

Notch in mechanotransduction – from molecular mechanosensitivity to tissue mechanostasis

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ABSTRACT

Tissue development and homeostasis are controlled by mechanical cues. Perturbation of the mechanical equilibrium triggers restoration of mechanostasis through changes in cell behavior, while defects in these restorative mechanisms lead to mechanopathologies, for example, osteoporosis, myopathies, fibrosis or cardiovascular disease. Therefore, sensing mechanical cues and integrating them with the biomolecular cell fate machinery is essential for the maintenance of health. The Notch signaling pathway regulates cell and tissue fate in nearly all tissues. Notch activation is directly and indirectly mechanosensitive, and regulation of Notch signaling, and consequently cell fate, is integral to the cellular response to mechanical cues. Fully understanding the dynamic relationship between molecular signaling, tissue mechanics and tissue remodeling is challenging. To address this challenge, engineered microtissues and computational models play an increasingly large role. In this Review, we propose that Notch takes on the role of a ‘mechanostat’, maintaining the mechanical equilibrium of tissues. We discuss the reciprocal role of Notch in the regulation of tissue mechanics, with an emphasis on cardiovascular tissues, and the potential of computational and engineering approaches to unravel the complex dynamic relationship between mechanics and signaling in the maintenance of cell and tissue mechanostasis.

KEY WORDS: Notch signaling, Mechanotransduction, Engineered model systems, Computational modeling, Cardiovascular mechanics

Introduction

Biological tissues constantly respond to their environment to obtain and maintain biological, chemical and mechanical homeostasis. Changes in the mechanical state lead to cellular responses promoting growth and/or remodeling to restore homeostasis. Loss of mechanical homeostasis can trigger the onset of mechanopathologies, including osteoporosis, myopathies, fibrosis and, ultimately, organ failure (Ingber, 2003; Humphrey et al., 2014; Pickup et al., 2014). Cardiovascular development and disease are textbook examples of processes intricately intertwined with changes in tissue mechanics.

Tissues emerge from the collective mechanical properties and behavior of individual cells, and cell fate decisions are at the core of tissue homeostasis. To understand tissue homeostasis, it is essential

to understand the processes that integrate the different mechanical and biomolecular cues driving cell fate decisions. The receptors for mechanical cues have been identified as an ensemble of membrane proteins, channels, structural cytoskeletal proteins and mechanosensory organelles, such as the cilium and the cell nucleus (Ferreira et al., 2019; Kirby and Lammerding, 2018). These protein complexes either actively probe the environmental mechanics by exerting force on their surroundings, or sense loads exerted on the cell by changing conformation (Hoffman et al., 2011). In doing so, they convert the mechanical signals into biochemical signals through the process of mechanotransduction (Kindberg et al., 2020; Martino et al., 2018). Understanding how mechanical signals affect cell and tissue functionality can enable the development of medical approaches to restore tissue homeostasis and advance strategies for tissue repair, engineering and regeneration (Drews et al., 2020; Stassen et al., 2017; Wissing et al., 2017). Several components of cellular mechanotransduction cascades have been identified, but a comprehensive picture of the molecular translation from distinct mechanical cues to cell fate decisions remains to be established. Novel mechanosensitive proteins are regularly discovered, such as the recent discovery of plexin D1 as a mechanosensitive receptor (Mehta et al., 2020), with more likely awaiting discovery.

The Notch signaling pathway has an important role in cell fate decisions (Artavanis-Tsakonas et al., 1999) and tissue organization (Sjöqvist and Andersson, 2017). Notch is evolutionary ancient, and has been proposed to have evolved from the fusion of several transmembrane proteins with functions in cell adhesion (Murata and Hayashi, 2016). This evolutionary heritage, combined with the traditional cell–cell signaling function, places Notch at a position to integrate cues coming from the extracellular matrix (ECM), mechanics and direct cellular neighbors (as reviewed by LaFoya et al., 2016). In this Review, we describe recent studies that have uncovered interactions between Notch and mechanical forces, and highlight the tools that might be adopted for the advancement of this field in the future. Given the central role of Notch and mechanics in cardiovascular development and disease, we emphasize cardiovascular tissues, although our conclusions can be generalized to other tissues.

Notch, cell fate and mechanics – a mechanotransducing feedback loop through reciprocal interactions

The canonical Notch signaling pathway has been thoroughly investigated (see Box 1 for an overview). Briefly, Notch ligands interact with Notch receptors on juxtaposed cells. This triggers cleavage of the Notch intracellular domain (NICD), which then translocates to the nucleus and regulates transcription (Bray and Gomez-Lamarca, 2018; Kopan and Ilagan, 2009). Recently, it has been uncovered that mechanical forces regulate Notch signaling via direct or indirect interactions (Gordon et al., 2015; Mack et al., 2017). Conversely, Notch regulates transcriptional programs that are involved in cell fate, including ECM homeostasis, cell and tissue morphogenesis, and cell and tissue mechanics (Lloyd-Lewis et al., 2019; Loerakker et al., 2018). This gives rise to a

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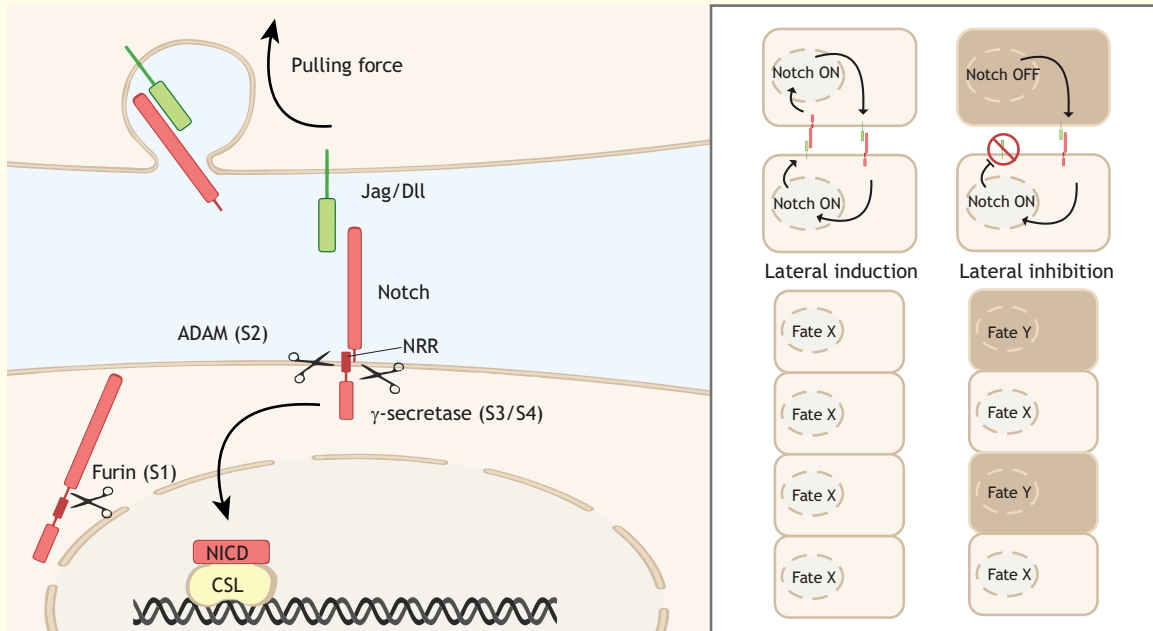
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Box 1. Brief overview of Notch signaling regulation

The canonical signal carried by Notch is linear – a ligand on a cell membrane binds a receptor on the membrane of a neighboring cell, activating a transcription factor complex in the signal-receiving cell (see left-hand panel of the figure). In humans, the pathway consists of the receptor proteins Notch1, Notch2, Notch3 and Notch4, and the ligand proteins delta-like-ligand (Dll)1, Dll3, Dll4, jagged 1 (Jag1) and jagged 2 (Jag2) (Dll/Jag in left-hand panel) (Mašek and Andersson, 2017). Both receptors and ligands consist of an extracellular domain, a transmembrane domain and an intracellular domain. The receptor precursor is cleaved (S1 cleavage) into a heterodimer by a furin-like-peptidase before insertion into the cell membrane (Logeat et al., 1998). Ligand-receptor binding can activate the receptor by exposing a hidden extracellular site in the negative regulatory region (NRR) to peptidases. ADAM10 or ADAM17 cleaves this site (S2 cleavage) to form the Notch extracellular truncation, which is subsequently recognized by the γ -secretase complex that cleaves inside the transmembrane domain and releases the Notch intracellular domain (NICD) from the membrane (S3 and S4 cleavage) (Kopan and Ilagan, 2009; Shah et al., 2005). The released NICD can then bind transcription factor complexes through CSL [an abbreviation based on the names in different species; CBF1/RBPJ κ , Su(H), Lag-1], and either drive or inhibit transcription (Bray and Gomez-Lamarca, 2018), thereby either inducing similar cell fate (lateral induction), or alternating cell fate (lateral inhibition) (right-hand panel of the figure).

The activity and output of Notch signaling is regulated at many levels (Antfolk et al., 2019; Kovall et al., 2017). Availability of ligands and receptors is determined through expression (Chakravarti et al., 2017; Shah et al., 2017), protein stability (Varshney and Stanley, 2018) and trafficking towards the membrane for functional presentation (Itoh et al., 2003; Shao et al., 2017; Takeuchi et al., 2017). The activating cleavage steps are regulated through the control over the peptidases involved in the cleavage and the availability of the S2 target domain, as further discussed in the main text. Apart from its cleavage, the regulation of the NICD also affects signal output, which is influenced by stability of the NICD and available cofactors in the nucleus (Bray and Gomez-Lamarca, 2018).



reciprocal interplay between Notch and mechanics with feedback mechanisms in place, enabling Notch to be an important regulator of mechanical homeostasis.

The most direct interaction between mechanical force and Notch signaling is at the stage of receptor activation, that is, a set of cleavage steps to release the NICD from the membrane. During this process, a hidden peptidase-binding site in the extracellular negative regulatory region of Notch is exposed by a conformational unfolding step (Fig. 1) (Chowdhury et al., 2016; Gordon et al., 2015; Morsut et al., 2016). Unfolding is induced by a pulling force acting on the receptor that is mediated by ligand endocytosis after receptor binding. This endocytic pulling force is dependent on dynamin, epsin and actin (Parks et al., 2000; Seugnet et al., 1997). Epsin targets ubiquitylated ligands for clathrin-mediated endocytosis and contributes to membrane bending (Langridge and Struhl, 2017). Dynamin forms a helical multimer that generates force to pinch off vesicles, whereas actin is essential in generating sufficient force to drive endocytosis of the ligand–receptor assembly (Meloty-Kapella et al., 2012). The ability of the ligand–receptor pair to withstand the pulling force depends on

their molecular affinity and load-bearing capacity. Interestingly, the Notch ligands jagged 1 (Jag1) and delta-like ligand 4 (Dll4) have distinct mechanical characteristics. The binding of both Jag1 and Dll4 to Notch1 gives rise to a catch bond, a type of bond that strengthens when it is put under tension (often compared to a Chinese finger trap). However, the tension required for Notch activation is different for the two ligands (4 pN for Dll4 and 12 pN for Jag1) and has been suggested to be due to an increased binding rate of Dll4 (Luca et al., 2017). These two ligands are known to have different effects and roles, for example in angiogenesis, inner ear development and airway differentiation (Benedito et al., 2009; Petrovic et al., 2014; Stupnikov et al., 2019). The different force magnitudes required for Notch activation could thereby differentiate between the ligands.

Recent findings also indicate that there is a direct interaction between cytoskeletal or contractile forces and Notch activation. Inhibition of non-muscle myosin II to reduce contractility in signal-sending cells reduces Notch activation *in vivo* (Hunter et al., 2019). This reduction is independent of the presentation of ligands or receptors on the cell surface, and it is additive with the effect from

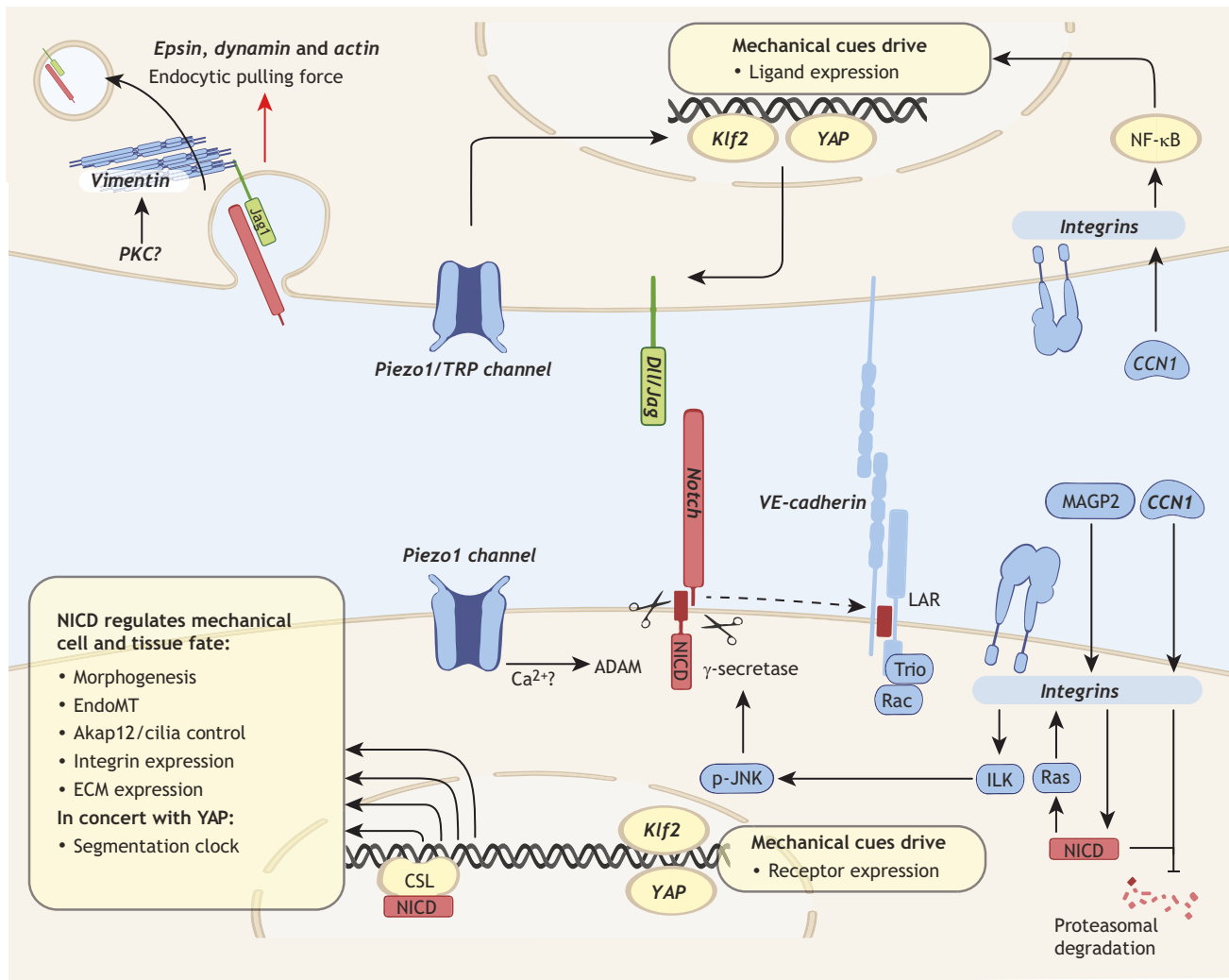


Fig. 1. Direct and indirect links between Notch signaling and mechanical signaling. Several mechanotransductive proteins (bold italic in the figure) regulate the expression of Notch receptors or ligands. In addition, Notch reciprocally influences several mechanosensitive pathways, mechanical cell fates and morphogenesis. The top cell represents main interactions between mechanics and Notch ligands, Delta-like ligand (Dli) or Jagged (Jag). Mechanical loading sensed by integrins and mechanosensitive Piezo and transient receptor potential (TRP) channels result in YAP-, Klf2- and NF-κB-mediated ligand transcription, regulating Notch signaling. Proteins responsible for generating endocytic forces (epsin, dynamin and actin) unfold the hidden receptor cleavage site for ADAM10/17. Phosphorylation of vimentin by mechanosensitive kinases, possibly PKC, increases Jag1 endocytosis. The bottom cell represents the main interactions between mechanics and Notch receptors. Integrins are activated by a combination of extracellular matrix (ECM) composition, e.g. cellular CCN1 and MAGP2, and mechanical cues; this affects transcription, cleavage activity and Notch intracellular domain (NICD) stability. Ca²⁺ influx can regulate ADAM10 activity, possibly coupling Piezo1-mediated mechanosensing to Notch cleavage. The mechanosensitive transcription factors Klf2 and YAP also regulate Notch transcription, depending on cell context, and, in concert, Notch and YAP orchestrate the segmentation clock. Notch activation controls several mechanical fates as indicated. CSL, CBF1/RBPJκ, Su(H), Lag-1; LAR, leukocyte common antigen related.

dynamin or epsin inhibition, indicating separate contributions of contractile and endocytic forces (Hunter et al., 2019).

Similar to intracellular forces, external forces appear to have an activating effect on Notch. In particular, the timing of flow-induced activation of Notch also points towards a direct role for Notch in shear sensing, as the levels of NICD increase as early as 60 or 30 min after the onset of shear (Fang et al., 2017; Masumura et al., 2009). This rapid onset can however be blocked by inhibition of vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2; also known as KDR), which could indicate both involvement of a mechanosensitive complex or a canonical VEGF-dependent downstream signal (Coon et al., 2015; Lawson et al., 2002; Masumura et al., 2009). However, the quick activation still allows for transcriptional to post-translational regulation of Notch ligands

as the point of control (Shimojo et al., 2008). In fact, the mechanoresponsiveness of the Notch transmembrane domain was inhibited by Dll4 knockout or inhibition of endocytosis, suggesting that the regulation of Dll4 could control this mechanoreponse (Polacheck et al., 2017). Recent findings indicate that the mechanosensitive channel Piezo1 might tune the activity of Ca²⁺-sensitive ADAM10 in mediating the cleavage-dependent activation of Notch through Ca²⁺ ions, but uncoupling this increased S2-cleavage (see Box 1) from increased ligand production is challenging (Caolo et al., 2020). For these reasons, the evidence for a direct Notch activation by shear stress or stretch is still inconclusive.

Many interactions between Notch and mechanics are indirect. This includes the mechanical regulation of the production of ligands

or receptors, and the crosstalk between Notch and mechanosensors (Fig. 1). Yes1-associated transcriptional regulator (YAP; also known as YAP1) and WW domain-containing transcription regulator 1 (TAZ; also known as WWTR1) (collectively YAP/TAZ) are transcriptional regulators that respond to diverse mechanical cues, including extracellular matrix rigidity, cell shape and shear stress (Panciera et al., 2017). Despite recent evidence for a crosstalk between YAP/TAZ and Notch in angiogenesis (Neto et al., 2018), the specific role of this crosstalk is still under investigation. More details have been uncovered in other tissues. For example, during myogenesis, the onset of contractions induces YAP translocation to the nucleus, where it drives expression of Jag2 and subsequent Notch activation (Esteves de Lima et al., 2016), which is associated with the maintenance of the progenitor cell pool required for regeneration (Bröhl et al., 2012), whereas in epidermal stem cells, YAP/TAZ regulates the expression of Notch-inhibiting ligands (Totaro et al., 2017). In the segmentation clock, a molecular oscillator that times the proper segmentation of developing somites, YAP and Notch work in concert to integrate mechanical cues with molecular signals and synchronize cell behavior over the segmenting tissue (Hubaud et al., 2017). The integrins are also mechanosensors that interact with Notch (Fig. 1). Upon activation of integrin linked kinase (ILK), a phosphorylated c-Jun N-terminal kinase family protein (p-JNK) is translocated to the membrane where it activates the γ -secretase complex and increases NICD, whereas direct phosphorylation of NICD by ILK has been shown to decrease NICD half-life, pointing to ILK-mediated regulation of NICD dynamics (Miyagawa et al., 2019; Mo et al., 2007). Cellular communication network factor 1 (CCN1) and microfibril-associated glycoprotein 5 (MAGP2; also known as MFAP5) are matrix proteins that regulate Notch through integrins. CCN1 is a shear-responsive matricellular protein (Hsu et al., 2019). CCN1 acts on integrins, thereby inducing expression of Dll4 in endothelial cells (Chintala et al., 2015) and of Jag1 through nuclear factor κ B (NF- κ B) in pancreatic ductal carcinoma and the hepatic duct (Haque et al., 2012; Kim et al., 2015). Further substantiating the link between integrin activation and NICD dynamics, is the increase in NICD levels seen upon CCN1 signaling inhibiting proteasomal degradation (Haque et al., 2012). In contrast, the ECM protein MAGP2 binds integrins to phosphorylate NICD through Src, reducing NICD transcriptional activity and half-life (Deford et al., 2016; LaFoya et al., 2018). Vice versa, Notch influences integrin activation through both transcriptional and non-transcriptional mechanisms; in fact NICD can directly activate R-RAS, a small GTPase that activates integrins (Hodkinson et al., 2007). Therefore, Notch is involved in crosstalk with other mechanosensitive signals, which enlarges the range of mechanical cues influencing this important signaling pathway in diverse tissues. In the context of cardiovascular tissues, these interactions might enable a fine-tuning of the Notch response to cell contractility (e.g. via YAP) or blood shear stress (e.g. via CCN1).

The production of Notch receptors and ligands is influenced by mechanics in several tissues. One of the most striking examples is the flow-induced increase or decrease in the levels of Notch receptors and ligands in endothelial cells (ECs) during development (Choi et al., 2017; Samsa et al., 2015; Volz et al., 2015). This is essential for the proper development of the heart, as shown in zebrafish, where heart contraction and subsequent blood flow induce *notch1b* transcription in the endocardium (Samsa et al., 2015). Similarly, the expression of Jag1 in the coronary artery endothelium depends on the onset of flow (Volz et al., 2015). Flow-induced transcription of Notch signaling components is both cell

and tissue dependent. For example, in lymph vessels, flow activates the Ca^{2+} channel *Orai1*, which mediates Notch degradation, subsequently allowing lymph vessel sprouting (Choi et al., 2017).

Notch determines cell fate, which in turn strongly influences tissue mechanics. Since an increase or decrease in cell number leads to a change in mechanical stresses within tissues, one way Notch affects mechanics is by driving or inhibiting proliferation and apoptosis. The exact role of Notch in these processes depends on the specific tissue context and the specific Notch receptor and ligand (Dang, 2012; Gao et al., 2013; Liu et al., 2009). Notch influences tissue mechanics by regulating ECM expression in diverse tissues, including cartilage (Mirando et al., 2013), skin (Dees et al., 2011) and heart (Del Monte-Nieto et al., 2018). In the endocardium, this depends on the ligand expressed, as Jag1 regulates the production of ECM, while Dll4 mediates cell adhesion (Torregrosa-Carrión et al., 2019). A bidirectional relationship between Notch and mechanics also is revealed for cilia, as shear induced cilia activation induces Notch1b, Jag1b and Dll4 production in zebrafish, while cilia length and proper mechanosensing are controlled by Notch activation through regulation of A-kinase anchoring protein 12 (Akap12) (Chen et al., 2017; Mukherjee et al., 2020). The fact that Notch is influenced by, and in turn adapts tissue mechanics to attain mechanical homeostasis suggests that Notch could be a key component of the cellular ‘mechanostat’. The term mechanostat was introduced in the context of bone remodeling, describing an equilibrium between the load typically exerted on a bone and the minimal strength of a bone across organisms (Frost, 1987). The concept that any tissue needs to be strong enough to withstand the forces it is typically exposed to, makes the mechanostat a likely ubiquitous mechanism, with Notch as a central candidate.

Notch and mechanics in cardiovascular tissues

Cardiovascular tissues develop and function under mechanical load. From 3 weeks after fertilization until the heart stops beating, there are approximately three billion heartbeats during a human lifetime. The heart and vasculature are constantly exposed to forces derived from blood flow and cellular contractions. Cardiovascular tissues develop to exert and withstand these forces, and they remodel when mechanical homeostasis is perturbed. Mechanically induced remodeling of cardiovascular tissues has an important role in physiology and pathology and is controlled by many different mechanotransduction pathways, as excellently reviewed previously (Baratchi et al., 2017; Hahn and Schwartz, 2009).

The cardiovascular system is exposed to a broad range of dynamic stretches and fluid shears. Small vessels never experience stretch, but the aorta can experience a circumferential stretch of 10%, whereas heart valves can experience up to 30% stretch (Morrison et al., 2009; Oomen et al., 2016; Sacks and Yoganathan, 2007). The ECs, the cells lining the inside of the cardiovascular system, are the main cells exposed to wall shear stress (WSS) from blood flow, and experience from 0.1 Pa in the venous system to 1 to 7 Pa in arterial vessels, depending on the location in the vascular tree (Malek et al., 1999). Physiological WSS elicits several physiological responses, including alignment of ECs, formation of the vascular barrier (Polacheck et al., 2017), vascular remodeling (Lucitti et al., 2007) and vasodilation or vasoconstriction to regulate blood flow (Pyke and Tschakovsky, 2005). Disturbed shear stress (DSS) is an important factor in various diseases, including atherosclerosis, aneurysms and collateral vessel formation (Eitenmüller et al., 2006; Hahn and Schwartz, 2009; Humphrey et al., 2015). Cellular forces are also important, as contractility and migration of cells are essential for tissue development and homeostasis, such as in the case of sprouting

angiogenesis, the process of blood vessels growing new branches (Vaeyens et al., 2020).

During cardiac development, Notch integrates with other signaling pathways to shape the architecture and functionality of the heart and heart valves (MacGrogan et al., 2018). The role of Notch in cardiovascular development makes it a potential therapeutic target in cardiovascular disease (Box 2) and cardiovascular regeneration (Gálvez-Santisteban et al., 2019; Kefalos et al., 2019; Münch et al., 2017). In vascular development, Notch signaling is crucial for the regulation of both vasculogenesis (i.e. *de novo* generation of novel blood vessels) and angiogenesis (Alabi et al., 2018; Hellström et al., 2007), as well as hemogenesis, arteriovenous specification and maturation of the vessels (Chen et al., 2017; Gama-Norton et al., 2015; Lawson et al., 2001; Manderfield et al., 2012; Porcheri et al., 2020; Volz et al., 2015). The importance of the interplay between Notch and mechanics is evident at several stages of cardiac development. Early in cardiac development, Notch has an important role in regulating the endothelial–mesenchymal transition (EndoMT) of the endocardium; it initiates the process of trabeculation, the formation of myocardial protrusions into the ventricle that are compacted again later in development, and triggers migration of the endocardium to form heart valves (Grego-Bessa et al., 2007; Timmerman et al., 2004). This process requires the onset of blood flow and endothelial primary cilia, as ablation of either component reduces the expression of *notch1b*, *dll4*, *jag1*, and *jag2* and prevents trabeculation in zebrafish (Samsa et al., 2015). Interestingly, the different oscillatory and pulsatile flow profiles

caused by the flow disturbance of the trabecula determine Notch activity at different sites of the endocardial lining, leading to different cell fate decisions (Fig. 2B) (Lee et al., 2018). In zebrafish heart valve development, oscillatory flow occurs before heart valves have formed. This activates the mechanosensitive channels Trpv4, Trpp2 and Piezo1 that antagonistically regulate Krüppel-like factor 2 (Klf2), a hallmark shear-responsive factor upstream of Notch expression (Fig. 2B) (Duchemin et al., 2019; Heckel et al., 2015). This flow-induced Notch expression and activation subsequently controls valvular cell EndoMT (Pestel et al., 2016; Timmerman et al., 2004). Damaging valves, and thus disturbing flow patterns, induces Notch reactivation and regeneration of valves (Kefalos et al., 2019). In adulthood, Notch is involved in preventing valve calcification, both in valvular endothelial cells and valvular interstitial cells. Loss of a Notch copy is sufficient to sensitize the cells to mechanical stimulation and calcification (Chen et al., 2015; Theodoris et al., 2015).

Notch is a marker for arterial vessels (Swift and Weinstein, 2009). Arterial vessels have a thicker vascular wall, due to a prominent layer of vascular smooth muscle cells (vSMCs), which serve to regulate blood flow and to withstand arterial pressures. Notch signaling occurs both among neighboring ECs and neighboring vSMCs, as well as between ECs and vSMCs (Fig. 2). There are multiple reports about the link between shear stress, Notch activation and arteriovenous fate. This functional link depends both on the organism and organ (Buschmann et al., 2010; Deng et al., 2013; Quillien et al., 2014). In chicken yolk, blood flow determines arteriovenous specification in an interplay with Notch, which is essential for expression of ephrin B2, a hallmark arterial gene (Buschmann et al., 2010; le Noble et al., 2004). In contrast, arteriovenous specification in zebrafish takes place before the onset of flow; here, Notch determines a base pattern of 60% arterial and 40% venous vessels in the zebrafish trunk, which is further balanced to a 50:50 ratio by flow (Geudens et al., 2019; Isogai et al., 2003; Weijts et al., 2018). However, the importance of shear- and strain-regulated expression of Notch for arteriovenous specification and maintenance remains to be further clarified (Gore et al., 2012). Notch signaling is required for Foxc1b-mediated vSMC recruitment (Chen et al., 2017) and differentiation towards a contractile phenotype (Ando et al., 2019; Manderfield et al., 2012). Expression of Notch3 and Jag1 in the wall is reduced by exposure to stretch (Loerakker et al., 2018; Morrow et al., 2005), and this mechanism could play a mechanostatic role by determining the arterial wall thickness in a Notch- and mechanics-driven feedback loop (Loerakker et al., 2018).

Distinct Notch responses, depending on cell type and mechanical cues, occur in the vessel wall. In ECs, different ligands and receptors display distinct responses to shear stress. Jag1 transcription increases under laminar shear of 1 Pa, whereas this downregulates Dll4 (Driessen et al., 2018). Shear stress also induces a reorganization of both Notch1 and Jag1 in the cell, with Notch1 accumulating on the membrane downstream of the shear direction, whereas Jag1 appears in submembranous foci (Fig. 2) (Driessen et al., 2018; Mack et al., 2017). Ligand-mediated Notch activation is also increased by shear stress (Masumura et al., 2009; Polacheck et al., 2017). Dll4 and Jag1 are known to have distinct roles in regulating cell fate in angiogenesis (Benedito et al., 2009), valvular development (MacGrogan et al., 2016), ventricular development (D'Amato et al., 2016) and hemogenesis (Porcheri et al., 2020). Therefore, the distinct effects of shear stress on the expression and localization of different Notch ligand and receptor proteins, in combination with the specific force magnitudes required for Notch

Box 2. Notch mutations and deregulation in cardiovascular disease

Mutations or misregulation in Notch pathway genes can lead to congenital and acquired cardiovascular malformations (Mašek and Andersson, 2017). Defects in *NOTCH1* can cause aortic valve disease (AoVD) (Garg et al., 2005). Mutations in *JAG1* or *NOTCH2* can give rise to Alagille syndrome, with cardiac symptoms similar to tetralogy of Fallot (McDaniell et al., 2006; Spinner et al., 2001). Mutations in *RBPJ* (*CSL*), *NOTCH1*, *DLL4*, and *EOGT* (a Notch-modifying N-acetylglucosamine transferase) can lead to Adams–Oliver syndrome, with 20% of patients having cardiac defects (Chapman et al., 2020; Meester et al., 2015). Mutations in *NOTCH3* have been shown to cause cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), but the pathogenesis is under intensive investigation (Joutel et al., 1996; Rajani et al., 2019; Rutten et al., 2019). Mutations in mindbomb1 (*MIB1*), a regulator of receptor and ligand endocytosis, lead to left ventricular non-compaction cardiomyopathy, where trabeculae are not compacted (Luxán et al., 2013). Among the acquired cardiovascular defects is pulmonary arterial hypertension, during which Notch1 and Notch3 control pathogenic proliferation of ECs and vascular smooth muscle cells (vSMCs), respectively (Dabral et al., 2016; Li et al., 2009; Miyagawa et al., 2019). Owing to vascular wall instability in thoracic/abdominal aortic aneurysm, Notch is likely to have a role, although this is still not clear (Li and Kong, 2020). Dll4 binding to Notch3 and Jag1–Notch1 interactions have been implicated in diabetic vasculopathy and diabetic retinopathy (Miloudi et al., 2019; Wimmer et al., 2019). In cerebral cavernous malformations, Notch also plays a role in the aberrant EndoMT transition and vascular malformations (Kar et al., 2016). Similarly, in arteriovenous malformations, where shunts form between arteries and veins, Notch is upregulated in human patients, and upregulation of Notch1 or Notch4 causes the disease in mouse models (Murphy et al., 2009; Nielsen et al., 2016). In vascular conditions with prominent inflammatory components, Notch ligands also determine cell fate, such as in large vessel vasculitis, vascular graft lesions, or arteriovenous graft remodeling (Guo et al., 2020; Koga et al., 2015; Wen et al., 2017).

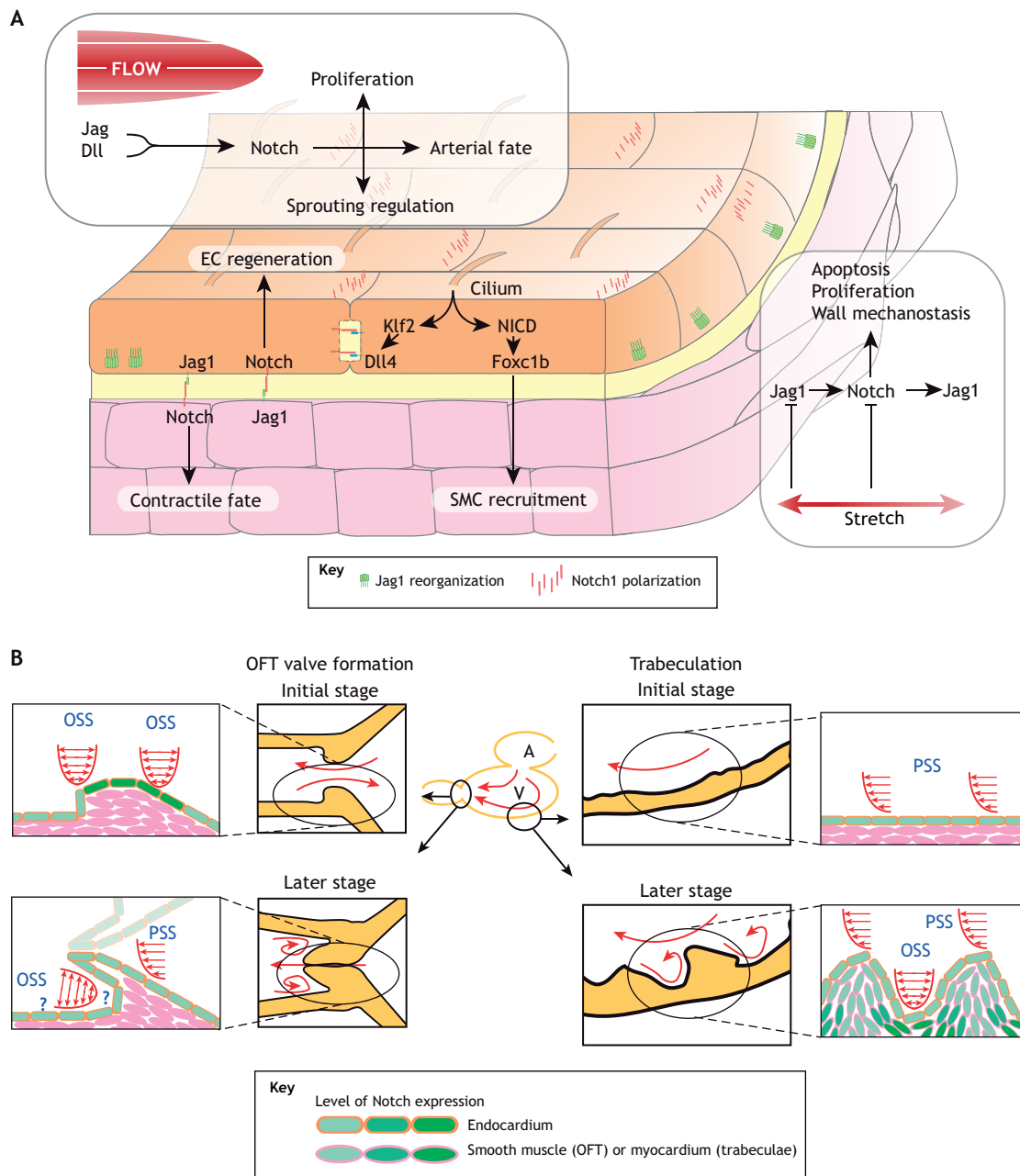


Fig. 2. Interactions of vascular mechanics with Notch signaling and subsequent vascular cell fate effects. (A) Effect of hemodynamic forces on Notch and vascular cell fate. Blood flow induces ligand transcription, partially through cilia, and Notch activation in endothelial cells (ECs) through Klf2, subsequently leading to Notch activation in neighboring ECs or vascular smooth muscle cells (vSMCs). Notch1 and Jag1 reorganize in the cells, with currently unknown function. In ECs, a balance of Jag1- and Dll4-mediated Notch signaling leads to decisions on sprouting, proliferation, regeneration and arterial fate adoption. Notch activation leads to Foxc1b-mediated vSMC recruitment to the vessel and Jag1-mediated contractile fate adoption in vSMCs. Stretch induces proliferative fate through Jag1 inhibition, possibly as a mechanism for restoring homeostasis upon loss of mechanical balance. (B) Localized hemodynamic forces in zebrafish heart induce Notch and morphogenesis. In zebrafish cardiac development, blood flows from the atrium ('A') to the ventricle ('V'), to the bulbus, or outflow tract (OFT), influencing Notch and morphogenesis. During heart valve formation, pulsatile and oscillating shear stress (PSS and OSS) induce Notch activation, which is required for heart valve morphogenesis (shown on the left). In the ventricle, blood flow initiates Notch mediated trabeculation (shown on the right). The local shear patterns induce a specific Notch response required for morphogenesis. Cells shown in pink are not described to have active Notch in these stages of development. The schemes illustrating trabeculation have been republished with permission of JCI Insight from Lee et al., 2018; permission conveyed through Copyright Clearance Center, Inc.

activation by these ligands (Luca et al., 2017), are further lines of evidence that mechanical cues are a major regulator of Notch signaling and thus cell fate and function.

Recent discoveries have provided more insight into the molecular mechanisms responsible for Notch mechanosensitivity to blood flow. For example, a novel flow-dependent Notch signal

transduction pathway has been shown to be involved in the maintenance of the vascular barrier (Polacheck et al., 2017). Exposure to flow induces ligand-mediated cleavage of the Notch receptor, with S2-cleavage releasing the Notch extracellular truncation and S3-cleavage releasing the NICD, while the short transmembrane domain in between these two cleavages remains in

the membrane (Fig. 1). This transmembrane domain forms a complex with the tyrosine phosphatase LAR (PTPRF), the Rac1 guanine exchange factor (GEF) Trio and VE-cadherin, stabilizing cell–cell junctions and the vascular barrier (Fig. 1) (Polacheck et al., 2017).

Recently, we have demonstrated that the mechanosensitive intermediate filament protein vimentin modulates Notch signaling in vascular cells (Antfolk et al., 2017; van Engeland et al., 2019). Vimentin binds the intracellular domain (ICD) of Jag1, but not Dll4, increasing the Jag1 signal sent to neighboring cells. In fact, Jag1 endocytosis and signaling strength are reduced in vimentin-knockout mice, resulting in Dll4 signaling being dominant in the endothelium, consequently reducing sprouting. Vimentin deletion also drives vSMC fate to the proliferative state, indicating that both EC–EC and EC–vSMC signaling is hampered (Antfolk et al., 2017; van Engeland et al., 2019). Vimentin phosphorylation on serine 38 is increased after exposure to shear stress, and phosphorylation increases the signal-sending capacity of Jag1 (van Engeland et al., 2019). Introduction of the mechanoresponsive protein kinase C (PKC), known to target vimentin (Nakayama et al., 2003; Eriksson et al., 2004), or introduction of the vimentin variants phosphomimicking PKC target sites, both enhance Notch transactivation (Antfolk et al., 2017; van Engeland et al., 2019). This raises the interesting possibility that vimentin responds to shear stress by interacting with PKC and, once phosphorylated, increases the Jag1-mediated Notch activation, thereby forming a mechanosensitive cell-fate-controlling axis.

As described above, Notch interaction with mechanics plays a key role in heart development, heart valves and vascular tissues. Despite recent discoveries, a comprehensive understanding of how mechanics and Notch are integrated in cardiovascular tissues at the molecular level still needs to be fully achieved. This will require technologies that allow for analyses of cell signaling and cell fate under controlled hemodynamic environments in real time, as well as methods to translate molecular-level information on mechanosignaling into tissue-level effects on form and function.

Engineered systems to addressing open questions of Notch signaling

Many biophysical tools to mechanically or spatially control cells or proteins, including optical and magnetic tweezers, microfluidics and microcontact printing, have been key in attaining novel discoveries in mechanobiology (Monteiro et al., 2018; Qin et al., 2010). Some of these tools have already been implemented to investigate Notch signaling as previously reviewed (Lovendahl et al., 2018), while other techniques have the potential to lead to breakthroughs in some of the long-standing questions in the Notch field (highlighted in Box 3). In particular, engineered tools can help to control mechanical, molecular and cellular variables, thereby enabling the investigation of the effects of these variables on Notch, while keeping other variables approximately unchanged. For example, identifying the contribution of the different Notch components to the mechanosensitivity of this pathway will require mechanical stimulation combined with cellular engineering or synthetic biology to separate the mechanosensitivity of different molecular components. The importance of dynamics in Notch signaling is emerging, as evidenced by the role of Notch oscillations in stem cell maintenance, the dose dependence of cell fate decisions, and specific receptor–ligand combinations resulting in different temporal activation curves (Nandagopal et al., 2018; Shimojo et al., 2008; Theodoris et al., 2015). To couple dynamic molecular activity to tissue morphogenesis requires real-time following of systems

Box 3. Main questions pertaining to Notch in mechanotransduction

- How sensitive is Notch to the dosage and dynamics of mechanical cues?

This is essential for the proposed role as a mechanostat. If Notch is sensitive to mechanical load dosage and its temporal dynamics, then it can trigger compensating responses in the cell.
- What is the systemic result of several mechanical cues acting on different Notch components and cell types interacting together?

Answering this question will increase our understanding of the integration of mechanics and cell fate signaling for tissue morphogenesis and homeostasis.
- Do the different Notch components have distinct mechanotransductory properties?

Dll4 and Jag1 require different force magnitudes to activate Notch (Luca et al., 2017). Additional differences among Notch components might fine-tune Notch mechanosensitivity.
- Are the peptidases that are involved in Notch cleavage mechanosensitive?

Piezo-mediated Ca^{2+} influx might influence ADAM10 activity, while membrane tension may affect γ -secretase activity, and these mechanisms might be important in physiology and pathology.
- Which mechanosensitive proteins interact with Notch?

Further enlarging the set of mechanosensitive pathways cross-talking with Notch and identifying the specific crossroads will potentiate pharmaceutical targeting.
- Are there mechanosensitive processes influencing Notch protein trafficking and polarization and, if so, what is their role in cell fate?

In ECs, Notch1 and Jag1 reorganize under shear stress (Driessen et al., 2018; Mack et al., 2017). Similar mechanisms might be present in other cell types and for Notch components involving different molecular machineries, and might have consequences in cell fate and tissue morphology.
- Are the dynamics of Notch signaling integrated with mechanical dynamics?

Notch temporal dynamics, signal strength and oscillations are crucial for cell fate decisions and morphogenesis. Mechanical cues are inherently dynamic, and therefore they might play a role in establishing Notch dynamics.
- Is the mechanotransductory role of Notch specific to different cell types, and what conveys this specificity?

Cell type specificities with respect to Notch mechanosensitivity might inform new therapeutic options that are more tailored to the target tissue.

instead of end-point analyses, and engineered culture systems enable these studies.

Elucidating the molecular machinery around Notch activation that contributes to its mechanosensitivity (Caolo et al., 2020; Mo et al., 2007) will require more screening approaches for molecular interactors. To achieve this, cells must be exposed to forces, either at the macroscopic or microscopic scale, with various methods available (Box 4). Identification of mechanosensitive interactors of Notch signaling might be possible through high cellular yield shear approaches, for example, using an orbital shaker (Driessen et al., 2020). To separate the effect of mechanical loading on the molecular receptor–ligand interaction from the cellular regulation of these components, techniques with higher temporal and spatial resolution of Notch readout, such as luminescent constructs (Ilgan et al., 2011), are required in conjunction with mechanical stimulation.

Verifying that Notch functions as a mechanostat will require engineered systems with optimal control over mechanical loading, or with force gradients built into the system, for example, a Y-shaped fluidic channel (Mack et al., 2017). In fact, to function as a

Box 4. Engineered approaches to study mechanics in cell biology

Many of the questions on Notch mechanotransduction can be addressed using engineered approaches. These allow the study of diverse aspects, such as cellular transcriptional responses, cell–cell interactions, morphogenesis, and cell–matrix interactions under mechanical cues. Mechanical cues that can be presented to cells include fluid-flow-induced shear stress, stretch, compression or viscoelasticity of the environment. Shear stress is typically imposed on cells by using parallel plate geometries (Driessen et al., 2018; Mack et al., 2017), cone-plate shear systems (Kunnen et al., 2017) or orbital shakers (Driessen et al., 2020), depending on the requirements (e.g. amount of cells for analysis and level of control over the fluid flow). Variations of the orbital shaker, using annular shaped dishes or seeding patterns, have recently opened novel possibilities by enhancing control over flow patterns (Driessen et al., 2020; Ghim et al., 2018). Cells can be stretched by culturing them on cyclically stretched flexible membranes (Loerakker et al., 2018; Morrow et al., 2005) or in deformable 3D microtissues (Foolen et al., 2012; Gould et al., 2012). Compression is applied by culturing cells in a gel or in a well and compressing the gel or culture medium (DiFederico et al., 2017; Manokawinchoke et al., 2017). Viscoelasticity can be controlled by culturing cells on hydrogels or lipid bilayers (Chaudhuri et al., 2020).

There is also a rapid development of bioengineered systems that more closely resemble (micro)physiological conditions, although they typically offer less control over mechanical variables. Many of these systems provide a 3D environment, resulting in different behavior of signaling pathways compared to that seen in 2D systems (Cukierman et al., 2001; Duval et al., 2017). Spatial constraints in 3D systems perturb mechanical cues, thereby possibly affecting cell behavior (Chen et al., 2018), although the effects of these constraints are difficult to control and quantify. Different types of bioengineered systems include organoids (Sato et al., 2009) and predefined or self-organizing vascular systems in gels (Polacheck et al., 2017; Wimmer et al., 2019). The microfluidic vessel-wall-on-a-chip offers control over independent cellular and mechanical variables in cell–cell contact situations (Huh et al., 2010; van Engeland et al., 2018).

mechanostat, Notch should either have a mechanical set point encoded in the pathway and its interactors, or a very high sensitivity to signaling dosage. To understand the effect and regulation of signaling dynamics (Nandagopal et al., 2018; Shimojo et al., 2008) and the interactions of mechanical forces with temporal dynamics, it is necessary to develop systems with temporal control over mechanical loading, as well as Notch readout. Microfluidic chips have been implemented before to pace the rate of oscillations in Notch (Sonnen et al., 2018), and could form a way to investigate the effect of periodicity in mechanical loading, as imposed by the heart beat (Ubezio et al., 2016).

Studying mechanics at the cellular and molecular level is challenging as there is overlap with topographical, or spatial, variables, as occurs for example in integrin and ephrin signaling, where specific spatial organization is essential for the signaling output (Westerfield and Barrera, 2020). Topographical control of ligand presentation can enable a more detailed investigation of Notch activation by mechanical forces. An early study showed that surface-immobilized Dll1 could activate Notch, whereas soluble ligands could not (Varnum-Finney et al., 2000). In contrast, Jag1-derived peptides did not activate Notch in a surface-immobilized form, possibly due to being sterically unavailable to the receptor, but activated Notch when they were self-assembled in solution (Putti et al., 2019). Making use of microcontact printing techniques, which allow the deposition of proteins in predefined patterns, has proven useful to spatially control cell fate (Tiemeijer et al., 2018),

and if applied at subcellular resolution could help in resolving the effect of polarized Notch activation.

The distinct Notch responses to flow and stretch and the different cell types making up mechanically activated tissues, can result in interactions that are challenging to understand, but which are still fundamental for the physiology of cardiovascular tissues. To characterize these system-level outcomes it is necessary to allow separate control of flow and stretch and to separately manipulate and monitor different cell populations, to obtain mechanistic insight. A device that enables this is the recently developed vessel-wall-on-a-chip (Box 4), based on a lung-on-a-chip design (Huh et al., 2010), as it allows two cell populations to communicate via Notch through pores, mimicking the myoendothelial projections that are sites of EC–vSMC Notch signaling *in vivo* (McCallinhardt et al., 2020), while separating interacting ECs and vSMCs, which can thus be subjected to different levels of flow and stretch (van Engeland et al., 2018).

While microfluidic devices can be employed to separate variables influencing Notch and mechanics, tissue-engineered constructs are a step closer towards mimicking the complexity of multicellular tissues *in vitro*. These constructs might be employed both on a level of fundamental understanding and for more translational purposes, such as towards the development of regenerative medicine approaches. These engineered tissues can be used to study how the long-term interplay between Notch and mechanics regulates cell fate and morphogenesis. For example, a bioreactor with a controlled pressurized tissue system has been recently developed to study the long-term effect of constant strain versus constant stress on tissue growth (van Kelle et al., 2017). We expect that these controlled engineered systems will have a great impact on the field, especially when coupled with computational models, as discussed next.

Computational models coupling Notch and mechanics

Despite recent developments, it is unlikely that engineered systems will ever fully control all variables influencing Notch and mechanics. Computational models can help in overcoming this limitation, owing to their potential to predict and isolate the effects of single parameters, and to carefully analyze cell and tissue mechanics. Several computational models of Notch have been proposed and have demonstrated their impact in testing hypotheses and generating new theories based on model predictions (Binshtok and Sprinzak, 2018). For example, computational simulations of sprouting angiogenesis accounting for the crosstalk between Notch and VEGF were fundamental to suggest that ECs shuffle at the top of angiogenic sprouts (Bentley et al., 2008), a computational prediction that was later verified *in vivo* (Jakobsson et al., 2010). In the context of Notch mechanosensitivity, computational models can enable the investigation of the effects of this phenomenon at a systemic level, as they can be adopted to analyze cell and tissue mechanics, growth, and remodeling, while accounting for Notch signaling among different cell types.

A direct relationship between Notch and mechanics was first included in a computational model of collective epithelial cell migration (Riahi et al., 2015). Because the authors observed *in vitro* that cells leading the migration exhibit high Dll4 levels and that perturbation of cell contractility affects the number of leading cells, they hypothesized that stress downregulates Notch1 and Dll4 and that this feature is crucial for the regulation of the spatial distribution of leading cells. To test this hypothesis, building on previous computational models of Notch–Delta lateral inhibition (Cohen et al., 2010; Collier et al., 1996; Sprinzak et al., 2010), they developed a computational model accounting for Notch downregulation in response to stress (Riahi et al., 2015). In particular, they assumed that Notch1 and Dll4 production decreases with the cell

distance from the migration leading edge, where lower stress levels had been experimentally observed. The experimental finding that Dll4-expressing cells appear at the leading edge could be computationally replicated only when Notch mechanosensitivity was considered, thereby supporting the notion that this phenomenon contributes to regulating the distribution of leading cells during epithelial cell migration (Riahi et al., 2015). Therefore, computational modeling was used here to verify the hypothesis that Notch mechanosensitivity played a role in the observed biological phenomenon.

Although hypothesis verification via modeling is useful, the added value of computational models is more evident when a full cycle of interplay between experiments and simulations is implemented, with simulations suggesting new theories and experiments. Recently, we have shown the potential of coupling experiments and simulations to study the systemic effects of the interplay between Notch and mechanics in arterial walls (van Engeland et al., 2019; Loerakker et al., 2018; Ristori et al., 2020). Using *in vitro* experiments, we first showed that cyclic strain downregulates Notch3 and Jag1 expression in vSMCs (Loerakker et al., 2018). To understand the implication for arterial remodeling, we extended a previous model of Notch that accounted for Notch–Jagged lateral induction (Boareto et al., 2015), present in vSMCs (Manderfield et al., 2012), by assuming that the production of Jag1 and Notch3 decreases with increasing strain (Loerakker et al., 2018). Coupled with mechanical analysis of arteries with different thickness, the new model predicted that vSMCs in relatively thin arteries experience high strain and thus have low Notch activation, corresponding to proliferative vSMCs and growing arteries. The strain decreased with arterial thickness and, when the arterial wall thickness reached a specific value, the model predicted that Notch signaling induces a collective switch of vSMCs from proliferative to contractile, corresponding to homeostatic non-growing arteries. The thickness for which the model predicted cell fate switching for arteries at different locations of the arterial tree corresponded well with their *in vivo* homeostatic thickness (Loerakker et al., 2018). Therefore, the model identified a new mechanism by which Notch mechanosensitivity might regulate arterial thickness and homeostasis. In a subsequent study, the same model was crucial for screening of the possible molecular mechanisms causing *in vivo* arterial thickening after vimentin knockout (van Engeland et al., 2019). In particular, the new simulations suggested that vimentin depletion disrupts Jag1-mediated Notch activation in vSMCs, which was later confirmed by additional *in vivo* experiments (van Engeland et al., 2019). More recent simulations of the interplay between Notch and mechanics in arteries suggest that Notch lateral induction is key in ensuring the collective switch of vSMCs from proliferative to contractile in growing arteries (Ristori et al., 2020), a computational prediction that hopefully will be verified in future experimental studies. Taken together, these studies therefore demonstrate the potential of computational models in translating findings from *in vitro* experiments to a higher level of complexity, such as *in vivo* cell behavior, and in generating new hypotheses about the underlying molecular mechanisms.

In these previous computational studies accounting for Notch mechanosensitivity, the local tissue mechanics was only approximated. Future studies should aim at overcoming this common limitation by adopting concepts from computational biomechanics to increase the predictive and analytical potential of computational models. Recently, a few computational modeling approaches have been proposed to analyze the local tissue mechanics and the consequential mechanosensitive behavior of interacting cells (Nolan and Lally, 2018a,b; Rouillard and Holmes, 2014; Thorne

et al., 2011; Zahedmanesh and Lally, 2012). Although these approaches have not been applied to Notch signaling yet, we anticipate that they might be promising in further elucidating the fundamental role of Notch mechanosensitivity in cardiovascular tissue development and disease.

Conclusions

Despite the increasing amount of studies linking mechanical forces to Notch signaling, mechanistic detail is lacking. One pressing question in the Notch field is the distinct role of the different ligands and receptors, and how the combinatorial communication through distinct receptors and ligands gives rise to different outcomes in different tissues. Knowing which components of the Notch pathway are specifically sensitive to mechanical cues and determining which mechanosensitive proteins interact specifically to regulate Notch, will help researchers to understand this combinatorial code. This potentiates the development of directed therapies for Notch-related diseases and mechanopathologies. Examples of avenues where further mechanistic detail might emerge are the mechanosensitivity of regulatory microRNAs targeting Notch, as these are closely involved in tuning Notch activity and generating its oscillatory dynamics (Roese-Koerner et al., 2017), or the effect of mechanics on NICD stability and transcriptional activity through post-translational modifications (Antila et al., 2018; Wiedermann et al., 2015). The axis involving the mechanosensor PECAM, VE-cadherin, VEGFR, vimentin, Jag1 and Notch proteins is likely of importance in vascular biology (Conway et al., 2013; Coon et al., 2015; van Engeland et al., 2019; Polacheck et al., 2017), but the hierarchy of the signals remains to be clarified. In general, the role of the cell membrane, membrane transport and endo- and/or exocytotic processes in response to mechanical forces is a relevant field of study (Boulant et al., 2011; Shi et al., 2018), since membrane dynamics are intrinsically important for both mechanotransduction and Notch signaling, with a recently identified lipid-interacting domain on Notch ligands with as of yet unknown function of particular interest (Suckling et al., 2017). The direct and indirect reciprocal signaling between Notch and mechanics, both at the cellular and the tissue level, places Notch as an integrating hub candidate that is essential for the maintenance of mechanostasis. Systemic approaches, including computational modeling, *in vivo* experimental designs and advanced engineered culture systems, will prove essential to further unraveling this mechanosensitive signaling hub and establishing its role as a mechanostat.

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Competing interests

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