The EMBO Journal (2012) 31, 3871–3884 | © 2012 European Molecular Biology Organization | All Rights Reserved 0261-4189/12
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THE EMBO JOURNAL

E2F7 and E2F8 promote angiogenesis through transcriptional activation of VEGFA in cooperation with HIF1

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The E2F family of transcription factors plays an important role in controlling cell-cycle progression. While this is their best-known function, we report here novel functions for the newest members of the E2F family, E2F7 and E2F8 (E2F7/8). We show that simultaneous deletion of E2F7/8 in zebrafish and mice leads to severe vascular defects during embryonic development. Using a panel of transgenic zebrafish with fluorescent-labelled blood vessels, we demonstrate that E2F7/8 are essential for proper formation of blood vessels. Despite their classification as transcriptional repressors, we provide evidence for a molecular mechanism through which E2F7/8 activate the transcription of the vascular endothelial growth factor A (VEGFA), a key factor in guiding angiogenesis. We show that E2F7/8 directly bind and stimulate the *VEGFA* promoter independent of canonical E2F binding elements. Instead, E2F7/8 form a transcriptional complex with the hypoxia inducible factor 1 (HIF1) to stimulate *VEGFA* promoter activity. These results uncover an unexpected link between E2F7/8 and the HIF1-VEGFA pathway providing a molecular mechanism by which E2F7/8 control angiogenesis.

The EMBO Journal (2012) **31,** 3871–3884. doi:10.1038/emboj.2012.231; Published online 17 August 2012
Subject Categories: cell & tissue architecture; development
Keywords: angiogenesis; E2F; HIF; VEGF; zebrafish

Introduction

The E2F family of transcription factors consists of eight family members and is divided into activators (E2F1–3) and repressors (E2F4–8) predominantly based on *in vitro* experiments (DeGregori and Johnson, 2006; Chen *et al*, 2009). However, a picture is beginning to emerge in which E2F family members can function as either activators or repressors of transcription, depending on cellular context, target gene and cofactors (Moon *et al*, 2005; Chong *et al*, 2009; Lee *et al*, 2011). The classical E2Fs (E2F1–6) contain one DNA-binding domain and form heterodimers with DP proteins, whereas the atypical family members, E2F7 and E2F8 (E2F7/8), possess two DNA-binding domains, form homodimers or heterodimers and regulate transcription in a DP-independent manner (Lammens *et al*, 2009). The textbook view of E2Fs suggests a critical role for these factors in control of cell-cycle regulation; however, this paradigm is increasingly under challenge as recent studies show that activator E2Fs are dispensable for cell division and serve critical functions beyond that (Chen *et al*, 2009; Chong *et al*, 2009; Wenzel *et al*, 2011). In line with these cell cycle-independent functions of E2Fs, we recently showed that deletion of *E2f7/8* in mice results in death by embryonic day E11.5 without proliferation defects (Li *et al*, 2008). Instead, $E2f7^{-/-}E2f8^{-/-}$ mice display massive apoptosis and vascular defects at E10.5. Intriguingly, apoptosis but not the vascular defects were rescued upon additional deletion of *E2f1* or *p53* in $E2f7^{-/-}E2f8^{-/-}$ mice, indicating that E2F7/8 regulate vascular integrity through an alternative mechanism.

The development of a functional vasculature consists of two phases: vasculogenesis, the formation of *de-novo* blood vessels by migration, differentiation and coalescence of angioblasts into a primitive network and a second phase, angiogenesis, in which the primitive network is remodelled by sprouting and pruning of endothelial cells into a complex vascular bed. The formation of a functional vascular system depends on the correct generation of a concentration gradient of the secreted mitogen, vascular endothelial growth factor A (VEGFA; Gerhardt *et al*, 2003). VEGFA acts as a chemoattractant that binds as a homodimer to receptor tyrosine kinase 1 and 2 (respectively FLT1 and FLK1/KDR) found on endothelial cells (Olsson *et al*, 2006). FLK1/KDR signalling stimulates endothelial cell proliferation and migration along the VEGFA gradient (Cross *et al*, 2003). Deletion of a single allele of *Vegfa* in mice results in embryonic lethality and display severe vascular defects showing that VEGFA is critical for angiogenesis (Ferrara *et al*, 2003). The major regulator of VEGFA in the context of angiogenesis is the hypoxia induced factor 1 (HIF1; Pages and Pouyssegur, 2005; Liao and Johnson, 2007). HIF1 activity is regulated both by oxygen-dependent and -independent mechanisms (Pouyssegur *et al*, 2006; Semenza, 2009). The presence of oxygen stimulates degradation of HIF1α through the PHD/VHL pathway, resulting in induced HIF1 activity in response to oxygen deprivation (hypoxia) (Semenza, 2009). Growth factor signalling on the other hand stimulates HIF1α

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Received: 8 March 2012; accepted: 26 July 2012; published online: 17 August 2012

translation independent of the oxygen levels, leading to increased HIF activity, even under normoxic conditions (Pouyssegur *et al*, 2006; Semenza, 2009). The importance of HIF1 for vascular development is demonstrated by the observation that mice lacking *Hif1* die around E10.5 with severe vascular defects (Maltepe *et al*, 1997; Iyer *et al*, 1998; Ryan *et al*, 1998).

In this study, we demonstrate that E2F7 and E2F8 regulate primary angiogenesis via transcriptional control of *VEGFA*. This is achieved by the formation of an *E2F7/8–HIF1α* transcriptional complex that directly binds and stimulates the *VEGFA* promoter, whereby E2F7/8 act through a non-canonical E2F-BS and require the presence of HIF1. This study underlines that the function of atypical E2Fs is not solely restricted to cell-cycle regulation, and that their classification as repressors does not meet their sophisticated biological function.

Results

E2f7 and E2f8 are essential for angiogenesis

To investigate E2f7/8 function in angiogenesis, we studied segmental artery formation in transgenic zebrafish embryos with fluorescent-labelled endothelial cells (*Tg(kdrl:gfp)*). Primary angiogenesis in zebrafish embryos starts at 22 h post fertilization (h.p.f.) in the trunk. Sprouts emerge bi-laterally from the dorsal aorta at every somite and migrate dorsally to form the intersegmental arteries (ISAs). At the most dorsal side of the trunk ISAs T-branch and form the dorsal longitudinal anastomotic vessel (DLAV). To investigate the role of *e2f7* and *e2f8* during segmental artery formation, we used morpholino oligomers (MOs) that interfere with the splicing of *e2f7* and *e2f8* (Supplementary Figure S1A). Sequencing of MO induced mis-spliced *e2f7* and *e2f8* mRNA, revealed the presence of frameshifts or intron insertions upstream of the DNA-binding domains (ranging from exons 3 to 6), which are crucial for proper transcriptional activity. Zebrafish treated with these MOs, showed ISAs that prematurely stalled at the horizontal myoceptum (HM) and failed to connect to the DLAV (Figure 1A). This angiogenic defect was observed in embryos injected with an MO against *e2f7* (15% of ISA) or *e2f8* (8% of ISA), but not with a scrambled MO (Figure 1B). A more severe angiogenic phenotype affecting 43% of ISAs was detected in embryos co-injected with both *e2f7* and *e2f8* (*e2f7/8* MOs) (Figure 1B), consistent with the redundant functions for E2F7 and E2F8 observed in mice (Li *et al*, 2008). These *e2f7/8*-deficient zebrafish embryos contained multiple ISA that completely failed to migrate from the dorsal aorta. Gross morphology of these embryos and initial formation of main axial vessels were unaltered. We confirmed specificity of the *e2f7/8* MOs by co-injecting an *in vitro*-transcribed mature *e2f7* and *e2f8* mRNAs that are not recognized by the MOs. Restoring the *e2f7/8* mRNA levels by ectopic expression resulted in a partial but significant rescue of the vascular phenotype, whereas injection of *e2f7* and *e2f8* mRNA alone had no effect on ISA and DLAV formation (Figure 1A and B). Previously, we reported that $E2f7^{-/-}E2f8^{-/-}$ mice suffer from severe apoptosis in the head region, branchial arch, somites and neural tube, which could be rescued by additional deletion of *p53* by generating $E2f7^{-/-}E2f8^{-/-}p53^{-/-}$ triple knockout mice (Li *et al*, 2008). In zebrafish embryos injected with *e2f7/8* MOs, we also observed apoptosis in the neural tube and head region, shown by TdT-mediated dUTP nick end-labelling (TUNEL; Supplementary Figure S1B). To determine whether the vascular defects are a consequence of the apoptotic phenotype, we co-injected a *p53* MO along with *e2f7/8* MOs. Similarly to our previous mouse studies, we were able to rescue the apoptotic phenotype in *e2f7/8/p53* MO-injected zebrafish embryos, but the vascular phenotype persisted to the same extent (Supplementary Figure S1B), demonstrating that the vascular defects are not a sequel of the multifocal cell death observed in *e2f7/8*-deficient zebrafish embryos.

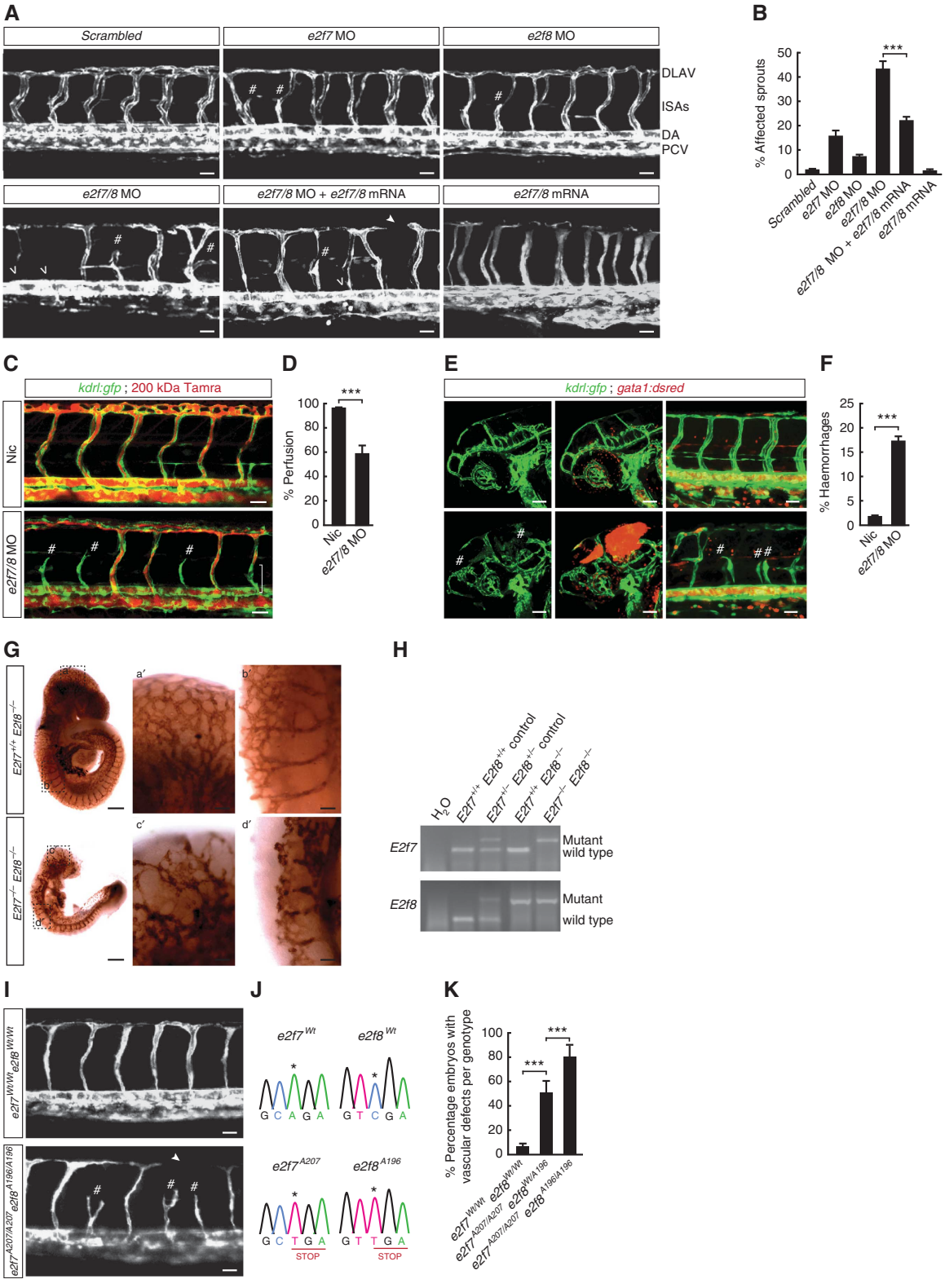
Since $E2f7^{-/-}E2f8^{-/-}$ mice display multifocal haemorrhages and blood vessel dilation (Li *et al*, 2008), we investigated whether *e2f7/8* are required for blood vessel integrity. To this end, we examined blood vessel perfusion and leakage in zebrafish embryos. First, we injected a heavy weight fluorescent protein (200 kDa Tamra) directly into the duct of Cuvier of *Tg(kdrl:gfp)* embryos and analysed the number of completely perfused vessels. Non-injected embryos had almost 100% blood vessel perfusion, in contrast *e2f7/8* MO-injected embryos showed an average perfusion rate of 58% (Figure 1C and D).

Second, we knocked down *e2f7/8* in transgenic zebrafish embryos expressing GFP in endothelial cells and dsRED in red blood cells *TG(kdrl:gfp;gata1:dsred)*, and monitored blood flow. In 18% of *e2f7/8* MO-treated embryos, we found extravascular blood accumulation in the head and eye region (Figure 1E and F). In addition, formation of head and eye vessels of *e2f7/8*-deficient embryos was impaired and often showed a dilated phenotype (Figure 1E). Conformingly, we found severe vascular defects of head and inter somatic vessels in E9.5 mouse fetuses deficient for *E2f7/8* visualized by whole mount immunohistochemistry with an antibody directed against platelet endothelial cell adhesion molecule-1 (PECAM-1; Figure 1G).

To complement the morpholino studies on the role of E2f7/8 in angiogenesis, we identified in a zebrafish

Figure 1 E2f7 and E2f8 are essential for angiogenesis. (**A**) Lateral images of *TG(kdrl:gfp)* zebrafish embryos 48 h.p.f. Scale bars are 50 μm. (**B**) Quantification of angiogenesis defect, all ISAs that showed abnormalities were counted ($n > 150$ per condition). (**C**) *TG(kdrl:gfp)* embryos were injected with fluorescent 200 kDa Tamra (red) to visualize blood vessel perfusion. Scale bars are 50 μm. (**D**) Quantification of perfused ISAs (non-injected control (NIC) $n = 10$; *e2f7 + 8* MO $n = 15$). (**E**) *TG(fli1a:gfp;gata1:dsred)* embryos, in which blood vessels are marked with green and red blood cells in red. Scale bars are 50 μm. (**F**) Quantification of haemorrhages (NIC $n = 43$; *e2f7 + 8* MO $n = 62$). (**G**) CD-31 (PECAM-1) staining of E9.5 mouse embryos. Scale bars are 500 and 100 μm for a′, b′, c′ and d′. (**H**) Genotyping using allele-specific PCR primers on DNA from presented embryos. (**I**) Lateral images of *wild-type TG(kdrl:gfp)* and $e2f7^{A207/A207}$; $e2f8^{A196/A196}$; *TG(kdrl:gfp)* zebrafish 48 h.p.f. Scale bars are 50 μm. (**J**) Genotyping was performed by sequencing, wild-type and mutated nucleotide are indicated. (**K**) Quantification of angiogenesis defect, all embryos that showed vascular abnormalities were selected and genotyped ($n = 90$). Abbreviations: dorsal longitudinal anastomotic vessel (DLAV), intersomatic arteries (ISAs), dorsal aorta (DA, blue bar) and posterior cardinal vein (PCV, red bar) are labelled. For all images applies that # depicts ISAs that are stalled at HM, additionally in (**C**) it indicates stalled ISA at the HM that are unperfused. Open arrow heads indicate ISAs that failed to sprout from the DA and closed arrow heads depicts the absence DLAV. Bracket shows unperfused part of ISA. All quantified data are presented as the average (± s.e.m.) compared to the control condition in three independent experiments (***$P < 0.001$).

mutagenesis library generated by targeted induced local lesions in the genome (TILLING; Wienholds *et al*, 2003), an *e2f7* (*e2f7*^{A207}) and *e2f8* (*e2f8*^{A196}) mutant zebrafish. Both mutants harbour a mutation within the highly conserved first DNA-binding domain, resulting in a stop codon (Supplementary Figure S1C). Transient expression of myc-tagged versions of zebrafish *e2f7*^{A207} and *e2f8*^{A196} mutants in mouse embryonic fibroblasts (MEFs) prevented full-length translation of these atypical E2Fs (Supplementary Figure S1C). Initial analysis of double mutant embryos showed a similar defect in angiogenesis and apoptosis compared to *e2f7/8* MO-treated embryos, thereby confirming *e2f7/8* function to control angiogenesis and specificity of *e2f7/8* MOs (Figure 1I–K).



Collectively, these findings demonstrate that E2f7/8 are required for sprouting angiogenesis and formation of ISAs in zebrafish and mice, without affecting vasculogenesis.

E2f7 and E2f8 regulate angiogenesis through stimulation of vegfA expression

To determine the mechanism how atypical E2Fs control angiogenesis, we searched for angiogenic factors that would be directly regulated by atypical E2Fs. We have previously taken an unbiased approach of chromatin immunoprecipitation in combination with sequencing (ChIP-seq) to identify target genes of E2F7 (Westendorp *et al*, 2011). Remarkably, this genome-wide analysis revealed that E2F7 was significantly enriched on the *VEGFA* promoter, whereas no other angiogenic factors were identified in this screening. Since primary sprouting of ISAs in zebrafish is largely dependent on *vegfA* (Ferrara *et al*, 2003), we investigated if *e2f7/8* control angiogenesis through regulation of *vegfA* expression.

We first determined whether *e2f7/8* expression pattern during zebrafish development coincides with tissues involved in secretion of *vegfA*, which is expressed predominantly within the somites, head and eye region. Previously, we showed ubiquitous *E2f7/8* expression at E9.5 in mouse embryos (Li *et al*, 2008) and found, by *in situ* hybridization, a similar ubiquitous expression of *e2f7/8* during the early stages of development, followed by a more specific expression in the head region in later stages (50 h.p.f.) (Figure 2A). In addition, neural tube, eye, brain and caudal vein appear to have a more pronounced expression. The expression pattern of *e2f7/8* overlaps in multiple tissues with the expression of *vegfA*, in particular in the brain and eye regions (Figure 2B).

Next, we investigated by *in situ* hybridization and quantitative PCR whether *vegfA* was the only vegf family members to be affected by the knockdown (KD) of *e2f7/8*. The zebrafish *vegfA* gene has been duplicated during evolution, resulting in two different isotypes, *vegfAa* and *vegfAb*. *VegfAa* transcribes the two most potent isoforms, $vegfAa_{165}$ and $vegfAa_{121}$, which are indispensible and predominantly expressed during development (Bahary *et al*, 2007). We found that expression of *vegfAa* was reduced about 40% in *e2f7/8* MO-injected embryos, whereas *vegfAb, B and C* showed no apparent difference in expression (Figure 2B and C). Notably, *vegfD* expression was upregulated, although it is expressed exclusively in the tailbud and little is known about its function during angiogenesis. In addition, *e2f7* and *e2f8* double mutant zebrafish embryos had reduced *vegfAa* mRNA levels compared to *wild-type* littermates (Supplementary Figure S2A and B). In contrast, *e2f1* a known target gene for E2f7/8 (Li *et al*, 2008; Zalmas *et al*, 2008), was upregulated in *e2f7/8* mutant zebrafish (Supplementary Figure S2B). Given the above observations, we hypothesize that the angiogenic defects observed in zebrafish and mice deficient for atypical E2Fs result from decreased VegfAa expression.

We took two experimental approaches to test this possibility, first we determined whether reduction of *vegfAa* levels enhances the ISAs defect of *e2f7/8*-deficient embryos. To this extend, *Tg(kdrl:gfp)* embryos were co-injected with *vegfAa* MO along with *e2f7/8* MOs. Single injection of *e2f7/8* MOs or *vegfAa* MO at subcritical concentrations did not result in any apparent ISAs defect (Figure 2D upper panel; Figure 2E). Importantly, co-injection of *e2f7/8* and *vegfAa* MOs at these subcritical levels resulted in an angiogenic phenotype in which 19% of the ISAs stalled at the horizontal myoseptum. We also injected *e2f7/8* and *vegfAa* MOs at a dosage where each MO injection alone causes an angiogenic defect and observed a more severe sprouting defect compared to embryos with single MO injections (Figure 2D lower panel; Figure 2E).

In a second approach, we tested whether the vascular phenotype of *e2f7/8*-deficient zebrafish embryos can be rescued by ectopic expression of *vegfAa* mRNA. First, we co-injected *vegfAa* MO with *vegfAa* mRNA and observed a 50% reduction in the *vegfAa* MO induced phenotype (Figure 2F and G). However, we never observed a complete rescue, which might be due to the fact that ectopically expressed *vegfAa* mRNA is not able to produce a proper *vegfAa* gradient. Next, we co-injected *e2f7/8* MO together with *vegfAa* mRNA and found a 70% reduction of the vascular phenotype (Figure 2F and G). Moreover, we ectopically expressed *e2f7/8* mRNA in *vegfAa* MO-treated embryos and also found here a significant reduction (30%) of the vascular phenotype (Figure 2F and G). Together, these findings provide strong evidence that the vascular defect in *e2f7/8*-deficient embryos is caused through reduced VegfAa expression.

Since VegfAa is a potent growth factor for endothelial cells, we furthermore tested if E2f7/8 can stimulate VegfAa expression and regulate endothelial cell numbers. For this purpose, we used transgenic embryos (*Tg(fli1:negfp;flk1:mCherry)*, which express a nuclear localized eGFP and cytoplasmic mCHERRY in endothelial cells. At 24 h.p.f., most ISA in control *Tg (fli1:negfp;flk1:mCherry)* embryos contained three or four cells as reported previously (Siekmann and Lawson, 2007; Supplementary Figure S2C and D). Embryos injected with *e2f7/8* MOs displayed reduced cell numbers down to one nucleus per sprout, and these cells often failed to migrate beyond the horizontal myoseptum (Supplementary Figure S2C and D). Consistently, segmental arteries of *VegfAa* MO-injected embryos phenocopied the loss of E2f7/8 in zebrafish (Supplementary Figure S2C and D). Injection of *e2f7/8* mRNA on the other hand resulted in sprouts containing increased cell numbers with up to seven cells without affecting sprouting and migration (Supplementary Figure S2C and D). The changes in number of cells were not only restricted to the ISAs, but were also present in the caudal vein area (Supplementary Figure S2C, not quantified). Importantly, the increased endothelial cell numbers upon injection *e2f7/8* mRNA were associated with increased *vegfAa* mRNA levels (Supplementary Figure S2E). From these results, we conclude that E2f7/8 stimulate endothelial cell numbers through induction of VegfAa expression.

E2F7/8 directly control VEGFA expression

To investigate how E2F7/8 regulate VEGFA expression at the molecular level, we used two human cell lines (U2OS and HeLa) that have been successfully applied to study E2F7/8 function (Zalmas *et al*, 2008). In addition, ChIP and western blot grade E2F7 antibodies are currently only available for human and mouse, but not for zebrafish E2F7. Expression analysis of E2F7/8 in these cells revealed that U2OS cells express exceedingly low E2F8 levels (transcript levels are at

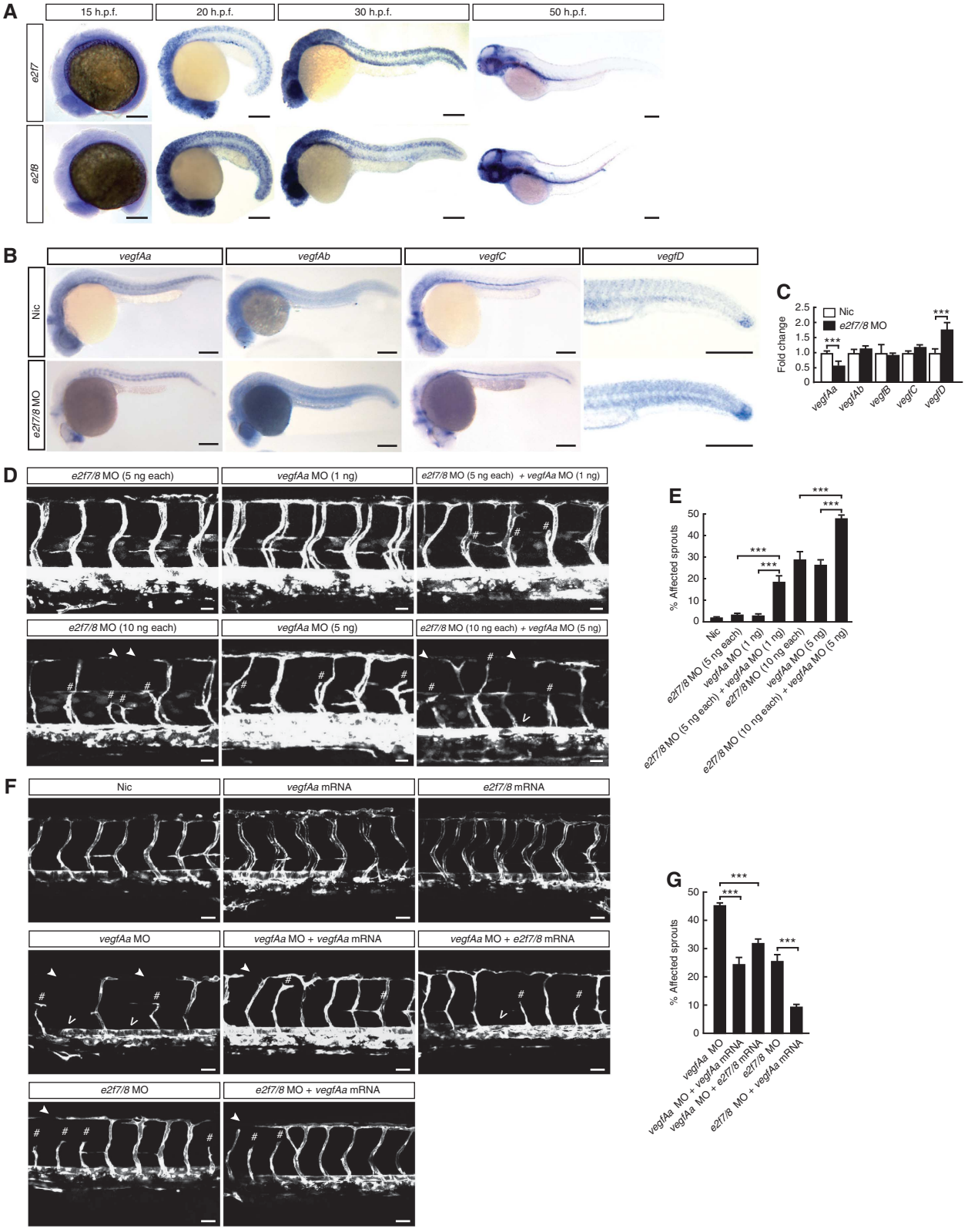


Figure 2 E2f7 and E2f8 regulate angiogenesis through stimulation of vegfA expression. (**A**) Expression of *e2f7/8* at four developmental stages visualized by *in situ* hybridization (ISH). Scale bars are 250 μm. (**B**) ISH and (**C**) qPCR of vegf ligands at 24 h.p.f. in control and e2f7/8 MO-treated embryos ($n = 10$). Scale bars are 250 μm. (**D**) Indicated MOs were injected in *TG(kdrl:gfp)* embryos at subcritical (upper row) or normal concentrations (lower row). Scale bars are 50 μm. (**E**) Graph represents the percentage of affected sprouts ($n > 55$ per condition). (**F**) Representative images of *TG(kdrl:gfp)* embryos (co-)injected with *e2f7/8* MO (10 ng each), *vegfAa* (5 ng) MO, *e2f7/8* (200 pg each) and *vegfA* mRNA (200 pg). Scale bars are 50 μm. (**G**) Percentage of affected sprouts was quantified, at least $n = 54$ embryos per condition were counted. For all images applies that an # indicates stalled ISA at the HM, open arrow head points towards ISAs that failed to sprout from the DA and closed arrow heads show the absences of the DLAV. All quantified data are presented as the average (± s.e.m.) compared to the control condition in two independent experiments (***$P < 0.001$).

least 1000-fold lower compared to E2F7), whereas HeLa cells express E2F7 and E2F8 to a similar level (Supplementary Figure S3A). To test whether E2F7/8 regulate *VEGFA* expression in human cells, we used RNAi-mediated knockdown (KD) for E2F7 in U2OS cells and both E2F7 and E2F8 in HeLa cells (validation of siRNA is shown in Supplementary Figure S3B). KD of *E2F7* in U2OS cells significantly reduced *VEGFA* expression, while *E2F1* expression was enhanced as expected (Figure 3A). In HeLa cells, simultaneous KD of *E2F7/8* resulted in a comparable decrease of *VEGFA* expression (Supplementary Figure S4A).

To investigate on the other hand if induction of E2F7/8 activity would stimulate *VEGFA* expression, we used HeLa cells that express E2F7-EGFP under control of a doxycycline-inducible promoter (Westendorp *et al*, 2011). After 4 h of doxycycline administration, *VEGFA* mRNA levels were induced and continued to accumulate at 8 and 12 h (Figure 3B). The E2F7/8-repressed target gene *E2F1* is regulated with similar kinetics (Figure 3B), suggesting that E2F7/8 control VEGFA, like E2F1, through direct promoter regulation. This was indeed confirmed by our previous reported genome-wide screen for E2F7-binding sites (Westendorp *et al*, 2011), in which we found binding of E2F7 to the proximal *VEGFA* promoter (Figure 3C). These data suggest that atypical E2Fs induce *VEGFA* expression through direct promoter regulation.

E2F7/8 cooperate with HIF1 to transcriptionally activate the VEGFA promoter both under normoxic and hypoxic conditions

Given the direct control of *VEGFA* expression by E2F7/8 (Figure 3), we computationally analysed the proximal *VEGFA* promoter region identified by ChIP sequencing (Figure 3C) for E2F-binding sites (E2F-BS). This revealed the presence of two putative E2F-BS in a 1173-bp fragment that corresponds to −1312/−140 of the human *VEGFA* promoter (Figure 4A). Interestingly, these E2F-BS are in close proximity of binding sites for HIF1, a key transcriptional regulator of *VEGFA*, that is regulated by both oxygen-dependent and -independent mechanisms (Pouyssegur *et al*, 2006; Semenza, 2009). Therefore, we hypothesized that HIF1 and E2F7/8 cooperate in transcriptional control of *VEGFA*. First, we tested whether HIF1 contributes to normoxic *VEGFA* expression given the *VEGFA* promoter binding and the

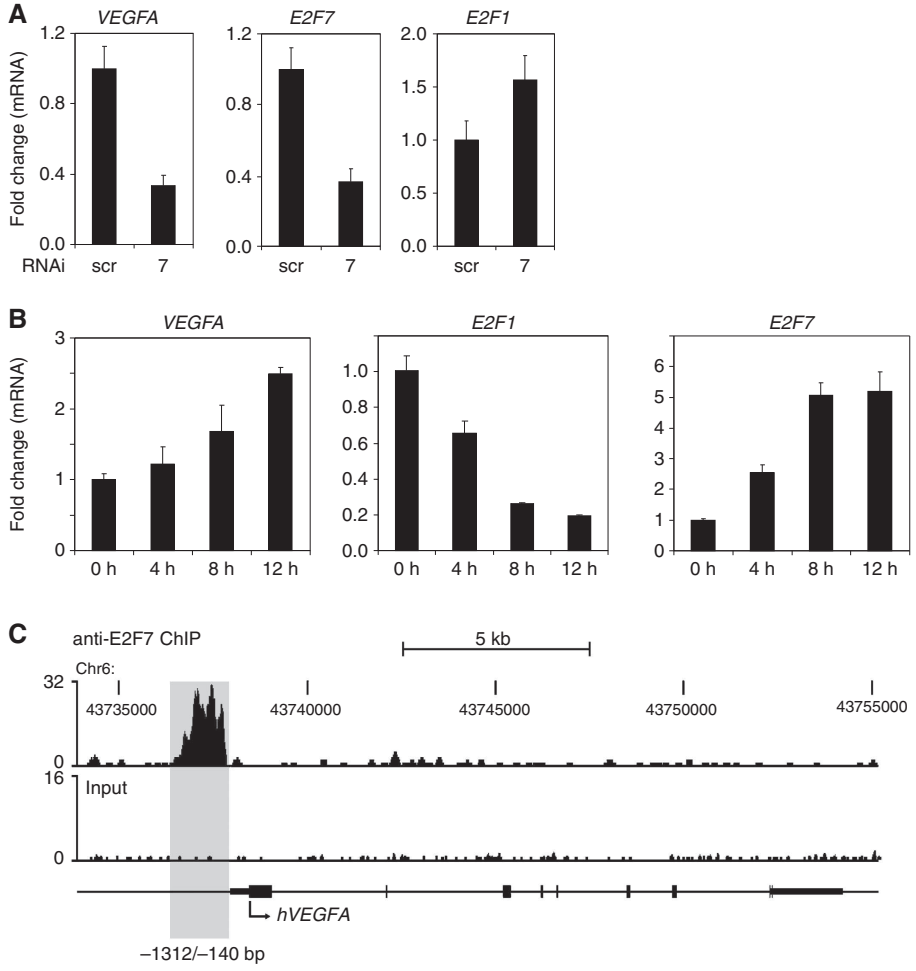


Figure 3 E2F7/8 bind the *VEGFA* promoter and positively control VEGFA expression. (**A**) Effect of E2F7 (7) or control (scr.) siRNA on indicated transcripts in U2OS cells. (**B**) S-phase synchronized HeLa cells expressing E2F7-EGFP (doxycycline inducible). Indicated transcripts were determined by qPCR in 0, 4, 8 and 12 h doxycycline-treated versus vehicle-treated cells. (**C**) Upper panel, ChIP sequencing revealed E2F7 binding (black peaks) to the proximal region (−1312/−140; grey box) of the human *VEGFA* promoter. Lower panel, sequencing of representative input sample is shown as a negative control. Gene organization of *VEGFA* is shown at the bottom. Boxes indicate exons. All quantified data are presented as the average (± s.d.) compared to the control condition in three independent experiments.

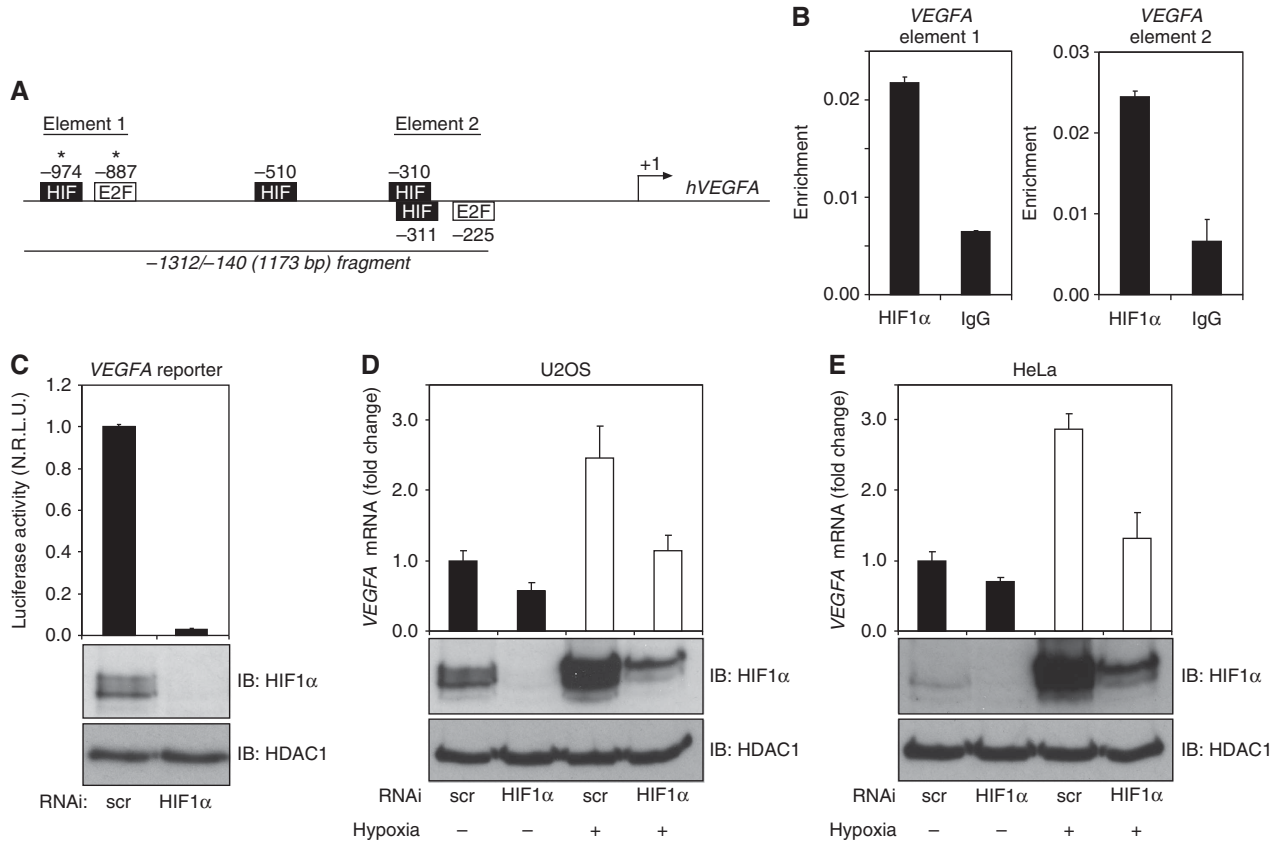


Figure 4 HIF1 binds and stimulates the *VEGFA* promoter in normoxia. (**A**) Schematic presentation of a human *VEGFA* promoter, −1312/−140 (1173 bp) corresponds to the region identified by ChIP sequencing (Figure 3C). Potential E2F and HIF binding sites (BS), identified by Matinspector software, are indicated. Positioning is based on the transcriptional start site (bent arrow). Two promoter elements (indicated as element 1 or 2) contain closely located E2F- and HIF-BS. Sites indicated with asterisks are 100% identical between human and mouse. (**B**) HIF1α ChIP (*VEGFA* promoter) performed on normoxic HeLa cells. (**C**) Upper panel, activity of a 47-bp (−985/−939) *VEGFA* reporter plasmid, containing the −974 HIF-BS, co-transfected with HIF1α or scrambled (scr) siRNA in normoxic U2OS cells. Lower panels, western blot for HIF1α and loading control HDAC1. (**D**) U2OS cells transfected with HIF1α or scr siRNA, grown under normoxic (black bars) or hypoxic conditions (white bars). Upper panel shows *VEGFA* mRNA levels and lower panel shows western blot for HIFα and HDAC1 (loading). (**E**) Similar to (**D**) but now for HeLa cells. All quantified data are presented as the average (±s.d.) compared to the control condition in three independent experiments.

regulation of angiogenesis by E2F7/8 under normal conditions. By performing ChIP assays, we found significant enrichment of HIF1α to *VEGFA* promoter element 1 and 2 under normoxia (Figure 4B). To test if HIF1 contributes to *VEGFA* promoter activation under these conditions, we performed reporter assays using a 47-bp *VEGFA* promoter element surrounding the reported HIF responsive site −974 (Forsythe *et al*, 1996). KD of HIF1α reduced promoter activity of the −974 element about 30-fold (Figure 4C). In addition, we analysed if HIF1 stimulates normoxic *VEGFA* mRNA expression. U2OS and HeLa cells were transfected with a control or HIF1α siRNA and cultured in normoxia or hypoxia. Although normoxic U2OS and HeLa express HIF1α to a different extend, HIF1α expression was efficiently suppressed (Figure 4D and E). This resulted in reduced *VEGFA* mRNA levels both in normoxia in and hypoxia (Figure 4D and E). Together, these data show that HIF1, similar to E2F7, also binds and stimulates the *VEGFA* promoter in normoxia.

Next, we examined if E2F7/8 cooperate with HIF on hypoxic induction of VEGFA expression. For this purpose, we ablated E2F7 and HIF1 alone or in combination by RNAi in U2OS cells. Remarkably, KD of E2F7 or HIF1α reduced hypoxia-induced *VEGFA* expression to a similar extent, which was further reduced upon their simultaneous KD (Figure 5A). Inactivation of E2F7/8 in HeLa cells and MEFs also resulted in significantly reduced hypoxic VEGFA expression (Supplementary Figure S5A and B). These data show that both E2F7/8 and HIF are required for hypoxic *VEGFA* induction, and suggest functional cooperation between atypical E2Fs and HIF1. Furthermore, E2F7 did not affect expression of NIX (Figure 5A) and FLT1 (Supplementary Figure S4B), two other HIF target genes. This suggests that E2F7/8 do not affect HIF-regulated gene expression in general, but rather acts in a promoter-specific manner.

We then investigated if simultaneous activation E2F7 and HIF1 would cooperate on stimulation of *VEGFA* mRNA expression. Therefore, we induced E2F7 using a previously reported doxycycline-inducible E2F7 HeLa cell line (Westendorp *et al*, 2011) in the presence of normoxia or hypoxia (HIF activation). As shown in Figure 5B, induction of E2F7-EGFP stimulated *VEGFA* expression under normoxia (~3-fold, Figure 5B, lane 6), whereas no effect was observed in the control cell line (lane 2). Notably, activation of E2F7 in combination with hypoxia synergistically potentiated *VEGFA*

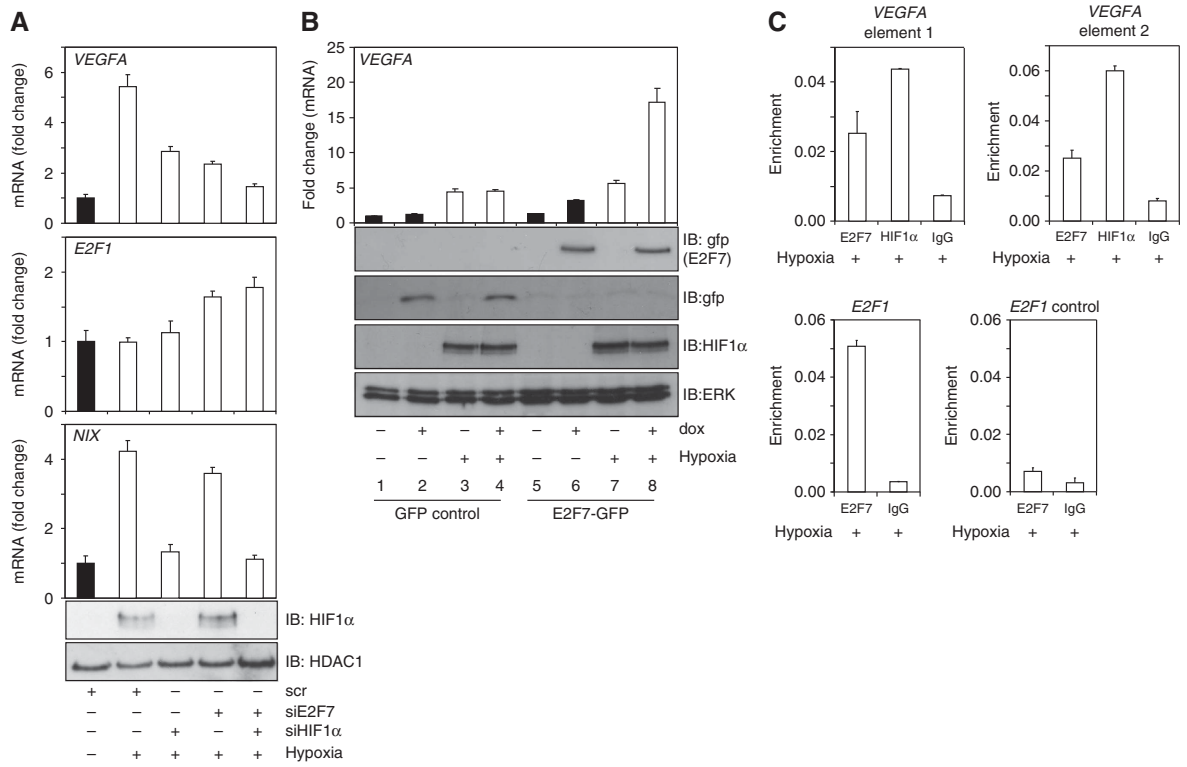


Figure 5 E2F7/8 and HIF1 are required for hypoxic *VEGFA* expression. (**A**) U2OS cells were transfected with a total of 150 pmol siRNA: 75 pmol *E2F7* plus *HIF1α*, or 75 pmol *E2F7* or *HIF1α* plus 75 pmol scr. Transfected cells were cultured in normoxia or hypoxia. qPCR was performed for indicated transcripts. Lower two panels show HIF1α and HDAC1 (loading) protein levels. (**B**) Doxycycline-inducible E2F7-EGFP or control doxycycline-inducible EGFP cells were treated with 20 h dox, 4 h hypoxia, 20 h dox of which the last 4 h in combination with hypoxia, or left untreated. Upper panel shows qPCR analysis of *VEGFA* mRNA. Lower panels display western blot for GFP, HIF1α or ERK (loading). (**C**) E2F7 and HIF1α ChIPs were performed on hypoxic HeLa cells. ChIP on the *E2F1* promoter served as a positive control. A non-specific region 700 basepairs upstream of the *E2F* binding site in the *E2F1* promoter served as a negative control. All quantified data are presented as the average (± s.d.) compared to the control condition in three independent experiments.

expression (16-fold; Figure 5B, lane 8). Additional ChIP assays performed in hypoxic HeLa cells showed both E2F7 and HIF binding to elements 1 and 2 on the *VEGFA* promoter (Figure 5C). Since we were able to distinguish a previously reported *E2F* site in the *E2F1* promoter (Li *et al*, 2008; Zalmas *et al*, 2008) from a non-specific region 700 bp upstream (Figure 5C, lower panels), we conclude that the resolution of our ChIP assays is sufficient to state that E2F7 and HIF1 bind to both elements 1 and 2 (Figure 5C). Taken together, our data show that E2F7/8 and HIF1 cooperate on transcriptional activation of *VEGFA* in normoxia and hypoxia.

E2F7 and E2F8 form a transcriptional complex with HIF1α

Given that atypical E2Fs and HIF1 bind to the same regions of the *VEGFA* promoter and both cooperate in regulating VEGFA transcription, we tested whether these proteins interact with each other. Co-immunoprecipitation assays using U2OS cells co-expressing myc-tagged E2F7 and flag-tagged HIF1α showed that E2F7 interacts with HIF1α (Figure 6A). Under these conditions, we also confirmed the previously reported interaction between E2F7 and E2F8 (Figure 6A; Li *et al*, 2008; Zalmas *et al*, 2008). Furthermore, we could not detect an interaction between HIF1α and FOXO3a (Supplementary Figure S6A), an indirect regulator of HIF1α (Tang and Lasky, 2003; Bakker *et al*, 2007). Using a similar approach, we could also detect an interaction between E2F7 and HIF2α (Supplementary Figure S6B). Importantly, we also detected an interaction between zebrafish E2f7 and E2f8 and human HIF1α (Figure 6B), showing the conserved nature of the interaction. To confirm the E2F7/8–HIF1 interaction at the endogenous level, we tested and verified that endogenous HIF1α interacts with myc-tagged E2F7 (Figure 6C) and endogenous E2F8 (Figure 6D). The latter co-immunoprecipitation assays were performed in *wild-type* MEFs and Cre-deleted *E2f7/8*-deficient MEFs, which served as an additional negative control. To investigate which HIF1α domain is required for atypical E2F binding, we overexpressed myc–E2F7 in combination with different flag–HIF1α deletion mutants. E2F7 binds to wild-type HIF1α, consisting of 826 amino acids, but not to an N-terminal deletion mutant (543–826) that lacks amino acids 1–542 (Figure 6E). To further narrow down the N-terminal HIF1α interaction domain, we examined E2F7 binding to different N-terminal HIF1α mutants consisting of amino acids 1–80, 1–170 or 1–300. We found that E2F7 specifically binds to the 80 N-terminal located amino acids of HIF1α (Figure 6E), a domain required for dimerization and DNA binding (Metzen and Ratcliffe, 2004; Semenza, 2007). Consistent with our data, the N-terminal part of HIF1α is known to be regulated in an oxygen-independent manner (Liu and Semenza, 2007).

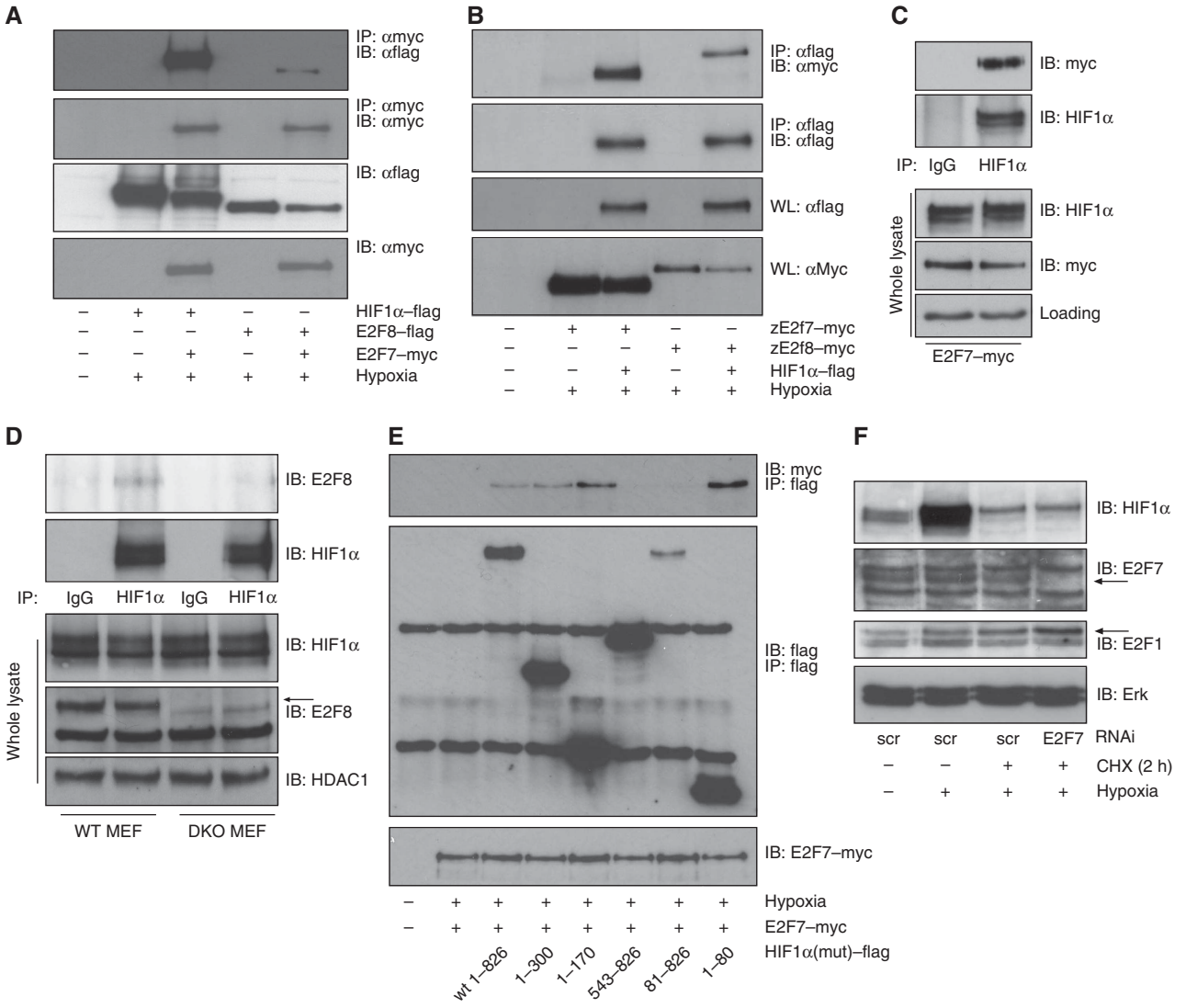


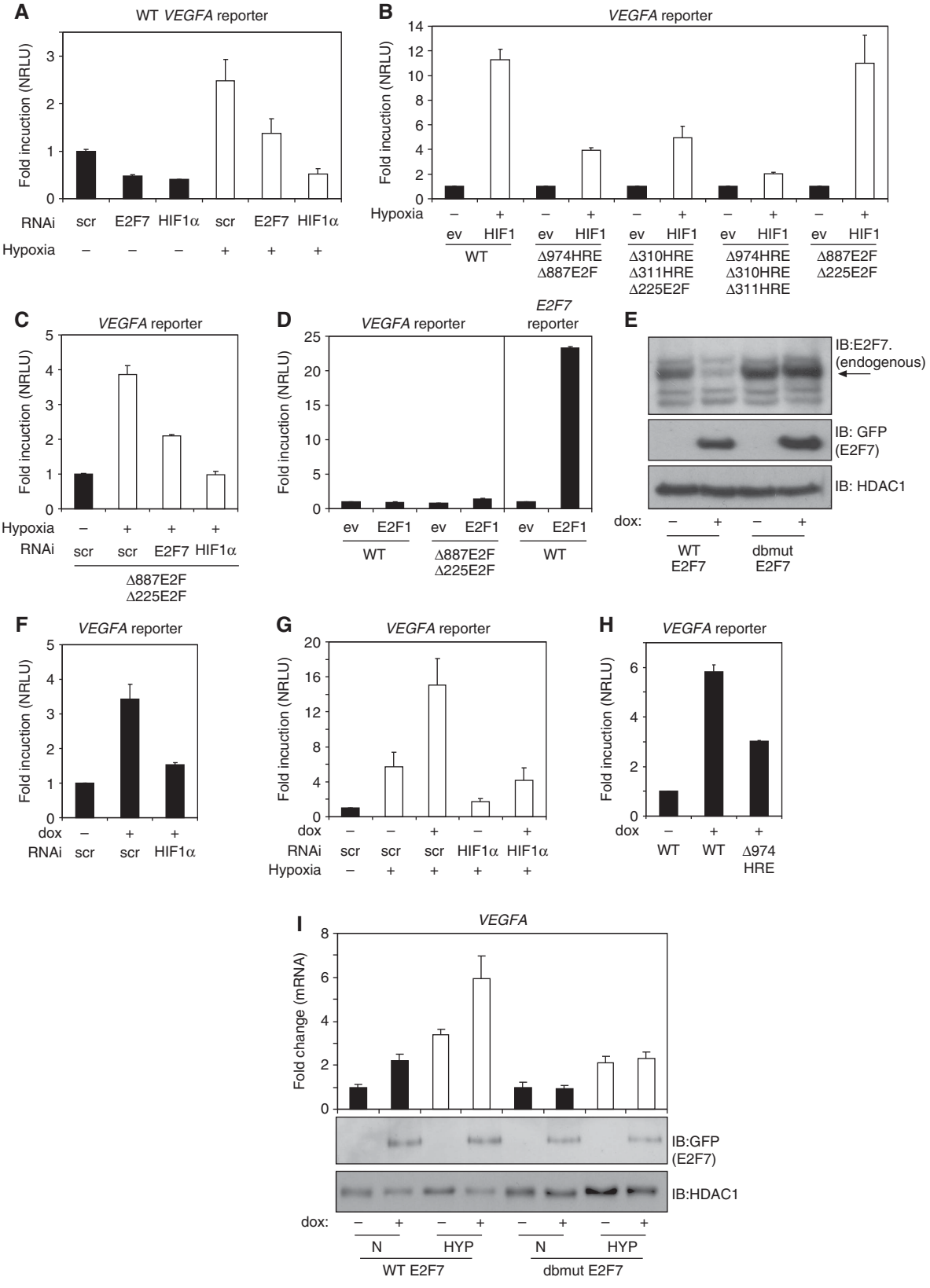
Figure 6 The N-terminal 80 amino acids of HIF1α are required for E2F7/8 binding. (**A**) Lysates of U2OS cells transfected with flag-tagged HIF1α or E2F8 in combination with an empty vector (−) or myc-tagged E2F7 were maintained in hypoxic conditions except for empty vector. Immunoprecipitation (IP) was performed using a myc antibody. Precipitation was immunoblotted (IB) using a flag antibody. To rule out non-specific precipitation, myc antibody and beads were added to all lysates. (**B**) Lysates from hypoxic U2OS cells transfected with myc-tagged versions of zebrafish E2f7 or E2f8, in combination with empty vector (−) or flag-tagged human HIF1α (WL: whole cell lysates). (**C**) U2OS cells were transfected with myc-tagged E2f7 and cultured in hypoxia. Endogenous HIF1α was immunoprecipitated (IP). IP efficiency and co-precipitating E2F7-myc are shown in the upper panel. IgG serves as a negative control. Lower panel shows expression levels for HIF1α and E2F7-myc in whole lysates. A non-specific band shows equal loading. (**D**) Endogenous Hif1α was immunoprecipitated from *wild-type* and *E2f7/8* double knockout (Cre-deleted) MEFs cultured in hypoxia. Upper panels show co-precipitating endogenous E2F8, as well as Hif1α backstaining to show efficient IP. Lower panel shows proteins levels for Hif1α, E2f8 and Hdac1 (loading). (**E**) Flag-tagged wild-type and HIF1α deletion mutants were co-transfected with wild-type E2F7-myc in U2OS cells. All conditions with HIF1α were cultured in hypoxia. IP was performed using a flag antibody. (**F**) U2OS cells were transfected with scr or *E2F7* siRNA, and treated with or without cycloheximide (CHX). Whole lysates were immunostained for HIF1α, E2F7, E2F1 and ERK (loading).

Additionally, we tested if E2F7/8 regulate HIF protein stability. However, KD of E2F7 did not change the rate of HIF1α degradation when HIF1α synthesis was blocked by cycloheximide (Figure 6F). Consistently, increasing or decreasing E2F7/8 expression levels by ectopic overexpression (Figures 5B and 6A; Supplementary Figure S6A and B) or siRNA mediated KD (Figures 5A and 6F; Supplementary Figure S7A) did also not affect HIF1α protein levels. These findings show that E2F7/8 form a transcriptional complex with HIF1α without affecting overall HIF1α protein stability, consistent with our conclusion above that E2F7/8 cooperate with HIF1 in a promoter-specific manner.

E2F7/8 directly bind and stimulate the VEGFA promoter independent of canonical E2F-BS and in cooperation with HIF1

To investigate through which binding sites the E2F7/8–HIF1 transcriptional complex regulates the *VEGFA* promoter, we cloned the highly conserved −1312/−140 human *VEGFA* promoter region, containing elements 1 and 2 (Figure 4A) to which both E2F7 and HIF bind, and performed luciferase reporter assays. First, we confirmed whether E2F7 and HIF1 regulates VEGFA expression through this promoter region by transfecting U2OS cells with the −1312/−140 reporter construct in combination with a control, E2F7 or HIF1α siRNA.

KD of E2F7 or HIF1α reduced *VEGFA* promoter activity in normoxia and hypoxia (Figure 7A, confirmation of protein KD in the reporter assay is shown in Supplementary Figure S7A). To analyse the role of the HIF-BS and putative E2F sites in *VEGFA* promoter regulation by the E2F7/8–HIF1 complex, we mutated the HIF-BS and E2F-BS (as indicated in Figure 7B and Supplementary Figure S7B). First, we tested the ability of HIF1 to activate these promoters. HIF1 induced WT promoter activity 11-fold. This was reduced to 4- to 5-fold when the E2F and HIF sites in element 1 or 2 were mutated, and was almost completely abolished upon simultaneous deletion of all HIF-BS (Figure 7B). Notably, mutation of both E2F sites



(−887/−225) had no effect on *VEGFA* promoter activation by HIF1 (Figure 7B). To investigate if E2F7 on the other hand requires the −887 and −225 E2F-BS to activate the *VEGFA* promoter, U2OS cells were transfected with the −1312/−140 *VEGFA* reporter construct mutant for both E2F-BS in combination with scrambled and E2F7 siRNA. Surprisingly, KD of E2F7 still reduced ΔE2F-mutant *VEGFA* promoter activity (Figure 7C), showing that E2F7 stimulates the *VEGFA* promoter independent of the putative E2F-consensus sites. Because the putative E2F-BS −887 and −225 resemble the classical E2F binding consensus (Supplementary Figure S7B) we also tested if an activator E2F could stimulate *VEGFA* promoter through the putative E2F-BS. However, ectopic E2F1 expression could not activate the wild-type or ΔE2F mutant *VEGFA* promoter, while it induced an E2F7 reporter construct (Figure 7D), supporting our conclusion that the putative E2F sites in the *VEGFA* promoter are not functional. We then analysed whether E2F7 requires the presence of HIF1 to activate the *VEGFA* promoter. The doxycycline-inducible E2F7 HeLa cell line was transfected with the previously reported HIF1-responsive 47 bp *VEGFA* promoter region surrounding the −974 HIF-BS, in combination with an HIF1α or control siRNA. Activation of E2F7 in these cells (Figure 7E) induced the *VEGFA* reporter, whereas KD of HIF1α almost completely inhibited this response (Figure 7F). As a control, induction of E2F7-repressed endogenous E2F7 expression (Figure 7E). In addition, ectopic expression of E2F7 in hypoxia enhanced the hypoxic induction of a −1007/−828 *VEGFA* reporter up to 16-fold, which was abrogated upon KD of HIF1α (Figure 7G). In an alternative approach, the doxycycline-inducible E2F7 HeLa cells were transfected with the wild-type 47 bp *VEGFA* reporter or a mutant reporter in which the −974 HIF-BS is mutated. Activation of E2F7 induced the WT −974 reporter about six-fold which was reduced to three-fold upon mutation of the −974 HRE (Figure 7H). These data show that E2F7 activates the *VEGFA* promoter in cooperation with HIF1 and suggest that E2F7/8 do so independent of their DNA binding ability. To investigate this possibility, we tested if a doxycycline-inducible, DNA binding-deficient E2F7 mutant (Figure 7E) could stimulate endogenous *VEGFA* expression. As before (Figure 5B), induction of wild-type E2F7 induced *VEGFA* mRNA expression in both normoxia and hypoxia, which was lost in the DNA-binding mutant (Figure 7I). Together, these data show that E2F7/8 directly bind to the *VEGFA* promoter through a non-canonical E2F-BS, and that the presence of HIF1 is required for full activation of the *VEGFA* promoter.

Discussion

In this study, we show that E2f7 and E2f8 are strictly required for angiogenesis during embryonic development in mice and zebrafish. We provide strong evidence, from both *in vitro* and *in vivo* experiments, that the observed vascular defects in these *E2f7/8*-deficient embryos are caused by reduced expression of *vegfAa*. *In situ* hybridization and qPCR analysis revealed that *VegfA* expression is strongly reduced in embryos and cell lines deficient for atypical E2fs, while induction of atypical E2fs results in enhanced *VegfA* expression. Moreover, we show that inactivation of *vegfAa* phenocopied the sprouting defects of *e2f7/8*-deficient embryos and that ectopic expression of *vegfAa* mRNA can partially rescue the vascular phenotype of *e2f7/8*-deficient zebrafish embryos. From these findings, we conclude that inactivation of atypical E2fs in zebrafish impairs VegfAa expression, a critical regulator of angiogenesis (Ferrara *et al*, 2003).

Contrary to many other studies demonstrating that atypical E2Fs function as transcriptional repressors (Chen *et al*, 2009; Lammens *et al*, 2009), our data propose a model in which E2F7/8 can function as activators of transcription when acting through non-canonical E2F-BS in cooperation with a transcriptional activator. In the context of the *VEGFA* promoter this activator is HIF1.

Interestingly, two other recent studies reported a similar activator role for E2F7/8. In the liver, E2F7 binds and stimulates the *TERT* promoter, while E2F8 binds and activates the *CYCLIN D1* promoter (Deng *et al*, 2010; Sirma *et al*, 2011). How atypical E2Fs enhance promoter activity in these studies is not understood, although it was suggested that E2F7/8 act in a dominant-negative manner by blocking the binding of other E2F repressor complexes (Deng *et al*, 2010). Based on protein information, the activator function of atypical E2Fs is unpredicted, as they lack a transactivation domain (Lammens *et al*, 2009). However, here we provide new insights into the transcriptional activator role of atypical E2Fs. We show that activation does not occur through direct interaction with a canonical E2F-BS, but through direct interaction with non-canonical E2F-BS, and in cooperation with a transcriptional activator. Interestingly, E2F1, although classified as an transcriptional activator, inhibits tumour vascularization through repression of the *VEGFA* promoter which was also suggested to occur independent of canonical

Figure 7 E2F7/8 stimulation of *hVEGFA* promoter region −1312/−140 requires direct promoter binding and the presence of HIF1. (**A**) U2OS cells were transfected with a scr, E2F7 or HIF1α siRNA in combination with a reporter plasmid containing region −1312/−140 (1173 bp, Figure 4A) of the *VEGFA* promoter under normoxia or hypoxia. (**B**) U2OS cells were transfected with wild-type −1312/−140 *VEGFA* reporter or different combinations of HIF and E2F binding site mutants. In addition, these cells were co-transfected with an HIF1α expression or control (ev) plasmid. (**C**) Similar as in (**A**) with the difference that now the effect of indicated siRNAs was analysed on the E2F-BS (Δ887/Δ225) −1312/−140 *VEGFA* promoter mutant. (**D**) U2OS cells were transfected with WT or E2F-BS mutant (Δ887/Δ225) *VEGFA* promoter, or a 2-kb *E2F7* promoter (de Bruin *et al*, 2003) together with an E2F1 expression or control (ev) vector. (**E**) Western blot of inducible E2F7 HeLa cells cultured in the absence (−) or presence (+) of doxycycline. Both an inducible wild-type (WT) and a DNA-binding mutant (dbmut) E2F7 cell line are shown. Staining for E2F7-EGFP, endogenous E2F7 and HDAC1 (loading) are shown. (**F**) Inducible WT-E2F7 HeLa cells were transfected with a 47-bp *VEGFA* promoter region (−985/−939) containing −974 (and not −887 E2F-BS), in combination with scr or HIF1α siRNA (all in normoxia) and treated overnight with doxycycline. (**G**) As in (**F**) with the difference that cells were transfected with the −1007/−828 *VEGFA* reporter (containing −974 HIF-BS) and maintained in hypoxia (overnight) as indicated. (**H**) As in (**F**) with the difference that cells were now transfected with the wild-type (WT) or 974 HIF-BS mutant (Δ974HRE) 47 bp (−985/−939) *VEGFA* reporter (Forsythe *et al*, 1996). (**I**) HeLa cells with inducible WT or DNA-binding mutant E2F7 were treated (as in Figure 5B) with doxycycline in normoxia or hypoxia as indicated. Upper panel shows *VEGFA* mRNA levels. Lower two panels show western blot for E2F7-GFP (WT and mut) and HDAC1 (loading). All quantified data are presented as the average (± s.d.) compared to the control condition in three independent experiments.

E2F-BS but instead in cooperation with p53 through SP1-BS (Qin *et al*, 2006). These data together with our findings suggest that certain E2Fs may have both a positive and negative effect on transcription. This may be dictated by the presence of canonical or non-canonical E2F-BS, as well as on the cooperation with other transcriptional regulators. These effects are thus promoter specific and are consistent with the finding that E2F7/8 do not regulate overall HIF1 protein levels. Indeed we find that E2F7/8 and HIF1 cooperate to induce VEGFA expression, whereas other HIF targets like NIX and FLT1 are not regulated by this transcriptional complex.

In summary, we propose that although E2F7/8 repress target genes through canonical E2F sites during S/G2 phase of the cell cycle (Chen *et al*, 2009; Lammens *et al*, 2009; Westendorp *et al*, 2011), they can also enhance promoter activity through non-canonical E2F-BS and by cooperating with other transcription factors.

Although the role of Hif in zebrafish is poorly understood, the importance of the Vhl/Hif pathway in controlling angiogenesis in zebrafish was shown by a recent study in which hif induction by genetic inactivation of *vhl* results in *vegfAa*-dependent hyperbranching of ISA, head and eye vasculature (van Rooijen *et al*, 2010). Given the small size of zebrafish embryos, oxygen can, to a certain extent, diffuse freely into the tissue, suggesting that the E2f7/8–Hif complex regulates angiogenic sprouting in zebrafish mainly through an oxygen-independent manner. This is not unlikely knowing that the highly proliferative capacity of the developing embryo will enhance HIF activity through growth factor induced *Hif* translation (Pouyssegur *et al*, 2006; Semenza, 2009). A role for HIF during normal development is furthermore supported by the fact that fast growing tissues such as embryos and tumours experience local areas of hypoxia due to high oxygen consumption (Semenza, 1999; Simon and Keith, 2008; Dunwoodie, 2009). These data, together with our observation that E2F7/8 cooperate with HIF1 on normoxic and hypoxic VEGFA expression, provide substantial evidence that E2f7/8 and Hif1 are critical to control VegfAa-mediated angiogenesis during zebrafish development.

Materials and methods

Cell culture and hypoxia

The osteosarcoma (U2OS) and the cervical cancer (HeLa) cell lines were cultured in DMEM (Invitrogen, 41966-052) supplemented with 10% FBS (Lonza, DE14-802F). *Wild-type* and *E2f7/8* double knockout MEFs, and the inducible E2F7-GFP (WT, dbmut) and control cell lines (HeLa) were generated and maintained as described previously (Westendorp *et al*, 2011). For hypoxia treatment, cells were incubated in the H35 Hypoxystation (Don Whitley Scientific) at 1% O_2.

siRNA transfection

Cells were grown to confluence and reseeded (HeLa 250 k/well; U2OS 200 k/well) in six well plates (Greiner). Next day, cells were transfected as specified by the manufacturer using 5 μl/well Lipofectamine 2000 (Invitrogen) and a final concentration of 50 or 75 nM of siRNA as indicated in the legends. Transfected cells were grown overnight in normoxia or hypoxia and next day harvested. For harvesting, cells were washed twice with cold PBS on ice, scraped in cold PBS supplemented with protease inhibitors (Roche), and pelleted by centrifugation (2600 *g*, 2′, 4°C). Protein samples were lysed in 60 μl of lysis buffer (0.05 M sodium phosphate pH 7.3, 0.3 M NaCl, 0.1% NP-40, 10% Glycerol). Cell pellets for RNA isolation were frozen in liquid nitrogen and stored in −80°C. RNAi used in this study: hHIF1α (L-004018-00-0005; Thermo Scientific), non-targeting siRNA #2 (D-001210-02; Thermo Scientific), hE2F7 (HSS135118, HSS135119, HSS175354; Invitrogen), hE2F8 (HHS128758, HSS128759, HSS128760; Invitrogen), Negative control medium and high GC (Invitrogen; 12935-300 and 12935-400, respectively).

SDS–PAGE and western blot

Cells were harvested (as described under siRNA transfection). Cell lysates were subjected to standard ECL reagents as described by the manufacturer (GE Healthcare, RPN2106). Used antibodies are E2F7 (Santa Cruz, sc-66870), E2F8 (Abnova, H00079733-M01; Abcam AB109596), HIF1α (BD Biosciences, 610959), HDAC1 (sc-7872), E2F1 (sc-193), Mouse IgG HRP-linked whole Ab (GE Healthcare, NA931) and Rabbit IgG HRP-linked whole Ab (GE Healthcare, NA934).

Chromatin immunoprecipitation

ChIP was performed according to the EZ ChIP protocol (Upstate, 17-371) using protein G agarose beads (Millipore, 16-266) coated overnight in 0.1% BSA (Sigma, A3294). The following antibodies were used: ChIP grade HIF1α (Abcam, ab2185), E2F7 (sc-66870), E2F1 (sc-193). De-crosslinked DNA was purified over a column (Qiagen, 28106) and eluted in 50 μl H_2O of which 2 μl was used for quantitative PCR.

Immunoprecipitation

U2OS cells were transfected according to manufacturer's instructions with a total of 5 μg DNA using Superfect (Qiagen, 301305) and incubated in normoxia or hypoxia conditions for 16 h. Cells were scraped on ice in cold PBS supplemented with protease inhibitors (Roche). Cells from two plates were pooled and lysed in 1 ml lysis buffer. Lysates were precleared by incubation with 20 μl prot A/G beads (Calbiochem, IP05) for 30 min at 4°C. Immunoprecipitations (IPs) were performed with 1 μg of antibody for 2 h at 4°C. Protein complexes were precipitated by incubation with prot A/G beads for 30 min at 4°C. Precipitates were washed three times in lysis buffer and resuspended in 20 μl sample buffer. Expression constructs used: wild-type HIF1α, and HIF mutants 1–300, 543–826 were generously provided by Marc Vooijs (Maastricht University, The Netherlands). The 1–80, 1–170 and 81–826 HIF mutants were generated using the primers listed in Supplementary Table S1.

RNA isolation, cDNA synthesis and quantitative PCR

Total RNA was extracted according to manufacturers' instructions using the RNeasy Mini Kit (Qiagen, cat #74106). cDNA was synthesized with random hexamer primers according to manufacturers' instructions (Fermentas, cat#K1622). Quantitative PCR was performed on a MyiQ cycler (Bio-Rad) using SYBRgreen chemistry (Bio-Rad). In our *in vitro* studies, two reference genes were used (ACTB and RPS18) and for zebrafish samples three reference genes were used (TBP, EF1α and β-actin). MIQE standards were applied to our protocols (Bustin *et al*, 2009).

Luciferase reporter assay

U2OS cells (60–70% confluent) were transfected using Superfect transfection reagent (Qiagen) following manufacturer's instructions. Per well (six well plate) we used 2.5 μg reporter plasmid, 100 ng TK renilla, and 0.5 μg expression or control plasmid and 10 μl Superfect per well (six well plate). TK was used for normalization of the data. For co-transfection of cells with plasmid DNA together with siRNA, we used Lipofectamine 2000 (Invitrogen) according to manufacturer's guidelines. Cells were transfected with 500 ng reporter plasmid, 100 pmol siRNA (50 nM final concentration), 100 ng TK renilla, and 5 μl Lipofectamine 2000 (per well (six well plate)). Reporter activity was measured using the Dual-Luciferase Reporter Assay System (Promega, E1910) on a microplate luminometer (Centro LB 960) 48 h after transfection.

Zebrafish and mice

All mice experiments were approved by the Utrecht University Animal Ethics Committee and performed according to institutional and national guidelines.

All zebrafish strains were maintained in the Hubrecht Institute (Utrecht Medical Center, The Netherlands) under standard

husbandry conditions. Animal experiments were performed in accordance with the rules of the Animal Experimentation Committee of the Royal Netherlands Academy of Arts and Sciences (DEC). Published transgenic lines used were Tg(kdrl:gfp)^{s843} (Jin *et al*, 2005), Tg((kdrl:gfp)^{s843} (gata1:dsred)) (Traver *et al*, 2003) and Tg((fli1:negfp)^{y7} (kdrl:mCherry)) (Roman *et al*, 2002; Hogan *et al*, 2009b).

Morpholino

The following morpholino oligonucleotides (Genetools) were used. *E2f7* splice donor morpholino targeting exon 2–intron 2-3: 5′-ATA AAGTACGATTATCCAAATGCAC-3′; *E2f8* splice donor morpholino targeting exon 2–intron 2-3: 5′-CTCACAGGTATCCGAAAAAGTCA TT-3′; *VegfAa* ATG morpholino: 5′-ATGAACTTGGTTGTTTATTTGAT AC-3′ targeting both vegfAa$_{121}$ and vegfAa$_{165}$ (Lee *et al*, 2002).

Imaging

Embryos were mounted in 0.5–1% low melting point agarose (Invitrogen) dissolved in E3 buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) on a culture dish with a glass coverslip replacing the bottom. Imaging was performed with a Leica SP2 confocal microscope (Leica Microsystems) using a $\times 10$ or $\times 20$ objective with digital zoom.

Plasmid constructs

Full-length *Danio rerio e2f7* (Ensembl accession number ENSDART00000140760) or *e2f8* (Ensembl accession number ENSDART00000128488) was cloned into the pCS^{2+} Myc-tag plasmid. mRNA from these plasmids was *in vitro* synthesized (SP6 mMessage mMachine kit; Ambion). VegfAa$_{121}$ and VegfAa$_{165}$ constructs were present in pCS^{2+} plasmid and used as described previously (Habeck *et al*, 2002).

In situ hybridization

In situ hybridization was performed as previously described (Bussmann *et al*, 2007). The *vegfAa*, *vegfAb*, *vegfC* and *vegfD* probes have been described previously (Bahary *et al*, 2007; Hogan *et al*, 2009a). *e2f7* and *e2f8* probes were synthesized by *in vitro* transcription from the respectively *SacI*- or *SacII*-digested full-length cDNA in pCS2+ using T7 RNA polymerase (Promega).

Immunohistochemistry

Whole mount tissues were fixed in 20% DMSO in methanol and blocked with 20% goat serum (Sigma)/2% low fat dry milk (LFDM) in TBST prior antibody staining. Tissues were incubated with rat anti-mouse PECAM-1 antibody (1:50; BD Pharmingen) in 20% goat serum/2% LFDM/TBST for at least 12 h at 4°C. Secondary labelling was done with a goat anti-rat biotinylated antibody (1:100 Instruchemie) in 20% goat serum/TBST o/n 4°C. Tissues were stained with Vectastain ABC kit (Vector Laboratories). Images were captured using the Zeiss Axiophoto microscope.

Statistical analysis

For statistical analysis of two groups, unpaired *t*-test, or in case of unequal variances, Mann–Whitney *U*-test were used. For statistical analysis of multiple groups, one-way ANOVA, or in case of unequal variances, Kruskal–Wallis test was used. Dunns *post hoc* test were used to compare between selected groups. *P*-values <0.05 were considered significant. Statistical analysis was performed using SPSS 20 (IBM).

Supplementary data

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

Acknowledgements

We thank Dr Marc Vooijs (Maastricht University) for generously providing us with expression constructs for wild-type HIF1α, and HIF1α mutants 1–300, 543–826, and G Semenza for the 47-bp (−974) *VEGFA* promoter element. This work was financially supported by grants from the Dutch Cancer Society (UU2009-4353) and from the Association of International Cancer Research (09-0718) to WJB.

Author contributions: BGMWW carried out all *in vivo* experiments. WJB discovered the presented molecular mechanism and carried out most *in vitro* experiments assisted by K-HL, FHS, CACMTdW, MP, CLGJS and PWAC. PWAC performed ChIP and real-time PCR experiments. AdB, LK and GL generated the *E2f7*$^{-/-}$ and *E2f8*$^{-/-}$ mice. BW generated inducible E2F7 overexpression cell lines and performed ChIP-seq analysis. WJB and AdB designed the *in vitro* experiments. BGMWW, AdB and SS-M designed the zebrafish experiments. BGMWW, WJB and AdB wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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