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Mechanical regulation of the Notch signaling pathway

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Abstract

The mechanical regulation of Notch signaling is an emerging area of interest in cell biology. Notch is essential in many physiological processes in which mechanical stress plays an important role. This review provides an overview of the mechanoregulation of Notch signaling in multiple steps of the pathway. First, we discuss the current knowledge on the direct mechanoregulation of Notch receptor maturation and localization to the membrane and the effect of mechanical stress on the Notch components. Next, we explore how ligand-receptor interactions and membrane dynamics are possible subjects to mechano-regulation, emphasizing the role of cytoskeletal interactions, membrane stiffness, and endocytic complex formation. We further delve into the necessity of tension generation for negative regulatory region (NRR) domain unfolding, facilitated by ligand endocytosis and other microforces. Additionally, we examine the indirect mechanoregulation of S2 and S3 cleavages. Finally, we discuss the mechanoregulation of the Notch intracellular domain (NICD) trafficking and nuclear entry and the impact of mechanical stress on heterochromatin dynamics and nuclear NICD interactions. This review aims to draw attention to the intricate interplay between mechanical cues and Notch signaling regulation, offering novel insights into the multifaceted nature of cellular mechanobiology.

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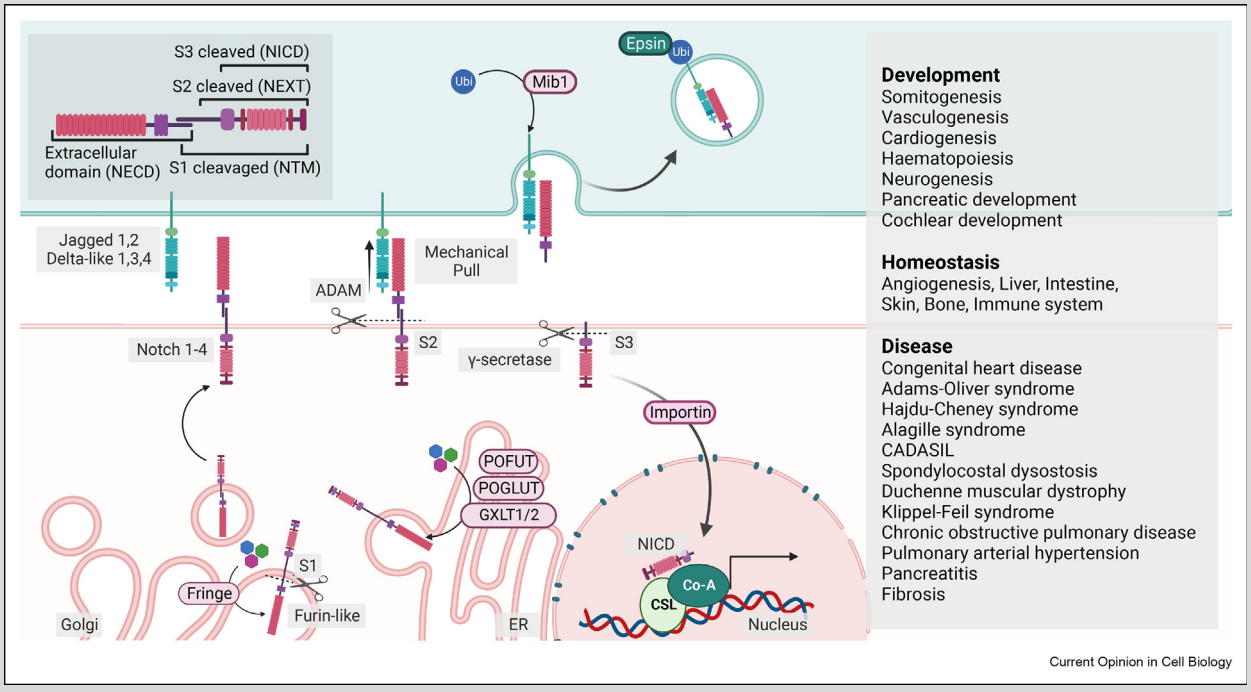
Introduction

Notch is a cell–cell signaling pathway that regulates cell fate decisions in many biological processes during development, homeostasis, and disease, and it can elicit different effects on cell behavior depending on the biological context [1]. Notch signaling is initiated between neighboring cells that express transmembrane ligands (Jagged1,2 and Delta-like 1,3,4) and receptors (Notch1-4). Following receptor-ligand interactions, the Notch receptor undergoes a series of cleavages releasing its intracellular domain (NICD), which translocates to the nucleus and induces transcription of target genes (Box 1). In this sense, the Notch receptor is a transmembrane transducer that can directly convert external input into transcriptional response without the need for additional enzymatic amplification, with such sensitivity that even small variations in the amount of NICD can lead to significant cellular response [2]. The transcriptional output of the Notch signal is controlled by the concentration and the temporal pattern of NICD [3,4]. For example, different ligands can produce distinct NICD signals over time, which can selectively regulate different target genes [3]. Therefore, the pathway outcome is likely sensitive to variations in the NICD signal caused by environmental stimuli. There is growing consensus that Notch signaling is subject to and involved in mechanoregulation. Notch signaling not only works in concert with other mechanosignaling machineries [5], but is also directly dependent on physical forces during its activation [6,7]. The recent line of direct and indirect findings suggests that mechanical forces affect how the Notch signal created at the membrane is conveyed to the nucleus and converted to transcriptional response (Figure 1). The following text will focus on the influence of mechanical forces in the creation and transmission of the Notch signal.

Post-translational regulation and processing

Not much is known about the way mechanical stress regulates the processing of Notch full-length (NFL). NFL processing starts in the endoplasmic reticulum

Box 1. Summary of the ligand-dependent Notch signaling.

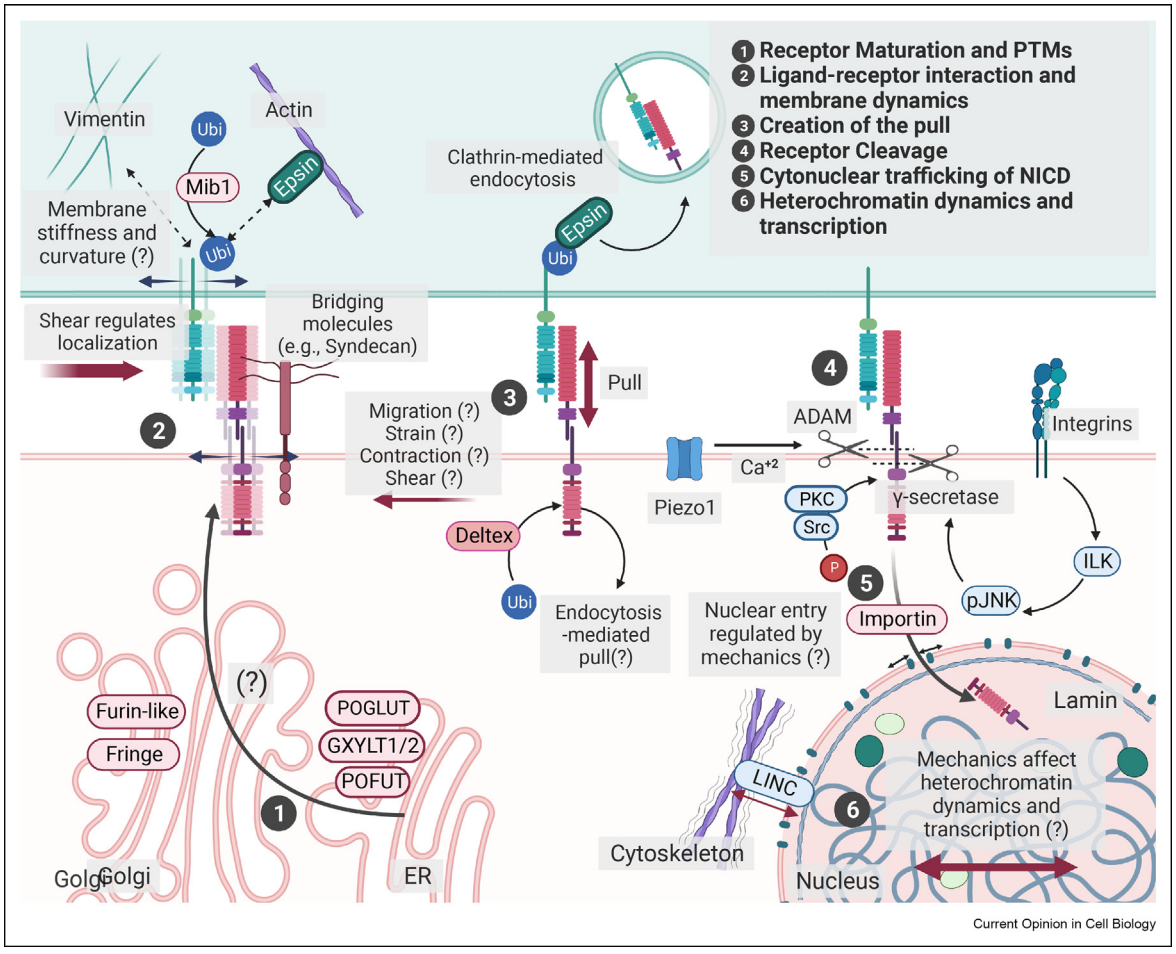


Notch is a contact-dependent pathway, that involves two neighboring cells expressing the Notch receptors (Notch1-4) and ligands (Jagged1,2 and Delta-like 1,3,4). The full-length Notch receptor (NFL) is synthesized as a single polypeptide and modified with sugar groups by e.g., POFUT, POGLUT and GXYLT in the endoplasmic reticulum. In the Golgi, the receptor is further modified by fringe glycosyl-transferases and goes through its first cleavage, S1, catalyzed by furin-like convertase yielding two fragments, the Notch transmembrane-intracellular domain (NTM), and Notch extracellular domain (NECD). On the membrane, once the receptor interacts with a ligand presented by a neighboring cell, it is subjected to a mechanical pull, which leads to two consecutive proteolytic cleavages, S2 and S3, on the NTM. These are catalyzed by metalloprotease ADAM and the γ-secretase complex (NTM) and yield the Notch extracellular truncated domain (NEXT) and the Notch intracellular domain (NICD), respectively. NICD translocates to the nucleus, where it interacts with CSL (CBF1, Suppressor of Hairless, Lag-1) and other transcriptional factors and drives gene transcription. The conversion of the NICD signal into transcription is dependent on its complex interactions with the hetero-chromatin. In the ligand-presenting cell, the ligand is endocytosed together with the receptor extracellular domain (NECD) and processed for further signaling or targeted for degradation. Notch signaling regulates development, homeostasis and diseases as reviewed by Siebel and Lendahl [1], some of which are directly related to mechanical stress [5].

(ER), where the addition of different sugar groups to the receptors will affect the ability of Notch to signal [8,9]. NFL is fucosylated by O-fucosyltransferases (POFUT) and glycosylated by O-glycosyltransferases (POGLUT) both necessary for ligand recognition, trafficking and interaction [8,9]. Some of these sugars will be further elongated with the addition of xylose groups by xylosyltransferases like GXYLT1 and GXYLT2, which affects Notch activation [10], trafficking [11] and ligand cis-inhibition [12]. Both this process and Notch signaling are regulated by ER stress [13,14]. At the same time, ER stress is influenced by mechanical stress [15–17]. This connection between mechanical and ER stress could modulate the Notch activation potential.

At the Golgi, N-Acetylglucosamine groups are added to the NFL by another group of glycosyltransferases named Fringes. This process is known to be critical for ligand-receptor binding [18] and specificity [19]; but its link to mechanical stress has not yet been elucidated. Before being transported to the membrane, a Furin-like convertase catalyzes the cleavage at site-1 (S1). Protein levels of similar enzymes to Furin-like convertase, like Furin, are also regulated by mechanical stress [20,21]. Finally, Notch is subject to post-translational modifications (PTMs), like phosphorylation by well-known mechanoregulated kinases like Src, Akt, ILK, and PKC. Antfolk and colleagues provide an exhaustive list of PTMs modification of Notch [22].

Figure 1



Possible mechano-regulated events during the transduction of the ligand-dependent Notch signaling. Ligand-dependent Notch signaling is subjected to mechanoregulation during multiple steps. **1)** Despite indications from other proteins, it is not known whether there is direct mechano-regulation of the Notch receptor maturation. **2)** Ligand and receptor interaction and membrane dynamics might be mechano-regulated. Limiting the lateral mobility of the ligand, likely through cytoskeletal interactions, membrane stiffness, formation of the endocytic complex, clustering or localization at membrane protrusions, leads to increased receptor signaling. Syndecans, which enforce Jagged1-Notch3 signaling through direct interactions, are mechano-regulated. Shear stress regulates the localization of the Notch receptors and ligands. **3)** Tension must be created to pull on the receptor-ligand complex and unfold the NRR domain. Ligand endocytosis by the signal-sender cell can create the sufficient pulling force. However, provided that the ligand mobility is limited, it is likely that endocytosis by the receiving cell, as well as other microforces arising from migration, strain, cellular contraction and shear stress, can also facilitate the pulling. **4)** Both the S2 and the S3 cleavages following the NRR unfolding are indirectly mechano-regulated. Calcium flux mediated by mechano-sensor Piezo1 regulates ADAM activity. γ-secretase complex activity is regulated downstream of integrin signaling in response to the mechanical cues from the extracellular matrix. **5)** Following the receptor cleavage, the NICD signal is still fine-tuned during its trafficking, e.g., by mechano-regulated kinases, such as PKC and Src. The nuclear entry of NICD is regulated by importins, which have been suggested to be mechano-sensitive. This process is also likely regulated by mechanical coupling of the nucleus to the cytoskeleton through the LINC complex, which might affect parameters, such as nuclear pore aperture in response to mechanical stress. **6)** Additionally, mechanical stress affects heterochromatin dynamics, which could impact how the nuclear NICD interacts with other transcriptional factors and target enhancers. This way mechanical stress might further fine-tune the Notch transcriptional readout also within the nucleus.

Localization of Notch receptors and ligands

Shear stress has been found to be an important factor to guide the localization of Notch receptors and to tune Notch signaling activity. Notch1 localizes downstream of flow, and Notch1 activity scales with shear stress magnitude. Notch1 maintains junctional integrity and serves an atheroprotective role [23]. Furthermore, shear stress triggers Dll4-mediated proteolytic cleavage of Notch1 to expose the transmembrane domain which regulates adherence junctions and the endothelial barrier in a non-canonical, transcription-independent fashion [24]. Shear stress also affects the localization, and expression of Notch ligands. Shear stress increases expression, intracellular re-organization, and the Notch

activation potential of Jagged1 in surrounding vascular smooth muscle cells [25,26], which has been shown to be important for arterial remodeling [26,27]*. On the other hand, low oscillatory shear increases Jagged1-Notch4 signaling in the endothelium and drives atherosclerosis [28]**. Dll1 and Dll4 have also been shown to be upregulated by shear stress to modulate arteriogenesis [29]. Even though a response to shear stress seems to be common among members of the Notch family, the specific mechanisms and consequence of the response differ which is likely related to shear stress magnitude and flow pattern as well as endothelial cell heterogeneity. Recently it was found that oscillatory shear stress induces nuclear localization of Notch 3 and not Notch 1 or 2 in microvascular endothelial cells during endothelial to mesenchymal transition in cerebral arteriovenous malformations [30]**.

Receptor-ligand interactions

While the exact mechanism of how mechanical forces act at the time of the receptor-ligand interaction is yet to be understood, evidence from other systems points to membrane−level interactions as a possible subject of mechanoregulation. For example, membrane-tethered molecules may need outward bending of the interacting membrane patches and reorganization and compression of longer membrane proteins, such as the components of the glycocalyx [31]. Such mechanical tension was suggested to partake in ligand discrimination by transmembrane receptors, such as T-cell antigen receptors [31,32]. In the case of Notch signaling, ligands Dll4 and Jagged1 both form catch-bonds with Notch1, which strengthen with applied tension despite their relatively low initial affinity for the Notch receptor. Thus, with applied force, the ligands can prolong the unfolded state of the negative regulatory region (NRR). The two ligands also have different affinities for Notch1 and have different tension thresholds to activate it [33,34]. Additionally, it is important to note that Notch ligands contain N terminal C2 domains facilitating lipid binding, which is important for their activity [35], and Jagged1 and 2 are much longer than delta-like ligands. Notch components can also interact with proteoglycans of the glycocalyx, one of the earliest identified mechanosensing complexes [36]. Proteoglycans, and in particular syndecans, are components of the glycocalyx known to regulate Notch signaling by directly binding to Notch and affecting its ability to signal [37]. Altogether, mechanical factors could have an important impact on the ligand-receptor kinetics in the intramembranous space and possibly play a role in ligand discrimination also by Notch receptors.

Dependence on the pulling forces and their creation

Notch ligands need to be membrane-tethered to activate the receptor. Soluble ligands can also bind the receptors but elicit an inhibitory effect on the Notch signaling [38*,39] with the exception that secreted ligands can activate Notch signaling in *C. elegans* [40]. The necessity for ligand tethering is partly explained by the autoinhibitory conformation of the Notch extracellular fragment, where the NRR folds over the S2 cleavage site to shield it from metalloproteases. Therefore, the ligand must mechanically pull on the receptor to unfold the NRR and expose the cleavage site [6,7], which has gained Notch its fame as a mechanoreceptor (Box 2). By modifying the NRR, it is possible to increase the force threshold, and render the receptor refractory to ligand-mediated activation [41]**. Consistently, when simply pulled on N-terminal SNAP-tagged Notch receptors with benzyl-guanine as a ligand, 9 pN of force alone suffices to activate the Notch receptor and overwrites ligand identity [42].

The exact mechanism of how the tension on the receptor-ligand complex is initiated remains unclear. The current line of evidence suggests that endocytosis of the ligand potentiates the necessary pulling for the NRR unfolding. During endocytosis, Mind bomb (Mib1) ubiquitinates the ligand ICD, availing epsin binding, which links the ligand ICD to the polymerizing actin filaments and allows its endocytosis through the clathrin-mediated pathway [43]. Indeed, the forces necessary for NRR unfolding and those created by ligand endocytosis match, ranging between 3 and 10 pN [42,44,45]. Complementary to the link between endocytosis and NRR unfolding, recent work showed that *C. elegans* lacks a structural element, “leucine plug”, which stabilizes the autoinhibitory conformation of

Box 2. Is Notch a mechanoreceptor?

The force requirement for Notch cleavage and the changes in localization and activation in response to mechanical stress underscores the importance of mechanical stress in regulating Notch signaling. That is why the constant reference of Notch as a mechanoreceptor is not surprising. Nonetheless, the authors contend that this label could be misleading. A mechanoreceptor is a protein that converts mechanical signals from the environment into chemical signals inside the cell. In contrast to better-characterized mechanoreceptors, such as Piezo proteins, integrins, the VEGFR2-PECAM-VE-Cadherin complex, and more recently, the receptor Plexin D1, Notch has not yet been demonstrated to directly detect and transmit the mechanical forces from the environment. Further investigation is needed to determine the precise involvement of the Notch receptor in the detection and transmission of mechanical stress. In the meantime, we propose using any of the following terms:

Mechanoregulated: A protein that requires mechanical stress to change its activation state.
Mechanoresponsive: A protein whose behavior is modulated by mechanical stress.
Mechanosensitive: A protein whose response is sensitive to specific levels of mechanical stress.

NRR. This eliminates the dependence on epsin-dependent endocytosis by tuning down the force required for receptor activation [46]**. Notably, substrate stiffness reversely correlates with NECD trans-endocytosis, but not overall endocytosis, suggesting an additional level of mechanoregulation at the sender cell level [47]*.

Despite the evident link between ligand endocytosis and receptor activation, other force-generating mechanisms are likely involved in creating the pulling force. *In vitro*, immobilization of the ligands on a culture substrate alone can activate the receptor [48]. Similarly, restricting the lateral diffusion of recombinant DLL4 on supported lipid membranes leads to increased Notch reporter activity [49]. In this scenario, micro-movements of the signal-receiving cell could create tension on the spatially restricted ligand-receptor complex in the absence of endocytosis by a signal-sender cell. For example, endocytosis of the receptor by the signal-receiving cell or simply its migration could pull on the tethered ligand-receptor complex. Indeed, Deltex4 has been shown to mediate endocytosis of the receptor, which is then cleaved by ADAM10 in an intracellular compartment [50]. Additionally, Jagged1, but not Delta-like, was shown to interact with vimentin [51]. Cytoskeleton anchoring could possibly restrict the lateral diffusion of the ligand and even conduct the cellular contractions through the receptor-ligand complex for additional pulling. The necessity for additional force-generating mechanisms for Jagged1 would make sense since Jagged1 requires a higher pulling force than Dll4 to activate the Notch1 [33]. The Notch receptor might also be subjected to similar factors, such as restricted lateral diffusion and cytoskeleton linkage, which could convert the cellular micromovements to pulling forces. Altogether, these mechanisms could impact the Notch signaling (e.g., by contributing to the ligand discrimination) in different tissue contexts, where the cytoskeleton composition, as well as the mechanical factors, such as extracellular matrix/tissue stiffness, shear stress, and strain vary significantly.

Mechanical regulation of Notch cleavage following NRR unfolding

As summarized in Stassen et al. [5], the proteins responsible for the next two cleavages of Notch are directly related to mechanosignaling pathways. After interacting with the ligand and the S2 cleavage site, the metalloproteinase ADAM is allowed to cut and induce conformational changes important for further Notch signaling transduction. ADAM is sensitive to changes in Ca^{2+} levels, and its ability to cleave Notch is regulated by the mechanoreceptor Piezo1, which consequently regulates the expression of Notch target genes [52]. This process has been shown to be important for outflow track valve development in zebrafish [53]. Subsequently, the Notch receptor undergoes S3 cleavage, which is mediated by the multi-subunit protease complex γ-secretase. The activation of this complex can be induced by the integrin-linked kinase (ILK) through c-Jun N-terminal kinase (p-JNK) [54]. ILK is known to be activated by integrins in response to mechanical cues from the extracellular matrix [55,56]. Recently it was discovered that γ-secretase recruitment, ligand-receptor engagement, and proteolytic cleavage are spatially and mechanically regulated by adherent junctions in membrane microdomains [57]**. This cleavage releases the Notch intracellular domain (NICD) from the membrane leaving a transmembrane domain embedded in the plasma membrane.

Notch cyto-nuclear trafficking and transcription

Studies on the mechanoregulation of Notch signaling have focused mostly on the events up until the release of NICD. In later events, NICD must transverse the nuclear envelope and form a complex with its co-factors and interact with the heterochromatin to regulate transcription. Growing evidence beckons at the nucleus as a hub for mechanosignaling. Mechanical stress can induce changes in the nuclear envelope architecture, the nuclear pores, and the deposition of epigenetic marks and heterochromatin dynamics [58,59]. Thus, Notch signaling might be subjected to mechanoregulation both around and within the nuclear envelope. There is currently limited evidence on the regulation of NICD nuclear transport. NICD bears nuclear localization signals (NLS) that are recognized by nuclear transport molecules importins, which aid its nuclear entry [60]. Additionally, nuclear pore components, nucleoporin 88 and 214, have been shown to repress Notch signaling by ejecting RBPJ from the nucleus [61]. While it is not clear whether these processes are subject to mechanoregulation, a recent study suggests that importin 7 mediates the nuclear import of YAP and that YAP competes with other cargo such as Smad3 [62]. Therefore, it would not be surprising if common mechanosensitive mechanisms regulate the nuclear entry of the NICD and its binding partners, considering that e.g., NICD was shown to directly interact with YAP [63].

In the nucleus, the conversion of the Notch signal to transcription is regulated by the DNA-binding dynamics of the NICD/CSL complex and its interactions with other transcription factors. NICD-mediated transcription is stochastic and bursty, but the binding of tissue-specific transcription factors to target enhancers can mediate robust and sustained conversion of NICD to transcriptional response [4]. Considering this intricate machinery, it is likely that Notch signaling readout is sensitive to mechanically induced changes on the

Box 3. Open questions

What is the role of the different Notch receptors and ligands in mechanotransduction?

- Do different Notch receptors have different force requirements for activation?
- Is force an important parameter in ligand preference of different Notch receptors?
- Are the Notch ligands and receptors differentially regulated by different magnitudes and types of mechanical stress?
- Does mechanical stress affect ligand-independent Notch signaling?

Does mechanical stress regulate Notch receptor-ligand interactions and modifications ?

- Are cis-activation and cis-inhibition regulated by mechanical stress?
- Are receptor- and ligand-specific posttranslational modifications regulated by mechanical stress?

How do mechanics regulate Notch receptor and ligand trafficking and membrane organization?

- Is the interaction between Notch components and trafficking proteins regulated by mechanical stress?
- Is the organization and localization of Notch receptors and ligands on the membrane regulated by mechanical stress?

How is Notch maturation regulated by mechanical stress?

- Does mechanical stress regulate NFL sugar modification?
- Can PTMs work as memory markers of mechanical stress in Notch?
- Does mechanical stress regulate Furin expression, localization or activity?

Through which mechanisms does the ligand exert force on the receptor?

- Is endocytosis the main driver of the pulling force?
- What role does the cytoskeleton play in facilitating ligand pulling of the receptor?
- How are cellular protrusions such as filopodia involved in the formation of force-dependent activation domains at the membrane?
- Can other mechanisms such as receptor or ligand clustering override the force requirement?

How is S2 and S3 cleavage regulated by mechanical stress?

- How do different patterns and sources of mechanical stress affect the formation of Notch proteolytic domains?
- Are the proteolytic hotspots regulating Notch activation dependent on cytoskeletal organization?
- Can mechanics affect the Notch signal dosing and transcriptional output through cleavage regulation?

Does mechanical stress regulate NICD transcriptional activity?

- Is nuclear translocation of NICDs regulated by mechanics?
- Is NICD binding to CSL regulated by mechanics?
- Does mechanical stress affect the ability of NICD to bind to DNA through changes in the heterochromatin availability?

What approaches can be used to manipulate Notch mechanosignaling?

- Can we control Notch's response to mechanical stress in a predictive manner by modifying mechanosensitive processing or post-cleavage events?
- Is it possible to manipulate Notch sensitivity to mechanical stress by modifying other domains of the Notch receptor outside of the NRR?
- Can engineered molecules, such as fusion proteins, short peptides and antibodies, targeting the NRR be used to control S2 cleavage in response to mechanical stress?
- Can we engineer ligands and ligand presentation methods that can fine-tune Notch activation through mechanics with spatiotemporal specificity?

heterochromatin. Indeed, it was shown that mechanical stress arising from gastrulation leads to pronounced transcriptional activity by some Notch-responsive enhancers. This effect was not explained by increased receptor activation or nuclear NICD import, but was altered when the LINC complex (the linker of nucleoskeleton and cytoskeleton) was disturbed, arguing that it is instead directly coupled to the mechanical stress on the nucleus–cytoskeleton axis [64]**.

Conclusion

From the processing of NFL in the ER to the transcriptional activation by NICD, we discuss links between mechanical stress and Notch signaling. Mechanical forces

regulate more than just the S2 cleavage of the Notch receptor. From the production of the Notch receptor and its localization and activation at the membrane to the transcriptional dynamics of free NICD, many steps of the pathway are possible subjects for mechanoregulation. At the same time, Notch regulates many proteins and pathways necessary for a homeostatic response to mechanical stress. Although the link between mechanics and Notch signaling is evident, there are still many remaining open questions, as summarized in Box 3. The answers to these questions might help clarify "context-dependent" consequences of Notch in different tissues, where the physical niche, the transcriptional circuitry, and the expression of Notch components are inherently different. A better understanding of the interplay between mechanical stress and Notch signaling could thus shed light on how Notch signaling steer cell fate decisions in many processes, such as morphogenesis, and cancer development, where cellular forces are predictory. Furthermore, mechanics will likely be an important design consideration in therapeutic and regenerative applications targeting Notch signaling. It is clear that Notch is essential for mechanosensing and mechanotransduction. The field of Notch in mechanobiology is ever-growing, and more research is still needed to elucidate the most obscure parts of the pathway.

Methods

Figure 1 and Box 1 were created with BioRender.com under agreement number WO25DPB13E and BW25DUU1YB respectively.

Author contribution

Conceptualization and writing: FSR, SS, and CS; Reference management: FSR; visualization: SS; supervision and funding acquisition: CS.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

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