Sequential Functions of CPEB1 and CPEB4 Regulate Pathologic Expression of VEGF and Angiogenesis in Chronic Liver Disease

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Abbreviations: ARE, AU-rich element; Aurora kinase A, AurKA; BDL, common bile duct ligation; CPE, cytoplasmic polyadenylation element; CPEB, cytoplasmic polyadenylation element-binding protein; EC, endothelial cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLA, β -galactosidase; iKO, inducible knockout mice; iNOS, inducible nitric oxide synthase; PAS, polyadenylation site; PPVL, partial portal vein ligation; SHAM, sham-operation; shRNA, short hairpin RNA; TNF α , tumor necrosis factor alpha; UTR, untranslated regions; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor; WT, wild-type.

Author contributions: VC performed *in vitro* studies in H5Vs, 3'UTR processing analyses, immunoblotting in KO mice and *in vivo* angiogenesis assays. JG conducted immunoblotting, immunohistochemistry and RT-PCR in human and rodent samples, animal surgery, *in vivo* angiogenesis assays and *in vivo* hemodynamic studies. VC and JG contributed to experimental design

and manuscript preparation. GFM performed wound healing and *in vivo* angiogenesis assays. CM performed partial hepatectomy and liver regeneration studies in mice. GFM with CM performed CPEB KO generation, cell growth curves, validation of antibodies for immunohistochemistry, subcellular fractionation and leptomycin B experiments. EGP and MM contributed to all rodent experiments. AB performed immunohistochemistry in human livers, collected clinical data and participated in statistical analysis. FAB performed experiments in My-Z cells. AAU and MG performed cell migration, spheroids and aortic ring assays and studies using the mouse retina model of angiogenesis. PN contributed to KO generation. JB supplied human samples. MF and RM directed the study and wrote the manuscript.

ABSTRACT

Background & Aims: Vascular endothelial growth factor (VEGF) regulates angiogenesis, yet therapeutic strategies to disrupt VEGF signaling can interfere with physiologic angiogenesis. In a search for ways to inhibit pathologic production or activities of VEGF without affecting its normal production or functions, we investigated the post-transcriptional regulation of VEGF by the cytoplasmic polyadenylation element-binding proteins CPEB1 and CPEB4 during development of portal hypertension and liver disease.

Methods: We obtained transjugular liver biopsies from patients with HCV-associated cirrhosis or liver tissues removed during transplantation; healthy human liver tissue was obtained from a commercial source (control). We also performed experiments with male Sprague-Dawley rats and CPEB-deficient mice (C57BL6 or mixed C57BL6/129 background) and their wild-type littermates. Secondary biliary cirrhosis was induced in rats by bile duct ligation and portal hypertension was induced by partial portal vein ligation. Liver and mesenteric tissues were collected and analyzed in angiogenesis, reverse transcription PCR, polyA tail, 3'RACE, southern blot, immunoblot, histologic, immunohistochemical, immunofluorescence, and confocal microscopy assays. CPEB was knocked down with small interfering RNAs in H5V endothelial cells, and translation of luciferase reporters constructs was assessed.

Results: Activation of CPEB1 promoted alternative nuclear processing within non-coding 3'-untranslated regions of VEGF and CPEB4 mRNAs in H5V cells, resulting in deletion of translation repressor elements. The subsequent overexpression of CPEB4 promoted cytoplasmic polyadenylation of VEGF mRNA, increasing its translation; the high levels of VEGF produced by these cells led to their formation of tubular structures in Matrigel assays. We observed increased levels of CPEB1 and CPEB4 in cirrhotic liver tissues from patients, compared with control tissue, as well as in livers and

mesenteries of rats and mice with cirrhosis or/and portal hypertension. Mice with liver-specific knockdown of CPEB1 or CPEB4 did not overexpress VEGF or have signs of mesenteric neovascularization, and developed less-severe forms of portal hypertension following portal vein ligation.

Conclusions: We identified a mechanism of VEGF overexpression in liver and mesentery that promotes pathologic, but not physiologic, angiogenesis, via sequential and non-redundant functions of CPEB1 and CPEB4. Regulation of CPEB4 by CPEB1 and the CPEB4 auto-amplification loop induces pathologic angiogenesis. Strategies to block the activities of CPEBs might be developed to treat chronic liver and other angiogenesis-dependent diseases.

KEY WORDS: portal hypertension; CPEB; cytoplasmic polyadenylation; alternative RNA processing

INTRODUCTION

Chronic liver diseases, including liver cirrhosis, are major causes of death worldwide, but their therapeutic options still remain severely limited.¹ Pathological angiogenesis triggered by vascular endothelial growth factor (VEGF) overproduction is central for liver disease progression and for development and maintenance of portal hypertension (PH),²⁻⁵ the most devastating complication that develops in cirrhotic patients.⁶ Hence, therapeutic targeting of angiogenesis has been proposed as a promising strategy.²⁻⁵ However, the clinical benefit of antiangiogenic drugs is restricted because of significant adverse effects, including collapse of normal vasculature, vascular leakage and bleeding, as exemplified in patients with hepatocellular carcinoma and cirrhosis.⁷ Most of these limitations arise from the fact that current anti-VEGF approaches are not selective for pathological VEGF production, but instead inhibit also physiological VEGF required for vascular homeostasis of healthy vessels and formation of vascular supply in many physiological settings.^{8,9} Deciphering mechanisms that regulate VEGF expression is therefore critical to achieve specific inhibition of pathological generation of VEGF without affecting its physiological production.

Although the prevailing paradigm of VEGF regulation focuses on its transcriptional activation,⁹ independent research lines have revealed an equally important posttranscriptional mechanism mediated by RNA-binding proteins and microRNAs, operating on cis-regulatory elements within VEGF mRNA untranslated regions (UTRs).¹⁰ For example, AU-rich elements, microRNA-target sites and a specific element recruiting the GAIT complex are negative regulators that have been implicated in VEGF mRNA silencing when VEGF synthesis is not needed, by repressing translation and/or reducing transcript half-life.¹¹⁻¹⁴ But, upon angiogenic stimuli and to support the high VEGF levels that drive pathological angiogenesis, this translationally silent VEGF mRNA needs to be readenylated to achieve full activation. The mechanism mediating this VEGF transcript reactivation upon pathological angiogenic stimulation remains unclear.

A possible explanation arises from the presence of cytoplasmic polyadenylation elements (CPEs) within non-coding VEGF 3'UTR.¹⁵ CPEs are bound by CPE-binding proteins (CPEBs), a family of four members that recognize the same cis-acting element, but are regulated by different signal transduction pathways and have specific cellular functions.¹⁶⁻²² Both CPEB1 and CPEB4 promote cytoplasmic-polyadenylation and translational activation of CPE-containing mRNAs when phosphorylated (activated), respectively, by the serine/threonine kinase Aurora kinase-A (AurKA) and by a yet unidentified kinase.^{23,24} In addition to this cytoplasmic function, we have recently found that CPEB1 also shuttles to the nucleus, where it binds to CPE-containing pre-mRNAs and directs the use of alternative-polyadenylation sites and subsequent shortening of 3'UTRs, thereby modulating their translation efficiency in the cytoplasm.²⁵ Because we have also found that CPEB4 is required for xenografted-tumor growth and neovascularization in pancreatic ductal adenocarcinoma and glioblastoma,²⁶ we propose that CPEBs could regulate cirrhosis and PH-associated pathological VEGF expression and angiogenesis. Our studies identify a new molecular mechanism where sequential and coordinated functions of CPEB1 and CPEB4 regulate posttranscriptionally the "pathologically-augmented" VEGF levels, but not the basal levels required for normal physiological angiogenesis, suggesting that CPEBs could be clinically attractive therapeutic targets for pathological angiogenesis in chronic liver disease and potentially other angiogenesis-dependent diseases.

MATERIALS AND METHODS

Patients and animals

Studies were approved by Research Ethic Committees of Hospital Clinic (Protocol #2011/6723), and Barcelona University and Scientific Park. Human samples of HCV-related cirrhotic liver were obtained from transjugular liver biopsies or from explanted organs during transplantation, after informed consent signed by each patient. Human normal liver tissue was commercially obtained (AMS Biotechnology). Male Sprague-Dawley rats and CPEB-deficient mice on a C57BL6 or mixed C57BL6/129 background

were used along with corresponding age-matched wild-type mice. Generation of tamoxifen-inducible CPEB1 and CPEB4 knockout (iKO) mice, and induction of secondary biliary cirrhosis (bile duct ligation, BDL) and portal hypertension (partial portal vein ligation, PPVL) are described in Supplementary Methods.

Molecular and functional analysis

Details of angiogenesis assays, translation of luciferase reporters in H5V cells, CPEB knockdown cells, luciferase assay, polyA tail assay, 3'RACE, RT-PCR, Southern-blotting, immunoblotting, histological analysis, immunohistochemistry, immunofluorescence and confocal microscopy are provided in Supplementary Methods.

Statistical analysis

Data are shown as mean $\pm$ SEM, mean $\pm$ SD, or mean $\pm$ range. Kolmogorov Smirnov test was used to determine whether data were normally distributed ($P>0.05$) or not ($P<0.05$). Normally-distributed results were compared with parametric statistical procedures [Student t test and two-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons]. Non-normally-distributed results were compared with non-parametric tests (Kruskall-Wallis one-way analysis of variance and Mann-Whitney U test). Significance was accepted at $P<0.05$.

RESULTS

CPEB1 and CPEB4 are required for VEGF expression and angiogenesis

Because angiogenesis inhibition in CPEB4-depleted pancreatic adenocarcinoma-xenografted tumors pointed to a defect in secreted factor rather than to endothelial cell defect,²⁶ we performed "in silico" search for potential CPE-regulated mRNAs encoding proangiogenic secreted factors. We found that VEGF 3'UTR contains various CPE elements, making it susceptible of being regulated by CPEBs.

Interestingly, we also identified several putative polyadenylation sites (PAS) in VEGF 3'UTR, being the more 5' (PAS1) flanked by 2 CPEs (Figure-1A), suggesting that CPEB could mediate alternative 3'UTR processing leading to shorter VEGF mRNA isoforms by alternative-polyadenylation.

Alternative-polyadenylation usually occurs by activation of "weaker" proximal PAS located 5' of "stronger" distal PAS, the latter resulting in mRNAs containing the longer form of the 3'UTR and tending to be used by default.²⁵ This alternative-polyadenylation generates mRNAs containing 3'UTRs of shorter length, displaying increased translation compared with longer transcripts, because they lack sequences that promote deadenylation, translational repression and mRNA destabilization, such as AU-rich elements (AREs).²⁵ Interestingly, we found that the VEGF 3'UTR fragment between proximal PAS1 and distal PAS2 harbors multiple AREs (Figure-1A). Identical arrangements of cis-regulatory motifs (CPEs, AREs and PAS) were identified in human, mouse and rat VEGF mRNA 3'UTRs by comparative sequence analysis (Figure-1A).

To test whether CPEBs could regulate VEGF mRNA processing and translation, we used the murine immortalized H5V endothelial cell line as an appropriate model of "pathological" or "activated" endothelial cell.²⁷ H5Vs are transformed and tumorigenic endothelioma cells that differ considerably from their normal counterparts, being highly proliferative and constitutively expressing CPEB1, CPEB4 and VEGF. When cultured in Matrigel, H5Vs stop proliferation and self-organize into "tubular" structures. H5V migration and cell attachment is dependent on the presence of VEGF in the media, which is secreted by H5Vs themselves in an "autocrine-like" manner. Thus, inhibition of VEGF synthesis by H5Vs results in the inability to generate "tubular" structures. We transduced H5Vs with recombinant lentiviruses expressing isopropylthio- β -galactoside-inducible small hairpin shRNAs against CPEB1 or doxycycline-inducible shRNAs against CPEB4 to silence CPEB1 and CPEB4 respectively (Figure-1B,C), and compared to parental untransfected endothelial cells or to endothelial cells expressing control shRNAs not induced with isopropylthio- β -galactoside or doxycycline. According to previous results,¹⁹ CPEB1 depletion reduced CPEB4 protein and mRNA levels (Figure-

1B,C). Knockdown of either CPEB1 or CPEB4 also decreased VEGF protein expression (Figure-1B; Supplementary-Figure-1A-C), but not VEGF mRNA (Figure-1C), and dramatically diminished the ability of H5Vs to form "tubular-like" structures on growth factor-reduced Matrigel (Figure-1D,E). This effect was specifically caused by VEGF downregulation as the "angiogenic" capacity of CPEB knockdowns was restored when either conditioned medium from non-induced H5Vs or recombinant VEGF was added to CPEB-depleted H5Vs (Figure-1D,E). CPEB4 shRNA specificity was confirmed by overexpressing a non-targetable CPEB4 variant that rescued "tubular" structure formation (Supplementary-Figure-1D,E). Due to cytotoxic effects of CPEB1 overexpression, it is not possible to perform a similar rescue experiment, but we confirmed CPEB1 shRNA specificity by repeating the experiment with another shRNA, which reproduced the same results (Supplementary-Figure-1F-H). CPEB depletion had no effect on cell viability (Supplementary-Figure-2A). These findings demonstrate that CPEB1 and CPEB4 are necessary for VEGF synthesis and subsequent "angiogenesis", presumably through a direct effect as both CPEBs specifically coimmunoprecipitated VEGF and CPEB4 mRNAs (Figure-2A,B).

CPEBs regulate VEGF mRNA processing and cytoplasmic polyadenylation

To further dissect the posttranscriptional level at which CPEB regulatory functions were exerted, we determined changes in 3'UTR processing and polyadenylation of VEGF mRNA upon CPEB depletion in H5Vs. As described above, we have recently shown that CPEB1 directs alternative-polyadenylation of transcripts containing CPEs near to "weak" polyadenylation sites (PAS1), upstream of the strong or default PAS.²⁵ This is the case of VEGF 3'UTR, which contains two CPEs flanking the more 5' PAS1 (Figure-2C). Using 3'RACE followed by Southern-blotting, we detected 3'UTRs generated by use of PAS1 and PAS2 (Figure-2D). Existence of these VEGF 3'UTR variants, obtained by alternative use of PAS1-2-3, has been confirmed in multiple mammalian tissues.¹⁴ However, how the choice of polyadenylation sites is regulated, or even if it is regulated, was unknown. It was also not known how

the different 3'UTRs modulate VEGF protein synthesis. Here, we addressed these issues and found that CPEB1 depletion led to decreased use of CPE-dependent PAS1 and converse increase in PAS2, which is used by default in a CPEB-independent manner. This alternative-polyadenylation caused 3'UTR lengthening and consequent inclusion of AREs and microRNA-binding sites, which, in turn, decreased translation in luciferase-reporter assays (Figure-2E). CPEB4 depletion, on the other hand, had no effect on PAS selection (Figure-2D).

When we measured changes in polyA tail length upon CPEB depletion, we found that CPEB4 was required to maintain a long polyA tail (120 residues) in the short 3'UTR variant of VEGF mRNA, and, therefore, required for VEGF translation (Figure-2F, *right*).¹⁵ The long VEGF 3'UTR variant already contained a short polyA tail (40 residues), even in presence of both CPEBs. This short polyA tail probably originated in presence of AREs and microRNA-binding sites as a result of competition between polyadenylation and deadenylation machinery activities. Because this short polyA tail is not sufficient to support translation of the harboring transcript,¹⁵ the further reduction in length observed upon CPEB4 depletion is most likely irrelevant for VEGF mRNA translation. Conversely, CPEB1 depletion had no effect on VEGF mRNA length (Figure-2F, *left*). However, in absence of CPEB1, VEGF mRNA was expressed mainly with long 3'UTR (obtained by use of PAS2) and, as a result of "weak" polyadenylation, was poorly translated. Further corroboration of these results was obtained by luciferase reporter assays. When wild-type (WT) H5Vs were transfected with synthetic chimeric mRNAs containing the Firefly luciferase coding sequence fused upstream of short (PAS1) or long (PAS2) VEGF 3'UTR, the mRNA presenting the longer 3'UTR, containing AREs, showed $\approx 50\%$ reduced translational activity (Figure-2E).

Together, these results indicate that both CPEB1 and CPEB4 are required for VEGF synthesis, controlling two sequential steps of its posttranscriptional regulation. First, CPEB1 regulates nuclear alternative VEGF pre-mRNA processing, generating shorter 3'UTRs that exclude translation-inhibitory elements (i.e., AREs and microRNA-binding sites). In that way, CPEB1 "takes off the brakes" of VEGF

mRNA. And then, CPEB4 promotes cytoplasmic-polyadenylation of VEGF mRNA, which is in turn required for translation stimulation. These two events are coordinated through CPEB1-mediated activation of CPEB4 pre-mRNA alternative processing (due to the presence of CPE elements in the 3'UTR of CPEB4), which also results in shorter CPEB4 3'UTR that excludes AREs and microRNA-binding sites (Supplementary-Figure-2B-D). Moreover, the finding that CPEB4 binds its own mRNA (Figure-2B) suggests a possible positive feedback-loop of translational regulation.¹⁹ Furthermore, subcellular localization analysis of CPEB proteins in H5Vs demonstrated that CPEB1 was located in nucleus and cytoplasm (Supplementary-Figure-2E), and was retained in the nucleus upon nuclear export inhibition with Leptomycin- B (Supplementary-Figures-2F,G), consistent with CPEB1 being a nucleo-cytoplasmic shuttling protein with dual function in pre-mRNA processing,²⁵ and translational control.^{16,17} In contrast, CPEB4 expression was restricted to cytoplasm (Supplementary-Figure-2E), as expected for its role in translational control and cytoplasmic-polyadenylation.¹⁹

CPEBs and VEGF are overexpressed in humans and rodents during PH and cirrhosis

To underscore the importance of CPEBs as regulators of VEGF expression *in vivo* in a pathological context, we characterized CPEBs and VEGF expression profiling in liver and mesentery, scenarios in which pathological angiogenesis is clinically relevant during PH and cirrhosis.²⁻⁵ In healthy human liver, CPEB1 and CPEB4 were uniformly expressed at very low levels. By contrast, CPEBs were strongly overexpressed in liver from patients with HCV-related cirrhosis (Figure-3A,B; Supplementary-Table-1; see controls in Supplementary-Figures-3-4), predominantly in hepatocytes within regeneration micronodules and at the interface between liver parenchyma and fibrous septa, following a similar localization pattern to VEGF (Figure-3B). Expression of both CPEBs and VEGF was also found in endothelium of numerous neovessels observed inside fibrotic areas of cirrhotic livers (Figure-3C,D). Given that VEGF is a secreted factor, its release from hepatocytes into surrounding, highly vascularized fibrotic microenvironment can paracrinically facilitate endothelial cell recruitment,

proliferation and neovascularization, whereas VEGF produced in neovascular endothelium may autocrinically act to reinforce endothelial cell angiogenic phenotype. Of note, VEGF protein overexpression was not associated with VEGF mRNA upregulation (Figure-3E), indicating that it was not transcriptionally-derived. Similar changes were seen in cirrhotic BDL rat livers (Supplementary-Figures-5-6A).

We also characterized the spatiotemporal expression pattern of CPEBs and its relationship with VEGF and with the time course of neovascularization in the mesentery of portal hypertensive PPVL rats. Although CPEB1 and CPEB4 were poorly expressed in sham-operated control animals, we found them to be abundant in preexisting vessels and neovessels following PH induction, overlapping with VEGF (Figure-4A,B; see controls in Supplementary-Figures-3-4) and correlating with neovascularization (Supplementary-Figures-6B and 7A-C). CPEB-VEGF overlapping was not restricted to endothelium, but also extended to pericytes and adventitial cells (Supplementary-Figure-7D,E; see controls in Supplementary-Figure-3C), known to modulate angiogenesis.^{8,9,28} Again, temporal changes in VEGF protein expression did not correlate with parallel mRNA expression changes (Figure-4C), suggesting that enhanced transcription is unlikely to be responsible for VEGF protein upregulation in PH-associated pathological angiogenesis.

Notably, mesenteric upregulation of phosphorylated activated CPEB1 was obvious earlier than maximal VEGF overexpression and neovascularization (Figure-4A), supporting the idea that VEGF could increase in response to PH-associated CPEB upregulation. This CPEB1 phosphorylation corresponds to activatory residues Thr171 and Ser176 (Figure-4A), and is known to be mediated by AurKA.^{23,24,29} AurKA activity is in turn dependent on its autophosphorylation at Thr288 within the kinase activation loop.²⁹ So, we determined the expression of this Thr288-phosphorylated AurKA by immunoblotting and found that it was significantly increased very early after PH induction in rat mesentery, preceding CPEB and VEGF upregulation (Figure-4D). Upregulation of phospho-AurKA was also observed in the liver of cirrhotic patients (Figure-3A). These results suggest that the

activatory phosphorylation of AurKA may be a key inductive factor for CPEB1 phosphorylation and activation. In turn, activation of CPEB1 causes the synthesis of CPEB4,¹⁹ and subsequent VEGF overexpression in PH and liver cirrhosis.

CPEBs cooperate to regulate pathological VEGF expression *in vivo*

The above results prompted us to study whether the CPEB-dependent alternative 3'UTR processing and cytoplasmic-polyadenylation that we found *in vitro* in transformed H5V endothelioma mouse cells also controlled VEGF expression *in vivo* under pathological conditions. We first analyzed VEGF mRNA processing and polyadenylation in mesentery of PH rats. As a control, we used intestinal mucosa, which is unaffected by VEGF changes during disease progression (Supplementary-Figure-8). VEGF mRNA was indeed coimmunoprecipitated with CPEB1 in PH mesenteries (Figure-5A). Moreover, PH induction resulted in preferential use of PAS1 over PAS2, generating the shorter VEGF 3'UTR variant and polyA tail elongation in mesentery, but not in intestinal mucosa (Figure-5B,C), in which CPEB1 was not readily detectable. Interestingly, same mechanisms were confirmed in cirrhotic liver of HCV-infected patients (Figure-5D-E). Thus, in healthy human liver (where CPEB1 is not expressed) the long VEGF 3'UTR generated by the use of PAS2 is the predominant form, whereas in cirrhotic liver (where CPEB1 is overexpressed), the use of PAS1 is increased to generate a shorter 3'UTR (Figure-5D). Concomitantly, this short 3'UTR is polyadenylated in cirrhotic liver (where CPEB4 is overexpressed), but not in healthy liver (where CPEB4 is not overexpressed) (Figure-5E). Similar conclusions derived from knockout mouse studies (described below). These results indicate that human, rat and mouse VEGF 3'UTRs are subjected to the same nuclear alternative processing and cytoplasmic-polyadenylation events in response to pathological angiogenic stimuli, and that mechanistic conclusions derived from animal models and *in vitro* are extrapolable to human disease.

CPEBs silencing limits pathological angiogenesis and VEGF overexpression and protects against disease progression

To further explore the functional implications of CPEBs in controlling pathological VEGF expression, we generated tamoxifen-inducible CPEB1 and CPEB4 iKO mice (Supplementary-Figure-9A-F) and subjected them to PPVL to induce portal hypertension. As expected, CPEB1 and CPEB4 protein expressions were almost absent in iKO mice (Figure-6A; Supplementary-Figure-9G; see controls in Supplementary-Figures-3,4).

Importantly, deficiency of either CPEB1 or CPEB4 significantly prevented PH-induced VEGF overexpression (Figures-6B,C) and mesenteric neovascularization (Figure-6C,D; Supplementary-Figure-10A), translating into amelioration of major hallmarks of PH,^{6,30-32} such as portosystemic collateral vessel formation (Figure-6E) and mesenteric arterial hyperdynamic circulation (Supplementary-Figure-10B), and improvement of other surrogate markers of disease severity, such as increased von Willebrand factor (vWF) plasma levels (Supplementary-Figure-10C), and splenic enlargement and hyperactivation (Supplementary-Figure-10D,E).

Remarkably, CPEB suppression specifically targeted pathological but not homeostatic, physiological VEGF functions. Thus, CPEB depletion in PPVL mice neither completely abrogated VEGF levels in preexisting vessels (Figure-6B,C) nor affected preexisting normal vasculature density (Figure-6C,D). In addition, in none of the iKO mice did we observe any wound complication after abdominal surgery for PPVL, or other adverse effects such as tissue injury (i.e., inflammation and oxidative stress) (Supplementary-Figure-11A,B), reduced body weight gain (Supplementary-Figure-11C), diarrhea, or hemorrhage. Furthermore, the absence of CPEB did not affect physiological angiogenesis associated with healing skin wounds (Figure-7A), liver regeneration after partial hepatectomy (Figure-7B,C), and the normal intraretinal vascular development that occurs postnatally in rodents (Figure-7D,E). Physiological developmental angiogenesis was also unaffected by CPEB

deficiency, as shown by the lack of embryonic lethality and the presence of normal vasculature in constitutive KO mice.

Of note, CPEB1 depletion *in vivo* in iKO mice led to preferential use of PAS2 *versus* PAS1, generating a longer 3'UTR that included AREs, whereas CPEB4 depletion caused shortening of polyA tail of the shorter 3'UTR (Figure-6F), collectively revealing that the same mechanism of VEGF expression control by CPEBs operates in different *in vivo* species (mouse, rat and human) and animal models (BDL and PPVL), and in two distinct scenarios (liver and mesentery) during PH.

Finally, we also found that the defective angiogenic response seen in CPEB KO mice was non-cell-autonomous since it was restored in presence of VEGF in *in vivo* Matrigel plug assays (Supplementary-Figure-12A-D), mouse lung endothelial cell sprouting and migration assays (Supplementary-Figure-12E-G), and aortic ring cultures (Supplementary-Figure-12H), indicating that it was originated from an autocrine or paracrine defect in VEGF synthesis.

DISCUSSION

Results presented herein unveil a new mechanism of regulation of pathological VEGF expression and angiogenesis through sequential, non-redundant functions of CPEB1 and CPEB4. This mechanism, elucidated in transformed H5V endothelioma cells, is activated also in PH animal models and HCV-infected cirrhotic patients, where CPEBs are overexpressed and required for VEGF mRNA processing and translation to generate high levels of VEGF and pathogenic neovascularization. Importantly, this CPEB-mediated mechanism is essential for pathological angiogenesis but dispensable for physiological neovascularization, as we have demonstrated through several corroborating observations, including low CPEB expression in healthy organs but robust CPEB overexpression during PH-associated angiogenesis; decreased VEGF overexpression and "pathological" angiogenesis after CPEB depletion *in vitro* in knockdown H5Vs or *in vivo* in KO mice, but preservation of basal VEGF levels and preexisting vasculature density; and lack of effect of CPEB suppression on

physiological angiogenesis associated with wound healing, liver regeneration after partial hepatectomy, postnatal intraretinal vascular development, and embryonic development. Furthermore, CPEB deficiency in KO mice did not induce tissue injury or any other detrimental effect. In addition, the viability of CPEB depleted cells by shRNA expression was demonstrated in vitro by cell growth analysis and by the possibility to rescue their phenotype using conditioned medium or VEGF. Consistently, previous studies in CPEB KO mice have also reported no deleterious effects in normal conditions.^{33,34}

From a translational point of view, our studies highlight that CPEBs could be promising angiogenesis-disrupting targets in disease. Thus, targeting CPEBs could lead to safer treatment outcomes by specifically reducing excessive pathological VEGF production instead of indiscriminately perturbing both pathological and physiological VEGF synthesis, minimizing potential adverse side-effects. Reduction of pathological angiogenesis in early disease stages could also prevent further disease progression and reduce the risk for developing overt liver cirrhosis. In support of this idea, we have found that CPEB suppression in experimental models of PH markedly ameliorated portosystemic collateral vessel formation, mesenteric neovascularization and splanchnic hyperdynamic circulation, which are crucial disturbances in PH and the main causes of most serious complications in cirrhosis.^{6,30-32} CPEB depletion also attenuated other clinical surrogate markers of disease progression,^{6,30-32} such as splenomegaly, splenic hyperactivation, and VEGF and vWF overexpression.

Based on these studies, we propose a model (Figure-7F) whereby in non-angiogenic tissues, in which activated (phosphorylated) CPEB1 is absent, VEGF and CPEB4 pre-mRNAs are processed using “default” distal polyadenylation site PAS2 with consequent formation of the longest possible 3'UTR variant, harboring multiple ARE- and microRNA-binding sites. These negative regulatory sequences and their binding factors inhibit cytoplasmic translation and/or shorten half-life of mature transcripts. Upon pathological angiogenesis stimulation, CPEB1 becomes overexpressed and

activated by early phospho-AurKA-mediated phosphorylation, while shuttling between nucleus and cytoplasm. In PH, this could be related with variations of vascular oxygen tension and/or mechanical forces due to increased blood pressure and flow.^{1,3} Nuclear CPEB1 promotes the use of more proximal polyadenylation sites (PAS1) during VEGF and CPEB4 pre-mRNA processing (due to the presence of CPE elements in the 3'UTR of both VEGF and CPEB4), shortening VEGF and CPEB4 3'UTRs and excluding translation-inhibitory elements (AREs- and microRNAs-binding sites) from mature transcripts, thus derepressing the transcripts. For CPEB4, elimination of these repressive motifs triggers transcript stabilization and translational activation. The resulting CPEB4 then binds to CPE elements present on its own transcript, generating an autoamplification positive feedback loop that further enhances CPEB4 levels. CPEB4, now present at high concentration, binds to and favors VEGF mRNA cytoplasmic-polyadenylation, which in turn activates translation. Therefore, CPEB1 is required for nuclear alternative 3'UTR processing of VEGF and CPEB4 mRNAs, while CPEB4 is required for subsequent translational activation by cytoplasmic-polyadenylation. Both functions are coordinated through pre-mRNA processing and cytoplasmic-polyadenylation loops connecting CPEB1 and CPEB4. Besides this mechanism, VEGF mRNA levels could be regulated in experimental conditions such as hypoxia through ARE-binding proteins and microRNAs, which target mRNA stability but do not stimulate translation.¹⁴ In early PH stages, however, we detect VEGF mRNA translation activation without any change in mRNA levels. Moreover, this CPEB-regulated translational activation is mediated by a 3'UTR shortening event that eliminates the aforementioned AREs and concomitantly promotes cytoplasmic-polyadenylation, which in turn augments recruitment of eIF4F translation initiation complex.^{16,17} Notably, this mechanism does not exclude an additional transcriptional regulation at latter times. This coordination is not unusual for "key-proteins", such as p53, which is regulated at multiple levels including transcription, translation, posttranslational modifications and protein degradation. In the case of VEGF and its regulation by CPEBs, the

translational regulation either precedes or dominates over transcriptional control, as CPEB-KO mice fail to upregulate VEGF to the levels required to support PH-associated angiogenesis.

Together, these studies strongly suggest the key role of CPEB1 and CPEB4 in pathological angiogenesis, and thus, underscore CPEBs as attractive targets for PH and chronic liver disease therapy. These findings may also have broader implications in other pathological conditions involving dysregulated angiogenesis including cancer,^{16,17} as suggested by our previous work in pancreatic adenocarcinoma and glioblastoma.²⁶ Further, we show here that CPEBs are highly expressed in regenerative cirrhotic micronodules potentially linked with hepatocellular carcinoma.² The precise role of CPEBs in cancer certainly merits further and dedicated future studies.

FIGURE LEGENDS

Figure-1: CPEBs are required for VEGF expression and "angiogenesis"

(A) Comparative analysis of VEGF 3'UTR in mouse, rat and human. CPE (cytoplasmic-polyadenylation elements) and PAS (polyadenylation-signals) are indicated in white (consensus sequences) or grey polygons (non-consensus sequences). PAS1 indicates proximal PAS, and PAS2 and PAS3 distal PAS. Several AU-rich elements (AREs) are illustrated using only one ellipse for simplification. Single-headed arrows indicate position of primers for 3'RACE analysis of PAS1 (solid), and PAS2 or PAS3 (dashed) usage. Double-headed arrows indicate 3'UTR length. Black, white and grey arrowheads indicate cleavage sites regulated by PAS1, PAS2 and PAS3, respectively. Rectangles indicate position of probes used for Southern-blotting to detect PAS1 (black) and PAS2 or PAS3 3'UTRs (white). **(B)** Representative immunoblotting (from 3 biological replicates) demonstrating effective silencing of CPEB1 and CPEB4 in CPEB1 (*left*) and CPEB4 (*right*) knockdown H5Vs, respectively. CPEB1 or CPEB4 knockdown H5Vs also had decreased VEGF protein expression. **(C)** CPEB1, CPEB4 and VEGF mRNA expression (mean $\pm$ SEM of 3 independent experiments) in CPEB1 (*left*) and CPEB4 (*right*) knockdown and control cells, demonstrating effective silencing of CPEB1 or CPEB4 mRNAs, respectively, but not decrease in VEGF mRNA. *P* values (*versus* non-induced cells) for *C-left*: ***P*=0.0012 (CPEB1), ***P*=0.0099 (CPEB4); for *C-right*: ***P*=0.0016. **(D,E)** *In vitro* angiogenesis assay and quantification (mean $\pm$ range of 5 independent biological replicates) in H5Vs seeded on Matrigel. CPEB1 and CPEB4 knockdown cells have diminished ability to form tubular structures compared with non-transfected cells and non-induced cells. This ability was restored by adding conditioned medium or 40ng/ml VEGF. ****P*<0.001, *****P*≤0.0001 *versus* non-induced cells. Scale bars=200μm (CPEB1), 400μm (CPEB4). See **Supplementary-Figure-1**

Figure-2: CPEBs regulate VEGF mRNA processing and cytoplasmic polyadenylation

(A-left,B-left) CPEB1 and CPEB4 immunoprecipitation (IP) analyzed by immunoblotting in H5Vs. IgG was used as control. Relative input amount: 10% of cell lysate used in IP. Both CPEBs specifically coimmunoprecipitated VEGF and CPEB4 mRNAs. **(A-right,B-right)** VEGF and CPEB4 PCR using reversed-transcribed immunoprecipitated mRNAs. GAPDH was used as control. Data in A-B are representative of 3 biological replicates. **(C)** Mouse VEGF 3'UTR representation (see Figure-Legend-1A). **(D)** Representative VEGF 3'UTR cleavage analysis by 3'RACE/Southern-blotting (from 3 biological replicates) in WT *versus* CPEB1 (*left*) or CPEB4 (*middle*) knockdown H5Vs. *Right*: PCR of VEGF and β -galactosidase (GLA) as control. **(E)** *Left*: Translational activity (mean $\pm$ SEM) in H5Vs transfected with synthetic chimeric mRNAs containing Firefly luciferase coding sequence fused upstream of short (PAS1) or long (PAS2) VEGF 3'UTRs. Renilla luciferase activity was used for normalization. $**P=0.0021$ *versus* PAS1. *Right*: Translational activity (mean $\pm$ SEM of 3 independent biological replicates) normalized against relative Firefly and Renilla mRNA levels. $P=0.0022$. **(F)** Representative polyadenylation analysis (from 3 independent biological replicates) of short (PAS1) and long (PAS2) VEGF 3'UTRs in control and CPEB1 (*left*) or CPEB4 (*right*) knockdown cells. PolyA tail length (arrows) was calculated by subtracting the length of the oligodT/RNase-H PCR product (from which polyA tail was removed by RNase-H digestion) from the length of the oligodT/RNase-H non-treated products, as described in Supplementary Methods. See **Supplementary-Figure-2**

Figure-3: CPEBs, VEGF and P-AurKA are overexpressed in human cirrhotic liver

(A) CPEBs, VEGF and phospho-AurKA immunoblotting and protein expression quantification (mean $\pm$ SEM) in healthy human liver and HCV-related cirrhotic liver. $*P=0.003$ (CPEB1), $P=0.012$ (CPEB4), $P=0.002$ (VEGF) and $P=0.006$ (P-AurKA) *versus* healthy liver. **(B)** Representative immunostainings in serial human liver sections (from 5 healthy and 12 cirrhotic independent livers). CPEBs and VEGF expression was low in healthy livers (a-c) but strong in cirrhotic livers (d-f), especially in micronodules (g-i; arrowheads) and at the interface between fibrous septa and

parenchyma (*j-l*; arrowheads). Scale bars=100 μ m (*a-f*), 50 μ m (*g-i*), 25 μ m (*j-l*). **(C)** CPEBs and VEGF immunostaining was also detected in vascular wall of neovessels within fibrous septa (arrowheads). Scale bar=25 μ m. **(D)** H&Es illustrating healthy (*a,b*) and cirrhotic (*c,d*) human liver. Scale bars=200 μ m (*a,c*), 100 μ m (*b,d*). **(E)** Human liver VEGF mRNA expression (mean $\pm$ SEM). See **Supplementary-Figures-3-6, Supplementary-Table-1**.

Figure-4: CPEBs, VEGF and P-AurKA are overexpressed in PH rat mesentery

(A) CPEBs and VEGF immunoblotting and protein expression quantification (mean $\pm$ SEM) in mesentery at different time points after PPVL. * P <0.05 *versus* SHAM rats. **(B)** Representative immunostainings showing CPEBs and VEGF overexpressions in preexisting vessels (*e-h*) and neovessels (*i-l*) in mesentery of PH (PPVL day 3; n =8) *versus* sham-operated control rats (*a-d*; n =6). Scale bars=50 μ m (*a-h*), 15 μ m (*i-l*). Neovessels have a notoriously smaller caliber than preexisting vessels, a thinner vascular wall and are found only in PH mesenteries, but rarely in controls. Preexisting vessels in PH rats also have a typical perivascular hypercellularity compared with equivalent vessels in control rats. **(C)** Mesenteric VEGF mRNA expression (mean $\pm$ SEM). Findings were confirmed with at least 3 independent biological replicates. **(D)** Phospho-AurKA and CPEBs immunoblotting and protein expression quantification (mean $\pm$ SEM) in mesentery at different time points after PPVL. See **Supplementary-Figures-3-7**

Figure-5: CPEBs cooperate to regulate pathological VEGF expression

(A) VEGF PCR using reversed-transcribed immunoprecipitated mRNAs from PH rat mesentery. GAPDH was used as control. Quantitative RT-PCR (arbitrary units) showed a significant enrichment of VEGF mRNA in anti-CPEB1 *versus* pre-immune IgG immunoprecipitate. * P =0.03 **(B)** *Left*: VEGF 3'UTR cleavage analysis by 3'RACE/Southern-blotting in rat mesentery (where VEGF is overexpressed in PH) and intestinal mucosa (where VEGF expression is unaffected by PH). *Right*:

PCR of VEGF and GLA used as control of total VEGF mRNA and general gene expressions for experiment in *B-Left*. **(C)** Polyadenylation analysis of short (PAS1) VEGF 3'UTR in SHAM and PPVL rat mesentery. PolyA tail length is indicated. **(D)** VEGF 3'UTR cleavage analysis by 3'RACE/Southern-blotting in healthy and cirrhotic human livers. **(E) Top:** Polyadenylation analysis of short (PAS1) VEGF 3'UTR in healthy and cirrhotic human livers. Lane corresponding to healthy liver is also shown at higher exposure (asterisk) to compensate the difference in PAS1 usage shown in *D*. PolyA tail length is indicated. *Bottom:* PCR of VEGF and GLA used as control of total VEGF mRNA and general gene expressions for experiment in *E-Top*. Findings were confirmed in 2-3 experimental replicates. See **Supplementary-Figure-8**

Figure-6: CPEB silencing limits VEGF overexpression and pathological angiogenesis

Tamoxifen-treated CPEB1 (n=9) or CPEB4 (n=12) iKO and WT (n=12) mice were subjected to PPVL and studied 8 days later. **(A)** Immunoblotting demonstrating effective CPEB1 depletion, both dimeric and monomeric protein forms,³⁵ in CPEB1 iKO mice, and CPEB4 depletion in CPEB4 iKO mice. **(B)** Immunoblotting and protein expression quantification (mean±SEM), showing mesenteric VEGF protein decrease after CPEBs depletion in PPVL mice. **P*=0.030 (CPEB1 iKO) and *P*=0.028 (CPEB4 iKO) versus WT mice. ECV: ECV304 cell lysate (spontaneously transformed cell line derived from a Japanese HUVEC culture), as positive control for VEGF expression. **(C)** Representative VEGF and von Willebrand factor (vWF; endothelial cell marker) immunostainings in mesentery of WT and iKO mice after PPVL. Arrowheads indicate neovessels. PV, preexisting vessels. Scale bar=50µm **(D)** Quantification of vascular density (vWF+ vessels/mm²; mean±SEM) of neovessels (*left*) and preexisting vessels (*right*) in mouse mesentery. Neovessels (arrowheads in C) readily distinguished from preexisting vessels (PV in C) by their small size and thin vascular wall. **P*<0.001 versus WT mice. **(E)** Extent of portosystemic collateral vessels (%). *P*=0.030 (CPEB1 iKO) and *P*=0.14 (CPEB4 iKO) versus WT mice. **(F) Left:** VEGF 3'UTR cleavage analysis by 3'RACE/Southern-blotting in

mesentery of WT and CPEB1 iKO mice after PPVL. *Middle*: Polyadenylation analysis of short (PAS1) VEGF 3'UTR in mesentery of WT and CPEB4 iKO mice after PPVL. PolyA tail length is indicated. Bands at 90, 110, 240 and 320 nucleotides correspond to polyadenylated VEGF mRNA variants, which disappear when treated with oligodT+RNaseH and are not detected in (-/-) duplicated samples. *Right*: PCR of VEGF and GLA as control of total VEGF mRNA and general gene expressions for experiments in *F-Left* and *F-Middle*. Data in *A*, *B*, *F* were confirmed with at least 2-3 independent biological replicates. See **Supplementary-Figures-3,4,9,10**

Figure-7: CPEB depletion does not affect physiological angiogenesis associated with wound healing, liver regeneration after partial hepatectomy and postnatal development of retinal vasculature. Proposed model

(A) Ratio of wound healing in WT and CPEB4 iKO mice demonstrating that CPEB4 is not required for physiological wound healing. Area was calculated using the formula: $A = \pi \times (D/2) \times (d/2)$ where D=main diagonal and d=minor diagonal. n=number of wounds. Representative images at indicated days after the 4-mm wound are also shown. **(B)** Representative immunostainings for the endothelial cell marker CD31 in paraffin-embedded WT and CPEB4 KO mice liver sections, 48h after partial hepatectomy. Scale bars=200 μ m (*a,c*), 50 μ m (*b,d*). **(C)** *Left*: Quantification of CD31+ area (mean $\pm$ SEM; n=3; $P=0.888$), 48h after partial hepatectomy, showing that physiological angiogenesis associated to liver regeneration after partial hepatectomy was unaffected by CPEB absence in iKO mice. *Middle*: Quantification of liver weight (mean $\pm$ SEM; n=3; $P=0.516$), 48h after partial hepatectomy. *Right*: Quantification of BrdU+ cells (mean $\pm$ SEM; n=3; $P=0.797$), 48h after partial hepatectomy. **(D)** Whole-mount isolectin B4 (grey) labeling of the retinal vasculature of postnatal day 5 (P5) pups, showing reduced vascular branching, but no differences in radial expansion in CPEB4 iKO mice, compared to WT mice. Scale bar=100 μ m. **(E)** *Left*: Quantification of the number of branch points in retinal vasculature (mean $\pm$ SD; n=9; $*P<0.003$). *Right*: Quantification of retinal radial expansion (mean $\pm$ SD;

n=7; * $P=0.1282$), showing that the normal intraretinal vascular development (physiological angiogenesis) that occurs postnatally in rodents was unaffected by CPEB depletion in KO mice. (F) Proposed model: Representation of nuclear alternative (*left*) and cytoplasmic-polyadenylation (*right*), in absence (*top*) and presence (*bottom*) of phospho-CPEB1. CPSF, cleavage-and-polyadenylation-specificity-factor; GLD-2, defective-in-germline-development-2. See **Supplementary-Figures-11,12**

REFERENCES

- 1-Schuppan D, Afdhal NH. Liver cirrhosis. *Lancet* 2008;371:838-851.
- 2-Fernandez M, Semela D, Bruix J, et al. Angiogenesis in liver disease. *J Hepatol* 2009;50:604-620.
- 3-Fernandez M. Molecular pathophysiology of portal hypertension. *Hepatology* 2015;61:1406-1415.
- 4-Mejias M, Garcia-Pras E, Tiani C, et al. Beneficial effects of sorafenib on splanchnic, intrahepatic, and portocollateral circulations in portal hypertensive and cirrhotic rats. *Hepatology* 2009;49:1245-1256.
- 5-Fernandez M, Vizzutti F, Garcia-Pagan JC, et al. Anti-VEGF receptor-2 monoclonal antibody prevents portal-systemic collateral vessel formation in portal hypertensive mice. *Gastroenterology* 2004;126:886-894.
- 6-Sanyal AJ, Bosch J, Blei A, et al. Portal hypertension and its complications. *Gastroenterology* 2008;134:1715-1728.
- 7-Llovet JM, Bruix J. Testing molecular therapies in hepatocellular carcinoma: the need for randomized phase II trials. *J Clin Oncol* 2009;27:833-835.
- 8-Potente M, Gerhardt H, Carmeliet P. Basic and therapeutic aspects of angiogenesis. *Cell* 2011;146:873-887.
- 9-Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003;9:669-676.
- 10-Chang SH, Hla T. Gene regulation by RNA binding proteins and microRNAs in angiogenesis. *Trends Mol Med* 2011;17:650-658.
- 11-Du M, Roy KM, Zhong L, et al. VEGF gene expression is regulated post-transcriptionally in macrophages. *FEBS J* 2006;273:732-745.
- 12-Onesto C, Berra E, Grepin R, et al. Poly(A)-binding protein-interacting protein 2, a strong regulator of vascular endothelial growth factor mRNA. *J Biol Chem* 2004;279:34217-34226.
- 13-Ray PS, Fox PL. A post-transcriptional pathway represses monocyte VEGF-A expression and angiogenic activity. *EMBO J* 2007;26:3360-3372.

- 14-Arcondeguy T, Lacazette E, Millevoi S, et al. VEGF-A mRNA processing, stability and translation: a paradigm for intricate regulation of gene expression at the post-transcriptional level. *Nucleic Acids Res* 2013;41:7997-8010.
- 15-Pique M, Lopez JM, Foissac S, et al. A combinatorial code for CPE-mediated translational control. *Cell* 2008;132:434-448.
- 16-Fernandez-Miranda G, Mendez R. The CPEB-family of proteins, translational control in senescence and cancer. *Ageing Res Rev* 2012;11:460-472.
- 17-D'Ambrogio A, Nagaoka K, Richter JD. Translational control of cell growth and malignancy by the CPEBs. *Nat Rev Cancer* 2013;13:283-290.
- 18-Weill L, Belloc E, Bava FA, et al. Translational control by changes in poly(A) tail length: recycling mRNAs. *Nat Struct Mol Biol* 2012;19:577-585.
- 19-Igea A, Mendez R. Meiosis requires a translational positive loop where CPEB1 ensues its replacement by CPEB4. *EMBO J* 2010;29:2182-2193.
- 20-Novoa I, Gallego J, Ferreira PG, et al. Mitotic cell-cycle progression is regulated by CPEB1 and CPEB4-dependent translational control. *Nat Cell Biol* 2010;12:447-456.
- 21-Afroz T, Skrisovska L, Belloc E, et al. A fly trap mechanism provides sequence-specific RNA recognition by CPEB proteins. *Genes Dev* 2014;28:1498-1514.
- 22-Belloc E, Mendez R. A deadenylation negative feedback mechanism governs meiotic metaphase arrest. *Nature* 2008;452:1017-1021.
- 23-Mendez R, Hake LE, Andresson T, et al. Phosphorylation of CPE binding factor by Eg2 regulates translation of c-mos mRNA. *Nature* 2000;404:302-307.
- 24-Mendez R, Murthy KG, Ryan K, et al. Phosphorylation of CPEB by Eg2 mediates the recruitment of CPSF into an active cytoplasmic polyadenylation complex. *Mol Cell* 2000;6:1253-1259.
- 25-Bava FA, Eliscovich C, Ferreira PG, et al. CPEB1 coordinates alternative 3'-UTR formation with translational regulation. *Nature* 2013;495:121-125.

- 26-Ortiz-Zapater E, Pineda D, Martinez-Bosch N, et al. Key contribution of CPEB4-mediated translational control to cancer progression. *Nat Med* 2012;18:83-90.
- 27-Williams RL, Risau W, Zerwes HG, et al. Endothelioma cells expressing the polyoma middle T oncogene induce hemangiomas by host cell recruitment. *Cell* 1989;57:1053-1063.
- 28-**Grunewald M, Avraham I**, Dor Y, et al. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell* 2006;124:175-189.
- 29-Sarkissian M, Mendez R, Richter JD. Progesterone and insulin stimulation of CPEB-dependent polyadenylation is regulated by Aurora A and glycogen synthase kinase-3. *Genes Dev* 2004;18:48-61.
- 30-Berzigotti A, Erice E, Gilabert R, et al. Cardiovascular risk factors and systemic endothelial function in patients with cirrhosis. *Am J Gastroenterol* 2013;108:75-82.
- 31-Mejias M, Garcia-Pras E, Gallego J, et al. Relevance of the mTOR signaling pathway in the pathophysiology of splenomegaly in rats with chronic portal hypertension. *J Hepatol* 2010;52:529-539.
- 32-La Mura V, Reverter JC, Flores-Arroyo A, et al. Von Willebrand factor levels predict clinical outcome in patients with cirrhosis and portal hypertension. *Gut* 2011;60:1133-1138.
- 33-**Alexandrov IM, Ivshina M**, Jung DY, et al. Cytoplasmic polyadenylation element binding protein deficiency stimulates PTEN and Stat3 mRNA translation and induces hepatic insulin resistance. *PLoS Genet* 2012;8:e1002457.
- 34-**Tsai LY, Chang YW, Lin PY**, et al. CPEB4 knockout mice exhibit normal hippocampus-related synaptic plasticity and memory. *PLoS One* 2013;8:e84978.
- 35-Lin CL, Huang YT, Richter JD. Transient CPEB dimerization and translational control. *RNA* 2012;18:1050-1061.

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Sequential Functions of CPEB1 and CPEB4 Regulate Pathologic Expression of VEGF and Angiogenesis in Chronic Liver Disease

SUPPLEMENTARY INFORMATION

- Supplementary Figure Legends
- Supplementary Table
- Supplementary Materials and Methods
- Supplementary References

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: CPEBs are required for VEGF expression and angiogenesis

H5V cells constitutively expressing control shRNA, CPEB1 shRNA or CPEB4 shRNA were generated as described in "Supplementary Materials and Methods". **(A)** Expression of VEGF splicing forms by RT-PCR in H5V cells, mouse mesentery, rat mesentery and human liver. Different splicing isoforms are indicated with arrowheads. PCR (35 cycles; 65°C annealing temperature; 1 min elongation time) was performed using previously described mouse, rat and human VEGF primers.³⁶⁻³⁸ **(B)** Primers used for the RT-PCR shown in A. **(C)** Immunoblotting in sh- and CPEB1sh+ or CPEB4sh+ H5V cells, with 10 min film exposure. Size of bands corresponding to VEGF₁₂₀ and VEGF₁₆₄ is consistent with RT-PCR results shown in A. Both VEGF₁₂₀ and VEGF₁₆₄ isoforms are downregulated in absence of CPEBs. VEGF₁₂₀ is visible only upon overloading and overexposure, meaning that its abundance is most likely negligible compared with VEGF₁₆₄. **(D)** Characterization of the effects of mutated-CPEB4 expression in CPEB4 knockdown H5V cells. CPEB4 and VEGF protein expression (*left*) and relative quantification (*right*) in control and CPEB4 iKO H5V cells. Tubulin was used as loading control. **(E)** *In vitro* angiogenesis assay (*left*) and its quantification (*right*) performed seeding CPEB4 knockdown H5V cells on grow factor reduced (GFR) Matrigel-coated dishes. Not induced cells were used as control. Tube formation scoring was performed as described in "Supplementary Materials and Methods". *P* values *versus* control shRNA: **P*=0.016; ***P*=0.0053. **(F)** *In vitro* characterization of CPEB1 depletion effects in H5V cells. CPEB1 and VEGF protein expression in control and CPEB1 knockdown cells, analyzed by immunoblotting. Tubulin is used as loading control. **(G)** CPEB1 and VEGF mRNA expression levels measured in control and CPEB1 knockdown cells. *P* value *versus* control shRNA: ***P*=0.0017. **(H)** *In vitro* angiogenesis assay (*left*) and its quantification (*right*) performed in CPEB1 knockdown H5V cells seeded on GFR Matrigel-coated dishes. Cells transfected with control shRNAs were used as control. Tube formation scoring was performed as described in "Supplementary

Materials and Methods". *P* value versus control shRNA: *** $P < 0.001$. Data in *D-H* are representative of 3 independent experiments and are shown as mean $\pm$ range (in *E* and *H*) and mean $\pm$ SEM. (in *G*). Scale bars, 200 μ m.

Supplementary Figure 2: CPEB1 and CPEB4 regulate VEGF mRNA processing and cytoplasmic polyadenylation

(A) Cell growth analysis in mouse embryonic fibroblasts (MEFs),³⁹ WT or CPEB1 or CPEB4 KOs, demonstrating that depletion of CPEB1 or CPEB4 does not affect cell viability. Data are mean $\pm$ SEM. For statistical analysis, ANOVA test was performed. **(B)** Simplified representation of mouse CPEB4 3'UTR. CPE (Cytoplasmic Polyadenylation Elements) and PAS (Poly Adenylation Signal) are indicated in white polygons (consensus sequences) or grey polygons (not consensus sequences). PAS1 and PAS2 indicate proximal and distal Poly Adenylation Signals, respectively. miR-26 and miR-92 binding sites are indicated with arrowheads. Single headed arrows indicate the position of the primers used for quantitative PCR analysis. Dashed arrows indicate the position of the primer used for 3'RACE analysis. Black and white rectangles indicate the primers used as radiolabeled probes for Southern blotting. **(C)** Analysis of CPEB1 depletion effects on CPEB4 3'UTR formation in H5V cells. Relative expression levels of CPEB4 mRNA measured in control and CPEB1 knockdown H5V cells (*top*). Relative expression levels of long CPEB4 3'UTR (obtained using PAS2) normalized by total CPEB4 mRNA levels (*bottom*). *P* values (obtained by Student *t* Test) versus not induced cells: ** $P = 0.0045$ (*top*), ** $P = 0.0091$ (*bottom*). Data, corresponding to 3 independent experiments, are mean $\pm$ SEM. **(D)** Analysis of the effects of CPEB1 depletion on CPEB4 3'UTR formation in H.D. My-Z cells. CPEB4 3'UTR cleavage analysis performed by 3'RACE/Southern blotting in CPEB1 knockdown H.D. My-Z cells. **(E)** CPEB1 and CPEB4 immunofluorescence in H5V cells, 12 and 24 h after plating. DNA staining (Dapi) in blue, CPEB1 or CPEB4 in green, and the merged signal are shown. CPEB1 shows both nuclear and cytoplasmic localization while CPEB4 is present only in the

cytoplasm of the cells. Scale bar, 10 μm . **(F)** Nuclear localization of CPEB1. H5V cells were transfected with a vector to express a Flag-tagged version of CPEB1 and treated or not with the nuclear export inhibitor leptomycin B (LMB) at 10 ng/ml for 20 hours. Immunofluorescence staining with anti-CPEB1 (Santa Cruz H300 and Abcam) and -Flag antibodies of CPEB1-overexpressing cells, showing nuclear retention of CPEB1 upon LMB treatment. Scale bars: 10 μm . **(G)** Nuclear localization of CPEB1. Subcellular fractionation and western blotting of the same cells treated with LMB described in panel F. Nuclear and cytoplasmic fractions were prepared using the NE-PER® Nuclear and Cytoplasmic Extraction reagents (Thermo Scientific) as per manufacturer's instructions. Flag-CPEB1 and endogenous CPEB1 are present in the cytoplasmic and nuclear fractions. Histone H3 serves as nuclear marker. T: total protein extract; C: Cytoplasmic fraction; N: Nuclear fraction; *: unspecific band.

Supplementary Figure 3: Negative controls for immunohistochemistry and immunofluorescence

(A) Negative controls for immunohistochemistry: Representative micrographs of human and rat liver incubated with rabbit IgG as a control for non-specific binding of primary antibodies. Images show no staining. Scale bars: 25 μm (human liver) and 50 μm (rat liver). **(B)** Negative controls for immunohistochemistry: Representative micrographs of rat and mouse mesentery incubated with rabbit or mouse IgG as a control for non-specific binding of primary antibodies. Images show no staining. Scale bars: 100 μm (rat mesentery/rabbit IgG) and 50 μm (rat mesentery/mouse IgG and mouse mesentery/rabbit IgG). **(C)** Negative controls for immunofluorescence: Representative micrographs of rat mesentery incubated with fluorescent secondary antibodies Alexa 647 and Alexa 555 together, rabbit IgG with Alexa 647 and mouse IgG with Alexa 555. Images show no fluorescent signal. Scale bar: 15 μm .

Supplementary Figure 4: Validation of CPEB1 and CPEB4 antibodies for immunohistochemistry and specificity of anti-phospho-CPEB1 antibody

(A) Immunodetection of CPEB1 in testicles of 12 wks-old mice. CPEB1 is expressed in Leydig and germ cells. (B) Immunodetection of phospho-CPEB1 in testicles of 12 wks-old mice. Phospho-CPEB1 expression is restricted to spermatocytes (arrows). (A,B) Testis from knock-out mice were used as negative controls. Scale bar: 50 μ m in panels and 25 μ m in insets. (C) Immunodetection of CPEB4 protein in the nervous system (ganglia) of embryos at E13.5. Knock-out embryos were used as negative controls. Scale bars: 50 μ m in panels and 25 μ m in inset. (D) Specificity of anti-phospho-CPEB1 antibody: The antibody against phosphorylated CPEB1 (483B9_{d4d12}) is a monoclonal antibody that we raised using RGSRLDT(p)RPILDS(p)RSC peptide as antigen. Then, the clones were selected for supernatants that, in ELISA, recognized this peptide, but not the unphosphorylated counterpart RGSRLDTRPILDSRSC. The antibody does not recognize recombinant CPEB1 expressed in bacteria. However, it recognizes a CPEB1 comigrating band in H5V cells (where CPEB1 is phosphorylated and active), but not in RPE cells, where CPEB1 acts as a translational repressor (i.e., is not phosphorylated). A monoclonal antibody that recognizes both the phosphorylated and unphosphorylated peptides detects CPEB1 in both H5V and RPE cells. The phosphorylation of CPEB1 corresponds to activatory residues Thr-171 and Ser-176, which are phosphorylated by Aurora Kinase A.^{23,24,29}

Supplementary Figure 5: CPEBs and VEGF are overexpressed in rat cirrhotic liver

(A) Histopathological features of the BDL model of cirrhosis. Sirius red staining in formalin-fixed paraffin-embedded liver sections from BDL rats at days 7, 14 and 28 after BDL, and sham-operated control animals (SHAM) (n=6 per group), showing the progressive increase in fibrosis and collagen deposition in the injured liver with disease development. Scale bars, 200 μ m. (B) Representative photomicrographs from H&E-stained cirrhotic rat liver (BDL day 28) displaying a robust disruption in

architecture of the entire liver with extensive bile ductular proliferation and formation of parenchymal micronodules of regenerating hepatocytes (arrows in micrograph at the bottom). Scale bars, 25 μm (*bottom*), 50 μm (*top*). **(C)** CPEB1, CPEB4 and VEGF immunoblotting in the rat liver at different time points after BDL (n=6 at each time point) and in the liver from SHAM control rats (n=6). GAPDH is used as a loading control. Densitometric quantification (mean $\pm$ SEM) of protein expression relative to GAPDH is also shown. These time courses show that CPEB1, CPEB4 and VEGF proteins increase early during disease progression after bile duct obstruction, in a stage in which fibrosis, but not yet cirrhosis, is fully developed (A). Therefore, it is likely that CPEBs also regulate VEGF expression in the course of fibrogenesis, consistent with the notion that fibrogenesis and angiogenesis are two closely linked pathological processes associated with liver disease. * $P < 0.05$ versus control rats. **(D)** Representative CPEB1, CPEB4 and VEGF immunohistochemistry in rat control (SHAM; n=6) liver and in liver from cirrhotic rats at day 28 after BDL (n=6). Micrographs from SHAM rats (*a-c*) show the normal liver parenchyma, with plates of hepatocytes radially disposed in the hepatic lobule surrounding a central vein. In the cirrhotic liver (*d-f*), numerous rounded micronodules of regenerating hepatocytes are detected, which strongly express CPEB1, CPEB4 and VEGF proteins. Micrographs *g-i* illustrate a low magnification area of the cirrhotic liver. Micrographs *j-l* show a higher magnification of the areas defined by a rectangle in micrographs *g-i*, focusing on the new blood microvessels formed in the liver in response to cirrhosis induction and demonstrating expression of CPEB1, CPEB4 and VEGF in the endothelium of newly formed vessels. *n*: blood neovessels. Scale bars, 25 μm (*j-l*), 50 μm (*a-f*), 100 μm (*g-i*). **(E)** Quantification (mean $\pm$ SEM) of relative VEGF mRNA expression by RT-PCR showing that, at least at the initial stages of angiogenesis, VEGF overexpression in the cirrhotic liver did not correlate with parallel changes in VEGF mRNA. In advanced disease, at day 28 after BDL, both VEGF protein and mRNA were elevated. These results suggest that VEGF overexpression in cirrhotic rat liver is post-transcriptionally regulated during first disease stages, but later on, in advanced cirrhosis, both transcriptional and post-transcriptional mechanisms may coordinately regulate VEGF

expression. * $P < 0.05$ versus control rats.

Supplementary Figure 6: Morphological differences between preexisting and new vessels in liver and mesentery

(A) Liver: Representative photomicrographs of VEGF immunostaining in cirrhotic rat liver (BDL day 28). Micrograph "a" illustrates a low magnification area of the cirrhotic liver (scale bar: 100 μm), with an inset area that is magnified in micrograph "b" (scale bar: 50 μm). Micrograph "c" shows a higher magnification (scale bar: 25 μm) of the area defined by a rectangle in micrograph "b", focusing on the new blood microvessels formed in the liver in response to cirrhosis induction and demonstrating expression of VEGF in the endothelium of newly formed vessels. In the liver, we differentiate neovessels from preexisting vessels based on their distinct vessel size (comparison between preexisting central vein or portal vein and the small caliber neovessels). Further, these small intrahepatic neovessels are present in the rat livers upon cirrhosis induction (closely associated with portal tracts and fibrous septa), but not in the control liver. **(B) Mesentery:** Photomicrographs of H&E-stained mesentery sections showing extensive mesenteric neovascularization in portal hypertensive rats, which is evident already at day 3 after inducing portal hypertension. Micrograph "a" is a high magnification photograph of the picture shown in Supplementary Figure 7A "Portal hypertension". Micrographs "b" and "c" are in turn high magnification photographs of the area defined by a rectangle in micrograph "a", focusing on neovessels. Scale bars: 50 μm in micrograph "a", 25 μm in micrograph "b" and 10 μm in micrograph "c". In the mesentery, we differentiate neovessels from preexisting vessels based on: First, their different vessel caliber. This vascular size is notoriously higher in the preexisting arterioles and venules of the mesentery than in the newly formed microvessels upon portal hypertension/cirrhosis induction. Second, the morphology and relative thickness of the vascular wall of newly formed blood vessels can be easily differentiated from that of mature preexisting blood vessels. This difference is even more prominent in the context of portal hypertension/cirrhosis because of the

perivascular hypercellularity that surrounds preexisting mesenteric vessels during the pathological angiogenic process associated with disease progression. Third, after induction of portal hypertension/cirrhosis, the mesentery is populated by many new microvessels, which are not present in the normal mesentery, and which as stated above are smaller and with thinner walls than the preexisting vasculature.

Supplementary Figure 7: *In vivo* studies in portal hypertensive rat mesentery

(A) Photomicrographs of H&E-stained mesentery sections showing preexisting vessels (preexisting arteriole and preexisting venule) and also extensive mesenteric neovascularization (arrowheads point to neovessels) in portal hypertensive rats, which is evident already at day 3 after inducing portal hypertension by partial portal vein ligation (PPVL) (micrograph at the right), compared with sham-operated control rats (micrograph at the left). These neovessels are functional and perfused, as shown by their vascular integrity and the presence of erythrocytes in their lumina. Additionally, previous studies from our group have demonstrated that the mesenteric blood flow increases in parallel with the development of mesenteric neovasculature, and that inhibition of VEGF-dependent angiogenesis in the mesenteric vascular bed of portal hypertensive and cirrhotic animals translates into a marked and significant decrease in mesenteric blood flow,^{4,5} further demonstrating that the neovasculature developed in the mesentery upon portal hypertension/cirrhosis induction is functional and contributes to the pathological increase in mesenteric blood flow. Scale bar, 100 μ m. **(B)** Quantification of the magnitude of mesenteric neovascularization, performed by morphometric analysis, over an observation period of 20 days after inducing portal hypertension, and compared with control rats. Angiogenic activity in the mesentery increases early after portal hypertension induction, remains maximal during the first week, and then reaches a plateau. The time course of mesenteric neovascularization progression is therefore consistent with the kinetics of VEGF (and CPEBs) overexpression in the portal hypertension model (Figure 4A). Thus, the promoting factor for

angiogenesis VEGF (and CPEBs) are predominant in the first disease stages, coincident with the period of more active growing of new blood vessels in the mesentery, further documenting the importance of VEGF overexpression as an inducing signal for turning on the initial pathologic angiogenic switch. In advanced disease stages, VEGF (and CPEBs) levels decrease, although they are still higher than in normal, corresponding with a period in which angiogenesis plateau and portal hypertension is already well established. Results are expressed as number of vessels per square millimeter (mean $\pm$ SEM.). * P <0.05 *versus* sham-operated control rats. **(C)** Mesenteric neovessel fate in portal hypertensive rats. Representative photomicrographs from immunohistochemical analyses in mesenteric sections from portal hypertensive rats at day 3 (micrographs at the top) or day 20 (micrographs at the bottom) after PPVL, showing expression of von Willebrand factor (vWF, a classic endothelial cell marker) and alpha-smooth muscle actin (α SMA, smooth muscle cell marker) in the vascular wall of preexisting vessels and neovessels. These findings demonstrate that the newly formed vessels upon portal hypertension induction present vascular integrity (vascular walls with endothelial cells covered by pericytes). Masson's trichrome staining also shows that neovessels were surrounded by collagen extracellular matrix, further demonstrating that the vascular wall of the neovessels was properly developed. Immunostaining for the cell proliferation marker Ki67 shows increased cell proliferation in the angiogenic endothelium of newly formed vessels at day 3 after portal hypertension induction (arrowheads point to Ki67+ proliferating endothelial cells in the vascular wall of neovessels), consistent with the finding that during the first week after PPVL, there is a high angiogenic activity in the mesentery with neovessels actively developing (B). At day 20 after PPVL, however, Ki67 immunostaining shows almost complete absence of cell proliferation in preexisting mesenteric vessels and neovessels at this advanced disease time point, consistent again with the kinetic of angiogenic activity. Scale bars, 25 μ m (neovessels), 50 μ m. **(D)** Double-immunofluorescence and confocal microscopy showing cells double-expressing CPEB1 proteins (green) and VEGF (red) in the vascular wall of preexisting mesenteric vessels and neovessels after

portal hypertension induction in rats (PPVL day 3; n=6). Cell nuclei were visualized by DAPI (4',6'-diamidino-2-phenylindole) (blue). Photographs show the merge of CPEB1, VEGF and DAPI stainings. Photograph at the left show a preexisting vessel and presence of small neovessels in the vascular periphery (scale bar, 50 μ m). Photograph at the right show a high magnification of a representative neovessel. Note the presence of cells co-expressing CPEB1 and VEGF mainly in the endothelium and adventitia of preexisting vessels. In the neovessels, CPEB1 and VEGF coexpression was observed in endothelial cells and pericytes. **(E)** Double-immunofluorescence and confocal microscopy showing cells double-expressing CPEB4 proteins (green) and VEGF (red) in the vascular wall of preexisting mesenteric vessels and neovessels in the mesenteric vascular bed of portal hypertensive rats (PPVL day 3; n=6). Cell nuclei were visualized by DAPI (blue). Photographs show the merge of CPEB4 proteins with VEGF and DAPI stainings. Photograph at the left show a preexisting vessel and presence of small neovessels in the vascular periphery (scale bar, 50 μ m). Photograph at the right show a high magnification of the area defined by a rectangle in the micrograph at the left, focusing on new blood microvessels. Note the presence of cells co-expressing CPEB4 and VEGF in the endothelium and adventitia of preexisting vessels, and in the vascular wall of neovessels.

Supplementary Figure 8: Lack of CPEB upregulation in intestinal mucosa, in which pathological VEGF overexpression is also not observed in portal hypertension

Immunoblotting of CPEB1, CPEB4 and VEGF proteins in the intestinal mucosa (and in the mesentery as control) at different time points after PPVL.

Supplementary Figure 9: Generation of CPEB1 and CPEB4 conditional KO mice

(A) Schematic representation of Cpeb1 alleles used in this study. The mouse Cpeb1 locus encoding CPEB1 contains 12 exons (boxes), including noncoding (open boxes) or protein-coding (grey boxes) sequences. *loxP* (black triangles) and FRT (white triangles) sites were used to flank exon4 or the neo-

resistance cassette (black). The neo cassette was deleted by expressing FlpO in the recombinant ES cells.²² Further excision of exon4 was achieved by Cre mediated DNA recombination leading to a frame-shift and loss of function $Cpeb1^{-}$ or Δ allele. Inducible activation of Cre by tamoxifen turns the conditional $Cpeb1^{lox}$ allele into the deleted $Cpeb1^{\Delta}$ allele. HR, homologous recombination. **(B)** Southern blot analysis of recombinant embryonic stem (ES) cells showing a clone that underwent HR. DNA was digested with XhoI and KpnI and hybridized with the 5' and 3' probes, respectively. Position of both probes is shown in A. **(C)** PCR amplification of WT (+), conditional (lox) and null (-) $Cpeb1$ alleles using oligonucleotides represented by arrows in A. **(D)** Schematic representation of the $Cpeb4$ alleles used in this study. The mouse $Cpeb4$ locus, encoding CPEB4, contains 9 exons (boxes) including noncoding (open boxes) or protein-coding (grey boxes) sequences. loxP (black triangles) and FRT (white triangles) sites were used to flank exon2 or the β geo cassette (blue), which encodes for β -galactosidase and the neo-resistance gene. The β geo cassette contains polyA sequences for termination of transcription and is preceded by a spliced acceptor so that, expression of β -galactosidase and the neo-resistance gene is driven by the CPEB4 regulatory elements. The β geo cassette was subsequently deleted by mating $Cpeb4^{+/loxfrt}$ with Flp mice.⁴⁰ Further excision of exon2 was achieved by Cre mediated DNA recombination leading to a frame-shift and loss of function $Cpeb4^{-}$ or Δ allele. Inducible activation of Cre by tamoxifen turns the conditional $Cpeb4^{lox}$ allele into the deleted $Cpeb4^{\Delta}$ allele. HR, homologous recombination. **(E)** Southern blot analysis from genomic DNA of $Cpeb4^{+/loxfrt}$ mice. DNA was digested with AvrII and hybridized with the 5' and 3' probes. The position of both probes is shown in D. **(F)** PCR amplification of WT (+), conditional (lox) and null (-) $Cpeb4$ alleles using oligonucleotides represented by arrows in D. **(G)** Representative photomicrographs of mesentery sections immunostained for CPEB1 (*top*) or CPEB4 (*bottom*) from WT sham-operated control mice (WT-SHAM), from WT portal hypertensive mice, 8 days after partial portal vein ligation (WT-PPVL), and from CPEB1 and CPEB4 iKO mice (CPEB1 iKO-PPVL and CPEB4 iKO-PPVL). Scale bars, 50 μ m.

Supplementary Figure 10: Effects of CPEB depletion on disease progression

Tamoxifen-treated CPEB1 (n=9) or CPEB4 (n=12) iKO and WT (n=12) mice were subjected to PPVL to induce portal hypertension and studied 8 days later. **(A)** Immunoblotting for the endothelial cell marker CD34 in the mesentery of CPEB4 KO PPVL mice and WT PPVL mice. GAPDH is used as a loading control. Densitometric quantification (mean±SEM) of protein expression relative to GAPDH is also shown. * $P<0.05$. **(B)** Blood flow in the superior mesenteric artery (ml/min/10 g body weight), as an estimation of splanchnic blood flow, in CPEB4 KO PPVL mice and WT PPVL mice. $P=0.18$. **(C)** Immunoblotting for the surrogate of portal hypertension severity,³² von Willebrand factor (vWF) in the plasma of CPEB1 KO PPVL mice, CPEB4 KO PPVL mice and WT PPVL mice. Ponceau staining is used to check gel loading. Densitometric quantification (mean±SEM) of relative protein expression is also shown. **(D)** Effects of CPEB depletion on splenomegaly. Measurement of the spleen weight per body weight ratio, which is a measure of spleen size and an indirect assessment of portal hypertension,^{30,31} in WT mice, and CPEB1 and CPEB4 iKO mice, 8 days after PPVL, and also in WT mice subjected to a sham operation (SHAM). WT mice exhibited spleen enlargement after PPVL, compared with WT-SHAM mice. The increased splenic to body weight ratio of PPVL mice was significantly prevented by CPEB1 or CPEB4 depletion. Data are as mean±SEM. * $P=0.005$ versus WT-SHAM, ** $P=0.01$ versus WT-PPVL, *** $P=0.008$ versus WT-PPVL. **(E)** Effects of CPEB depletion on splenic immunological function. Immunoblotting showing that the expression of lymphocyte-derived immunoglobulin G-class antibodies, which is known to be increased in portal hypertension due to enlargement and hyperactivation of the splenic white pulp,³¹ was reduced in the spleen of PPVL mice after CPEB4 depletion. GAPDH is used as loading control. Densitometric quantification (mean±SEM) of protein expression relative to GAPDH is also shown. $P=0.17$.

Supplementary Figure 11: CPEB depletion does not induce tissue injury in portal hypertensive mice

Tamoxifen-treated CPEB1 (n=9) or CPEB4 (n=12) iKO and WT (n=12) mice were subjected to PPVL to induce portal hypertension and studied 8 days later. **(A) Left:** Immunoblotting showing that the expression of the potent proinflammatory cytokine tumor necrosis factor- α (TNF α) is not significantly increased in the mesentery of portal hypertensive mice (PPVL), compared with sham-operated control mice (SHAM). *Middle:* Immunoblotting showing that CPEB1 depletion does not induce overexpression of TNF α in mouse mesentery after PPVL, compared with wild-type (WT) PPVL mice. *Right:* Immunoblotting showing that CPEB4 depletion does not induce overexpression of TNF α in mouse mesentery after PPVL, compared with WT PPVL mice. Densitometric quantifications (mean $\pm$ SEM) of protein expression relative to β -actin or GADPH are also shown. **(B) Micrographs in the left column:** H&E staining in mouse mesentery indicating that CPEB1 and CPEB4 depletion does not induce proinflammatory cell infiltration (inflammatory cells identified with hematoxylin blue staining on H&E) after PPVL, compared with WT PPVL mice. *Micrographs in the middle column:* Immunohistochemistry for inducible nitric oxide synthase (iNOS), which is a a major source of oxidative stress, showing that CPEB1 and CPEB4 depletion does not induce detectable iNOS overexpression in PPVL mouse mesentery, compared with WT PPVL mice. *Micrographs in the right column:* Magnifications of the areas outlined in micrographs at the middle. **(C)** Measurement of the body weight (mean $\pm$ SEM) in WT mice, and CPEB1 and CPEB4 iKO mice, 8 days after PPVL, and also in WT mice subjected to a sham operation (SHAM; n=4). CPEB depletion did not induce reduced body weight gain in mice.

Supplementary Figure 12: *In vivo* and *in vitro* assays in CPEB constitutive KO mice

(A) Representative H&E staining (a-r), immunofluorescence for the endothelial cell marker CD31 (d-s), and DAPI staining of cell nuclei (e-t) in sections from VEGF-containing (f-t) or non-containing (a-e)

Matrigel plugs injected subcutaneously into WT mice (*a-j*; *n*=5) and CPEB1 (*k-o*; *n*=5) and CPEB4 (*p-t*; *n*=5) constitutive KO mice. Higher magnification of the boxed areas in *a*, *f*, *k* and *p* are shown in *b*, *g*, *l* and *q*, respectively. Line in photographs *a*, *f*, *k* and *p* indicates the boundary between flank muscle and explanted Matrigel. Scale bars, 20 μm (*c,h,m,r*), 50 μm (*a,b,f,g,k,l,p,q*), 100 μm (*d,e,i,j,n,o,s,t*). **(B)** CD31 expression was analyzed with fluorescence microscopy and Image J software, and expressed as the strength of fluorescence in Matrigel plug of 5 independent experiments (mean $\pm$ SEM). **P* values versus control (Matrigel plugs non-containing VEGF injected into WT mice): *P*=0.004 (WT+VEGF), *P*=0.048 (CPEB1 KO+VEGF), *P*=0.0012 (CPEB4+VEGF). **(C)** CPEB1 and CPEB4 immunoblotting in testicles from WT, CPEB1 and CPEB4 constitutive KO mice demonstrating effective depletion of both CPEB1 and CPEB4 proteins in the CPEB1 and CPEB4 constitutive KO mice, respectively, and reduction of CPEB4 expression in the absence of CPEB1 in the CPEB1 KO mice. **(D)** Representative H&E staining in sections from Matrigel plugs, non-containing VEGF, injected subcutaneously into CPEB1 (*n*=5) and CPEB4 (*n*=5) constitutive KO mice. As expected, the *in vivo* plug assay did not show any vascularization in absence of VEGF regardless of CPEB levels. Scale bar, 50 μm . **(E)** Sprouting assay. Representative bright field images of VEGFA-stimulated sprouts outgrowth and quantification (mean $\pm$ SEM) in spheroids of WT, CPEB1 KO and CPEB4 KO mouse lung endothelial cells, showing no significant difference between groups. Scale bar, 50 μm . **(F)** Immunoblotting for the endothelial cell marker VE-cadherin in spheroids of WT, CPEB1 KO and CPEB4 KO mouse lung endothelial cells from the sprouting assays shown in panel E. We found no significant differences between groups, in agreement with the observed absence of significant changes in the quantification of VEGFA-stimulated sprouts outgrowth in these spheroids (*E*). **(G)** Cell migration assay. Representative images and quantification (mean $\pm$ SEM) of migrated mouse lung endothelial cells after 16 and 24 h showing that CPEB1 and CPEB4 are not required for endothelial cell migration. Scale bar, 100 μm . **(H)** Aortic ring assay. Representative micrographs of VEGFA-stimulated microvessel outgrowth and quantification (mean $\pm$ SEM) in mouse aortic ring explants of WT, CPEB1 KO and

CPEB4 KO mice, showing no significant differences between groups. Scale bar, 50 μ m

SUPPLEMENTARY TABLE

Supplementary Table 1: Clinical data of the patients at the moment of liver sampling. Liver samples were obtained from different patients (n=12) with hepatitis C virus-related cirrhosis and portal hypertension. The following table summarizes the main characteristics of the patients [HVPG, hepatic venous pressure (equivalent of portal pressure gradient in cirrhosis)].

Gender and age	Source of liver sample	Stage of the disease and Child-Pugh score	HVPG	Histology description
Female, 62 y/o	Liver biopsy	Compensated cirrhosis; no esophageal varices; Child A5	7.5 mmHg	Micronodular cirrhosis with mild parenchymal and periseptal necroinflammatory activity
Male, 60 y/o	Liver biopsy	Compensated cirrhosis; no esophageal varices; Child A5	9 mmHg	Micronodular cirrhosis with parenchymal necroinflammatory activity and mild macrovesicular steatosis
Female, 62 y/o	Liver biopsy	Compensated cirrhosis; small esophageal varices; Child A5	15 mmHg	Micronodular cirrhosis with mild parenchymal necroinflammatory activity and mild macrovesicular steatosis
Female, 55 y/o	Explanted liver on transplantation	Decompensated cirrhosis; large esophageal varices; Child B9	18 mmHg	Micro- and macronodular cirrhosis with focal parenchymal necroinflammatory activity
Female, 58 y/o	Explanted liver on transplantation	Decompensated cirrhosis; no varices; Child C11	24 mmHg	Micronodular cirrhosis with minimal parenchymal necroinflammatory activity
Female, 59 y/o	Explanted liver on transplantation	Compensated cirrhosis; no varices; Child B9	N/A	Macronodular cirrhosis with marked parenchymal necroinflammatory activity and mild macrovesicular steatosis. Concomitant hepatocellular carcinoma
Male, 63 y/o	Explanted liver on transplantation	Decompensated cirrhosis; large esophageal varices; Child B9	18.5 mmHg	Micronodular cirrhosis without parenchymal necroinflammatory activity; marked perinodular biliary duct proliferation
Male, 50 y/o	Explanted liver on transplantation	Decompensated cirrhosis; small esophageal varices; Child C10	20.5 mmHg	Cirrhosis predominantly micronodular without parenchymal necroinflammatory activity; moderate lymphocytic periseptal infiltrate
Male, 63 y/o	Explanted liver on transplantation	Decompensated cirrhosis; large esophageal varices; Child B9	18.5 mmHg	Micronodular cirrhosis without parenchymal necroinflammatory activity; marked perinodular biliary duct proliferation
Male, 50 y/o	Explanted liver on transplantation	Decompensated cirrhosis; small esophageal varices; Child C10	20.5 mmHg	Cirrhosis predominantly micronodular without parenchymal necroinflammatory activity; moderate lymphocytic periseptal infiltrate
Male, 56 y/o	Explanted liver on transplantation	Decompensated cirrhosis; small esophageal varices; Child B7	13 mmHg	Micronodular cirrhosis with marked periseptal and parenchymal necroinflammatory activity

Male, 42 y/o	Explanted liver on transplantation	Decompensated cirrhosis; small esophageal varices; Child C11	12 mmHg	Micronodular cirrhosis with absence of necroinflammatory activity
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SUPPLEMENTARY MATERIALS AND METHODS

Animal models of cirrhosis and portal hypertension

Secondary biliary cirrhosis was induced in male Sprague-Dawley rats by common bile duct ligation (BDL).⁴ While each animal was under anesthesia (100-mg/kg ketamine plus 5-mg/kg midazolam), a midline abdominal incision was made. The common bile duct was isolated, and doubly ligated with 5-0 silk. The first ligature was made below the junction of the hepatic ducts and the second ligature was made above the entrance of the pancreatic ducts. The portion of the bile duct between the two ligatures was resected to avoid repermeabilization.

Portal hypertension was induced in male Sprague Dawley rats and C57BL6 or mixed C57BL6/129 mice by partial portal vein ligation (PPVL).^{4,5} Under anesthesia (ketamine plus midazolam in rats, isoflurane in mice), a midline abdominal incision was made. The portal vein was dissected free of surrounding tissue, and a loose ligature of silk suture (size 3-0 for rats, and 5-0 for mice) was guided around it. A blunt-end needle (20-gauge in rats, and 27-gauge in mice) was placed alongside the portal vein, and the suture was tied snugly around the portal vein and needle. The needle was subsequently removed to yield a calibrated constriction of the portal vein.

In SHAM animals, bile duct or portal vein were similarly manipulated but not ligated.

Generation of CPEB1 and CPEB4 conditional knockout (KO) mice

CPEB1-targeted mice. Conditional targeting vector was assembled by flanking exon 4 of murine *Cpeb1* locus with loxP sequences (Supplementary-Figure-9A-C). The vector was electroporated in mouse W4 ES cells derived from 129S6/Sv strain. Positive recombinant ES cells were identified by Southern blotting and microinjected in developing blastocysts. Resulting chimeric mice were crossed with C57BL6/J mice, and mouse colony was maintained in mixed (129/Sv x C57Bl/6J) background.

CPEB4-targeted mice. Mouse ES cells carrying a β geo [β -galactosidase gene fused to neomycin

resistance gene] cassette in Cpeb4 intron 1 (clone EPD0060_4_E10; Sanger Institute) were microinjected in developing blastocysts (Supplementary-Figure-9D-F). Resulting chimeric mice were crossed with C57BL6/J mice, and mouse colony was maintained in pure C57BL6 background.

Conditional mice for CPEB1 and CPEB4 were generated upon expression of FlpO recombinase in recombinant ES cells,⁴⁰ or by mating with Tg.pCAG-Flp mice.⁴¹ To obtain tamoxifen-inducible mouse line, conditional mice were crossed with Tg.Ubc-CreERT2 mice.⁴² For systemic deletion, 4-wk-old conditional mice received tamoxifen-enriched food for 30 days.

CPEB knockdown cells

CPEB4 knockdown H5V cells were generated as described,²⁰ and cultured for 5 days in DMEM supplemented with 10% FBS, 2-mM L-glutamine and 1% penicillin/streptomycin, in presence of 5- μ l/ml PBS or 5- μ g/ml doxycycline to induce expression of shRNAs directed against sequence 5'GCTGCAGCATGGAGAGATAGA3'. CPEB1 knockdown H5V cells were generated as described,²⁰ and transduced with pLKO IPTG Lac0 lentivector carrying a sequence for CPEB1 shRNAs expression (Sigma). Virus production was done as indicated in <http://tronolab.epfl.ch/>. CPEB1 knockdown H5V cells were cultured for 4 days in DMEM supplemented with 10% FBS, 2-mM L-glutamine and 1% penicillin/streptomycin, in presence of 1- μ l/ml PBS or 5-mM IPTG to induce expression of shRNAs directed against sequence 5'AGGCGTTCCTTGGGATATTAC3'. All cells were then used for RNA and protein extractions and Matrigel tube formation assay. CPEB4 knockdown H5V cells were transduced with pLV-mutated-CPEB4 lentivector for stable expression of CPEB4 mRNA not target of shRNAs. Virus production was done as indicated in <http://tronolab.epfl.ch/>. To generate CPEB1 knockdown H5V cells, H5V cells were transduced with pLKO1 lentivector carrying a sequence for constitutive expression of CPEB1 shRNAs or control shRNAs (Sigma). CPEB1 shRNAs targeted the sequence 5'CCATCTTGAATGACCTATTTG3'. Cells were cultured in DMEM supplemented with 10% FBS, 2-mM L-glutamine, and 1% penicillin/streptomycin and were used for RNA and protein extraction and

Matrigel tube formation assay. H.D. MyZ cells were produced as described.²⁵ After thawing, cells were tested for mycoplasma (every 2 wks) and cultured for not longer than one month.

***In vitro* Matrigel tube formation assay**

Twenty-four hours before conducting the tube formation assay, GFR Matrigel (BD Biosciences) was incubated at 4°C for slow thawing. Liquid Matrigel was added to 24-well plate (300µl/well) previously chilled at -20°C. Plate was then incubated (37°C, 1h) to allow Matrigel solidification. H5V cells (80,000/well for CPEB1sh-/+ cells and 110,000/well for CPEB4sh-/+ cells) were incubated (37°C, 5% CO₂, 15h) in fresh medium (DMEM, 10% FBS, 2mM L-glutamine, 1% penicillin/streptomycin) or fresh medium supplemented with either 40ng/ml VEGFA (Sigma) or conditioned medium [ratio 1:1; obtained by incubation (48h) in absence of doxycycline and IPTG, in DMEM supplemented with 10% FBS, 2mM L-glutamine, 1% penicillin/streptomycin, centrifugation (300g, 2min), and collection of supernatant]. Medium was removed and endotubes rinsed with PBS and fixed with 4% paraformaldehyde. Endotube formation was evaluated by inverted microscopy and scored as previously described:⁴³ 0-individual cells, well-separated; 1-cells begin to migrate and align themselves; 2-capillary tubes visible, no sprouting; 3-sprouting of new capillary tubes visible; 4-closed polygons begin to form; 5-complex mesh-like structures develop.

***In vivo* mouse Matrigel-plug assay**

In vivo mouse Matrigel-plug assay was performed as previously described,⁴⁴ with some modifications. WT (n=5), CPEB1 (n=5) or CPEB4 (n=5) KO mice were injected sc in each dorsolateral flank with 400 µl of GRF Matrigel supplemented with 150 ng VEGF per ml of Matrigel. WT mice injected with Matrigel in absence of VEGF were used as negative control (n=5). After 9 days, Matrigel plugs were extracted, fixed in 10% buffered formalin solution, and embedded in paraffin. Successive 2 µm sections were then obtained and prepared for CD31 immunofluorescence and H&E. For immunofluorescence, slides

were blocked with 5% horse serum for 1 h, incubated with anti-CD31 primary antibody (1:50; SantaCruz) and visualized with Alexa A488-conjugated anti-rabbit secondary antibody (Invitrogen). Fluorescence was quantified by measuring integrated density with Image J.

Determination of the extent of portosystemic collateral vessels in mice

Under anesthesia with isoflurane (3.5% for induction; 1.5% for maintenance), the extent of portosystemic collateral vessels was quantified by injection of colored polystyrene latex microspheres (15- μ m diameter, Dye-trak, Triton Technologies, San Diego, CA) into the spleen of mice. The animals were then sacrificed and the spheres were recovered from lung and liver by digestion and subsequent micro-filtration. The dyes were then recovered from the spheres within a known volume of solvent and their concentrations determined by spectrophotometry. This allows us to quantify the degree of collateral formation in a 0% to 100% scale,^{4,5} by the equation: collateralization (%)=(lung microspheres)/(lung microspheres+liver microspheres)x100.

Determination of mesenteric arterial blood flow in mice

Under anesthesia with isoflurane (3.5% for induction; 1.5% for maintenance), a tracheostomy was performed and a polyethylene tubing (Clay-Adams Inc, New York, NY) was inserted into the trachea to ensure a patent airway. Then, an abdominal midline incision was performed. The superior mesenteric artery was dissected free from connective tissue, and a nonconstrictive perivascular ultrasonic flowprobe (Transonic Systems, New York, NY) was placed around this vessel close to its aortic origin. The ultrasonic flowprobe was connected to a small animal T206 blood flow meter (Transonic Systems) to measure blood flow in the superior mesenteric artery (ml/min/10 g body weight) by the ultrasonic transit-time technique using ADI Chart software. This method involves a perivascular flowprobe, which contains two ultrasonic transducers that emit a plane wave of ultrasound back and forth, alternatively intersecting the flowing blood in upstream and downstream directions. The flowprobe subtracts the

downstream from the upstream integrated transit times, and this difference is a measure of the volume of blood flow (ml/min). Rectal temperature was maintained at $37\pm0.5^{\circ}\text{C}$ throughout the study.⁴

Partial hepatectomy

Male mice (8-12 wk old) were anesthetized (isoflurane) and 3 liver lobes including the gall bladder were removed. After surgery, mice received 0.1 mg/kg buprenorphine for analgesia. 48 h after partial hepatectomy, mice were euthanized (CO_2 inhalation), and the remaining liver was harvested for further analysis. 250 $\mu\text{g/g}$ BrdU was injected i.p. 2 h before killing the mice.

Postnatal retinal angiogenesis model

Acute depletion of CPEB4 in neonatal mice was achieved by intragastric injection of 50 μg of tamoxifen (Sigma) once daily from P1 to P4 in WT ($\text{Cpeb4}^{\text{lox/lox}}$) and CPEB4 iKO ($\text{Cpeb4}^{\text{lox/lox}}$ Ubc-CreERT2) littermates. At P5, retinas were fixed and prepared as previously described.⁴⁵

Sprouting assay

To analyze VEGFA-stimulated sprout outgrowth in spheroids of WT, CPEB1 KO and CPEB4 KO mouse lung endothelial cells, these cells were isolated, purified and cultured as previously described.⁴⁶ At passage 4, mouse lung endothelial cells (1000 cells) were incubated for 24 h in hanging drops in F12 medium supplemented with 20% FBS and ECGS (promoCell) containing 0.25% (w/v) methylcellulose (Sigma) to form spheroids. Spheroids were then embedded in collagen gel and overlaid with 100 μl of OptiMEM with VEGFA (30 ng/ml). 24 h later, sprouts overgrowth was visualized by phase contrast microscopy and quantified.⁴⁷

Cell migration assay

Cell migration assays were done using 8- μ m pore, 6.5-mm diameter transwell filters (Corning Costar). At passage 3, mouse lung endothelial cells were stained with CellTracker Green CMFDA (Molecular Probes) and grown in low-serum (0.1% FBS) medium for 24 h. Cells (2.5×10^4) suspended in low-serum medium were then seeded on the upper surface of filters and allowed to migrate toward 10% FBS-containing media in the bottom compartment. 16 or 24 h later, cells on the upper surface were wiped off with a cotton swab and cells that migrated to the underside of transwell filters were fixed with 4% paraformaldehyde. Green-CMFDA⁺ cells were visualized by phase contrast microscopy. Green signal indicates cells that have migrated by chemotaxis from the upper to the bottom compartment of the transwell. Ratio of cells that have migrated after 16 and 24 h was quantified.

Aortic ring assay

Mouse aortic ring assay was performed as previously described.⁴⁸ Thoracic aortic rings (1 mm) explants from WT, CPEB1 KO and CPEB4 KO mice were embedded on top of 50 μ l rat collagen and overlaid with 100 μ l of OptiMEM with VEGFA (30 ng/ml). VEGFA-stimulated microvessel outgrowth was visualized by phase contrast microscopy and number of vessels growing from each aortic ring was counted on day 10. Micrographs and quantification of WT, CPEB1 KO and CPEB4 KO aortic ring microvessel outgrowth in collagen in presence of VEGFA were obtained.

Translation of luciferase reporters in H5V cells

For RNA synthesis, plasmids were linearized by restriction enzyme digestion, and transcription reaction was performed using mMACHINE® T7 Kit (Ambion). Firefly and Renilla mRNAs were co-transfected (ratio 5:1) into H5V cells using Metafectene Pro (Biontex). Luciferase activity was measured using Dual-Luciferase reporter assay (Promega) and normalized against mRNA levels by quantitative PCR.

Subcellular fractionation

Nuclear and cytoplasmic fractions were prepared using the NE-PER® Nuclear and Cytoplasmic Extraction reagents (Thermo Scientific) as per manufacturer's instruction.

Leptomycin B treatment

H5V cells were treated with the nuclear export inhibitor Leptomycin B (Sigma) at 10 ng/ml for 20 hours.

RNA extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated and purified using RNeasy Mini Kit (GE Healthcare) and additionally treated with TURBO DNA-free Kit (Ambion). RNA quality was verified using Agilent's 2100 Bioanalyzer. For human and rodent samples, RNA was reverse transcribed to cDNA using QuantiTect Reverse Transcription kit (Qiagen). cDNA templates were amplified by real-time TaqMan PCR on an ABI Prism 7900 sequence Detection System (Applied Biosystems). VEGF expression was analyzed using predesigned gene expression assays (Applied Biosystems), and reported relative to endogenous control 18S. All PCR reactions were performed in duplicate, using nuclease-free water as no template control. For H5V cells, RT-PCR was performed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). qPCR was carried out in a LightCycler 480 (Roche) using SYBRGreen I Master (Roche), 50 ng cDNA per sample and primers indicated in Expanded View Materials and Methods. All quantifications were normalized to endogenous control (GLA) using LightCycler 480 Software. For mouse genotyping, 35 cycles of PCR were performed using 100 ng of tail genomic DNA and primers described in "Oligonucleotides". Primer annealing temperature was 60°C. Annealing and elongation times were 30 sec and 60 sec, respectively.

PolyA tail assay

PolyA tail assay (RNA-ligation-coupled RT-PCR) was performed as previously described,²² with some

modifications. Total RNA was extracted using RNeasy Mini Kit, treated with TURBO DNA-free Kit and precipitated with lithium chloride at 2.5M final concentration. Total RNA (6 μ g) was incubated (30min, 37°C) with an oligonucleotide oligodT (20 μ M) and 0.2U RNase-H (New England Biolabs) or H₂O, and purified by phenol/chloroform extraction. All reactions were prepared in duplicate, one with the addition of oligodT and RNase-H, the other without. RNA (4 μ g) was ligated to SP2 anchor primer (0.4 μ g) in a 10 μ l reaction using T4-RNA-ligase (New England Biolabs). The whole 10 μ l RNA ligation product was used in a 50 μ l reverse transcription (RevertAid™ First Strand cDNA Synthesis Kit) using ASP2T antisense primer (0.4 μ g). cDNA (1 μ l) was used for 50 μ l PCRs using BioTaq Polymerase (Bioline). PCR products were resolved in 2% agarose gel and subjected to Southern blotting. Because RNase-H is an endoribonuclease that specifically hydrolyzes the phosphodiester bonds of RNA in RNA:DNA duplexes, this enzyme removed the polyA tail on the oligodT:polyA duplex. RNase does not degrade single-stranded or double stranded RNA or DNA. The size of a polyA tail on a given mRNA can thus be determined by running reactions with and without RNase-H side by side on a gel and comparing the electrophoretic mobilities of the two samples. PolyA tail length was calculated by subtracting the length of the oligodT/RNaseH-treated PCR product (from which polyA tail was removed by RNase-H digestion) from the length of the oligodT/RNaseH non-treated products using molecular marker running together with PCR products by electrophoresis. Since sequence of primers and full length of human, rat and mouse 3'UTR (Database) are available (described later in the text), it is possible to calculate the exact polyA tail length.

3'RACE (Rapid Amplification of cDNA ends)

Reverse transcription was performed as indicated above, starting with 1 μ g of total RNA and 3'RACE antisense primer. 3'RACE was performed using sense primers that specifically amplified VEGF 3'UTR obtained by usage of PAS1 or PAS2 and T7 antisense primer. PCR products were resolved in 1.8% agarose gel and subjected to Southern blotting. Total RNA from H5V cells or murine and human

tissues was extracted and retrotranscribed to cDNA using 3'RACE AS as reverse primer. This primer is composed by a known sequence of ≈ 20 nucleotides followed by a stretch of 25 T and the degenerated nucleotide VN (where V can be any nucleotide less than T and N can be any nucleotide) 5'-TAATACGACTCACTATAGGGCGGATCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3'. Presence of the stretch of T, together with the VN, allows this oligonucleotide to anneal to the last 2 nucleotides of 3'UTR and the first 25 adenosines (A) of polyA tail. Then, a PCR is performed using specific forward primers located in VEGF 3'UTR, and the universal reverse oligonucleotide T7 AS, that anneals to the 3'RACE primer used for RT. The PCR products are run in an agarose gel and submitted to southern blot to achieve specificity. After identification of the bands, the site of cleavage and polyadenylation was calculated by subtracting the length of the 3'RACE primer from the length of the amplicon using the molecular marker running together with the PCR products by electrophoresis. Since the sequence of both forward and reverse primers using for this experiment are available in "Oligonucleotides" and the sequence of the full length of human, rat and mouse 3'UTR are available in database, it is possible to calculate the exact cleavage site of VEGF 3'UTR in the different mammalian species used in this work.

RNA immunoprecipitation (RIP) RT-PCR

RIP was performed as described before,²⁶ with some modifications. H5V cells were cultured in DMEM supplemented with 10% FBS up to 90% confluence, rinsed twice with 10 ml PBS and incubated with FBS-free DMEM and 1% formaldehyde, for 10 min at room temperature under constant soft agitation to crosslink RNA-binding proteins to target RNAs. Crosslinking reaction was quenched adding glycine to final concentration of 0.25M and incubating cells for 5 min at room temperature under constant soft agitation. Cells were washed twice with 10 ml PBS, lysated with scraper and RIPA buffer [25 mM Tris-Cl, pH 7.6, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 100 mM EDTA, 150 mM NaCl] containing complete EDTA-free protease inhibitor cocktail, RNase inhibitors (200 U/ml; Termo

Scientific), and sonicated for 10 min at low intensity with Standard Bioruptor Diagenode. After centrifugation (10 min, max speed, 4°C), supernatants were collected, precleared and immunoprecipitated (4 h, 4°C, on rotation) with 10 µg anti-CPEB4 antibody (Abcam), anti-CPEB1 antibody (ProTein Tech) or rabbit IgG (Sigma) bound to 50 µl of Dynabeads Protein A (Invitrogen). Beads-immunoprecipitated complexes were washed 10 times with cold RIPA buffer supplemented with RNase inhibitors, resuspended in 200 µl proteinase K buffer, 70 µg proteinase K (Roche) and incubated 40 min at 30°C. RNA was extracted using RNA microextraction kit (Qiagen) and retrotranscribed as indicated above, using random primers (Roche). After PCR, amplification products were resolved in 2% agarose gel. To perform RIP RT-PCR in rat tissues, samples were homogenized using an all-glass homogenizer in ice-cold lysis buffer containing 10 mM HEPES, pH 7.0, 100 mM KCl, 5 mM MgCl₂, 250 mM saccharose, 0.5% NP40, 1 mM DTT, protease inhibitor cocktail (Roche), 100 U/ml RNase inhibitor (Fermentas) and 2 mM vanadyl ribonucleoside complexes (Sigma). Cellular debris were pelleted (15,700g, 15 min, 4°C) and protein concentration was determined by the Bradford Protein assay (BioRad). Supernatants were precleared and immunoprecipitated (overnight, 4°C, on rotation) with 2 µg anti-CPEB1 antibody (Abcam) or rabbit IgG (Sigma) bound to 20 ml protein G PLUS (SantaCruz). Beads-immunoprecipitated complexes were washed 10 times with cold lysis buffer supplemented with RNase inhibitors, treated (10 min, 37°C) with DNase I-RNase-free (Roche), resuspended in 200 µl proteinase K buffer, 35 µg proteinase K (Roche), and incubated 30 min at 50°C. Protein-bound RNAs were purified by phenol-chloroform extraction and used for retrotranscription, with 30 Race primer and MluV reverse transcriptase (Fermentas). After PCR, amplification products were resolved in 2% agarose gel. RIP quantification was performed by real-time TaqMan PCR. Fold enrichment of target sequences in immunoprecipitated (IP) *versus* input fractions was calculated using the comparative Ct (cycle number required to reach a threshold concentration) method with equation $2^{Ct(IP)-Ct(Ref)}$. Values were normalized considering 1 the value obtained for VEGF IP with CPEB1.

Southern blotting

Agarose gels used in 3'RACE analysis or polyA tail assay were incubated in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 30 min under constant soft agitation and neutralized by incubation with neutralization solution (1 M Tris, 1.5 M NaCl pH 7.4) for 30 min under soft agitation. After overnight transfer, DNA was cross-linked (254 nm; 0.12 J) to nylon membrane (0.45 μ m, Pall Corporation). The membrane was pre-hybridized with Church buffer for 3 h, hybridized with 32 P-labelled probe (indicated in "Oligonucleotides") for 12 h, rinsed with washing buffer (SSC 1X, 0.1% SDS) until background signal removal, and exposed to Phosphorimager screen.

Plasmid construction

The plasmid pFlag-hCPEB4-CMV2, previously described,²⁰ was used to subclone the DNA corresponding to the CDS (coding domain sequence) of CPEB4 into pLV plasmid. The nucleotide sequence of CPEB4 sh binding site 5'GCTGCAGCATGGAGAGATAGA3' was mutated to 5'GCCGCAGCGTGGAGGGATAGG3' using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). The mutagenesis affected neither the CDS frame nor the amino acid sequence. This new construct is called pLV-mutated-CPEB4. The sequence corresponding to the first 1220 nucleotides of mouse VEGF mRNA 3'UTR was subcloned downstream of the Firefly luciferase CDS in PLuc cassette plasmid. Using this first clone we generated, by PCR, another construct carrying the first 414 nucleotides of mouse VEGF mRNA 3'UTR subcloned downstream of the Firefly luciferase CDS in PLuc cassette plasmid. The previously described pBSK-Renilla plasmid was used for normalization.²⁰

Immunoblotting

Tissue or plasma samples were homogenized using an all-glass homogenizer in ice-cold lysis buffer

(20 mM Tris, pH 7.5, 150 mM NaCl, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 40 µg/ml phenylmethylsulfonyl fluoride, 10 µM sodium pyrophosphate, 20 µM sodium fluoride, 1% Triton X-100, 1 mM sodium orthovanadate and 0.1 µM okadaic acid). Cellular debris were pelleted (15,700g, 15 min, 4°C) and protein concentration was determined by the Bradford Protein assay (BioRad). Equal amounts of proteins were heated (95°C, 5 min) in SDS-and β-mercaptoethanol containing sample buffer and separated by SDS-polyacrylamide gel electrophoresis. After transfer onto a nitrocellulose or PVDF membrane, specific proteins were labeled with the corresponding primary antibodies against CPEB1 (1:500), phospho-CPEB1 (1:10), CPEB4 (1:500), VEGF (1:1000), phospho-Aurora A (1:1000), von Willebrand factor (vWF; 1:1000), CD34 (1:200), TNFα (1:1000) and IgG (1:5000). Loading accuracy was evaluated by membrane rehybridization with antibodies against GAPDH (1:1000) or β-actin (1:1000). Proteins were then detected using horseradish peroxidase-conjugated secondary antibodies (Stressgen) and an enhanced chemiluminescence detection system. Quantification of protein signals was performed using computer-assisted densitometry. For immunoblotting in H5V cells, cell lysis was performed by scraping, using RIPA buffer [25 mM Tris-Cl, pH 7.6, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl] containing EDTA-free protease inhibitor cocktail (Roche), 100 mM PMSF and 1 mM DTT. Lysates were sonicated 5 min at medium intensity with Standard Bioruptor Diagenode. After centrifugation (10 min, 4°C, max speed), supernatants were collected, quantified, boiled with Laemmli buffer and resolved by 10% SDS-PAGE. Proteins were transferred onto a PVDF membrane (Immobilon-P, Millipore), 2 h at 250 mA for CPEB1 and VEGF or Nitrocellulose (Whatman, GE Healthcare) and 1 h at 100 V for CPEB4. Membranes were blocked in TBST, 5% dry milk and then incubated with antibodies against CPEB1 (1:200), CPEB4 (1:2000), VEGF (1:500) and tubulin (1:3,000) overnight at 4°C. Immunocomplexes were revealed using ECL Western blotting detection reagents (GE-Healthcare) after exposure to Hyperfilm ECL (GE-Healthcare).

Histological analysis and immunohistochemistry

Tissues were fixed in 10% buffered formalin solution and embedded in paraffin. Successive 2- μ m sections were obtained and prepared for histological H&E and modified Massons' Trichrome standard stainings. For immunostaining, 2- μ m paraffin sections were deparaffinized in xylol and rehydrated in graded alcohol series. Endogenous peroxidase was inhibited using 3% H₂O₂ (10 min at room temperature). Sections were then washed in distilled water and heated in a pressure cooker for epitope retrieval (in 10 mM citrate buffer, pH 6.0, 5 min). Slides were blocked with 5% goat serum and then incubated (1 h at room temperature, or overnight at 4°C) with primary polyclonal antibodies against VEGF (1:500), CPEB1 (1:500), CPEB4 (1:500), von Willebrand factor (vWF; 1:1000) and inducible nitric oxide synthase (iNOS; 1:100); and monoclonal antibodies against P-CPEB1 (1:50), Ki67 (1:25) and α SMA (1:1000). Sections were then washed with TBST and incubated with Dako Real EnVision Detection System (HRP mouse/rabbit secondary antibody; 30 min at room temperature). Antibody binding was revealed using H₂O₂ as a substrate, and diaminobenzidine as a chromogen. Hematoxylin was used as a counterstain. For negative control, primary antibody was omitted and then sections were incubated with corresponding secondary antibodies and detection systems. Stained sections were visualized with a Zeiss microscope. Images from several regions of the tissue sections were then acquired using an AxioCam camera (Carl Zeiss Vision). Analysis of the digitalized images was performed with computerized imaging system (AxioVision and Image J).

Immunofluorescence and confocal laser microscopy

Tissue sections (2 μ m) were deparaffinized, and antigen retrieval and blocking were performed as described above for immunohistochemistry. The following primary antibodies were used: Polyclonal antibodies against CPEB1 (1:100), and CPEB4 (1:200), and monoclonal antibody against VEGF (1:50). For fluorescence visualization of antibody reactions, primary antibodies were detected using secondary antibodies labeled with the fluorochromes Alexa Fluor (Alexa anti-mouse A555 and Alexa

anti-rabbit A647) from Invitrogen. To detect cell nuclei, paraffin-embedded tissues were deparaffined and mounted on Vectashield mounting medium for fluorescence with DAPI (Vector). Negative controls were run omitting the primary antibody and incubating with secondary antibodies labeled with the fluorochromes Alexa Fluor. Confocal microscopy was performed at the Advanced Light Microscopy Unit (Scientific and Technological Centers) from the University of Barcelona. Confocal images were acquired using a TCS SP5 laser scanning confocal microscope (Leica) equipped with a DMI6000 inverted microscope. DAPI, Alexa Fluor 555 and Alexa Fluor 647 images were acquired sequentially using 405, 561 and 633 laser lines, AOBS (Acoustic Optical Beam Splitter) as beam splitter and emission detection ranges 415-525 nm, 570-650 nm and 650-750 nm, respectively, and the confocal pinhole set at 1 Airy units. All images were acquired with an APO 63x oil (NA 1.4) immersion objective lenses, digitized into TIFF format of 1024×1024 pixels and 12 bit depth (4096 fluorescence intensity levels of information). Electronic zoom (x3) was used for stronger magnification and better image resolution. Images were taken in the X-Y plane and/or the Z axis. Stacks of 1 µm-thick serial optical slices were taken in Z axis. Immunofluorescence analysis in H5V cells was performed as previously described.²⁵

Quantification of tissue vascularization

To quantify vascularization on tissue sections, blood vessels were first detected by immunostaining for the endothelial cell marker vWF or by histological staining with H&E. Digital images of an average of 40 different microscopic fields (at x200 final magnification) of each mesenteric tissue, obtained from five individual animals per group, were then acquired using a Zeiss microscope and an AxioCam colour digital camera (Carl Zeiss Vision). Zeiss Axio Vision image analysis system (Zeiss) and IPLab software (BioVision Technologies) were used for computerized quantification of immunostained vascular structures. Results were expressed as number of vessels per square millimeter. All immunohistochemistry sections were examined independently by 2 blinding investigators, experts in

the field, who were unaware of the samples' profiles.

Antibodies

Following polyclonal antibodies were used: CPEB4 serum (prepared in R. Mendez's laboratory), CPEB4 (ab83009, Abcam), CPEB1 (13274-1-AP from Protein Tech and Abcam ab15917 from Abcam), vWF (Dako or F3520 Sigma), VEGF (ab46154, Abcam), iNOS (ab15323, Abcam), CD34 (AF4117, R&D), and IgG (A9044, Sigma). Following monoclonal antibodies were used: VEGF (ab1316 and ab46154, Abcam), phospho-Aurora kinase A (Thr288; C39D8, Cell Signaling), α SMA (A2547, Sigma), Ki67 (M7248, Dako), α -tubulin (T9026, Sigma), β -actin (A2228, Sigma) and GAPDH (sc-32233, SantaCruz). Antibody against phosphorylated CPEB1 (483B9_{d4d12}) was a monoclonal antibody that we raised using RGSRLDT(p)RPILDS(p)RSC peptide as antigen. Then, the clones were selected for supernatants that, in ELISA, recognized this peptide, but not the unphosphorylated counterpart RGSRLDTRPILDSRSC. The antibody, produced in Abyntek Biopharma, does not recognize recombinant CPEB1 expressed in bacteria. However, it recognizes a CPEB1 comigrating band in H5V cells (where CPEB1 is active), but not in RPE cells, where CPEB1 acts as a translational repressor (Supplementary Figure 3A). CPEB1 phosphorylation corresponds to activatory residues Thr-171 and Ser-176, which are phosphorylated by Aurora Kinase A.^{23,24,29}

Oligonucleotides

For PCR:

mouse CPEB4 5'TTGTTTCCGATGGAAGATGG3' (sense)

5'TCAATATCAGGAGGCAATCCA3' (antisense)

mouse VEGF 5'GGTTCAGAAAGGAGAGGAG3' (sense)

5'GCATTACATCTGCTGTGCT3' (antisense)

mouse GAPDH 5'CCCTTCATTGACCTCAACTAC3' (sense)

5'AAGGCCATGCCAGTGAG3' (antisense)

mouse GLA 5'ACCAGCAGGTGACACAGATG3' (sense)

5'GAAACAGTAGCCCTGCTTGC3' (antisense)

rat VEGF 5'GGCCTCTGAAACCATGAACT3' (sense)

5'CGTACTAGACGTATCACTGC3' (antisense)

human VEGF 5'GAGACCCTGGTGGACATCTT3' (sense)

5'TGGTGATGTTGGACTCCTCA3' (antisense)

human GLA 5'GGCGAAATTTTGCTGACATT3' (sense)

5'CATATCTGGGTCATTCCAACC3' (antisense)

mouse VEGF₁₂₀₋₁₆₄₋₁₈₈ 5'CAGGCTGCTGTAACGATGAA3' (sense)

5'CACCGCCTTGGCTTGTACA3' (antisense)

rat VEGF₁₂₀₋₁₆₄₋₁₈₈ 5'GACCCTGGTGGACATCTTCCAGGA3' (sense)

5'GGTGAGAGGTCTAGTTCCCGA3' (antisense)

human VEGF₁₂₁₋₁₆₅₋₁₈₉ 5'GAGATGAGCTTCCTACAGCAC3' (sense)

5'TCACCGCCTCGGCTTGTACAT3' (antisense)

For quantitative PCR:

mouse CPEB1 5'CCTCCTCTGCCCTTTCTTTC-3' (sense)

5'TCCAAGAAGGTCCCAAGATG3' (antisense)

mouse CPEB4 5'TTGTTTCCGATGGAAGATGG3' (sense)

5'TCAATATCAGGAGGCAATCCA3' (antisense)

mouse VEGF 5'CATGCGGATCAAACCTCAC3' (sense)

5'GCATTACATCTGCTGTGCT3' (antisense)

mouse GLA 5'ACCAGCAGGTGACACAGATG3' (sense)

5'GAAACAGTAGCCCTGCTTGC3' (antisense)

mouse CPEB4 3'UTR PAS1 5'CTCTCGTGTCAGTCAAACAG3' (sense)

5'TTAGATCCTCTTGGCCTCCA3' (antisense)

mouse CPEB4 3'UTR PAS2 5'AAAGTCATTTTCACGTAAAGTTCC3' (sense)

5'TCCACTAGCACTTCAACAAATGA3' (antisense)

Firefly luciferase 5'TGATTTTTCTTGCCTCGAGTT3' (sense)

5'GTTTTGGAGCACGGAAAGAC3' (antisense)

Renilla luciferase 5'GATAACTGGTCCGCAGTGGT3' (sense)

5'ACCAGATTTGCCTGATTTGC3' (antisense)

For 3'RACE:

3'RACE: 5'TAATACGACTCACTATAGGGCGGATCCTTTTTTTTTTTTTTTTTTTTTTTVN3'
(antisense)

PAS1 of mouse VEGF 3'UTR: 5'CCTCAGGGTTTCGGGAACC3' (sense)

PAS2 of mouse VEGF 3'UTR: 5'AAGAAGAGGCCTGGTAATGG3' (sense)

T7: 5'GTAATACGACTCACTATAGGGC3' (antisense)

PAS1 of rat VEGF: 5'GGAAGATTAGGGAGTTTTGTTTC3' (sense)

PAS2 of rat VEGF: 5'AAGAAGAGGCCTGGTAATGG3' (sense)

PAS1 of human VEGF: 5'GCGCAGAGCACTTTGGGTC3' (sense)

PAS2 of human VEGF: 5'CCGAGGCACAGAGAGACAGG3' (sense)

PAS1 of human CPEB4: 5'GATTCTTGTGCTCACTGCAAAC3' (sense)

PAS2 of human CPEB4: 5'CATCACTTCAATTCACCAAGC3' (sense)

For PolyA tail assay:

SP2: 5'-P-GGTCACCTCTGATCTGGAAGCGAC-NH₂-3' (sense)

ASP2: 5' GTCGCTTCCAGATCAGAGGTGACCTTTTT3' (antisense)

PAS1 of mouse VEGF: 3'UTR 5'AAGATTAGGGTTGTTTCTGG3' (sense)

PAS2 of mouse VEGF: 3'UTR 5'AAGAAGAGGCCTGGTAATGG3' (sense)

PAS1 of rat VEGF: 5'GGAAGATTAGGGAGTTTTGTTTC3'

PAS1 of human VEGF: 5'GCGCAGAGCACTTTGGGTC3'

For Southern blotting probes of 3'RACE:

PAS1 of mouse VEGF 3'UTR: 5'AGTCCTTAATCCAGAAAGCC3' (sense)

PAS2 of mouse VEGF 3'UTR: 5'GGTACAGCCCAGGAGGACCT3' (sense)

PAS1 of human CPEB4: 5'GGAAGTTTGGATCCTCTTGG3' (sense)

PAS2 of human CPEB4: 5'GCACTTCAACAAATGAACTC3'

PAS1 of rat VEGF: 5'TGGGATTCTGTAGACACAC3' (sense)

PAS2 of rat VEGF: 5'TGATCAGACCATTTGAAACCAC3' (sense)

PAS1 of human VEGF: 5'CGGTCCCTCTTGGAATTGG3' (sense)

PAS2 of human VEGF: 5'GATCCACGTGCCCATTGTG3' (sense)

PAS2 of human VEGF: 5'GATCCACGTGCCCATTGTG3' (sense)

For Southern blotting probes of PolyA tail assay:

PAS1 of mouse VEGF 3'UTR: 5'GATTCCTGTAGACACACCCACC3' (sense)

PAS2 of mouse VEGF 3'UTR: 5'GGTACAGCCCAGGAGGACCT3' (sense)

PAS1 of rat VEGF: 5'TGGGATTCTGTAGACACAC3' (sense)

PAS1 of human VEGF: 5'CGGTCCCTCTTGGAATTGG3' (sense)

Genotyping of KO mice:

CPEB1 KO mice 5'TCTGGTGGTCAGTCATCCTG3' (sense)

5'CAGAACACTGACAAGATGCTCA3' (antisense)

CPEB4 KO mice 5'GTGAGGAAAGCAGTATCTAGGGTC3' (sense)

5'ACATAGTCACTAACAACTTGAGG3' (antisense)

SUPPLEMENTARY REFERENCES

36. Halder JB, Zhao X, Soker S, et al. Differential expression of VEGF isoforms and VEGF(164)-specific receptor neuropilin-1 in the mouse uterus suggests a role for VEGF(164) in vascular permeability and angiogenesis during implantation. *Genesis* 2000;26:213-224.
37. Kim NH, Jung HH, Cha DR, et al. Expression of vascular endothelial growth factor in response to high glucose in rat mesangial cells. *J Endocrinol* 2000;165:617-624.
38. Medford AR, Douglas SK, Godinho SI, et al. Vascular endothelial growth factor (VEGF) isoform expression and activity in human and murine lung injury. *Respir Res* 2009;10:27.
39. Todaro GJ, Green H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J Cell Biol* 1963;17:299-313.
40. Raymond CS, Soriano P. High-efficiency FLP and PhiC31 site-specific recombination in mammalian cells. *PLoS One* 2007;2:e162.
41. Rodriguez CI, Buchholz F, Galloway J, et al. High-efficiency deleter mice show that FLP is an alternative to Cre-loxP. *Nat Genet* 2000;25:139-140.
42. **Ruzankina Y, Pinzon-Guzman C**, Asare A, et al. Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss. *Cell Stem Cell* 2007;1:113-126.
43. **Bahlmann FH, De Groot K**, Spandau JM, et al. Erythropoietin regulates endothelial progenitor cells. *Blood* 2004;103:921-926.
44. Yoshihara T, Takahashi-Yanaga F, Shiraishi F, et al. Anti-angiogenic effects of differentiation-inducing factor-1 involving VEGFR-2 expression inhibition independent of the Wnt/ β -catenin signaling pathway. *Mol Cancer* 2010;9:245-259.
45. Pitulescu ME, Schmidt I, Benedito R, et al. Inducible gene targeting in the neonatal vasculature and analysis of retinal angiogenesis in mice. *Nat Protoc* 2010;5:1518-1534.
46. Soler A, Serra H, Pearce W, et al. Inhibition of the p110 α isoform of PI3-kinase stimulates nonfunctional tumor angiogenesis. *J Exp Med* 2013;210:1937-1945.

47. Graupera M, Guillermet-Guibert J, Foukas LC, et al. Angiogenesis selectively requires the p110alpha isoform of PI3K to control endothelial cell migration. *Nature* 2008;453:662-666.
48. Korff T, Kimmina S, Martiny-Baron G, et al. Blood vessel maturation in a 3-dimensional spheroidal coculture model: direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness. *FASEB J* 2001;15:447-457.

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