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Transcription regulation by TBX18 in smooth muscle cells is essential for normal aortic development and homeostasis

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Aims This study investigated whether TBX18, a transcription factor known for its critical roles in cardiovascular and urogenital development, but never previously studied in the context of the aorta, contributes to the normal development and homeostasis of this major artery.

Methods and results Histological analyses revealed *Tbx18* expression in smooth muscle cells (SMCs) of the adult and embryonic aorta. Following this observation, transgenic mouse models were used to promote ablation of *Tbx18* in SMCs from early embryogenesis or in adulthood. Phenotypes were assessed by quantitative imaging and histological analyses. Embryonic conditional ablation of *Tbx18* resulted in severe aortic malformations and lethality, whereas adult ablation resulted in milder phenotypes. However, when adult *Tbx18* ablation was combined with a Marfan-causing mutation, it promoted degradation of aortic ultrastructure, aortic root dilation, and lethality. Multiomics analyses at the transcriptomic and translatomic levels revealed up-regulation of immediate early genes encoding critical transcription factors such as EGR1, FOS, and JUNB in SMCs from Marfan aortae, a response further exacerbated by concomitant ablation of *Tbx18*. ChIP-seq in primary human aortic SMCs revealed that TBX18 directly binds to several of the genes that were misexpressed in mutant aortae, suggesting direct regulation. Finally, transcriptomic and histological analyses of human patient samples revealed that *TBX18* expression was down-regulated in aneurysms, with the extent of down-regulation correlating with lesion severity.

Conclusion These results demonstrate that TBX18 in SMCs is essential not only for normal aortic development, but also for preventing gene expression programmes linked to adverse remodelling in adulthood. These findings enhance our understanding of the function of this transcription factor and of molecular mechanisms underlying aneurysm formation, a pathology responsible for a significant number of fatalities in developed countries.

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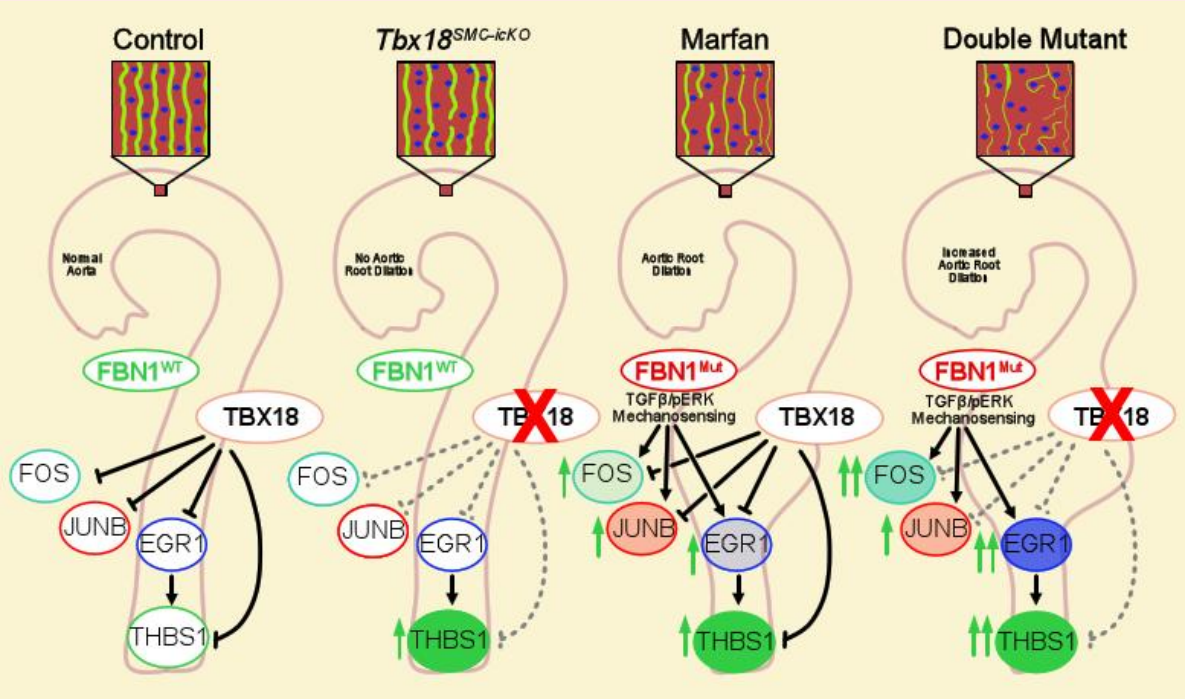
Graphical Abstract



Keywords Aortic homeostasis • Aneurysms • Transcription regulation • Vascular smooth muscle cells (vSMCs) • Multiomics

1. Introduction

To fulfil its critical pressure reservoir function,[1] the aorta has a highly specialized architecture in which distinct cell types (endothelial cells, smooth muscle cells, and adventitial fibroblasts) assemble concentric layers separated by rings of elastin-rich extracellular matrix (ECM).[2] During ventricular systole, vascular smooth muscle cells (vSMCs) relax and elastic fibres stretch, enabling expansion of the aortic volume. During ventricular diastole, elastic fibres recoil and vSMCs contract, pushing blood at a steady flow to downstream vessels.[1] Malfunctioning of one or more of these structural specializations frequently leads to aortic aneurysms (AA)—localized dilations of the aorta that are life-threatening due to the risk of rupture.[3,4] Aneurysms can affect the thoracic (TAA) or abdominal (AAA) aorta, with distinct underlying cellular and molecular mechanisms.[3,5,6] Despite being treatable by open surgery or endovascular repair, AA are estimated to account for 1–2% of deaths in industrialized countries because they typically develop asymptomatically and frequently are detected when it is too late to intervene.[3] Achieving a deep understanding of molecular mechanisms operating in the distinct cell types of the aortic wall to maintain its homeostasis will facilitate more efficient identification of individuals at risk, allowing for early clinical intervention and new therapies.[3,4]

Familial TAAs are frequently caused by mutations in ECM components, with the most notorious examples being Marfan syndrome, caused by mutations in *FBN1* (encoding Fibrillin-1),[7] and cutis laxa caused by mutations in *EFEMP2* (encoding Fibulin-4).[8] The involvement of ECM genes in these familial forms led to an initial view of aneurysms as lesions driven by structural defects that are, by nature, extremely difficult to correct.[3] However, it is currently accepted that aneurysmogenesis goes beyond the structural perspective and involves a re-wiring of intracellular signalling pathways, a process that is potentially reversible, therefore opening new therapeutic opportunities.[3] In addition to their structural role, ECM proteins regulate the bioavailability of multiple cytokines. Fibrillin-1 limits transforming growth factor β (TGFβ) availability via interaction with latent TGFβ-binding proteins (LTBPs), and disruption of this function is a central component in aneurysm formation in Marfan syndrome.[9–11] Further supporting a central role for TGFβ in aortic homeostasis, mutations in molecules involved in TGFβ signalling cause Loeys–Dietz syndrome, another hereditary aneurysm syndrome.[12,13] In the case of Fibulin-4 deficiency, aneurysm formation is thought to be based, at least in part, on the activation of the mechanosensor transcription factor EGR1 (early growth response 1)[14] and of its target thrombospondin-1,[15,16] an ECM molecule also known to regulate TGFβ signalling via conversion of latent TGFβ precursors to the biologically active form.[17]

vSMCs are central players in aneurysmal development, and pathological aortic remodelling involves a switch from a normal contractile vSMC phenotype to a synthetic phenotype with increased production of ECM molecules that represents a non-productive attempt to rescue defective vSMC-to-ECM anchoring.[18] Despite this, our current understanding of transcriptional mechanisms operating in vSMCs to regulate their gene expression programmes remains limited.[3]

Transcription factors (TFs) of the TBX family share a similar DNA-binding domain, the T-box, and play critical roles in organogenesis by binding to consensus T-box elements (AGGTGTGAAA) in the promoters of target genes.[19] Certain TBX TFs, such as TBX5 and TBX20, have been extensively studied and their cell type-specific effects and mechanisms of action are well characterized, thanks to a multitude of conditional knockouts and next-generation sequencing (NGS) datasets.[19] However, this sort of comprehensive knowledge is not available for several members of this family, including TBX18. TBX18 is essential for the normal development of the kidneys, skeleton, and cardiac pacemaker node.[20–22] However, as *Tbx18* null mice die shortly after birth due to defects in lung inflation,[20] we currently lack any information as to potential roles of this transcription factor in adulthood. Additionally, the lack of conditional knockout models precludes the study of TBX18 cell type-specific effects. Mechanistically,

TBX18 has been reported to function primarily as a transcriptional repressor via interaction with Groucho corepressors;[23] however, the lack of ChIP-seq datasets and the few transcriptomics datasets assessing cell type-specific consequences of TBX18 manipulation limit our understanding of TBX18.

Following our previous observation that *Tbx18* is robustly expressed in vSMCs,[24] we used transgenic mouse strains to generate conditional knockout models that revealed TBX18 is essential in aortic SMCs both in embryogenesis and in adulthood. Mechanistic analyses revealed activation of immediate early genes (IEGs) as a major event in early pathologic aortic remodelling, and ChIP-seq studies in primary human aortic SMCs revealed that TBX18 directly targets these genes. *TBX18* expression was strongly down-regulated in human aneurysmal aortae, suggesting that altered TBX18 activity might also be involved in human disease progression. Taken together, these data clearly place TBX18 as a relevant factor in aortic development and homeostasis, strengthening our understanding of the complex cell type-specific molecular mechanisms operating to maintain a functional aorta.

2. Methods

Detailed methods are presented in Supplementary material online, Material.

2.1 Animal models

All procedures involving transgenic mouse models conformed to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the NIH Guide for the Care and Use of Laboratory Animals. Experiments performed were authorized by the competent local authorities: Regierungspräsidium Darmstadt, Hessen, Germany (project FU/2008) or the IACUC of the University of California, San Diego (UCSD, protocols S04150 or S00138). All transgenic alleles were kept on an outbred background (CD1, Black-Swiss, or a mix of both). Humane euthanasia of research animals was performed using a combination of anaesthesia with isoflurane followed by cervical dislocation. For echocardiographic measurements, mice were initially anaesthetized in an induction chamber using 2% isoflurane and 1 L/min 100% oxygen. Once the animal lost its righting reflex, it was laid supine on a heated platform with its nose enveloped in a nosecone with 1.5–2% isoflurane. For aortic angiography, mice were anaesthetized via intraperitoneal injection with a solution of ketamine (100 mg/kg) and xylazine (10 mg/kg). Tamoxifen induction, tissue harvesting, and all histological techniques were performed as previously described.[24] TRAP-seq and aortic angiography were performed following modified versions of previously published protocols.[25,26]

2.2 Human subjects

All analyses involving human samples followed the principles outlined in the Declaration of Helsinki. Written consent was obtained from all individuals prior to the collection of tissues or inclusion in the study. RNA-seq of human AAA was performed at the Technical University Munich. Adherent abdominal aortic fragments collected during renal transplantation were used as controls. RNAscope analyses on histological sections of TAA (genetic and sporadic) or flanking non-dilated tissue (both collected during surgical aneurysm repair) were performed in Centro Cardiologico Monzino (Milan, Italy).

3. Results

3.1 TBX18 in smooth muscle cells is required for normal development of the aorta

Histological analyses of tissues from *Tbx18-H2B:GFP* mice[27] revealed that, in the adult aorta, *Tbx18* expression was observed in all vSMCs (αSMA+ cells) of the medial layer, but not in intimal or adventitial cells, consistent with our previous findings[24] (*Figure 1A* and *B*). Scattered *Tbx18-H2B:GFP* + nuclei could be observed in peri-aortic tissues and corresponded to pericytes in peri-aortic fat,[24] as well as the layer of mesenchymal cells that envelops this adipose depot. *Tbx18* expression was also observed in the embryonic aorta, but with an asymmetric pattern in which positive vSMCs were more abundant in the dorsal region of the vessel (see Supplementary material online, *Figure S1*). These expression patterns suggested that gene regulation by TBX18 in vSMCs might be important for normal aortic development and homeostasis. Even though *Tbx18* expression was also observed in other types of SMC, including in the ureter, activity of this transcription factor does not seem to be a requisite for the acquisition of an SMC phenotype, as *Tbx18* was not expressed in SMCs of the gastrointestinal system and SMCs of the lungs or liver (*Figure 1A* and *B*). Specificity of αSMA immunostaining was validated via IgG control stainings in adjacent histological sections (see Supplementary material online, *Figure S2*).

To specifically explore the function of TBX18 in vSMCs, we ablated a floxed *Tbx18* allele[24] using PDGFRβ-Cre.[28] Albeit not being entirely restricted to mural cells,[24] PDGFRβ-Cre promoted robust recombination in the aorta and coronary vasculature (including *Tbx18*+ vSMCs, arrows in *Figure 1C*), without hitting other relevant populations of *Tbx18*-expressing cells, such as the epicardium or ureteric smooth muscle (arrowheads in *Figure 1C*). Conditional mutants (from here on designated as $Tbx18^{PDGFR\beta\text{-}cKO}$) were born at expected Mendelian ratios, but displayed an early lethal phenotype, with more than half of $Tbx18^{PDGFR\beta\text{-}cKOs}$ dying by 4 months and the longest-lived $Tbx18^{PDGFR\beta\text{-}cKO}$ dying before 1 year of age (*Figure 1D*). Phenotypic analyses revealed that, consistent with the lack of PDGFRβ-Cre activity in ureteric smooth muscle, the ureters of $Tbx18^{PDGFR\beta\text{-}cKOs}$ attached normally to the kidney, unlike what happens in *Tbx18* global knockouts, in which ureters attach to the posterior apex of the kidney.[21] However, due to the activity of PDGFRβ-Cre in bone, $Tbx18^{PDGFR\beta\text{-}cKOs}$ displayed skeletal anomalies similar to those observed in *Tbx18* global knockouts,[20] that resulted in lower body weights than those of littermate controls (*Figure 1E*). $Tbx18^{PDGFR\beta\text{-}cKO}$ also displayed a moderate, but statistically significant, increase in systolic blood pressure (*Figure 1F*). Aortic angiography in young adults revealed evident anomalies in $Tbx18^{PDGFR\beta\text{-}cKOs}$ (*Figure 1G* and *H*). Whereas control animals displayed a normal aorta in which the arch was followed by a straight vessel, $Tbx18^{PDGFR\beta\text{-}cKOs}$ showed aortic tortuosity throughout the thoracic and abdominal aorta (*Figure 1G* and *H*). This tortuosity is highly similar to that observed in *Fibulin-4* and *Fibulin-5* mutants, two models with defective elastic fibre assembly during arteriogenesis.[26,29] Masson trichrome analyses in animals that survived until 5 months of age revealed an evident dilation of the aortic root in $Tbx18^{PDGFR\beta\text{-}cKOs}$ (*Figure 1I* and *J*). At the microscopic level, this phenotype was accompanied by disruption of normal aortic architecture, with $Tbx18^{PDGFR\beta\text{-}cKOs}$ displaying broken elastic laminae, thickened SMC layers, and disruption of adventitial collagen fibres, all of which are typical features of AA.[3,10,11,15] These observations suggested that the lethality observed in $Tbx18^{PDGFR\beta\text{-}cKOs}$ might be caused by ruptured aneurysms. Consistent with this hypothesis, analyses of bodies from deceased animals revealed blood accumulation in the thoracic (4/6) or abdominal (1/6) cavities.

Altogether, these analyses clearly revealed that TBX18 in vSMCs is necessary for normal aortic development, with the absence of this transcription factor resulting in macroscopic and ultrastructural defects that severely compromise aortic stability, resulting in early lethality.

3.2 Adult ablation of *Tbx18* in aortic smooth muscle exacerbates the Marfan phenotype

The fact that vSMC-specific expression of *Tbx18* persists in adulthood (*Figure 1A*) prompted us to test if, in addition to a role in normal aortic development, this transcription factor might also be required for maintenance of aortic homeostasis. To assess this, we generated conditional mutants in which ablation of the floxed *Tbx18* allele was driven by the tamoxifen-inducible, SMC-specific *Myh11-CreERT2* (*or smMHC-CreERT2*).[30] In *Myh11-CreERT2; Tbx18*$^{fl/fl}$ animals (from here on simply designated as $Tbx18^{SMC\text{-}icKOs}$), SMC-specific ablation of *Tbx18* only takes place upon tamoxifen administration, which was performed in adulthood, allowing for undisturbed aortic development (*Figure 2B*). As $Tbx18^{PDGFR\beta\text{-}cKOs}$ displayed phenotypic manifestations resembling those seen in Marfan patients (aortic root dilation, disorganization of aortic ultrastructure, *Figure 1I* and *J*), we also assessed if

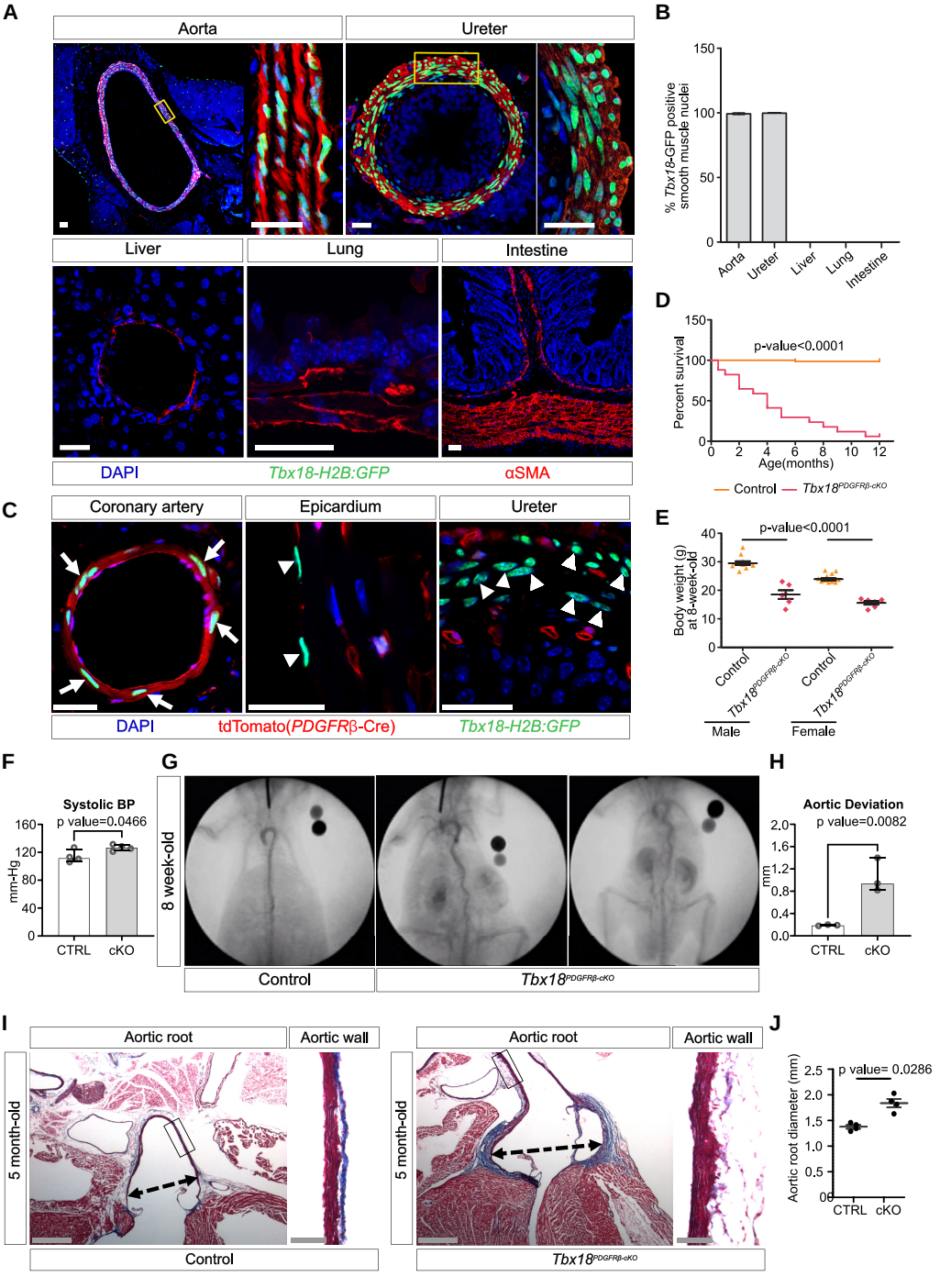


Figure 1 Mural cell-specific ablation of *Tbx18* from early embryogenesis results in vascular abnormalities and lethality. (*A*, *B*) Histological analyses and respective quantification showed that *Tbx18* was specifically expressed in SMCs (αSMA+) of the aorta and ureter, but was completely absent from SMCs of the liver, lung, and intestine ($N = 3$). (*C*) PDGFRβ-Cre promoted recombination in the vasculature throughout the body, including endothelial cells and *Tbx18*-expressing mural cells in the heart (arrows). This same Cre did not hit other relevant populations of *Tbx18*-expressing cells, including epicardial cells and ureteric SMCs (arrowheads). (*D*) Kaplan–Meier plot showing that ablation of *Tbx18* using PDGFRβ-Cre resulted in a lethal phenotype with more than half of the mutants dying prior to 6 months of age (*N*: $Tbx18^{PDGFRβ\text{-}cKOs} = 17$, controls = 63, log-rank Mantel-Cox test). (*E*) $Tbx18^{PDGFRβ\text{-}cKOs}$ showed reduced body weight compared with gender-matched controls (a minimum of seven animals were analysed for each genotype and sex, one-way ANOVA). (*F*) $Tbx18^{PDGFRβ\text{-}cKOs}$ showed a moderate but significant increase in systolic blood pressure ($N = 4$, unpaired *t*-test). (*G*) Aortic angiographies in young adults revealed that $Tbx18^{PDGFRβ\text{-}cKOs}$ had evident aortic tortuosity. (*H*) Quantification of aortic tortuosity in $Tbx18^{PDGFRβ\text{-}cKOs}$ and littermate controls ($N = 3$, unpaired *t*-test). (*I*) $Tbx18^{PDGFRβ\text{-}cKOs}$ that survived until 5 months of age displayed dilation of the aortic root (arrows in low magnification images) and severe disorganization of the aortic wall (high magnification images showing boxed areas). (*J*) Quantification of aortic root dilation ($N = 4$, Mann–Whitney test). Scale bars = 10 μm in *A*; 30 μm in *B* and *C*; 500 μm (low magnification) or 100 μm (higher magnification of boxed areas) in *I*. In *G*, scale spheres diameter: small = 3.96 and large = 4.33 mm. In *B*, *E*, *F*, *H*, and *J*, data are represented as mean ± s.e.m.

combining SMC-specific ablation of *Tbx18* with a heterozygous *Fbn1-C1039G* mutation widely used as a murine model of Marfan syndrome[31] resulted in exacerbated phenotypes. Therefore, as shown in *Figure 2A*, our experimental design comprised four distinct genotypes: a control group, animals with conditional *Tbx18* ablation alone ($Tbx18^{SMC\text{-}icKOs}$), animals with a heterozygous *Fbn1-C1039G* mutation alone (Marfan), and animals combining conditional *Tbx18* ablation with the Marfan-causing mutation (designated as double mutants for simplicity, *Figure 2A*). To ensure comparability between experimental groups, all animals were positive for the *Myh11-CreERT2* allele, and all animals were subjected to the same tamoxifen induction regimen (*Figure 2B*).

A time-course analysis of aortic root diameter using non-invasive echocardiography (*Figure 2C–F*) showed that, prior to tamoxifen injection (when all animals were wild type for *Tbx18*), Marfan mice already exhibited significantly increased mean aortic root diameter (1.774 ± 0.132 mm) compared with control littermates (1.47 ± 0.06 mm, *Figure 2D*), revealing that aortic root dilation consequent to *Fbn1* mutation is already detectable in young adults. With aging, all genotypes displayed a trend to increased aortic root diameter (*Figure 2E* and Supplementary material online, *Figure S3*). During the first 6 months post-tamoxifen, this progressive enlargement took place at a comparable rate in all genotypes. However, between 6 and 8 months post-tamoxifen, there was a peak of dilation in double mutants that was not observed in any of the other groups (*Figure 2E* and *F* and Supplementary material online, *Figure S3*). The exacerbated aortic root dilation in double mutants was further validated by histological analyses (*Figure 2G* and *H*). The accentuated root dilation between 6- and 8-months post-tamoxifen coincided with a wave of lethality amongst the double mutant group, without any lethality being observed in the other groups (*Figure 2I*). To investigate if the sudden death of double mutants could be a consequence of aortic anomalies, we performed a histological time course to assess aortic wall structure at distinct time points post-tamoxifen administration. Analyses performed 1-month post-tamoxifen revealed a very high efficiency of *Myh11-CreERT2*-driven recombination (ranging from 95.2 to 97.5%) in vSMCs of all experimental groups, but mild phenotypic manifestations (see Supplementary material online, *Figure S4*). On the other hand, similar analyses performed 10 months post-induction revealed strong histological differences between groups (*Figure 3A*). Whereas wildtype controls had concentric layers of vSMCs separated by intact elastin rings, aortae from animals of the $Tbx18^{SMC\text{-}icKO}$ and Marfan groups displayed areas with evident thinning and occasional breaks of elastic laminae (arrows in *Figure 3A*). Double mutants showed an exacerbated phenotype, with areas of severe disorganization of elastic laminae (*Figure 3A*). Specificity of elastin immunostaining was validated via IgG control stainings in adjacent histological sections (see Supplementary material online, *Figure S2B*). Quantification of histological data confirmed enhanced loss of elastic lamina integrity in the double mutant group, with a reduction in the number of distinguishable SMC layers and increased average thickness of individual SMC layers (*Figure 3B–D*). These observations showed that combining a Marfan-causing *Fbn1* mutation with SMC-specific ablation of *Tbx18* resulted in a severely fragile aortic structure, potentially predisposing to lethality due to rupture or dissection. This perspective was further supported by the analysis of aortae from deceased double mutants, revealing areas of dilation with even more marked defects than those seen in surviving animals (see Supplementary material online, *Figure S5*). Taken together, these findings revealed that transcriptional control by TBX18 in vSMCs is not only necessary for proper arteriogenesis during development, but also for maintenance of aortic homeostasis in adulthood.

3.3 Up-regulation of immediate early genes in aneurysm-predisposed aortae

To gain insight into gene expression networks underlying the observed phenotypes, we conducted RNA-seq analyses on aortae of the four experimental groups. As we were interested in identifying gene expression changes underlying early disease-causing events rather than secondary transcriptional alterations taking place in later stages of aortic remodelling, these analyses were conducted 4 weeks post-tamoxifen induction (*Figure 4A*). Animals used in these experiments also carried a copy of the *Rosa26-Ribo-GFP* allele, enabling the complementation of RNA-seq with SMC-specific translatomics.[32] Prior to Cre activity, all cells from *Rosa26-Ribo-GFP* animals have normal ribosomes. Following *Myh11-CreERT2*-driven excision of a floxed stop cassette, SMCs begin expressing a GFP-fused version of the ribosomal protein L10a (*Figure 4B*). This enables translating ribosome affinity purification sequencing (TRAP-seq), a translatomics technique in which SMC ribosomes are immunoprecipitated from whole tissue lysates using anti-GFP antibodies. The associated, actively translated mRNAs (ribosome-bound) are then isolated and processed for sequencing[25,32] (*Figure 4C*). Comparison of translatomics and transcriptomics datasets for the control group (*Figure 4D* and Supplementary material online, *Table S1*) revealed that the TRAP-seq datasets were enriched in SMC-specific transcripts (*Actg2*, *Myl9*) and de-enriched in transcripts of endothelium, immune cells, and adventitial fibroblasts (*Pecam1*, *Cdh5*, *Ptprc*, *Pdgfra*), confirming that TRAP-seq employing genetic models is a valid strategy to assess translation events taking place specifically in vSMCs.

From the three mutant groups, when compared with wild-type controls, $Tbx18^{SMC\text{-}icKO}$ aortae displayed the lowest number of differentially expressed genes (DEGs, RNA-seq) and differentially translated transcripts (DTTs, TRAP-seq) (*Figure 4E* and *F* and Supplementary material online, *Table S1*). In RNA-seq, $Tbx18^{SMC\text{-}icKO}$ aortae exhibited down-regulation of genes involved in muscle contraction (including multiple ion channels). On the other hand, in TRAP-seq, a major observation in $Tbx18^{SMC\text{-}icKOs}$ was the increased translation of multiple thrombospondins (*Thbs1*, *Thbs2*, and *Thsd7b*; grouped in the reactome category '*O*-glycosylation of TSR domain-containing proteins', Supplementary material online, *Table S2*). THBS1 is an ECM protein that plays a crucial role during aneurysm formation in *Fibulin-4* KO mice,[15] suggesting that *Thbs1* up-regulation might be an important component of the aortic phenotypes observed upon conditional ablation of *Tbx18*. Marfan aortae exhibited more pronounced transcriptional and translational differences than $Tbx18^{SMC\text{-}icKOs}$ (*Figure 4E* and *F* and Supplementary material online, *Table S1*). From RNA-seq analyses, Marfan animals had in common with the $Tbx18^{SMC\text{-}icKO}$ the down-regulation of genes involved in muscle contraction. Marfan aortae also showed up-regulation, both at transcriptional and translational levels, of multiple IEGs (*Egr1*, *Egr3*, *Arc*, *JunB*) that, in reactome functional annotation, were grouped in the category 'NGF-stimulated transcription'. In addition, at the translational level, vSMCs of the Marfan group showed down-regulation of transcripts involved in Ephrin signalling (*Epha1*, *Ephb3*), as well as up-regulation of transcripts associated with Neuropilin signalling (*Nrp1*, *Nrp2*) and a selection of ECM proteins (*Col4a3, Col4a4, Sdc3, Fn1, Tnc, Ntn4, Thbs1*, Supplementary material online, *Table S2*), the latter indicating conversion to a secretory vSMC phenotype.[5,18] Consistent with the exacerbated phenotype, double mutants had the highest number of DEGs and DTTs (*Figure 4E* and *F*), and, as expected, shared functional annotation categories with the single mutants, including the down-regulation of molecules involved in muscle contraction and up-regulation of even more IEGs than Marfan vSMCs (*Egr1*, *Egr2*, *Egr3*, *Fos*, *JunB,* and *Arc*– reactome category 'NGF-stimulated transcription', Supplementary material online, *Tables S1* and *S2*).[33] A surprising observation was the down-regulation, at the transcriptional and translational levels, of critical components of the circadian clock (*Npas2*, *Arntl,* and *Clock*) that was not present in either of the single mutants alone and could not be attributed to practical aspects of experimental design, as samples from the different groups were harvested at the same time of the day. The increased translation of ECM molecules (already observed in *Fbn1* mutants) was exacerbated in double mutants. In addition to the molecules modulated in vSMCs from Marfan mice, these animals also showed increased translation of multiple additional collagens (*Col5a1*, *Col6a6*, *Col8a1*, *Col12a1*, and *Col16a1*), indicating an even stronger transition to a secretory vSMC phenotype than that observed in Marfan alone (*Figure 4F*).

Altogether, these multiomics analyses provided a unique level of mechanistic detail into early events in the molecular cascade leading to aneurysm formation. Down-regulation of muscle contraction genes was a feature observed in all genotypes, with multiple genes being shared between

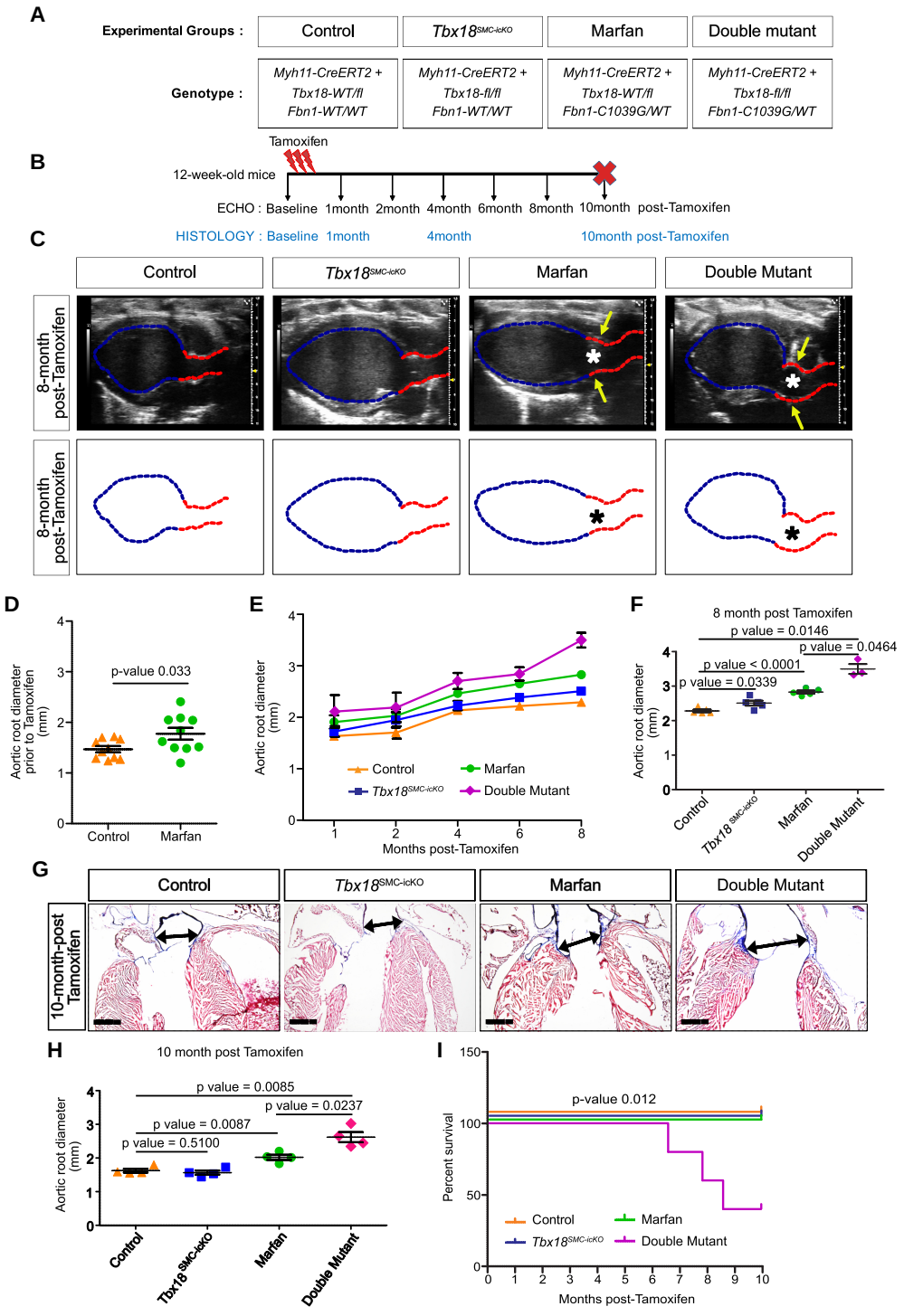


Figure 2 SMC-specific ablation of *Tbx18* in adulthood exacerbates the Marfan phenotype. (*A*) Experimental groups and respective genotypes of animals included in our experiments of *Tbx18* ablation in adult SMCs. (*B*) Diagram summarizing the experimental time course. (*C*) Representative ultrasound long-axis view images of the diastolic heart 8 months post-tamoxifen. Dashed lines delineate the left ventricular chamber and the beginning of the ascending aorta. Asterisks highlight aortic root dilation. Scale bars in millimetres. (*D*) Quantification of maximal aortic root diameter prior to tamoxifen administration (data represented as mean ± s.e.m; $N = 10$ per group, unpaired *t*-test with Welch's correction). (*E*) Graphical representation of aortic root diameter (assessed by echocardiography) for the different groups over the course of the experiment (data are represented as mean ± s.e.m; $N = 5$ per group). (*F*) Statistical analysis of differences in aortic root diameter (assessed by echocardiography) observed between groups at 8 months post-tamoxifen. (*G*, *H*) Histological analysis (*G*) and corresponding quantification (*H*, data presented as mean ± s.e.m; $N = 4$ per group) assessing aortic root diameter in the different groups at 10 months post-tamoxifen. (*I*) Kaplan–Meier plot showing a wave of lethality in the double mutant group that temporarily coincided with the period of accelerated dilation of the artic root ($N = 5$, statistical significance assessed with log-rank Mantel-Cox test).

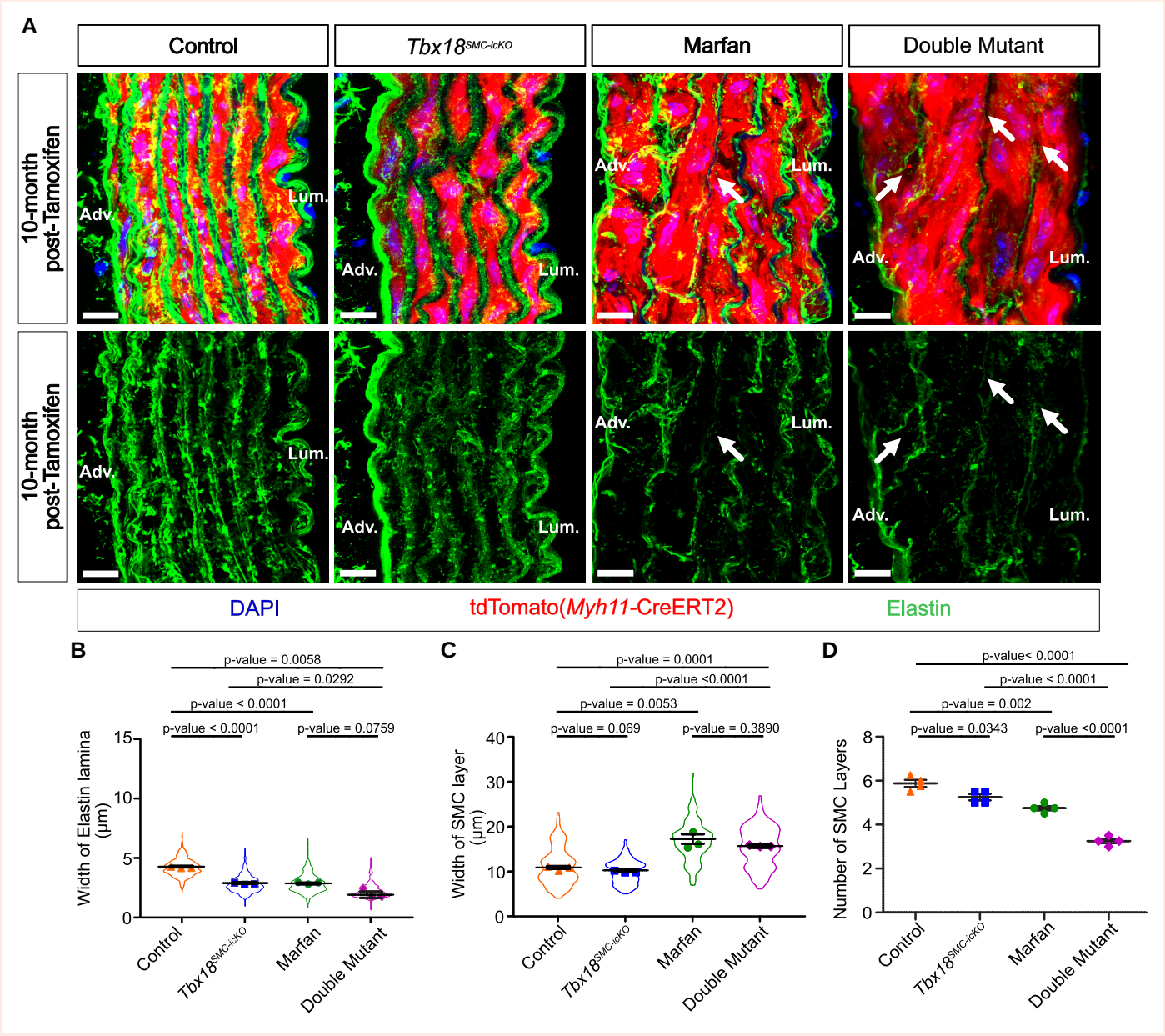


Figure 3 Adult ablation of *Tbx18* in SMCs compromises aortic wall integrity. (*A*) Representative confocal microscopy images showing anomalies in the aortic ultrastructure at 10 months post-tamoxifen. Merged images of elastin immunostaining and endogenous tdTomato fluorescence (reporter of Cre activity) are shown in the top row, whereas the bottom row shows elastin immunostaining only. The severe thinning or complete disappearance of elastin layers (arrows) observable in the Marfan group was exacerbated in double mutants. (*B–D*) Quantification of histological data 10-month post-tamoxifen showing reduction of elastic laminae thickness in all mutant groups (*B*), thickening of individual vSMC layers in Marfan and double mutant aortae (*C*), and reduced number of distinguishable vSMC layers in aortae of all mutant groups (*D*). Elastin disruption and reduction of vSMC layers were more pronounced in double mutants than in any other group. Data presented as mean ± s.e.m ($N = 3$ in *B*, *C* and 4 in *D*), statistical significance estimated using an unpaired *t*-test. In *B* and *C*, violin plot lines represent all the individual measurements done for each experimental group. Scale bars = 10 μm.

experimental groups. From these, only one—*Kcne4*—was down-regulated in all three genotypes, both at the transcriptional and translational levels. KCNE4 is known to be expressed in arteries,[34] where it acts in vSMCs to control contractility via promoting the activity of the voltage-gated potassium channel Kv7.4.[35] Previous studies have shown that altered vSMC contractility is a functional consequence of aneurysm-causing mutations (including the Marfan-causing *Fbn1-C1039G* model we employed),[36,37] and it is conceivable that altered expression of ion channels and their regulators (including but not restricted to *Kcne4*), is involved in this process. Another common trend was the up-regulation of IEGs ('NGF-stimulated transcription'),[33] both at the transcriptional and translational levels, in Marfan and double mutant aortae. This reactome category was mainly driven by TFs of the EGR and AP-1 (Activator Protein 1—*Fos*, *Junb*) families and did not emerge as functionally enriched in *Tbx18*$^{SMC\text{-}icKO}$ aortae. Nonetheless, TBX18 seems to be involved in regulating the expression of these genes, as simultaneous ablation of *Tbx18* amplified the effects already observed in Marfan aortae, with double mutants displaying a higher number of modulated genes and higher fold changes. Notably, IEGs are known to be mechanosensors that can be activated by altered anchoring of cells to the surrounding ECM or by altered vSMC contractility,[14]

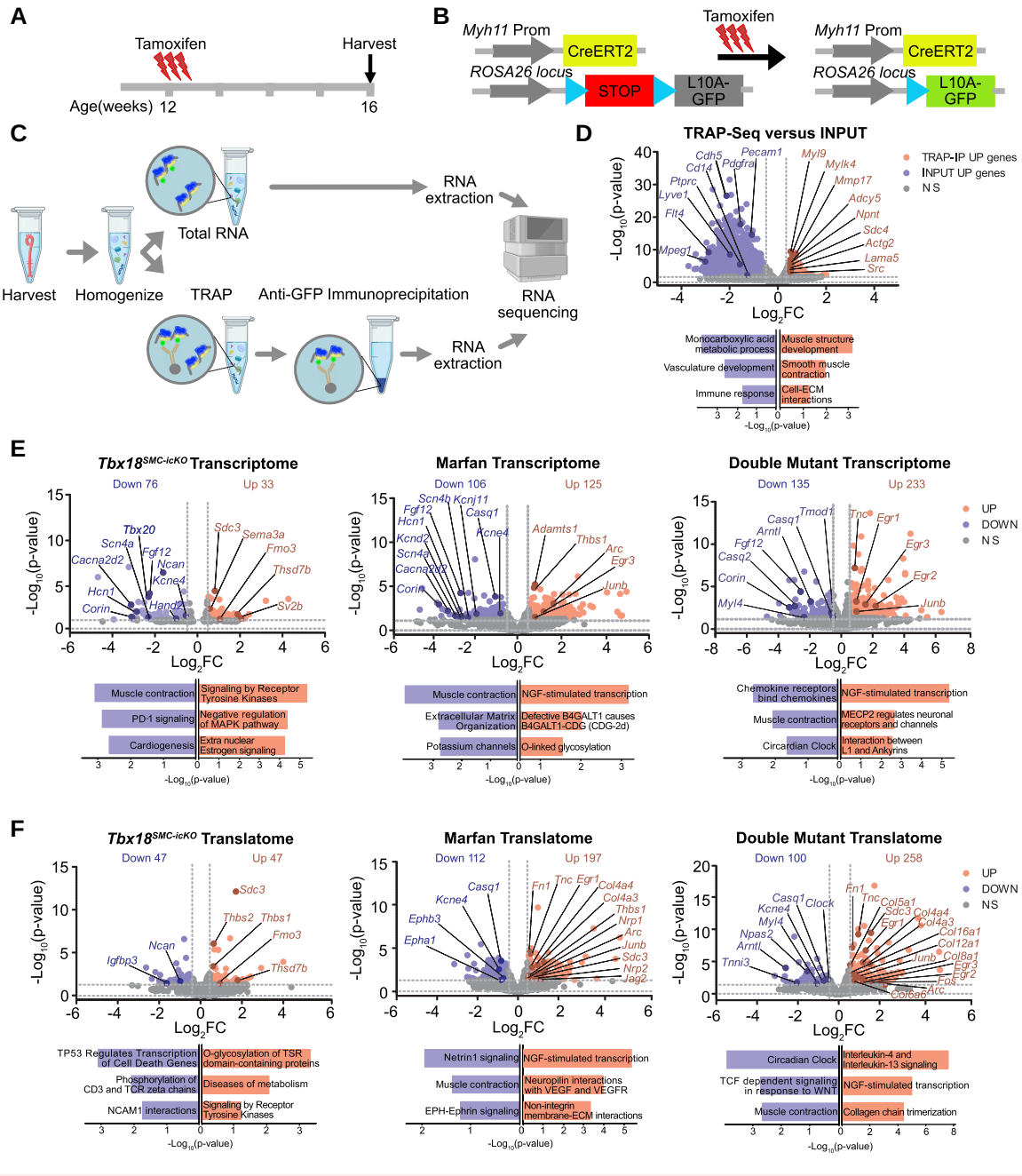


Figure 4 Early gene expression alterations in aneurysm-predisposed aortae. (*A*) Tamoxifen induction took place at 12 weeks of age, and harvest of aortae for transcriptomics and translatomics was done 4 weeks after. (*B*) Schematic representation illustrating Cre-dependent activation of Ribo-GFP (L10A-GFP) in SMCs. (*C*) After homogenization of harvested aortae, an aliquot was used for bulk RNA-Seq, and the remaining lysate was used for TRAP-seq of vSMC ribosomes ($N = 3$ per group). (*D*) Volcano plot comparing results from TRAP-seq and bulk RNA-Seq in control aortae. (*E*) Volcano plots and corresponding functional annotation analyses representing the top differently expressed genes (DEGs) in RNA-Seq datasets of the different mutant groups vs. controls. (*F*) Volcano plots and corresponding functional annotation analyses showing the top differently translated transcripts (DTTs) in TRAP-seq datasets of the different mutant groups vs. controls.

providing a link between two biological processes commonly modulated across different genotype groups. Importantly, EGR1, one of the consistently up-regulated IEGs, has been previously identified as a central player in aneurysmal development consequent to *EFEMP2*/Fibulin-4 loss-of-function,[29] suggesting that two of the most widely studied familial aneurysm syndromes (those caused by *Fbn1* and *Fibulin-4* mutations) might share similar molecular mechanisms.

3.4 TBX18 directly regulates critical genes in aortic homeostasis

Our current knowledge as to genes directly targeted by TBX18 is very limited due to the lack of datasets assessing genome-wide patterns of TBX18-DNA binding. To circumvent this limitation, we performed ChIP-seq in primary human aortic smooth muscle cells (haSMCs)

expressing a FLAG-tagged version of the human TBX18 transcript (*Figure 5A*). Immunoprecipitation using a ChIP-grade anti-FLAG antibody followed by NGS allowed the identification of 11 667 peaks significantly enriched over input (see Supplementary material online, *Table S3*). From these, the majority overlapped with promoters or intronic sequences (*Figure 5B* and Supplementary material online, *Table S3*). Consistent with a role for TBX18 in aortic homeostasis, using GREAT[38] to probe for human phenotypes associated with the enriched peaks revealed that the top categories were aortic dilation/aneurysm and mitral valve prolapse (*Figure 5C*). Using HOMER[39] to assess transcription factor binding motifs enriched in TBX18 ChIP-seq peaks produced a ranking in which the top 10 positions were dominated by the AP-1 complex (heterodimer of FOS and JUNB, motif present in 20.5% of peaks) and other bZIP TFs that bind to the same conserved motif (*Figure 5D*). Amongst the top enriched motifs, there was also a canonical TBX binding motif (AGGTGTGAAA, matching the TBX21 binding sequence determined by the ENCODE consortium via ChIP-seq in GM12878 cells) present in 14% of peaks, as well as a degenerate TBX binding motif containing a central position without nucleotide preference (GGTG*TGAAA) previously identified via TBX20 ChIP-seq in murine cardiomyocytes.[40]

We subsequently performed combined analyses of ChIP-seq and TRAP-seq to identify direct targets of TBX18 regulation (genes modulated when *Tbx18* is ablated in aortic SMCs and containing a TBX18 ChIP-seq peak overlapping their promoter, Supplementary material online, *Table S4*). From the 111 transcripts differentially translated in $Tbx18^{SMC\text{-}icKOs}$, 14 were bound by TBX18, suggesting direct regulation (*Figure 5E*). Consistent with the view of TBX18 as a transcriptional repressor, the majority of these genes corresponded to transcripts up-regulated in $Tbx18^{SMC\text{-}icKOs}$ (*Thbs1, Flrt2, Gabre*) that, from reactome analyses, were associated with functional categories such as diseases of metabolism and platelet activation (*Figure 5E* and Supplementary material online, *Table S5*). Although less abundant, there were also examples of genes bound by TBX18 and down-regulated when this TF was ablated, suggesting that, in addition to its recognized repressive functions, TBX18 might also work as a transcriptional activator. In the double mutant group, 67 of the 387 modulated genes (16 down-regulated and 51 up-regulated) were directly bound by TBX18 (*Figure 5E*). Amongst direct TBX18 targets up-regulated in double mutants (therefore negatively regulated by TBX18), in addition to those aforementioned for the $Tbx18^{SMC\text{-}icKO}$ group, there were immediate early TFs *Egr1*, *Fos*, *Junb*, included in the reactome category 'Cytokine Signalling in Immune system' (*Figure 5E* and *G* and Supplementary material online, *Table S5*). TBX18 also negatively regulated genes involved in ECM degradation (*Figure 5E*), including ADAMTS1, a protein thought to be involved in TAA progression.[41] On the other side of the scale, amongst direct TBX18 targets down-regulated in double mutants (therefore positively regulated by TBX18), there were two central elements of the circadian clock (*Npas2* and *Clock*), as well as molecules involved in ion transport regulation (*Kcne4* and *Kcnj15*, reactome categories muscle contraction and neuronal system, *Figure 5E* and *F* and Supplementary material online, *Table S5*).

Altogether, these results suggested that TBX18 plays a central role in safekeeping the transcriptional landscape of aortic vSMCs by repressing expression not only of a set of effector proteins known to play an important role in aneurysm formation (THBS1, ADAMTS1), but also of upstream TFs up-regulated in early steps of aneurysm development (EGR1, JUNB, FOS). Some TBX18 targets were immediately modulated in $Tbx18^{SMC\text{-}icKOs}$, whereas others only showed altered expression after a second hit (Marfan-causing *Fbn1* mutation). *Thbs1* was amongst those immediately modulated in $Tbx18^{SMC\text{-}icKOs}$, which, together with its known involvement in aneurysm formation,[15] places it as a central target. Therefore, we further investigated the abundance of the THBS1 protein in the different genotype groups via western blot (*Figure 5H* and Supplementary material online, *Figure S6*). Quantitative analyses (*Figure 5I*) revealed a trend for increased THBS1 levels in $Tbx18^{SMC\text{-}icKO}$ and Marfan aortae without reaching statistical significance. On the other hand, double mutant aortae showed an exacerbated and statistically significant up-regulation of THBS1, which is consistent with the exacerbated phenotype observed in this group.

3.5 *TBX18* expression is reduced in aneurysmal aortae of human patients

Results from our *in vivo* and *in vitro* models placed TBX18 as an important gatekeeper of normal aortic vSMC gene expression programmes. To investigate the clinical relevance of these findings, we assessed whether TBX18 down-regulation might be involved in human AA. Transcriptomic analyses of a cohort of AAA samples (*Figure 6A*) revealed that *TBX18* was significantly down-regulated in aneurysmal specimens and even further reduced in ruptured aneurysms (*Figure 6B*). In the same cohort, multiple genes that our study identified as direct targets of TBX18 were also significantly modulated following the expected pattern of regulation (up-regulation of negative TBX18 targets—*FOS*, *JUNB*, *EGR1*, and *THBS1*—and down-regulation of positive TBX18 targets, *Figure 6B*). Altered *TBX18* expression in human aneurysms was further validated in a cohort of TAA histological specimens containing both sporadic and genetic (Marfan) aneurysms (*Figure 6C*). RNAscope analyses on tissues collected during surgical TAA repair revealed a significant down-regulation of *TBX18* transcript abundance in the aneurysm vs. the flanking non-dilated thoracic aorta (*Figure 6D–F*). Altogether, these results support the conclusion that reduced TBX18 activity affects aortic aneurysm development and disease progression.

4. Discussion

Multiple effector proteins whose mutation or dysregulation can lead to aneurysm formation have been described.[3,4] However, our understanding of the transcriptional mechanisms underlying physiological and pathological aortic states remains limited. Work here reported sheds light on this theme, placing TBX18 as a transcription factor required in vSMCs to prevent gene expression programmes associated with pathological aortic remodelling.

Ablation of *Tbx18* in mural cells from early embryogenesis resulted in a severe aortic phenotype, revealing that TBX18 is required for normal aortic development. Adult ablation of *Tbx18* alone produced milder phenotypes, with occasional disorganization of the aortic ultrastructure without any associated lethality or aortic root dilation. However, combining adult ablation of *Tbx18* in SMCs with a second hit (a Marfan-causing *Fbn1* mutation) led to an aggravation of the Marfan phenotype. Mechanistic studies revealed that a major alteration in Marfan aortic vSMCs in early phases of adverse remodelling (prior to any major histological phenotypes) was the up-regulation of IEGs encoding evolutionary conserved TFs (EGR and AP1) that determine cellular responses to environmental stimuli.[42] Consistent with the exacerbated phenotype, IEG up-regulation was further increased in double mutants. In addition to being a known regulator of the cell cycle, AP-1 also modulates transcriptional events downstream of TGFβ[43] and silencing of AP-1 is sufficient to inhibit aortic elastolysis in Marfan.[44] EGR1, another of the up-regulated IEGs, is a mechanosensor[14] that drives aneurysm formation in cutis laxa caused by mutations in the gene encoding Fibulin-4, via up-regulation of its direct target *Thbs1*.[15,16] *Thbs1* itself was up-regulated in all our mutant groups (including $Tbx18^{SMC\text{-}icKO}$) and THBS1 protein levels were particularly elevated in double mutant aortae, placing it as a likely mediator of observed phenotypes. Notably, single-cell RNA-seq studies assessing the mechanistic bases of a completely distinct aneurysmal model (angiotensin infusion) also revealed up-regulation of EGR1 and AP-1 TFs in clusters of proliferative or ECM-producing vSMCs associated with pathological remodelling.[45] This convergence of signalling events in three distinct forms of aneurysm (Marfan, cutis laxa, and angiotensin infusion), suggests that activation of IEGs might be a conserved step in early aneurysmal development and that targeting these transcriptional regulators could be a strategy to prevent adverse aortic remodelling across a wide spectrum of aneurysmal diseases. Importantly, our mechanistic results are also in line with previous reports of increased TGFβ activity in Marfan syndrome.[9,10] TGFβ drives aneurysm formation via activation of the extracellular regulated kinase (ERK) pathway,[11] a known activator of *EGR1* expression.[46] At the same

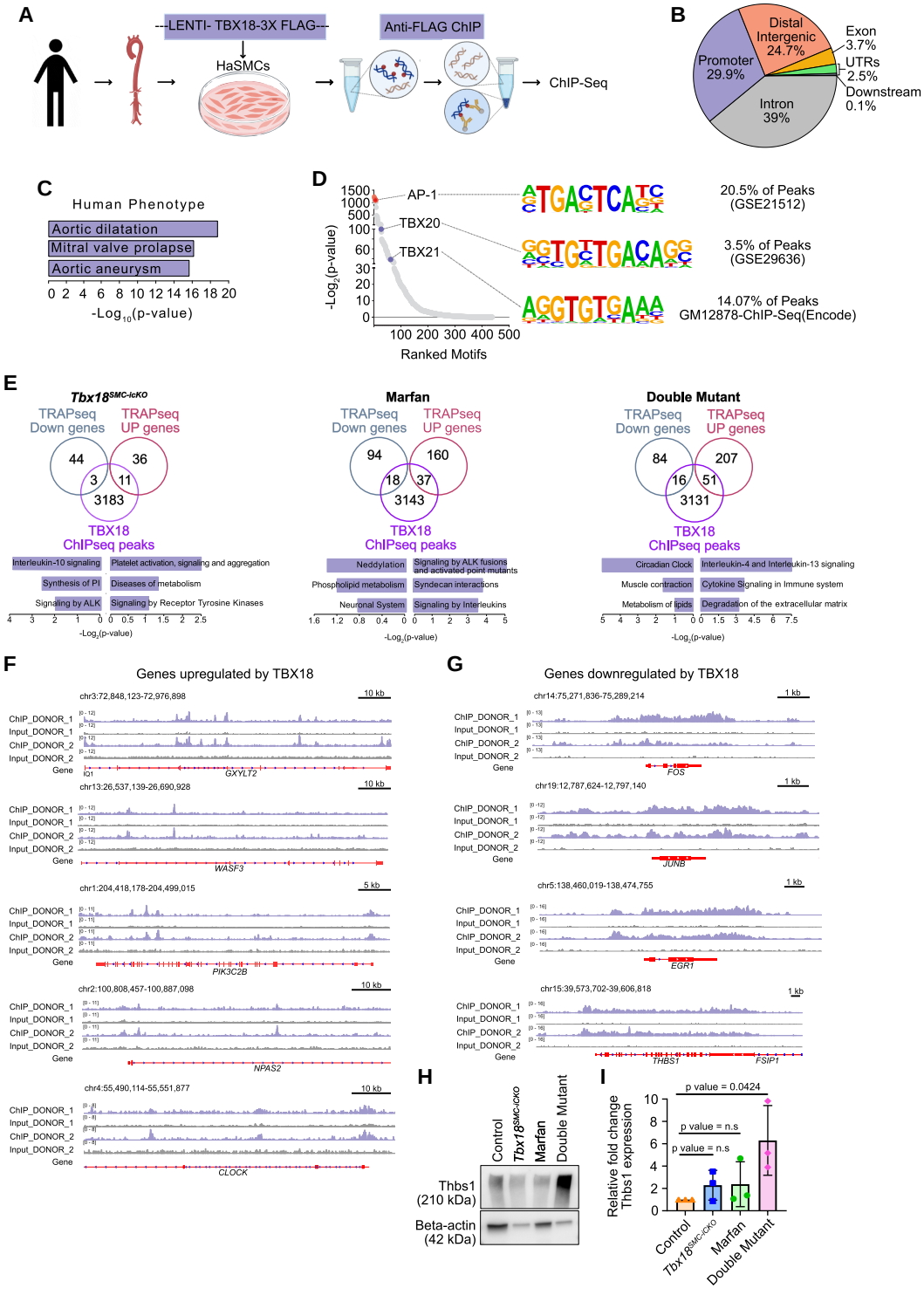
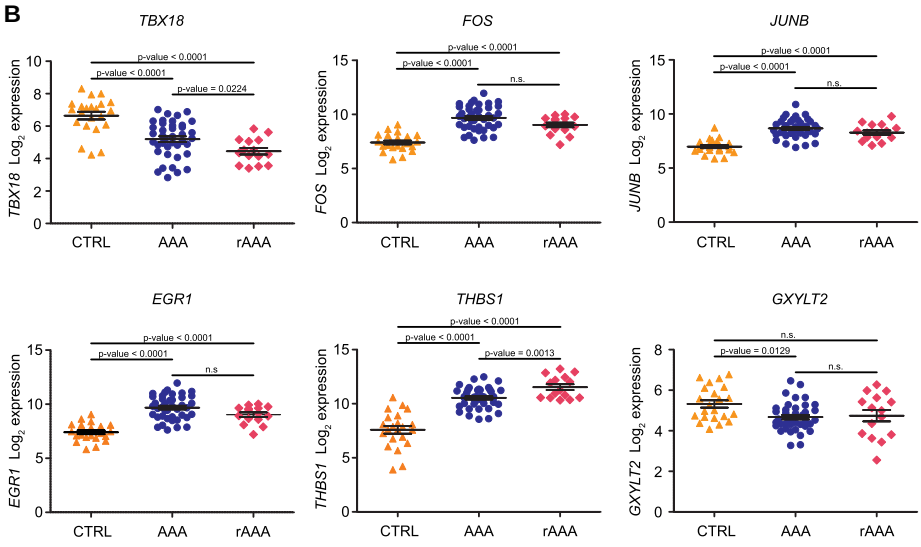


Figure 5 Determining direct targets of TBX18. (*A*) Primary human aortic SMCs (haSMCs) were transduced with lentiviral vectors driving expression of TBX18 fused with a 3xFLAG tag, enabling ChIP-Seq ($N = 2$, male donors) using an anti-FLAG antibody. (*B*) Pie chart displaying the genomic distribution of TBX18 ChIP-seq peaks. (*C*) Analysis of TBX18 ChIP-Seq peaks with GREAT revealed enrichment in human phenotypes associated with aortic pathologies. (*D*) Motif analysis using HOMER revealed an enrichment of AP1 and TBX binding motifs amongst TBX18 ChIP-seq peaks. (*E*) Intersection of ChIP-seq and TRAP-seq datasets for the identification of likely direct targets of TBX18 in aortic vSMCs. Functional annotation plots refer to the genes intersected in the corresponding Venn diagram. (*F*) Genome browser tracks showing examples of ChIP profiles in genes positively regulated by TBX18. (*G*) Genome browser tracks showing examples of ChIP profiles in genes negatively regulated by TBX18, including *EGR1* and its target *THBS1*. (*H*, *I*) Representative immunoblot of aortal lysates (*H*) and respective quantification (*I*), showing a significant increase in THBS1 protein levels in double mutants 1 month post-tamoxifen. Data presented as mean ± s.e.m ($N = 3$ per group), statistical significance estimated using an unpaired *t*-test.

A

	Average Age	Females	Males	Diameter(mm)
Elective	68,3	5	37	61,7
Ruptured	74,5	5	12	82,1
Control	47,7	11	11	–



C

Type of TAA	Average Age	Gender		
		Females	Males	no info
Sporadic TAA	71,2	3	2	--
Marfan TAA	31,7	1	2	2

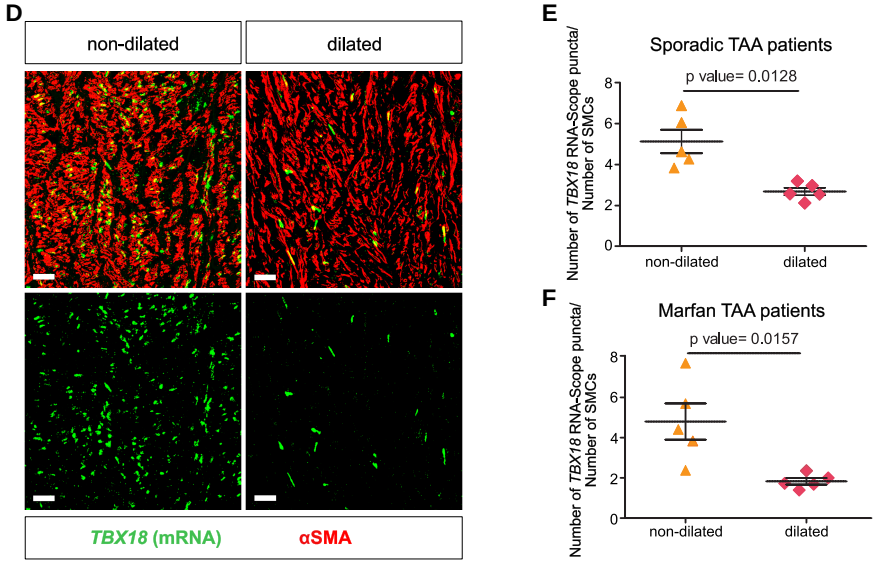


Figure 6 Modulation of TBX18 and its targets in human aneurysms. (*A*) Characteristics of the cohort of AAA patients and controls included in analyses assessing *TBX18* expression in human aneurysmal disease. (*B*) Transcriptomics analyses showing down-regulation of *TBX18* expression in human abdominal aortic aneurysms (AAA) ($N = 42$) vs. control aortae ($N = 23$). The down-regulation of *TBX18* was further exacerbated in ruptured aneurysms (rAAA) ($N = 15$). Multiple of the direct targets of TBX18 identified in our mechanistic studies were also modulated following a pattern consistent with the nature of regulation (negative or positive) by TBX18. Statistical significance was estimated using one-way ANOVA with Bonferroni's multiple comparison test. (*C*) Characteristics of the cohort of TAA patients included in analyses assessing *TBX18* expression via histological techniques. (*D–F*) Imaging of RNAscope analyses (*D*) and respective quantifications (*E, F*) showing decreased abundance of *TBX18* transcripts in SMCs in the aneurysmal area vs. flanking non-dilated tissue (both collected during surgical TAA repair). Data presented as mean ± s.e.m ($N = 5$ per group), statistical significance estimated using an paired *t*-test. Scale bars = 50 μm.

time, THBS1, a positive target of EGR1,[16] contributes to activation of latent TGFβ ligands,[17] thereby creating a positive feedback signalling loop.

ChIP-seq analyses and their integration with translatomics revealed that TBX18, a transcription factor that acts predominantly as a repressor,[23] directly binds to and inhibits several of the aforementioned genes (*Egr1*, *Fos*, *Junb*, *Thbs1*), placing TBX18 as a gatekeeper that acts in aortic vSMCs to prevent gene expression programmes associated with pathologic remodelling. Interestingly, the motif recognized by the AP-1 complex was the most enriched in TBX18 ChIP-seq peaks, which could suggest TBX18 partners with AP-1 to co-regulate the expression of target genes. However, this seems unlikely as attempts to identify the interactome of TBX18 did not reveal any partnership with proteins of the AP-1 complex.[47] A seemingly more likely explanation is that TBX18 might compete with AP-1 for DNA binding, suggesting that TBX18 negatively regulates AP-1 at two levels: repression of genes encoding AP-1 subunits (*Fos*, *Junb*) and competition for binding to target loci. Intriguingly, our results also suggested that TBX18 directly binds to and promotes expression of critical elements of the circadian clock (*Clock* and *Npas2*). NPAS2 and CLOCK are important for the maintenance of a peripheral vascular circadian clock;[48] however, it remains unclear if disturbance of this mechanism can contribute to aneurysm formation.

In addition to studies in murine models, a role for TBX18 in preventing adverse aortic gene expression profiles was also supported by analyses in samples from human AAA and TAA patients. The AAA cohort contained different stages of aneurysm progression (dilated vs. ruptured), which revealed an increasing degree of *TBX18* down-regulation during aneurysm progression. A previous study assessing tunica-specific transcriptional alterations in AAA had also identified *TBX18* as one of the top down-regulated TFs in the tunica media of aneurysmal aortae.[49] This consistent pattern of *TBX18* down-regulation in three completely independent sample cohorts (involving both TAA and AAA) is highly indicative that disrupted TBX18 activity is involved in human aneurysms. Despite its central role in aortic vSMCs, TBX18 seems dispensable in other SMC populations (gut, liver, intestine). It is possible that the transcriptional effects TBX18 exerts in the aorta are not required in these populations. Alternatively, it is conceivable that in these populations those same functions are executed by another transcription factor.

In addition to improving our understanding of transcriptional mechanisms maintaining aortic homeostasis, this study also generated datasets valuable to the community. AA have been previously analysed at transcriptomics, proteomics, and metabolomics levels,[50] but, to our knowledge, our TRAP-seq datasets are the first translatomics analyses in the context of an aneurysmal disease. Translatome datasets revealed differences that were not detected at the transcriptome level, including up-regulation of multiple ECM molecules in vSMCs from the Marfan and double mutant groups (indicating transition to a synthetic vSMC phenotype), or decreased Ephrin and increased Neuropilin signalling in Marfan vSMCs. This can have two distinct explanations: the modulation of these pathways might have been missed at the transcriptomics level because the samples used for RNA-seq contained all cell types of the aortic wall, while TRAP-seq is vSMC-specific, or it might indicate that these processes are regulated at the ribosome level, rather than at the nuclear expression level.[51] The TBX18 ChIP-seq dataset we produced might also be of interest to laboratories studying this transcription factor in different contexts, although it is not currently known whether TBX18 genomic occupancy patterns are preserved across distinct cell types.

This study also has limitations that should be kept in consideration. The absence of an antibody specifically recognizing TBX18 does not allow us to perform analyses assessing levels of TBX18 protein in murine or human samples. It also makes it impossible to immunoprecipitate the endogenous protein. To circumvent this, for ChIP-seq assays, we had to resort to a FLAG-tagged version of TBX18 overexpressed in primary aortic SMCs using viral vectors. Despite being a strategy widely used by multiple laboratories, including the ENCODE project, we cannot exclude the possibility that the overexpression might have produced ectopic ChIP-seq peaks that do not necessarily reflect binding patterns of the endogenous protein. The aortic phenotype observed upon embryonic ablation of TBX18 was much stronger than the one observed when TBX18 was ablated in adulthood. This might reflect a strong requirement for TBX18 during aortic organogenesis, but might also be a consequence of the fact that embryonic cKOs had a moderate increase in blood pressure, a known risk factor for aneurysm development. As most of our results were generated in murine models, we acknowledge that interspecies differences may limit the direct translatability of some findings to human biology.

In conclusion, in addition to placing TBX18 as a relevant player in aortic transcriptional control, our work also increases our understanding of molecular events taking place in early phases of pathologic aortic remodelling, suggesting potentially shared mechanisms between distinct types of aneurysms. Given these observations, it will be of future interest to investigate a potential involvement of TBX18 in other forms of vascular disease (for example, aneurysms caused by mutations in the gene encoding Fibulin-4) and assess whether mutations in *TBX18* or genomic elements regulating its aortic expression might contribute to human vascular pathologies.

Translational perspective

Using preclinical models and patient samples, we demonstrate that the transcription factor TBX18 functions in aortic smooth muscle cells (SMCs) to prevent transcriptional programmes associated with adverse remodelling. Among the targets repressed by TBX18 in SMCs were several immediate early genes, whose activation, based on our findings and existing literature, appears to be a common early event in distinct models of aortic aneurysm. In addition to improving our understanding of transcriptional processes operating to maintain aortic homeostasis, these findings also highlight upstream regulators of SMC properties that could be targeted by pharmacological or biological approaches to prevent aneurysm progression.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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Data availability

Raw data are available at: https://zenodo.org/records/15642466.

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