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The Wnt antagonist Dickkopf-1 is regulated by Bmp signaling and c-Jun and modulates programmed cell death

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Dickkopf-1 (Dkk-1) has been shown to be a potent inhibitor of Wnt/β-catenin signaling in a variety of assays and organisms. In this study, we show that expression of *Dkk-1* overlaps significantly with the sites of programmed cell death in normal as well as mutant vertebrate limb development, and identify several of its upstream regulators, one of which is Bmp-4. Interestingly, Bmp-4 only activates *Dkk-1* when it concomitantly induces apoptosis. Moreover, *Dkk-1* is heavily up-regulated by UV irradiation and several other genotoxic stimuli. We further show that normal expression of *Dkk-1* is dependent on the Ap-1 family member c-*Jun* and that overexpression of *Dkk-1* enhances Bmp-triggered apoptosis in the vertebrate limb. Taken together, our results provide evidence for an important role of Dkk-1-mediated inhibition of Wnt/β-catenin signaling in response to different stress signals that all converge on the activation of c-*Jun in vivo*.

Keywords: apoptosis/Bmp/c-Jun/Dkk-1/limb development

Introduction

Secreted glycoproteins of the Wnt family of signaling molecules are important regulators of cell growth and survival. Wnts are expressed in a tissue-specific manner, and mice harboring mutations in genes, the products of which are involved in Wnt signal transduction, show severe defects in the development of a variety of organs (for a review see Cadigan and Nusse, 1997). Wnts bind to a receptor complex consisting of a member of the Frizzled family of seven transmembrane proteins and the LDL-receptor-related proteins Lrp-5 or Lrp-6 (for a review see Bejsovec, 2000). In the canonical Wnt/β-catenin pathway, receptor activation leads to a stabilization of β-catenin, which accumulates and translocates into the nucleus to activate target gene expression in concert with transcription factors such as Tcf and Lef. Recently, a biochemical link between the receptor complex and the cytoplasmic components that rapidly degrade β-catenin in the absence of a Wnt signal has been established by the finding that Lrp-5 binds to Axin (J.Mao *et al*., 2001). The ability of many Wnts to stabilize β-catenin seems to be the basis for their proliferation-promoting effect. This proto-oncogenic potential is reflected in frequently found mutations in components of the Wnt pathway in human cancers (for a review see Polakis, 2000). Dickkopf-1 (Dkk-1) is a secreted protein that specifically inhibits Wnt/β-catenin signaling by interacting with the co-receptor Lrp-6 (B.Mao *et al*., 2001; Zorn, 2001). Dkk-1 was originally identified as a strong head inducer in *Xenopus*, due to its potent anti-Wnt effect (Glinka *et al*., 1998). Analyses carried out in zebrafish and chick embryos showed that the Wnt-antagonizing function of Dkk-1 is conserved in other vertebrates (Shinya *et al*., 2000; Marvin *et al*., 2001). However, less is known about the regulation of *Dkk-1* expression and possible functions outside head development.

Vertebrate limb development provides a paradigm for developmental apoptosis. Programmed cell death (PCD) occurs in well-defined domains and sculpts the shape of the limb by eliminating cells between the differentiating cartilages (Hurle *et al*., 1996). In the early chicken limb bud, the most prominent sites of apoptosis are located in the anterior (ANZ) and posterior (PNZ) necrotic zones and in the apical ectodermal ridge (AER). Later, massive cell death takes place in the mesodermal web (interdigital necrotic zone, INZ) separating the digits. The pattern of PCD is very similar in mouse limb development, but ANZ and PNZ are less pronounced compared with in chick. Bone morphogenetic proteins (Bmp) have been identified as important signals triggering cell death in these zones (Yokouchi *et al*., 1996; Pizette and Niswander, 1999). In sharp contrast, Bmps also promote the formation of bone (Duprez *et al*., 1996; Buckland *et al*., 1998). These opposing activities reside in close vicinity to each other in the developing limb, namely in the interdigits versus the digital rays. The downstream mechanisms exerting this dual function are poorly understood. Bmps, as members of the transforming growth factor (TGF)-β superfamily, transmit their signal via at least two distinct pathways. One involves Smad-1, -5 or -8, which are phosphorylated by activated type I Bmp-2/4 receptors and then associate with a common signaling mediator, Smad-4. The heteromeric complex translocates into the nucleus and activates target genes together with different co-factors (for a review see Massagué *et al*., 2000). A mitogen-activated protein kinase (MAPK) cascade represents an alternative way for Bmp signal transduction. Several MAPKs like, for example, Jnk can be activated depending on cell type and experimental conditions (for a review see Massagué *et al*., 2000). The Jnk protein kinases phosporylate serine residues 63 and 73 of the c-Jun activation domain leading to increased Ap-1 transcriptional activity (Derijard *et al*., 1994; Kyriakis *et al*., 1994). Accumulating evidence suggests that the MAPKKK family member TAK-1 provides the biochemical link between the TGF-β receptor and the MAPK pathway (Takatsu *et al*., 2000). Smads can interact *in vitro* with the Jnk substrate c-Jun, indicating that Bmps might simultanously activate the Smad and

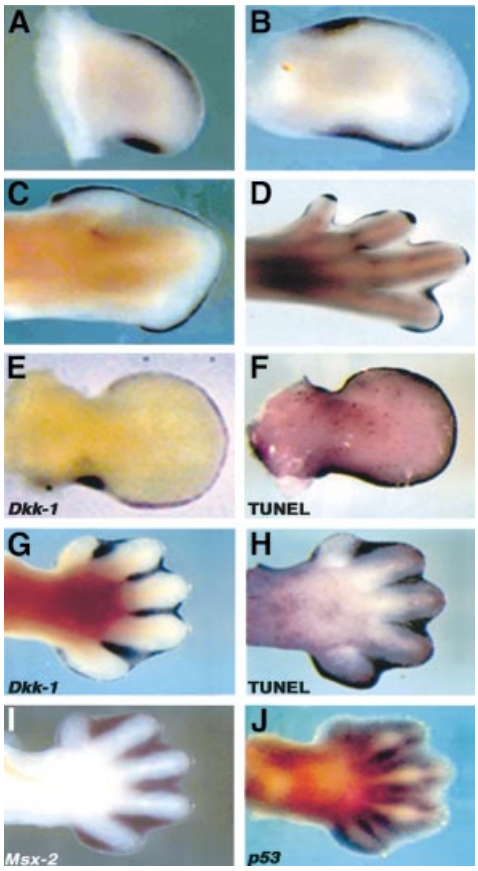

Fig. 1. *Dkk-1* is expressed at sites of apoptosis during vertebrate limb development. (A–D) Chick limb buds of different stages, (**A**) HH23 wing, (**B**) HH25 wing, (**C**) HH32 wing and (**D**) HH32 leg, show *Dkk-1* transcripts in the ANZ, PNZ, the AER and INZ and the developing joints. (**E**) *Dkk-1* expression in an E11.5 mouse forelimb bud in the AER and posterior mesenchyme and (**F**) whole-mount TUNEL staining of an age-matched forelimb bud indicates the co-localization of *Dkk-1* transcripts and the sites of apoptosis. (**G**) At E13.5, *Dkk-1* expression is confined to the interdigital mesenchyme, where massive apoptosis takes place as the TUNEL staining of an E13.5 limb bud indicates (**H**). At this time point *Dkk-1* is co-expressed with the pro-apoptotic genes *Msx-2* (**I**) and *p53* (**J**).

MAPK pathways, which then physically converge on target genes (Zhang *et al*., 1998; Wong *et al*., 1999). Recent evidence suggests that these two pathways can also counteract each other (Kimura *et al*., 2000; Pessah *et al*., 2001), raising the possibility that the balance of these two intracellular pathways is a key to co-ordinated cellular response to Bmp in the physiological context.

We have previously shown that *Dkk-1* is expressed in a dynamic pattern during mouse limb development (Grotewold *et al*., 1999). Here, we show that these expression domains significantly overlap with the sites of PCD, indicating a potential function of *Dkk-1* in this process. Another implication for *Dkk-1* in PCD comes from a recent study providing evidence for *Dkk-1* being a target of p53, a checkpoint protein controling cell cycle progression and apoptosis (Wang *et al*., 2000). Therefore, we were interested to determine whether Dkk-1 might be involved in controling PCD in development. Furthermore, we asked whether signals triggering apoptosis, like Bmp signaling, are involved in the regulation of *Dkk-1* expression.

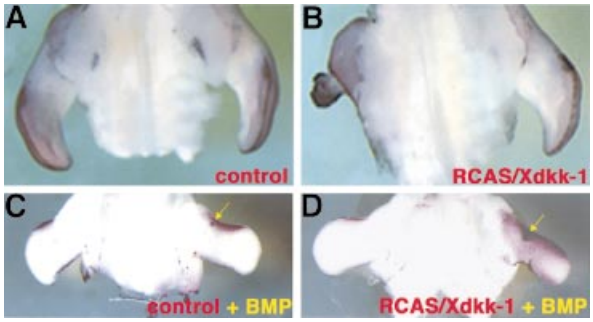

Fig. 2. *Dkk-1* promotes apoptosis in limb buds. (A and B) TUNEL stainings of RCAS-infected wing buds, ventral view. (**A**) Control wing infected with RCAS/AP showing the normal pattern of PCD in the ANZ and AER. (**B**) Dramatic truncation of a wing bud infected with RCAS/Xdkk-1. Note the massive apoptosis in the truncated wing compared with the contralateral control wing. (C and D) Dorsal views of wing buds infected with RCAS/AP (C) and RCAS/Xdkk-1 (D), which additionally received a bead soaked in rhBMP-4 (arrows). (**C**) Twenty hours after bead implantation the ectopic Bmp signal leads to an increased PCD in a region surrounding the bead as shown by TUNEL. (**D**) The overexpression of *Dkk-1* dramatically enhances this effect of Bmp. The region of cells undergoing PCD covers nearly the complete wing mesoderm.

Results

Expression pattern of Dkk-1

We have recently described the dynamic expression pattern of *Dkk-1* in mouse limb development (Grotewold *et al*., 1999). We extended this expression study to the chicken embryo and observed a very similar pattern. At HH23, *Dkk-1* is expressed in a posterior and an anterior mesenchymal domain, as it is at HH25 (Figure 1A and B). At later stages it starts to be expressed in the AER and weakly in the interdigital mesenchyme (Figure 1C and D). At HH32 transcripts can also be detected in the developing joints (Figure 1D). These sites of expression overlap to a high degree with the sites of PCD, as exemplified in Figure 1E–H for mouse limb buds (data not shown for the chick). At embryonic day (E) 11.5, *Dkk-1* expression is confined to the AER and a posterior mesenchymal domain (Figure 1E) corresponding to the PNZ. TUNEL staining of an age-matched limb bud reveals that these areas undergo massive PCD at this time point (Figure 1F). A similar coincidence is observed at E13.5 (Figure 1G and H). In the interdigital mesenchyme, *Dkk-1* is also co-expressed with the pro-apoptotic genes *Msx-2* (Figure 1I), which has been suggested to mediate Bmp-triggered PCD (Marazzi *et al*., 1997), and *p53* (Figure 1J).

Dkk-1 promotes apoptosis in limb buds

Given the correlation between *Dkk-1* expression and the sites of PCD, we wanted to investigate whether overexpression of *Dkk-1* might be sufficient to induce apoptosis. To this end, we injected a recombinant retrovirus containing the *Xenopus Dkk-1* coding sequence (RCAS/Xdkk-1) into the mesoderm of HH18 wing buds. Viral spread was monitored by whole-mount *in situ* hybridization using a probe that detects viral message. Thirty-six hours after infection we observed viral transcripts in the whole wing mesoderm (data not shown). After 60–72 h, distal truncations of variable degree could be observed (42% of injected embryos, Figure 2B shows a dramatically truncated wing bud representing 29% of

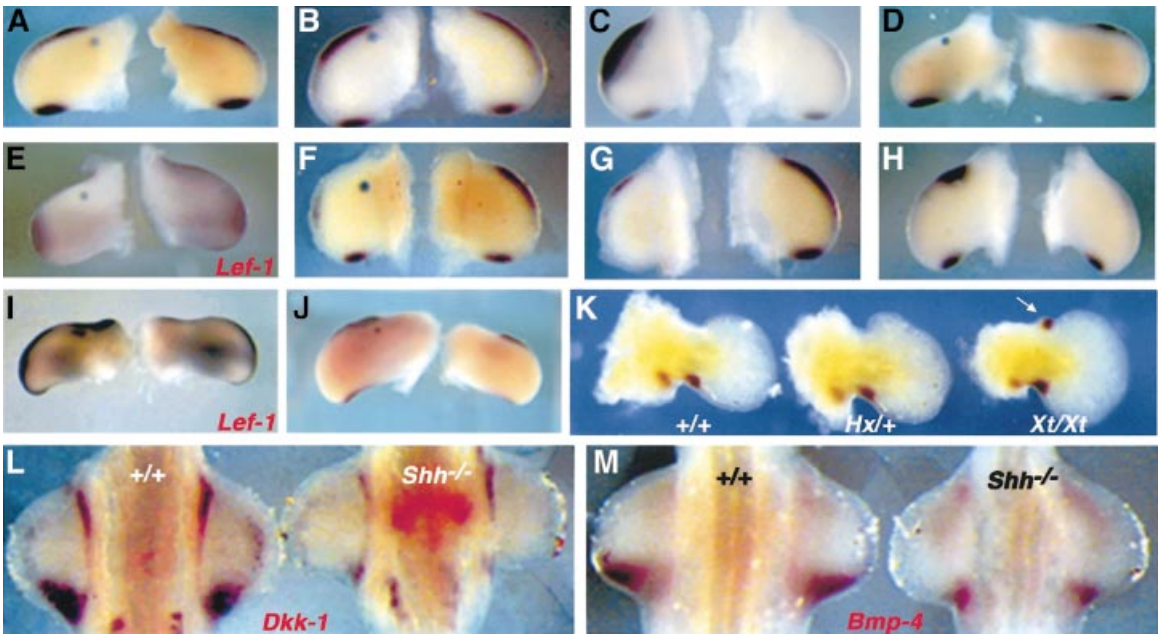

Fig. 3. Regulation of *Dkk-1* by Bmp, Fgf and Shh. Surgical manipulations of wing buds in (A)–(K) were performed between HH20 and HH23. Operated wings (left) and contralateral controls (right) are presented in each panel. (**A**) A BSA-soaked control bead or (**B**) a bead soaked in 1 µg/ml rhBMP-4 does not affect *Dkk-1* expression after 8 h. (**C**) Massive ectopic expression of *Dkk-1* around a Bmp bead (100 µg/ml) 2 h post-implantation. Note that *Dkk-1* is only induced between the bead and the AER. (**D**) Twenty hours after application of 100 µg/ml rhBMP-4, *Dkk-1* expression is completely down-regulated. (**E**) *Lef-1* expression is drastically reduced 8 h after the same treatment. (**F**) Loss of *Dkk-1* transcripts in the vicinity of a bead soaked in 1 mg/ml Noggin after 8 h. (**G**) Removal of the AER leads to a decline of *Dkk-1* transcripts in the subjacent mesenchyme after 6 h. (**H**) *Dkk-1* is induced by 1 mg/ml Fgf-8 (8 h). (**I**) *Lef-1* expression is also clearly enhanced following this treatment. (**J**) Shh (2.5 mg/ml) has no effect on *Dkk-1* expression, even after 24 h. Note the increased size of the operated wing, which indicates that the Shh protein provided by the bead is functional. (**K**) *Dkk-1* expression in E12.0 mouse forelimb buds. Wild type (left) and *Hx*/+ (middle) show the same pattern, whereas a strong ectopic expression domain of *Dkk-1* is detectable in the anterior mesenchyme of *Xt*/*Xt* (right) limb buds (arrow). (**L**) *Dkk-1* expression is drastically reduced in $Shh^{-/-}$ forelimb buds (right) at E10.5 (left side shows an age-matched wild-type control). (**M**) *Bmp-4* is also down-regulated in $Shh^{-/-}$ limb buds (right) compared with wild type (left) at E10.5.

infected limbs) in the infected wings. TUNEL staining revealed massive ectopic PCD in the distal part of the truncated limbs (Figure 2B). As a control, we infected early wing buds with a virus expressing human *alkaline phosphatase* (RCAS/AP). Normal wing buds developed in 100% of these embryos, which also showed the normal pattern of PCD 60–72 h post-infection (Figure 2A). This shows that the observed phenotype after RCAS/Xdkk-1 infection is not due to non-specific cytotoxic effects of the virus.

Overexpression of RCAS/Xdkk-1 resulted in only a very limited increase in TUNEL-positive cells before the drastic truncations occured (data not shown). Thus, we could not exclude the possibility that the massive PCD in these specimens (Figure 2B) occured secondary to the truncations. In order to address the question of whether *Dkk-1* modulates PCD, we used another strategy. Implantation of beads soaked in Bmp protein into the undifferentiated limb mesoderm induces PCD within 20 h in a region surrounding the bead (Figure 2C; Macias *et al*., 1997; Zou *et al*., 1997; Pizette and Niswander, 1999). We asked whether the overexpression of *Dkk-1* would have an effect on the extend of cell death induced by Bmp. For this purpose, wing buds were infected with RCAS/Xdkk-1 or RCAS/AP at HH18. Twenty-four hours later, we implanted a bead soaked in 100 µg/ml recombinant human BMP-4 (rhBMP-4) and analyzed the extent of PCD a further 20 h later by TUNEL staining. As shown in Figure 2D, the ectopic expression of *Dkk-1* prior to the ectopic Bmp signal significantly enhanced the PCD-inducing effect of Bmp.

These results present strong evidence that *Dkk-1* promotes PCD in the developing vertebrate limb.

Regulation of Dkk-1 by Bmp, Fgf and Shh

As the expression domains of *Dkk-1* are remarkably similar to those of some members of the *Bmp* family and their target genes (Figure 1I) and *Dkk-1* enhances the PCD-triggering effect of Bmp-4 in the vertebrate limb, we started to analyze the effects of enhanced Bmp signaling on *Dkk-1* transcription. For this purpose, we implanted beads soaked in rhBMP-4 into the mesenchyme of HH20–23 limb buds. Control bovine serum albumin (BSA) beads and beads soaked in 1 µg/ml rhBMP-4 did not affect *Dkk-1* expression levels (n = 8/8 and 5/5, respectively; Figure 3A and B). When we increased the rhBMP-4 concentration to 100 µg/ml, massive ectopic *Dkk-1* expression was induced within 2 h (n = 6/6; Figure 3C), which was still evident after 8 h (n = 8/8; data not shown). To investigate whether this massive up-regulation leads to an enhanced inhibition of Wnt/β-catenin signaling, we monitored the expression of the Wnt target gene *Lef-1*. Indeed, *Lef-1* was drastically reduced in a wide area around the bead after 8 h (n = 6/6; Figure 3E). In order to analyze whether Bmp signaling is necessary for *Dkk-1* expression, we implanted beads soaked in Noggin

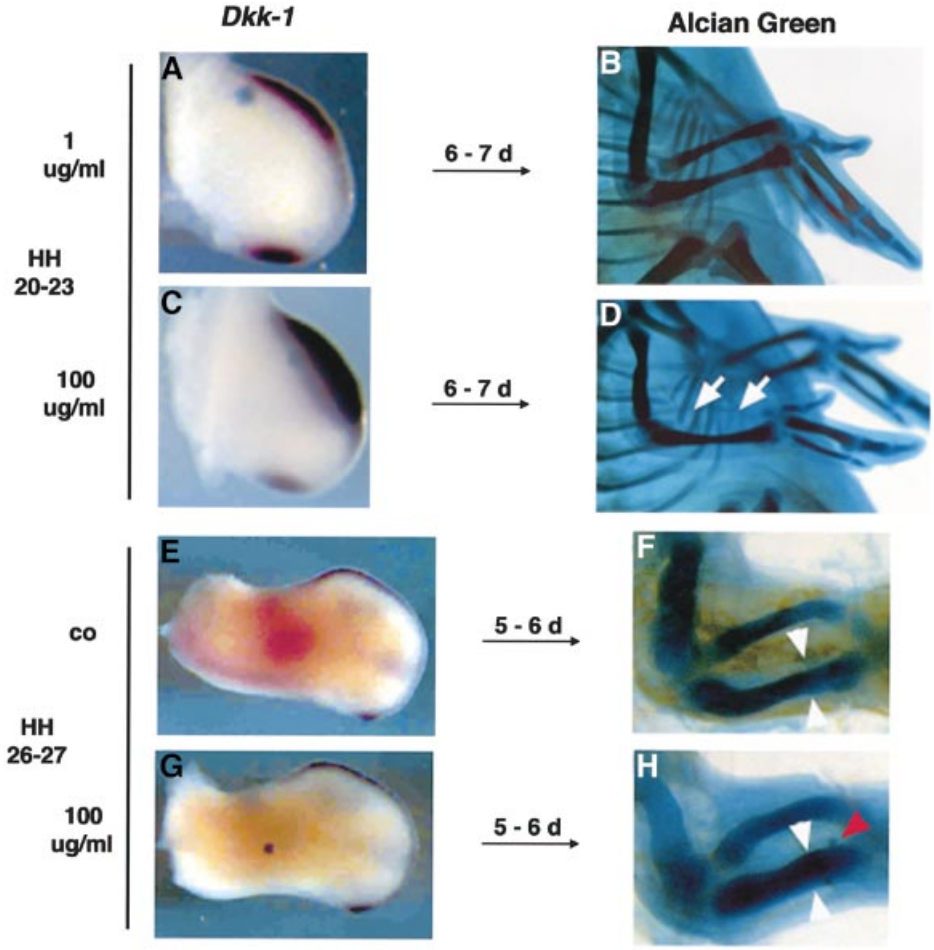

Fig. 4. Bmp-4 concomitantly induces *Dkk-1* expression and apoptosis. The bead implantations in (A)–(D) have been carried out between HH20 and HH23, and those in (E)–(H) at HH26/27. (**A**) A control bead soaked in 1 µg/ml rhBMP-4 does not affect *Dkk-1* expression. (**B**) Wild-type skeletal pattern of a wing treated the manner described in (A) after 10 days of incubation. (**C**) rhBMP-4 100 µg/ml rapidly induces *Dkk-1* expression, resulting in the complete absence of the radius after 10 days [(**D**), arrows)]. (**E**) Control (co) wing shows the wild-type pattern of *Dkk-1* expression at HH26. (**F**) Wild-type size of radius and ulna in an E10 embryo. (**G**) A Bmp bead (100 µg/ml) implanted into the center of a HH26/27 wing does not induce *Dkk-1* expression after 8 h. (**H**) This treatment leads to a significant increase in bone mass of radius and ulna after 10 days. White arrowheads have the same distance as in (F), the red arrowhead points to the implanted bead.

(1 mg/ml), a potent Bmp antagonist, into the anterior mesenchyme of HH20–23 wing buds. Six, 8 and 15 h after this treatment we observed a complete loss of the endogenous *Dkk-1* expression domain in vicinity to the bead (n = 16/16; shown for 8 h in Figure 3F). Only at a distance from the bead was a faint staining for *Dkk-1* transcripts detectable in the very distal mesenchyme. Thus, Bmp signaling is essential for normal *Dkk-1* expression. Twenty hours after implantation of a bead soaked in 100 µg/ml rhBMP-4, *Dkk-1* transcription was completely abolished or drastically reduced at the implanted site (n = 7/8; Figure 3D). As this treatment induces massive ectopic cell death after 20 h (see Figure 2C), loss of these cells might provide an explanation for this down-regulation. Alternatively, this might indicate that *Dkk-1* expression depends on signals from the AER, the structure and function of which has been shown to be negatively regulated by Bmp under these experimental conditions (Pizette and Niswander, 1999). In order to test for the latter possibility, the AER was surgically removed from HH20–23 limb buds. This manipulation resulted in a severe decrease of *Dkk-1* transcripts in the underlying mesoderm within 6 h (n = 6/6; Figure 3G). As members of the fibroblast growth factor (Fgf) family are regarded as the main mediators of AER function, we implanted beads soaked in Fgf-8 protein to analyze whether this is sufficient to induce *Dkk-1* expression. We found that *Dkk-1* is up-regulated at 8 and 20 h after the surgery (n = 15/15; Figure 3H; data not shown). In spite of the forced *Dkk-1* expression, ectopic Fgf did not result in an inhibition but rather in an activation of Wnt/β-catenin signaling as judged by enhanced *Lef-1* transcription (n = 8/8; Figure 3I). These results show that *Dkk-1* is positively regulated by Bmp and Fgf signaling.

Shh is expressed in the posterior mesenchyme of the limb bud and is essential for proper limb development (Chiang *et al*., 1996). A recent study also implicated Shh in the regulation of PCD (Sanz-Ezquerro and Tickle, 2000). As the expression domains of *Dkk-1* and *Shh* initially overlap and then become separated (Grotewold *et al*., 1999), we wanted to investigate possible effects of this signaling molecule on *Dkk-1* mRNA levels. Beads

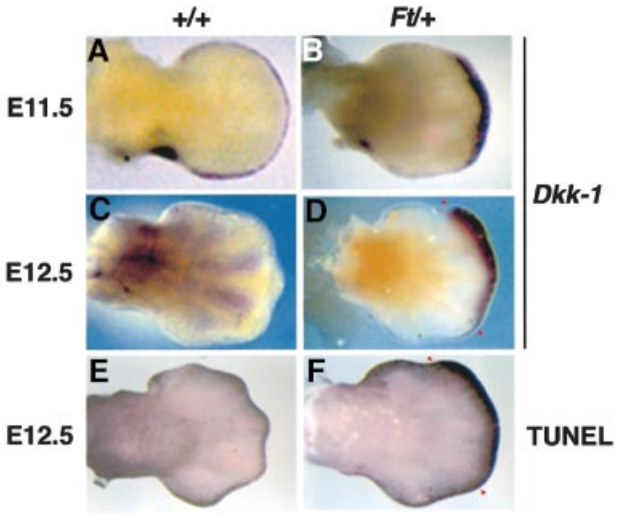

Fig. 5. *Dkk-1* expression and PCD in *Ft*/+ forelimb buds. (**A**) E11.5 wild-type limb bud shows *Dkk-1* transcripts in the PNZ and AER. (**B**) Ectopic expression of *Dkk-1* in the distal-most mesenchyme of an E11.5 *Ft*/+ limb bud. (**C**) *Dkk-1* expression in the interdigital mesenchyme of an E12.5 wild-type limb bud. (**D**) *Dkk*-1 is ectopically activated at E12.5 in *Ft*/+ forelimb buds (red arrowheads). (**E**) TUNEL staining shows the wild-type pattern of PCD at E12.5. Apoptotic cells are restricted to the AER and the distal-most interdigital mesenchyme. (**F**) High ectopic cell death in *Ft*/+ forelimb bud (red arrowheads) in a region that significantly overlaps with that of ectopic *Dkk-1* transcription [compare with (D)].

soaked in 2.5 mg/ml Shh failed to induce ectopic activation of *Dkk-1* at all time points investigated (4 h, *n* = 5/5; 10 h, *n* = 4/4; 24 h, *n* = 5/5; Figure 3J). Thus, Shh seems not to be sufficient to activate the *Dkk-1* promoter. This finding in the chick is consistent with data we obtained from expression analyses in two different polydactylous mouse mutants, *Extra-toes* (*Xt*) and *Hemimelic extratoe* (*Hx*). Although *Shh* is strongly expressed in the anterior mesenchyme of *Hx*/+ limb buds (Büscher and Rüther, 1998), no ectopic activation of *Dkk-1* could be observed at all developmental stages examined (E11.0–12.5; Figure 3K). Thus, also in the mouse, ectopic *Shh* expression is not sufficient to induce *Dkk-1* expression. In the anterior mesenchyme of *Xt*/*Xt* forelimb buds, however, *Dkk-1* is ectopically expressed at E12.0 (Figure 3K). In order to analyze whether Shh is necessary for the normal expression of *Dkk-1*, we performed *in situ* hybridizations on $Shh^{-/-}$ embryos at E10.5. Figure 3L shows that *Dkk-1* is expressed in those embryos, albeit at a dramatically decreased level. This shows that Shh is not required for the initial induction of *Dkk-1*, but is for the maintenance of *Dkk-1* expression. In addition, *Bmp-4* is drastically down-regulated in $Shh^{-/-}$ mutant embryos (Figure 3M). These results uncover a complex regulation of *Dkk-1* in the molecular network of limb development.

Bmp concomitantly induces Dkk-1 expression and apoptosis

As shown in Figure 2C, a Bmp-bead implanted into the undifferentiated mesenchyme between HH20 and HH23 induces massive apoptosis within 20 h. Some embryos treated this way were allowed to develop until E9–10 to observe the morphological effects of increased cell death. Figure 4D shows that a complete loss of the radius could be detected in all of these embryos (*n* = 8/8). The increased cell death is preceeded by a dramatic up-regulation of *Dkk-1* in the same region (Figure 4C). As shown previously, implantation of a Bmp bead of the same concentration at HH26–27 leads to the completely opposite phenotype, namely an increase in bone mass (Buckland *et al*., 1998). This was also the case in our experiments (*n* = 9/12; compare Figure 4F with H). Importantly, in none of these cases (*n* = 12/12) could an up-regulation of *Dkk-1* expression be observed at 4–10 h around the bead (Figure 4G). This suggests that the concomitant activation of Bmp and the inhibition of Wnt/β-catenin signaling leads to apoptosis in limb mesodermal cells. Our laboratory has previously characterized the syndactylous mouse mutant *Fused toes* (*Ft*) heterozygous embryos, which exhibit ectopic activation of the Bmp signaling pathway in the anterior/distal mesenchyme of the limb (Heymer and Rüther, 1999). We were interested in whether this physiological expression of *Bmps* also influenced the expression of *Dkk-1* and the extent of PCD. Remarkably, at E11.5–12.0, when the ectopic Bmp activation can be first observed, *Dkk-1* is ectopically expressed in the distal mesenchyme of *Ft*/+ forelimb buds (Figure 5B). At this timepoint, no ectopic cell death could be observed by whole-mount TUNEL staining (data not shown). At E12.5, when *Dkk-1* is expressed in the interdigital mesenchyme in the wild type (Figure 5C), *Dkk-1* is still ectopically expressed in the region of the future fusion of the digits in *Ft*/+ embryos (Figure 5D). At this point, massive ectopic cell death could be detected in the *Dkk-1*-expressing region (compare Figure 5D with F). Thus, during normal as well as mutant limb development, *Dkk-1* is expressed in regions of high Bmp signaling and apoptosis. Strikingly, *Dkk-1* expression preceeds the appearance of apoptosis and is not induced by a Bmp signal that leads to enhanced chondrogenesis. This implies an important function of Dkk-1 in the modulation of Bmp-induced PCD.

Dkk-1 is induced by several genotoxic signals

We were interested in whether *Dkk-1* might also be activated by other apoptotic stimuli as *Dkk-1* has recently been suggested to be a target of the tumor-suppressor gene *p53*, one of the most important regulators of PCD (Wang *et al*., 2000). Therefore, we exposed chick embryos of different developmental stages to ultraviolet (UV) light as UV-induced DNA damage results in a stabilization of p53, which then activates the cell death or cell cycle arrest program (for a review see Vogelstein *et al*., 2000). Figure 6A shows that this treatment induces massive cell death after 10 h (*n* = 16/16) in the irradiated territories as judged by Nile Blue staining. When we looked for *Dkk-1* expression in these irradiated embryos, we observed high ectopic expression of the gene in a salt-and-pepper-like fashion in the whole mesenchyme as well as ectoderm (*n* = 12/12 after 8 h; Figure 6B), which closely resembled the pattern of apoptosis (cf. Figure 6A). Thus, *Dkk-1* is induced by the UV-activated cell death pathway *in vivo*. As mentioned above, Bmp-4 is a strong inducer of *Dkk-1* in apoptotic responses. Therefore, we wished to investigate whether the UV-induced *Dkk-1* activation might be mediated by Bmp-4 or whether the two pathways are rather independent. Figure 6C shows that *Bmp-4* is

normally expressed in irradiated chick embryos at various time points after the treatment (1 h, $n = 3/3$; 4 h, $n = 5/5$; 8 h, $n = 8/8$; 16 h, $n = 6/6$). In the next set of experiments, we implanted beads soaked in staurosporin, a protein kinase inhibitor that has been shown to induce apoptosis when delivered to the chick limb bud (Sanz-Ezquerro and Tickle, 2000). When we monitored *Dkk-1* expression following this treatment, we found a strong up-regulation of its expression 2 h ($n = 5/5$; Figure 6D) and 4 h ($n = 6/6$; Figure 6E) after bead implantation. Thus, we conclude that several different apoptotic stimuli lead to an up-regulation of *Dkk-1* expression.

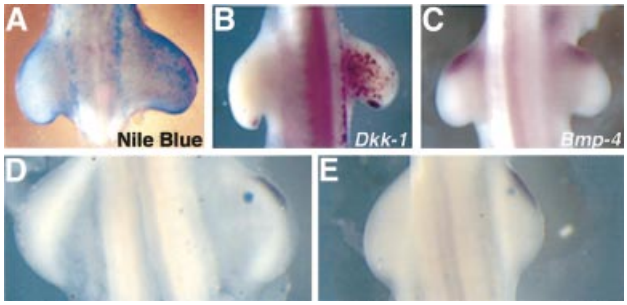

Fig. 6. *Dkk-1* is induced by UV irradiation and staurosporin. (**A**) Nile Blue staining of a chick embryo 10 h after UV irradiation. Note the massive cell death in the right irradiated limb bud. The non-irradiated left side served as an internal control in all of these experiments. (**B**) *Dkk-1* is ectopically expressed in a salt-and-pepper-like fashion 8 h after UV irradiation. (**C**) *Bmp-4* expression is not altered after 8 h. (D and E) Beads soaked in staurosporin induce *Dkk-1* expression after 2 h (**D**) and 4 h (**E**).

Dkk-1 is regulated by c-Jun

As very different stress stimuli lead to the induction of *Dkk-1*, we sought to determine whether these signals might activate a common transcription factor to induce *Dkk-1*. A good candidate for such a role was c-*Jun*, which has been shown to be required for UV-induced apoptosis (Shaulian *et al*., 2000). In addition, staurosporin was suggested to induce apoptosis via Jnk-mediated increase in Ap-1 activity (Chae *et al*., 2000). We performed *in situ* hybridizations on UV-irradiated chicks and found a massive up-regulation of chicken c-*Jun* after 1 h ($n = 9/9$; Figure 7A). This up-regulation was transient as it was no longer detectable after 4 h (data not shown). Next we were interested in determining whether Bmp can activate c-*Jun* expression under conditions where *Dkk-1* is also induced. We could not test this by bead implantations because c-*Jun* is induced by embryonic wounding (L.Grotewold, unpublished observation); therefore, we decided to switch to a cell culture assay. We applied rhBMP-4 to mouse embryonic fibroblasts (MEF) and could strongly induce *Dkk-1* expression after 30 min, as determined by RT–PCR (Figure 7B). Under these conditions, c-*Jun* was also strongly induced (Figure 7B). c-*Jun* has been shown to be regulated by serum factors and induced by several

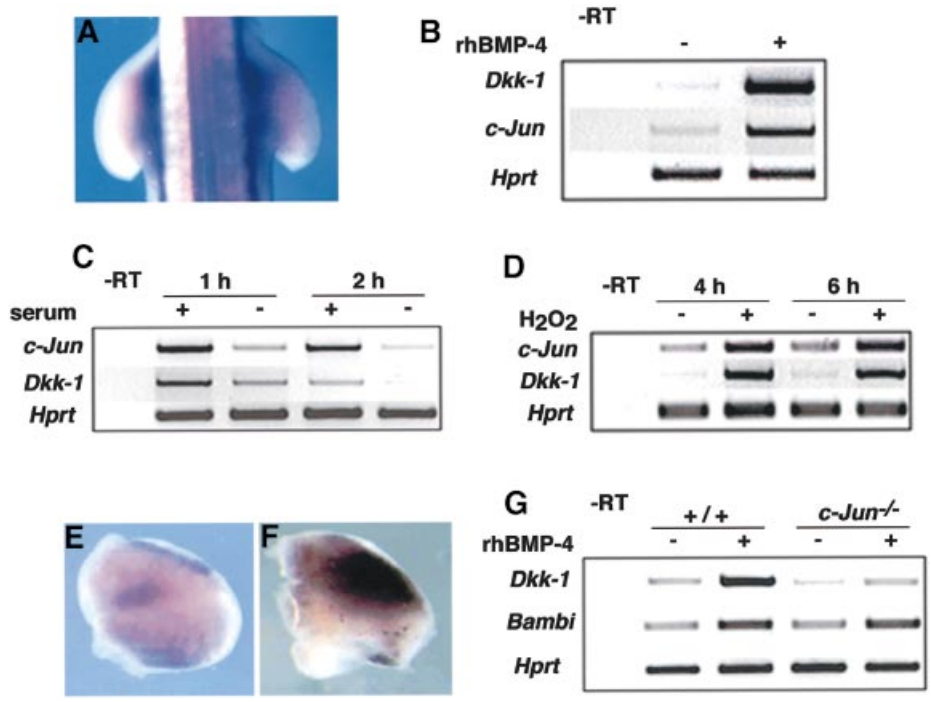

Fig. 7. *Dkk-1* is regulated by c-Jun. (**A**) c-*Jun* is up-regulated 1 h after UV irradiation. Note the intense c-*Jun* expression in the right wing bud and the flank compared with the non-irradiated left side of the embryo. (**B**) *Dkk-1* and c-*Jun* are co-induced by application of rhBMP-4 to MEF after 30 min. *Hprt* was used for standardization in all RT–PCR experiments. (**C**) c-*Jun* and *Dkk-1* are down-regulated in MEF, 1 and 2 h after serum withdrawal. (**D**) H_2O_2 strongly up-regulates c-*Jun* and *Dkk-1*. (E and F) Limb bud explants electroporated with a control vector (**E**) and a c-*Jun* expression plasmid (**F**). Note the intense up-regulation of *Dkk-1* expression 6 h after electroporation of the c-*Jun* expression vector (F). (**G**) *Dkk-1* expression is reduced by 40% in c-$Jun^{-/-}$ MEF compared with wild type (compare +/+ and c-$Jun^{-/-}$ without rhBMP-4). After the application of rhBMP-4 *Dkk-1* is 5.2-fold induced in wild-type MEF, but only 1.7-fold in c-$Jun^{-/-}$ cells. *Bambi* is normally expressed and induced by the same factor in c-$Jun^{-/-}$ as in wild-type MEF. –RT, control without reverse transcriptase.

different stress stimuli. If *Dkk-1* was regulated by c-*Jun* we should expect a concomitant switch of *Dkk-1* expression under the respective conditions. Figure 7C shows that c-*Jun* and *Dkk-1* expression are rapidly down-regulated after serum withdrawal, indicating that *Dkk-1* expression depends on serum factors and/or c-*Jun* expression. Apoptosis triggered by oxidative stress involves c-*Jun* induction in different cell types (Janssen *et al*., 1997; Richter-Landsberg and Vollgraf, 1998). We applied H_2O_2 to MEF and observed an induction of c-*Jun* and *Dkk-1* expression (Figure 7D). These results show that c-*Jun* and *Dkk-1* are co-regulated under several experimental conditions. The question emerged whether c-*Jun* might be sufficient to induce *Dkk-1* expression. To analyze this, we used the *ex vivo* electroporation system to deliver a c-*Jun* expression plasmid to the limb bud. After 6 h in culture, the explants were processed for *in situ* hybridization. Whereas the endogenous expression pattern of *Dkk-1* was nicely recapitulated in the control limb buds, those electroporated with a c-*Jun* expression vector expressed *Dkk-1* at high levels at ectopic sites (Figure 7E and F). This clearly indicates that c-*Jun* is able to induce *Dkk-1* expression *in vivo*. To analyze whether c-Jun might also be essential for *Dkk-1* expression and the Bmp-induced *Dkk-1* up-regulation, we applied rhBMP-4 to MEF derived from either wild-type or c-*Jun*$^{-/-}$ embryos. Figure 7G shows that the endogenous expression of *Dkk-1* (without application of rhBMP) was already severely reduced in c-*Jun*$^{-/-}$ cells. When rhBMP-4 was added to the cultures, *Dkk-1* was up-regulated 5.2-fold after 4 h in wild-type MEF. In c-*Jun*$^{-/-}$ MEF, *Dkk-1* was still induced by rhBMP-4, albeit at much reduced levels (only by a factor of 1.7 compared with c-*Jun*$^{-/-}$ without rhBMP-4; Figure 7G). Another gene that we have previously shown to be inducible by rhBMP-4 in this system is *Bambi* (Grotewold *et al*., 2001). The basal level of *Bambi* expression and its induction by rhBMP-4 are unaltered in c-*Jun*$^{-/-}$ MEF (Figure 7G), demonstrating that these cells do not harbor a general defect in Bmp signal transduction. These results show that c-*Jun* is indispensable for the normal expression of *Dkk-1* as well as for the full induction of *Dkk-1* transcription in response to Bmp-4.

Discussion

Expression of Dkk-1 at sites of PCD

We show that during mouse and chicken limb development the sites of *Dkk-1* expression significantly overlap with the areas of PCD. We found this co-localization in the early phase of limb development, where the most prominent apoptotic activity resides in the ANZ, PNZ and in the AER, and during the growth and differentiation phase, when the interdigital mesenchyme undergoes extensive PCD. Here, *Dkk-1* expression overlaps with the expression of the pro-apoptotic genes *Bmp-4*, *Msx-2*, *p53* and also c-*Jun*. In the late limb bud, the developing joints represent another prominent site of co-expression of *Dkk-1* and c-*Jun*. Interestingly, the domains of *Bmp-4*, *Dkk-1* and c-*Jun* also overlap at other sites in the embryo, including the otic vesicle and branchial arches, where apoptosis plays a pivotal role to elaborate the shape of the corresponding structure (Wilkinson *et al*., 1989; Sanz *et al*., 1999; data not shown). This indicates that the molecular cascade for Bmp-triggered apoptosis we propose (see below) is not limited to the developing limb but might be of general relevance during embryonic development.

Interaction of Bmp, Fgf and Wnt: the role of Dkk-1

Besides the Bmp, Fgf and Wnt pathways, Shh is also crucial for patterning the vertebrate limb. Our results from bead implantations in the chick embryo as well as from gene expression analyses in different mouse mutants, however, show that Shh plays only a minor role in *Dkk-1* expression. The up-regulation of *Dkk-1* expression in *Xt/Xt* limb buds preceeds the ectopic activation of *Shh*, as it can already be detected at E10.5 (data not shown), i.e. before the ectopic *Shh* domain emerges (Büscher and Rüther, 1998). Thus, the up-regulation of *Dkk-1* in *Xt/Xt* limb buds is independent of *Shh*. Shh is neither sufficient nor necessary to activate *Dkk-1*; however, it seems to be needed for the maintenance of *Dkk-1* transcription. This effect of Shh on *Dkk-1* is likely to be mediated by Bmp-4, which is down-regulated in *Shh*$^{-/-}$ limb buds, as we show here that *Dkk-1* is rapidly induced by Bmp *in vivo*. Application of a bead soaked in Bmp to the undifferentiated mesenchyme induces *Dkk-1* within 2 h and thereby leads to a subsequent down-regulation of the Wnt/β-catenin target gene *Lef-1*. In the corresponding region, massive PCD is induced, resulting in the loss of the radius. In addition, Mukhopadhyay *et al*. (2001) recently reported the activation of *Dkk-1* transcription by BMP-2. Further evidence implicating an important endogenous function of Dkk-1 in modulating Bmp-triggered apoptosis comes from its ectopic expression and the ectopic PCD in *Ft*/+ mutant limb buds.

Dkk-1 also underlies positive regulation by Fgf signals from the AER. Fgf released from a bead led to a rapid induction of *Dkk-1*. In contrast to Bmp and consistent with previous studies (Montero *et al*., 2001 and references therein), Fgf treatment caused a significant mesodermal outgrowth during the first 8 h after its application. Thus, although *Dkk-1* has the ability to induce apoptosis in the limb bud, the Fgf treatment did not cause apoptosis even after 18 h (data not shown). Strikingly, Fgf application led to increased transcriptional activity of the Wnt/β-catenin target gene *Lef-1* in our experiments. Thus, the strong proliferative signal of increased Fgf and Wnt/β-catenin signaling overrides the pro-apoptotic effect of Dkk-1, leading to a net activation of Wnt signaling following this treatment. Fgfs have classically been regarded as factors promoting mesodermal proliferation (Martin, 1998). However, a recent paper by Montero *et al*. (2001) now provides evidence that Fgfs are also required for PCD in limb development. Fgf beads implanted in a comparable way to in the present study inhibited physiological cell death in the first 12 h; however, this was followed by an increase of PCD after 24 h and later. Their results further suggest that Bmps are not capable of inducing apoptosis in the absence of Fgf signals. The implication of Fgfs in apoptosis appears to result from their requirement for the expression of genes of the PCD cascade, such as *Msx-2*. A similar mechanism might hold true for the regulation of *Dkk-1*. By the implantation of Noggin beads we could show that Bmp signaling is necessary for *Dkk-1* expression. The fact that *Dkk-1* is rapidly down-regulated after

surgical removal of the AER indicates a requirement of Fgf for *Dkk-1* activation. Consistent with this, the Bmp-induced ectopic expression domain of *Dkk-1* is always located between the bead and the AER. Thus, the greater distance from an Fgf source might explain why a Bmp bead implanted into the center of a HH26 limb bud did not induce *Dkk-1* expression, which suggests that both Bmp and Fgf signaling are essential for *Dkk-1* activation. This might indicate that the inhibition of Wnt/β-catenin signaling and the presence of Fgf are required for Bmp to induce apoptosis. In conclusion, concomitant activity of Bmp and Wnt/β-catenin pathways should lead to a completely different reaction in a cell. Indeed, Wnt/β-catenin signaling is implicated in the promotion of chondrogenesis in the chick limb bud (Hartmann and Tabin, 2000) and we have shown that *Dkk-1* is not activated by Bmp when it promotes bone formation *in vivo*. This suggests that the co-ordinated regulation of the Bmp, Wnt/β-catenin and Fgf pathways is critical for the genetic program activated in limb mesodermal cells to undergo either apoptosis or chondrogenesis.

Mediation of the Bmp signal: a crucial role for c-Jun

As outlined above, Bmps can use at least two different intracellular pathways, the mutual interaction of which leads to the activation of a particular genetic program. We propose that the c-Jun-mediated activation of a Wnt/β-catenin inhibitor is fundamental for Bmp-induced apoptosis. The cytoplasmic kinase TAK-1 has been reported to be essential for Bmp-2-induced apoptosis, and Bmp-4 can also directly activate this kinase (Kimura *et al*., 2000 and references therein). There are several further implications for TAK-1 in apoptosis. Importantly, overexpression of TAK-1 in the *Drosophila* visual system leads to ectopically induced apoptosis mediated by JNK (Takatsu *et al*., 2000). Enhanced apoptosis has also been observed in transgenic frogs and mice overexpressing TAK-1 (Shibuya *et al*., 1998; Zhang *et al*., 2000). TAK-1 activates Jnk signaling, which in turn activates c-Jun. Transcription of c-*Jun* is then autoregulated by the c-Jun protein (Angel *et al*., 1988), the overexpression of which is sufficient to induce apoptosis (Bossy-Wetzel *et al*., 1997). This cascade might provide the link between Bmp and the induction of c-*Jun* that we report in this study. In our electroporations, c-*Jun* was sufficient to induce *Dkk-1* expression, a potent inhibitor of the Wnt/β-catenin signaling pathway. Consistently, the TAK-1–MAPK-like pathway was recently shown to counteract the Wnt/β-catenin pathway (Ishitani *et al*., 1999). Based on our finding that *Dkk-1* expression and induction by Bmp is severely hampered in c-*Jun*$^{-/-}$ cells, we show that c-*Jun* is also necessary for the full expression of *Dkk-1*, although the residual expression in these cells indicates that additional transcription factors contribute to *Dkk-1* activation. However, our result that the induction of *Dkk-1* is reduced to 22% of the wild-type level in c-*Jun*$^{-/-}$ MEF in response to Bmp highlights the importance of c-*Jun* for *Dkk-1* activation by Bmp. The residual part of activation by Bmp could be mediated by Smads or other Ap-1 family members that are also activated by Jnk. As we were not able to detect *Dkk-1* transcripts in *Jnk-1*$^{-/-}$ MEF by RT–PCR (data not shown), we conclude that the Bmp/Smad pathway plays, if any, only a minor role for transcription of *Dkk-1*. The Bmp-induced activation of *Dkk-1* leads to an inhibition of Wnt/β-catenin signaling as shown by the dramatic down-regulation of *Lef-1* transcription. Interestingly, increased Jnk activity has also been reported to cause a destabilization of β-catenin accompanied with increased apoptosis in several cell lines (Neo *et al*., 2000). We suggest that the predominant activation of a particular intracellular signaling cascade downstream of the Bmp receptor also contributes to the different effects that Bmps have on limb mesodermal cells. According to our model, Bmp would induce apoptosis when the Bmp/Jnk pathway dominates the Bmp/Smad pathway to activate certain genes, as is the case for *Dkk-1*. Further support for our model comes from a study showing that the distortion of positional information determined by dpp and wg signaling gradients leads to the activation of the DJNK apoptotic pathway, which subsequently induces cell death in the *Drosophila* wing (Adachi-Yamada *et al*., 1999). The authors suggested that this pathway is latent in normal wing development, but is activated upon abnormal dpp signaling to maintain proper development. Whether c-Jun activity is necessary for the physiological cell death occuring during vertebrate limb development is hard to judge, as c-*Jun*$^{-/-}$ embryos die at E13.5 (Hilberg *et al*., 1993) when the most prominent PCD in the interdigits of the limb starts. However, a recent study provided genetic evidence that c-Jun N-terminal phosphorylation is not essential for the developmental regulation of apoptosis, since it is not impaired in mice harboring a c-*Jun* allele that cannot be phosphorylated at serines 63 and 73 (Behrens *et al*., 1999). Thus, we cannot rule out the possibility that this pathway might only be used upon inappropriate signaling.

Regulation by stress signals

c-Jun activity is rapidly induced by exposure of cells to a variety of stress signals that commit a cell to apoptosis. In fact, c-*Jun* induction is the hallmark of the mammalian UV response and cells lacking c-*Jun* are less sensitive to UV-induced apoptosis (Shaulian *et al*., 2000). We now show that c-*Jun* is also immediately induced in chick embryos exposed to UV light. UV irradiation results in a rapid accumulation of p53, which leads to either cell cycle arrest or apoptosis. Recently, it was shown that constitutive expression of c-*Jun* is the critical event regulating p53-induced apoptosis upon UV irradiation (Shaulian *et al*., 2000). Remarkably, *Dkk-1* has been suggested to be a p53 target gene, as it is induced by DNA damage in a p53-dependent manner in cultured cells (Wang *et al*., 2000). These findings, together with our findings that *Dkk-1* is activated upon UV irradiation in chicken embryos and that *Dkk-1* is a c-Jun target gene, provide strong evidence for an important role of Dkk-1-mediated Wnt/β-catenin inhibition downstream of p53. Recently, two papers revealed another way of linking genotoxic injury to the destruction of β-catenin. It was shown that the p53-inducible Siah-1 mediates β-catenin degradation independent of β-TrCP (Liu *et al*., 2001; Matsuzawa and Reed, 2001). In summary, these results suggest that blocking Wnt/β-catenin signaling might be a general requirement in apoptotic responses, as multiple ways exist

to ensure degradation of β-catenin upon inappropriate stimuli.

Although *p53* can induce *Dkk-1* expression, three lines of evidence indicate that it is not required for it. First, staurosporin, which has been shown to induce apoptosis independently of p53 (Rocha *et al*., 2000), also strongly activates *Dkk-1* expression. Secondly, the apoptosis-inducing ability of TGF-β family members also seems not to proceed via p53 (Selvakumaran *et al*., 1994; Yamamoto *et al*., 1996; Francis *et al*., 2000). Thirdly, *Dkk-1* is expressed normally in limb buds of $p53^{-/-}$ embryos (data not shown), indicating that *p53* is also dispensable for the normal expression of *Dkk-1*. On the other hand, the UV-induced expression of *Dkk-1* is likely to be independent of Bmp signaling, as *Bmp-4* is not transcriptionally induced in our assay. Thus, we conclude that *Dkk-1* can be activated by multiple independent apoptotic stimuli. In contrast to p53, however, c-Jun is pivotal for the physiological expression of *Dkk-1* and an important transcription factor for its induction by Bmp-4. Moreover, staurosporin-, H_2O_2- and UV-induced apoptosis also involve Jnk-mediated activation of Ap-1 activity and c-*Jun* induction (Buschmann *et al*., 2000; Chae *et al*., 2000; Shaulian *et al*., 2000; this study). Hence, we propose that multiple apoptotic pathways converge on c-Jun activation, resulting in increased *Dkk-1* transcription.

In our experiments, overexpression of *Dkk-1* with a retroviral vector led to severe distal truncations of the infected wing buds. These results are in line with those of Mukhopadhyay *et al*. (2001), who recently reported that ectopic *Dkk-1* expression with an adenoviral vector in the chick limb bud produced distal truncations similar to those reported here. This phenotype might not result from enhanced PCD as we could not detect a significant increase in apoptotic cells before truncations were rather advanced. An alternative explanation would be that the *Dkk-1* overexpression interferes with normal AER function, as Wnt/β-catenin signals have been shown to be essential for AER maintenance (Kawakami *et al*., 2001). This might impair distal outgrowth of the *Dkk-1*-infected wing buds, which then leads to more PCD.

By showing that *Dkk-1* clearly promotes Bmp-triggered apoptosis, however, we provide direct evidence for the pro-apoptotic capability of *Dkk-1*. As *Dkk-1* is induced by Bmp-4 under PCD-inducing conditions, this might suggest that antagonism of Wnt/β-catenin signals by Dkk-1 is even necessary for Bmp-triggered apoptosis to occur in limb mesodermal cells. In consequence, the loss of *Dkk-1* function should lead to decreased PCD in areas where Bmps are known to be important endogenous regulators of PCD like, for example, the interdigital mesenchyme. Indeed, a strong argument in favor of this interpretation comes from the phenotype of $Dkk\text{-}1^{-/-}$ mice. The fusion of the digits observed in these mice is likely to result from decreased PCD (Mukhopadhyay *et al*., 2001).

It will be interesting to determine whether Dkk-1, as a secreted protein, exerts its pro-apoptotic function in a cell-autonomous or non-cell-autonomous manner. In conclusion, our study unravels a putative important function of Wnt/β-catenin antagonism by Dkk-1 in developmental and adaptive apoptosis. Furthermore, we show that the Ap-1 family member c-Jun is pivotal for *Dkk-1* transcriptional activation.

Materials and methods

Details on Materials and methods are available at *The EMBO Journal* Online as Supplementary data, or on request.

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