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Patterning and Differentiation of the Vertebrate Spine

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One of the most striking features of the human spine is its periodic organization. This so-called "segmental" arrangement of the vertebrae along the anteroposterior body axis is established during embryonic development. Structures called somites, which contain the precursors of the vertebrae, form in a rhythmic fashion at the posterior end of the embryo during the process of somitogenesis. Somites are sequentially added to the growing axis, thus establishing the characteristic periodic pattern of the future vertebral column. The primary segmentation of the vertebrate embryo displayed by somitic organization also underlies much of the segmental organization of the body, including muscles, nerves, and blood vessels. In amniotes, somites are the major component of the paraxial mesoderm that form bilaterally along the nerve cord as a result of primitive streak and tail bud regression during body axis formation. Somites bud off from the anterior presomitic mesoderm (PSM) as epithelial spheres surrounding a core of mesenchymal cells called the somitocoele. The dorsal portion of the somite remains epithelial and forms the dermomyotome, which differentiates into muscle and dermis while its ventral moiety undergoes an epithelio-mesenchymal transition, leading to the formation of the sclerotome. The sclerotome gives rise to the skeletal elements of the vertebral column: the vertebrae, ribs, intervertebral disks, and tendons. Most of our understanding of amniote somitogenesis at the morphogenetic and molecular levels results from studies involv-

ing the chicken (*Gallus gallus*) and the mouse (*Mus musculus*). In this chapter, we essentially focus on the patterning and development of the spine in amniote species such as chickens, mice, and humans.

SEGMENTAL PATTERNING OF THE VERTEBRAL COLUMN: THE CLOCK AND WAVEFRONT SYSTEM

Origin of the Vertebral Precursors: The Paraxial Mesoderm

Together with the head mesoderm, somites form the paraxial mesoderm, which appears as bilateral strips of tissue that flank the neural tube and notochord, and are bound laterally by the intermediate and lateral plate mesoderm. In the chicken, mouse, and human embryos, the first somite lies immediately posterior to the otic vesicle (Huang et al. 1997; Spörle and Schughart 1997; O'Rahilly and Müller 2003). Anterior to this somite, the paraxial mesoderm is referred to as the head or cephalic mesoderm, and it contributes to the skeletal muscles and bones of the head (see Chapter 4 by Le Douarin and Creuzet). The paraxial mesoderm is generated when cells located in a defined region of the epiblast (the superficial layer of the embryo) ingress into the primitive streak during gastrulation (Waddington 1952; Rosenquist 1966; Nicolet 1971; Tam and Beddington 1987; Schoenwolf et al. 1992; Hatada and Stern 1994; Tam and Trainor 1994; Psychoyos and Stern 1996; Pourquié 2004; Iimura et al. 2007).

At the beginning of gastrulation, the presumptive territory of the paraxial mesoderm in the epiblast is located bilaterally to the forming primitive streak that defines the future anteroposterior axis of the embryo (Fig. 1A). During primitive streak formation, these territories converge toward the streak and begin to ingress while undergoing an epithelio-mesenchymal transition. The first paraxial mesoderm precursors to ingress form the head mesoderm, which lies at the anterior tip of the embryo. Then, the primitive streak begins to shrink and regress, and its anterior tip, which corresponds to the Spemann organizer of amniotes called Hensen's node or the node, moves posteriorly. This regression lays in its wake the forming body axis and progressively forms more posterior levels of the paraxial mesoderm (Fig. 1B). Fate maps of the early embryo show that the paraxial mesoderm precursors are located in the anterior portion of the streak and in the adjacent epiblast, as well as in the node region where the notochord precursors are located (Fig. 1A,B) (Waddington 1952; Rosenquist 1966; Nicolet 1971; Tam and Beddington 1987; Selleck and Stern 1991; Schoenwolf et al. 1992; Hatada and Stern

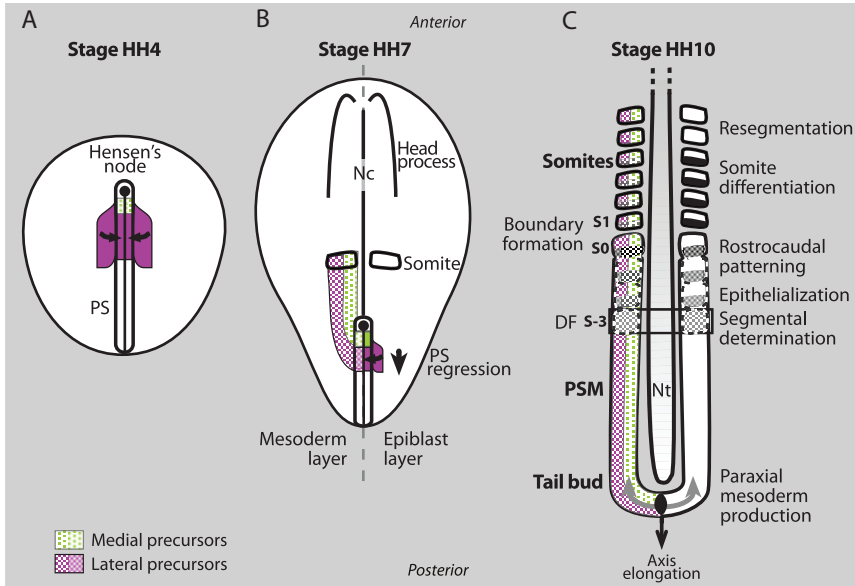

Figure 1. Paraxial mesoderm formation and segmentation in the chicken embryo. (*A*) Hamburger and Hamilton Stage 4 (Stage HH4) chicken embryo (Hamburger and Hamilton 1992). At this stage, the primitive streak (PS), which corresponds to the amniote blastopore, has reached its maximal length. Presumptive territories of somites are located in the superficial epiblast just below Hensen's node (medial precursor population, green) and in two symmetrical domains located on both sides of the PS (lateral precursor population, purple). These cells are ingressing (arrows) through the PS to form the paraxial mesoderm. (*B*) Stage HH7 chicken embryo. The PS and node have begun their posterior regression (arrowhead), leaving in their wake the embryonic axis comprising the head process anteriorly and the notochord (Nc) axially. Epiblast cells (purple) continue to ingress in the PS (arrow) and join the descendents of a population of resident stem cells located in the anterior primitive streak (green) to generate the paraxial mesoderm that forms two strips of tissue bilaterally to the notochord. The mesodermal layer is represented on the *left* side without the superficial epiblast. Medial and lateral precursors are derived from the streak stem cells and the epiblast layer, and contribute to the medial and lateral compartments of the forming somites, respectively. (*C*) Stage HH10 chicken embryo. Somitogenesis progresses posteriorly in concert with axis elongation (arrow). Paraxial mesoderm cells are produced at the tail bud level and undergo a maturation process in the presomitic mesoderm (PSM), leading to the periodic formation of new pairs of somites (S0). Dorsal views, anterior to the top. (Nt) neural tube, (DF) determination front. (Presumptive somite nomenclature according to Pourquié and Tam 2001).

1994; Tam and Trainor 1994; Psychoyos and Stern 1996; Iimura et al. 2007).

Whereas the primitive streak contributes to the formation of the most anterior somites, more posteriorly, the paraxial mesoderm is formed from the tail bud (see Fig. 1C). In this structure, gastrulation movements, similar to those observed in the primitive streak, are still observed (Pasteels 1937; Gont et al. 1993; Catala et al. 1995; Kanki and Ho 1997; Knezevic et al. 1998; Davis and Kirschner 2000; Cambray and Wilson 2002, 2007; Stern 2006). In the tail bud, precursors of the paraxial mesoderm are located immediately posterior to the chordo-neural hinge region (Charrier et al. 1999; Cambray and Wilson 2007; McGrew et al. 2008). During axis elongation, newly ingressed paraxial mesoderm cells are deposited bilaterally to the node or chordo-neural hinge and the axial structures, resulting in the formation of the two strips of paraxial mesoderm tissue (Fig. 1C) (Selleck and Stern 1991; Schoenwolf et al. 1992; Hatada and Stern 1994; Psychoyos and Stern 1996).

The origin of the paraxial mesoderm during gastrulation can be traced to two distinct populations of precursors (Rosenquist 1966; Nicolet 1970; Bellairs 1986; Selleck and Stern 1991; Iimura et al. 2007). These two populations exhibit different fates, forming the future medial and lateral somitic compartments (Fig. 1, green and purple, respectively). The medial compartment gives rise to the epaxial muscles, the vertebral column, and the dermis of the back; whereas the lateral compartment produces essentially the ribs and the hypaxial muscles that include intercostals and limb muscles (Ordahl and Le Douarin 1992; Olivera-Martinez et al. 2000). In the chicken embryo, medial somites derive from a population of precursors that exhibit a stem-cell-like behavior and are located in the anteriormost primitive streak and Hensen's node, while the lateral somite precursors derive from the epiblast adjacent to the anterior primitive streak (Fig. 1) (Bellairs 1986; Selleck and Stern 1991; Iimura et al. 2007). A stem-cell-like population, located in the primitive streak and contributing to somites, has also been identified in the mouse (Nicolas et al. 1996; Eloy-Trinquet and Nicolas 2002). The self-renewal capacity of these stem cells has been revealed by lineage analysis using various cell-labeling strategies and by serial transplants in chicken and mouse embryos (Selleck and Stern 1991; Nicolas et al. 1996; Cambray and Wilson 2002, 2007; McGrew et al. 2008). The precursors of the medial somites control the segmentation process and impose their segmental pattern on the lateral somite (Freitas et al. 2001). This dual origin of paraxial mesoderm precursors is likely conserved across vertebrates (Iimura et al. 2007).

A Molecular Oscillator Involved in Establishing the Periodicity of Vertebrae

In all vertebrate species, pairs of somites bud off periodically at the anterior tip of the PSM at a specific pace (for example, every 90 minutes in chickens, 120 minutes in mice, and between 4–8 hours in humans) (Romanoff 1960; Tam 1981; Palmeirim et al. 1997; William et al. 2007; Aulehla et al. 2008). Microsurgical experiments in the chicken embryo have revealed that segmentation of the PSM is a tissue-autonomous process and is independent of the surrounding embryonic tissues (Menkes and Sandor 1969; Christ et al. 1974; Packard 1978; Palmeirim et al. 1998; Jouve et al. 2000). The rhythmic production of somites from the PSM has inspired theoretical models such as the "clock and wavefront" model (Cooke and Zeeman 1976). This model proposed that the periodicity of somites results from the action of a molecular oscillator (called the clock) traveling along the embryonic axis. In this model, the periodic segment formation is triggered during a defined (permissive) phase of the oscillation, while the oscillator is constantly displaced posteriorly by a wave of maturation (called the wavefront), hence, ensuring the spacing of the response to the oscillator. A number of subsequent theoretical models have been proposed, many of which have also relied upon the conversion of a temporal oscillation into a spatial periodic pattern (for reviews, see Dale and Pourquié 2000; Kulesa et al. 2007).

The first evidence of the existence of an oscillator coupled to somitogenesis was provided by the periodic expression of the transcription factor *hairy1* mRNA in the chicken embryo PSM (Palmeirim et al. 1997). During the formation of each somite, the PSM is swiped by a dynamic wave of *hairy1* mRNA expression. During each somitogenesis cycle, *hairy1* is first activated in the posterior PSM and then progressively in more anteriorly located cells, giving the illusion of a traveling wave of gene expression (Fig. 2A–D). These *hairy1* transcriptional oscillations were proposed to reflect the existence of a molecular oscillator called the segmentation clock (Palmeirim et al. 1997). Subsequently, it has been shown in the fish, frog, chicken, and mouse that several members of the *Hes/Her/Hairy* family of the basic helix-loop-helix (bHLH) transcriptional repressors exhibit such a cyclic behavior, indicating that the oscillator is conserved in vertebrates (Palmeirim et al. 1997; Holley et al. 2000; Jiang et al. 2000; Jouve et al. 2000; Bessho et al. 2001; Dunwoodie et al. 2002; Li et al. 2003b). In the chicken, the genes *hairy1, hairy2*, and *Hey2*, and in the mouse, the genes *Hes1*, *Hes5, Hey1*, and *Hes7* oscillate in the PSM (Palmeirim et al. 1997; Jouve et al. 2000; Leimeister et al. 2000;

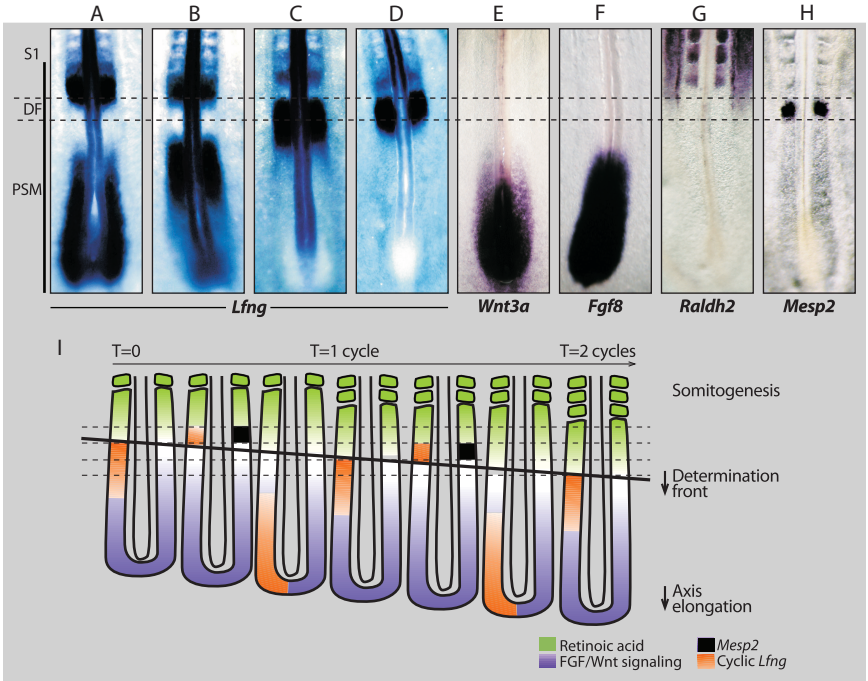

Figure 2. Clock and wavefront model of somitogenesis. (*A–H*) Expression pattern comparison of key components of the clock and wavefront system during somitogenesis in chicken embryos. (*A–D*) Wave of expression of the cyclic gene *Lfng* during one somite formation. All embryos have 18 somites and have been hybridized with the *Lfng* probe. A similar expression pattern dynamic is observed for the *hairy1* gene. (*E*) *Wnt3a,* (*F*) *Fgf8*, (*G*) *Raldh2,* (*H*) *Mesp2* expression by in situ hybridization. (S1) last formed somite, (DF) determination front (dotted lines). (*I*) Clock and wavefront segmentation model. Antagonistic gradients of Fgf/Wnt signaling (purple) and retinoic acid signaling (green) position the determination front (thick, black line). The periodic signal of the segmentation clock is shown in orange (represented on the *left* side only). As the embryo extends posteriorly, the determination front moves caudally. Cells that reach the determination front are exposed to the periodic clock signal, initiating the segmentation program and activating gene expression, such as *Mesp2* (black stripes, represented on the *right* side only), in a stripe that prefigures the future segment. This establishes the segmental pattern of the presumptive somites. Dorsal views, anterior to the top.

Bessho et al. 2001; Dunwoodie et al. 2002; Dequeant et al. 2006). In several developmental processes, the *Hes/Her/Hairy* genes are targets of the Notch pathway, but in the PSM, their oscillatory expression does not seem to absolutely require Notch signaling (Kageyama et al. 2007; Niwa et al. 2007; Ozbudak and Lewis 2008). In amniotes, other classes of cyclic genes have been identified based on their rhythmic expression pattern in

the PSM (Fig. 2A–D). These cyclic genes belong, in their vast majority, to the Notch, Wnt, and fibroblast growth factor (Fgf) signaling pathways (Dequeant and Pourquié 2008). Their dynamic expression sequence has now been imaged in real time in live mouse embryos or in isolated PSM cells using luciferase or green fluorescent protein-based methods (Masamizu et al. 2006; Aulehla et al. 2008). Cyclic expression at the protein level has been demonstrated only for a subset of cyclic genes that includes *Hes7* and *Lunatic fringe* (*Lfng*) (Bessho et al. 2003; Dale et al. 2003). As discussed below, analysis of the mutation and misexpression of the characterized cyclic genes demonstrate that many are important for proper somitogenesis.

Periodic Notch Activation Is Associated with Somite Formation

Notch signaling has been shown to play an essential role at different stages of somitogenesis (for review, see Pourquié 2001; Weinmaster and Kintner 2003; Rida et al. 2004; Gridley 2006; Lewis and Ozbudak 2007; Ozbudak and Pourquié 2008). Notch receptors (Notch 1–4 in mammals) are single-pass transmembrane proteins that undergo regulated, sequential proteolysis that is required for proper membrane presentation, as well as for signal transduction (Fig. 3A). Three cleavages have been identified and are mediated sequentially: first, by a Furin-like protease in the Golgi complex; next, at the cell surface by members of the ADAM (A disintegrin and metalloprotease) family of metalloproteases (such as ADAM17/TACE and ADAM10/Kuz); and finally, by the Presenilin/γ-secretase complex (cleavage S3) upon ligand binding to release the Notch intracellular domain (NICD) (Kopan and Ilagan 2004; Bray 2006). The originality of the Notch signaling pathway resides in the absence of a secondary messenger, since NICD acts directly as a transcription factor (Fig. 3A). Notch receptors are activated by transmembrane ligands of the Delta/Serrate/Lag-2 (DSL) family, namely, Delta-like 1, 3, and 4 (Dll1, -3, and -4) and Jagged/Serrate 1 and 2 (Jag1 and -2) in mammals. Notch signaling is mediated by the CBF1/RBP-Jk, Suppressor of Hairless Su(H)/Lag1 (CSL) transcription complex that associates with NICD. In the absence of NICD, CSL acts as a repressor. Upon nuclear translocation of NICD and binding to CSL, histone acetylases (HAT) and other components, such as Mastermind-like proteins (MAML1-3), are recruited and lead to transcriptional activation of specific target genes (Fig. 3A) (Baron 2003; Fryer et al. 2004).

Notch signaling has been implicated in a large array of developmental processes, where it can act as a switch that controls cell fate or regu-

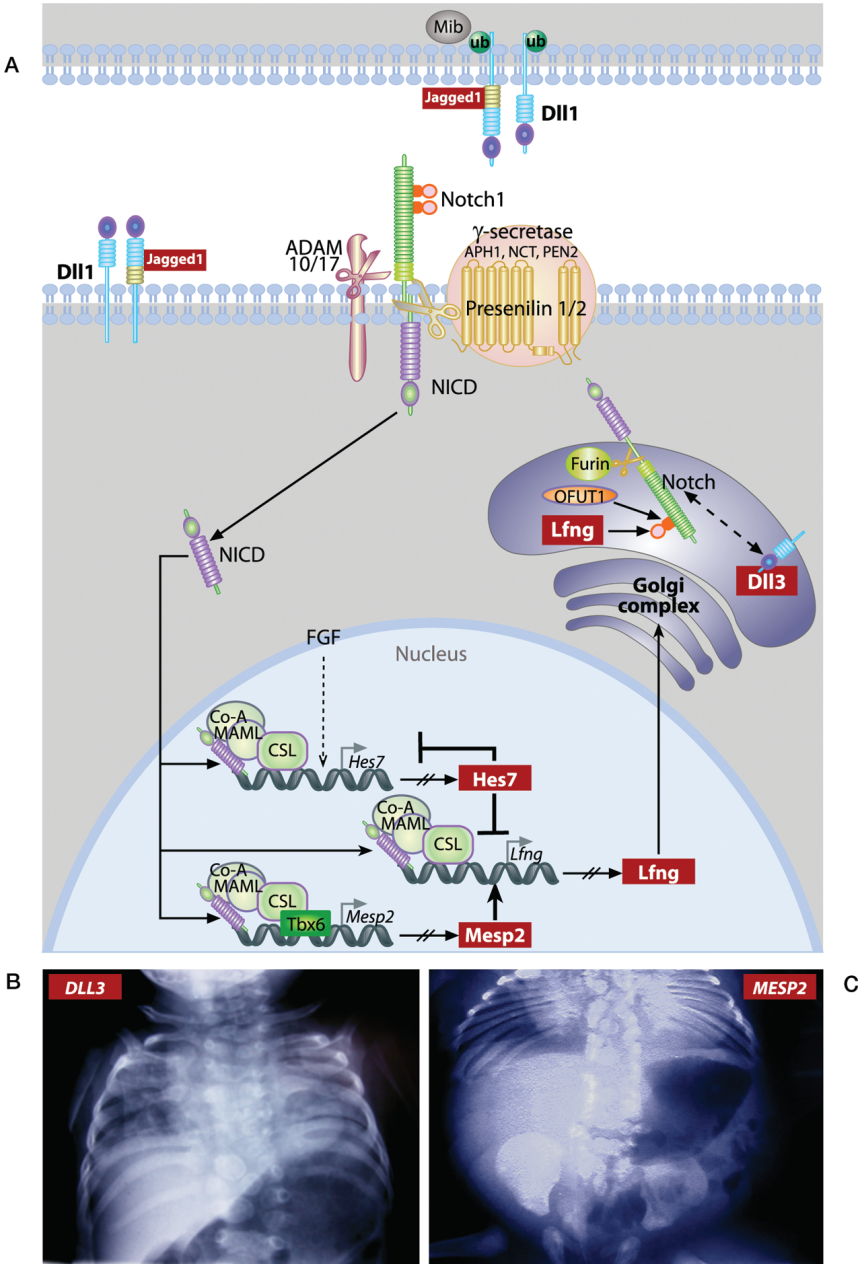

Figure 3. (*See facing page for legend.*)

lates tissue patterning (Artavanis-Tsakonas et al. 1999). In the mouse PSM, the activation of the Notch1 receptor is periodic and parallels the rhythm of somite production. Rhythmic cleavage of NICD can be detected using a specific antibody that recognizes the cleaved form of the Notch1 receptor (Huppert et al. 2005; Morimoto et al. 2005). In mice and zebrafish, mutation of *Notch1* (*Notch1* in mice and *Notch1a/des* in zebrafish) results in defects in somite formation, suggesting that it acts as the major Notch receptor involved in somite segmentation (Conlon et al. 1995; Holley et al. 2002). While *Notch2* is also expressed in the mouse PSM, the *Notch2* null embryo does not exhibit segmentation defects (Hamada et al. 1999). However, the double *Notch1* and *-2* null embryo exhibits more severe segmentation defects than the single *Notch1* mutant, suggesting some functional redundancy between the two receptors (Huppert et al. 2005). Mice mutant for *Presenilins* or for the other components of the γ-secretase complex (e.g., *Nicastrin* [*Nct*] or *Aph-1a*) exhibit a Notch-like segmentation defect, and double *PS1* and *-2* mutants do not form somites (Fig. 3A) (Shen et al. 1997; Wong et al. 1997; Donoviel et al. 1999; Herreman et al. 2000; Koizumi et al. 2001; Li et al. 2003a; Ma et al. 2005). Moreover, in the chicken and zebrafish, pharmacological inhibition of γ-secretase to block Notch signaling results in segmentation defects (Geling et al. 2002; Dale et al. 2003; Mara et al. 2007; Riedel-Kruse et al. 2007; Ozbudak and

Figure 3. Notch signaling pathway and human vertebral abnormalities. (*A*) The Notch signaling pathway. The transmembrane Notch receptor is activated by the binding of Delta and Jagged ligands from neighboring cells, leading to proteolytic cleavage and release of the Notch intracellular domain (NICD). Notch cleavage involves the γ-secretase complex. NICD translocates to the nucleus and interacts with the DNA-binding CSL protein, recruiting Mastermind (MAML) and leading to the activation of transcription of target genes, such as *Hes7*, *Lfng*, and *Mesp2*. The ubiquitination (Ub) by the ubiquitin ligase Mib controls the endocytosis of Notch ligands, which is necessary for proper signaling. During Notch receptor trafficking to the membrane, Notch undergoes sequential proteolytic cleavages by Furin and ADAMs protease. Notch undergoes sequential glycosylation by OFUT1 and Lfng. Dll3 is located principally in the Golgi complex where it interacts in *cis* to modulate Notch signaling. Notch pathway components found to be mutated in association with human vertebral segmentation abnormalities are shown in red boxes. (*B*) Radiograph of a patient with Spondylocostal dysostosis (SCDO1) showing the severe axial skeletal malformations associated with a mutation in the *DLL3* gene. (Radiograph provided by Peter Turnpenny, M.D.) (*C*) Radiograph of patient with spondylothoracic dysostosis (STD) illustrating severe vertebral and rib malformations associated with a mutation in the *MESP2* gene. (Radiograph provided by Albert Cornier, M.D., and reprinted from Cornier et al. 2008 [© Elsevier].) (Co-A) Coactivator.

Lewis 2008). After nuclear translocation, NICD activates transcription of target genes, such as *Lfng* or *Notch-regulated ankyrin repeat protein* (*Nrarp*) (see Fig. 3A) (Krebs et al. 2001; Lamar et al. 2001; Cole et al. 2002; Morales et al. 2002; Dale et al. 2003; Pirot et al. 2004). Periodic activation of transcription downstream of Notch is indicated by the rhythmic waves of *Nrarp* and *Lfng* expression in the PSM (see Fig. 2A–D) (Forsberg et al. 1998; McGrew et al. 1998; Aulehla and Johnson 1999; Dequeant et al. 2006). Mice mutant for *RBP-Jk* (Oka et al. 1995; del Barco Barrantes et al. 1999) and *Lfng* (Evrard et al. 1998; Zhang et al. 2002) exhibit marked defects in somitogenesis with few anterior somites formed and an overall disruption of posterior segmentation.

The rhythmic activation of Notch signaling in the PSM could be triggered by the periodic expression of the Notch ligands. In amniotes, *Dll1*, *Dll3*, and *Jag/Serrate1*, genes are expressed in the PSM (Bettenhausen et al. 1995; Henrique et al. 1995; Lindsell et al. 1995; Myat et al. 1996; Dunwoodie et al. 1997). Genes coding for Notch ligands exhibit cyclic expression in the mouse (*Dll1*) and zebrafish (*deltaC*) PSM (Jiang et al. 2000; Maruhashi et al. 2005). Moreover, mice mutant for *Dll1* and *Dll3* (*Pudgy*), and zebrafish mutant for *deltaD* (*aei*) and *deltaC* (*bea*) exhibit somitogenesis defects (Hrabě de Angelis et al. 1997; Kusumi et al. 1998; van Eeden et al. 1998; Barrantes et al. 1999; Jiang et al. 2000; Jouve et al. 2000; Dunwoodie et al. 2002; Jülich et al. 2005). In the mouse, Dll1 (which is expressed at the surface of PSM cells) activates Notch signaling; whereas, Dll3 (a highly divergent Notch ligand that is localized primarily in the Golgi complex) acts intracellularly through an unknown mechanism (see Fig. 3A) (Geffers et al. 2007). In vitro experiments and analysis of mice mutant for *Dll3* indicate that Dll3 modulates Notch signaling in a cell-autonomous fashion through a mechanism that is independent of Presenilin (Takahashi et al. 2003; Ladi et al. 2005). In addition, several ubiquitin ligases modulate Notch signaling by regulating Notch ligand endocytosis (Le Borgne 2006). In mice and zebrafish, mutations in genes coding for E3 ubiquitin ligases (e.g., *Mind bomb* [*Mib1*] and *Neuralized* [*Neur1/2*]) result in somitogenesis defects (see Fig. 3A) (Itoh et al. 2003; Chen and Casey Corliss 2004; Barsi et al. 2005; Koo et al. 2005; Zhang et al. 2007).

Alternatively, periodic Notch activation could also be triggered by intracellular negative feedback loops. *Hes7* codes for a bHLH transcriptional repressor that can act downstream of Notch and can repress its own transcription, as well as that of the negative feedback inhibitor *Lfng* (see Fig. 3A) (Bessho et al. 2001, 2003; Dale et al. 2003; Morimoto et al. 2005; Niwa et al. 2007). *Hes7* is periodically expressed in the mouse PSM,

and in mutant mice, its inactivation results in an arrest of *Lfng* oscillations and in an up-regulation of *Hes7* transcription due to the lack of *Hes7* repressive activity, resulting in somitogenesis defects (see Fig. 3A) (Bessho et al. 2003). Thus, Hes7 was proposed to play a role in the control of cyclic gene oscillations in the PSM (Kageyama et al. 2007). In zebrafish, homologs of the *Hes7* gene—*Her1* and *Her7*—display a cyclic expression in the PSM, and their overexpression or their knockdown leads to segmentation defects (Takke and Campos-Ortega 1999; Holley et al. 2000, 2002; Henry et al. 2002; Oates and Ho 2002; Gajewski et al. 2003; Lewis 2003; Rida et al. 2004; Oates et al. 2005a; Giudicelli et al. 2007; Holley 2007; Ozbudak and Lewis 2008). It has been proposed that a simple *Her1/Her7*-based, delayed negative feedback mechanism generates oscillations and, thus, acts as the clock pacemaker (Lewis 2003). In zebrafish, it has been proposed that the role of Notch signaling is restricted to the synchronization of oscillations between individual PSM cells and, therefore, coordinates the waves of cyclic gene expression along the PSM (Jiang et al. 2000; Horikawa et al. 2006; Mara et al. 2007; Riedel-Kruse et al. 2007; Ozbudak and Lewis 2008). Evidence is still lacking for such a role for Notch signaling in amniotes.

A second negative feedback loop relying on Lfng and regulating Notch activation has been described in amniotes (Dale et al. 2003; Morimoto et al. 2005). Lfng is a glycosyltransferase that modifies the Notch extracellular domain during its trafficking to the cell surface (see Fig. 3A) (Haines and Irvine 2003). In the PSM, Lfng periodically inhibits Notch signaling and participates in a negative feedback loop involved in the control of Notch pathway oscillation and somite boundary positioning (Dale et al. 2003; Serth et al. 2003; Huppert et al. 2005; Morimoto et al. 2005; Shifley et al. 2008). Lfng-modification activity is dependent upon the priming of the Notch receptor by the O-fucosyltransferase (OFUT1), which is expressed ubiquitously and is essential for mesoderm segmentation in mice (see Fig. 3A) (Shi and Stanley 2003). In the PSM, *Lfng* cyclic expression is controlled at the transcriptional level and is observed only in amniotes (see Fig. 2A–D) (Forsberg et al. 1998; McGrew et al. 1998; Aulehla and Johnson 1999; Leve et al. 2001; Cole et al. 2002; Morales et al. 2002; Qiu et al. 2004).

Oscillations of Canonical Wnt and of Fgf Signaling in the Mouse Embryo

Wnt ligands are soluble proteins that bind to a receptor complex composed of Frizzled (Fz) and low-density-lipoprotein receptor-related pro-

tein 5/6 (LRP5/6). In the absence of Wnt signaling, the protein β-catenin is sequestered in a "destruction complex" that contains the scaffolding proteins Adenomatosis polyposis coli (APC), Disheveled (Dsh), Axin1 and -2, and the kinases Glycogen synthase kinase 3 (GSK3) and Casein kinase 1 (CK1) that phosphorylate β-catenin, targeting it for proteosomal degradation. Wnt binding to its receptor complex leads to the release of β-catenin from the destruction complex and, thus, prevents its phosphorylation (Fuerer et al. 2008; Klaus and Birchmeier 2008). This stops β-catenin degradation and allows it to translocate to the nucleus and to bind to the lymphoid enhancer factor/T-cell-specific factor (LEF/TCF) transcriptional complex. In the absence of Wnt signaling, LEF/TCF (*Lef1* and *Tcf1, -3,* and *-4* in mammals) binds Groucho-related proteins and acts as a repressor. Upon activation of the pathway, the complex, formed from LEF/TCF and β-catenin, is turned into a transcriptional activator that drives the expression of a specific set of target genes (Nusse 2005; Hoppler and Kavanagh 2007). *Axin2* is a classical target of the Wnt canonical pathway that has been shown to be expressed periodically in the mouse embryo PSM (Jho et al. 2002; Leung et al. 2002; Aulehla et al. 2003). This implicated the Wnt/β-catenin pathway as a novel player in the mouse segmentation clock (Aulehla et al. 2003). While mice mutant for the cyclic gene *Axin2* do not exhibit mesodermal defects, mice mutant for *Axin1* (*Fused*) exhibit segmentation defects (Zeng et al. 1997; Yu et al. 2005).

In addition to *Axin2*, several other cyclic Wnt targets have been identified in a microarray screen aimed at identifying all cyclic genes in the mouse PSM transcriptome (Dequeant et al. 2006). These genes include several negative feedback inhibitors of the pathway, including: *Dickkopf homolog 1 (Dkk1)*, *Dapper homolog 1* (*Dact1*), and *Naked cuticle 1* (*Nkd1)* (Ishikawa et al. 2004; Dequeant et al. 2006; Suriben et al. 2006). Inactivation of several of these inhibitors, such as *Dkk1* and *Dact1*, results in segmentation defects (Mukhopadhyay et al. 2001; MacDonald et al. 2004). Wnt ligands are distributed in a graded but steady fashion along the PSM. Thus, as proposed for Notch oscillations, despite the presence of a constant (nonperiodic) Wnt signaling input in the PSM, oscillations of Wnt target transcription could be triggered by the periodic activation of unstable negative feedback inhibitors (Goldbeter and Pourquié 2008). However, as discussed further, periodic expression of cyclic genes, such as *Dkk1*, is still detected in mouse mutants that express a constitutively stable version of β-catenin in the PSM, thus, arguing against the role of periodic β-catenin destabilization in the clock pacemaker (Aulehla et al. 2008; Dunty et al. 2008).

The Fgf pathway constitutes a third signaling pathway activated periodically in the mouse and chicken PSM. Upon binding by Fgf ligands,

the Fgf receptors (*FgfR1–4* in mammals) dimerize and through transphosphorylation, initiate several transduction cascades that include: (1) MAP kinase or ERK kinase (MEK), Extracellular-signal-regulated kinase/Mitogen-activated protein kinase (ERK/MAPK) cascade, (2) the PI3 Kinase cascade, and (3) the Phospholipase Cγ- (PLCγ) cascade, ultimately leading to the activation of specific target genes (Bottcher and Niehrs 2005). Cyclic expression of the Fgf targets *Snail homolog 1* and *2* (*Snail1* and *-2*), *Sprouty homolog 2* (*Spry2*), and *Dual specificity phosphatase 4* and *6* (*Dusp4* and *-6*) suggests that the Fgf signaling pathway is activated periodically in the posterior PSM (Dale et al. 2006; Dequeant et al. 2006; Niwa et al. 2007). This periodic regulation of Fgf signaling is further supported by the dynamic phosphorylation of ERK in the mouse PSM (Niwa et al. 2007). As for the Wnt and Notch pathways, many of the Fgf pathway cyclic genes are negative feedback inhibitors. Fgf signaling has been proposed to control the initiation of *Hes7* oscillations in the tail bud, while Notch signaling would maintain these oscillations in the PSM (see Fig. 3A) (Niwa et al. 2007). Thus, in the posterior PSM, Fgf-dependent *Hes7* expression controls the cyclic activation of the Fgf inhibitor *Dusp4,* hence, potentially driving the periodic inhibition of this pathway (Niwa et al. 2007).

Does the Cyclic Gene Network Act as the Clock Pacemaker?

The complex epistatic relationships and multiple cross talks between the Notch, Fgf, and Wnt signaling pathways in the PSM make the analysis of their respective contributions to the segmentation clock mechanism particularly challenging. Global analysis of cyclic gene expression reveals that Notch- and Fgf-related cyclic genes oscillate mostly in antiphase to Wnt-cyclic genes, suggesting a cross talk between these signaling pathways (Dequeant et al. 2006; Goldbeter and Pourquié 2008).

Oscillations of *Axin2* and *Spry2* are maintained in the mouse *RBP-Jk* mutants and in transgenic mouse embryos constitutively activating Notch in the PSM (Aulehla et al. 2003; Hirata et al. 2004; Dequeant et al. 2006; Feller et al. 2008). In contrast, *Lfng* and *Axin2* oscillations are disrupted in the mouse *Wnt3a* hypomorphic *vestigial tail* (*vt*) mutant (Aulehla et al. 2003). Together, these findings argue that Notch does not act as the clock pacemaker and that Wnt signaling acts upstream of the Notch oscillations.

The identification of many cyclic Wnt pathway negative feedback inhibitors raises the possibility that the periodic β-catenin destabilization, which should result from the action of these negative feedback loops,

could act as the clock pacemaker that controls oscillations of the Notch and Fgf pathways. However, using a transgenic reporter mouse in which the *Lfng* promoter is fused to the yellow fluorescent protein Venus, *Lfng* has been shown to oscillate with the same periodicity in β-catenin gain-of-function mutants and in wild-type mice (Aulehla et al. 2008). Therefore, periodic β-catenin production is not required to control the rhythmicity of Notch activation in the PSM.

Conditional deletion of *FgfR1* in the PSM abolishes oscillations of *Hes7, Lfng, Axin2*, and *Spry2* (Niwa et al. 2007; Wahl et al. 2007). Similarly, in vitro treatment of mouse embryos with the Fgf inhibitor SU5402 leads to a rapid arrest of *Axin2* and *Spry2* oscillations; whereas, *Hes7* and *Lfng* oscillations cease only after a delay of more than one cycle (Niwa et al. 2007; Wahl et al. 2007). These results are consistent with a role for Fgf signaling in the control of the Notch and Wnt oscillations. However, introducing a constitutively stable version of β-catenin in a mutant mouse embryo, in which *FGFR1* is conditionally deleted in the PSM, restores the formation of the *Lfng* stripes of expression (Aulehla et al. 2008). These embryos show constitutive nuclear β-catenin expression and lack Fgf signaling in the PSM; yet, they appear to be capable of producing *Lfng* oscillations. Therefore, this result argues against a role for an Fgf-based negative feedback loop in the pacemaker of the segmentation clock.

Taken together, these data support the argument that none of the three signaling pathways periodically activated in the PSM individually appear to act as a global clock pacemaker. Hence, this raises doubts about the current models in which the periodic gene expression associated with the segmentation clock is presented as resulting from the dynamic properties of the cyclic gene network (Dequeant and Pourquié 2008). In amniotes, it is possible that each subnetwork has the capacity to generate its own oscillations independent of the oscillations of the other subnetworks—while coupling among the subnetworks entrain them to each other (Goldbeter and Pourquié 2008; Ozbudak and Pourquié 2008). Alternatively, it is possible that the network of cyclic genes that underlie the segmentation clock is entrained by an outside pacemaker that remains to be identified.

Defects in the Segmentation Clock Lead to Congenital Scoliosis in Humans

Congenital abnormalities in vertebral segmentation occur in a wide variety of rare but well-characterized disorders, encompassing many diverse and poorly understood phenotypic patterns (Turnpenny et al. 2007). Congenital forms of scoliosis involve structural malformations of the spine

that are visible on radiographs and include segmental abnormalities such as hemivertebrae, wedge-shaped vertebrae, vertebral fusions, and bars. These observations in patients with congenital scoliosis are suggestive of various abnormalities that may occur during early developmental patterning (see Fig. 3B,C). A new nomenclature, developed by the International Consortium for Vertebral Anomalies and Scoliosis (ICVAS), has been created to better describe these conditions (Turnpenny et al. 2007).

Manifestations leading to a diagnosis of congenital scoliosis include: (1) generalized vertebral segmentation abnormalities as observed in patients with spondylocostal dysostosis (SCD) or spondylothoracic dysostosis (STD), (2) regionalized conditions, such as Klippel-Feil syndrome (which affects only the cervical region), and (3) conditions that involve only one or two vertebrae. Congenital scoliosis can also be associated with anomalies in other organ systems, most frequently involving the renal, cardiac, or neural systems. In some cases, patients with congenital scoliosis with abnormalities of the chest wall present a major surgical challenge. A better understanding and increased knowledge of the disease mechanism(s) will aid in improving the prediction of the clinical course of the disease, particularly in children.

Although most cases of congenital scoliosis were previously thought to be sporadic, recent evidence suggests that a considerable genetic component may be involved. Strikingly, thus far, the four mutations associated with congenital scoliosis in humans have been identified in genes associated with the segmentation clock mechanism (see Fig. 3A). These mutations result in monogenic autosomal recessive forms of SCD (Turnpenny et al. 2007; Sparrow et al. 2008). Homozygosity mapping and linkage analysis in consanguineous Arab-Israeli and Pakistani pedigrees with a particular form of SCD (SCDO1 [MIM 277300]) led to the discovery of multiple mutations in the Notch ligand *DLL3* (Bulman et al. 2000). These *DLL3* mutations result in abnormal vertebral segmentation throughout the entire spine with all vertebrae losing their normal form and regular three-dimensional shape (see Fig. 3B). A second, milder form of SCD (SCDO2 [MIM 608681]) has been associated with a 4-base-pair duplication in the *MESP2* gene that is essential for normal segmentation (discussed later in this chapter) (Whittock et al. 2004). A mutation in *LFNG* was identified in members of one family with SCD (SCDO3 [MIM 609813]) (Sparrow et al. 2006). A mutation in the *HES7* gene was also found to be associated with a case of SCD (SCDO4) (Sparrow et al. 2008). The identified mutation impairs HES7 protein to heterodimerize with the E47 cofactor. In addition, a recessive null mutation in the *MESP2* gene was also identified in patients with STD (Jarcho-Levin syndrome)

(see Fig. 3C) (Cornier et al. 2008). Also, various mutations of the Notch ligand *JAGGED1* have been associated with the autosomal dominant Alagille syndrome in which misshaped vertebrae (butterfly vertebrae) are observed (AGS [MIM 118450]) (Li et al. 1997; Oda et al. 1997; Joutel and Tournier-Lasserve 1998). Together, these data strongly support the argument that human somitogenesis also relies on molecular mechanisms similar to those identified in mouse and chicken embryos.

The Wavefront: Translating the Periodic Signal into Repeated Segments

The Determination Front Controls PSM Maturation

The concept of a "wavefront," as originally proposed by Cooke and Zeeman, suggests that a transitional zone exists in the PSM where cells that reach this level undergo a "catastrophe" corresponding to a sudden change in cell properties and leading to somite formation (Cooke and Zeeman 1976). Several studies have demonstrated the existence of such a transitional region in the PSM, called the determination front (Dubrulle et al. 2001; Sawada et al. 2001). This region is located at approximately the S-3/S-2 level in chicken embryos (presumptive somite -3/-2; nomenclature according to Pourquié and Tam [2001]) and corresponds to an important transition in gene regulation and cellular properties (see Figs. 1C and 2). Grafting experiments in chicken embryos reveal that the segmental pattern is established only anterior to the determination front (Dubrulle et al. 2001).

The determination front is positioned by antagonistic gradients of Fgf, Wnt, and retinoic acid (RA) signalings, and regresses posteriorly as the embryo elongates along the anteroposterior axis (see Fig. 2E–G,I) (Aulehla and Herrmann 2004; Dubrulle and Pourquié 2004a; Moreno and Kintner 2004). Mathematical modeling of this system led to the proposal that the determination front is associated with a bistability window defined by the mutually antagonistic gradients of Fgf and RA (Goldbeter et al. 2007; Dequeant and Pourquié 2008). In this window, cells are poised to switch abruptly from one steady state to the other in response to a triggering signal, such as the signaling pulse delivered by the segmentation clock. This abrupt transition could explain the sudden rhythmic appearance of stripes of gene expression at the determination front level. Molecularly, segmental genes (such as *mesoderm posterior 2* [*Mesp2*]) become derepressed at the determination front level (Delfini et al. 2005), and stripes of *Mesp2* gene expression form in a rhythmic sequence in response to the clock sig-

nal (see Fig. 2H,I) (Saga et al. 1997). As we shall see, the stripes of *Mesp2* expression provide the template from which the morphological segments—the somites—will form (Morimoto et al. 2005; Saga 2007).

Fgf/Wnt Signaling Gradients Control the Maturation of the PSM

Parallel posterior-to-anterior gradients of Fgf and Wnt signaling (evidenced by graded phosphorylated ERK and nuclear β-catenin, respectively) are established in response to the graded expression of secreted ligands, such as Fgf8 and Wnt3a, along the PSM (see Fig. 2E,F) (Dubrulle et al. 2001; Sawada et al. 2001; Aulehla et al. 2003; Dubrulle and Pourquié 2004b; Delfini et al. 2005; Aulehla et al. 2008). In the posterior PSM, cells are exposed to a high level of Fgf and Wnt activity, and are maintained in an immature, undifferentiated state (Dubrulle et al. 2001; Sawada et al. 2001; Aulehla et al. 2008; Dunty et al. 2008). The transition from the immature to the competent state of PSM cells can be visualized by the down-regulation of the *Mesogenin1* (*Msgn1*) gene at the level of the determination front (Buchberger et al. 2000; Yoon et al. 2000).

Molecular Mechanisms Generating the Signaling Gradients. The posterior gradients and thus the determination front move posteriorly in the wake of axis elongation. This posterior displacement of the *Fgf8* gradient has been studied and shown to rely on an original mechanism involving *Fgf8* mRNA decay (Dubrulle and Pourquié 2004b). Transcription of the *Fgf8* mRNA is restricted to the PSM precursors in the tail bud, and it ceases when their descendents enter the posterior PSM. Thus, as the axis elongates, cells gradually become located more anteriorly in the PSM, and their *Fgf8* mRNA content progressively decays (see Fig. 2F). This results in the establishment of an *Fgf8* mRNA gradient that is converted into a graded ligand distribution pattern and in graded Fgf activity along the PSM (Sawada et al. 2001; Dubrulle and Pourquié 2004b; Delfini et al. 2005). A similar mechanism is assumed to be responsible for establishing the Wnt gradient (Aulehla et al. 2003). As a result of the progressive decay of the *Fgf/Wnt* mRNA and proteins in PSM cells, the determination front is constantly displaced posteriorly (Goldbeter et al. 2007). In the zebrafish, chicken, mouse, and snake, the regression speed of the determination front (marked by the anterior boundary of the *Msgn1* expression domain) during somitogenesis is similar to the speed of somite formation (Gomez et al. 2008). Thus, during embryogenesis, this original gradient formation mechanism ensures a tight coupling between axis elongation and segmentation.

Fgf Signaling and Maturation of the PSM. The role of the Fgf signaling gradient in positioning the determination front was first demonstrated by experiments challenging the slope of the signaling gradient in chicken embryos by grafting FGF8-soaked beads next to the PSM or by overexpressing an *Fgf8*-expressing construct in the PSM by electroporation (Dubrulle et al. 2001). This resulted in an anterior extension of posterior PSM markers (such as *Brachyury*), in down-regulation of segmentation and differentiation markers (such as *Paraxis*, *Mesp2*, or *myogenic differentiation 1* [*MyoD*]), and in the formation of smaller somites (Dubrulle et al. 2001; Delfini et al. 2005). Conversely, inhibition of Fgf signaling was achieved by treating chicken embryos with pharmacological inhibitors (Dubrulle et al. 2001). This resulted in a posterior shift of the anterior boundary of genes associated with a posterior PSM identity (such as *Fgf8*), and in the formation of larger somites. Together, these data suggested that high levels of Fgf signaling are required to maintain the posterior identity of PSM cells (Dubrulle et al. 2001). This further led to the idea that the progressive decrease in Fgf signaling activity along the PSM defines a specific threshold below which the cells become competent to respond to the signaling pulse delivered by the segmentation clock. Changing the position of the threshold by acting on Fgf levels leads to a change in the position of the somite boundary position, resulting in smaller or larger somites. The position of this threshold was proposed to correspond to the determination front (Dubrulle et al. 2001).

Fgf8 and *FgfR1* have emerged as key players during embryonic axis elongation and somitogenesis, with conserved expression domains and function in the vertebrate PSM (see Fig. 2F) (Deng et al. 1994; Yamaguchi et al. 1994; Crossley and Martin 1995; Sun et al. 1999; Dubrulle et al. 2001; Sawada et al. 2001; Hoch and Soriano 2006; Niwa et al. 2007; Wahl et al. 2007). Fgf signaling in the PSM activates the ERK/MAPK and PI3K-Akt cascade (Yamaguchi et al. 1992, 1994; Deng et al. 1994; Crossley and Martin 1995; Ciruna et al. 1997; Sawada et al. 2001; Dubrulle and Pourquié 2004b; Delfini et al. 2005; Lunn et al. 2007; Niwa et al. 2007; Wahl et al. 2007). In mice mutant for *Fgf8* and *FgfR1*, loss of function of Fgf signaling results in severe gastrulation defects (Deng et al. 1994; Sun et al. 1999). Conditional deletion of *Fgf8* in the PSM does not lead to abnormal somitogenesis, suggesting that the *Fgf3*, *-4*, and *-17* ligands, which are also expressed in the mouse tail bud and PSM, probably act redundantly with *Fgf8* to pattern the paraxial mesoderm (Bottcher and Niehrs 2005; Perantoni et al. 2005; Wahl et al. 2007). In contrast, *FgfR1* is the only Fgf receptor expressed in the mouse PSM (Wahl et al. 2007). Conditional deletion of *FGFR1* in the PSM results in severe segmenta-

tion defects and axis truncation, suggesting that Fgf signaling in the PSM is mediated essentially by FgfR1 (Niwa et al. 2007; Wahl et al. 2007).

Wnt Signaling and Maturation of the PSM. In amniotes, Wnt signaling in the posterior PSM involves *Wnt3a* and *-5a*, and the *Frizzled1*, *-2*, and *-7* receptors (see Fig. 2E) (Takada et al. 1994; Yamaguchi et al. 1999a; Cauthen et al. 2001; Aulehla et al. 2003). Loss of function of Wnt signaling in mice mutant for *Wnt3a* or its downstream effectors *Lef1* and *Tcf1* causes severe axis truncation immediately following the forelimb level (Takada et al. 1994; Galceran et al. 1999). Similarly, axis truncations are observed when β-catenin function is deleted in the PSM (Aulehla et al. 2008; Dunty et al. 2008). Mice bearing the *Wnt3a* hypomorphic mutation *vt* are truncated at the tail level, whereas mice bearing the *vt* allele and the *Wnt3a* knockout allele exhibit a more severe truncation phenotype, and a variable number of lumbar (but not caudal) vertebrae are formed (Greco et al. 1996). Interestingly, mutations of *Wnt3a, Lef1/Tcf1*, or their targets *T* and *Tbx6*, also cause the posterior paraxial mesoderm to switch to a neural tube fate (Takada et al. 1994; Yoshikawa et al. 1997; Chapman and Papaioannou 1998). These results clearly indicate that Wnt3a, acting through the canonical Wnt signaling pathway, is required for production of the posterior paraxial mesoderm. In humans, abnormalities of this gradient system could result in congenital anomalies such as caudal agenesis, a rare condition that occurs sporadically but is more specific to pregnant women with diabetes mellitus (Catala 2002).

In mice, nuclear β-catenin forms a clear gradient parallel to the *Wnt3a* mRNA gradient in the posterior PSM (Aulehla et al. 2008). β-catenin conditional loss- and gain-of-function studies in mouse embryos demonstrate that the level of nuclear β-catenin controls PSM cell maturation (Aulehla et al. 2008; Dunty et al. 2008). Gain of function of Wnt/β-catenin signaling leads to an anterior expansion of the expression domain of posterior genes, such as *Brachyury, Msgn1*, or *Tbx6*. Furthermore, in these experiments, both segmentation and the onset of the differentiation program in the PSM are blocked (Aulehla et al. 2008; Dunty et al. 2008). The most striking effect in the gain-of-function mutant is an anteroposterior extension of the oscillatory domain that results in a multistripe, oscillatory expression pattern in the enlarged PSM (Aulehla et al. 2008; Dunty et al. 2008). These results imply that Wnt/β-catenin controls the size of the oscillatory domain and maintains the immature state of posterior cells and, thus, controls the onset of segmentation and differentiation in the PSM (Aulehla et al. 2008). Therefore, Wnt signaling also plays a major role in the positioning of the determination front. Whether Wnt acts in par-

allel or synergistically with Fgf in this process is not understood.

Wnt signaling is regulated at multiple levels in the PSM. Secreted WNT antagonists (such as Dkk1 and Secreted frizzled-related proteins [Sfrps]), are implicated in axis elongation and somitogenesis regulation (Hoang et al. 1998; Baranski et al. 2000; Ladher et al. 2000; Terry et al. 2000; Kawano and Kypta 2003). Dkk1 interacts with the coreceptor LRP6 by competitive binding with WNT ligands (Sëmenov et al. 2001; Satoh et al. 2006). Mice mutant for *Dkk1* and *LRP6* exhibit segmentation defects (Mukhopadhyay et al. 2001; Kokubu et al. 2004; MacDonald et al. 2004). Sfrps function by sequestering WNT ligands (Hsieh et al. 1999; Kawano and Kypta 2003; Satoh et al. 2006). Mice compound mutant for *Sfrp1, -2*, and *-5* exhibit defects in mesoderm segmentation and a shortening of the anteroposterior axis (Satoh et al. 2006, 2008).

Cross Talk between Fgf and Wnt Signaling. The interactions between Fgf and Wnt signaling in the PSM remain unclear. In mice, *Fgf8* expression is absent from the *Wnt3a* hypomorph *vt* mutants, suggesting that Wnt signaling acts upstream of Fgf signaling in the tail bud (Aulehla et al. 2003, 2008). However, gain of function of β-catenin in the PSM of mouse embryos results only in a partial gain of function of Fgf signaling, suggesting that Wnt signaling is necessary but insufficient for Fgf signaling in the PSM (Aulehla et al. 2008).

In addition, the Wnt and Fgf pathways have been shown to simultaneously control the expression of a number of transcription factors—*Brachyury/T*, *Tbx6, Gbx2, Cdx1, -2, and -4*, and *Msgn1* genes—that in turn control different aspects of PSM maturation (Pownall et al. 1996; Yamaguchi et al. 1999a,b; Arnold et al. 2000; Ciruna and Rossant 2001; Lohnes 2003; Bussen et al. 2004; Hofmann et al. 2004; Pilon et al. 2006, 2007; Wahl et al. 2007; Wittler et al. 2007; Wardle and Papaioannou 2008). Mutations of several of these genes cause similar truncation phenotypes as those observed in mutations in the Fgf or Wnt pathways (Herrmann et al. 1990; Schulte-Merker et al. 1994; Wilson et al. 1995; Chapman et al. 1996; Chapman and Papaioannou 1998; White et al. 2003).

How Fgf and Wnt signalings elicit a response, which is graded for some genes and cyclic for others, is not understood. Additionally, the control of the surface distribution of the Fgf and Wnt receptors (FgfR and Fz, respectively) allows spatial restriction of the signaling along the PSM. *Shisa* is a conserved gene family (*Shisa1-3*) that encodes endoplasmic reticulum-localized chaperone proteins (He 2005; Yamamoto et al. 2005; Filipe et al. 2006; Nagano et al. 2006; Furushima et al. 2007). *Shisa2* is expressed in the anterior PSM and acts as a negative feedback inhibitor

of Fgf and Wnt signalings by regulating the chaperone machinery to prevent FgfR and Fz maturation and cell-surface expression (Yamamoto et al. 2005; Nagano et al. 2006). In Xenopus, *Shisa2* knockdown results in an anterior shift of the determination front, resulting in delayed PSM maturation (Nagano et al. 2006). Thus, several distinct molecular mechanisms can modulate the Fgf/Wnt signaling gradients in the PSM.

Other Signaling Pathways Involved in PSM Patterning. The noncanonical Wnt pathway has also been implicated in the control of paraxial mesoderm differentiation. In the mouse embryo, *Wnt5a* is expressed in a caudal-to-rostral gradient in the PSM, and mice mutant for *Wnt5a* exhibit a shorter PSM, skeletal defects, and axial truncation (Yamaguchi et al. 1999a; Schwabe et al. 2004). This phenotype is observed also in mice double mutant for *Dsh1* and *-2,* which are downstream transducers of Wnt signaling (Hamblet et al. 2002; Wang et al. 2006). In humans, disorders such as the Robinow syndrome (RRS [MIM 268310]), are associated with autosomal recessive mutations in the receptor tyrosine kinase orphan receptor *ROR2*, resulting in spine abnormalities that include hemivertebrae (Afzal et al. 2000; van Bokhoven et al. 2000; Patton and Afzal 2002). ROR2 has been shown to interact with Wnt5a (Oishi et al. 2003). Among the identified human *ROR2* mutations, some have been shown to affect specifically the targeting of the ROR2 receptor to the cell surface (Chen et al. 2005). In amniotes, *Ror2* is expressed in the PSM (Al-Shawi et al. 2001; Matsuda et al. 2001), and mice mutant for *Ror2* or double mutant for *Ror1* and *-2* exhibit several defects including vertebral malformations reminiscent of RRS (DeChiara et al. 2000; Takeuchi et al. 2000; Nomi et al. 2001; Schwabe et al. 2004; Raz et al. 2008).

Other evidence suggests that the transforming growth factor-β (TGF-β) signaling, in addition to being essential for mesoderm specification (Miura et al. 2006; Shen 2007), may be implicated in positioning the determination front. In the mouse, *Smad interacting protein1* (*SIP1*) is expressed segmentally in the anterior PSM, and mice mutant for *SIP1* exhibit a shortened axis and form only a few anterior somites (Maruhashi et al. 2005).

Progressive Epithelialization of the PSM Accompanies Somite Formation

The posterior PSM is a loose mesenchymal tissue. As the cells reach the anterior PSM, they undergo a progressive mesenchymal-to-epithelial transition that involves changes in cell shape, cell substrate, and cell–cell interactions (Fig. 4A–C) (Christ et al. 2007). As the PSM cells epithelial-

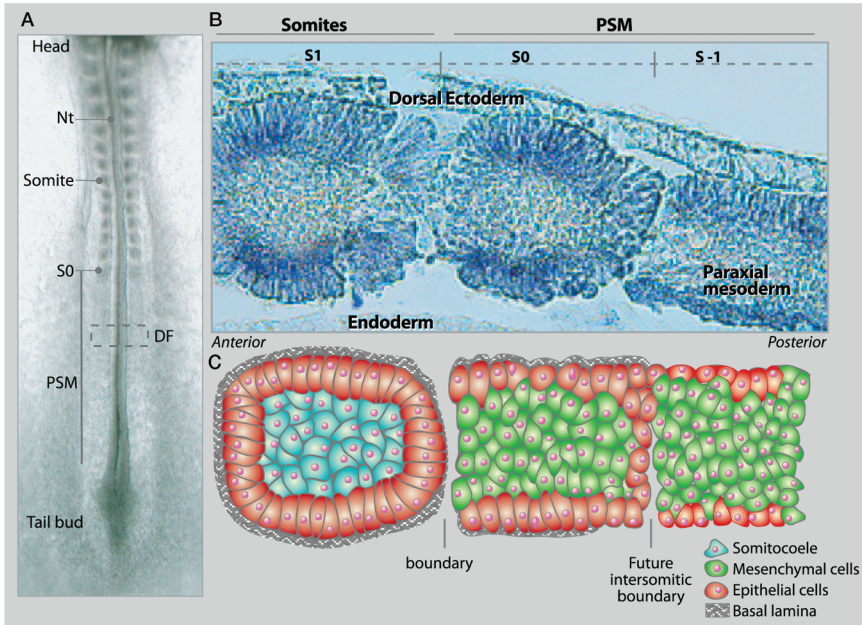

Figure 4. The mesenchymal-to-epithelial transition during somite formation. (*A*) Dorsal view of a two-day-old chicken embryo highlighting the distribution of the epithelial somite pairs, bilaterally to the neural tube (Nt). The posterior PSM is a loose mesenchymal tissue produced by the tail bud. The PSM initiates epithelialization around the level of the determination front (DF, boxed), and eventually forms an epithelial pair of somites (S0) at its anterior extremity. (*B*) Saggital section of the paraxial mesoderm showing the cellular polarization associated with the transition of PSM to somites. Tissue section stained for *N-cadherin* expression by in situ hybridization. (*C*) Corresponding schematic representation of the cellular epithelialization during somite boundary formation. Epithelialization is associated with the deposition of a basal lamina on the outer surface of the forming somite. Somitocoele cells do not epithelialize. (S-1) Next somite to form, (S0) forming somite, (S1) newly formed somite (according to Pourquié and Tam 2001).

ize, they adopt an elongated and polarized shape, and their basolateral side comes in contact with the nascent basal lamina on the outer surface of the forming somite while their apical domain establishes adherent junctions with somitocoele cells (Fig. 4B,C). In the chicken embryo, the first signs of epithelialization become evident around the determination front level, with the cell nuclei aligning dorsally and ventrally in the PSM (Duband et al. 1987).

The *Snail* gene family, coding for zinc-finger transcriptional repressors, has been implicated in the control of the mesenchymal state of sev-

eral cell types by negatively regulating the expression of cadherins (Cano et al. 2000). In the mouse and chicken, these genes are expressed in a periodic fashion in the posterior mesenchymal PSM under the control of Fgf signaling (Dale et al. 2006). In chicken embryos, overexpression of *Snail2* blocks segmentation and epithelialization in the anterior PSM, suggesting that down-regulation of *Snail* genes is required in the anterior PSM for cells to become epithelial (Dale et al. 2006).

In the PSM, the expression of the conserved bHLH transcription factor *Paraxis* (*Tcf15*) begins around the level of the determination front and, hence, coincides with the onset of PSM epithelialization. *Paraxis* expression is maintained in epithelial somites, where it becomes restricted to the differentiating dermomyotome (Quertermous et al. 1994; Blanar et al. 1995; Burgess et al. 1995; Barnes et al. 1997; Shanmugalingam and Wilson 1998; Carpio et al. 2004; Tseng and Jamrich 2004; Wilson-Rawls et al. 2004). The dorsal ectoderm has been shown to be required for proper PSM epithelialization, possibly via *Paraxis* activation in the PSM (Sosic et al. 1997; Palmeirim et al. 1998; Correia and Conlon 2000; Schmidt et al. 2004; Linker et al. 2005). Mouse embryos mutant for *Paraxis* show specific defects in somite epithelialization, and newborn mutant mice exhibit vertebral fusions and chondrogenesis defects (Burgess et al. 1996; Takahashi et al. 2007a). Thus, *Paraxis* controls the morphological transition from mesenchyme to epithelium during somite formation, as well as later steps of spine development (Takahashi et al. 2007a). In addition to their role during somite differentiation (discussed later in this chapter), the transcription factors *Mesenchyme homeobox 1* and *2* (*Meox1* and *-2*) and *Paired box gene 3* (*Pax3*) regulate the epithelialization of the forming somite (Schubert et al. 2001; Mankoo et al. 2003).

The RA Signaling Gradient Antagonizes Fgf/Wnt Signaling

RA Signaling and Maturation of the PSM. RA is a Vitamin A derivative that exhibits pleiotropic effects during embryonic development (Niederreither and Dollé 2008). Signal transduction requires direct RA binding to its nuclear receptor, formed by a heterodimer of RA and Retinoid X receptors (RARs and RXRs). These receptors act as ligand-dependent transcriptional activators of genes that contain RA-response elements (RAREs) (Niederreither and Dollé 2008). RA signaling is regulated by controlling the amount of biologically active RA. While the retinal dehydrogenases RALDH1-4 and CYP1B1 enzymes synthesize RA from precursors, the CYP26 cytochrome p450 family of proteins (*Cyp26a1, -b1*, and *-c1*) is implicated in RA catabolism in the vertebrate

embryo (Chambers et al. 2007; Vilhais-Neto and Pourquié 2008). Importantly, gene expression and loss-of-function studies demonstrate that *Raldh2* (*Aldh1a2*), which catalyzes the last step of RA biosynthesis, is expressed in somites and is the major source of RA during early embryogenesis (see Fig. 2G). Other enzymes, such as RDH10 and CYP1B1, have been implicated in RA production in the embryo (Chambers et al. 2007; Sandell et al. 2007), but their role in paraxial mesoderm patterning has not been established.

In amniotes, RA signaling forms a decreasing rostrocaudal gradient that is opposite to the Fgf and Wnt gradients in the PSM (Begemann and Meyer 2001; Begemann et al. 2001; Diez del Corral et al. 2003; Moreno and Kintner 2004; Sirbu and Duester 2006). In the trunk region, *Raldh2* is expressed in the anteriormost PSM and segmented region, and is excluded from the tail bud and posterior PSM (see Fig. 2G) (Niederreither et al. 2002b). Using a RARE-LacZ reporter mouse, RA signaling was found to be restricted to the anterior PSM and segmented region, and was absent from the posterior PSM and tail bud where *Cyp26a1* is expressed downstream of Fgf (Rossant et al. 1991; Abu-Abed et al. 2001; Vermot et al. 2005). Analysis of mice deficient for RA production (null for *Raldh2*) or of Vitamin A-deficient (VAD) quail embryos reveals that perturbation of RA signaling results in mesoderm segmentation defects (Niederreither et al. 1999; Diez del Corral et al. 2003; Vermot et al. 2005). Moreover, mice or zebrafish mutant for *Cyp26a1* exhibit axis truncation, suggesting that the regulation of the amount of RA in the embryo is critical for proper axis elongation (Abu-Abed et al. 2001; Sakai et al. 2001). Interestingly, mice mutant for *Cyp26a1* exhibit down-regulation of Wnt signaling targets such as *T*. Moreover, ectopic neural structures form in place of the paraxial mesoderm, a phenotype also observed in a number of Wnt signaling mutants (Sakai et al. 2001). Thus, in the PSM, increased RA signaling correlates with a loss of Wnt signaling.

In the chicken embryo, treatment of posterior PSM explants with RA agonists can down-regulate *Fgf8* expression, while a graft of an FGF8-soaked bead in the PSM represses *Raldh2* expression (Diez del Corral et al. 2003). Furthermore, in mice mutant for *Raldh2* and in chicken or quail embryos deprived of RA, the *Fgf8* domain is extended along the PSM (Diez del Corral et al. 2003; Vermot and Pourquié 2005). Experiments in chicken and Xenopus embryos indicate that RA can activate transcription of key segmentation genes, such as the *Mesp2* homologs, either directly or by counteracting Fgf signaling that represses their expression (Moreno and Kintner 2004; Delfini et al. 2005). Together, this led to the proposal that the mutual inhibition of the Fgf and RA gradients is

involved in positioning the determination front (see Fig. 2). Nevertheless, somites do form in the mouse *Raldh2* mutant in which RA signaling is not detected (Niederreither et al. 2002a). Furthermore, in the *FgfR1* conditional knockout, no significant posterior shift of the RARE-lacZ domain is observed, suggesting that Fgf is not the only antagonist to the RA gradient (Wahl et al. 2007). Whether the posterior Wnt gradient, by itself, can antagonize RA signaling remains to be investigated.

RA Signaling Controls Somite Bilateral Symmetry. Whereas the spine is a symmetrical structure, obvious left–right asymmetries are observed in the positioning and structure of vertebrate internal organs, such as the heart and liver (Shiratori and Hamada 2006). Establishment of these asymmetries is downstream of a left–right signaling pathway that is active during gastrulation (Shiratori and Hamada 2006). The secreted factor Nodal acts on the left side of the embryo to activate the expression of specific genes, such as *Paired-like homeodomain transcription factor 1* (*Pitx1*), in the left lateral plate (Shiratori and Hamada 2006; Speder et al. 2007). These initial asymmetries control the subsequent and specific left–right development of the internal organs. Strikingly, in normal embryos, the asymmetric signals produced by the node cross the forming paraxial mesoderm without affecting the perfect symmetry of paraxial mesoderm patterning and development (Yokouchi et al. 1999). During early somitogenesis, RA signaling has been shown to play a role in maintaining the bilateral coordination of paraxial mesoderm development (Kawakami et al. 2005; Vermot et al. 2005; Vermot and Pourquié 2005; Sirbu and Duester 2006). Thus, mice mutant for *Raldh2* or chicken embryos treated with the compound Disulfiram (in which RA synthesis is blocked) exhibit asymmetric somite formation at the cervical level. This asymmetry of somite formation is downstream of the left–right machinery, as it can be reversed by changing the situs of the embryos (Vermot et al. 2005; Vermot and Pourquié 2005). Thus, RA signaling acts in the paraxial mesoderm by buffering the asymmetric signal generated by the left–right machinery. While this appears to be a conserved feature among vertebrates, the underlying molecular mechanisms remain unknown (Kawakami et al. 2005; Vermot et al. 2005; Vermot and Pourquié 2005; Sirbu and Duester 2006). In humans, a majority of patients with idiopathic scoliosis exhibit a spine curvature toward the right, suggesting an underlying defect in the left–right symmetry (Ahn et al. 2002). While the molecular mechanisms underlying these diseases have not been identified, pathways controlling the left–right symmetry of the spine during embryogenesis, such as the RA pathway, are indeed attractive candidates.

Segmental Patterning and Somite Boundary Specification

The segmentation process is initiated at the determination front level when a segment-wide domain acquires a distinct genetic identity that isolates it from the posterior PSM (see Figs. 1C and 2). This change can be visualized, by activation of the transcription of the *Mesp* gene family, as a striped domain that prefigures the future segment (see Fig. 2H). *Mesp* family genes (*Mesp1* and *-2* in the mouse and *Meso1* and *-2* in the chicken) code for bHLH transcription factors, which show a conserved expression pattern and function during somitogenesis (Saga et al. 1996, 1997; Buchberger et al. 1998, 2002; Oginuma et al. 2008). *Mesp1* and *Mesp2* are first expressed as a dynamic stripe located at the determination front level and then become restricted to the future rostral somitic compartment in more anterior regions of the PSM. *Mesp1* and *Mesp2* gene expression becomes down-regulated when the somite forms. While high Fgf signaling in the posterior PSM inhibits *Mesp2* expression, Tbx6 and Notch signaling synergize to induce *Mesp2* expression at the determination front level (see Figs. 3A and 5) (Takahashi et al. 2003; Delfini et al. 2005; Yasuhiko et al. 2006; Wahl et al. 2007). Furthermore, *Mesp2* gene activation requires the transcription factors *Forkhead box c1* (*Foxc1*) and *Forkhead box c2* (*Foxc2/Mfh1*) that are expressed anterior to the determination front (Miura et al. 1993; Winnier et al. 1997; Kume et al. 2001).

An important role for *Mesp2* is to position the future somitic boundary (Morimoto et al. 2005). As described earlier, Notch signaling oscillations in the posterior PSM generate waves of NICD production that control *Lfng* expression in the mouse (see Fig. 2A–D). When the NICD/*Lfng* wave reaches the determination front level, *Mesp2* becomes activated in the future segmental domain where it takes over *Lfng* regulation, thus stabilizing its expression in the *Mesp2* expression domain (see Figs. 2D,H and 5). Because Lfng negatively regulates Notch activation, this results in the creation of an interface between a *Mesp2*-positive domain (the future somitic domain) in which Notch activation is suppressed, and an adjacent posterior *Mesp2*-negative domain in which Notch is activated. This interface marks the level of the future somitic boundary (Fig. 5). This model is supported by grafting experiments in the chicken, which suggest that Lfng activity at the posterior border of the interface could instruct PSM fissure formation (Sato et al. 2002). However, mutant mouse embryos that overexpress NICD throughout the PSM still generate stripes of *Mesp2* expression and do form somites, although they exhibit rostrocaudal polarity defects (Feller et al. 2008). Similarly, overexpression of NICD in the zebrafish PSM does not prevent

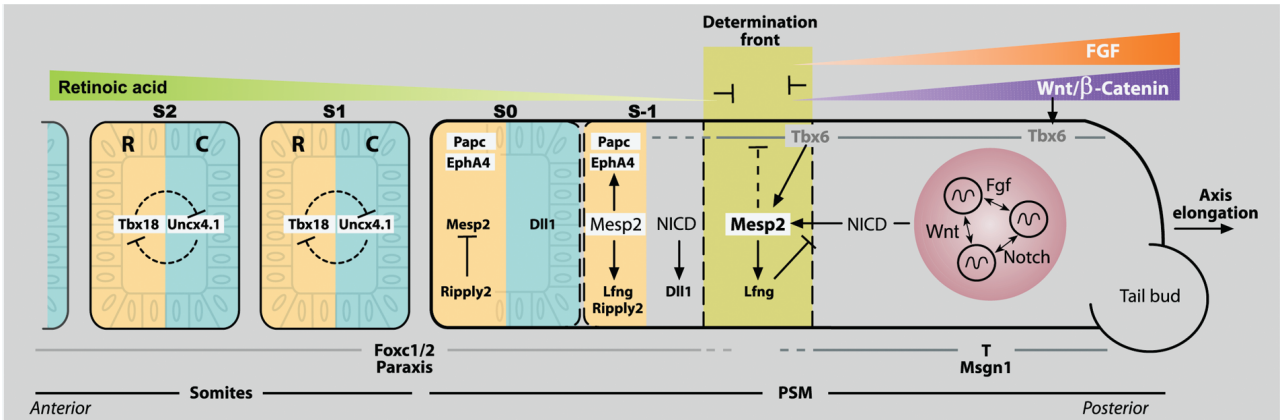

Figure 5. The Notch/*Mesp2* genetic network involved in segment formation and somite rostrocaudal patterning. The system of opposing gradients of Fgf/Wnt and RA plays a key role in positioning the determination front. The posterior PSM expresses a specific set of transcription factors including *Brachyury (T)*, *Tbx6*, and *Msgn1*, and undergoes periodic activation of the Notch, Wnt, and Fgf signaling pathways driven by the segmentation clock. At the determination front level, synergistic action of *Tbx6* and of the pulse of Notch signaling (NICD) downstream of the clock activates *Mesp2* in cells that have reached the determination front during the preceding oscillation cycle, in a striped pattern. Mesp2 activates *Lfng* in the future segmental domain, creating an interface between a domain where Notch is activated (gray) and a domain where Notch is inhibited (green). This interface marks the presumptive somite boundary. Rostrocaudal somite polarity is subsequently established in the newly specified segment by repressing *Mesp2* expression in the future caudal compartment (which reactivates Notch and expresses *Dll1*), while maintaining *Mesp2* in the rostral compartment. Mesp2 then activates downstream targets such as *EphA4* and *Papc*. *Ripply2* activation by *Mesp2* results in the termination of the segmentation program by a negative feedback loop mechanism. Concomitantly, PSM cells progressively acquire epithelial characteristics after the determination front. The anterior PSM expresses a distinct set of transcription factors including *Paraxis* and *Foxc1* and *-2*. The rostrocaudal polarity of the newly formed somite is maintained by the antagonism between *Tbx18* and *Uncx4.1*. (R) Rostral, (C) caudal. (Somite nomenclature according to Pourquié and Tam 2001).

the formation of somite boundaries (Ozbudak and Lewis 2008), arguing that more than an interface between NICD-positive and -negative domains is necessary for somite boundary specification.

The *Ripply1* and *-2* genes code for transcription factors that interact with the Groucho-related family of corepressors (Kawamura et al. 2005; Chan et al. 2006, 2007; Biris et al. 2007; Morimoto et al. 2007). *Ripply* genes are expressed in the anterior PSM downstream of *Mesp2* and *Tbox* genes (Kawamura et al. 2005; Morimoto et al. 2007; Hitachi et al. 2008). In the mouse, *Ripply2* acts as a negative feedback inhibitor, turning off *Mesp2* expression once the segment is specified (see Fig. 5) (Morimoto et al. 2007). Therefore, *Ripply* genes are an essential component in the termination of the segmentation program, allowing subsequent somite differentiation to proceed (Takahashi et al. 2005, 2007b).

Morphogenesis of the Epithelial Somite

Somite boundary morphogenesis primarily involves a localized medio-lateral fissure that forms across the epithelialized anterior PSM tissue (see Fig. 4B,C). This process has been examined in vivo using time-lapse video microscopy that reveals complex cellular rearrangements (Kulesa and Fraser 2002). It has been proposed that boundary formation involves cell repulsion and differential cell adhesion mechanisms driven by large families of conserved transmembrane molecules, such as Cadherins, Integrins, Eph/ephrins, Immunoglobulin-like Cell Adhesion Molecules (CAMs), and by cytoskeleton remodeling factors such as the Rho small GTPases.

The Cadherins are transmembrane, calcium-dependent, cell-adhesion proteins required for cell sorting, boundary formation, epithelial integrity, and cell movements during embryo morphogenesis (Halbleib and Nelson 2006). Cell–cell contacts and adhesion in epithelial cells are primarily mediated by adherens junction complexes in which classical cadherins are the major cell–cell-connecting elements (Gumbiner 2005; Halbleib and Nelson 2006). At the cell membrane, cadherins are tethered to the actin cytoskeleton by a complex of proteins including α-catenin, β-catenin, and several actin filament-binding proteins (Gumbiner 2005). In amniotes, mesodermal tissues are devoid of E-cadherin and express predominantly N-cadherin (*Cdh2*) (Duband et al. 1987; Horikawa et al. 1999; Cano et al. 2000). N-cadherin is essential for proper somite formation, and the mouse null mutants exhibit significant defects in somite morphogenesis (Duband et al. 1987; Radice et al. 1997; Linask et al. 1998). Furthermore, in the double *N-cadherin* and *Cadherin11* mouse mutant, somites disaggregate into small cell clusters (Horikawa et al. 1999). These results suggest a functional

overlap and compensation mechanism among the cadherin family members (Kimura et al. 1995; Horikawa et al. 1999).

The Integrin-extracellular matrix (ECM) interactions play a major role in cell-substrate adhesion. Integrins are transmembrane heterodimers that act as receptors for major components of the ECM, such as fibronectins, laminins, and collagens (Juliano 2002). The interactions between PSM cells and the fibronectin matrix are essential for proper somitogenesis (see Fig. 4C) (Ostrovsky et al. 1983; Lash et al. 1984; Lash and Yamada 1986; Duband et al. 1987; George et al. 1993; Georges-Labouesse et al. 1996). Mice mutant for *integrin* α*5* and *fibronectin1* exhibit severe defects in mesoderm formation and cell migration, resulting in disruption of somite formation (George et al. 1993; Georges-Labouesse et al. 1996; Goh et al. 1997; Yang et al. 1999). In the chicken embryo, fibronectin is produced mainly by the dorsal ectoderm, a tissue required for proper epithelialization of somites (Palmeirim et al. 1998; Correia and Conlon 2000; Rifes et al. 2007). Other integrin ligands, such as laminin1 and collagen IV, form the main components of somitic basal lamina (see Fig. 4C) (Duband et al. 1987). The anchoring of ECM components to the cell by integrins leads to the formation of focal adhesion sites that act as cell-adhesion and cell-signaling centers (Mitra et al. 2005). Mice mutant for *focal adhesion kinase* exhibit general mesoderm development defects that are similar to those observed in mice mutant for *laminin* (Ilic et al. 1995; Miner and Yurchenco 2004). Thus, multiple interactions between cell-surface integrins and the ECM components are essential for proper PSM and somite morphogenesis.

The small GTPases of the Rho family are major regulators of the actin cytoskeleton and of the endocytic traffic (Ellis and Mellor 2000). Among the Rho GTPases, Ras-related C3 botulinum substrate 1 (Rac1), cell division cycle 42 homolog (cdc42), and RhoA have been shown to control cytoskeletal dynamics, cell polarity, and motility (Malbon 2005). In chicken embryos, the effect of *Rac1* and *cdc42* overexpression suggests that these Rho GTPases control the epithelial versus mesenchymal status of cells in the anterior PSM (Nakaya et al. 2004). Thus, these small GTPases likely integrate Cadherin- and Integrin-mediated signals during somite morphogenesis.

FROM SOMITES TO VERTEBRAE

Resegmentation of Somites Generates the Vertebrae

As originally proposed by Remak (1850), one vertebra does not directly derive from one somite, but rather, from the fusion of the rostral com-

partment of one sclerotome to the caudal compartment of the following sclerotome through a process called resegmentation (Fig. 6) (Bagnall et al. 1988; Goldstein and Kalcheim 1992; Aoyama and Asamoto 2000; Christ et al. 2007). In contrast, the myotome maintains its original somitic segmentation that results in the intervertebral musculature connecting two successive vertebrae. While the concept of resegmentation has been challenged by some authors (Verbout 1976), experimental evi-

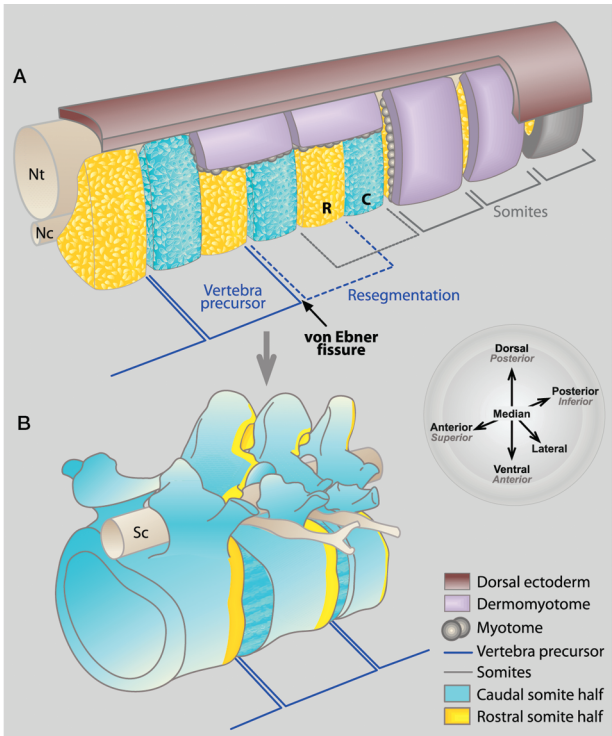

Figure 6. Resegmentation of the sclerotome and its contribution to vertebrae. (*A*) Schematic temporal sequence of sclerotome resegmentation (side view). Sclerotome rostral and caudal compartments are separated by the von Ebner fissure. The rostral compartment of one somite/sclerotome (yellow) fuses to the caudal compartment of the consecutive somite/sclerotome (blue) to form one vertebra. Thus, the somites and the vertebrae are out of register by one half of a segment. The dorsal ectoderm (brown) and dermomyotome that do not resegment (purple) have been removed to visualize the underlying sclerotome. (*B*) Fate of the rostral and caudal sclerotome compartments projected onto adult human vertebrae. Respective contribution of the somite caudal and rostral compartments is shown. The orientation of the embryonic axes is indicated in black bold in the circle and the corresponding medical terminology is shown in gray italics. (R) Rostral somite sclerotome compartments. (C) caudal somite sclerotome compartments, (Nt) neural tube, (Nc) notochord, (Sc) spinal cord.

dence largely supports this hypothesis (Bagnall et al. 1988, 1989; Ewan and Everett 1992). For example, quail somites grafted into a chicken embryo host (Bagnall et al. 1988, 1989; Huang et al. 2000a) or retroviral labeling of single somites (Ewan and Everett 1992) clearly indicate a contribution of each somite to two consecutive vertebrae.

The early rostrocaudal organization of the somite, first established in the anterior PSM, is maintained in the sclerotome in which the rostral and caudal compartments become separated by the von Ebner fissure (Figs. 6 and 7B). The rostral and caudal sclerotome differ notably by their extracellular matrix and cell membrane composition. Thus, the caudal sclerotome has been shown to specifically express molecules such as T-cadherin, versican, collagen type XI, peanut agglutinin binding molecules, and chondroitin-6-sulphate-proteoglycan (Bagnall and Sanders 1989; Oakley and Tosney 1991; Ranscht and Bronner-Fraser 1991; Landolt et al. 1995; Ring et al. 1996; Henderson et al. 1997). The sclerotome compartments also differ in the expression of neural guidance molecules, such as Eph/ephrins, F-sponding, neuropilin2, or Semaphorin3F (Debby-Brafman et al. 1999; Baker and Antin 2003; Kuan et al. 2004; Gammill et al. 2006). As a result, neural crest cells and motor axons are repelled from the caudal sclerotome and their migration is confined to the rostral compartment of the sclerotome (Keynes and Stern 1984; Rickmann et al. 1985). Hence, the rostrocaudal subdivision of the sclerotome controls the segmentation of the peripheral nervous system (Fig. 6) (for review, see Kuan et al. 2004).

Fate mapping studies of the rostral and caudal somite compartments using lineage tracing and quail-chicken chimeras indicate that the rostral somite/sclerotome compartment gives rise to the caudal half of the vertebral body and a small part of the neural arch (Figs. 6 and 7B,D). Conversely, the caudal somite/sclerotome compartment gives rise to the rostral half of the vertebral body, to the pedicles and most of the neural arch (Figs. 6 and 7B,D) (Bagnall et al. 1988, 1989; Ewan and Everett 1992; Goldstein and Kalcheim 1992; Huang et al. 1994, 1996, 2000c; Aoyama and Asamoto 2000). The intervertebral discs are contributed by the somitocoele cells, which integrate the caudal sclerotome compartment (Figs. 6 and 7B,D) (Christ et al. 2007). The ribs also derive from the fusion of two consecutive somites and thus exhibit resegmentation (Chevallier 1975; Evans 2003; Aoyama et al. 2005).

Establishment of Somite Rostrocaudal Polarity

In amniotes, newly formed somites can be subdivided into a rostral and a caudal compartment (Saga and Takeda 2001). The future rostral and caudal compartments of the somite first acquire their identity in the ante-

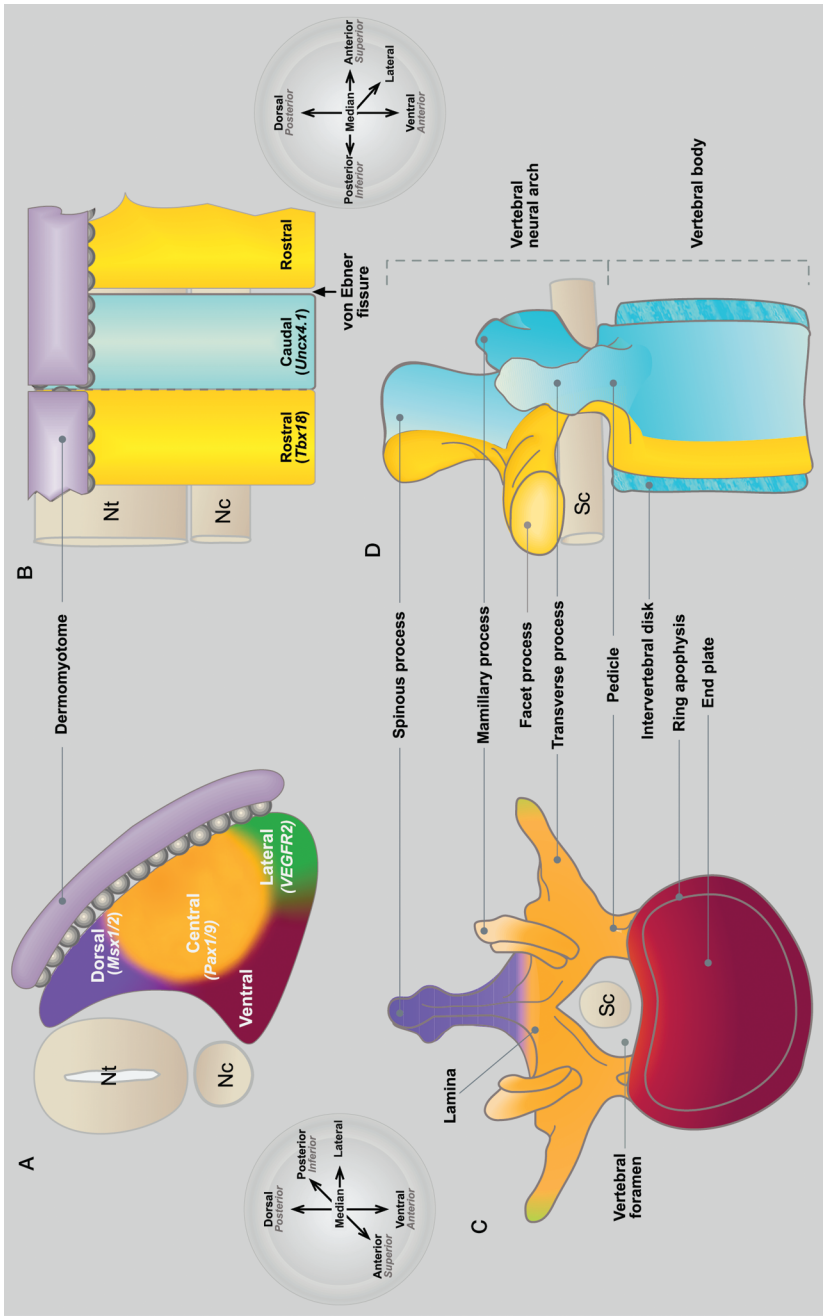

Figure 7. (*See facing page for legend.*)

rior PSM immediately after segment determination and before morphological somite individualization (see Fig. 1C). Rostrocaudal identity of the newly formed segment is materialized by stripes of gene expression restricted either to the future rostral or caudal compartments (see Fig. 5) (Saga and Takeda 2001). In amniotes, *Dll1* is initially expressed in a broad domain of the posterior PSM under the control of Wnt, Notch, and Tbx6 (Galceran et al. 2004; Hofmann et al. 2004; White and Chapman 2005; White et al. 2005). *Dll1* expression becomes down-regulated by Mesp2 in the newly specified segmental domain, and its expression is reactivated in the caudal domain of the forming somite in response to Notch activation (see Fig. 5) (Takahashi et al. 2000). Hence, *Dll1* expression becomes restricted to the caudal compartment of the forming somite, while Mesp2 becomes restricted to the rostral domain (Takahashi et al. 2003). The functional significance of the caudal restriction of *Dll1* expression in somites, however, is unclear since overexpression of *Dll1* throughout the PSM does not alter somite polarity (Serth et al. 2003; Teppner et al. 2007).

Analysis of mice mutant for components of the Notch/*Mesp2* genetic network reveals striking vertebral abnormalities involving specific absence of structures derived from either the rostral or the caudal sclerotome. Such phenotypes can be interpreted as resulting from specific defects in rostrocaudal polarity. Thus, in mice, mutations leading to a loss of Notch activity, such as null mutations for *Notch1*, *Dll1*, *Ofut1, PS1, RBP-Jk*, and *Ripply2,* exhibit rostralized paraxial mesoderm (Evrard et al. 1998; Kusumi et al. 1998; Zhang and Gridley 1998; del Barco Barrantes et al. 1999; Koizumi et al. 2001; Takahashi et al. 2003; Morimoto et al. 2007; Feller et al. 2008). Conversely, mice harboring mutations that interfere with Mesp1/2 functions (such as null mutants for *Mesp1/2*, *Dll3*, *Lfng Hes7, Tbx6*, or constitutive expression of NICD) exhibit only caudal sclerotome derivatives (Chapman et al. 1996; Saga et al. 1997; Takahashi et al. 2000, 2003, 2005; Bessho and Kageyama 2003; Bussen et al. 2004;

Figure 7. Sclerotome compartments and their contribution to vertebrae. (*A*, *B*) Schematic representation of the sclerotome compartments as defined by molecular markers and fate map. (*A*) transversal view. (*B*) lateral view, dermomyotome has been removed to visualize the underlying sclerotome. (*C*, *D*) Color-coded projection of the sclerotomal compartments (shown in *A*, *B*, respectively) onto an adult human lumbar vertebra. (*C*) Transversal (superior) view; (*D*) Lateral (right) view. The orientation of the embryonic axes is indicated in black bold in the circle and the corresponding medical terminology is shown in gray italics. (Nt) Neural tube, (Nc) notochord, (Sc) spinal cord.

Cordes et al. 2004; Morimoto et al. 2005, 2006; Yasuhiko et al. 2006; Oginuma et al. 2008). Such genetic evidence argues that *Mesp2* promotes the rostral identity of somites, while Notch activity is required to specify their caudal identity (see Fig. 5). In amniotes, this Notch/*Mesp2* system is downstream of the Foxc1 and -2 transcription factors in the anterior PSM. Mice double mutant for *Foxc1* and *-2* do not exhibit any rostrocaudal polarity (Kume et al. 2001). Whereas both the somite boundary formation and the establishment of the rostrocaudal somitic identities are concommitent and use similar regulatory loops and downstream effectors, the two processes can be separated genetically (Nomura-Kitabayashi et al. 2002; Feller et al. 2008). Thus, somite boundaries can form in the absence of defined rostrocaudal polarity.

In humans, *MESP2* mutations have been found to be associated with congenital scoliosis (SCD02 [MIM 608681]) or with Spondylothoracic dysostosis/Jarcho-Levin Syndrome. STD has been characterized as an autosomal recessive disorder in which patients exhibit severe disruption of the spine segmentation. These patients are short in stature due to a short rigid neck and thorax, with a "crab like" chest, resulting from the fusion of the ribs at the costovertebral junctions (see Fig. 3C) (Cornier et al. 2004). This phenotype is strikingly similar to the mouse *Mesp2* null mutation in which the axial skeleton is caudalized (Saga et al. 1997). Sequencing the *MESP2* genes in these patients led to the identification of a recessive null mutation in this gene, suggesting that a similar mechanism controls the establishment of the rostrocaudal identity of somites in mice and humans (Cornier et al. 2008).

The downstream cellular effectors of the Notch/*Mesp2* system are still poorly characterized. It has been shown that in mice, *Mesp2* controls the segmental expression of the ephrin ligand *EphA4* (see Fig. 5) (Nakajima et al. 2006). Eph-ephrins are the largest family of membrane-bound tyrosine kinase receptors (RTKs), with 14 to 16 Eph receptors and eight to nine ephrin ligands identified in mammals (Kullander and Klein 2002). Receptor/ligand associations result in cell–cell attraction, repulsion, or modulation of cell adhesion (Durbin et al. 1998; Xu et al. 1999; Barrios et al. 2003; Cooke et al. 2005). The *ephrin-B2* receptor is expressed in the anterior PSM in a conserved segmental and complementary manner to *EphA4* (Bergemann et al. 1995; Flenniken et al. 1996; Irving et al. 1996; Baker and Antin 2003). While alteration of Eph/ephrin signaling in zebrafish leads to segmentation defects, in mice mutant for *EphA4* or *EphrinB2,* somitogenesis is normal, suggesting the existence of compensatory mechanisms in amniotes (Dottori et al. 1998; Adams et al. 1999; Barrios et al. 2003).

Protocadherins are a large family of cadherin-like molecules that act as putative signaling receptors rather than adhesion molecules (Sano et al. 1993; Redies et al. 2005). *Paraxial protocadherin* (*Papc*) is implicated in several biological processes, including convergent-extension movements during gastrulation (Kim et al. 1998; Yamamoto et al. 1998; Hukriede et al. 2003; Medina et al. 2004; Unterseher et al. 2004) and paraxial mesoderm patterning in *Xenopus*, zebrafish, and mice (Kim et al. 1998, 2000; Rhee et al. 2003). During somite formation, *Papc* has been shown to be regulated by *Mesp2*, *Ripply*, and paraxial mesoderm-specific *T-box* genes (see Fig. 5) (Kim et al. 2000; Sawada et al. 2000; Nomura-Kitabayashi et al. 2002; Rhee et al. 2003; Kawamura et al. 2005; Oates et al. 2005b; Muyskens and Kimmel 2007). While interfering with PAPC expression results in somitogenesis defects, the mouse *Papc* null mutant, however, is viable without major developmental defects, suggesting a functional redundancy with other protocadherins in amniotes (Yamamoto et al. 1998, 2000; Kim et al. 2000; Rhee et al. 2003). Therefore, Ephrin receptors and ligands, as well as protocadherins, are potentially involved in both the process of boundary establishment and the acquisition of rostrocaudal identities downstream of the *Mesp2* regulatory network.

Maintenance of Somite Rostrocaudal Identity by *Tbx18* and *Uncx4.1*

The two transcription factors *Tbx18* and *Uncx4.1* act downstream of the Notch/*Mesp2* system to maintain the rostral and the caudal identities of somites, respectively (see Figs. 5 and 7B). *Tbx18* expression is initiated at the level of the forming somite and is restricted to the rostral compartment (Kraus et al. 2001; Bussen et al. 2004; Haenig and Kispert 2004; Tanaka and Tickle 2004). Mice mutant for *Tbx18* exhibit severe rib fusions and vertebral malformations that result from somite caudalization (Bussen et al. 2004). Somite polarity is primarily established in the mutant, but is then subsequently lost. In addition, ectopic expression of *Tbx18* is sufficient to confer rostral identity to somite derivatives; thus, *Tbx18* appears necessary and sufficient for somite rostral identity (see Fig. 5) (Bussen et al. 2004). *Uncx4.1,* a paired-related homeobox gene, is expressed in the caudal compartment of formed somites (see Fig. 5) (Rovescalli et al. 1996; Saito et al. 1996; Mansouri et al. 1997). Mice mutant for *Uncx4.1* exhibit skeletal malformations of the spine due to sclerotome condensation defects. They lack the caudal sclerotome compartment derivatives, including the pedicles and transverse processes, as well as the proximal ribs, while the initial segmental pattern remains

unaffected (see Fig. 7B,D) (Leitges et al. 2000; Mansouri et al. 2000; Schrägle et al. 2004). While *Tbx18* and *Uncx4.1* emerge as key factors in somite rostrocaudal identity, their downstream targets remain unknown. The segmental pattern of the axial skeleton also requires maintenance of the sclerotome boundaries, a process involving the TGF-β type II receptor (*Tgfbr2*) (Baffi et al. 2006).

The Ventral Somite Forms the Vertebral Precursors of the Sclerotome

Sclerotome Compartments and Their Derivatives

Newly formed somites are epithelial spheres that rapidly subdivide into the ventral mesenchymal sclerotome and the dorsal epithelial dermomyotome. Cells of the newly formed somites are not yet committed with respect to their future differentiation (Aoyama and Asamoto 1988). The commitment of somite cells to the osteo-chondrogenic lineage is progressively determined in response to local inducers (Aoyama and Asamoto 1988; Dockter and Ordahl 1998; Dockter 2000). Patterning of the epithelial somites is controlled by inductive signals produced by the adjacent structures, namely the notochord, neural tube, dorsal ectoderm, and the lateral plate (Watterson et al. 1954; Strudel 1955; Hall 1977; Brand-Saberi et al. 1993; Pourquié et al. 1993, 1995, 1996; Sosic et al. 1997; Dockter 2000). In response to this combinatorial signal, the sclerotome becomes subdivided into a ventral, a central, a lateral, and a dorsal compartment (see Fig. 7A) (Marcelle et al. 1997; Brand-Saberi and Christ 2000; Brent and Tabin 2002; Kalcheim and Ben-Yair 2005; Christ et al. 2007).

Schematically, the ventral part of the sclerotome expresses predominantly the *Paired box transcription factor* (*Pax1*) and forms the vertebral bodies and intervertebral disks (see Fig. 7A,C). The neural arch essentially derives from the central sclerotome, which also contributes to the proximal ribs. The spinous process and the dorsal neural arch are contributed by the *Msx1* and *-2*-positive dorsal compartment of the sclerotome (see Fig. 7A,C) (Monsoro-Burq 2005). The lateral sclerotome, which strongly expresses *Vascular endothelial growth factor receptor 2* (*VEGFR2*) and *Sim1*, gives rise to the distal ribs, tendons, and endothelial cells (Eichmann et al. 1993; Pourquié et al. 1996). Another small compartment, the syndetome, lies at the interface between the central sclerotome and the dermomyotome. This compartment is marked by *Scleraxis* expression and forms the tendons linking the segmental muscles to the vertebrae (Cserjesi et al. 1995; Schweitzer et al. 2001; Brent et al. 2003). Fate mapping studies have demonstrated that the somitocoele forms a compartment called the

arthrotome, which contributes to intervertebral joints (synovial joints) and disks, as well as to the proximal ribs (Huang et al. 1996; Christ et al. 2004; Mittapalli et al. 2005). The sclerotome also contributes to both the endothelium and smooth muscles of the aorta (Wiegreffe et al. 2007; Pouget et al. 2008). Interestingly, while the pelvic girdle is derived entirely from the lateral plate mesoderm, some skeletal elements of the pectoral girdle are contributed by the somites (Chevallier et al. 1977). Thus, in the chicken, the long blade of the scapula is formed by somite-derived precursors that express *Pax1* and exhibit a segmental organization (Huang et al. 2000b; Wang et al. 2005; Christ et al. 2007).

The Sclerotome Is Patterned by Diffusible Signals from Surrounding Structures

The somite ventral compartment differentiates in the sclerotome in response to diffusible signals produced by the notochord and the floor plate (Fig. 8) (Watterson et al. 1954; Strudel 1955; Hall 1977; Brand-Saberi et al. 1993; Pourquié et al. 1993; Dockter 2000). Major components of this ventralizing signal are the proteins Sonic hedgehog (Shh) (Fan and Tessier-Lavigne 1994; Johnson et al. 1994; Fan et al. 1995) and the BMP antagonist, Noggin (Nog) (Hirsinger et al. 1997; Marcelle et al. 1997; McMahon et al. 1998). These signals induce expression of sclerotome-specific transcription factors, such as *Pax1* and *-9*, which initiate the skeletal differentiation program (Fig. 8). In response to these signals, the ventral somite undergoes an epithelio-mesenchymal transition to generate the mesenchymal sclerotome. This transition involves the down-regulation of N-cadherin and requires the MMP-2 metalloprotease activity (Hatta and Takeichi 1986; Duband et al. 1987; Duong and Erickson 2004). Subsequently, the sclerotome cells form the peri-notochordal tissue that gives rise to the vertebral centra and then to the vertebral bodies and intervertebral discs (see Figs. 7A,C, 8C, and 9C) (Christ et al. 2007). *Pax3*, which is expressed in all cells of the anterior PSM, becomes down-regulated in the sclerotome cells in response to Shh (Dietrich et al. 1993; Fan and Tessier-Lavigne 1994). *Pax3* is maintained in cells of the somite dorsolateral compartment, which retains an epithelial structure and differentiates into the dermomyotome (Fig. 8A) (Schmidt et al. 2004; Christ et al. 2007). The dermomyotome subsequently gives rise to the skeletal musculature of the trunk and the dorsal dermis (Buckingham et al. 2006).

Mice mutant for *Shh* lack the entire axial skeleton, but initiate *Pax1* expression in the ventral somite (Chiang et al. 1996). However, this weak induction of *Pax1* in the ventral somite appears to result from Indian

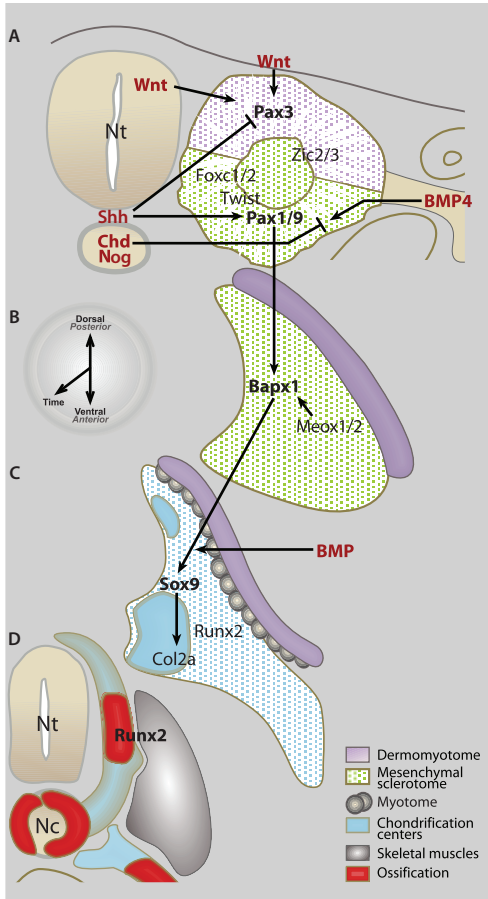

Figure 8. Signals patterning the sclerotome and transcriptional program underlying sclerotome differentiation. Temporal sequence from the differentiation of the epithelial somite to the ossification of the vertebra (signaling factors are shown in red). (*A*) Epithelial somites are patterned in two compartments by diffusible signals produced by adjacent structures. The sclerotome is primarily specified by "ventralizing signals," such as Shh produced by the notochord and the floor plate, and BMP antagonists Chordin (Chd) and Noggin (Nog) produced by the notochord. While Chd and Nog antagonize BMP4 (from the lateral plate mesoderm), Shh signaling activates the expression of the transcription factors *Pax1* and *-9*. The dermomyotome is specificied by "dorsalizing" signals, namely Wnts, produced by the dorsal ectoderm and neural tube, which activate *Pax3* and antagonize the action of Shh. (*B*) Pax1 and -9 control a cascade of transcription factors that are responsible for sclerotomal differentiation. Thus, Pax1 and -9 activate the key chondrogenesis factor Bapx1 (Nkx3.2) in synergy with Meox1 and -2. (*C*) Bapx1 allows the sclerotome to respond to BMP signals by triggering the expression of Sox9. This initiates the chondrogenesis program leading to the expression of *Col2a* and *Runx2*. (*D*) The final stage of sclerotomal differentiation consists of the ossification of the cartilaginous vertebral model, controlled by the Runx2 genetic network. The orientation of the embryonic axis and time axis is indicated in black bold and the corresponding medical terminology is indicated in gray italics.

hedgehog (Ihh) activity produced by the dorsal endoderm (Zhang et al. 2001). In mice mutant for the Shh coreceptor *Smoothened* (*Smo*) or for both *Shh* and *Ihh*, induction of sclerotome markers, such as *Pax1* or *Nkx3.2* (*Bapx1*), is not observed, suggesting that Hedgehog signaling is an absolute requirement for sclerotome induction (Zhang et al. 2001). In the ventral somite, Shh activates the expression of its receptor *Patched,* the *Gli2* and *-3* genes, and the Wnt antagonist *Sfrp2* (Goodrich et al. 1996; Hahn et al. 1996; Borycki et al. 1998; Motoyama et al. 1998; Buttitta et al. 2003). The Gli proteins are zinc-finger transcription factors acting downstream of Hedgehog signaling in vertebrates. *Gli1*, *-2*, and *-3* are expressed in somites, and mice mutant for *Gli* genes exhibit skeletal defects (Hui et al. 1994; Mo et al. 1997; Platt et al. 1997; Borycki et al. 1998). Shh signaling in the ventral somite antagonizes the dorsalizing influence from the neural tube and the dorsal ectoderm mediated by Wnt signals (see Fig. 8A) (Fan and Tessier-Lavigne 1994; Fan et al. 1995). In addition to its patterning function, Shh is also required as a trophic factor for sclerotomal cells to promote their survival and proliferation (Borycki et al. 1998; Teillet et al. 1998; Marcelle et al. 1999).

The secreted BMP4 growth factor is expressed by the lateral plate at the time of somite formation (Pourquié et al. 1996). The right level of BMP signaling along the medio-lateral axis is critical to maintain paraxial mesoderm and sclerotome identity (Hirsinger et al. 1997; Tonegawa et al. 1997). At this stage, BMP4 induces lateral markers, such as *Sim1,* in the somite and it antagonizes *Pax1* expression, contributing to its restriction to the medial somite domain (Balling et al. 1996; Pourquié et al. 1996; Tonegawa et al. 1997). The BMP signal is counteracted by the axial structures that produce the noggin (Nog) antagonist (Fig. 8A) (Hirsinger et al. 1997; Marcelle et al. 1997; Reshef et al. 1998; Tonegawa and Takahashi 1998). In addition, other BMP antagonists (such as Chordin [Chd], Cerberus, and Follistatin) may counteract the BMP4 effects (Patel et al. 1996; Belo et al. 1997; Biben et al. 1998; Shawlot et al. 1998). Therefore, sclerotome specification involves antagonism between the Shh and the BMP4/Wnt signaling pathways.

Cross talk between the myotome and sclerotome compartments involving platelet-derived growth factor (PDGF) signaling is also required for proper sclerotomal development. While the receptor *PDGFRα* is expressed mostly in the sclerotome, PDGFA and C ligands are produced by the myotome (Orr-Urtreger et al. 1992; Orr-Urtreger and Lonai 1992; Ding et al. 2000; Aase et al. 2002). Mice mutant for *PDGFRα* exhibit spina bifida and fusion of skeletal elements along the axis (Soriano 1997; Klinghoffer et al. 2002). PDGF signaling is required for regulating chon-

drogenesis and sclerotomal cell migration, leading to formation of the neural arches (Hoch and Soriano 2003; Pickett et al. 2008).

A Transcriptional Network Underlying Sclerotome Development

A network of transcription factors that acts downstream of Shh and BMP signaling, controls sclerotomal fate, and cartilage and bone differentiation. Shh induces *Pax1* and *Pax9,* which are essential for sclerotomal cell proliferation and differentiation in the ventral somite (see Fig. 8A) (Balling et al. 1996; Müller et al. 1996; McMahon et al. 1998; Peters et al. 1999). *Pax1* expression precedes the epithelio-mesenchymal transition that characterizes the sclerotome formation, while *Pax9* is expressed later during sclerotomal differentiation (Goulding et al. 1993; Love and Tuan 1993). While mice mutant for *Pax9* are essentially normal, *Pax1* mutants (*Undulated-short-tail* [*Uns*]) lack vertebral bodies and intervertebral disks (Wallin et al. 1994). Mice double mutant for *Pax1* and *Pax9* form only neural arches, demonstrating the key role played by these factors in vertebral body formation (see Figs. 7A,C, and 8) (Peters et al. 1999). *Pax1* and *Pax9* (and also *Meox1* and *-2*; discussed later in this chapter) directly activate expression of the homeobox-containing transcription factor *Nkx3.2* (*Bapx1*) in the sclerotome (see Fig. 8B) (Lettice et al. 1999; Tribioli and Lufkin 1999; Akazawa et al. 2000; Herbrand et al. 2002; Rodrigo et al. 2003, 2004). As in the *Pax1* and *-9* double mutant, mice mutant for *Nkx3.2* lack vertebral bodies and intervertebral disks (Lettice et al. 1999; Tribioli and Lufkin 1999; Akazawa et al. 2000). The induction of *Nkx3.2,* downstream of Shh and *Pax1* and *-9,* is a key step in the sclerotome determination process as it endows sclerotomal cells with the competence to respond to the BMP signal by activating *Sox9* (see Fig. 8C) (Murtaugh et al. 2001). *Nkx3.2* forms with *Sox9*, a BMP-dependent, autoregulatory loop that promotes sclerotomal chondrogenesis in amniotes (Murtaugh et al. 2001; Zeng et al. 2002). Subsequently, *Sox9* activates the chondrogenic program that leads to the formation of the vertebral cartilaginous template (see Fig. 8C,D) (Healy et al. 1996, 1999; Bell et al. 1997; Bi et al. 1999, 2001). During later stages, the Runx2 transcriptional network (which controls bone formation) is activated during vertebral ossification (see Fig. 8D). The role of Sox9 or Runx2 in the control of cartilage and bone development is discussed extensively in other chapters of this book and is not detailed here.

Several other transcription factors have been implicated in sclerotome differentiation. *Nkx3.1* is a gene closely related to *Nkx3.2*, which is also strongly expressed in the sclerotome (Herbrand et al. 2002). Whereas the *Nkx3.1* null mutant does not show an axial skeleton phenotype, the

double *Nkx3.1* and *3.2* null mutant exhibits more severe skeletal defects compared to the single *Nkx3.2* mutant, suggesting that the two genes play partly redundant roles (Herbrand et al. 2002). The *Meox 1* and *2* homeobox transcription factors are expressed in somites of vertebrate embryos (see Fig. 8B) (Candia et al. 1992; Candia and Wright 1996; Mankoo et al. 2003; Reijntjes et al. 2007). *Meox1* and *-2* have partially redundant functions in mice. While *Meox1* mutants present defects in sclerotomal derivatives (such as fusion of vertebrae) and *Meox2* mutants do not exhibit an axial skeleton phenotype, the double *Meox1/2* null mutants present a severe disruption of somite organization and differentiation (Mankoo et al. 2003). *Foxc2* is expressed in the sclerotome under the control of Shh and is required to promote sclerotomal cell proliferation (see Fig. 8A) (Winnier et al. 1997; Furumoto et al. 1999). In addition, it has also been demonstrated that *Twist,* coding for a bHLH transcription factor activated by Shh and Fgf signaling, controls sclerotomal cell viability and proliferation, while inhibiting bone formation (see Fig. 8A) (Chen and Behringer 1995; Bialek et al. 2004; Hornik et al. 2004). *Zic1-3* (zinc-finger transcription factors) are expressed in differentiating somites, and mice mutant for these transcription factors exhibit skeletal defects (Aruga et al. 1999; Carrel et al. 2000; Klootwijk et al. 2000; Nagai et al. 2000; Purandare et al. 2002; Merzdorf 2007). *Zic2* and *-3* are specifically expressed in the PSM and newly formed somites, and have been shown to act cooperatively during paraxial mesoderm segmentation (see Fig. 8A) (Inoue et al. 2007). Since the somite boundaries and rostrocaudal polarity are normal in the compound mutant mice, *Zic2* and *-3* are more likely to be implicated in the maintenance and differentiation of the sclerotome (Inoue et al. 2007). The transcription factor *Jun*/*AP-1* has been implicated in the differentiation of intervertebral disks by controlling degeneration of the notochordal cells (Behrens et al. 2003).

Differentiation of the Vertebrae from the Sclerotome

The sclerotome subsequently differentiates into a cartilaginous model that becomes ossified and then forms the definitive vertebra (Fig. 9C–E) (Bono et al. 2006; Christ et al. 2007). In humans, cartilage-producing centers (identifiable by the deposition of Collagen type 2) form at approximately the 6th week in the embryo (see Figs. 8C and 9C) (Bareggi et al. 1993; Nolting et al. 1998). Primary chondrification centers appear in the neural arches, and they subsequently fuse over the midline dorsally and with the centrum ventrally (see Figs. 8D and 9D). Failure of the neural arch to fuse dorsally is associated with spina bifida. Two primary chondrification cen-

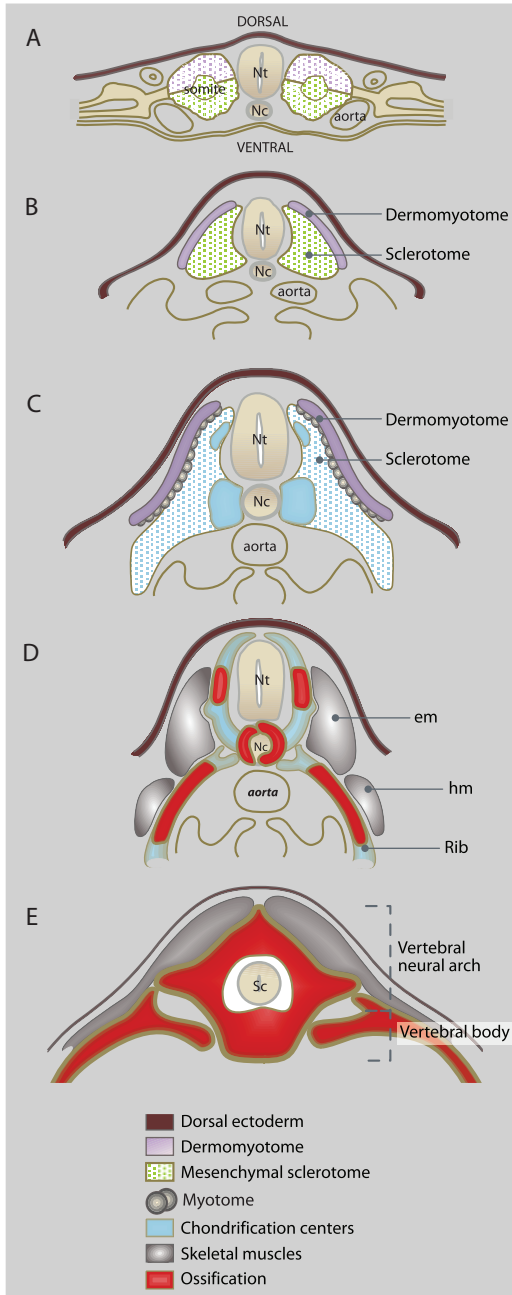

Figure 9. (*See facing page for legend.*)

ters also form on the left and right sides of the regressing notochord and eventually fuse to form the cartilaginous centrum (Fig. 9C). Subsequently, cartilaginous transverse and spinous processes form from the neural arch. In between forming centra, regions of loosely packed cells surrounding the remnant of the notochord will form the cartilaginous intervertebral disks. The disk center, consisting of the *nucleus pulposus*, is produced by a remnant of the notochordal cells, while it is surrounded by the *annulus fibrosus*, formed by sclerotomal cells (Christ et al. 2007).

The cartilaginous template then becomes progressively ossified (endochondral ossification) (Fig. 9D,E) (Bareggi et al. 1993; Nolting et al. 1998). In the human embryo, this process begins around the 8th week when blood vessels invade the cartilaginous centrum (Bono et al. 2006). Three primary ossification centers are typically recognized: one in the centrum, and one on each side of the vertebral arch (Fig. 9D). In mice and humans, ossification of vertebrae follows a specific spatio-temporal sequence along the anteroposterior axis, beginning in the lower thoracic and upper lumbar regions (Bareggi et al. 1993). Five secondary ossification centers develop following the onset of puberty: (1) one at the tip of the spinous process, (2) one at the tip of each transverse process, and (3) one ring apophysis in the superior and inferior endplates (attachment surface disk-vertebrae) of the vertebral bodies (see Fig. 7C) (Bono et al. 2006).

Regionalization of the Axial Skeleton and the Role of the *Hox* Genes

Each vertebra exhibits a distinct morphology depending on its position along the anteroposterior axis. Based on shared morphological features,

Figure 9. Sclerotome differentiation and vertebra formation. Schematic temporal sequence of sclerotome differentiation, morphogenesis, chondrogenesis, and osteogenesis leading to the formation of the vertebra (thoracic level; transversal section). (*A*) Two compartments are specified from the epithelial somites: the dermomyotome dorsally and the sclerotome ventrally. (*B*) The epithelial sclerotome undergoes an epithelio-mesenchymal transition, while the dermomyotome maintains its epithelial structure. (*C*) The sclerotome initiates chondrogenesis in centers initially localized around the notochord ventrally and on both neural arches dorsally. Meanwhile, the dermomyotome produces the skeletal muscles and dermis. (*D*) Skeletogenesis further proceeds by the progressive ossification of the cartilaginous model of the vertebrae, in which vertebrae centra, neural arches, and costal elements (thoracic level) are identifiable. (*E*) Ossification centers eventually fuse to produce the final vertebra structure. (Nc) Notochord, (Nt) neural tube, (Sc) spinal cord, (Em) epaxial muscle mass, (Hm) hypaxial muscle mass.

five main regions of the amniote vertebral column can be distinguished: the cervical, thoracic, lumbar, sacral, and caudal (Fig. 10). In humans, the five sacral vertebrae fuse to form a triangular-shaped sacrum. The number of each type of vertebra defines the vertebral formula of a particular species and varies dramatically among vertebrate species (Burke et al. 1995; Burke 2000; Gomez et al. 2008). The acquisition of the distinct vertebral identities is controlled by a class of transcription factors called Hox proteins (for review, see Krumlauf 1994; Duboule 2007; Wellik 2007). In

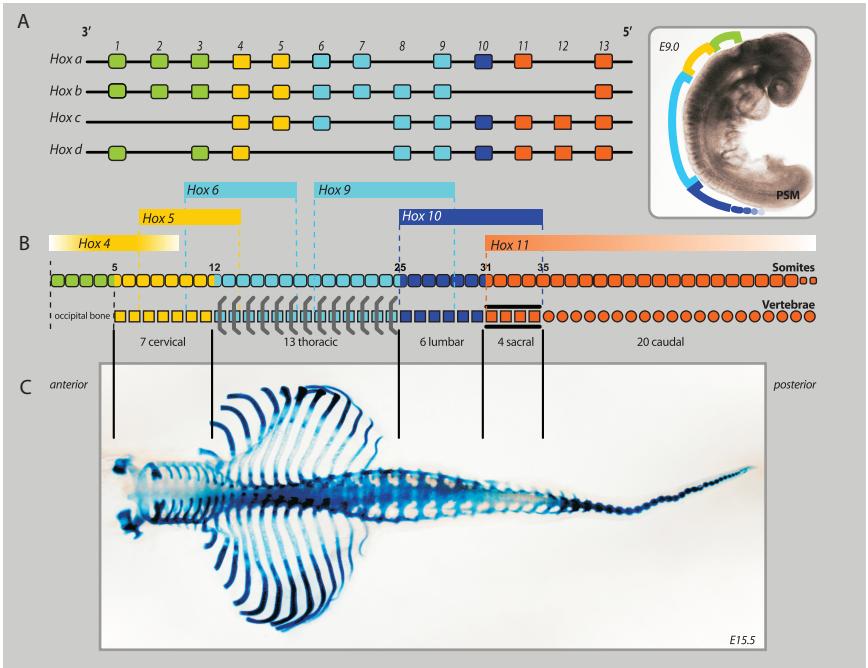

Figure 10. *Hox* gene expression domains and vertebral formula of the mouse embryo. (*A*) Representation of the genomic organization of the four mouse paralog *Hox* clusters. *Hox* gene combinatorial expression controls the vertebral identity along the anteroposterior axis. Genes are color-coded based on their expression and phenotypic domains (the skeletal domain that is affected when a particular *Hox* gene is mutated) along the anteroposterior axis. The inset panel (*upper right*) illustrates a mouse embryo (embryonic day 9.0) with approximate somite identity along the anteroposterior axis (lateral view). (*B*) Schematic representation of the somitic regions compared to the vertebral formula of the mouse. The somites and derived vertebrae are aligned and subdivided by color-coded domains corresponding to distinct vertebrae types and which correlate with specific *Hox* paralog expression (*A*). (*C*) The different regions of the axial skeleton are illustrated in a mouse embryo (embryonic day 15.5) stained with Alcian blue.

mice and humans, there are 39 *Hox* genes that are organized into four paralogous clusters (*Hox1–13*) (Fig. 10A) (Duboule 2007). The position of the genes along a cluster correlates with the order in which each gene will be expressed in time and space along the anteroposterior axis of the embryo, a property called collinearity (Kmita and Duboule 2003). *Hox* genes control the timing of ingression of paraxial mesoderm precursors into the primitive streak (Iimura and Pourquié 2006). Thus, precursors expressing more "posterior" genes (i.e., genes located 5′ in the *Hox* cluster) will ingress later than those expressing more "anterior" (genes located 3′) and, hence, will be positioned more posteriorly along the anteroposterior axis. This mechanism results in a collinear or nested arrangement of the *Hox* gene expression domains along the anteroposterior axis. This nested arrangement of the *Hox* expression domains defines a specific combination of genes expressed for each somite (Kessel and Gruss 1991). The combination of *Hox* genes that are expressed in each somite is involved in the control of the specification of vertebral identities (Fig. 10) (Wellik 2007). However, although the *Hox* gene expression domains often extend from the posterior end of the embryo to their anterior expression limit in the somites, their action is essentially restricted to their anteriormost expression domain. Thus, the identity of a segment is controlled by the posteriormost *Hox* genes expressed in this segment, a property called posterior prevalence in vertebrates (Duboule and Morata 1994; Burke et al. 1995). Grafting experiments in chicken embryos support the idea that vertebral axial identity is already determined at the level of the PSM, before somite individualization (Kieny et al. 1972; Nowicki and Burke 2000). *Hox* loss of function or ectopic expression in the paraxial mesoderm can change vertebral identities, leading to homeotic transformations in the axial skeleton (see Wellik 2007 and references therein). The function and targets of *Hox* genes in the developing spine are virtually unknown.

Several factors involved in the regulation of *Hox* genes in the patterning of the axial skeleton have been identified. In the mouse, the TGF-β family secreted factor, GDF11 (which is expressed in the tail bud), acts upstream of *Hox* genes. Mice mutant for *Gdf11* show homeotic transformation of the vertebral column and tail truncation, correlating with disruption of *Hox* expression domains (McPherron et al. 1999; Nakashima et al. 1999). These patterning defects are partially phenocopied in mice mutant for the GDF11 proprotein convertase *Pcsk5*, specifically expressed in the PSM, as well as TGF-β type I receptor (*Alk5*) and type II receptors (*ActRIIA/B* [*Acvr2/b*]) (Oh and Li 1997; Rancourt and Rancourt 1997; Oh et al. 2002; Andersson et al. 2006; Essalmani et al. 2008; Szumska et al. 2008).

The caudal transcription factors (*Cdx)* play a conserved role in *Hox* regulation during anteroposterior axis formation (van den Akker et al. 2002; Houle et al. 2003a; Chawengsaksophak et al. 2004; Copf et al. 2004; Pilon et al. 2007). In mice, *Cdx* gene expression (*Cdx1, -2*, and *-4*) is initiated at the primitive streak stage and is later restricted to the posterior part of the embryo. *Cdx* gene expression is regulated by Wnt, Fgf, and RA signalings (Isaacs et al. 1994; Pownall et al. 1996; Houle et al. 2000, 2003b; Ikeya and Takada 2001; Prinos et al. 2001; Bel-Vialar et al. 2002; Lohnes 2003; Deschamps and van Nes 2005; Pilon et al. 2006, 2007). Cdx factors act by regulating a subset of *Hox* genes in a dose-dependent manner (van den Akker et al. 2002). Mice mutant for *Cdx1, -2*, and *-4* exhibit severe axis truncation (van den Akker et al. 2002; Chawengsaksophak et al. 2004; van Nes et al. 2006). Other transcription factors, such as *Plzf* (*Zfp145*) and Hox nuclear cofactors such as *Pbx1*, also regulate *Hox* gene-dependent patterning along the anteroposterior axis (Barna et al. 2000; Selleri et al. 2001; Capellini et al. 2008). Importantly, *Hox* gene expression in the axial skeleton also depends on the conserved families of chromatin remodeling proteins of the Polycomb and Trithorax groups, which negatively and positively regulate *Hox* expression, respectively. Mutation of several genes of these families results in homeotic transformations of the axial skeleton (Kim et al. 2006).

The anterior boundary of any given *Hox* gene expression domain in the precursors of the vertebral column is always positioned at the same somitic level, indicating that *Hox* gene expression is tightly coupled to somitogenesis (see Fig. 10B). Several *Hox* genes have been shown to be expressed in a dynamic fashion (similar to *Mesp2*) in the anterior PSM, suggesting that their regulation might be taken over by the segmentation machinery (Zákány et al. 2001). In the chicken embryo, changing the somite size by treating embryos with Fgf results in a corresponding change in the position of Hox expression boundaries (Dubrulle et al. 2001). Interestingly, in the mouse, altering the signals that control the positioning of the determination front—namely Fgf (*FgfR1*), Wnt (*Wnt3a*), and RA (RA, *RAR* receptors, *Cyp26*)—also results in homeotic transformations (Kessel 1992; Lohnes et al. 1994; Partanen et al. 1998; Abu-Abed et al. 2001; Ikeya and Takada 2001; Sakai et al. 2001; Mallo et al. 2008).

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