Atypical E2Fs, old acquaintances with new faces

A novel role for E2F7 and E2F8 in angiogenesis and lymphangiogenesis

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Cover: Confocal image of zebrafish *Tg(fl1a:gfp)* blood vessels, derived from the subintestinal vein, that have grown to the place of xenografted mouse embryonic fibroblasts. Tumor size is shown by UV spotting (glossy).

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Een nieuwe functie voor E2F7 en E2F8 in angiogenese en lymphangiogenese

(met samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 10 juli 2013 des middags te 4.15 uur

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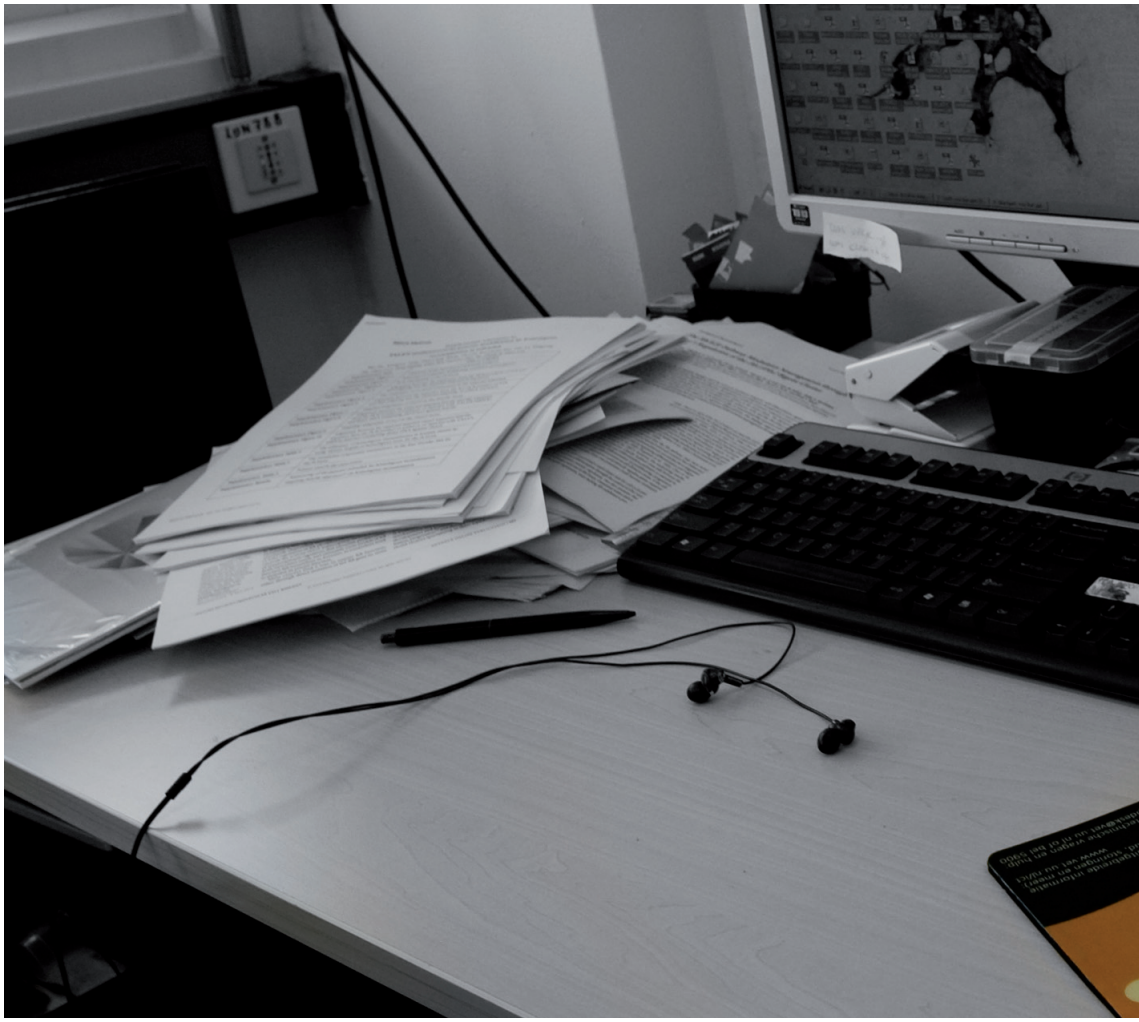


Chapter 1

THE E2F FAMILY

The E2F family of transcription factors is a highly conserved group of proteins within eukaryotic cells and can be subdivided into transcriptional activators and repressors. Dependent on the species, the E2F family has underwent extreme expansion during the course of evolution, with a single activator and a single repressor in flies to three activators and five repressors in mammals[1]. Interestingly, many E2Fs are expressed in a cell cycle dependent manner, which means that their levels oscillate when cells progress through the cell cycle. Moreover, individual members, like E2f1, are able to modulate the expression of other members like E2f7 and E2f8 or even compensate for the loss of a family member[1-3], arguing for a complex regulation with positive and negative feedback loops. Because of their cell cycle related expression pattern, the E2F family has been extensively investigated in relation to proliferation and differentiation. Although their functioning seems crucial for proper regulation and entering of the cell cycle, it has recently become apparent that the E2Fs possess the potential to regulate processes that reach beyond cell cycle regulation.

E2F7 and E2F8, the atypical members of the family

The completion of the whole genome sequencing of the flowering plant *Arabidopsis thaliana* and somewhat later *Homo sapiens*, initiated the search for new E2F family members. This search yielded for both plants and humans in a new class of E2Fs and where designated E2Fe-f (DEL1-3) in plants[4] and E2F7 and E2F8 in humans[5, 6]. In general, the E2F family is characterized by a DNA binding domain, through which they bind a specific sequence present in the promoter of E2F target genes. Moreover, to actually bind the DNA, E2F1-6 need to acquire a second DNA binding domain, which is achieved by dimerizing and formation of hetero-dimers with an dimerization partner (DP) protein through the dimerization domain[7]. These two main domains, DNA and dimerization domain, are seen as the minimal criteria for a protein to be designated as typical E2F (Figure 1). In addition, E2F1-5 also possess a pocket protein binding domain, which is an essential domain that regulates the E2F transcriptional activity (Figure 1)[1]. Although it appeared that the overall amino sequence similarity of the newly identified E2Fs was rather low in respect with the typical E2Fs, they possessed a highly conserved DNA binding domain also found in typical E2Fs[9]. Interestingly, this DNA binding domain appeared to be duplicated, whereas the dimerization and pocket protein domain were lacking (Figure 1). Due to these characteristics, these newly identified E2Fs were designated as atypical.

For proper functioning of E2F7 and E2F8, hereafter referred to as E2F7/8 or atypical E2Fs, both DNA binding domains need to be intact[10-12]. Although E2F7/8 lacked the characteristic dimerization domain found in typical E2Fs, some dimerization domain residues were found within the DNA binding domain[9]. Interestingly, E2F7/8 appeared to form homo- and hetero-dimers through these residues, with an increased affinity for dimerization with E2F7, suggesting that E2F7 is more important than E2F8[13]. In line with the suggestion that E2F7 is

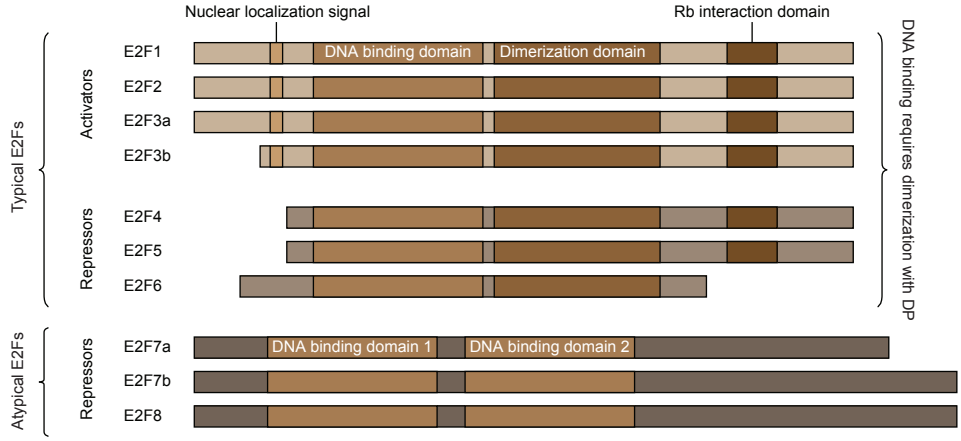


Figure 1 I The mammalian E2F family of transcription factors can be subdivided into two main groups, transcriptional activators (E2F1-3) and repressors (E2F4-8). However, evidence for such classification is mainly derived from *in vitro* studies. The main feature that shapes the E2F family is the highly conserved DNA binding domain. The typical E2Fs (E2F1-6) posses a single DNA binding domain and need to dimerize with their dimerization partner (DP) to be able to bind DNA and exert their transcriptional effect on the promoter of E2F target genes. In contrast, the recently identified E2F7 and E2F8 posses two DNA binding domains, for which they were designated atypical. Moreover, due to the double DNA binding domain, the atypical E2Fs lack the necessity to dimerize with DP to bind DNA. The typical E2Fs contain two other important domains that are essential for proper functioning of the E2Fs. The RB interaction domain mediates interaction between the E2Fs and retinoblastoma protein (RB). RB regulates the transcriptional activity of E2F1-5 by repressing the transcriptional activity of the E2Fs. Furthermore, the activator E2Fs (E2f1-3) contain a nuclear localization signal, that mediates the transport of E2Fs into the nucleus.

Adapted from Chen et. al. 2009[8]

less dispensable than E2F8, it was shown that *E2f7*$^{+/-}$*E2f8*$^{-/-}$ embryos were born and lived to old age, while *E2f7*$^{-/-}$*E2f8*$^{+/-}$ mice lived until 3 months of life and *E2f7*$^{-/-}$*E2f8*$^{-/-}$ embryos died *in utero*[13].

E2F7 and E2F8, indispensable for development

As mentioned above, *E2f7/8* double knock out embryos die *in utero* around embryonic day 11.5 (E11.5)[13]. These embryos displayed a severe apoptotic phenotype in the embryo and, in addition, the placenta was poorly developed[13]. Expression pattern analysis of E2f7/8 revealed a ubiquitous expression pattern around E9.5 both in the embryo and placenta[13, 14], while in adults they are expressed in skin, thymus, spleen, intestine and testis[5, 6]. These organs are all known for their proliferative state or potential. Indeed, many defects found in plants and mice deficient for E2f7/8 are related to defects in the cell cycle. To this extent it was shown that plants lacking the atypical E2F DEL1, were decreased in size due to a decreased DNA ploidy content in cells[15]. To a similar fashion, the trophoblast giant cells, found in the placenta, also displayed a decrease ploidy when *E2f7/8* were deleted[2].

Interestingly, *E2f7/8* double knock out embryos also displayed vascular defects at E10.5, seen as a dilated umbilical cord and hemorrhages in the embryo proper[13]. Importantly, the

previous mentioned apoptotic phenotype, seen in *E2f7/8* double knock out embryos, was not an underlying cause of these vascular defects[13], although, the placenta phenotype contributed significantly to the severity of the vascular phenotype found in the embryo proper[14]. Supplying *E2f7/8* double knock out embryos with an wildtype placenta carried these embryos to term, although they still died shortly after birth due to a still unknown cause[14]. Moreover, expression analysis of these *E2f7/8* double knock out embryos with wildtype placentas at E11.5 revealed that many genes essential for vascular development were deregulated[16].

E2F7 and E2F8, new players in building the vascular tree?

As discussed above, the deletion of *E2F7/8* during embryonic development results in premature death of the embryo. The distinct vascular phenotype that these double knock out embryos displayed argues for a role of the atypical E2Fs in vascular development. Investigating this phenotype in more detail in mice will be very challenging because of the poor integrity, among others due to the placenta defects, of these embryo. Therefore, we used the zebrafish as model to study the function of E2f7/8 in the formation of the blood vessels during embryonic development. Next, there will be a brief overview about the vascular system in general and how the zebrafish functions as a model for vascular development.

THE VASCULAR TREE

The formation of a closed circulatory system is one of the main characteristics of all vertebrate species. This tube like system can be divided into a pump, the heart, that circulates the blood through the piping, which are specialized tubes. The arteries transport oxygen rich blood towards the tissues and veins transport the oxygen poor blood back to the heart. Besides the difference in blood composition between arteries and veins, they also differ structurally from each other. Because the arteries need to cope with a much higher blood pressure, due to the contractions of the heart, they developed a thick vascular smooth muscle layer, while veins lack such a thick layer. In contrast to arteries, veins contain valves that help the blood to stream “upwards” to the heart. To provide all tissues and organs with, among other, the necessary oxygen and nutrients, the vascular system needs to branch from big tubes to very thin tubules. This branching looks very similar to a tree, with the stem as the main vessels, aorta and veins, while the thicker branches and the very tiny nerves in the leafs resemble the arterioles/venules and the capillaries, respectively (Figure 2). The branching towards very thin tubes enables the vascular system to cover a big area with a lot of surface contact, which ensure optimal gas and molecule exchange.

As the blood circulates, fluid carrying food to the cells and waste products back to the bloodstream, leaks from the capillaries. Although most fluid drains back into the bloodstream, the remaining fluid that is not able to drain into the bloodstream is scavenged by a second circulatory system, the lymphatic system. The lymphatic system is closely related to and

derived from the vascular system (Figure 2) and carries a clear fluid called lymph[18]. The lymph is eventually collected in the main lymphatic vessels, (left and right) thoracic duct, and then drained back into the venous system. Next to fluid homeostasis, the lymphatic system fulfills also an important function in fatty acid and vitamin resorption from the intestine and immune cell trafficking. The latter is of crucial importance for proper functioning of the immune system.

Building the vascular tree

The building blocks of the vascular tree are endothelial cells, specialized cells that line the interior surface of blood and lymphatic vessels. Instructive signals are needed to guide and direct these building blocks to the correct position within the tree. Although there are a broad range of signals that influence the behavior of the endothelial cells, the most important class of instructive signals are the secreted vascular endothelial growth factors (VEGF) and their corresponding receptors (VEGFR)[19]. To shape the vascular tree, the instructive signals need to be switched ON or OFF at the right time and with the right amounts. This kind of regulation is achieved by modulating the production and the longevity of the instructive signals and takes place on the level of the DNA (transcription), RNA (translations) and protein (destruction). One major regulator for triggering the production of VEGF is hypoxia, e.g. shortage in oxygen. Hypoxia will lead to a cascade of events in which VEGF is produced *de novo* by transcription and secreted by cells experiencing these decreased amounts of oxygen[20]. Secreted VEGF will guide endothelial cells and thereby the formation of blood vessels towards the hypoxic region, resulting in reoxygenation of the hypoxic area.

Zebrafish as model for vascular development

Understanding how the vascular tree is built under normal conditions is of great importance for unraveling the underlying cause and treatment of diseases related to or dependent on the formation of blood vessels. Recently it has become clear that using the vertebrate species *Danio rerio*, commonly known as the zebrafish, as a model for vascular development offers many advantages above other species used in research[21]. The main advantage of the zebrafish is their transparency during embryonic development, which makes it possible to visualize and follow the formation of the blood vessels in real-time. Moreover, they develop *ex utero* without the support of a placenta, are relative cheap to keep and can produce a large amount of offspring. Although there are some difference between the development of the mammalian and zebrafish vascular system, it has been proven that the genetic regulation of blood vessel formation is greatly conserved between these species.

Primary angiogenesis, formation of arteries

The formation of the vascular system in the zebrafish is initiated by the formation of the main axial vessels. Endothelial cell precursors called angioblasts arise from lateral plate mesoderm with an anterior population that makes up the head vasculature and a posterior population

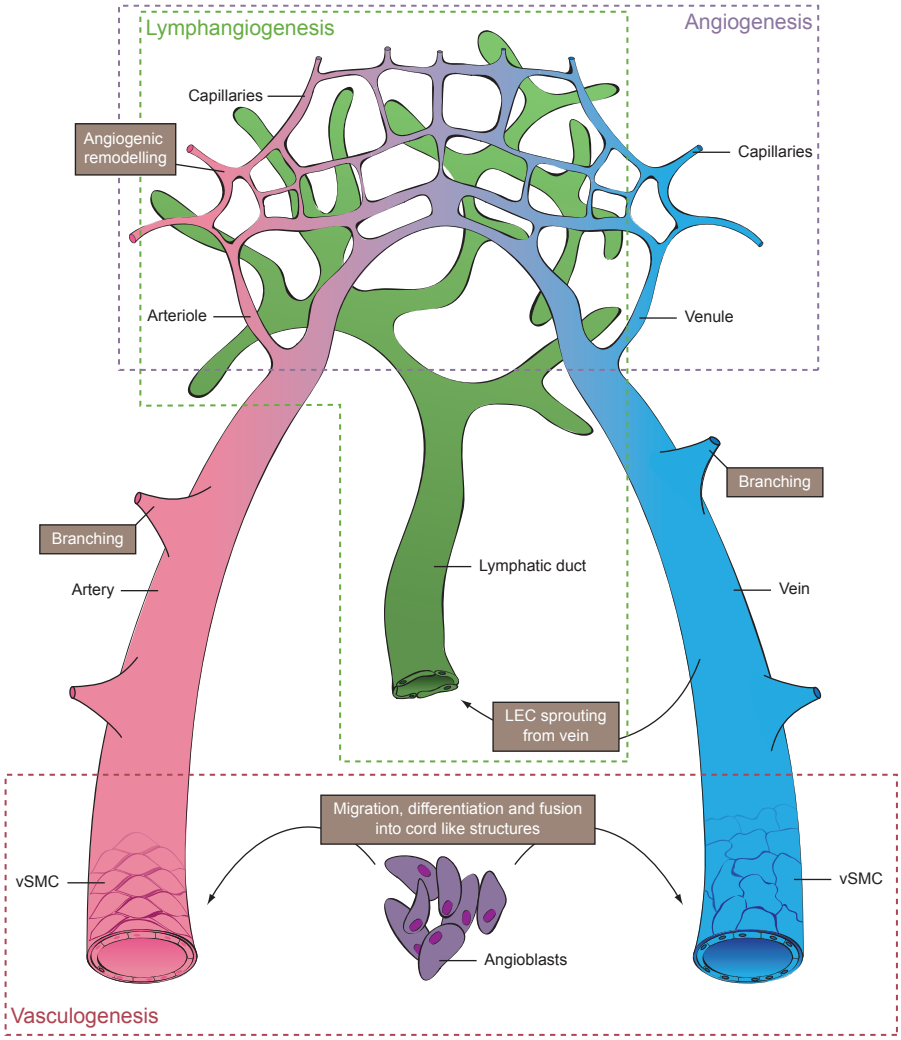


Figure 2 I Early during development, endothelial precursors (angioblasts) migrate, differentiate and coalescence to form the main axial blood vessels, the aorta and the cardinal vein, in a process called vasculogenesis. In a process called angiogenesis, endothelial cells from pre-existing blood vessels start to sprout and form a dense network of arterioles/venules and capillaries to ensure oxygen and nutrient supply to the tissues and organs. The first lymphatic precursors arise and migrate away from the vein to form the initial lymphatic structures. In process called lymphangiogenesis, a blunt end network of lymphatic vessels is formed.

Adapted from Adams & Alitalo 2009[17]

that makes up the trunk vasculature (Figure 3A)[22]. In a process called vasculogenesis, *de novo* synthesis of blood vessels, these angioblasts migrate, coalescence and differentiate to form the first blood vessels in the trunk of the embryo, the dorsal aorta and posterior cardinal vein. The dorsal aorta and the posterior cardinal vein make up the first circulatory loop in the trunk and is situated in the ventral part of the trunk (Figure 3B). Next, the dorsal part of the trunk is vascularized by a process called primary or arterial angiogenesis, in which arteries arise from the existing dorsal aorta. Single endothelial cells from the dorsal aorta are selected and referred to as sprouts. These sprouts grow out of the aorta in a bi-lateral fashion between the somite

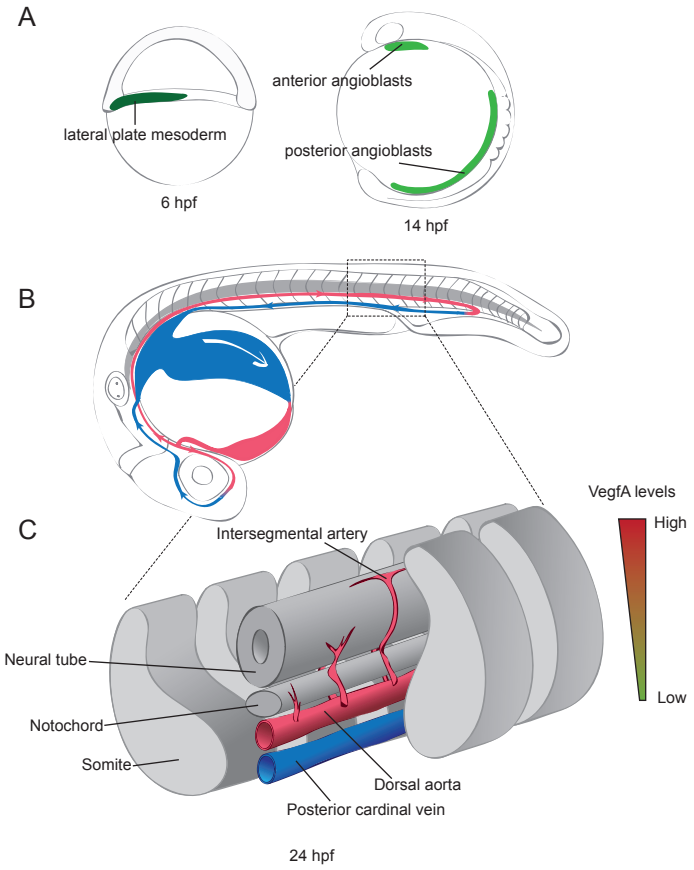
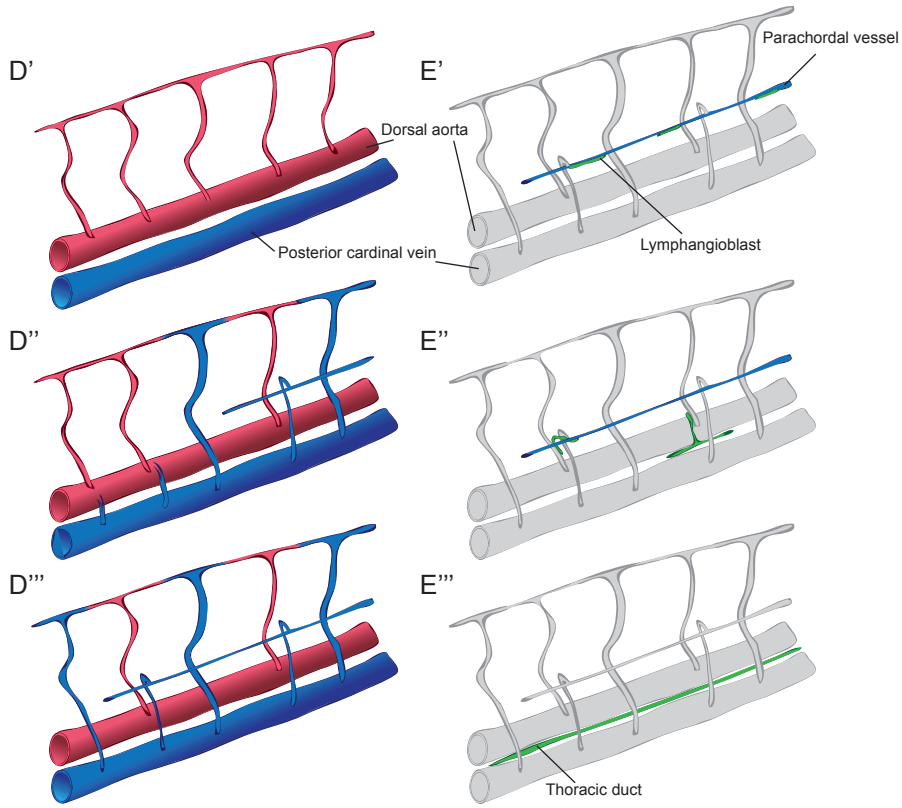


Figure 3 I Zebrafish as model for angiogenesis and lymphangiogenesis. **A** Around 6 hours post fertilization (hpf) the endothelial precursors (angioblasts) originate from the lateral plate mesoderm and form, around 14 hpf, an anterior population that forms the head vasculature and a posterior population that forms the trunk vasculature. Under influence of growth factors, endothelial cells start to sprouts exclusively from the dorsal aorta. Vascular endothelial growth factor A (VegfA) is the main driver of this process, which is secreted in a gradient pattern with high levels at the dorsal side of the trunk. Endothelial cells sense and migrate towards this VegfA gradient thereby forming the intersegmental vessels, which are all of a arterial identity. →

boundaries to the dorsal side of the trunk, where they T-branch and interconnect to form the dorsal longitudinal anastomotic vessel (DLAV) (Figure 3C)[23].

VegfA plays an indispensable role in the process of sprouting and migrating of the endothelial cells towards the dorsal part of the trunk. More specific, VegfA, a chemoattractant, is secreted in such a way that it forms a gradient with decreasing levels from dorsal to ventral that serves as a path for endothelial cells to follow[24, 25]. Endothelial cells sense VegfA with Vegf receptor 1 and 2, present on the surface membrane of these cells[26-28]. In the end this process yields in blood vessels, called intersegmental vessels, with an exclusive arterial identity that run between the somite boundaries and are interconnected with each other at the dorsal side of the trunk (Figure 3D').



D' - D''' The trunk vaculature, which is generated during primary (arterial) angiogenesis is exclusively arterial (red color). In a second wave of angiogenesis, secondary (venous) angiogenesis (blue color), cells sprout and migrate solely from the posterior cardinal vein. About half of these venous sprouts connect and convert the arterial intersegmental vessels into venous intersegmental vessels. The other half of venous sprouts migrate further to the horizontal myoseptum and shape the parachordal vessel. **E' - E'''** A subset of cells, the lymphatic precursors, start to migrate exclusively alongside arteries from the parachordal vessel to the space between the dorsal aorta and the posterior cardinal vein, where they form the thoracic duct, the main collection duct of the lymphatic system.

Secondary angiogenesis, formation of veins, lymphatic precursors and first lymphatic vessels

To establish a functional circulation in the trunk it is essential to connect, at least a portion, of the arterial intersegmental vessels to the venous system. A second wave of angiogenesis that is exclusively venous derived ensures the connection of arterial intersegmental vessels to the venous system. To this extent, endothelial cells from the posterior cardinal vein sprout and migrate towards the dorsal aorta. Interestingly, at this point these venous cells have two possible fates; 1, the venous fate, about half of the cells that sprout from the posterior cardinal vein connect and convert arterial intersegmental vessels to venous intersegmental vessels, thereby establishing venous connections; or II, the lymphatic fate, the reminder of the venous cells migrate further to the horizontal myoseptum without altering the fate of the arterial intersegmental vessels and are now referred to as parachordal lymphangioblasts (Figure 3D'-D'"). A longstanding question in the field of lymphangiogenesis is: How do the venous cells "know" whether to choose for the venous fate or the lymphatic fate?. Several theories can be postulated regarding this question. One may speculate whether the venous cells that sprout from the posterior cardinal vein have an already preprogrammed fate. A second theory might be that the dorsal aorta and/or arterial intersegmental vessels provide cues that determine whether these venous sprouts become part of the vascular or lymphatic system. It is also not excluded that it is a combinatory mechanism of both signals from the venous cells and the arterial system.

To shape the lymphatic system, the parachordal lymphangioblasts that populate the horizontal myoseptum start to migrate, in a process called lymphangiogenesis, dorsally to form the intersegmental lymphatic vessels and ventrally towards the space between the dorsal aorta and posterior cardinal vein. Remarkably, migration occurs specifically alongside arterial intersegmental vessels, which they use as guidance (Figure 3E'-E")[29, 30]. Between the space of the dorsal aorta and posterior cardinal vein, these lymphangioblasts proliferate and fuse together to form the thoracic duct, the main lymphatic collection duct (Figure 3E'")[31, 32].

To date, little is known about the underlying processes that drives venous sprouting and thereby lymphangiogenesis. Although several key players have been identified that appear to be indispensable. Where VegfA and its receptor Vegfr1 and 2 play a crucial role in arterial angiogenesis, the same important role has VegfC and its receptor Vegfr3 in venous angiogenesis[32, 33]. Loss of either VegfC or Vegfr3 leads to a decreased number of cells sprouting from the posterior cardinal vein, consequently leading to a predominantly arterial identity of the intersegmental vessels in the trunk and near absence of lymphatic structures[32, 33]. Recently it was shown that, Collagen and calcium binding EGF domains 1 protein (Ccbe1) enhances the biological effect of Vegfc, thereby playing an indispensable role in venous sprouting and lymphangiogenesis[34, 35]. Furthermore, it was shown that Delta like 4 (Dll4), a Notch receptor ligand, represses the output of VegfC mediated activation of Vegfr3[30, 36]. Interestingly, these studies showed that loss of Dll4 resulted in an increased venous fate of cell budding from the

posterior cardinal vein, resulting in a predominantly venous identity of the intersegmental vessels in the trunk. This finding might argue that Dll4 itself or the effect of Dll4 on VegfC-Vegfr3 signaling determines whether the venous sprouts adapt a venous fate or lymphatic fate. Moreover, expression pattern analysis of *dll4* by *in situ* hybridization revealed an almost exclusive expression in the arterial intersegmental vessels and dorsal aorta[36], suggesting that the signal determining the fate of venous sprouts is derived from the aorta and/or arterial intersegmental vessels. The above discussed factors involved in venous sprouting and lymphangiogenesis show that the VegfC-Vegfr3 axis has a pivotal role in this process, although it remains poorly understood how these factors are regulated, i.e. turned ON and OFF. Fully understanding how the process of venous sprouting and lymphangiogenesis is regulated in development is essential for developing treatment for diseases that are due to or dependent on the vascular or lymphatic system.

Tumor (lymph)angiogenesis

Cancer is a well-known disease that depends on the vasculature for survival. Although the word cancer is used to describe a broad varieties of diseases, there are several characteristics that connect these diseases with each other, with uncontrolled cell growth as main characteristic. A series of events that are caused by a variety of factors, like genetic predisposition[37], environment[38] and diet[39], compromises the cell's safeguard to uncontrolled cell division and moreover the potential to "commit suicide", a controlled process of cell death called apoptosis[40, 41]. An individual cell that bypasses these safeguards starts to grow uncontrollably and forms a tumor. Initially this tumor can be provided with nutrients and oxygen by simple diffusion, however, when the tumor exceeds the physical properties of diffusion it will need to attract blood vessels for supply of essential nutrients and oxygen. Likewise normal development, cells in a hypoxic environment start to secrete angiogenic factors with VegfA as the most important and potent angiogenic factor[42]. As a result, endothelial cells derived from existing blood vessels start to sprout and migrate towards the source of secreted factors, in this case the tumor (Figure 4). By gaining access to the blood stream, the tumor not only ensures a constant supply of oxygen and nutrients, but also enables the possibility to metastasis to distal organs and tissues. Due to the uncontrolled and unorganized nature of tumor growth, the angiogenesis response is also poorly controlled, which often results in unstable and torturous intratumoral blood vessels that often lack a lumen[44].

Next to the blood stream as a way to disseminate to distal organs, the lymphatic vessels provide an alternative route for metastasis. Of all solid tumors found, 80% metastasize via the lymphatic vessels rather than through the blood stream[45]. There are two possibilities for tumor cells to enter the lymphatic system; first, attracting new lymphatic vessels by inducing lymphangiogenesis from existing lymphatic vessels; or second, cells enter existing lymphatic vessels due to the high interstitial pressure within the tumor[46]. The finding that tumors often overexpress several pro-lymphangiogenesis factors, like VegfC and D, and contain

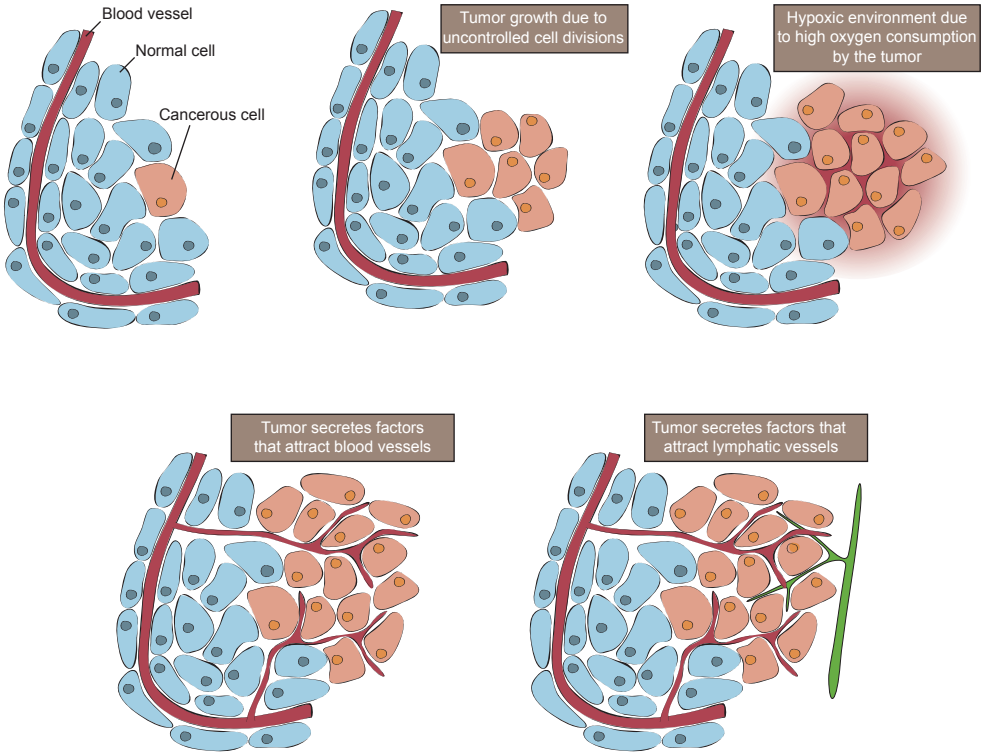


Figure 4 I Exposure to chemicals , radiation, tobacco, genetic predisposition, infections and diet are all factors that can lead to (additional) mutations in genes essential for controlling cell proliferation. Loss of so called tumor suppressor genes leads aberrant cell proliferation and as a consequent, more mutations are gained which influence the behaviour of the tumor cells. Nutrients and oxygen are initially provided by simple diffusion. However, when tumor size exceeds the physical capacity of diffusion, the tumor environment becomes hypoxic (low oxygen concentration). Hypoxia will trigger the release of angiogenic growth factors from the tumor cells and induce an angiogenic response from the blood vessels. Ultimately, blood vessels invade the tumor to ensure oxygen en nutrient supply, moreover, these blood vessels provide a way to access the circulation and thereby the possibility to metastasis to distal organs. In a similar fashion, the tumor also attracts lymphatic vessels.

Adapted from Bergers & Benjamin 2003[43]

intratumoral lymphatic vessels, favor a model in which tumors attract new lymphatic vessels via lymphangiogenesis[47, 48]. In addition, it has been shown that intratumoral lymphatic vessels, like intratumoral blood vessels, are of poor quality and often lack a lumen due to the high intratumoral interstitial pressure[48].

The E2F family, old acquaintance in tumor formation, but new players in tumor (lymph) angiogenesis

The retinoblastoma protein (Rb) is, besides one of the main regulators of E2F1-5 activity, also an important tumor suppressor gene. In various human cancers Rb is found to be deregulated

directly due to mutations in the *Rb* gene or due to phosphorlyation, which in both cases results in deregulation of the E2F family[49, 50]. Next to loss of this major E2F regulator in cancer, it has also been shown that in several human hepatocellular carcinomas the *E2F1* or *E2F3* gene locus is amplified, resulting in overexpression of these genes[51]. The repressive arm of the E2Fs (E2F4-8) has been postulated as tumor suppressors, however, they are not frequently mutated, deleted or silenced in human cancers, arguing for a role in tumor progression rather than inhibition[8]. Recently is has become evident that the E2F family of transcription factors also controls cell cycle independent process, like angiogenesis[52], adipogenesis[53] and migration[54]. These findings indicate that the E2Fs might not only be essential for tumor proliferation, but could also potentially regulate tumor angiogenesis, migration and differentiation, thereby greatly affecting tumor progression and malignancy. Together, there is a substantial amount of evidence that in a wide range of tumors the E2F family is directly or indirectly deregulated, however the exact contribution of this family in tumor initiation, progression and malignancy is currently unknown.

SCOPE AND OUTLINE OF THIS THESIS

In this thesis we studied the role of the most recently identified members of the E2F family, E2F7 and E2F8, in the formation of blood vessels during embryonic development and tumor formation. These studies provide new insights in E2F7/8 functioning. Furthermore, our studies also contribute to the general understanding of the processes of angiogenesis and lymphangiogenesis. First, in ***Chapter 2*** we analyze in detail whether the vascular defects found in *E2f7/8* double knock mice embryos is a specific phenotype due to the loss of E2F7/8. Because these mice die early during development, we used the zebrafish as model to study the role of E2f7/8 in vascular development. In ***Chapter 3*** we discuss the finding that E2F7/8 regulate angiogenesis. We speculate whether there is a common mechanism(s) that connects the atypical E2Fs with the regulation of genes essential for angiogenesis. In ***Chapter 4*** we actually provide substantial evidence that E2F7/8 regulates additional genes that are indispensable for proper angiogenesis. Because, (venous)angiogenesis and lymphangiogenesis are inextricably linked to each other, we describe here a phenotype that includes defects in the formation of lymphatic precursors and the therefrom derived lymphatic vessels. The phenotypes described in Chapter 2 and 4 together with the knowledge from the literature that the E2F family plays an important role in tumor formation, let to the investigation in ***Chapter 5*** whether E2F7/8 play a role in tumor formation with a detailed focus on the formation of intratumoral blood vessels. In ***Chapter 6*** we collaborated with Prof. Dr. Jeroen den Hertog and Dr. Suma Choorapoikayil to investigate the angiogenesis defect observed in Pten knock out zebrafish. In ***Chapter 7*** we discuss data presented in this thesis and, moreover, additional data are presented to support specific topics that are being discussed.

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

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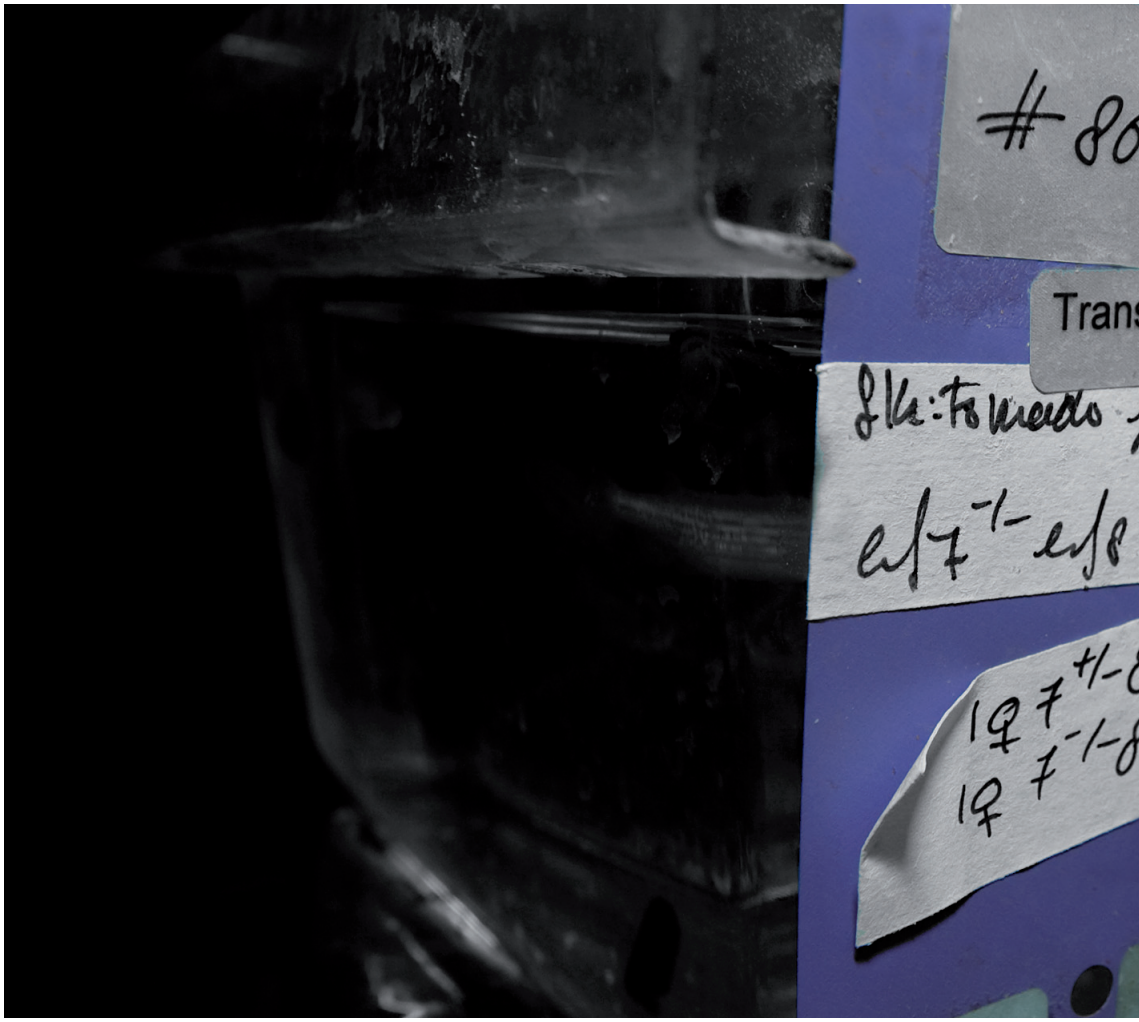


Chapter 2

ABSTRACT

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 labeled 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.

INTRODUCTION

The E2F family of transcription factors consists of 8 family members and is divided in activators (E2F1-3) and repressors (E2F4-8) predominantly based on *in vitro* experiments[1, 2]. 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[3-5]. 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[6]. 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[2, 3, 7]. 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[8]. 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 remodeled 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)[9]. 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[10]. FLK1/KDR signaling stimulates endothelial cell proliferation and migration along the VEGFA gradient[11]. 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[12]. The major regulator of VEGFA in the context of angiogenesis is the hypoxia induced factor 1 (HIF1)[13, 14]. HIF1 activity is regulated both by oxygen dependent and independent mechanisms[15, 16]. The presence of oxygen stimulates degradation of HIF1α through the PHD/VHL pathway, resulting in induced HIF1 activity in response to oxygen deprivation (hypoxia)[15]. Growth factor signaling on the other hand stimulates HIF1α translation independent of the oxygen levels, leading to increased HIF activity, even under normoxic conditions[15, 16]. The importance of HIF1 for vascular development is demonstrated by the observation that mice lacking *Hif1* die around E10.5 with severe vascular defects[17-19].

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 labeled endothelial cells (*Tg(kdrl:gfp)*). Primary angiogenesis in zebrafish embryos starts at 22 hours post fertilization (hpf) 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 (MO) that interfere with the splicing of *e2f7* and *e2f8* (Supplemental Figure S1A). Sequencing of MO induced miss-spliced *e2f7* and *e2f8* mRNA, revealed the presence of frame-shifts or intron insertions upstream of the DNA-binding domains (ranging from exon 3 to 6), which are crucial for proper transcriptional activity. Zebrafish treated with these MOs, showed ISAs that prematurely stalled at the horizontal myoceptum and failed to connect to the DLAV (Figure 1A). This angiogenic defect was observed in embryos injected with a 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[8]. 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[8]. 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-labeling (TUNEL) (Supplemental 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. Similar to our previous mouse studies we were able to rescue the apoptotic phenotype in *e2f7/8/p53* MOs injected zebrafish embryos, but the vascular phenotype persisted to the same extent (Supplemental 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 hemorrhages and blood vessel dilation[8], 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 analyzed the number of completely perfused vessels. Non injected embryos had almost 100% blood vessel perfusion, in contrast *e2f7/8* MOs injected embryos showed an average perfusion rate of 58% (Figure 1C and D).

Secondly, 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* MOs 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 mutagenesis library generated by targeted induced local lesions in the genome (TILLING)[20], an *e2f7* (*e2f7*^{A207}) and *e2f8* (*e2f8*^{A196}) mutant zebrafish. Both mutants harbor a mutation within the highly conserved first DNA binding domain, resulting in a stop codon (Supplemental Figure S1C). Transient expression of myc-tagged versions of zebrafish *e2f7*^{A207} and *e2f8*^{A196} mutants in mouse embryonic fibroblasts (MEF) prevented full length translation of these atypical E2Fs (Supplemental 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[21]. 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*[12], 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

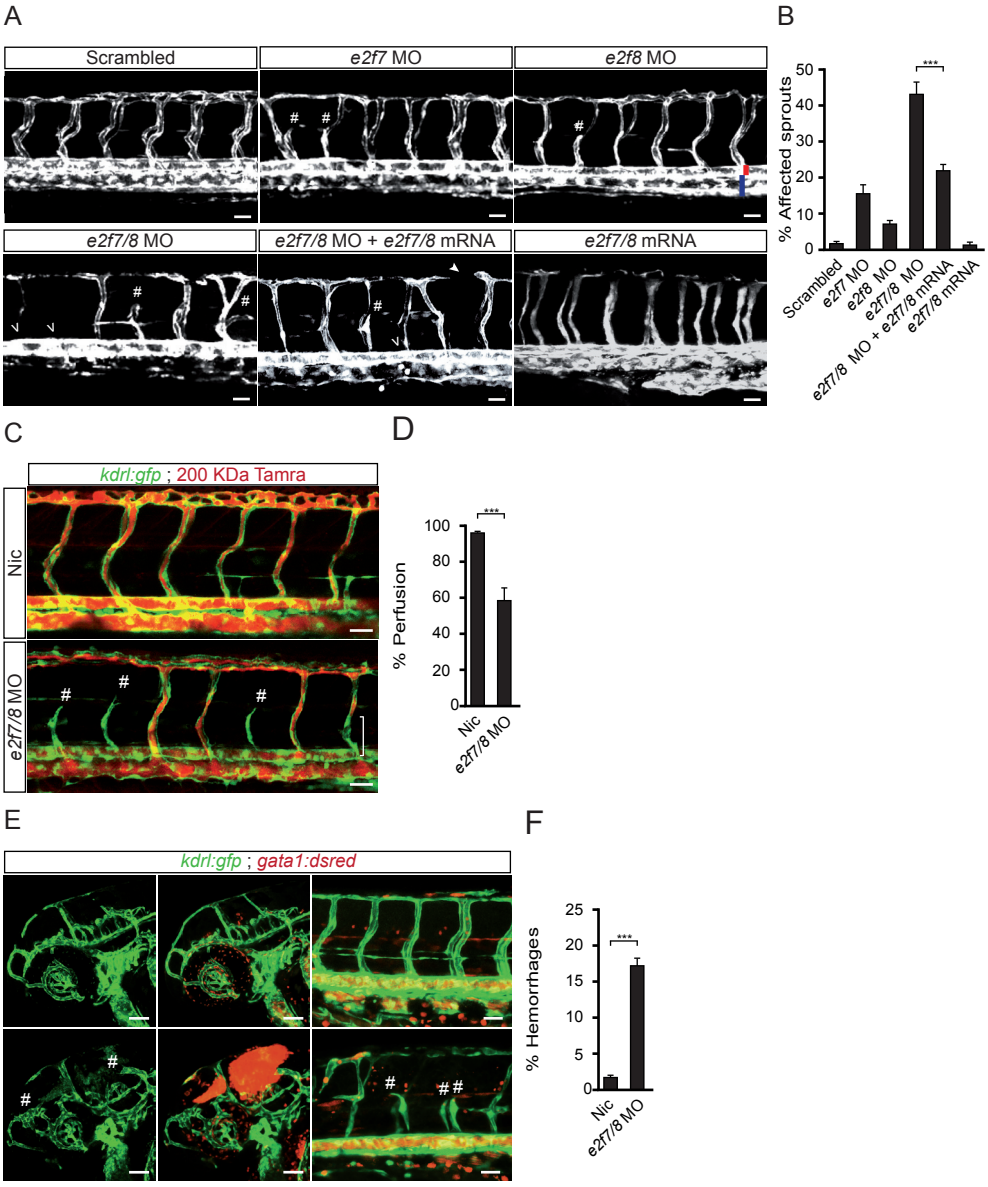
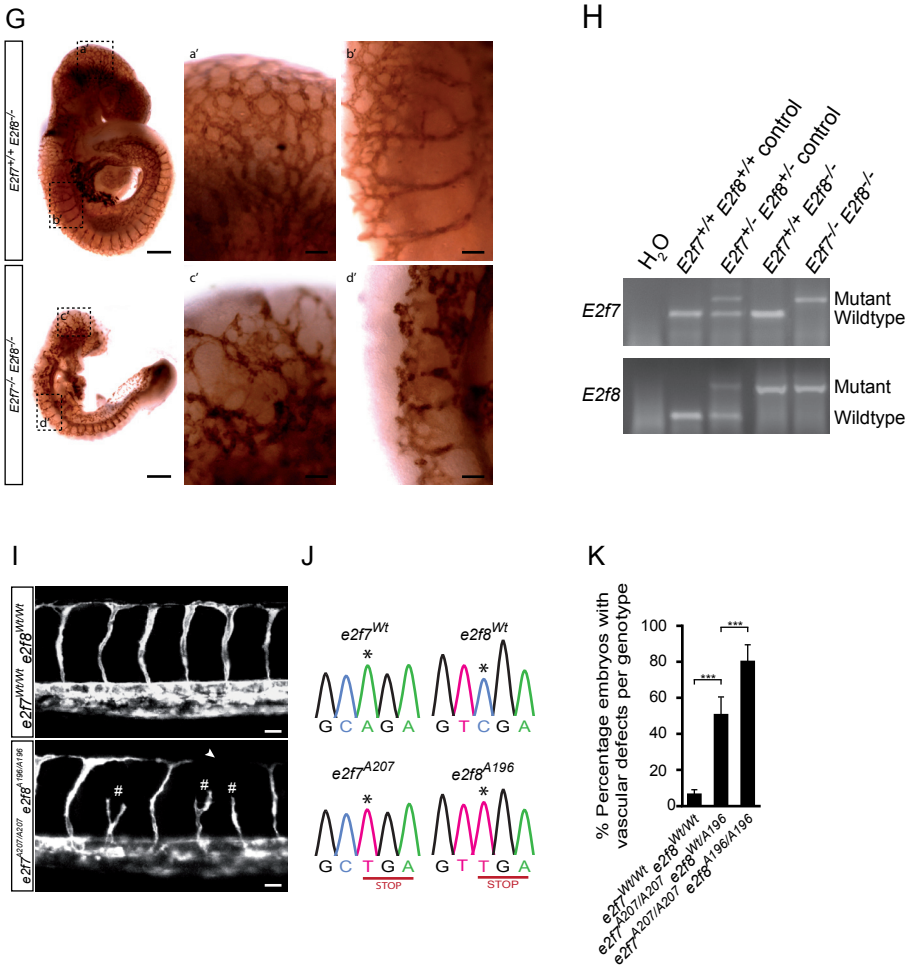


Figure 1 I E2f7 and E2f8 are essential for angiogenesis. **A** Lateral images of *Tg(kdrl:gfp)* zebrafish embryos 48 hpf. Scale bars are 50 µm. **B** Quantification of angiogenesis defect, all ISAs that showed abnormalities were counted (n > 150 per condition). **C** *Tg(kdrl:gfp)* embryos were injected 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 hemorrhages (NIC n=43; *e2f7+8* MO n=62). **G** CD-31 (PECAM-1) staining of E9.5 mouse embryos. Scale bars are 500 µm 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 *wildtype Tg(kdrl:gfp) and* $e2f7^{A207/A207}$; $e2f8^{A196/A196}$; *Tg(kdrl:gfp)* →



zebrafish 48hpf. Scale bars are 50 µm. **J** Genotyping was performed by sequencing, wildtype 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 labeled. 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, closed arrow heads depicts the absence DLAV. Bracket shows unperfused part of ISA. All quantified data are presented as the average (± SEM) compared to the control condition in three independent experiments (***$P < 0.001$).

the somites, head and eye region. Previously, we showed ubiquitous *E2f7/8* expression at E9.5 in mouse embryos[8] 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 hpf) (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* hybridisation and quantitative PCR whether *vegfA* was the only vegf family members to be affected by the knock down 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[22]. 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 tailbut and little is known about its function during angiogenesis. In addition, *e2f7* and *e2f8* double mutant zebrafish embryos had

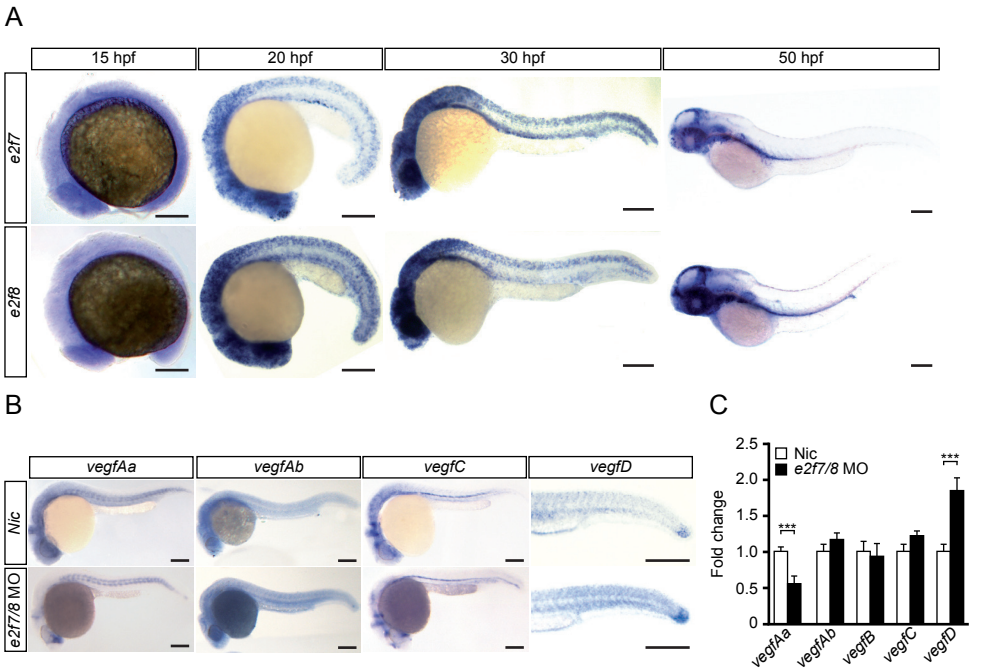
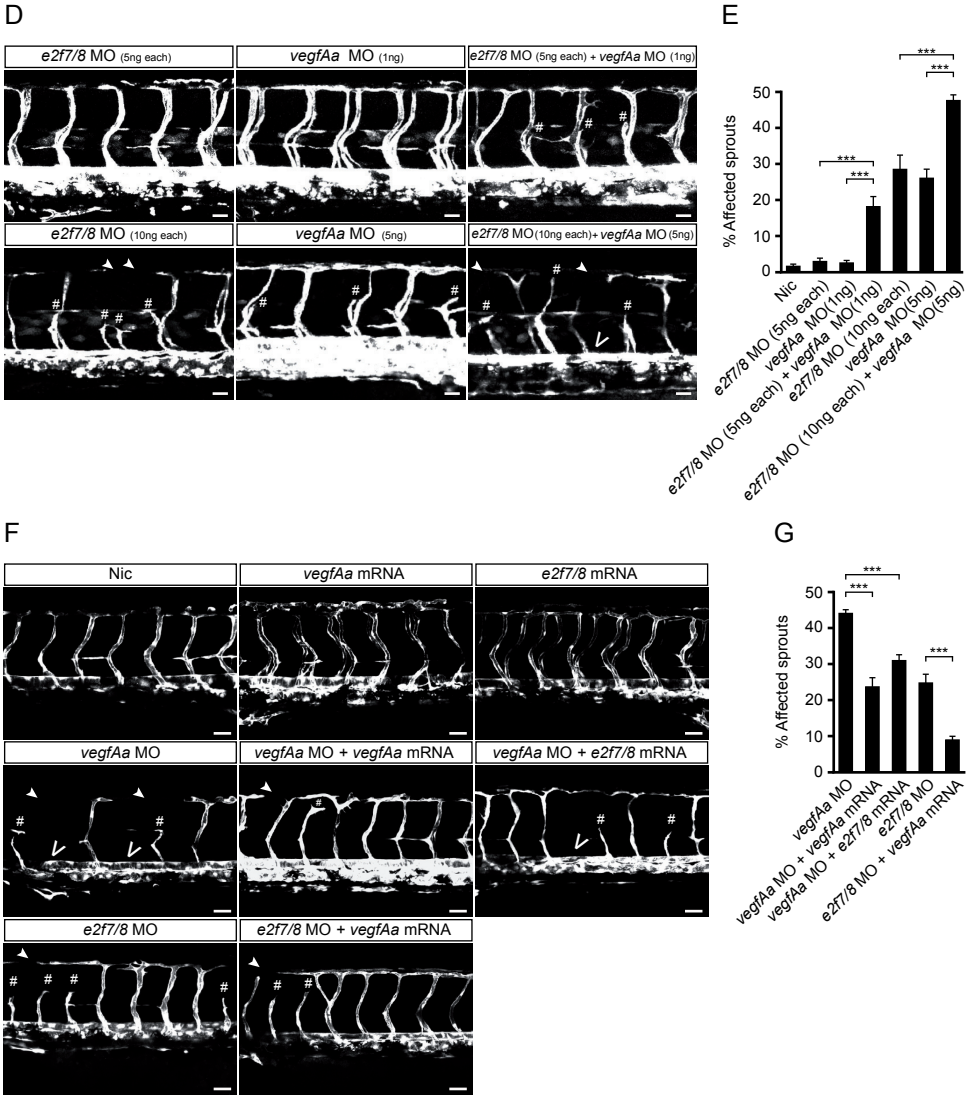


Figure 2 I E2f7 and E2f8 regulate angiogenesis through stimulation of *vegfA* expression. **A** Expression of *e2f7/8* at four developmental stages visualized by in situ hybridization (ISH). Scale bars are 250 µm. **B** ISH and **C** qPCR of vegf ligands at 24 hpf in control and *e2f7/8* MO treated embryos (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 (10ng each), *vegfAa* (5ng) MO, *e2f7/8* (200pg each) and *vegfA* mRNA (200pg). 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 (± SEM) compared to the control condition in two independent experiments (***P < 0.001).

reduced *vegfAa* mRNA levels compared to *wildtype* littermates (Supplemental Figure S2A and B). In contrast, *e2f1* a known target gene for e2f7/8[8, 23], was upregulated in *e2f7/8* mutant zebrafish (Supplemental 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 extent, *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,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,E).

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 are 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 hpf most ISA in control *Tg (fli1:negfp;flk1:mCherry)* embryos contained three or four cells as reported previously[24] (Supplemental 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 (Supplemental Figure S2C and D). Consistently, segmental arteries of *VegfAa* MO injected embryos phenocopied the loss of E2f7/8 in zebrafish (Supplemental 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 (Supplemental 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 (Supplemental Figure S2C, not quantified). Importantly, the increased endothelial cell numbers upon injection *e2f7/8* mRNA was associated with increased *vegfAa* mRNA levels (Supplemental 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[23]. 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 least 1000-fold lower compared to E2F7), whereas HeLa cells express E2F7 and E2F8 to a similar level (Supplemental Figure S3A). To test whether E2F7/8 regulate *VEGFA* expression in human cells we used RNAi mediated knock down for E2F7 in U2OS cells and both E2F7 and E2F8 in HeLa cells (validation of siRNA is shown in Supplemental Figure S3B). Knockdown (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 (Supplemental Figure S4A).

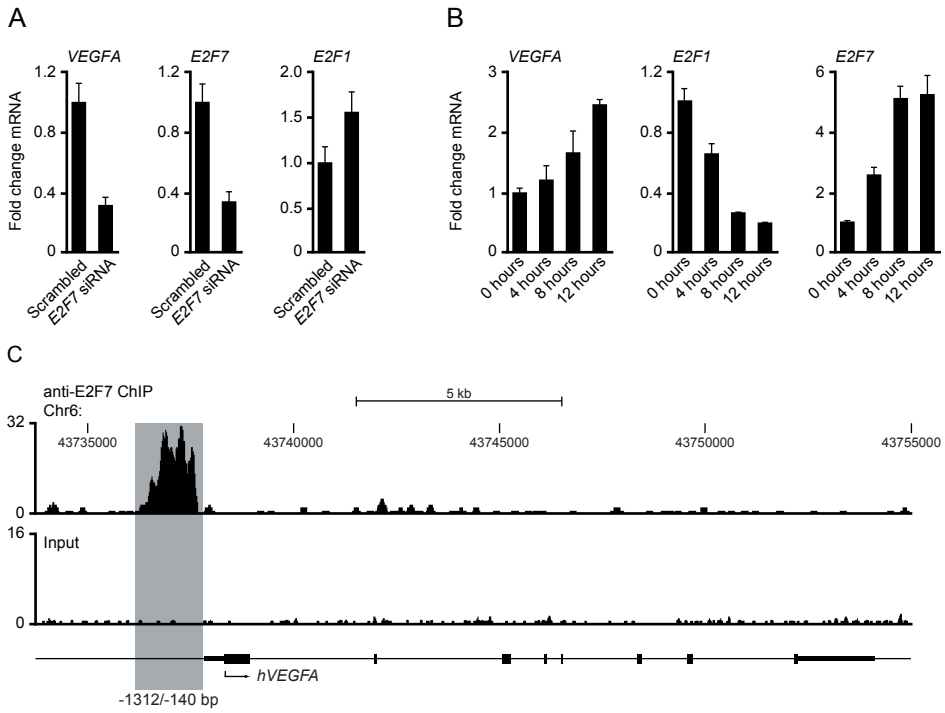


Figure 3 I E2F7/8 bind the *VEGFA* promoter and positively control *VEGFA* expression. **A** Effect of E2F7 or control (Scrambled) siRNA on indicated transcripts in U2OS cells. **B** S-phase synchronized HeLa cells expressing E2F7-EGFP (doxycycline inducible). Indicated transcripts were determined by qPCR in 0h, 4h, 8h, and 12h dox-treated versus vehicle treated cells. **C** Upper panel, ChIP-sequencing revealed E2F7 binding (black peaks) to the proximal region (-1312/-140; gray 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 (± SD) compared to the control condition in three independent experiments.

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[21]. After 4 hours of doxycycline administration *VEGFA* mRNA levels were induced and continued to accumulate at 8 and 12 hours (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[21], 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 analyzed the proximal *VEGFA* promoter region identified by ChIP-sequencing (Figure 3C) for E2F binding sites. This revealed the presence of two putative E2F binding sites in an 1173 bp fragment that corresponds to -1312/-140 of the human *VEGFA* promoter (Figure 4A). Interestingly, these E2F-binding sites (E2F-BS) are in close proximity of binding sites for HIF1, a key transcriptional regulator of *VEGFA*, that is regulated both by oxygen dependent and independent mechanisms[15, 16]. 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 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 47bp *VEGFA* promoter element surrounding the reported HIF responsive site -974[25]. KD of HIF1α reduced promoter activity of the -974 element about 30 fold (Figure 4C). In addition, we analyzed 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 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 mouse embryonic fibroblasts (MEF) also resulted in significantly reduced hypoxic VEGFA expression (Supplemental 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* (Figure S4B), two other HIF target genes. This suggests that E2F7/8 do no 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[21] 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

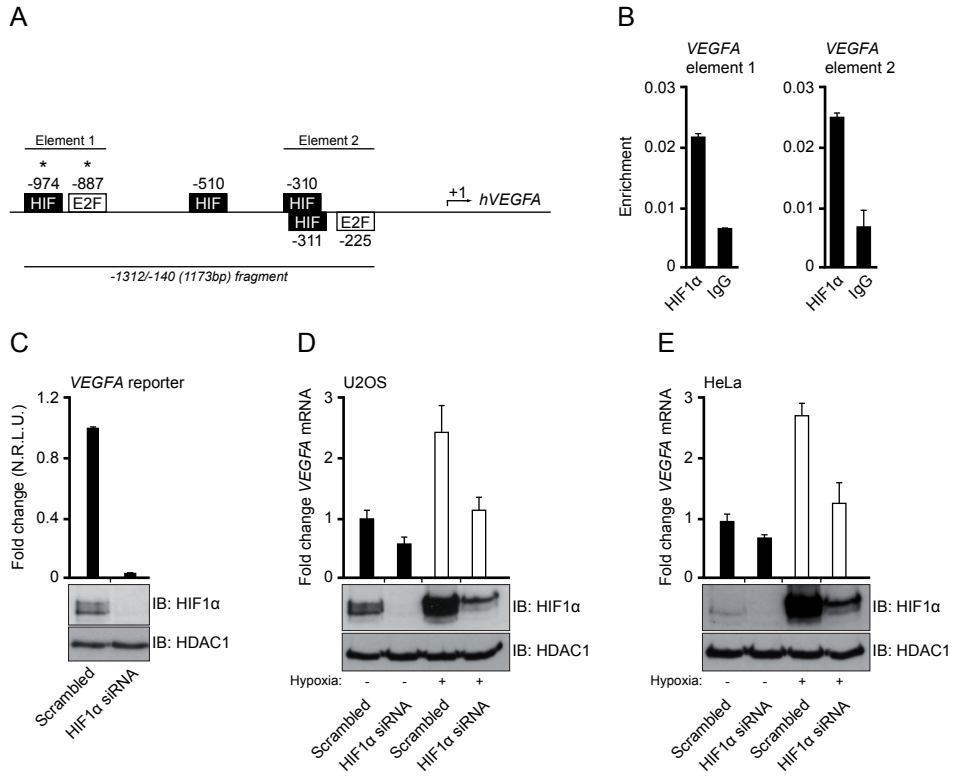


Figure 4 I HIF1 binds and stimulates the VEGFA promoter in normoxia. **A** Schematic presentation of a human *VEGFA* promoter, -1312/-140 (1173bp) corresponds to the region identified by ChIP-sequencing (Figure 3C). Potential E2F and HIF binding sites (BS), identified by Matinspector software, are indicated. 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 47bp (-985/-939) *VEGFA* reporter plasmid, containing the -974 HIF-BS, co-transfected with HIF1α or scrambled siRNA in normoxic U2OS cells. Lower panels, western blot for HIF1α and loading control HDAC1. **D** U2OS cells transfected with HIF1α or scrambled siRNA, grown under normoxic (black bars) or hypoxic conditions (white bars). Upper panel shows *VEGFA* mRNA levels, 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 (± SD) compared to the control condition in three independent experiments.

VEGFA expression (16-fold, Figure 5B lane 8). Additional ChIP assays performed in hypoxic HeLa cells showed both E2F7 and HIF binding to element 1 and 2 on the *VEGFA* promoter (Figure 5C). Since we were able to distinguish a previously reported *E2F* site in the *E2F1* promoter[8, 23] from a non-specific region 700bp upstream (Figure 5C, lower panels), we conclude that the resolution of our ChIP assays is sufficient to state that E2F7 and HIF1 bind both to element 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.

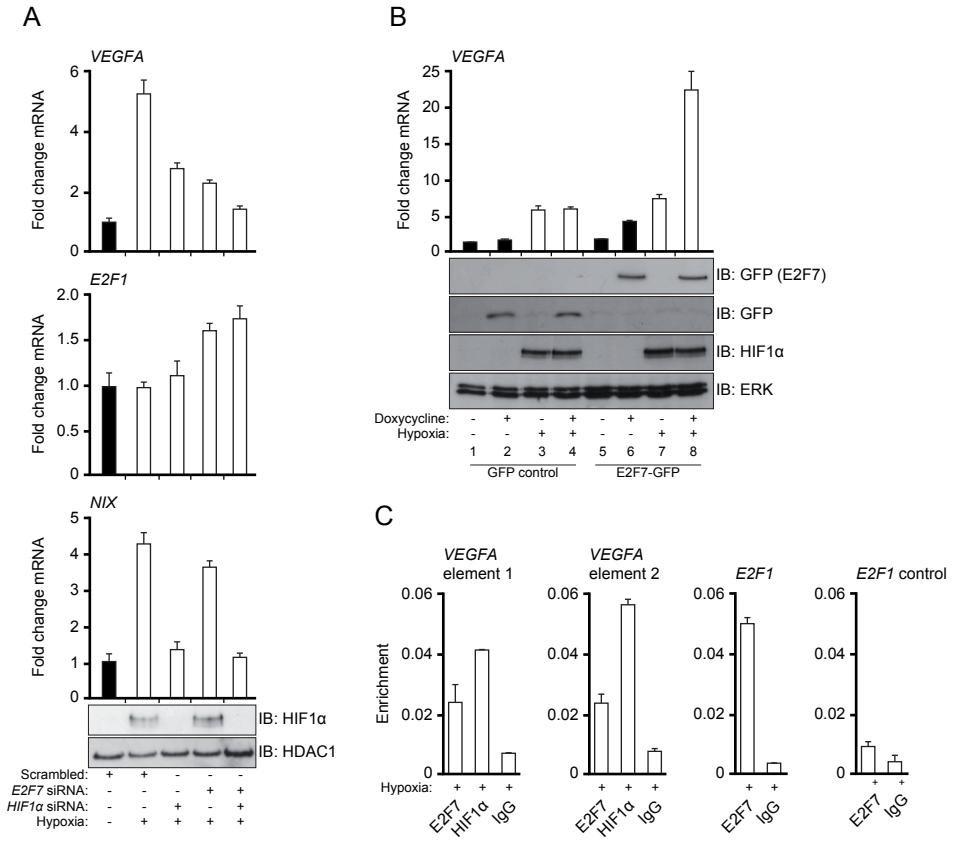


Figure 5 I E2F7/8 and HIF1 are required for hypoxic *VEGFA* expression. **A** U2OS cells were transfected with a total of 150pmol siRNA: 75 pmol E2F7 plus HIF1α, or 75 pmol E2F7 or HIF1α plus 75 pmol Scrambled. Transfected cells were cultured in normoxia or hypoxia. qPCR was performed for indicated transcripts. Lower two panels show HIF1α and HDAC1 (loading) protein levels. **B** Doxycycline-inducible E2F7-EGFP or control doxycycline-inducible EGFP cells were treated with 20h dox, 4h hypoxia, 20h dox of which the last 4h 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 (± SD) compared to the control condition in three independent experiments.

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)[8, 23]. Furthermore, we could not detect an interaction between HIF1α and FOXO3a (Supplemental Figure S6A), an indirect regulator of HIF1α[26, 27]. Using a similar approach we could also detect an interaction between E2F7 and HIF2α (Supplemental 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[28, 29]. Consistent with our data, the N-terminal part of HIF1α is known to be regulated in an oxygen-independent manner[30].

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 expression levels by ectopic overexpression (Figure 5B, 6A, S6A and B) or siRNA mediated KD (Figure 5A, 6F and 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 element 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

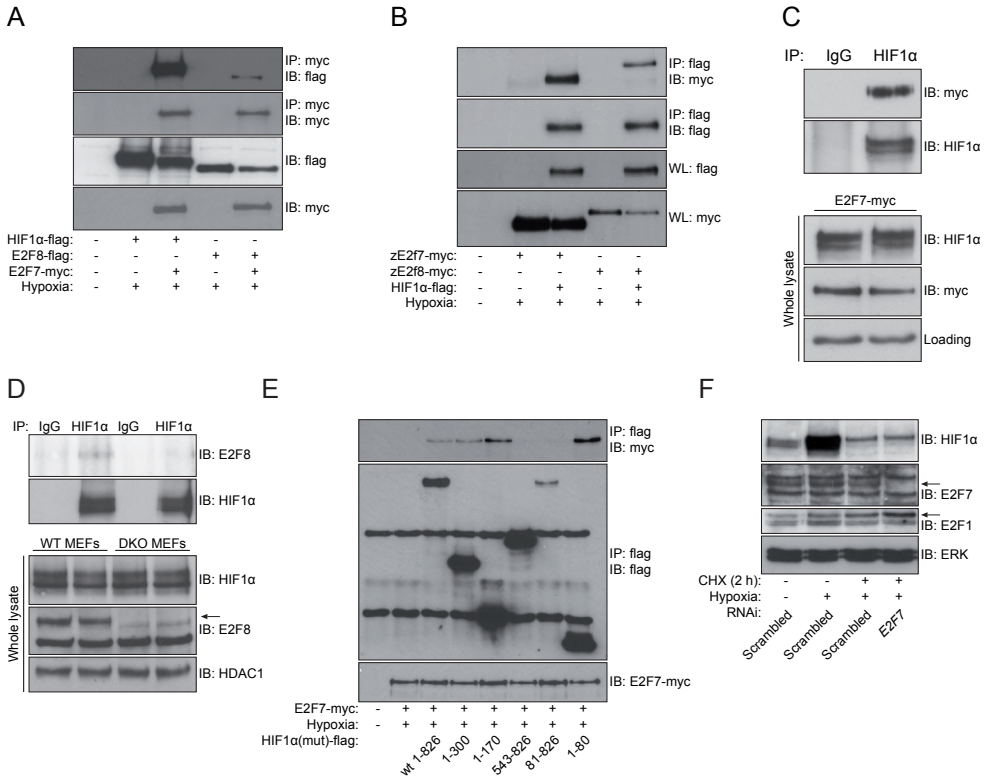


Figure 6 I The N-terminal 80 amino acids of HIF1α are required for E2F7/8 binding. **A** Lysates of U2OS cells transfected with flag-tagged HIF1α or E2F8 in combination with an empty vector (-) or myc-tagged E2F7 were maintained in hypoxic conditions except for empty vector. Immunoprecipitation (IP) 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. **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 E2F7myc 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 an 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).

VEGFA promoter activity in normoxia and hypoxia (Figure 7A, confirmation of protein KD in the reporter assay is shown in Supplemental Figure S7A). To analyze 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 Supplemental Figure S7B). First we tested the ability of HIF1 to activate these promoters. HIF1 induced wt promoter activity 11 fold. This was reduced to 4-5 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 (Supplemental 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 analyzed 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 47bp *VEGFA* promoter region surrounding the -974 HIF-BS, in combination with a 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 wildtype 47bp *VEGFA* reporter or a mutant reporter in which the -974 HIF-BS is mutated. Activation of E2F7 induced the WT -974 reporter about 6 fold which was reduced to 3-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 doxycyclin-inducible, DNA-binding deficient E2F7 mutant (Figure 7E) could stimulate endogenous *VEGFA* expression. As before (Figure 5B), induction of wildtype E2F7 induced *VEGFA* mRNA expression both in 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.

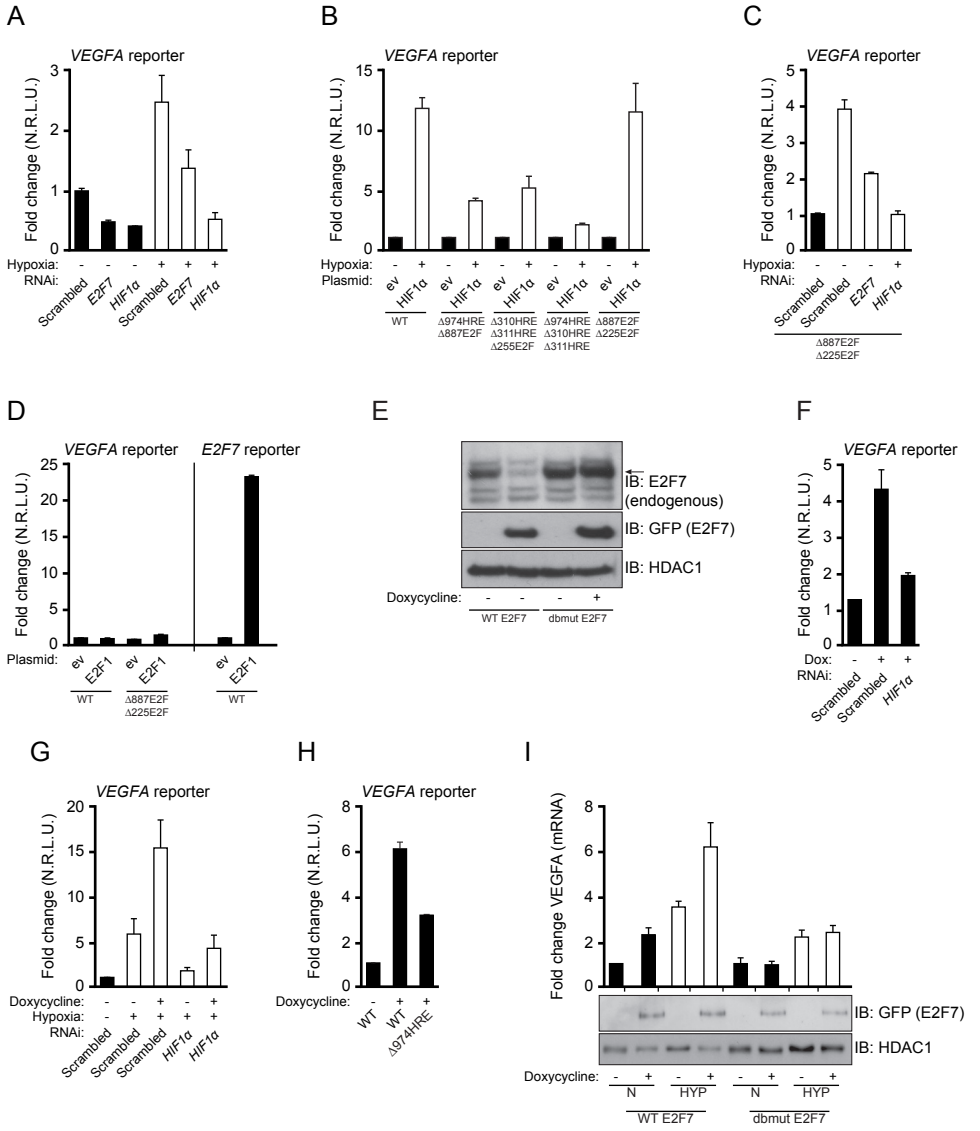


Figure 7 I E2F7/8 stimulation of hVEGFA promoter region -1312/-140 requires direct promoter binding and the presence of HIF1. **A** U2OS cells were transfected with a Scrambled, E2F7 or HIF1α siRNA in combination with a reporter plasmid containing region -1312/-140 (1173bp, Figure 4A) of the VEGFA promoter 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 a HIF1α expression or control (ev) plasmid. **C** Similar as in (A) with the difference that now the effect of indicated siRNAs was analyzed 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 2kb *E2F7* promoter together with an E2F1 expression or control (ev) vector. **E** Western blot of inducible-E2F7 HeLa cells cultured in the absence (-) or presence (+) of doxycyclin. Both an inducible wildtype (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 47bp *VEGFA* promoter region →

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[12].

Contrary to many other studies demonstrating that atypical E2Fs function as transcriptional repressors[2, 6], our data proposes 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[31, 32]. 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[32]. Based on protein information, the activator function of atypical E2Fs is unpredicted, as they lack a transactivation domain[6]. 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 binding site, 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 tumor vascularization through repression of the *VEGFA* promoter which was also suggested to occur independent of canonical E2F-BS but instead in cooperation with p53 through SP1-BS[33]. These data together with our findings, suggest that certain E2Fs may have both a positive or negative

(-985/-939) containing -974 (and not -887 E2F-BS), in combination with scrambled 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 wildtype (WT) or 974 HIF-BS mutant (Δ974HRE) 47bp (-985/-939) *VEGFA* reporter25. **I** HeLa cells with inducible WT or DNA-binding mutant E2F7 were treated (as in Figure 5B) with doxycyclin 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 (± SD) compared to the control condition in three independent experiments.E2F7 were treated (as in Figure 5B) with doxycyclin 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 (± SD) compared to the control condition in three independent experiments.

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[2, 6, 21], 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 hifα induction by genetic inactivation of *vhl* results in *vegfAa* dependent hyperbranching of ISA, head and eye vasculature[34]. 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[15, 16]. A role for HIF during normal development is furthermore supported by the fact that fast growing tissues such as embryos and tumors experience local areas of hypoxia due to high oxygen consumption[35-37]. 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.

ACKNOWLEDGEMENTS

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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). Wildtype and *E2f7/8* double knockout mouse embryonic fibroblasts (MEFs), and the inducible E2F7-GFP (WT, dbmut)

and control cell lines (HeLa) were generated and maintained as described previously[21]. 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 re-seeded (HeLa 250k/well; U2OS 200k/well) in 6 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 50nM 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 xg, 2', 4^{0}C). Protein samples were lyzed in 60µl of lysisbuffer (0.05M sodium phosphate pH7.3, 0.3M NaCl, 0.1% NP40, 10% Glycerol). Cell pellets for RNA isolation were frozen in liquid nitrogen and stored in -80^{0}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, 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: 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), Rabbit IgG HRP-linked whole Ab (GE Healthcare, NA934).

Chromatin immunoprecipitation (ChIP)

ChIP was performed according the EZ ChIP protocol (Upstate, 17-371) using protein G agarose beads (Milipore, 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 (IP)

U2OS cells were transfected according manufacturer's instructions with a total of 5 µg DNA using Superfect (Qiagen, 301305) and incubated in normoxia or hypoxia conditions for 16h. Cells were scraped on ice in cold PBS supplemented with protease inhibitors (Roche). Cells from two plates were pooled and lyzed in 1ml lysis buffer. Lysates were precleared by incubation with 20 µl prot A/G beads (Calbiochem, IP05) for 30 min at 4^{0}C. IPs were performed with 1 µg of antibody for 2h at 4^{0}C. Protein complexes were precipitated by incubation with prot A/G beads for 30 min at 4^{0}C. Precipitates were washed three times in lysis buffer and resuspended in 20 µl sample buffer. Expression constructs used: wildtype HIF1 , and HIF mutants 1-300, 543-826

were generously provided by Marc Vooijs (Maastricht University, 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 (Biorad) using SYBRgreen chemistry (Biorad). In our *in vitro* studies two reference genes were used (ACTB, RPS18) and for zebrafish samples three reference genes were used (TBP, EF1α, β Actin). MIQE standards were applied to our protocols[38].

Luciferase reporter assay

U2OS cells (60-70% confluent) were transfected using Superfect transfection reagent (Qiagen) following manufacturer's instructions. Per well (6 well plate) we used 2.5 µg reporter plasmid, 100ng TK renilla, and 0.5 µg expression or control plasmid and 10 µl Superfect per well (6 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 500ng reporter plasmid, 100pmol siRNA (50nM final concentration), 100ng TK renilla, and 5 µl Lipofectamine 2000 (per well (6 well plate). Reporter activity was measured using the Dual-Luciferase Reporter Assay System (Promega, E1910) on a microplate luminometer (Centro LB 960) 48 hours 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, 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} [39], Tg((kdrl:gfp)^{s843}(gata1:dsred))[40], and Tg((fli1:negfp)^{y7} (kdrl:mCherry))[41, 42].

Morpholino

The following morpholino oligonucleotides (Genetools) were used. *E2f7* splice donor morpholino targeting exon 2–intron 2-3: 5'-ATAAAGTACGATTATCCAAATGCAC-3'; *E2f8* splice donor morpholino targeting exon 2-intron 2-3: 5'-CTCACAGGTATCCGAAAAAGTCATT-3'; *VegfAa* ATG morpholino: 5'-ATGAACTTGGTTGTTTATTTGATAC-3' targeting both vegfAa$_{121}$ and vegfAa$_{165}$ [43].

Imaging

Embryos were mounted in 0.5-1% low melting point agarose (Invtrogen) 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 cover slip replacing the bottom. Imaging was performed with a Leica SP2 confocal microscope (Leica

Microsystems) using a 10x or 20x objective with digital zoom.

Plasmid constructs

Full length Danio *rerio e2f7* (Ensembl accession number ENSDART00000140760) or *e2f8* (Ensembl accession number ENSDART00000128488) were cloned into the pCS^{2+} Myc-tag plasmid. mRNA from these plasmids were *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[44].

In situ hybridization

In situ hybridization was performed as previously described[45]. The *vegfAa*, *vegfAb*, *vegfC* and *vegfD* probes have been described previously[22, 46]. *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 hours at 4ºC. Secondary labeling was done with a goat anti-rat biotinylated antibody (1:100 Instruchemie) in 20% goat serum/TBST o/n 4ºC. Tissues were stained with Vectastain ABC kit (Vector Laboratories). Images were captured using the Zeiss Axiophoto microscope.

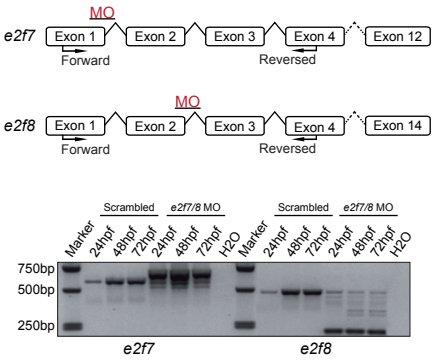
Statistical analysis

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, 1-way ANOVA, or in case of unequal variances, Kruskal-Wallis test was used. Dunns posthoc test were used to compare between selected groups. *P* values < 0.05 were considered significant. Statistical analysis was performed using SPSS 20 (IBM).

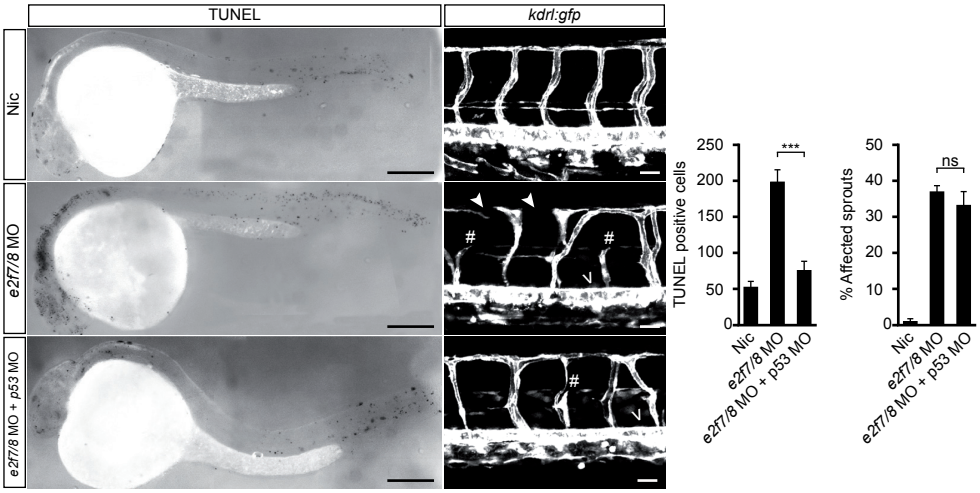
Table S1: Oligonucleotide sequences

Primer	Technique; species	Forward (3' - 5')	Reversed (3' - 5')
vegfAa	qPCR; Zebrafish	AAAAGAGTGCGTGCAAGACC	GACGTTTCGTGTCTCTGTCG
tbp	qPCR; Zebrafish	TCACCCCTATGACGCCTATC	CAAGTTGCACCCCAAGTTT
ef1α	qPCR; Zebrafish	GATTGTTGCTGGTGGTGTTG	TGTATGCGCTGACTTCCTTG
β-actin	qPCR; Zebrafish	CGTCTGGATCTAGCTGGTCGTGA	CAATTTCTCTTTCGGCTGTGGTG
e2f7 MO check	RT-pcr; Zebrafish	GCTTGGTGTCAGAACACTGG	GCATCAGAGACTCCAGCACA
e2f8 MO check	RT-pcr; Zebrafish	AAGTCCATCAAAGGCAACATC	TGTCGTAGATGCGACGTCTT
E2F7	qPCR; Human	CTCCTGTGCCAGAAGTTTC	CATAGATGCGTCTCCTTTCC
E2F8	qPCR; Human	AATATCGTGTTGGCAGAGATCC	AGGTTGGCTGTCGGTGTC
E2F1	qPCR; Human	GACCACCTGATGAATATCTG	TGCTACGAAGGTCCTGAC
ACTB	qPCR; Human	GATCGGCGGCTCCATCCTG	GACTCGTCATACTCCTGCTTGC
VEGFA(1)	qPCR; Human	ACCTCCACCATGCCAAGTG	TCTCGATTGGATGGCAGTAG
VEGFA(2)	qPCR; Human	CAGAGCGGAGAAAGCATTTG	GAGATCTGCAAGTACGTTCG
CDC6	ChIP; Human	AAACCCGATCCCAGGCACAG	AGGCAGGGCTTTTACACGAGGAG
RSP18	ChIP; Human	AGTTCCAGCATATTTTGCGAG	CTCTTGGTGAGGTCAATGTC
NIX	ChIP; Human	CGTACCATCCTCATCCTCCA	TGTGGCGAAGGGCTGTCAC
CDC6	ChIP; Human	AAAGGCTCTGTGACTACAGCCA	GATCCTTCTCACGTCTCTCACA
E2F1	ChIP; Human	AGGAACCGCCGCCGTTGTTCCCGT	CTGCCTGCAAAGTCCCGGCCACTT
E2F1 control	ChIP; Human	CGCCCAGACGCCACTTCATC	TTCATTCCCTCACTCATTCAACAA
VEGFA element 1	ChIP; Human	GGTCCTCTTCCCTCCCAGTC	GCAGACATCAAAGTGAGCGG
VEGFA element 2	ChIP; Human	CACTTTCCTGCTCCCTCCTCG	GGACGCTCAGTGAAGCCTGG
VEGFA element 3	ChIP; Human	GCAAAGTGAGTGACCTGCTT	GTCAGCGCGACTGGTCAG
E2F7 element 1	ChIP; Human	GGTTCATGTGTCACACCAGCG	AGGACAGGAAAGCAGATGGGG
E2F7 element 2	ChIP; Human	TCGCTCTCCCTTCCCGATGC	CAATTCCCGCCTCCCCACTG
E2F7 element 3	ChIP; Human	CCCTCAAAGCCGAATTCC	AGTCGGAAGGGTGTTGGG
-974 HIF-BS	Mutagenesis; Human	gactccacagtgcatacataggctccaacagg	cctgttggagccTATGtatgcactgtggagtc
-887 HIF-BS	Mutagenesis; Human	agctccacaaacttgTTaCCGAAttcttctcccctggg	cccaggggagaagaaTTCGGtAAcaagtttgtggagct
-510 HIF-BS	Mutagenesis; Human	CTTCGAGAGTGAGGACATATGTGTCTGTGTGGGT	ACCCACACAGACACATATGTCCTCACTCTCGAAG
-311 HIF-BS	Mutagenesis; Human	TCAGGCCCTGTCCATATGTAACCTCACTTTCCTG	CAGGAAAGTGAGGTTACATATGGACAGGGCCTGA
-225 HIF-BS	Cloning; Human	ggggcggatgggtaaTTTCCAAGTtgtgaaccttgg	ccaaggttcacaACTTGGAAAttacccatccgcccc
-1312/-140 VEGFA promoter	Cloning; Human	gagaCTCGAGtgtgagcctggagaagtagcc	gagaAAGCTTctctgcg gacgccca gtgaagcct
-1007/-828 VEGFA promoter	Cloning; Human	gagactcgagCCCTTTGGGTTTTGCCAGACTC	gagaaagcttAGTGACTGGGGTCCTTTGGGAA

S1A



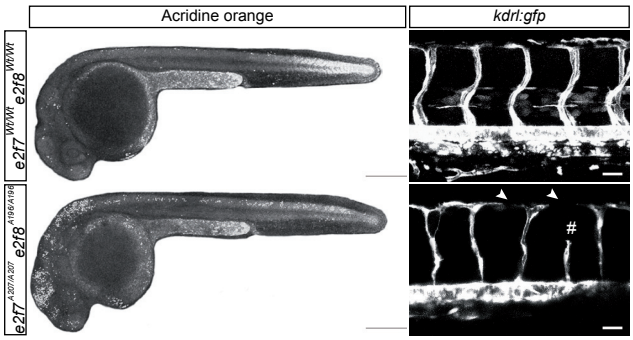
S1B



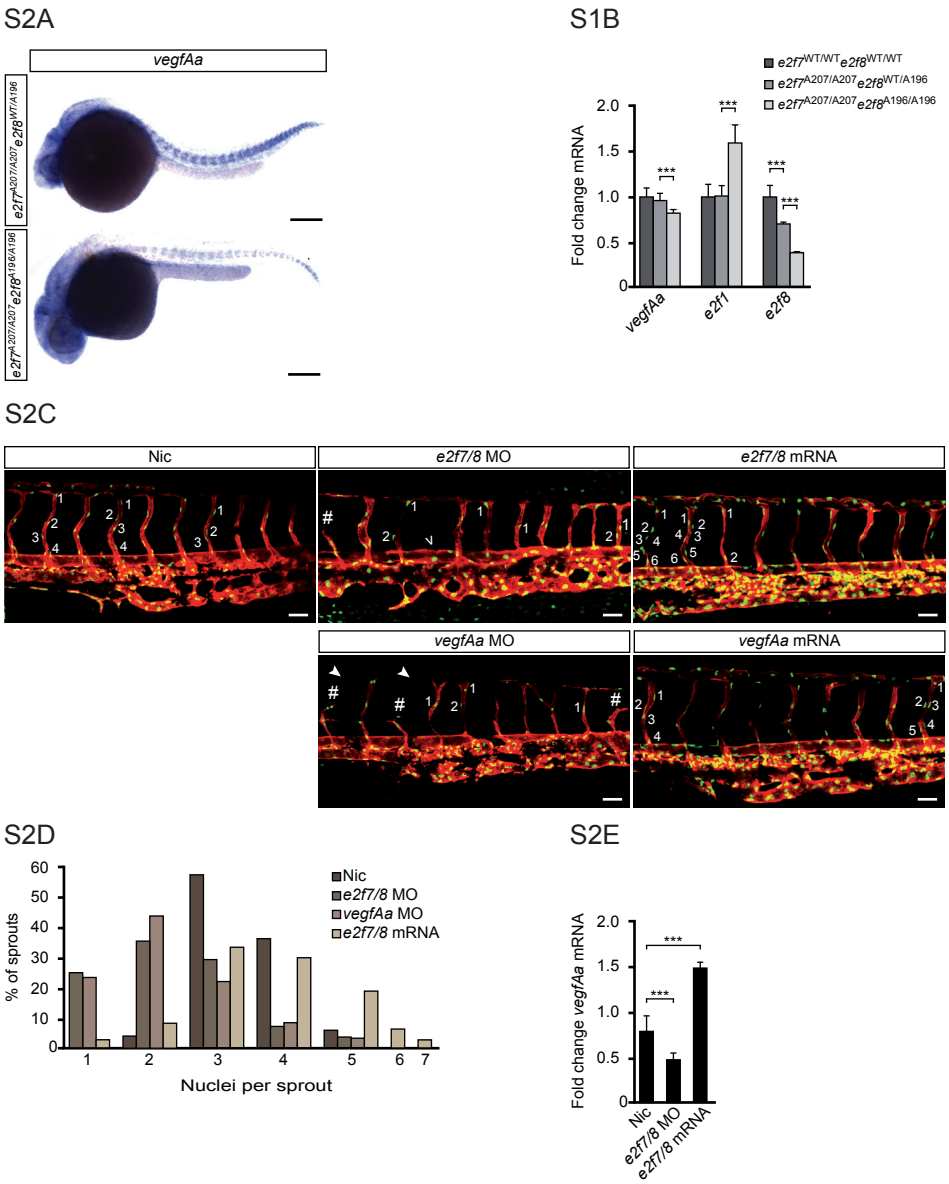
Supplemental Figure S1 I E2f7 and E2f8 are essential for angiogenesis. **A** Schematic drawing of the *e2f7* and *e2f8* zebrafish gene. Morpholino target site is marked with MO and forward and reversed primers are marked with black arrows. DNA-binding domains of both *e2f7/8* range from exon 3 to 6. All major bands were extracted from gel and analyzed by sequencing to investigate the splicing effect of the morpholinos on the mRNA. **B** TdT-mediated dUTP nick end-labeling (TUNEL) in *Tg(kdrl:gfp)* control, *e2f7/8* and *e2f7/8/p53* MO treated embryos, representative images of ISAs are shown for each condition. Quantification of TUNEL positive cells and vascular defect from 2 different experiments with at least n=60. →

S1C

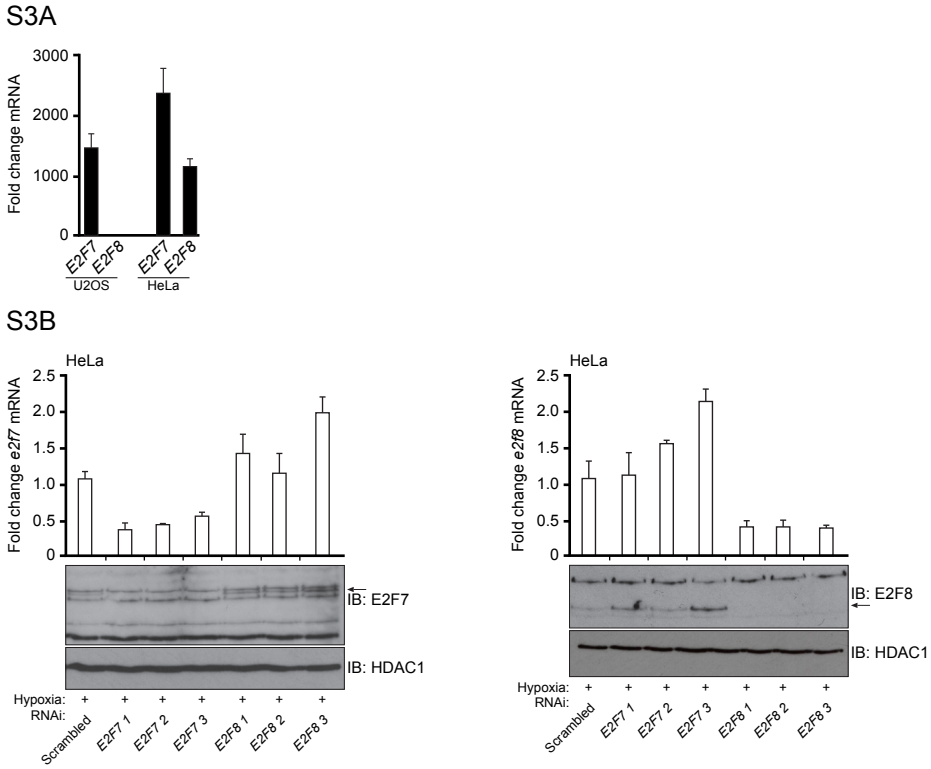
S1D



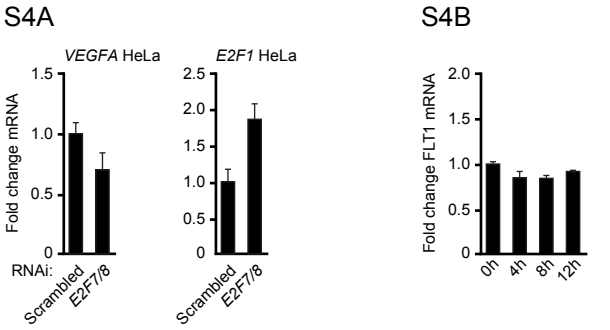
C Multiple protein sequence alignment of the first DNA binding domain of both E2f7 and E2f8 showing conservation of mutation, E2f7 arginine 207 and E2f8 arginine 196. Western blot showing ectopic expression of Myc tagged zebrafish E2f7, E2f7A207, E2f8, E2f8A196 and empty vector. **D** Apoptotic cells stained by acridine orange in Tg(kdrl:gfp) mutant zebrafish, representative images of ISAs are shown.
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. (*) $P < 0.001$ Kruskal-Wallis.



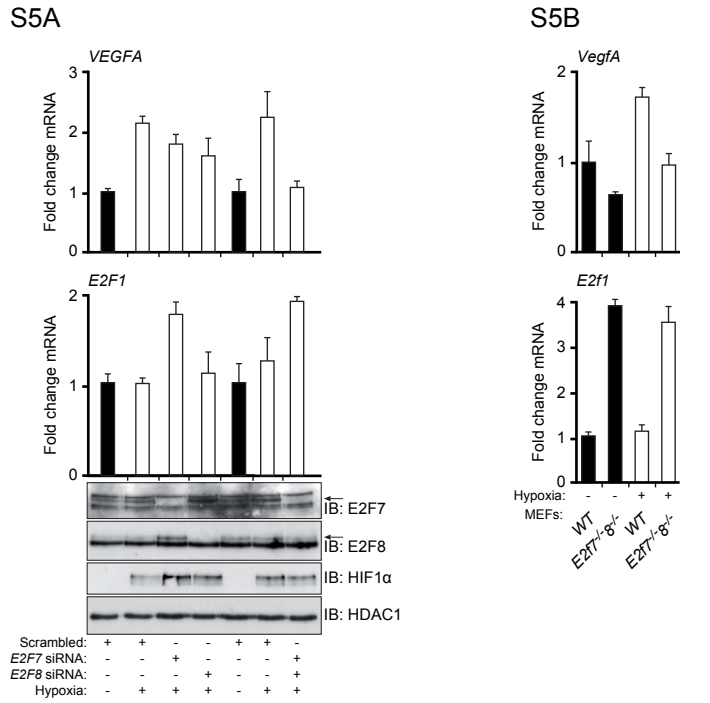
Supplemental Figure S2 I E2f7 and E2f8 regulate angiogenesis through stimulation of *vegfA*a expression. **A** *vegfAa* ISH in mutant zebrafish at 24 hpf, after imaging embryos were subjected to genotyping. **B** qPCR of *vegfAa*, *e2f1* and *e2f8* at 24 hpf in control and mutant embryos. RNA was extracted from single embryos at least n=30 per condition in 2 different experiments. Samples were normalized against 4 reference genes (elongation factor-1 alpha, tata box binding protein, beta 2-microglobulin and 18S ribosomal RNA). **C** Representative images of *e2f7/8* (10ng each) or *vegfAa* (5ng) MO treated and ectopic *e2f7/8* (200pg each) or *vegfA* mRNA (200pg) expressing *Tg(fli1a:negfp;flk1:mCherryRas)* embryos that were used to quantify number of nuclei present in ISAs. **D** Quantification nuclei distribution (n=137 sprouts). **E** *vegfAa* mRNA levels measured by qPCR at 24 hpf. (n=10 per condition). (*) P < 0.001 Mann-Whitney U (B,E)



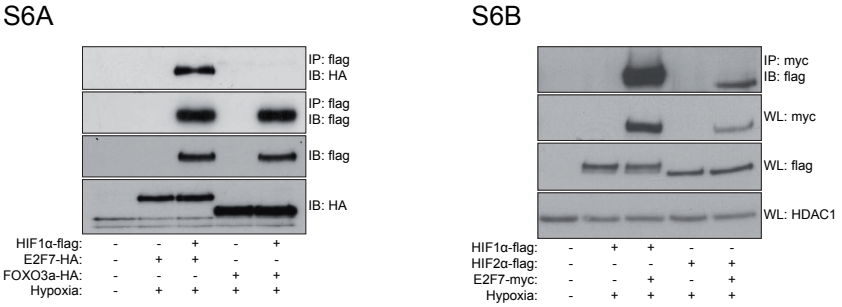
Supplemental Figure S3 I E2F7/8 mRNA levels in U2OS and HeLa cells determined by qPCR, and validation of E2F7 and E2F8 siRNA. **A** For both cell lines 1 µg of total RNA was used for cDNA synthesis. The relative fold change in mRNA was calculated to the low level of *E2F8* mRNA expression in U2OS cells. Data are presented as the average fold change (± SD) in samples analyzed in triplicate. **B** Validation of E2F7/8 siRNA in HeLa cells expressing both *E2F7* and *E2F8*. Cells were transfected with control siRNA (Scrambled) or a single siRNA for E2F7 or E2F8. Three different siRNAs for E2F7 and E2F8 were tested. Cells were harvested 24 hour after transfection. KD was confirmed by qPCR (upper panel) and Western blot (two lower panels). Left panel shows analysis for E2F7, right panel for E2F8. KD of E2F7 (left panel) is efficient with all three different siRNAs. *E2F7* mRNA level reduction (50-60%) result in absence of E2F7 protein (arrow indicates E2F7 band (middle of the three bands)). KD of E2F8 results in increased expression of *E2F7* (shown by increased E2F7 mRNA and protein levels, left panel). HDAC1 immunostaining as well as background bands in E2F7 blot show equal loading. Validation of *E2F8* (right panel) shows that 60-70% KD on mRNA level (upper panel), results in the absence of E2F8 protein (E2F8 protein is indicated by arrow, middle panel). Consistent with the repression of *E2F7* expression by E2F8 (left panel), KD of E2F7 derepressed *E2F8* expression (shown by increased E2F8 mRNA and protein, right panel). This negative feedback regulation between E2F7 and E2F8 has not been reported.



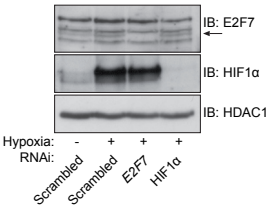
Supplemental Figure S4 I E2F7/8 stimulate *VEGFA* expression in HeLa cells. **A** HeLa cells were transfected with siRNA for E2F7 and E2F8 (7/8, 50 pmol each), or a scrambled siRNA (scr, 100pmol). 24 hr after transfection cells were harvested. The transcript levels of VEGFA and *E2F1* in these samples were determined by qPCR. Data presented as average fold change (± SD) compared to controls (Scrambled) in samples analyzed in triplicate. **B** E2F7 does not affect expression of the HIF target gene *FLT1*. This figure is related to Figure 3B now showing *FLT1* mRNA in these samples. Transcript levels of *FLT1* was determined in 0h, 4h, 8h, and 12h dox-treated cells by realtime PCR. Data are presented as the average fold change (± SD) compared to 12h vehicle treated cells in samples analyzed in triplicate.



Supplemental Figure S5 I E2F7/8 stimulate *VEGFA* expression in HeLa and MEF cells. **A** In HeLa cells the simultaneous KD of *E2F7/8* is required to affect *VEGFA* expression, caused by functional compensation between E2F7 and E2F8 (through redundancy and negative feedback control of E2F7 by E2F8 and vice versa (Supplemental Figure S3B). HeLa cells were transfected with a total of 100 pmol (50nM, lane 1-4) or 150 pmol siRNA (75 nM, lane 5-7). For the double E2F7 and E2F8 siRNA transfection (lane 8) we used 75 pmol of each siRNA. qPCR was performed to determine *VEGFA* and *E2F1* mRNA levels. Data are presented as average fold change (± SD) compared to controls (Scrambled) in samples analyzed in triplicate. Western blot analysis with antibodies specific for E2F7 (middle band of triplet indicated by arrow. Similar as in supplemental Figure S3B), E2F8 (upper band indicated by arrow) and HIF1α validated efficient knockdown, and HDAC1 immunoblotting served as a loading control. **B** Deletion of *E2f7* and *E2f8* in mouse embryonic fibroblasts (*E2f7*$^{-/-}$*E2f8*$^{-/-}$ MEFs) results in decreased *VegfA* expression and derepression of *E2f1*. Wildtype (WT) or *7/8*$^{-/-}$ cells were treated for 16h with hypoxia (white bars) or left untreated (normoxia, black bars). Data are presented as average fold change (± SD) compared to normoxic wilt type cells (WT), in samples analyzed in triplicate.



Supplemental Figure S6 I E2F7 binds both to HIF1α and HIF2α. **A** To test the specificity of the E2F7-HIF1α interaction we analyzed HIF1α binding to FOXO3a, an indirect regulator of HIF1[27]. U2OS cells were transfected with E2F7-HA and HIF1α–Flag or vector (-), or with FOXO3-HA and HIF1α–Flag or vector as indicated. Transfected cells were grown overnight in hypoxia, whereas vector transfected cells (-) were cultured under normoxia. Immunoprecipitations (IP) were performed with a αFlag specific antibody which was added to all conditions. E2F7 did co-IP with HIF1α, while FOXO3a did not (analyzed by αHA immunoblot (IB), upper panel). HIF1α IP were also backstained (IP and IB αFlag antibody) to show successful IP. **B** To investigate if E2F7 both binds to HIF1α and HIF2α, U2OS cells were transfected with HIF1α-flag or HIF2α-flag in combination with vector (-) or myc-tagged E2F7 (as indicated). Cells transfected with vector (-) alone were maintained in normoxia, all other conditions in hypoxia. E2F7-myc was immunoprecipitated (IP) using a myc-specific antibody, and co-precipitating HIF1α-flag or HIF2α-flag was detected by immunoblotting (IB) using a flag-specific antibody. Myc antibody and beads were added to all lysates ruling out non-specific precipitation of HIF1α. To show IP of E2F7-myc, IPs were backstained with myc antibody. WL: whole cell lysates.



S7B

-887 E2F-BS (+)	
E2F consensus	T T T S S C G C
E2F7 consensus	T T C C C G C
-887 E2F-BS WT	T T T G G C A C
-887 E2F-BS Mut.	T T C G G T A A
-225 E2F-BS (-)	
E2F consensus	T T T S S C G C
E2F7 consensus	T T C C C G C
-225 E2F-BS WT	T T T C A G G C
-225 E2F-BS Mut.	T T C C A A G T

-974 HIF-BS (+)	
HIF consensus	R C G T G
-974 HIF-BS WT	A C G T G
-974 HIF-BS Mut.	A C A T A
-311 HIF-BS (+)	
HIF consensus	R C G T G
-311 HIF-BS WT	A C G T A
-311 HIF-BS Mut.	A T G T A
-311 HIF-BS (-)	
HIF consensus	R C G T G
-311 HIF-BS WT	A C G T G
-311 HIF-BS Mut.	A C A T A

Supplemental Figure S7 I Validation of KD of E2F7 and HIF1α in a reporter assay performed in U2OS cells. **A** Cells were transfected with a combination of reporter plasmid DNA and siRNA as indicated. E2F7 protein band is indicated by arrow (middle of triplet of bands). Immunostaining of samples with an HDAC specific antibody was used as loading control. **B** Mutation of HIF and E2F binding sites present in the -1312/-140 *VEGFA* promoter. The HIF and putative E2F binding sites (BS) were identified using Matinspector software. BS located in the positive strand are indicate by (+), localization in the negative strand by (-). The -887 and -225 E2F-BS are aligned to the classic E2F consensus sites TTTSSCGC, as well as to the E2F7 consensus site (TTCCCGCC) recently identified[21]. HIF-BS -974, -311 and -310 were aligned to the HIF consensus RCGTG (Semenza, 2009).S represents a G or C, R represents an A or G according to the IUPAC code. Mutated bases are underlined.

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HIF proteins connect the RB-E2F factors to angiogenesis

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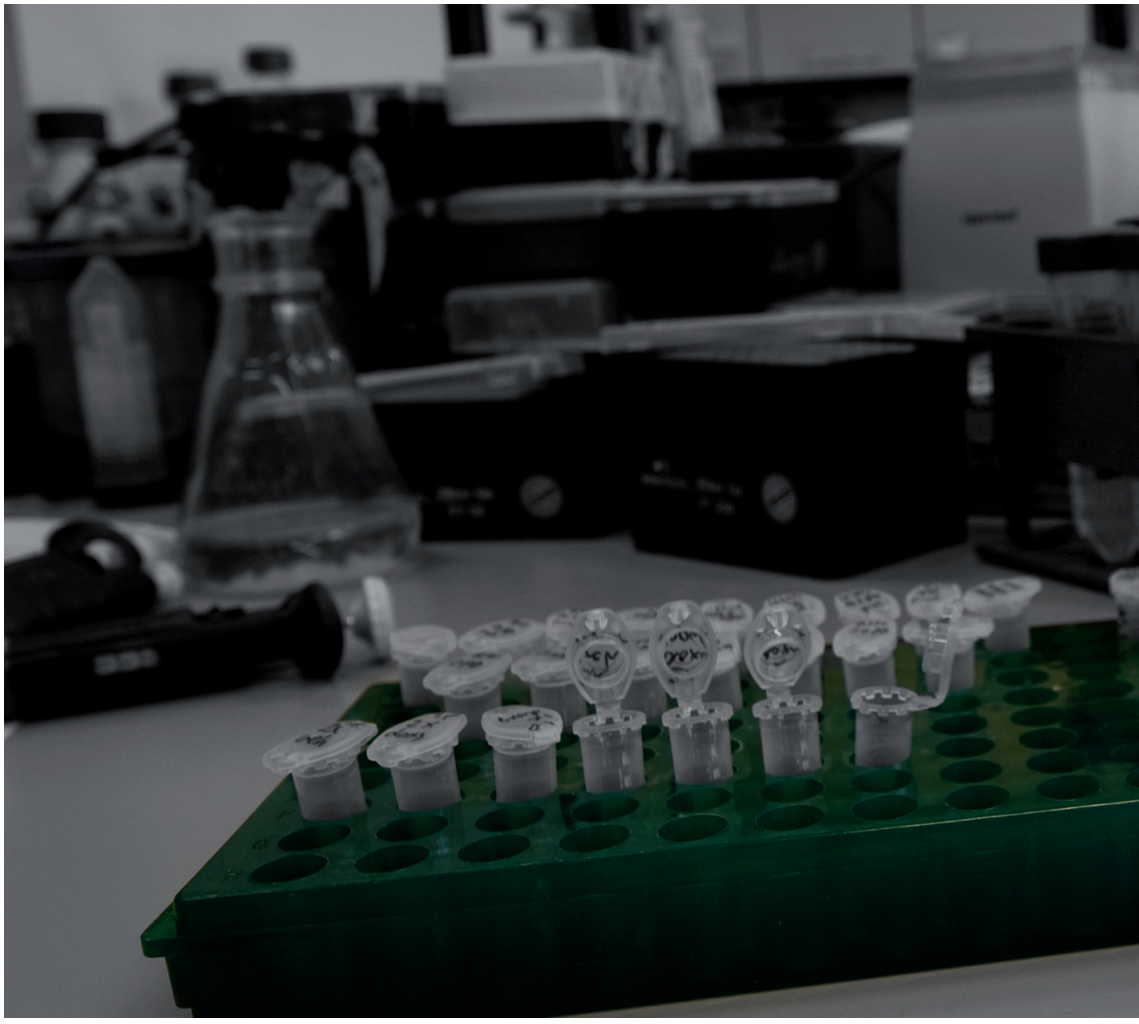

Chapter 3

ABSTRACT

Recently we showed that E2F7 and E2F8 (E2F7/8) are critical regulators of angiogenesis through transcriptional control of *VEGFA* in cooperation with HIF[1]. Here we investigate the existence of other novel putative angiogenic E2F7/8-HIF targets, and discuss the role of the RB-E2F pathway in regulating angiogenesis during embryonic and tumor development.

E2F7/8 function as novel and critical regulators of angiogenesis

Two decades after the identification of E2F1 as the factor through which the Retinoblastoma (RB1/RB) protein controls the adenoviral E2 promoter, the RB-E2F pathway has been proven as a key regulator of cellular proliferation[2, 3].Today, eight E2F family members have been cloned which are generally classified as activators (E2F1-3) or repressors (E2F4-8). The most recently identified E2F members E2F7 and E2F8 (E2F7/8) are referred to as atypical E2Fs because they harbor two instead of one DNA binding domain and regulate gene transcription independent of DP and RB proteins[2-6]. Although E2F factors were long understood as essential regulators of cellular proliferation, recent *in vivo* studies using gene-targeting knockout strategies showed that E2Fs are dispensable for proliferation. Mice lacking all three activator E2Fs (*E2f1-3*)[7, 8] or both atypical E2Fs (*E2f7/8*)[9] die during mid-gestation without any obvious effects on cellular proliferation. These studies indicate that E2F1-3 and E2F7/8 are essential for embryonic survival through control of critical functions other than proliferation. Indeed, combined deletion of the *E2f7* and *E2f8* genes in mice causes lethality around embryonic day 10.5, resulting from widespread apoptosis and vascular defects[9]. Interestingly, although apoptosis was rescued upon additional deletion of *E2f1*, vascular defects and embryonic lethality (E10.5) were not rescued in these *E2f7/8/1* triple knockout mice[9], suggesting that E2F7/8 are essential for angiogenesis during development. In a follow up study we could indeed confirm a critical role for E2F7/8 in angiogenesis: inactivation of E2F7/8 in mice or zebrafish causes disorganized angiogenic sprouting resulting in an instable and leaky vasculature[1]. Mechanistically we found that E2F7/8 form a transcriptional complex with hypoxia-inducible factor (HIF1) and mediate angiogenesis through control of vascular endothelial growth factor (*VEGFA*) expression[1], a key regulator of vascular development. To our surprise we found that E2F7/8, although classified as repressors, stimulate *VEGFA* transcription. This activator role for E2F7/8 in transcription is probably promoter context dependent. In the case of the *VEGFA* promoter E2F7/8 stimulate transcription because they cooperate with the transcriptional activator HIF and act through a HIF binding site (HIF-BS) instead of an E2F-BS[1], through which they in general repress transcription[2, 3]. The importance of the E2F7/8-HIF interaction is furthermore underlined by the observation that similar to *E2f7/8* knockout mice, mice lacking *Hif1α*, *Hif2α* or *Hifβ* (*Arnt*) also die around embryonic day 10.5 due to vascular defects[10]. Moreover, specific deletion of *E2f7/8* in the extra-embryonic trophectoderm results in a poorly formed placental vascular network[11], a phenotype also observed in mice deficient for *Hif1α*, *Hif2*α or *Hifβ*[10]. Interestingly, both conventional deletion of *E2f7/8* as well as trophectoderm (placenta)-specific deletion of *E2f7/8* results in embryonic death around embryonic day E10.5, whereas mice are born alive when *E2f7/8* are deleted only in the embryo and not in the placenta[11]. These data show that regulation of placental development is an essential function of E2F7/8, a function that they likely perform in cooperation with HIF. Furthermore, these *in vivo* studies also suggest that the vascular defects in the placenta are also responsible for the embryonic lethality observed in *Hif*$^{-/-}$ mice. The placenta might additionally serve as a suitable model to further explore the

molecular mechanism of the E2F7/8-HIF interaction *in vivo*.

Identifying novel E2F7/8-HIF targets involved in angiogenesis

Because the E2F7/8-HIF complex plays an essential role in angiogenesis[1] we screened for novel putative E2F7/8-HIF angiogenic targets. We first analyzed the promoters of genes within the GO cluster angiogenesis (AmiGO GO:0001525) for the presence of conserved HIF- and/or E2F-BS with DAVID (Functional Annotation Bioinformatic Microarray Analysis). The GO cluster angiogenesis contains 354 genes of which 54 genes contain only a conserved HIF-BS in their promoter, while 128 have both a conserved HIF- and E2F-BS in their promoter, and 81 genes carry only an E2F-BS in their promoter (Figure 1A). In addition, functional annotation within DAVID offers the possibility to look at *in silico* tissue expression, providing additional information whether these genes are expressed in specific tissues/organs (DAVID; UniProt_tissue expression (UPte)). Interestingly, analysis of all 354 genes form the GO angiogenesis showed the 2nd most strong correlation to the placenta (behind the UPte category Plasma), providing support to study angiogenesis not only in the embryo but also in the placenta.

Next, we used recently published microarray data in which *E2f7/8* had been specifically deleted in either the mouse embryo or the placenta[11], to identify E2F7/8-regulated angiogenic genes (Figure 1B). Because we recently showed that the E2F7/8-HIF complex regulates *VEGFA* through a HIF-BS[1], we focused our further analysis only on target genes having at least a HIF-BS in their promoter (Figure 1, cluster 1 and 2). Because E2F7/8 in general repress transcription when bound to an E2F-BS[12] we expected to find a higher percentage of E2F7/8 regulated genes to be up-regulated in cluster 2 (in which genes contain an E2F-BS besides a HIF-BS in their promoter) compared to cluster 1 genes (in which genes only contain a HIF-BS in their promoter). However, both clusters have a comparable percentage of up-regulated genes, suggesting that the presence of an E2F-BS does not seem to be predictive for the mode (up or down) of regulation (Figure 1B). Instead, we assume HIF to be an important determinant for the repressive or activating transcriptional character of E2F7/8. In the case of cluster 1 genes, E2F7/8 probably depend on the presence of HIF in order to regulate gene expression, as we show for *VEGFA*[1]. However in the case of cluster 2 genes, E2F7/8 may repress expression in the absence of HIF (through the E2F-BS), whereas the presence of HIF may turn E2F7/8 in transcriptional activators. Furthermore it must be mentioned that in both cluster 1 and 2 loss of *E2f7/8* results in more down- than up-regulated genes in the placenta suggesting that the E2F7/8-HIF complex predominantly functions as an activator of angiogenic genes in the placenta. Our analysis also identifies several described HIF targets genes (indicated with an asterisks, Figure 1B), including *Vegfa*, which is down-regulated in the absence of E2F7/8 as we previously reported[1]. This strengthens the validity of our approach. Further studies are required to verify that these angiogenic factors are indeed regulated by the HIF-E2F7/8 complex.

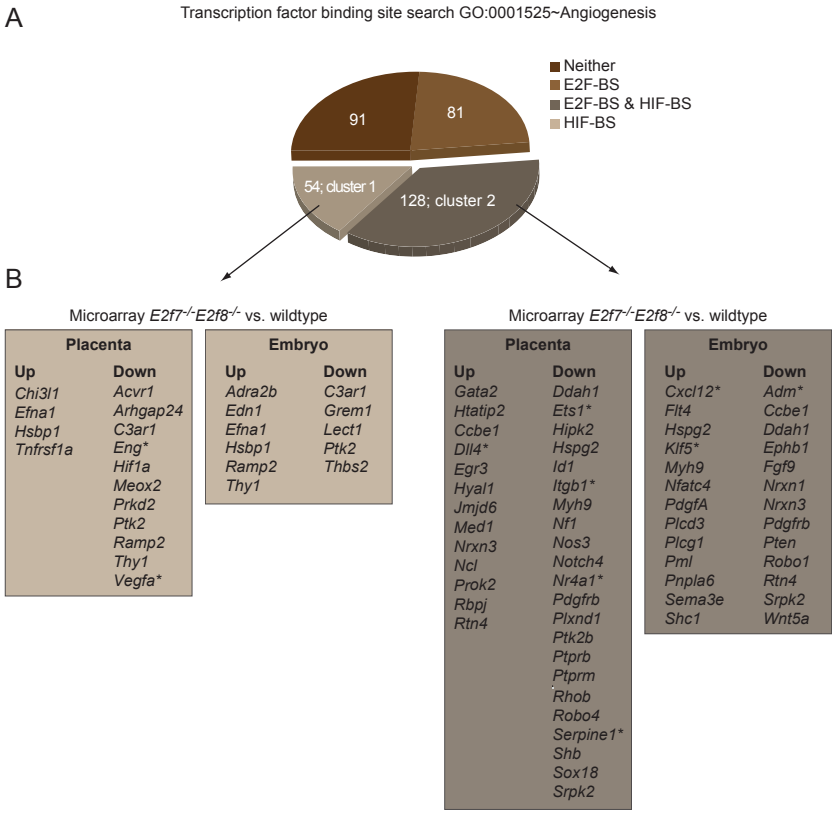

Figure 1 I Exploring novel putative angiogenic targets of the E2F7/8-HIF complex. **A** HIF binding site (HIF-BS) and E2F binding site (E2F-BS) analysis in promoters of genes contained by the ontology cluster angiogenesis (AmiGO term GO:0001525, 354 genes). The UCSC TFBS function within the DAVID software was used to search in each of these genes for conserved (between human, mouse and rat) binding sites within a region up to 5kb upstream of the transcription start site. **B** Identification of deregulated angiogenesis transcripts in embryos or placentas lacking E2f7/8. Data analysis was performed on a public dataset (GSE30488), using Flexarray 1.6.1. After background correction with RMA, Empirical Bayes estimation (Wright&Simon) was performed. Sox2-Cre; E2f7-/-E2f8-/- embryos were compared with wild type embryos; and Cyp19-Cre; E2f7-/-E2f8-/- placentas with wild type placentas. Gene lists represent transcripts from angiogenesis genes with an adjusted P value <0.05 versus wild type, subdivided according to presence of HIF-BS or HIF-BS+E2F-BS as found in (A). Asterisks indicate described HIF target genes.

RB-E2F factors control angiogenesis through regulation of VEGFA and its receptors

Typical E2F function (E2F1-5) is tightly regulated by the pocket proteins RB1/p105, RBL1/p107 and RBL2/p130. When bound to these pocket proteins, E2Fs act as transcriptional repressors[2, 3]. An intriguing question that needs to be addressed is whether the RB-E2F pathway in general regulates vascular development and whether such a role would depend on a cooperation with HIF factors. With regard to RB1 the literature indeed suggests that RB1 like E2F7/8 regulates

vascular development in cooperation with HIF. Specifically, RB1 has been shown *in vitro* to stimulate HIF-dependent transcription by forming a direct interaction with HIF[13], like we show for E2F7/8[1]. Furthermore, RB1 is enriched on the *VEGFA* promoter, possibly through its interaction with HIF[13] and regulates *VEGFA* expression[14]. In line with the observation that deletion of *E2f7/8* or *Hif* in mice results in vascular defects in the placenta, loss of *Rb1* also results in vascular defects in the placenta[15]. Deletion of *Rb1* in the trophectoderm not only results in a disruption of the labyrinth architecture because of excessive trophoblast proliferation, but also results in a reduced number of fetal capillaries[15]. Based on the interaction between RB-HIF, HIF-E2F7/8 and the similar vascular phenotypes in placentas of individual *Rb1*$^{-/-}$, *Hif*$^{-/-}$, and *E2f7/8*$^{-/-}$ mice, we suggest that the interaction between RB-E2F and HIF pathway plays an important role in regulating the expression of angiogenic factors in the placenta. Although RB1, E2F7/8 and HIF may cooperate on gene transcription in a common transcriptional complex, it is unlikely that RB1 and E2F7/8 directly interact. Namely, unlike E2F1-5, E2F7/8 do not harbor a RB-binding domain[2, 3]. HIF1, however may facilitate an indirect interaction between E2F7/8 and RB1 because both proteins interact with HIF1α through different domains: E2F7/8 bind to the N-terminal 80 amino acids of HIF1α[1], while RB1 binds to amino acids 530-694[13]. E2F7/8 and RB1 may thus regulate angiogenesis as part of shared transcriptional complex through their direct but independent interaction with HIF1 (Figure 2). Furthermore, E2F7/8 and RB1 may also functionally interact in their control of angiogenesis. Similar to the reported synergistic function of E2F8 and RB1 in controlling erythropoiesis[16], E2F7/8 and RB1 may also synergistically regulate angiogenesis. For example, simultaneous deletion of E2F7/8 and RB1 may result in ectopic activator E2F activity which may lead to deregulation of angiogenic E2F targets such as *VEGFA*.

Further support that the RB-E2F pathway controls angiogenesis is provided by *in vivo* studies using *E2f1*$^{-/-}$ mice that display enhanced angiogenesis, endothelial cell proliferation and reperfusion in a hind limb ischemia model, resulting from enhanced *Vegfa* expression[17]. Mechanistically, E2F1 was proposed to repress the *VEGFA* promoter in cooperation with p53 (*Tp53*)[17], although another study reported that E2F1 can also repress *VEGFA* transcription independent of p53[18]. Importantly, recent studies demonstrated that E2Fs not only regulate *VEGFA* transcription in cells that secrete angiogenic factors, but also regulate expression of VEGF receptors in endothelial cells. Specifically, VEGFA stimulation of endothelial cells results in inactivation (hyperphosphorylation) of RB1 leading to E2F1 induced transcriptional activation of *VEGFR1/FLT1* and *VEGFR2/KDR*[19] (Figure 2). Interestingly, in zebrafish we observed that inactivation of *e2f7/8* potently induces the expression of *vegfr1/2* in vascular endothelial cells (unpublished observations), suggesting that E2F7/8 also regulate angiogenesis on the level of endothelial cells and that E2F activators and E2F7/8 repressors balance the expression of *VEGFR1/2*. Notably, *VEGFR1*[20] and *VEGFR2*[21] have also been described as HIF target genes, raising the possibility that E2F7/8 regulate the expression of these factors in cooperation with HIF. Combined with our observations[1] a model emerges in which the E2F7/8-HIF complex induces *VEGFA* expression in hypoxic cells. Secreted VEGFA subsequently binds to VEGF-receptors

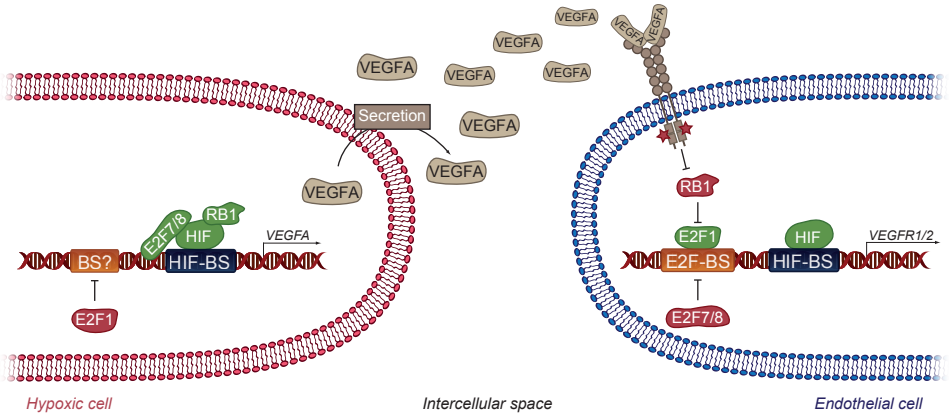

Figure 2 I RB-E2F factors control angiogenesis through regulation of VEGFA and its receptors. In hypoxic cells HIF proteins stimulate VEGFA expression. RB1 and E2F7/8 have both been reported to interact with HIF and co-stimulate transcriptional activation of VEGFA. E2F1 down-regulates VEGFA transcription through a yet unknown mechanism. The VEGFA protein is secreted by hypoxic cells, migrates through the intercellular space and binds to VEGFR2 on endothelial cells which in turn activates a downstream signaling cascade leading to the inactivation (hyper-phosphorylation) of pocket proteins such as RB1. As a result, E2F1 is activated and subsequently stimulates expression of VEGFR1/2, thereby regulating endothelial cell function. E2F7/8 on the other hand repress transcription of VEGFR1/2.

on endothelial cells, and stimulates transcription of *VEGFR1/2* through activation of E2F1 (Figure 2). E2Fs may thus function in a feedback loop to control VEGFA signaling in endothelial cells. Together these data suggest that regulation of angiogenesis may be a general function of RB-E2F proteins in which the cooperation with HIF may play an essential role.

A role for RB-E2F factors in regulating tumor angiogenesis

It was recently suggested that RB-E2F factors regulate tumor development independent of their ability to control cell proliferation[2]. Regulation of tumor angiogenesis by the RB-E2F pathway presents such an unanticipated E2F function. For example, E2F1 represses neo-angiogenesis in a xenograft tumor model. Specifically, injection of cancer cells into *E2f1*$^{-/-}$ mice results in more highly vascularized and hemorrhagic tumors compared to wild type mice which was suggested to result from increased *Vegfa* expression upon deletion of in *E2f1*[17]. Another study also showed that E2F1 repressed tumor angiogenesis by repressing *VEGFA* expression[18]. Interestingly, the ability of E2F1 to either inhibit or promote tumor angiogenesis may depend on the status of p53. Although E2F1 represses VEGFA-induced tumor angiogenesis[17, 18] possibly in cooperation with wild type p53[17], a transcriptional complex consisting of E2F1 and mutant p53 was reported to stimulates angiogenesis by increased transcription of *ID4*[22]. Together these studies suggest that in the tumor-microenvironment an E2F1-p53 complex inhibits angiogenesis through

decreased *VEGFA* expression, while in the tumor mutant p53 may cooperate with E2F1 to stimulate angiogenesis.

In addition to E2F1, there is experimental evidence that RB1 also regulates tumor vascularization. Loss of *p53* but not *Rb1* in the skin results in spontaneous squamous cell carcinomas[23]. Interestingly, combinatorial deletion of *p53* and *Rb1* in the skin accelerates the formation of squamous cell carcinomas and augmented tumor angiogenesis[23]. In line with these studies, infection of keratinocytes with the papillomavirus E6 and E7 oncoproteins, which inactivate p53 and RB1, also results in an pro-angiogenic transcriptional response including increased expression of *VEGFA*[24]. Furthermore, *Rb1*$^{+/-}$ mice develop spontaneously highly vascularized pituitary adenocarcinomas[25]. Enhanced Id2 activity in these *Rb1*-deficient tumors was shown to stimulate tumor angiogenesis by increasing *Vegfa* expression[25]. Finally, the role of RB1 in in regulating tumor angiogenesis can also be regulated through its interaction with the RAF1 kinase. RAF1 directly binds and inhibits RB1, while specific disruption of this interaction significantly reduces tumor angiogenesis[26, 27]. Why RB1 stimulates angiogenesis in the placenta but represses angiogenesis during tumor development is currently unclear. In the placenta, the prominent pro-angiogenic activity of HIF may switch RB1 into a pro-angiogenic factor through their direct interaction. In addition, loss of RB1 in the placenta may also activate E2F1, leading to E2F1-induced inhibition of angiogenesis. In tumors the angiogenic function of RB1 may also be determined by HIF. In hypoxic tumors HIF may switch RB1 into a pro-angiogenic factor, whereas in more oxygenated tumors RB1 could function as an anti-angiogenic factor. Alternatively, deletion of RB1 in tumors could also lead to ectopic E2F1 activity which may stimulate angiogenesis in cooperation with mutant P53[22], as mentioned before.

These studies show a role for RB1 and E2F1 in tumor angiogenesis. It will be interesting to investigate under which conditions they function as pro- or anti-angiogenic factors, and if their capacity to regulate angiogenesis is shared by other RB or E2F family members, especially E2F7/8. Because hypoxia is a hallmark of solid tumor development and HIF factors are critical for promoting tumor angiogenesis[28-30], we expect that E2F7/8 and RB1 regulate tumor angiogenesis through their interaction with HIF.

Concluding remarks and future outlook

There is strong evidence that RB and E2F factors regulate normal and tumor angiogenesis. Future studies are required to determine if and how the RB-E2F pathway regulates the formation of blood vessels in general, and to which extend they depend on HIF for this function. Downstream of RB and E2F factors a major pathway begins to emerge. The above mentioned studies clearly identify the VEGFA signaling pathway through which E2F7/8, E2F1 and RB1 control angiogenesis. However, future experiments will determine if the identified putative E2F7/8-HIF angiogenic targets (Figure 1B) present novel downstream targets through which E2f7/8, and possibly the RB-E2F pathway in general, controls angiogenesis during embryonic and tumor development.

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Atypical E2fs control lymphangiogenesis through transcriptional regulation of Ccbe1 and Flt4

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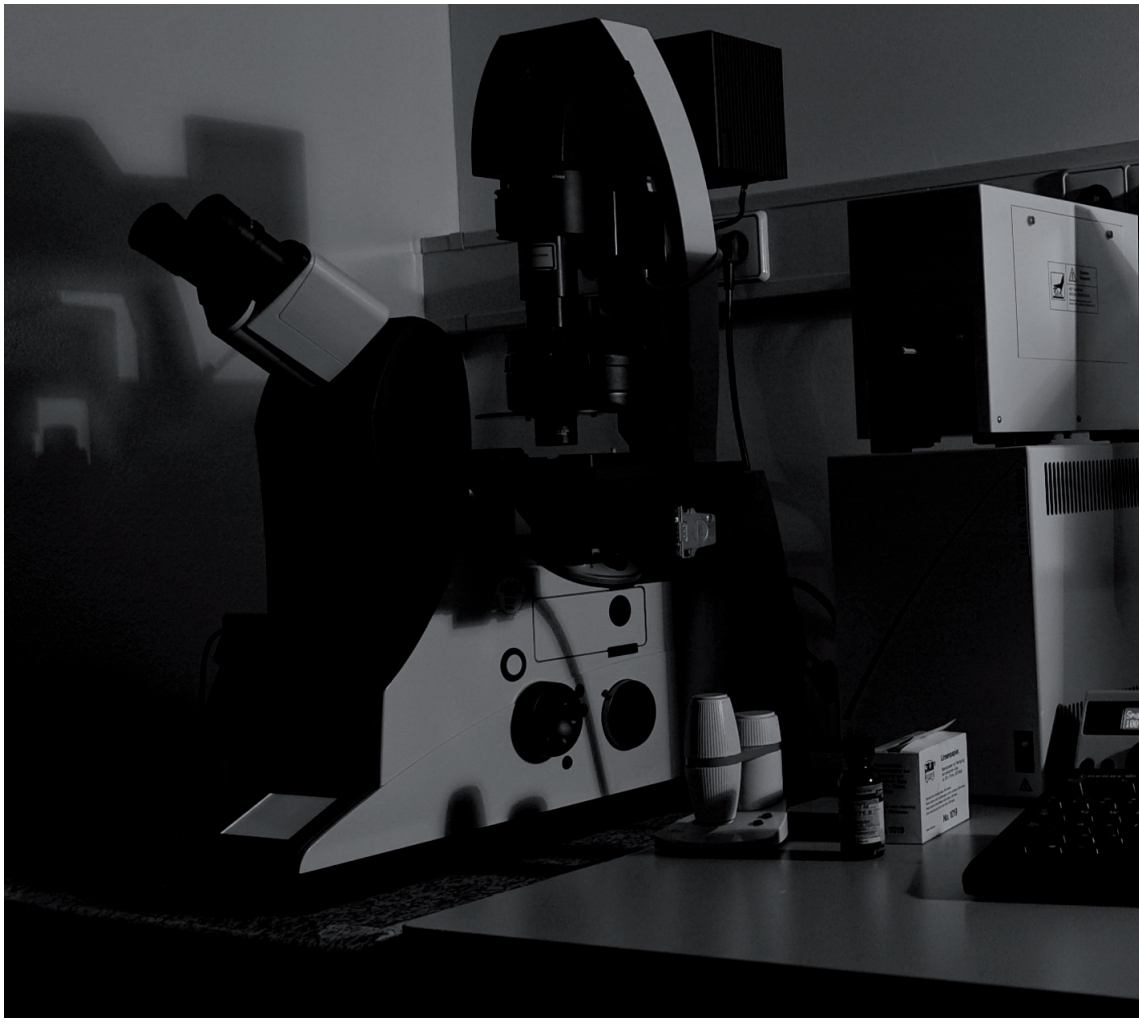
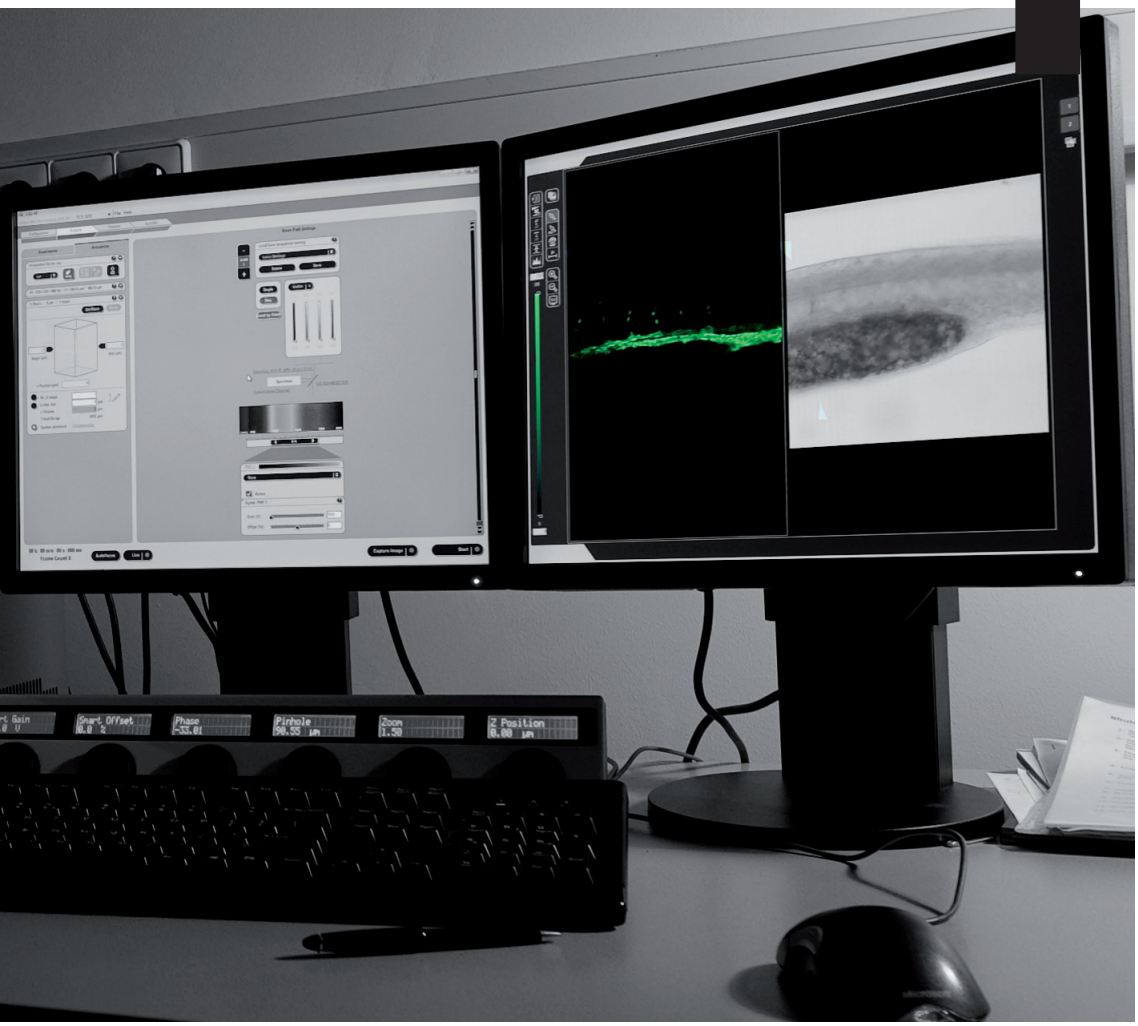


Chapter 4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ABSTRACT

Lymphatic vessels are derived from venous endothelial cells and their formation is governed by the Vascular endothelial growth factor C (VegfC)/Vegf receptor 3 (Vegfr3; Flt4) signaling pathway. Recent studies show that Collagen and Calcium Binding EGF domains 1 protein (Ccbe1) enhances VegfC-dependent lymphangiogenesis. Both Ccbe1 and Flt4 have been shown to be indispensable for lymphangiogenesis. However, how these essential players are transcriptionally regulated remains poorly understood. In the case of angiogenesis, atypical E2fs (E2f7 and E2f8) however have been recently shown to function as transcriptional activators for VegfA. Using a genome-wide approach we here identified both CCBE1 and FLT4 as direct targets of atypical E2Fs. E2F7/8 directly bind and stimulate the *CCBE1* promoter, while recruitment of E2F7/8 inhibits the *FLT4* promoter. Importantly, inactivation of *e2f7/8* in zebrafish impaired venous sprouting and lymphangiogenesis with reduced *ccbe1* expression and increased *flt4* expression. Remarkably, over-expression of *e2f7/8* rescued Ccbe1- and Flt4-dependent lymphangiogenesis phenotypes. Together these results identified E2f7/8 as novel *in vivo* transcriptional regulators of *Ccbe1* and *Flt4,* both essential genes for venous sprouting and lymphangiogenesis.

INTRODUCTION

The lymphatic vascular system is a specialized capillary network of blind ended vessels that are essential for maintaining interstitial fluid balance, macro-molecular uptake and immune cell trafficking. One of the main drivers behind lymphangiogenesis is the Vascular endothelial growth factor C (VegfC) – Vegf Receptor 3 (Vegfr3; Flt4) pathway[1-4]. Tight regulation of VegfC-Flt4 signaling is of fundamental importance for proper lymphangiogenesis. It has been shown that Delta like ligand 4 (Dll4) suppresses VegfC-Flt4 signaling while Collagen- and Calcium-binding EGF domains 1 (Ccbe1) enhances the biological effect of VegfC, thereby regulating the lymphangiogenic response in opposing ways[2, 5]. Besides these important findings, it remains currently unclear how these factors are regulated at the transcriptional level.

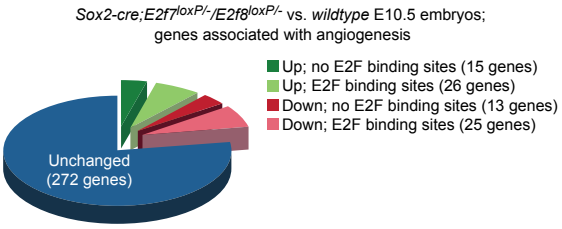
The atypical E2fs, E2f7 and E2f8, form homo- or heterodimers, possess two DNA binding domains and form thereby an unusual duo within the E2F family[6-8]. E2f7/8 function predominantly as transcriptional repressor of cell cycle genes involved in DNA replication, DNA metabolism, DNA repair, mitosis and cytokinesis[9, 10]. However, we recently showed that E2f7/8 can also function as a transcriptional activator of VegfA, thereby promoting blood vessel formation [11]. The aim of this study was to determine whether E2f7/8 modulate lymphangiogenesis through transcriptional regulation of lymphangiogenic factors. We report here that Flt4 and Ccbe1 are directly regulated by E2f7/8 and thereby show that these atypical E2Fs are essential modulators of lymphangiogenesis *in vivo*.

RESULTS

E2F7/8 directly regulate CCBE1 and FLT4 expression

Recently, we showed that E2f7/8 regulate VegfA dependent angiogenesis in zebrafish[11]. To determine whether E2f7/8 also control other angiogenenic or lymphangiogenic factors besides VegfA, we searched in a recently published genome-wide microarray analysis on *E2f7/8*-deficient E10.5 mouse fetuses (*Sox2-cre;E2f7*$^{loxP/-}$*;E2f8*$^{loxP/-}$)[12] for de-regulated expression of genes associated with the AmiGO gene ontology (GO) term (lymph)angiogenesis (Figure 1A). This analysis revealed that among the genes that have been shown to be indispensable for lymphangiogenesis, only *Ccbe1* and *Flt4* were deregulated and contained canonical *E2f* binding sequences within their proximal promoter (Figure 1A, B) [2, 13]. To investigate whether these genes are indeed bound and regulated by E2F7/8, we first performed chromatin immunoprecipitation (ChIP) experiments in HeLa cells and found that both E2F7 and E2F8 bound strongly to the *CCBE1* promoter (Figure 1C). E2F8 was also strongly enriched on the *FLT4* promoter, while E2F7 showed only weak binding (Figure 1C), which might be due to the overall lower affinity of the E2F7 antibody. We used a previously reported *E2F* binding site within the *E2F1* promoter and a non-specific site upstream as controls (Figure 1C)[11]. Next we tested whether ectopic expression of E2F7 was

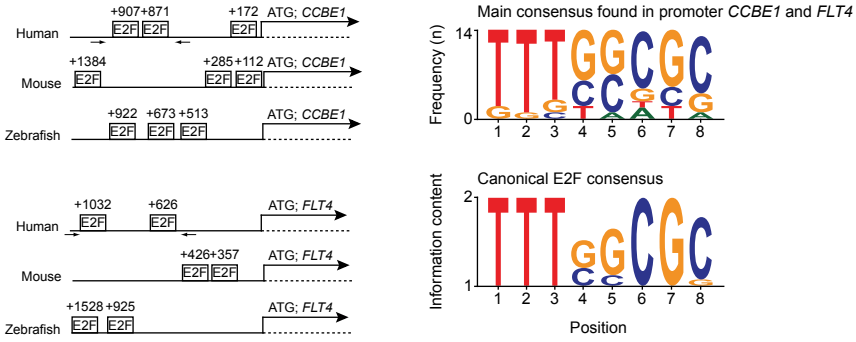
A



GO term lymphangiogenesis (GO:0001946)

Up; E2F binding sites		Down; E2F binding sites	
Gene	Fold change	Gene	Fold change
Foxc2	1.22	Prox1	0.84
Flt4	1.21	Bmpr2	0.85
Acvr2b	1.19	Ccbe1	0.89
Ptpn14	1.18		
Foxc1	1.11		

B



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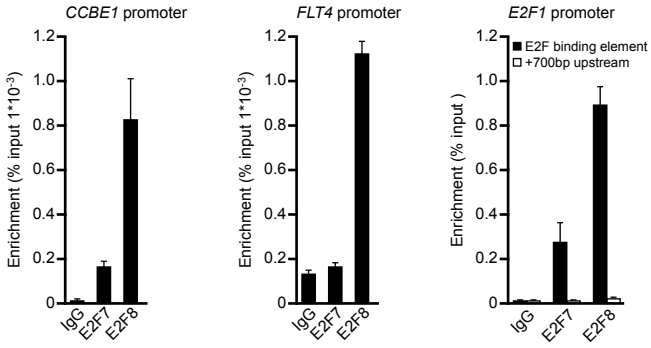
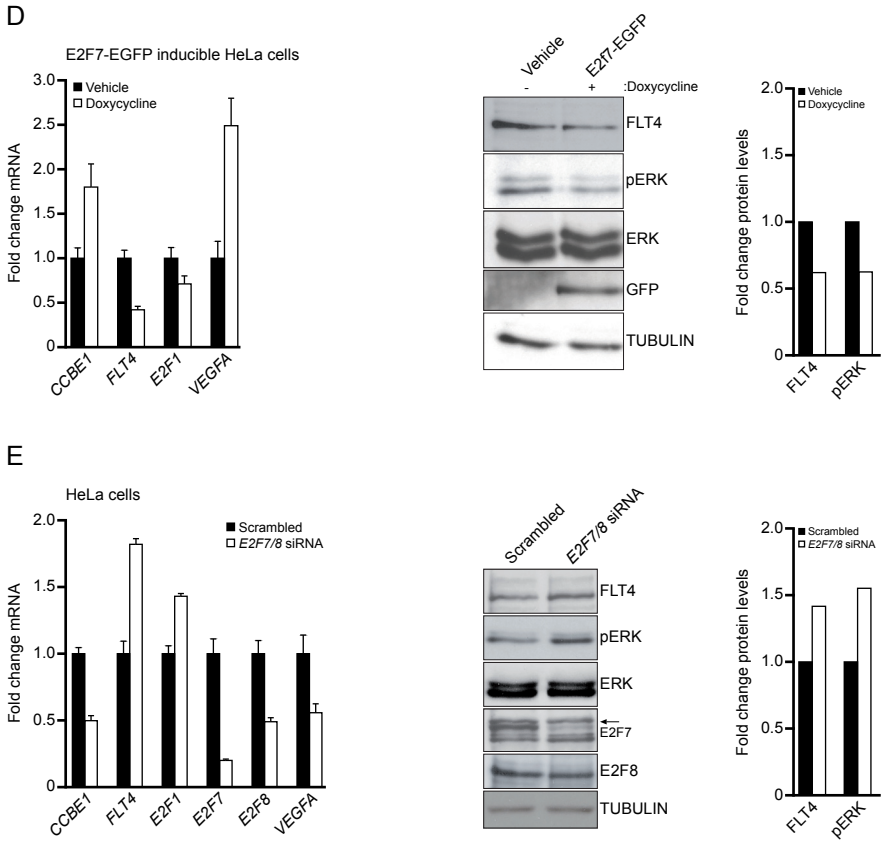


Figure 1 I E2F7/8 directly regulate CCBE1 and FLT4 expression. **A** Genes associated with the gene ontology (GO) term angiogenesis were extracted from the *Sox2-cre;E2f7*$^{loxP/-}$*E2f8*$^{loxP/-}$ vs. wildtype E10.5 mouse embryos (P<0.05) database (GEO: GSE30488) and additionally analyzed for E2F binding sites and their presence in GO lymphangiogenesis (GO:0001946). **B** E2F binding elements within the *CCBE1* and *FLT4* promoter. Average and canonical E2F binding consensus. **C** E2F7 and E2F8 ChIP performed on *CCBE1* and *FLT4* promoters in HeLa cells, arrows in (B) indicate primers used for ChIP. A previous reported E2F site within the *E2F1* promoter and a non-specific site upstream (+700bp) served as controls. →

able to modulate the expression of *CCBE1* and *FLT4*. To this extent we used HeLa cells which express E2F7-eGFP upon administration of doxycycline[10]. Induction of E2F7 showed an increase in *CCBE1* mRNA and a decrease in FLT4 mRNA and protein levels (Figure 1D). Additionally, phosphorylation of extracellular-signal-regulated kinase (pERK), a downstream factor of FLT4 signaling, showed a decrease while total ERK levels were unchanged (Figure 1D). As controls, two previously described atypical E2F target genes, E2F1 and VEGFA, were used (Figure 1D)[11]. Conformingly, E2F7/8 siRNAs caused a decrease in *CCBE1* mRNA levels, while *FLT4* mRNA and protein levels were increased. Consistently, downstream phosphorylation or ERK was increased upon deletion of *E2F7/8* (Figure 1E).



D mRNA and protein levels of E2f7-EGFP inducible HeLa cells. **E** mRNA and protein levels of HeLa cells treated with scrambled or E2f7/8 siRNAs. Data presented as the average (±s.e.m.) compared to the control condition in three independent experiments.

Next we investigated whether E2F7/8 regulate *CCBE1* and *FLT4* in cell types that reflect their *in vivo* expression pattern, As *ccbe1* is strongly expressed in mesenchymal cells in zebrafish (30hpf)[13], we used mouse embryonic fibroblasts (MEFs). In addition, *flt4* shows a strong endothelial specific expression in zebrafish[2], therefore, we used human umbilical vein endothelial cord cells (HUVECs) to investigate *Flt4* expression. Moreover, previous analysis on the spatio-temporal expression at the onset of lymphangiogenesis, revealed a ubiquitous pattern for atypical E2fs in mice (E9.5) and zebrafish (30 hours post fertilization (hpf))[6, 11]. In line with these *in vivo* expression patterns, we found a 4000 times higher expression of *Ccbe1* in MEFs and a 230 times higher expression of *FLT4* in HUVECs, while atypical E2Fs display comparable expression levels in all three cell lines (Supplementary Figure S1A). Furthermore, we analyzed publically available CHIP-sequencing data (ENCODE) of the trimethylated Lys4 mark on histone H3, a post-translational modification that is exclusively linked to transcriptional activation[14]. Comparing the levels of H3K4me3 between HUVECs, HeLa cells and fibroblasts confirmed that the active transcription status of *E2F7, E2F8, CCBE1 and FLT4* promoters correlates with the cell-type specific expression for CCBE1 in mesenchymal cells and FLT4 in endothelial cells (Supplementary Figure S1A). To test whether E2F7/8 are capable of modulating CCBE1 and FLT4 expression in MEFs and HUVECs to a similar extent as we described for HeLa cells (Figure 1D and E), we inactivated E2F7/8 in these cell lines utilizing a conditional deletion approach in *E2f7*$^{loxP/loxP}$ *E2f8*$^{loxP/loxP}$ MEFs[6] and siRNA technology in endothelial cells. As expected, knockdown of atypical E2fs in MEFS and HUVECS decreased *CCBE1* expression and increased the expression of *FLT4*, (Supplementary Figure S1B).

Together, our results show that E2F7/8 directly bind and modulate *CCBE1* and *FLT4* expression in cell types that reflect their *in vivo* expression. Moreover, comparison of *in vitro* expression and H3K4me3 CHIP-sequence data suggests that ubiquitous expressed E2F7/8 do not provide an ON/OFF switch for tissue specific expression pattern of CCBE1 and FLT4, but rather fine-tune the expression levels of these lymphangiogenesis factors. Nevertheless, loss of E2F7/8 function results in phenotypic changes (see below).

Loss of *e2f7/8* impaired venous sprouting and lymphangiogenesis

To investigate E2f7/8 function in lymphangiogenesis *in vivo*, we injected zebrafish embryos at the one-cell stage with full length *e2f7/8* mRNA or used morpholino oligomers (MOs) to prevent correct splicing of *e2f7/8*. As described previously, sequencing of MO induced mis-spliced *e2f7* and *e2f8* mRNA revealed the presence of frameshifts or intron insertion uptream of DNA binding domain[11]. Consistent with our *in vitro* studies, KD of E2F7/8 resulted in decreased *ccbe1* and increased *flt4* levels, while forced expression of *e2f7/8* mRNA had the opposite effect (Figure 2A, B). Furthermore, knockdown (KD) of *e2f7/8* in a transgenic line that drives YFP expression under the control of the *flt4* promoter showed 2-fold increased YFP expression, mainly apparent in the ISVs (Figure 2C).

In zebrafish, lymphangiogenesis starts around 36 hpf[13, 15] with cells that sprout from the posterior cardinal vein (PCV). A subset of these cells connects to the arterial intersegmental

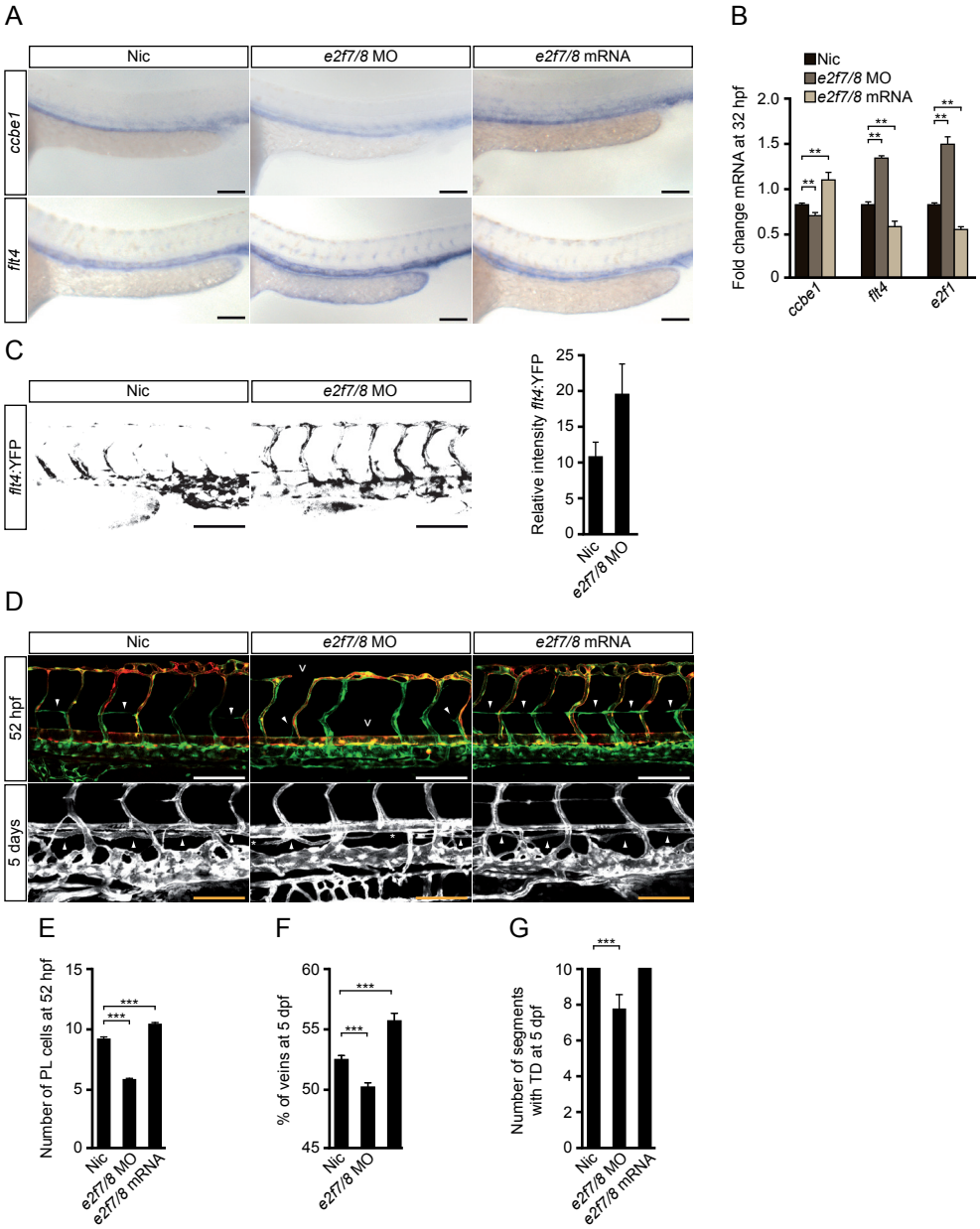


Figure 2 I Loss of E2f7/8 impaired venous sprouting and lymphangiogenesis. **A** *In situ* hybridisation and **B** qPCR (** $P<0.05$; 2 independent experiments) for *flt4* and *ccbe1* in zebrafish embryos 32hpf, un-injected control (Nic) or injected with *e2f7/8* MOs or mRNA. **C**, *Flt4*:YFP transgene level of 36hpf embryo, lateral view. **D** Lateral images of *Tg(fli1a:gfp;flt1^{enh}:rfp)* imaged at 52hpf or 5dpf. **E**, **F**, **G** Quantification of the indicated parameters. Open arrow heads indicate missing intersegmental vessels or dorsal longitudinal anastomotic vessel. Closed arrow heads indicate PLs (upper panel) or the TD (lower panel). Stars indicate missing TD fragments. All scale bars are 100 µm. Data presented as the average (±s.e.m.) compared to the control condition in three independent experiments (*** $P<0.001$).

vessels (aISVs) and remodels them to venous ISVs (vISVs), which consequently determines the ratio of arteries and veins within the trunk vasculature. The "non-connecting" cells migrate further dorsally and populate the horizontal myoseptum (HM) and are referred to as parachordal lymphangioblasts (PLs)[13]. Next, these PLs start to migrate ventrally and dorsally from the HM exclusively alongside aISVs[15] and give rise to the embryonic lymphatic structures, including the thoracic duct (TD)[13].

We assessed E2f7/8 function during lymphangiogenesis by using *Tg(fli1a:gfp;flt1^{enh}:rfp)* zebrafish embryos, in which endothelial cells (ECs) are labeled green and arterial ECs have an additional red color[15]. At 52 hpf, KD of *e2f7/8* resulted in a decreased number of PLs at the HM (Figure 2D; upper panel and E). As described earlier, *E2f7/8* morphants also display defects or missing aISVs (Figure 2D)[11].

At 5 days post fertilization (dpf) we found a decreased number of vISVs in *e2f7/8* morphants. This, with the addition that we quantified a reduced number of PLs at the HM, indicates that the total number of cells initially sprouting from the PCV was reduced in *e2f7/8* morphants (Figure 2F). Furthermore, the formation of the TD was impaired in *e2f7/8* morphants (Figure 2D; lower panel and G). In contrast, ectopic expression of *e2f7/8* mRNA resulted in increased number of both, PLs and vISVs, without any TD phenotype (Figure 2D-G).

Together these results suggest that *e2f7/8* regulate the number of cells emerging from the PCV during venous sprouting.

Induction of E2f7/8 restored Ccbe1-dependent venous sprouting and lymphangiogenesis.

Considering that *e2f7/8* mRNA induced *ccbe1* expression (Figure 2A,B), we hypothesized that *e2f7/8* are able to rescue the *ccbe1* morphant phenotype, characterized by reduced venous sprouting, which consequently leads to a decreased number of veins, absence of PLs and loss of TD (Figure 3A-D)[13]. Consistent with the data that loss of E2F7/8 results in a decreased expression of CCBE1, injection of *e2f7/8* MOs together with ccbe1 MOs resulted in no apparent improvement of the *ccbe1* MO induced phenotype (Figure 3A-D). However, as hypothesized, co-injecting *e2f7/8* mRNA or *ccbe1* mRNA in *ccbe1* morphants resulted in partial restoration of the number of PLs at the HM and reappearance of TD fragments in 55% of the morphants (Figure 3A,B,D). Although we could only rescue 55% of the embryos co-injected with *ccbe1* MO and *e2f7/8* mRNA on the level of the TD, we found in almost all embryos a rescue in the number of veins to the same extent as with *ccbe1* mRNA, (Figure 3C).

These findings suggest that E2F7/8 promote venous sprouting and lymphangiogenesis through transcriptional activation of *ccbe1*.

E2f7/8 modulate Flt4-dependent venous sprouting, lymphangiogenesis, and arterial hyperbranching.

During venous sprouting, VegfC signaling specifically drives the budding of (venous) ECs from the PCV, whereas ECs from the DA and aISVs seem to be non-responsive to VegfC. Recently, it has been shown that Dll4, which is expressed in the DA and aISVs, maintains arterial ECs in

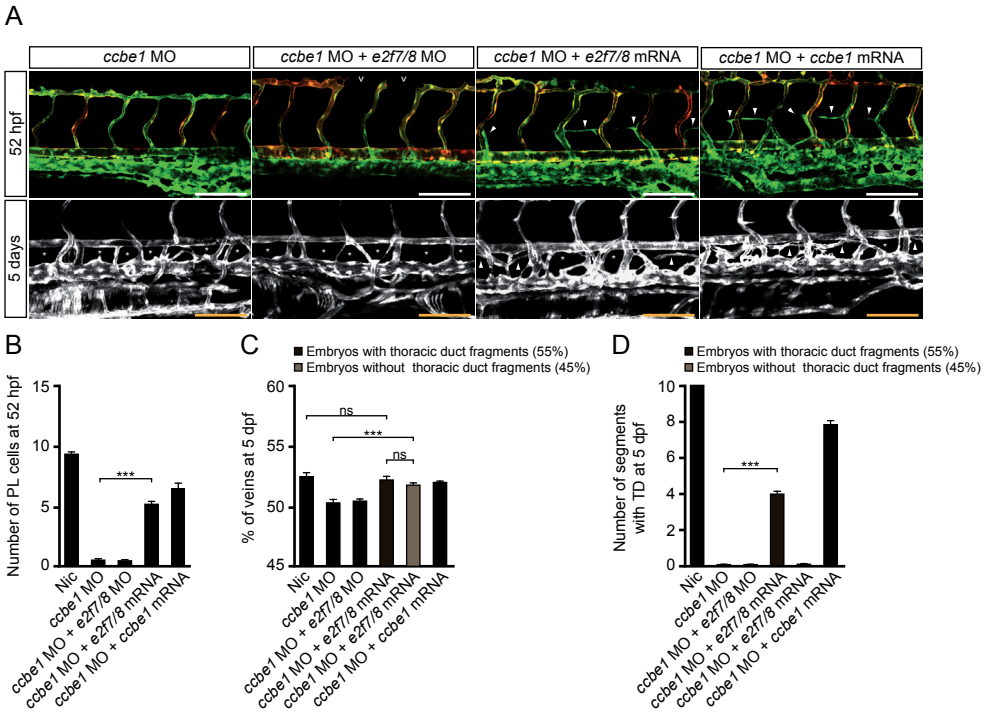


Figure 3 I E2f7/8 rescued Ccbe1dependent lymphangiogenesis phenotype. **A** Representative images of *Tg(fli1a:gfp;flt1^{enh}:rfp)* un-injected control embryos (Nic) or injected as indicated. **B**, **C**, **D** Quantification of the indicated parameters. Open arrow heads indicate in (A; upper panel) missing dorsal longitudinal anastomotic vessels. Closed arrow heads indicate (upper panel in A) PLs or (lower panel, A) presence of the TD. All scale bars are 100 µm. Stars indicate missing TD fragments. Data presented as the average (±s.e.m.) compared to the control condition in three independent experiments (*** P<0.001).

a quiescent state during venous sprouting. More specific, it was reported that Dll4 represses VegfC-Flt4 signaling, without affecting *flt4* and *vegfC* mRNA levels, in arterial cells and loss of Dll4 therefore resulted in hyperbranching of aISVs (Figure 4A; open arrowheads)[2]. We used this hyperbranching phenotype during the onset of venous sprouting in *dll4* morphants to assess the transcriptional repression of E2f7/8 on Flt4 signaling *in vivo*. In line with our finding that *e2f7/8* mRNA injections leads to decreased *flt4* levels, we found that ectopic expression of *e2f7/8* mRNA partially suppressed the hyperbranching phenotype in *dll4* morphants (Figure 4A, B). These results indicate that Dll4 modulates Flt4 signaling in a transcriptional independent manner[2], while E2f7/8 regulate Flt4 signaling on the transcriptional level. Furthermore, analysis of *dll4* expression in *e2f7/8* mRNA injected embryos by *in situ* hybridization, showed a decrease in *dll4* mRNA (data not shown), excluding the possibility that E2f7/8-mediated suppression of the hyperbranching phenotype of *dll4* morphants occurs through upregulation of *dll4* expression.

In addition to the hyperbranching phenotype, *dll4* morphants also displayed an almost complete absence of PLs and loss of TD (Figure 4A,C,D,E)[2]. Moreover, ISVs of *dll4* morphants have a predominantly venous identity, suggesting that venous sprouting is not completely lost, but that cells budding from the PCV are pre-dominantly programmed to connect to aISVs rather than migrating to the HM. To investigate if these phenotypes were also due to loss of *dll4* suppression on Flt4 signaling and whether E2f7/8 are able to rescue these phenotypes by modulating *flt4* expression, we first co-injected *dll4* MO and *e2f7/8* MOs. In line with the results that KD of *dll4* leads to the loss of repression of VegfC-Flt4 signaling and loss of *e2f7/8* would only lead to a further increase of signaling by increased amounts of *flt4,* we found no improvement of the phenotype in *e2f7/8* and *dll4* double-morphants (Figure 4B, C, D). In contrast, ectopic expression of either *e2f7/8* mRNA or *dll4* mRNA in *dll4* morphants, increased the number of PLs to almost *wild-type* levels. Moreover, the TD was partially restored at 5 dpf.

As mentioned above, the vasculature of *dll4* morphants consists out of almost 90% vISVs (Figure 4D) and in line with our previous data, injection of *e2f7/8* mRNA or *dll4* mRNA in *dll4* morphants reduced the number of veins (Figure 4D). However, injection of *e2f7/8* MOs in *dll4* morphants also significantly reduced the number of veins in *dll4* morphants (Figure 3D). In regard with our previous data, loss of E2f7/8 in *dll4* morphants leads, next to the increased expression of *flt4*, also to a decreased expression of *ccbe1* (Figure 2A, B), resulting in reduced venous sprouting and consequently to a lower number of vISVs.

Together, these data provide strong evidence that E2f7/8 can compensate for the loss of repression on Flt4 signaling in *dll4* morphants by transcriptional repression of *flt4*, thereby leading to the partial rescue of the hyperbranching phenotype, the arterial-venous patterning defects and the impaired formation of PLs and TD.

DISCUSSION

In this study, we show that two important players of lymphangiogenesis, Flt4 and Ccbe1, are directly regulated by atypical E2Fs. We demonstrate that inactivation of E2f7/8 in zebrafish impairs venous sprouting and lymphangiogenesis accompanied by deregulated expression of Ccbe1 and Flt4. Moreover, we show that E2f7/8 induction can rescue lymphangiogenesis defects caused by loss of Ccbe1 or by enhanced Flt4 signaling due to the loss of Dll4. From these findings we conclude, that E2f7/8 are required for lymphangiogenesis through fine-tuning Ccbe1 and Flt4 expression.

We recently showed that ablation of *e2f7/8* in zebrafish resulted in a *vegfAa* dependent angiogenesis phenotype [11]. However, several studies indicated that venous sprouting and, thereby, lymphangiogenesis is a VegfA independent process in zebrafish. It was shown that VegfA dependent phosphorylation of Vegf receptor 2 (KDR/Flk-1) acts mainly through downstream signaling via the phospholipase C-γ (PLC-γ) - protein kinase C (PKC) - Raf-mitogen activated protein (MAP) kinase pathway[16, 17]. Zebrafish mutant for PLC-γ or injected with PLC-γ

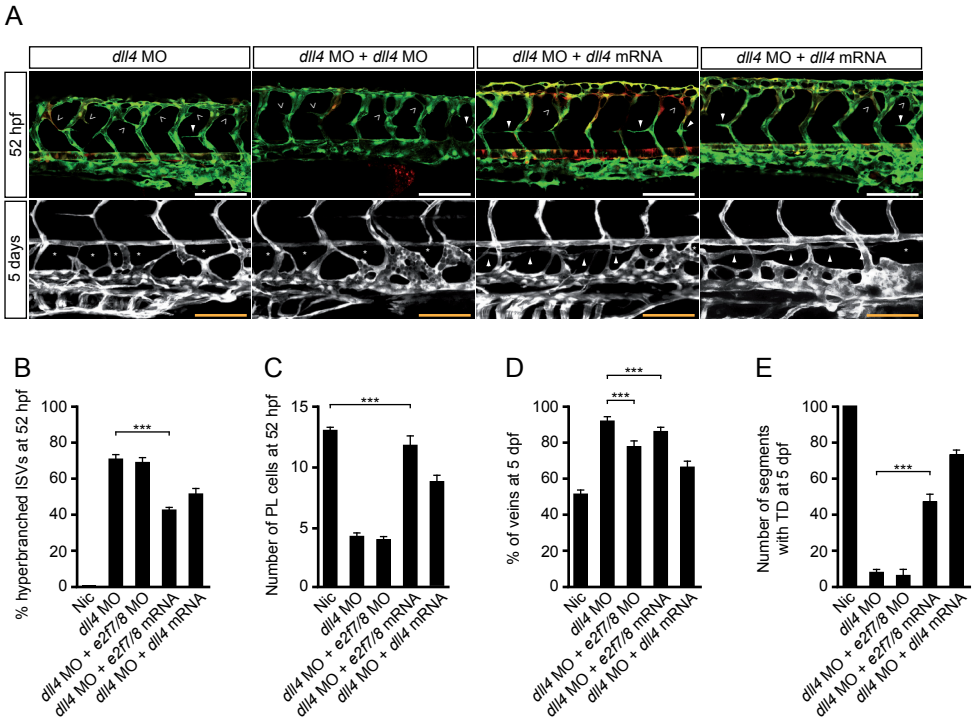


Figure 4 I E2f7/8 rescued Flt4-dependent lymphangiogenesis phenotypes **A** Representative images of *Tg(fli1a:gfp;flt1^{enh}:rfp)* un-injected control embryos (Nic) or injected as indicated. **B**, **C**, **D**, **E** Quantification of the indicated parameters. Open arrow heads indicate in (A; upper panel) hyper-branching of intersegmental vessels. Closed arrow heads indicate (upper panel in A) PLs or (lower panel, A) presence of the TD. All scale bars are 100 µm. Stars indicate missing TD fragments. Data presented as the average (±s.e.m.) compared to the control condition in three independent experiments (*** P<0.001).

morpholino oligomers showed specific defects in the formation of arteries, but not veins or parachordal lymphangioblasts[13, 18]. These findings indicate that the lymphatic phenotype observed in *e2f7/8* deficient zebrafish is likely not due to decreased *vegfAa* expression.

Atypical E2fs are known to function predominantly as transcriptional repressors[10], which is consistent with the inhibition of Flt4 expression. In contrast, Ccbe1 expression is induced by E2f7/8, indicating that the transcriptional activity of atypical E2fs is promoter dependent. Interestingly, three other studies reported a similar activator role for atypical E2fs by binding and stimulating the promoters of *TERT*, *CYCLIN D1*, and *VEGFA* [11, 19, 20].

Comparison of the spatio-temporal expression pattern between atypical E2fs, Flt4 and Ccbe1[2, 6] indicates that E2F7/8 do not function as the ON/OFF switch for the tissue-specific expression of Flt4 and Ccbe1. Instead our findings support a model where E2F7/8 fine-tune the expression levels of Flt4 and Ccbe1 to modulate venous sprouting and thereby lymphangiogenesis.

The dual role of atypical E2Fs in regulating cell cycle and lymphangiogenesis is particularly interesting in the context of cancer. Atypical E2Fs are highly expressed in many types of tumors (Oncomine). Furthermore, dissemination of tumor cells often occurs via the lymphatic system and can be blocked by interfering with FLT4 signaling[21, 22]. Thus, it would be of great interest to investigate whether E2F7/8 not only control CCBE1 and FLT4 during developmental lymphangiogenesis but also during tumor dissemination.

Our previous finding that *e2f7/8* regulate angiogenesis[11], provided strong evidence that atypical E2fs possess functions that reach beyond cell cycle control. In support, we show here that E2f7/8 regulate Ccbe1 and Flt4, two indispensable genes for lymphangiogenesis *in vivo*.

ACKNOWLEDGEMENTS

We would like to thank Bart Westendorp for providing comments.

METHODS

Ethics statements

Animal experiments were performed in accordance with the rules of the Animal Experimentation Committee of the Royal Netherlands Academy of Arts and Sciences (DEC).

Zebrafish

All zebrafish strains were maintained in the Hubrecht Institute (Utrecht Medical Center, Netherlands) under standard husbandry conditions. Transgenic lines used Tg(fli1a:gfp)^{y1} [23] and Tg(flt1^{enh}:rfp)[15].

Quantification

Parachordal lymphangioblasts[13, 15] and thoracic duct were quantified in zebrafish embryos as follows: the space between two intersegmental vessels was considered as 1 segment. 10 segments anterior from the end of the yolk sac extension were quantified at the indicated time points.

Micro-array analysis

Data from Gene Expression Omnibus (GEO) database number GSE30488 was analyzed by FlexArray [24]. All significant genes associated with AmiGO gene ontology lists, angiogenesis (GO:0001525) were extracted and analyzed for E2F binding elements within their proximal promoters with DAVID (http://david.abcc.ncifcrf.gov/ [25, 26]). Additionally, genes with E2F binding elements that showed a deregulation (up or down) were further analyzed for their association with GO term lymphangiogenesis (GO:0001946). GO terms were retrieved from (http://www.geneontology.org/[27]). Promoter analysis was performed with Consite

(http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite/).

Morpholino

The following morpholino oligonucleotides (Genetools) were used. E2f7 splice morpholino targeting exon 2–intron 2-3: 5'-ATAAAGTACGATTATCCAAATGCAC-3' [11]; e2f8 splice morpholino targeting exon 2-intron 2-3: 5'-CTCACAGGTATCCGAAAAAGTCATT-3' [11]; dll4 splice morpholino targeting exon 4 – intron 4-5: 5'- TGATCTCTGATTGCTTACGTTCTTC-3' [2]; ccbe1 ATG morpholino: 5'- CGGGTAGATCATTTCAGACACTCTG-3' [13].

Imaging

Embryos were mounted in 0.5-1% low melting point agarose (Invtrogen) 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 cover slip replacing the bottom. Imaging was performed with a Leica SP2 confocal microscope (Leica Microsystems) using a 10x or 20x objective with digital zoom.

Plasmid constructs

E2f7 [11] and e2f8 [11] pCS^{2+} plasmids were linearized with NotI and mRNA was synthesized using the SP6 RNA polymerase and the mMachine kit (Ambion).

In situ hybridization

In situ hybridization was performed as previously described [28]. The ccbe1 [13] and flt4 [29] probes have been described previously. Probes were synthesized by in vitro transcription using T7 RNA polymerase (Promega).

Cell culture, overexpression and siRNA transfection

Cervical cancer (HeLa) cell lines were cultured in DMEM (Invitrogen, 41966-052) supplemented with 10% FBS (Lonza, DE14-802F). Cells were transfected as specified by the manufacturer using 5 µl/p60 dish (Greiner) Lipofectamine 2000 (Invitrogen) with a final concentration of 25nM of siRNA and grown for 24 hours. Alternatively, overexpression was induced by adding 0.2 µg ml^{-1} doxycycline (Sigma) to the cell culture medium, treatment duration was 12 hours. After incubation of siRNAs or doxycycline with the indicated duration, cells were washed with PBS and trypsinized (lonza; cc-5012), pelleted by centrifugation (2600 xg, 2', 4^{0}C), snap freezed in liquid nitrogen and stored in -80^{0}C for RNA or protein isolation. Protein was isolated by disrupting cells in lysis buffer (0.05M sodium phosphate pH7.3, 0.3M NaCl, 0.1% NP40, 10% Glycerol) supplemented with protease inhibitors (Roche). Total RNA was extracted according to manufacturers' instructions using the RNeasy Mini Kit (Qiagen, cat #74106).

RNAi used in this study: scrambled siRNA #2 (D-001210-02, Thermo Scientific). hE2F7 (HSS175354, Invitrogen), hE2F8 (HSS128760, Invitrogen).

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: E2F7 (Santa Cruz Biotechnology, sc-66870), E2F8 (Abcam, AB109596), FLT4 (Santa Cruz Biotechnology; sc-321), ERK (Santa Cruz Biotechnology; sc-94), pERK (Santa Cruz Biotechnology; sc-7383), Mouse IgG HRP-linked whole Ab (GE Healthcare, NA931), Rabbit IgG HRP-linked whole Ab (GE Healthcare, NA934).

Chromatin immunoprecipitation (ChIP)

ChIP was performed according the EZ ChIP protocol (Upstate, 17-371) using protein G agarose beads (Milipore, 16-266) coated overnight in 0,1% BSA (Sigma, A3294). The following antibodies were used: E2F7 (Santa Cruz Biotechnology; sc-66870) and E2F8 (Abcam, AB109596). 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.

RNA isolation, cDNA synthesis and quantitative PCR

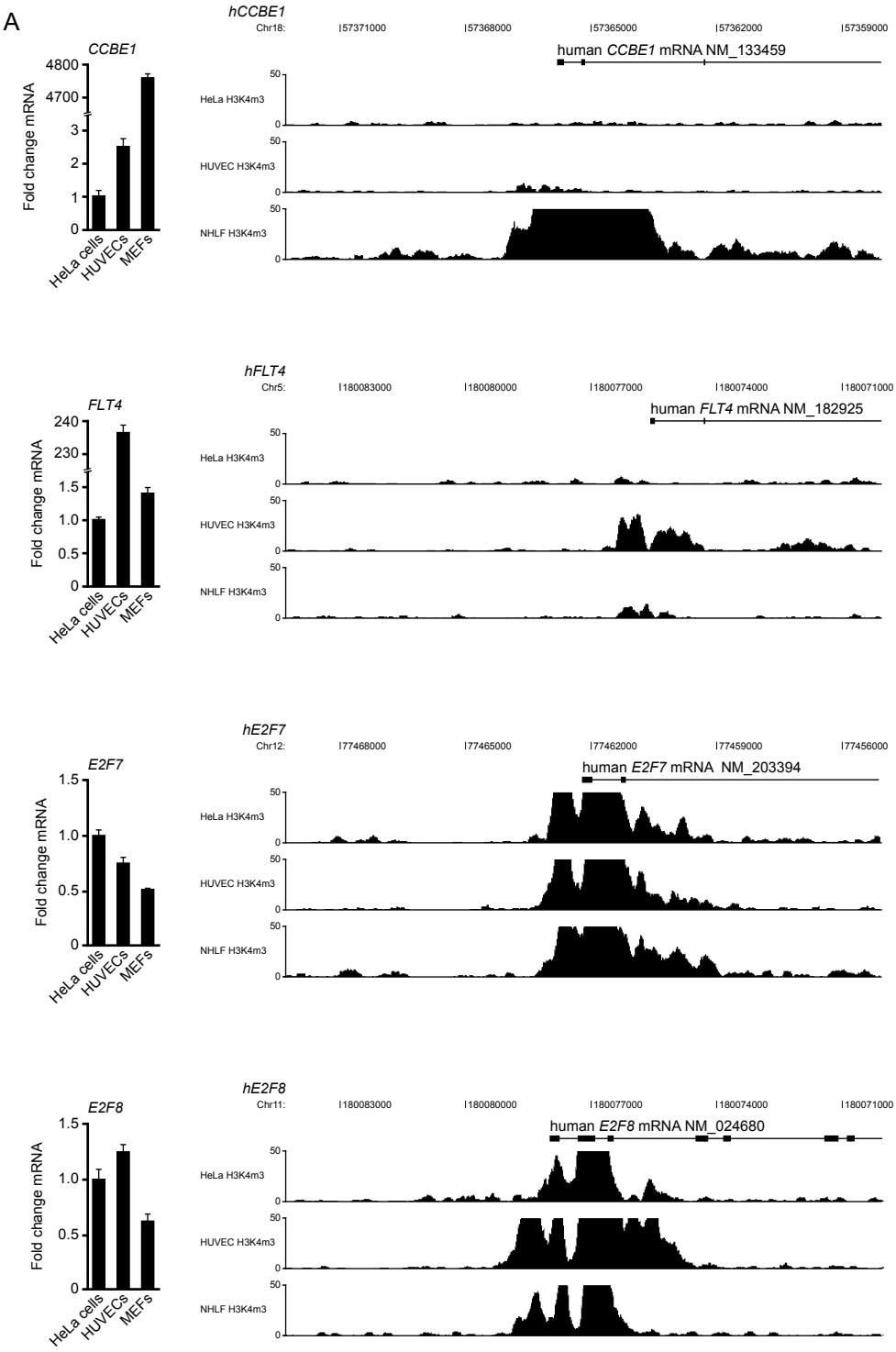
cDNA was synthesized with random hexamer primers according to manufacturers' instructions (Fermentas, cat#K1622). Quantitative PCR was performed on a MyiQ cycler (Biorad) using SYBRgreen chemistry (Biorad). In our in vitro studies two reference genes were used (ACTB, RPS18) and for zebrafish samples three reference genes were used (TBP, EF1α, β Actin). MIQE standards were applied to our protocols [30].

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).

Table S1: Oligonucleotide sequences

Primer	Technique; species	Forward (3' - 5')	Reversed (3' - 5')
ccbe1	qPCR; Zebrafish	AATCGCAACGACGAAGTACC	CCGGCACACACATCATAATC
flt4	qPCR; Zebrafish	TGCACCAGTATGCCACATTT	TGCTTCCATTGCTTTGACTG
e2f1	qPCR; Zebrafish	ACGCATCTACGACATCACCA	CTCCGTCAGCTCAGAAACCT
tbp	qPCR; Zebrafish	TCACCCCTATGACGCCTATC	CAAGTTGCACCCCAAGTTT
ef1α	qPCR; Zebrafish	GATTGTTGCTGGTGGTGTTG	TGTATGCGCTGACTTCCTTG
β-actin	qPCR; Zebrafish	CGTCTGGATCTAGCTGGTCGTGA	CAATTTCTCTTTCGGCTGTGGTG
CCBE1	qPCR; Human	CCCTCCTCCGTTTTCTTGTT	TTGTCCTGAGCGGCTTTAAT
FLT4	qPCR; Human	TTGGAGAGAGCTGGTAGTGG	CCTGTAATCCCAGCTTCTCG
E2F7	qPCR; Human	CTCCTGTGCCAGAAGTTTC	CATAGATGCGTCTCCTTTCC
E2F8	qPCR; Human	AATATCGTGTTGGCAGAGATCC	AGGTTGGCTGTCGGTGTC
E2F1	qPCR; Human	GACCACCTGATGAATATCTG	TGCTACGAAGGTCCTGAC
VEGFA	qPCR; Human	ACCTCCACCATGCCAAGTG	TCTCGATTGGATGGCAGTAG
ACTB	qPCR; Human	GATCGGCGGCTCCATCCTG	GACTCGTCATACTCCTGCTTGC
RSP18	qPCR; Human	AGTTCCAGCATATTTTGCGAG	CTCTTGGTGAGGTCAATGTC
CCBE1	ChIP; Human	CCCTCCTCCGTTTTCTTGTT	TTGTCCTGAGCGGCTTTAAT
FLT4	ChIP; Human	TTGGAGAGAGCTGGTAGTGG	CCTGTAATCCCAGCTTCTCG
E2F1	ChIP; Human	AGGAACCGCCGCCGTTGTTCCCGT	CCTCTGCCTGCAAAGTCCCGGCCACTT
E2F1 control	ChIP; Human	CGCCCAGACGCCACTTCATC	TTCATTCCCTCACTCATTCAACAA


A
CCBE1
Fold change mRNA
4800
4700
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2
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HeLa cells
HUVECs
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hCCBE1
Chr18:
57371000
57368000
57365000
57362000
57359000
human CCBE1 mRNA NM_133459
HeLa H3K4m3
HUVEC H3K4m3
NHLF H3K4m3
50
0
FLT4
240
230
1.5
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hFLT4
Chr5:
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Chr12:
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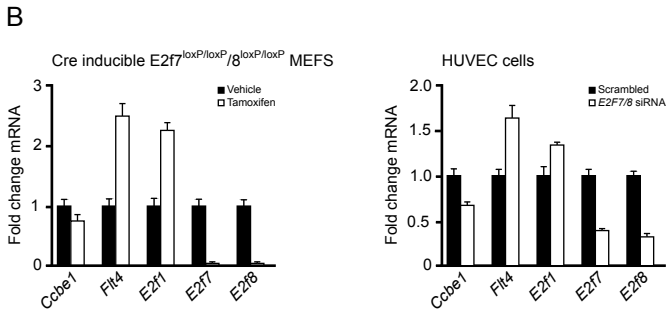

Supplementary Figure S1 I E2F7/8 directly regulate CCBE1 and FLT4 expression. **A** Relative expression of indicated gene in HeLa, HUVECs and MEFs. Additional, *in silico* analysis of the trimethylated Lys4 mark on histone H3 in HeLa, HUVECs and normal human lung fibroblasts (NHLF). **B** indicated mRNA levels in Cre inducible *E2f7*$^{loxP/loxP}$*E2f8*$^{loxP/loxP}$ MEFs and E2F7/8 siRNA treated HUVECs. Data presented as the average (±s.e.m.) compared to the control condition in two independent experiments.

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E2f7 and E2f8 repress intratumoral blood vessel branching

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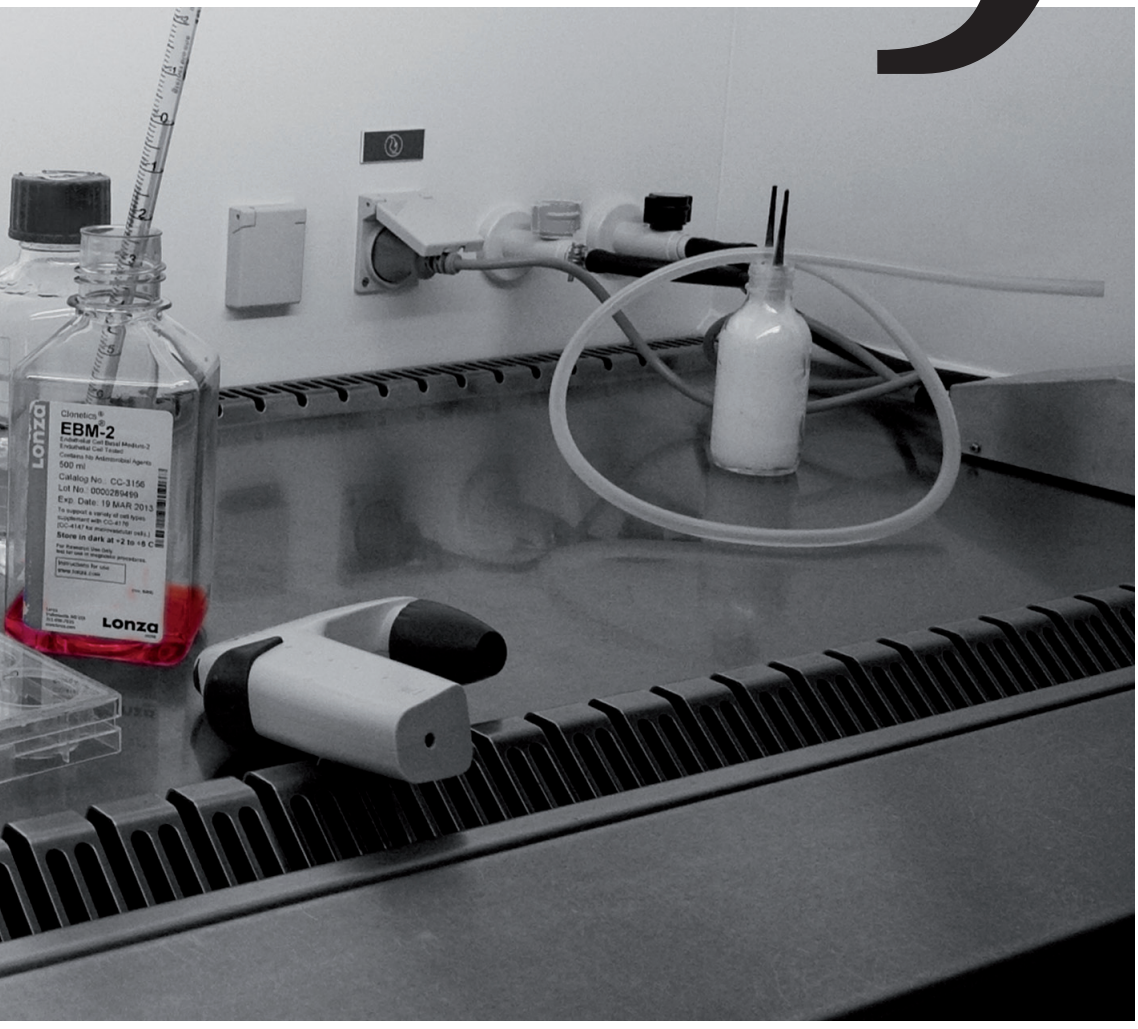
Chapter 5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ABSTRACT

Vascularization of tumors is an essential step in tumor progression, which involves a myriad of factors. Although the architecture of the tumor vasculature is severely compromised, it still provides the tumor with essential nutrients and a way to disseminate to distal organs and tissues. Preventing tumor angiogenesis proves to be a necessary step in tumor treatment and often involves the targeting of key players in angiogenesis like vascular endothelial growth factor A (VEGFA). However little is known how these key players in angiogenesis become deregulated during the formation of cancer. The E2F family of transcription factors is well known for their regulation of the cell cycle and moreover their deregulation during the formation of tumors. Recently, we showed that the atypical members of the E2F family, E2F7 and E2F8, regulate developmental angiogenesis by regulating VEGFA. Here we show that loss of the atypical E2Fs during tumor formation results in increased vascularization of subcutaneous engrafted tumors consisting of Myc/Ras-transformed mouse embryonic fibroblasts. Secretion of key angiogenic factors is decreased in E2F7/8 deficient tumors. In contrast, we show that the increased vascularization is due to an increased branching phenotype within the tumor. We speculate that this branching phenotype is being mediated by a decreased Delta-like 4 (Dll4) expression, a key component of the Notch signaling pathway. Concluding, E2F7 and E2F8 regulate tumor angiogenesis by modulating the branching potential of intratumoral blood vessels. .

INTRODUCTION

The attraction and formation of blood vessels is for most types of cancers a crucial step to gain access to the blood circulation, which provides not only oxygen and nutrients, but is also a way to disseminate to distal organs. The formation of new blood vessels (vasculogenesis) and blood vessels derived from pre-existing blood vessels (angiogenesis) during development is a tightly regulated process which results in a quiescent stable vasculature consisting of arterioles, venules and capillaries. In contrast, the vasculature found in solid tumors is unstable, leaky, hemorrhaging and highly unorganized. These characteristics are mainly due to an overabundance of vascular growth factor A (VEGFA) and a decreased number pericytes, which are in addition loosely attached[1]. Normally, these pericytes provide vascular endothelial cells with proliferation inhibitory signals through gap junctions and are therefore tightly connected with vascular endothelial cells[2].

Initial tumor growth can be supported by diffusion of local nutrients and oxygen. However, when the tumor size surpasses the boundaries of diffusion, a hypoxic microenvironment within the tumor ensures the initiation of angiogenesis, largely through the stabilization and expression of hypoxia inducible factors (HIFs). In addition, HIF stabilization and expression can also be influences by gaining mutations in genes normally involved in degrading HIF by polyubiquitylation, like Von Hippel Lindau (VHL) or activation of pathways enhancing HIF expression, like increased signaling through PI3K and protein kinase C pathways[3-5]. As HIF is the major regulator of VEGFA expression, the stabilization and increased expression of HIF subsequently leads to increased secretion of VEGFA [4, 6, 7]. VEGFA secreted by tumor cells acts as an autocrine signal promoting tumor survival and invasiveness, in addition to its well described chemoattractant function on endothelial cells [8].

Under physiological conditions, VEGFA will initiate angiogenesis by the selective sprouting of endothelial cells. This process results in a leading endothelial cell, the so-called tip-cell, that starts to migrate from the pre-existing blood vessel towards the VEGFA source. To ensure that not all endothelial cells in the blood vessel start to act on the VEGFA signal, the endothelial cells communicate with each other among others by the Delta-like 4 (Dll4) – Notch pathway. During developmental angiogenesis, Dll4-Notch signaling is needed to limit excessive angiogenesis by inhibiting sprouting of endothelial cells[9, 10]. In a similar fashion, Dll4-Notch signaling influences the amount of tumor angiogenesis in solid tumors[11, 12]. More specific, tumor cells that ectopically express Dll4 have a decreased vasculature, whereas tumors expressing a dimerized soluble form of Dll4, that acts as a presumed blocker, are characterized by increased angiogenesis and a decreased tumor volume[12]. Interestingly, blood vessels within solid tumors start to express Dll4, whereas the surrounding normal blood vessels do not[12].

The atypical E2Fs, E2F7 and E2F8 (E2F7/8), are two unique members of the E2F family of transcription factors. Their uniqueness lies within the structural difference regarding to the other members, in which they contain two DNA binding domains, lack a retinoblastoma (Rb) and Dimerization Partner (DP) domain and form hetero- or homo-dimers[13]. Since their discovery

in 2003, just a handful of reports have been published on their functions. Moreover these initial reports mainly focus on their developmental role or their *in vitro* effect on proliferation[14-16]. Recently, we have shown that the atypical E2Fs modulate VEGFA expression on transcriptional level during developmental angiogenesis[17]. Although atypical E2Fs have been shown to mainly repress transcription[18], we showed that E2F7/8 are able to induce VEGFA transcription *in vitro* and *in vivo*[17]. More specific, we showed that E2F7/8 cooperate with HIF, a strong transcriptional activator, to enhance VEGFA transcription[17]. Regarding the classical view of E2F7/8 being repressors of transcription and proliferation, one could expect them to act as tumor suppressors. However, recent insights suggest that E2F functioning might be dependent on spatio-temporal expression and recruitment of other transcriptional modifying proteins[17, 19-23]. Therefore, we investigated the function of E2F7/8 in tumor growth and angiogenesis.

In this study we show that in a xenograft model with transformed MEFs the ablation of E2F7/8 results in increased angiogenesis, whereas tumor growth is decreased. Interestingly, detailed analysis of the vasculature present in the solid tumor mass in a zebrafish xenograft model revealed structural differences between the vasculature in wildtype vs *E2f7/8* double knock out (DKO) tumors. More specific, the vasculature in E2F7/8 DKO tumors contained more endothelial cells, and, moreover the tumor vasculature showed a hyper-branching phenotype. This phenotype seems to be in correlation with *Dll4* expression, as KO of *Dll4* has been associated with increased angiogenesis and decreased tumor growth[12]. To this extent, KO of *E2f7/8* results in decreased expression of *Dll4* both in engrafted and cultured MEFs. These results suggest that loss of E2F7/8 during the formation or progression of cancer is not beneficial regarding its effect on angiogenesis, however tumor size is reduce upon loss of E2F7/8. Together, these findings would designate E2F7/8 as tumor suppressors.

RESULTS

E2F7 and E2F8 modulate growth and tumor angiogenesis in a mouse xenograft model

To assess the role of E2F7/8 in tumor angiogenesis, we investigated the angiogenic potential of Myc/Ras-transformed wildtype (Wt) and *E2f7/8* double-knockout (DKO) mouse embryonic fibroblasts (MEFs) grafted subcutaneously in athymic mice. MEFs were grafted with a total of 1 million cells, and tumors were harvested after 8 days of incubation, when the first tumors reached a diameter of 1 cm (Figure 1A). Strikingly, *E2f7/8* DKO tumors were significantly smaller that Wt tumors, whereas gross morphology did not differ (Figure 1B). Some grafts showed invasion in the surrounding muscular layer, however this phenomenon occurred in tumors of both genotypes, at similar frequency (data not shown). To investigate whether the size difference between *E2f7/8* DKO and Wt tumors was due to altered proliferation, we quantified phospho-histone 3 (PH3) -positive cells in the outer border zone of the tumors. Surprisingly, the proliferation rate was not altered between the two genotypes (Figure 1B). In addition, we found no difference in growth rate, reflected by similar growth curves and BrdU incorporation

under normal cell culture conditions (Figure 1C). To investigate whether difference in tumor size could be due to an increased cell death in *E2f7/8* DKO grafts, we quantified TUNEL-positive cells. Remarkably, we could also not find any differences in apoptosis between Wt and *E2f7/8* DKO grafts (Figure 1B).

To investigate the amount of angiogenesis within the grafts, we assessed the influx of endothelial cells from the host into solid tumor mass. To this extent, endothelial cells were labeled with fluorescent IsolectinB4, an endothelial specific marker and quantified by confocal microscopy. Unexpectedly, we found a marked increase in numbers of endothelial cells in E2f7/8 grafts (Figure 1D).

Together these results suggest that E2F7/8 regulate tumor growth. However interestingly, *in vitro* we were not able not detect any growth difference between Wt and *E2f7/8* DKO MEFs. Moreover, ablation of E2F7/8 in MEFs xenografts unexpectedly promoted tumor angiogenesis, reflected by the number of endothelial cells within the grafts.

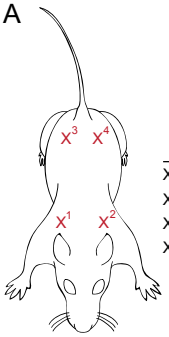
Deletion of *E2f7* and *E2f8* reduces growth factor secretion *in vitro* and *in vivo*

It has been shown that overexpression of growth factors, among other Vascular Endothelial Growth Factor A (VEGFA)[24], Platelet-Derived Growth Factor (PDGF)[25, 26] and Fibroblast Growth Factor (FGF)[27]), in tumor cells increases the amount of tumor angiogenesis. To test whether the difference in tumor angiogenesis between Wt and *E2f7/8* DKO MEFs was due to an altered secretion of growth factors, we assessed the "wound healing" potential of either *E2f7/8* DKO or Wt MEFs-conditioned medium in an *in vitro* wound healing assay. To this extent, we measured the closure rate of a scratch made in a confluent layer of human umbilical cord vein endothelial cells (HUVECs). HUVECs cultured in *E2f7/8* DKO-conditioned medium showed a decreased closure of the scratch versus HUVECs cultured in non-conditioned normal serum medium or Wt MEFs conditioned medium (Figure 2A). Moreover, HUVECs cultured in Wt MEF-conditioned medium showed a minor advantage versus non-conditioned normal serum medium, indicating that Wt MEFs secrete growth factors that stimulate endothelial cell proliferation and migration (Figure 2A). In addition, similar results were obtained when HUVECs were replaced with Wt MEFs or mouse embryonic endothelial cells (MEECs) in this "wound healing" assay (data not shown).

Conformingly, we detected a decreased or unchanged amount of mRNA levels of the most potent growth factors, *VegfA*, *VegfC, PDGF* and *FGF* in *in vitro* cultured *E2f7/8* DKO MEFs (Figure 2B), whereas *E2f1* and *Cdc6*, genes known to be repressed by E2F7/8 were upregulated (Figure 2B)[13, 18]. To test whether these factors were also decreased in the xenografts, we extracted mRNA from the solid tumor mass. Despite the variation between tumors, the expression of *VegfA*, *VegfC, PDGF* and *FGF* was decreased or unchanged, similar to the *in vitro* expression analysis (Figure 2C).

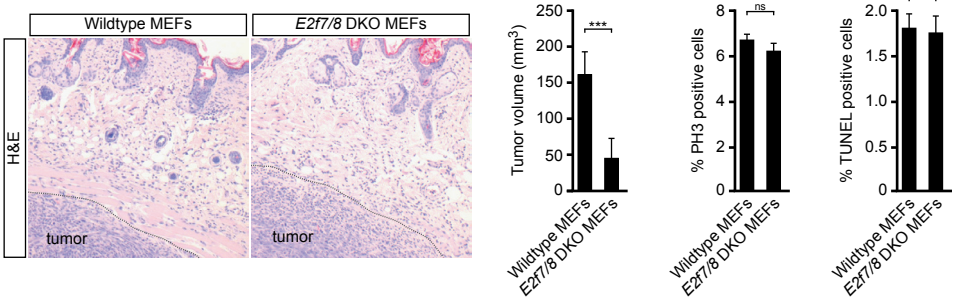
Transferring cells from an *in vitro* culturing system to an engrafted environment results in a dramatic stress response. To mimic some of these conditions *in vitro*, we cultured the MEFs under high confluency, hypoxic state and cell adhesion to a matrigel layer. Although secretion

A



	Mouse 1	Mouse 2	Mouse 3	Mouse 4
X^1	DKO clone 8	Wt clone 2	DKO clone 1	Wt clone 1
X^2	Wt clone 2	DKO clone 8	Wt clone 1	DKO clone 1
X^3	DKO clone 8	Wt clone 2	DKO clone 1	Wt clone 1
X^4	Wt clone 2	DKO clone 8	Wt clone 1	DKO clone 1

B



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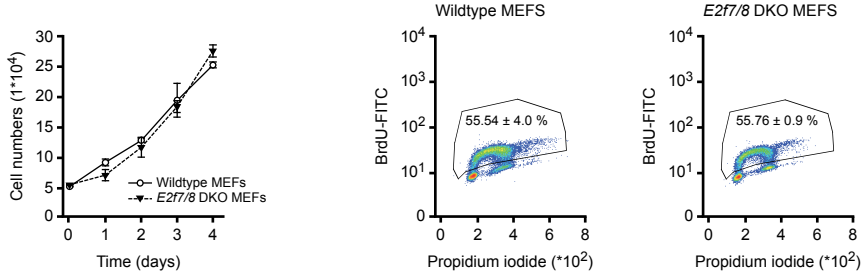

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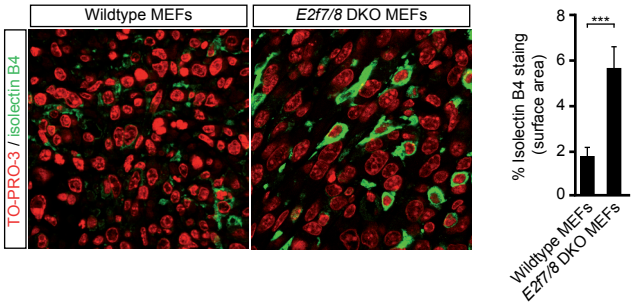


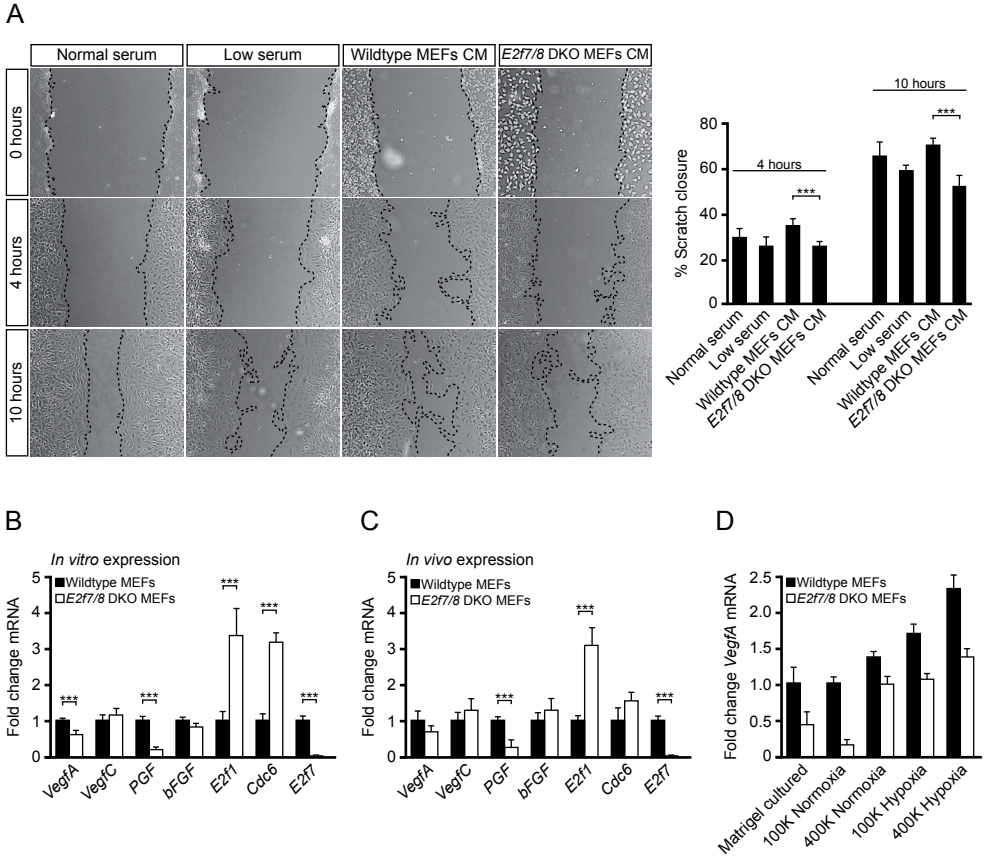


Figure 2 I Loss of *E2F7/8* in MEFs leads to decreased secretion of growth factors. **A** Representative images and quantification of a scratch assay in a confluent layer of HUVECs, cultured in normal serum, low serum or medium conditioned by wildtype or *E2f7/8* DKO MEFs. **B** Indicated mRNA levels measured in wildtype or *E2f7/8* DKO MEFs cultured under standard conditions. **C** Indicated mRNA levels measured in mouse xenografts of wildtype (8 tumors) or *E2f7/8* DKO MEFs (5 tumors). **D** mRNA levels of *VegfA*, measured in wildtype or *E2f7/8* DKO MEFs cultured on matrigel or under normoxic and hypoxic conditions in two different densities. Data presented as the average (±s.e.m.) compared to the control condition in two independent experiments.

← **Figure 1 I** Deletion of *E2F7/8* in mouse xenograft tumors leads to increased vascularisation of the tumor. **A** Schematic representation of xenograft positions and experimental setup. **B** Hematoxylin and eosin staining, tumor volume, proliferation and apoptosis of wildtype and *E2f7/8* DKO tumors. Dashed line indicates tumor border **C** *In vitro* proliferation behaviour of wildtype and *E2f7/8* DKO MEFs. **D** Staining and quantification of intratumoral endothelial cell (Isolectin B4) and nuclei (Tropo3).

of different factors differs between the several conditions, we did not observe increased secretion of pro-angiogenic factors in E2F7/8 DKO MEFs (Figure 2D; only *VegfA* shown).

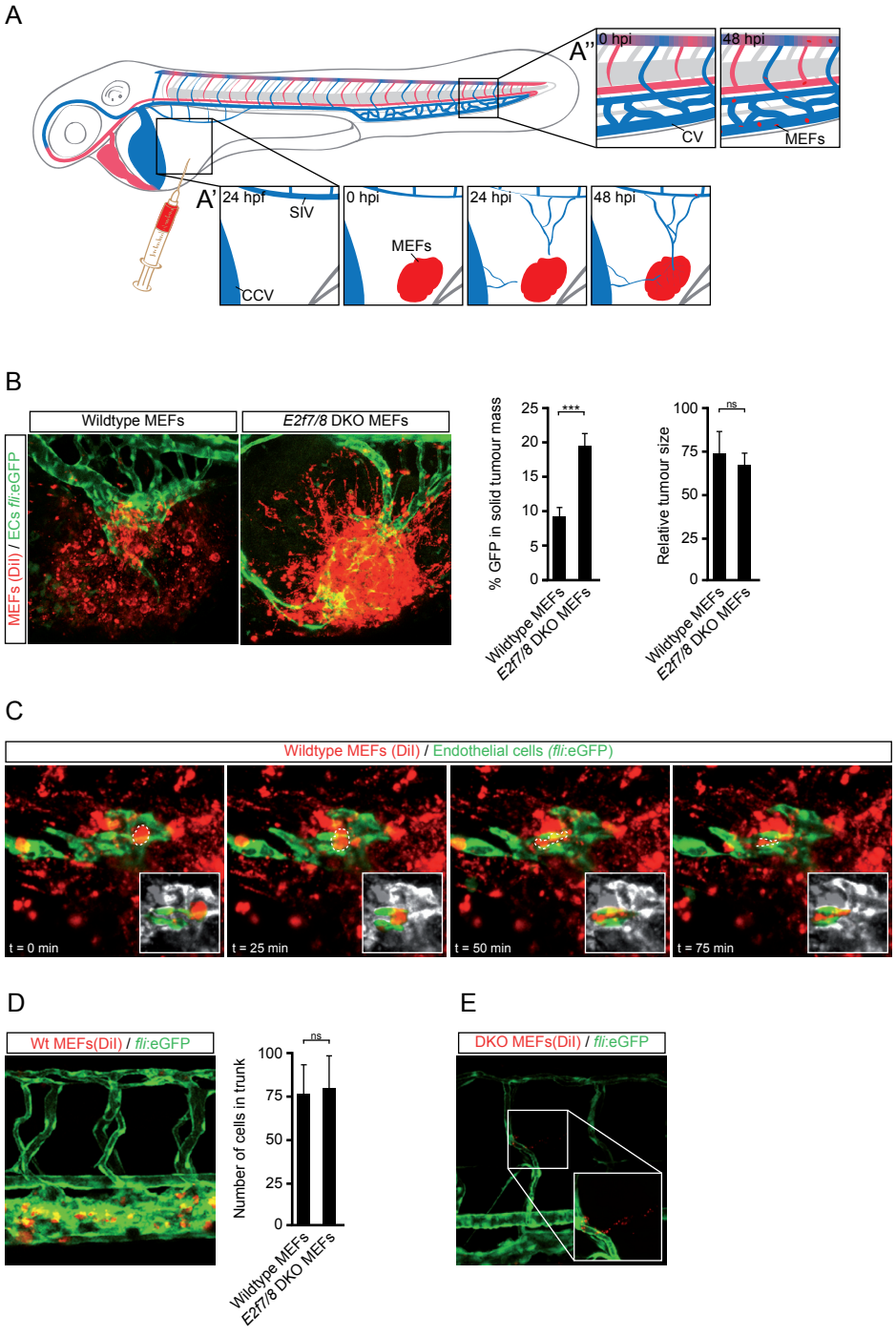
In sum, loss of atypical E2Fs *in vitro* and in engrafted cells results in a decreased secretion of growth factors, consequently leading to a decreased endothelial proliferation and migration. These results indicate that enhanced vascularization of E2F7/8 DKO tumors is independent of enhanced angiogenesis due to increased growth factor secretion.

E2f7 and E2f8 repress hyperbranching of intratumoral blood vessels in a zebrafish xenograft model

The use of athymic mice as host for the xenograft experiments has some limitations. First, tumor progression, initiation of tumor angiogenesis and influx of endothelial cells is difficult to assess over time, as detailed *in vivo* imaging is technically challenging. Second, dissemination and metastasis of tumor cells to distal organs or tissues is hard to detect , especially in short term experiments in which metastatic cells have limited time to expand. These disadvantages can be surpassed by using zebrafish as graft hosts[28, 29]. Thus to investigate the short term "real-time" tumor angiogenesis response, MEFs of either genotype were injected in *Tg(fli1:eGFP)* zebrafish, in which endothelial cells (ECs) are labeled green, at 24 hours post fertilization (hpf). Grafts were injected in the perivitellin space on the yolk sac, ventrally of the sub intestinal vein (SIV) and posterior of the common cardinal vein (CCV or duct of Cuvier) (Figure 3A). Grafts tend to evoke an angiogenic response from the SIV, but also frequently from the CCV (Figure 3A'). Additionally, it has been described that cells from the injection site are able to enter the circulation via the vessels formed by tumor angiogenesis and often attach or simply get stuck in the caudal vein (CV) region (Figure 3A")[30]. MEFs were 24 hours before injection labeled with the lipophilic dye DiI or DiD, with emission spectra of 565 nm and 665 nm respectively, to visualize them with confocal microscopy.

In line with the athymic mouse xenografts, *E2f7/8* DKO MEFs grafts triggered an increased influx of endothelial cells, quantified as the amount of GFP signal within the boundaries of the tumor 48 hours post injection (hpi) (Figure 3B). Tumor size measurements revealed that *E2f7/8* DKO MEFs grafts were smaller in size, however not significant (Figure 3B). Timelaps imaging showed a continuous movement of cells within the graft and, additionally, cells entering endothelial cell (GFP positive) tube-like structures (Figure 3C). Metastatic cells were detected throughout the fish 48 hpi, but within the existing circulatory system and with an increased density in the CV (Figure 3D). However, quantification of the number of cells present in the

Figure 3 I Deletion of *E2F7/8* in zebrafish xenograft tumors leads to increased vascularisation of the tumor. **A** Schematic representation of xenograft positions and experimental setup. **B** Representative image of wildtype (Red; DiI; n=10) and *E2f7/8* DKO tumors (Red; DiI; n=10) injected in *Tg(fli1a:gfp)* zebrafish, which harbour GFP (green) in vascular endothelial cells. **C** Timelapse serie that shows metastasising cells from the tumor into the vasculature. **D** Representative image and quantification of MEFs metastasis in the trunk region. **C** Example of extravasation of MEFs into the surrounding tissue. **Abbreviations:** CV, caudal vein; CCV, common cardinal vein; hpi, hours post injection →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CV
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B
Wildtype MEFs
E2f7/8 DKO MEFs
MEFs (DiI) / ECs fli:eGFP
% GFP in solid tumour mass
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Wildtype MEFs
E2f7/8 DKO MEFs
Relative tumour size
ns
C
Wildtype MEFs (DiI) / Endothelial cells (fli:eGFP)
t = 0 min
t = 25 min
t = 50 min
t = 75 min
D
Wt MEFs(DiI) / fli:eGFP
Number of cells in trunk
ns
E
DKO MEFs(DiI) / fli:eGFP

CV 48 hpi showed no difference in metastatic rate between *E2f7/8* DKO MEFs and Wt MEFs (Figure 3E). Of note, we sporadically detected cells outside the vasculature (Figure 3E). These cells could give rise to metastases, although we cannot exclude the possibility that these DiI- or DiD-positive cells are macrophages that have scavenged fluorescent cell debris or phagocytized MEFs.

Together, these data show that the results obtained from the zebrafish xenograft model show high similarity with the results from the mouse xenograft model, i.e. *E2f7/8* DKO MEFs grafts show decreased growth and increased angiogenesis. In addition, the data obtained from these zebrafish experiments also show that there is no difference in growth speed of host derived endothelial cells towards the tumor.

Tumors deficient for E2f7 and E2f8 display a hyperbranching phenotype

Blood vessels invading solid tumors interact extensively with the tumor cells. To investigate the effect of endothelial-tumor cell interaction, we quantified the number of intratumoral blood vessel branching points. Interestingly, blood vessels in *E2f7/8* DKO tumors show an almost four times higher number of branching points versus Wt tumors (Figure 4A). As blood vessel branching during developmental and tumor angiogenesis is mainly controlled by the Dll4-Notch pathway[12, 31], we tested *in vitro* and in engrafted tumors whether components of the Dll4-Notch pathway were deregulated after ablation of *E2f7/8*. In line with the known repressive function on blood vessel branching, we found both *in vitro* and *in vivo* decreased expression of the Notch ligand Delta-like 4 (Dll4), while the Notch receptor 1 and downstream target of Notch1, Hey1 was unchanged (Figure 4B and C).

Together these data indicate that the increased angiogenic phenotype in *E2f7/8* DKO tumors arises due to an increased intratumoral branching of blood vessels. Moreover, there is strong correlation of hyperbranching with the expression levels of *Dll4*, a Notch ligand known to be a repressor of hyperbranching during developmental and tumor angiogenesis. However, the decreased expression of *Dll4* seems to be an indirect effect of loss of *E2f7/8*.

DISCUSSION

In this study we show that loss of E2f7/8 during tumor formation results in increased vascularization of the tumor. However, this did not provide E2f7/8 DKO tumors with a growth advantage, reflected by reduced sizes. Moreover, angiogenic factors that have been show to enhance tumor angiogenesis, like Vascular Endothelial Growth Factor A (VEGFA)[24], Platelet-Derived Growth Factor (PDGF)[25, 26] and Fibroblast Growth Factor (FGF)[27]), showed no change or a decreased expression in *E2f7/8* DKO MEFs or tumors. In contrast, blood vessels in *E2f7/*8 DKO tumors showed an increased intratumoral branching. Moreover, the expression of *Dll4*, a Notch ligand and known for its repressive function on blood vessels branching, is decreased both *in vitro* and *in* vivo.

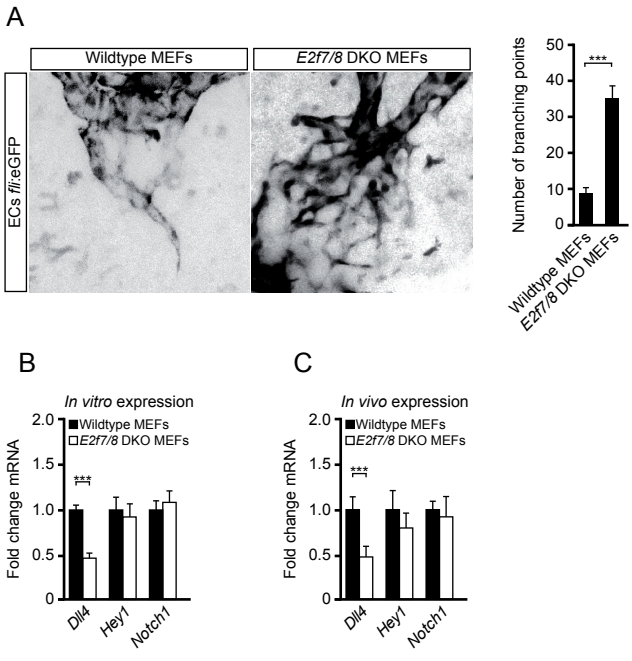

Figure 4 I Loss *E2F7/8* in xenografted MEF tumors in zebrafish leads to increased intratumoral blood vessel branching. **A** Representative image and quantification of hyperbranching in wildtype and *E2f7/8* DKO tumors injected in *Tg(fli1a:gfp)* (black color; endothelial cells). **B** Indicated mRNA levels measured in wildtype or *E2f7/8* DKO MEFs cultured under standard conditions. **C** Indicated mRNA levels measured in mouse xenografts of wildtype (8 tumors) or *E2f7/8* DKO MEFs (5 tumors).

The control of blood vessel branching is an essential step in the formation of a functional vasculature. During developmental angiogenesis, the so-called endothelial tip cells starts to grow out of pre-existing blood vessels under influence of VegfA. Expression of *Dll4* in the tip-cell signals through the Notch1 receptor present on neighboring cells, thereby preventing other cells from adapting a tip-cell fate[9, 10]. Loss of *Dll4* leads to uncontrolled tip-cell formation and thereby an hyperbranching phenotype in mice and zebrafish[10, 31]. To a similar fashion, loss of *Dll4* in solid tumors leads to increased intratumoral blood vessel branching and decreased tumor volume[11, 12]. Moreover, it was shown that these intratumoral blood vessel were of poor quality with a low perfusion rate, thereby suggesting that growth inhibition might be due to decreased nutrients, waste removal and oxygen delivery in the tumor[11, 12]. Importantly, these results also suggest that Dll4 expressed by and present on the tumor cells is able to modulate the morphology of invading blood vessels, indicating close contact between tumor cells and endothelial cells.

Interestingly, the expression of *Dll4* is positively correlated, and moreover induced by, *VegfA*[32, 33]. To this extent, it was shown that chemical blockage of VegfA in tumor bearing mice resulted in decreased *Dll4* expression[12] and in addition, *in vitro* treatment of endothelial cells with VegfA consequently led to increased *Dll4* expression[34]. These findings suggest that the phenotype presented here is a consequence of decreased VegfA expression due to loss of E2f7/8, which in turn results in decreased *Dll4* expression, thereby suggesting that the decrease in *Dll4* expression is an indirect results of loss of *E2f7/8* in tumor cells. In line with an

indirect regulation of Dll4, it was recently shown that the deletion of all three pocket proteins (Rb, P107 and P130) in the liver results in E2F1 and 3 mediated activation of the Notch pathway, while *Dll4* expression was unaltered[35]

Currently, there is little to no information about the role of atypical E2Fs in the process of tumor formation and progression, although, atypical E2Fs are highly expressed in many types of tumors (Oncomine). Based on the results that E2f7/8 act mainly as transcriptional repressors and prevent cells from (re-)entering the cell cycle, one may suggest that E2F7/8 act as tumor suppressors. However, the data presented here together with the finding that E2f7/8 directly induce *VegfA* expression[17], suggest that loss of E2f7/8 within tumor cells could potentially inhibit tumor growth and interfere the formation of a fully functional tumor vasculature. Because of the current lack of E2f7/8 function in tumor formation and progression, it would be worthwhile to investigate tumor samples for E2f7/8 status, e.g. expression and protein levels. Moreover, it would be of interest to determine E2f7/8 mutation rate to examine whether dysfunctional E2f7/8 play a role in carcinogenesis.

METHODS

Mice and zebrafish

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, 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). Transgenic lines used *Tg(fli1a:gfp)*^{y1} [36]

Cell culture

E2f7$^{loxP/loxP}$ *E2f8*$^{loxP/loxP}$ mouse embryonic fibroblasts were isolated and maintained as described previously [37]. Immortalization with retroviral constructs containing Myc and Ras^{61L} was performed as described previously[13, 38]. The immortalized lines were then treated with retrovirus containing the Cre recombinase to generate *E2f7/8* double knockout cells, according to standard methods [39]. Mouse embryonic fibroblast were (MEFs) were cultured in DMEM (Invitrogen, 41966-052) supplemented with 10% FBS (Lonza, DE14-802F). For hypoxia treatment, cells were incubated in the H35 Hypoxystation (Don Whitley Scientific) at 1% O2.

Tumor xenograft

MEFs were re-suspended to a final volume of 1 million cells per 100µl DMEM, and injected subcutaneously into Rj:NMR1-*Foxn1*^{nu}/*Foxn1*^{nu} athymic nude mice. Tumor diameters were monitored every two days by measuring in 3 dimensions with a caliper. After 8 days, when the first tumors reached a diameter of 1 cm, all mice were euthanized, and tumors were harvested.

Immunohistochemistry

Tumors were deparaffinized and rehydrated. Slides were treated with warm citrate buffer prior antibody incubation. Slides were incubated with anti Isolectin B4 (Vector Laboratories; # B-1205) or anti pH3 (Millipore; #06-570). Next slides were incubated with an Alexa 488 conjugated secondary anti-body (Invitrogen; #A1006).

Imaging

Embryos were mounted in 0.5-1% low melting point agarose (Invtrogen) 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 cover slip replacing the bottom. Imaging was performed with a Leica SP2 confocal microscope (Leica Microsystems) using a 10x or 20x objective with digital zoom.

Chromatin immunoprecipitation (ChIP)

ChIP was performed according the EZ ChIP protocol (Upstate, 17-371) using protein G agarose beads (Milipore, 16-266) coated overnight in 0,1% BSA (Sigma, A3294). The following antibodies were used: E2F7 (Santa Cruz Biotechnology; sc-66870) and E2F8 (Abcam, AB109596). De-crosslinked DNA was purified over a column (Qiagen, 28106) and eluted in 50 µl H2O of which 2 µl was used for quantitative PCR.

RNA isolation, cDNA synthesis and quantitative PCR

cDNA was synthesized with random hexamer primers according to manufacturers' instructions (Fermentas, cat#K1622). Quantitative PCR was performed on a MyiQ cycler (Biorad) using SYBRgreen chemistry (Biorad). In our *in vitro* studies two reference genes were used (ACTB, RPS18). MIQE standards were applied to our protocols [40].

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).

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Loss of Pten promotes angiogenesis and enhanced *vegfAa* expression in zebrafish

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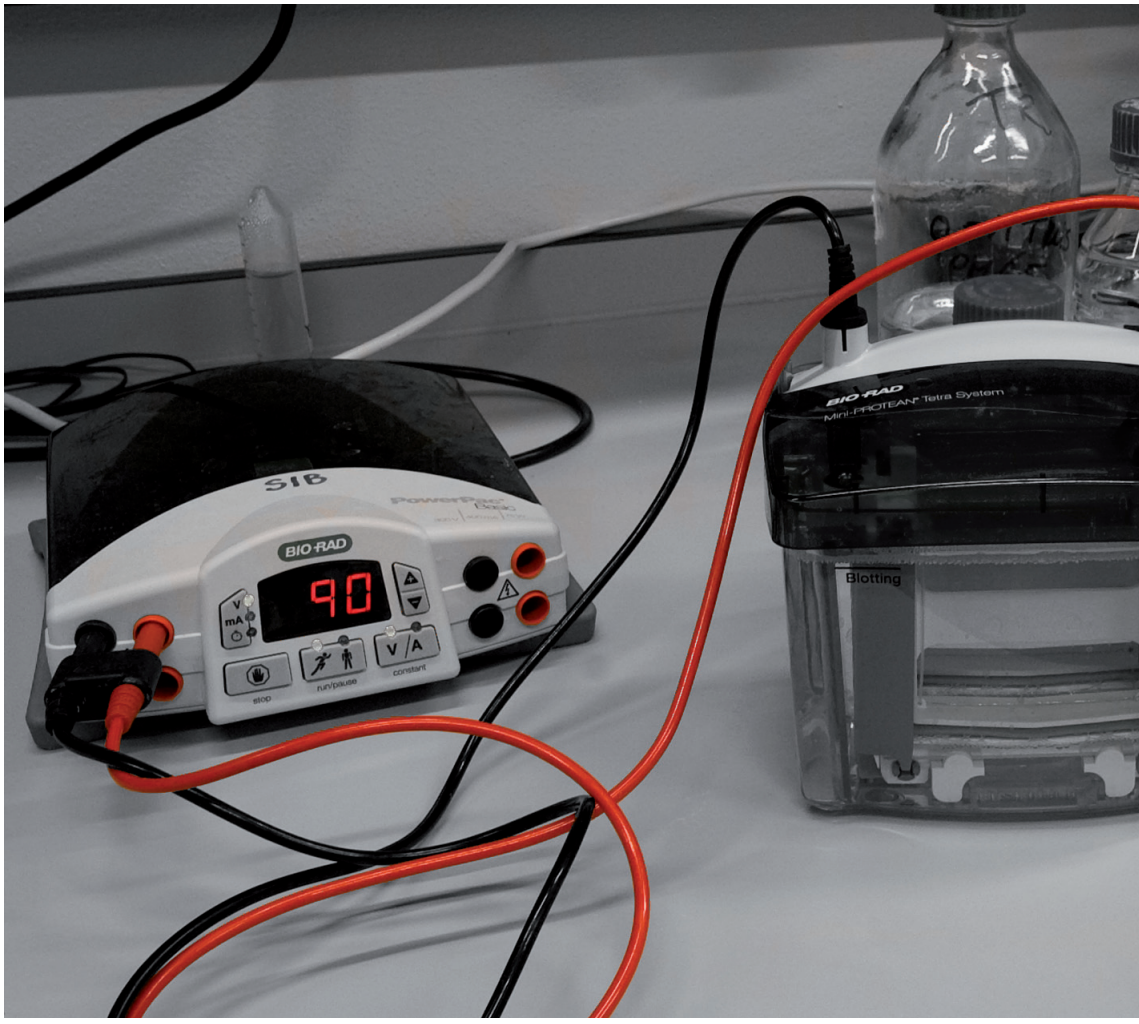
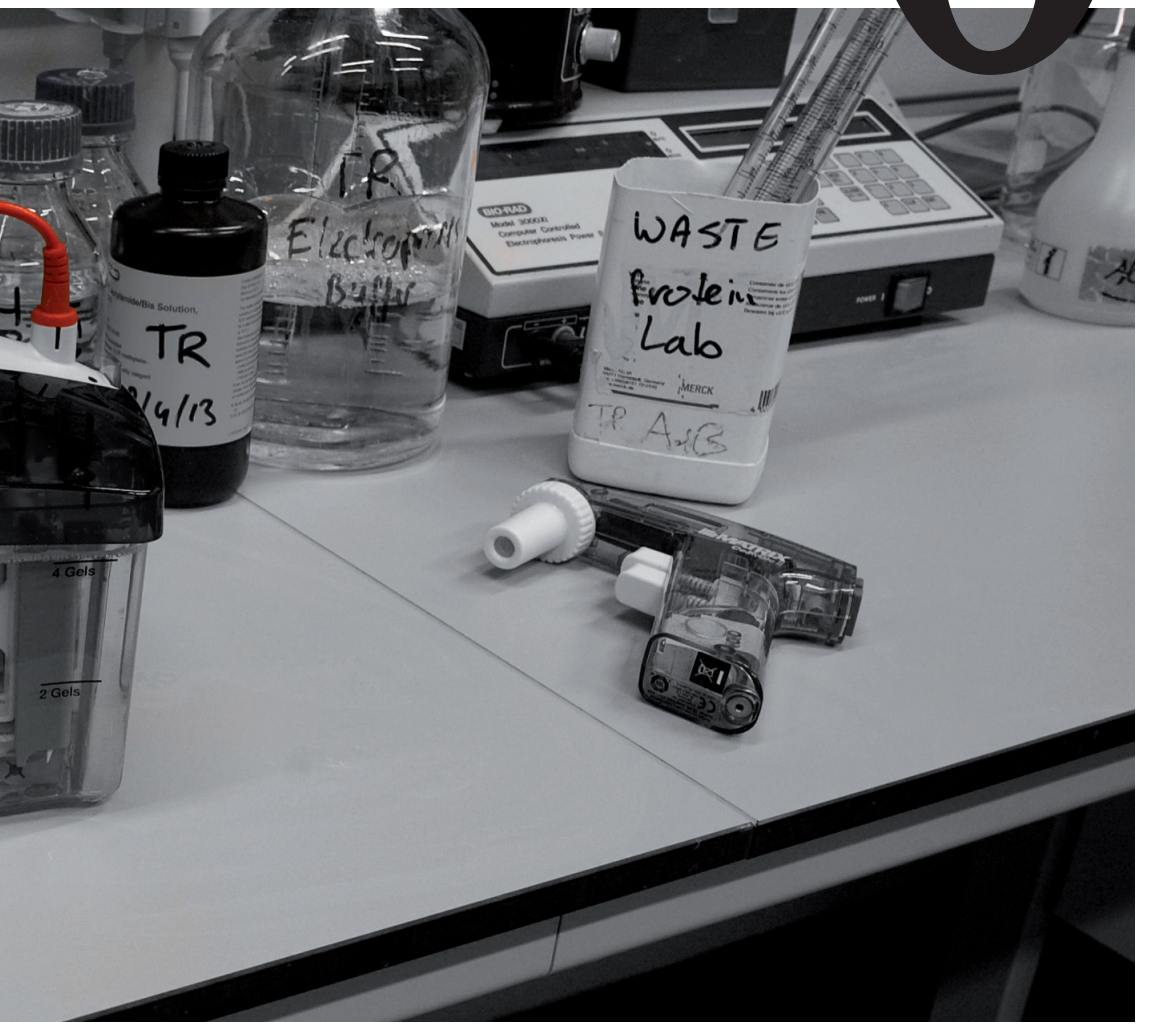
Disease Models and Mechanisms (accepted)



Chapter 6



ABSTRACT

Angiogenesis, the emergence of vessels from an existing vascular network, is pathologically associated with tumor progression and is of great interest for therapeutic intervention. PTEN is a frequently mutated tumor suppressor and has been linked to the progression of many types of tumors, including hemangiosarcomas in zebrafish. Here, we report that mutant zebrafish embryos lacking functional Pten exhibit enhanced angiogenesis, accompanied by elevated levels of pAkt. Inhibition of Phosphatidylinositol-3Kinase (PI3K) and angiogenesis by LY294002 and Sunitinib, respectively, suppressed enhanced angiogenesis in *Pten* mutants. VegfAa has a crucial role in angiogenesis and *vegfAa* expression was upregulated in embryos lacking functional Pten. Interestingly, *vegfAa* expression was also upregulated in hemangiosarcomas from haploinsufficient adult zebrafish *Pten* mutants. Elevated *vegfAa* expression in mutant embryos lacking functional Pten was suppressed by LY294002. Surprisingly, Sunitinib treatment dramatically enhanced *vegfAa* expression in *Pten* mutant embryos, which may account for tumor relapse in human patients who are treated with Sunitinib. Combined treatment with suboptimal concentrations of Sunitinib and LY294002 rescued enhanced angiogenesis in *pten* mutant embryos without the dramatic increase in *vegfAa* expression, suggesting a new approach for therapeutic intervention in VEGFR signaling-dependent tumors.

INTRODUCTION

PTEN is one of the most frequently mutated tumor suppressor genes found in cancer[1]. Somatic deletion of PTEN leads to tissue specific tumor formation and germ line deletion of PTEN is associated with syndromes, known as Cowden's disease, Bannayan-Zonana and Lhermitte-Duclos disease[2-4]. Patients affected with those syndromes share pathological features, including the formation of benign tumors and enhanced susceptibility to malignant cancer. PTEN, a lipid and protein phosphatase, antagonizes the PI3K-Akt (also called PKB) pathway by balancing the cellular phosphatidylinositol-(3,4,5)triphosphate (PIP3) level[5, 6]. Loss of PTEN increases PIP3 levels, resulting in constitutive activation of Akt signaling. Cell survival and proliferation are linked to activated Akt and thus uncontrolled activation of Akt leads to enhanced cell survival and proliferation, the hallmarks of cancer.

The zebrafish genome encodes two *pten* genes, designated *ptena* and *ptenb*[7, 8]. Single mutants are viable and fertile, suggesting redundant function during development. Concomitant loss of Ptena and Ptenb results in embryonic lethality[8] reminiscent of loss-of-function of PTEN in mice[9], *C. elegans*[10] and *Drosophila*[11]. We recently reported that haploinsufficiency of Pten in zebrafish, *ptena*$^{+/-}$*ptenb*$^{-/-}$ respectively *ptena*$^{-/-}$*ptenb*$^{+/-}$, results in hemangiosarcoma formation during adult life[12]. The mechanism underlying uncontrolled endothelial growth resulting in hemangiosarcoma is not understood.

In vitro studies showed that inhibition of endogenous PTEN in cultured endothelial cells enhances vascular endothelial growth factor (VEGF) signaling[13]. VEGFs, secreted ligands binding to VEGF receptors (VEGFRs), are key players in vasculogenesis and angiogenesis. VEGF signaling promotes proliferation and differentiation of endothelial cells. The human VEGF family consists of five related growth factors, VEGFA, VEGFB, VEGFC, VEGFD and PIGF (placental growth factor). From these five secreted ligands, VEGFA was shown to be the main factor during angiogenesis that functions as a mitogen, acting specifically on endothelial cells[14]. It has been demonstrated that VEGFB promotes fatty acid uptake in endothelial cells[15, 16] and the role of VEGFB during angiogenesis is not fully elucidated yet. VEGFC is together with VEGFD critical for lymphangiogenesis and has a minor role in vasculogenesis and angiogenesis[14].

We set out to study the function of Pten in endothelial cells *in vivo*. To this end, we investigated angiogenesis during embryonic development in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants. Here we report that *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants displayed ectopic vessel growth. Inhibition of PI3K signaling suppressed hyperplasia of endothelial cells. Treatment of *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants with Sunitinib, a Receptor Tyrosine Kinases (RTKs) inhibitor, also reduced enhanced angiogenesis. We found that elevated overall pAkt levels in embryos were suppressed by PI3K inhibitors, and to a lesser extent by Sunitinib. *VegfAa* expression was upregulated in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants and inhibition of PI3K abolished upregulation of *vegfAa*. Suprisingly, *vegfAa* expression was dramatically upregulated by Sunitinib treatment. Combining PI3K inhibitors and Sunitinib cooperatively suppressed hypervascularization in *ptena*$^{-/-}$*ptenb*$^{-/-}$ zebrafish embryos, revealing a tentative therapeutic approach to combat neovascularization in cancer.

RESULTS

Ptena$^{-/-}$ptenb$^{-/-}$ mutants display enhanced angiogenesis

Haploinsufficiency of Pten leads to uncontrolled proliferation of endothelial cells resulting in formation of hemangiosarcomas in zebrafish[12]. To investigate how loss of Pten supports tumor growth and in particular how loss of Pten affects endothelial cells, we visualized the vasculature in zebrafish *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutant embryos using the *Tg(kdrl:eGFP)*[17], in which all vascular endothelial cells express GFP. The anatomy of the vasculature in the trunk was monitored between 2 and 4 dpf. We observed excessive sprouting of endothelial cells of the intersegmental vessels, in that these cells developed excessive filopodia at 72 hpf (Figure 1), resulting in ectopic vessel growth at 4 dpf (Figure 2A, B). Using confocal microscopy we observed that newly formed vessels are perfused at 3 and 4 dpf (data not shown). Mutants retaining one wildtype allele, *ptena*$^{+/-}$*ptenb*$^{-/-}$ or *ptena*$^{-/-}$*ptenb*$^{+/-}$, do not display any detectable malformations in vasculogenesis or angiogenesis during embryogenesis (Supplemental Figure 1). Taken together, we found that angiogenesis was enhanced in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants, resulting in hypervascularization.

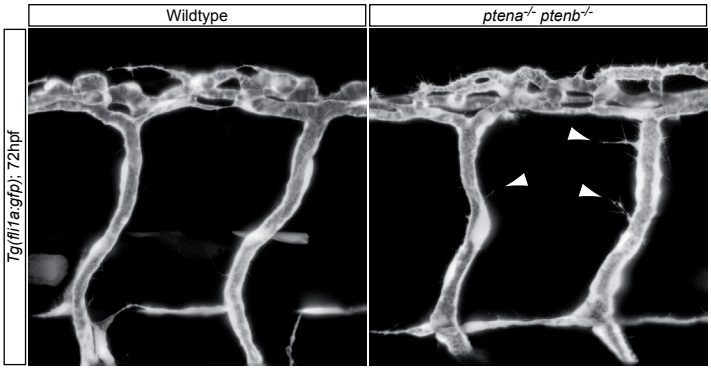


Figure 1 I Loss of Ptena and Ptenb leads to excessive filopodia formation in endothelial cells at 72 hpf. Endothelial cells in living wildtype and *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants were visualized using *Tg(kdrl:eGFP)* and confocal imaging was performed at 70-72 hpf. Intersegmental vessels along the trunk in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants show excessive filopodia formation (arrowheads) whereas no filopodia were observed in wildtype embryos. Anterior to the left, 40 x + 1.5 zoom, 0.5 µm step size.

Hypervascularization in ptena$^{-/-}$ptenb$^{-/-}$ mutants is rescued by LY294002 and Sunitinib

To investigate signaling underlying hypervascularization in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutant embryos, we performed rescue experiments. Microinjection of *ptena* mRNA in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants at the one cell stage suppressed the enhanced angiogenic phenotype at 4 dpf (Figure 2d). A similar suppression was observed with microinjection of *ptenb* mRNA (data not shown). Ectopic expression of moderate amounts of Ptena in wildtype embryos did not affect the vasculature (Figure 2c). Morphological analysis revealed that at 4 dpf wildtype embryos injected with *ptena* mRNA displayed mild defects in body length (Supplemental Figure 2a, c). Expression of exogenous *ptena* in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutant embryos largely suppressed their overall morphology (Supplemental Figure 2b, d).

To investigate whether enhanced PI3K signaling is associated with enhanced angiogenesis in loss of Pten mutants, we treated embryos with the PI3K inhibitor, LY294002, from the earliest timepoint we observed defects (70-72 hpf) onwards. Earlier treatment with LY294002 induces severe defects in the vasculature[18] as well as defects as early as gastrulation[19]. The overall morphology and vasculature of treated embryos was examined at 4 dpf. Wildtype embryos displayed mild defects in head size and body length was reduced compared to non-treated embryos (Supplemental Figure 2a, e). Consistent with our previous report[8], the morphological phenotype of *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants was largely rescued by LY294002 treatment (Supplemental Figure 2f). In addition, the excessive sprouting phenotype in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants is largely rescued at 4 dpf after treatment with LY294002 (Figure 2f). Wildtype embryos treated with LY294002 displayed mild defects in vessel morphology suggesting that endothelial cells are highly responsive to altered PI3K/Akt levels (Figure 2e). Thus, antagonizing the PI3K pathway suppressed ectopic vessel growth in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants, indicating that PI3K signaling has a central role in angiogenesis.

Next, we investigated whether inhibition of angiogenesis in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants suppressed the phenotype. To this end, we used the angiogenesis inhibitor, Sunitinib that selectively inhibits RTKs[20], including VEGFRs in embryos. Wildtype embryos that were treated from 70-72 hpf onwards with Sunitinib displayed no morphological malformation in the vasculature (Figure 2g). Examination of the vasculature in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants at 4 dpf revealed that enhanced angiogenesis was suppressed by Sunitinib treatment (Figure 2g, h). Our results suggest that signaling by Sunitinib-sensitive RTKs has a critical role in hypervascularization in Pten mutants.

Elevated pAkt level in ptena$^{-/-}$ptenb$^{-/-}$ mutants is suppressed by LY294002 and to a lesser extent by Sunitinib

Pten antagonizes PI3K signaling, upstream of the Akt pathway and consequently, loss of Pten leads to constitutive activation of Akt. We assessed pAkt levels by immunoblotting of individual embryos at 4 dpf. As expected, *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants display dramatically enhanced levels of pAkt compared to wildtype at 4 dpf (Figure 3). Whereas pAkt levels varied from embryo

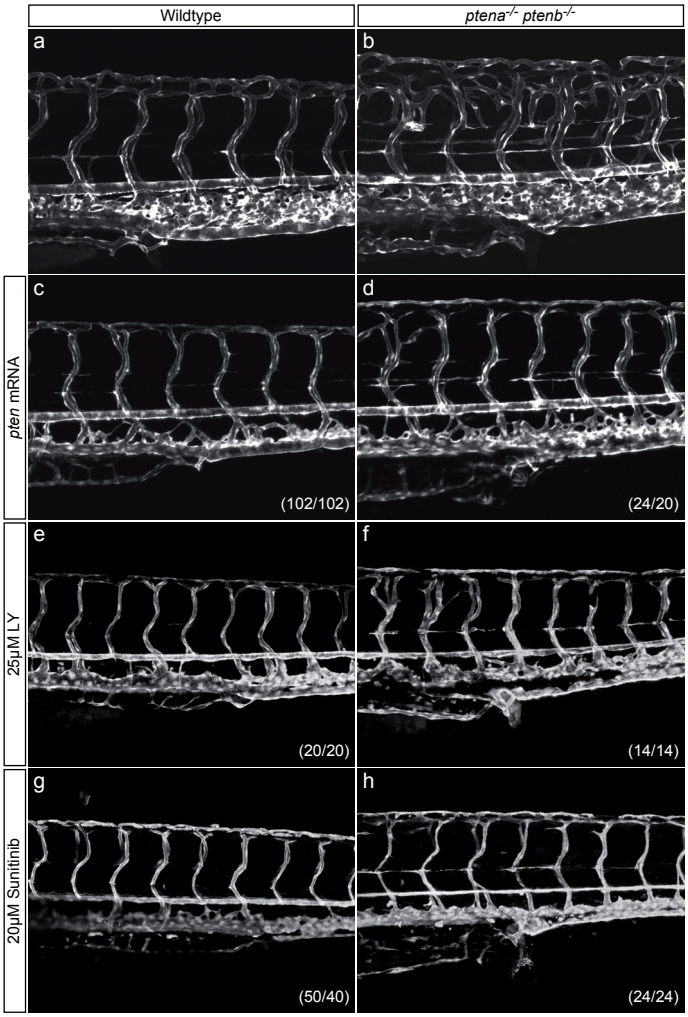


Figure 2 I Rescue of enhanced angiogenesis in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants by exogenous *ptena* mRNA, LY294002 and Sunitinib. The transgenic line, *Tg(kdrl:eGFP)*, was used to visualize the vasculature at 4 dpf in wildtype (a,c,e,g) and ptena$^{-/-}$ptenb$^{-/-}$ embryos (b,d,f,h). (**a**,**b**) *Ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants display ectopic vessel growth compared to wildtype embryos. (**c**,**d**) 10 pg synthetic *ptena* mRNA was injected at the one cell stage into wildtype and *ptena*$^{-/-}$*ptenb*$^{-/-}$ embryos. (**e**,**f**) 25 μM LY294002 (LY) was applied from 70-72 hpf onwards. (**g**,**h**) 20 μM Sunitinib was applied from 70-72 hpf onwards. Images were taken using confocal microscope with 20x. The numbers in the bottom right corner represent the total number of embryos treated/ the number of embryos showing the phenotype depicted here. Anterior to the left, 20x, 2 μm step size.

to embryo, pAkt levels were consistently elevated in *ptena*$^{-/-}$*ptenb*$^{-/-}$ embryos, compared to wildtype embryos. Re-expression of Ptena resulted in down regulation of elevated pAkt in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutant embryos (Figure 3). Similarly, we observed suppressed levels of pAkt in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutant embryos upon treatment with the PI3K inhibitor, LY294002 (Figure 3). Sunitinib treatment reduced elevated pAkt levels in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants to a much lesser extent than Ptena expression or LY294002 treatment (Figure 3). pAkt levels were also reduced in wildtype embryos by expression of Ptena and LY294002 or Sunitinib treatment (Figure 3). In summary, elevated pAkt levels in Pten mutants were suppressed by expression of Ptena and by treatment with LY294002 and Sunitinib.

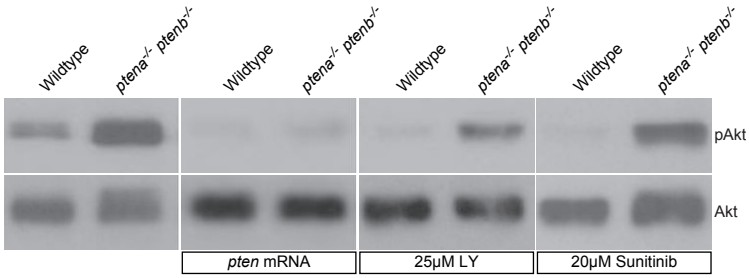


Figure 3 I Elevated pAkt level in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants is suppressed by LY294002 and to a lesser extent by Sunitinib. Wild type and *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutant embryos were left untreated, were injected at the one-cell stage with *ptena* mRNA, or were treated with 25 μM LY294002 or 20 μM Sunitinib from 72 hpf onwards. Single embryos were lysed at 4 dpf and the protein from individual embryos was isolated. The proteins were run on a denaturing SDS-polyacrylamide gel and transferred to PVDF membranes. After blocking the blot was probed with phosphospecific pAkt antibody (directed against pSer473), stripped and probed with Akt-specific antibody as a loading control. Representative blots are depicted here.

Ptena$^{-/-}$ptenb$^{-/-}$ mutants display enhanced expression of vegfAa

VEGF signaling, in particular *vegfAa*, is indispensable for angiogenesis. To address if VEGF signaling is involved in enhanced angiogenesis in Pten mutants, we examined *vegfAa* expression levels at 4 dpf by quantitative PCR. *VegfAa* expression was dramatically upregulated (8-fold) in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants compared to wildtype (Figure 4A). To assess at which developmental stage *vegfAa* expression is elevated in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants, we performed time course analysis at 1, 2 and 3 dpf. At 1 and 2 dpf of development, no difference in expression was detected between mutants and wildtypes. We found that *vegfAa* is significantly upregulated (3-fold) from 3 dpf onwards (Figure 4B), which coincides with the onset of enhanced filopodia formation in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutant embryos (*cf.* Figure 1). Next, we addressed if the rescued angiogenic phenotype in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants after re-expression of Pten is associated with downregulation of *vegfAa*. We found that restoring Ptena expression in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants significantly downregulated the elevated *vegfAa* level (from 8-fold to 2.5-fold) (Figure 4a). Similarly, we found that *vegfAa* expression was significantly downregulated in *ptena*$^{-/-}$*ptenb*$^{-/-}$

mutants by LY294002 (from 8-fold to 2-fold) (Figure 4a). Surprisingly, *vegfAa* expression was dramatically enhanced by Sunitinib in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants (from 8-fold to 40-fold, compared to untreated wildtype). In wildtype embryos, Sunitinib treatment induced a modest increase in *vegfAa* expression (4-fold) (Figure 4a). Taken together, loss of Pten led to elevated *vegfAa* expression, which was rescued by inhibition of PI3K. Inhibition of angiogenesis using Sunitinib greatly enhanced *vegfAa* expression in wildtype and *ptena*$^{-/-}$*ptenb*$^{-/-}$ embryos, suggesting a feedback loop.

Combined LY294002 and Sunitinib treatment abolished enhanced vegfAa expression and reduced hypervascularisation

Sunitinib is a widely used anti-angiogenic compound to prevent neo-vascularization[20]. Our results demonstrate that Sunitinib treatment led to increased *vegfAa* expression, particularly in *ptena*$^{-/-}$*ptenb*$^{-/-}$ embryos (Figure 4). LY294002 treatment rescued elevated *vegfAa* expression to some extent. We hypothesized that LY294002 treatment might suppress Sunitinib-induced *vegfAa* expression and the two inhibitors might cooperate to suppress enhanced angiogenesis. To test this, we combined LY294002 and Sunitinib at suboptimal doses. A suboptimal concentration of LY294002 (5 µM) did not fully repress enhanced angiogenesis (Figure 5b-e), but suppressed enhanced *vegfAa* expression in *ptena*$^{-/-}$*ptenb*$^{-/-}$ embryos (Figure 5a), suggesting that *vegfAa* expression is tightly regulated by PI3K signaling. A suboptimal concentration of Sunitinib (5 µM) did not fully repress enhanced angiogenesis in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutant embryos (Figure 5g) and still led to an 8-fold increase in *vegfAa* expression (Figure 5a), indicating that a slight modification of VEGFR signaling still has a dramatic effect on *vegfAa* expression. Concomitant application of suboptimal concentrations of LY294002 and Sunitinib suppressed *vegfAa* expression and fully inhibited hypervascularization in *ptena*$^{-/-}$*ptenb*$^{-/-}$ embryos (Figure 5a, i). Our data indicate that simultaneous inhibition of PI3K and VEGFR signaling cooperatively suppressed enhanced angiogenesis in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutant embryos.

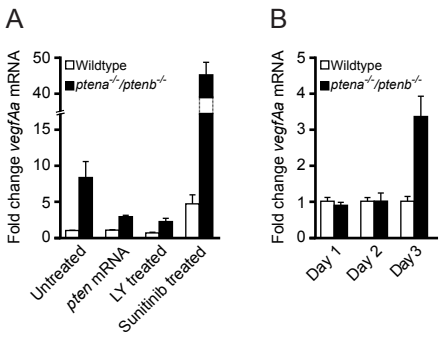


Figure 4 I Upregulated *vegfAa* expression in *ptena*$^{-/-}$*ptenb*$^{-/-}$ is diminished by LY294002 and enhanced by Sunitinib. **A** Quantitative PCR was performed to determine *vegfAa* expression levels in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants compared to wildtype at 4 dpf. Rescue experiments were done by microinjection of *ptena* mRNA at the one cell stage or by treatment with 25 µM LY294002 (LY) or 20 µM Sunitinib from 72 hpf onwards. **B** Quantitative PCR was performed to determine *vegfAa* expression levels in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants compared to wildtype at 4 dpf and at 1, 2 and 3 dpf. Wildtype control was set to one and all values were determined relative to the wildtype control at 3 dpf. Statistical analysis (Kruskall-Wallis with Dunns post-hoc test) of duplicates or triplicates was performed. Note that the y-axis is discontinuous to accommodate the 40-fold increase in *vegfAa* expression upon Sunitinib treatment of *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants.

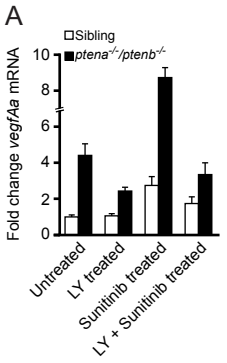
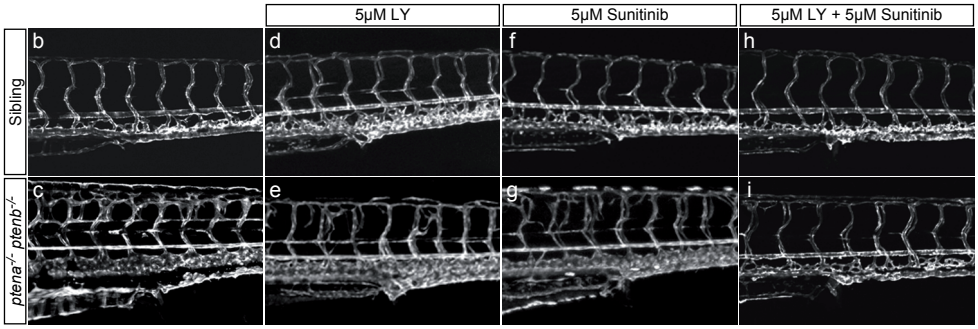


Figure 5 I Combined treatment with LY294002 and Sunitinib rescued hypervascularization. **A** Quantitative PCR was performed to determine *vegfAa* expression at 4 dpf in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants compared to siblings following treatment with suboptimal concentrations of LY294002 (LY, 5 μM) and Sunitinib (5 μM) or both from 72 hpf onwards. Relative amounts were determined with wildtype untreated set to 1.0. Statistical analysis was performed using Excel. (**b**-**i**) Vasculature of wildtype and *ptena*$^{-/-}$*ptenb*$^{-/-}$ embryos at 4 dpf was imaged in *Tg(kdrl:eGFP)* line by confocal microscope with 20 x. The embryos were treated with suboptimal concentrations of LY294002 (LY) and Sunitinib or both as indicated. Representative embryos are depicted; anterior to the left.



Hemangiosarcoma formation in Pten haploinsufficient fish is accompanied by elevated vegfAa expression

Ptena$^{+/-}$*ptenb*$^{-/-}$ *and ptena*$^{-/-}$*ptenb*$^{+/-}$ mutant adult fish are prone to develop hemangiosarcomas during their life time[12]. We have established that these hemangiosarcomas are preferentially formed in the *rete mirabile*, a highly vascularized tissue that is connected to the eye bulb. In general, hemangiosarcomas are associated with the vasculature and consist of perfused endothelial lumens. We investigated if *vegfAa* expression was enhanced in hemangiosarcomas of *pten* mutant adult fish by quantitative PCR. We isolated RNA from the tumors and from contralateral tissue of the same animals and as a control, we isolated RNA from roughly the same tissue in wildtype zebrafish. *VegfAa* expression was 3-fold higher in the hemangiosarcoma than in wildtype tissue. *VegfAa* expression in the contralateral tissue from the tumor-bearing fish was not significantly different from *vegfAa* expression in wildtype (Figure 6a). *In situ* hybridization of tumor sections revealed enhanced *vegfAa* expression compared to contralateral tissue (Figure 6b,c). Taken together, we show that *vegfAa* expression is enhanced in hemangiosarcomas which may enhance tumor growth.

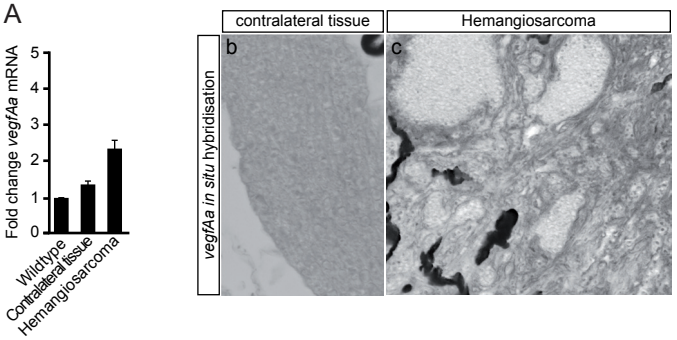


Figure 6 I Elevated expression of *vegfaa* in hemangiosarcoma. **A** Hemangiosarcoma tumor material of *ptena*$^{+/-}$*ptenb*$^{-/-}$ mutants (n=3) was isolated. Contralateral tissue from the tumor-bearing fish and tissue from the same area in wildtype adult fish were isolated as control. RNA was isolated and quantitative PCR was performed to establish *vegfAa* expression. Statistical analysis was performed using Excel and fold change values were determined with wildtype set to 1.0. A *ptena*$^{+/-}$*ptenb*$^{-/-}$ mutant fish with an apparent hemangiosarcoma was fixed and *in situ* hybridization was performed on paraffin sections using a *vegfAa*-specific probe. Representative areas of the same section showing contralateral tissue **b** and the hemangiosarcoma **c** are depicted here.

DISCUSSION

PTEN is one of the most frequently mutated tumor suppressor genes in cancer. Concomitant loss of both *pten* genes in zebrafish leads to hyperplasia and dysplasia, resulting in embryonic lethality by 5 dpf[8]. Mutants that retain one wildtype *pten* allele, *ptena*$^{+/-}$*ptenb*$^{-/-}$ respectively *ptena*$^{-/-}$*ptenb*$^{+/-}$, are prone to develop endothelial derived hemangiosarcomas later in life. Here, we investigated angiogenesis in the absence of functional Pten during zebrafish embryogenesis and found a dramatic hypervascularisation in the dorsal trunk area. We observed enhanced sprouting from 3 dpf onwards, resulting in the formation of ectopic blood vessels at 4 dpf. The absence of defects in vasculature at earlier timepoints may be due to maternally provided Pten. However, this is unlikely because immunoblotting demonstrated that maternally contributed Pten was not detectable anymore from 60 hpf onwards (data not shown), well before the stage that we observed enhanced angiogenesis. Perhaps Pten is not essential for vasculogenesis, *i.e. de novo* formation of blood vessels, and it only has a role in angiogenesis. Interestingly, it has been reported that PI3K signaling is essential for angiogenesis in mouse and fish development. Mouse mutant embryos with a homozygous mutation in the PI3K catalytic subunit (p110α$^{D933A/D933A}$) show regular heartbeat and blood flow in central vessels till E10.5, indicating that vasculogenesis is normal. However, at E12.5, phosphorylation of Akt in p110α$^{D933A/D933A}$ mutants is absent and embryos are lethal, exhibiting primary angiogenic remodeling defects[21]. We conclude that the loss of Pten induced defects in angiogenesis,

not vasculogenesis.

Hypervascularization was not limited to the trunk area. We have also observed massive increases in blood vessels in other areas of the embryo, including the head, by imaging of *ptena*$^{-/-}$*ptenb*$^{-/-}$ and wildtype embryos in the *Tg(kdrl:eGFP)* line (data not shown). However, we focused on hypervascularisation in the trunk and we investigated the molecular basis for upregulated endothelial proliferation in Pten mutant embryos by treatment with the inhibitors , LY294002 and Sunitinib. Treatment of *ptena*$^{-/-}$*ptenb*$^{-/-}$ embryos with the PI3K inhibitor, LY294002, from 72 hpf onwards rescued the hypervascularization phenotype at 4 dpf, indicating that these defects were caused by enhanced PI3K-Akt signaling. Consistent with this notion is that elevated pAkt levels in Pten mutant embryos were suppressed by LY294002 treatment. The morphological defects in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants were also largely rescued by LY-treatment, which is consistent with our earlier report in which we treated embryos from 2 dpf onwards[8]. Inhibition of PI3K at very early stages induced severe gastrulation defects[19], which precludes a full rescue of the loss of Pten phenotype by early treatment with LY294002.

Sunitinib treatment led to a full rescue of hypervascularisation at 4 dpf. Yet, Sunitinib only marginally affected enhanced pAkt levels. Sunitinib selectively inhibits a subset of RTKs, including the angiogenic VEGFR1, VEGFR2 and PDGFRβ[20]. PI3K-Akt signaling downstream of other RTKs is not affected by Sunitinib. Therefore, it is not surprising that Sunitinib treatment did not fully suppress pAkt levels in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants. Apparently, inhibition of the angiogenic RTKs by Sunitinib fully rescued hypervascularisation in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants.

It appears that endothelial cells are particularly sensitive to loss of Pten. This may be due to an intrinsic sensitivity of endothelial cells to loss of Pten. However, the finding that *vegfAa* expression is enhanced in *ptena*$^{-/-}$*ptenb*$^{-/-}$ embryos may contribute to enhanced sensitivity of endothelial cells to loss of Pten, as these cells express VEGFRs, providing positive feedback. Upregulation of VEGF expression in response to deletion of Pten is not unprecedented. siRNA-mediated knockdown of PTEN in a panel of pancreatic cell lines led to upregulation of VEGF expression[22]. Moreover, ectopic expression of PTEN in the chronic myelogenous leukemia cell line, K562, led to reduced expression of VEGF[23], which is consistent with our data in zebrafish. Elevated *vegfAa* expression in *ptena*$^{-/-}$*ptenb*$^{-/-}$ zebrafish embryos is suppressed by treatment with LY294002, indicating that upregulation of *vegfAa* expression in *ptena*$^{-/-}$*ptenb*$^{-/-}$ embryos is dependent on PI3K signaling. Sunitinib treatment led to a dramatic increase in *vegfAa* expression, particularly in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutant embryos, suggesting a feedback mechanism. Inhibition of VEGFR1 and VEGFR2 and a subset of other RTKs enhanced expression of the VEGFR-ligand, VegfAa. The mechanism underlying transcriptional regulation of *vegfAa* in Pten mutants and in response to inhibitors remains to be determined.

VEGF signaling is crucial for vascular development during embryogenesis. Elevated levels of *vegfAa* mRNA expression were detected from 72 hpf onwards, which is concomitant with the onset of enhanced angiogenesis, suggesting a causal relation. To address directly whether elevated *vegfAa* expression induced enhanced angiogenesis, we used morpholinos to knockdown VegfAa expression. Unfortunately, VegfAa knockdown induced massive

defects in vasculature in wildtype embryos, consistent with previous reports[24, 25], precluding assessment of the effect of VegfAa knockdown on angiogenesis in *ptena*$^{-/-}$*ptenb*$^{-/-}$ embryos. Elevated expression of *vegfAa* was not limited to *ptena*$^{-/-}$*ptenb*$^{-/-}$ embryos, but we also observed significant upregulation of *vegfAa* expression in hemangiosarcomas that were isolated from adult zebrafish mutants that retained one wildtype allele of *pten*. Hemangiosarcomas are tumors that consist of endothelial cells and exhibit constitutive expression of Vegfr2/kdrl[26]. Elevated *vegfAa* expression will result in a positive feedback loop, which may account for the hyperpoliferation of endothelial cells in the hemangiosarcoma and hence contribute to tumor growth.

Sunitinib is commonly used as an anti-angiogenic drug to prevent (tumor) angiogenesis. Clinical reports describe cases where after administration of Sunitinib tumor relapse occurred with severe growth and increased metastatic behavior[27-29]. Here we discovered that applying Sunitinib to embryos led to upregulation of *vegfAa* in wildtype embryos and to a further upregulation of *vegfAa* expression in mutant embryos lacking Pten, which may explain severe relapses following Sunitinib treatment. Transcriptional upregulation of *VEGFA* expression in response to Sunitinib in patients will result in long-term enhanced VEGFA expression. By the time Sunitinib has lost its potency, VEGFA expression is still elevated, leading to hyperactivation of VEGFRs, resulting in hyperproliferation of endothelial cells, hence explaining the tumor relapse after Sunitinib treatment. Treatment with suboptimal concentrations of LY294002 and Sunitinib did not lead to dramatic increases in *vegfAa* expression in zebrafish embryos, yet it fully rescued the hypervascularization phenotype. These results suggest that combined treatment may represent a novel approach for therapeutic intervention.

METHODS

Zebrafish husbandry

Ptena$^{-/-}$*ptenb*$^{-/-}$ and *Tg(kdrl:eGFP)*[17] were maintained, crossed, raised and staged as described[30, 31]. All procedures involving experimental animals were approved by the local animal experiments committee and performed in compliance with local animal welfare laws, guidelines and policies, according to national and european law.

Immunoblotting

Single embryo lysates were obtained from wildtype and *ptena*$^{-/-}$*ptenb*$^{-/-}$ at 4 dpf using lysis buffer (50 mM HEPES, pH 7.4, 15 mM NaCl, 1 mM $MgCl_2$, 10 % glycerol , 1 % Triton X-100, 1 % sodium orhtovanadate and protease inhibitors, including 5 mM betaglyecrophosphate, 1 µg/ml aprotinin, 5 mM NaF, 1 mM Na_3VO_4 and 1 µg/ml leupeptin. Samples were mixed with 2 x Laemmli sample buffer, boiled for 5 min and proteins were run on SDS-polyacrylamide gels. Immunoblotting was performed according to standard procedures, using p-Ser473-Akt (1:2000,

Cell Signaling) and Akt (1:1000, Cell Signaling) antibodies.

Confocal and brightfield microscopy

Fluorescence images of transgenic embryos were acquired using TCS-SPE and processed with Image J. Embryos were anesthesized with Tricaine and mounted on a glass cover dish with 0.7 % low melting agarose and covered with standard E3 medium. Whole mount bright field images were taken with a Leica DC 300F stereomicroscope.

RNA isolation, cDNA synthesis and quantitative PCR

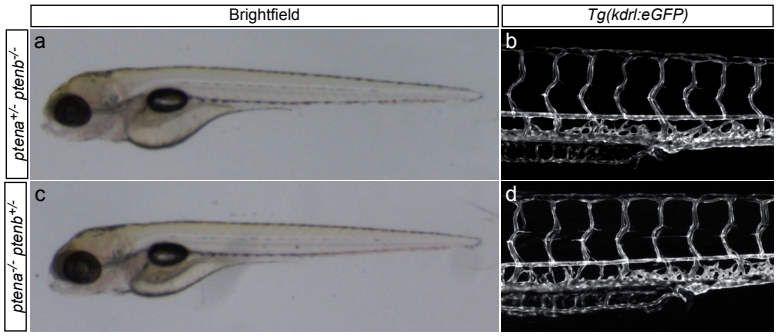
Total RNA was extracted according to manufacturers' instructions using the RNeasy Mini Kit (Qiagen). cDNA was synthesized with random hexamer primers according to manufacturers' instructions (Fermentas). Quantitative PCR was performed on a MyiQ cycler (Biorad) using SYBRgreen chemistry (Biorad). Three reference genes were used: tata box binding protein (TBP), elongation factor 1 α (EF1α) and β Actin). Sequences of oligonucleotide are listed in table 1. MIQE standards were applied to our protocols[32].

LY294002 and Sunitinib treatment, pten RNA injection

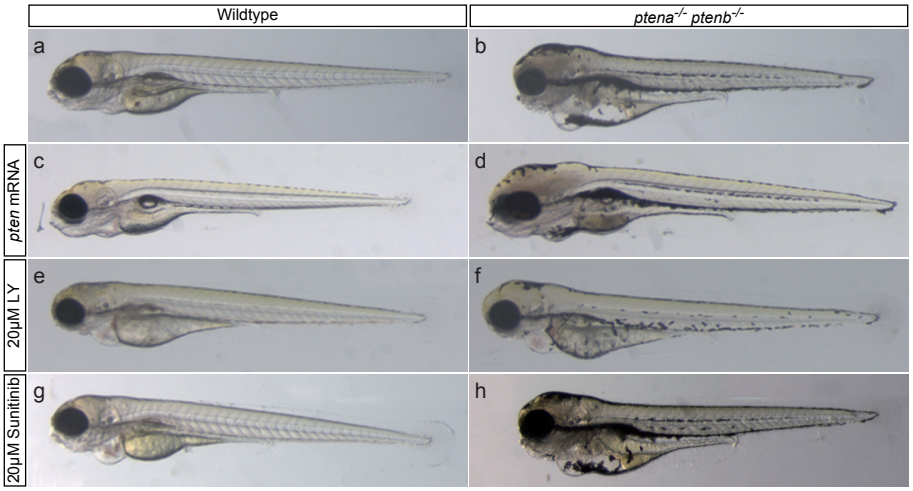
Embryos were treated from 70-72 hpf onwards with 25 μM LY294002 (Calbiochem), or 20 μM Sunitinib Malate (Sigma), unless stated otherwise. Control embryos were mock treated with DMSO and presence of *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutations were confirmed by genotyping as described. Embryos were kept in the dark during treatment. Ptena and Ptenb cDNA was cloned in pCS2+. 5' capped sense RNA was synthesized using the mMessage mMachine kit from Ambion according to manufacturer's instructions and 10 pg/nl injected at one cell stage.

In situ hybridization

In situ hybridization was performed as described elsewhere[33] using *vefgaa* probe[34].



Supplemental Figure S1 I Vasculogenesis and angiogensis is not perturbed in *ptena*$^{+/-}$*ptenb*$^{-/-}$ **a**,**b** or *ptena*$^{-/-}$*ptenb*$^{+/-}$ **c**,**d** mutants 4 days post fertilisation. The vasculature was visualized in *ptena*$^{+/-}$*ptenb*$^{-/-}$ and *ptena*$^{-/-}$*ptenb*$^{+/-}$ mutants using *Tg (kdrl:eGFP)*. Up to 4 dpf no defects were observed in mutants. Anterior to the left, 20x, 2 µM step-size.



Supplemental Figure S2 I Rescue of morphological defects in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants by exogenous Ptena, LY294002 and Sunitinib. At 4 dpf *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants display severe morphological defects compared to wildtype **a**, **b**: head and eye edema, wider set eyes, enlargement of yolk sac, reduced body length. Expression of exogenous Ptena did not cause malformation in development of wildtype embryo, but largely rescued the developmental defects in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants **c**, **d**. Treatment of embryos from 72 hpf onwards with 25 µM LY294002 resulted in mild growth defects in the head region in wildtype embryos and almost complete rescue of developmental defects in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants **e**,**f**. Treatment with 20 µM Sunitinib from 72 hpf onwards did not induce obvious morphological defects in wildtype embryos and largely rescued the morphological malformations in *ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants **g**,**h**.

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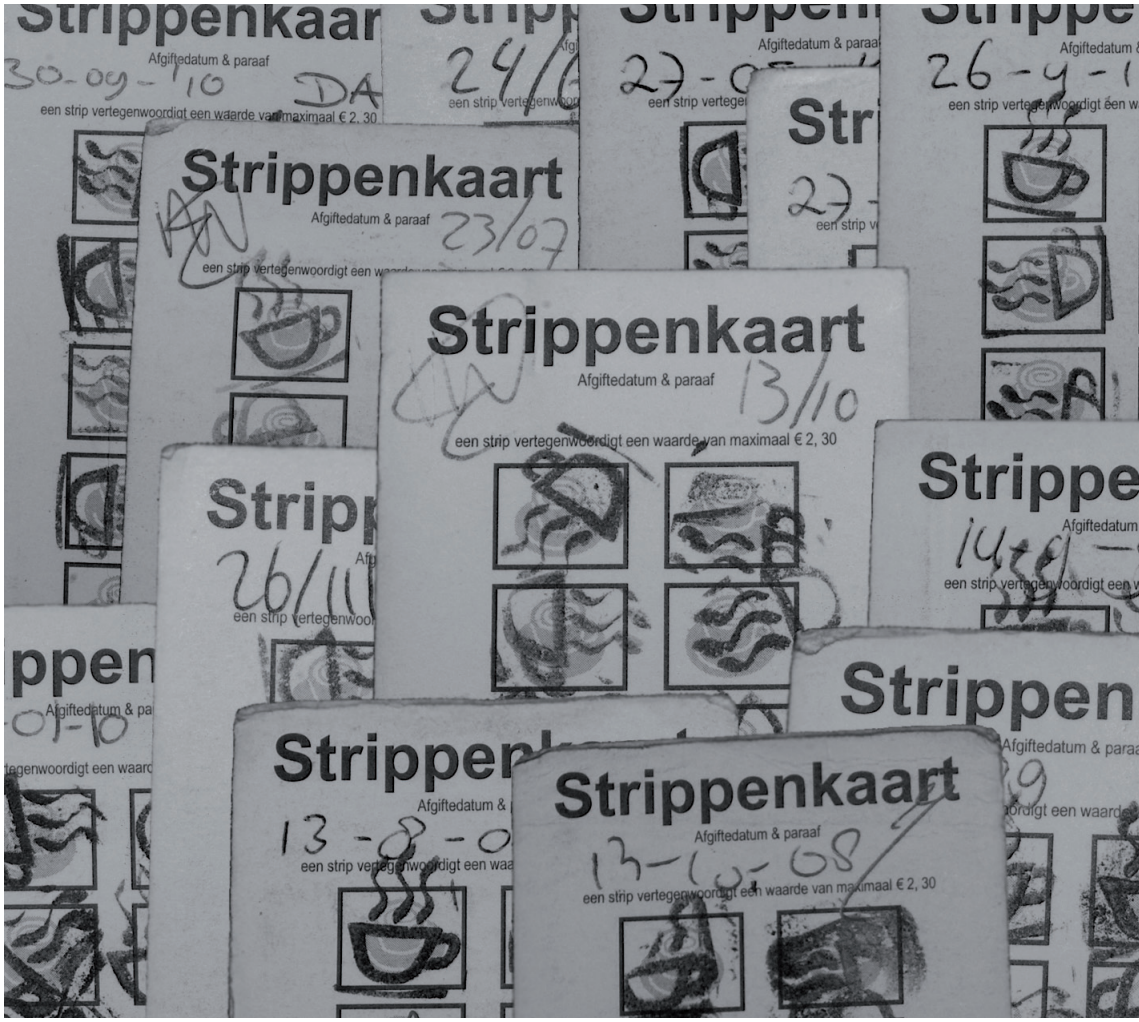
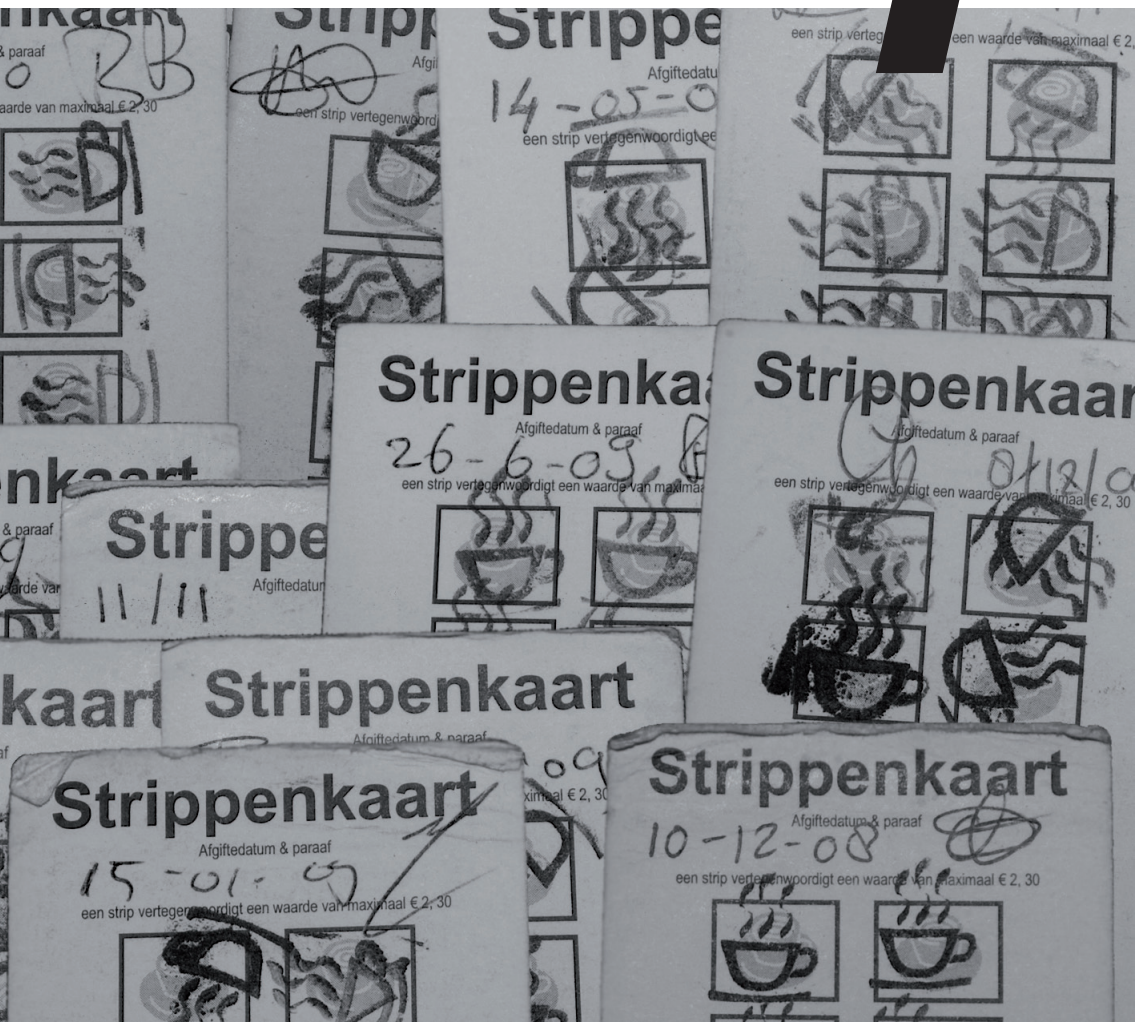
General Discussion



Chapter 7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In this thesis, we aimed to provide a better understanding of the functions of the atypical E2Fs, E2F7 and E2F8 (E2F7/8), in the process of (lymph)angiogenesis and tumor angiogenesis. In Chapter 2 and 4, we showed that E2F7/8 transcriptionally regulate key players of angiogenesis and lymphangiogenesis. Loss of E2F7/8 in respect to these two processes resulted in a decreased formation and quality of blood and lymphatic vessels. Interestingly, in Chapter 5 we showed that loss of E2f7/8 in tumors resulted in increased formation of blood vessels in the solid tumor mass. These contradicting results might be explained by the nature of the processes. Unlike developmental angiogenesis, tumor angiogenesis is a highly disorganized process in which angiogenic factors are secreted not only by tumor cells but also by stromal cells and invading inflammatory cells. Although the atypical E2Fs have been mainly investigated in a cell cycle setting, the presented angiogenesis data in this thesis clearly shows that the atypical E2Fs possess functions that reach beyond cell cycle control. Furthermore it becomes apparent in this thesis that the atypical E2Fs are not solely transcriptional repressor, but can function under certain circumstances as transcriptional activators. Although we provided mechanistic insights into how E2F7/8 can activate transcription, it remains unclear whether this mechanism is a common route for E2F7/8 to positively regulate genes. In addition, it is yet unexplored whether transcriptional activation by E2F7/8 is exclusive for certain processes like angiogenesis.

This thesis answers many questions previously unknown to the E2F and angiogenesis field. However, providing answers will automatically raise new questions. Therefore, we would like to discuss some of the questions raised by this thesis. Moreover, we would like to discuss new insights, which are partially supported with novel findings.

The transcriptional activator potential of E2F7 and E2F8

In Chapter 2, we provided evidence that E2F7/8 may function as a transcriptional activator. In this specific case, E2F7/8 recruited hypoxia inducible factor 1α (HIF1α), a strong transcriptional activator, and this transcriptional complex induced expression of VegfA. Although HIF proteins are well known for their transcriptional activity under low oxygen concentrations (hypoxia), there is also substantial evidence that HIF induces expression under normoxic conditions, arguing for a common transcriptional complex independent of oxygen status[1-3]. The recruitment of additional transcription factors has been reported for several members of the E2F family. To this extent it has been shown that the transcriptional repressor E2F6, which is similar to E2F7/8 not regulated by Rb, recruits the transcription factors Mga and Max[4]. Most interestingly, the transcriptional repressor Mga is converted to a transcriptional activator by the recruitment of Max[5]. In addition, the E2F6-Mga-Max transcriptional complex does not only bind to the E2F consensus motif, which is mediated by E2F6, but also Myc- and Brachyury binding sites, mediated through Mga and Max. Moreover, histon modifiers, heterochromatin protein 1 (HP1) and Polycomb group (PcG) proteins, were also found in this transcriptional complex, indicating that this complex is able to modify the chromatin to silence or enhance transcription[6, 7]. Similar to the E2F6-Mga-Max complex, the recruitment of HIF1α by E2F7/8 gives this complex the potential to bind to HIF binding sites and the possible switch from a transcriptional repressors towards a transcriptional activators. The classical repressor function of E2F7/8 requires the ability to interact with the DNA through their DNA binding domains[8, 9]. An interesting question arising is whether E2F7/8 need to interact with the DNA to exert their positive transcriptional effect. In Chapter 2, we showed that ectopic expression of an E2F7 DNA binding mutant was not able to induce *VEGFA* transcription. Moreover, upon mutation of the E2F binding consensus within the *VEGFA* promoter, E2F7 was still able to induce *VEGFA* transcription. Thus, E2F7/8 need the ability to interact with the DNA to exert their transcriptional effect on VEGFA, but apparently independent of the canonical E2F binding sites. However, verification whether the mutated E2F binding sites completely had lost their ability to bind E2F7 is lacking.

In Chapter 4 we showed that E2F7/8 induced the expression of Collagen and Calcium Binding EGF domains 1 (CCBE1). In addition, we identified several canonical E2F binding elements within the proximal promoter of *CCBE1*, which were bound by both E2F7 and 8 (Chapter 4, Figure 1A). Interestingly, we find, in a similar fashion as for the *VEGFA* promoter, several canonical HIF sites within the *CCBE1* promoter that are in the close proximity of the E2F sites (Figure 1A). Moreover, we described in the same Chapter that FLT4 is being repressed by E2F7/8 and remarkably we found no HIF sites within the close proximity (<300bp) of the E2F sites in the proximal promoter of FLT4 (Figure 1A). These observations might suggest that E2F7/8 activate *CCBE1* expression in a similar way as we have shown for VEGFA. Although we find for both VEGFA and CCBE1 canonical E2F sites, it remains unclear how they contribute to the activation of transcription. Alternatively, it has been shown that E2Fs are also able to bind DNA independently of the canonical E2F consensus[10], suggesting that E2F7/8 might interact through

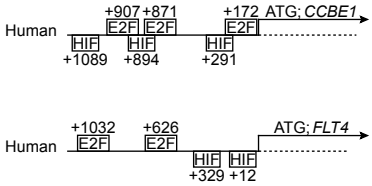


Figure 1 I Position of E2F and HIF binding elements within the human *CCBE1* and *FLT4* promoter.

an alternative binding site to activate transcription whereas the classical repressor function might be exerted through the canonical E2F sites.

Additional evidence that E2F7/8 might function as activators can be found within some recently published data. Westendorp *et al*. investigated the role of E2F7 during the cell cycle. To this extent they performed E2F7 chromatin immunoprecipitation experiments followed by sequencing (ChIP-seq) and correlated these results with microarray results from cells that overexpress mouse E2F7 tagged with eGFP[11]. As initial studies have shown that E2F7/8 are expressed in a cell cycle dependent manner and cells blocked and released form HU showed peak levels of E2F7 around mid S-phase, they performed their experiments at maximal E2F7 protein levels[11-13]. In line with the repressive function of E2F7 they found that from the 648 genes with a peak in the 5kb proximal promoter, only 89 genes (12%) were actually downregulated in the micro-array experiment. This low overlap between the Chip-seq and micro-array experiment might be partially explained by the use of their experimental setup, the comparison of an endogenous E2F7 ChIP-seq versus an ectopic expression of E2Ff7-eGFP in their micro-array. Forced expression of E2F7 might occupy and regulate genes that under normal conditions are not accessible. Approaching the micro-array as a separate experiment, the analysis of the significantly upregulated genes revealed that about 55% of the in total 80 upregulated genes, possess a canonical E2F binding consensus (Figure 2A). Although the upregulation of these genes might occur through an indirect mechanism as suggested by Westendorp *et. al.*, alternatively E2F7 could act as a direct transcriptional activator for these 44 genes. In addition, analysis of the significantly downregulated genes from the same set, revealed that 60% of the in total 334 genes downregulated, contained a canonical E2F binding consensus (Figure 2A). Thus, both up- and downregulated genes in the micro-array, about 50% of the genes contain a canonical E2f binding site. Although the total number of downregulated genes exceeds the total of upregulated genes by far, we should take into account that these experiments were specifically conducted in a cell cycle dependent setting, which may indicate that E2F7 mainly acts as a transcriptional repressor during the S-phase.

Additional evidence that E2F7/8 might act as transcriptional activators was found in a recently publication of Ouseph *et al.,* in which they used a *Sox2-Cre* conditional knock out system to investigate the role of E2F7/8 specifically in the embryo proper. In contrast to the conventional *E2f7*$^{-/-}$*E2f8*$^{-/-}$ knockout, deletion of *E2f7/8* only in the embryo carried these embryos to term[14]. By micro-array analysis they investigated the effect of *E2f7/8* deletion on gene expression (E10.5). We re-analyzed these public available data with the question whether the deletion of *E2f7/8* results in the downregulation of genes, which might be due to the loss of activation by E2F7/8. Using a 1.5 fold increase or 0.66 decrease with an additional $P<0.05$, we

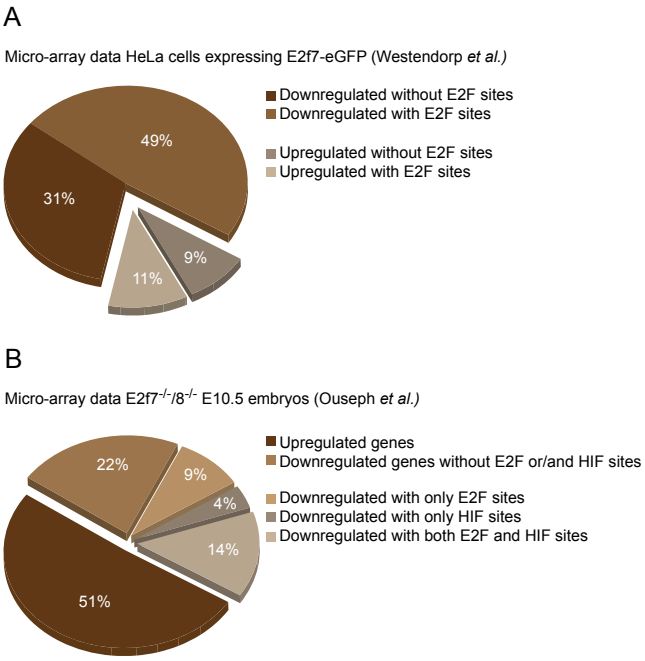


Figure 2 I Analysis of public available micro-array data of E2F7/8. **A** Pie chart showing genes up/down regulated in HeLa cells overexpressing E2F7 for 8 hours. **B** Micro-array analysis on mice embryo's (E10.5) with *E2f7/8* specific deleted in the embryo proper

found an almost equal portion of genes up- or downregulated (Figure 2B). Further analysis of these genes revealed that 42 (17%) of these genes are downregulated in *Sox2-Cre E2f7/8* DKO mice have both an HIF and E2F site within their proximal promoter (Figure 2B). Indicating that the E2F7/8-HIF transcriptional complex might activate these genes under normal conditions in E10.5 embryos. Off note, this analysis did not take into account the distance between the E2F and HIF sites, which we argued earlier to be of importance.

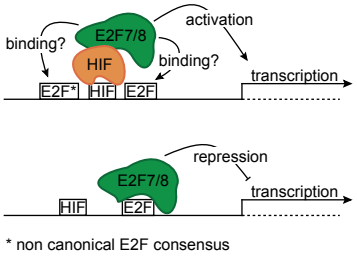


Figure 3 I Possible model how E2F7/8 activate transcription. E2F7/8 interact with hypoxia inducible factor (HIF) to positively regulate transcription. It remains unclear whether in this situation E2F7/8 bind to a canonical or non canonical E2F site.

In sum, against the current paradigm that E2F7/8 act exclusively as transcriptional repressors, we provide substantial evidence that E2F7/8 are able to activate transcription of genes. It appears that E2f7/8 gain, at least in part, their transcriptional activation properties by interacting with other transcription factors, like HIF1α. In addition, the presence of E2F and HIF binding sites within close proximity of each other in the proximal promoters of genes might be decisive whether this gene is positively regulated by E2F7/8. However, it remains unclear which role the canonical E2F binding sites play in this setting (Figure 3).

E2F7 and E2F8, partners in crime?

E2f7 and E2f8 (E2f7/8) are often mentioned in one breath, as if they are one and the same. Indeed, one allele of either *E2f7* or *E2f8* has been shown to be sufficient to carry *E27*$^{+/-}$*8*$^{-/-}$ or *E2f7*$^{-/-}$*8*$^{+/-}$ embryos to term, thereby suggesting overlapping functions[15]. The potential to compensate for each other is supported by the finding that E2f7 and E2f8 form homo- and hetero- dimers, in which apparently E2f7 has a higher tendency to form homo-dimers than hetero-dimers[15]. Interestingly, the transcriptional effect on E2f targets of these different dimers, E2f7 homo-dimer, E2f7/8 hetero-dimer or E2f8 homo-dimers, is unknown. The expression pattern of *E2f7/8* mRNA *in vivo*, which showed a ubiquitous pattern during embryonic development for both genes in mice and zebrafish[15] (Chapter 2 Figure 2A), suggests that most cells expressed both *E2f7* and *E2f8* at the same time. Interestingly, E2f7 mRNA levels seem to peak later than E2F8 in synchronized mouse embryonic fibroblasts (MEFs), 18 and 12 hours respectively[16, 17], although we need to keep in mind that mRNA levels might not reflect the protein levels. As mentioned previously, both E2F7 and E2F8 modulate each other's expression levels and knock down of either gene results in derepression of the other, although the amount of derepression seems to differ between cell lines and tissues[18] (data not shown). To some extent this might be due to the levels of E2F7 or E2F8 present in the cells or tissues. For hepatocytes it has been shown that E2f7 levels are hardly detectable, whereas E2f8 is abundantly present[19]. In this respect, loss of *E2f8* leads to an increased expression of *E2f7*, whereas loss of *E2f7* does not lead to an increased *E2f8* expression. Based on these data and the current knowledge from the literature we would like to postulate the hypothesis that cells going through the cell cycle first express E2f8 (peak at 12 hours), which in turn is down regulated upon expression of E2f7 (peak at 18hours), thereby indicating that, next to their overlapping function, E2f7/8 have distinct properties (Figure 4). Some support for this hypothesis might be found in the proximal promoter region of *E2f7/8*. Analysis of the promoter region revealed that from the in total 144 binding elements (1500bp promoter; matrix similarity 1-0.8) *E2f7/8* share 44% of the elements. Further ontology on these shared elements shows enrichment for negative regulation of expression, differentiation and placenta development, which is line with the strong expression of atypical E2Fs in trophblasts and their essential role for placental development , recently shown by Ouseph *et. al.*. In contrast, elements only present in *E2f7* (33%), show an enrichment for sensory organ development, cell fate commitment and neuron differentiation, whereas *E2f8* (23%) show an enrichment

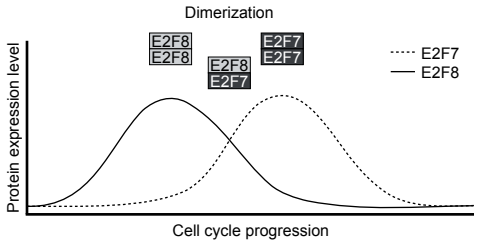
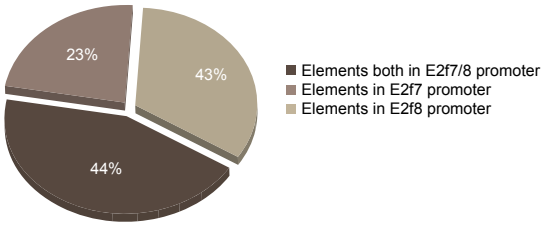


Figure 4 I E2F7 and E2F8 form hetero- and homo-dimers, with an increased preference for E2F7 rather than E2F8 homo-dimers. During cell cycle progression E2F8 expression, which is expressed earlier, is repressed by E27. Due to the difference in affinity, E2F8 is scavenged by E2F7, ensuring a quick shift towards a gene expression program driven by E2F7.



E2f7 promoter

Element		Function
Gata3/5		Transcriptional activator for erythroid development
Hnf4A	Hepatocyte nuclear factor 4A	Essential for development of the liver, kidney and intestine
Lmx1A/1B	LIM homeobox	Positive regulator of insulin gene transcription; development of dopamine producing neurons
Nanog		Regulator of inner cell mass and embryonic stem (ES) cells proliferation and self-renewal
Brachyury		Required for mesoderm formation and differentiation.
Pax6	Paired box 6	Eye and neural development

E2f8 promoter

Element		Function
Dlx3	Distal-less homeobox 3	Forebrain and craniofacial patterning and morphogenesis
Irf6	Interferon regulatory factor 6	Keratinocyte proliferation-differentiation switch; mammary epithelial cell proliferation
Pparg	Peroxisome proliferator-activated receptor gamma	Adipocyte differentiation and glucose homeostasis; involved in obesity, diabetes and cancer
Tcf7	Transcription factor 7	T-cell differentiation; survival of $CD4^+$ $CD8^+$ immature thymocytes
Tlx1	T-cell leukemia homeobox 1	Spleen development; neuronal specification; linked to T-cell acute lymphoblastic leukemias
Srf	Serum response factor	Cell cycle regulation, apoptosis, cell growth and cell differentiation

E2f7 and E2f8 promoter

Element		Function
Arnt	Aryl hydrocarbon receptor nuclear translocator	HIF1A and EPAS1/HIF2A hetero-dimer in response to hypoxia
E2f2/3/4		Cell cycle and DNA replication
Irf1	Interferon regulatory factor 1	Hematopoiesis, immune responses and cell proliferation and differentiation, apoptosis
NFAT5	nuclear factor of activated T-cells 5	Immune response; osmotic stress
Sox2	SRY (sex determining region Y)-box 2	Neural stem cell self-renewal; supresses neuronal differentiation
Sox9	SRY (sex determining region Y)-box 9	Skeletal development

Figure 5 I Next to overlapping functions, E2F7 and E2F8 might possess unique functions. Promoter analysis, 1500bp upstream of the ATG start site, of E2F7 and E2F8 revealed that both genes share elements conform their overlapping functions, but also posses unique elements. These unique elements might drive spatiotemporal expression of either E2F7 or E2F8.

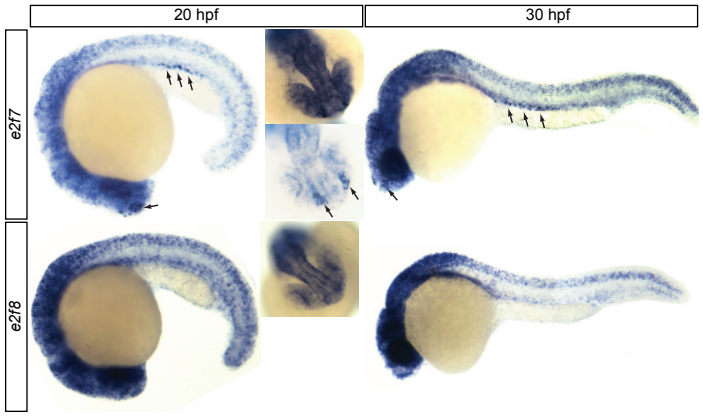


Figure 6 I Expression of *e2f7* and *e2f8* shown by *in situ* hybridisation in zebrafish embryos. At 20 and 30 hours post fertilization (hpf) both genes have an ubiquitous expression pattern with an increased intensity in the eye, brain and in the dorsal and ventral side of the trunk. Arrows depict intense expression of E2F7 in cells in the olfactory placode and renal duct.

for embryonic axis specification (anterior/posterior, ventral/dorsal and proximal/distal), tube development and limb morphogenesis (Figure 5).

Currently, there is limited evidence for such distinct function between E2f7/8. However, *in situ* hybridization (ISH) of *e2f7/8* in zebrafish at 20 and 30 hours post fertilization (hpf) showed a ubiquitous expression pattern with an increased expression in the brain region, ventral and dorsal part of the trunk (Figure 6). Interestingly, ISH of *e2f7* showed an additional strong staining in what appeared to be cells in the renal duct and cells in the olfactory placodes both at 20 and 30hpf (Figure 6; arrows). The olfactory placodes give rise to the olfactory neurons that in turn transfer odor information to the central nervous system. These placodes develop out of cells derived from the neural plate[20]. Remarkably, this increased expression in the olfactory placodes coincides with the finding that the *E2f7* proximal promoter contains elements that promote expression in neural and sensory organs (Figure 5).

In sum, there is, although limited, evidence for distinct functions between E2f7/8. Moreover, the fact that E2f7/8 can form homo- or hetero-dimers might favor the hypothesis of overlapping functions, although it might also provide a mechanism of transition from a predominantly E2f8 dimer towards E2f7 dimers during the cell cycle (Figure 4). In addition, it will be interesting to investigate the functional consequence of the dimer composition on transcription of E2f7/8 target genes. Likewise, it would be interesting to investigate the tissue specific expression of *E2f7/8* and the consequence thereof on the transcriptional program.

In theory, E2F7 and E2F8 can be classified as tumor suppressors

In general, tumor suppressors are genes that protect cells against uncontrolled proliferation and (de-)differentiation, thereby preventing tumor formation and progression. In 1971, Knudsen showed that retinoblastomas were linked with the mutations in the retinoblastoma gene (Rb), a well-known tumor suppressor [21]. He proposed a two-hit hypothesis, in which the first hit results in inactivation of one allele, often acquired by germline transmission. The second hit, acquired in somatic cells, results in the complete inactivation of the gene and thereby the likely onset of tumor formation. Regarding the classical view that atypical E2Fs, E2F7 and E2F8 (E2F7/8), repress genes essential for S-phase progression and thereby control cell proliferation, indicates that E2F7/8 are potential tumor suppressors[11]. Indeed, conditional inactivation of E2f7/8 in the mouse liver results in spontaneous hepatocellular carcinomas in aging mice (Pandit & de Bruin unpublished data). In addition, specific knock out of *E2f7/*8 in keratinocytes, by the use of *K14-Cre,* in a two-stage carcinogenesis skin cancer model showed that *E2f7*$^{-/-}$*8*$^{-/-}$ keratinocytes develop more tumors with an increased malignancy (Martinez & de Bruin unpublished data). Moreover, in Chapter 5 we showed that subcutaneously engrafted *E2f7*$^{-/-}$*8*$^{-/-}$ mouse embryonic fibroblasts (MEFs) show an increased angiogenic response (Chapter 5 Figure 1B). In this perspective, recruitment of blood vessels is an essential step for tumor dissemination and metastasis and contributes thereby to the tumor malignancy. Together these data favor a model in which E2F7/8 act as tumor suppressors. However, it is apparent that E2F7/8 functions are still

not well understood and several Chapters in this thesis showed contradicting results regarding the classical view. To this extent, we also found evidence that argues against the hypothesis that E2F7/8 act as tumor suppressors.

In Chapter 2 & 4 we used ectopic expression of *e2f7/8* mRNA in zebrafish embryos to investigate the role of these atypical E2fs in angiogenesis and lymphangiogenesis. Strikingly, we found that 5% of the *e2f7/8* mRNA injected embryos displayed a phenotype that closely resembled the formation of melanomas (Figure 7A; personal communication with Elizabeth Patton). In some cases these lesions on the skin showed an accumulation of cells surrounded by melanocytes (pigmented cells) (Figure 7A; white and black arrowheads). Moreover, it appeared that these lesions induced an angiogenesis response from the parachordal vessel, shown by *fli1a*:gfp positive endothelial cells (Figure 7B).

Interestingly, the fish as a model organism has a pivotal role in melanoma research. The *Xiphophorus maculatus* is a pigmented platy fish and because of its close relation to the non-pigmented *Xiphophorus hellerii* these fish can be successfully intercrossed. The F1 genetic hybrids of this interbreed develop spontaneous melanomas from specialized melanin-containing cells[22]. Moreover, *Xiphophorus* melanomas transplanted in immune-compromised nude mice proliferate and induce angiogenesis while maintaining expression of fish antigens[23]. Genetically, several studies identified *Xiphophorus* melanoma receptor kinase (Xmrk) as the oncogene responsible for the spontaneous formation of these melanomas in F1 hybrids. The Xmrk gene is a result of a gene duplication event of the *Xiphophorus* epidermal growth factor receptor b (Egfrb) gene and possesses a distinct flanking region that results in expression in pigmented cells[24]. Xmrk was shown to activate the MAP kinase pathway leading to strong proliferation. Moreover, it was shown that F1 hybrids had decreased levels of a cyclin-dependent

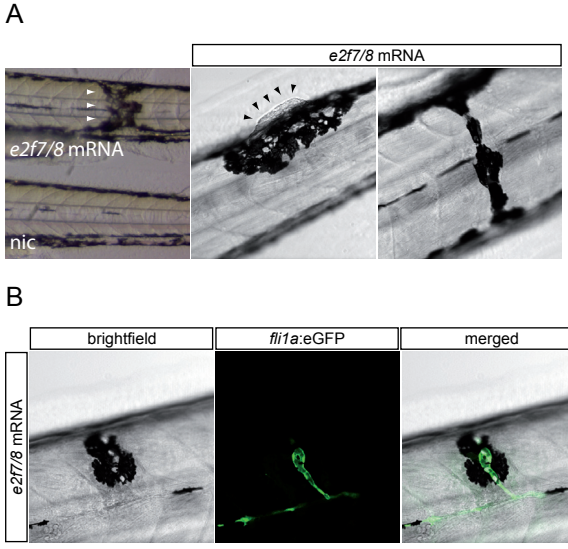


Figure 7 I Ectopic expression of *e2f7* and *e2f8* mRNA in zebrafish embryos induces melanoma like lesions. **A** Representative images of melanoma like lesions in the trunk of 5 day old zebrafish. Black and white arrows indicate a cellular mass at the site of lesion. **B** *E2f7/8* mRNA injected in *Tg(fli1a:gfp)* shows an angiogenic response towards the melanoma like lesion.

kinase inhibitor (CDKi) 2AB homolog due to the mixed genetic background and, additionally, decreased expression of CDKi2AB showed great correlation with Xmrk activity[25, 26]. Together the increased activity of the oncogene Xmrk and loss of CDKi2AB in F1 hybrids results in the loss of the G1/S checkpoint, consequently leading to uncontrolled proliferation of melanocytes and the formation of benign hyperplasia. Interestingly, a recent report shows that ectopic expression of *E2f7* in acute myeloid leukemia (AML) cells results in aberrant proliferation due to inhibition of cyclin-dependent kinase inhibitor p21$^{CIP1/WAF127}$. Together these results suggest that ectopic expression of *e2f7/8* mRNA might repress cyclin-dependent kinase inhibitors in pigment cells, for which fish, at least the *Xiphophorus*, are exceptional sensitive and consequently leads to spontaneous formation of melanomas.

Because ectopic expression of the "tumor suppressors" E2f7/8 in zebrafish embryos leads to melanoma like lesions, it would be of interest to investigate the underlying mechanism in more detail, with a main focus on the effect on the cell cycle and cyclin-dependent kinase inhibitor regulation. It would be of great help to increase the incidence of *e2f7/8* mRNA induced melanoma formation, for example by expressing *e2f7/8* mRNA specific in melanin producing cells, which can be achieved by placing *e2f7/8* expression under the control of the melanocortin receptor 1 (MCR1) promoter[28].

The development of a functional brain requires E2F7 and E2F8

In Chapter 2 we showed that E2f7/8 transcriptionally regulate VegfA during zebrafish primary angiogenesis. Next to this VegfA dependent angiogenesis phenotype, we also showed that *e2f7/8* morphants display severe apoptosis, by dUTP nick end labeling (TUNEL) and acridine orange staining, in the head and neural tube at 24 hours post fertilization (hpf) (Figure 8A). Additionally, at 48hpf the head region of *e2f7/8* morphants show enlargement, most likely due to edema (Figure 8B; upper panel; black arrowheads). To some extent, this edema might be due to a decreased or delayed formation of the head vasculature, which might be part of the VegfA phenotype as we found in the trunk region (Figure 8B, lower panel; white arrowheads).

Figure 6 shows a strong expression of *e2f7* and *8* in the brain of developing embryos. During development, the zebrafish brain undergoes remodeling and folding to form the brain ventricles, which are cavities in the brain that contain the cerebrospinal fluid (CSF). The CSF is believed to be essential for nutrition, protection, pressure equilibration and waste removal of the brain[29]. The zebrafish brain shows three distinct cavities, forebrain, midbrain and the hindbrain cavity[30]. During the formation of these cavities, the two brain parts are heavily folded between the mid- and hindbrain, the so called midbrain-hindbrain boundary (MHB) (Figure 9A). Initial opening of the cavities between 17 and 21hpf is independent of blood flow, although the expansion of the ventricles between 21 and 36hpf is blood flow dependent[30]. Furthermore, at 17 hpf 8% of the cells in the brain are proliferating with an ubiquitous distribution[30]. Interestingly, at 21hpf about 10% of the cells in the same region are proliferating, however this number is decreased by half in the MHB[30]. Chemical blockage of DNA synthesis between 17 and 24 hpf results in impaired formation of brain ventricles, indicating that cell proliferation plays a crucial

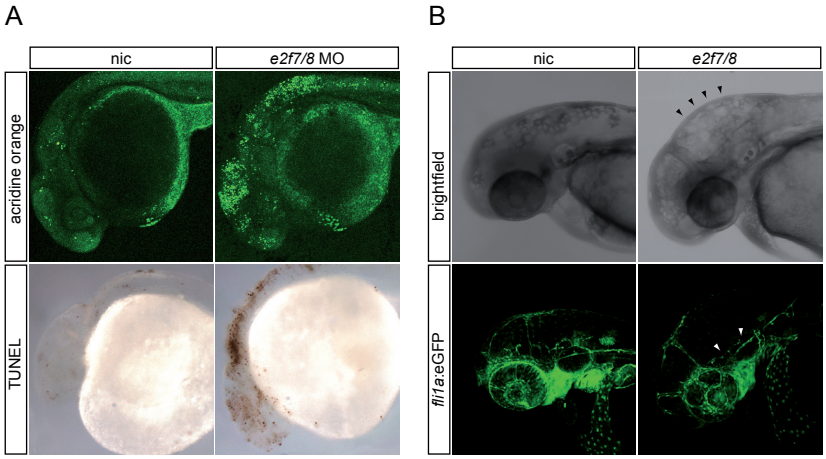


Figure 8 I *E2f7* and *e2f8* are important during zebrafish brain development **A** At 24 hours post fertilization (hpf), e2f7/8 morphants show increased apoptosis in the head region. **B** Around 48 hpf, the brain shows an edematic swelling, moreover, blood vessels in the brain are impaired and delayed.

role in formation of the brain ventricles[30]. Moreover, TUNEL staining revealed that there is no pattern or localized apoptotic cells present during the formation of the ventricles, suggesting that apoptosis does not play a role in the formation of the brain ventricles[30]. Together these data indicated that cell proliferation and the control thereof plays an important role in the development of the brain ventricles.

Between 21 and 24hpf, when the initial brain ventricles have emerged, *e2f7/8* morphants display severe apoptosis within the brain and neural tube, as discussed above (Figure 8; 1st and 2nd panel). To investigate whether the formation of the brain ventricles is impaired due to this apoptotic phenotype, we stained the brain with bodipy ceramide complexed to bovine serum albumin. Interestingly, at 28hpf the midbrain ventricle of *e2f7/8* morphants showed an enlargement, whereas the hind brain ventricle appears to be unchanged (Figure 10; upper panel). In addition, analysis of proliferating cells, stained with phospho-histone 3 (PH3), showed an remarkable increase in PH3 positive cells in the brain and neural tube at 24hpf (Figure 10; lower panel). As it has been shown that loss of *e2f7/8* leads to increased apoptosis mediated through the E2f1-Tp53 axis[15], we co-injected *tp53* together with *e2f7/8* morpholinos. Although apoptosis was greatly decreased in *e2f7/8tp53* morphants, the brain and neural tube still displayed an increased proliferation phenotype (data not shown). Together, these data might suggest that the loss of *e2f7/8* results in uncontrolled proliferation in neural tissue and thereby the enlargement of the mid brain ventricle.

Interestingly, several groups have shown that Rb, a major regulator of E2f functioning, is indispensable for neurogenesis[31-33]. Moreover, it has been show that Rb/E2f3 are essential for

neural migration[34] and, in addition, the *Dlx1/Dlx2* bi-gene cluster, essential for neural migration and differentiation, was shown to be repressed by E2f7[35]. These and several other reports show the importance of proper regulation of the E2fs during brain development. Because the zebrafish provides the opportunity to follow brain development in real-time it would be of great interest to investigate the effect of *e2f7/8*, or in general Rb-E2f signaling, in zebrafish brain development.

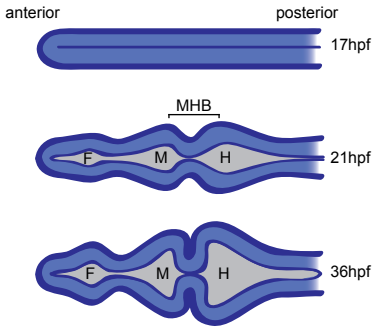


Figure 9 I Schematic representation of zebrafish brain ventricle development. Initially, the brain develops independently of circulation, upto 21 hours post fertilization. Furthermore, brain ventricles are shaped due to inflation by fluid accumulation and localized proliferation. **Abbreviations:** F, forebrain ventricle; M, midbrain ventricle; H, hind brain ventricle; MHB, midbrain-hindbrain boundary.

Adapted from Lowery & Sive 2005[30]

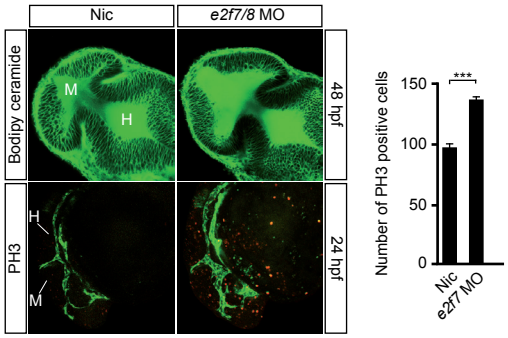


Figure 10 I Live-cell staining of the zebrafish brain with bodipy ceramide. *E2f7/8* morphants show an increased volume of the midbrain ventricle, while the hindbrain ventricle appears similar to wildtype. Phospho-histon 3 (PH3) was used to quantify and image proliferation in the brain. **Abbreviations:** F, forebrain ventricle; M, midbrain ventricle.

E2F7 and E2F8 knock out, a male thing

As shown in Chapter 2, we identified in a zebrafish mutagenesis library generated by targeted induced local lesions in the genome (TILLING; [36]), an e2f7 (*e2f7*^{A207}) and e2f8 (*e2f8*^{A196}) mutant zebrafish. Both mutations resulted in a premature stop codon in the first highly conserved DNA biding domain, which is essential for E2F7/8 functioning[11, 37] (Chapter 2 supplemental figure 1C). In contrast to *E2f7*$^{-/-}$*/8*$^{-/-}$ mice embryos that died between E9.5-10.5[15], zebrafish mutant for both *e2f7/8* (*e2f7*^{A207}*e2f8*^{A196}) were viable and live to old age. A great deal of this difference can be explained by the placental defects occurring in conventional *E2f7/8* knockout mice embryos, which, if supplied with a wildtype placenta, are carried to term[14]. Surprisingly however, the offspring of *e2f7*^{A207}*e2f8*^{A196} zebrafish consists solely out of males (Figure 11). Moreover, one allele of *e2f7* or *e2f8* was already sufficient to induce the "female program" (Figure 11).

The determination whether an individual becomes male or female can occur on the genetic level, genetic sex determination (GSD) or is influenced by environmental cues, environmental sex determination (ESD). Sex determination on the genetic levels mostly requires the presence of a sex chromosome. In mammals, fruit flies and medaka fish the females are homogametic for the X chromosome (XX) and the males are heterogametic (XY). In contrast, female birds, snakes and turbot are heterogametic (ZW) and the males are homogametic (ZZ). In mammals, Sex-determining region Y (Sry), located on the Y chromosome, has been identified as a genetic switch that triggers the initiation of the male pathway in the bipotential gonads[38]. Remarkably, the Sry gene does not exist beyond therian mammals (marsupials and placentals), thereby excluding the hypothesis that Sry is an universal male/female switch for all species. The search of other genetic factors that stimulate the bipotential gonads to become male or female has resulted in the identification of doubblesex and mab-3 related transcription factor 1 (Dmrt1) as a downstream factor in the male sex determination. In a similar fashion as for Sry, the absence of Dmrt1 in several species, also rules out this gene as a master switch for sex determination[39]. Several studies have attempted to identify dimorphic sex chromosomes and a sex determining switch in zebrafish, but as these studies were not successful, it was proposed that zebrafish sex determination is dependent on environmental cues. Indeed, several studies have shown that zebrafish male/female ratio can be influenced by altering the environment, including temperature, breeding density (number of fish per liter), food restriction and a reduced oxygen concentration in the water (hypoxia)[40-43]. However, most of these environmental cues resulted in an (mild) increased male to female ratio, suggesting that genetic sex determination in the zebrafish plays a more important role than environmental sex determination. Due to the increased resolution of the zebrafish genetic map, several recent studies have tried to identify loci that correlate with sex determination. One of these studies used a SNP based linkage map method and identified two sex-associated loci, one on chromosome 5 and one on chromosome 16[44]. Remarkably, the locus on chromosome 5 contained *dmrt1*, as discussed above, a gene associated with sex determination. Moreover, the locus on chromosome 16 contained *cyp21a2*, a gene that encodes for 21-hydroxylase and is involved in the hydroxylation of steroids that in turn influence sex-determination. In contrast with this study, another study that used the F2

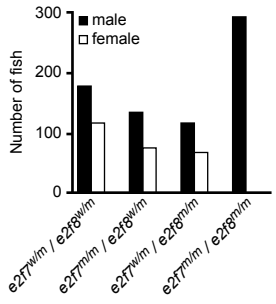


Figure 11 I Loss of *e2f7* and *e2f8* results masculinisation. In Chapter 1 Figure 1I-K we identify and characterize *e2f7* (*e2f7*$^{A07/A207}$) and *e2f8* (*e2f8*$^{A196/A196}$) double mutant zebrafish. Maintaining and breeding these fish revealed that loss of both *e2f7* and *e2f8* results in the development of only male zebrafish, also referred to as masculinisation. **Abbreviations:** m, mutant; w, wildtype.

offspring of Oregon and Nadia wildtype zebrafish found, by genome-wide linkage analysis, a sex-associated locus at the end of the long arm of chromosome 4 and an additional locus in the middle of chromosome 3[45]. Moreover, this study did not find any sex-association with *dmrt1*. Together these results rule out that there is one locus or gene in the zebrafish that determines whether the individual becomes a male or female, but rather suggest that the zebrafish has a polygenic sex determination system in which environmental cues play a minor but influential role.

In general, all developing zebrafish initially form a gonadal primordium that develops as an ovary containing immature oocytes, independent of their definitive sex[46]. The initiation of the "male program" is characterized by the degeneration of these oocytes (20-30 days post fertilization) and the acquisition of a testis morphology. The "female program" is characterized by oocytes that enter the meiosis, a specialized cell cycle program that results in four diploid cells with a high gene shuffling (recombination). Both male and female gametes arise from the primordial germ cells (PGCs), cells that are specified early in development and need to migrate to the somatic part of the gonads, which they populate and where they ultimately give rise to the gametes[47]. Interestingly, depleting the PGCs, among others by injection of the dead end (*dnd*) morpholino, results in the formation of a testis without gametes, thereby creating infertile males[48]. The exact cues that determine whether the oocytes degenerate or enter meiosis are yet poorly understood. However, recently it has been shown that mutations in the Fanconi genes *fancl* or *fancd1*(*brca2*) resulted in only male offspring due to increase PGC apoptosis mediated by Tp53[49]. Fanconi genes mediate homologous recombination DNA repair of double strand breaks and, furthermore, are important for genome stability[50]. Interestingly, some of the Fanconi genes have been identified as E2f7 targets[11]. Additionally, ablation of *E2f7/8* results in E2f1-Tp53 mediated apoptosis[15] and *E2f7/8* upregulation is required for a proper DNA damage repair response [37]. Together these results might suggest that, as in a similar fashion for the Fanconi genes, enhanced apoptosis of the PGCs might be the underlying cause of masculinization of $e2f7^{A207}e2f8^{A196}$ mutant zebrafish. To test this hypothesis, we crossed $e2f7^{A207}e2f8^{A196}$ with $tp53^{M214K}$ mutants, in which the *tp53* gene contains a point mutation that results in an amino acid substitution and consequently fail to induce apoptosis upon DNA damage[51]. Surprisingly,

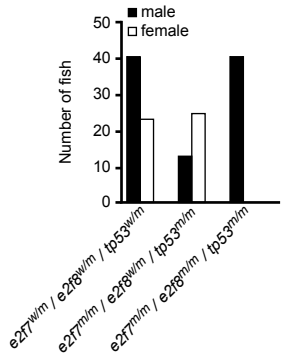


Figure 12 I Additional deletion of *tp53* in *e2f7/8* morphants does not rescue the masculinisation phenotype. Male gonads develop due to degeneration of oocytes, a process driven by apoptosis. As loss of *e2f7/8* leads to increased apoptosis via the E2f1-Tp53 axis, we inhibited apoptosis by mutating *tp53* ($tp53^{M214K/M214K}$). The lack of any female in the triple mutants (*e2f7/8/tp53*) excludes increased degeneration of oocytes as possible cause of the masculinisation phenotype. **Abbreviations:** m, mutant; w, wildtype.

e2f7^{A207}*e2f8*^{A196}*tp53*^{M214K} triple mutant fish are still all of a male sex (Figure 12), indicating that the loss of *e2f7/8* does not lead to increased genome instability/DNA damage that would consequently lead to apoptosis of PGCs. In addition, loss of PGCs leads to infertile males, while *e2f7*^{A207}*e2f8*^{A196} mutants are all fertile, which indicates that there is no defect in the PGCs. Moreover, these results also suggest that during normal development the degeneration of immature oocytes is not mediated by Tp53. As discussed above, environmental cues influence male/female ratio. *E2f7*^{A207}*e2f8*^{A196} mutants are grown under relative variable conditions with a breeding density of about 60 fish per tank 30/50 liter. As a consequence, the male/female ratio is increased towards a male bias. Decreasing the breeding to 20 fish per tank, often results in an increased number of females. However, this approach failed to induce any feminization in *e2f7*^{A207}*e2f8*^{A196} mutants, indicating that the underlying mechanism of masculinization in these mutants is of a genetic cause.

A first step towards unraveling the underlying mechanism, is a pathological examination of the gonads of *e2f7*^{A207}*e2f8*^{A196} mutant males. Currently, sex is determined by appearance, belly size. In case female fish lack the characteristic big belly due to absence of oocyte maturation, these females will consequently be phenotypically seen as males.

Lymphangiogenesis and arterial-venous ratio, a matter of Dll4 levels and Flt4 signaling

How do venous sprouts know whether they need to connect to arterial intersegmental vessels (aISVs) to convert them to veins (vISVs) or migrate further to the horizontal myoseptum (HM) to form the initial lymphatic structures, remains a longstanding question in the field of lymphangiogenesis in zebrafish. In more detail, vascular endothelial growth factor C (VegfC), which is secreted by the dorsal aorta (DA), binds to the vascular endothelial growth factor receptor 3 (Vegfr3; Flt4) expressed by the posterior cardinal vein (PCV), and in lower levels also by DA and intersegmental vessels (ISVs) (Figure 13A). Upon binding of VegfC to Flt4 present on endothelial cells in the PCV, a subset of cells start to bud and migrate from the PCV. These cells can behave in two ways: 1) these cells migrate towards the dorsal aorta (DA) and connect to the aISVs, converting this arterial connection into a venous one (Figure 13B); 2) budding cells from the PCV migrate past the DA and populate the HM as a string of cells and are thereby lymphatic pre-cursors (hereafter referred to as parachordal lymphangioblasts (PLs)) (Figure 13B). It remains unknown whether the budding cells already "know" whether they become part of the vascular or lymphatic system. Recently, it has been shown that the Notch ligand delta-like 4 (Dll4) is able to alter the fate of these budding venous cells. To this extent, the trunk vasculature of *dll4* morphants consists almost for 90% out of veins (Chapter 4 figure 4D). Although it appears that *dll4* morphants also show to some degree a decreased number of cells budding from the PCV, most of these cells seem to be pre-destined to connect to the aISVs[52, 53]. Interestingly, *in situ* hybridization (ISH) shows that *dll4* is exclusively expressed in arterial cells, DA and aISVs (Figure 13A)[52]. This expression pattern suggests that the DA and/or aISVs provide the signal whether venous sprouts connect to aISVs. Off note, expression of *dll4* in venous cells might be

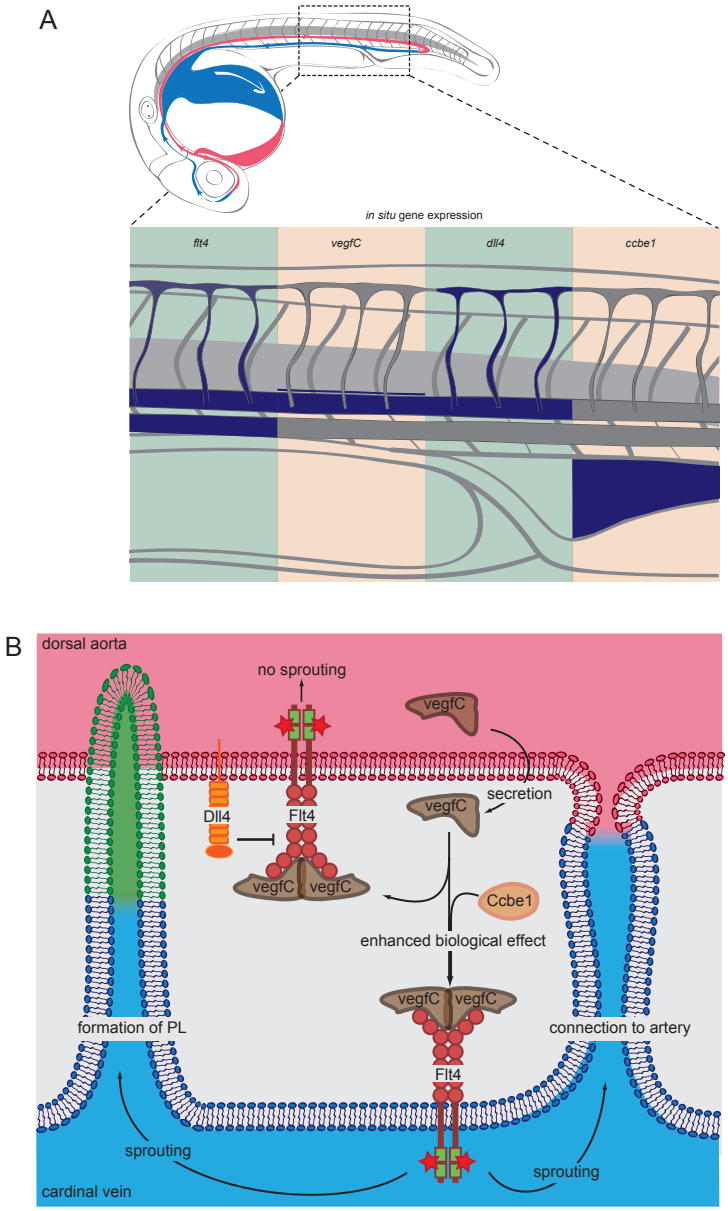


Figure 13 I Expression of lymphangiogenic factors and mechanism of lymphangiogenesis in zebrafish. **A** Tissue specific expression of *flt4*, *vegfC*, *dll4* and *ccbe1* at 24 hours post fertilization. **B** Venous sprouting is induced by VegfC, which is secreted from the dorsal aorta, and its effect on Flt4 is enhanced by Ccbe1. Arterial cells remain quiescent due to the inhibitory effect of Dll4 on Flt4-VegfC signaling. Venous cells can either connect to the dorsal aorta to establish an circulatory loop (blue sprout) or migrate further to differentiate into a parachordal lymphangioblast (PL; green sprout) that contributes to the formation of the lymphatic system.

not detected by ISH as the staining is heavily depending on the duration of staining and areas with low level of expression might be missed.

In Chapter 5 we showed that *e2f7/8* mRNA induces Collagen and Calcium Binding EGF domains 1 (*ccbe1)* expression (Chapter 4, Figure 3). Because it has recently been shown that CCBE1 enhances the biological effect of VEGFC dependent lymphangiogenesis[54] (Figure 13B), we wanted to investigate whether the increased *ccbe1* expression in *dll4* morphants injected with *e2f7/*8 mRNA could influence our analysis. To this extent, we ectopically expressed *ccbe1* mRNA alone, *dll4* mRNA alone or injected these mRNAs in *dll4* morphants. Ectopic expression of *dll4* alone resulted in an increased number of PLs at the HM, whereas the number of veins were decreased (Figure 14A-D). This observation again argues that *dll4* is important for fate determination of cells budding from the PCV. Consistent with previous data that CCBE1 protein promotes VEGFC dependent lymphangiogenesis [54], ectopic expression of *ccbe1* mRNA resulted in more PLs at the HM whereas the arterial-venous ratio was unchanged (Figure 14A-D). This

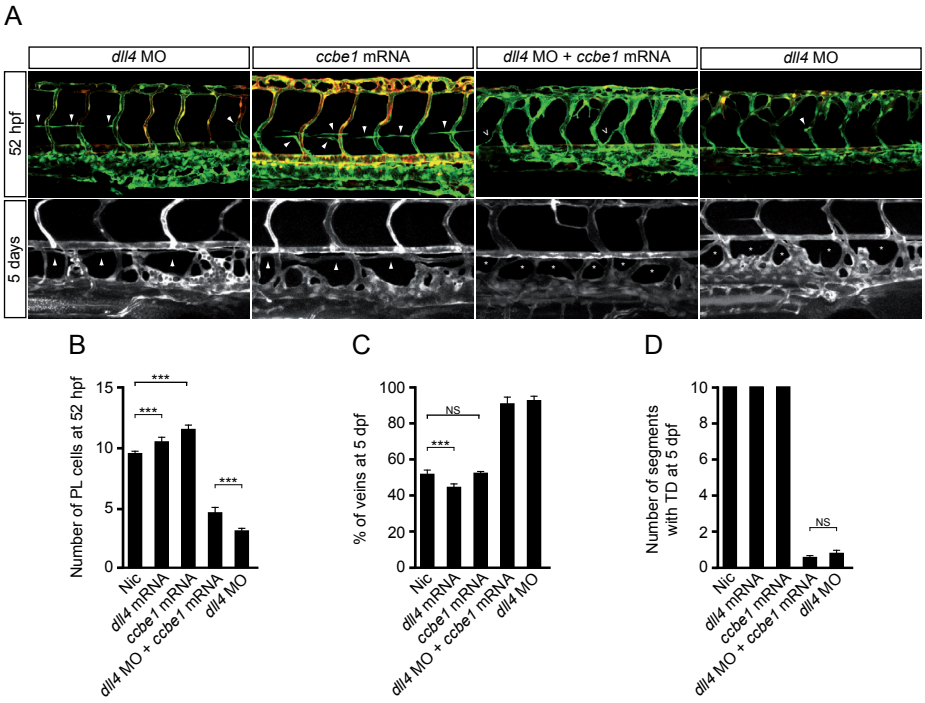


Figure 14 I Ectopic expression of *ccbe1* does not rescue Flt4-dependent lymphangiogenesis phenotypes **A** Representative images of *Tg(fli1a:gfp;flt1*^{enh}*:rfp)* un-injected control embryos (Nic) or embryos injected as indicated. **B**, **C**, **D** Quantification of the indicated parameters. Open arrow heads indicate in (A; upper panel) inter-connecting intersegmental vessels. Closed arrow heads indicate (A; upper panel) PLs or (A; lower panel) presence of the TD. All scale bars are 100 µm. Stars indicate missing thoracic duct fragments. Data presented as the average (±s.e.m.) compared to the control condition in three independent experiments (*** P<0.001).

result suggest that ectopic expression of *ccbe1* enhances the number of cells that bud from the PCV and, moreover, all these "extra" cells migrate towards the HM. Next, we co- injected *ccbe1* mRNA together with *dll4* morpholino oligomer (MO), which showed no improvement of the *dll4* phenotype (Figure 14A-D). However interestingly, it appeared that the "extra" cells induced by ectopic expression of *ccbe1* mRNA all connected to aISV (Figure 14A; upper panel open arrowheads).

Together these results strengthen the hypothesis that Dll4 might be a decisive factor whether venous sprouts connect to aISV or migrate further to the HM to form the initial lymphatic structures. In addition, ISH of *dll4* expression insinuates that this signal is derived from the DA and/or ISVs.

As discussed above, Dll4 plays an important function for the fate of cells that bud from the PCV, which is mediated by VegfC-Flt4 signaling. Moreover, Hogan *et al.* showed that Dll4 suppresses Flt4 signaling without affecting the expression levels of *flt4* or *vegfC*. So rephrasing the results discussed above would suggest that increased Flt4 signaling in *dll4* morphants, due to loss of suppression on Flt4, leads to the connection of budding cells from the PCV to aISVs. As shown in Chapter 4, E2F7/8 repress the expression of FLT4 *in vitro* and *in vivo* in zebrafish. Moreover, we showed that ectopic expression of *e2f7/*8 in *dll4* morphants rescues the *dll4* phenotype, i.e. number of veins, PLs and thoracic duct. We argue that the loss of repression of Flt4 signaling due to ablation of *dll4* was compensated by the increased repression of *flt4* expression. However interestingly, ISH revealed that ectopic expression of *e2f7/*8 also led to a decreased *dll4* expression, whereas *e2f7/8* knock down resulted in an increased *dll4* expression (Figure 15A). Thus, co-injecting *e2f7/*8 mRNA and dll4 MO results in: 1) decreased expression of *flt4* and *dll4* due to *e2f7/8* mRNA; 2) loss of *dll4* expression and repression on Flt4 signaling due to *dll4* MO, the sum of all effects on *dll4, flt4* and Flt4 signaling results a less severe phenotype in these *dll4* morphants (Figure 15B). Additionally, *flt4* is expressed in both venous (PCV) and in a lower dose also in arterial cells (DA and aISVs; Figure 13A), indicating that the "connective signal" might also be present in the budding cells themselves (Figure 13B). However we need to keep in mind that next to its effect on Flt4 signaling, Dll4 also plays a role in transcription and cell behavior, mainly driven by their corresponding Notch receptor[55]. To explore and further support the hypothesis that Flt4 determines the fate of venous sprouts, it might be informative to step-wise decrease *flt4* expression, by injecting *flt4* MO, in *dll4* morphants and analysis the arterial-venous ratio. Alternatively, a soluble Ig domain of human FLT4 that acts as a dominant negative inhibitor of Flt4 signaling by scavenging VegfC might be even more informative as this blocks VegfC signaling without affecting Flt4 expression [52, 56]. Moreover, the generation of a *dll4*-BAC transgenic fish, expressing a fluorescent dye under the control of the *dll4* promoter, might shed more reliable light on the spatio-temporal expression of *dll4*.

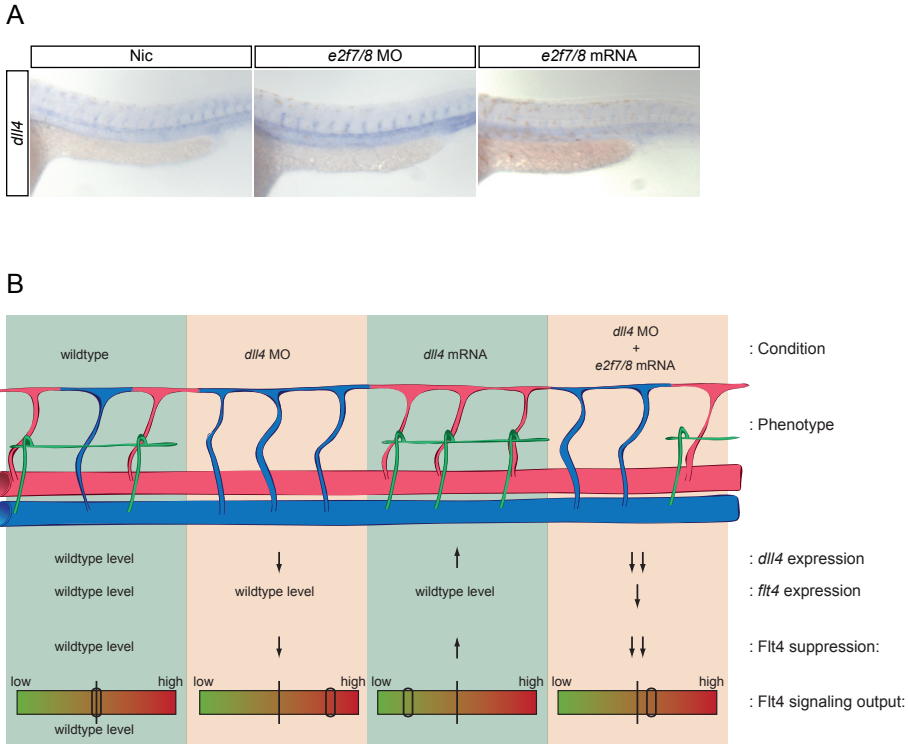


Figure 15 I Flt4 signaling determines the fate of venous sprouts. **A** *In situ* hybridisation for *dll4* in non-injected and *e2f7/8* MO or mRNA injected embryos. **B** Flt4 signaling is regulated on the transcriptional level by E2f7/8 and signaling activity is regulated by Dll4, without affecting *flt4* expression. Schematic representation of the net effect on Flt4 signaling under different conditions. The comparison of the net effect of Flt4 signaling during these different conditions suggests that the amount of Flt4 signaling determines whether a venous sprout becomes part of the circulatory system or the lymphatic system.

Conclusion

Since the discovery of the atypical E2Fs much progression has been made in understanding their function. In this thesis it has become apparent that the functions of E2F7/8 reach beyond cell cycle control. Although we investigated in this thesis the function of the atypical E2Fs in angiogenesis, the data discussed above clearly shows that other process are also regulated by the atypical E2Fs, like brain and reproductive system formation. Moreover, we discussed that the transcriptional effect of E2F7/8 on their targets is dependent on spatio-temporal expression and the ability to form complexes with other proteins. Another important aspect will be the role of the atypical E2Fs within the E2F family itself. In this respect, it has been shown that within the family there are negative and positive feedback loops and moreover individual members can compensate for the loss of other members. Understanding these aspects of atypical E2F biology will be essential to get the full picture how these atypical E2Fs work under different conditions. Because the E2Fs plays such a pivotal role in many basic processes, understanding their behavior will also increase the understanding of these basic processes.

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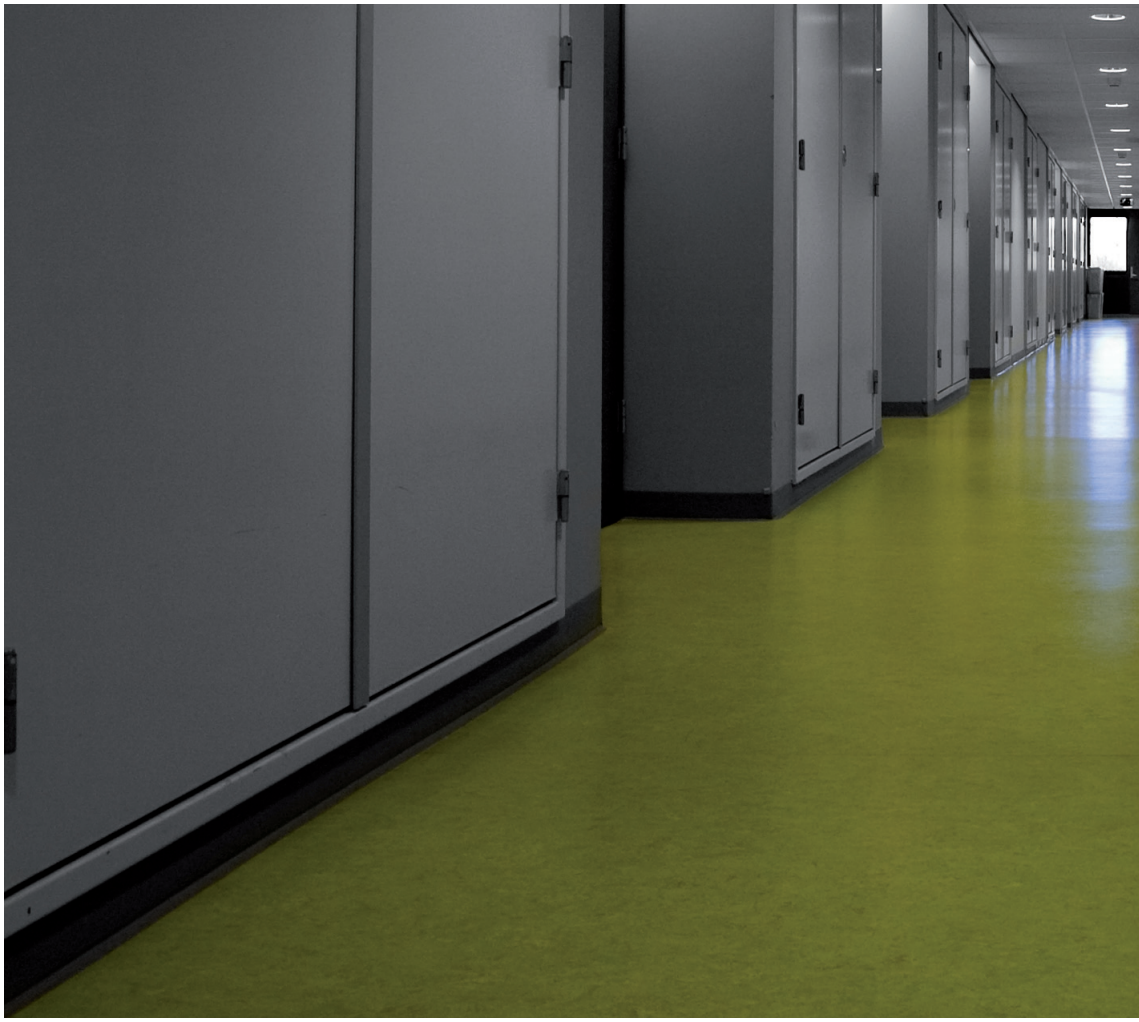

Addendum

SCIENTIFIC ABSTRACT

Proper functioning of the vertebrate body requires a system that efficiently transports oxygen, nutrients, hormones and circulating (immune) cells to all cells, tissues and organs. This task is carried out by two tree-like branched structures: the vascular and lymphatic system, which consists out of endothelial cells. The main axial vessels of the vascular system arise during embryonic development by coalescence and differentiation of endothelial cell progenitors, the angioblasts, in a process referred to as vasculogenesis. To form a branched like structure, endothelial cells from the main axial vessels start to sprout and migrate to form smaller blood vessels in a process called angiogenesis. At a certain developmental stage, endothelial cells specifically derived from the venous system start to sprout and differentiate into lymphatic endothelial cells and in a called lymphangiogenesis, these lymphatic endothelial cells branch into a blunt end system. Understanding how the process of angiogenesis and lymphangiogenesis during development is regulated on the physiological and genetic level is of great importance for developing treatments for diseases depending on or cause by the vascular or lymphatic system. The most well-known disease that depends on both angiogenesis and lymphangiogenesis is cancer, in which an individual cell starts to divide uncontrollably and ultimately forms a tumor. Initially, the tumor can be supplied with nutrients and oxygen by simple diffusion, however when tumor size exceeds the physical properties of simple diffusion, the lack of oxygen induces a genetic program within the tumor cells that ensures secretion of growth factors. These growth factors will induce the formation of blood and lymphatic vessels towards the tumor, thereby ensuring a constant supply of nutrients and oxygen. Moreover, the blood vessels also provide a way for the tumor cells to enter the blood stream and disseminate to distal organs. For this reason, it is of crucial importance to block angiogenesis and lymphangiogenesis during tumor formation. In this thesis we show that the transcription factors E2F7 and E2F8 are novel regulators of angiogenesis and lymphangiogenesis. More specific, we describe how E2F7 and E2F8 regulate vascular endothelial growth factor A (VEGFA), the most important driver behind angiogenesis. Furthermore, we show that E2F7 and E2F8 also regulate VEGFC, a close relative of VEGFA and indispensable for lymphangiogenesis. However, E2F7 and E2F8 regulate VEGFC not directly on the genetic level, but by modulating the expression of its receptor VEGF receptor 3 (FLT4). Moreover, E2F7 and E2F8 enhance the biological effect of VEGFC by regulating the expression of Collagen and Calcium-Binding EGF domains 1 (CCBE1), which has been shown to enhance the VEGFC-FLT4 signaling pathway. In sum, the results in this thesis link the E2F family with angiogenesis and lymphangiogenesis during development and cancer formation. Because E2F7 and E2F8 have been shown to be frequently deregulated in many human cancers, follow up studies should be directed to unravel whether E2F7 and E2F8 regulate the angiogenic potential of these human cancers. Ultimately, these results could contribute to improvement of current cancer therapies.

NEDERLANDSE SAMENVATTING

Ons lichaam bevat vele kilometers aan bloedvaten die ervoor moeten zorgen dat al onze cellen en organen worden voorzien van zuurstof en voedingsstoffen. Om al deze cellen en organen te bereiken vertakt ons bloedvatenstelsel van grote vaten naar steeds kleiner wordende vaten, hierbij wordt er vaak een vergelijking getrokken met het uiterlijk van een boom. In dit geval kun je de stam van de boom vergelijken met de aders, de kleinere takken met arteriolen en de nerven in het blad met de haarvaatjes. Omdat het van cruciaal belang is dat alle cellen kunnen worden voorzien van zuurstof en voedingsstoffen, zal de aanleg van bloedvaten, ook wel angiogenese genoemd, zeer strikt gereguleerd moeten worden. In eerste instantie worden tijdens de vroege ontwikkeling van de embryo de grote aders, waaronder de aorta, aangelegd. Tijdens dit proces clusteren gespecialiseerde cellen, de zogenaamde endotheel cellen, samen tot een lange holle buis. Om deze buis te modelleren tot een vertakt systeem van bloedvaten, beginnen individuele endotheel cellen zich los te maken en uit te groeien tot een vertakking. Dit proces wordt herhaald om een vertakt systeem van bloedvaten te creëren dat alle cellen en organen kan voorzien van zuurstof en voedingsstoffen, welke getransporteerd worden door het bloed.

Het bloed in ons lichaam bestaat uit verschillende componenten waaronder rode bloedcellen, immuun cellen, eiwitten, suikers, mineralen, hormonen en plasma. Tijdens het transport door de bloedvaten kunnen cellen, moleculen (suikers, hormonen, etc.) en plasma uit de bloedbaan treden om door de omliggende cellen opgenomen te worden. Gedeeltelijk wordt de uitgetreden vloeistof ook weer geabsorbeerd door de bloedvaten, maar het is niet in staat om alle uitgetreden vloeistof weer terug op te nemen, terwijl dit wel noodzakelijk is voor het correct functioneren van ons lichaam. Om de resterende vloeistof op te kunnen nemen en daarbij de vloeistofbalans op peil te houden, is er een tweede systeem van buizen aangelegd in ons lichaam, de lymfevaten. Deze lymfevaten ontstaan tijdens de embryonale ontwikkeling van de veneuze bloedvaten. Op een bepaald moment in de ontwikkeling zijn er endotheel cellen in de veneuze bloedvaten die een teken krijgen dat ze zich moeten losmaken van het bloedvat en verder moeten specialiseren tot lymf endotheel cellen. De lymf endotheel cellen maken op ongeveer dezelfde wijzen als bloedvaten een vertakt netwerk van lymfevaten. Naast het reguleren van de vocht balans, heeft het lymfe stelsel ook een belangrijke rol bij het correct functioneren van het immuunsysteem.

Zoals eerder aangegeven is het aanleggen van bloed- en lymfevaten een nauwkeurig gereguleerd proces, waarbij communicatie tussen cellen van groot belang is. Zo zullen cellen die niet genoeg zuurstof of voedingsstoffen krijgen signalen afgeven, zogenaamde groei factoren, die worden waargenomen door de endotheel cellen in de bloedvaten. In respons groeien de endotheel cellen in de richting van het signaal en op deze manier worden de cellen met een tekort aan zuurstof en voedingsstoffen voorzien van deze essentiële stoffen. Het afgeven van groei factoren wordt onder andere gereguleerd door transcriptie factoren. Transcriptie factoren kunnen in de cel de aanmaak en afgifte van groeifactoren AAN of UIT zetten en functioneren als

een zogenaamde AAN/UIT knop.

Transcriptie factoren worden op basis van hun uiterlijk of functie ingedeeld in families. In dit proefschrift hebben we gekeken naar de E2F-familie van transcriptie factoren, welke bestaat uit acht familieleden, E2F1 tot en met E2F8, waarvan E2F7 en E2F8 het meest recent zijn ontdekt. Doordat E2F7 en E2F8 qua uiterlijk niet erg veel lijken op de rest van de familieleden worden ze ook wel atypisch genoemd. De E2F-familie staat er om bekend vooral instructies te lezen in het DNA die bepalen of een cel zich mag vermenigvuldigen of dat de cel dit juist niet mag doen. Zo heeft men laten zien dat het wegnemen van E2F7 en E2F8 in een cel ertoe leidt dat een cel de instructies van niet delen verliest en zich juist gaat vermenigvuldigen. Een eerdere studie waarbij E2F7 en E2F8 werd weggenomen tijdens de ontwikkeling van muizen embryo's, liet zien dat deze embryo's vroegtijdig in de baarmoeder stierven door onder meer defecten aan de placenta en in de embryo. Waarbij de bloedvaten in de navelstreng sterk vergroot waren en er in de embryo op verschillende plekken bloedingen te zien waren. Gezien de toen bekende functie van E2F7 en E2F8 waren deze bloedvat defecten onverwacht en leidde deze resultaten tot de hoofd vraagstelling van dit proefschrift; zijn E2f7 en E2f8 betrokken bij de formatie van bloedvaten.

In dit proefschrift hebben we daarom gekeken of en hoe E2F7 en E2F8 de formatie van bloedvaten reguleren. Zoals eerder aangegeven, sterven muizen zonder E2F7 en E2F8 zeer vroeg tijdens de ontwikkeling in de baarmoeder. Mede hierdoor is het technisch zeer moeilijk om een gedetailleerde analyse van de bloedvaten te doen en om deze reden hebben we gekozen om te kijken naar de formatie van bloedvaten in zebravissen (*Danio rerio*). Deze 10 cm lange vissen komen origineel voor in de Himalaya regio en zijn populaire aquarium vissen. Omdat de zebravis makkelijk te houden is en zich zeer snel en in grote getalen voortplant is de zebravis de laatste jaren erg in trek als model voor verschillende wetenschappelijke studies. De zebravis is, net als de mens en de muis, een vertebraat (gewerveld) organisme waardoor veel processen zoals bloed- en lymfevat aanleg vergelijkbaar verlopen. Tijdens de embryonale ontwikkeling van de zebravis, welke zich buiten de baarmoeder afspeelt, is de embryo geheel transparant. Deze transparantie blijkt ontzettend goed van pas te komen bij het onderzoek naar de aanleg van de bloed- en lymfevaten. Door een artificiële aanpassing in het DNA van de zebravis, is het onderzoekers gelukt de bloed- en lymfevaten van de zebravis embryo te laten oplichten onder de microscoop waardoor de aanleg van bloed- en lymfevaten zeer gedetailleerd bestudeerd kan worden.

Zoals eerder beschreven, kunnen individuele endotheel cellen in een bloedvat een nieuwe vertakking aanleggen of zich verder specialiseren tot cellen die de lymfevaten vormen. Eén van de belangrijkste signalen die deze endotheel cellen kan aanzetten tot het maken van een nieuwe vertakking of zich verder te specialiseren tot lymfendotheel cel is afkomstig van een familie van groei factoren genaamd de Vasculaire Endotheliale GroeiFactor (VEGF). Van deze familie is de A-vorm (VEGFA) onmisbaar voor bloedvat aanleg en de C-vorm (VEGFC) onmisbaar voor lymfevat aanleg. VEGFA en VEGFC zijn voorbeelden van groei factoren die worden uitgescheiden door cellen die niet voldoende bevoorraad worden met zuurstof of voedingsstoffen. In dit

proefschrift laten we zien dat de transcriptie factoren E2F7 en E2F8 VEGFA reguleren in cellen die te weinig zuurstof krijgen. Wanneer we E2F7 en E2F8 weg halen uit de cel, is deze niet goed meer instaat om bloedvaten aan te trekken bij een te kort aan zuurstof. Opmerkelijk blijken E2F7 en E2F8 niet alleen de bloedvat formatie te reguleren, maar ook de formatie van lymfevaten. Verrassend vonden we dat E2F7 en E2F8 niet de directe AAN/UIT knop waren voor VEGFC, zoals we dat wel vonden voor VEGFA. In het geval van VEGFC, zetten E2F7 en E2F8 factoren AAN die de activiteit van VEGFC beïnvloeden en daarbij het potentieel van VEGFC om lymfevaten aan te maken. Samengevat, laten wij in dit proefschrift zien dat wanneer E2F7 en E2F8 wordt weggehaald uit de cel tijdens de embryonale ontwikkeling, VEGFA en VEGFC niet goed meer gereguleerd worden. Als consequentie ontvangen de bloed- en lymfevaten niet meer genoeg signalen om vertakkingen te maken. Uiteindelijk leidt dit ertoe dat bepaalde delen van de embryo niet genoeg zuurstof en voedingsstoffen meer krijgen en afsterven. Deze bevinding laat een nieuw licht schijnen niet alleen op E2F7 en E2F8, maar op de hele E2F-familie. Lang werd de E2F-familie vooral bestudeerd in het proces van celdeling, maar de bevindingen in dit proefschrift laten een hele nieuwe kant van de familie zien waardoor het interessant wordt om de E2F familie verder te bestuderen in processen en ziektes die veroorzaakt worden door of afhankelijk zijn van bloed- en lymfevaten formatie.

Zoals eerder genoemd, is de E2F familie vooral bekend om hun regulatie en controle van de celdeling. Het verlies van controle over de celdeling leidt in vele gevallen tot wildgroei van deze cellen, ook wel kanker genoemd. In eerste instantie verliest één cel de controle over de celdeling, wat kan gebeuren doordat een cel constant wordt bloot gesteld aan stress zoals, UV straling, chemicaliën of rook van tabak. Hierdoor kunnen er veranderingen (mutaties) in het DNA plaats vinden. Deze mutaties zorgen ervoor dat de AAN/UIT functie van transcriptie factoren niet goed meer functioneerd. Het is bekend dat bepaalde mutaties leidt tot het ongecontroleerd AAN zetten van allerlei factoren door de E2F familie, wat er uiteindelijk toe leidt dat de cel ongecontroleerd gaat delen en er kanker ontstaat. In eerste instantie zal de wildgroei aan cellen leiden tot een klein klompje cellen, ook wel tumor genoemd, welke verder geen problemen veroorzaak op de plek waar het zich bevindt. Naarmate de tumorcellen zich vermeerderen en de tumor verder groeit, zal ook de vraag naar zuurstof en voedingsstoffen door deze tumorcellen flink toenemen. De tumor zal blijven doorgroeien totdat er een gebrek aan zuurstof ontstaat, waardoor deze groeifactoren gaat uitscheiden, zoals VEGFA en VEGFC. Het uitscheiden van deze factoren leidt ertoe dat bloedvaten richting de tumor groeien. Het aantrekken van bloedvaten door de tumorcellen verzekerd hun niet alleen van zuurstof en voedingsstoffen, maar biedt ook de mogelijkheid om zich los te maken van de tumor en via de bloedbaan door het hele lichaam te verspreiden. Een belangrijke strategie in het bestrijden van kanker is het verhinderen van deze bloedvat formatie, wat leidt tot "uithongering" en uiteindelijk afsterven van de tumorcellen. Hiertoe worden patiënten onder andere behandeld met middelen die VEGF blokkeren.

Onze bevinding dat E2F7 en E2F8 de bloedvat formatie reguleren tijdens de embryonale ontwikkeling, samen met het hier boven genoemde feit dat de E2F familie een belangrijke rol

spelen in de formatie van kanker, deed ons speculeren of E2F7 en E2F8 ook een rol zouden kunnen spelen in de formatie van bloedvaten die naar de tumor groeien. Alhoewel we het precieze mechanisme nog niet hebben kunnen achter halen, lijkt het er wel op te duiden dat E2F7 en E2F8 ook de formatie van bloedvaten reguleerd gedurende de formatie van tumoren.

Samenvattend laten we in dit proefschrift zien dat de transcriptie factoren E2F7 en E2F8 nieuwe spelers zijn in de regulatie van bloed- en lymfevat formatie. We laten zien dat dit gebeurt tijdens de embryonale ontwikkeling, maar ook tijdens de formatie van tumoren. Het begrijpen hoe bloedvaten worden aangelegd tijdens de embryonale ontwikkeling en tijdens ziekte processen zoals kanker is van cruciaal belang om nieuwe strategieën en therapieën te ontwikkelen voor ziektes en aandoeningen die gerelateerd zijn aan de bloedvaten. De resultaten beschreven in dit proefschrift kunnen bijdragen aan het ontwikkelen van nieuwe of verbeteren van bestaande therapieën in de strijd om kanker.

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If everybody had an ocean
Across the U.S.A.
Then everybody'd be surfin'
Like californ-I-A
You'd see 'em wearin' their baggies
Huarachi sandals, too
A bushy bushy blonde hairdo
Surfin' U.S.A.

You'd catch **me** surfin at **Del Mar**

Beach Boys – Surfin U.S.A.

CURRICULUM VITAE

Bart Weijts was born on January 30th, 1979 in Sittard. In 2001 he started his study Biology at the Utrecht University, the Netherlands. As part of his study he worked on a 9 months project at the department of sport medicine, Utrecht Medical Centre, under supervision of Dr. Sandor Schmikli and Dr. Maria Zonderland. During this period he investigated the overtraining syndrome in endurance athletes. He also completed a 9 month laboratory project in the lab of Prof. Dr. Leon de Windt under supervision of Dr. Meriem Bourajjaj. In this project he studied the effect of Nuclear Factor of Activated T-cells 2 (NFATc2) during cardiac hypertrophy and heart failure. After receiving his Master's degree, he started his PhD training in the lab of Prof. Dr. Alain de Bruin at the Veterinary Medicine faculty at the Utrecht University. During this period he mainly focused on the role of E2F7 and E2F8 during angiogenesis, lymphangiogenesis and tumorangiogenesis. The results obtained during this period are presented in this thesis.

LIST OF PUBLICATIONS

B. Weijts, A. van Impel, S. Schulte-Merker, A de Bruin. E2f7 and E2f8 regulate Flt4 and Ccbe1 during lymphangiogenesis. *Submitted*

S. Choorapoikayil*, B. Weijts*, A. de Bruin, J. den Hertog. Loss of Pten promotes angiogenesis and enhanced *vegfAa* expression in zebrafish. Disease Models and Mechanisms (*accepted)*

D. Mans*, J. Vermaat*, B. Weijts, E. van Rooijen, J. van Reeuwijk, K. Boldt, L. Daenen, P. van der Groep, B. Rowland, R. Roepman, E. Voest, P. van Diest, M. Verhaar, A. de Bruin, R. Giles. Regulation of E2F1 by the von Hippel-Lindau tumor suppressor protein predicts survival in renal cell cancer patients. *Submitted*

W. Bakker, B. Weijts, B. Westendorp, A. de Bruin. HIF proteins connect the RB-E2F factors to angiogenesis. Transcription. 2013; 4(2):1-5

B. Weijts*, W. Bakker*, P. Cornelissen, K. Liang, F. Schaftenaar, B. Westendorp, C. de Wolf, M. Paciejewska, C. Scheele, G. Leone, S. Schulte-Merker, A. de Bruin. E2F7 and E2F8 promote angiogenesis through transcriptional activation of VEGFA in cooperation with HIF1. EMBO Journal. 2012; 31(19):3871-84

M. Bourajja, A. Armand, P. da Costa Martins, B. Weijts, R. van der Nagel, S. Heeneman, X. Wehrens, L. de Windt. NFATc2 is a necessary mediator of calcineurin-dependent cardiac hypertrophy and heart failure. J Biol Chem. 2008; 283(32):22295-303

** equal contribution*

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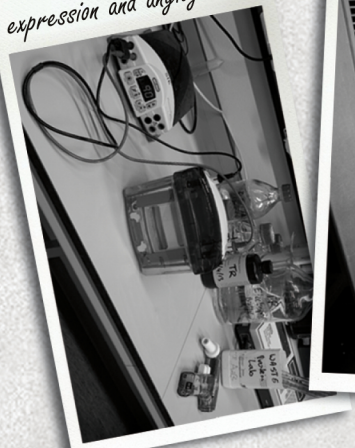
Pten promotes vegfAa expression and angiogenesis



Atypical E2Fs repress tumorangiogenesis

Atypical E2Fs regulate flt4 and ccbe1 in lymphangiogenesis

HIF connects E2FS and Rb to angiogenesis

Atypical E2Fs cooperate with HIF during angiogenesis