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Impaired intervertebral disc formation in the absence of *Jun*

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SUMMARY

Jun is a major component of the heterodimeric transcription factor AP-1 and is essential for embryonic development, as foetuses that lack *Jun* die at mid-gestation. Ubiquitous mosaic inactivation of a conditional *Jun* allele by cre/LoxP-mediated recombination was used to screen for novel functions of *Jun* and revealed that its absence results in severe malformations of the axial skeleton. More-specific *Jun* deletion by collagen2a1-cre demonstrated the essential function of Jun in the notochord and sclerotome. Mutant notochordal cells showed increased apoptosis, resulting in hypocellularity of the intervertebral discs. Subsequently, fusion of vertebral bodies caused a scoliosis of the axial skeleton. Thus, *Jun* is required for axial skeletogenesis by regulating notochord survival and intervertebral disc formation.

Key words: *Jun*, Notochord, Skeleton, cre/loxP, Mouse

INTRODUCTION

The vertebrate body is supported by the vertebral column, a series of segmental skeletal elements that provide both stability and mobility. The segmental units of the vertebrate axial skeleton are generated by division of the unsegmented paraxial mesoderm into paired epithelial somites, which subsequently give rise to the metameric pattern of vertebrae and intervertebral discs (Gossler and Hrabe de Angelis, 1998; McGrew and Pourquie, 1998).

The development of the axial skeleton is a multi-step process, starting with the formation of somites from the unsegmented paraxial mesoderm on both sides of the neural tube. Shortly after formation, the somites compartmentalise to generate dermomyotomes and sclerotomes, the latter forming skeletal elements of the vertebral column and ribs. Vertebral column development requires the co-ordination of a series of cellular events. These include de-epithelialization of somites, proliferation and migration of sclerotomal cells, and establishment of anteroposterior polarity of the sclerotome (Gossler and Hrabe de Angelis, 1998; McGrew and Pourquie, 1998; Theiler, 1988). In mammals, a single vertebra is composed of a variety of components that perform different functions, depending on the level of the body axis. Thus, a fine-tuned regulation is required to coordinate the prepatterning of individual skeletal elements by region-specific mesenchymal growth and condensations that are finally replaced by cartilage and bone. The notochord has the most diverse functions during vertebrate development. The early notochord is a rod-like axial structure of mesodermal origin that plays an important role in the dorsoventral patterning of both the neural tube and the somitic mesoderm. In the neural tube, it induces the formation of the floor plate; in the somites, it induces the differentiation of the ventral somitic derivatives into the sclerotome (Fan and Tessier-Lavigne, 1994; Pourquie et al., 1993). After these inductive events, some sclerotomal cells migrate towards the notochord, where they form a continuous and initially unsegmented perichordal tube. Later on, this axial mesenchyme acquires a characteristic metameric pattern of condensed and noncondensed areas. While the regularly spaced condensations represent intervertebral disc rudiments and will give rise to the annulus fibrosus of the future intervertebral disc (IVD), the noncondensed perichordal cells form the cartilaginous primordia of the vertebral bodies (Theiler, 1988). During mammalian embryonic development, the inner part of the annulus fibrosus differentiates into hyaline-like cartilage, which, together with the vertebral bodies, forms an uninterrupted cartilaginous column around the notochord. Parallel with the ongoing chondrification, the notochord vanishes in areas where vertebral bodies develop but expands between the vertebrae to form the center of the IVD, where it forms the nucleus pulposus (Theiler, 1988).

Several transcription factors have been shown to be required for skeletogenesis (McGrew and Pourquie, 1998; Summerbell and Rigby, 2000; Wagner and Karsenty, 2001). Targeted inactivation of Sox9 results in skeletal defects due to impaired cartilage differentiation and absence of osteoblasts in *Cbfa1*-deficient mice (*Runx2* – Mouse Genome Informatics) mimics

the human heritable skeletal disorder cleidocranial dysplasia (Bi et al., 2001; Otto et al., 1997). In addition, the Pax family members Pax1 and Pax9, the proto-oncogene Myc, the homeobox gene *Bapx1* and several other transcription factors have been implicated in the genetic control of skeletogenesis (Gossler and Hrabe de Angelis, 1998; Lettice et al., 1999; Nagy et al., 1998; Peters et al., 1999; Tribioli and Lufkin, 1999). However, many of the pivotal signalling molecules regulating skeletal development are likely to still await identification.

The transcription factor AP-1 consists of a variety of dimers composed by members of the Fos and Jun families of proteins (Jochum et al., 2001). While the Fos proteins [Fos, Fosb, Fra1 (Fosl1 – Mouse Genome Informatics), Fra-2 (Fosl2 – Mouse Genome Informatics)] can only heterodimerise with members of the Jun family, the Jun proteins (Jun, JunB, JunD) can both homodimerise and heterodimerise with other Jun or Fos members to form transcriptionally active complexes (Angel and Karin, 1991). In addition to Fos proteins, Jun proteins can also heterodimerise efficiently with other transcription factors such as members of the ATF/CREB families (Hai et al., 1999). Jun is a major component of the AP-1 transcription factor complex and, together with JunB and JunD, forms the family of mammalian Jun proteins (Angel and Karin, 1991). Targeted disruption of the *Jun* gene results in embryonic lethality presumably because of defective hepatogenesis caused by increased apoptosis of hepatoblasts and hematopoietic cells (Hilberg et al., 1993; Johnson et al., 1993). In addition, $Jun^{-/-}$ embryos show malformations of the heart outflow tract (Eferl et al., 1999) that resemble the human disease truncus arteriosus persistens, a developmental defect probably caused by impaired neural crest cell function. Although the extent of functional conservation between individual members of the Jun family is not fully understood, knock-in experiments have demonstrated that Junb can substitute for some of the biological functions of Jun during embryogenesis (Passegue et al., 2002).

As the embryonic lethality of *Jun*-deficient foetuses was likely to prevent the identification of other biological functions of *Jun* at later stages of development, we decided to use a genetic strategy based on cre/loxP-mediated somatic recombination to screen for novel loss-of-function phenotypes.

MATERIALS AND METHODS

Mice

The Balancer1-cre, the human collagen2a1-cre transgenic lines and mice carrying a floxed *Jun* allele have already been described (Behrens et al., 2002; Betz et al., 1996; Haigh et al., 2000).

Histological analysis, BrdU immunohistochemistry, TUNEL assay, in-situ hybridisation and skeletal analysis

Embryos were dissected from timed matings of $Jun^{f/f}$ homozygous males with $Jun^{f/+}$ heterozygous females harbouring the Balancre1-cre or murine or human collagen2a-cre transgenes, respectively. Yolk-sac DNA was used for genotyping by PCR. Embryos for histological analysis sections were fixed in 4% paraformaldehyde and 5 μm sections were stained with Harris Haematoxylin and Eosin. BrdU immunohistochemistry was performed as described (Behrens et al.,

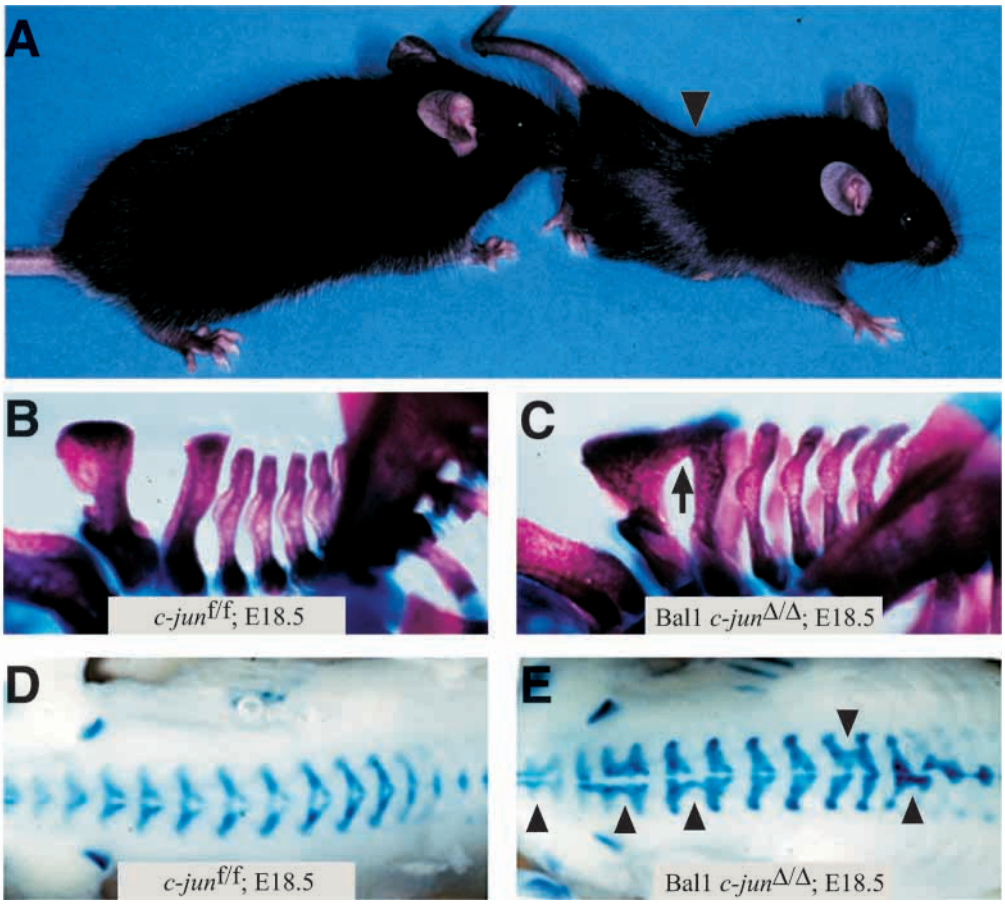

Fig. 1. *Jun* is required for axial skeletogenesis. (A) Skeletal phenotype of Bal1;$Jun^{\Delta/\Delta}$ mice. Photograph of an adult Bal1;$Jun^{\Delta/\Delta}$ and $Jun^{f/f}$ control littermate. Arrowhead indicates scoliosis. (B,C) Alcian Blue/Alizarin Red skeletal preparations of E18.5 Bal1;$Jun^{\Delta/\Delta}$-mutant (C) and $Jun^{f/f}$ control (B) embryos. Arrow in C indicates fused atlas and axis. (D,E) Dorsal view of Alcian Blue stained E16.5 Bal1;$Jun^{\Delta/\Delta}$-mutant (E) and $Jun^{f/f}$ control (D) mice. The epidermis and the neural tube were removed to visualise vertebral cartilage. Arrowheads in E indicate fusions of Alcian Blue-positive domains.

2000). For detection of apoptotic cells, histological sections prepared as above were used for TUNEL assay (Boehringer Mannheim) according to the manufacturer's instructions (Behrens et al., 1999). Skeletal analysis was carried out according to established procedures (Peters et al., 1999), whole-mount in situ hybridisation and non-radioactive in situ hybridisation on paraffin wax-embedded sections were performed as described previously (Haigh et al., 2000).

RESULTS

In order to identify additional biological processes regulated by *Jun*, we employed somatic cre/loxP-mediated gene inactivation in mice to screen for novel loss-of-function phenotypes of *Jun*. The Balancer1-cre transgenic (Bal1) line causes ubiquitous, but variable, recombination of a floxed target gene has been shown to reach 50-70% deletion in cells of a large number of adult organs (Betz et al., 1996). Significant cre-mediated recombination can first be detected at E10.5, and cre expression proceeds throughout embryogenesis and postnatal life. When combined with a homozygous floxed target gene, the Bal1 transgene generates mosaic mice, which contain a significant proportion of mutant cells in all organs (Betz et al., 1996). Owing to the mosaicism and variability of cre-mediated gene inactivation in individual mice, we reasoned that Bal1-mediated inactivation of a floxed *Jun* allele (Jun^{f}) could be used to overcome embryonic lethality and to uncover novel *Jun*-dependent biological functions.

Newborn $Jun^{f/f}$ mice carrying the Bal1 transgene (Bal1;$Jun^{\Delta/\Delta}$) were smaller and could be identified by open eyelids, suggesting a function of *Jun* in eyelid closure and providing the proof-of-principle for the feasibility of our genetic approach. The severity of the eyelid closure defect was variable, being bilateral or only unilateral in individual mice, presumably reflecting differences in Jun^{f} inactivation (data not

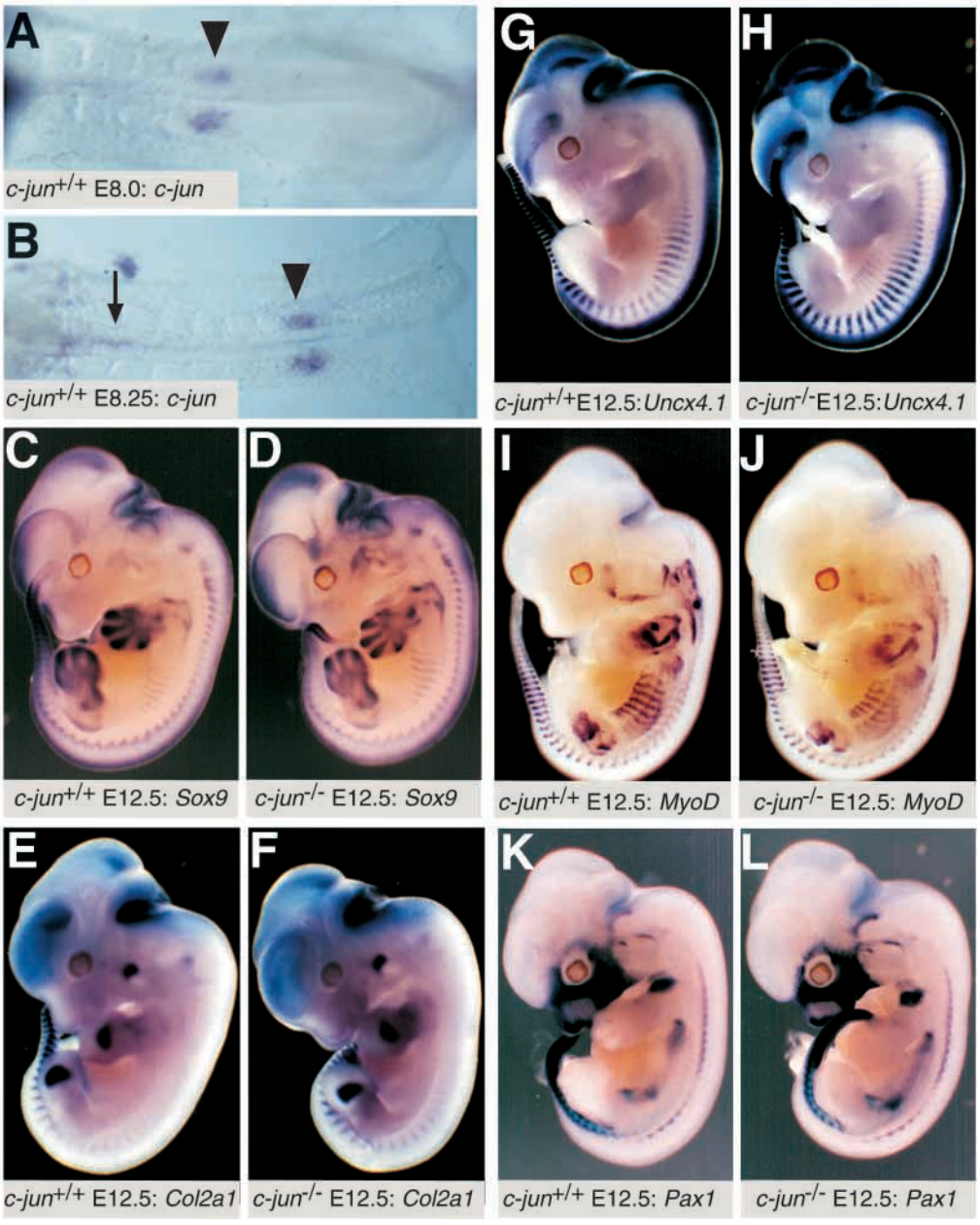

Fig. 2. *Jun* is dispensable for somitogenesis. (A,B) Expression of *Jun* is detected in the most recently formed somite. (A) Wild-type foetus with four somites; (B) wild-type foetus with seven somites. Arrowheads indicate *Jun* expression in the somite; arrow in B indicates midline expression of *Jun*. (C-L) *Sox9* (C,D), *Col2a1* (E,F), *Uncx4.1* (G,H), *Myod1* (I,J) and *Pax1* (K,L) expression detected by whole-mount in situ hybridisation is comparable between $Jun^{+/+}$ control (C,E,G,I,K) and $Jun^{-/-}$ (D,F,H,J,L) E12.5 embryos.

shown). Mutant mice were not present in numbers expected from Mendelian inheritance at birth, which could be due to Bal1-mediated recombination of *Jun* in the embryonic liver and heart, which are essential sites of *Jun* function (Eferl et al., 1999; Hilberg et al., 1993). In addition, Bal1;$Jun^{\Delta/\Delta}$ mice also had reduced viability before weaning age [4/186 survived until adulthood (2%) compared with 25% expected], indicating an essential, yet unidentified, function of *Jun* in postnatal development. All surviving Bal1;$Jun^{\Delta/\Delta}$ mice had a shortened body axis and showed scoliosis and kyphosis, an abnormal curvature and bending of the spinal column (Fig. 1A). To evaluate the nature of the skeletal abnormalities present in Bal1;$Jun^{\Delta/\Delta}$ mice, skeletal preparations of Bal1;$Jun^{\Delta/\Delta}$ E18.5 foetuses and littermate $Jun^{f/f}$ controls were made. Whereas $Jun^{f/f}$ controls showed a discrete metameric patterning of the spinal column, Bal1;$Jun^{\Delta/\Delta}$ foetuses displayed numerous defects, including fusions of the neural arches (Fig. 1B,C). At E16.5, in Bal1;$Jun^{\Delta/\Delta}$ embryos the chondrocytes of the spinal column, which will subsequently ossify and form the vertebral bodies, were not present in clearly demarcated domains, as in $Jun^{f/f}$ controls, but cell populations of adjacent segmental units were connected at several different levels along the entire longitudinal axis (Fig. 1D,E). Therefore, the genetic analysis using mosaic Bal1;$Jun^{\Delta/\Delta}$ mice has identified a role for *Jun* in the developing spinal column.

As the ubiquity and variability of Bal1-mediated cre expression did not allow the identification of the developmental stage and cell type requiring *Jun* function, we next investigated *Jun* expression during skeletogenesis. During somitogenesis, *Jun* mRNA is expressed in a dynamic pattern in presomitic mesoderm at the level of the most recently forming somite (Fig. 2A,B). *Jun* expression was also detected in the midline (arrow in Fig. 2B), and cross-sectioning revealed expression in the somitic mesoderm (data not shown). To examine a possible function of *Jun* during epitheliasation of the paraxial mesoderm, the expression of molecular markers of various cell lineages and derivatives of the somites were determined in the original *Jun* knockout mice and wild-type littermate controls (Hilberg et al., 1993). At E12.5, the expression pattern of *Sox9* and collagen 2a1 (*Col2a1*) was not affected by the absence of *Jun*, indicating that the initial phase of chondrogenesis had proceeded normally (Fig. 2C-F). Likewise, *Pax1*, *Myod1*, *Uncx4.1*, *Scx* and *Bapx1* expression, which characterise various cell populations of the developing axial skeleton (Gossler and Hrabe de Angelis, 1998; McGrew and Pourquie, 1998; Summerbell and Rigby, 2000), were unaltered in $Jun^{-/-}$ embryos (Fig. 2G-L and data not shown). We concluded that, until E12.5, skeletogenesis had proceeded normally in *Jun* mutants with proper anteroposterior patterning of the somites.

To assess *Jun* function in the notochord and sclerotome, Jun^{f} was inactivated using a transgenic line that expresses the cre recombinase under the transcriptional control of human collagen 2a1 promoter and enhancer sequences (Col2a1-cre). This line has been previously demonstrated to excise lox-P flanked reporter sequences in collagen 2-expressing cells, including the notochord and sclerotome (Haigh et al., 2000). At E12.5, Jun protein was strongly expressed in the floor plate and in the notochord, but weaker expression was also detected in the neural tube and in sclerotomal cells. Jun^{f} was deleted by Col2a1-cre in the notochord and sclerotome, but not in the floor

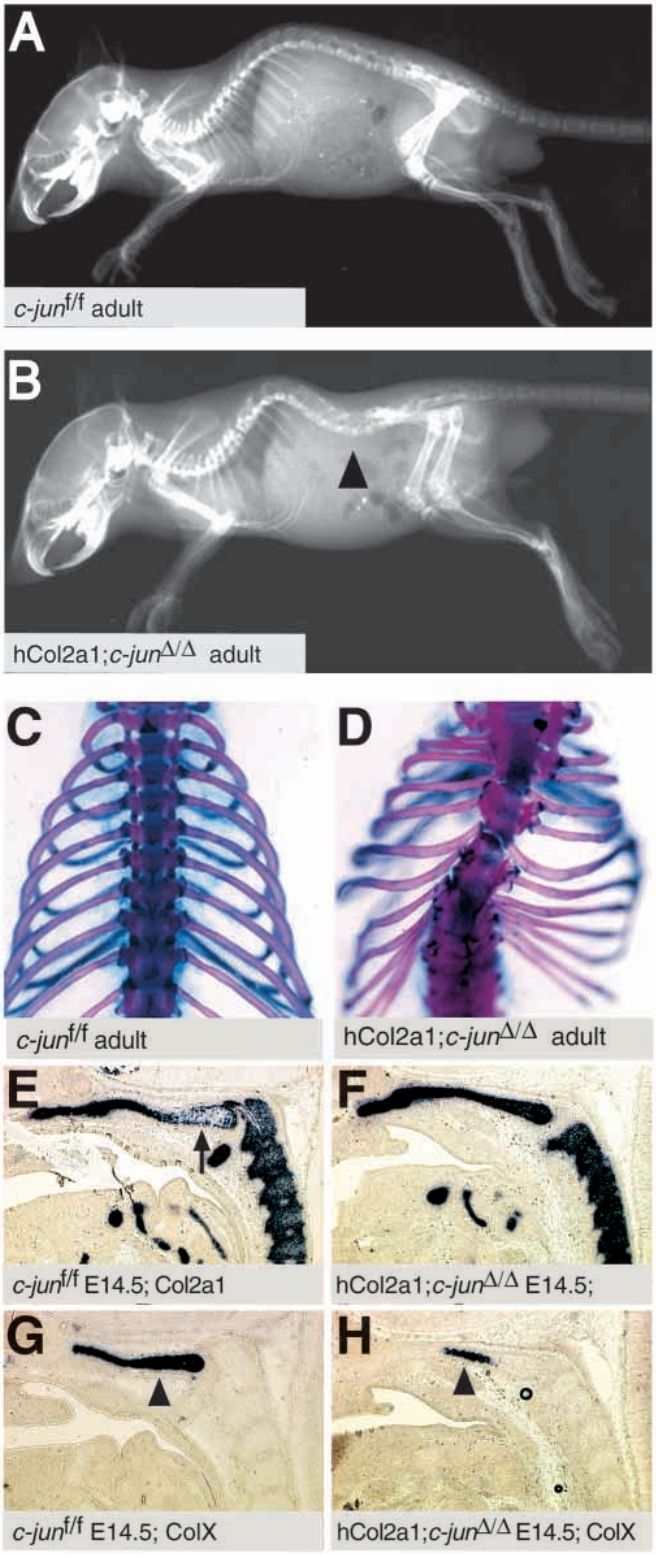

Fig. 3. *Jun* expression in the sclerotome and notochord is required for skeletogenesis. (A,B) Skeletal X-ray analysis of adult human Col2a1;$Jun^{\Delta/\Delta}$ (B) and $Jun^{f/f}$ control (A) mice. Arrowheads indicate scoliosis. (C,D) Alcian Blue/Alizarin Red skeletal preparations of the thoraxic region of adult Col2a1;$Jun^{\Delta/\Delta}$ (D) and $Jun^{f/f}$ control (C) mice. (E-H) mRNA in situ analysis of E14.5 Col2a1;$Jun^{\Delta/\Delta}$ (F,H) and $Jun^{f/f}$ control foetuses (E,G) using a *Col2a1* (E,F) and a collagen X (G,H) antisense probe.

plate (data not shown). The axial skeletal defects of both the Col2a1;$Jun^{\Delta/\Delta}$ mice resembled the Bal1;$Jun^{\Delta/\Delta}$ phenotype arguing for an essential function of *Jun* in the sclerotome and/or notochord. X-ray analysis and skeletal preparations of adult Col2a1;$Jun^{\Delta/\Delta}$ mice revealed a malformed, scoliotic vertebral column and abnormal morphogenesis of the rib cage (Fig. 3A-D). Interestingly, although Col2a1-cre is expressed and mediates recombination in growth plate chondrocytes of developing bones (Haigh et al., 2000), the length and overall gross morphology of the bones of the appendages appeared to be normal in adult Col2a1;$Jun^{\Delta/\Delta}$ mice (Fig. 3A,B and data not shown). We then analysed the expression of genes characterising the maturational stages of chondrogenesis at E14.5. In Col2a1;$Jun^{\Delta/\Delta}$ and $Jun^{f/f}$ control sclerotomal cells, *Col2a1*, a marker for proliferating chondrocytes, was highly expressed (Fig. 3E,F). Collagen X (*ColX*; *Col10a1* – Mouse Genome Informatics), indicative of hypertrophic chondrocytes, was not detected in the vertebral columns of embryos of either genotype (Fig. 3G,H). However, *ColX* was expressed in other skeletal structures at E14.5. In the developing baso-occipital bone, the *ColX* expression domain was broader at the expense of *Col2a1* expression in $Jun^{f/f}$ controls than in COL2A1;$Jun^{\Delta/\Delta}$ mutant foetuses (Fig. 3G,H; arrowheads). The increased expression of *ColX* was accompanied by decreased *Col2a1* expression in $Jun^{f/f}$ controls (Fig. 3E arrow). However, this delay in chondrocyte differentiation appeared to be transient as defects in adult Col2a1;$Jun^{\Delta/\Delta}$ mice were restricted to the axial skeleton and the formation of the baso-occipital bone was unaffected (Fig. 3A,B). Therefore, the absence of *Jun* did not have a severe effect on chondrogenesis and the skeletal defect of *Jun* mutant mice appears to be restricted to the developing spinal column, where Jun protein is most abundantly expressed in the notochord.

To understand the function of *Jun* during skeletogenesis, we performed a histological analysis of Col2a1;$Jun^{\Delta/\Delta}$ and littermate $Jun^{f/f}$ foetuses at various embryonic stages starting at E11.5. As expected from the normal expression of marker genes (Fig. 2C-L), sagittal sections of the developing spinal column of E11.5-E13.5 Col2a1;$Jun^{\Delta/\Delta}$ foetuses revealed no apparent alterations (Fig. 4A,B; data not shown), but at E14.5 Col2a1;$Jun^{\Delta/\Delta}$ embryos could be unambiguously identified histologically. Between E13.5 and E14.5, the sclerotomal cells of the prospective vertebral bodies had progressed from a proliferating to a hypertrophic chondrocyte morphology in $Jun^{f/f}$, but not in Col2a1;$Jun^{\Delta/\Delta}$ embryos. Secondly, a dramatic reduction in size of the nucleus pulposus, the central part of

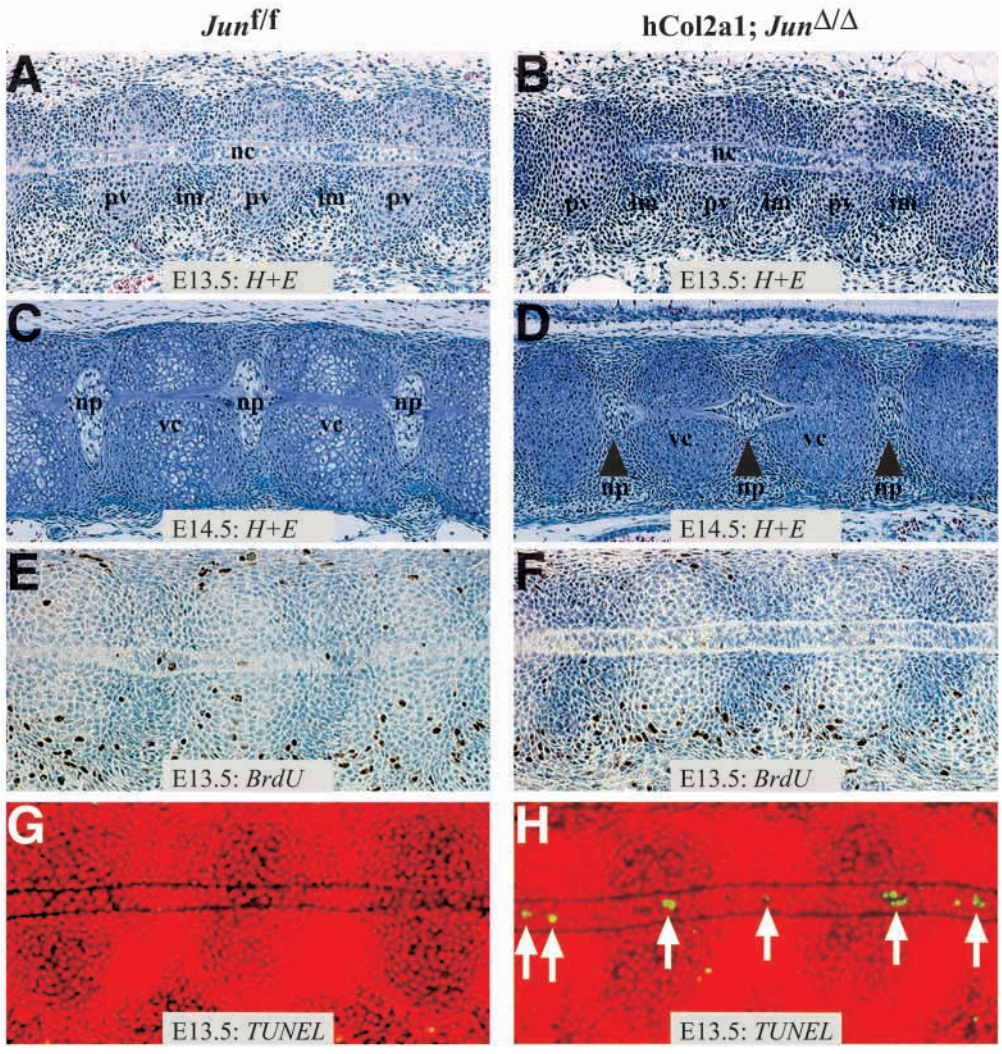

Fig. 4. *Jun* regulates the viability of notochordal cells. (A-D) Histology of sagittal sections of human Col2a1;$Jun^{\Delta/\Delta}$ (B,D) and $Jun^{f/f}$ control foetuses (A,C) at E13.5 (A,B) and E14.5 (C,D). Arrowheads in D indicate the nucleus pulposus; nc, notochord; pv, prevertebrae; im, intervertebral mesenchyme; vc, vertebral cartilage; np, nucleus pulposus. (E,F) Cell proliferation measured by BrdU incorporation in E13.5 Col2a1;$Jun^{\Delta/\Delta}$ (F) and $Jun^{f/f}$ control foetuses (E). (G,H) Apoptotic cell death measured by TUNEL staining (green) in E13.5 Col2a1;$Jun^{\Delta/\Delta}$ (G) and $Jun^{f/f}$ control foetuses (H). Sections were counterstained with propidium iodide (red). Arrows indicate TUNEL-positive cells.

the intervertebral disc anlage, was apparent in Col2a1;$Jun^{\Delta/\Delta}$ foetuses (Fig. 4C,D).

To investigate the reason for the inefficient formation of the nucleus pulposus, whose cells are derived from the notochord (Theiler, 1988), the extent of cellular proliferation and apoptosis was analysed at E13.5, before histological differences became apparent. Bromodeoxyuridine (BrdU) was injected into pregnant mothers and cells that had incorporated BrdU indicative of cellular proliferation were quantified by manual counting. 26±4 BrdU-positive cells/metameric segment were detected in the sclerotome of Col2a1;$Jun^{\Delta/\Delta}$ foetuses compared with 28±7 present in littermate $Jun^{f/f}$ controls. In the notochord 1.5±0.4 and 0.7±0.5 BrdU-positive cells were detected in $Jun^{f/f}$ and Col2a1;$Jun^{\Delta/\Delta}$ foetuses, respectively (Fig. 4E,F). Thus, the absence of *Jun* results in a reduced, albeit statistically insignificant, number of proliferating cells in both the sclerotome and the notochord, which appears to be unable to explain fully the failure of IVD development. By contrast, the absence of *Jun* had a profound effect on the extent of cell death. Whereas only a minimal number of TUNEL-positive cells were detected in E13.5 $Jun^{f/f}$ vertebral columns, a significant number of cells in the notochord, but not in the sclerotome, of Col2a1;$Jun^{\Delta/\Delta}$ foetuses were found to be undergoing apoptosis (Fig. 4G,H).

DISCUSSION

In this study we have identified an essential function for *Jun* in maintaining the viability of notochordal cells. The reduced cellularity of the nucleus pulposus resulted in defective intervertebral disc formation. Presumably as a secondary effect of the notochord defect, fusions of the vertebral bodies are observed.

In different biological processes, *Jun* can have both pro- and anti-apoptotic functions. During embryogenesis, Jun appears to elicit a crucial anti-apoptotic signal that is required for the survival of notochord cells. In addition, *Jun*-deficient embryos die at midgestation, displaying increased apoptosis of hepatoblasts and hematopoietic cells, and adult mice with a liver-specific *Jun* deletion show hepatocyte death during liver regeneration (Behrens et al., 2002; Eferl et al., 1999; Hilberg et al., 1993; Johnson et al., 1993). By contrast, Jun and Jun phosphorylation appears to be required for the execution of apoptosis in both neuronal cells and thymocytes (Behrens et al., 2001; Behrens et al., 1999). The molecular mechanism that underlies the dichotomy of *Jun* functions in apoptosis is not understood. The DNA-binding properties and sequence specificity of Jun can be altered by dimerisation with different interacting proteins. Jun/Fos heterodimers bind the DNA sequence 5′-TGA G/C TCA-3′, whereas the DNA motif 5′-TGACATCA-3′ is preferentially recognised by Jun/Atf2 complexes (Karin and Hunter, 1995). Possibly, different Jun-containing heterodimeric transcription factor complexes could be required for the transcriptional regulation of two classes of target genes, which are independently required for cell survival and apoptosis.

Reciprocal interactions between the notochord and the sclerotome are essential for axial skeleton development (Gossler and Hrabe de Angelis, 1998; McGrew and Pourquie, 1998). The notochord induces sclerotomal formation of the paraxial mesoderm and subsequently regulates the cartilagenous differentiation of sclerotomal cells mainly through production and secretion of the signalling molecule sonic hedgehog (*Shh*) (Fan and Tessier-Lavigne, 1994; Pourquie et al., 1993). However, *Pax1* expression in the developing sclerotome, which is dependent on and a mediator of the *Shh* signal (Fan and Tessier-Lavigne, 1994; Koseki et al., 1993), is unaltered in $Jun^{-/-}$ embryos (Fig. 2K,L). Moreover, the absence of *Jun* did not affect the induction of chondrogenic differentiation, as judged by marker gene expression (Fig. 3C-L). *Pax1*-mutant mice have provided evidence for a sclerotomal signal back to the notochord, as the absence of *Pax-1* expression in the sclerotome resulted in a hyperproliferation of notochordal cells (Wallin et al., 1994). Owing to the bidirectionality of the sclerotome-notochord interaction, the cell type that requires *Jun* function during skeletogenesis cannot be unambiguously identified. *Jun* is expressed and Jun^{f} is inactivated in both the notochord and sclerotome. The high expression of Jun in the notochord argues in favour of a cell-autonomous function, but we cannot exclude the possibility that Jun expression in the sclerotome is required for the generation of a sclerotomal survival signal for notochordal cells. The removal of *Jun* function by conditional gene targeting only in the notochord or only in the sclerotome, will be necessary to distinguish between these alternatives.

Jun becomes essential at the developmental stage when the notochord transforms into the centre of the vertebral disc anlagen. Initially, an uninterrupted longitudinal structure, the notochord, vanishes in areas where the vertebral bodies develop, but expands between the vertebrae to form the nucleus pulposus (Theiler, 1988). It is thought that the developing vertebral bodies exert a mechanical stimulus on the notochord that induces the migration of notochordal cells towards the presumptive intervertebral disc area (Aszodi et al., 1998; Rufai et al., 1995; Theiler, 1988). In *Jun*-mutant mice, the regionalisation of the notochord proceeds normally as notochordal cells are removed from the area of developing vertebral bodies, but notochordal cell number is reduced. Previously genes have been identified that are required for notochord survival at early stages of somitogenesis (Maatman et al., 1997), but *Jun* is the first factor shown to be essential for the survival of notochordal cells at a later stages of skeletogenesis. The *Jun*-mutant phenotype suggests that two distinct mechanisms control notochordal viability, a *Jun*-independent pathway during early development and a *Jun*-dependent pathway during later phases of axial skeletogenesis. The identification of *Jun* target genes required for notochord survival is expected to result in a better understanding of this new aspect of notochord biology.

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REFERENCES

Angel, P. and Karin, M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochem. Biophys. Acta* **1072**, 129-157.

Aszodi, A., Chan, D., Hunziker, E., Bateman, J. F. and Fassler, R. (1998). Collagen II is essential for the removal of the notochord and the formation of intervertebral discs. *J. Cell Biol.* **143**, 1399-1412.

Behrens, A., Sibilia, M. and Wagner, E. F. (1999). Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nat. Genet.* **21**, 326-329.

Behrens, A., Jochum, W., Sibilia, M. and Wagner, E. F. (2000). Oncogenic transformation by ras and fos is mediated by c-Jun N-terminal phosphorylation. *Oncogene* **19**, 2657-2663.

Behrens, A., Sabapathy, K., Graef, I., Cleary, M., Crabtree, G. R. and Wagner, E. F. (2001). Jun N-terminal kinase 2 modulates thymocyte apoptosis and T cell activation through c-Jun and nuclear factor of activated T cell (NF-AT). *Proc. Natl. Acad. Sci. USA* **98**, 1769-1774.

Behrens, A., Sibilia, M., David, J. P., Mohle-Steinlein, U., Tronche, F., Schutz, G. and Wagner, E. F. (2002). Impaired postnatal hepatocyte proliferation and liver regeneration in mice lacking c-jun in the liver. *EMBO J.* **21**, 1782-1790.

Betz, U. A., Vosshenrich, C. A., Rajewsky, K. and Müller, W. (1996). Bypass of lethality with mosaic mice generated by Cre-loxP-mediated recombination. *Curr. Biol.* **6**, 1307-1316.

Bi, W., Huang, W., Whitworth, D. J., Deng, J. M., Zhang, Z., Behringer, R. R. and de Crombrugghe, B. (2001). Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization. *Proc. Natl. Acad. Sci. USA* **98**, 6698-6703.

Eferl, R., Sibilia, M., Hilberg, F., Fuchsbichler, A., Kufferath, I., Guertl, B., Zenz, R., Wagner, E. F. and Zatloukal, K. (1999). Functions of c-Jun in liver and heart development. *J. Cell Biol.* **145**, 1049-1061.

Fan, C. M. and Tessier-Lavigne, M. (1994). Patterning of mammalian somites by surface ectoderm and notochord: evidence for sclerotome induction by a hedgehog homolog. *Cell* **79**, 1175-1186.

Gossler, A. and Hrabe de Angelis, M. (1998). Somitogenesis. *Curr. Top. Dev. Biol.* **38**, 225-287.

Hai, T., Wolfgang, C. D., Marsee, D. K., Allen, A. E. and Sivaprasad, U. (1999). ATF3 and stress responses. *Gene Exp.* **7**, 321-335.

Haigh, J. J., Gerber, H., Ferrara, N. and Wagner, E. F. (2000). Conditional inactivation of VEGF-A in areas of collagen2a1 expression results in embryonic lethality in the heterozygous state. *Development* **127**, 1445-1453.

Hilberg, F., Aguzzi, A., Howells, N. and Wagner, E. F. (1993). c-jun is essential for normal mouse development and hepatogenesis. *Nature* **365**, 179-181.

Jochum, W., Passegue, E. and Wagner, E. F. (2001). AP-1 in mouse development and tumorigenesis. *Oncogene* **20**, 2401-2412.

Johnson, R. S., van Lingen, B., Papaioannou, V. E. and Spiegelmann, B. M. (1993). A null mutation at the c-*jun* locus causes embryonic lethality and retarded cell growth in culture. *Genes Dev.* **7**, 1309-1317.

Karin, M. and Hunter, T. (1995). Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr. Biol.* **5**, 747-757.

Koseki, H., Wallin, J., Wilting, J., Mizutani, Y., Kispert, A., Ebensperger, C., Herrmann, B. G., Christ, B. and Balling, R. (1993). A role for Pax-1 as a mediator of notochordal signals during the dorsoventral specification of vertebrae. *Development* **119**, 649-660.

Lettice, L. A., Purdie, L. A., Carlson, G. J., Kilanowski, F., Dorin, J. and Hill, R. E. (1999). The mouse bagpipe gene controls development of axial skeleton, skull, and spleen. *Proc. Natl. Acad. Sci. USA* **96**, 9695-700.

Maatman, R., Zachgo, J. and Gossler, A. (1997). The Danforth's short tail mutation acts cell autonomously in notochord cells and ventral hindgut endoderm. *Development* **124**, 4019-4028.

McGrew, M. J. and Pourquie, O. (1998). Somitogenesis: segmenting a vertebrate. *Curr. Opin. Genet. Dev.* **8**, 487-493.

Nagy, A., Moens, C., Ivanyi, E., Pawling, J., Gertsenstein, M., Hadjantonakis, A. K., Pirity, M. and Rossant, J. (1998). Dissecting the role of N-myc in development using a single targeting vector to generate a series of alleles. *Curr. Biol.* **8**, 661-664.

Otto, F., Thornell, A. P., Crompton, T., Denzel, A., Gilmour, K. C., Rosewell, I. R., Stamp, G. W., Beddington, R. S., Mundlos, S., Olsen, B. R. et al. (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* **89**, 765-771.

Passegue, E., Jochum, W., Behrens, A., Ricci, R. and Wagner, E. F. (2002). JunB can substitute for Jun in mouse development and cell proliferation. *Nat. Genet.* **30**, 158-166.

Peters, H., Wilm, B., Sakai, N., Imai, K., Maas, R. and Balling, R. (1999). Pax1 and Pax9 synergistically regulate vertebral column development. *Development* **126**, 5399-5408.

Pourquie, O., Coltey, M., Teillet, M. A., Ordahl, C. and le Douarin, N. M. (1993). Control of dorsoventral patterning of somitic derivatives by notochord and floor plate. *Proc. Natl. Acad. Sci. USA* **90**, 5242-5246.

Rufai, A., Benjamin, M. and Ralphs, J. R. (1995). The development of fibrocartilage in the rat intervertebral disc. *Anat. Embryol.* **192**, 53-62.

Summerbell, D. and Rigby, P. W. (2000). Transcriptional regulation during somitogenesis. *Curr. Top. Dev. Biol.* **48**, 301-318.

Theiler, K. (1988). Vertebral malformations. *Adv. Anat. Embryol. Cell Biol.* **112**, 1-99.

Tribioli, C. and Lufkin, T. (1999). The murine Bapx1 homeobox gene plays a critical role in embryonic development of the axial skeleton and spleen. *Development* **126**, 5699-5711.

Wagner, E. F. and Karsenty, G. (2001). Genetic control of skeletal development. *Curr. Opin. Genet. Dev.* **11**, 527-532.

Wallin, J., Wilting, J., Koseki, H., Fritsch, R., Christ, B. and Balling, R. (1994). The role of Pax-1 in axial skeleton development. *Development* **120**, 1109-1121.