Cell. Mol. Life Sci. (2011) 68:2555–2567
DOI 10.1007/s00018-011-0695-5

Cellular and Molecular Life Sciences

REVIEW

The multiple roles of Notch signaling during left-right patterning

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Received: 31 December 2010 / Revised: 28 March 2011 / Accepted: 19 April 2011 / Published online: 5 May 2011
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Abstract The establishment of left-right (LR) asymmetry is regulated by intricate signaling mechanisms during embryogenesis and this asymmetry is critical for morphogenesis as well as the positioning of internal organs within the organism. Recent progress including elucidation of ion transporters, leftward nodal flow, and regulation of asymmetric gene expression contributes to our understanding of how the breaking of the symmetry is initiated and how this laterality information is subsequently transmitted to the organ primordium. A number of developmental signaling pathways have been implicated in this complex process. In this review, we will focus on the roles of the Notch signaling pathway during development of LR asymmetry. The Notch signaling pathway is a short-range communication system between neighboring cells. While Notch signaling plays essential roles in regulating the morphogenesis of the node and left-specific expression of *Nodal* in the lateral plate mesoderm, a hallmark gene in LR patterning, Notch signaling also suppresses the expression of *Pitx2* that is a direct downstream target of Nodal during later stages of development. This negative activity of Notch signaling towards left-specific activity was recently shown to be inhibited by the B cell lymphoma 6 (BCL6)/BCL6 co-repressor (BcoR) transcriptional repressor complex in a target-specific manner. The complex regulation of Notch-dependent gene expression for LR asymmetry will be highlighted in this review.

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Keywords Left-right asymmetry · Notch signaling pathway · BCL6 · BcoR · Nodal · Pitx2

Introduction

Although the external appearance of vertebrate body is bilaterally symmetric, left-right (LR) asymmetry is observed in both the structure and placement of internal organs as well as the organization of the circulatory system. The left side contains most of the heart and the spleen, while most of the liver and the gall bladder reside on the right side. This consistent asymmetry in the position of the body organs is a common feature of all vertebrates [1–3]. If establishment of LR asymmetry is disrupted, this leads to disorders of laterality including situs inversus and heterotaxy [4–7]. In particular, disorganization of the heart atria and ventricles by defective LR asymmetric establishment results in severe congenital heart diseases.

LR patterning is likely oriented according to the predetermined anterior-posterior (AP) and dorsal-ventral (DV) axes during early development [8, 9]. Brown and Wolpert proposed a chiral “F-molecule” that transports intracellular constituents to create an intracellular gradient when aligned with the AP and DV axes [8]. Aligning such a molecule with respect to the AP and DV axes allows the cell containing it to determine which direction is left or right. This laterality information appears to be translated into the posterior tilt of cilia (see below) in the main LR coordinator termed the node [also referred to as the posterior notochord (PNC)] [10, 11]. AP and DV axes are represented by the posterior tilt of the cilia. These posteriorly tilted nodal cilia produce the leftward flow of extracellular fluid across the LR coordinator in mice, zebrafish, and

Xenopus while no flow in chick has been reported thus far [12]. The left-specific activity produced by the leftward nodal flow is then transferred to the left lateral plate mesoderm (LPM), and the present midline structures form a physical barrier to prevent this left-specific activity from diffusing to the right side and vice versa. The laterality information is finally delivered to multiple developing internal organs. During these steps, asymmetrically expressed genes, including *Nodal*, *Lefty* and *Pitx2*, play essential roles for the maintenance of LR asymmetry as well as the determination of internal organ positioning. A dizzying number of intercellular signaling pathways including Notch, Nodal, Hedgehog, FGF, Wnt, and BMP have been implicated in the regulation of these asymmetrically expressed genes [3, 13–15].

Here we focus on recent advances, highlighting the molecular mechanisms of LR asymmetric determination in vertebrates and the roles of intercellular signaling pathways, particularly the Notch signaling pathway, in this process.

Left-right axis development in vertebrates

Ion flow during establishment of early LR asymmetry

Levin et al. reported that a domain of depolarization is observed within the left aspect of the primitive streak in chick embryos at Hamburger Hamilton (HH) stage 3 to 4 [15]. Pharmacological inhibition of an H^+/K^+-ATPase transporter led to a randomization of placement of internal organs [15], indicating that the H^+/K^+-ATPase functions to maintain a polarized membrane potential on the right side of the primitive streak. This polarized membrane potential is due to the net potassium current associated with the H^+/K^+-ATPase function [16]. H^+/K^+-ATPase inhibitors also randomized the expression of asymmetrically expressed genes in *Xenopus laevis* [15] and zebrafish [17], leading to the speculation that this ion flow mediated by the activity of H^+/K^+-ATPase during early LR asymmetric establishment may be an important feature in vertebrates, although no such feature has been observed in mice thus far. Interestingly, asymmetrically expressed transcripts of the H^+/K^+-ATPase α-subunit have been observed in *Xenopus* and are localized within right ventral blastomeres by the four-cell stage [15]. It is therefore plausible that an LR difference of H^+/K^+-ATPase activity may be regulated at the translational and/or post-translational level in other species. In addition, the H^+/V-ATPase in chick, *Xenopus* and zebrafish [18], and the Na^+/K^+-ATPase in zebrafish [19] have been identified as an additional ion transporter that is involved in early LR asymmetric determination.

How is this asymmetric H^+/K^+-ATPase activity transmitted to downstream LR asymmetric establishment remains unclear. An interesting model based on events characterized in *Xenopus* [20] is that the asymmetric difference in membrane voltage potential may regulate intercellular communication between cells via gap junction communication (GJC), which has been shown to be necessary for LR asymmetric determination in *Xenopus*, chick, and zebrafish [21, 22]. The asymmetric activities of ion transporters lead to a circuit establishing physiological asymmetries such as an increased pumping of positively charged ions outside of the right side cell, resulting in a difference in membrane potential between the left and right blastomeres across the ventral midline at the eight-cell stage. The blastomeres of the early embryo are then connected by means of open gap junctions, although only left-most and right-most ventral cells are not connected via gap junction. Subsequently, small charged molecules, such as serotonin [23], are driven toward the right-most ventral cell by an electrophoretic force and accumulate in the right-most ventral cell. The localization of small charged molecules to the right side of the embryo suppresses downstream expression of genes on the right side through undiscovered pathway(s), which contributes to the establishment of LR patterning. Although asymmetric H^+/K^+-ATPase activity remains the earliest known determinant for LR asymmetric establishment, this activity itself cannot be viewed as the initiating factor as a priori some factor simply must localize its mRNA or the activity of H^+/K^+-ATPase. This is an important question to address in the future.

Cilia and nodal flow during LR asymmetric determination

A crucial mechanism of LR asymmetric determination in vertebrates is the rotation of cilia, which can generate a leftward flow of extracellular fluid in mouse embryonic node [24–26] or its derivatives including *Xenopus* gastrocoel roof plate (GRP) [27], zebrafish Kupffer's vesicle (KV) [28, 29], and rabbit PNC [30]. Although no flow has been reported so far in chick, its observation in other major model vertebrates [31] strongly implicates that this leftward flow around the node is important for LR asymmetric establishment. Since this mechanism has been well studied in the mouse embryo, this vertebrate model will be discussed in this review. In mice, the concave triangular region, which is formed at the ventral midline surface of embryo during gastrulation, is called the node, and the cavity surrounding the node (referred to as the nodal pit) is filled with embryonic fluid. The ventral side of the nodal pit is lined with a few hundred monociliated cells (nodal pit cells). The node contains two distinct classes of cilia (nodal cilia), left-right dynein (lrd)-positive and -negative cilia, with 9 + 0 axonemes consisting of a ring of nine doublet

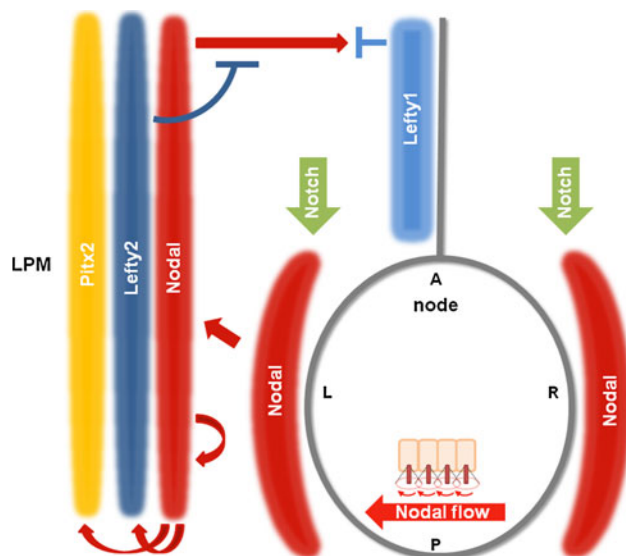

Fig. 1 Summarized schematic of left-right (LR) asymmetry cascades in the mouse embryo. The node is viewed from the dorsal side. Early symmetry breaking signals such as asymmetric ion transporter activities in *Xenopus*, chick, zebrafish, and possibly other vertebrates are transmitted to the node through unknown mechanisms. Notch signaling directly induces perinodal expression of *Nodal* (*red*), a TGF-β family ligand, which is necessary for expression of *Nodal* in the left lateral plate mesoderm (*LPM*). Simultaneously, the leftward nodal flow leads to the accumulation of intracellular Ca^{2+} that is presumably required for LR asymmetric establishment. The Nodal in the LPM up-regulates its own expression in a positive feedback loop and induces expression of *Lefty2* (*dark blue*) and *Pitx2* (*yellow*) in the LPM, and expression of *Lefty1* (*light blue*) in the left prospective floor plate, dorsal to the notochord. By antagonizing the activity of Nodal, Lefty1 limits the range of Nodal activity and Lefty2 inhibits the spread of Nodal signal from the left side to the right side. The Pitx2 maintains left-specific signals during organ morphogenesis. *A* anterior, *P* posterior, *R* right, *L* left

microtubules minus the central pair [32, 33]. While lrd-containing cilia are motile and generate leftward flow, non-lrd-containing cilia are immotile and function as mechanosensors [25, 33, 34]. The rotation of nodal cilia generates a leftward flow of extraembryonic fluid in the nodal pit and this flow determines LR asymmetry [25] (Fig. 1). Two recent studies revealed that the nodal cilia are tilted by 40 ± 10° [30] and 15–35° [35] to the posterior from the vertical angle, respectively. This rotational axis is further conserved among other vertebrates including zebrafish and rabbits [30, 36]. Interestingly, numerical modeling and simulation studies by Cartwright et al. suggested that the array of posteriorly orientated cilia can produce a leftward flow above cilia, but there is also a concomitant rightward flow below cilia [37]. However, this rightward flow was not observed below cilia in the nodal pit [30] because the shear resistance of node's stationary surface retards the movement of fluids, resulting in the rightward phase being less effective than the leftward phase in producing bulk fluid movement [30]. This model is also supported by other simulation studies that show that the effect of the surface is a major factor for converting the rotation of posteriorly tilted cilia into the unidirectional leftward flow [38–41]. These experimental and theoretical studies clarify how the leftward flow of extracellular fluid can be produced by the rotation of monocilia on nodal pit cells. Besides the tilt, positioning of cilia on nodal pit cells, which is regulated by the planar cell polarity (PCP) pathway, is also an essential factor for LR asymmetric establishment. Several studies in mice, *Xenopus*, zebrafish, and chick showed loss of PCP genes, Van Gogh (Vangl) 1/2 genes and Rho-associated coiled-coil containing protein kinase (Rock) 2b protein resulted in the failure of LR asymmetric determination [42–47]. In mice lacking all Vangl genes, ciliary positioning on the nodal pit cells was randomized and nodal flow becomes turbulent [43]. Similar defects were also observed by inhibition of non-canonical Wnt signaling, which regulates PCP pathway as described below [48].

Two models, the "chemical gradient model" [25, 26] and the "two-cilia model" [33, 49], have been proposed to explain how the leftward flow in the node initiates asymmetric differences in the mouse embryo. The chemical gradient model proposes that it is a concentration gradient of diffusible secreted chemicals (referred to as morphogens) such as proteins and lipids generated by nodal flow that is primarily responsible for producing LR asymmetry. Only 20–40 kDa proteins have been observed to generate a gradient concentration by nodal flow in the nodal pit [30], although proteinaceous morphogens still remain undiscovered. The identification of the morphogen transporter referred to as nodal vesicular parcels (NVPs) also supports the chemical gradient model [50]. NVPs are extracellular particulate materials containing multiple lipophilic granules. They are secreted from the node surface and transported to the left side by nodal flow. When they reach and contact with monocilia at the left periphery of the node, they are fragmented and subsequently absorbed by the cell surface. NVPs seem to carry proteins, lipoproteins, and other unidentified chemicals, and can generate a gradient concentration of morphogens across the node. However, the chemical gradient model remains unable to explain the differences in molecular phenotypes in mice harboring only immotile cilia that leads to abnormal spatial expression of left-specific genes [32, 51, 52], and mice devoid of all nodal cilia that results in bilateral or absent expression of left-specific genes [25, 53–55]. Therefore, alternative mechanisms are likely involved in establishing the signaling events downstream of nodal flow. Since the node contains two class of cilia, motile cilia and immotile mechanosensor cilia [33], the two-cilia model was proposed. A higher fluid pressure on the left side of the node generated by motile monocilia is sensed by immotile monocilia in the peripheral region of the node. When cilia

are immotile in mutants such as mice lacking lrd, random movements around the node can still be detected by the mechanosensory cilia and therefore in these mutants the expression of the left-specific genes becomes randomized, either activated on the left, on the right or on both sides [32, 51, 52]. When cilia are absent in mutants such as mice lacking KIF3A, KIF3B, or Polaris, there are no cilia to generate flow and sense movement fluctuations and in this situation bilateral or absent expression of the left-specific genes is observed [25, 53–55]. Left-specific activity stimulated by either model, the combination of these two models or unknown mechanisms in the node elevates intracellular Ca^{2+}concentration, which may be mediated by Ca^{2+}/CaM-dependent protein kinase (CaMK-II) [56] that contributes to the determination of LR asymmetry.

Downstream of nodal flow during LR asymmetric establishment

While it still remains unclear how transient LR asymmetric signaling events around the node such as the elevation of intracellular $Ca^{2+}$are translated into the left-specific expression of *Nodal* in the left LPM, it seems that a Nodal-like signal is required to induce expression of *Nodal* in the left LPM directly, because the FoxH1 transcriptional factor [57, 58], an intracellular transducer of the Nodal signaling pathway, and the epidermal growth factor-cripto/FRL-1/cryptic (EGF–CFC) family [59, 60], co-receptors of the Nodal signaling pathway, and growth differentiation factor 1 (GDF1) [61], a TGF-β superfamily, have been shown to be required for expression of *Nodal* in the left LPM. Indeed, symmetric expression of *Nodal* around the node [62] and *GDF1* [63] are required for expression of *Nodal* in the left LPM. However, mechanisms by which expression of *Nodal* specifically in the left LPM is activated still remains undeciphered.

Nodal in the left LPM directly induces the expression of left-specific genes such as *Lefty* [57, 58] and *Pitx2* [64]. Lefty1 and Lefty2 are members of the TGF-β superfamily and Nodal induces Lefty2 in the left LPM and Lefty1 in the left midline [57, 65] (Fig. 1). In Lefty1 and Lefty2 mutant mice, left-specific genes downstream of Nodal are inappropriately expressed on the right side [66, 67]. The Lefty1 and Lefty2 were therefore proposed to be involved in restricting Nodal signaling to a specific region of the LPM functioning as antagonists both locally and at a distance (Fig. 1). The paired-like homeodomain transcriptional factor Pitx2 mediates the stabilization of the left identity and regulates asymmetric organogenesis [68–70] (Fig. 1). Although the *Pitx2* is expressed in the left LPM similar to *Nodal* and *Lefty2*, its asymmetric expression is maintained until much later stages after expression of *Nodal* in the left LPM disappears. Indeed, while expression of *Pitx2* is initiated by Nodal, it is subsequently maintained by Nkx2 [64]. Interestingly, when the ion channel polycystic kidney disease 2 (PKD2) is mutated in mice [71], the serine protease Furin is deficient in mice [72] and the components of Notch signaling are mutated in mice [73, 74], expression of *Pitx2* is observed in the absence of Nodal function, indicating that expression of *Pitx2* is also induced in a Nodal-independent manner. While the Nodal-Pitx2 signal is highly conserved among vertebrates [31], it still remains unresolved as to how each organ primordium individually interprets the asymmetric information encoded by Pitx2.

Roles of signaling pathways during LR asymmetric determination

Besides Nodal signaling, other signaling pathways including those of FGF, hedgehog (HH), and Wnt have been reported to play roles in the establishment of LR asymmetry.

FGF8, a member of the FGF family, was found to induce expression of *Nodal* in the left LPM of mouse embryos but repressed *Nodal* expression in chick [75, 76]. How FGF signaling regulates LR asymmetric determination was not well understood until recently. In zebrafish, knockdown of FGF receptor 1 (FGFR1) or knockdown of FGF24 within an FGF8 mutant background resulted in defective expression of *spaw*, a homologue of Nodal, due to the presence of defective length cilia in KV [77]. Another group also demonstrated that loss of FGF8 and mediators of FGF signaling, Ier2, and FGF intracellular binding protein 1 (Fibp1), led to either a loss of cilia or cilia with a short length [78]. These studies together demonstrate that FGF signaling modulates ciliogenesis during LR asymmetric determination. Interestingly, pharmacological inhibition of FGF receptors in zebrafish also showed that inhibition of FGF signaling at early stages (shield stage) resulted in short cilia and defects of LR asymmetry but later treatment disrupted LR asymmetry without any obvious effects to ciliogenesis [77]. This likely indicates that FGF signaling has at least two distinct temporal roles during LR asymmetry establishment. In mice, expression of FGFRs on the cell surface and cilia in the ventral node is observed and pharmacological inhibition of FGFRs suppressed the normal elevation of intracellular concentration of Ca^{2+}at the left side of the ventral node [50], which presumably initiates the left specific activity in mice. In addition, FGF signaling functions to launch NVPs, which is transported to the left side by nodal flow from the surface of the node [50]. Taken together, FGF signaling is involved in both ciliogenesis and transmission of LR laterality information by nodal flow.

While the SHH signaling pathway is required to establish a physical barrier in the midline during later stages of LR asymmetric determination [75, 79], the HH signaling pathway itself generates LR asymmetry. Expression of

Nodal in the LPM was abolished in mutant smoothened (smo) mice, a G-protein-coupled receptor of HH signaling, as well as in double mutants of sonic hedgehog (SHH) and Indian hedgehog (IHH) in mice [80]. Furthermore, mis-expression of SHH in the right side of chick, *Xenopus* and zebrafish resulted in bilateral expression of *Nodal* in the LPM and defects of laterality [81–83]. Recently, two molecular mechanisms by which the HH signaling pathway regulates LR asymmetric establishment were uncovered. First, SHH and RA synergistically activate NVPs release from the surface of the node downstream of FGF signaling and are transported to the left side by NVPs to elevate intracellular Ca^{2+}concentration at the left periphery of the node [50]. Second, SHH and IHH signaling in the left LPM directly regulate expression of *Nodal* in the left LPM of mouse embryos through a Foxf1/BMP pathway [84].

The canonical and non-canonical Wnt signaling pathways have been shown to produce LR asymmetry. Knockout study of Wnt3a in mice revealed multiple laterality defects [85] and Wnt3a/β-catenin pathway (canonical Wnt signaling) regulated expression of asymmetrically expressed genes including *Nodal*, *Lefty* and *Pitx2* by regulating the symmetric expression of *Nodal* around the node [86]. On the other hand, non-canonical Wnt signaling is required for ciliogenesis in the node. Basal body on the nodal pit cells failed to shift posteriorly and unidirectional left flow was replaced by multiple vortical flows in Dishevelled (Dvl)-deficient mouse embryos ($Dvl1^{-/-}Dvl2^{+/-}Dvl3^{-/-}$and $Dvl1^{-/-}Dvl2^{-/-}Dvl3^{+/-}$) and by inhibition of Rac1, a downstream component of non-canonical Wnt signaling [48]. In addition, RNA-binding protein Bicaudal C (BicC), which can uncouple Dvl2 from the canonical Wnt pathway and stimulate the non-canonical pathway, is required for normal positioning of nodal cilia [87]. In zebrafish, knockdown of duboraya (dub), which is regulated by phosphorylation dependent manner via Frizzled2, which mediates non-canonical Wnt signaling, led to both defects of ciliogenesis in KV and randomization of LR asymmetry [88].

Although spatially and temporally intricate signaling pathways including these signals have been shown to play important roles in the determination of LR asymmetry, studies of these signals and their crosstalk are still necessary to deeply understand the mechanisms of LR patterning.

The Notch signaling pathway in LR asymmetric development

The Notch signaling pathway

Recent studies have shown that the Notch signaling pathway is required for expression of *Nodal* in the left LPM during early stages [73, 74, 89–91] and must be subsequently suppressed for maintenance of *Pitx2* expression in the left LPM during later stages [91]. The Notch signaling pathway is evolutionarily conserved and regulates various biological processes including cell-fate specification, self-renewal, differentiation, proliferation, and apoptosis [92–96]. Notch signaling is a local communication system between a cell expressing a membrane-associated ligand with a cell expressing a transmembrane receptor. The activation of Notch signaling upon the interaction between a ligand and a receptor leads to proteolytic cleavages of the Notch receptor and the subsequent release of the Notch intracellular domain (NICD). The NICD translocates into the nucleus and activates transcription of target genes by replacing co-repressors with co-activators interacting with the DNA-binding protein CBF1/RBPjκ/Su(H)/Lag-1 (CSL) (Fig. 2).

Notch receptors are single-pass transmembrane proteins and the extracellular domain of Notch receptors contains multiple epidermal growth factor (EGF)-like repeats that mediates interaction with the Notch ligand (Fig. 2a). EGF-like repeats are followed by a negative regulatory region (NRR) that consists of three cysteine-rich Lin12-Notch repeats (LNR) and a heterodimerization (HD) domain that prevents the activation of receptors in the absence of ligands [97] (Fig. 2a). The maturation of Notch receptors occurs by the cleavage of the site 1 (S1) within their HD domain by proteases of Furin family [98, 99] in the secretory pathway, and then the heterodimer of an extracellular subunit [Notch extracellular domain (NECD)] and a membrane-tethered intracellular subunit [Notch transmembrane and intracellular domain (NTMIC)] are held together by non-covalent interactions (Fig. 2a). The interaction of NECD with ligands and subsequent endocytosis of this complex in ligand-expressing cell appears to generate a mechanical force for dissociation of the NECD/NTMIC heterodimer, thereby exposing site 2 (S2) located ~12 amino acids before the transmembrane domain of NTMIC [100–102]. This conformational change of the Notch receptors appears to allow for the cleavage of the Notch receptor by the metalloprotease ADAM10 or ADAM17 at S2 [103], which creates a membrane-tethered intermediate [Notch extracellular truncation (NEXT)] (Fig. 2b). Finally, the γ-secretase complex that contains presenilin-1or -2, Aph1, Pen2, and Nicastrin [104, 105] cleaves NEXT first at site 3 (S3) and subsequently at site 4 (S4) within the transmembrane domain (TMD) and the NICD is released from membrane to activate transcription of Notch target genes (Fig. 2b). The NICD contains RBPjκ association a module (RAM) domain, seven ankyrin repeats (ANK), proline/glutamic acid/serine/threonine-rich motifs (PEST) and two separately located nuclear localization sequences (NLSs) (Fig. 2a). The RAM domain is defined as

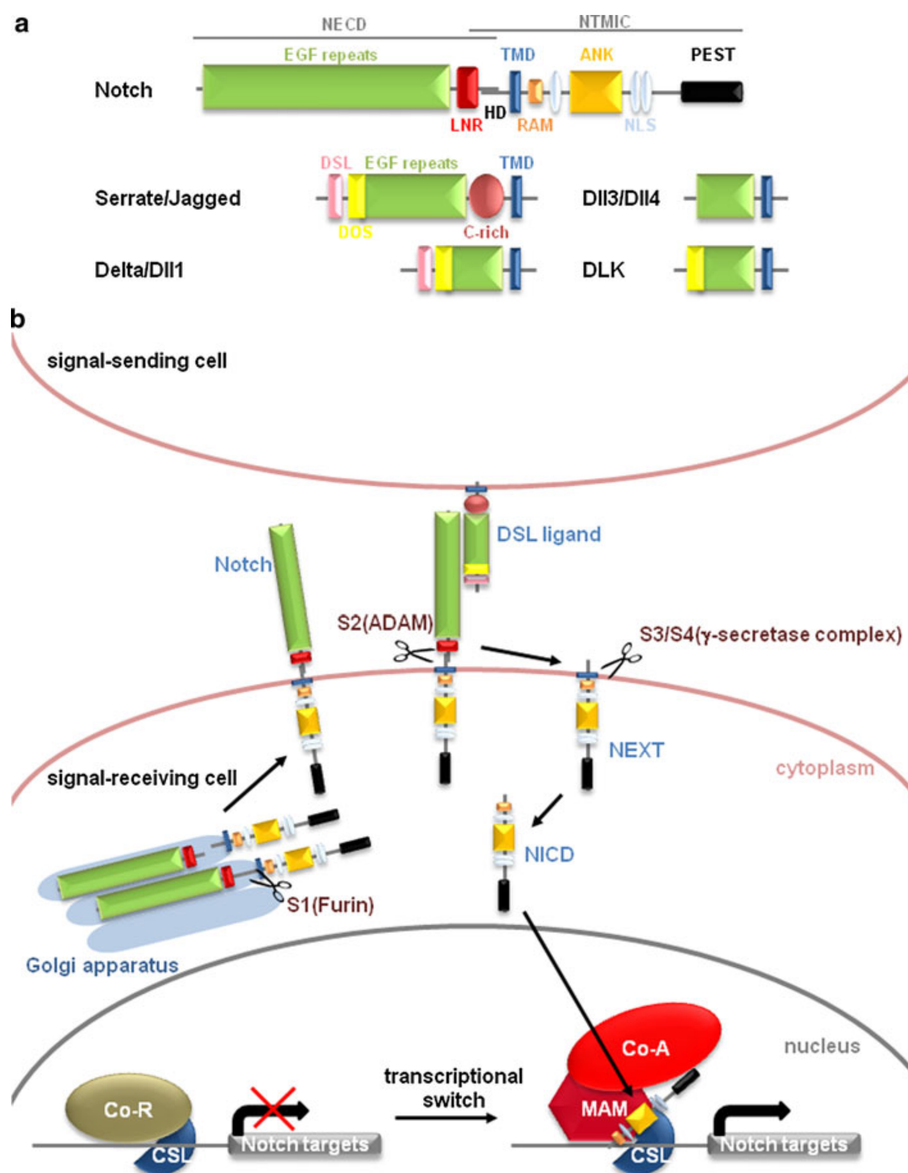

Fig. 2 The canonical Notch signaling pathway. **a** The structure of Notch receptor and Notch ligands. The Notch receptors are single transmembrane proteins that consist of multiple extracellular epidermal growth factor (*EGF*)-like repeats (29–36 repeats), cysteine-rich Lin12-Notch repeats (*LNR*) (three repeats), a heterodimerization (*HD*) domain, a transmembrane domain (*TMD*), a RBPjκ association module (*RAM*) domain, nuclear localization sequences (*NLS*s), seven ankyrin repeats (*ANK*) domain, and proline/glutamic acid/serine/threonine-rich motifs (*PEST*). The Delta/Serrate/LAG-2 (*DSL*) ligands are characterized by the presence or absence of a cysteine-rich (*C-rich*) domain, Serrate/Jagged or Delta/Delta-like (*Dll*), respectively. Classical DSL ligands contain a DSL domain, a Delta and OSM-11-like proteins (DOS) domain and EGF-like repeats. A subtype of DSL ligands lacking DSL and DOS domains, such as mouse Dll3 and 4, may act alone or along with DOS co-ligands such as DLK-1. **b** The core Notch signaling pathway. Translated precursor of Notch receptor is glycosylated and cleaved by Furin at site 1 (*S1*). Matured heterodimer receptor is targeted to the cell surface. The interaction between Notch receptors and ligands on neighboring cells results in the conformational change of receptor and the site 2 (*S2*) is exposed for the cleavage by ADAM metalloproteases. This cleavage creates the membrane-anchored Notch extracellular truncation (*NEXT*) fragment and γ-secretase complex then cleaves NEXT from site 3 (*S3*) to site 4 (*S4*) to release the Notch intracellular domain (*NICD*). The NICD translocates into the nucleus and binds to DNA-binding protein CBF1/RBPjκ/Su(H)/Lag-1 (*CSL*). The NICD/CSL complex is recognized by transcriptional co-activator Mastermind (*MAM*). This ternary complex recruits additional co-activators (*Co-A*) to activate transcription. In the absence of NICD, CSL protein associates with a variety of co-repressors (*Co-R*) and histone deacetylases to suppress transcription of target genes

an approximately 100-residue region between the γ-secretase cleavage site and ANK domain [106]. The ANK domain follows RAM domain, and is composed of seven canonical ankyrin repeats with a non-canonical eight repeats capping the N-terminal first repeat [107, 108]. The RAM and ANK domains are necessary for formation of the

ternary complex with CSL and the co-activator, Mastermind (MAM) [109]. The PEST domain is located at very C-terminus of Notch receptor and contributes to its instability and the degradation of NICD by Sel10 ubiquitin ligase [110, 111].

Several different isoforms of Notch ligands have been reported [112] (Fig. 2a). Most of them are type I transmembrane proteins that harbor EGF-like repeats in their extracellular domain. The major class of Notch ligands [referred to as Delta/Serrate/LAG-2 (DSL) ligands] consists of a DSL domain, a Delta and OSM-11-like proteins (DOS) domain, and EGF-like repeats. Serrate/Jagged family of DSL ligands additionally possess a cysteine-rich domain, and some DSL ligands such as Delta-like (Dll) 3 and Dll4 don't have DOS domain. Both DSL and DOS domains are required for the interaction between DSL ligands and Notch receptors [113, 114]. A recent study suggests that DSL ligands lacking the DOS domain such as Dll3 may employ DOS domain-containing proteins such as DLK-1 as a co-ligand to stimulate Notch activation [115]. In addition, non-canonical Notch ligands such as DNER, NB-3/Contactin6, MAGP1 and MAGP2, which lack DSL and DOS domains has been described [95, 116]. However, the physiological roles of these proteins are not completely understood.

CSL protein contains three domains; an N-terminal domain (NTD), a β-trefoil domain (BTD) and a C-terminal domain (CTD) [117]. The BTD is located between the NTD and CTD. The NTD and BTD work together to recognize and bind to either a high (YGTGRGAA) or low (RTGRGAR) affinity binding site in the promoters of Notch-target genes [118]. In the absence of NICD, CSL protein binds to DNA and recruits co-repressors including SMRT/NcoR, SHARP/MINT/SPEN, and CtBP in mammals and SMRTER (a homologue of SMRT), Spen (a homologue of SHARP), Groucho and CtBP in *Drosophila* [96]. Subsequently, the complex of CSL and its associated co-repressors recruit histone deacetylases (HDACs) [119] and additional cofactors including CBF1-interacting co-repressor (CIR), SIN3A and NF-κB activating protein (NKAP) [120, 121]. Once NICD translocates into the nucleus, the RAM domain of NICD first binds to the BTD of CSL through approximately the first 20 residues of RAM domain [108, 122, 123] (Fig. 2b). This high-affinity interaction increases the local concentration of the ANK domain, thereby facilitating the formation of low affinity interactions between the CTD of CSL and ANK domain [124]. Subsequently, the N-terminal helical region of MAM interacts with the CTD and ANK domains, and C-terminal helix of MAM can bind to NTD [108, 125, 126]. Finally, DNA-bound Notch ternary complex can recruit histone acetyltransferases, CBP/p300 and PCAF [127–129], components of the chromatin-remodeling complex, BRM and Nipped-A/TRA1/TRAPP, Ada2b and Domino, [130, 131] and components of the Mediator complex, CDK8 and MED220 [132]. The CDK8 recruited by MAM phosphorylates NICD and promotes its degradation by the nuclear ubiquitin ligase Sel10 [132].

Roles of Notch signaling during LR asymmetric determination

Studies in mice demonstrated that the Notch ligand Dll1 or Notch1 and Notch2 double mutant resulted in defects of laterality via suppression of symmetric *Nodal* expression in the periphery of the node [73, 74, 133]. The expression of *Nodal* around the mouse node is important for left-specific expression of *Nodal* in the LPM [62, 134]. Transcription of perinodal *Nodal* expression is regulated by a nodal-specific enhancer in the upstream region of *Nodal* gene (−9.5 to −8.7 kb), whereas the left-specific enhancer of the *Nodal* gene was found within an intron 1 [135, 136]. The nodal-specific enhancer contains two CSL-binding sites, and these binding sites are functionally important for the expression of *Nodal* around the node [73, 74]. Indeed, the expression of *Nodal* within the perinode was also not detected in RBPjκ-deficient mice [74]. These findings demonstrate that canonical Notch/CSL signaling directly regulates the symmetric expression of *Nodal* around the node. Involvement of Notch signaling in regulation of *Nodal* expression in the perinode has also been reported in other vertebrates including zebrafish [74] and *Xenopus* [91]. In zebrafish, the expression of two Nodal-related genes, *cyclops* and *spaw*, in the left LPM are regulated by Notch signaling, but these two genes appear to be controlled through different mechanisms. Over-expression of active Notch randomized both expression of *cyclops* and *spaw* in the LPM, but only *cyclops* expression in the perinode was increased [74, 137]. In contrast, inhibition of Notch signaling by a γ-secretase inhibitor suppressed the expression of *charon*, which is a Cerberus/Dan family secreted factor expressed in the posterior domain of KV and blocks the transfer of spaw from KV to the right side of LPM [138, 139], leading to randomized expression of *spaw* in the LPM [137]. Baf60c, a component of Swi/SNF-like BAF chromatin remodeling complex, binds to CSL protein and recruits the DNA-stimulated ATPase Brahma-related gene 1 (Brg1), a core component of the BAF chromatin remodeling complex, into the Notch-mediated transcriptional complex assembled on the nodal-specific enhancer [90]. In addition, a loss of Dll1 and Baf60c resulted in disruption of the organization of the node [73, 90, 133], indicating that Notch signaling may affect LR asymmetric establishment by at least two different ways, regulation of perinodal *Nodal* expression and morphogenesis of the node.

Dll1 is required for the expression of *Nodal* around the mouse node [73, 74, 133], but asymmetric expression of *Dll1* around the node is thus far observed only in chick but not other

vertebrates including mice and *Xenopus* [73, 89, 91]. This expression of *Dll1* is regulated by Wnt3a [86] and the T-box transcription factor Tbx6 [140] in mice, although curiously there is no genetic interaction between these two genes [140]. In chick, omeprazole, a pharmacological inhibitor of H^+/K^+-ATPase, inhibited asymmetric expression of *Dll1* and *Nodal* around Hensen's node, indicating that the activity of H^+/K^+-ATPase could be a responsible factor for the regulation of Notch signaling on the left side of Hensen's node [89].

A recent study in zebrafish showed that a mutant deltaD, a homologue of Dll1, led to short cilia within KV and a slow fluid flow velocity inside of KV [141]. Interestingly, hyperactivation of Notch signaling by over-expression of NICD and deltaD increased the length of cilia through foxj1a, a marker of KV motile cilia, demonstrating that Notch signaling can affect both an increase or a decrease the length of cilia [141]. However, the leftward fluid flow at the node of Dll1 null mouse embryos was normal [73]. Therefore, it remains unclear whether the role of Notch signaling during ciliogenesis is conserved in other vertebrates.

Besides the roles of Notch signaling in the node, a new function of Notch signaling in regulation of *Pitx2* expression in the left LPM has been reported. Knockdown of transcriptional repressor B cell lymphoma 6 (BCL6) or its co-repressor BCL6 co-repressor (BCoR) in *Xenopus* showed defects of LR asymmetry [91, 142]. The expression of *Pitx2* but not *Xnr1*, a homologue of Nodal, in the left LPM was suppressed in either BCL6 or BCoR knockdown embryos, because BCL6/BCoR complex blocks Notch signaling in the LPM by inhibiting transcription of selected Notch targets such as *ESR1*, a homologue of Hes5 [91]. BCL6 is recruited into promoters of Notch targets in a sequence-specific manner through an unknown mechanism and competes with Mastermind-like 1 (MAM1) away from ANK domain of NICD (Fig. 3). Although the canonical Notch signaling mediated by CSL protein and MAM1 in the LPM can activate other target genes such as *Hairy2*, its role in the LPM remains unclear. While the expression of selected Notch targets in specific tissues is activated by the cooperation of Notch and other transcriptional factors on neighboring enhancers [143–148], this inhibitory system by the BCL6/BCoR complex is likely a novel mechanism that allows only required Notch targets to be expressed in specific tissues.

Taken together, these studies have shown that Notch signaling plays multiple roles in the establishment of LR asymmetry and is a vital signal in this biological process. However, many questions still remain unanswered.

Conclusions

Our understanding of the mechanisms underlying LR asymmetric determination has progressed at a breath-taking pace over the past 15 years. However, we still have fundamental questions to address. How is symmetry breaking at the node initiated? Studies in *Xenopus* and chick showed that the cytoskeletal organization and intracellular microtubule-dependent motor protein function at very early embryonic stage are crucial for LR asymmetry [18, 20, 149–152], although the leftward flow by nodal cilia remains the earliest known mechanism for symmetry breaking in mice. Could this cytoskeletal organization be the origin of LR asymmetry and conserved in mammals? Next, how and when is the boundary between the left and right of the midline determined? Since the midline prevents asymmetric signals from leaking to the other side [153], the function of the midline clearly is important for LR asymmetric establishment. Third, what signals initiate expression of asymmetric genes such as *Nodal*, *Lefty* and *Pitx2*? The leftward flow by cilia in the node is an essential mechanism to induce expression of *Nodal* in the left LPM, but the loss of Wnt3a or Notch in mice resulted in suppression of asymmetric genes in the left LPM with normal nodal flow [73, 85, 86]. These data indicate that several different mechanisms likely cooperate to generate such asymmetric gene expression. The advancement of technology and development of model animal systems will no doubt provide further insights into these important questions.

The Notch signaling pathway is involved in two different steps during LR asymmetric determination in vertebrates. First, canonical Notch signaling mediated by CSL and MAM induces symmetric expression of *Nodal* in the perinode by directly activating transcription of the *Nodal* gene through a nodal-specific enhancer in its

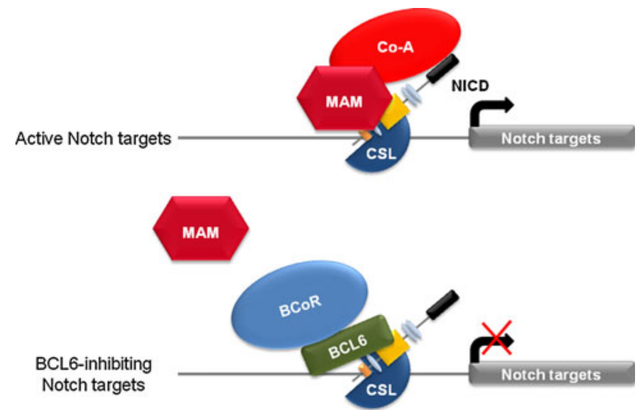

Fig. 3 The inhibitory mechanism of Notch signaling by BCL6/BCoR complex. Transcriptional repressor B-cell lymphoma 6 (*BCL6*) is recruited to promoters of selected Notch target genes in a sequence-specific manner through an unknown mechanism. Once BCL6 binds to promoters, BCL6 competes with MAM for ANK domain of NICD and blocks the interaction between NICD and MAM. Subsequently, BCL6 recruits BCL6 co-repressor (*BCoR*) and inhibits Notch-dependent transcription.

promoter, and canonical Notch signaling also controls morphogenesis of the node. In zebrafish, Notch signaling also determines the length of cilia. Second, a recent study in *Xenopus* showed that Notch signaling in the LPM can be inhibited by the BCL6/BCoR complex to maintain expression of *Pitx2* in the left LPM, although the role of Notch signaling in the LPM is poorly understood. This BCL6/BCoR complex directly binds to promoters of selected Notch targets in a sequence-specific manner and competes with MAM1 for ANK domain of NICD. This is the first reported selective inhibitory mechanism of Notch-mediated transcription. Although similar roles for the BCL6/BCoR complex have not been reported in mammals to date, it should be noted that mutations of human BCoR leads to the oculofaciocardiodental (OFCD) syndrome that includes defective laterality [142, 154]. Although these interesting functions of Notch signaling have been observed, it still remains unclear whether the regulation of Notch signaling on the left side of Hensen's node observed in chick is conserved in other vertebrates, whether the role of Notch signaling in zebrafish ciliogenesis is conserved in other vertebrates, how Notch signaling is involved in ciliogenesis and how ESR1, a Notch target, inhibits expression of *Pitx2* in the left LPM. Further studies will be necessary to better understand the roles of Notch signaling in the establishment of LR asymmetry.

Acknowledgments I thank Drs. Hiroshi Hamada and Raymond Habas for their helpful suggestions on the manuscript and current and former members of the Kato lab for assistance and discussion. Our work is supported by a grant from the National Institute of General Medical Sciences (GM087641).

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