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The transcription factor E2F1 and the SR protein SC35 control the ratio between pro- versus anti-angiogenic isoforms of VEGF-A to inhibit neovascularisation "in vivo"

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Running title: E2F1 controls VEGF-A alternative splicing

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Abstract

The transcription factor E2F1 plays a crucial role in the control of cell growth and has been shown to regulate neoangiogenesis in a p53-dependent manner through inhibition of the VEGF-A promoter activity. Besides transcription, VEGF-A is also highly regulated by pre-mRNA alternative splicing, allowing the expression of several VEGF isoforms with either pro-(VEGF_{xxx}) or anti-(VEGF_{xxx}b) angiogenic properties. Recently, we identified the SR protein SC35, a splicing factor, as a new transcriptional target of E2F1. Here, we demonstrate that E2F1, but not a mutant E2F1 unable to bind DNA, downregulates VEGF-A promoter activity in tumor cells independently of p53, leading to a strong decrease of VEGF_{xxx} mRNA levels. Strikingly, we further show that E2F1 alters the ratio between pro-VEGF_{xxx} versus anti-VEGF_{xxx}b angiogenic isoforms in favor of the anti-angiogenic ones, by a mechanism involving induction of SC35 expression. Finally, using lung tumor xenografts in nude mice, we provide evidence that E2F1 and SC35 increase the VEGF₁₆₅b/VEGF ratio as well as decrease tumor neovascularisation "in vivo". Overall, these findings highlight E2F1 and SC35 as two regulators of the VEGF_{xxx}/VEGF_{xxx}b angiogenic switch in human cancer cells, a role that could be crucial during tumor progression as well in tumor response to anti-angiogenic therapies.

Key Words: Alternative splicing, Angiogenesis, Cancer, E2F1, SC35, VEGF-A.

Introduction

Angiogenesis occurs physiologically during reproduction, embryonic development and wound repair. Furthermore, it also contributes to several pathological processes including arthritis, atherosclerosis, diabetes and cancer (Carmeliet and Jain, 2000; Folkman, 2007). Angiogenesis is stimulated by the expression of vascular growth factors, the most angiogenic of which is VEGF-A (Vascular Endothelial Growth Factor) which receptors (VEGF-R1 and VEGF-R2) are located on endothelial cells. In tumors, VEGF-A is upregulated by hypoxia (Shweiki *et al.*, 1992) or by overexpression of oncogenes (Rak *et al.*, 1995), and stimulates the proliferation and the migration of endothelial cells, the sprouting of blood vessels and the generation of new vessels resulting in sustained blood flow, oxygen supply and waste removal of the growing tumors (Ferrara *et al.*, 2003). In addition, VEGF-A regulates the function and physiology of some cancer cells themselves, by acting as an autocrine survival factor (Bachelder *et al.*, 2001; Bachelder *et al.*, 2002). The importance of VEGF-A in cancer is attested by the use of anti-VEGF antibodies that have demonstrated clinical benefits in colorectal, renal, breast or non small cell lung cancer (Hurwitz *et al.*, 2004 ; Hurwitz *et al.*, 2005).

VEGF-A exists as multiple isoforms generically referred to as VEGF_{xxx} which result from the pre-mRNA alternative splicing of eight exons (Robinson and Stringer, 2001). Alternative splicing of VEGF-A was initially shown to generate four different isoforms having respectively 121, 165, 189 and 206 amino acids (VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, VEGF₂₀₆). In 2002, a sister family of VEGF-A isoforms termed VEGF_{xxx}b has been identified (Bates *et al.*, 2002 ; Ladomery *et al.*, 2007). VEGF_{xxx}b isoforms are generated by the use of an alternative 3' splice acceptor site in the final exon 8 of VEGF-A, leading to the synthesis of proteins of

the same length as the VEGF_{xxx} isoforms but with distinct C-terminal six amino acids (Bates *et al.*, 2002). At the biological level, VEGF_{xxx} isoforms are pro-angiogenic and up-regulated in human tumors, whereas VEGF_{xxx}b isoforms are anti-angiogenic and downregulated in some tumors (Bates *et al.*, 2002 ; Pritchard-Jones *et al.*, 2007; Woolard *et al.*, 2004). It has been shown that VEGF₁₆₅b binds VEGFR-2 and inhibits its downstream classical signalling pathway (Kawamura *et al.*, 2008; Woolard *et al.*, 2004). Moreover, VEGF₁₆₅b overexpression inhibits the growth of prostate carcinoma, Ewing's sarcoma and renal cell carcinoma in xenografted mouse tumor models through inhibition of tumor-cell-mediated migration and proliferation of endothelial cells (Rennel *et al.*, 2008). In addition, VEGF₁₆₅b binds and inhibits Bevacizumab (Avastin^R), a humanised monoclonal antibody directed against VEGF-A that is currently used in clinic (Varey *et al.*, 2008). As a whole, these data indicate that the balance between pro-(VEGF_{xxx}) versus anti-(VEGF_{xxx}b) angiogenic splice variants of VEGF-A plays a critical role in both tumor progression and tumor cells response to anti-angiogenic therapies. However, while there has been some investigation of splicing mechanisms in epithelial cells (Cohen *et al.*, 2005 ; Nowak *et al.*, 2008; Nowak *et al.*, 2010), almost nothing is known about the proteins and signalling networks that control the splicing of VEGF-A pre-mRNA in cancer cells.

The transcription factor E2F1 belongs to the E2F family that comprises eight members. Initially, studies revealed that E2F1 determines the timely expression of many genes required for S phase entry and progression. However, it has become clear that transcriptional activation of S-phase-related genes is only a facet of E2F1 activity. Indeed, E2F1 functions in a wide range of biological processes including DNA replication, mitosis, DNA-damage checkpoints, DNA repair, differentiation and apoptosis (Polager and Ginsberg, 2008). In addition, E2F1 down-regulates VEGF promoter activity under hypoxic conditions via a p53-dependent

transcriptional mechanism, unraveling an unexpected function of E2F1 in the control of angiogenesis (Qin *et al.*, 2006). Recently, we established the first link between E2F1 and pre-mRNA processing events by identifying SC35, a member of the SR proteins family involved in constitutive and alternative pre-mRNA splicing, as a direct transcriptional target of E2F1 (Merdzhanova *et al.*, 2008). Based on these results, we questioned here whether E2F1 and/or SC35 proteins could control VEGF-A expression and/or pre-mRNA splicing in p53-deficient tumor cells.

Results

E2F1 down-regulates the expression of pro-angiogenic VEGF_{xxx} isoforms independently of p53

In a murine system, it has been previously demonstrated that E2F1 decreases VEGF protein level in a p53-dependent manner (Qin *et al.*, 2006). To test whether E2F1 could also control VEGF expression in human tumor cells deficient for p53, we took advantage of previously established stable E2F1-inducible clones in the H358 cell line derived from a human lung adenocarcinoma. In this system, E2F1 or an E2F1(E132) DNA-binding defective mutant is overexpressed upon doxycyclin treatment (Figure 1A, upper panel). As detected using RT-PCR analysis, a significant decrease of *VEGF*₁₈₉, *VEGF*₁₆₅ and *VEGF*₁₂₁ mRNA levels was found in cells overexpressing E2F1 (Figure 1A, middle panel). This effect required the DNA binding activity of E2F1 as it was not observed with the mutant E2F1(E132). Importantly, a decrease of both *VEGF*₁₆₅ and *VEGF*₁₈₉ mRNA levels was also observed in cells overexpressing E2F1 when RT-PCR analysis was performed using a couple of primers targeting exon 7 and exon 8a of *VEGF-A* that did not detect VEGF_{xxx}b isoforms (Bates *et al.*, 2006; Pritchard-Jones *et al.*, 2007; Varey *et al.*, 2008) (Figure 1A, lower panel). Therefore,

these data indicated that E2F1 down-regulates the expression level of pro-angiogenic VEGF_{xxx} transcripts. In order to test whether this effect was specific for VEGF_{xxx} transcripts, we analyzed the expression level of neuropilin-1, the co-receptor of VEGFR-2 recently identified as a new E2F1 transcriptional target gene (Jiang *et al.*, 2007). *neuropilin-1* mRNA level slightly increased upon E2F1 induction in these cells, while the expression of neuropilin-2 was not affected (Figure 1A, lower panel). These data indicated that E2F1 specifically decreases the expression of VEGF_{xxx} mRNAs. To confirm these results, we performed RT-qPCR experiments using primers detecting either all or each VEGF isoforms (Table 1) (Catena *et al.*, 2007). We found that *VEGF*₁₂₁, *VEGF*₁₆₅ and *VEGF*₁₈₉ mRNA levels significantly decreased upon E2F1 induction, whereas this was not the case with the mutant E2F1(E132) (Figure 1B). To extend these results at the protein level, we quantified the expression of VEGF in supernatants or in total cellular extracts from E2F1- or E2F1(E132)-overexpressing cells using ELISA assay or western blotting, respectively. We observed that wild-type E2F1 significantly decreased VEGF level both in culture medium (Figure 1C) and cell extracts (Figure 1D), as compared to mutant E2F1(E132) which did not have any effect. To confirm these results, we knocked-down E2F1 by using a pool of two distinct siRNAs in the H69 cell line that expresses high levels of E2F1 and a deficient p53. In these cells, the silencing of E2F1 (Figure 2A, left panel) was accompanied by a significant increase of VEGF₁₂₁, VEGF₁₆₅ and VEGF₁₈₉ mRNA levels as detected by using RT-qPCR (Figure 2A, right panel). These data were confirmed by ELISA assay and western blotting. Indeed, we found that neutralization of E2F1 increased VEGF level in culture supernatants (Figure 2B), as well as in cellular extracts (Figure 2C). Overall, these data demonstrate that E2F1 negatively controls VEGF_{xxx} mRNA and protein levels in tumor cells independently of p53 and in normoxic conditions.

E2F1 inhibits VEGF promoter activity in a p53-independent manner

Based on our data demonstrating that E2F1 affects the expression of VEGF_{xxx} isoforms independently of p53, we then questioned whether it could act by controlling VEGF-A promoter activity using a 2.4-kb VEGF promoter-luciferase construct (pVp-ecor; Figure 3A) (Zhang *et al.*, 2000). H1299 cells were transiently co-transfected with pVp-ecor and increasing amounts of plasmid encoding E2F1, and luciferase activity was measured 48 hours later. As shown in Figure 3B (left panel), E2F1 strongly inhibited VEGF promoter activity in a dose dependent manner, whereas it did not affect the activity of a control pGL3-luc vector. Similar results were obtained in another cellular model, the SAOS2 cells (Figure 3B, right panel). This effect of E2F1 on the *Vegf* promoter was direct. Indeed, by performing chromatin immunoprecipitation (ChIP) experiments in the H358/Tet-On/E2F1 cells cultured in the presence or absence of doxycyclin, we further provided evidence that E2F1 binds to the *Vegf* promoter whereas no binding was observed with the *Gapdh* promoter (Supplementary Figure S1). In order to confirm the effects of E2F1, we analyzed the consequences of its knock-down on VEGF promoter activity in the same cells co-transfected with either pVp-ecor or pGL3-luc and small interfering RNAs targeting E2F1. In both cellular models, the neutralization of E2F1 significantly increased VEGF promoter activity as compared to cells transfected with *mismatch* siRNA, without modifying pGL3-luc promoter activity (Figure 3C). Taken together, these results demonstrate that E2F1 can repress VEGF promoter activity independently of p53 and in normoxic conditions.

E2F1 upregulates the expression of anti-angiogenic VEGF_{xxx}b splice variants

Having demonstrated that E2F1 downregulates the expression of pro-angiogenic VEGF_{xxx} splice isoforms, we next examined whether anti-angiogenic VEGF_{xxx}b isoforms could be also affected. To do this, we performed RT-PCR experiments using intron-spanning primers

(Table 1) that detect VEGF₁₆₅b only, even in the presence of 1000x greater concentration of VEGF₁₆₅ mRNA (Bates *et al.*, 2002 ; Bates *et al.*, 2006 ; Pritchard-Jones *et al.*, 2007 ; Rennel *et al.*, 2008; Woolard *et al.*, 2004). Using primers complementary to exons 4 and 8b, we were able to amplify a fragment with a size consistent with VEGF₁₆₅b transcript (Figure 4B, right panel). We observed that expression of VEGF₁₆₅b transcript increased in response to E2F1 induction, a phenomenon which was not observed with the mutant E2F1(E132). Similar results were obtained using another set of primers complementary to exon 7 and exon 7/8b that allows the detection of VEGF₁₆₅b mRNA only (Figure 4B) (Pritchard-Jones *et al.*, 2007). Therefore, while E2F1 decreases VEGF_{xxx} mRNA and protein levels, it seems to affect the expression of VEGF_{xxx}b isoforms in an opposite way. To confirm these results, we designed primers to perform isoform-specific quantitative PCR (q-PCR) of VEGF₁₂₁b, VEGF₁₆₅b and VEGF₁₈₉b splice variants (Table 1 and Figure 4A). Using these specific sets of primer, we showed that E2F1 significantly increased both VEGF₁₆₅b and VEGF₁₈₉b mRNAs while it did not affect the expression level of VEGF₁₂₁b mRNA in our model (Figure 4C, left panel). Strikingly, in the same conditions, induction of mutant E2F1(E132) significantly decreased the expression of all three VEGF₁₂₁b, VEGF₁₆₅b and VEGF₁₈₉b variants (Figure 4C, right panel). Since a dominant negative effect of E2F1(E132) has been previously reported (Davis *et al.*, 2006), these results suggest that the binding of E2F1 to DNA is required for the expression of VEGF_{xxx}b splice variants. In order to confirm these results at the protein level, we performed western blot experiments using an anti-VEGF_{xxx}b antibody (Woolard *et al.*, 2004). The results showed that VEGF₁₆₅b protein level increased when expression of E2F1 was induced by doxycyclin treatment in H358 cells (Figure 4D, left panel). In these cells, we were not able to detect the VEGF₁₂₁b protein. By contrast, the knock-down of E2F1 in H69 cells correlated with a significant decrease of both VEGF₁₆₅b and VEGF₁₂₁b proteins as compared to *mismatch* transfected cells (Figure 4D, right panels). Overall, these results

demonstrate that E2F1 upregulates the expression of VEGF_{xxx}b variants. Therefore, E2F1 controls the switch between VEGF_{xxx}/VEGF_{xxx}b splice variants and favors the expression of the anti-angiogenic VEGF_{xxx}b isoforms.

The splicing factor SC35 is required for E2F1-induced VEGF₁₆₅b upregulation

We recently identified the splicing factor SC35, a member of the SR proteins family, as a direct transcriptional target of E2F1 (Merdzhanova *et al.*, 2008). These data led us to postulate that SC35 could be involved in the control of the VEGF_{xxx}b/VEGF ratio by E2F1. To test this hypothesis, two distinct siRNAs targeting *sc35* were transfected into H358/Tet-On/E2F1 cells cultured in the presence or absence of doxycyclin, and expression of VEGF_{xxx} and VEGF₁₆₅b splice variants was analyzed by RT-PCR (Figure 5A) and western blot (Figure 5B). In both experiments, the knock-down of SC35 strongly prevented the increase of VEGF₁₆₅b induced by E2F1 (Figures 5A&5B). Of note, we observed that the neutralization of SC35 "*per se*" decreased VEGF_{xxx} mRNA levels, while it did not significantly affect that of VEGF₁₆₅b. In order to mimic the induction of SC35 detected in E2F1-overexpressing cells, we then established SC35 stable inducible clones derived from H358/Tet-On cells. As shown in Figure 5C, induction of SC35 expression by doxycyclin strongly increased the VEGF₁₆₅b/VEGF ratio, indicating that SC35 accumulation recapitulates E2F1 overexpression. Taken together, these results demonstrate that SC35 is required for E2F1-mediated VEGF₁₆₅b upregulation in H358 human lung carcinoma cells and suggest that both E2F1 and SC35 proteins cooperate to favor the expression of VEGF_{xxx}b isoforms.

E2F1 and SC35 inhibit neovascularisation "in vivo" by modulating the VEGF_{xxx}b/VEGF ratio

Since we demonstrated that E2F1 affects the VEGF_{xxx}b/VEGF ratio "in vitro", we finally wondered whether this effect could be observed "in vivo". H358/Tet-On 4 (Tet-On 4 ; control cells) and H358/Tet-On/E2F1-6 (E2F1-6) cells were sub-cutaneously injected into immunodeficient nude mice and induction of E2F1 expression was obtained by adding doxycyclin (Dox +) in drinking water. In order to eliminate the potential effects of E2F1 induction on apoptosis and to focus our analysis on early events such as neoangiogenesis, mice were sacrificed 7 days after doxycyclin drinking. Tumors were weighed and total proteins were then extracted from whole tumoral samples. We observed that induction of E2F1 (E2F1-6, Dox +) diminished significantly the weight of the tumors ($p= 0.0362$), a phenomenon that was not observed in Tet-On 4 control tumors receiving doxycyclin (Tet-On 4, Dox +, Figure 6A). We also noticed that tumors overexpressing E2F1 (E2F1-6, Dox +) were less haemorrhagic than those derived from control cells (Tet-On 4, Dox +), as well as from E2F1-6 cells in which expression of E2F1 was not induced (E2F1-6, Dox -) (Figure 6B, upper panel). Consistent with this, the hemoglobin levels were significantly decreased upon E2F1 induction (E2F1-6, Dox +), as compared to uninduced E2F1-6 tumors (E2F1-6, Dox -) (Figure 6B lower panel ; $p= 0.007$). This effect was specific since doxycyclin did not affect hemoglobin level in tumors derived from Tet-On 4 control cells. Such inhibitory effect of E2F1 on tumor neovascularisation was further confirmed using CD31 immunostaining of murine endothelial cells. As shown in Figure 6C (upper and lower panels), tumors overexpressing E2F1 displayed significantly less infiltrating murine vessels, as compared to all other conditions ($p=0.0044$). In addition, murine stroma surrounding tumors appeared also less vascularised in E2F1-overexpressing tumors as compared to all other conditions (data not shown). Altogether, these results demonstrate that E2F1 prevents neovascularisation. To analyze

whether this could be linked to modifications of the VEGF_{xxx}b/VEGF ratio, we performed western blot analysis on tumor extracts using either anti-pan-VEGF or anti-VEGF_{xxx}b antibody. As illustrated, the expression of both VEGF and VEGF₁₆₅b isoforms remained relatively constant in control Tet-On 4 xenografts either in the presence (Dox +) or absence (Dox -) of doxycyclin (Figure 6D, upper panel). In contrast, overexpressing E2F1 significantly increased the VEGF₁₆₅b/VEGF ratio (Figure 6D, lower panel). To assess the role of SC35 in this setting, similar experiments were performed using the H358/Tet-On/SC35 cells. As shown in Figure 6E, tumor xenografts overexpressing SC35 (Dox+) displayed a higher amount of VEGF₁₆₅b protein (Figure 6E, left panel) and a lower amount of CD31-positive infiltrating murine endothelial cells (Figure 6E, right panel and Supplementary Figure S2), as compared to uninduced tumors. Of note, pan-VEGF protein level was not significantly affected in tumors overexpressing SC35. Taken together, these results demonstrate that E2F1 and SC35 proteins can modulate "in vivo" the ratio between the pro- and anti-angiogenic isoforms of VEGF in favor of the anti-angiogenic ones, leading to the inhibition of tumor neovascularisation.

Discussion

The transcription factor E2F1 was first identified as a crucial regulator of cell cycle progression. However, based on the growing number of studies published this last decade, it is now clear that E2F1 plays numerous other biological roles. Recently, a new function for E2F1 in a negative control of angiogenesis has been proposed, through the demonstration that mice deficient in E2F1 exhibit enhanced angiogenesis as a result of VEGF overproduction (Qin *et al.*, 2006). In this model, increased angiogenesis occurs during both recovery from ischemic injury and tumor growth and is dependent of wild-type p53. In this study, we show that E2F1

negatively affects VEGF-A promoter activity in p53-deficient tumor cell lines and in normoxic conditions, indicating that neither hypoxia nor p53 are required for E2F1-mediated VEGF repression in cancer cells. Interestingly, during the writing of this manuscript, it was also demonstrated that E2F1 cooperates with mutant p53-R175H to promote angiogenesis through the induction of pro-angiogenic soluble mediators (Fontemaggi *et al.*, 2009). Therefore, these and our data suggest that E2F1 could control angiogenesis in an opposite way depending on the status of p53.

An increasing amount of evidence now supports the fact that the balance between VEGF_{xxx} (pro-angiogenic) and VEGF_{xxx}b (anti-angiogenic) splice variants plays a crucial role in the control of tumor progression, as well as in the response of tumor cells to anti-VEGF therapies (Bates *et al.*, 2002 ; Rennel *et al.*, 2008 ; Varey *et al.*, 2008; Woolard *et al.*, 2004). Recently, it has been demonstrated that VEGF splicing is controlled by the SR proteins, ASF/SF2, SRp40 and SRp55 in primary epithelial cells (Nowak *et al.*, 2008; Nowak *et al.*, 2010). To date, nothing is known about the factors that control the switch between VEGF_{xxx} and VEGF_{xxx}b splice variants in cancer cells. In this study, we provide evidence that E2F1 controls the angiogenic switch between pro- and anti-angiogenic isoforms of VEGF and favors the expression of the anti-angiogenic ones in human lung carcinoma cell lines. What could be the molecular mechanisms involved in this process? We provide evidence that E2F1 decreases the activity of the VEGF-A promoter. Therefore, one possibility is that E2F1 could specifically affect the transcription of pro-angiogenic isoforms but not that of anti-angiogenic because of the use of a different promoter region. Another possibility is that E2F1 down-regulates the transcription of both pro- and anti-angiogenic VEGF isoforms but favors the use of the distal splice site selection within exon 8 inside pre-mRNA, thereby generating more anti-angiogenic isoforms. In favor of such hypothesis, we provide evidence that the SR

protein SC35 is absolutely required for VEGF₁₆₅b accumulation in response to E2F1, and show that overexpression of SC35 mimicks the positive effects of E2F1 on VEGF₁₆₅b as well as decreases total VEGF amount. Of note, SC35 did not down-regulate the activity of the VEGF-A promoter in our cellular models (data not shown). Therefore, these data highly suggest that the positive effects of E2F1 on the VEGF_{xxx}b/VEGF balance mainly reflect a splicing event through SC35 upregulation and the preferential choice of the distal splice site inside *VEGF-A* pre-mRNA. Of note, the role of SC35 might be dependent on its global amount since its knock-down in H358 cells (Figure 5A) but also in other carcinoma cell lines (data not shown) was actually found to decrease rather than increase VEGF amount. Interestingly, we also observed that E2F1 strongly increases VEGF₁₆₅b and VEGF₁₈₉b mRNA levels in H358 Tet-On/E2F1 cells, without affecting that of VEGF₁₂₁b. Therefore, besides its effects on splicing, we cannot exclude that E2F1 also regulates VEGF_{xxx}b mRNAs stability. This effect could be dependent on the cell type since we observed a strong decrease of VEGF₁₂₁b protein level in H69 cells upon E2F1 knock-down, while we never detected an accumulation of VEGF₁₂₁b protein in H358 cells overexpressing E2F1 (Figure 4D). Overall, these results suggest another level of complexity in the regulation of VEGF-A splicing pattern by E2F1 and SR proteins in human cancer cells, that could be dependent on the cell type, on the upstream signals, as well as on the total amount of various SR proteins.

Finally, using lung xenografts in nude mice, we demonstrate that inhibition of tumor growth by E2F1 correlates with a decrease in tumor neovascularisation, as well as with a significant increase of the VEGF₁₆₅b/VEGF balance "*in vivo*". Moreover, we provide evidence that tumors overexpressing SC35 also express higher amount of VEGF₁₆₅b protein and lower neovasculation as compared to control tumors. Therefore, as it was recently demonstrated that overexpressing VEGF₁₆₅b reduces the growth of renal carcinoma cells xenografts even in the

presence of VEGF₁₆₅ (Rennel *et al.*, 2008), these and our data highly suggest that the positive effect of E2F1 and SC35 proteins on VEGF_{165b} is involved in the reduction of tumor neovascularisation. E2F1-deficient mice exhibit an impaired apoptotic function, which was previously presumed to explain the increased incidence of cancer in these mice. The results presented here strongly suggests that this could also result from an increase in the vascular supply of tumors secondary to dysregulated VEGF pre-mRNA splicing. Therefore, besides its ability to control the alternative splicing of apoptotic regulators during apoptosis (Merdzhanova *et al.*, 2008), E2F1 also has the capacity to regulate VEGF-A pre-mRNA alternative splicing to modulate neo-angiogenesis. These data add further evidence that the regulation of pre-mRNA processing events is a key function of E2F1 that could contribute to its seemingly contradictory roles as both an oncogene and a tumor suppressor.

Material and Methods

Cell lines, plasmids, transfection and luciferase assays

H358, H1299, H69 and SAOS2 cell lines were cultured as previously described (Salon *et al.*, 2007). All these cell lines are defective for wild-type p53. The human lung adenocarcinoma H358/Tet-On/E2F1, Tet-On/E2F1(E132), Tet-On/SC35 inducible clones were obtained as previously described (Salon *et al.*, 2006). E2F1, E2F1(E132) or SC35 expression was induced upon 1 μ g/ml doxycyclin treatment. Transient transfections were carried out using Fugene 6 (Roche Diagnostic). Plasmids used in transient transfections were pcDNA3, pCMV-E2F1, pGL3-promoter vector and pVp-ecor encoding luciferase under the control of the whole human VEGF promoter [-2362 to +956 relative to the transcription start site (Zhang *et al.*, 2000)]. Luciferase assays were performed as previously described (Salon *et al.*, 2007).

ELISA assays

For the quantitative determination of human VEGF concentrations in cell culture supernatants, 3×10^5 cells were seeded in 6-wells plate and incubated for 24 or 48 hours before supernatants recovery. The determination of VEGF concentrations was performed by ELISA assay using Quantikine VEGF immunoassay (DVE00) from R&D Systems. Of note, this assay has been shown to measure all VEGF isoforms, although VEGF_{165b} at a lower efficiency according to Varey and colleagues (Varey *et al.*, 2008).

Antibodies and immunoblotting experiments

Antibodies used in this study were anti-panVEGF (MAB293) and anti-VEGF_{165b} (MAB3045) from R&D Systems, anti-panVEGF(A-20), anti-E2F1 (C-20) and anti-actin from Santa Cruz, anti-E2F1 (KH95) and anti-mouse CD31 from BD Biosciences, anti-SC35(4F11) from Euromedex. Immunoblotting experiments were performed as previously described (Salon *et al.*, 2006).

Transfection of siRNA oligonucleotides

The sequences designed to specifically target human *e2f1* RNA were as follow: (1): 5'-GUCACGCUAUGAGACCUCATT-3'; (2): 5'-ACAAGGCCCGAUCGAUGUUTT-3'. The sequences designed to specifically target human *sc35* RNAs were as follow: *sc35*(1): 5'-GCGUCUUCGAGAAGUACGGTT-3'; *sc35*(2): 5'-UCGUUCGCUUUCACGACAATT-3'. The scrambled siRNA oligonucleotides used as control for all RNA interference experiments were as follow: 5'-UCGGCUCUUACGCAUUCAATT-3' and 5'-CAAGAAAGGCCAGUCCAAGTT-3'. Cells were transfected with siRNA oligonucleotides duplex using Oligofectamine reagent (Invitrogen). Doxycyclin (1 μ g/ml) was added or not in

the culture medium 4 hours after transfection. The cells were analyzed 48 or 72 hours post-transfection.

RT/PCR and RT-qPCR analyses

All the primers used in this study for detection of VEGF_{xxx}, VEGF_{xxx}b, neuropilin-1 and neuropilin-2 mRNAs using either RT-PCR (Bates *et al.*, 2006; Cheung *et al.*, 1998; Lantuejoul *et al.*, 2003; Pritchard-Jones *et al.*, 2007) or RT-qPCR (Catena *et al.*, 2007; Elias and Dias, 2008) are detailed in Table 1. For RT-PCR analysis, total cellular RNAs were isolated using Trizol reagent (Invitrogen). In all conditions, 1 μ g of total RNA was reversed transcribed using oligo(dT) primer and MMLV reverse transcriptase (Invitrogen). For neuropilin-1 detection, the PCR conditions were 94°C for 2 min, 34 cycles (94°C for 45 s, 60°C for 1 min and 72°C for 1 min) and 72°C for 10 min. For neuropilin-2 detection, the PCR conditions were 95°C for 2 min followed by 39 cycles (94°C for 15 sec, 50°C for 30 sec, 72°C for 30 sec) and 72°C for 7 min. For VEGF_{xxx} amplification, the PCR conditions (Cheung *et al.*, 1998) were as follow: 94°C for 2 min, 29 cycles (94°C for 45 sec, 60°C for 1 min, 72°C for 1 min) and 72°C for 10 min. The detection of VEGF_{xxx} transcripts using an exon 7 forward primer and a reverse specific primer for exon 8a that did not detect VEGF_{xxx}b transcripts was performed as previously described (Pritchard-Jones *et al.*, 2007). To detect the anti-angiogenic VEGF_{xxx}b splice variants, PCR conditions were 94°C for 2 min, 35 cycles [(96°C for 30 sec, 65°C for 30 sec, 72°C for 1 min) or (96°C for 30 sec, 55°C for 30 sec, 72°C for 1 min)] for exon4/exon8b or exon7a/exon8b primer set, respectively, and 72°C for 10 min. Amplification of a cDNA fragment of G3PDH (Invitrogen) was performed in the same PCR reaction as an internal control. PCR products were run on a 2-3% agarose gel and visualized by ethidium bromide staining.

Quantitative real-time RT-PCR was performed on Stratagene MX3005P apparatus. 1 μ g of total RNA were subjected to cDNA synthesis with Superscript III First-Strand Synthesis SuperMix for qPCR (Invitrogen) and subsequently amplified during 40 PCR cycles (10 min at 95°C, cycles: 15 s at 95°C, 1 min at 60°C) using Power SYBR Green PCR Master Mix (Applied Biosystems). In parallel, RT-PCR detecting the reference gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was performed for each sample. Relative gene expression was calculated for each sample, as the ratio of target gene copy number to GAPDH mRNA copy number multiplied by 100, thus normalizing VEGF_{xxx} and VEGF_{xxx}b splice variants mRNA expression for sample to sample differences in RNA input. PCR efficiency was assessed for each set of primers and ranged from 106% to 110%.

"In vivo" studies

All animals experiments were conducted in agreement with the "Principles of laboratory animal care" (NIH publication n°86-23, revised 1985) and approved by a regional ethical committee onto femelle athymic Swiss nude mice purchased from Janvier (Le Genest-Isle, France) at 6-8 weeks of age. In all experiments, 20x10⁶ H358/Tet-On/E2F1, H358/Tet-On/SC35 or H358/Tet-On4 cells were injected subcutaneously into nude mice. One week after injection, the mice were randomized into two groups (7 mice per group), one receiving doxycyclin (2 mg/ml) in their drinking water and the second one receiving water only. 7 days later, mice were anesthetized and sacrificed by cervical dislocation, the tumors were extracted, weighed and divided before freezing. For western blot analysis, tumors were homogenized in RIPA buffer containing proteases and phosphatases inhibitors and immunoblotting was performed on the supernatants as previously described. The extent of vascularisation in tumors samples was assessed in RIPA extracts by measuring the amount of hemoglobin using the Drabkin's reagent (Sigma). Results were normalized relative to protein amount in each

sample. For CD31 immunodetection, frozen sections were fixed in 3,7% PFA and immunostained with a rat anti-mouse CD31 (PECAM-1) using a three-steps indirect immunoperoxydase technic.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary information is available at Oncogene's website.

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Legends of figures

Figure 1: Overexpression of E2F1 down-regulates VEGF_{xxx} isoforms in p53-deficient cells.

(A, B, C, D) H358/Tet-On/E2F1 or H358/Tet-On/E2F1(E132) cells were incubated for 48 hours in the presence (+) or absence (-) of 1 μ g/ml doxycyclin (Dox). (A) *Upper panel*: western blot analysis of E2F1 protein expression. Actin was used as a loading control. *Middle panel*: RT-PCR analysis of *Vegf₁₈₉*, *Vegf₁₆₅*, *Vegf₁₂₁*, *neuropilin 1* and *neuropilin 2* mRNAs. Amplified *g3pdh* was used as an internal control. *Lower panel*: RT-PCR analysis of pro-angiogenic VEGF_{xxx} isoforms using a forward primer targeting exon 7 and a reverse primer targeting exon 8a that detects specifically VEGF_{xxx} but not VEGF_{xxx}b transcripts. Amplified *g3pdh* was used as an internal control. L: ladder. (B) RT-qPCR analysis of *Vegf₁₂₁*, *Vegf₁₆₅*, *Vegf₁₈₉* and *Vegf_{all}* mRNA levels. Glyceraldehyde-3-Phosphate Dehydrogenase (*gapdh*) was used as a reference. Relative gene expression was calculated for each sample, as the ratio of *Vegf₁₂₁*, *Vegf₁₆₅*, *Vegf₁₈₉* or *Vegf_{all}* copy number (target gene) to *gapdh* mRNA copy number multiplied by 100, thus normalizing *Vegf_{xxx}* mRNAs expression for sample to sample differences in RNA input. For each splice variant, the ratio obtained in cells cultured without doxycyclin was arbitrarily assigned to 1. (C) Quantification of VEGF protein level in culture supernatants using ELISA assay. (D) Western blot analysis of E2F1 and pan-VEGF protein expression. Actin was used as a loading control.

Figure 2: Silencing of E2F1 upregulates VEGF_{xxx} splice variants.

H69 cells were transfected for 72 hours with either *mismatch* or *e2f1* siRNA. (A) *Left panel*: E2F1 protein level was detected by immunoblotting. Actin was used as a loading control. *Right panel*: RT-qPCR analysis of *Vegf₁₂₁*, *Vegf₁₆₅*, *Vegf₁₈₉* and *Vegf_{all}* mRNA levels.

Glyceraldehyde-3-Phosphate Deshydrogenase (*gapdh*) was used as a reference. Relative gene expression was calculated for each sample as above (Figure 1B). For each splice variant, the ratio obtained in cells transfected with *mismatch* siRNA was arbitrarily assigned to 1. **(B)** VEGF protein level was quantified in the culture supernatants using ELISA assay. **(C)** E2F1 and pan-VEGF protein levels were determined in cellular protein extracts by immunoblotting. Actin was used as a loading control.

Figure 3: E2F1 represses VEGF promoter activity independently of p53.

(A) Schematic representation of VEGF promoter-luciferase reporter pVp-ecor containing the upstream regulatory sequences and promoter region of the human *Vegf* gene (-2362 to +956 relative to the transcription start site) fused to the *luciferase* reporter gene in pGL3-basic vector. The HIF-1-, E2F1- and SP1- cis-binding elements are labeled. **(B)** H1299 (left panel) and SAOS2 (right panel) cells were co-transfected for 48 hours with 1 μ g of pVp-ecor or pGL3-luciferase plasmid and increasing amounts of a pCMV-E2F1 vector. The luciferase activity obtained in cells transfected with pVp-ecor or pGL3-luciferase plasmid alone was normalized to 1 and a relative luciferase activity was then calculated for each condition. **(C)** H1299 (left panel) and SAOS2 (right panel) cells were co-transfected for 72 hours either with mismatch (*mis*) or a pool of two distinct *e2f1* (*E2F1*) siRNA and 1 μ g pVp-ecor or pGL3-luciferase plasmid as indicated. The luciferase activity obtained in cells transfected with pVp-ecor or pGL3-luciferase vector was normalized to 1 and a relative luciferase activity was then calculated for each condition.

Figure 4: E2F1 increases the expression level of VEGF_{xxx}b variants.

Exon structure of the *VEGF* gene. The *VEGF* gene consists of eight exons. Differential terminal exon 8 splice site selection results in two mRNA isoform families. The pro-

angiogenic isoforms (VEGF_{xxx}, left) are generated by proximal splice site (PSS) selection in exon 8 and the anti-angiogenic family (VEGF_{xxx}b, right) from exon 8 distal splice site (DSS) choice. The set of primers used to analyze by RT-qPCR the expression level of Vegf₁₂₁b, Vegf₁₆₅b and Vegf₁₈₉b mRNAs are schematized by arrows. **(B, C, D)** H358/Tet-On/E2F1 or H358/Tet-On/E2F1(E132) cells were incubated for 48 hours in the presence (+) or absence (-) of 1 µg/ml doxycyclin (Dox). **(B) Left panel:** western blot analysis of E2F1 or E2F1(E132) protein expression. Actin was used as a loading control. *Right panel:* RT-PCR analysis of Vegf_{xxx}b, neuropilin-1 or neuropilin-2 mRNAs. Amplified *g3pdh* was used as an internal control. **(C)** RT-qPCR analysis of Vegf₁₂₁b, Vegf₁₆₅b and Vegf₁₈₉b mRNA levels in H358/Tet-On/E2F1 (left panel) or H358/Tet-On/E2F1(E132) (right panel) cells. Glyceraldehyde-3-Phosphate Dehydrogenase (*gapdh*) was used as a reference. Relative gene expression was calculated for each sample as previously described (Figure 1B). For each splice variant, the ratio obtained in cells cultured without doxycyclin (black bars) was arbitrarily assigned to 1. **(D) Left panel:** Western blot analysis of E2F1 and VEGF_{xxx}b proteins expression in H358/Tet-On E2F1. Actin was used as a loading control. *Right panels:* H69 cells were transfected for 72 hours with either *mismatch* or *E2F1* siRNAs and E2F1 and VEGF_{xxx}b protein levels were studied by immunoblotting. Actin was used as a loading control.

Figure 5: SC35 is required for E2F1-mediated VEGF₁₆₅b upregulation.

(A, B) H358 Tet-On/E2F1 cells cultured in the presence (+) or absence (-) of doxycyclin were transfected for 72h with either *mismatch* (*mis*) or *sc35* (*sc35*) siRNA. **(A)** RT-PCR analysis of Vegf₁₈₉, Vegf₁₆₅, Vegf₁₂₁, and Vegf₁₆₅b mRNAs. Amplified *g3pdh* was used as an internal control. **(B) Upper panel:** Western blot analysis of the indicated proteins using specific antibodies. Actin was used as a loading control. *Lower panel:* the VEGF₁₆₅b/VEGF ratio was calculated after densitometric analysis of specific signals using image J software and

normalization to actin signal. The value 1 was arbitrarily assigned to the ratio obtained in non induced cells transfected with *mismatch* siRNA. (C) *Upper panel*: H358 Tet-On/SC35 cells were cultured in the presence (+) or absence(-) of 1 μ g/ml doxycyclin for 24 hours. Western blot analysis of SC35, panVEGF and VEGF₁₆₅b protein expression. Actin was used as a loading control. *Lower panel*: the VEGF₁₆₅b/VEGF ratio was calculated after densitometric analysis of specific signals using image J software and normalization to actin signal. The value 1 was arbitrarily assigned to the ratio obtained in non induced cells.

Figure 6: E2F1 and SC35 proteins decrease tumor neovascularisation in nude mice.

(A, B, C, D) H358/Tet-On 4 (Tet-On 4) or H358/Tet-On/E2F1 (E2F1-6) cells were subcutaneously injected into nude mice. 7 days later, animals were treated with (Dox +) or without (Dox -) 2mg/ml doxycyclin for one week before sacrifice. (A) Tet-On 4 and E2F1-6 tumors weight. Bars represent the mean tumor weight in each condition. For each condition, the mean value for tumor weight (mg \pm SD) is indicated in brackets. NS: not significant; * $p < 0.05$ using a non parametric Mann-Whitney statistical test. (B) *Upper panel*: Tumors derived from mice injected with either Tet-On 4 or E2F1-6 cells and receiving (Dox+) or not (Dox-) doxycyclin in their drinking water. Notice that (E2F1-6, Dox +) tumors were less haemorrhagic than (E2F1-6, Dox -) tumors. *Lower panel*: Hemoglobin level (mg/ml) was quantified in tumors derived from Tet-On 4 or E2F1-6 cells in mice receiving (Dox +) or not (Dox -) doxycyclin. Bars represent the mean level in each case. NS: not significant ; ** $p < 0.01$ using a non parametric Mann-Whitney statistical test. (C) *Upper panel*: Murine CD31 immunohistochemical staining in Tet-On 4 or E2F1-6 xenografts. Overexpression of E2F1 (E2F1-6, Dox +) decreases the number of blood vessels per unit area, as compared to uninduced tumors. *Lower panel*: Quantification of microvascular density using CD31 staining. Seven tumors per treatment were examined. Each point represents the mean of 4

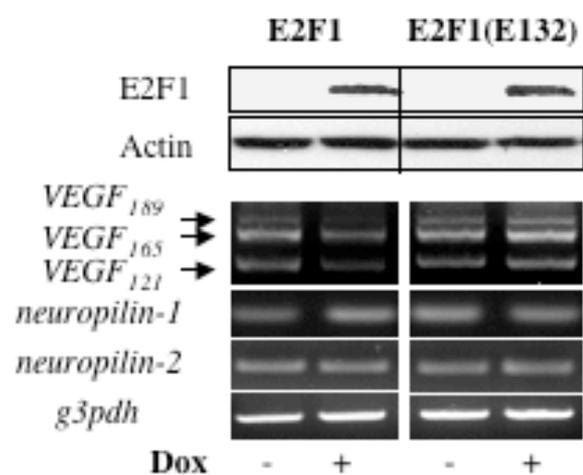
random analyzed fields. NS: not significant ; ** $p < 0.01$ using a non parametric Mann-Whitney statistical test. **(D)** Western blot analysis of E2F1, pan-VEGF and VEGF₁₆₅b protein levels in total protein extracts obtained from tumors derived from Tet-On 4 or E2F1-6 cells and treated (Dox +) or not (Dox -) with 2mg/ml doxycyclin, as indicated. Actin was used as a loading control. For tumors derived from E2F1-6 cells, histograms represent the VEGF₁₆₅b/VEGF ratio calculated after densitometric analysis of each specific signal using image J software normalized to that of actin. * $p < 0.05$ using a non parametric Mann-Whitney statistical test. **(E)** *Left panel:* western blot analysis of the indicated proteins in total protein extracts obtained from tumors derived from SC35-5 cells and treated (Dox +) or not (Dox-) with 2 mg/ml doxycyclin. Actin was used as a loading control. *Right panel:* Quantification of microvascular density using CD31 staining. Six tumors per treatment were examined. Each point represents the mean of 4 random analyzed fields. * $p < 0.05$ using a non parametric Mann-Whitney statistical test.

Merdzhanova Table 1

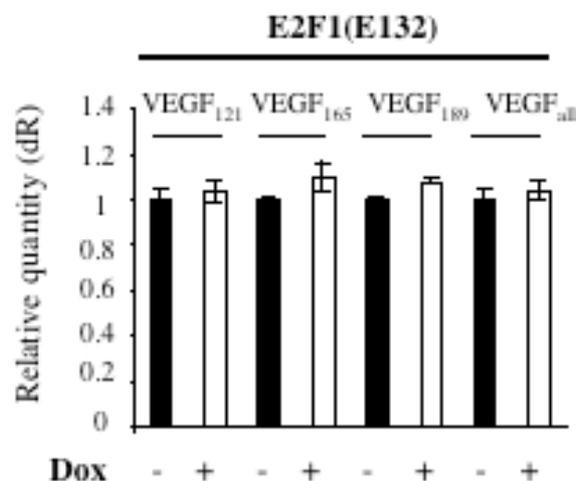
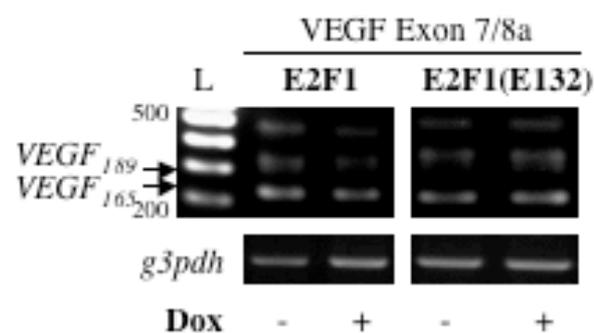
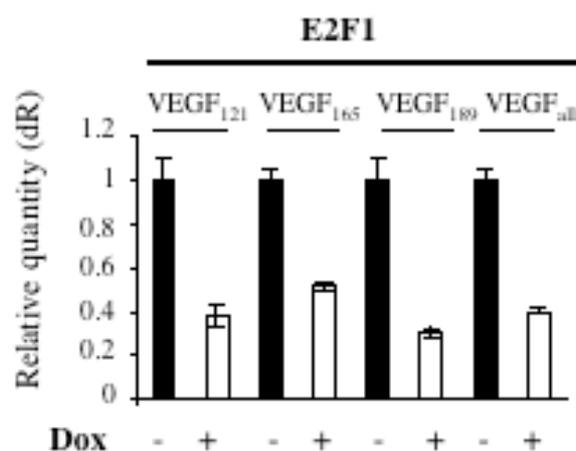
Table 1 : Primers used for detection of VEGF_{xxx} and VEGF_{xxx}b splice variants and neuropilin 1 or 2 mRNA by using RT-PCR or RT-qPCR.

Detection of VEGF_{xxx} by RT-PCR	
VEGF forward	5'-AGCTACTGCCATCCAATCGC-3'
VEGF reverse	5'-GGGCGAATCCAATTCCAAGAG-3'
VEGF exon 7 forward	5'-GTAAGCTTGTACAAGATCCGCAGACG-3'
VEGF exon 8a reverse	5'-TCACCGCCTCGGCTTGTACAT-3'
Detection of neuropilins NP1 and NP2 by RT-PCR	
NP1 forward	5'-ATCACGTGCAGCTCAAGTGG-3'
NP1 reverse	5'-TCATGCAGTGGGCAGAGTTC-3'
NP2 forward	5'-GGATGGCATTCCACATGTTG-3'
NP2 reverse	5'- ACCAGGTAGTAACGCGCAGAG-3'
Detection of VEGF_{xxx} by RT-qPCR)	
VEGF ₁₂₁ forward	5'-ATAGAGCAAGACAAGAAAAATG-5'
VEGF ₁₂₁ reverse	5'-ATCGTTCTGTATCAGTCTTTCCT-3'
VEG ₁₆₅ forward	5'-AGAGCAAGACAAGAAAAATCC-3'
VEGF ₁₆₅ reverse	5'-TACAAACAAATGCTTTTCTCC-3'
VEGF ₁₈₉ forward	5'-TATAAGTCCTGGAGCGTTC-3'
VEGF ₁₈₉ reverse	5'-TACACGTCTGCGGATCTTG-3'
VEGF _{all} forward	5'-CTTCCTACAGCACAAACAAT-3'
VEGF _{all} reverse	5'-GTCTTGCTCTATCTTTCTTTGG-3'
Detection of VEGF_{xxx}b by RT-PCR	
VEGF-exon4 forward	5'- GAGATGAGCTTCCTACAGCAC-3'
VEGF-exon8b reverse	5'-TCAGTCTTTCCTGGTGAGAGATCTGCA-3'
VEGF-exon7 forward	5'-GTAAGCTTGTACAAGATCCGCAGACG-3'
VEGF-exon8b reverse	5'-TCAGTCTTTCCTGGTGAGAGATCTGCA-3'
Detection of VEGF_{xxx}b mRNAs by RT-qPCR	
VEGF ₁₂₁ b forward	5'-AAGGCCAGCACATAGGAGAG-3'
VEGF ₁₂₁ b reverse	5'-TTCCTGGTGAGAGATTTTCTTG-3'
VEGF ₁₆₅ b forward	5'-AAGAAAATCCCTGTGGGCCTT-3'
VEGF ₁₆₅ b reverse	5'-TGGTGAGAGATCTGCAAGTACGTT-3'
VEGF ₁₈₉ b forward	5'-CTGGAGCGTTCCTGTGG-3'
VEGF ₁₈₉ b reverse	5'-TGGTGAGAGATCTGCAAGTACGTT-3'

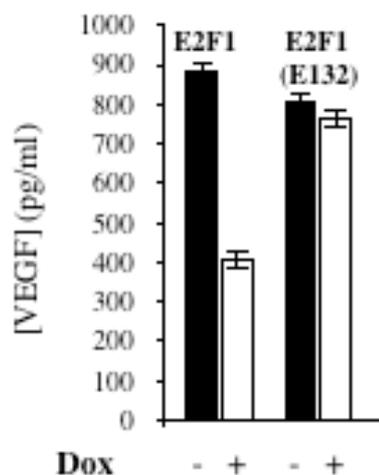
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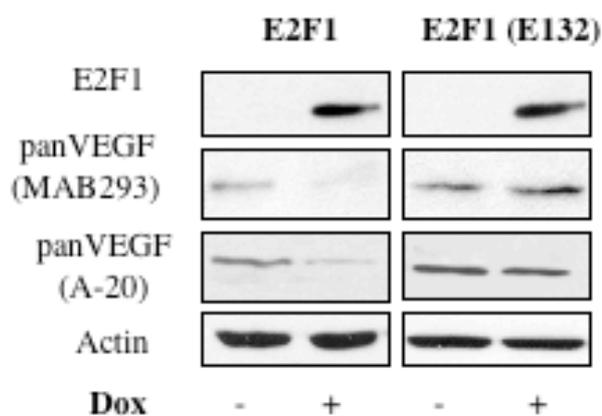
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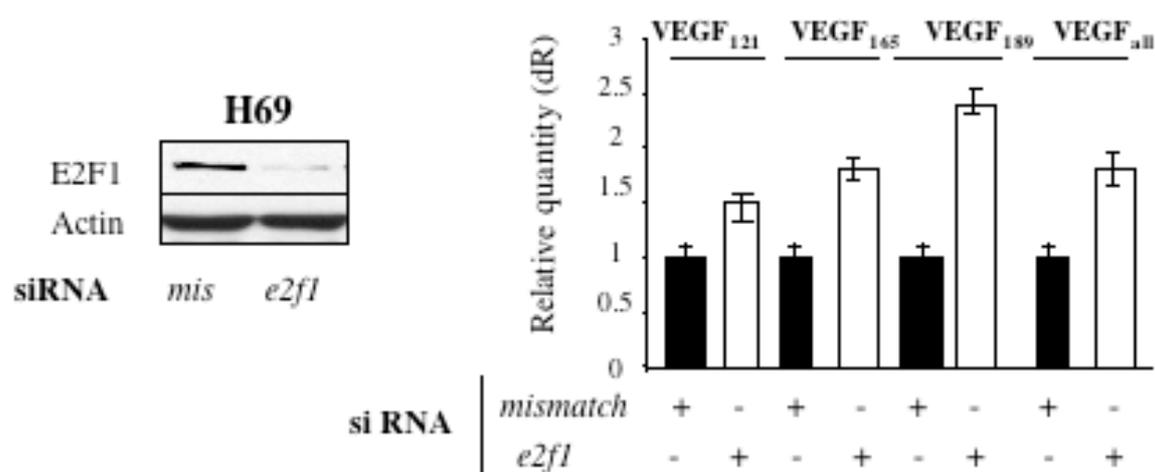
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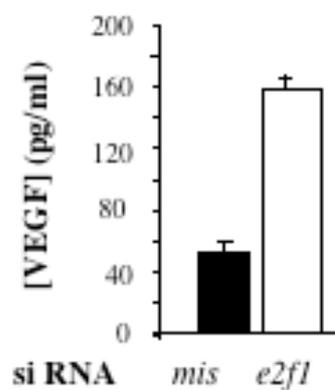
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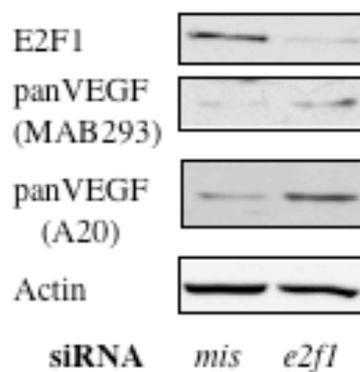
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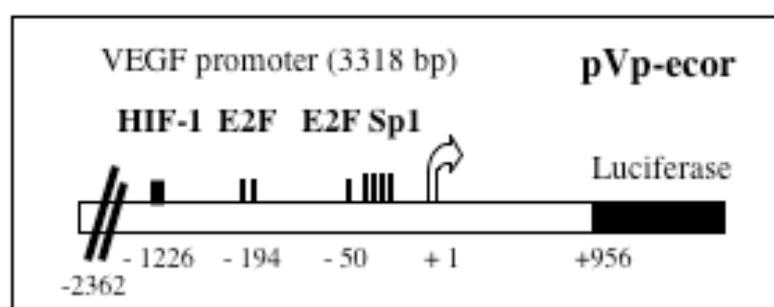
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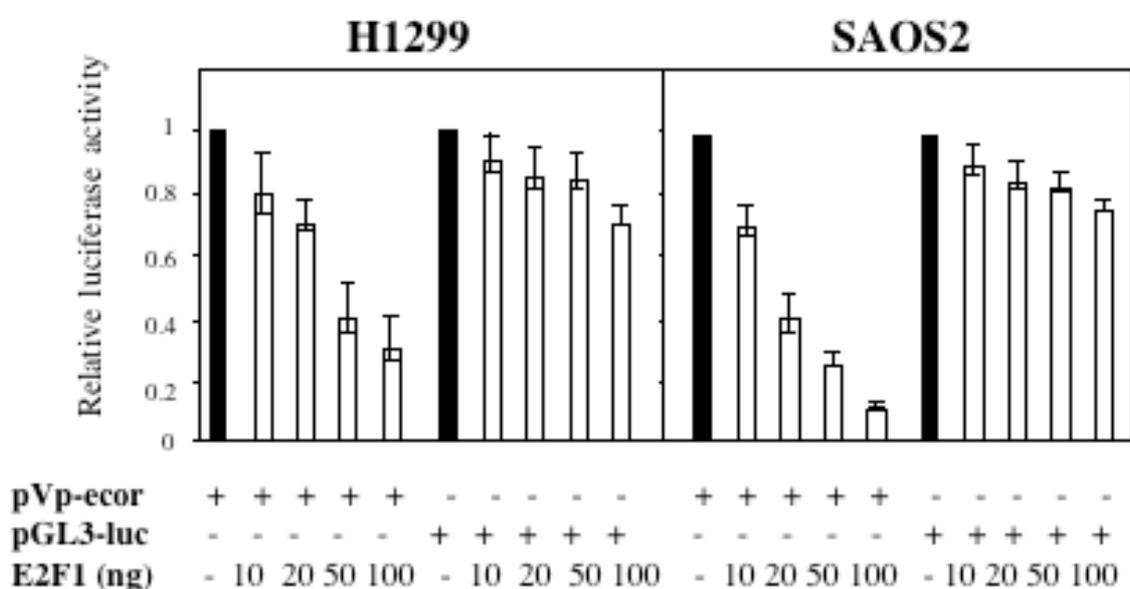
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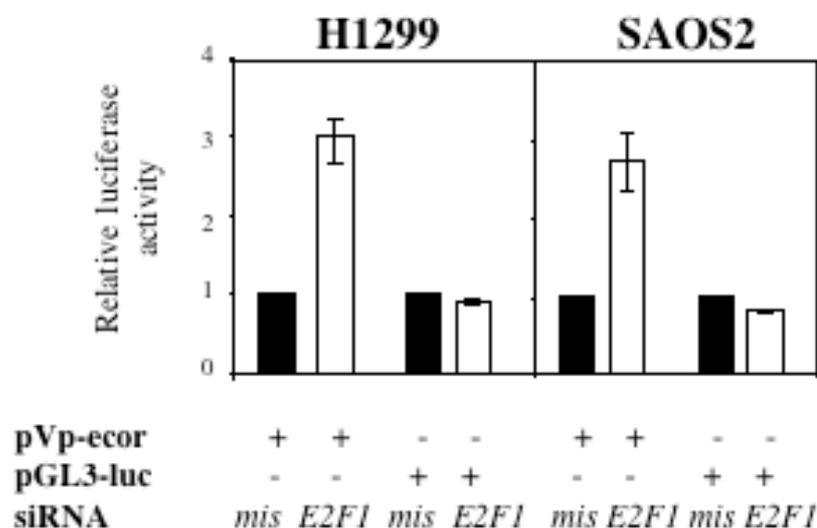
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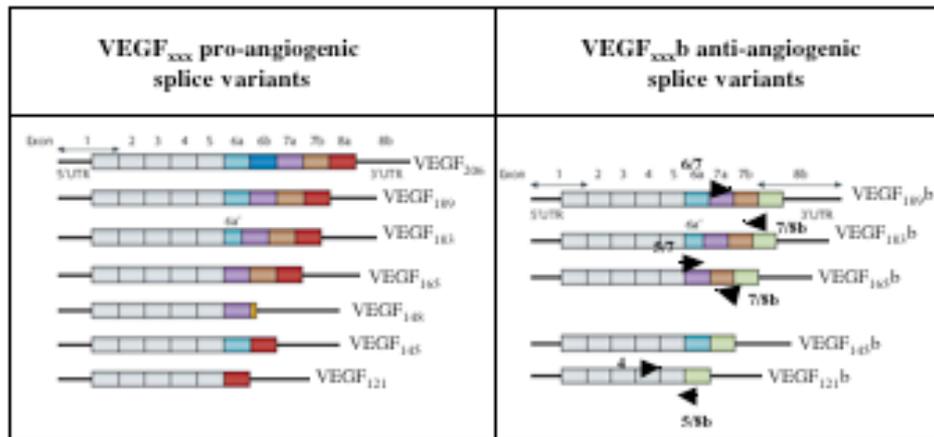
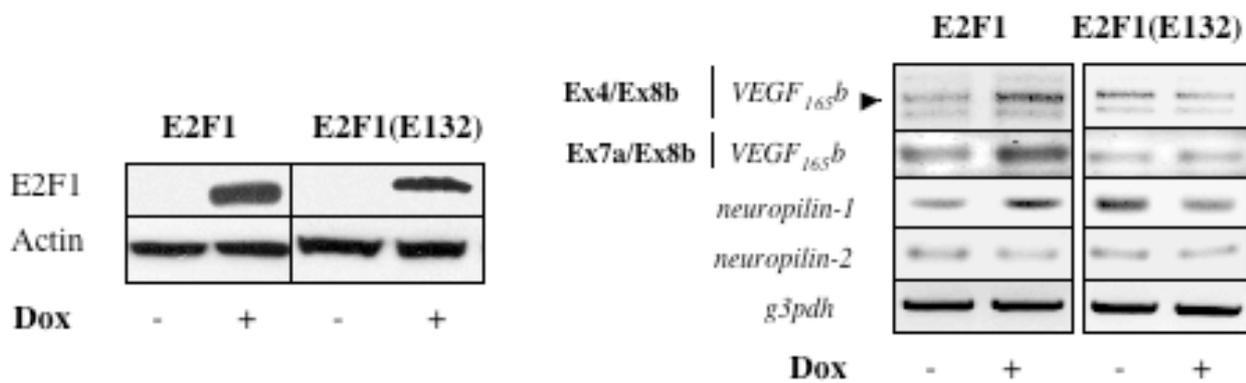
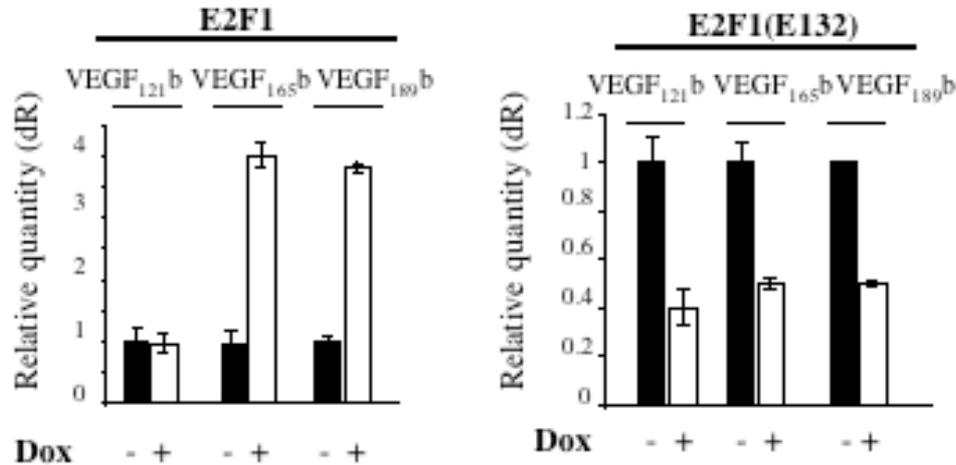
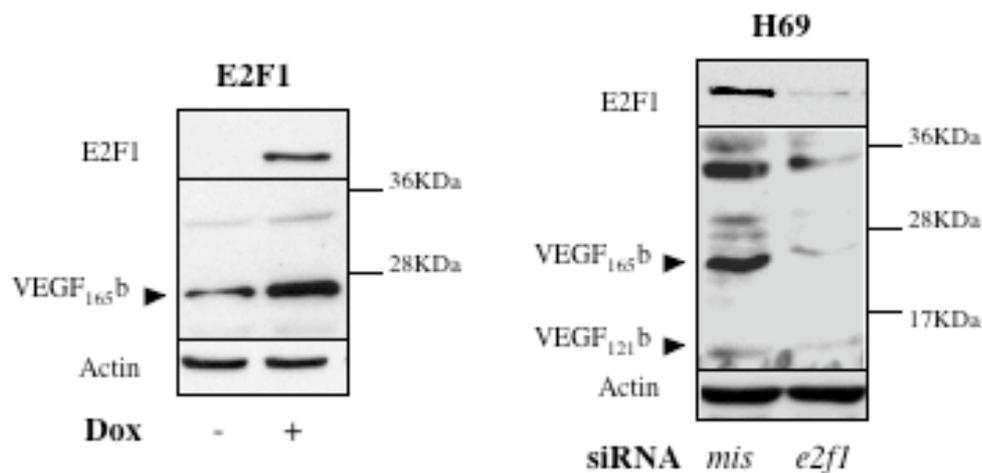


C

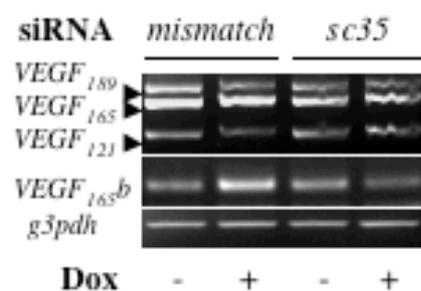


A

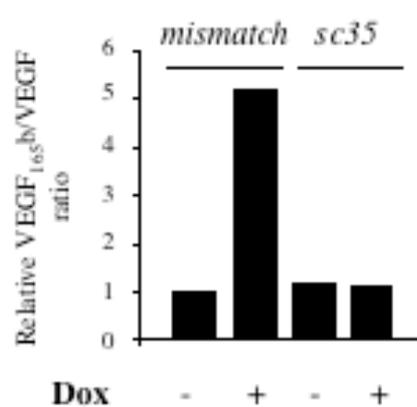
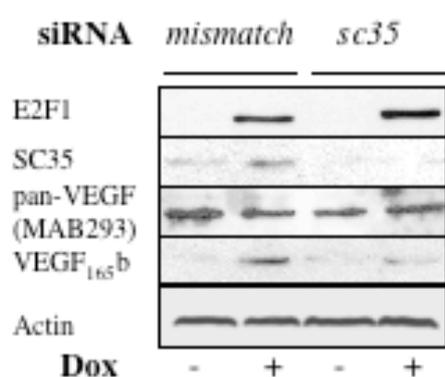
Merdzhanova Fig 4R

**B****C****D**

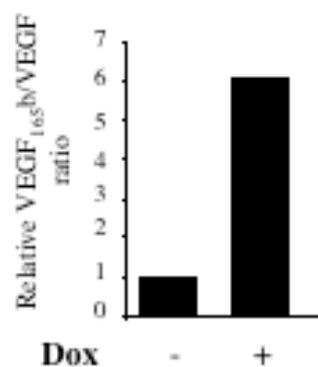
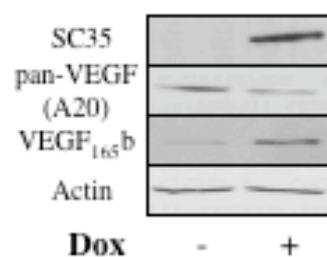
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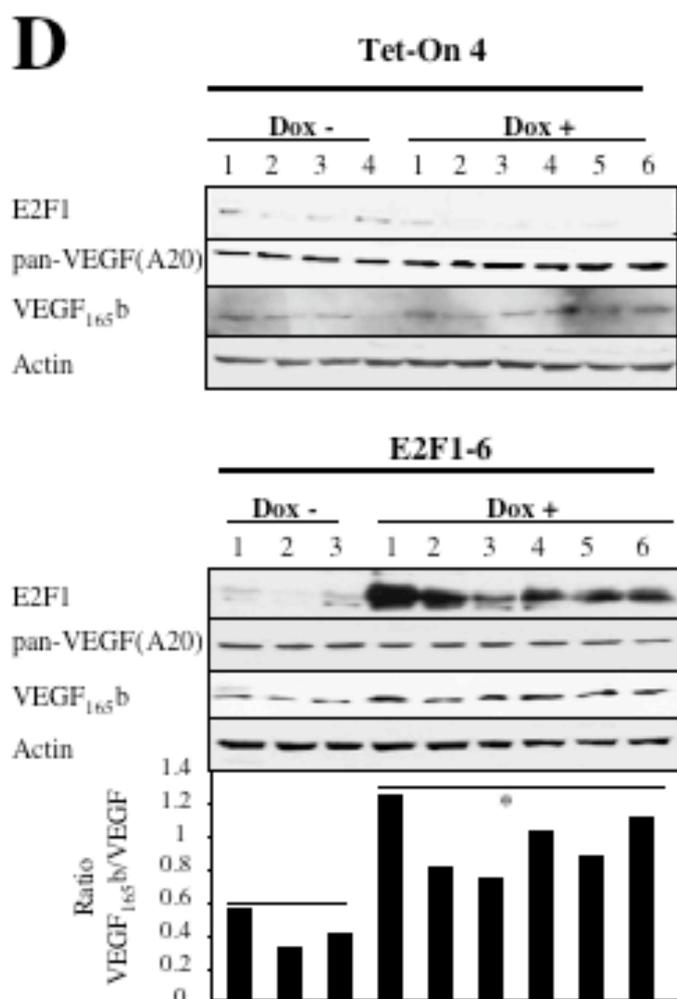
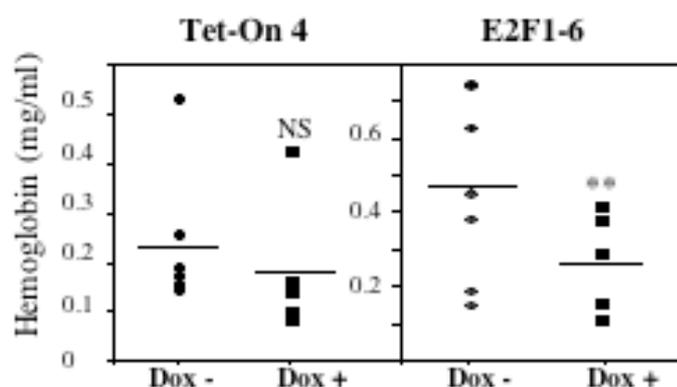
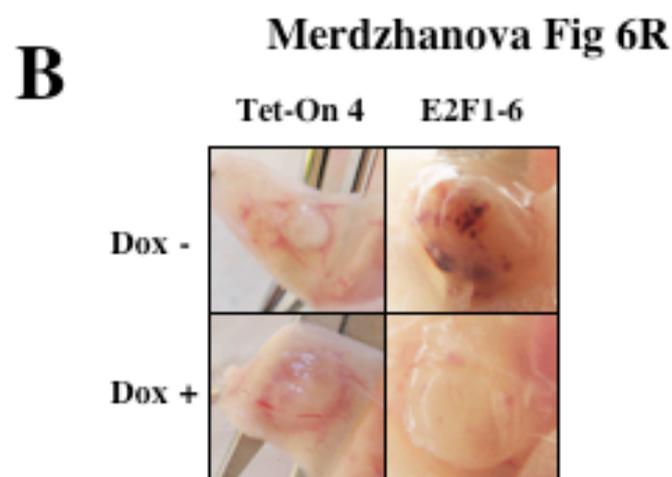
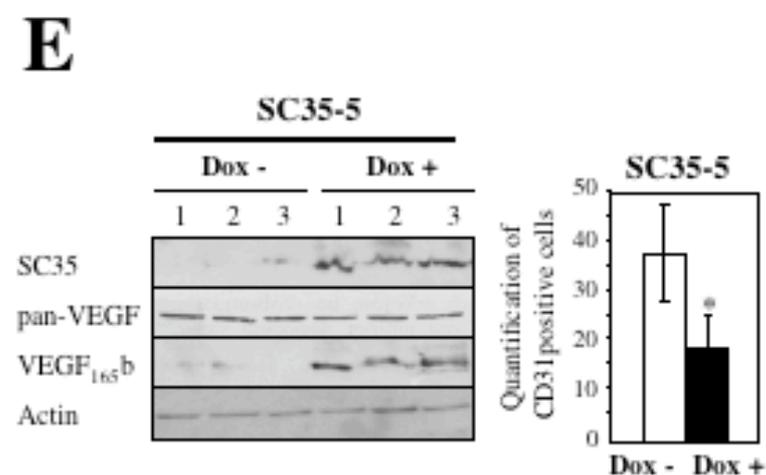
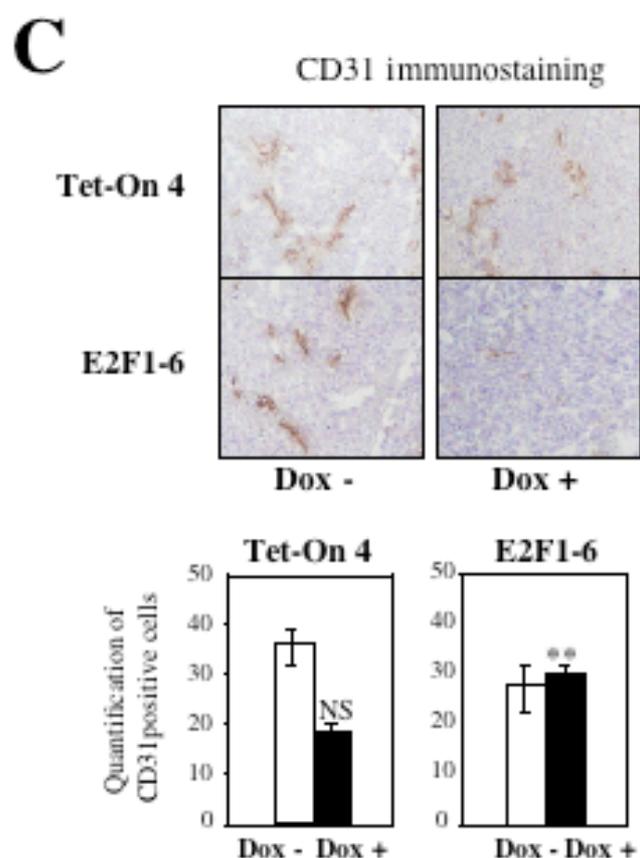
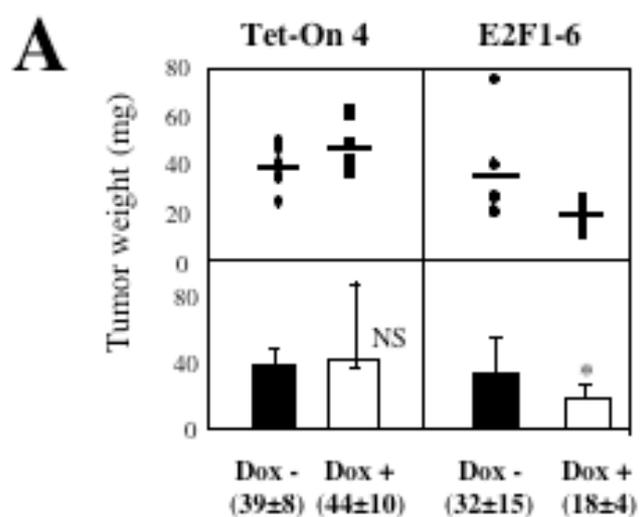


B

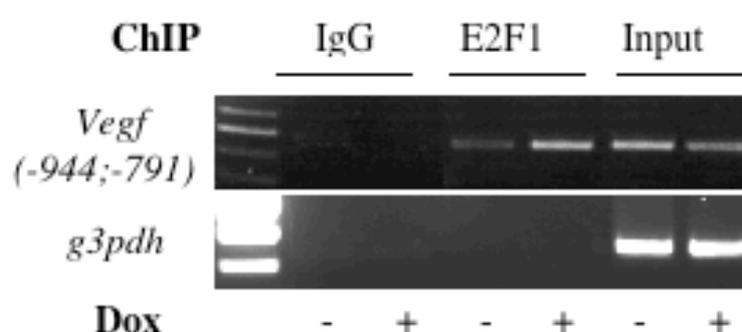


C



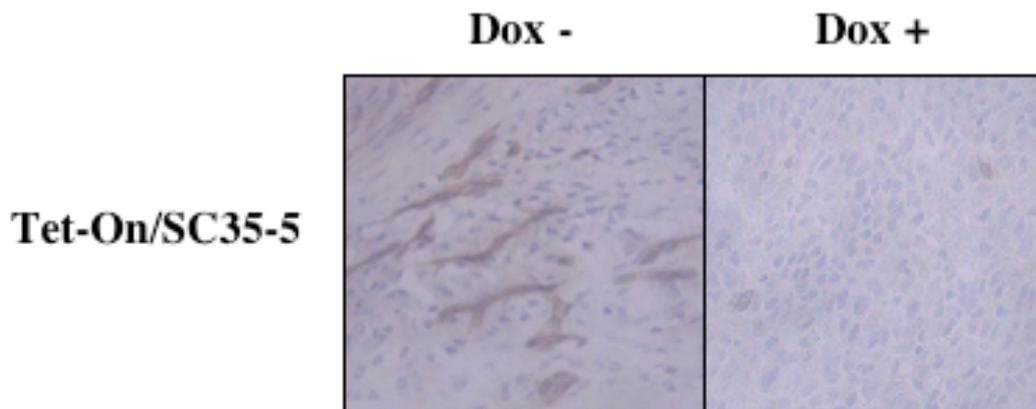


Merdzhanova Supplementary Fig S1



Supplementary Figure 1. E2F1 binds to the *Vegf* promoter in H358/Tet-On/E2F1 cells.

H358/Tet-On/E2F1 cells cultured in the presence (+) or absence (-) of 1 μ g/ml doxycyclin for 24 hours were processed for ChIP analysis using antibody for E2F1 (C-20 ; Santa Cruz). The co-precipitated chromatin DNA was analyzed by semi-quantitative PCR using pair of primers that amplify the human *Vegf* or *Gapdh* promoter respectively, as described in the Supplementary Materials and Methods section. IgG was used as an irrelevant antibody. Input lane represents 0.5% of the total chromatin immunoprecipitated for each sample. As compared with input amount, 0.22% and 3% of chromatin was immunoprecipitated by E2F1 antibody at the *Vegf* promoter level in uninduced and induced cells, respectively.



Supplementary Figure 2. SC35 inhibits tumor neovascularization *in vivo*. Murine CD31 immunohistochemical staining in Tet-On/SC35-5 xenografts was performed as described in the Materials and Methods section. Overexpression of SC35 (SC35-5, Dox +) decreases the number of blood vessels per unit area, as compared to uninduced tumors (SC35-5, Dox -).