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Review Article

Signalling dynamics in embryonic development

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In multicellular organisms, cellular behaviour is tightly regulated to allow proper embryonic development and maintenance of adult tissue. A critical component in this control is the communication between cells via signalling pathways, as errors in intercellular communication can induce developmental defects or diseases such as cancer. It has become clear over the last years that signalling is not static but varies in activity over time. Feedback mechanisms present in every signalling pathway lead to diverse dynamic phenotypes, such as transient activation, signal ramping or oscillations, occurring in a cell type- and stage-dependent manner. In cells, such dynamics can exert various functions that allow organisms to develop in a robust and reproducible way. Here, we focus on Erk, Wnt and Notch signalling pathways, which are dynamic in several tissue types and organisms, including the periodic segmentation of vertebrate embryos, and are often dysregulated in cancer. We will discuss how biochemical processes influence their dynamics and how these impact on cellular behaviour within multicellular systems.

Introduction

Communication between cells coordinates the self-organization process of embryonic development. Cell proliferation, differentiation, migration and death have to be regulated across scales — from single-cell to organism level — to allow the formation of an organism from a single cell. When cells communicate, an extracellular signal often induces an intracellular signal transduction cascade, which results in a cellular response encompassing, for instance, changes in cytoskeleton, metabolism or gene expression. Regulatory and feedback mechanisms at every level of such transduction cascades modulate pathway activity over time [1]. This leads to temporal variations in signalling, e.g. transient, pulsatile, ramping or oscillatory activity, collectively termed signalling dynamics [2]. Signalling oscillations are, for example, found in neural stem cells and the developing pancreas [3–5]. Dynamic changes in signalling can also occur upon changes in the levels of signalling molecules surrounding a cell, for instance when a cell moves through a spatial gradient of signalling molecules. Such gradients, also termed morphogen gradients, have been shown to organize the embryo into its anteroposterior (head to tail), dorsoventral (back to front), proximodistal and left-right axes [6,7].

Function of signalling dynamics

While feedback mechanisms might initially have evolved to keep signalling activity in check and prevent, for instance, overactivation, the resulting signalling dynamics have diverse functions in model systems found today [8,9].

Dynamic signal encoding

Often, signalling dynamics are used to encode and transmit biological information in the dynamics (Figure 1A). This has two consequences for the signalling process: (A) Encoding information in dynamics can make the signal more robust to noise and ensure proper transmission. Noise naturally occurs in a cell as a result of all biochemical reactions, leading to random fluctuations in, e.g. gene expression, protein–protein interactions, post-translational modifications and metabolic changes.
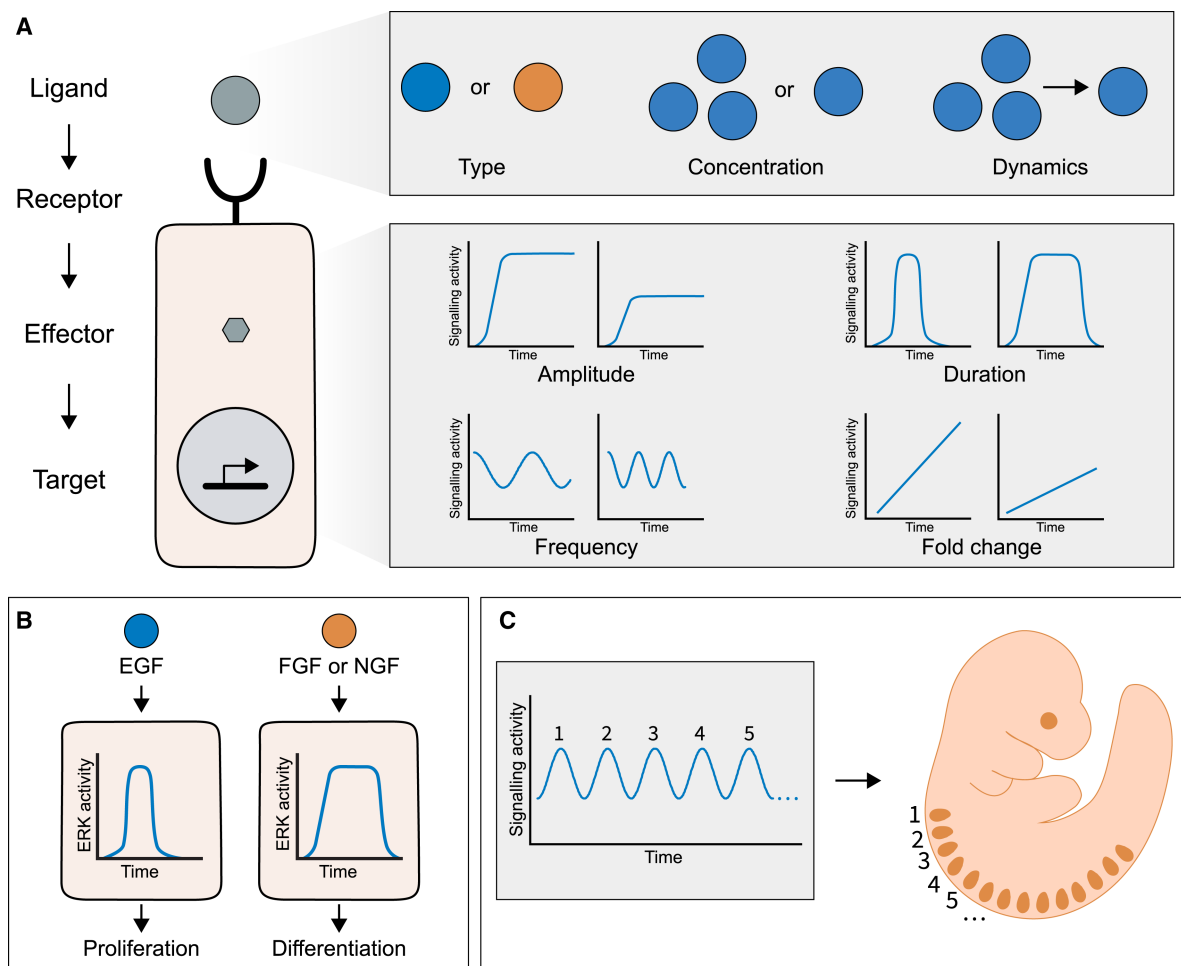
Figure 1. Cellular signalling dynamics.

(**A**) Signal transduction is induced when a ligand interacts with the receptor, which leads to the activation of effector proteins. Effectors exert various functions in cells, among which is the induction of gene expression. The type, concentration and dynamics of ligands can be encoded in signalling dynamics, such as the amplitude of a stable signal, the amplitude and duration of a transient signal and the amplitude, duration and frequency of an oscillatory signal or the fold-change of a ramping signal. (**B**) An example of dynamic signal encoding is the effect of growth factors on PC12 cells. EGF induces a transient signal of Erk activity, which results in proliferation, while FGF or NGF induce a sustained signal, which leads to differentiation. (**C**) Oscillatory signals can coordinate periodic events, such as the sequential segment formation in vertebrate embryos.

Therefore, information transmission is less accurate and precise when encoding information in the absolute signalling activity. Such information loss can be reduced or prevented by encoding information in the dynamics of ERK activity, Ca^{2+} concentration or nuclear localization of the transcription factor NFκB [10,11]. In Xenopus embryos, the fold-change in Wnt signalling activity controls development even with varying levels of baseline Wnt signalling [12]. (B) Encoding information in the dynamics increases the versatility of signalling pathways [13]. How a dozen of signalling pathways can transmit diverse information with high specificity in organisms has been a matter of investigation for decades. Besides employing distinct isoforms of pathway components, combinatorial use of multiple pathways and activation of specific transduction cascades, varying the dynamics is another means to increase the repertoire of signalling modalities [14]. A classic example is the stimulation of the rat PC12 (pheochromocytoma) cell line by growth factors. While epidermal growth factor (EGF) induces a transient ERK signal resulting in proliferation, nerve growth factor (NGF) or fibroblast growth factor (FGF) activate a sustained ERK response leading to neuronal differentiation (Figure 1B) [15–17].

Regulation of repetitive events

Apart from transmitting information in the dynamics of a signal, oscillations can control periodic events. This way, oscillations of the circadian clock adjust physiological processes of the body to the repeating day-night cycles [18]. In embryonic development, oscillations regulate the sequential segmentation of vertebrate embryos (Figure 1C) [19] and the consecutive steps of larval development in *Caenorhabditis elegans* [20].

Communication across tissues

Tissue-wide oscillation dynamics can synchronize cells within a multicellular system to regulate collective behaviour. It allows the efficient transfer of information from one side of a tissue to the other. This is exemplified by the coordination of tissue growth with segmentation in vertebrate embryos to ensure the formation of properly sized embryos [19].

How dynamics are decoded to induce specific cellular responses is still largely unknown. For dynamic signal encoding, the change in signal activity over time has to be detected and converted to an absolute signal, such as the expression of differentiation markers. In a ramping signal, information can be encoded in its derivative, while in an oscillatory signal, the frequency, amplitude or number of oscillations can encode information (Figure 1A) [2]. It has been shown that in mouse and human cell cultures, the number of NFκB oscillations is directly converted to a stepwise expression of different sets of target genes [21–23].

Signalling dynamics from a biochemical perspective

Intracellular signalling is initiated when a ligand from a signal-sending cell interacts with a receptor on a signal-receiving cell. Signalling can be autocrine, when signal-sending and -receiving cell coincide, paracrine, when neighbouring cells communicate with each other, or endocrine, when signal-sending and -receiving cells are spatially separated and signalling molecules travel via the circulation as in the case of hormones. An intracellular signalling event is initiated upon ligand–receptor interaction, entailing protein–protein interactions, post-translational modifications, proteolytic cleavages, protein translocations or lysosomal degradation of pathway components. These ultimately lead to changes in enzyme activities, which modulate e.g. metabolism or cytoskeleton, and to changes in gene expression. The dynamics of this signalling process directly depend on the thermodynamics of the system itself, the kinetics of the individual reactions and induced feedback mechanisms (Figures 1, 2A).

The expression levels of all involved signalling components and their isoforms can vary widely in a cell type- and developmental stage-dependent manner [24–27]. The concentrations of receptors on the cell surface and ligands and their respective binding affinities and geometry are critical determinants of the maximal and actual extent of pathway activation. In combination with the stoichiometry, affinity of protein–protein interactions, e.g. the ligand–receptor interaction, the concentration of all components and their interactions collectively influence the equilibrium of steady-state signalling. The intracellular signalling cascades often involve enzymatic reactions, such as post-translational modifications, proteolytic cleavages or degradation of inhibitory components via the proteasome [28] that occur at the second to minute time-scale. In most cases, this culminates in the activation of transcription factors (TFs) that shuttle to the nucleus, where they directly or indirectly modulate gene expression. When labelling these TFs with fluorescent proteins, nuclear accumulation can be detected within minutes after stimulating cells with a ligand [29,30]. In mice, the resulting gene expression, including transcription, splicing, mRNA export and translation, leads to the generation of proteins within at least 20 min in mice [31–33]. Thus, pathway stimulation can result in the appearance of new proteins within 25 to 30 min, but can also take significantly longer.

When ligand stimulation ends, the whole signal transduction cascade is reset to baseline. While post-translational modifications, e.g. protein phosphorylation, are reversible, protein cleavage and degradation cannot be reversed, which has immediate implications for the dynamics of a pathway. For instance, the MAPK (mitogen-activated protein kinase) pathway consists of a series of phosphorylation events. In many cell types, the effector kinase MAPK shows pulsatile cycles of activation/ inactivation with a period of a few minutes [34]. In contrast, dynamics of other pathways building on, for instance, proteolytic cleavage of pathway components take more time to reverse, since the active protein needs to be re-expressed.

Besides pathway inactivation by ligand–receptor dissociation or degradation, signalling activity of most pathways is actively modulated via feedback mechanisms. Negative feedback can result in the inactivation of the pathway at various levels of the cascade. For instance, the binding of ligands to their receptor can trigger the

Figure 2. Processes influencing the timing of dynamics.
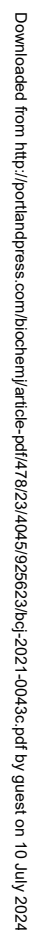
(**A**) Signalling is initiated when ligands interact with their receptors. Intracellular signalling can encompass various processes, which ultimately lead to the activation of effector proteins. These effector proteins have diverse functions, often modulating gene expression. Two potential signal transduction cascades and biochemical parameters that can influence the dynamics of a signalling pathway are indicated in the scheme. On the left-hand side, an effector protein is activated by dissociation from an inhibitory interaction partner. On the right-hand side, the effector protein is activated by phosphorylation. (**B**) A delayed negative feedback system can induce oscillations, for instance, if the pathway induces the expression of a negative regulator. Stable oscillations occur if parameters for activation and delayed inhibition are synchronized. (**C**) A positive feedback loop can induce a sigmoidal cellular response or even bistability under the right conditions. In developmental biology, such networks can guide cell fate transitions.

endocytosis of the signalling complex, which is followed either by the recycling of the receptor or degradation of the complex in the lysosome [35]. The extent of Wnt signalling in cells is regulated by receptor degradation mediated by a network of E3 Ubiquitin ligases and regulatory factors [36]. In addition, the expression of negative regulators upon pathway stimulation can reverse post-translational modifications, block protein degradation

or repress the expression of target genes of the pathway [37]. Negative feedback can result in adaptation, when continued pathway stimulation results only in a minimal response or baseline activity [1]. If the parameters for pathway activation and induced negative feedback are precisely coordinated regarding levels and timescales of activation, delay and inactivation, oscillatory activity can be induced (Figure 2B) [1]. In addition, network motifs containing positive feedback loops can, for instance, lead to bistability that controls developmental cell fate decisions or cell cycle progression (Figure 2C) [38]. In reality, the regulatory networks are usually more complex than this and can include both positive and negative feedback loops, which modulate signalling activity in complex ways [1].

Since the study of dynamic processes in the context of a multicellular organism is technologically challenging, our understanding of signalling dynamics in multicellular systems is limited, especially at the mechanistic level. With new technologies to analyse, visualize and perturb signalling activity in cells and embryos, signalling dynamics are found in more and more model systems and tissues, and their functions are being revealed. It seems that signalling dynamics is a common characteristic of signalling processes and that there might be general principles underlying the function of dynamic signalling in different tissues and organisms. Here, we review the current knowledge of the role of signalling dynamics in embryonic development and aim to make a conjunction with our understanding of the biochemical properties of the individual signalling pathways. Although pathways such as hedgehog, TGFβ or Hippo signalling are equally important in embryonic development, we will focus on Wnt, Notch and ERK signalling to highlight the complexities of dynamic signalling.

Notch signalling

Notch signalling is a highly conserved signalling pathway essential for embryonic development and the maintenance of adult tissues [39]. It mediates the communication between neighbouring cells by cell–cell contact. The ligands Delta/Jagged/Serrate and the receptor Notch are transmembrane proteins, with the ligand being expressed in signal-sending cells and the receptor in neighbouring signal-receiving cells (Figure 3A). Notch activation requires several proteolytic cleavage steps that occur with specific kinetics and therefore influence the kinetics of pathway activation. After translation, Notch is cleaved in the Golgi apparatus by a Furin protease (S1 cleavage), which results in the formation of a heterodimer consisting of the Notch extracellular domain (NECD) bound to a fragment containing a transmembrane domain and the Notch intracellular domain (NICD) [40]. Upon interaction of the ligand with the NECD, an intracellular signal transduction cascade is induced, whereby the Notch intracellular domain (NICD) is released by proteolytic cleavages and travels to the nucleus [41]. These Notch cleavage steps require mechanical pulling forces exerted by the ligand on the receptor, which opens a negative regulatory region (NRR) [42,43]. This allows proteolytic cleavage by ADAM metalloproteases (S2 cleavage) on the extracellular site [44]. This step releases the ligand-bound NECD and enables the final cleavages: γ-Secretase (a multi-subunit complex consisting of Nicastrin, Presenilin, PEN2 and APH1) exerts the S3 and S4 cleavages on the intracellular site, which releases NICD into the cytoplasm [45]. Due to the mechanical forces required for Notch activation, it has so far not been possible to develop a soluble receptor agonist for experimental activation of Notch signalling.

Within the nucleus, NICD interacts with the transcription factor CSL (CBF1/RBPJ, Su(H), Lag-1) and the co-activator mastermind (MAM) as well as others to induce expression of Notch target genes [46–48]. Among these are basic helix–loop–helix transcription factors of the Hes family, but also other genes controlling cellular behaviour such as myc, Cyclin D or Nrarp [49–53]. Notch signalling is terminated by phosphorylation of NICD in its PEST domain and degradation by the proteasome [54–56]. In contrast, the ligand-NECD complex is endocytosed by the signal-sending cell [57]. The combination of nuclear translocation, affinity to the CSL complex and the timing until phosphorylation for proteasomal degradation determines the lifetime of activated NICD. In human cell lines, NICD has a half-life of 180 min [54]. This half-life is further modified by the precision of the final Notch cleavage that results in the release of NICD. Depending on the resulting N-terminal amino acid, NICD half-life can vary in accordance with the N-end rule, which states that N-terminal amino acids are recognized by ubiquitin ligases with different efficiencies to mark the protein for degradation [58,59]. Such processes modulate the dynamics of Notch signalling in a cell type- and state-dependent manner, which should be taken into account when investigating the molecular mechanism of signalling dynamics.

Cis- and lateral inhibition

When the Notch receptor and ligands are expressed in the same cell, they interact *in cis*. Since such an interaction cannot generate the required pulling forces for Notch activation, signalling is essentially blocked, a
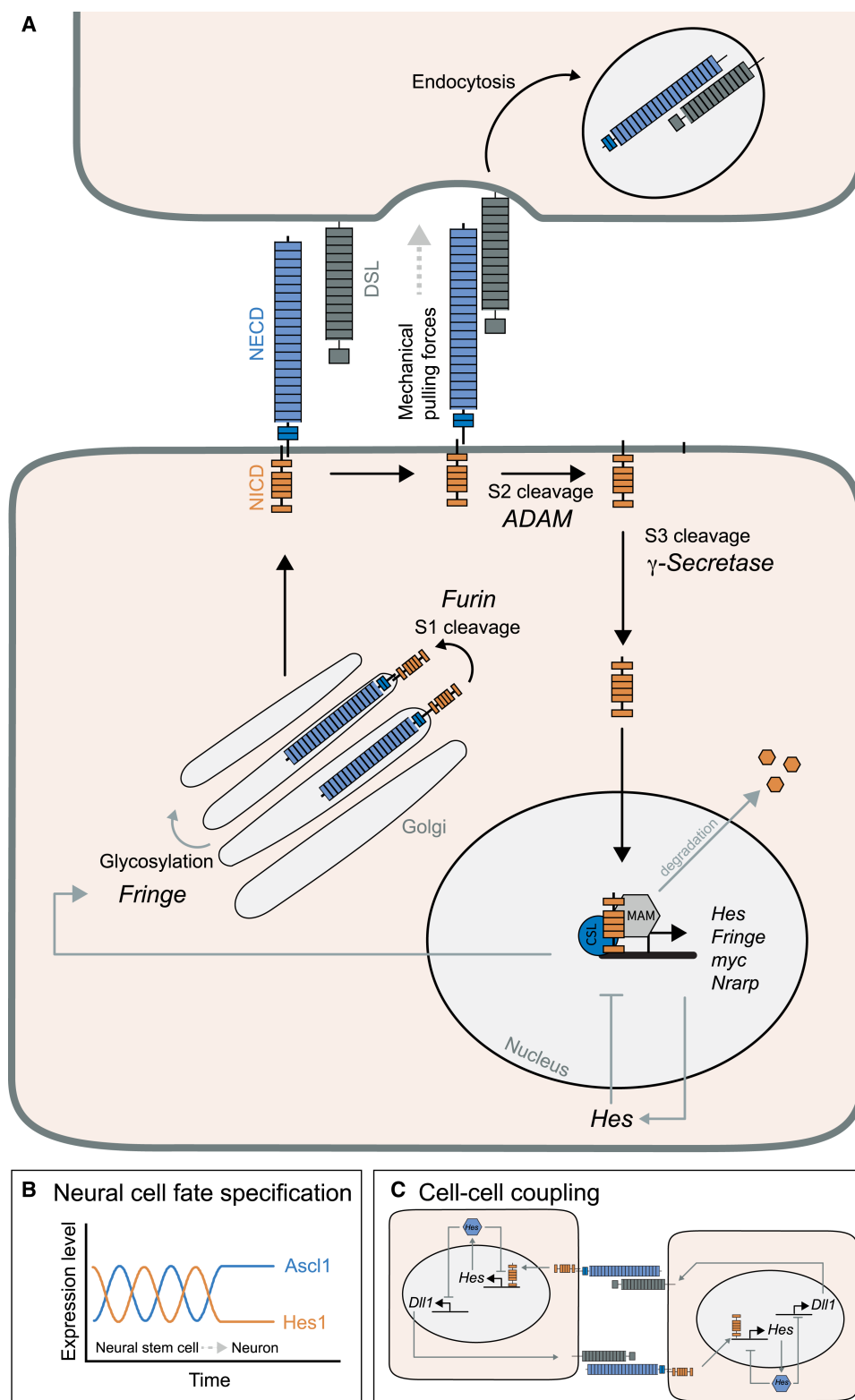
Figure 3. Dynamics of Notch signalling.   Part 1 of 2

(**A**) Notch is generated in the endoplasmic reticulum and travels to the plasma membrane via the Golgi apparatus. In the Golgi, the first proteolytic cleavage is mediated by Furin, which leads to the formation of a Notch heterodimer. There, Notch binding affinities can be modulated by post-translational modifications, e.g. glycosylation by Fringe. When the ligand from a

Figure 3. Dynamics of Notch signalling. Part 2 of 2

neighbouring cell interacts with Notch, a mechanical pulling force results in a conformational change in Notch. This allows the proteolytic cleavage of Notch by ADAM and then γ-Secretase, which finally releases NICD (Notch intracellular domain) into the cytoplasm. In the nucleus, NICD interacts with the transcription factor CSL and the co-activator Mam to induce the expression of Notch target genes. Among these are Hes genes, which initiate a delayed negative feedback loop, as well as Fringe and Nrarp, which both feed back onto Notch signalling. Finally, NICD is phosphorylated and degraded by the proteasome. (**B**) Due to the negative feedback loop of Hes proteins, Hes expression oscillates in many systems. In neural stem cells of the developing brain Hes1 oscillates alternatingly with pro-neural genes. When Hes1 oscillations cease and pro-neural proteins get stabilized, cells differentiate. (**C**) Neighbouring cells can couple via Notch signalling and synchronize their intracellular oscillations, for instance during periodic segmentation of vertebrate embryos. This is thought to be achieved by reciprocal activation of Notch signalling in neighbouring cells and induction of the ligand Dll1.

process termed *cis* inhibition [60,61]. Thus, within a tissue, Notch signalling is active, if ligand and receptor are expressed in different ratios or if ligand and receptor are spatially separated within a cell [62,63]. This auto-regulatory mechanism, in combination with the fact that Notch ligands themselves are downstream targets of the pathway, can result in lateral inhibition by which neighbouring cells induce each other to express either the ligand or the receptor and thereby assume different cell identities [64–66]. This way, a multicellular tissue can self-organize into a highly ordered structure with alternating, equally spaced cell types. For instance, a combination of *cis* inhibition and lateral inhibition allows the formation of regularly spaced sensory hairs in separate lines along the back of the fly [67]. In the mammalian gut epithelium, lateral inhibition coordinates the regular spacing of secretory and non-secretory cells along the gut lining. Secretory cells express Notch ligands, which block their neighbours to adopt the same cell fate [68,69]

Cellular Notch signalling oscillations

Several Notch target genes function as negative regulators of the pathway [50]. Prominent among these are members of the Hes transcription factor family, which can function as transcriptional repressors of their own expression as well as other genes [70,71]. Another negative regulator is Nrarp (Notch regulated ankyrin repeat protein), a Notch signalling target in Xenopus embryos [72]. It interacts with the NICD-CSL complex and induces its dissociation, resulting in NICD degradation via the proteasome [50]. Such delayed negative feedback loops induce oscillations of Notch target genes. Indeed, Hes oscillations have been observed in various tissue types, such as embryonic stem cells, neural stem cells and the developing pancreas (Figure 2B) [3,5,73]. In neural stem cells of the developing mouse brain, oscillatory expression of Hes1 and several pro-neural genes such as Ascl1 controls the balance between proliferation and differentiation [5,74]. When pro-neural genes are stabilized and Hes genes down-regulated, neural differentiation is initiated (Figure 3B). Similar oscillation dynamics of Hes5 have been observed in the developing spinal cord [75,76]. To test the functional significance of Hes dynamics in regulating cell behaviour, Imayoshi et al. generated an optogenetic system to modulate Ascl1 expression in cells by light pulses [4,77]. When oscillations of Ascl1 were induced in neural stem cells, cells proliferated and did not differentiate [4]. Conversely, sustained Ascl1 expression resulted in neuronal differentiation. This work highlights the importance of signalling oscillations in controlling the balance between proliferation and differentiation in stem cells; however, the molecular decoding mechanism remains to be investigated.

Spatiotemporal Notch dynamics

Owing to the intrinsic propensity of Notch signalling to oscillate, the fact that expression of both ligand and receptor is induced by the pathway itself as well as its paracrine nature, Notch signalling can generate tissue-wide oscillation or wave dynamics. One example is the periodic formation of segments or somites during vertebrate development, a process termed somitogenesis. Somites are transient embryonic structures and precursors of vertebrae, muscles and dermis [78]. Periodic segmentation of the growing tissue is regulated by signalling oscillations in Notch, Wnt and FGF signalling in the unsegmented tissue, the presomitic mesoderm (PSM) [19]. The network of oscillating signalling pathways is termed the *segmentation clock*. Each cell is thought to be an autonomous oscillator [79–81], but cell-to-cell communication via Notch signalling synchronizes neighbouring cells (Figure 3C) [82–84]. This way, kinematic waves of signalling activity are observed in tissues travelling

from the posterior tip of the tail to the side of segment formation. Since the segmentation clock also induces the expression of the Notch ligand Dll1, a model has been proposed in which oscillatory expression of Dll1 drives Notch oscillations and mediates cell–cell communication to form waves of Notch activity travelling through the segmenting tissue (Figure 3C, also see Figure 4B) [85]. In fact, Notch signalling seems to be central to generating these oscillations. Oscillations of the downstream target Hes7 have been detected in various model organisms such as zebrafish, mouse and human cells [84,86–89]. When the timing of Hes7 transcription was altered by the removal of several introns (see Figure 2), the oscillation period and, consequently, the timing of segmentation were shortened [90]. Interestingly, shortening gene expression timing resulted in damped oscillations, highlighting that the right parameters of the delayed negative feedback loop are essential for stable oscillations (Figure 2A,B). Another set of experiments underscoring the role of Notch signalling for oscillations was performed by Soza-Ried et al. who induced periodic overexpression of the ligand DeltaC in zebrafish, which could control segmentation oscillations [91].

Specificity in information transmission

Mammalian genomes encode for 4 Notch isoforms (Notch1 to Notch4) and 5 Notch ligands (Jagged1 (Jag1), Jag2, Delta-like 1 (DLL1), DLL3 and DLL4). However, even if cells express the same Notch receptors, different intracellular dynamics and effects can be activated. How different ligands induce different intracellular dynamics and result in different downstream effects is still a matter of investigation. One potential explanation comes from structural and biochemical analyses revealing that different Notch ligands bind their receptors with different affinities. For instance, DLL4 binds Notch 1 with a higher affinity than DLL1 [3,92–95]. In agreement with this, DLL4 has recently been found to induce sustained Notch activation, which results in expression of the downstream target Hey1, while Dll1 induces Notch pulses and expression of Hes1. It has been suggested that ligand–receptor clustering in the case of Dll4 results in sustained activation, which explains how ligand identity can be encoded in the dynamics of Notch signalling [96].

Ligand–receptor affinity is further fine-tuned by post-translational modifications such as glycosylation of the Notch receptors by several enzymes, including Fringe, POFUT1, POGLUT1 or EOGT1 [97–99]. Local modifications of Notch receptors in Drosophila embryos ensures spatiotemporal specificity of Notch signalling [97,98]. Interestingly, in mammalian cells, Lunatic Fringe (Lfng) is one of the cyclic genes whose expression is periodically induced during vertebrate segmentation [100,101]. Considering that the Notch receptor pool has to be replenished continuously to maintain Notch signalling, oscillatory expression of the glycosylating enzymes might further amplify signalling activity and thereby enhance the oscillation amplitude. It has been shown recently that Hes7 oscillations downstream of Notch signalling do not depend on the presence of Lfng. In contrast, Lfng is essential for coupling between cells, highlighting the importance for glycosylation for proper intercellular signalling [102].

How specific dynamics are decoded to induce a precise, cell type-specific effect is not understood. The architecture and affinity of CSL binding sites and the recruitment of other regulatory proteins seem to play a role, which is discussed in detail elsewhere [41]. In vitro systems, such as periodic induction of ligand expression [91] or the synthetic Notch system syn-Notch [103], allow defined external activation of Notch signalling to analyze the encoding and decoding process in detail.

Wnt signalling

The Wnt signalling pathway is an evolutionarily conserved signalling pathway essential for stem cell renewal, cell proliferation and cell differentiation during embryogenesis and adult tissue regeneration and repair. Deregulated Wnt signalling is implicated in various diseases, including cancer, developmental disorders, and degenerative diseases [104–106]. Multiple, functionally distinct Wnt signal transduction pathways have been identified [107]. Here, we will focus on the Wnt/β-catenin-dependent pathway, the *canonical Wnt pathway,* that regulates context-specific gene expression programs (Figure 4A).

The transcriptional co-activator β-catenin is a master regulator of this transduction pathway and is constantly synthesized. In the absence of Wnt ligands, it is rapidly degraded by ubiquitin-dependent proteasomal degradation [108]. This process is initiated by the β-catenin destruction complex, a multiprotein complex composed of the scaffold proteins adenomatous polyposis coli (APC) and Axin1 [109–112] the protein kinases casein kinase 1 (CK1) and glycogen synthase kinase-3 (GSK3), which phosphorylate β-catenin on key serines/threonines [113–115]. The phosphorylated motif is a docking site for the β-TrCP-containing E3 ubiquitin ligase that mediates ubiquitination and subsequent proteasomal degradation [116–118]. Wnt binding to the cell surface
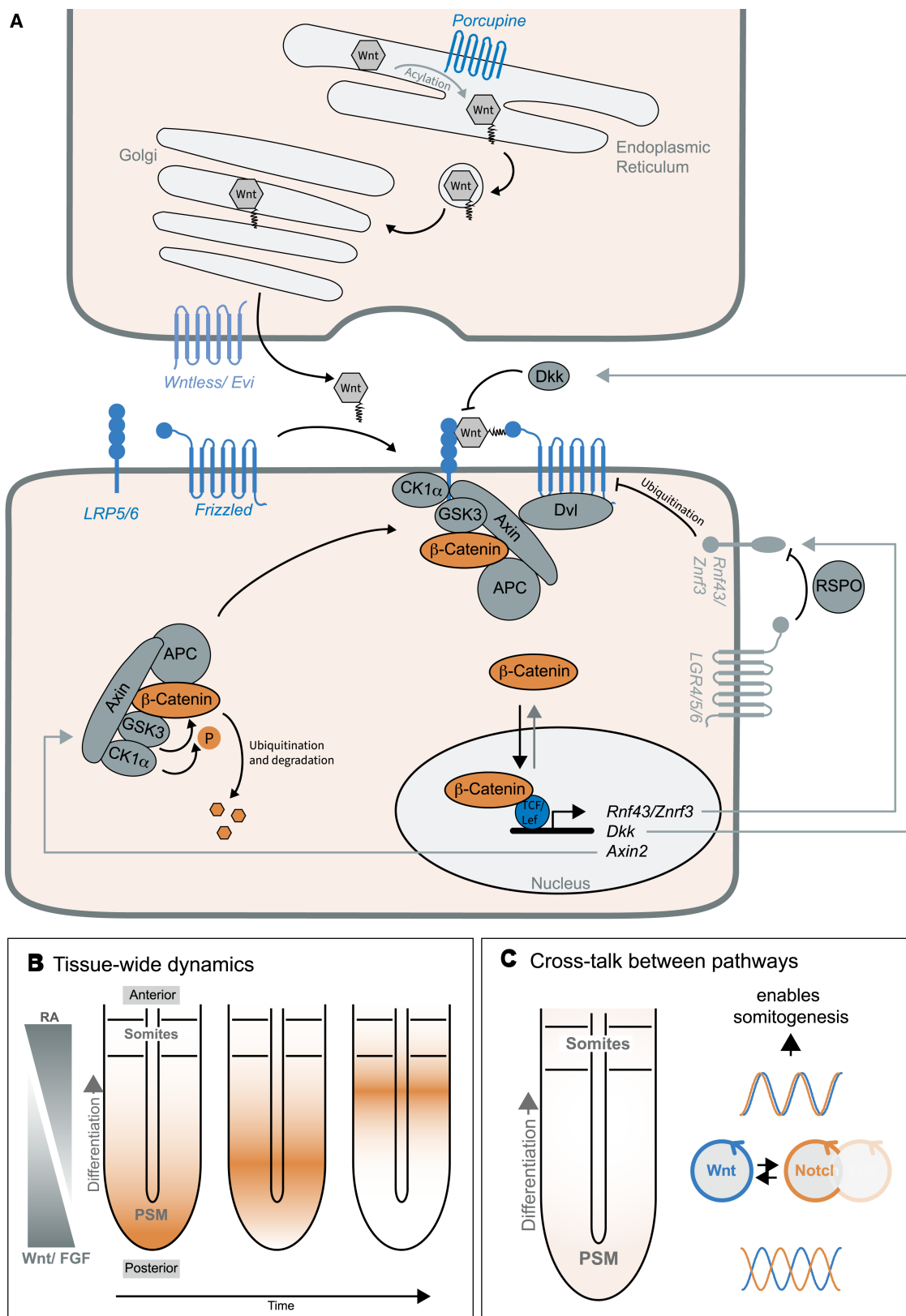
Figure 4. Dynamics of Wnt signalling.   Part 1 of 2

(**A**) Wnts are secreted proteins that are lipidated in the ER by Porcupine. Wntless/ Evi regulates Wnt secretion. In the

Figure 4. Dynamics of Wnt signalling. Part 2 of 2

signal-receiving cell, many Wnt ligands induce the β-catenin-dependent signalling cascade. In the absence of Wnt, β-catenin is constantly phosphorylated by the destruction complex and marked for degradation by the proteasome. When Wnt binds to its receptor Fzd and the co-receptor LRP5/6, Dvl and the destruction complex are recruited to the plasma membrane. This prevents phosphorylation and degradation of β-catenin, which results in the accumulation of hypo-phosphorylated β-catenin in the cytoplasm. β-Catenin travels to the nucleus to modulate gene expression by interacting with the transcription factor TCF/LEF. Several Wnt target genes function as negative regulators of the pathway, such as Axin2, Dkk and Rnf43/Znrf3. Rnf43/Znrf3 marks Fzd for proteasomal degradation. When RSPO binds to Lgr, this complex recruits and inhibits Rnf43/Znrf3. This double-negative regulation amplifies Wnt signalling activity. (**B**) Periodic segmentation of mammalian embryos or somitogenesis is regulated by oscillations of Wnt, Notch and Fgf signalling. Coupling between cells via Notch signalling leads to the formation of signalling waves (in orange) travelling from the posterior tip to the anterior side of the presomitic mesoderm (PSM), where segment formation is induced. In addition to signalling waves, the tissue is organized by antagonistic gradients of Wnt/Fgf and retinoic acid (RA). (**C**) In mouse somitogenesis, Wnt and Notch signalling oscillations are coupled and have different dynamics in the segmenting tissue: In the posterior, Wnt and Notch signalling oscillate out-of-phase, in the anterior they oscillate in phase. This change in phase-relationship is essential for proper segmentation.

receptors Frizzled (Fzd) and co-receptor low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6 triggers the recruitment of Dishevelled (Dvl) to the intracellular regions of Fzd [119–121], and of the Axin-GSK3 complex to the C-terminal tail of LRP5/6, promoting its phosphorylation by GSK3 [122,123]. This results in the inhibition of the β-catenin destruction complex, and the accumulation of hypo-phosphorylated β-catenin in the cytoplasm and the nucleus [124,125]. In the nucleus, β-catenin functions as a co-activator for transcription factors of the TCF/LEF family and modifies the expression of Wnt target genes [126–128], thereby leading to changes in key cellular processes including cell proliferation, cell fate determination and migration depending on cell type and cell state. Besides its function in Wnt signalling, β-catenin stably localizes to the plasma membrane as a component of adherens junctions [129]. The relevance of this β-catenin pool for signalling and the dynamics between the different pools is still controversial and might be context-dependent [130,131].

While the Wnt/β-catenin pathway and the molecular interactions of signalling components are extensively studied, only a few studies investigate the dynamics of Wnt signalling and how cells receive, process, and interpret different features of extracellular ligands, such as molecular identity, concentration, and combinations with other ligands to control specific cellular behaviour. However, many of the 'communication codes' identified in other signalling pathways could also be critical determinants for Wnt/β-catenin signalling dynamics and cell fate decisions.

Wnt gradient

Wnts are post-translationally lipidated by the O-acyl transferase porcupine in the ER [132,133] and further shuttled through the Golgi to the plasma membrane by the escort protein Wntless/Evi [134–136] (Figure 4A). After secretion, lipidation is essential for Wnt binding to the Fzd receptor [137] and to limit diffusion in the extracellular environment [132]. As outlined above, the Wnt gradient is critical for coordinated spatial and temporal activity (Figure 4B), but the molecular mechanism of gradient formation remains the subject of many ongoing studies. The current notion suggests that Wnts 'travel' away from the secreting cells when incorporated into particles, vesicles or exosomes [138,139] or bound to receptors, i.e. Fzd and Rnf43/Znrf3 through direct cell contact and cell division [140]. Furthermore, in many systems, the Wnt gradient appears to be fine-tuned by negative feedback mechanisms or counteracting gradients of Wnt antagonists. For instance, in the mouse embryo at gastrulation, the expression of Wnt3 in the posterior region opposes the expression of the antagonist dickkopf 1 (Dkk1) to control head morphogenesis [141].

Feedback mechanisms

Wnt pathway activity is carefully controlled in healthy cells by antagonists and potentiators, as well as positive and negative feedback loops that enhance, augment, dampen or terminate Wnt signalling (Figure 4A). The interactions of Wnts with its receptors are negatively regulated by numerous proteins, including Dkk1-4 [142–144], sclerostin/SOST [145], the Wnt inhibitory factor WIF [146] and Notum [147,148], several being Wnt target genes themselves (Figure 4A). In contrast with Axin1, Axin2 is a universal Wnt target gene, and increased Wnt

signalling enhances destruction complex formation and β-catenin degradation, thereby suppressing β-catenin-mediated gene expression [12,149,150]. Furthermore, Rnf43 and Znrf3 are Wnt target genes and transmembrane E3-ubiquitin-protein ligases that mediate Fzd degradation [151,152], leading to reduced Fzd cell surface density and decreased sensitivity of cells to Wnt ligands. This results in the attenuation or termination of the Wnt signal necessary for many biological processes. For instance, Znrf3 knockout mouse embryos die around birth and show a lack of lens development as a most obvious phenotype [151]. Conversely, Wnts also trigger the expression of leucine-rich repeat-containing G protein-coupled receptor family 5 (Lgr5) [152]. R-spondin ligands (RSPO) are expressed in many stem cell niches and engage Lgr4-6 and Rnf43/Znrf3, thereby triggering endocytosis of the ternary complex and blocking Rnf43/Znrf3-mediated Fzd degradation [151–153] (Figure 4A). This leads to a higher sensitivity of cells to Wnt ligand, markedly increased Wnt/β-catenin signalling levels and, in the small intestine, to the activation of the transcriptional programs required for stem cell proliferation and self-renewal [154].

Combinatorial use of Wnt ligands and receptors

Many developmental signalling pathways have evolved multiple ligand and receptor variants that interact promiscuously, and the Wnt pathway is no exception. The human genome encodes 19 Wnts, 10 Fzds, and two LRP5/6 co-receptors. Although loss-of-function of most Wnts result in characteristic phenotypes (http://web. stanford.edu/group/nusselab/cgi-bin/Wnt/), observations suggest that these are related to the localized expression, rather than intrinsic activities of different Wnt subtypes. It is generally accepted that Wnts/Fzds are highly cross-reactive. Biochemical and functional approaches that characterize interacting Wnt — Fzd pairs [155,156] have revealed broad Wnt — Fzd cross-reactivity, with some Wnts having broader specificity than others. Consistent with the broad Fzd specificity, Wnt3a is pleiotropic and can exert a broad range of biological activities, such as supporting the growth of organoids from many different epithelial tissues [157], exceeding what could be expected based on the Wnt3a expression profile. Nevertheless, it can be expected that the different Wnts are not functionally equivalent, meaning that their signalling outputs have different characteristics. This notion is supported by the observation that different Wnt subtypes synergistically activate Wnt/β-catenin signalling in multiple cell types [158].

Furthermore, biophysical parameters including stability, geometry and stoichiometry of the Wnt/Fzd/LRP5/6 signalling complex are likely to infer signalling dynamics. Despite some progress in recent years [137,159–162] studies to exploit ligand/receptor-induced Wnt signalling dynamics remain challenging owing to the complex nature of the receptors and the Wnts. To overcome these difficulties, several laboratories have developed Wnt mimetics, consisting of non-lipidated Fzd–LRP5/6 'heterodimerizers' that are structurally distinct from natural Wnts but recapitulate their activities with a tuneable activity [137,159,160,163,164]. Furthermore, ESCs and adult neural stem cells with light-inducible Wnt signalling have been generated by expressing the cytoplasmic tail of LRP6 fused to the blue-light photoreceptor Cryptochrome 2, allowing to probe the effect of dynamic Wnt signalling activation [165,166].

In contrast, the relevance of receptor abundance for tuning Wnt signalling dynamics is better established. RSPO ligands strongly potentiate Wnt signalling by inhibiting the Rnf43/Znrf3-mediated proteasomal degradation of Fzd, leading to increased receptor abundance on the surface of Wnt receiving cells [151,152]. Recently, it has been demonstrated that Wnts and RSPOs exert differential activities in the intestinal crypt stem-cell niche. While Wnts confer a basal competence of stem cells for Wnt signalling by maintaining the expression of Lgr5, RSPOs support the expansion of stem cells [154]. Hence, it would be interesting to elucidate the differential functions of Wnts and RSPOs and their association with diverse signalling dynamics in other systems and models. Besides attenuating or potentiating Wnt signalling, some co-receptors have been found to be required for correct Wnt signalling initiation. For instance, RECK is a selective Wnt7 receptor that, together with GPR124, is required for transducing a Wnt/β-catenin signalling to control embryogenic CNS angiogenesis and blood-brain barrier formation [167,168].

Fold change detection

Intracellularly, β-catenin is the master effector of the Wnt pathway critically involved in regulating gene expression in response to Wnt stimulation. A first mathematical model describing the functioning of the Wnt/ β-catenin pathway in time and space was developed by Kirschner, Heinrich, and colleagues and is based on measurements with Xenopus oocyte extracts, and the molecular reactions of the pathways' core effectors including Axin, APC, and β-catenin [169]. This model was subsequently modified regarding signalling characteristics,

refined interactions, functional diversity, compartmentalization and nuclear shuttling of β-catenin, and negative feedback loops [12,169–172]. An important observation was that the fold-change rather than a linear increase in β-catenin is the critical determinant read out by the downstream transcriptional system. While the absolute β-catenin levels induced by Wnt stimulation are very sensitive to relatively small, stochastic pathway perturbations, a fold-change is robustly buffered. Having the output of Wnt signalling sensed in fold-change allows the cells to detect ligand levels while cancelling out fluctuating noise. It requires that at least some Wnt target genes perceive fold-changes, rather than absolute levels, of β-catenin, giving rise to adaptive responses. Detection of the fold change may be accomplished by an incoherent feedforward loop of gene regulation [12,173]. This suggests that Wnt signalling, at least in the absence of Lgr5/RSPO contribution, is optimized for controlling transient events, such as cell fate decisions, rather than continuously transmitting information about extracellular ligand levels.

Adaption

A series of studies used quantitative microscopy to interrogate the dynamics of fluorescently labelled endogenous β-catenin, or β-catenin expressed at near endogenous levels, in response to Wnt signal activation and inhibition [174,175]. Stimulation of HEK293 cells with Wnt3a led to an increase in the total amount of β-catenin. However, the increase was initially faster in the nucleus than in the cytoplasm, resulting in a higher nucleus/cytoplasm protein ratio. The shuttling of β-catenin to the nucleus appears to be mediated by active transport alongside passive diffusion. While the dynamics of ß-catenin nuclear build-up, i.e. rates, levels, wave profile, varied substantially between individual cells, the total levels of accumulated β-catenin were similar in most cells, suggesting that the balance between accumulation and degradation affects the extent of β-catenin accumulation in individual cells. The expression kinetics of the Wnt target gene cyclin D paralleled the kinetics of the initial phase of β-catenin nuclear accumulation. Rate of change, and not actual protein levels, of nuclear β-catenin correlated with the transcription of cyclin D1 mRNA, especially during the early phase of Wnt stimulation [174].

However, β-catenin dynamics appear to be related to cell type, context, and fate. For instance, stimulation of hESCs with exogenous Wnt, resulted in an adaptive β-catenin response. β-catenin accumulated rapidly in both the nucleus and the cytoplasm but started to decline in the nucleus after four hours [176]. The level of adaptation depended on the concentration of the Wnt signal, being complete at a low dose of Wnt3a and partial at saturating concentration of Wnt3a. In contrast, the small-molecule GSK3ß inhibitor CHIR99021, commonly used as a Wnt substitute, induced a nonadaptive dose-dependent increase in nuclear β-catenin, indicating the mechanism that controls adaptation in hESCs is upstream of GSK3β. Interestingly, Wnt target genes showed various response profiles. While some genes were transiently activated in response to Wnt3a, others were sustained, further adding to the complexity of Wnt signalling dynamics and questions to address.

Wnt signalling oscillations

Besides fold-change detection and adaption, Wnt signalling can display complex oscillatory dynamics. The main example is the segmentation of mouse embryos, which is controlled by oscillatory pathway activity (see above, Figure 4B). The first cyclic genes identified in chicken, mouse and zebrafish embryos were downstream targets of Notch signalling [88,100] (Holley et al. 2000; Palmeirim et al. 1997). To identify other oscillatory genes and pathways, a transcriptomic screen was performed, in which one half of the PSM was used for transcriptome analysis [177]. The other half was stained for the cyclic gene Lfng, which allowed the authors to put the transcriptomic samples into temporal order based on the Lfng staining pattern. This revealed Wnt signalling as a new oscillatory component of the segmentation clock. Subsequently, staining of nascent Axin2 mRNA as downstream target of Wnt signalling and the quantification of dynamic Wnt signalling reporters confirmed oscillatory Wnt activity in models of mouse and human somitogenesis [87,178,179]. The regulatory network inducing Wnt signalling oscillations is not understood in detail. Modelling of the Wnt signalling network indicated that a delayed negative feedback loop via the negative regulator Axin2 could account for the oscillations [180,181]. However, other negative regulators such as Dkk are induced likewise (Figure 4A) and their function should be tested experimentally.

The cross-talk between and hierarchy of Wnt and Notch signalling in somitogenesis has been investigated and has remained inconclusive [178,182–185]. This stems in part from the presence of a Wnt signalling gradient in the tissue that adds to the complexity and makes the experimental dissection difficult [178,182]. When knocking out the transcriptional repressor Hes7, Notch signalling oscillations were not detected anymore,

indicating Hes7 as part of the oscillatory mechanism [90,184]. In contrast, Wnt signalling oscillations persisted in Hes7 knockout mice. To analyse a potential coupling between Wnt and Notch signalling oscillations, a microfluidic system was applied that allows the subtle modulation of signalling oscillations in segmenting tissue [179,186]. This showed that Wnt signalling oscillations followed, when Notch signalling oscillations were entrained, and vice versa, implying that Wnt and Notch signalling oscillations are indeed coupled (Figure 4C) [179]. Interestingly, Wnt and Notch signalling oscillations change phase-relationship from out-of-phase in the posterior tissue to in-phase in the anterior part of the PSM, where segments form [177–179]. When changing this phase relationship using microfluidics, segment formation was impaired, which highlights that phase shift carries critical information for somitogenesis (Figure 4C). How this phase-shift changes and how it controls segment formation is not understood. Furthermore, whether and how Wnt signalling oscillations regulate the development of other tissues has to be investigated in future studies.

MAPK signalling

One of the main signal transduction pathways activated by growth factors is MAPK signalling. Growth factors such as EGF or FGF are soluble ligands that bind to receptor tyrosine kinases (RTKs) to mediate diverse functions within organisms, e.g. cell proliferation, survival or modification of cell metabolism [187,188]. Upon ligand–receptor interaction and receptor dimerization, the intracellular RTK kinase domain is activated and several tyrosine residues of the intracellular domain are phosphorylated in trans (Figure 5A) [189–191]. Phosphorylated tyrosines serve as docking sites for the recruitment of Shc, Grb2 and Sos [192]. Sos functions as a guanine nucleotide exchange factor to activate the small G protein Ras by exchanging GDP with GTP [192,193]. Ras-GTP then initiates a kinase cascade by activating the kinase Raf. Raf (a *MAPK kinase kinase*) phosphorylates and thereby activates the kinase MEK (a *MAPK kinase*), which in turn activates ERK (a *MAPK*) by phosphorylation. ERK as effector kinase of the pathway phosphorylates diverse proteins within the cell to change cellular behaviour. It also travels to the nucleus to mediate gene expression of target genes by phosphorylating transcription factors [188]. The activation process of Raf and its dynamics are modulated by the combinatorial usage of ligands and receptors, post-translational modifications, subcellular localization and endocytosis of the receptor complex, all of which is dependent on cell type and developmental stage, discussed in detail elsewhere [187,188]. Here we focus on the kinase cascade itself and its implications for signalling dynamics.

Scaffold proteins in ERK signalling

Mechanistic details of Erk signalling have been studied extensively in human cell lines and *in vitro*. One regulatory component of MAPK signalling is the use of scaffold proteins, which consist of modular interaction domains that coordinate the three kinases of the MAPK cascade (Figure 5B) [194,195]. This has several consequences for ERK signalling: (1) By recruiting the kinases to the plasma membrane, in the vicinity to the activator Ras, the phosphorylation cascade can be activated efficiently. Furthermore, the cascade itself gets more specific and more efficient by bringing the right proteins in close proximity, as long as the ratio between scaffold and kinases is not too high. For instance, the scaffold protein KSR (kinase suppressor of Ras) binds Raf, MEK and ERK, all components of the ERK activation cascade [195]. Despite these advantages for cells, too stable interactions might limit the amplification of the MAPK cascade. (2) The scaffold has an allosteric effect on the phosphorylation process, for instance, on Raf kinase activation [196]. In addition, in a ternary complex consisting of KSR, B-Raf and MEK1 a conformational change in MEK1 promotes phosphorylation by a second B-Raf molecule [197]. (3) Scaffold proteins influence feedback mechanisms of the signalling pathway. For instance, when ERK bound to KSR gets activated, ERK phosphorylates both KSR and Raf, which induces the dissociation of KSR and Raf thereby inhibiting further amplification of the signal within short timeframes [198]. How scaffold proteins contribute to ERK signalling dynamics is not known. It is however conceivable that such proteins modulate the parameters of dynamics, such as timing, amplitude and duration dependent on the cell type and developmental stage. Their expression and dynamics should therefore be studied in the cellular context of ERK signalling dynamics.

ERK signalling dynamics

ERK signalling is driven and regulated by post-translational modifications. Not only phosphorylation, but also others such as ubiquitination, modify activity and stability of the involved proteins [199,200]. These modifications are largely reversible, since both modifying and reversing enzymes are expressed in the same cells, giving
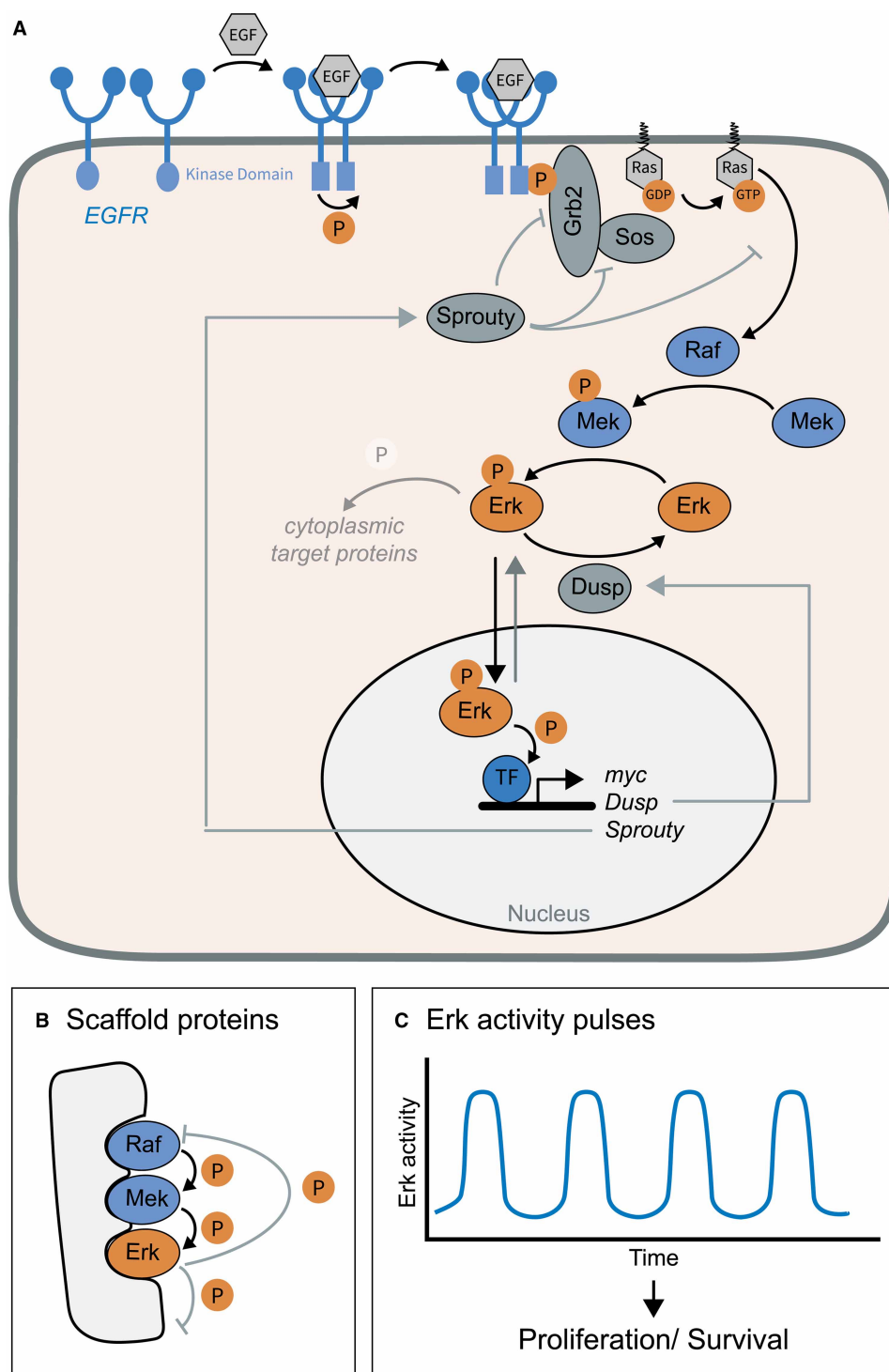
Figure 5. Erk signalling dynamics.   Part 1 of 2

(**A**) When EGF interacts with a dimer of its receptor EGFR, a conformational change is induced, which results in the activation of the intracellular kinase domain. Phosphorylation in trans allows the recruitment of the adaptor Grb2 and the GEF Sos. Sos promotes the exchange of GDP with GTP in the small G protein Ras, which then activates the kinase Raf. Raf then phosphorylates and activates the kinase Mek, which in turn phosphorylates and activates the effector kinase Erk. Erk phosphorylates various cytoplasmic proteins and travels to the nucleus to activate the expression of target genes. Among these are the negative regulators Sprouty and the phosphatase Dusp. (**B**) Erk signalling is modulated by scaffold proteins,

Figure 5. Erk signalling dynamics.   Part 2 of 2

which make the kinase cascade more specific and efficient. In addition, feedback mechanisms involving the scaffold protein regulate the cascade further. For instance, the activated effector kinase phosphorylates and both the scaffold protein and Raf, which results in dissociation of the two proteins. (**C**) Erk activity pulses have been observed in various tissue types ranging from ∼15 to 90 min in period. In these tissues, pulses induce cell proliferation and promote cell survival.

rise to complex signalling dynamics. In addition, the expression of activating and inactivating enzymes is induced by the ERK pathway, which generates positive and negative feedback loops [201]. Dynamics in ERK signalling have first been described in the PC12 cell line, in which both growth factors EGF and NGF activate ERK signalling, but with different dynamics and lead to different cellular outcomes, i.e. cell proliferation or differentiation, respectively (see above, Figure 1B) [17,202]. Using an optogenetic tool, Toettcher *et al.* induced ERK signalling by light with different dynamics, which confirmed that sustained signalling resulted in neuronal differentiation [203]. It has been suggested that EGF and NGF activate different signalling network topologies, thereby leading to different dynamics: EGF binds to the EGF receptor EGFR, while NGF binds to TrkA (Tropomyosine receptor kinase A). This way, EGF mainly induces a negative feedback loop. NGF induces a positive feedback loop via additional activation of PKC (protein kinase C), leading to sustained ERK signalling [204,205]. In fact, single-cell analyses indicated that the relationship between activated ERK and PKB (protein kinase B)/AKT determines cellular outcome [206–208].

ERK activity pulses

Over the last decade, several highly versatile ERK activity reporters have been generated that are based on the phosphorylation of fluorescent reporter constructs, which mediate a change in Foerster resonance energy transfer (FRET) or subcellular localization [209–211]. Such reporters have allowed the identification of ERK dynamics in various organisms and tissue types within their multicellular context. It turns out that ERK signalling is often pulsatile within a range of several minutes, presumably owing to the periodic phosphorylation and dephosphorylation dynamics (Figure 5C) [212]. In skin cells the period of ERK pulses varied between ∼30 min and 1.5 h in both mice and human cells, which was dependent on cell type and cultivation time [212]. The generation of pulses was dependent on the dual specificity phosphatases (DUSP) Dusp6 and Dusp10 [212]. A screen using more than 400 kinase inhibitors indicated the complex network and crosstalk between different signalling pathways influencing ERK dynamics [203,213]. Pulsatile ERK activity correlates with cell proliferation in skin cells [212]. In the context of blocked endogenous EGF signalling with an EGFR inhibitor, induced ERK pulses using optogenetics could recapitulate this proliferative effect [213]. Since continuous activation of ERK activity had a similar effect, it has to be investigated in future studies whether the dynamics or the cumulative activity of ERK determine cellular behaviour. Indeed, similar optogenetic modulation of ERK activity in *Drosophila* embryos showed that the cumulative dose of ERK activity defines the induced cell fate [214,215]. Moreover, in a two-dimensional layer of the canine kidney cell line MDCK, a wave of ERK activity is initiated around apoptotic cells, which promotes survival of surrounding cells, a phenomenon also found in *Drosophila* [216,217]. Optogenetic induction of ERK activity pulses showed that pulses with a period of at least 3–4 h could prevent apoptosis [217], which indicates that maintaining the cellular effect of ERK constantly at a sufficiently high level has a pro-survival effect. Similar ERK dynamics have been detected in other tissues such as intestinal organoids and lung cells [209,218,219], indicating that this might be a common phenomenon in epithelial tissue. The functional significance for embryonic development and tissue maintenance as well as the decoding mechanism have to be investigated in future studies.

ERK signalling oscillations

The periodic segmentation of mouse embryos is mediated by a network of oscillatory Wnt, Notch and FGF signalling (see above, Figure 4B,C). Several downstream targets of FGF/ERK signalling have been shown to be oscillatory with a period of 2.5 h in mice and 5 h in humans [87,177]. Periodic absence of FGF signalling in the region of segmentation with the concomitant presence of Notch activity allows the formation of a new somite [183]. Using a luciferase reporter based on the downstream target DUSP4, signalling waves of FGF signalling in the developing mouse embryo were observed [183]. In contrast, the dynamics of ERK activity itself have not been quantified using live reporters yet. However, oscillations of phosphorylated ERK (P-ERK) were

detected by immunohistochemistry [183]. In the future, it will be important to analyse ERK dynamics within single cells at high temporal resolution using dynamic ERK activity reporters to determine whether ERK activity is also pulsatile in the segmenting embryo. This will allow the investigation of how ERK dynamics are translated into an oscillatory expression of ERK target genes.

Species-specific differences in signalling dynamics

Even though embryonic development of different mammalian organisms is strikingly similar in its molecular processes, developmental time greatly differs between species [220]. In two recent studies, this has been attributed to differences in biochemical parameters of the molecular processes and signalling events [33,221]. Pluripotent stem cells (PSCs) were either differentiated along neuromesodermal progenitors towards neural tube cells [221] or along oscillating PSM towards somitic cells [33]. *In vitro* formation of oscillating PSM cells confirmed previous findings that mouse cells oscillate with a period of ∼2.5 h, while in human cells the period is ∼5 h (Figure 6A) [33,80,84,87,222]. By swapping the human and mouse version of the cyclic gene Hes7, it was ruled out that the difference lay in the gene locus. In contrast, it turned out that several biochemical parameters of the delayed negative feedback loop generating oscillations differed (Figure 6B). Transcription, splicing and translation as well as protein degradation take approximately double the amount of time in human compared with mouse cells [33]. Equivalent differences in protein stability were found when differentiating PSCs towards neural tube cells [221]. The cause of this divergence in the timing of cellular processes remains elusive.
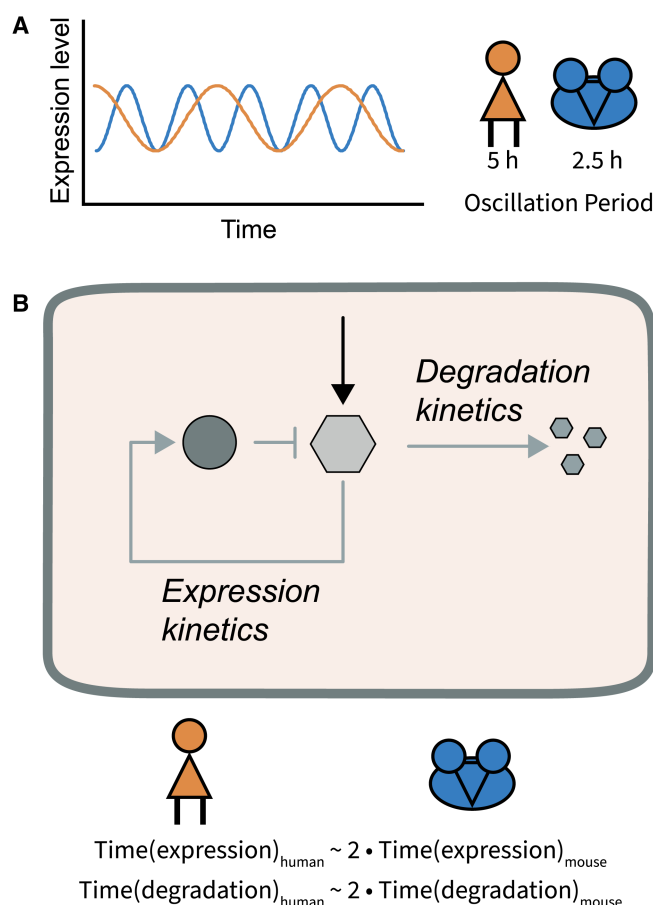

Figure 6. Species-specific differences in the timing of dynamics.
(**A**) Oscillations of the segmentation clock have a period of ∼5 h in humans and 2.5 h in mice. (**B**) Segmentation clock oscillations are thought to be generated by a delayed negative feedback loop, involving the induction of the expression of a negative regulator. It has been shown that the timing of expression (transcription, splicing and translation) and protein degradation take 2× longer in humans than in mice.

The question of whether all biochemical reactions are equally changed — or only a subset of processes — will have direct implications on signalling dynamics in the different model organisms. For instance, ERK dynamics are mediated by periodic post-translational modifications on the one hand and gene expression of negative regulators on the other hand. A direct comparison of ERK dynamics can be performed by quantifying equivalent reporters for both ERK activity [209,211] and target gene expression [183] in equivalent mouse and human cells.

Perspective

Signalling pathways are intrinsically dynamic. Such dynamics have diverse functions in organisms, ranging from the control of cell proliferation and differentiation to the coordination of periodic events during embryonic development. Here, we have discussed the relevance and mechanisms of dynamics in Notch, Wnt and MAPK signalling. Other signalling pathways are equally important in embryonic development and dynamics have been detected in various contexts [10,223,224]. This indicates that signalling dynamics might be a common characteristic of cellular signalling.

Oscillatory signalling activity occurs in various pathways, model systems and tissue types. These oscillations seem to maintain cells in an undifferentiated and proliferative state. This suggests that signalling oscillations might keep cells in an undecided, but responsive state and allows cells to quickly adapt to changes. Only upon sustained expression of transcription factors, differentiation seems to be initiated. Some questions that emerge from this are the following: Are Erk pulses a general property of epithelial cells to control proliferation? How do dynamics change in the process of epithelial-to-mesenchymal transition and *vice versa*? How do tumorigenic mutations affect these dynamics [218,219]? During embryogenesis periodic segmentation is a common process often occurring in both vertebrates and invertebrates. Dynamic and oscillatory activity are found in several other segmenting species, the best-studied invertebrate being the beetle *Tribolium* [225–227]. Whether the mechanism of tissue-wide communication and induction of segments are similar remains to be determined.

With new technological developments, the mechanistic investigation of the role of signalling dynamics becomes amenable. (1) *In vitro* models of embryonic development and adult tissue homeostasis, including stem cell-based embryo-like structures and organoid models, that can be generated in high numbers, enable the study of signalling dynamics in accessible and simplified systems [228,229]. (2) Signalling reporters allow the spatiotemporally resolved quantification of signalling dynamics. Due to the functionally critical cellular heterogeneity within tissues and organisms, it is essential to analyse signalling within single cells. Signalling reporters either report on the transduction cascade itself, e.g. nuclear translocation or change in abundance of the effector protein [203], or are based on the expression of target genes [179]. To get a more comprehensive understanding of the signalling complexity, the different components of a pathway should be analysed in the same model system. This will allow the analysis of ligand–receptor interaction, the signal transduction cascade, cellular effects, target gene expression and feedback loops. For visualizing newly generated proteins, fast maturing fluorescent proteins or tagging systems should be used that are not dependent on fluorescent protein maturation, such as SNAP-tag or Llama-tag [230,231]. In addition, the correlation between mRNA and protein expression of target genes will be critical to account for species-to-species and isoform-to-isoform variations. In combination with advanced fluorescence microscopes that induce only low phototoxicity, signalling can be quantified at a high spatiotemporal resolution within multicellular systems [232,233]. (3) To reveal the complete picture of cellular signalling dynamics, it is essential to understand the abundance of various signalling components within a cell, as well as their interaction partners. With advances in omics approaches, this indeed becomes feasible. Bulk and single-cell transcriptomics have revealed cyclic genes in vertebrate segmentation [87,177,234]. However, since the transcriptome does not necessarily reflect protein expression, the proteome and phosphoproteome, ideally at a single-cell resolution, should be analysed in addition to revealing the signalling components in cells [24,235–237]. Such approaches will start to give a clear picture of how signalling dynamics are generated and how they control cellular behaviour. (4) To understand the function of signalling dynamics, we need tools to specifically modulate dynamics without altering overall signalling activity. The establishment of optogenetic and microfluidic approaches have enabled changing dynamics with high precision [179,203,238]. Systems to externally induce the degradation of selected proteins [239–241] will allow the dissection of how specific proteins, for instance negative feedback regulators, affect signalling dynamics. (5) Synthetic biology and bioengineering approaches then allow researchers to build intercellular signalling networks *in vitro*, modulate them and test predictions on the functionality of these networks [159,160,242].

To conclude, dynamic signalling is a common phenomenon in the animal kingdom. With technological advances we have started to reveal the functional significance of signalling dynamics in multicellular systems. This will allow us to study the role in different model systems to reveal general principles of its functioning. To dissect the molecular mechanism of how dynamics are generated and how they control cellular behaviour, it will be essential to transfer our understanding of signal transduction cascades at sub-cellular and molecular levels to multicellular systems and model systems of embryonic development. Moreover, the same signalling pathways, governing embryonic development, also control adult tissue homeostasis and mutations in signalling pathways induce and promote pathological conditions such as cancer [209,219,243,244]. It will be intriguing to compare how signalling dynamics modulate embryonic development, tissue homeostasis and cancer biology.

Competing Interests

C.Y.J is a cofounder of Surrozen, Inc. K.F.S. has no conflict of interest to declare.

Abbreviations

APC, adenomatous polyposis coli; CSL, CBF1/RBPJ, Su(H), Lag-1; DUSP, dual specificity phosphatases; EGF, epidermal growth factor; FGF, fibroblast growth factor; GSK3, glycogen synthase kinase-3; KSR, kinase suppressor of Ras; LRP5, lipoprotein receptor-related protein 5; MAM, mastermind; MAPK, mitogen-activated protein kinase; NECD, Notch extracellular domain; NGF, nerve growth factor; NICD, Notch intracellular domain; PSCs, pluripotent stem cells; PSM, presomitic mesoderm; RSPO, R-spondin ligands; TFs, transcription factors.

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