44 and CD25, with the following developmental sequence: DN1

(CD44⁺ CD25⁻); DN2 (CD44⁺ CD25⁺); DN3 (CD44⁻ CD25⁺); and, DN4 (CD44⁻ CD25⁻). By the DN3 stage, thymocytes are fully committed to the T cell lineage and their further differentiation requires the expression of the pre-T cell receptor (TCR), a critical developmental checkpoint termed β -selection (Michie and Zuniga-Pflucker 2002). This results in the generation of CD4 and CD8 (double positive, DP) expressing cells, which rearrange their TCR- α gene loci, to produce an $\alpha\beta$ -TCR, which following interactions with major histocompatibility complex (MHC)-class-I or MHC-class-II molecules may lead to the selection of CD8⁺ or CD4⁺ single positive (SP) cells (Ciofani and Zuniga-Pflucker 2007; Petrie and Zuniga-Pflucker 2007).

$\gamma\delta$ T cells are able to develop from the DN1-DN3 stages, and do not undergo selection checkpoints in the same manner as $\alpha\beta$ developing thymocytes. Instead it seems that the strength of $\gamma\delta$ -TCR signals directs the adoption of this lineage outcome (Hayes et al. 2005; Wong and Zuniga-Pflucker 2010).

There are many factors that are involved in the process of thymocyte differentiation, including cytokines, transcription factors, and genes that are important in determining cell fate and patterning in the developing embryo, such as Hh, BMPs, and Notch. All these factors act in conjunction with each other and their interplay is important in the production of a successful pool of T cells that exit the thymus. The reader is referred to recent reviews (Ciofani and Zuniga-Pflucker 2007; Thompson and Zuniga-Pflucker 2011; Varas et al. 2003). The focus of this review will be the role of components of the Notch signaling pathway in early T cell development, in particular concentrating on Notch ligands and Notch ligand endocytosis, and recent advances in this field in relation to T cell development.

2 The Notch Signaling Pathway

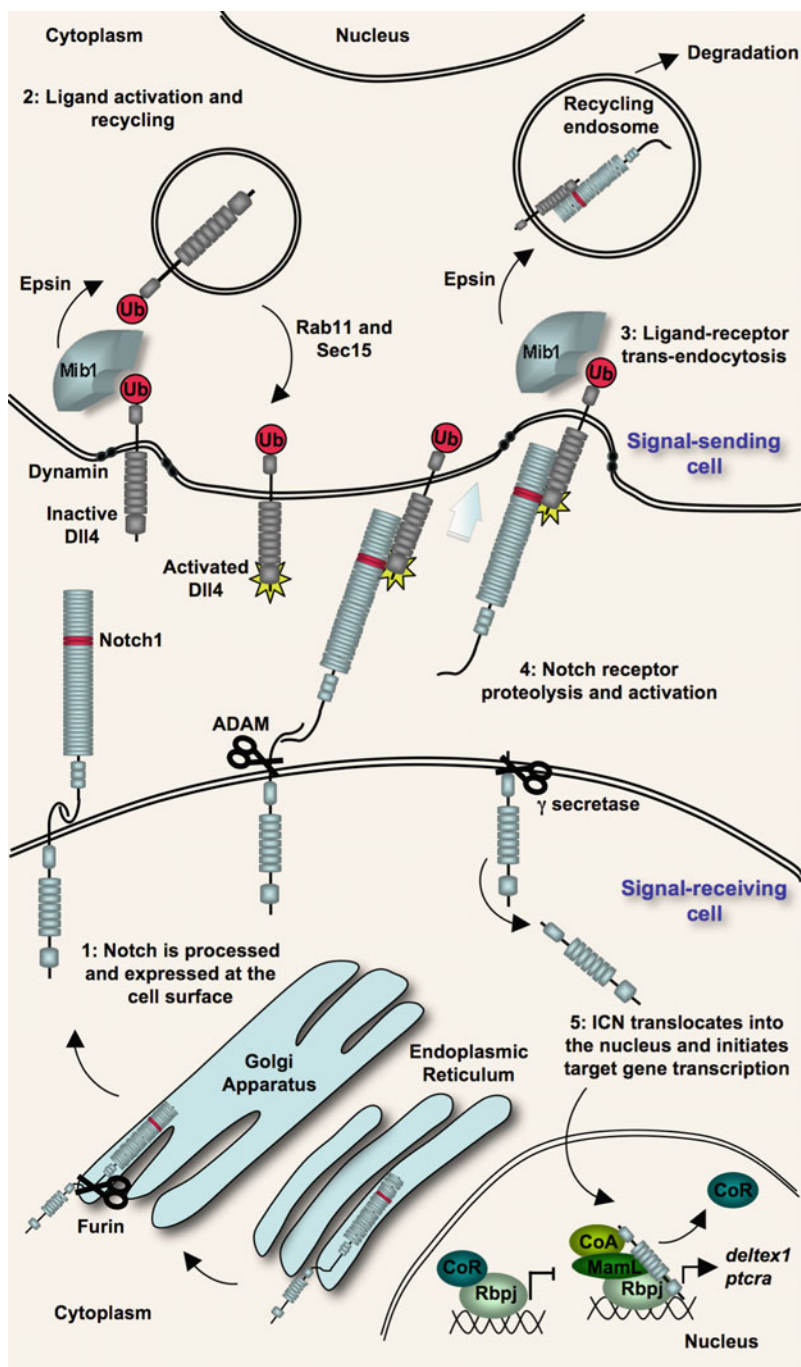
Notch was first identified in *Drosophila*, after the observation of a heterozygous loss-of function mutation that exhibited a ‘notched’ wing margin phenotype (Morgan 1917). Notch was cloned in 1983 (Artavanis-Tsakonas et al. 1983) and since then a plethora of work has identified that it is highly conserved in many species. The Notch receptor is activated by Delta-Serrate-Lag2 (DSL) ligands, of which there are two families: Delta and Serrate (Bray 2006; Tien et al. 2009). In mammals, there are four Notch receptors (Notch-1, -2, -3, and -4) (DeL Amo et al. 1992; Ellisen et al. 1991; Lardelli et al. 1994; Lardelli and Lendahl 1993; Uyttendaele et al. 1996; Weinmaster et al. 1992), which bind and interact with two Serrate family homologues (Jagged-1 and -2), and three Delta family homologues (Delta-like-1, -3, and -4) (Bettenhausen et al. 1995; Dunwoodie et al. 1997; Lindsell et al. 1995; Shawber et al. 1996; Shutter et al. 2000). As illustrated in Fig. 1, upon ligand-receptor interactions the heterodimeric Notch receptor undergoes an S2 cleavage in its extracellular domain, mediated by a disintegrin and metalloprotease (ADAM). The transmembrane associated Notch is then further cleaved by a γ -secretase multiprotein complex (S3 cleavage) consisting

of presenilin, nicastrin, APh-1, and PEN-2 proteins, releasing the active intracellular Notch (ICN) into the cytoplasm (Kopan and Ilagan 2009). The ICN translocates into the nucleus, and interacts with the helix-loop-helix transcription factor CSL (CBF-1 in humans, Suppressor of Hairless in *Drosophila*, Lag in *C. elegans*)/RBPJ (recombination binding protein joining-kappa) in mice, displacing corepressors such as silencing mediator of retinoid and thyroid receptor (SMRT) and Msx interacting nuclear target protein (MINT) (Kao et al. 1998; Kuroda et al. 2003; Oswald et al. 2005), and recruits Mastermind-like (MamL) proteins, which then aid in recruiting coactivators p300 and histone acetyl transferases, thus promoting target gene transcription (Kovall 2007; Nichols et al. 2007; Wu et al. 2000). There are three mammalian homologues of MamL, which are able to bind to all four Notch receptors (Fryer et al. 2002; Wu et al. 2002). Target genes of Notch signaling include: *hairy enhancer of split-1* (*hes-1*), *deltex1*, and tissue-specific targets such as: *pre-T α* and *cd25* (Defetos et al. 2000; Jarriault et al. 1995; Reizis and Leder 2002).

3 Notch Receptors

In mammals, the Notch receptor is produced as a single protein, which is proteolytically cleaved by a furin-like convertase (S1 cleavage) in the cytoplasm producing a heterodimeric transmembrane receptor, consisting of non-covalently associated extracellular and intracellular domains that are expressed at the cell surface (Blaumueller et al. 1997). The structure of the extracellular domain includes a series of 29–36 epidermal growth factor (EGF)-like repeats, important for ligand binding, in particular EGF repeats 11–12 are important for Notch ligand interactions (Rebay et al. 1991), a negative regulatory region (NRR) which is composed of three cysteine-rich LIN-12 Notch repeats (LNR), important for preventing ligand-independent activation and a heterodimerization domain. The intracellular portion consists of an RBPJ association module (RAM) domain, two nuclear localization signals (NLS), and seven ankyrin repeats (ANK), of which the RAM and the ANK repeats are both involved in binding to CSL/RBPJ and MamL (Tamura et al. 1995). There is also a transactivation domain (TAD) and a polypeptide enriched in proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST) domain, which regulates protein stability (Kopan and Ilagan 2009). Proteolytic processing of Notch receptors is carried out by ADAM proteases (S2 cleavage) and γ -secretase activity of the presenilin multiprotein complex (S3 cleavage) (Kopan and Ilagan 2009).

The EGF repeats on the Notch receptor can undergo O linked-glycosylation, O linked-glucose modifications are mediated by the glycosyltransferase Rumi in flies (Acar et al. 2008) and are thought to improve binding of Notch to its ligand. O-fucose modifications are mediated by the fucosyltransferase Pofut in mammals (Ofut in flies) (Okajima and Irvine 2002; Sasamura et al. 2003; Shi and Stanley 2003). Fringe proteins then extend the O-fucose modified EGF repeats further



◀ **Fig. 1** *Schematic of Notch1 receptor activation by Delta-like 4 in the thymus.* Activation of the Notch signaling pathway is initiated by receptor-ligand interactions. The biosynthesis, processing, and activation required for Notch1 and Dll4 interactions are depicted as indicated. (1) The Notch1 receptor is produced as a single protein in the ER that is proteolytically cleaved by a furin-like convertase within the Golgi, generating a heterodimeric receptor that is non-covalently expressed on the cell surface of thymocytes, the ‘signal-receiving cells’. Notch1 can also undergo O-linked glycosylation and Fringe modifications in the cytoplasm (not shown). (2) Dll4 expressed by the thymic epithelial cells, the ‘signal-sending cells’, may require activation as suggested by the ‘Recycling model’—in which ligands are produced and expressed in an inactive form on the cell surface, followed by internalization mediated by Mib1 ubiquitinylation, which is thought to promote the “activation” of the ligand prior to its reexpression on the cell surface, via a process that also involves Dynamin, Epsin, Rab11, and Sec15. (3) The DSL domain of Dll4 and the EGF repeats (11–12) on the Notch1 receptor are important for ligand-receptor interactions. The ‘Mechanotransduction model’ suggests that when Notch1 binds Dll4, the Mib1-ubiquitinylated Dll4 undergoes endocytosis, which “pulls” on Notch1 to induce a conformational change in the Notch receptor that leads to the trans-endocytosis of the Notch ECD into the signal-sending cell and it is then targeted for degradation. (4) Following the removal of Notch ECD by Dll4, an ADAM cleavage site is exposed on the Notch receptor. The ADAM-cleaved Notch receptor becomes internalized (not shown), and then undergoes γ -secretase processing, releasing ICN into the cytoplasm, allowing it to translocate into the nucleus where it binds to RBPI, displacing corepressors, recruiting MamL and co-activators, and inducing target gene transcription, such as: *deltex1* and *ptrca*

(Panin et al. 1997). There are three mammalian homologues of Fringe: Lunatic Fringe, Manic Fringe, and Radical Fringe which work in a cell autonomous manner, to potentiate signaling by Delta family members and restrict signaling by Serrate family members (Johnston et al. 1997).

4 DSL Notch Ligands

DSL Notch ligands are transmembrane proteins, which directly interact with Notch receptors at their cell surface. As mentioned earlier there are two families of ligands, the delta family, in mammals: Delta-like-1 (Dll1), Dll3, and Dll4, and the Serrate family, in mammals: Jagged1 (Jag1) and Jag2. The extracellular domain of all DSL ligands consist of an N-terminal (NT) domain, followed by a DSL domain and a series of EGF repeats (Parks et al. 2006; Skwarek et al. 2007). The N-terminal region can be further subdivided into two regions, based on its richness in cysteine, the N1 region: is cysteine-rich and the N2 region: is cysteine free (Parks et al. 2006). Jagged ligands have an additional cysteine-rich region not present in Dll ligands (Vitt et al. 2001). Recently Hamel et al., identified a conserved glycosphingolipid (GSL)-binding motif within the N2 region that may regulate membrane association and ligand endocytosis in *Drosophila* (Hamel et al. 2010). In addition a conserved motif called the Delta and OSM-11-like proteins (DOS) domain has been identified as part of the first two EGF repeats proceeding the DSL domain and is thought to contribute to Notch receptor binding and signaling (Cordle et al. 2008; Komatsu et al. 2008; Parks et al. 2006; Shimizu et al. 1999). Interestingly Dll3 and Dll4 both lack this DOS domain, and

therefore it has been proposed that proper activation of the Notch pathway by these ligands would require an additional DOS domain-containing partner (D'Souza et al. 2010; Komatsu et al. 2008). The highly conserved histidine-268 in the second EGF repeat in Jag1 has also been shown to be important for Notch binding and activation of Notch signaling (Hansson et al. 2010). Following the transmembrane domain, the intracellular domain of Notch ligands is the most dissimilar portion of the whole protein, between the different homologues (Pintar et al. 2007). In this region, they contain multiple lysine residues, which are important for ligand endocytosis and a PSD-95/Dlg/ZO-1-ligand (PDZL) motif that is important for Notch-independent signaling and has been predicted for Dll1, Dll4, and Jag1 (Pintar et al. 2007). Mutations of the lysine residues or deletions of the intracellular domain reduce Notch signaling activity in flies and mammalian cells (Glittenberg et al. 2006; Nichols et al. 2007; Parks et al. 2006). Recently, it has been shown that mutation of lysine-613 in the intracellular domain of Dll1 is important for enabling interactions with Notch. Surprisingly this one mutation in the intracellular domain caused a reduction in multiubiquitylation of Dll1, reduced Notch1 binding and decreased Notch target gene activation in a transcellular assay (Zhang et al. 2011). It would be interesting to see if the other Notch ligands also have this conserved lysine residue in their intracellular domain, and if it too is important for allowing efficient Notch receptor binding.

Dll3 is the most divergent of all the mammalian Notch ligands it does not have any intracellular lysine residues with which to mediate activation of the Notch receptor (Geffers et al. 2007). Furthermore, Dll3 does not bind Notch (Ladi et al. 2005) and is unable to substitute for the loss of Dll1 in mice (Geffers et al. 2007) suggesting that Dll1 and Dll3 are not functionally compatible.

Similar to Notch receptors, Notch ligands can also undergo proteolytic cleavage, both by ADAM metalloproteases and γ -secretase activity, producing an extracellular cleavage product that could modulate Notch signaling and an intracellular cleavage product that is thought to be involved in inhibiting Notch signaling (D'Souza et al. 2010; Jung et al. 2011).

In addition to the canonical ligands described above, several studies have identified non-canonical ligands that are able to activate the Notch signaling pathway. These proteins do not contain the DSL domain, but are glycosylphosphatidylinositol (GPI)-linked or secreted proteins. In addition, some contain the recently identified DOS domain, and it has been suggested that they cooperate or compete with DSL containing ligands to bind Notch (D'Souza et al. 2010; Komatsu et al. 2008).

5 Notch Ligand Endocytosis

Endocytosis of the Notch ligands, has been suggested to be critical to activate Notch signaling; however, the mechanism of how ligand engagement leads to proteolysis of the Notch protein is not well understood. Several molecules involved in ligand endocytosis have been discovered in *Drosophila*, *Xenopus* and Zebrafish.

5.1 Neuralized and Mindbomb

Neuralized (Neur) was first identified in *Drosophila*, and its loss caused a neurogenic phenotype similar to that seen in Notch and Delta mutants (Lehmann 1983). It was originally thought that Neur-enhanced Notch signaling by relieving *cis*-inhibition by Delta (Deblandre et al. 2001); however, expression of Neur is enhanced in signal sending cells, by Neur-induced endocytosis of the Notch ligand and thus stimulating ligand signaling activity (Le Borgne and Schweisguth 2003b). Neur contains a C-terminal ‘really interesting new gene’ (RING) finger domain, important for its ubiquitin ligase activity, and two Neuralized homology repeats (NHR)-1 and NHR2 (Deblandre et al. 2001; Lai et al. 2001; Pavlopoulos et al. 2001; Yeh et al. 2001). The NHR1 domain is required for binding and trafficking of Delta from the cytoplasm to the plasma membrane (Commisso and Boulianne 2007). Neur also binds phosphoinositol phosphates (PIPs) though a polybasic region in its N terminus, and is required for Neur-mediated Delta endocytosis (Skwarek et al. 2007). The activity of Neur can be further modified by the action of Bearded proteins: Bearded and Twin of m4 (Tom), are negative regulators of Neur function (Bardin and Schweisguth 2006; He et al. 2009). In mammals there are two homologues of Neuralized: Neuralized1 and Neuralized2, both have been found to interact with and ubiquitinylate Delta (Ruan et al. 2001; Song et al. 2006; Vollrath et al. 2001). Despite the strong neurogenic phenotypes in *Drosophila*, Neur1^{-/-} mice do not display any obvious defects in Notch signaling (Ruan et al. 2001; Vollrath et al. 2001). These data suggested that there might be more E3 ubiquitin ligases that could modify DSL ligands in their intracellular domains.

To this end, studies in Zebrafish identified, Mindbomb1 (Mib1) (Chen and Casey Corliss 2004; Itoh et al. 2003). Similar to Neur, Mib binds, and ubiquitinylates Delta, and in the absence of Mib, mutant cells exhibit an accumulation of Delta on their cell surface (Itoh et al. 2003). There are two Zebrafish homologues: ZMib1 and ZMib2. ZMib1 contains three RING finger domains, where its ubiquitin ligase activity resides, and ANK repeats, which are important for internalization (Chen and Casey Corliss 2004). ZMib2 has two RING finger domains and is able to compensate for the absence of ZMib1 (Zhang et al. 2007). There are two homologues in mammals: Mindbomb1 and Mindbomb2 (Koo et al. 2005a, b). Mib1 has been shown to interact with all the Notch ligands (Koo et al. 2005a), whereas Mib2 has been shown to interact with Jag2 only (Takeuchi et al. 2005).

Although several homologues of Neur and Mib have been isolated from several species, and both show a functional similarity in *Drosophila*, Neur and Mib may have evolved differently in vertebrates. *Drosophila* has one Neur gene (with two splice variants (Commisso and Boulianne 2007)), and two Mib1 homologues, both of which are able to bind and ubiquitinylate Delta and Serrate, thus stimulating ligand endocytosis and signaling activities and for the most part these two proteins are also functionally redundant as seen by gene rescue experiments (Lai et al. 2001, 2005; Pitsouli and Delidakis 2005). In contrast, mammalian Neur1 and Neur2 are dispensable for normal development (Koo et al. 2007; Ruan et al. 2001;

Vollrath et al. 2001), as is Mib2 (Koo et al. 2007), whereas deletion of Mib1 produces an embryonic lethal phenotype, and defects in neural tube and brain development and reduced Notch target gene expression (Barsi et al. 2005; Koo et al. 2005a, 2007). Mib2 is not expressed during embryonic development, and thus may explain its inability to compensate for the absence of Mib1 (Koo et al. 2007). Recent studies in *Drosophila* have identified three distinct motifs in the intracellular domain of Delta that are important for Mib1 and Neur docking. Physical interaction with Delta is a prerequisite for its ubiquitinylation, lysine-742 is important for Neur-mediated ubiquitinylation, whereas Mib1 does not seem to prefer any specific lysine residue, this of course may lead to differential trafficking behavior (Daskalaki et al. 2011). The DSL-Mib1 motifs were found to be conserved in other species, and therefore may play an important role in vertebrates too, whereas there was less conservation for the Neur motifs, which supports the lack of functional conservation of Neur in vertebrates (Daskalaki et al. 2011).

Previous reports have also found conserved Mib1 and Neur binding motifs in Serrate (Fontana and Posakony 2009; Glittenberg et al. 2006). Thus, Neur and Mib proteins appear to have similar roles in *Drosophila*, but not necessarily functionally equivalent roles in mammals. Neur2 and Mib1 display different subcellular localization and therefore may have different, yet complementary roles in mammals (Song et al. 2006). It is thought that Mib plays a more direct role in ligand endocytosis in mammals, whereas Neur has been suggested to direct internalized proteins for degradation (Song et al. 2006). It has not been identified whether these differences are due to differential ubiquitinylation states of the Notch ligand intracellular domains. Notch ligands contain several lysine residues (Pintar et al. 2007) that could be mono-, multi-, or polyubiquitinylated. While polyubiquitinylation leads to proteosomal degradation of a protein, mono-, or multiubiquitinylation, leads to endocytosis and intracellular trafficking of the protein (Staub and Rotin 2006). Recently Zhang et al., showed that a specific lysine residue (K613) in the cytoplasmic tail of Dll1 is important for multi-ubiquitinylation, when this lysine was mutated, multi-ubiquitinylation was attenuated and Dll1 only underwent mono-ubiquitinylation, but surprisingly this was not due to the inability of the mutant to interact with Mib1 (Zhang et al. 2011).

5.2 Other Molecules Involved in Endocytosis

Prior to the discovery of Mib and Neur proteins, there was genetic evidence that endocytosis played an important role during Notch signaling. Dynamin (encoded by *Shibre*) is required in both the signal-receiving as well as the signal-sending cells in *Drosophila*. The GTPase Dynamin is important for ‘pinching off’ endocytic vesicles from the plasma membrane (Seugnet et al. 1997). In addition to Dynamin, other molecules found to be involved in ligand endocytosis are: Auxilin, Rab11, and Clathrin (Banks et al. 2011; Eun et al. 2008; Kandachar et al. 2008). Epsin also seems to play an important role in Notch ligand endocytosis.

Liquid facets (*Lqf*), which encodes *Drosophila* Epsin, binds to Clathrin and other ubiquitinated cargo (Overstreet et al. 2004). There are three Epsin homologues in mammals, two of which, Epsin 1 and 2 have been deleted in mice, exhibiting an embryonic lethal phenotype and Notch signaling-related defects (Chen et al. 2009).

5.3 Models Proposed for Ligand Endocytosis

There are two main models that have been proposed to explain the purpose of Notch ligand endocytosis and its significance and requirement in inducing Notch receptor activation.

5.3.1 The Mechanotransduction Model

The ‘Mechanotransduction’ or ‘Pulling’ model, was first proposed after the observation of the Notch extracellular domain (NECD) in vesicular structures in *Drosophila* signal-sending cells (Nichols et al. 2007; Parks et al. 2000). Losses in Notch ligand endocytosis result in the absence of NECD in the signal-sending cell (Nichols et al. 2007; Parks et al. 2000). The fact that endocytosis is necessary for the activation of the Notch signaling pathway and the above observation, led Parks et al., to suggest that the interaction of the Notch receptor with DSL Notch ligands, was to induce a conformational change in the non-covalent interactions of the heterodimeric Notch receptor that leads to proteolysis by ADAM metalloproteases (Parks et al. 2000). This would release the extracellular portion of the Notch receptor, thus allowing it to be taken up by the signal-sending cell, leaving the transmembrane associated intracellular portion of Notch to undergo γ -secretase mediated cleavage. This process of NECD uptake by the signal-sending cell, is termed ‘trans-endocytosis’ (Klueg et al. 1998). These observations were confirmed in mammalian cells for Dll1 and Jag1 (Nichols et al. 2007; Parks et al. 2000), blocking of the S2 cleavage did not affect Notch trans-endocytosis, suggesting that Notch ligand endocytosis must create a physical force in order to expose the site for ADAM-mediated cleavage (Hansson et al. 2010; Nichols et al. 2007). Furthermore, structural studies using X-ray crystallography, have shown that the S2 cleavage site is buried deep within the heterodimerization domain and is protected by three LNR domains, suggesting that Notch ligand endocytosis is definitely required to ‘stretch’ the Notch protein and expose the cleavage site for proteolysis (Gordon et al. 2007, 2008, 2009). In addition, atomic force microscopy has shown that Notch and DSL ligands have a strong binding force (Ahimou et al. 2004). This force would presumably have to be created in the signal-sending cell and involve other proteins, such as Epsin, Dynamin, and Clathrin, which have all been implicated in generating a mechanical force (Liu et al. 2010; McMahon and Gallop 2005) and shown to have a role in Notch ligand endocytosis. Notch receptor

binding to ligand induces ubiquitinylation of the Notch ligand, and recruitment of Epsin (Overstreet et al. 2004; Tian et al. 2004; Wang and Struhl 2004). Ligand clustering would presumably increase this assembly of multiple Epsin molecules, and generate a mechanical force to induce endocytosis. By creating a complex that induces Notch ligand endocytosis, and therefore NECD trans-endocytosis, Notch signaling is activated in the signal-receiving cell.

To support this theory proteolysis of Notch ligands causes the release of possible signaling fragments into the extracellular milieu (Mishra-Gorur et al. 2002); however, most secreted forms of DSL ligands are unable to induce Notch signaling, presumably due to the absence of a pulling force not being generated. The ability of Notch ligands to signal when immobilized, cross-linked, or clustered, suggests that a force is being generated to induce Notch activation (Hicks et al. 2002; Varnum-Finney et al. 2000).

5.3.2 The Recycling Model

The Recycling model is based on the observation that bulk endocytosis of Delta is unaffected in *Drosophila* Epsin mutants (*Lqf*[−]), yet Delta is unable to signal, the *Lqf*[−] mutant exhibits phenotypes similar to the loss of Notch, Delta, and Serrate, and therefore is thought to be required by the signal-sending cell (Wang and Struhl 2004). Wang and Struhl suggested that the newly synthesized Delta was not able to signal as it had not been modified, and that it required internalization and sorting, a process that required ubiquitinylation and then trafficking back to the plasma membrane, after having been post-translationally modified or relocated to a more favorable location for signaling. This model suggests that internalization and recycling cause the ligand to become ‘activated’ and having acquired its signaling capabilities, is better able to interact with the Notch receptor (Wang and Struhl 2004). Removal of the intracellular domain of Delta inhibits endocytosis; however, replacing this domain either with the intracellular domain of the low density lipoprotein (LDL) receptor, which is known to be internalized by endocytosis, or an extendable ubiquitin (Ub), which can be polyubiquitinated, promotes internalization and signaling activity in *Drosophila* (Wang and Struhl 2004), whereas a non-extendable Ub signals more weakly (Wang and Struhl 2004).

In mammalian cells, mutation of all 17 lysine residues in Dll1 (Dll1K17), reduces the ability of this construct to internalize, and transendocytose the Notch receptor, in addition it renders this mutant unable to recycle back to the cell surface, (Heuss et al. 2008). These authors suggest that the process involves localization to the lipid rafts and that recycling increases the affinity of Dll1 for Notch binding (Heuss et al. 2008). In contrast, Zhang et al., show that mutation of a single lysine, K613, in the intracellular domain of Dll1 enhances lipid raft localization (Zhang et al. 2011), suggesting that different lysine residues may be important for different functions with regards to ligand localization and endocytosis.

Initially several Notch loss-of-function phenotypes and Delta recycling defects were identified that seem to support this notion: Sec15, a component of the exocyst

complex in *Drosophila*, and a dominant-negative form of Rab11 in mammalian cells, lead to recycling defects (Emery et al. 2005; Jafar-Nejad et al. 2005); however, recently it has been shown that Sec15, which functions with Rab11, is not required in every Notch signaling event (Banks et al. 2011; Eun et al. 2008; Jafar-Nejad et al. 2005; Windler and Bilder 2010).

If the recycling model is the method of endocytosis that activates the Notch signaling pathway, then further questions as to what leads to an ‘activated’ Notch ligand need to be identified, suggestions include: clustering of ligands, trafficking into lipid microdomains, proteolytic cleavages, and post-translational modifications (Chitnis 2006; Le Borgne and Schweisguth 2003a; Wang and Struhl 2004).

The Mechanotransduction model and Recycling models are not necessarily mutually exclusive. It has been suggested that perhaps two ligand internalization events are required, the first to activate the ligand, and the second to activate the receptor and induce Notch signaling in the signal-receiving cell. Heuss et al., showed that mutation of all 17 lysine residues in Dll1 (Dll1K17), reduces the ability of this construct to internalize, and recycle back to the cell surface (Heuss et al. 2008). These authors suggest that the process involves localization to the lipid rafts and that recycling increases the affinity of Dll1 for Notch binding (Heuss et al. 2008); however, the same group also showed that this may not be entirely due to the lack of ubiquitylation, as a lysine-less intracellular domain of Dll3, when fused to the extracellular domain of Dll1 (Dll1-3chimera), is still able to undergo internalization and recycling, but is unable to activate Notch signaling, despite its ability to bind Notch, suggesting other mechanisms are important in Notch ligands signaling capabilities (Heuss et al. 2008). Studies using a mutant form of Jag1, also lend support to both models. A mutant form of Jag1, Jag^{Ndr}, which has a missense mutation in the extracellular domain at position histidine-268 located in the second EGF repeat, has lost the ability to bind and activate Notch, providing support for the Mechanotransduction model. In this report, Jag1 and NECD were targeted for degradation, whereas Jag^{Ndr} expressing cells fail to trans-endocytose NECD. In the absence of Notch, trafficking and interaction with Mib1 were similar to WT Jag1. siRNA-mediated inhibition of MIB1 function led to a loss of receptor activation, but not receptor binding by WT Jag1, providing support for the Recycling model (Hansson et al. 2010).

The role of Jag2 and Dll4 in activating the Notch signaling pathway via its endocytosis has yet to be fully determined, although deletion of the intracellular domain of Dll4 is unable to activate Notch signaling in a functional assay (Abe et al. 2010). Further analysis through deletions and mutations of Notch ligands will help to answer this question.

6 Modulators of Notch Signaling

Notch signaling is regulated at multiple levels in the pathway. Notch receptors can be modified by the action of Fringe glycosyl transferases, which can influence ligand specificity (Stanley and Okajima 2010). In the cytoplasm, proteins such as

Numb interact with Notch and promote its ubiquitinylation and degradation (Guo et al. 1996; Rhyu et al. 1994; Spana and Doe 1996; Uemura et al. 1989; Zhong et al. 1997). The mechanism underlying this inhibition involves the E3 ubiquitin ligase, Itch (McGill and McGlade 2003), which also interacts with and ubiquitinylates Notch (Fang and Kerppola 2004). In the nucleus, Sel-10/Fbw7 is an E3 ubiquitin ligase and member of the F box family of proteins, that interacts with a cyclin dependent kinase 8 (CDK8) phosphorylated nuclear form of Notch, and targets it for ubiquitinylation and degradation by the proteasome (Fryer et al. 2004; Gupta-Rossi et al. 2001; Oberg et al. 2001). Deltex (Matsuno et al. 2002), Nrarp (Notch-regulated ankyrin repeat protein) (Lamar et al. 2001), and MINT (also known as SHARP) (Kuroda et al. 2003) are also involved in Notch regulation, and together may serve to fine-tune the activity of Notch within the signal-receiving cell.

7 Notch Signaling and T Cell Development

7.1 Notch Receptor Expression in the Thymus

Notch-1, -2, and -3 receptor proteins are all expressed in thymocytes (Felli et al. 1999; Fiorini et al. 2009; Hasserjian et al. 1996; Shi et al. 2011). Notch1 expression is differentially expressed throughout T cell development. The use of novel monoclonal antibodies, against Notch-1 and Notch-2 confirmed that Notch1 expression is highest in DN and immature single positive (ISP) CD8⁺ thymocyte subsets in adult and embryonic stage 16 (E16), and decreases steadily in DP cells in adults (Fiorini et al. 2009), with intermediate to low expression in SP cells (Fiorini et al. 2009; Hasserjian et al. 1996). Notch2 expression was found to be highest in DN1 and DN2 subsets of adult thymocyte preparations (Fiorini et al. 2009). In addition, Notch1 and -2 are expressed in $\gamma\delta$ T cells isolated from adult mice (Fiorini et al. 2009). Notch3 mRNA and protein expression is found in DN and DP thymocyte subsets, with the highest levels in DN3 cells (Shi et al. 2011).

It is well established that Notch signaling is critically required for the T vs. B cell lineage decision (Han et al. 2002; Izon et al. 2002; Maillard et al. 2004; Pui et al. 1999; Radtke et al. 1999). Continuous Notch signals are required for the commitment of the earliest thymic progenitor cells to the DN3 stage of T cell development (Schmitt et al. 2004), VDJ recombination (Wolfer et al. 2002) and the β -selection checkpoint (Ciofani et al. 2004; Maillard et al. 2006; Tanigaki et al. 2004), for its functional outcomes, including: survival, proliferation, and differentiation (Ciofani et al. 2004; Ciofani and Zuniga-Pflucker 2005). Notch signaling is also required at the $\alpha\beta$ and $\gamma\delta$ lineage decision (Ciofani et al. 2006; Robey et al. 1996; Washburn et al. 1997; Wolfer et al. 2001, 2002). Its role in the CD4 vs. CD8 lineage decision remains controversial (Robey et al. 1996; Wolfer et al. 2001); however, the Notch and TCR signaling pathways may work together to influence

positive selection and CD4/CD8 T cell development as evidenced in mice conditionally deleted for Presenilin1/2 (Laky and Fowlkes 2007).

Although much is known about the role of Notch1 in T cell development, recent studies have focused on the role of Notch3. Recent data has found that Notch3 is dispensable for T cell development (Shi et al. 2011; Suliman et al. 2011), although previous studies had suggested a role for Notch3 in T cell development (Bellavia et al. 2000). Notch3 was found to have a lower binding affinity for Dll4 (Suliman et al. 2011) and therefore presumably does not interact with Dll4 in the thymus. However, Petrie and colleagues examined the same mice, and found that Notch3 gene trapped mice had slightly smaller thymi, although this was not statistically significant. They reasoned that the mild phenotype observed may be due to a compensatory role by Notch1, however, this was not the case, as deletion of Notch3 did not exacerbate the Notch1 phenotype (Shi et al. 2011).

7.2 Notch Ligand Expression in the Thymus

In vivo, analysis of Notch ligand expression has revealed that Dll1, Dll4, Jag1, and Jag2 are expressed in the day 16 embryonic thymus, with Dll4 exhibiting the highest level of expression (Mohtashami et al. 2010). In adults, Dll4 is most highly expressed in cortical thymic epithelial cells (cTECs) (Hozumi et al. 2008; Koch et al. 2008), in particular in the subcapsular and outer cortical areas of the thymus (Mohtashami et al. 2010), which are incidentally where DN subsets express high levels of Notch1 and are undergoing β -selection. Dll1 expression was found to be absent from inner cortical or medullary regions, and confined to the blood vessels, the point of entry of blood-borne progenitors; (Koch et al. 2008) however, other reports have found Dll1 expressed in the cortex and pericortex (Mohtashami et al. 2010; Schmitt et al. 2004). Recently, Dll3 expression was observed in DN, CD4⁺, and CD8⁺ thymocytes, with the highest expression in DN3 subsets (Hoyne et al. 2011). In adults, Jag1 is expressed at low levels throughout the thymus, whereas Jag2 is expressed most highly in the inner cortex and outer medulla (Hozumi et al. 2008; Koch et al. 2008).

Despite the expression of several Notch ligands in the thymus, it is now clear that Dll4 is the relevant physiological ligand within the thymus (Hozumi et al. 2008; Koch et al. 2008). Conditional deletion of Dll4 abrogated T cell development and led to the development of B cells in the thymus (Hozumi et al. 2008; Koch et al. 2008), in contrast, conditional deletion of Dll1 did not inhibit T cell development, and presumably Dll4 was able to compensate for its absence (Hozumi et al. 2004). Jag1^{−/−} mice and Jag2 mutant mice, exhibit normal $\alpha\beta$ T cell development (Jiang et al. 1998; Mancini et al. 2005), however, Jag2 mutant mice have reduced numbers of $\gamma\delta$ thymocytes (Jiang et al. 1998). Interestingly Hoyne et al. have recently reported a cell autonomous role for Dll3 in the thymus, with Dll3^{−/−} mice exhibiting increased *hes5* transcription at the DP stage, thus suggesting an inhibitory role in Notch signaling (Hoyne et al. 2011).

The transcription factor (TF) FoxN1, which is required for TEC differentiation (Su et al. 2003), also seems to regulate the expression of Dll1, Dll4, and Jag2 expression in TECs. Mice expressing a hypomorphic allele of FoxN1, exhibited decreased mRNA expression of these Notch ligands (Xiao et al. 2008).

Although Dll4 is the physiological ligand, *in vitro* studies have shown that T cell development can be supported from the other Notch ligands too, such as with the OP9-DL1 system (Zuniga-Pflucker 2004), the results of which are presumably precluded *in vivo*, by the presence of Dll4 (Hozumi et al. 2004). Several labs have shown that T cell development can be supported from over expression of Dll1 *in vitro* (Besseyrias et al. 2007; Jaleco et al. 2001; Schmitt and Zuniga-Pflucker 2002). In addition, experiments carried out by Radtke and colleagues showed that analysis of Notch1 conditionally deleted BM derived precursors, which are unable to give rise to T cells *in vivo*, were able to give rise to T cells *in vitro* when cultured on OP9-DL1 stromal layers, indicating that this was a Notch-2-mediated event. While, Notch1/Notch2 conditionally deleted BM-derived precursors were not able to give rise to T cells when grown on OP9-DL1 or OP9-DL4, indicating that Notch2 is only able to interact with Dll1, whereas, Notch1 is able to interact with both Dll1 and Dll4 (Besseyrias et al. 2007).

To assess whether Dll1 and Dll4 are equally capable at supporting T cell development, Mohtashami et al., recently showed that Dll4 is better able to support T cell development *in vitro* compared to Dll1. These authors directly compared the ability of these two proteins to induce and support T cell development, when expressed in OP9 cells (Mohtashami et al. 2010). Similar to previous reports, when Dll1 and Dll4 are expressed at high levels in OP9 cells, both proteins are able to induce and support $\alpha\beta$ and $\gamma\delta$ T cell development (Besseyrias et al. 2007; Schmitt and Zuniga-Pflucker 2002). However, when Dll4 is expressed at lower levels in OP9 cells, but similar to the levels on thymic epithelial cells, Dll4 is better able to support T and myeloid cell development and induce Notch- and myeloid-specific gene transcription, from fetal liver (FL)-derived progenitors, compared to Dll1, which exhibited an increase in the development of myeloid and B cell lineages (Mohtashami et al. 2010).

Overexpression of Jag1 in stromal cells was unable to give rise to $\alpha\beta$ T cells, but was able to support $\gamma\delta$ T cell and NK cell lineages (Lehar et al. 2005), suggesting a role for Jagged proteins in supporting $\gamma\delta$ T cell development. Recent data from Habu's group confirms that Jag1 is unable to support T cell development, but they did show that Jag2 is able to support T cell development from FL-derived HSCs, *in vitro*, to the DP stage, when cocultured on transfected PA6 cell lines, and that this was dependent on the magnitude of the signal available (Abe et al. 2010).

These various *in vivo* and *in vitro* studies highlight differences in Notch ligands and their ability to support T cell development, and these differences may be related to their structure and their ability to induce Notch receptor activation. One of the proposed methods of Notch activation lies in the ability of the endocytosis of these Notch ligands, suggesting that this could explain some of the differences described above. Notch ligands have the least similarity in their intracellular domain (Pintar et al. 2007). The intracellular domain is the target for Mib and

Neur-mediated ubiquitinylation, inducing endocytosis of the Notch ligands and activation of the Notch signaling pathway (D'Souza et al. 2010; Yamamoto et al. 2010). Recent studies focusing on endocytosis of Notch ligands and other molecules involved in this process have shed some light on this area, with regards to T cell development.

7.3 Notch Ligand Endocytosis and Role in T Cell Development

In vitro studies have primarily focused on the role of Dll1 endocytosis in T cell development. Heuss et al., showed that when Dll1, that has been mutated at all 17 potential lysine residues in its intracellular domain (Dll1K17), is overexpressed in OP9 cells, it is unable to support T cell development from FL-derived progenitors to the DP stage, producing 12-fold lower DP cells than OP9 cells expressing wild-type Dll1 (Heuss et al. 2008). These results were also observed with a Dll1-Dll3 chimeric construct, which is unable to be ubiquitinated. Both constructs were unable to trans-endocytose NECD efficiently (Heuss et al. 2008), indicating that ubiquitinylation is a requirement for ligand endocytosis and thus Notch activation in developing progenitors to differentiate and adopt the T cell lineage. Further studies that investigate the role of the intracellular domain of Notch ligands come from Habu's group. Here, Habu and colleagues showed that the Dll4 and Jag1 intracellular domains are necessary, but interchangeable and that the signaling capabilities of these two Notch ligands lie in the interactions of their extracellular domains with Fringe-modified Notch receptors (Abe et al. 2010). FL HSCs cocultured on transfected PA6 cells with Jag1 extracellular domain fused to Dll4 intracellular domain (J1d-D4), were unable to support T cell development, whereas when the Dll4 extracellular domain fused with the Jag1 intracellular domain is overexpressed in either NIH-3T3 or PA6-derived transfectants, T-lineage cells are able to develop in these cultures (Abe et al. 2010). These authors also reported that overexpression of a Dll4 construct that lacks its intracellular domain, is unable to support T cell development (Abe et al. 2010). Swapping of the DSL domain of Dll4 or Jag2 onto Jag1 did not confer their T cell inducing capabilities onto Jag1, and suggested that additional sequences within the Jag2 protein were needed for effective signaling to promote T cell development (Abe et al. 2010).

Studies focused on the E3 ubiquitin ligases which ubiquitinylate the intracellular domain of the Notch ligands (Daskalaki et al. 2011; Itoh et al. 2003), show that *mib1*, *mib2*, *neur1*, and *neur2* are all expressed in both the thymic stromal and CD45⁺ hematopoietic compartments (Song et al. 2008), with Mib1 and Mib2 exhibiting the highest level of expression. Conditional deletion of Mib1 by Cre under the control of the MMTV promoter caused a reduction in Mib1 expression in the thymic stromal cells. Deletion of *Neur1*, *Neur2*, or *Mib2* did not affect T cell development (Song et al. 2008); however, conditional deletion of Mib1 by Cre under the MMTV promoter or Mx1, interferon inducible promoter, led to the

abrogation of T cell development in these mice (Song et al. 2008), indicating that Mib1 is the relevant physiological E3 ubiquitin ligase in the thymus. These mice exhibited a reduction in total cellularity, decreased ETP, DP, and SP cell populations, and an increase in B220⁺ B-lineage cells in the DN compartment of these mice. To confirm that Mib1 acts in a non-cell autonomous manner, WT BM cells were unable to reconstitute the Mib1^{-/-} conditionally deleted thymus, whereas Mib1 deficient BM cells were able to reconstitute a WT thymus, showing that Mib1's activity resides in the thymic epithelial cells (Song et al. 2008). Dll1 endocytosis was normal in Neur1^{-/-}, Neur2^{-/-}, and Mib2^{-/-} thymi, whereas Dll1 accumulated on cortical thymic epithelial cells in the Mib1 conditionally deleted thymus, indicating its importance. Furthermore, siRNA knockdown of Mib1 in OP9 stromal cells that overexpressed Dll1 caused a block in development of BM LSKs or FL LSKs at the DN1 stage, indicating that Mib1 can interact with Dll1 (Song et al. 2008).

The ubiquitinylation of Dll ligands is a requirement for endocytosis and currently there is little data for the ubiquitinylation of Dll4 and its role in T cell development. As Dll4 is the physiological ligand, it would give an insight into its regulation. In particular, in light of Mib1 being the functional E3 ubiquitin ligase in the thymus, it would be interesting to investigate the role of Mib1 on Dll4, presumably Mib1 interacts with and ubiquitinylates Dll4 in vivo.

7.4 Notch Modulators and T Cell Development

Mice conditionally deleted for Lunatic Fringe (Lfng) under the Lck promoter, exhibit a decrease in the transition from DN3 to DP cell development (Yuan et al. 2011), whereas overexpression of Lfng in the thymus led to a decrease in the generation of T cells in the thymus, and the development of intrathymic B cells (Koch et al. 2001). The same group showed that DN3 and DN4 cells were found to exhibit increased proliferation and self-renewal when Lfng was overexpressed, thereby increasing the amount of time that β -selected pre-DP cells were exposed to OP9-DL induced proliferation (Yuan et al. 2011). Overall these studies show that Lfng-mediated glycosylation directly increases Notch1 binding affinity for Dll1 and Dll4, and recently also Jag2, but decreases their affinity for Jag1 (Van de Walle et al. 2011).

Inactivation of Numb or Numb-like in T cells indicates that they are dispensable for T cell development (Anderson et al. 2005; Wilson et al. 2007). This is in contrast to other studies, in which overexpression of Numb specifically in T cells causes a reduction in Notch activity (French et al. 2002); however, recent studies using overexpression of full length Numb resulted in a larger thymus and loss of pre-TCR signaling, whereas overexpression of a dominant-negative Numb resulted in a smaller thymus and enhanced pre-TCR signaling and differentiation (Aguado et al. 2010) indicating that the role of Numb in T cell development still requires further analysis.

ADAM proteases are required for both Notch receptor and ligand proteolytic processing. Several ADAM proteases are expressed in the thymus and some have demonstrated a role in T cell development (Haidl et al. 2002; Li et al. 2007). Recently data from several labs show that the absence of ADAM8 causes an accumulation of mature thymocytes and increased medulla size, due to increased proliferation, decreased apoptosis and impairment in thymocyte migration (Gossens et al. 2010). Mice conditionally deleted for ADAM10 by Cre under the control of the Lck promoter showed a reduction in total thymic output, a reduction in DP and SP thymocytes and decrease in the Notch target gene *deltex1* (Tian et al. 2008). Furthermore, overexpression of ADAM10 in early lymphocyte progenitors under the H-2 Kb promoter and the IgH enhancer region, exhibited a smaller thymus, reduced DN and DP thymocyte populations and other B and myeloid specific defects (Gibb et al. 2011). In contrast, proteolytic processing of Notch ligands is not critical during T cell development. Using an uncleavable Dll1 overexpressed in OP9 cells, T cell development was analyzed and found to progress normally (Gravano and Manilay 2010).

8 Summary

A complex interplay of various factors is required to initiate and support the development of T cells. Inherent to this complexity is the requirement for Notch signaling. Recent studies have highlighted the importance of Notch ligand endocytosis in activating the Notch signal, and importantly have identified some of the key physiological players within the thymus. The task now is to determine the precise interactions that some of these proteins have in the thymus, in particular in the regulation and interaction between the Notch ligand Dll4 and the E3 ubiquitin ligase Mib1. Several questions remain: Does Dll4 require endocytosis for Notch activation during T cell development? Is it regulated in the same manner as the other Notch ligands? Which lysine residues serve as potential ubiquitinylation sites on Dll1 and Dll4 by Mib1? Is a lysine-613-like residue also important for Dll4? Does it also affect Notch binding of Dll4? What other DOS-containing ligands are expressed in the thymus? Are any of these important in potentiating the Notch signal by canonical DSL Notch ligands? Which RING finger domain in Mib1 is important for interacting with the Notch ligands in the thymus? Further studies will certainly begin to address these questions to better understand the role of Notch ligand endocytosis in the thymus that predicates the induction and support of T-lineage commitment and differentiation.

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