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# Muscle Contraction Is Necessary to Maintain Joint Progenitor Cell Fate

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## SUMMARY

**During embryogenesis, organ development is dependent upon maintaining appropriate progenitor cell commitment. Synovial joints develop from a pool of progenitor cells that differentiate into various cell types constituting the mature joint. The involvement of the musculature in joint formation has long been recognized. However, the mechanism by which the musculature regulates joint formation has remained elusive. In this study, we demonstrate, utilizing various murine models devoid of limb musculature or its contraction, that the contracting musculature is fundamental in maintaining joint progenitors committed to their fate, a requirement for correct joint cavitation and morphogenesis. Furthermore, contraction-dependent activation of β-catenin, a key modulator of joint formation, provides a molecular mechanism for this regulation. In conclusion, our findings provide the missing link between progenitor cell fate determination and embryonic movement, two processes shown to be essential for correct organogenesis.**

## INTRODUCTION

Spontaneous embryonic movement commences soon after the first contact between motor axons and presumptive muscle cells, as early as 4–5 days of incubation in chicks (Bekoff, 1981; Bennett et al., 1983; Hamburger and Balaban, 1963). Similar movement is seen in murine embryos at a comparable developmental stage (E12.5) (Carry et al., 1983; Suzue, 1996). This stage also marks the point of initial muscle formation (Ontell et al., 1993), at which time spontaneous waves of highly rhythmic activity originating in the spinal cord are observed (Hanson and Landmesser, 2003).

The involvement of embryonic movement and muscle contraction in skeletogenesis was reported as early as 1901. In particular, their role in joint formation was cited by Herbest, where E.H Weber found ankylosed joints in a newborn calf lacking both the spinal cord below the cervical region and muscles in the posterior half of the body (Herbest, 1901). The contribution of embryonic movement and muscle contraction to joint formation was mostly studied on chemically paralyzed chick embryos (Drachman and Sokoloff, 1966; Fell and Canti, 1934; Hamburger and Waugh, 1940; Lelkes, 1958; Mikic et al., 2000; Mitrovic, 1982; Murray and Drachman, 1969; Osborne et al., 2002; Ruano-Gil et al., 1980) and to a lesser extent on murine embryos (Hasty et al., 1993; Pai, 1965; Tremblay et al., 1998). Yet, the mechanism that underlies the role of muscle contraction in joint formation still remains to be elucidated.

Synovial joints are specific structures within the limb that separate adjacent opposing skeletal elements from each other and facilitate smooth articulation between them. The mature joint structure is comprised of articular cartilage, synovial fluid, ligaments, and a fibrous capsule, which together function to enable it to transmit biomechanical loads (Khan et al., 2007; Pacifici et al., 2005).

Joint development involves the initial specification of progenitor cells in the condensation of the forming limb skeleton at the site of the future joint. This region, known as the interzone, is characterized by densely packed flattened cells (Mitrovic, 1977). These cells adopt a nonchondrogenic phenotype, as indicated by the loss of chondrogenic markers such as *Sox9* and collagen type II (*Col2a1*), and instead express new sets of genes including *Gdf5*, *Wnt4*, and *Wnt9a* (Hartmann and Tabin, 2001; Storm et al., 1994).

Joint specification is followed by joint cavitation, a process whereby adjacent cartilaginous elements physically separate to form two distinct articulating surfaces, with the synovial cavity in between. This is followed by morphogenetic processes that produce the articular cartilage, capsule, synovium, and other joint structures, culminating in the formation of a mature joint (Khan et al., 2007; Pacifici et al., 2005). Fate mapping has shown that various structures of the mature joint, such as articular chondrocytes, are derived from interzone cells (Koyama et al., 2008; Pacifici et al., 2006; Rountree et al., 2004).

A major signaling pathway shown to be involved in early joint formation is the Wnt/β-catenin pathway. Canonical Wnt signaling is transduced through stabilization and nuclear accumulation of

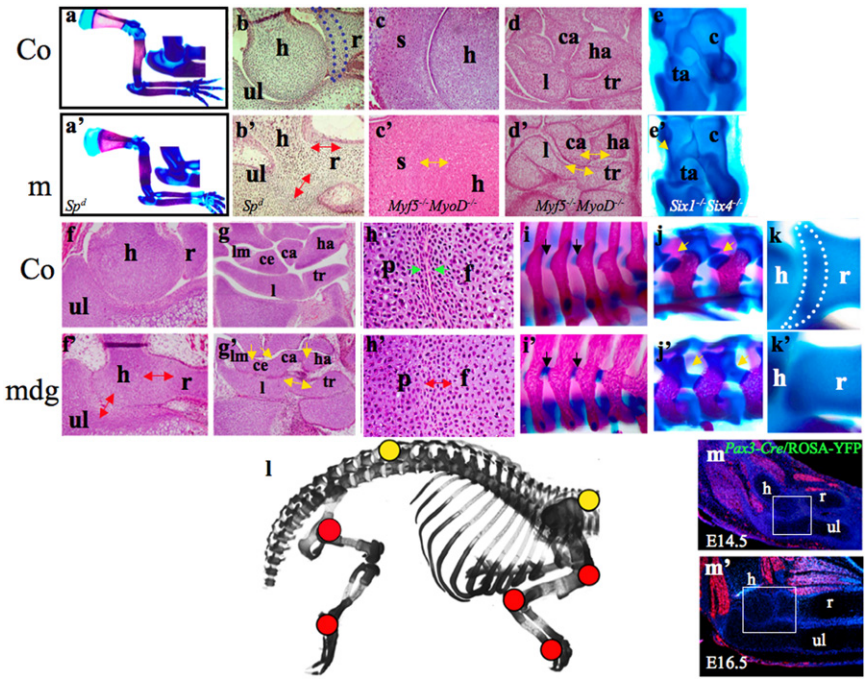


**Figure 1. Joint Loss in the Absence of Muscle Contraction**

Alcian blue and Alizarin red staining of control (a) and *Sp*$^{d}$ mutant (a′) forelimbs indicates the loss of the elbow joint in the *Sp*$^{d}$ mutant. H&E-stained sections and Alcian blue and Alizarin red skeletal preparations of various joints from control (Co; [b–k]) versus representative muscleless mutants (m; [b′–e′]) or *mdg* embryos (f′–k′) at E18.5. Joint loss at the humerus (h)–radius (r) and humerus (h)–ulna (ul) intersections in the elbow of *Sp*$^{d}$ mutant embryo (b′); dotted lines indicate the concave-convex structure at humeroradial joint (HRJ) (b), which is absent in the mutant (b′). Absent joints (as indicated by arrows) of *Myf5*$^{-/-}$*MyoD*$^{-/-}$ mutants at the scapula (s)–humerus (h) intersection (c′) and at indicated carpal elements: capitate (ca) to hamate (ha) and lunate (l) to triangular (tr) (d′). Fusion of the talus (ta) to the calcaneus (c) in *Six1*$^{-/-}$*Six4*$^{-/-}$ embryos (e′). All three muscleless mouse strains exhibited similar joint losses. Loss of joints at the elbow (f′), the carpals (g′), lesser multangular (lm), centrale (ce), and the hip (h′) of *mdg* mutants. Alcian blue and Alizarin red staining indicates loss of joints between the cervical (i′) and the lumbar (j′) vertebrae. Alcian blue and Alizarin red staining indicates lack of concave-convex structure in *mdg* HRJ (k′), which is present in control HRJ (dotted line in [k]). (l) A general scheme summarizing the joints that were lost in all three muscleless mutants and in the *mdg* mutant mice (red dots) or only in the *mdg* mutant (yellow dots). Immunofluorescence staining with anti-GFP on sections of E14.5 (m) and E16.5 (m′) control embryos demonstrates staining of limb muscles; boxed areas indicate the unstained elbow joint region.

cytoplasmic β-catenin where, in conjunction with the LEF/TCF binding proteins, it acts as a transcriptional activator. This pathway plays a key role in joint development by maintaining joint cell fate and preventing their differentiation to chondrocytes (Guo et al., 2004; Hartmann and Tabin, 2001; Spater et al., 2006).

Retaining progenitor cells committed to their designated fate is a prerequisite for correct organ development. How these progenitors are maintained properly committed is a key question.

In this study, we demonstrate a mechanism that maintains joint progenitor cell fate by the involvement of muscle contraction. We analyzed joint formation in three mouse models that lack limb musculature and a fourth that lacks muscle contractility. In these mice, the normal joint differentiation sequence was interrupted, as cells at presumptive joint sites in the limb ceased to express joint markers and, instead, expressed chondrogenic markers, resulting in joint loss. Cell fate mapping experiments unambiguously established that these chondrocytes were descendants of the joint progenitor pool, whose joint cell identity was lost. Our finding that the musculature regulates, at least in part, the β-catenin signaling pathway provides a mechanistic explanation for the failure in joint formation in paralyzed limbs.

## RESULTS

### The Absence of Muscle Contraction in Developing Murine Limbs Results in Joint Loss

We examined joint formation in three mutant mouse models in which limb musculature fails to develop (in the following referred to as muscleless), namely: splotch delayed mutation (*Sp*$^{d}$), where a point mutation in the *Pax3* gene leads to a defect in migration of muscle progenitor cells to the developing limb (Franz et al., 1993; Tremblay et al., 1998); *Six1*, *Six4*-double-deficient embryos, also with a defect in muscle progenitor cell delamination and migration from the somite, a consequence of *Six1* and *Six4* control of *Pax3* expression (Grifone et al., 2005); and *Myf5*, *MyoD*-double-deficient embryos (Rudnicki et al., 1993) that were later demonstrated to also lack the expression of the *Mrf4* gene, in which no myoblasts are formed (Kassar-Duchossoy et al., 2004).

Histological analysis of E18.5 limb skeletons revealed similar abnormalities in several limb joints in the *Sp*$^{d}$, *Six1*$^{-/-}$*Six4*$^{-/-}$ and *Myf5*$^{-/-}$*MyoD*$^{-/-}$ muscleless mutants, not seen in their control littermates (Figure 1). All three mutant mouse models exhibited the same phenotype of failure in joint formation, with a 100% penetrance. Specifically, in the forelimb we identified missing joints in the elbow, in the humeroradial and humeroulnar articulations (Figures 1a′ and 1b′), as well as in the shoulder, between the humerus and scapula (Figure 1c′). Partial joint loss was also observed between indicated carpal elements in the wrist (Figure 1d′). In the hind limb, we identified absent joints between the talus and calcaneus (Figure 1e′) and in some of the metacarpals, as well as a loss of the hip joint (data not shown).

Failure in joint formation in all three mutated mouse models could result either directly from the absence of musculature or from the lack of muscle contraction. To distinguish between these two possibilities, we examined skeletons of paralyzed E18.5 *mdg* mutant embryos. These mutants are characterized

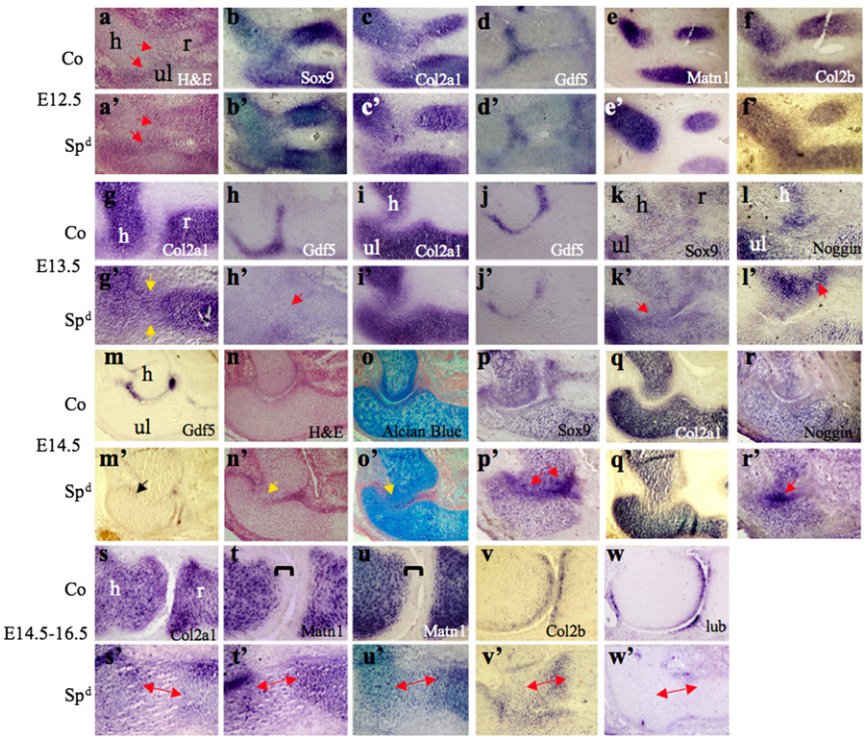


**Figure 2. Aberrant Expression of Joint Markers in $Sp^d$ Mutant Embryos**

Serial sections through control (Co; [a–w]) and $Sp^d$ mutant (a′–w′) forelimbs: Normal joint specification is visualized by H&E staining (a, a′), as *Sox9* (b, b′), *Col2a1* (c, c′), *Gdf5* (d, d′), matrilin 1 (*Matn1*) (e, e′), and *Col2b* (f, f′) expression in E12.5 elbow indicates the emergence of distinct humerus (h), radius (r), and ulna (ul) separated by a presumptive joint region (arrows). Sections of control (g–l) and $Sp^d$ (g′–l′) forelimbs at E13.5 show *Col2a1*-positive chondrocytes instead of *Gdf5*-positive joint cells within the elbow of mutants (arrows in [g′]); *Gdf5* expression is absent from the humeroradial intersection (arrow in [h′]) and reduced at the humeroulnar intersection (j′) of mutant embryos, when compared to control embryos (j). Increased *Sox9* (k′) and noggin (l′) expression is seen in joint region of mutant embryos relative to control ([k] and [l], respectively). Serial sections of control (m–r) and $Sp^d$ (m′–r′) humeroulnar joint at E14.5: no *Gdf5* expression is observed in humeroulnar region of $Sp^d$ (arrow in [m′]) in contrast to control joint region (m). Arrows indicate joint loss in the mutant as visualized by H&E staining (n′), Alcian blue staining (o′) *Sox9* (p′), and *Col2a1*-positive chondrocytes (q′); noggin is upregulated (r′) relative to control joint region (r). In situ hybridization on HRJ of E14.5 control (s–t) and $Sp^d$ (s′–t′) embryos showing *Col2a1*- and *Matn1*-positive chondrocytes in the middle of the joint region of mutants (double-headed arrows), absent from control embryos (indicated area), where the interzone is emerging. E16.5 HRJ of control (u–v) and $Sp^d$ (u′–v′) embryos showing expression of *Matn1* and *Col2b* across the articular region of presumptive mutant joints (arrow), with concomitant loss of lubricin expression in mutant (w′), when compared to control (w).

by the lack of excitation-contraction coupling, leading to an absence of skeletal muscle contraction and resulting in paralysis. We noted a lack of cavitation of the shoulder joint, as was previously described in these mice (Pai, 1965). In addition, and similarly to the muscleless embryos, the elbow (Figure 1f′), the midcarpal joints (Figure 1g′), and the hip joint (Figure 1h′) were absent. This failure in joint formation had a full penetrance.

The abnormal skeletal development in the $Myf5^{-/-}MyoD^{-/-}$ embryos (Braun et al., 1992) and the failure in muscle development restricted to the limb in the $Sp^d$ and $Six1^{-/-}Six4^{-/-}$ mutants prevented us from studying the involvement of the musculature in the formation of synovial joints other then in the limb. The complete paralysis of the *mdg* embryos allowed us to demonstrate the involvement of the musculature in the formation of the joints between the cervical and the lumbar vertebrae (Figures 1i′ and 1j′).

Another common feature of bones of both muscleless and paralyzed embryos was an abnormal joint morphogenesis. For example, normally in interlocking bones, one bone acquires a convex shape, while the reciprocal bone acquires a concave shape. This morphogenic process was missing in the mutated mice (Figures 1b, 1b′, 1k, and 1k′).

These histological and morphological data strongly imply that in mice, muscle contraction is required for joint formation.

To validate the above conclusion, it was important to exclude the possibility of a direct involvement of the mutated genes in joint formation. Since our subsequent analyses were to be performed mostly on the $Sp^d$ mutants, we assessed the contribution of *Pax3*-positive cells and their descendants to the forming joints by a genetic lineage analysis on *Pax3-Cre* mice crossed with ROSA-YFP reporter mice (Engleka et al., 2005; Srinivas et al., 2001). Examination of sections of *Pax3-Cre,* ROSA-YFP heterozygous embryos at E14.5 and E16.5 revealed, as expected, a robust YFP expression in limb muscles; in contrast, the joints were YFP-negative (Figures 1m and 1m′). These results clearly indicate that *Pax3*-positive cells and their descendants do not contribute to the forming joints.

## The Presumptive Joint of $Sp^d$ Mutant Embryos Is Occupied by Chondrocytes

A key step in deciphering the mechanism leading to aberrant joint formation in limbs with defective musculature is the identification of the stage at which joint formation is disrupted. To identify this time point, we examined the elbow joint of the $Sp^d$ mutant. Histological and gene expression analyses of the E12.5 elbow joint revealed, both in control and in $Sp^d$, the emergence of interzone cells separating three cartilaginous rudiments: radius, humerus, and ulna (Figures 2a–2c′). The expression of *Gdf5* by interzone cells of $Sp^d$ limbs (Figure 2d′) clearly indicated that initially, joint forming cells were specified even in the absence of limb musculature. However, 1 day later, *Gdf5* expression was absent from the humeroradial intersection and decreased in the humeroulnar intersection of the mutant embryos (Figures 2h′ and 2j′); by E14.5, *Gdf5* expression was lost altogether (Figure 2m′). At that stage in control embryos, *Gdf5* expression was maintained and initiation of joint cavitation

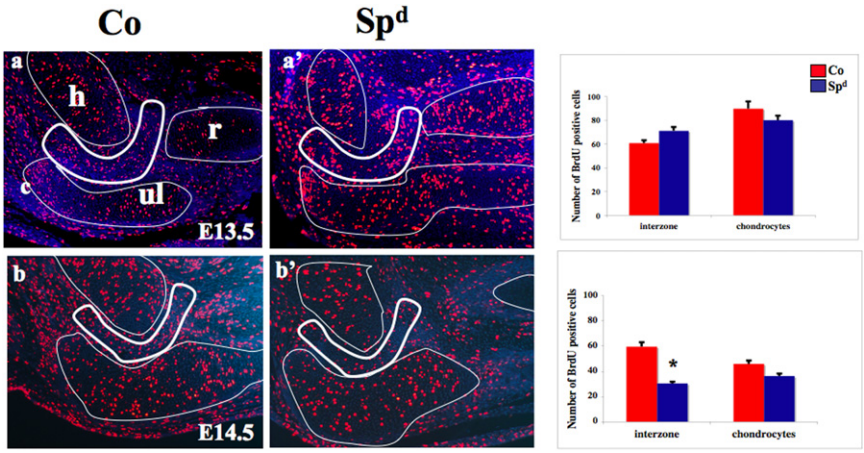


**Figure 3. Reduced Proliferation at Presumptive Joints of *Sp*$^d$ Mutants**

The number of BrdU-positive cells in the histologically defined interzone region of E13.5 control (a) and *Sp*$^d$ (a′) embryos revealed no significant difference in cell proliferation (control, 60.83 ± 2.31; *Sp*$^d$, 70.1 ± 3.22). In contrast, by E14.5 there was a 2-fold reduction in the number of dividing cells in the mutant interzone (b′) relative to control littermates ([b]; control, 59.6 ± 3.45; *Sp*$^d$, 30.4 ± 1.36; $p < 0.004$, $n = 4$). No significant differences were observed in chondrocyte proliferation in adjacent cartilaginous anlagen (E13.5: control, 89 ± 6; *Sp*$^d$, 80 ± 3.7; E14.5: control, 46 ± 2.5; *Sp*$^d$, 36.3 ± 1.8). Error bars represent the standard deviation from the mean.

could be observed (Figures 2m and 2n). In contrast, in mutant embryos, joint cavitation was missing (Figure 2n′); instead, at the presumptive joint, we observed cells that stained positively for Alcian blue and expressed *Sox9, Col2a1* and noggin (Figures 2o′–2r′).

The expression of chondrogenic markers by cells in the presumptive joint region of mutant limbs prompted us to determine the differentiation state of these cells. To this end, we used the following markers: matrilin 1 (*Matn1*), which is excluded from articular chondrocytes (Murphy et al., 1999); *Col2b*, a splice variant of *Col2a1* initially expressed by prechondrocytes and later in permanent articular cartilage (Nalin et al., 1995); and lubricin, a key molecule of joint lubrication that is expressed by superficial chondrocytes at the articular surface (Swann et al., 1985).

The expression of *Matn1* and *Col2b* in E12.5 forelimbs was comparable between control and *Sp*$^d$ mice (Figures 2e, 2e′, 2f, and 2f′). In E14.5 control embryos, we detected a strong *Matn1* expression in chondrocytes, except in sharply demarcated bands of articular chondrocytes on both sides of the joint (Figure 2t). In contrast, throughout the presumptive joint region in mutants, the cells were *Matn1* positive (Figure 2t′), an expression pattern that was perpetuated at E16.5 (Figures 2u and 2u′). In E16.5 control limbs, the expression of *Col2b*, which was initially expressed by all prechondrogenic cells (Figure 2f), could only be observed in a region that overlapped the *Matn1*-negative bands at the articulating surface (Figure 2v). In *Sp*$^d$ embryos, the population of *Col2b*-positive cells at the presumptive joint corresponded to the *Matn1*-positive region (Figure 2v′). In control embryos at E16.5, lubricin expression was indeed restricted to a subset of articular chondrocytes bordering the joint cavity, whereas its expression was completely absent from presumptive joints of mutant littermates (Figures 2w, and 2w′).

A parallel analysis of the *mdg* mutant elbow joint yielded similar results; however, we observed a 1 day delay in the expression sequence of the various markers (see Figure S1 available online).

These data suggest that interzone specification is initially accomplished in the absence of contracting muscles. However, as development proceeds, the presumptive joint is occupied by cells that fail to express the normal sets of joint markers and instead express chondrogenic markers.

## Decreased Cell Proliferation at the Presumptive Joints of *Sp*$^d$ Mutant Embryos

One possible explanation for the noticeable increase in chondrogenic cells in the presumptive joint of the *Sp*$^d$ elbow is the loss of committed joint cells by apoptosis combined with increased proliferation of chondrocytes that flank the forming joint. This would lead to cartilage overgrowth and, subsequently, to joint loss. Tunnel analysis did not reveal any significant cell death in either *Sp*$^d$ or control elbow joints (data not shown). BrdU incorporation into dividing cells revealed no significant differences in cell proliferation at E13.5 in the interzone regions and the surrounding chondrocyte anlagen of *Sp*$^d$ forelimbs, compared to control littermates (Figures 3a and 3a′). However, 1 day later (E14.5), we observed a 2-fold decrease in BrdU-positive cell count at the presumptive *Sp*$^d$ joint, whereas in the surrounding anlagen, no significant difference was noted when compared to their control littermates (Figures 3b and 3b′).

The absence of significant cell death and the decrease in cell proliferation at the presumptive *Sp*$^d$ joint indicate that the increase in chondrogenic cells and subsequent joint loss cannot be explained by cartilage overgrowth.

## Cells at Presumptive Joints of *Sp*$^d$ Embryos Lost Their Normal Differentiation Sequence

The observed decrease in cell proliferation and the changes in gene expression seen at the presumptive joint region of mutant embryos (namely, the loss of *Gdf5* concomitantly with an increase in *Col2a1*) may arise from distinct cell populations or, alternatively, may represent an alteration in the normal differentiation program of a single cell population. The latter would lead cells in the *Sp*$^d$ presumptive joint to coexpress both markers. We therefore performed a double fluorescent in situ hybridization for *Gdf5* and *Col2a1* on E12.5-E13.5 forelimbs. Our analysis of both control and *Sp*$^d$ E12.5 developing elbow regions revealed cells that simultaneously expressed *Gdf5* and *Col2a1* (Figures 4Ac and 4Ac′). However, by E13.5, in control forelimbs a clear segregation could be observed between *Col2a1* and *Gdf5* expressing cells (Figures 4Af and 4Ag, enlarged box region), although some overlap was apparent at the interface between the two populations. In contrast, at the presumptive joint of *Sp*$^d$ forelimbs, we observed a population of cells that simultaneously expressed *Gdf5* and *Col2a1* (Figures 4Af′ and 4Ag′, enlarged box region).

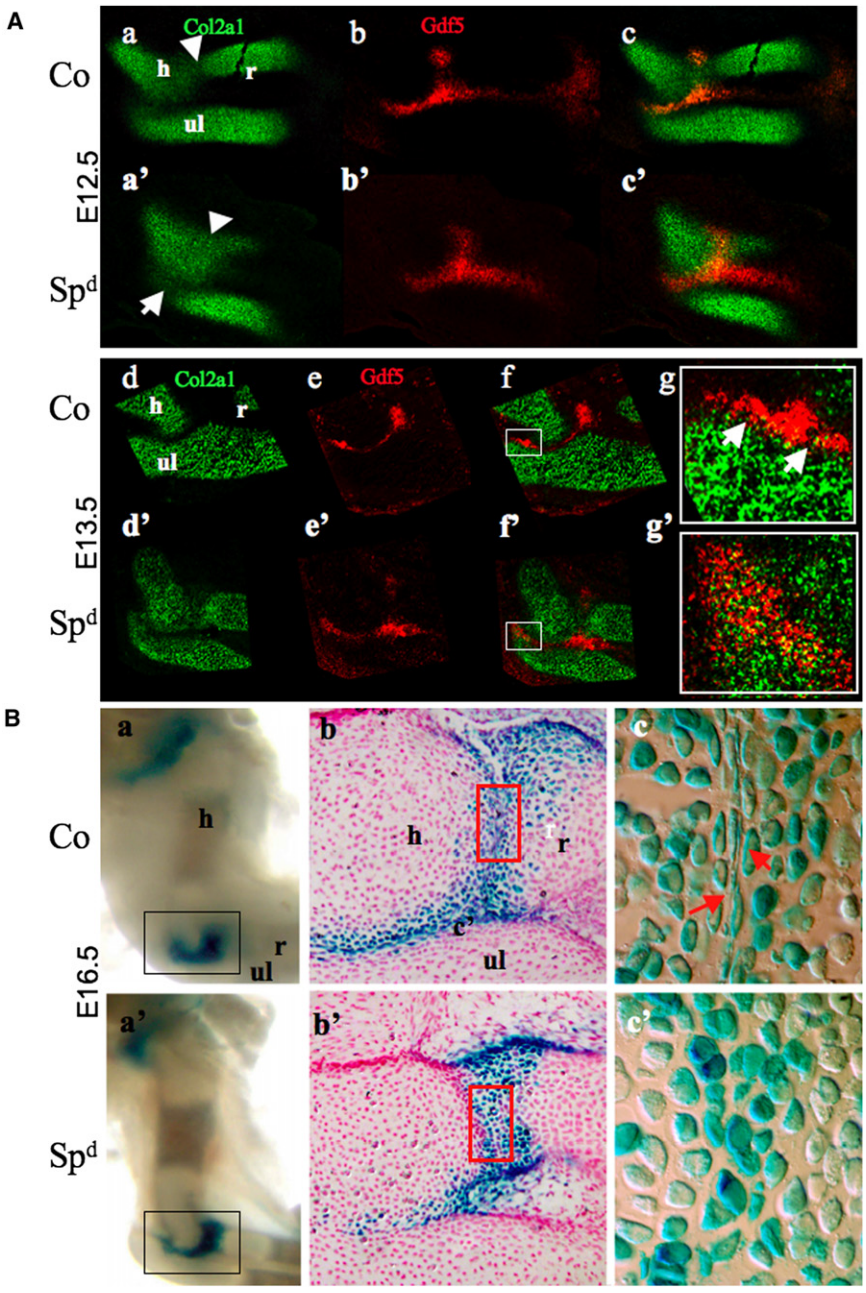


**Figure 4. Chondrocytes in Presumptive Joints of *Sp*$^d$ Mutants Are Descendants of *Gdf5*-Positive Cells**

(A) Coexpression of joint and chondrocyte markers in mutant joints. Fluorescent in situ hybridization for simultaneous detection of *Col2a1* (green) and *Gdf5* (red) at presumptive elbow region of E12.5 (upper panel) and 13.5 (lower panel) control (a–g) and *Sp*$^d$ (a′–g′) embryos. Higher magnification of the boxed area in (f, f′) (merged image) illustrates the distinct segregation between joint marker- and chondrocyte marker-expressing cells in E13.5 control embryos (g), versus coexpression of these markers in mutants (g′).

(B) Chondrocytes in presumptive joints of *Sp*$^d$ mutants are descendants of *Gdf5*-positive cells. Whole mount and section β-gal staining of E16.5 limbs of control (*Gdf5-Cre*, R26R-*lacZ* embryos; [a–c]) and *Sp*$^d$, *Gdf5-Cre*, R26R-*lacZ* embryos (a′–c′) demonstrates β-galactosidase activity in the elbow region of both phenotypes ([b, b′]: sections through the boxed region in [a, a′], respectively). Higher magnification of the HRJ shown in the boxed areas in (b, b′) indicates the presence of flattened, elongated interzone-like cells in control embryos ([c], arrow heads), which are absent from this area in *Sp*$^d$ embryos (c′).

Our observation that cells at the presumptive joint of the *Sp*$^d$ mutant cease to proliferate and, concurrently, coexpress both joint and chondrogenic markers supports our hypothesis that joint progenitor cells of *Sp*$^d$ embryos lose their normal sequence of differentiation and proliferation and, instead, differentiate into chondrocytes.

To directly test this hypothesis, we examined the origin of cells occupying the presumptive joint of *Sp*$^d$ limbs by applying a genetic lineage analysis, using *Gdf5-Cre* mice crossed with R26R-*lacZ* reporter mice (Rountree et al., 2004; Soriano, 1999). This system was previously utilized to label joint progenitor cells and their descendants (Koyama et al., 2008; Rountree et al., 2004). Examination of *Sp*$^d$ embryos at E16.5, when joints were clearly lost, revealed *lacZ* expression in the presumptive joint regions of mutant embryos, similar to their control littermates (Figures 4Ba–4Bc′). Sections through control joint region revealed two distinct morphologies of *lacZ*-positive cells: articular cells and elongated cells of the superficial layer. The latter cell population was absent in mutants, where all cells exhibited a round, chondrogenic-like morphology that was less organized (Figures 4Bb and 4Bb′ and Figures 4Bc and 4Bc′; enlarged box regions of Figures 4Bb and 4Bb′). Similar results were obtained when analyzing joint progenitor cell fate in the *mdg* mutant (data not shown).

These results clearly indicate that cells in the fused joints of *Sp*$^d$ and *mdg* embryos are descendants of *Gdf5*-positive cells and are initially fated to form joint tissue. However, in the absence of muscle contraction, these cells lose their designated fate and differentiate into chondrocytes.

## Decreased β-catenin Activation in the Presumptive Joint Region of Mutant Embryos

Evidence for the involvement of the β-catenin signaling pathway in maintaining joint progenitor cell fate (Guo et al., 2004; Hartmann and Tabin, 2001; Spater et al., 2006) prompted us to determine whether β-catenin activation is part of the mechanism by which the musculature regulates joint progenitor cell fate. To

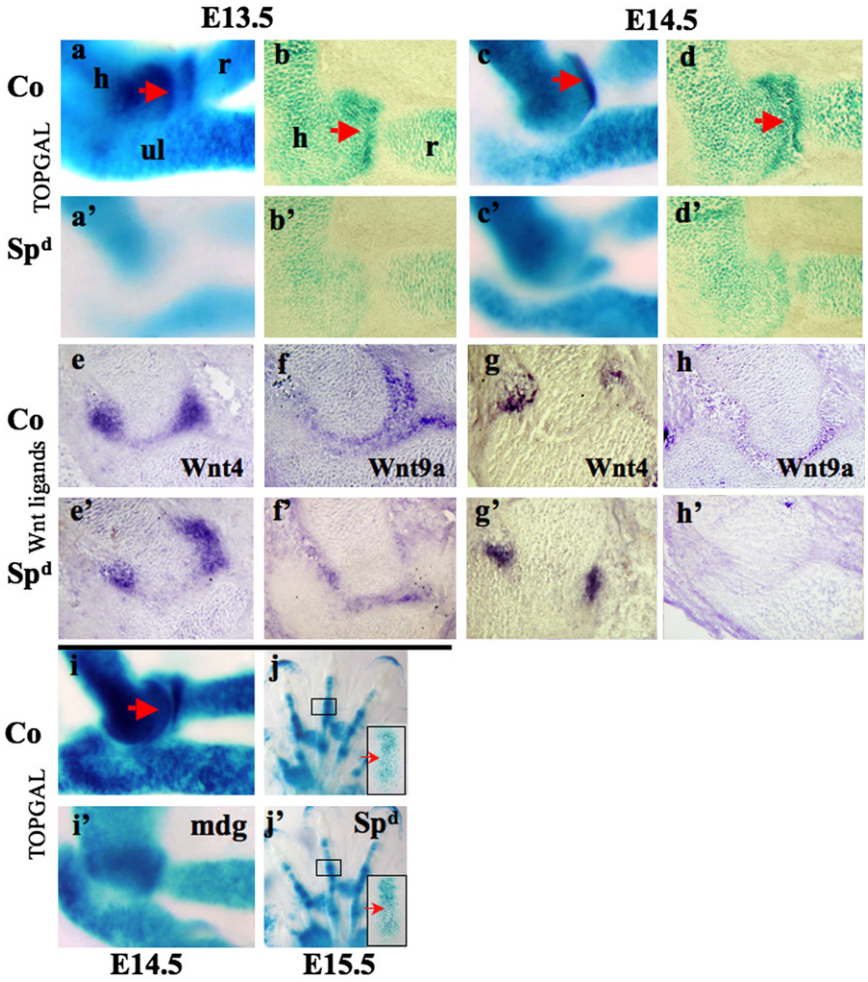


**Figure 5. Decreased β-catenin Activation in Presumptive Joints of Mutants**

Whole mount and section β-gal staining demonstrates β-catenin activity in forming joints of E13.5–E14.5 control embryos (a–d, i), which is reduced in presumptive joints of *Sp*$^{d}$ (a′–d′) or *mdg* (i′) mutants. *Wnt4* and *Wnt9a* expression at E13.5 is comparable between the *Sp*$^{d}$ (e′,f′) and control joints (e, f). By E14.5, *Wnt4* expression is maintained (g, g′), whereas *Wnt9a* expression is lost in the *Sp*$^{d}$ joint (h′). Comparable β-gal staining (j, j′) at E15.5 indicates no change in β-catenin signaling in the autopods of *Sp*$^{d}$ embryos relative to control.

this end, we utilized the TOPGAL mouse strain as a reporter for β-catenin activation (DasGupta and Fuchs, 1999). Whole mount and sections of E13.5-E14.5 control limbs stained for X-gal demonstrated β-catenin activation at the joint articular region (Figures 5a–5d); this activation was reduced in *Sp*$^{d}$ embryos (Figures 5a′–5d′). We further examined β-catenin activation in *mdg* mutant embryos. Here, too, we observed a reduction of specific X-gal staining relative to control at the articular regions of E14.5 forelimbs (Figures 5i and 5i′).

The reduction in β-catenin activation in *Sp*$^{d}$ embryos led us to examine the expression of *Wnt9a* and *Wnt4*, ligands of the Wnt/β-catenin signaling that are known to be expressed in the developing joints. At E13.5, both *Wnt4* and *Wnt9a* were expressed in the *Sp*$^{d}$ presumptive joint (Figures 5e′ and 5f′), although by that stage β-catenin activation in *Sp*$^{d}$ joint was reduced. By E14.5, while *Wnt4* expression was maintained in the *Sp*$^{d}$ presumptive joint (Figure 5g′), *Wnt9a* expression was lost (Figure 5h′). The expression of *Wnt9a* and *Wnt4* in the *Sp*$^{d}$ presumptive joint at a stage when β-catenin activation was reduced suggests that, in addition to its dependence on Wnt signaling for its activation, β-catenin signaling in joint forming cells is, at least in part, regulated by muscle contraction.

This finding prompted us to examine β-catenin signaling in *Sp*$^{d}$ nonaffected joints; for example, in the knee and the fingers. Our goal was to differentiate between two hypotheses: normal β-catenin signaling in the knee or finger joints would imply that, in different joints, β-catenin signaling is differently regulated. Alternatively, alteration in the activity of the β-catenin signaling in *Sp*$^{d}$-nonaffected joints would indicate a compensation by a different signaling pathway, which does not occur in the affected joints. Using the TOPGAL mice as a reporter for β-catenin signaling in the autopod, we did not observe any differences in the activation of β-catenin signaling in *Sp*$^{d}$ embryos relative to the control (Figures 5j and 5j′ and Figure S2). These results support the first supposition that β-catenin signaling is regulated differently in different joints.

In conclusion, these findings provide a mechanistic explanation for the failure in maintaining joint progenitor cell fate and consequent absence of joint formation in paralyzed limbs.

## DISCUSSION

In this study, we describe a role for embryonic movement and muscle contraction in joint formation. In the absence of contracting musculature, we observed failure of joint formation, which we ascribe to the inability of joint progenitor cells to maintain their designated fate and their consequent differentiation to chondrocytes. We further identify muscle contraction as a participant in the regulation of β-catenin activation in the forming elbow joint, thereby revealing new mechanistic insight into how the musculature maintains joint progenitor cell identity.

### Joint Formation: Embryonic Movement and Regulation of Progenitor Cell Fate

Understanding the mechanisms that regulate differentiation and proliferation of organ progenitor cells is fundamental. While the majority of studies emphasize the significance of soluble molecules such as growth factors and cytokines in regulating the differentiation process, a role for movement-induced mechanical stimuli in the regulation of progenitor cells was suggested by numerous in vitro studies (Altman et al., 2002; Elder et al.,

2000, 2001; Feron et al., 1999; McAllister et al., 2000; Simmons et al., 2003; Thomas and el Haj, 1996). At this time, data on the in vivo contribution of embryonic movement to this process are meager.

Embryonic movement plays a fundamental role in the normal in utero developmental process. This is demonstrated by the human syndrome fetal akinesia deformation sequence (FADS [OMIM 208150]). In this syndrome, restriction of embryonic mobility may lead to polyhydramnios, intrauterine growth retardation, pulmonary hypoplasia, craniofacial and limb anomalies, multiple joint contractures, short umbilical cord, and lethality, depending on the level of restriction (Hall, 1986). Of particular relevance to the present study is the requirement for embryonic movement in normal joint formation as seen in the congenital disorder arthrogryposis multiplex congenita (AMC). This disorder is characterized by multiple joint contractures found throughout the body at birth as a result primarily of fetal akinesia (i.e., decreased fetal movements) due to fetal abnormalities (e.g., neurogenic, muscle, or connective tissue abnormalities; mechanical limitations to movement) (Hall, 1997).

That embryonic movement, myogenesis, and joint formation commence at parallel developmental stages in the murine embryo (E12.5) (Ontell et al., 1993; Suzue, 1996) supports a role for muscle contraction in early joint formation. By examining joint development in various murine models devoid of normal muscle contraction, we provide evidence for the essential role of embryonic movement in the early stages of joint formation.

Prior to the current study, the mechanisms that underlie the contribution of movement to this developmental process were mostly missing. Previous studies, primarily in avian embryos, on the role of embryonic movement and muscle contraction in joint formation have concluded that while these factors have no role in joint specification, they are necessary for joint cavitation (Craig et al., 1987; Dowthwaite et al., 1998; Edwards et al., 1994; Pitsillides et al., 1995) by effecting local synthesis and retention of extracellular matrix (ECM) components such as hyaluronan (HA), shown to be involved in the cavitation process. Our study is in general agreement with these previous studies, as we show that despite the absence of muscle contraction, interzone formation remains intact, and that the interzone cells originally possess a joint identity, indicating that indeed the musculature and its contraction have no role in joint specification. However, diverging from previous studies, we demonstrate here that muscle contraction affects joint development even prior to joint cavitation. This occurs at an intermediate stage that bridges between joint specification and the cavitation process, a stage at which joint progenitors are not yet committed and are probably most sensitive to environmental queues. During this stage, joint progenitor cells need to maintain their plasticity and proliferative abilities, most likely to enable their differentiation to the various lineages that would eventually form the different components of the mature joint. Indeed, at this critical stage in joint development, we observed cells coexpressing joint and chondrogenic markers in both control and mutant presumptive elbow regions. Normally, as joint development proceeds, chondrogenic markers such as *Col2a1* and *Sox-9* are downregulated, with simultaneous perpetuation of joint markers such as *Gdf5* and *Wnt9a*. However, we revealed that in the absence of muscle contraction, this dual expression of joint and chondrogenic markers is maintained and prolonged beyond the initial developmental stage. Eventually, the joint markers are lost, whereas the expression of chondrogenic markers such as *Sox-9*, *Clo2a1*, *Matn1*, and *Col2b* is maintained, culminating in joint fusion.

This observation strongly suggests that muscle contraction regulates the fate of the newly specified joint progenitor cells, thus providing the answer to a decade-old question of how the musculature affects joint formation; namely, by maintenance of joint progenitor cell identity and suppression of chondrogenesis.

In addition to its role in maintaining joint progenitor cell fate, our findings also demonstrate the involvement of muscle contraction in joint morphogenesis, as witnessed by the loss of the stereotypical convex-concave shape in many of the joints of mutated embryos. These derangements and the lack of cavitation could result, at least in part, from a reduction in interzone cell proliferation, as observed in $Sp^d$ mutated mice.

## Differential Mechanisms Regulating Joint Formation

The synovial joint is a key component of the musculoskeletal system, as it enables the skeleton to provide both structural support and mobility. From an evolutionary point of view, the advantage of the regulation of joint progenitor cell fate by the musculature is not absolutely clear. Mechanical stimuli could regulate the size of the progenitor cell pool required for articular cartilage maintenance.

In view of this hypothesis, it is puzzling that the failure in joint formation was not observed in all joints of mutant embryos; the knee joint and the fingers joints, for example, were intact. One plausible explanation is that in some joints, the lack of musculature was compensated for by other components in the genetic program that regulates joint development. This conjecture is supported by our finding that β-catenin activation is regulated differently in different joints. β-catenin is a key molecular regulator shown to be involved in suppression of chondrogenesis in the joint region (Spater et al., 2006). We show that muscle contraction regulates β-catenin activation in the $Sp^d$ elbow, whereas in the fingers, the absence of muscle contraction had no effect on β-catenin activation, and therefore the joints remained intact. Interestingly, at the elbow joint, the expression of *Wnt9a* and *Wnt4*, ligands of the β-catenin signaling pathway, was comparable between mutant and control embryos.

While we cannot eliminate the possibility that some of the modulation may come through the expression of the *Wnt* ligands, these results suggest that in the elbow region, the musculature is involved in β-catenin activation. This claim was supported by Spater et al., who observed that despite the widespread expression of *Wnt9a* and *Wnt4* in forming joints, *Wnt4*:*Wnt9a* double-mutant mice revealed only specific joint loss such as certain carpal and tarsal elements, with only minor joint abnormalities in the elbow (Spater et al., 2006). In line with our data, demonstrating the ability of the musculature to regulate β-catenin activation is a recent work in *Drosophila*, where mechanical deformations associated with embryonic morphogenetic movements triggered nuclear translocation of Armadillo/β-catenin (Desprat et al., 2008).

Further support for the existence of different modes of regulation in different joints is provided by various mutations in both humans and mice that affect only a subset of joints. For example,

deletion of *Tgfbr2* in early limb mesenchyme results only in interphalangeal joint fusion without affecting other joints such as the elbow, despite its high, specific expression in these joints (Seo and Serra, 2007; Spagnoli et al., 2007). Similarly, although *Gdf5* is expressed in all synovial joints, only a subset of joints such as carpal, certain phalanges, and tarsals are disrupted by *Gdf5* null mutations (Storm and Kingsley, 1996). These findings, together with the findings in this paper, strongly suggest that there may not be one mechanism regulating β-catenin expression in the joints and consequent joint formation, but rather that different mechanisms are needed to regulate the formation of different joints, one of these mechanisms being muscle contraction. A possible explanation for the need for alternative mechanisms in different joints is the requirement for the formation of variable joint structures, facilitating different functions.

The regulation of progenitor cell differentiation and embryonic movement are two developmental processes shown to be essential for correct organogenesis. However, until now, no direct connection in vivo has been reported between them. Our finding, in a murine model, that in the absence of muscle contraction, joint progenitor cells lose their normal differentiation program strongly supports the paradigm that movement-induced mechanical stimuli play a key role in the regulation of organ progenitor cells during development and thus underscores the importance of movement in embryonic development in general.

## EXPERIMENTAL PROCEDURES

### Mouse Strains

Heterozygous $Sp^{d}$ mice (Dickie, 1964) were received from the laboratory of Clifford J. Tabin, Harvard Medical School. Mice heterozygous for the mutation muscular dysgenesis (*mdg*) (Pai, 1965) were obtained from the laboratory of George Kern, Innsbruck, Austria. As control, we used heterozygous $Sp^{d}$ and *mdg* embryos. The generation of $Six1^{-/-}Six4^{-/-}$ (Grifone et al., 2005) was previously described; as control, we used heterozygous *Six1,Six4* embryos. The generation of *MyoD*, *Myf5*-deficient embryos (Rudnicki et al., 1993) was previously described; as control, we used heterozygous *MyoD*, *Myf5* embryos. *Pax3-Cre* mice (Srinivas et al., 2001), ROSA-YFP reporter mice (Engleka et al., 2005), and TOPGAL mice [Tg(fos-lacZ)34Efu/J line] (DasGupta and Fuchs, 1999) were purchased from Jackson Laboratory (Bar Harbor, ME). For genetic lineage analysis, *Gdf5-Cre,* $Sp^{d+/-}$ mice were crossed with R26R-*lacZ,* $Sp^{d+/-}$ mice, and limbs from *Gdf5-Cre,* R26R-*lacZ,* $Sp^{d+/-}$ embryos were compared with *Gdf5-Cre,* R26R-*lacZ,* $Sp^{d-/-}$ limbs (Rountree et al., 2004; Soriano, 1999). In all timed pregnancies, plug date was defined as E0.5. For harvesting of embryos, timed-pregnant female mice were sacrificed by $CO_2$ intoxication. The gravid uterus was dissected out and suspended in a bath of cold PBS and the embryos were harvested after amnionectomy and removal of the placenta. Tail genomic DNA was used for genotyping.

We have analyzed three different embryos from the $Six1^{-/-}Six4^{-/-}$ and $Myf5^{-/-}MyoD^{-/-}$ mutant lines and dozen of $Sp^{d}$ and *mdg* mutants, all of which manifested abnormalities in the elbow joint. Therefore, we conclude that the penetrance at the elbow is indeed 100%.

### Skeletal Preparations

Cartilage and bones in whole mouse embryos were visualized after staining with Alcian blue and Alizarin red S (Sigma) and clarification of soft tissue with potassium hydroxide (McLeod, 1980).

### Immunofluorescence

For section immunofluorescence, embryos were fixed overnight in 4% PFA/PBS, dehydrated to 100% EtOH, embedded in paraffin and sectioned at 7 μm. Samples were incubated overnight at 4°C with the primary antibody biotinilated anti-goat GFP (Abcam), diluted 1 in 50 in blocking solution.

### Histology and In Situ Hybridization

Embryos were fixed in 4% paraformaldehyde at 4°C overnight. Fixed samples were embedded in paraffin and sectioned at 7 μm thickness. Section in situ hybridizations were performed as described previously (Murtaugh, 1999; Riddle et al., 1993). All probes are available by request. Hematoxylin and eosin (H&E) staining was performed following standard protocols. For Alcian blue staining, sections were incubated in Alcian blue solution (pH 2.5) for 5 min, washed. and counterstained with nuclear fast red solution for 1 min. Double fluorescent in situ hybridizations on paraffin sections were performed using biotin- and DIG-labeled probes. After hybridization, slides were washed, quenched, and blocked. Probes were detected by incubation with streptavidin-HRP (Perkin Elmer, diluted 1 in 1500) and anti-DIG-HRP (Roche, dilute 1 in 50), followed by Cy2- or Cy3-tyramide-labeled fluorescent dyes (according to instructions of the TSA Plus Fluorescent Systems Kit, Perkin Elmer).

### BrdU Assay

Mice were injected intraperitoneally with 100 mg/kg body weight BrdU labeling reagent (Sigma) and sacrificed two hours later. Forelimbs were fixed in 4% paraformaldehyde for 24 hr at 4°C and embedded in paraffin. Serial sagittal sections (7 μm) traversing the entire joint from the lateral to medial side were collected on Fisherbrand Superfrost Plus slides and used for histochemical staining. A BrdU staining kit (Zymed Laboratories Inc.) was used according to the manufacturer's specifications to stain for BrdU-positive cells within the interzone (defined by histological analysis) and in a demarcated 220 square μm chondrocytic area of the embryos. To quantify the rate of cell proliferation, serial images of the same joints were collected and BrdU positive (red) and negative (gray) cells in the joint region were counted.

A total of eight embryos were used from two different litters: four control and four $Sp^{d}$. Six different sections from each embryo were analyzed. Statistical significance was determined by student's t test.

### Section and Whole-Mount β-Galactosidase Staining

Embryos were fixed for 1 hr in 4% PFA at 4°C, washed three times in rinse buffer containing 0.01% deoxycholate, 0.02% NP-40, 2 mM $MgCl_2$, and 5 mM EGTA at room temperature and stained for 3 hr at 37°C in rinse buffer supplemented with 1mg/ml X-gal, 5 mM $K_3$ $Fe(CN)_6$ and 5 mM $K_4$ $Fe(CN)_6$. Limbs were washed with PBS, cleared in 1.8% KOH, and transferred to glycerol for long-term storage.

For histological examination, stained whole-mount limbs were fixed in 4% PFA overnight, dehydrated, embedded in paraffin, and used to generate 7 μm thick sections, which were collected on Fisherbrand Superfrost Plus slides, dehydrated, and cleared in xylene.

## SUPPLEMENTAL DATA

Supplemental Data include two figures and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00175-0.

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