

Critical role for NF- κ B-induced JunB in VEGF regulation and tumor angiogenesis

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Regulation of vascular endothelial growth factor (VEGF) expression is a complex process involving a plethora of transcriptional regulators. The AP-1 transcription factor is considered as facilitator of hypoxia-induced VEGF expression through interaction with hypoxia-inducible factor (HIF) which plays a major role in mediating the cellular hypoxia response. As yet, both the decisive AP-1 subunit leading to VEGF induction and the molecular mechanism by which this subunit is activated have not been deciphered. Here, we demonstrate that the AP-1 subunit *junB* is a target gene of hypoxia-induced signaling via NF- κ B. Loss of JunB in various cell types results in severely impaired hypoxia-induced VEGF expression, although HIF is present and becomes stabilized. Thus, we identify JunB as a critical independent regulator of VEGF transcription and provide a mechanistic explanation for the inherent vascular phenotypes seen in JunB-deficient embryos, *ex vivo* allantois explants and *in vitro* differentiated embryoid bodies. In support of these findings, tumor angiogenesis was impaired in *junB*^{-/-} teratocarcinomas because of severely impaired paracrine-acting VEGF and the subsequent inability to efficiently recruit host-derived vessels.

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Introduction

The cardiovascular system is the first organ formed in the gastrulating embryo (Weinstein, 1999). The initiation of blood vessel formation requires basic fibroblast growth factor

as well as the paracrine-acting vascular endothelial growth factor (VEGF) (Risau and Flamme, 1995). Mice lacking a single copy of the VEGF gene die *in utero*, indicating that tightly controlled VEGF levels are necessary to maintain blood vessel integrity (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). VEGF and its two receptors (flt-1 and flk-1) are key regulators of vasculogenesis and angiogenesis, as loss of any of these molecules severely affects vascular development (Carmeliet, 2000). VEGF promotes embryonic development, is important in wound healing and is the key regulator of angiogenesis in cancer. VEGF expression is induced by growth factors, oncogenes and hypoxia. Cellular adaptations to hypoxia include the induction of genes implicated in glucose transport and metabolism, and in mammalian oxygen homeostasis, such as *erythropoietin* and VEGF (Bunn and Poyton, 1996). Key regulators of VEGF expression in response to hypoxia have been identified as members of the helix-loop-helix-PAS family of transcription factors called hypoxia-inducible factor (HIF). Yet, HIF appears not to be sufficient to induce maximal levels of VEGF upon hypoxia. In addition to HIF, multiple transcription factor-binding sites for AP-1, Sp1, NF- κ B and CREB have been identified within the VEGF promoter (Pages and Pouyssegur, 2005). Despite numerous reports, the requirement of AP-1 in VEGF regulation is not resolved conclusively with regard to the contribution of the individual AP-1 subunits. Similarly, the mechanism by which the decisive AP-1 subunits are activated in response to hypoxia remains to be fully elucidated. The AP-1 family is composed of dimeric protein complexes formed by products of the *jun*, *fos* and *ATF* gene families (Shaulian and Karin, 2002; Eferl and Wagner, 2003; Hess *et al.*, 2004) and is a major nuclear target of mitogen and stress-induced signal transduction cascades. Intensive investigation of AP-1 function in tissue culture cells and *in vivo* by targeted inactivation of individual members led to the well-accepted perception that the individual AP-1 subunits may have independent functions as tissue-specific and signal-specific activators of AP-1-dependent genetic programs (Jochum *et al.*, 2001; Eferl and Wagner, 2003; Hess *et al.*, 2004). Furthermore, recent work supports the common assumption that alterations in the ratio of the two subunits c-Jun and JunB may be indicative for either cell proliferation or differentiation (Bakiri *et al.*, 2000; Szabowski *et al.*, 2000; Andrecht *et al.*, 2002). Despite the widespread expression pattern of Jun members, only the loss of JunB affects vascular development *in vivo* (Schorpp-Kistner *et al.*, 1999).

Here, we report on the molecular mechanism responsible for this critical role of JunB in vascular development and tumor angiogenesis. JunB is a hypoxia-responsive transcription factor that acts independently of HIF but is activated by hypoxia-induced NF- κ B. Moreover, JunB is essential for maximal transcriptional induction of VEGF. The absolute requirement of JunB for VEGF expression and thus for angiogenesis could be confirmed in a murine *in vivo* tumor angiogenesis model. In line with the critical role of VEGF in

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tumor angiogenesis, teratocarcinomas with *junB*^{-/-} genotype display a growth-retarded phenotype owing to strongly diminished *VEGF* expression in JunB-deficient tumor cells and concomitant impaired angiogenesis.

Results

Vascular failure in *junB*^{-/-} embryoid bodies due to limiting amounts of VEGF

Mouse embryos lacking JunB display a placentation defect in concert with impaired angiogenesis of the yolk sac owing to a failure in remodeling the primary vascular plexus to a highly organized vascular network (Schorpp-Kistner *et al*, 1999). The use of *in vitro* systems to study blood vessel formation, such as allantois explants and differentiation of *junB*^{-/-} ES cells towards embryoid bodies (EBs), revealed that in both systems *junB*^{-/-} cells failed to develop a hierarchically organized vascular plexus (Figure 1A). Thus, the vascular defect observed in the *junB*^{-/-} yolk sacs (Schorpp-Kistner

et al, 1999) is inherent to *junB*^{-/-} EBs and allantois explants. Based on previous reports that *VEGF* is a downstream target of AP-1 (Damert *et al*, 1997; Bobrovnikova-Marjon *et al*, 2004; Pages and Pouyssegur, 2005), we aimed to rescue the phenotype of *junB*^{-/-} EBs by ectopic application of recombinant human VEGF (Figure 1B). CD31 expression pattern was used as criteria to stage vascular development and to classify vascular phenotypes as reported (Vittet *et al*, 1997). Of the examined wild-type EBs, 28 and 41 % displayed an organized vascular plexus with well-defined cord-like vascular structures on days 10 and 11 of differentiation, respectively and those percentages reflect the usual range achieved under optimal conditions (Vittet *et al*, 1997). In contrast, *junB*^{-/-} EBs showed marked defects, as on day 10 only 3% of the differentiated *junB*^{-/-} EBs were able to form organized vascular structures. This percentage increased up to 8% on day 11 but was still significantly reduced (three-fold) in comparison to the wild-type EBs. Addition of VEGF to the culture medium of differentiating *junB*^{-/-} ES cells resulted in

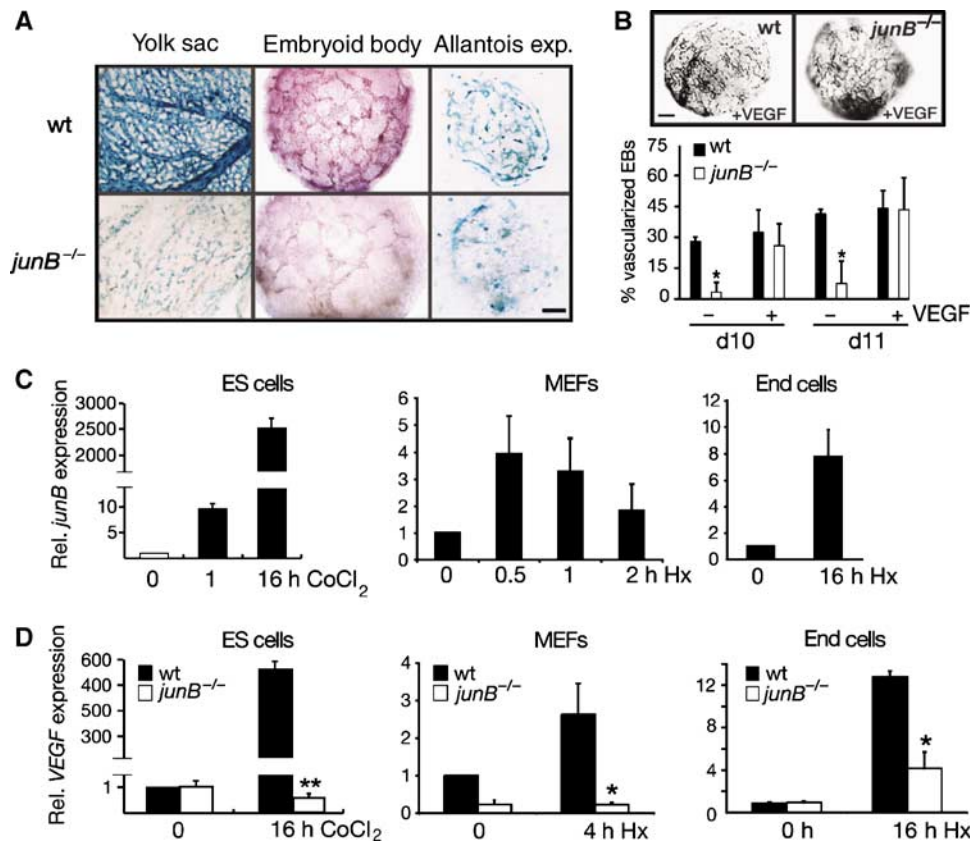


Figure 1 Inherent vascular defects in *junB*^{-/-} tissues due to impaired *VEGF* expression. (A) Analyses of vascular network formation of wild-type and *junB*^{-/-} yolk sacs (E 9.5), differentiated EBs (day 11) and allantois explants (40 h) from early headfold-stage embryos by β -galactosidase staining for yolk sacs and allantois explants or whole mount immunohistochemical staining for CD31. Size bar corresponds to 120 μ m for the yolk sacs, 70 μ m for EBs and 40 μ m for wild-type and *junB*^{-/-} allantois explants, respectively. (B) Ectopic VEGF can rescue the phenotype of *junB*^{-/-} EBs. Top, black and white photographs of CD31-stained EBs derived from wild-type or *junB*^{-/-} ES cells differentiated for 11 days. Every second day, 50 ng/ml recombinant human VEGF had been added to the cultures. Size bar corresponds to 40 μ m. Bottom, quantification of EBs differentiated for 10 and 11 days. EBs that display an elaborate organized vascularization according to the criteria of Vittet *et al* (1997) were counted. Error bars represent s.d. values of at least 30 different EBs analyzed for each time point. $P=0.022$ (d10) or $P=0.021$ (d11) versus wild type. (C) Quantitative RT-PCR analysis of *junB* mRNA from wild-type ES cells and MEFs and End cells. ES cells were treated with 75 μ M CoCl₂, and MEFs and End cells were incubated under hypoxic conditions (Hx, 1.5% O₂) for the indicated time points; before hypoxia induction, MEFs were starved for 36 h (0.5% FCS). Relative gene expression is given; expression of normoxic wild-type cells was set to 1. (D) Quantitative RT-PCR analysis of *VEGF* mRNA from wild-type and *junB*^{-/-} ES cells; MEFs and End cells treated as described in (C). Enhanced *VEGF* transcript levels in hypoxic cells (black bars) are indicated as fold difference compared to the expression in normoxic wild-type cells (white bars), which was set to 1. Error bars in (C) and (D) show s.e.m. values of at least three independent experiments with $P=0.0289$ (End cells) or $P=0.0005$ (ES cells), $P=0.0448$ (MEFs) for *junB*^{-/-} versus wild-type cells.

a highly organized network of capillary-like structures that resembled the one of wild-type EBs (Figure 1B). Thus, ectopic application of VEGF rescued the JunB phenotype with regard to the formation of an organized vascular plexus.

JunB is induced in response to hypoxia and is required for VEGF expression

As VEGF levels apparently represent the limiting factor responsible for the observed phenotype, we next asked whether JunB is directly involved in the VEGF response to hypoxia. To address this question, we analyzed different *junB*^{-/-} cell types, that is, ES cells, mouse embryonic fibroblasts (MEFs) and endothelioma cells (End cells) cultured under hypoxic conditions. Measuring mRNA levels by quantitative RT-PCR revealed that under hypoxic conditions *junB* transcription was rapidly induced in ES cells, MEFs and End cells (Figure 1C). Maximal levels of *junB* mRNA were reached in ES and End cells at 16 h and in wild-type MEFs at 0.5 h under hypoxic conditions (Figure 1C), and correlate with or precede VEGF induction with maximal levels at 16 h in wild-type ES cells and End cells or, at 4 h in MEFs, (Figure 1D, wt). Whereas in serum-starved MEFs, basal expression of VEGF was already reduced to 23–33%, hypoxia-induced VEGF mRNA levels were severely impaired in the absence of JunB in all cell types analyzed (Figure 1D, *junB*^{-/-}) from approximately 562-fold in ES cells, 2.6-fold in MEFs and 12.7-fold in End cells to no induction (ES cells and MEFs) or only a 4.1-fold induction (End cells) in JunB-deficient cells (Figure 1D).

Moreover, in serum-starved MEFs, VEGF regulation upon mitogenic stimulation, represented by treatment with the phorbol ester TPA, also depends strictly on JunB. Whereas basal VEGF mRNA was barely detectable, VEGF expression levels reached upon TPA induction were strongly diminished in *junB*^{-/-} MEFs (Supplementary Figure S1). Thus, in MEFs, JunB is essential for basal and for maximal mitogen- and hypoxia-induced VEGF transcription.

HIF-independent hypoxia response of JunB

As HIF plays a major role in hypoxia-induced transcriptional activation of VEGF and as AP-1, in particular the subunits c-Jun and c-Fos has been proposed to cooperate with HIF, it was necessary to study whether loss of JunB affects the

expression and/or stabilization of these regulators. Thus, we analyzed JunB-deficient MEFs for expression of AP-1 and HIF subunits under hypoxia. Importantly, loss of JunB did not cause a reduction in expression of c-Jun, phosphorylated c-Jun and c-Fos (Figure 2A). Similarly, ablation of JunB did neither affect stabilization of HIF-1 α in the presence of the hypoxia-mimicking agent CoCl₂ nor alter expression levels of the HIF subunits HIF-1 β /ARNT and HIF-2 α (Figure 2B) known to be constitutively upregulated but transcriptionally inactive in MEFs (Park *et al*, 2003). As expression or activation and stabilization of these well-known VEGF regulators were not altered, JunB may act independent of HIF and may be directly implicated in the transcriptional control of VEGF.

To determine whether *junB* is a target of HIF, we analyzed HIF-1 α -deficient MEFs for JunB expression. Yet, hypoxia-dependent JunB induction was not impaired in HIF-1 α -deficient cells excluding a role for HIF in hypoxia-dependent *junB* regulation (Figure 2C).

Hypoxia-mediated induction of junB via NF- κ B

A wide variety of stimuli can induce transcriptional upregulation of *junB*, which is, like all AP-1 members, a preferred downstream target of mitogen-activated protein kinase (MAPK) signaling cascades. MAP kinases have indeed been reported as transducers of hypoxic signaling although the oxygen-sensing mechanism upstream of MAPK signaling cascades remains unclear (Minet *et al*, 2001a, b). However, we could exclude an involvement of MAP kinase signaling as it was absent or occurred only at a later time point when transcriptional activation of *junB* by hypoxia was already completed (Supplementary Figure S2). As the transcription factor NF- κ B has been shown to activate *junB* expression in response to LPS in B cells (Brown *et al*, 1995; Krappmann *et al*, 2004) and has been reported as hypoxia-mediating transcription factor (Faller, 1999; Harris, 2002), the involvement of NF- κ B was investigated. First, we determined NF- κ B translocation to the nucleus in response to hypoxia. Indeed, within 20 min under hypoxic conditions, NF- κ B becomes translocated to the nucleus of wild-type and also HIF-1 α -deficient MEFs (Figure 3A) where it is able to transactivate potential target genes. Moreover, a transiently cotransfected reporter gene flanked by *junB* regulatory sequences is

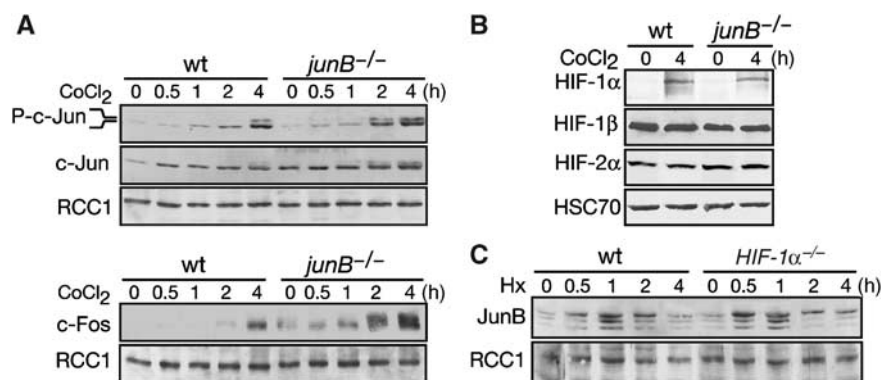


Figure 2 Hx responses of c-Fos, c-Jun and HIF are not affected in JunB-deficient MEFs, and the JunB induction is independent of HIF. (A–C) Immunoblotting was performed for the various proteins as indicated on the left of each panel. Fifty micrograms of nuclear extracts for AP-1 members or total extract for HIF members, respectively, was used, which were isolated from wild-type and *junB*^{-/-} MEFs kept under hypoxia mimicked by treatment with 100 μ M CoCl₂ for the indicated time points. RCC1 or HSC70 served as a control for equal quality and loading of nuclear extracts or total extracts, respectively.

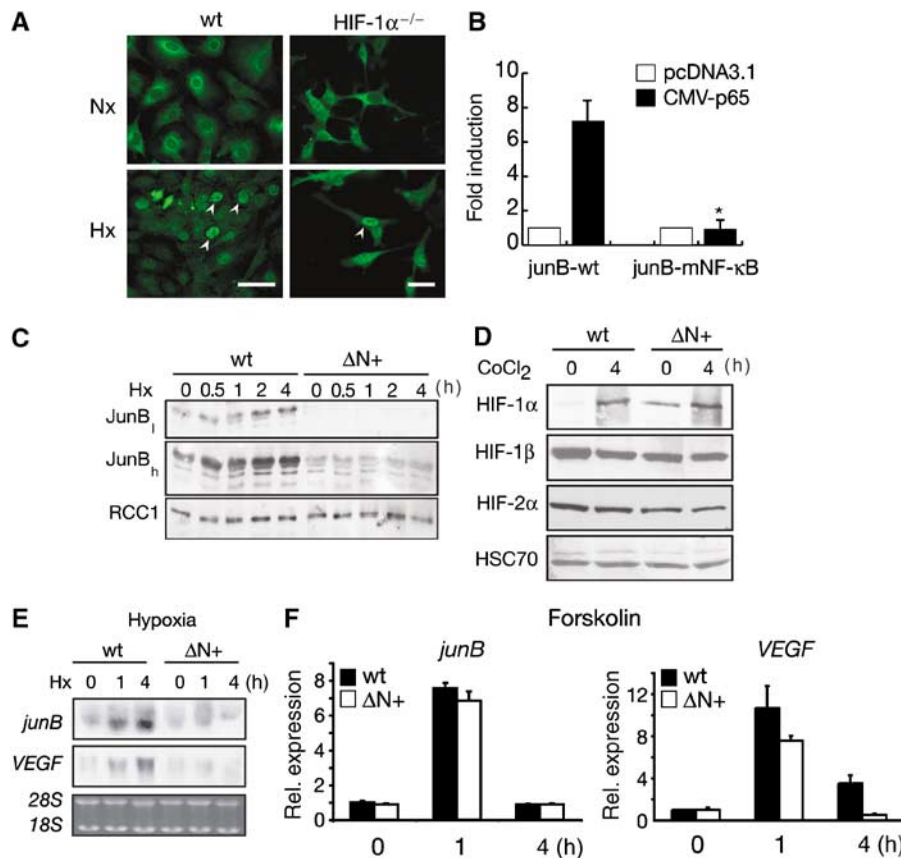


Figure 3 *junB* induction is achieved via NF- κ B and is independent of HIF. (A) NF- κ B is rapidly translocated to the nucleus in response to hypoxia. Immunofluorescence staining for p65 on wild-type (wt) or HIF-1 $\alpha^{-/-}$ MEFs kept under normoxic (Nx) or hypoxic conditions (Hx, 1.5% O₂) for 20 min. White arrows (Hx) depict NF- κ B-positive nuclei. Size bar, 25 and 12.5 μ m for wt and HIF-1 $\alpha^{-/-}$ cells, respectively. (B) F9 cells were cotransfected with *junB* reporter constructs, *junB-wt* and *junB-mNF- κ B*, and p65 expression vector (CMV-p65, black bars) or control vector (*pGL3*, white bars). Promoter activity 16 h post-transfection is shown in relation to the luciferase activity of the vector control, which was set to 1. A cotransfected *Renilla* luciferase reference gene was used for normalization. Error bars of at least three independent experiments show s.d. values. * $P=0.0028$ for mutant versus *junB-wt*. (C) Protein levels of JunB in wild-type or Δ N+ cells kept under hypoxia for the indicated time points were determined by immunoblot analysis using 50 μ g of nuclear extract. Two different exposures (low and high) are given to show basal as well as induced JunB levels. RCC1 served as a control for equal quality and loading. Hx, hypoxia. (D) HIF protein levels are not affected by loss of NF- κ B. Immunoblotting was performed for HIF-1 α , HIF-1 β and HIF-2 α by using 50 μ g of total protein from wild-type and Δ N+ MEFs kept under hypoxia mimicked by treatment with 100 μ M CoCl₂ for the indicated time points. HSC70 served as a control for equal quality and loading of extracts. (E) Hx-mediated *junB* and *VEGF* induction is strongly diminished in fibroblasts with repressed NF- κ B activity (Δ N+). Northern blot analyses of wild-type MEFs or cells with suppressed NF- κ B activity exposed to hypoxia (Hx). Ethidium bromide-stained agarose gel is shown as loading control. (F) *junB* and *VEGF* can be induced by Forskolin despite the absence of NF- κ B. Quantitative RT-PCR analyses of wild-type MEFs or cells with suppressed NF- κ B activity treated with 10 μ M Forskolin for the indicated time points were performed using specific primers for *junB* or *VEGF*.

strongly transactivated in the presence of coexpressed p65, which is abolished upon mutation of four putative NF- κ B-binding sites in the *junB* 3' flanking sequences of the reporter (Figure 3B). Finally, we analyzed MEFs overexpressing a transdominant negative I κ B α mutant protein, a so-called super-repressor that impairs NF- κ B activation (Schmidt-Ullrich *et al*, 2001) and its subsequent translocation to the nucleus. In these cells, hypoxia-dependent induction of JunB protein and its mRNA (Figure 3C and E) but also of *VEGF* transcripts was severely diminished (Figure 3E), although HIF-1 α was already slightly stabilized under normoxic conditions and was further inducible under hypoxic conditions (Figure 3D). As we were still able to induce *junB* transcription in these cells via the protein kinase A (PKA) signaling pathway elicited by increase in intracellular cAMP levels through Forskolin treatment (Figure 3F), we conclude that the hypoxia response on *junB* transcription is fully mediated via NF- κ B. Most importantly, despite reduced basal VEGF mRNA levels

in the absence of NF- κ B (Supplementary Figure S3), Forskolin treatment led in parallel to a similar induction of VEGF when compared to wild-type cells (Figure 3F).

***JunB* contributes to hypoxia-induced AP-1 binding and is able to transactivate the VEGF promoter**

Owing to the presence of several recognition sites for AP-1, so-called TREs (TPA, (12-*O*-tetradecanoylphorbol-13-acetate)-responsive elements) and CREs (cAMP-response elements) present within the *VEGF* promoter (Figure 4A), it is very likely that JunB can positively act on the promoter as it has been described previously for other genes (Chiu *et al*, 1989; Passegue and Wagner, 2000; Andrecht *et al*, 2002). Thus, we applied electromobility shift assays (EMSA) using *in vitro*-translated JunB dimerized to c-Fos. JunB:Fos heterodimers bound preferably to the TRE located at position -1093 to -1086 of the murine *VEGF* promoter (Figure 4A, left panel) but also with much lower preference to a TRE close to the HIF

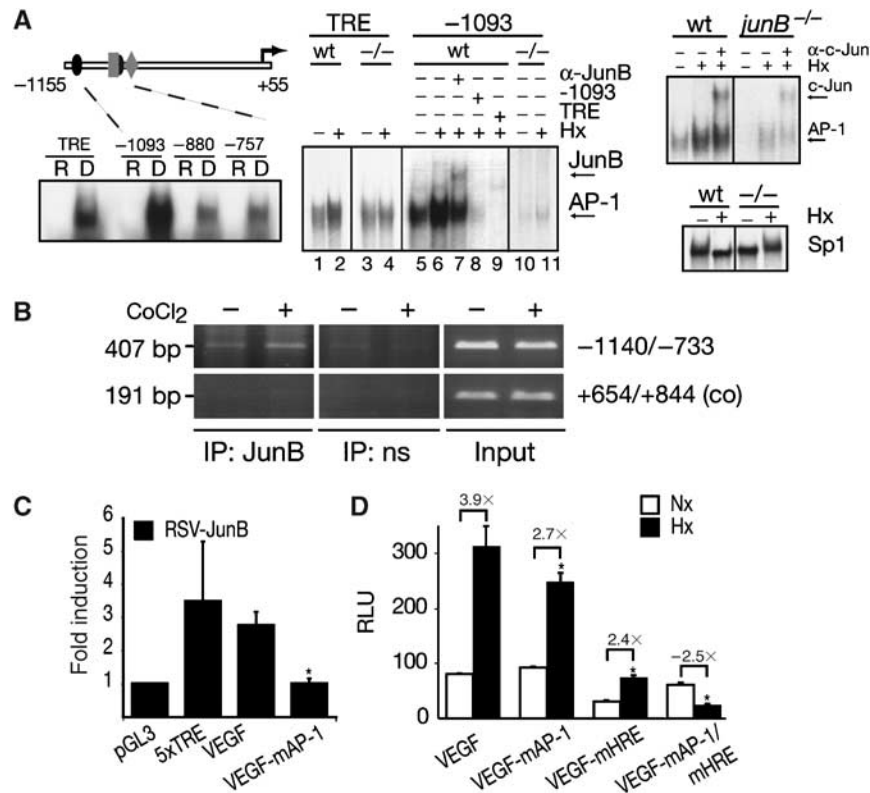


Figure 4 JunB binds to and activates the murine *VEGF* promoter. (A) Left panel, top: schematic representation of the murine *VEGF* promoter indicating the positions of the relevant consensus-binding sites for AP-1 (black ellipse for AP-1 and gray diamond for CRE) and HIF (gray bar). Left panel, bottom: EMSA of complexes formed by *in vitro*-translated JunB:Fos dimers (D) on the consensus AP-1 sites (TRE) and different potential AP-1-binding sites within the *VEGF* promoter. Location of these sites is indicated on the top. For control, the DNA elements were incubated with reticulocyte extract only (R). Middle panel: EMSAs of complexes formed with the consensus AP-1 site (TRE) and the *VEGF*-AP-1 element located at -1093 using nuclear extracts from wild-type (wt) and *junB*^{-/-} fibroblasts grown under normoxic or hypoxic (Hx) conditions for 16 h. For cross-competition experiments, a 50-fold excess of non-labeled competitor TRE or -1093 , as indicated on the top, was used. For supershift analysis, extracts were preincubated with a specific antiserum recognizing JunB (α -JunB). Arrows depict specific complexes. No specific protein–DNA complexes were obtained with an oligonucleotide representing a mutated version of the *VEGF*-AP-1 site (data not shown). Individual lanes were numbered for convenience. Right panel, top: residual AP-1-binding activity in *junB*^{-/-} nuclear extracts from wild-type (wt) and *junB*^{-/-} fibroblasts grown under normoxic or hypoxic (Hx) conditions for 16 h. For supershift analysis, extracts were preincubated with a specific antiserum recognizing c-Jun (α -c-Jun). Right panel, bottom: in parallel, EMSAs with an Sp1 element were performed for control for equal quality and amount of extracts used. (B) ChIP analysis of the *VEGF* promoter was performed using an antibody specific for JunB. Specific primer sets were used to discriminate between the promoter region harboring the proximal TREs (-1140 to -733) and a control region (co) in the 5'UTR that does not contain any TRE ($+654$ to $+844$). JunB binding to the *VEGF* promoter region between -1140 and -733 was detected and was enhanced in End cells treated with $200 \mu\text{M}$ CoCl₂ for 4 h. (C) F9 cells were cotransfected with a JunB expression vector and luciferase reporters containing no promoter (pGL3), a JunB target ($5 \times \text{TRE}$), wild-type *VEGF* or *VEGF* containing the mutated AP-1 site at -1093 (VEGF-mAP-1) as indicated. The fold induction upon transactivation was calculated as described in Figure 3B. (D) Hypoxia-dependent transactivation of the wild-type and *VEGF* reporters with mutated AP-1 (VEGF-mAP-1), mutated HIF (VEGF-mHRE) or both mutated binding sites (VEGF-mAP-1/mHRE) was measured in transiently transfected HepG2 cells. Twenty-four hours post-transfection, cells were incubated for 12 h under normoxic or hypoxic (Hx) conditions. Relative luciferase activities (RLU) are given. Fold activation of reporter plasmid under hypoxia over normoxia is depicted on top of the columns. Error bars of at least three independent experiments show s.d. values. * $P = 0.0001$ (C) and $P < 0.01$ (D) for mutant versus wild-type *VEGF* reporter, respectively.

recognition site at -880 to -873 , and to the CRE at -757 to -749 . The high-affinity site at -1093 was used to analyze DNA-binding activities of nuclear extracts from wild-type and *junB*^{-/-} MEFs grown under normoxic or hypoxic conditions (Figure 4A, middle panel). A hypoxia-inducible complex was observed with a consensus TRE (lane 2) or the -1093 *VEGF*-AP-1 site (lane 6), using extracts of wild-type cells. The complex at the -1093 site could be competed by both the unlabeled oligonucleotide (lane 8), and the consensus TRE (lane 9), and was partially supershifted by preincubation with a specific antibody directed against JunB (lane 7). When nuclear extracts from *junB*^{-/-} cells were used, complex formation on the TRE (lane 3) was comparable to that seen with normoxic wild-type extracts (lane 1). However, complex

formation was no longer hypoxia-inducible (lane 4) and was severely reduced on the -1093 *VEGF*-AP-1 site (lanes 10 and 11). The remaining, hypoxia-induced complex (lane 11) is most likely due to c-Jun present in the extract (Figure 4A, right panel, top). Gel shifts performed with the consensus site for Sp1 did not reveal any differences in overall binding activities between wild-type and *junB*^{-/-} extracts (Figure 4, right panel, bottom). To prove physical interaction of JunB with the *VEGF* promoter, chromatin immunoprecipitation (ChIP) analysis was performed in End cells in the absence or presence of CoCl₂. Primers were designed to amplify the region of the *VEGF* promoter (-1140 to -733) containing both TREs for potential binding of JunB. We found weak basal JunB binding to this promoter region (Figure 4B) which

was enhanced in CoCl₂-treated cells. In contrast, no interaction was observed at a control region containing no TREs (+654 to +844). To determine the transactivation potential of promoter-bound JunB, activation of a murine *VEGF* promoter luciferase reporter was analyzed. Coexpression of JunB resulted in strong transactivation of the wild-type -1155/+55 murine *VEGF* promoter (Figure 4C, VEGF) comparable to that obtained with the typical artificial *junB* reporter (Figure 4C, 5 × TRE). Mutation of the high-affinity AP-1 site (-1093 to -1086) within the murine promoter fragment resulted in a complete suppression of luciferase activity (Figure 4C, VEGF-mAP-1), suggesting that JunB acts primarily through this element. When hypoxia-dependent transactivation of these reporters was analyzed, a robust induction of the reporter carrying the *VEGF* wild-type sequences -1155/+55 was observed (Figure 4D). Mutation of the AP-1 site at position -1093 to -1086 resulted in a significant impairment of the hypoxia inducibility from 3.9- to 2.7-fold. By comparison, mutation of the HIF recognition site HRE compromised

hypoxia-dependent reporter gene expression to a slightly higher extent revealing a residual inducibility of 2.4-fold. Reporter gene expression was barely detectable when both AP-1 and HIF sites were mutated (Figure 4D).

Compromised tumor growth and angiogenesis in the absence of JunB

Hypoxia-driven *VEGF* expression is considered to be an important factor governing tumor angiogenesis and growth. Reduced expression of *VEGF* caused by the loss of JunB is therefore expected to affect also tumor angiogenesis that was analyzed by generating ES-cell derived teratocarcinomas (germ cell tumors) in *nu/nu* mice. Indeed, macroscopic analysis of wild-type teratocarcinomas revealed a red and hemorrhagic phenotype with bleeding upon isolation. In contrast, the *junB*^{-/-} tumors were pale and poorly hemorrhagic (Figure 5A).

To quantify the influence of JunB ablation on tumor progression, tumors that had a minimal size of 100 mm³ at

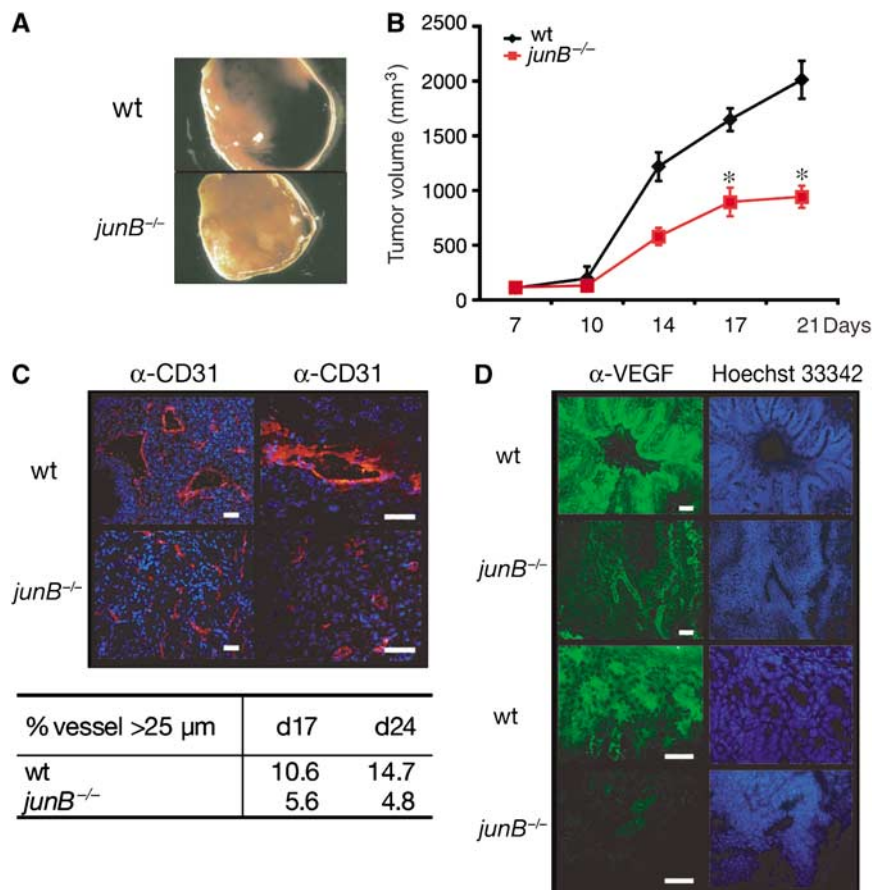


Figure 5 Reduced tumor volume of JunB-deficient teratocarcinomas, lack of large blood vessels and impaired VEGF expression. (A) Photographs of a *junB*^{-/-} and a wild-type (wt) teratocarcinoma showing severe hemorrhaging in wild-type but not in the *junB*^{-/-} teratocarcinomas. (B) Tumor progression of wild-type (wt) and *junB*^{-/-} teratocarcinomas monitored for the indicated time points post injection by measuring the tumors in two dimensions (width by length) and subsequent calculation of the volumes. Error bars of at least three tumors of each genotype show s.d. values. **P*=0.001 (d17 and d21). (C) CD31 (α-CD31, red signal) immunofluorescence staining on cryosections of two different tumor areas derived from wild-type (wt) and *junB*^{-/-} ES cells. To determine the vessel sizes, three independent tumors of wild-type and *junB*^{-/-} genotype, and of two stages, day 17 and day 24, as indicated in table, were analyzed by measuring the vessel size using the software Lucia Archive (Nikon). Five randomly chosen optical fields of each tumor were analyzed. Table shows the percentage of vessels being larger than 25 μm in diameter. Wilcoxon's rank sum test comparing all vessel diameters of day 24 wild-type and *junB*^{-/-} teratocarcinomas reveals that diameters of JunB-deficient vessels differ significantly from those of wild-type vessels (*P*=0.006). (D) VEGF (α-VEGF, green signal) immunofluorescence staining on cryosections of two different tumor areas derived from wild-type (wt) and *junB*^{-/-} ES cells. For CD31 and VEGF, stainings of wild-type and *junB*^{-/-} tumors isolated at day 24 are shown. Nuclei were counterstained with Hoechst 33342 (blue signal). Size bar, 100 μm.

day 7 after injection were followed up. Up to 10 days after injection of ES cells, there was no difference in the growth behavior of wild-type and *junB*^{-/-} tumors. Interestingly, tumor growth was attenuated in *junB*^{-/-} teratocarcinomas (Figure 5B) day 14 onwards. Between days 21 and 24, 37.5% of wild-type tumors had reached a size no longer tolerable from point of view of the animal's welfare, thus, mice had to be killed. When tumors were analyzed for the presence of blood vessels, different sizes of vessels were present throughout the whole wild-type tumors. By contrast, *junB*^{-/-} tumors showed predominantly small capillaries; large vessels were very rarely present or completely absent (Figure 5C, α -CD31). To quantify the vascular phenotype, the vessel size per optical field was measured for three independent tumors of each stage and genotype. Remarkably, teratocarcinomas derived from *junB*^{-/-} ES cells never contained blood vessels larger than 55 μ m in diameter in contrast to wild-type tumors that developed large vessels and blood vessel lakes larger than 80 μ m in diameter (Figure 5C). Importantly, VEGF was expressed at high levels in wild-type tumors, whereas *junB*^{-/-} teratocarcinomas revealed only marginal VEGF expression for all tumor stages and diversely differentiated tumor areas analyzed (Figure 5C, α -VEGF). Thus, in addition to embryonic development, tumor angiogenesis represents an additional *in vivo* condition where the lack of JunB affects VEGF expression resulting in impaired neovascularization.

Discussion

Our data obtained from three independent JunB-deficient cell model systems and various stimuli strongly suggest that JunB is a critical player in VEGF regulation affecting VEGF transcription in response to mitogens and hypoxia. Moreover, *junB* itself is a target of the hypoxia-induced gene program. For MEFs, we could identify the molecular mechanism responsible for *junB* induction, which involves NF- κ B signaling. Hypoxia influences the formation of initial placental structures guaranteeing oxygen supply of the embryo (Land, 2003) and subsequently serves as important stimulus for angiogenesis (Giaccia *et al*, 2004). Thus, failure in both hypoxia-mediated placentation and angiogenesis may represent the underlying molecular mechanism responsible for the phenotype of JunB-deficient embryos displaying various failures in trophoblast function, yolk sac and placental labyrinth vascularization (Schorpp-Kistner *et al*, 1999).

Besides the master regulatory transcription factor HIF mediating the stimulatory effect of hypoxia on gene expression (Semenza, 2000; Bacon and Harris, 2004; Poellinger and Johnson, 2004), several other transcription factors were reported to be activated in response to lowered oxygen pressure (Faller, 1999; Harris, 2002). These include CREB (the cyclic AMP-response element-binding protein), EGR-1 (early growth response-1), metal-transcription factor-1, NF- κ B and AP-1. Although NF- κ B and AP-1 are at the receiving end of different signaling pathways, they are often activated by the same stimuli and regulate simultaneously common target genes implicated in important physiological and pathological processes such as proliferation, differentiation, apoptosis, inflammation and tumorigenesis (Eferl and Wagner, 2003; Greten and Karin, 2004; Hess *et al*, 2004). Previous studies suggested that NF- κ B regulates VEGF gene expression at the transcriptional level (Huang *et al*, 2001; Kiriakidis *et al*,

2003; Fujioka *et al*, 2004). Here, we provide a direct mechanistic link between NF- κ B and the AP-1 subunit JunB in the hypoxia-mediated induction of VEGF. We found that JunB is required for basal and hypoxia-induced transcriptional activation of VEGF and that JunB itself is induced in response to low oxygen levels via NF- κ B. In cells lacking NF- κ B activity, *junB* as well as VEGF induction in response to hypoxia is severely diminished, whereas expression and stabilization of HIF were not altered. In line with previous reports (Huang *et al*, 2001; Kiriakidis *et al*, 2003; Fujioka *et al*, 2004), we observed a diminished basal expression of VEGF in MEFs lacking NF- κ B activity. However in these cells, *junB* and its target VEGF are still inducible via the PKA signaling pathways. By contrast, VEGF expression in response to hypoxia is hampered in different cell types, lacking JunB. As loss of JunB has no negative influence on NF- κ B induction and activity in response to hypoxia (Supplementary Figure S3), we conclude that despite the presence of active NF- κ B as well as stabilized HIF, JunB is absolutely required for VEGF regulation in a cell-type-independent manner.

In endothelial cells, an induction of AP-1 transcription factor activity and concomitant activation of target genes such as *endothelin-1* and *PDGF-B* by hypoxic environment has been shown (Ausserer *et al*, 1994; Bandyopadhyay *et al*, 1995). Yet, in certain cell types, transcriptional induction of AP-1 members, such as *c-jun*, did not result in concomitant increased DNA binding and subsequent reporter gene activation (Ausserer *et al*, 1994; Bandyopadhyay *et al*, 1995), suggesting cell-specific signals or additional factors or different AP-1 subunits involved in the AP-1-mediated hypoxia regulation. Previous reports have already underlined the functional importance of AP-1-binding sites within the human VEGF promoter (Damert *et al*, 1997) and described a functional cooperativity of c-Jun and HIF-1 (Alfranca *et al*, 2002; Salnikow *et al*, 2002) and an HIF-1 α dependency of c-Jun in chronic hypoxia (Laderoute *et al*, 2002). Previous studies were concentrated on c-Jun and recently on c-Fos (Fujioka *et al*, 2004); however, experimental tools were used that do not necessarily discriminate among the different Jun members, such as antibodies for supershift assays that recognize all three Jun members (Damert *et al*, 1997), as well as the transdominant-negative mutants TAM67 (Alfranca *et al*, 2002) and A-Fos (Bobrovnikova-Marjon *et al*, 2004; Fujioka *et al*, 2004) that will simultaneously inhibit all Jun members. Based on our data and a recent report on NF- κ B-mediated VEGF regulation via the AP-1 subunit c-Fos (Fujioka *et al*, 2004), we propose that a JunB:c-Fos heterodimer participates in VEGF transcription as c-Fos function requires a Jun dimerization partner. Yet, whereas c-Fos is only indirectly regulated by NF- κ B through the activation of Elk-1 (Fujioka *et al*, 2004), a member of the ternary complex factor (TCF), well known for its prominent function in *c-fos* transcriptional regulation (Sassone-Corsi and Verma, 1987; Hipkind *et al*, 1991; Marais *et al*, 1993), we found that *junB* is a direct target of NF- κ B in the hypoxia regulation. The nuclear translocation of NF- κ B within 20 min in response to hypoxia correlates with the rapid transcriptional induction of *junB*. Interestingly, in a recent *in vitro* study, post-translational hydroxylation of ankyrin repeats in I κ B proteins by the HIF asparaginyl hydroxylase factor inhibiting HIF could be demonstrated (Cockman *et al*, 2006), which may provide a potential explanation for the induction of NF- κ B signaling by

hypoxia. Finally, by applying coexpression studies, we were able to confirm previous findings that *junB* is a direct target of NF- κ B (Brown *et al*, 1995; Krappmann *et al*, 2004) and extend these findings to the physiological stimulus hypoxia.

The absence of a vascular or angiogenic phenotype in mice with the genetic ablation of c-Jun, JunD or c-Fos (Eferl and Wagner, 2003; Hess *et al*, 2004) favors JunB as the primary AP-1 subunit in the regulation of *VEGF* expression *in vivo*. This is also supported by the direct and hypoxia-increased binding of JunB to the *VEGF* promoter, a strongly diminished hypoxia-inducible binding activity at the VEGF-AP-1 site in *junB*^{-/-} cells as well as the JunB-dependent transactivation of the *VEGF* reporter in coexpression studies. Importantly, JunB ablation severely affected basal expression and inducibility of *VEGF* in response to TPA but did not hamper the expression of c-Jun and c-Fos. Our data imply that JunB regulation of *VEGF* in MEFs is a very early but essential event at least in MEFs. The slower induction kinetics in both ES and End cells may either be due to cell-type-specific differences in basal levels of transcription factors or due to different thresholds of ES cells and endothelial cells versus MEFs for sensing hypoxia. The JunB response can be separated from the known regulatory function of AP-1 subunits presently regarded as hypoxia-responsive factor regulating VEGF. Our observation is in line with a previous report that c-Jun expression is even increased in JunB-deficient granulocytes (Passegue *et al*, 2001), and supports the concept that JunB can act as an antagonist of c-Jun (Chiu *et al*, 1989; Szabowski *et al*, 2000). Here, we could identify with *VEGF* a protagonist for a putative subset of AP-1 target genes controlled by HIF-1 α , c-Jun and NF- κ B via c-Fos and JunB in concert (Figure 6). Applying a cDNA microarray on wild-type and *junB*^{-/-} endothelial cells, we very recently identified in addition to *VEGF*, only a few other JunB-dependent hypoxia-regulated targets, of which core-binding factor β is required for endothelial cell morphogenesis (Licht *et al*, 2006). Undisputedly, there is an increasing body of evidence that AP-1 subunits play an important role in angiogenesis or tumor angiogenesis (Folkman, 2004). Thus, it has been shown that JunD regulates genes involved in antioxidant defense and H₂O₂ production and, also protects cells from oxidative stress and exerts an anti-angiogenic effect (Gerald

et al, 2004). By contrast, DNazyme-mediated suppression of c-Jun inhibited corneal neovascularization stimulated by VEGF and also significantly reduced tumor growth in an *in vivo* mouse model (Zhang *et al*, 2004). Our data suggest that suppression of JunB may have a similar, if not even more prominent effect on tumor angiogenesis. JunB-deficient experimental teratocarcinomas were smaller and less vascularized than wild-type tumors. As proliferation and apoptosis were not affected in *junB*^{-/-} teratocarcinomas as measured by BrdU incorporation and TUNEL staining (Supplementary Figures S4 and S5), we conclude that the reduced tumor growth was most likely due to failure in hypoxia-mediated *VEGF* induction, as VEGF protein was barely detectable in tumor tissue of JunB-deficient teratocarcinomas. In line with this assumption, we found *junB*^{-/-} teratocarcinomas harbor more hypoxic areas than wild-type tumors as shown by increased expression of glucose transporter GLUT-1 (Supplementary Figure S6), a protagonist for hypoxia-induced gene products.

JunB can be activated by a plethora of stimuli, including inflammatory cytokines, and thus, JunB could represent a potential link between inflammation and oncogenesis. Such a link has previously been suggested for HIF-1 α shown to be upregulated via an NF- κ B-Cox2 pathway (Jung *et al*, 2003).

Our approach provides exciting novel insights into a remarkably complex network of transcriptional regulation involving JunB as a novel critical factor acting in response to NF- κ B and in concert with HIF, and the other AP-1 subunits c-Jun and c-Fos as critical mediators of *VEGF* response to hypoxia (Figure 6) and of tumor angiogenesis. For the future, it will be important to investigate whether a combinatorial inhibitory approach for these essential components of a hypoxia-dependent transcriptional network will be useful for anti-angiogenic tumor therapies.

Materials and methods

Cell lines and culture conditions

Generation and culture of ES cells and *in vitro* differentiation of D3 ES cells was performed as described (Vittet *et al*, 1996; Schorpp-Kistner *et al*, 1999; Andrecht *et al*, 2002). Allantois explants from early headfold-stage (E7.75–E8.75) embryos were derived from timely mated heterozygous *junB* mice carrying a *Tie2-lacZ* transgene (Schlaeger *et al*, 1997) and cultivated as described (Downs *et al*, 2001). As β -galactosidase is under the control of the *Tie-2* promoter and enhancer, positive β -galactosidase staining is predominantly indicative for vascular endothelial cells (Schlaeger *et al*, 1997), and thus, was used for analysis of yolk sacs and allantois explants. Primary NF- κ B super-repressor cells and respective wild-type cells (Schmidt-Ullrich *et al*, 2001) were immortalized as described (Andrecht *et al*, 2002). *HIF-1 α* ^{-/-} and wild-type MEFs (Ryan *et al*, 2000) as well as End cells (Licht *et al*, 2006) were reported elsewhere. F9 teratocarcinoma and HepG2 cells were transiently transfected as described (Andrecht *et al*, 2002).

Construction of reporter genes

For further details, see Supplementary data.

Electrophoretic mobility shift assays

Preparation of nuclear extracts from wild-type and *junB*^{-/-} MEFs and gel retardation assays were performed as described previously (Andrecht *et al*, 2002).

Chromatin immunoprecipitation assay

A ChIP kit (Millipore) was used according to the instructions of the manufacturer. Antibody used for JunB was c11 (Santa Cruz). For primer sequences for amplification of the *VEGF* gene, see

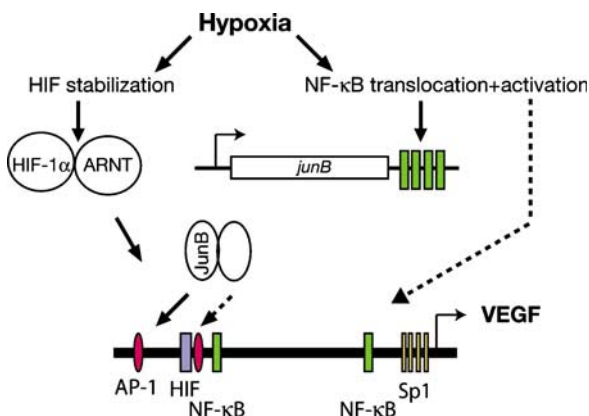


Figure 6 Model for hypoxia-induced transcription factors HIF, JunB and NF- κ B acting in concert on the murine *VEGF* promoter to confer maximal *VEGF* expression. The model is based on previous reports and our own findings; c-Fos or c-Jun may represent the most likely dimerization partners of JunB.

Supplementary data. The CHIP Assay was performed as described previously (Licht *et al*, 2006).

Immunoblot

Whole-cell extracts or nuclear extracts were prepared as described (Dignam *et al*, 1983; Andrecht *et al*, 2002). Proteins (50 μ g) were separated by SDS-PAGE, blotted onto nitrocellulose, and immunodetection was performed with an enhanced chemiluminescence system (Perkin Elmer LAS GmbH, Germany). For further details on antibodies used, see Supplementary data.

Induction of teratocarcinomas, histological analysis of tumors and immunofluorescence

For tumor formation, 2×10^6 wild-type and *junB*^{-/-} ES cells were injected subcutaneously at the left and right sides of the dorsal area into *nu/nu* mice. The procedures for performing animal experiments were in accordance with the principles and guidelines of the ATBW (authority for animal welfare) and were approved by the Regierungspräsidium Karlsruhe, Germany. For further details, see Supplementary data.

RNA isolation and RT-PCR

Total RNA was isolated using TRIzol[®] reagent (Invitrogen GmbH, Karlsruhe, Germany) according to the standard protocols followed by treatment with DNase I for 30 min at 37°C. Quantitative differences in gene expression were determined by real-time RT-PCR using the Absolute[™] QPCR SYBR[®] Green Fluorescein Mix (ABgene, Surrey, UK) and a thermal cycler controlled by the

MyiQ Real Time Detection System software (BioRad, Munich, Germany). All experiments were performed at least in triplicate.

Statistics

Unless described differently, the Student's *t*-test was used to assess statistical significance.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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