Notch signaling; linking embryonic lung development and asthmatic airway remodeling

Musaddique Hussain, Chengyun Xu, Mashaal Ahmad, Youping Yang, Meiping Lu, Xiling Wu, Lanfang Tang, Ximei Wu

Department of Pharmacology, School of Medicine, Zhejiang University, Hangzhou City, 310058, China

The Key Respiratory Drug Research Laboratory of China Food and Drug Administration, School of Medicine, Zhejiang University, Hangzhou City, 310058, China

The Second People’s hospital of Wenling, Wenling City, Zhejiang Province, 317503, China.

Department of Respiratory Medicine, the Affiliated Children Hospital, School of Medicine, Zhejiang University, Hangzhou City, 310006, China.
Running title: Notch signaling and asthmatic airway remodeling

Corresponding author: Prof. Ximei Wu (M.D., Ph.D.) Department of Pharmacology, School of Medicine, Zhejiang University, 866 Yuhangtang Road, Hangzhou, 310058, China. Tel/Fax: +86-571-8898-1121. E-mail: xiwu@zju.edu.cn

Number of text pages: 83
Number of tables: 3
Number of figures: 3
Number of references: 206
Number of words in Abstract: 143
Number of words in Introduction: 651
Number of words in Discussion: 224

Non-standard Abbreviations: NICD, Notch intracellular domain; NECD, Notch extracellular domain; EDF, epidermal growth factor; LNR, Lin-12-Notch repeats; ADAM, a disintegrin and metalloprotease; RAM, RBP-jκ-associated molecule; HD, heterodimerization domain; TMD, transmembrane domain; ANK, ankyrin repeats; TAD, transactivation domain; PEST, proline-glutamic acid-serine-threonine-rich motifs; DSL, Delta-Serrate-LAG-2; DOS, Delta and OSM-11-like proteins; vWF, von Willebrand Factor; MNNL, N-terminal domain of Notch ligands; ADAM, a disintegrin and metalloprotease; NEXT, Notch extracellular truncation; CSL, CBF1/RBPjκ/Su(H)/LAG-1; HIF-1α, hypoxia-inducible factor-1α; MAML, mastermind-like; ED, embryonic days; p, postnatal days; ECM, extracellular matrix
Abstract:

Lung development is mediated by assorted signaling proteins, and orchestrated by complex mesenchymal-epithelial interactions. Notch signaling is an evolutionarily conserved cell-cell communication mechanism that exhibits a pivotal role in lung development. Notably, both aberrant expression and loss of regulation of Notch signaling are critically linked to the pathogenesis of various lung diseases, in particular, pulmonary fibrosis, lung cancer, pulmonary arterial hypertension, and asthmatic airway remodeling; implying that precise regulation of intensity and duration of Notch signaling is imperative for appropriate lung development. Moreover, evidence suggests that Notch signaling links the embryonic lung development and asthmatic airway remodeling. Herein, we summarized all-recent advances associated with the mechanistic role of Notch signaling in lung development, consequences of aberrant expression or deletion of Notch signaling in linking early-impaired lung development and asthmatic airway remodeling, and all possible recent potential therapeutic strategies to treat asthmatic airway remodeling.
Introduction:

Lung development originates from the anterior foregut and is orchestrated by complex mesenchymal-epithelial interactions that coordinate the temporal and spatial expression of multiple regulatory factors for normal development (Morrisey and Hogan, 2010). Lung development occurs through following sequential stages: embryonic stage (week 3-7 in humans; embryonic day (ED) ~8.0-9.5 in mice), pseudoglandular stage (week 7-17; ED 9.5-16.5), canalicular stage (week 17-27; ED 16.5-17.5), saccular stage (week 27-36; ED, 17.5 to postnatal day 5 (P5) in mice) and alveolar stage (week 36 to ~7-10 years in humans; P5 to ~P30 in mice) (Schittny; Warburton et al., 2010). Stages of lung development and expression of Notch signaling are depicted in figure 1. Later in gestation, epithelial progenitor are differentiated into basal cells, ciliated cells, goblet cells, and neuroendocrine cells (in proximodistal axis), submucosal glands consisting of mucous and serous cells (in the proximal region) and ciliated and Clara cells (in bronchioles which are distal to the trachea and bronchi). Alveoli, most distal part of the lungs, are layered with flattened type-I pneumocytes and cuboidal surfactant-producing type-II pneumocytes (Rawlins and Hogan, 2006). Similar airway epithelium exists in mouse and human lung but there are some differences. For instance, a pseudostratified epithelial layer including basal cells is merely originated in the main stem bronchus and trachea in mouse lung whereas, in human lung, basal cells are distributed in the terminal bronchioles. Further, large numbers of Clara cells are thoroughly distributed in mouse lung while in the human lung, Clara cells are only found in the bronchiolar epithelium. Human lung contains extensive branches in the bronchial tree as compared to mouse lung. Branching pattern is also different in both mouse and human lung (Metzger et al., 2008). Alveolar development starts from the saccular stage (ED 17.5) in mouse while in human lung, alveolar expansion starts from the late
canalicular stage (~24 weeks). Nevertheless, precisely coordinated signaling pathways regulate lung developmental processes; whereas disruptions of these signaling pathways could result in neonatal respiratory disorders such as lung cancer, pulmonary fibrosis, chronic obstructive pulmonary disease (COPD), asthma and airway remodeling.

Asthmatic airway remodeling, first described in 1922 (Huber and Koessler, 1922), is characterized by structural changes in the airway walls such as thickened epithelium with mucous gland hypertrophy (mucus hypersecretion), thickened subepithelial basement membrane, deposition of extracellular matrix, fibrosis, angiogenesis, neovascularization, hyperplasia and hypertrophy of airway smooth muscle cells and increased airway vascularity (Elias et al., 1999; Fehrenbach et al., 2017; James et al., 2012; Prakash, 2013). Asthmatic airway remodeling mediates the airway hyperresponsiveness, airway obstruction, pulmonary dysfunction and infiltration of inflammatory cells, followed by chemokines, cytokines and growth factors, under the influence of various signaling proteins, also known as morphogens, such as Wnt/β-catenin, TGF-β/BMP, fibroblast growth factor, epidermal growth factor, Sonic hedgehog and Notch.

Notch, discovered in 1913 (Morgan, 1917), is an exceedingly conserved signaling system. Molecular mechanism of Notch signaling activation and transduction is multifarious; however, fundamentally, after ligand binding, γ-secretase cleaves the released NICD then this NICD translocates to the nucleus. The NICD interacts with the DNA-binding transcriptional repressor (CBF-1/RBP-jκ) and converts it into a transcriptional activator complex (NICD-CBF-1/RBPjκ-MAML) that induces the transcription of target genes (Figure 2). Notch regulates the differentiation, development, proliferation, and apoptosis (Amsen et al., 2009; de la Pompa, 2009; McCright, 2003). Mutation in Notch components results in genetic disorders, such as spondylocostal dysostosis, T-cell acute lymphoblastic leukemia, alagille syndrome, cerebral
MOL #110254

dominant autosomal arteriopathy and schizophrenia as well as the commencement of multiple tumors (Mašek and Andersson, 2017). Moreover, aberrant expression of Notch has been implicated in the pathogenesis of lung diseases. In this review, we briefly discussed the molecular basis of Notch signaling, its role related to lung development, and the consequences of aberrant expression of Notch signaling in the initiation and progression of asthmatic airway remodeling. Moreover, we also emphasized the relevant potential therapeutic targets to regulate/inhibit the Notch signaling to facilitate the appropriate treatment of asthmatic airway remodeling.

**Regulation of Notch signaling:**

The taxonomy and structural features of involved molecules and commencement mechanism of Notch receptors are different among *D. melanogaster*, *C. elegans*, and mammals. Owing to this reason, we discussed the Notch signaling pathway in mammalian cells. Four Notch receptors (Notch-1, Notch-2, Notch-3, and Notch-4) and five major ligands (three Delta-family ligands (Delta-like ligand; Dll-1, Dll-3, and Dll-4) and two Serrate-family ligands (Jagged; Jag-1 and Jag-2)) have been recognized in mammals (Guruharsha et al., 2012; Kopan and Ilagan, 2009) (table 1). Although all Notch receptors exhibit same structures but show the considerable differences in the protein domains. Notch receptors are synthesized as large precursors in the endoplasmic reticulum (ER), travel to the plasma membrane through the exocytic pathway, and then undergo various post-translational alterations. During maturation process, in the endoplasmic reticulum, the sequence of signals located at N-terminus is cleaved off, and the extracellular domain of Notch is subjected to modification mediated by glycosyltransferases (Rana and Haltiwanger, 2011). For instance, O-fucosylation by protein O-fucosyltransferase 1 (*Pofut-1*) and consequent elongation of chain with O-linked β-D-N-acetylglucosamine (O-
GlcNAc) by fringe genes (radical, manic and lunatic fringe) regulate the ligand specificity in Golgi complex (Bruckner et al., 2000; Kovall et al., 2017; Okajima et al., 2003). Moreover, O-glucosylation by protein-o-glucosyltransferase (Poglut) in the ER is essential for activation of the receptor (Acar et al., 2008), while further elongation of chain with O-xylose negatively regulates Notch receptor (Lee et al., 2013). In Golgi complex, Notch extracellular domain undergoes its first proteolytic cleavage by a Furin-like proprotein convertase at site S1 (Logeat et al., 1998), whereas S1 cleavage is not necessary for signal activation but facilitates exocytosis (Gordon et al., 2009) (figure 2). After cleavage, Notch receptors are presented as heterodimers of a large, modular, N-terminal portion of the plasma membrane, which is exposed to extracellular spaces, known as Notch extracellular domain (NECD). NECD is noncovalently attached to C-terminal part of the multidomain intracellular portion that contains transcriptional activity, and referred as Notch intracellular domain (NICD); hence, Notch receptors contain large extracellular and small intracellular domains. The extracellular domain of Notch-1 and Notch-2, each, includes 36 epidermal growth factor-like repeats (EGF), whereas Notch-3 and Notch-4 contain 34 and 29 respectively (Radtke and Raj, 2003) that bind to calcium ions and demonstrate the necessary role in the receptor-ligand binding (Cordle et al., 2008) as well as in signaling efficacy (Raya et al., 2004). Generally, NECD is acknowledged as inhibitory because EGF-like repeats are followed by a negative regulatory region (NRR), which comprises of three cysteine-rich Lin-12-Notch repeats (LNR) and Notch heterodimerization region that demonstrates the crucial role in inhibiting the inappropriate Notch activation (Kopan and Ilagan, 2009; Malecki et al., 2006) while LNR modulates the communications between NECD and NICD (Greenwald, 1994). Transmembrane domain of Notch receptor (NICD) consist of recombination binding protein for immunoglobulin kappa J region (RBP-jκ) associated molecule (RAM) domain, a nuclear
localization signal, seven ankyrins (ANK) repeats, a transactivation domain (TAD) and degradation domain (glutamine-rich repeat (OPA)/proline-glutamic acid-serine-threonine-rich motifs (PEST) to regulate the stability (Bigas et al., 2013) (figure 2). Classical TAD is present in Notch-1 (strong TAD) and Notch-2 (weak TAD) excluding Notch-4 (Ong et al., 2006) while Notch-3 contains potent but specific TAD for activation of the HES-5 promoter. It has been revealed that RAM domain exhibit a crucial role in NICD and RBP-jκ interaction, whereas ankyrin repeats are also critical for the transcriptional activation complex formation and recruitment of Mastermind-like-1 (MAML-1) (Nam et al., 2006). Besides, NICD contains nuclear localization sequences (NLS) and target sites for ubiquitination (Fryer et al., 2004) phosphorylation and hydroxylation (Ramain et al., 2001). A list of proteins that interact with NICD by phosphorylation, ubiquitination, and hydroxylation are described in table 2.

In mammals, Notch ligands are type-1 transmembrane proteins with relatively small intracellular domain and large extracellular domain. Synthesis, trafficking, and exocytosis of the Notch ligands are same as Notch Receptors. In Notch ligands, three interconnected structural patterns are described: EGF-like repeats (variable numbers), Delta and OSM-11-like proteins (DOS) domain (a specialized tandem EGF-repeats) and Delta-Serrate-LAG-2 (DSL) domain (a cryptic EGF-like repeat); DOS and DSL domains, both, are implicated in receptor binding (Kopan and Ilagan, 2009) (figure 2). Nevertheless, because of the presence or absence of a cysteine-rich domain, Notch ligands can be more classified into Delta-like group or Jagged/Serrate (D'souza et al., 2008). Notch signaling pathway components are depicted in table 1.
Canonical Notch signaling

Importantly, Notch signaling initiates upon interaction of signal sending (Notch ligand containing) and signal receiving (Notch receptor containing) cells, that results in initiation of transendocytosis process and binding of ligands to the extracellular domain of Notch receptor. For ligand endocytosis, an E3 ubiquitin ligase, Mindbomb-1 (Mib-1), exhibits the critical role in the mono-ubiquitination of the intracellular domains of ligands in mammals (Koo et al., 2007) while Notch signal activation fails in the absence of ligand ubiquitination. After ligand binding and endocytosis, ligand induces the conformational change on ECD of Notch, to expose the S2 proteolytic cleavage site, operated by A disintegrin and metalloproteinase (ADAM) at site S2 (Weinmaster and Fischer, 2011) (figure 2). S2 cleavage is a key step for Notch activation, but some uncertainties still exist regarding enzymes responsible for cleavage. Indeed, ADAM10 is generally considered to be the protease that is responsible for ligand-inducible cleavage while ADAM17 is responsible for ligand-independent cleavage (Kopan and Ilagan, 2009). However, cleaved S2 (second cleavage) is yet entrenched in the membrane and known as Notch extracellular truncated form (NEXT). NEXT acts as a substrate of the γ-secretase complex; n intramembrane protease (Jorissen and De Strooper, 2010). In turn, Notch exposes the S3 cleavage site to γ-secretase complex, which is composed of Presenilin 1 (PSEN-1), anterior pharynx-defective 1 (APH-1), Presenilin enhancer 2 (PEN-2), and Nicastrin (NCSTN), undergoes S3 cleavage (third cleavage), and eventually releases the NICD from the membrane (Bray, 2006) (table 1). The exact subcellular location of γ-secretase cleavage is still controversial, i.e., γ-secretase cleavage occurs at multiple sites within the transmembrane domain, however, most probably occurs at the plasma membrane and in the endocytic vesicles (Yamamoto et al., 2010). The pharmacological effects of Notch signaling can be minimized by
targeting the S3 cleavage via γ-secretase inhibitors (GSIs). NICD then enters the nucleus and interacts with DNA-binding protein, to accumulate a transcriptional complex comprising of NICD, CSL (C promoter-binding factor-1 (CBF-1) also known as RBP-jκ in mammals; suppressor of hairless, Su(H) in D. melanogaster; longevity-assurance gene-1, LAG-1 in C. elegans) and co-activator of mastermind (MAML-1, MAML-2, MAML-3 in mammals and MAM in Drosophila) through ANK and RAM domains of NICD and converts it into transcriptional activator to induce transcription of target genes. In the absence of Notch, CSL, mainly CBF-1/RBP-jκ in mammals, recruits transcriptional co-repressors such as Ski-interacting protein (SKIP), Hairless (H), C-terminal-binding protein-1 (Ctbp-1), SPEN (also known as SHARP/MINT; SMRT/HDAC-1 associated repressor protein (SHARP) in human and mouse homologues MINT; Msx2 interacting nuclear target), RBP-jκ interacting and tubulin associated (RITA) protein, Groucho to recruit histone deacetylases (HDAC), a silencing mediator for retinoid or thyroid hormone receptors (SMRT) and further repressive cofactors to depressingly control the expression of Notch target genes (Chen and Evans, 1995; Oswald et al., 2005).

Whilst in the presence of NICD, SKIP collaborate with ankyrin repeat domain of NICD to assist NICD function by dissociating the repressor complex (Zhou et al., 2000) and recruiting the CBF-1 transcriptional coactivators, like chromatin remodeling complexes and histone acetyltransferases (HATs) (Kurooka and Honjo, 2000), to create short-lived transcriptional activation complex, NICD-CBF-1/RBP-jκ-MAML, which then activates downstream target gene via the addition of extra co-activator, such as p-300 (Fryer et al., 2002; Wallberg et al., 2002) (figure 2). The best-known Notch targets are the components of basic-helix-loop-helix (bHLH) family genes such as Hairy and enhancer-of-split (HES) family genes (HES-1, -3, -5 and -7) and Hairy and enhancer-of-split-related with a YRPW motif (HEY) family genes (HEY-1, HEY-2
and HEY-L) (Chen et al., 2014; Ranganathan et al., 2011). These components act as transcription repressor either by direct binding to E and N region, to attach co-repressor (Groucho) or by a unique mechanism still mysterious but independent of direct protein binding. In addition to HES and HEY family genes, Cyclin-D1 (cell-cycle promoter), c-MyC (proliferation-related gene), Bcl-2 (anti-apoptotic gene), the gene for HER-2, Deltex-1, p21Cip1/Waf1, Notch-regulated ankyrin repeat protein (Nrarp) and the pre-T-cell receptor gene have also been described as Notch target genes (Takebe et al., 2014; Wakabayashi et al., 2015). Mostly Notch-mediated processes need a transient activation, but few procedures required prolong activation. Hence, continuous signal activation is controlled by regulation point that shut off the Notch signaling via phosphorylation of NICD within PEST domain by kinases such as cyclin-dependent kinase-8 (CDK8) (Fryer et al., 2004), and targeted for poly-ubiquitination via E3 ubiquitin ligases such as SEL10/FBXW7 that results in proteasome-mediated degradation and termination of Notch signaling, and resets the cells for the next round of signaling (Guo et al., 2016; Kovall et al., 2017). It yet needs to recognize whether CDK8 and FBXW7 are common mediators of NICD degradation because some other kinases and E3 ubiquitin ligases are considered to contribute to NICD regulation in context-dependent circumstances. Additionally, Notch activity can also be determined by the ubiquitylation status of the receptor. Thereby phosphatases, ubiquitin ligases, and kinases remain to be identified and characterized. List of proteins that interact with NICD and influence the output of signaling pathway are described in table 2.

Non-canonical Notch signaling

Several recent studies have revealed the existence of numerous modes of Notch signaling, in addition to canonical Notch signaling, commonly referred as non-canonical Notch signaling. Non-canonical Notch signaling is related to other different transcription factors, such as hypoxia-
inducible factor-1α (HIF-1α), β-catenin, estrogen receptor (ERα), Yin and Yang 1 (YY1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), instead of CSL (CBF1, suppressor of hairless, and Lag-1) transcription factor (figure 2). Interestingly, canonical Notch signaling is associated with various normal cellular processes such as early development of embryo, lungs and other physiological processes, while non-canonical Notch signaling is mainly concerned with potentially pathological conditions like cancer and activation of the immune system. Hence, blockade of non-canonical Notch signaling may possibly establish new opportunities to inhibit pathological conditions without harming normal physiological processes; more detailed description of non-canonical Notch signaling pathway can be found elsewhere (Andersen et al., 2012) and is beyond our topic of discussion.

**Regulatory role of Notch signaling in fetal lung development:**

**Early proximodistal cell fates**

It is challenging to evaluate the participation of Notch signaling in normal lung development owing to Notch receptor/ligand interactions complexity, but the experimental data revealed that Notch signaling exhibited the crucial role in regulating the patterning and proximodistal cell fate in the early lung development (Tsao et al., 2008). Genetic ablation of RBP-jκ or Pofut-1 from developing epithelium or mesenchyme demonstrated the substantial differentiation defect, including complete ablation of the Club cell secretory lineage, and overpopulation of ciliated cells and neuroendocrine cells, in proximal airways; same defects were also found in the DAPT (γ-secretase inhibitor)-treated proximal airways explants (Morimoto et al., 2010; Tsao et al., 2009).

In addition, expression patterns of Notch receptors, ligands, and its other constituents (ED 11.5 to maturity) also recommend the participation of Notch signaling in regulating
proximodistal cell fates. For instance, Notch-1 is persistently expressed in the distal lung endoderm, Notch-2 and Notch-3 both are articulated in fetal lung mesenchyme, and Notch-4 is endothelial specific although Notch-3 can be articulated in mesenchyme and endothelial cells of the airway (Post et al., 2000). During budding, Dll-1 is restricted to the proximal area, while Jag-1 and Jag-2 are found in the distal region (Kong et al., 2004). Jag-1 is highly expressed in proximal airways (ED 16.5) (Zhang et al., 2013a) and Jag-2 expression (ED 13) is observed in peripheral lung mesenchyme (Post et al., 2000). Dll-1 is first expressed in the secondary bronchi (ED 13.5) and gradually rises till birth within bronchioles and branch points (Post et al., 2000). Postnatally, Dll-1 expression is identified as endothelial cells lining the lung vasculature throughout the lung tissue (Beckers et al., 1999; Post et al., 2000). Hence, Dll-1 is well located to relate a subset of Notch proteins expressed in SPNC cells/Club-like cell that surrounds neuroepithelial bodies (Guha et al., 2014; Morimoto et al., 2010). Dll-4 expression is restricted to endothelial cells in the lung (Yoneya et al., 2001). Further, HES-1 expression starts from the early pseudoglandular stage (ED 12) and gradually rises till birth, which can also be promptly detected in non-endocrine airway epithelial cells in the fetal lung (Ito et al., 2000). Predominantly, HES-1-reactive cells are differentiated into Club cells in distal airway epithelium (ED 17 at low levels), and remains continue up to fetal lung development (Hackett et al., 1992). HEY-1 is notably expressed in developing lung while HEY-2 expression is low (Steidl et al., 2000). HEY-L is expressed in lung vasculature (Leimeister et al., 2000).

Differentiation of pseudostratified epithelium

Notch signaling regulates the differentiation of pseudostratified epithelial layer, including basal cells, mainly toward secretory lineages in the airway (Rock et al., 2011) while genetic and pharmacological inhibition of Notch-3 led to the aberrant expansion of basal cells, and altered
MOL #110254

pseudostratification (Mori et al., 2015). Further, Notch is required to maintain the Club cells (Pardo-Saganta et al., 2015) while conditional deletion of HES-1 or RBP-jκ or Pofut-1 showed the remarkable elevation of ciliated cells count and attenuation of Club cell count; in fact, in the dearth of such signaling, cells are generally intended to turn into ciliated cells through a default program (Morimoto et al., 2010; Tsao et al., 2009). Transgenic misexpression of the Notch-1 intracellular domain in distal epithelial cells demonstrated ectopic expression of Club cells (Guseh et al., 2009). Furthermore, Jag-1 actively participates in controlling Notch-dependent differentiation of Club cells during lung development because spatial-temporal deletion of Jag-1 in the airway epithelium caused the aberrant cell fate specification with excess ciliated cells at the expense of Club cells, a phenotype correlated with downregulation of HES-1 (Zhang et al., 2013a). Moreover, mouse trachea and human airway basal cells undergo both self-renew and generate ciliated and Club cell lineage (Engelhardt et al., 1995; Rock et al., 2009). In the Scgb1a1-CreER knockin mouse, the majority of Club cell in bronchioles undergo both self-renew and generate ciliated cells while in the trachea, Club cells generate ciliated cells but do not undergo the self-renew (Rawlins et al., 2009). Taking together, differentiation of airway epithelium into the secretory, ciliated, Club and neuroendocrine cell types in the developing and adult murine lung is regulated by Notch signaling but little human data is available such as Notch-1 and Notch-3 pathways regulate the differentiation of basal cells into ciliated and secretory cells (Gomi et al., 2015) via Jag-1 mediation (Gomi et al., 2016).

Pulmonary neuroendocrine cell differentiation

Pulmonary neuroendocrine cells (PNEC) differentiation is regulated by Notch signaling (Ito et al., 2000). HES-1 and Mash-1 are expressed in nonneuroendocrine cells and pulmonary neuroendocrine cells (and neuroepithelial bodies in lungs) respectively. However, analyses of
both Mash-1 and HES-1 knockout mice proposed a significant correlation between Notch/Notch-ligand pathways and the regulation of bHLH proteins in the cell fate decision in developing lung. An elevated level of Mash-1 mRNA and pulmonary neuroendocrine cells has been observed in HES-1 deficient mice, whereas lack of pulmonary neuroendocrine cells has been noticed in Mash-1 deficient mice (Borges et al., 1997; Ito et al., 2000). Nevertheless, the exclusive interaction between HES-1 and Mash-1 is the consequences of direct suppression of Mash-1 promoter by HES-1, and the ability of Notch-1, HES-1-independent, to promote degradation of Mash-1 (Chen et al., 1997; Sriuranpong et al., 2002). Hence, Notch-1 might control the expression of HES-1 and inhibition of fate of pulmonary neuroendocrine cells, in view of the fact that antisense oligonucleotides of Notch-1 promoted the differentiation of PNEC and transgenic expression of Notch-1 intracellular domain blocks the differentiation of PNEC (Kong et al., 2004; Shan et al., 2007). Expression of the Dll-1 in neuroendocrine cells, and Notch-1, -2 and -3 in nonneuroendocrine cells further authenticated this model (Xu et al., 2010). Consequently, Dll-1-mediated activation of Notch could significantly encourage the expression of HES-1 and suppression of neuroendocrine differentiation via lateral inhibition (Noguchi et al., 2015).

Importantly, Jag-1 deletion resulted in an increased number of PNEC (Zhang et al., 2013a). On the other hand, conditional removal of RBP-jκ in lung endoderm has no burly influence on the number of PNECs or expression of HES-1. Moreover, an involvement of non-canonical Notch signaling in neuroendocrine cell fate selection is determined by Notch-1, -2, -3 triple knockout which shows a massive expansion of neuroepithelial bodies (Morimoto et al., 2010; Morimoto et al., 2012). Hence, under normal physiologic conditions, most probably, a non-canonical pathway might compel inhibition of pulmonary neuroendocrine cells fate and expression of HES-1.
Although such mechanisms could utilize different signaling from JAK, Alk5, FGFR, or ERK kinases (Niwa et al., 2007; Xing et al., 2010; Yoshiura et al., 2007).

**Pulmonary goblet cell fate**

Notch signaling also regulates the fate of pulmonary goblet cells, but it is complicated to evaluate the precise physiological participation of Notch signaling in this context owing to a few goblet cells in murine airway epithelium. It has been exposed that Dll-4 augmented the number of Muc5AC goblet cells in both human airway cell cultures and murine tracheal explants studies (Guseh et al., 2009). Interestingly, mice with conditional inactivation of RBP-jκ or Pofut-1 via Tgfb3-Cre could survive to adulthood with aberrant airway phenotype characterized by obvious goblet cell metaplasia, increased ciliated cells numbers and attenuated Club cells count (Tsao et al., 2011). Moreover, goblet cells metaplasia is observed in adult Jag-1 deleted mice lungs, telling that Jag-1 contributes to repression of mucin production and goblet cell fate, accompanied by downregulation of Dll-1, HES-1, and HES-5 (Zhang et al., 2013a). Recently, it has been demonstrating that Notch signaling particularly Notch-2 directly promoted the pulmonary goblet cells while anti-Notch-2 antibodies prevented the IL-13 as well as allergen-driven goblet cell metaplasia (Danahay et al., 2015; Lafkas et al., 2015).

**Alveolar and microvasculature development**

Alveolar development takes place because of coordinated proceedings of endothelium, epithelium and mesenchyme stroma in the distal lung. Notch signaling exhibits a pivotal role in the cell differentiation and cell fate specification in the vascular and parenchymal compartments to coordinate the alveolar and microvasculature development. Constitutive expression of Notch in the peripheral (Dang et al., 2003) and distal (Guseh et al., 2009) lung epithelium in mice
caused perinatal lethality due to aberration and/or inhibition of differentiation of alveolar cells into type-I and type-II cells. Additionally, dilated cysts, appeared on proximal airway epithelium, were lacking some markers particularly SP-C, keratin-5, β-tubulin, transformation-related protein 63 (p63), Club cell 10 kDa protein (CC10), but not all markers (Guseh et al., 2009). In contrast, conditional deletion of *RBP-jκ* or *Pofut-1* in lung epithelium exhibited no negative impact on the differentiation of alveolar epithelial cells and formation of alveolar saccules (Morimoto et al., 2010; Tsao et al., 2009). Interestingly, failure of alveolar septation and defective alveolar development have been reported in Notch-2+/−/Notch-3−/− compound mutant (*lfng*) knockout mouse due to flawed differentiation and recruitment of myofibroblast cells rather than alveolar epithelial cells while conditional deletion of RBP-jκ (ED 14.5 to 18.5) impaired the myofibroblast differentiation without affecting the alveolar epithelial cells (Xu et al., 2010).

Notch-deficient mice, such as *Jag-1* or *lfng* mutants, that survive postnatally helped to explore the Notch-mediated events in alveolar development. Conditional deletion of *Jag-1* in lung epithelium disrupted alveolar septation showed no effect on differentiation and maturation of alveolar epithelial cells (Zhang et al., 2013a). Moreover, overexpression of *lfng* in distal lung epithelium did not affect the spatial or temporal expression of HES-1 and Mash-1, and expression of proximal ciliated, nonciliated, distal epithelial cell and mesenchymal cell marker (vWF, α-SMA, PECAM-1) (van Tuyl et al., 2005). However, Tsao and his group members used the epithelial Notch-2 null mice and explored that epithelial Notch signaling regulates the alveologenesis by triggering the paracrine activation of PDGF receptor-α signaling in alveolar myofibroblast progenitors while overexpression of stimulated Notch-2 reversed the negative effect of Notch inhibition (Tsao et al., 2016).
Of note, infiltration of growing microvascular into emerging alveolar walls is necessary for the alveolar development. This infiltration event is strongly coordinated with alveolar epithelium development to facilitate lung physiology at birth. In lung vasculature, Notch genes expression is gradually rising from early to late lung developmental stages, suggesting that Notch pathway exhibits significant role during alveolar microvasculature development (Post et al., 2000; Taichman et al., 2002; Xu et al., 2010). This suggestion is consistent with the identified role of Notch signaling in whole body vascular development (Holderfield and Hughes, 2008; Siekmann et al., 2008). Mostly mutants, with entire deletion of Notch ligand and receptor gene such as Notch-1, Notch-2, Dll-1, and Jag-1, die during the early embryonic developmental process, therefore the appropriate role of Notch gene in microvasculature development of distal lung is yet unclear. Nevertheless, some facts supporting the role of Notch signaling in peripheral lung microvasculature development arises from Foxf-1 heterozygous mutants, where Foxf-1 haploinsufficiency mutant showed abnormal morphogenesis of lung microvasculature and lethality of neonates, due to disruption of pulmonary expression of Notch-2 signaling and HES-1, even though disrupted Notch-2 expression and directly affected cell type, by Foxf-1 haploinsufficiency, remains unclear (Kalinichenko et al., 2004). Notch-3 and Notch-4 null mice demonstrated almost normal lung physiology and were viable (Domenga et al., 2004; Krebs et al., 2000) while aberrant activation of Notch-4 in vascular endothelium exhibited pulmonary arteriovenous shunts due to vessel sprouting inhibition and overgrowth of the vessel at capillary bed interface (Miniati et al., 2010). The requirements of Notch and its transcription factors during lung development are substantially depicted in table 3.
**Pulmonary vascular development**

Pulmonary vascular system, at birth, is subjected to a striking switch from a low-flow and low-pressure fetal status to a high-flow and higher-pressure postnatal status. The essential roles of Notch signaling in the vascular development have been supported by the fetal lethality of Notch signaling deficiency. For instance, either knockout of Notch-1, Dll-4, Jag-1, Notch-1 plus Notch-4, HERP-1 plus HERP-2, presenillin-1, or continuous Notch-4 expression in mice or zebrafish led to embryonic death owing to vascular remodeling defects (Gridley, 2007; Gridley, 2010; Miniati et al., 2010; Roca and Adams, 2007) but its exact role in coordination of pulmonary vascular development is yet ambiguous because of limited data.

Notch-3 receptor in embryonic pulmonary vascular smooth muscle cells is activated from an intracellular compartment (Ghosh et al., 2010). Defects in lung vascular smooth muscle have been reported in Notch-3 deficient mice and being viable throughout adulthood without other apparent lung abnormalities (Li et al., 2009). In this context, Ghosh and colleagues reported apparently similar morphology in Notch-3 deficient and WT (ED 18.5) while at postnatal day 3, vascular smooth muscle cells (VSMCs) were non-cohesive, dysmorphic, and vacuolated with disordered α-SMA distribution in Notch-3 deficient mice. They reported that Notch-3 is expressed primarily in pulmonary artery VSMCs-derived Jag-1, which is activated from late fetal to early postnatal life, and exhibit the role in maintaining the morphological characteristics and gene expression profile of the pulmonary artery after birth (Ghosh et al., 2011). Moreover, Notch-3 activation promotes proliferation of pulmonary vascular smooth muscles cells HES-1/p27Kip1 signaling pathway (Song et al., 2015).
Notch signaling as a potential biomarker in asthmatic airway remodeling:

**Immunological role of Notch signaling in allergic asthmatic airway remodeling**

Allergic asthmatic airway remodeling is characterized by infiltration of many inflammatory cells such as lymphocytes (T-lymphocytes), macrophages, neutrophils, mast cells, and eosinophils (Pascual and Peters, 2005). These inflammatory cells exhibit defensive role in the pathogenesis of asthma. Activation of CD4$^+$ T cells or Th-cells into Th-1 and Th-2 cell types, and Th-1/Th-2 imbalance (particularly an elevated level of Th-2) exhibit the important role in the pathogenesis of chronic asthma (Elias et al., 1999). Interleukins (ILs), such as IL-4, IL-5, IL-13 and IL-20 released as a result of Th-2 reactions, drive the asthmatic airway remodeling by interacting with airway smooth muscle cells and epithelial cells (Gong et al., 2014).

Notch/RBP-$j_k$ in CD4$^+$ T cells exhibit the pivotal role in the induction of allergic asthma (KleinJan et al., 2013) because Notch is involved in the differentiation of CD4$^+$ T cells into Th-1/Th-2 (Zhang et al., 2013b) while γ-secretase inhibitor reduced allergic asthma and goblet cell metaplasia by attenuating the Th-2 cytokines production (Kang et al., 2009). Elicitation of Th-2 cells requires GATA-3 (transcription factor) (Tanigaki et al., 2004). Notch directly governed the expression of GATA-3, while Notch and GATA-3 synergistically activated the IL-4 expression and the Th-2 cell responses (Fang et al., 2007); this was further confirmed by the inhibition of Notch/GATA-3 signaling pathway that resulted in the prevention of deterioration owing to allergic asthma (Chong et al., 2014). Further, intravenous immunoglobulin ameliorated allergic asthma by enhancing the Dll-4 levels, and by decreasing the GATA-3 levels and Jag-1 expression; suggesting the suppression of expected Th-2 response (Kaufman et al., 2011). Moreover, in asthma models, Notch and phosphoinositide-3-kinase (PI3K) coordinately regulated the activation and differentiation of T-lymphocytes via down-regulation of p27$^{kip1}$ of
CD4⁺ T lymphocytes and upregulation of cyclin D-1 of lymphocytes (Zhang et al., 2013b), whereas CD4⁺ T lymphocytes exhibited the crucial role in regulation of asthmatic airway remodeling (Foster et al., 2002). Besides CD4⁺ T-cell, regulatory T-cell (Treg) and Th-17 cells also exhibit role in asthma (Shi et al., 2011). For instance, γ-secretase inhibitor (L685, 458) alleviated inflammation induced allergic asthma in mice by down-regulating the differentiation of Th-17 cells (Zhang et al., 2014). In addition, in vivo study has recently shown that Notch signaling in T cells, but not Jag-1 or Jag-2 on dendritic cells (DCs), is important for the induction of house dust mites-mediated Th-2 responses, eosinophilia, and airway hyperreactivity (Tindemans et al., 2017).

Eosinophils also exhibit the vital role in allergic asthma (Gaurav et al., 2014), and Notch regulates the differentiation and maturation of eosinophils via ERK pathway (Kang et al., 2005; Kang et al., 2007) while histone deacetylation of T lymphocytes and γ-secretase inhibitors inhibited the eosinophilic airway inflammation (Kang et al., 2009; Zhang et al., 2015). In granulocyte-macrophage-colony-stimulating factor (GM-CSF)-stimulated eosinophils, activation of Notch receptors and subsequent transcription of HES-1 has been noticed (Liu et al., 2015; Radke et al., 2009). Total ablation of eosinophils lineage in mice resulted in elevated mucus secretion and airway hyperresponsiveness similar to wild-type (acute and chronic phases) while eosinophil-deficient mice were considerably protected from deposition of airway smooth muscle cells and peribronchiolar collagen (Humbles et al., 2004). Various studies have demonstrated that eosinophils contributed to asthmatic airway remodeling through the secretion of cytokines (TGF-β) and eosinophil cationic protein as well as through interaction with epithelial cells and mast cells (Kay et al., 2004; Venge, 2010). More detailed studies are required to evaluate the remarkable role of Notch in airway remodeling in response to allergic asthma.
Mucous/goblet cell metaplasia

Mucous/goblet cell metaplasia is the hallmark of airway diseases in epithelial remodeling. Mucus, comprising of Muc5AC and Muc5B, is secreted by the goblet cells and submucosal glands of the lung through epidermal growth factor receptor (EDFR) signaling and STAT6 activation, via IL-4, IL-5, and IL-13, during allergic disease particularly in asthma (Boucherat et al., 2013; Justice et al., 2002). The role of Notch signaling in the goblet cell metaplasia and mucus overproduction remains somewhat controversial. For instance, ablation of Notch signaling either by conditional inactivation of Pofut-1 or by deletion of RBP-jκ in mice resulted in striking goblet cell metaplasia and mucus metaplasia associated with significant downregulation of bHLH transcription repressor HES-5 (Tsao et al., 2009; Tsao et al., 2011), while Jag-1 deletion induced mucous metaplasia is mainly accompanied by downregulation of HES-1 and HES-5 (Zhang et al., 2013a). Another study has reported that Notch signaling directly downregulated the expression of Muc5AC via HES-1 dependent mechanism (Ou-Yang et al., 2013). These findings suggest an inhibitory function of Notch in mucus overproduction and goblet cell metaplasia in asthmatics. However, in contrast, various studies have proposed that Notch signaling directly promotes the mucous metaplasia and goblet cell metaplasia (Danahay et al., 2015; Lafkas et al., 2015; Rock et al., 2011). Activation of Notch signaling in entire lung epithelium (Guseh et al., 2009) or in airway basal cells (Rock et al., 2011) resulted in an elevated count of goblet cells and mucous cells. Most importantly, Notch ligands treated STAT6-null cultured tracheal epithelial cells were able to cause mucous metaplasia, and unchanged expression of FOXA2 was noticed as well; demonstrating that Notch signaling acts via the STAT6-independent pathway, probably Notch and STAT6 may operate in parallel, to promote goblet cell metaplasia (Guseh et al., 2009). FOXA2-independent goblet cell metaplasia was
noticed in Hoxa-5 deficient mice accompanied by an increased activity of Notch signaling while γ-secretase inhibitor attenuated the goblet cell metaplasia (Boucherat et al., 2012). Besides, forced expression of the Notch-1 intracellular domain in lung epithelial cells induced both epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinases (ERK) phosphorylation with a Muc5AC expression even in the absence of epidermal growth factor. The phosphorylation of ERK induced by exogenous NICD was inhibited by a γ-secretase inhibitor (L-685,458) or introduction of small interfering RNA directed against Notch-1 that antagonizes EGFR activity; indicating that Notch signaling induced Muc5AC expression by activating the EGFR pathway (Kang et al., 2011). Consistent with previous studies, antagonizing the Notch prevented and reversed the IL-13 and allergen-driven goblet cell metaplasia (Danahay et al., 2015; Guseh et al., 2009; Kang et al., 2009; Lafkas et al., 2015) as well as overturned the human airway epithelium disorder (Gomi et al., 2016), providing evidence to support the involvement of Notch signaling in goblet cell metaplasia and asthmatic airway remodeling. Taken together, controversies regarding the role of Notch in mucous/goblet cell metaplasia might be due to the disparate function of Jagged1 and Dll-4 in the pathogenesis of allergic asthma (Huang et al., 2017).

**Extracellular matrix production and subepithelial fibrosis**

Subepithelial fibrosis, most distinctive pathological characteristic of asthmatic airway remodeling (Elias et al., 1999), is characterized by unnecessary deposition of extracellular matrix (ECM). Type-I collagen, tenascin-C (TN-C), periostin and hyaluronan are the imperative components of ECM that serve as biomarkers of asthma and scaffolds for continuing airway remodeling. Type-I collagen, heterotrimer of two α1 (col1α1) and one α2 collagen (col1α2) subunits, becomes most abundant during subepithelial fibrosis. Recently, *in vitro* study both in
human (MRC-5 cells) and mice (L929 cells) lung fibroblast cell lines demonstrated that Notch signaling might regulate the expression of col1α1 and col1α2 via HES1-dependent mechanism (Hu et al., 2014). Accordingly, in vivo study on asthmatic mouse models revealed excessive expression of type-I collagen whereas KyoT2, a negative regulator of Notch signaling, alleviated the subepithelial fibrosis through HES-1 dependent mechanism (Hu et al., 2015). TN-C is involved in the pathogenesis of bronchial asthma (Rogers et al., 2012) while its deficiency attenuated the allergen-induced bronchial asthma (Nakahara et al., 2006). Notch signaling regulates the endogenous TN-C expression via RBP-jκ-induced target gene (Sivasankaran et al., 2009). Recently, Sarkar and colleagues have shown that integrin α2β1 might be a direct link between TN-C and Notch signaling (Sarkar et al., 2017). It will be important to study the involvement of TN-C regulation in Notch-deficient asthmatic mice to clarify the relationship between TN-C/Notch and asthmatic airway remodeling.

Hyaluronan, polysaccharide of ECM, is found within the peribronchial and perialveolar spaces of the healthy lung. During inflammation, smaller fragments of hyaluronan, proinflammatory in nature, are produced, which affect ECM components including collagen I and II and are potentially associated with airway remodeling, hyperresponsiveness and clinical symptoms (Garantziotis et al., 2016). Hyaluronan regulates the expression of Notch genes because expression of Notch-1 mRNA was decreased while Notch-3 mRNA was increased significantly compared to controls when normal human epidermal keratinocytes were incubated in dishes coated with sulfated hyaluronan (Nagira et al., 2007). Periostin is a multifunctional protein that is up-regulated in response to IL-4 and IL-13 in airway epithelial cells and lung fibroblasts of asthmatics. Periostin is involved in many aspects of asthma, such as eosinophil recruitment, airway remodeling, development of a Th2 phenotype, and increased expression of inflammatory
mediators (Li et al., 2015). Interestingly, Notch-1 is downregulated in periostin deficient mice, suggesting that periostin could directly interact with Notch-1 precursor to stabilize the Notch-1 expression and subsequent signaling. Moreover, periostin directly interacts with type-I collagen (Norris et al., 2007), fibronectin and TN-C (Kii et al., 2010). Through these interactions, periostin seems to induce the airway remodeling via modulating the ECM production. To date, limited data are available to support the participation of Notch in EMC, and its components, production and airway epithelium and/or subepithelial fibrosis in asthmatics. Hence, more profound studies are needed.

**Myofibroblast differentiation**

All of Notch receptors, except Notch-4, are accomplished with the regulation of myofibroblast differentiation. Notch-1/CSL activation induced the differentiation of myofibroblast through direct regulation of smooth muscle α-actin (α-SMA) (Noseda et al., 2006) while α-SMA is normally used a molecular marker for myofibroblasts. Knockout mice study has demonstrated that Notch-1 exhibited a distinct role in stimulating the α-SMA gene expression (Liu et al., 2009) whereas Notch-2 inhibited the TGF-β-induced collagen-I and α-SMA gene expression via down-regulating the Notch-3 in myoblasts (Ono et al., 2007). However, in 10T1/2 fibroblasts, overexpression of activated Notch-3 blocked the TGF-β1-dependent smooth muscle-specific genes, such as α-SMA, by inhibiting the activation of Smad-3 and p38 mitogen-activated protein kinase (Kennard et al., 2008) whereas TGF-β1 decreased the Notch-3 expression but unexpectedly *HES-1* was up-regulated. It is also suggested that fibroblast proliferation is inhibited by Notch-1 and is mediated through Wnt-1-independent but Wnt-11-dependent Wnt-1-inducible signaling pathway protein 1 expression (WISP-1) (Liu et al., 2012). In contrast, Notch-1 actively
induced the differentiation of myofibroblast of alveolar epithelial cells through a TGF-β-Smad-3 pathway that activates α-SMA gene transcription in a TGF-β control element (TCE)-dependent manner and SRF-binding site [CC(A/T)6GG, also termed as CArG box]-dependent or CArG-dependent manner (Aoyagi-Ikeda et al., 2011). Interestingly, physiological differentiation of myofibroblast during lung alveogenesis required the involvement of IENG-mediated Notch signaling (Xu et al., 2010). The Notch in alveolar type 2 cells induced the PDGF-A expression to expand the cell population of myofibroblast (Tsao et al., 2016). Hence, the consequence of Notch signaling in fibrosis (Kavian et al., 2012) might be due to activating effects of Notch signaling on myofibroblast differentiation via endothelial-mesenchymal transition and epithelial-mesenchymal transition (EMT).

**Epithelial-mesenchymal transition**

Dysregulation of epithelial barrier function is a pathological hallmark of asthmatic airway remodeling that leads to transdifferentiation of epithelial cells to attain mesenchymal characteristics, such as the production of extracellular matrix, α-SMA, vimentin, and enhanced motility, in a process known as epithelial-mesenchymal transition (EMT). Transcription factors including Snail-1, Slug (Snail-2), Twist-1/2 and zinc finger E-box-binding homeobox-1/2 (ZEB-1/2) serve as phenotypic markers of EMT. Slug, Snail, SIP1/ZEB, Twist, and E-47 negatively control the E-cadherin expression. Notch cross-talks with numerous transcription and growth factors related to EMT, such as Snail, Slug, PDGF, FGF, and TGF-β (Gonzalez and Medici, 2014). Notch up-regulated the Snail and Slug that subsequently led to transcriptional repressors of CDH1 (E-cadherin gene), alleviation of the cell-cell junction, and destabilization of epithelial structure (primary step in EMT) (Leong et al., 2007). Notch-1 knockdown in intestine epithelial
cells contributed to decreased barrier function, the expression of intercellular tight junction protein Claudin-5 was declined, and increased intercellular permeability (Mathern et al., 2014). Overexpression of Notch alone up-regulated the Snail expression and downregulated the E-cadherin whereas inactivation of Notch decreased the Snail expression and attenuated the down-regulation of E-cadherin expression, suggesting the participation of Notch signaling in EMT induction via Snail (Xie et al., 2012). Notch signaling induced EMT could trigger the upregulation of mesenchymal markers (Snail, Slug) and down-regulation of epithelial markers (E-cadherin) in the cardiac valve and cushion formation, human kidney epithelial cells, and non-small cell lung cancer (NSCLC) cell lines. Importantly, Loffredo and colleagues recently identified Notch-2, among other recognized genes, as novel marker central to dysregulation of epithelial-mesenchymal signaling in asthmatics; suggests that EMT process, concerned with asthma pathogenesis, is a manifestation of an underlying profound and persistent suppression of epithelial differentiation because significant suppression of epithelial differentiation was demonstrated in the scarcity of Notch signaling (Loffredo et al., 2017). Moreover, inactivation of Notch signaling by a γ-secretase inhibitor could reverse the EMT process; highlighting the direct involvement of Notch signaling in EMT induction, making them attractive targets for new therapeutics.

Of note, EMT in NSCLC is mediated by TGF-β that promotes the interface of SMAD to the promoters of Snail. Crosstalk between Notch and TGF-β is imperative for induction of EMT because Notch signaling is required to maintain TGFβ-induced expression of HEY-1 while pharmacological inactivation of Notch and/or knockdown of HEY-1 or jag-1 could inhibit the TGF-β induced EMT; demonstrating the key role of Notch signaling in TGF-β-induced EMT (Zavadil et al., 2004). Similarly, in alveolar epithelial cells, Notch-1 activated α-SMA gene in
MOL #110254

TGF-β control element (TCE)-dependent manner and CArG-dependent manner through TGF-β-Smad-3 Pathway (Aoyagi-Ikeda et al., 2011). Meanwhile, pharmacological inhibition of Notch signaling could notably inhibit the TGF-β-induced expression of α-SMA, signifying Notch-induced EMT via TGF-β-Smad-3 pathway (Matsuno et al., 2012). Nevertheless, Notch prevented TGF-β-mediated EMT in cultured cells through Smad-7 induction (Tsai et al., 2014). Collectively, these studies indicate that the crosstalk between Notch signals and TGF-β play an insightful role in EMT, and more detailed studies are needed to evaluate the role of Notch signaling in EMT-induced asthmatic airway remodeling.

**Pulmonary vascular remodeling**

Pulmonary vascular remodeling, including increased subepithelial vascularity and microvascular leakage, is significantly increased in asthmatics with the severity of asthma (Shifren et al., 2012). It involves multiple factors including inflammatory mediators (pro-inflammatory mediators such as IL-6 and -8, TNF-α, TGF-β), extracellular matrix proteins, bioactive extracellular matrix fragments (matrikines), growth factors (FGF family, PDGF, TGF-β, TNF-α and IL-1family) and proteases (MMPs and ADAMS) (Harkness et al., 2015). The enhanced vasculogenic effect in pulmonary tissue is determined by the Th-1 and Th-2-dependent selection predisposition in T-cells which act as chemotactic substances for endothelial precursor cells, such as VEGF-A, to induce Th-2 type inflammatory responses that are proinflammatory and leads to the angiogenic switch.

Notch-3/Jagged controls the differentiation and expansion of Treg (Anastasi et al., 2003), and Treg exhibits the angioregulatory response (Asosingh et al., 2007). So, Notch signaling demonstrated a crucial role in angiogenesis and vasculogenesis (Iso et al., 2003). Activation of Notch-HES-1 axis via TGF-β exhibited Treg-mediated immunosuppression (Ostroukhova et al.,
Injection of sensitized Treg cells in chronic mouse asthma model alleviated the airway remodeling by reducing the pulmonary vasculature via induction of apoptosis through a Dll-4/Notch signaling pathway (Huang et al., 2009). Dll-4 and Jag-1 surprisingly demonstrated the different responses in asthma models (Huang et al., 2017). Dll-4 alleviated the airway hyperresponsiveness by suppressing neo-vasculature airway remodeling via Treg (Huang et al., 2009) while Jag-1 initiated allergic asthma by stimulating the Th-2 differentiation and IL-4 production, leading to airway inflammation, airway hyperresponsiveness and asthmatic airway remodeling (Okamoto et al., 2009).

As discussed, Notch signaling is imperative for regulation of proximodistal cell fates, pseudostratified epithelium differentiation, pulmonary neuroendocrine cell differentiation, pulmonary goblet cell fate, and alveolar and pulmonary vasculature development. While deletion and/or deterioration of Notch signaling during early lung development leads to abnormal enlargement of alveolar spaces, disturbance of integrity of bronchial and epithelial smooth muscle layer (Tsao et al., 2016), widespread cellular death (Hamada et al., 1999; Swiatek et al., 1994), aberrant maturation of vascular smooth muscular cells (Krebs et al., 2000), vascular remodeling defects (Gale et al., 2004; Roca and Adams, 2007), hypoplastic lungs with attenuated Club cells count (Morimoto et al., 2010; Tsao et al., 2009), and goblet cells metaplasia (Danahay et al., 2015; Lafkas et al., 2015; Tsao et al., 2011). These pathological changes elaborate the underlying causes for abnormal lung functioning. Moreover, Notch genes participating in fetal lung development may assist to clarify the molecular processes involved in lung function impairment, but clinical data to support the role of Notch signaling in linking lung development and asthmatic airway remodeling is yet missing. However, murine model studies suggest that abnormal in utero expression of Notch genes, concerned with healthy lung development, can
result in impaired lung physiology after birth. Gathering all data suggests that Notch signaling exhibits a distinct role in linking lung development and asthmatic airway remodeling but mysterious, thereby detailed experimental and genetic studies are needed to investigate the underlying philosophy.

**Notch signaling as a potential therapeutic target:**

As, Notch signaling exhibits a potential role in the regulation of asthmatic airway remodeling; thereby direct or indirect targeting Notch pathway at signal sending-signal receiving sites, intracellular signaling levels, transcriptional/post-transcriptional levels, specific co-factors action sites, and crosstalk with other pathways would facilitate to develop new effective therapeutic approaches to control/reverse the asthmatic airway remodeling (figure 3).

**Targeting Notch signaling via γ-secretase inhibitors**

γ-secretase inhibitors, non-specific Notch pathway inhibitors, inhibit the S3 cleavage that subsequently inhibits the production of NICD from membrane-tethered Notch receptors and transcription of target genes. γ-secretase inhibitors are divided into three main classes: peptide isosteres, azepines, and sulfonamides. L685458, MW167 (gamma-secretase inhibitor II) and DAPT (also known as GSI-IX and Compound 3) are reversible peptide-based γ-secretase inhibitors. From DAPT, LY-411575 (Compound 5; 100-fold stronger than DAPT), LY-450139 (Compound 6) and RO-4929097 (Roche, Nutley, NJ, USA) have been developed that are even more effective. DBZ (dibenzazepine or YO-01027) and JLK6 are irreversible azepines-based γ-secretase inhibitors. MK-0752 and Compound 18 are sulfonamide-based γ-secretase inhibitors. So far, various clinical trials are ongoing to explore how and whether targeting Notch signaling via γ-secretase inhibitor can alleviate different ailments, particularly in oncology while in the
case of asthmatic airway remodeling few approaches have been performed via antagonizing γ-secretase.

In asthma model, L 685458 alleviated allergic asthma phenotypes via downregulating the Th-2 cytokine production (Kang et al., 2009) and antagonizing the differentiation of Th-17 cells (Zhang et al., 2014). L 685458 vitiated the airway goblet cell metaplasia in a Hoxa-5 mutant mouse model (Boucherat et al., 2012). In asthma models, MW167 attenuated the Th-2 polarization, airway inflammation and the levels of IL-4 and IL-5 in BALF and serum as compared to PBS treated (Zhou et al., 2015). Furthermore, DAPT reversed the pathological remodeling of airway epithelium (Gomi et al., 2015), and attenuated the accumulation of eosinophils in allergic airways (Liu et al., 2015). In human airway epithelial cells, DBZ attenuated the IL-13 induced airway mucous metaplasia either by independent of STAT-6 or by downstream of the STAT-6 pathway (Guseh et al., 2009). Treating CD4+ T cells with DBZ resulted in inhibition of Th-2 differentiation, IL-4 production, airway hyperresponsiveness and eosinophilic airway inflammation (Okamoto et al., 2009). Importantly, like other key growth factors and signaling pathways, appropriate Notch level is needed for normal airway development and homeostasis. Non-specific inhibition of Notch pathway via γ-secretase inhibitors may cause many unpredictable negative effects. Therefore, more specific γ-secretase inhibitors for a bench-to-bedside therapeutic application are needed to develop.

**Targeting Notch signaling other than γ-secretase inhibitors**

Fringe (fucose-β1, 3-N-acetyl glucosaminyl transferases) regulates the Notch signaling via ligand-receptor binding while Notch signaling regulates the development and differentiation of T helper cells. An OVA-sensitized asthmatic rat model has shown significantly low expression of both manic fringe (mfng) and lunatic fringe (lfng) as compared to radical fringe (rfng) (Gu et al.,
Over-expression of \textit{lfng} in CD4$^+$ T cells in asthmatic rats, using \textit{lfng} plasmid, inhibited the Th-2 cytokine production via Notch-dependent manner (Gu et al., 2012); suggesting that overexpression of \textit{lfng} may be helpful to treat Th-2 cytokine-associated asthma. In contrast, an increased expression of \textit{lfng} has been reported in respiratory syncytial virus (RSV)-induced asthma models. Accordingly, STAT-5-dependent \textit{lfng} expression in Th-2 development augmented the Dll4-Notch-mediated IL-4 release during RSV-induced allergic asthma while genetic deletion of \textit{lfng} inhibited the Dll4-mediated Notch activation, Th-2 cytokines production and IL-4 release (Mukherjee et al., 2014). These studies suggest that fringe could be a new curative approach for the impediment of allergic asthma and airway remodeling, so cannot be discounted. Thereby, more studies are needed to elucidate the exact role of fringe in the pathogenesis of asthma and to explore whether or not the interference of fringe is a feasible treatment.

Dll-4 expressed on dendritic cells, influence the differentiation of T helper cells toward Th-1 and Th-2 or Treg (Amsen et al., 2009). Dll-4 neutralization exacerbated the IL-5 and IL-13 production, Th-2 cytokine production, and airway hyperresponsiveness during RSV infection (Jang et al., 2010; Schaller et al., 2007; Ting et al., 2017) while Dll-4 activation during differentiation sustained Treg cell phenotype and function to control RSV infection (Ting et al., 2017). Moreover, intravenous administration of OVA-pulsed plus Dll4-pretreated DCs in Th-2 dominant asthmatic murine models notably increased the production of IL-10, reduced the production of pro-inflammatory cytokines, decreased the severity of airway hyperresponsiveness and alleviated the expression levels of OVA-specific IgE; indicating that Dll-4 altered the DCs activation (Huang et al., 2013). In contrast, \textit{in vivo} neutralization of Dll-4 remarkably decreased the Th-2 cytokines production, mucus production, and airway hyperreactivity whereas Dll4-
mediated Notch activation during viral exacerbation further augmented the Ifng-dependent Th-2 cell activation (Mukherjee et al., 2014). It would be reasonable to hypothesize that allergen-induced Th-2 inflammatory surroundings are utilized by virus to further intensify the allergic circumstance most probably due to RSV-induced pro-Th-2 factors (IL-25) (Kaiko et al., 2010), and thymus and activation-regulated chemokine (TARC) (Monick et al., 2007) that ultimately amplify the development and recruitment of Dll4-mediated Th-2 cytokine in the lung. Hence, these observations suggest that Dll-4 could be an effective therapeutic target for modulating airway hyperresponsiveness and chronic asthma but it is difficult to justify the exact role of Dll-4-mediated Notch signaling due to either complexity of Notch receptor-ligand interactions or participation of non-canonical Notch pathways. Nonetheless, continuous attention is required to explore the exact role of Dll4-mediated Notch signaling to treat the asthmatic airway remodeling.

Natural regulatory T-cells (nTreg; CD4^+ CD25^+) or induced regulatory T Cells (iTreg) can effectively attenuate the features of allergic airway inflammation and inhibit the development of airway remodeling (Kearley et al., 2008; Lan et al., 2012) while Notch/Jagged controlled the differentiation and expansion of Treg cells (Hoyne et al., 2000); particularly constitutive Notch-3 activation enhanced the Treg cells generation (Anastasi et al., 2003). Adoptive transfer of iTreg (Xu et al., 2012) and Dll4-expressing Treg (Huang et al., 2009) to OVA-sensitized chronic asthma mouse model effectively alleviated the airway remodeling. Moreover, administration of Dll4-expressing antigen-presenting cells in OVA-induced murine asthma model ameliorated allergic asthma via induction of Treg-mediated regulatory pathway whereas blockage of Dll-4 impaired the Treg differentiation, and resulted in exaggerated asthma phenotypes (Huang et al., 2017). Hence, Treg needed to be further investigated in established airway remodeling for the important therapeutic approach.
The pseudostratified airway epithelium is layered by the balanced proportion of secretory and ciliated cells, accompanied by undifferentiated basal cells, but differentiated to basal stem cells (goblet cell hyperplasia) during asthma. Adult trachea study has shown that persistent activation of Notch impeded the ciliogenesis and endorsed the luminal differentiation of the basal cell into secretory cells (Rock et al., 2011). Consistent with this, STAT-3 activated by IL-6, produced in response to injury, promoted multi-ciliogenesis and regeneration of airway multiciliated cells from basal cells through upregulation of ciliogenesis genes, such as Foxj1 and Mcidas, and down-regulation of Notch-1 (Tadokoro et al., 2014). Interestingly, signaling pathway between Toll-like receptor 4 (TLR-4) and Notch-1 might coordinate with STAT-3 because expression levels of TLR-4 and Notch-1, and phosphorylation of STAT-3 in asthma models was increased while decreased after Cornuside, natural secoiridoid glucoside, treatment (Zheng, 2016). Hence, modulation of ciliogenesis by IL-6/STAT-3, via direct regulation the Notch-1, might prove helpful to prevent/treat the asthmatic airway remodeling.

RBP-jκ along another transcriptional complex (NICD-CBF-1/RBP-jκ-MAML) induces the transcription of target genes whereas targeting/inactivation of RBP-jκ inhibits the RBP-jκ-mediated transcription and Notch pathway. KyoT2, the negative regulator of Notch pathway, inhibits RBP-jκ mediated transcriptional activation (Collins et al., 2014). Hu and colleagues showed that KyoT2 downregulated asthmatic airway remodeling by alleviating the subepithelial fibrosis and airway hyperresponsiveness through the HES1-dependent mechanism as well as improved the lung function (Hu et al., 2015); suggesting that targeting/inactivation of RBP-jκ might act as a potential clinical treatment strategy for asthmatic airway remodeling in future.

The epigenetic study revealed that asthma is linked with epigenetic changes of the Notch-1 promoters, such as abnormal histone acetylation and methylation (Cui et al., 2013). Cui and
colleagues investigated the histone modification of asthmatic lung CD4+ T cells and revealed that histone acetylation and trimethylation levels of Notch-I gene promoter, P300, and pCAF activities were remarkably elevated as compared to control. While histone acetyltransferases (HATs) activity, expression level Notch-I and HES-I, and asthmatic parameters such as IL-4, IL-5, and IL-13 were significantly decreased after the intervention of asthmatic lung CD4+ T cells with garcinol, a potent natural inhibitor of HATs, p300, and pCAF (Cui et al., 2013); suggesting that targeting histone acetylation might be a new therapeutic strategy for the appropriate regulation of Notch signaling pathway and for the treatment of asthmatic airway remodeling.

In summary, our ever-increasing understanding of Notch signaling pathway offers great hope that specific targeting Notch signaling may represent a promising alternative complementary therapeutic strategy to treat asthmatic airway remodeling in future.

Side effects associated with Notch pathway inhibition:

Although preclinical studies, on Alzheimer's disease but not on asthmatic airway remodeling, have shown that long-term interference of Notch signaling by γ-secretase inhibitors led to severe gastrointestinal toxicity, such as vomiting, nausea, diarrhea, decreased appetite, and inflammation due to full inhibition of Notch pathway (Hsu et al., 2013). Importantly, Notch drives gastrointestinal precursor cells toward an epithelial fate and away from a secretory cell fate while Notch inhibition results in an imbalance of this process and leads to secretory goblet cells hyperplasia (van Es et al., 2015). Additionally, inhibition of Notch pathway leads to skin cancer (Demehri et al., 2009) and disruption of the immune system (Wong et al., 2004). Consequently, a dose-dependent effect of LY-411575 (γ-secretase inhibitor) on the intestine, spleen, and thymus of mice has been reported (Wong et al., 2004) because LY-411575 at high
and low dose concentrations attenuated and enhanced the β-amyloid respectively by modulating γ-secretase complex at different binding sites. These toxicities might be challenging for any Notch inhibitor. Therefore, some γ-secretase modulators sparing the Notch cleavage have been developed (Chávez-Gutiérrez et al., 2012). Further, NSAIDs as therapeutic agents for reducing inflammation by targeting γ-secretase are under research (Saura, 2010). In order to minimize these side effects, two possible strategies have been proposed: the co-administration of γ-secretase inhibitors with glucocorticoids such as dexamethasone (Real et al., 2009) and recurrent dosing plans of γ-secretase inhibitors (Purow, 2009). Both strategies have been shown to mainly spare the gut toxicity while maintaining the desired effect.

**Concluding remarks**

Although major research has been conducted to understand the role of Notch signaling in malignancies and fibroproliferative disorders but clinical data to support the role of Notch signaling in asthmatic subjects are yet lacking. Evidence obtained from animal models support the crucial role of Notch signaling in embryonic lung development, initiation and progression of asthmatic airway remodeling, and linking impaired fetal lung development either with asthmatic airway remodeling directly or through other pathways. Moreover, currently identified Notch targets can be counted on a few fingers, but even in those cases, we have little knowledge. γ-secretase inhibitors and other described approaches might be effective to overcome both aberrant expression and overproduction of Notch signaling, but are associated with unwanted effects due to pleiotropic nature of Notch signaling; therefore further detailed Genome-wide association studies are needed to develop more specific γ-secretase inhibitors and modulators that could easily differentiate between wellness and illness. In this regard, better understanding the communication of canonical and non-canonical Notch signaling, considering how and why
different target genes are activated according to cell type and time, exploration of Notch signaling cross-talk with other pathways, and apposite targeting of Notch signaling will open new therapeutic era. In particular, future studies aimed at delineating the precise cellular and molecular mechanisms underpinning Notch-mediated asthmatic airway remodeling suppression will be important for developing potential therapeutic strategies.
Acknowledgments

We gratefully acknowledge Higher Education Commission (HEC), Pakistan (Ref: OS-II/Batch-6(China)/PM/2015)

Author’s contributions

Participated in conception and design of manuscript: Hussain, Xu, and Ximei Wu.

Contributed to the writing of the manuscript: Hussain, Xu, and Ximei Wu.

Tables and figures preparation: Ahmad, Lu, and Xiling Wu.

Supervision of drafted manuscript: Yang, Tang, and Ximei Wu.

Declarations of interest

All authors have no conflicts of interest.
References:


MOL #110254


MOL #110254


MOL #110254


MOL #110254


MOL #110254


MOL #110254


Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proc Natl
Acad Sci* **106**: 12771-12775.

Rogers NK, Clements D, Harrison TW, Shaw D and Johnson SR (2012) S11 Expression of
Tenascin-C Regulates Airway Smooth Muscle Derived Matrix Metalloproteinase-1 in

Activation of NOTCH signaling by tenasin-C promotes growth of human brain tumor-


Schaller MA, Neupane R, Rudd BD, Kunkel SL, Kallal LE, Lincoln P, Lowe JB, Man Y and
Lukacs NW (2007) Notch ligand Delta-like 4 regulates disease pathogenesis during


cell differentiation in cell lines and in transgenic mice. *Am J Physiol Lung Cell Mol

Coexistence of Th1/Th2 and Th17/Treg imbalances in patients with allergic asthma. *Chin


MOL #110254


Footnotes:

This work was supported by National Natural Science Foundation of China [81170016, 81170787, 81200022, 81200023, 81270067, 31571493, 81571928]; and Natural Science Foundation of Zhejiang Province [LY13H150002, LY12H16005].
Figure Legends:

Figure 1. Notch signaling and lung developmental stages in humans and mice

During embryonic stage (ED ~8.0-9.5 in mouse, ~3-7 weeks in human), lung development is initiated by the emergence of lung buds from foregut endoderm for the onset of lung specification. In the pseudoglandular stage (ED 9.5-16.5 in mouse, 7-17 weeks in human), numerous Notch ligands, receptors, and extracellular modulators are expressed within proximal-distal airways and surrounding mesenchyme that coordinate the proximal-distal patterning of branching morphogenesis. Most mesenchymal and epithelial cells start to form during this stage.

In the canalicular stage (ED 16.5-17.5 in mouse, 17-27 weeks in human); Notch signaling regulates the balance of proliferation and differentiation of the distal airway epithelium and results in the appearance of AECI/II and formation of blood capillary. In the saccular stage to alveolar stage (ED 17.5 to postnatal day 5 in mouse, 27-36 weeks in human), Notch signaling may organize the vascularization and alveolarization (sac-like structures), which is accompanied by the enlargement of lymphatic and capillary networks, and production of surfactant. In mouse, the alveolar stage starts postnatally (P5 to ~P30) whereas, in humans, it starts in utero (36 weeks to ~7-10 years).

Abbreviations: ED, embryonic days; p, postnatal days; ECM, extracellular matrix; ( → ) Arrows are representing the expression of Notch and its components at different stages of lung development.

Figure 2. Notch signaling (canonical and non-canonical), Notch receptor and Notch ligand

Notch receptors and ligands are single-pass transmembrane receptors. Notch receptors contain 29–36 EGF-like repeats, followed by three LNRs while all Notch ligands, except for Dll-3, contain variable numbers of EGF-like repeats, followed by DOS domain and DSL domains. The
newly translated Notch receptor protein is glycosylated to mature receptor after proteolytic cleavage by furin at site 1 (S1) and then targeted to the cell surface as a heterodimer. Membrane-bound Notch receptor is activated by binding with ligand on a neighboring cell, which results in S2 cleavage, at site 2 to generate the membrane-anchored NEXT fragment, by ADAM; followed by S3/S4 cleavage, at site 3 (S3) to site 4 (S4) to release stable form of NICD and Nβ Peptide with γ-secretase. The NICD contains the RAM domain, two nuclear localization signals, six ANK repeats and a PEST sequence that regulates NICD degradation. Released NICD then translocates to the nucleus where it associates with the DNA-binding protein CSL. In the absence of NICD, CSL may associate with ubiquitous co-repressor (Co-R) proteins and histone deacetylases to repress transcription. In the presence of NICD, allosteric changes may occur in CSL which displaces the transcriptional repressors then MAML recognizes the NICD/CSL interface to recruits additional co-activators (Co-A) for the transcription. Non-canonical Notch signaling may occur via the membrane-bound, uncleaved Notch receptor or via the NICD. Non-canonical Notch signaling is independent of CSL and allows for interaction with PI3K/AKT/mTORC2, Wnt/β-catenin, IKKα/β, NFκB, YY1, and HIF1α pathways at the cytoplasmic and/or nuclear level.

**Abbreviations:** NICD, Notch intracellular domain; NECD, Notch extracellular domain; EDF, epidermal growth factor; LNR, Lin-12-Notch repeats; ADAM, a disintegrin and metalloprotease; RAM, RBP-jκ-associated molecule; HD, heterodimerization domain; TMD, transmembrane domain; ANK, ankyrin repeats; TAD, transactivation domain; PEST, proline-glutamic acid-serine-threonine-rich motifs; DSL, Delta-Serrate-LAG-2; DOS, Delta and OSM-11-like proteins; vWF, von Willebrand Factor; MNNL, N-terminal domain of Notch ligands; ADAM, a
disintegrin and metalloprotease; NEXT, Notch extracellular truncation; CSL, CBF1/RBPjκ/Su(H)/Lag-1; HIF-1α, hypoxia-inducible factor-1α; MAML, mastermind-like.

**Figure 3. Therapeutically effective components of Notch pathway to treat asthmatic airway remodeling**

There are several therapeutically effective nodes in the Notch signaling pathway but some are putative targets for new drug intervention. Cleavage by γ-secretase (a), ADAM secretase (b) and furin (c) can be targeted pharmacologically and are under preclinical studies to treat asthmatic airway remodeling. Interfering the interaction between NICD and MAML (d) or CSL (or RBPjκ) (e) nullify Notch signaling. Interfering with fucosylation, glycosylation (f) or other EGF-specific modifications (g) by inhibiting fringe activity may modulate specific Notch pathways. Similarly, Notch antibodies targeting Notch ligands (h) or receptors (i) would exclusively target individual receptor pathways. Finally, Notch ligand fusion peptides (j) can be used as an effective target.

**Abbreviations:** NECD, Notch extracellular domain; NICD, Notch intracellular domain; ADAM, a disintegrin and metalloprotease; dnMAML, dominant-negative MAML; MAML, Mastermind-like; CSL, CBF1/RBPjκ/Su(H)/Lag-1
Table 1: Notch pathway components in vertebrates and mammals

<table>
<thead>
<tr>
<th>Component</th>
<th>Types</th>
<th>Vertebrates and mammals</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptor</td>
<td>Notch-1, Notch -2, Notch -3, Notch -4</td>
<td>(Guruharsha et al., 2012; Kopan and Ilagan, 2009; Koval et al., 2017)</td>
<td></td>
</tr>
<tr>
<td>Ligand</td>
<td>DSL/DOS</td>
<td>Dll-1, Jagged-1 and Jagged-2</td>
<td>(Kopan and Ilagan, 2009; Koval et al., 2017)</td>
</tr>
<tr>
<td></td>
<td>DSL only</td>
<td>Dll-3 and Dll-4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DOS Coligands</td>
<td>DLK-1, DLK-2/EGFL9</td>
<td></td>
</tr>
<tr>
<td>Noncanonical</td>
<td>DNER, NB 3/Contactin6, F3/Contactin1, MAGP-1 and MAGP-2, (Andersen et al., 2012)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receptor proteolysis</td>
<td>Furin convertase (site 1 cleavage)</td>
<td>Furin, PC5/6</td>
<td>(Logeat et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Metalloprotease (site 2 cleavage)</td>
<td>ADAM-17/TACE, ADAM-10/Kuzbanian</td>
<td>(Kopan and Ilagan, 2009; Weinmaster and Fischer, 2011)</td>
</tr>
<tr>
<td></td>
<td>γ-secretase (site 3/site 4 cleavage)</td>
<td>PEN-2, Nicastrin, APH-1a-c, PSEN-1 and -2</td>
<td>(Bray, 2006; Jorissen and De Strooper, 2010)</td>
</tr>
<tr>
<td>Glycosyltransferase modifiers</td>
<td>O-fucosyl-transferase</td>
<td>Pofut-1</td>
<td>(Bruckner et al., 2000; Kovall et al., 2017)</td>
</tr>
<tr>
<td></td>
<td>β1,3-GlcNAc-transferase</td>
<td>Lunatic, Manic, and Radical Fringe</td>
<td></td>
</tr>
<tr>
<td><strong>Endosomal sorting/membrane trafficking regulators</strong></td>
<td><strong>Ring Finger E3 Ubiquitin ligase</strong>&lt;br&gt;(ligand endocytosis)</td>
<td><strong>Skeletrophin, Mindbomb, Neuralized 1-2</strong>&lt;br&gt;(Okajima et al., 2003)</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Ring Finger E3 Ubiquitin ligase</strong>&lt;br&gt;(receptor endocytosis)</td>
<td><strong>Deltex-1, Deltex-2, Deltex-3, Deltex-4</strong>&lt;br&gt;(Bray, 2006; Guo et al., 2016)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>HECT Domain E3 Ubiquitin ligase</strong>&lt;br&gt;(receptor endocytosis)</td>
<td><strong>Itch/AIP4, Nedd4</strong>&lt;br&gt;(Chapman et al., 2006)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Negative regulator</strong></td>
<td><strong>Numb, Numb-like, ACBD3</strong>&lt;br&gt;(Chapman et al., 2006)</td>
<td></td>
</tr>
<tr>
<td><strong>NICD degradation</strong></td>
<td><strong>F-Box Ubiquitin ligase</strong></td>
<td><strong>Fbw-7/SEL-10</strong>&lt;br&gt;(Kopan and Ilagan, 2009)</td>
<td></td>
</tr>
<tr>
<td><strong>Nuclear effectors</strong></td>
<td><strong>CSL DNA-binding transcription factor</strong></td>
<td><strong>RBP-jκ/CBF-1</strong>&lt;br&gt;(Bigas et al., 2013; Kovall et al., 2017)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Transcriptional Coactivator</strong></td>
<td><strong>MAML-1, MAML-2, MAML-3</strong>&lt;br&gt;(Mukherjee et al., 2014)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Transcriptional Corepressors</strong></td>
<td><strong>KyoT2, NCoR/SMRT,</strong>&lt;br&gt;<strong>Mint/SHARP/SPEN</strong>&lt;br&gt;(Chen et al., 1997; Ostroukhova et al., 2006)</td>
<td></td>
</tr>
<tr>
<td><strong>Canonical target</strong></td>
<td><strong>bHLH</strong></td>
<td><strong>HES/ESR/HEY</strong>&lt;br&gt;(Chen and Evans, 1995; Rana and Haltiwanger, 2007)</td>
<td></td>
</tr>
</tbody>
</table>
Repressor genes

Abbreviations: NICD, Notch intracellular domain; DSL, Delta/Serrate/LAG-2 motif; DOS, Delta and OSM-IL-like proteins motif; AP2, adaptor protein-2; E3, ubiquitin ligase; bHLH, basic-helix–loop–helix; CSL, CBF1/RBP-jκ/Su(H)/Lag-1.
Table 2: Various proteins interacting with NICD

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Protein</th>
<th>interface with NICD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Regulation of NICD by phosphorylation</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>GSK3β</strong></td>
</tr>
<tr>
<td></td>
<td>Glycogen synthase kinase 3</td>
<td>Phosphorylates Notch, which can lead to degradation or stabilization</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>CycC</strong></td>
</tr>
<tr>
<td></td>
<td>Cyclin C</td>
<td>Target NICD along with CDk8 for phosphorylation that subsequently acts as substrate for ubiquitylation and degradation</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>CDK8</strong></td>
</tr>
<tr>
<td></td>
<td>Cyclin-dependent kinase 8</td>
<td>Phosphorylates NICD with CycC that subsequently acts as substrate for ubiquitylation and degradation</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>G-CSF</strong></td>
</tr>
<tr>
<td></td>
<td>Granulocyte colony-stimulating factor</td>
<td>Phosphorylation of NICD that subsequently leads to its inactivation</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Regulation of NICD by ubiquitylation</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Fbxw7/Cdc4</strong></td>
</tr>
<tr>
<td></td>
<td>F-box/WD-repeat protein 7</td>
<td>Ubiquitylates the NICD and leading to its degradation</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Numb</strong></td>
</tr>
<tr>
<td></td>
<td>Numb homolog</td>
<td>Suppresses Notch signaling by recruiting E3 ubiquitin ligases to ubiquitylate Notch</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Dtx1-4</strong></td>
</tr>
<tr>
<td></td>
<td>Deltex 1-4</td>
<td>Controls Notch ubiquitylation, processing, and internalization</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Itch</strong></td>
</tr>
<tr>
<td></td>
<td>Itchy, E3 ubiquitin protein ligase</td>
<td>Promotes ubiquitylation of NICD</td>
</tr>
</tbody>
</table>
### Regulation of NICD by hydroxylation

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIH</td>
<td>Factor inhibiting HIF1α</td>
<td>Hydroxylates the Notch and represses Notch HIF1α</td>
</tr>
<tr>
<td>HIF1α</td>
<td>Hypoxia-inducible factor 1, alpha subunit</td>
<td>Stabilizes the NICD and synergizes with it in transcription of Notch target genes</td>
</tr>
</tbody>
</table>

### Miscellaneous proteins interacting with NICD

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axin</td>
<td>Axin</td>
<td>Synergies with NICD to regulate the stability of β-catenin and controls the trafficking of NICD in association with adenomatous polyposis coli</td>
</tr>
<tr>
<td>Apc</td>
<td>Adenomatous polyposis coli</td>
<td>Control the trafficking of Notch</td>
</tr>
<tr>
<td>CSL/RBP-jκ</td>
<td>CBF1, Su(H) and LAG-1/RBP-jκ</td>
<td>Key canonical transcriptional co-factor for NICD</td>
</tr>
<tr>
<td>Ctnnb1</td>
<td>β-catenin</td>
<td>Synergies with NICD/CSL on Notch target gene</td>
</tr>
<tr>
<td>Dab</td>
<td>Disabeled</td>
<td>Acts as link to Abelson tyrosine kinase (Abl) protein in non-canonical Notch axon guidance</td>
</tr>
<tr>
<td>Dvl/Dsh</td>
<td>Dishevelled</td>
<td>Dishevelled regulates ligand-dependent Notch trafficking and inhibits canonical Notch signaling</td>
</tr>
<tr>
<td>MAML-1/2</td>
<td>Master mind like-1/2</td>
<td>Co-activator of NICD/CSL</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor of kappa light</td>
<td>NICD blocks NF-κB transcription of NF-κB target genes through binding to</td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Function/Effect</td>
</tr>
<tr>
<td>-------</td>
<td>------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>polypeptide gene enhancer in B-cells</td>
<td>p50/cRel</td>
<td></td>
</tr>
<tr>
<td>Nrarp</td>
<td>Notch-regulated ankyrin repeat protein</td>
<td>Nrarp binds and inhibits the NICD/CSL</td>
</tr>
<tr>
<td>p73α (TA)</td>
<td>Tumor protein p73 alpha (transactivating form)</td>
<td>Binds NICD and inhibits the NICD/CSL-mediated transcription</td>
</tr>
<tr>
<td>RITA/C12OR F52</td>
<td>RBP-jκ interacting and tubulin associated</td>
<td>Shuttles NICD between the nucleus and cytoplasm on tubulin networks</td>
</tr>
<tr>
<td>SMAD</td>
<td>Smad family members</td>
<td>Smads augment Notch signaling, Notch fine-tunes signaling via Smads</td>
</tr>
<tr>
<td>SNW1/SKIP/NCOA-62</td>
<td>SNW domain-containing protein 1/Ski-interacting protein/Nuclear receptor co-activator NCoA-62</td>
<td>Can bind both NICD and co-repressor SMRT, but these are mutually exclusive; forms multimers with NICD and MAML, which then associates with CSL to activate transcription</td>
</tr>
<tr>
<td>Tacc3</td>
<td>Transforming, acidic coiled-coil containing protein 3</td>
<td>Binds NICD and blocks transcription from Notch target promoters; can be reversed by over-expression of CSL</td>
</tr>
<tr>
<td>Trio</td>
<td>Triple functional domain (PTPRF interacting)</td>
<td>A guanine nucleotide exchange factor (GEF) for Rho GTPases that acts as link to Abelson tyrosine kinase (Abl) proteins in non-canonical Notch axon guidance</td>
</tr>
</tbody>
</table>
Abbreviations: NICD, Notch intracellular domain; E3, ubiquitin ligase; SMRT, a silencing mediator for retinoid or thyroid hormone receptors; CSL, CBF1/RBP-jκ/Su(H)/Lag-1
Table 3: Notch pathway gene knockout and lung phenotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Lung phenotype</th>
<th>Remarks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch-1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Developmental retardation, and widespread cellular death</td>
<td>Embryonic lethality at ED 10</td>
<td>(Conlon et al., 1995; Swiatek et al., 1994)</td>
</tr>
<tr>
<td>N1ICD&lt;sup&gt;Δ/Δ&lt;/sup&gt;</td>
<td>Alveolar hyperplasia and apoptosis</td>
<td>Viable</td>
<td>(Allen et al., 2011)</td>
</tr>
<tr>
<td>Notch-2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Developmental retardation, and widespread cellular death</td>
<td>Embryonic lethality at ED 11.5</td>
<td>(Hamada et al., 1999)</td>
</tr>
<tr>
<td>Notch-2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Abnormal enlargement of alveolar spaces, inhibition of Type II cells activation, decreased PDGF-A expression, and disturbance of integrity of bronchial and epithelial smooth muscle layer of distal airways</td>
<td>Viable (if stimulated by Notch-2 overexpression).</td>
<td>(Tsao et al., 2016)</td>
</tr>
<tr>
<td>Notch-3&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>No arterial defects</td>
<td>viable</td>
<td>(Domenga et al., 2004; Krebs et al., 2000)</td>
</tr>
<tr>
<td>Notch-3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Apparently normal lung morphology with aberrant maturation of vascular smooth muscle cells (arterial</td>
<td>Viable and fertile</td>
<td>(Domenga et al., 2004; Krebs et al., 2000; Li et al., 2009)</td>
</tr>
<tr>
<td>Genotype</td>
<td>Phenotype</td>
<td>Outcome</td>
<td>Reference</td>
</tr>
<tr>
<td>----------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>--------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Notch-3(^{+/\Lambda})</td>
<td>Dramatic abnormalities of lung morphogenesis, delayed development of type II pneumocyte and inhibition of type I pneumocyte differentiation, ductal formation and dilated sacculi</td>
<td>Perinatal lethality</td>
<td>(Dang et al., 2003)</td>
</tr>
<tr>
<td>Notch-2(^{+/\Lambda}) + Notch-3(^{-})</td>
<td>Alveolar septation failure, defective alveolar development, and flawed differentiation and recruitment of myofibroblast cells</td>
<td>Perinatal lethality</td>
<td>(Xu et al., 2010)</td>
</tr>
<tr>
<td>Notch-4(^{-})</td>
<td>No obvious mutant phenotype.</td>
<td>Fertile and viable</td>
<td>(Krebs et al., 2000)</td>
</tr>
<tr>
<td>Notch-1(^{+/\Lambda}) + Notch-4(^{-})</td>
<td>Vascular remodeling defects</td>
<td>Embryonic lethality</td>
<td>(Roca and Adams, 2007)</td>
</tr>
<tr>
<td>Notch-4(^{+/\Lambda})</td>
<td>Vascular remodeling defects, arteriovenous shunting, lung hemorrhages, and respiratory insufficiency</td>
<td>Viable but lethal around 6-7 weeks</td>
<td>(Miniati et al., 2010)</td>
</tr>
<tr>
<td>Pofut-1(^{-})</td>
<td>Complete ablation of Club cell secretory lineage, airways overpopulated with ciliated cells, goblet cell</td>
<td>Neonatal lethality</td>
<td>(Tsao et al., 2009; Tsao et al., 2011)</td>
</tr>
<tr>
<td>Trait</td>
<td>Description</td>
<td>Outcome</td>
<td>References</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>--------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td><strong>metaplasia and attenuation of epithelium in airway and bronchioles</strong></td>
<td>RBP-jk&lt;sup&gt;-/-&lt;/sup&gt; Remarkably similar phenotype characterized by Pofut-1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Neonatal lethality</td>
<td>(Tsao et al., 2009; Tsao et al., 2011)</td>
</tr>
<tr>
<td><strong>Epithelium lacking Club, ciliated and goblet cells in conducting airway epithelium</strong></td>
<td>Sox-2&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>Viable</td>
<td>(Tompkins et al., 2009)</td>
</tr>
<tr>
<td><strong>Delayed-type I pneumocyte differentiation and defective alveolar septation and elastogenesis</strong></td>
<td>lfn&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Viable</td>
<td>(Xu et al., 2010)</td>
</tr>
<tr>
<td><strong>No obvious mutant phenotype</strong></td>
<td>lfn&lt;sup&gt;−/−&lt;/sup&gt; in distal lung epithelium</td>
<td>Viable</td>
<td>(van Tuyl et al., 2005)</td>
</tr>
<tr>
<td><strong>Severe fusions of right lung lobes with pulmonary hemorrhage, severe defects in peripheral lung saccules development, and abnormal microvasculature development</strong></td>
<td>Low Foxf-1&lt;sup&gt;+/−&lt;/sup&gt; (diminished Notch-2)</td>
<td>Postnatal lethality</td>
<td>(Kalinichenko et al., 2004)</td>
</tr>
<tr>
<td><strong>Normal microvascular development</strong></td>
<td>High Foxf-1&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>viable</td>
<td>(Kalinichenko et al., 2004)</td>
</tr>
<tr>
<td>Gene</td>
<td>Phenotype</td>
<td>Lethality</td>
<td>Reference</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Foxf-1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Defects in extraembryonic mesoderm development</td>
<td>Embryonic lethality at ED 8</td>
<td>(Kalinichenko et al., 2001)</td>
</tr>
<tr>
<td>HES-1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Hypoplastic lungs with attenuated Club cells count and Notch-1 mRNA, and increased count of PNEC and Mash-1 mRNA</td>
<td>Peri- or postnatal lethality</td>
<td>(Ishibashi et al., 1995; Ito et al., 2000; Tsao et al., 2009)</td>
</tr>
<tr>
<td>Mash-1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Hypoplastic lungs with disappeared Dll-1 mRNA and decreased count of PNEC</td>
<td>Perinatal lethality due to hypoventilation</td>
<td>(Guillemot et al., 1993; Ito et al., 2000)</td>
</tr>
<tr>
<td>Jag-1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Hemorrhage during early embryogenesis</td>
<td>Embryonic lethality at ED 10</td>
<td>(Xue et al., 1999)</td>
</tr>
<tr>
<td>Jag-1&lt;sup&gt;−/−&lt;/sup&gt; in airway epithelium</td>
<td>Flawed alveologenesis, goblet cell metaplasia and aberrant cell fate specification with excess ciliated cells at the expense of Club cells same as HES-1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>viable</td>
<td>(Zhang et al., 2013a)</td>
</tr>
<tr>
<td>Jag-2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Impaired differentiation</td>
<td>Perinatal lethality</td>
<td>(Xue et al., 1999)</td>
</tr>
<tr>
<td>Dll-1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Patterning defects</td>
<td>Embryonic lethality at ED 12</td>
<td>(de Angelis et al., 1997)</td>
</tr>
<tr>
<td>Genotype</td>
<td>Phenotype</td>
<td>Adult Phenotype</td>
<td>Reference</td>
</tr>
<tr>
<td>------------</td>
<td>----------------------------------</td>
<td>-----------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Dll-4&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Vascular remodeling defects</td>
<td>Embryonic lethality at ED 10.5</td>
<td>(Gale et al., 2004)</td>
</tr>
<tr>
<td>PSEN -2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Vascular remodeling defects</td>
<td>Embryonic lethality</td>
<td>(Herreman et al., 1999)</td>
</tr>
<tr>
<td>HERP-1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Vascular remodeling defects</td>
<td>Embryonic lethality</td>
<td>(Doi et al., 2005)</td>
</tr>
</tbody>
</table>

Abbreviations: ED, embryonic day; +/-, deletion of single allele (heterozygous); -/-, Null allele (homozygous); Δ/Δ, over-expression of specific gene
Figure 1

Embryonic period → Fetal Period → Birth and postnatal growth

Weeks ~ 3-7 → Weeks 7-17 → Weeks 17-27 → Weeks 27-36 → Weeks 36-~7-10 years

Embryonic
- Lung bud formation
- Trachea and bronchi differentiation

Pseudoglandular
- Formation of conducting airways
- Terminal bronchioles
- Immature neural network
- Appearance of Type-II pneumocytes

Canalicular
- Lung periphery formation
- Increased vascularization
- Appearance of Type-I pneumocytes
- Formation of air-blood interface

Saccular
- Alveolar sacculae formation
- Surfactant detectable in amino fluid
- ECM formation

Alveolar
- Mature alveoli formation
- Proliferation and expansion of capillaries, nerve and gas exchange areas
- ECM formation

DB-1 → Hey-1 → Hey-5 → Jag-1
Proximal airways
- Notch-1
- Notch-3
- HES-1

Jag-1 → Jag-2 → HES-1
Distal tips
- DB-1
- Mash-1

Mesenchyme
- Notch-2
- Notch-3
- Jag-1
- Jag-2

Endothelial cells
- Notch-3
- Notch-4
- Jag-1
- DB-1

Mesoderm
- DB-1
Figure 2
Figure 3