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Alagille, Notch, and robustness: why duplicating systems does not produce redundancy

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Abstract

The mammalian kidney forms from several populations of progenitors that only persist during embryogenesis. The epithelial nephron progenitors reside in the cap mesenchyme (CM), whereas mesangial and endothelial cell progenitors reside in the neighboring stromal mesenchyme (SM). After a ureteric bud (UB) signal induces mesenchymal to epithelial transition of some CM cells, they form a nascent epithelial ball (a renal vesicle or RV) that require signals mediated by Notch receptors to separate proximal from distal fates. Two Notch receptors (*Notch1* and *Notch2*) and two ligands (*Jagged1* and *Delta1*) are expressed in the RV. Notably, instead of providing sufficient redundancy to ensure that losing any one allele will be inconsequential to human health, a reduction in the dose of one ligand (*Jagged1*) or one receptor (*Notch2*) is causally associated with a rare developmental syndrome (Alagille syndrome, ALGS) affecting eye, kidney, liver, and cranio-facial development. Here we will discuss our current understanding of the molecular basis for the non-redundant role of *Notch2* in this process, and the avenue for new therapeutic strategies that these insights provide.

Keywords

Notch; Alagille; Jagged; Dll1; Kidney; nephron

Introduction

The intermediate mesoderm forms discrete cellular populations that interact to produce the metanephric kidney

The metanephric kidney is derived entirely from the intermediate mesoderm (IM). The dorsal IM will form the nephric duct (ND) or Wolffian duct [1], which extends in a rostral to caudal direction during development until it reaches the pelvis, connecting to the urethra. In contrast, the developmental potential of cells in the rostral/ventral IM becomes progressively restricted; at the level of the hind limb they form a bean-shaped territory called the metanephric mesenchyme (or MM) around E10. At that time the MM contains distinct populations of mesenchymal progenitors for the epithelial [2, 3], stromal [4] and vascular [5] components of the metanephric (adult) kidney. The epithelial progenitors begin to secrete GDNF, FGF10 and other inductive signals that activate receptor tyrosine kinases (RTKs) in the ND, triggering pseudostratification and evagination of a single ureteric bud (UB) from the ND. UB evagination is led by tip stem cells experiencing high levels of Ret activity [6, 7]. Continuous Ret activation triggered by MM signals causes the UB to extend and branch repeatedly, contributing to the future collecting duct system. At the same time, UB signals

back to the MM to induce their differentiation into nephrons. Detailed discussion of the molecular mechanisms involved in ND and UB formations are discussed elsewhere [8, 9].

Wnt signals induce epithelia in a reiterative process

Two Wnt ligands, WNT9b and WNT4, are sequentially required to induce differentiation of nephron progenitors into renal epithelia. The UB produces WNT9b, which signals to the CM to perform two functions: first, Wnt9b supports expansion of the progenitors [10, 11]. Then, in cells conditioned by BMP7-mediated SMAD signaling [12], Wnt9b initiates mesenchymal to epithelial transition [13] and activates target genes including *Fgf8* and *Wnt4*. Wnt4 then drives epithelialization [14] and *Fgf8* drives proliferation leading to the formation of the renal vesicles (RV) [15, 16].

Genetic and pharmacological manipulations suggested that β -catenin/Lef/TCF signaling pathway is activated by both Wnt9b and Wnt4 signaling during CM differentiation [10, 11, 17, 18]. CM specific removal of β -catenin activity completely blocked the formation of RVs and abolished expression of genes such as *Fgf8*, *Pax8* and *Wnt4*. In contrast, ectopic expression of stabilized β -catenin in the CM leads to differentiation of CM *in vitro* and rescued some differentiation defects in *Wnt9b* and *Wnt4* mutants *in vivo*. Together, these results indicate that canonical Wnt signaling mediates the majority of signaling activity of Wnt9b and Wnt4 during nephrogenesis. However, non-canonical Wnt signals are also contributing [19], perhaps by antagonizing the canonical signal: only transient, low-level stabilization of β -catenin could induce epithelialization [11, 20], whereas continuous signal or a higher dosage blocks this process [18]. This highlights the requirement for tightly regulated Wnt activity for proper nephron differentiation.

Nephron segmentation: the separation of proximal fates from mostly distal-fated epithelia

In order to form functional nephrons, newly formed epithelia need to acquire a proximal-distal axis (PDA) in a process called segmentation and elongate giving rise to more than 15 different cell types [21] within the mature nephron (Fig. 1C). Morphologically, the progenitor niche remains at the surface of the developing metanephric kidney, while the nascent RVs always appear under the UB branch until the last day of nephrogenesis [22]. Beginning as an un-polarized cell cluster, RV quickly acquires apical-basal polarity to create a lumen [23]. At this stage, differential gene expression along the PDA in the RV suggests that nephron segmentation has already been initiated. Notch ligands (*Jag1*, *Dll1*) and Wnt signaling components (*Wnt4*, *Dkk1*, and *Ccnd1*) are all expressed in the distal RV, while Notch receptor (*Notch1*, *Notch2*) and transcription factor *Wt1* are highly expressed on the proximal RV. The RV PDA is always aligned with the duct tip at the distal end, facilitating fusion with the duct that occurs before a burst of proliferation that contorts the nascent tube into an S-shaped body [24].

The earliest molecular evidence of a PDA include changes in the expression of *Pax2* and *Wt1* which are seen even in mutants that fail to establish a proximal domain [25]. This PDA alignment is reminiscent of a process involving a morphogen gradient, with Wnt as a candidate morphogen produced by the UB [26]. A morphogen gradient would be sufficient to establish a PD axis in other system, but not here: a signal exchanged between RV cells is required to establish the emerging proximal domain to generate the proximal tubule, podocytes, and partial epithelium lining the bowman capsule. This process requires a signal generated by Notch receptors [27, 28].

The Notch signaling pathway

Notch proteins are large, single-pass molecules with an extracellular domain containing 29–36 EGF repeats and a negative control region (NRR) that folds into a structure unique to

Notch proteins (Fig. 2; for reviews, see [29, 30]). Activation occurs when ligand binding exerts a force that unfolds the NRR [31], exposing a hidden target (called S2) to the protease ADAM10 [29, 32] which cleaves ligand-bound Notch receptors shedding their extracellular domains. This cleavage generates a truncated Notch substrate for the enzyme -secretase [33] which cleaves the Notch transmembrane domain at multiple peptide bonds, starting near the inner plasma membrane leaflet at S3 (Fig. 2B,D). Notch intracellular domain (NICD) fragments containing a Val or Met residues at their amino terminus are longer-lived and translocate to the nucleus, where they bind the protein RBPjk and recruit the adaptor Mastermind-like (MAML), p300, the mediator complex, and RNA polymerase II [29]. The Notch pathway is used throughout development and in adult tissue to enable cells to choose one of two cellular options in coordination with their neighbors [34].

The two Notch receptors expressed in the RV are not equivalent

In the RV, two of four mammalian Notch receptors (*Notch1* and *Notch2*) are co-expressed. Despite the great degree of conservation between *Notch1* and *Notch2* and overlap in their expression domain, the two receptors are not functionally equivalent: in mice, loss of both *Notch1* alleles is tolerated, but loss of two *Notch2* alleles is not; in humans, loss of one *Notch2* allele leads to Alagille syndrome [25, 35–38]. In the mouse, Alagille-like symptoms were seen only when the dose of *Notch2* and the ligand *Jag1* are simultaneously reduced [35]. In contrast, no *Notch1* or *Dll1* mutations have been linked to Alagille. Notch1 protein does provide some activity during RV segmentation, but this can only be demonstrated in a sensitized background in which *Notch2* levels are greatly reduced [39]. Given that all NICD proteins contain highly conserved RBPjk binding domain and Ankyrin repeats, and that all bind to RBPjk with the same affinity [40–42] without changing RBPjk sequence preferences [43], a nagging question remained: why is Notch1 incapable of rescuing loss of one *Notch2* allele? And, given that that loss of one *Notch2* allele causes Alagille syndrome, what can be done to elevate Notch1 activity in affected organs without causing other systemic problems?

To address the mechanism behind Alagille, we created two new mouse strains harboring genes we call *Notch12* and *Notch21* (Fig. 3). These alleles were created by swapping the genomic sequences coding for the signaling element (the intracellular domains or ICDs) between the two receptors, leaving the promoter, regulatory elements, the extracellular domain, the transmembrane domain and the UTR of the original locus intact [44]. Thus, expression domains, transcript stability and protein levels, ligand binding and cleavage are all controlled by the locus (the *Notch1* locus controlling *Notch12* and the *Notch2* locus controlling *Notch21*) but the signaling entity released after γ -secretase cleavage has been swapped. Given that the few amino acids distinguishing N1ICD from N2ICD are all on exposed surfaces of the molecule (Fig. 4), this experiment will ask whether these differences can drive the assembly of different transcription complexes by Notch1 vs Notch2. If the two ICD are equivalent, it may help identify other elements within the locus that establish Notch2 as the main signaling receptor during renal development.

Analysis of these mice has just been completed, and the results are unequivocal [44]. We detected no differences in mRNA abundance or in the overall levels of Notch1 and Notch2 protein expression in nascent renal epithelia. The surface distribution of the Notch1 and Notch12 proteins was identical, as was the surface levels of Notch2 and Notch21 indicating the domain swap did not affect cellular expression, stability, or transport. Only Notch2 was expressed in CM cells but we have established previously that the CM does not use Notch signaling to self renew or to execute MET [25, 28, 45]. Importantly, as long as it was released from a Notch21 protein, a single Notch21 allele producing N1ICD was sufficient to support the formation of glomeruli domains in every single nephrons, whereas two alleles of Notch12, releasing N2ICD, could not support the formation of even one glomerulus. In

short, the Notch1 and Notch2 ICD are interchangeable. The difference is due to other elements in the Notch1 and Notch2 locus.

Two differences between Notch21 and Notch12 did emerge. First, two different pairs of antibodies to the Notch extracellular domains (ECDs) established that Notch2 was between 2 and 3 fold more abundant than Notch1 on the cell surface of nascent renal epithelia. Second, when we measured how many NICD entered the nucleus in cells containing equivalent surface levels of Notch1 and Notch21, we noticed again a 2 fold advantage to Notch21 proteins activated by either Dll1 or Jag1 ligands. These small differences could synergize to create a significant overall difference in the amounts of NICD present in the nucleus, reaching a threshold necessary for nephron segmentation only when Notch2 molecules are present.

What was the molecular basis of these differences between Notch1 and Notch2? Since overall expression levels were the same, and the swap of ICD did not alter the surface distribution of Notch1 relative to Notch12 or of Notch2 relative to Notch21 (Fig. 3 in [44]), the ECD holds the key. Not only does the Notch 2 ECD contain signals that promote exocytosis but it is also better at unfolding the NRR in response to ligand. Notch exocytosis involves passage through a furin-containing compartment, which cleaves Notch at an unstructured loop protruding from the folded NRR at S1 (see [46–48] for NMR and crystal structures of the Notch1 and Notch2 NRRs). This cleavage generates a form of Notch1 or Notch2 that is longer-lived at the surface than the un-processed, full-length protein [49, 50]. Interestingly, when the S1 containing loop was removed, no structural changes in the NRRs were detected but surface expression of Notch1 lacking the S1 loop was reduced and its response to ligand was reduced as well [46]. In contrast, Notch2 lacking the S1 loop reached the surface and accumulated there to a similar degree with WT Notch2, and had an improved response to ligand. It thus appears safe to speculate that the NRR may regulate differential exocytosis as well as ligand responsiveness, and that the details of transporting Notch molecules to the surface will explain the functional differences between the two proteins.

The two Notch ligands expressed in the RV are also not equivalent

Similar to what has been observed with the receptors, the Notch ligands *Jag1* and *Dll1* are co-expressed in the developing RVs yet their functions are not redundant. In human, whereas 94% of Alagille patients harbor *Jag1* mutations, not one *Dll1* mutation has been identified so far [51]. Consistently, deletion of *Jag1* in mouse results in dramatic reduction of nephron numbers, while deletion of *Dll1* produced a milder phenotype [44]. Clearly, *Jag1* ligand and *Notch2* receptor produce the key signals during RV segmentation in both mouse and human kidneys.

Why does Jag1 play a more important role than Dll1 in this process? After all, Dll1 is a dominant ligand, activating Notch1 during the maintenance of gut stem cells [52]. It is indispensable for the activation of Notch2 during marginal zone B cell development in the spleen [53, 54]. The answer lies in the modification status of the Notch extracellular domains by Fringe glycosyltransferases. The three related enzymes (lunatic fringe (Lfng), manic fringe (Mfng) and radial fringe (Rfng)) mediate glycosylation of specific, fucosylated Ser/Thr residues on Notch receptors (Fig. 2A and [55]) and result in qualitatively different interaction between the Notch receptors and their ligands. In general, these modifications enhance the response to Dll ligands but suppress the response to Jag ligands; however, these effects are highly cell-context specific. At least in one system the activation of Notch2 by Jag1 was enhanced, not suppressed, by Lfng modification and subsequent glycosylation [56].

In the developing kidney, Lfng is expressed in the same expression domain of Notch receptors and ligands [44]. More importantly, tests in the human embryonic kidney 293 cell line show that Lfng expression significantly potentiates the response of Notch1 to Dll1, inhibits its response to Jag1, but enhances the responses of Notch2 to both ligands. These data suggest that Lfng modification underlie the unequal contributions of different receptor-ligand pairs during nephrogenesis.

Implications for the clinic

At present, several Notch antagonists have entered clinical trials, mostly targeting T-cell acute lymphoblastic leukemia and other tumors [57]. No agonists have been developed with the exception of EDTA [58]. Agonists will not present a meaningful intervention in Alagille because of the many untoward effects anticipated with elevating Notch activity in all the developmental processes in which Notch is involved, which may literally be too many to count. However, the observation that differential transport differentiates Notch2 from Notch1 opens a possible avenue for therapeutic intervention. We know that not all cells display the same transport bias, and it may be that the tissues affected in Alagille are all the ones favoring Notch2 transport over Notch1. Investigation into the mechanism preventing Notch1 from efficiently reaching the surface in these cells but not in others may lead to new strategy for augmenting the surface presence of Notch1 without a negative impact on other tissues.

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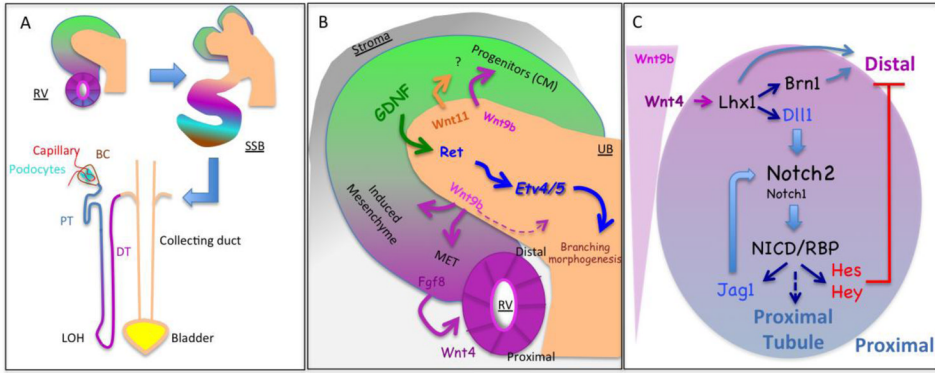


Figure 1. Schematic representation of steps in nephrogenesis. (A) Renal vesicles (RV) form beneath the ureteric bud (UB) tip near the periphery of the developing kidney. These polarized epithelial ball elongate into an S shape body (SSB) that will give rise to the entire nephron. Proximal-distal axis (PDA) organization in the RV and SSB seems to be preserved in the nephron with the most proximal [Bowman capsule (BC) and podocytes] followed by intermediate structures [proximal tubules (PT), loop of Henle (LOH)] and distal one [distal tubule (DT), connecting segment and collecting duct]. (B). GDNF/Ret/Etv4-5 signaling controls UB branching whereas Wnt signaling controls progenitor expansion in the cap mesenchyme (CM) as well as their differentiation via mesenchymal to epithelial transition (MET). Wnt4 is produced by the epithelia. (C) Wnt9b could create a morphogen gradient initiating the formation of PDA but Notch signaling is required to fix the PDA and allow proximal fate selection. The role of the genes listed in C is reviewed elsewhere [8, 26]

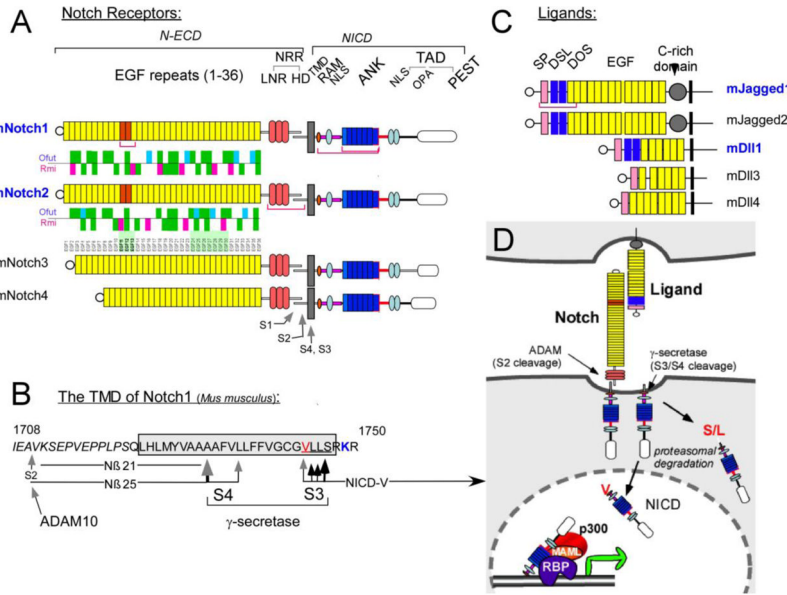


Figure 2. Type I proteins belonging to the mammalian Notch receptor and ligand families. A) Notch proteins contain multiple extracellular EGF-like repeats. Repeats 11–12 (orange) mediate interactions with ligands. 4 mammalian Notch receptors differ in the number of repeats (29–36). EGF repeats are followed by three cysteine-rich Lin12-Notch repeats (LNR-A, B and C) and a heterodimerization domain (HD) which together fold into one globular domain, the NRR. Notch proteins are cleaved by furin-like convertases at site 1 (S1). EGF repeats may contains consensus motifs for fucosylation by O-Fut1 followed by glycosylation by Fringe; Rumi, another glycosyltransferase, has its own conserved sites. The distribution of potential fringe (Cyan) and rumi (magenta) sites in the extracellular domain are shown for mNotch1 and mNotch2 (Green- same in by both receptors). Note the ligand binding regions differ in their modification patterns. B) Details of mouse Notch1 proteolysis. After ligand binding, cleavage at S2 is followed by cleavage at the S3 region. Multiple scissile bonds are cleaved but only peptides initiating at Val 1744 evade Notch-end rule degradation. Cleavage then proceeds towards the cell surface until the short Nβ peptides can escape the lipid bilayer; most are 21 amino acids long. Two amino acid substitutions (V1744G and K1749R, colorized) change the choice of scissile bonds. Only antibodies that recognize the underlined sequence detect the active form of Notch1. C) Ligands of Notch receptors can be divided into two groups based on the length and subtype of EGF-like repeats they contain (DSL, DOS and EGF motifs; see [29]). Red brackets mark domains that have been crystallized alone or in combination with binding partners. Abbreviated domain names are: SP- signal peptide. DSL- delta, serrate, lag1. DOS- Delta and OSM-11-like. D) a schematic diagram of the Notch activation mechanism. Explained in the text and in [29].

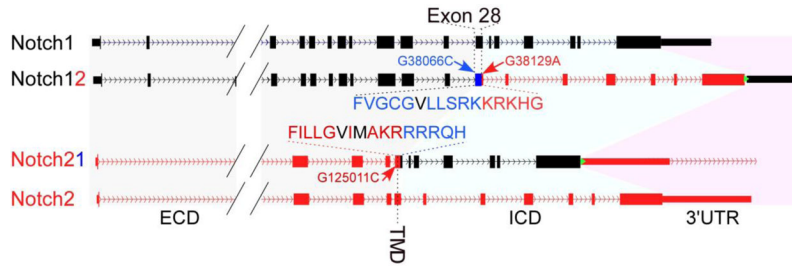


Figure 3.

The Notch12 and Notch21 alleles. (A) Schematic illustration of Notch1 (black) and Notch2 (red) loci before and after the ICD swap. The swapped fragments between the Notch1 and Notch2 loci range from after the transmembrane domain in Exon 28 to the stop codon in Exon 34. The *Notch1* ICD encompasses 5,926bp on chromosome 2, ranging from nucleotide +38,103 to +44,028 (numbering the A in ATG as +1) and encoding amino acid 1,750 to 2,531; for *Notch2*, the ICD encompasses 8,699bp on chromosome 3, ranging from nucleotide +125,048 to +133,746 and encoding amino acid 1,705 to 2,473. G38066C, G38129A and G125011C denote silent single nucleotide mutations introduced into the targeting constructs for pyrosequencing-based ES cell screening and mRNA comparisons. Amino acids in black denote the S3 cleavage sites.

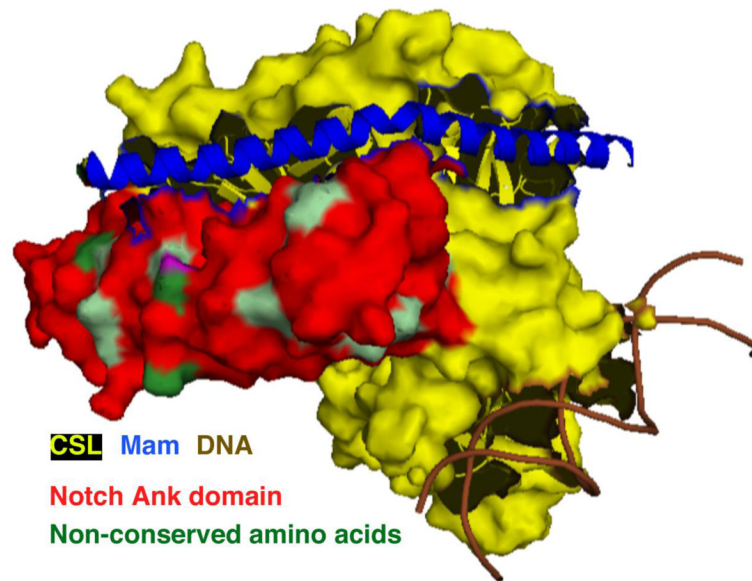


Figure 4. The superimposed crystal structure of Notch1, Notch2, RBP, Mastermind-like (MAML) and DNA shows that non-conserved amino acids (light and dark green) between Notch1 and Notch2 ICDs exist at the surface of the transcription complex, which might serve to recruit different binding partners and determine specificity of target genes.