LOSS OF ERK1/2 ACTIVITY IN BAV TRIGGERS AP-1 DEGRADATION AND AORTIC WALL INSTABILITY

Stephanie Wales Tobin[1], Ren-Ke Li[1, 2]
[1]Division of Cardiovascular Surgery, Toronto General Hospital Research Institute, University Health Network, Toronto, Canada
[2]Division of Cardiac Surgery, Department of Surgery, University of Toronto, Toronto, Canada

Authors and Contributions:
Stephanie W. Tobin, PhD, First Author: Analyzed microarray data (Bioinformatics, Heatmap, Cytoscape Plot and Volcano Plot); identified novel patterns in microarray data and validated these findings in patient samples using RT-qPCR (mRNA expression) and western blot (protein analysis); wrote the manuscript
Jie Wu, MD, PhD: Analyzed data, performed real time qPCR experiments and analyzed data
Shu-Hong Li, MD, MSc: helped with tissue collection, RNA extraction and helped with real time qPCR experiments
Jian Guo, MD, PhD: sample preparation and microarray data analysis
Azadeh Yeganeh, PhD: Prepared tissue sections for staining
Katherine Tsang, MN: patient consent, tissue collection
Laura Tumiati, BSc: helped with tissue collection
Jagdish Butany, MBBS: helped with tissue collection
Rodolfo Rocha, MD: Provided patient information for correlation analysis
Terrence M. Yau, MD, MSc: helped with tissue collection
Maral Ouzounian, MD, PhD: helped with tissue collection
Tirone E. David, MD: helped with tissue collection
Richard D. Weisel, MD: Senior Scientist
Ren-Ke Li, MD, PhD: Senior Scientist

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Introduction

Bicuspid aortic valve (BAV) is a congenital abnormality present in 1-2% of the population in which the aortic valve is composed of two leaflets instead of three[1]. Those born with BAV are more likely to develop an aortic aneurysm that leads to aortic rupture than those born with a tricuspid aortic valve (TAV), therefore, understanding the mechanisms responsible for this phenomenon is important to providing efficient treatment.

BAV intrinsically changes the direction and velocity of blood flow in the ascending aorta, leading many to believe that hemodynamics is a major cause of aneurysm[2]. Several genetic components have also been implicated in BAV but none have been shown to be causative in aortic dilatation. For example, degradation of the extracellular matrix (ECM) is enhanced in BAV due to an upregulation in the activity of matrix metalloproteinases (MMPs) and downregulation of Tissue inhibitors of metalloproteinases (TIMPs)[3]. This unbalanced TIMP:MMP ratio is also seen in Marfan syndrome, an inheritable disease of the connective tissue caused by a mutation in Fibrillin-1[4]. Patients with Marfan syndrome are also prone to aortic aneurysms as loss of Fibrillin-1 enhances extracellular TGF-β. This induces activation of the canonical (Smad) and non-canonical (ERK1/2 and JNK) TGF-β pathways leading to transcriptional changes in matrix remodelling programs including MMP expression[4]. TGF-β has also been implicated in BAV aneurysm progression but its role is controversial as different groups have demonstrated that both precocious and low levels of TGF-β contribute to BAV aortic dilatation[5–8].

As aortic aneurysm in BAV patients is progressive, a comparison of diseased tissue from the proximal (severely dilated) and distal (less dilated) regions within a patient sample could provide insight into the molecular changes that drive this disease. Using this strategy we have previously identified that miR-17, an inhibitor of TIMP-1 translation, is more highly expressed in the distal region of the diseased aorta from BAV patients[3]. There is still much unknown about the molecular changes that initiate aortic remodelling, and there has been insufficient biochemical exploration into novel pathways that may promote aortic dilatation. To this end, we have completed microarray analysis of the proximal and distal regions of the dilated ascending aorta from six patients with BAV. We observed that several members of the Activator Protein 1 (AP-1) transcription factor family were upregulated in the distal region of BAV aneurysmal tissue. This induction was not detected within paired Marfan or TAV aneurysmal tissue. Further downstream biochemical analysis identified dysregulation of ERK1/2 activity in BAV aneurysmal tissue, an upstream regulator of AP-1 activity and stability, warranting further examination of the ERK1/2-AP-1 signaling axis in BAV aortic dilatation.

Results

Identification of differentially expressed genes in diseased BAV tissue

Microarray gene expression analysis was performed on the proximal (severely dilated) and distal (less dilated) regions of dilated aorta from patients with BAV. A total of 1616 differentially expressed probes were identified. Figure 1A depicts the resultant probes in a heatmap using hierarchical clustering and demonstrates a distinction between the gene expression profiles of the distal and proximal regions of six BAV patients. We next assessed KEGG pathways enriched in our microarray analysis (Figure 1B). Many of the identified pathways were related to inflammation (e.g. T cell receptor signaling pathway), DNA damage (e.g. Non-homologous end-joining) or metabolism (e.g. Starch and sucrose metabolism). VEGF signaling pathway (9 genes) and MAPK signaling (21 genes) were also enriched.

The log2 Fold Change in gene expression between the Distal and Proximal regions was mapped using a Volcano plot to identify the most differentially expressed probes (Figure 1C). Fosb, a member of the Activator Protein-1 (AP-1) transcription factor family was the most upregulated gene. Several secreted and extracellular factors that have been identified as prognostic markers of disease were significantly downregulated in the distal aorta compared to the proximally dilated area such as Itln1 (Intelectin), Prg4 (proteoglycan 4), Msln (Mesothelin), and Bche (Butyrylcholinesterase).

We performed qRT-PCR analysis to validate the expression pattern of a subset of genes that showed the most dynamic expression in the microarray analysis (Figure 1D). Compared to the proximal (severely dilated) region, several putative secreted or extracellular biomarkers were downregulated in the distal (less dilated) portion of the aorta. Conversely, many transcription factors were upregulated in the distal region: Nr4a2, KLF4, Egr1, Atf3 and three members of the AP-1 transcription factor family Jun, Fos and Fosb.

Expression of Fosb, Fos, Atf3 and Jun in aortic disease

AP-1 can complex as a Jun-Jun, Jun-Fos or Jun-Atf dimer. We hypothesized that upregulation of Fosb, Fos, Atf3, and Jun indicated an important role for AP-1 complexes in aortic disease progression in other aneurysmal tissue in addition to BAV. To test this we compared the proximal:distal expression of these factors from patients with BAV to normal aortic tissue and dilated aortic tissue from patients with Marfan syndrome or TAV. One patient presented as BAV-Marfan and another as TAV-Loeys-Dietz, a heritable mutation within the TGF-β signaling pathway. qRT-PCR analysis of the distal and proximal regions of dilated BAV aorta confirmed differential expression of Fosb, Fos, Atf3 and Jun however, we did not observe differences within Marfan or TAV aortic tissue (Figure 2A). Compared to normal aortic tissue, Fosb and Jun also showed variable expression in BAV tissue (Figure 2A). Atf3 was significantly downregulated in Distal Marfan aortic tissue. The BAV-Marfan patient presented an AP-1 expression pattern similar to that of BAV patients. Patients with aneurysmal TAV showed high variability in AP-1 expression, and one sample was removed from the analysis as they presented inconsistently higher levels of AP-1 expression. The patients with TAV-Loeys-Dietz also had overall higher levels of AP-1 expression.

AP-1 protein is differentially regulated in BAV aneurysmal tissue

We next assessed changes in AP-1 protein levels in BAV aneurysmal tissue. We focused on Jun and Fos since they are the classic AP-1 heterodimer. Western blot analysis revealed that several bands were detected by our Fos and Jun antibodies, potentially representing post-translational protein modification or degradation. Quantification of the predicted molecular weight of Fos (62 kDa) and Jun (39 kDa) demonstrated that these AP-1 factors are downregulated at the protein level in the distal portion of aneurysmal BAV tissue, in contrast to mRNA data (Figure 2B). To determine the location of AP-1 expression we stained for Jun in distal BAV aneurysmal aortic tissue. Smooth muscle actin (SMA) was used to determine the location of smooth muscle cells, but the autofluorescence generated by the collagen fibers identified cell boundaries in green. We observed that Jun was virtually unexpressed in the medial layer and instead localized to the periphery of the aortic tissue in the intima and adventitia (Figure 2C). Next, anterior and posterior sections of the distal aorta were stained. Interestingly the distal anterior aorta contained cytoplasmic Jun throughout the tissue layers, including in the medial layer (Figure 2D).

Reduced ERK1/2 activity in BAV aortic tissue and putative transcriptional consequences

Compared to BAV aneurysmal samples, the additional Jun and Fos protein bands observed at approximately 26 kDa and 50 kDa, respectively, were absent in normal aortic tissue (Figure 3A). AP-1 protein stability depends on ERK1/2 activity therefore we investigated whether ERK1/2 activity is altered in aneurysmal aortic tissue. Compared to normal aortic tissue, ERK1/2 activity in BAV-3 (BAV Patient #3) and TAV-1 (TAV Patient #1) was lower within both regions of the aorta (Figure 3B). ERK1/2 activity in Marfan tissue was comparable to normal aorta. Fos protein (62 kDa) was detected in all samples, however a smaller Fos protein of approximately 55 kDa was detected most strongly in BAV when ERK1/2 activity was low. Next we compared only the distal regions of aneurysmal tissue in four BAV, Marfan and TAV samples and two normal aorta samples. Three out of the four BAV samples showed dramatic reduction in ERK1/2 activity (Figure 3C). ERK1/2 activity in Marfan samples and normal aorta were comparable. TAV aortic samples showed high variability in ERK1/2 activity, similar to the variation of AP-1 mRNA expression.

To determine the downstream consequences of changes in AP-1 activity we used *in silico* analysis to predict AP-1 target genes that were differentially expressed in our microarray dataset. Sixty-four genes contained AP-1 consensus sites (TGACTCA) within 5kb of the transcription start site and showed greater than ±1.5 Fold Change (Distal:Proximal). Seventeen of these genes are found in the extracellular region and thus may have a paracrine effect on dilatation. The majority of these putative extracellular target genes were downregulated in the distal region of the aorta (Figure 3D). Many of the putative AP-1 target genes are related to inflammatory pathways such as IL8 (CXCL8) and CCL3L1 or are involved in endothelial cell function such as Cyr61 (aka CCN1), Esam (endothelial cell-specific adhesion molecule), Apold1 (aka Verge) and Bmper (BMP-binding endothelial regulator).

Discussion/Conclusion

Using microarray analysis of paired BAV aneurysmal tissue we have identified that AP-1 transcription factors are differentially expressed in the distal and proximal regions of the ascending aorta. BAV samples also demonstrated reduced ERK1/2 activity, an upstream AP-1 regulator. This may be a consequence of hemodynamic stress as this is not conserved in Marfan or TAV aneurysmal samples.

Both ERK1/2 activity and AP-1 expression can be altered in the endothelium by shear stress *in vitro* [9,10] therefore it is possible that induction of AP-1 in BAV is driven by hemodynamic stress. BAV patient samples, however, fail to induce ERK1/2 activity by an unknown mechanism (Figure 3E). Together, high shear stress and reduced ERK1/2 activity would lead to enhanced AP-1 transcription, but rapid AP-1 protein turnover (Figure 3E). Restoration of ERK1/2 activity could therefore have a role in limiting dilatation. AP-1 can regulate proliferation and inflammation including direct regulation of TIMP and MMP expression [11,12]. Indeed we observed that Timp1 is downregulated in distal BAV aortic tissue, and this could be driven by loss of AP-1 (Figure 3C). We have also identified several other secreted factors that may have a role in aortic dilatation or that could serve as potential biomarkers for disease progression.

We have demonstrated a novel molecular feature that is unique to human aortic BAV aneurysmal tissue. With this data, ERK1/2 and downstream transcription factors targets such as AP-1 should be considered in future research into the treatment of BAV aortic aneurysm progression.

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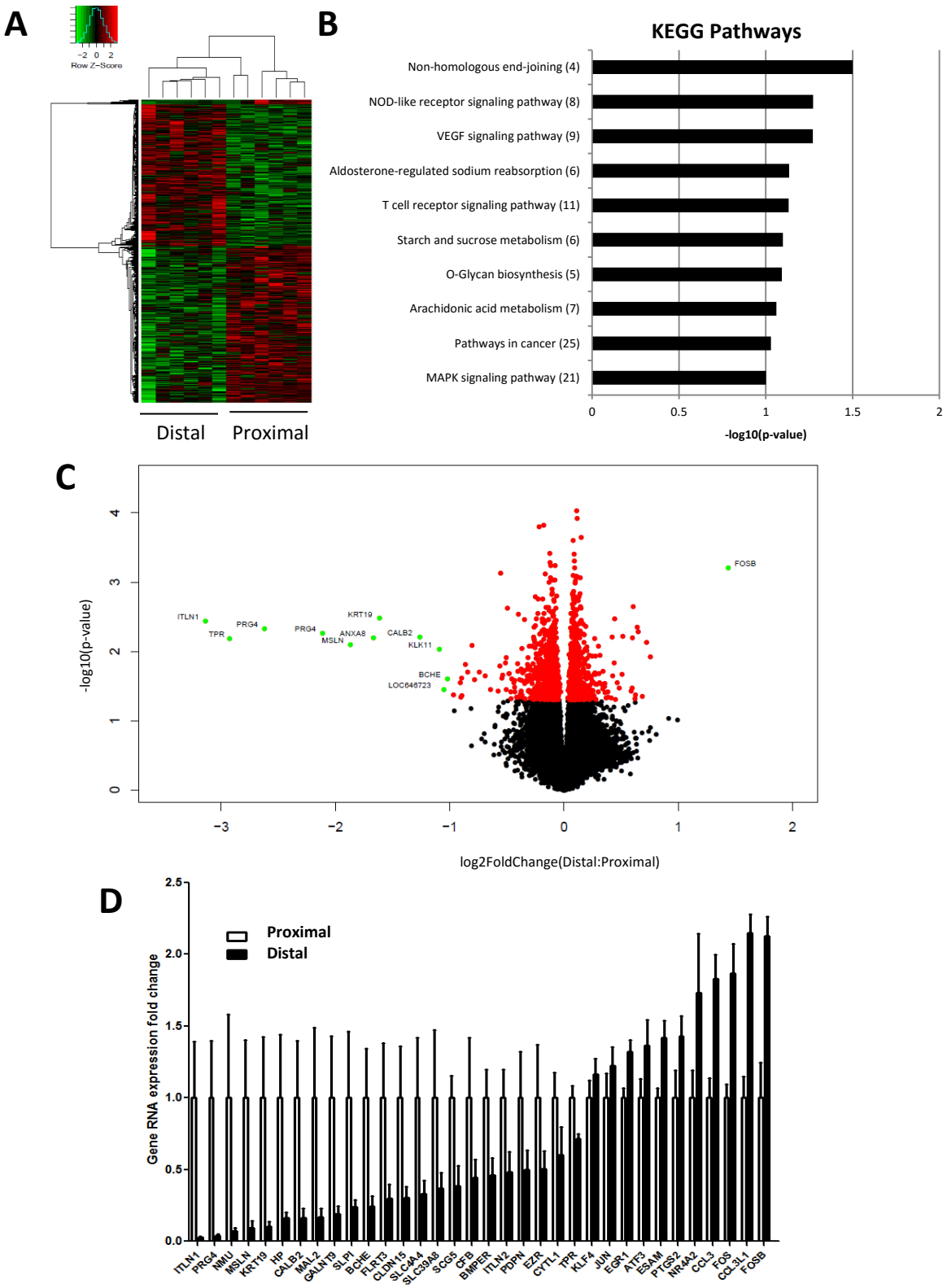

Figure 1. The AP-1 transcription factor family is highly expressed in the distal aortic segment of BAV patients. **A)** Heatmap depicting 1614 differentially expressed probes. Paired distal and proximal regions of aneurysmal aorta from patients with BAV were prepared for microarray analysis (n=6, two-tailed unpaired t-test p<0.05). **B)** KEGG Pathway analysis of differentially expressed genes. The number of genes associated with each annotation is indicated in parentheses. **C)** Volcano plot depicting the most up and downregulated genes. **D)** qRT-PCR validation of the most up and downregulated genes (n=6). Four AP-1 factors (Fosb, Fos, Atf3 and Jun) were upregulated in the Distal portion of the ascending aorta. Expression was normalized to Gapdh

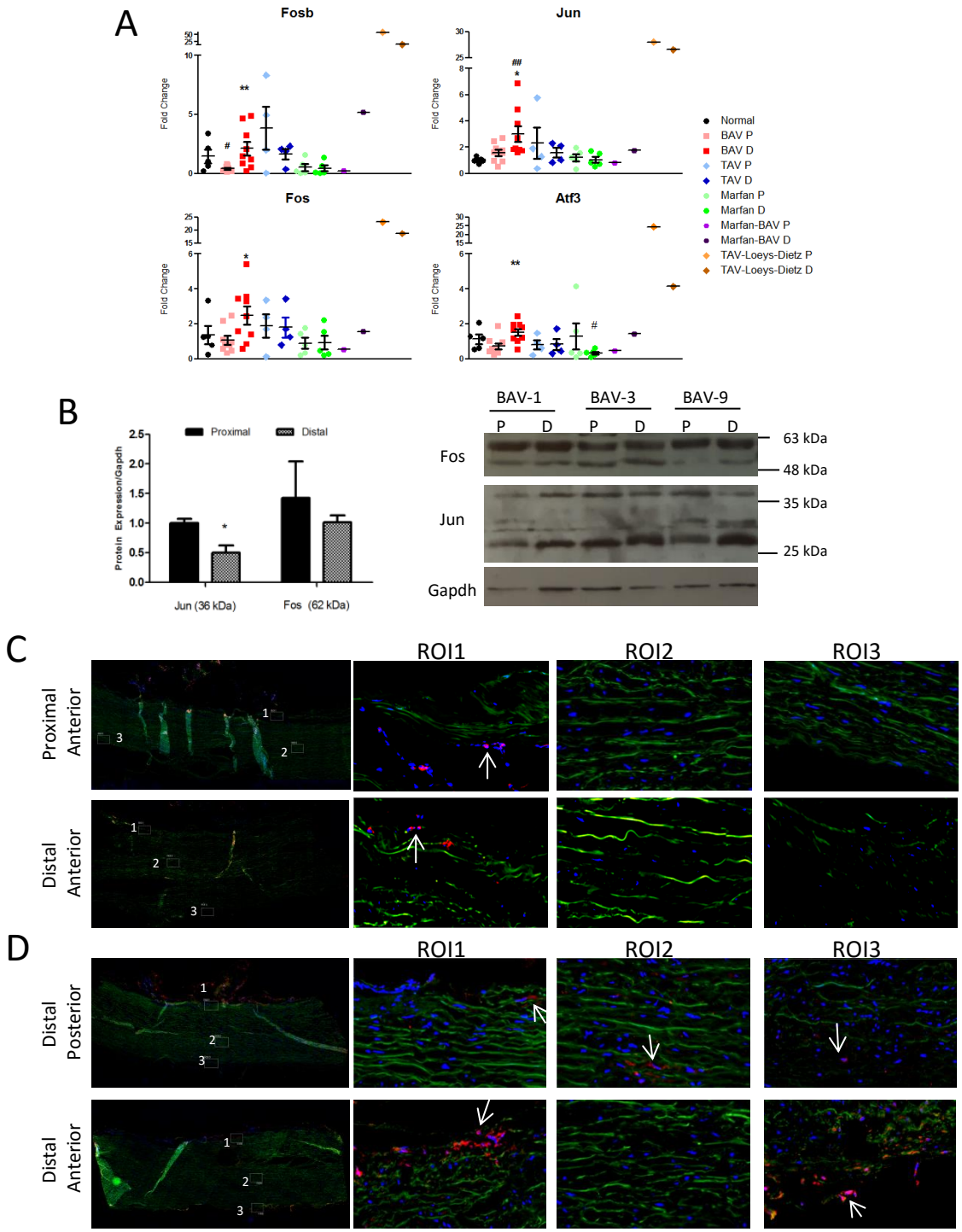

Figure 2. Expression of *Fosb, Fos, Atf3* and *Jun* in dilated BAV, TAV and Marfan patient samples. A) Distal (D) and Proximal (P) portions of the tissue were separated for qRT-PCR analysis. Normal n=5; BAV n=9; TAV n=4; Marfan n=5; BAV-Marfan n=1; TAV-Loeys-Dietz n=1. Data are expressed as the relative fold change to normal aortic tissue. Mean±SEM. # P<0.05 and ## P<0.01 compared to Normal Control. *P<0.05 and **P<0.01 represent Proximal vs Distal statistical significance. Statistical significance was calculated using a two-tailed unpaired t-test. Expression was normalized to Gapdh. **B)** Fos and Jun expression in Proximal and Distal BAV aneurysmal tissue. The aneurysmal tissue was divided into proximal and distal sections and analyzed by western blot with the respective antibodies. The predicted molecular weight of Jun and Fos is 39 and 62 kDa, respectively. Jun: n=4, *p<0.05; Fos: n=3. **C)** Jun expression is localized to the periphery of the aorta of paired (same patient) distal and proximal BAV aneurysmal samples. Three regions of interest (ROI) were taken from the interior, medial and outer layers of the aorta. Blue: Dapi; Red: Jun; Green: Smooth Muscle Actin (SMA). Note that SMA staining resulted in auto-fluorescence of collagen. Arrows point to cells positive for Jun expression. **D)** Jun is cytoplasmic within the distal posterior aortic segment of paired (same patient) distal anterior and posterior BAV aneurysmal samples. Samples were analyzed as in Figure 2C. Note that Distal Posterior ROI2 contains cytoplasmic Jun in the medial layer that is absent in all other images of the medial aorta.

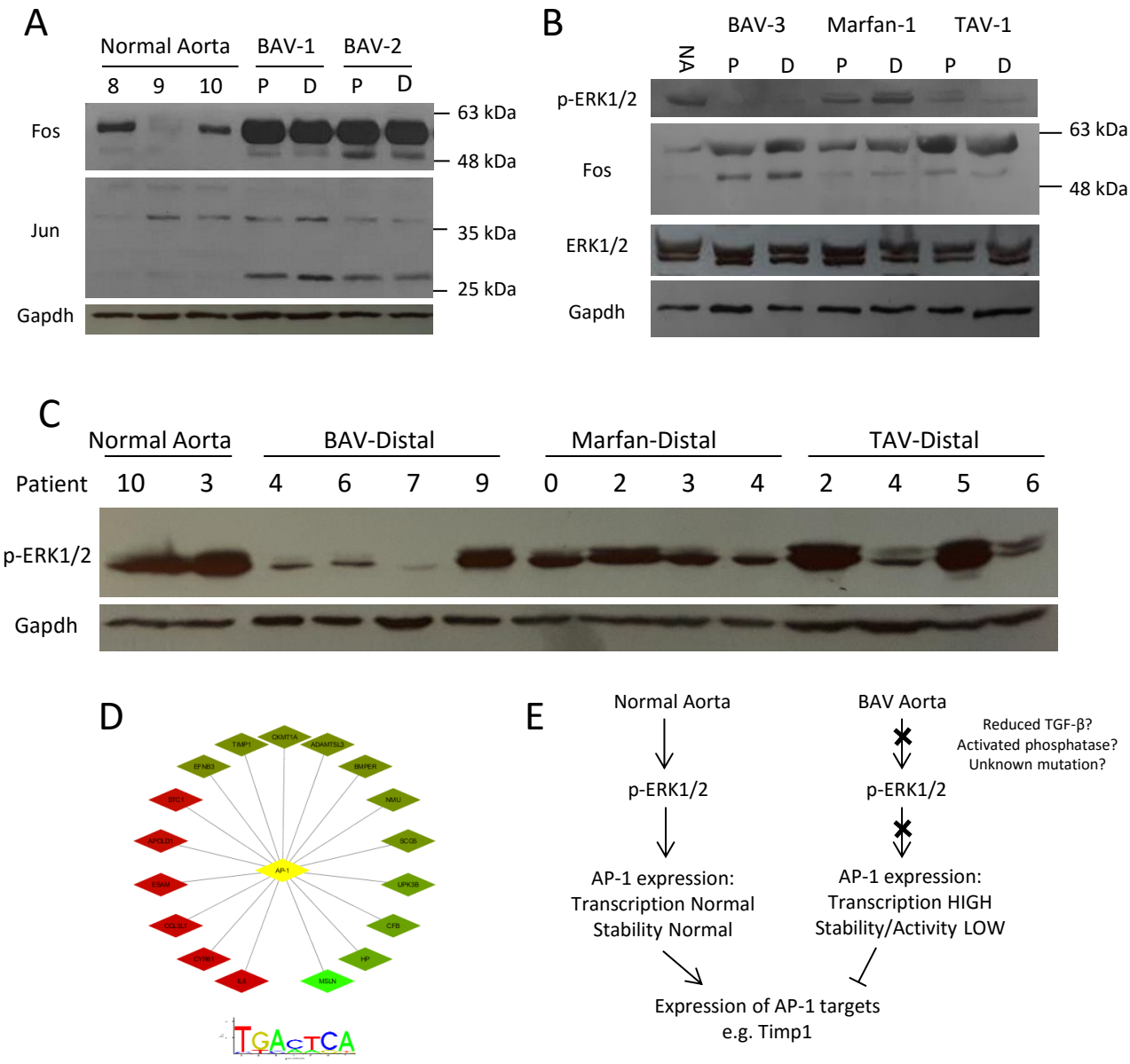

Figure 3. ERK1/2 activity is reduced in BAV aneurysmal tissue. **A)** Alternate forms of Jun and Fos are present within BAV aneurysmal tissue that are absent in normal aorta. Additional Jun and Fos protein bands of approximately 26 kDa and 50 kDa, respectively, were detected in BAV aneurysmal tissue but not normal aortic tissue. **B)** Fos expression and ERK1/2 activity in Proximal and Distal BAV, Marfan and TAV aneurysmal tissue. The aneurysmal tissue was divided into proximal and distal sections and analyzed by western blot with the respective antibodies. The expression of the higher mobility form of Fos (approximately 50 kDa) in highest when ERK1/2 activity is low. **C)** ERK1/2 activity in the distal positions of aneurysmal tissue from BAV, Marfan or TAV aorta were analyzed by western blot. **D)** Predicted AP-1 targets identified in microarray analysis that are found in the extracellular space. Opposum3.0 was used to identify differentially expressed genes with AP-1 consensus sequences ±5kb from the transcription start site. Sixty-four genes fit this criteria that had a fold change greater than ±1.5. From this, 17 genes were found to function in the extracellular space and depicted in relation to AP-1. Putative target genes were colored red or green based on the level of Distal:Proximal fold change in BAV aneurysmal samples. Red: Upregulated; Green: Downregulated. The consensus sequence of AP-1 is TGACTCA. **E)** Proposed mechanism. Those with BAV aorta have reduced ERK1/2 activity via an unknown mechanism. This results in reduced AP-1 protein stability and/or reduced transcriptional activity which leads to lower expression of AP-1 target genes, such as Timp1, and promotes aortic wall instability.