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# ATF-2 and Tpl2 regulation of endothelial cell cycle progression and apoptosis

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27 **ABSTRACT**

28 Cells respond to soluble and membrane-bound factors to activate signalling  
29 cascades that control cell proliferation and cell death. Vascular endothelial  
30 growth factor A (VEGF-A) is a soluble ligand that modulates a variety of cellular  
31 responses including cell proliferation and apoptosis. It is not well understood  
32 how VEGF-A signalling pathways regulate cell proliferation and cell death. To  
33 address this, we examined VEGF-A-regulated signalling pathways in the  
34 cytosol and nucleus and functional requirement for such cellular responses.  
35 The VEGF-A-regulated transcription factor, ATF-2, is required for cell cycle  
36 proteins such as p53, p21 and Cyclin D1. A cytosolic serine/threonine protein  
37 kinase (Tpl2) modulates ATF-2-regulated effects on the endothelial cell cycle.  
38 Such regulatory effects impact on endothelial cell proliferation, cell viability and  
39 apoptosis. These cellular effects influence complex cell-based organisation  
40 such as endothelial tubulogenesis. Our study now provides a framework for  
41 incorporating VEGF-A-stimulated signalling events from the cytosol to the  
42 nucleus which helps to understand how cell proliferation and apoptosis are  
43 controlled.

44 **(158 words)**

45

46 **Keywords:** Signal transduction, Cell proliferation, Apoptosis, ATF-2, p53, Tpl2

47

## 48 **1. Introduction**

49 Eukaryote organisms sense changes in the extracellular environment  
50 and modulate signal transduction pathways that control different aspects of cell  
51 physiology and animal function. An important feature of cellular physiology is  
52 to tightly regulate cell proliferation and programmed cell death (apoptosis). One  
53 question is how membrane receptors bind extracellular ligands and trigger  
54 such signalling pathways that modulate cell proliferation and apoptosis [1]. In  
55 higher eukaryotes, a wide variety of membrane receptors exist with ubiquitous  
56 and tissue-specific regulatory functions. One such class of proteins are the  
57 receptor tyrosine kinase (RTK) family which bind extracellular ligands and  
58 transduce signals into the cellular interior, thus modulating cell behaviour and  
59 function [2, 3]. It is well known that the RTK activation modulates cell cycle  
60 progression in a wide variety of cells and tissues. RTKs are thus useful models  
61 to better understand how complex eukaryotes integrate signalling pathways  
62 with cell cycle progression and apoptosis.

63 The vascular endothelial growth factor receptor (VEGF) family binds to a  
64 subset of receptor tyrosine kinases (VEGFRs) and have provided valuable  
65 insights into vascular physiology in health and disease states [4, 5]. The  
66 founding member of the VEGF family (i.e. VEGF-A) binds to the pro-angiogenic  
67 receptor, VEGFR2, to regulate many aspects of endothelial function including  
68 cell migration, proliferation and angiogenesis [6]. The role of VEGF-A and  
69 VEGFR2 in promoting signal transduction which impacts on cell migration and  
70 endothelial tubule formation (tubulogenesis) is well-characterised. However,  
71 the effect of VEGF-A on endothelial cell proliferation although clear-cut, is  
72 relatively mild. Interestingly, VEGF-A regulates both cell cycle progression and  
73 metabolic control, indicating an ability to simultaneously modulate different  
74 biochemical pathways [7].

75 To answer how VEGF-A regulates biochemical events that contributes to  
76 choices between cell proliferation and apoptosis, we examined the functional

77 roles of components of signalling pathways regulated by VEGF-A. Different  
78 studies have identified both cytosolic and nuclear proteins that regulate VEGF-  
79 A-stimulated signal transduction, metabolism and gene expression [6, 8]. We  
80 focused on the link between the proto-oncogene and serine/threonine protein  
81 kinase, Tpl2 (MAP3K8), the tumour suppressor and cell cycle regulator, p53,  
82 and the nuclear transcription factor, ATF-2. Our findings in this study link Tpl2-  
83 regulated signal transduction impacting on ATF-2 and p53 levels to regulate  
84 cell proliferation and apoptosis which impacts on endothelial tubulogenesis.

## 85 **2. Materials and Methods**

### 86 *2.1. Materials*

87 Antibodies used in this study are goat anti-VEGFR2 (R&D Systems,  
88 Minneapolis, USA), mouse anti-Cyclin D1 (DCS6), mouse anti-p21, rabbit anti-  
89 ATF-2, rabbit anti-phospho-ATF-2 (pT71), rabbit anti-cleaved and total  
90 caspase 3, rabbit anti-phospho-VEGFR2-Y1175 (pY1175) (Cell Signaling  
91 Technology, Danvers, USA), mouse anti-p53, mouse anti-Cyclin A2, mouse  
92 anti-Bax, mouse anti-Cyclin B (BD Transduction Laboratories, Oxford, UK),  
93 mouse anti- $\alpha$ -tubulin, mouse anti-actin (Sigma-Aldrich, Poole, UK). Endothelial  
94 cell growth medium (ECGM) and recombinant human VEGF-A<sub>165</sub> and VEGF-  
95 A<sub>121</sub> were from PromoCell (Heidelberg, Germany).

96

### 97 *2.2. Immunoblotting and analysis*

98 Human umbilical vein endothelial cells (HUVECs) were cultured and  
99 grown as previously described [9]. Cells were seeded into 6-well plates until  
100 ~80% confluent, cells were washed twice with PBS and starved overnight in  
101 MCDB131 + 0.2% (w/v) BSA and 2 mM thymidine, to stimulate cell cycle arrest  
102 (G1/S). Starvation media was aspirated and cells stimulated in ECGM + 25  $\mu$ M  
103 2-deoxycytidine, containing (0.25 nM) VEGF-A<sub>165</sub> or VEGF-A<sub>121</sub> if required.  
104 Cells were then lysed and processed for immunoblot analysis as previously  
105 described [10]. Membranes were imaged using a G:BOX XT4 Chemi imaging  
106 system (Syngene, Cambridge, UK). Band intensity was determined using 2-D  
107 densitometry running on dedicated image analysis software (Syngene).

108

### 109 *2.3. Quantitative real-time PCR analysis*

110 HUVECs were subjected to control or ATF-specific siRNA duplex  
111 treatments as previously described. Cells were then starved for 2 h in minimal

112 media, before stimulation with VEGF-A (25 ng/ml) for 2 h followed by cell lysis  
113 and processing for qRT-PCR analysis. Total cellular RNA was reverse  
114 transcribed into cDNA using random hexamer primers included in the High-  
115 Capacity cDNA Archive Kit (Life Technologies) [11]. PCR reactions were  
116 performed in a LightCycler apparatus using the LC-FastStart DNA Master  
117 SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) as described  
118 previously [12]. 2 µg of total RNA were used for first-strand cDNA synthesis  
119 with Superscript II reverse transcriptase and oligo d(T)12–18 primers according  
120 to the manufacturer's protocol (Life Technologies). Primer sequences used for  
121 qRT-PCR are: human Tpl2 forward primer: 5'- CGC AAG AGG CTG AGT A-3'  
122 and human Tpl2 reverse primer: 5'-TTC CTG TGC ACG AAG AAT CA-3'. All  
123 PCR reactions were optimised at the same annealing temperature of 60°C and  
124 thermocycling for each reaction was subsequently performed in a final volume  
125 of 20 µl containing 2 µl of cDNA sample, 4 mM MgCl<sub>2</sub>, 0.5 µM of each primer  
126 and 2 µl of LC-FastStart DNA Master SYBR Green I. Samples were initially  
127 denatured for 8 min at 95°C, followed by 45 cycles of denaturation (95°C for  
128 15 sec), annealing (60°C for 5 sec), elongation (72°C for 10 sec), and a short  
129 temperature increase to 82°C for 3 sec (for fluorescence measurements). PCR  
130 products were quantified relative to a housekeeping gene encoding β-actin.  
131 Expression levels of all other genes are given relative to the expression levels  
132 of β-actin by evaluation of their crossing-over points of product accumulation  
133 curves relative to the standard curve of β-actin. All PCR products were checked  
134 by melting point analysis and by agarose gel electrophoresis to verify that  
135 products were of correct length.

136

#### 137 *2.4. Fucci immunofluorescence analysis*

138 The fluorescent ubiquitinated cell cycle indicator (Fucci) has been  
139 previously described [13]. Briefly, a novel puromycin-resistant single co-

140 expression construct was generated as follows. Plasmid pLV-eGFP (Addgene  
141 #36083; a gift from Pantelis Tsoulfas) was digested with BamHI and Sall to  
142 remove the eGFP open reading frame. PCR primers were used to amplify  
143 sequences encoding mKO2-hCdt1 (30/120) (5'-TAG AAG ACA CCG ACT  
144 CTA GAG GAT CCA TGG TGA GTG TGA TTA AAC-3' and 5'-ACG TCG CCG  
145 CAG GTC AGC AGG CTG CCT CTG CCC TCG CCG CTG CCG ATG GTG  
146 TCC TGG TCC TGC GC-3') and mAG-hGem (1/110) (5'-CAG CCT GCT GAC  
147 CTG CGG CGA CGT GGA GAA CCC CGG CCC CGT GAG CGT GAT CAA  
148 GCC CGA G-3' and 5'-ACG TCT CCA CAT GTC AGG CTT CCT CTT CCT  
149 TCT CCG CTT CCC AGC GCC TTT CTC CGT TTT TCT G-3'), and Pac (5'-  
150 AAG CCT GAC ATG TGG AGA CGT GGA AGA AAA CCC TGG GCC CGC  
151 CAC CGA GTA CAA GCC CAC G-3' and 5'-TAA TCC AGA GGT TGA TTG  
152 GCT AGC TCA GGC ACC GGG CTT GCG GGT C-3'), from the FUCCI  
153 plasmid templates [13] and pGIPZ, respectively. Gibson assembly was used to  
154 create pLV-FUCCI, which encodes a T2A peptide linking mKO2-hCdt1  
155 (30/120) with mAG-hGem (1/110), and a P2A peptide linking mAG-hGem  
156 (1/110) with Pac. To overcome difficulties caused by high GC-content in  
157 overlapping regions, the three inserts were joined using splicing by overlap  
158 extension (2) before addition to the assembly reaction. Supernatants  
159 containing lentiviral particles were generated in HEK293T cells using a 3<sup>rd</sup>  
160 generation packaging system obtained from Addgene. Endothelial cells were  
161 incubated with lentivirus carrying the FUCCI reporter and a puromycin marker  
162 and cultured for up to 3-4 weeks in ECGM containing puromycin (3 µg/ml).

163 Following transduction with high-titre viral solutions generated in  
164 HEK293T cells, endothelial cells were transfected with specific siRNA duplexes  
165 for 48 h prior to being starved overnight in MCDB131 + 0.2% (w/v) BSA and 2  
166 mM thymidine (G1/S cell cycle arrest). Cells were subsequently trypsinised and  
167 reseeded into 96-well, black-walled plates at  $2.5 \times 10^3$  cells per well in ECGM +  
168 25 µM 2-deoxycytidine and imaged at 8 h and 30 h post release following

169 addition of Hoechst 33342 to visualise nuclear DNA. Image acquisition was  
170 carried out using either a BD Pathway 435 imager or Olympus X81  
171 immunofluorescence microscope equipped with 405 nm, 488 nm, and 543 nm  
172 light-source lines. Image analysis was performed using ImageJ and  
173 Metamorph 6 software (Universal Imaging, Media, PA) on between 400-2000  
174 cells at each time-point per siRNA duplex treatment.

175

## 176 *2.5. Cell viability assays*

177 3000 HUVECs were seeded per well of a 96-well plate, cultured in 90  $\mu$ l  
178 ECGM for 16-20 h, before adding 10  $\mu$ l of MTS reagent (CellTiter 96 AQueous  
179 Non-Radioactive Cell Proliferation Assay, Promega, Madison, USA). After  
180 further incubation for 4 h, colour change caused by reduction of the yellow  
181 tetrazolium compound (MTS) by metabolically active cells to brown formazan  
182 was monitored at 490 nm using a Tecan Sunrise plate reader (Mannedorf,  
183 Switzerland).

184

## 185 *2.6. Annexin V apoptosis assay*

186 20000 HUVECs were seeded into 24-well plates and left overnight for  
187 16-20 h. Media was removed and cells were subjected to RNAi and protein  
188 knockdown as previously described. Cells were gently detached from the  
189 substratum using previously described protocol for processing for flow  
190 cytometry. Before this was carried out, cells were centrifuged at 1000 g for 5  
191 mins and resuspended in 0.5 ml of Annexin V binding buffer using a kit (Sigma-  
192 Aldrich, Poole, UK). Annexin V-FITC conjugate (to stain apoptotic cells) and  
193 propidium iodide (to stain DNA of dead or dying cells) was added as  
194 recommended by manufacturer. Samples were then examined using a  
195 Fortessa flow cytometer (Becton Dickinson, UK) and >10000 labelled cells

196 were analysed per experiment. Early apoptotic cells show labelling with the  
197 Annexin V-FITC conjugate alone. Live cells show no labelling with either the  
198 propidium iodide or Annexin V-FITC. Necrotic cells are labelled by both  
199 propidium iodide and Annexin V-FITC.

200

## 201 *2.7. Cell proliferation assay*

202 2000 HUVECs were seeded per well of a 96-well plate and left overnight  
203 for 16-20 h. Media was removed and replaced with serum-free medium for 3 h,  
204 then stimulated with 0.25 nM VEGF-A in 100  $\mu$ l for 24 h. 10  $\mu$ M BrdU was  
205 added per well at this point. Cell proliferation ELISA was used according to  
206 manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Colour  
207 change was developed using 3,3',5,5'-tetramethylbenzidine solution and the  
208 reaction quenched with 1 M H<sub>2</sub>SO<sub>4</sub>. Absorbance was measured at 450 nm  
209 using a variable wavelength Tecan Sunrise plate reader (Tecan, Mannedorf,  
210 Switzerland).

211

## 212 *2.8. Tubulogenesis assay*

213 Primary human foreskin fibroblasts (Promocell) were cultured in 48-well  
214 plates until confluent, before seeding 7500 endothelial cells per well onto the  
215 fibroblast monolayer and left overnight for 20-24 h essentially as previously  
216 described [9]. Briefly, media was aspirated and replaced with fresh media  
217 supplemented with VEGF-A (0.25 nM) every 2-3 days for 7 days. Co-cultures  
218 were fixed in 200  $\mu$ l 10% (v/v) formalin for 20 min at room temperature,  
219 quenched and labelled with mouse anti-human PECAM-1 (CD31) and donkey  
220 anti-mouse Alexa Fluor 594 conjugate as previously described [9]. Processed  
221 samples containing stained endothelial tubules were visualized using an  
222 EVOS-fl inverted digital microscope (ThermoFisher). 3 random fields were  
223 imaged per well. Total tubule length was then quantified from each

224 photographic field using the open source software AngioQuant  
225 ([www.cs.tut.fi/sgn/csb/angioquant](http://www.cs.tut.fi/sgn/csb/angioquant)) and values averaged. For a more detailed  
226 methods see elsewhere [9].

227

## 228 *2.9. Statistics*

229 We used one-way analysis of variance (ANOVA) followed by Tukey's  
230 post-hoc test or two-way ANOVA followed by Bonferroni multiple comparison  
231 test using GraphPad Prism software (La Jolla, USA). Significant differences  
232 between control and test groups were evaluated with  $p$  values less than 0.05  
233 (\*), 0.01 (\*\*), 0.001 (\*\*\*) and 0.0001 (\*\*\*\*) indicated on the graphs or  
234 histograms. Error bars denote mean $\pm$ SEM.

### 235 **3. Results**

#### 236 *3.1. ATF-2 modulates p21, p53 and Cyclin D1 levels*

237 In primary endothelial cells VEGF-A regulates signal transduction and  
238 cell proliferation [14] partially through recruitment of nuclear transcription  
239 factors, including c-fos and other AP-1 components [15]. We have previously  
240 identified a signalling nexus between VEGFR2, the MAP3K family member  
241 Tpl2, and a transcription factor, ATF-2. In previous studies on VEGF-A-  
242 stimulated and ATF-2-regulated endothelial gene expression on VCAM-1 [15]  
243 and Tpl2 [16], we find that maximal VEGF-A<sub>165</sub>-stimulated, ATF-2-regulated  
244 VCAM-1 synthesis occurs 8 h after VEGF-A addition. Under such conditions,  
245 there is complexity in decreased VEGFR2 levels caused by increased  
246 ubiquitination, endocytosis and degradation [15]. However, there is also a rise  
247 in new VEGFR2 synthesis [15], which can also substantially add to VEGFR2  
248 activation in the presence of excess exogenous VEGF-A. However, the  
249 influence of this pathway on endothelial cell cycle progression and survival was  
250 unknown. To ascertain whether different VEGF-A isoforms regulate cytosolic  
251 and nuclear signalling events which impact on the endothelial cell cycle, we  
252 probed the biochemical state of key regulatory proteins associated with such  
253 events (Fig. 1). As previously reported [17], two different VEGF-A isoforms  
254 (VEGF-A<sub>165</sub> and VEGF-A<sub>121</sub>) show differential ability to activate VEGFR2,  
255 revealed by varied phospho-VEGFR2 (Y1175) levels (Fig. 1A). Such signalling  
256 further impacts on gene expression, as shown by increased levels of VCAM-1  
257 in response to VEGF-A<sub>165</sub> stimulation for 8 h (Fig. 1A). As ATF-2 is implicated  
258 in the VEGF-A-stimulated (after 8 h) VCAM-1 increase, concomitant with a  
259 decrease in VEGFR2 levels [17], we depleted ATF-2 levels using RNA  
260 interference (RNAi) (Fig. 1A, 1B). Knockdown of ATF-2 levels resulted in ~50%  
261 decrease in this essential nuclear regulator (Fig. 1B). However, ATF-2  
262 knockdown did not affect levels of either Cyclin A2 or Cyclin B, critical drivers  
263 of cell proliferation (Fig. 1A). Unexpectedly, depletion of ATF-2 caused

264 elevated levels of two other cell cycle regulators, Cyclin D1 and the cyclin-  
265 dependent kinase inhibitor, p21 (Fig. 1A). The latter is a major transcriptional  
266 target of the tumour suppressor p53, suggesting p53 involvement in the  
267 process.

268 There is link between VEGF-A-stimulated ATF-2 phosphorylation and  
269 ATF-2 levels [15, 16]. Activation of the p53 tumour suppressor is known to  
270 increase levels of p21, a negative cell cycle regulator, thus potentially  
271 impacting additional cell cycle modifiers including Cyclin D1 [18-20]. We  
272 explored further links between ATF-2 and p53 by knockdown of either protein  
273 alone or together (Fig. 1C). As expected, ATF-2 knockdown abrogated the  
274 VEGF-A-stimulated increase in VCAM-1 expression (Fig. 1C, 1D). ATF-2  
275 knockdown elevated p21 levels substantially. Importantly, p53 levels were also  
276 elevated under such conditions (Fig. 1C, 1E). The >2-fold increase in p53  
277 levels caused by ATF-2 knockdown were reversed by p53 or combined ATF-  
278 2/p53 knockdown (Fig. 1E). Under these conditions, ATF-2 knockdown caused  
279 ~3.5-fold rise in Cyclin D1 levels (Fig. 1F). Furthermore, the increased p21 and  
280 cyclin D1 expression evident upon ATF-2 depletion were dependent on the  
281 presence of p53, as co-depletion of ATF-2 and p53 abolished the increases  
282 (Fig. 1C-F). However, VCAM1 levels were not rescued by depletion of both  
283 ATF-2 and p53 (Fig. 1D).

284

### 285 3.2. *Tpl2* dependence on ATF-2 and impact on p53 and Cyclin D1

286 The MAP3K family member and oncoprotein, Tpl2, which is implicated in  
287 lung carcinogenesis [21] regulates VEGF-A-stimulated angiogenesis [22] and  
288 transduces signals from the cytosol to the nucleus in endothelial cells [16]. To  
289 assess whether Tpl2 is functionally linked to levels of ATF-2 and cell cycle  
290 regulators such as p53, we asked whether RNAi altered protein expression  
291 (Fig. 2A). Knockdown of Tpl2 caused ~60% reduction in protein levels (Fig.

292 2B). Tpl2 depletion also suppressed ATF-2 expression to levels similar to those  
293 observed after direct ATF-2 knockdown (Fig. 2A). Surprisingly, although ATF-  
294 2 knockdown caused >2-fold increase in p53, and Tpl2 depletion also reduced  
295 ATF-2 expression, Tpl2 RNAi had no substantial effect on p53, p21, or Cyclin  
296 D1 protein levels (Fig. 2A, 2D, 2E). However, combined depletion of both ATF-  
297 2 and Tpl2 caused p53 and cyclin D1 protein levels, to return to baseline (Fig.  
298 2A, 2D, 2E). Depletion of Tpl2 caused >60% reduction in steady-state Tpl2  
299 levels (Fig. 2B).

300 ATF-2 depletion elevates Tpl2 protein expression (Fig. 2A), indicative of  
301 a functional link between the two proteins. To explore this further we carried  
302 out qRT-PCR to analyse the link between ATF-2 and *Tpl2* expression (Fig. 2C).  
303 In a comparison between control and VEGF-A-stimulated conditions, we found  
304 ~15-fold increase in *Tpl2* mRNA levels (Fig. 2C). Depletion of ATF-2 levels  
305 caused reduction in *Tpl2* mRNA to background levels (Fig. 2C). ATF-2  
306 knockdown elevates cell cycle regulator levels (cyclin D1, p53, p21) but Tpl2  
307 depletion reverses such effects (Fig. 2D, 2E), suggesting Tpl2 expression is  
308 required for mediating the p53 increase evident upon ATF-2 knockdown. The  
309 presence of ATF-2 might act as a mitotic accelerator or enabler by dampening  
310 the expression of p53 and its downstream cell cycle inhibitors, including p21,  
311 via a mechanism dependent on Tpl2 activity.

312 The eukaryote cell cycle is regulated by a combination of protein kinases,  
313 cyclins and transcription factors [20, 23, 24]. The previous data in this study  
314 suggested that ATF-2 levels influences cell cycle proteins. To assess whether  
315 the endothelial cell cycle is affected by ATF-2, we used the fluorescent  
316 ubiquitinated cell cycle indicator (FUCCI) [13] which is a dual reporter system  
317 used to monitor progression through the cell cycle in living cells, tissues and  
318 animals [25]. A lentiviral FUCCI construct was used to transduce primary  
319 endothelial cells and cell synchronisation (see Materials and Methods) before  
320 monitoring of fluorescent reporter proteins, mKO2-hCdt1 (red) for non-cycling

321 G1 stage cells, and mAG-hGem (green) for proliferating G2/M stage cells  
322 (green) (Fig. 3A). Under control conditions, stimulation with VEGF-A caused  
323 transient increase in the G1:G2/M ratio after 8 h which is more than 2-fold  
324 higher than that that observed after 30 h (Fig. 3B), indicating a rise in the  
325 number of cells in G2/M phase within soon after VEGF-A stimulation. Here,  
326 depletion of either ATF-2 or p53 had no effect on the G1:G2/M ratio compared  
327 to controls (Fig 3B). However, combined ATF-2/p53 caused a significant >2-  
328 fold increase in the proportion of cells in G2/M after 30 h (Fig 3B). Furthermore,  
329 knockdown of Tpl2 caused a dramatic rise in proportion of cells in the G2/M  
330 phase both at the 8 h and 30 h time points (Fig. 3B). However, the relative  
331 difference between the 8 h and 30 h time points was a 2-3-fold change in Tpl2-  
332 depleted cells, similar to controls (Fig. 3B). Interestingly, combined ATF-2/Tpl2  
333 knockdown showed little change between the 8 h and 30 h time points (Fig.  
334 3B), suggesting substantial effects on the endothelial cell cycle.

335 Cells that are senescent or have exited the cell cycle are in G0, and p53  
336 is a key regulator of cellular senescence [19]. One possibility is that depletion  
337 of cell cycle regulatory factors results in increased endothelial cell senescence  
338 or cell cycle exit. We evaluated the proportion (%) of the endothelial cell  
339 population lacking staining for both mAG and mKO indicative of G0 (Fig. 3C).  
340 This was substantially higher in the cells depleted of ATF-2 (Fig. 3C). However,  
341 this effect was rescued upon combined knockdown of either ATF-2/p53 or ATF-  
342 2/Tpl2 (Fig. 3C). Furthermore, we found that in addition to cell cycle arrest,  
343 ATF-2 knockdown also resulted in ~40% decrease in endothelial cell viability  
344 (Fig. 3D).

345

### 346 *3.3. ATF-2, Tpl2 and p53 modulation of apoptosis*

347 One question arising from these data is how endothelial cells integrate  
348 complex signal transduction pathways with nuclear gene expression to

349 influence programmed (i.e. apoptosis) and general (i.e. necrosis) cell death. To  
350 address this, we used flow cytometry (see Materials and Methods) to identify,  
351 necrotic, early and late apoptotic endothelial cell populations (Fig. S1). We  
352 used controls (Fig. S1) to evaluate early and late apoptotic cells compared to  
353 necrotic cells under conditions of specific protein depletion (Fig. S1B).  
354 Quantification of the AnxV-positive population revealed ~4-fold rise in such  
355 endothelial cells upon ATF-2 depletion (Fig. 4A). Tpl2 depletion also caused  
356 >2-fold rise in AnxV-labelled cell population compared to control (Fig. 4A). A  
357 similar profile was observed when the relative cell population was analysed  
358 with early apoptotic cell population marked increased >2-fold upon either ATF-  
359 2 or Tpl2 depletion (Fig. 4B). In the analysis of late apoptotic cells, ATF-2  
360 depletion caused a >4-fold rise in apoptotic cells, and only a ~2-fold rise in Tpl2-  
361 depleted cells (Fig. 4C). Considering the total apoptotic cell population, the  
362 ATF-2 or Tpl2-depleted cells all show significant 2-3-fold rise in apoptosis (Fig.  
363 4D). In these flow cytometry experiments, simultaneous depletion of ATF-  
364 2/Tpl2 or ATF-2/p53 causes a reversal in the effects observed with ATF-2  
365 depletion alone (Fig. 4A-4D).

366 To explore the molecular basis for increased endothelial apoptosis  
367 caused by ATF-2 depletion, we assessed the biochemical status of key  
368 apoptotic regulators (Fig. 4E). Immunoblotting of control (scrambled) and ATF-  
369 2-depleted endothelial cells revealed an increase in cleaved pro-apoptotic  
370 Caspase 3 (Fig. 4E). There were also increased levels of p53 and pro-apoptotic  
371 Bax proteins in ATF-2-depleted cells (Fig. 4E). Quantification of these data  
372 showed >2-fold rise in cleaved Caspase 3 upon ATF-2 depletion (Fig. 4F).

373

### 374 *3.4. ATF-2, Tpl2 and p53 involvement in VEGF-A-regulated endothelial cell* 375 *responses*

376 VEGF-A promotes multiple endothelial responses including cell viability,  
377 cell proliferation and tubule formation, key features of angiogenesis [4, 5]. We  
378 compared endothelial cell viability upon knockdown of specific cytosolic and

379 nuclear factors: ATF-2 knockdown caused ~50% decrease in VEGF-A-  
380 stimulated cell viability (Fig. 5A). Tpl2 knockdown also caused ~30% decrease  
381 in VEGF-A-stimulated cell viability (Fig. 5A). However, simultaneous combined  
382 knockdown of either ATF-2/p53, or ATF-2/Tpl2 caused cell viability to return to  
383 baseline levels (Fig. 5A). Depletion of p53 alone had no effect on VEGF-A-  
384 stimulated endothelial cell viability (Fig. 5A).

385 As VEGF-A is well-known to stimulate cell proliferation, we asked  
386 whether this response was affected by knockdown of components of signal  
387 transduction pathways involving ATF-2 and Tpl2 (Fig. 5B). ATF-2 knockdown  
388 caused ~30% decrease in endothelial cell proliferation; however, knockdown  
389 of p53 or Tpl2 had no effect (Fig. 5B). However, simultaneous knockdown of  
390 ATF-2/p53 or ATF-2/Tpl2 returned cell proliferation to control baseline levels  
391 (Fig. 5B).

392 One unique feature of VEGF-A is the capacity to stimulate endothelial  
393 cells to proliferate, migrate and form biological tubes [26]. We used the *in vitro*  
394 endothelial-fibroblast co-culture assay to evaluate VEGF-A-stimulated  
395 tubulogenesis (Fig. 5C). VEGF-A stimulation causes ~6-fold increase in  
396 average endothelial tubule length under control conditions (Fig. 5D). In  
397 contrast, ATF-2 knockdown caused ~50% decrease in endothelial tubule  
398 length, (Fig. 5D). Although Tpl2 knockdown caused ~4-fold increase in VEGF-  
399 A-stimulated tubule length, there was ~35% reduction in tubulogenesis  
400 compared to control (Fig. 5D). Again however, combined knockdown of either  
401 ATF-2/p53 or ATF-2/Tpl2 caused return to baseline levels of VEGF-A-  
402 stimulated endothelial tubulogenesis (Fig. 5D). Lack of ATF-2 hampers  
403 endothelial tubule formation through upregulation of p53 expression and  
404 induction of cell cycle arrest, all dependent on the presence of Tpl2.

405

#### 406 **4. Discussion**

407 One important aspect feature of primary cells and tissues is the balance  
408 between cell survival, proliferation and different types of cell death. Complex  
409 regulatory mechanisms exist to ensure correct timing of cell proliferation in  
410 healthy and diseased states. In this study, we investigated how endothelial  
411 cells balance cytosolic and nuclear signalling which impacts on cell cycle  
412 progression and cell death. Our study suggests a model where Tpl2 receives  
413 signals and modulates the activity of downstream factors which regulate gene  
414 expression and cell cycle progression (Fig. 6). A balance between Tpl2  
415 signalling and ATF-2 nuclear activity is required for normal endothelial  
416 responses (Fig. 6A). Reduction in ATF-2 activity impacts on the endothelial  
417 commitment to cell cycle progression and apoptosis (Fig. 6B). This function  
418 appears dependent on the presence of Tpl2.

419 The evidence for this model is based on 4 lines of evidence. Firstly, ATF-  
420 2 depletion promotes a rise in p53, p21 and Cyclin D1 levels, but other cyclins  
421 are not affected. This suggests that under such conditions, these endothelial  
422 cells have entered a pro-apoptotic phase coincident with cell cycle arrest.  
423 Secondly, reduction in ATF-2 levels also causes a rise in the proportion of cell  
424 population in senescence (G0), and this coincides with decreased endothelial  
425 cell viability. Thirdly, a reduction in Tpl2 levels cause a corresponding decrease  
426 in ATF-2 levels, although this was not accompanied by elevated p53, p21, or  
427 Cyclin D1 expression, suggesting a functional link between these two proteins,  
428 where Tpl2 may be viewed as a master regulator. Importantly, ATF-2 depletion  
429 blocks the VEGF-A-stimulated rise in Tpl2 levels, suggesting ATF-2 regulates  
430 gene transcription of the *Tpl2* locus. Moreover, loss of ATF-2 is reversed by  
431 simultaneous depletion of either Tpl2 or p53, suggesting a regulatory pathway  
432 linking all three proteins (Fig. 6). Finally, such regulatory effects are manifested  
433 in VEGF-A-stimulated endothelial tubulogenesis, which requires coordination  
434 of cell proliferation and migration for biological tube formation. Reduction in

435 ATF-2 levels also reduces the efficiency of endothelial tubulogenesis; however,  
436 such effects are reversed by simultaneous depletion of either Tpl2 or p53. This  
437 regulatory pathway (Fig. 6) thus impacts on the VEGF-A-stimulated formation  
438 of biological tubes.

439 The tumour progression locus 2 (Tpl2/Cot/MAP3K8) gene product was  
440 originally identified as a serine/threonine protein kinase in T-cells which  
441 regulated signal transduction and cellular responses [27]. Tpl2 is also a proto-  
442 oncoprotein, conferring resistance to Raf kinase inhibition [28], promoting  
443 breast cancer [29], keratocanthoma and squamous cell carcinoma [30].  
444 However, its role in the vascular system is less well-studied, but reports  
445 suggest that it can regulate angiogenesis [22] and diabetic retinopathy [31].  
446 Our studies now suggest that cytosolic Tpl2 and the nuclear transcription factor  
447 ATF-2, are part of a pathway that controls cell cycle progression and apoptosis.  
448 The communication between Tpl2 and ATF-2 may occur directly:  
449 hyperphosphorylation of ATF-2 is implicated with increased signalling through  
450 the MAPK pathway [15]. Recent studies suggest that pharmacological  
451 inhibition of p38 MAPK, JNK and AKT signal transduction pathways had little  
452 or no effect on ATF-2 phosphorylation in endothelial cells [15]. One possibility  
453 is that signalling through the canonical MAPK pathway leads to Tpl2  
454 phosphorylation, activation and translocation into the nucleus in a manner  
455 analogous to ERK1/2. It has been reported that Tpl2 activity is required for  
456 phosphorylation of nuclear factors including ATF-2 [16, 32], implying a  
457 functional role in nuclear gene expression. Importantly, a direct nuclear role of  
458 Tpl2 has also been reported [30]. This raises the possibility of Tpl2 itself  
459 responding to VEGFR2 activation, phosphorylation via canonical (MAPK) or  
460 non-canonical pathways which enables nuclear translocation, transcription p  
461 factor phosphorylation and modulation of nuclear gene transcription. In  
462 endothelial cells, VEGF-A-stimulated signal transduction through a non-  
463 canonical signal transduction pathway involving the MEK5-ERK5 axis impacts

464 on a range of vascular responses [33], which could have relevance to Tpl2  
465 regulation in this context. In tumour cells, there is evidence that Tpl2 activity is  
466 linked to ERK5 status [34].

467 There is a notable relationship between Tpl2 and ATF-2 levels.  
468 Decreased Tpl2 levels also cause a substantive decrease in ATF-2 levels;  
469 combined ATF-2/Tpl2 knockdown reverses effects caused by ATF-2 depletion  
470 alone. However, decreased ATF-2 levels alone, increase Tpl2, p53, cyclin D1  
471 and p21 levels; this is not evident when Tpl2 is depleted in isolation, despite  
472 ATF-2 levels also falling. Tpl2 expression or activity, appears necessary to  
473 allow ATF-2 depletion to stabilise or activate p53, and promote cell cycle arrest.  
474 One explanation is that decreased ATF-2 phosphorylation, due to loss of  
475 upstream Tpl2, increases post-translational modifications on ATF-2 leading to  
476 increased proteolysis and clearance. The loss of ATF-2 leading to increased  
477 Tpl2 (Fig. 6B) could be explained by a possible role for ATF-2 in repressing  
478 *TPL2* nuclear gene expression. Alternatively, ATF-2 could activate  
479 transcription of genes encoding ubiquitin ligases; such enzymes could target  
480 components of the Tpl2-regulated pathway. Protein phosphorylation and  
481 ubiquitination are strongly linked in many signal transduction pathways to  
482 control signalling and protein levels. As p53 undergoes complex post-  
483 translational modifications including phosphorylation and ubiquitination [35],  
484 one explanation is that decreased ubiquitination due to reduced ATF-2 activity  
485 could drive increased p53 levels. Indeed, Tpl2 has been shown to regulate the  
486 activity of the main p53 E3 ubiquitin ligase, HDM2 [30]. ATF-2 loss may activate  
487 Tpl2 to reduce HDM2 activation or expression, allowing p53 to escape  
488 proteasomal degradation and undergo nuclear accumulation. Furthermore,  
489 both p21 and Cyclin D1 are also regulated by ubiquitination and proteolysis,  
490 suggesting that ATF-2-regulated gene expression could have impact in this  
491 context.

492 In conclusion, our study shows that in primary endothelial cells, basal and  
493 ligand-activated signalling pathways are tightly regulated to ensure that signals  
494 from the cytosol impact on nuclear gene expression and nuclear protein  
495 function. A major finding is that a signalling axis involving Tpl2 serine/threonine  
496 protein kinase and ATF-2 transcription factor with a wider impact on key cell  
497 cycle regulators such as p53, p21 and Cyclin D1. This work also raises new  
498 questions on the mechanisms of gene expression and the nature of post-  
499 translational modifications that govern the levels of key cell cycle regulators.

500

### 501 **Conflict of interest**

502 The authors have no conflict of interest.

503

### 504 **Contributors**

505 G. W. Fearnley designed research, designed and performed  
506 experiments, interpreted results, wrote and revised the manuscript. A. M.  
507 Latham performed experiments and provided data. A. F. Odell designed  
508 research, designed and performed experiments, interpreted results and  
509 revised the manuscript. M. Hollstein helped to design experiments and carry  
510 out the work. S. Ponnambalam designed research, interpreted results, wrote  
511 and revised the manuscript.

512

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518 **References**

- 519 [1] Proud CG, Cold Spring Harbor Persp Biol. 2019; 11:a033050.
- 520 [2] Lemmon MA, Schlessinger J, Cell. 2010;141:1117-1134.
- 521 [3] Lemmon MA, Freed DM, Schlessinger J, Kiyatkin A, Cell. 2016;164:1172-1184.
- 522 [4] Bates DO, Beazley-Long N, Benest AV, Ye X, Ved N, Hulse RP, Barratt S, Machado MJ,  
523 Donaldson LF, Harper SJ, Peiris-Pages M, Tortonese DJ, Oltean S, Foster RR, Comp  
524 Physiol. 2018;8:955-979.
- 525 [5] Apte RS, Chen DS, Ferrara N, Cell. 2019;176:1248-1264.
- 526 [6] Simons M, Gordon E, Claesson-Welsh L, Nat Rev Mol Cell Biol. 2016;17:611-625.
- 527 [7] Smith GA, Fearnley GW, Harrison MA, Tomlinson DC, Wheatcroft SB, Ponnambalam S,  
528 J Inherit Metab Dis. 2015;38:753-763.
- 529 [8] Smith GA, Fearnley GW, Tomlinson DC, Harrison MA, Ponnambalam S, Biosci Rep.  
530 2015;35:e00253.
- 531 [9] Fearnley GW, Smith GA, Odell AF, Latham AM, Wheatcroft SB, Harrison MA, Tomlinson  
532 DC, Ponnambalam S, Meth Enzymol. 2014;535:265-292.
- 533 [10] Fearnley GW, Wheatcroft SB, Ponnambalam S, Meth Mol Biol. 2015;1332:49-65.
- 534 [11] Uhrig M, Ittrich C, Wiedmann V, Knyazev Y, Weninger A, Riemenschneider M,  
535 Hartmann T, PLoS One. 2009;4:e6779.
- 536 [12] Ernst T, Hergenhausen M, Kenzelmann M, Cohen CD, Bonrouhi M, Weninger A, Klaren  
537 R, Grone EF, Wiesel M, Gudemann C, Kuster J, Schott W, Staehler G, Kretzler M, Hollstein  
538 M, Grone HJ, Am J Pathol. 2002;160:2169-2180.
- 539 [13] Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, Osawa H, Kashiwagi  
540 S, Fukami K, Miyata T, Miyoshi H, Imamura T, Ogawa M, Masai H, Miyawaki A, Cell.  
541 2008;132:487-498.
- 542 [14] Wu LW, Mayo LD, Dunbar JD, Kessler KM, Baerwald MR, Jaffe EA, Wang D, Warren  
543 RS, Donner DB, J Biol Chem. 2000;275:5096-5103.
- 544 [15] Fearnley GW, Odell AF, Latham AM, Mughal NA, Bruns AF, Burgoyne NJ, Homer-  
545 Vanniasinkam S, Zachary IC, Hollstein MC, Wheatcroft SB, Ponnambalam S, Mol Biol Cell.  
546 2014;25:2509-2521.
- 547 [16] Fearnley GW, Abdul-Zani I, Latham AM, Hollstein MC, Ladbury JE, Wheatcroft SB,  
548 Odell AF, Ponnambalam S, Biol Open. 2019;8:bio034215.
- 549 [17] Fearnley GW, Bruns AF, Wheatcroft SB, Ponnambalam S, Biol Open. 2015;4:731-742.
- 550 [18] Klein EA, Assoian RK, J Cell Sci. 2008;121:3853-3857.
- 551 [19] Biegging KT, Mello SS, Attardi LD, Nat Rev Cancer. 2014;14:359-370.
- 552 [20] Engeland K, Cell Death Differ. 2018;25:114-132.
- 553 [21] Gkirtzimanaki K, Gkouskou KK, Oleksiewicz U, Nikolaidis G, Vyrla D, Lontos M,  
554 Pelekanou V, Kanellis DC, Evangelou K, Stathopoulos EN, Field JK, Tsihliis PN, Gorgoulis  
555 V, Liloglou T, Eliopoulos AG, Proc Natl Acad Sci USA. 2013;110:E1470-1479.
- 556 [22] Lee WJ, Lan KH, Chou CT, Yi YC, Chen WC, Pan HC, Peng YC, Wang KB, Chen YC,  
557 Chao TH, Tien HR, Sheu WH, Sheu ML, Neoplasia. 2013;15:1036-1048.
- 558 [23] Dominguez-Brauer C, Brauer PM, Chen YJ, Pimkina J, Raychaudhuri P, Cell Cycle.  
559 2010;9:86-89.

560 [24] Gordon EM, Ravicz JR, Liu S, Chawla SP, Hall FL, Mol Clin Oncol. 2018;9:115-134.  
561 [25] Sakaue-Sawano A, Miyawaki A, Cold Spring Harbor Prot. 2014:pdb.prot080408.  
562 [26] Chung AS, Ferrara N, Annu Rev Cell Dev Biol. 2011;27:563-584.  
563 [27] Xu D, Matsumoto ML, McKenzie BS, Zarrin AA, Pharmacol Res. 2018;129:188-193.  
564 [28] Johannessen CM, Boehm JS, Kim SY, Thomas SR, Wardwell L, Johnson LA, Emery  
565 CM, Stransky N, Cogdill AP, Barretina J, Caponigro G, Hieronymus H, Murray RR, Salehi-  
566 Ashtiani K, Hill DE, Vidal M, Zhao JJ, Yang X, Alkan O, Kim S, Harris JL, Wilson CJ, Myer  
567 VE, Finan PM, Root DE, Roberts TM, Golub T, Flaherty KT, Dummer R, Weber BL, Sellers  
568 WR, Schlegel R, Wargo JA, Hahn WC, Garraway LA, Nature. 2010;468:968-972.  
569 [29] Kim G, Khanal P, Kim JY, Yun HJ, Lim SC, Shim JH, Choi HS, Mol Carcinogenesis.  
570 2015;54:440-448.  
571 [30] Lee JH, Lee JH, Lee SH, Do SI, Cho SD, Forslund O, Inn KS, Lee JS, Deng FM,  
572 Melamed J, Jung JU, Jeong JH, Cancer Res. 2016;76:6712-6722.  
573 [31] Lai DW, Lin KH, Sheu WH, Lee MR, Chen CY, Lee WJ, Hung YW, Shen CC, Chung  
574 TJ, Liu SH, Sheu ML, Circ Res. 2017;121:e37-e52.  
575 [32] Kanellis DC, Bursac S, Tsihchlis PN, Volarevic S, Eliopoulos AG, Oncogene.  
576 2015;34:2516-2526.  
577 [33] Roberts OL, Holmes K, Muller J, Cross DA, Cross MJ, J Cell Sci. 2010;123:3189-3200.  
578 [34] Wang X, Gocek E, Novik V, Harrison JS, Danilenko M, Studzinski GP, Cell Cycle.  
579 2010;9:4542-4551.  
580 [35] Meek DW, Biochem J. 2015;469:325-346.  
581  
582

583 **FIGURE LEGENDS**

584 **Figure 1. VEGF-A-regulated gene expression exhibits dependence on**  
585 **ATF-2 and p53.** (A) Endothelial cells subjected to treatment with siRNA  
586 duplexes (scrambled or ATF-2) were treated under serum-free (SF), normal  
587 growth media or VEGF-A<sub>165</sub> or VEGF-A<sub>121</sub> isoforms for 8 h followed by lysis and  
588 immunoblotting (see Materials and Methods). Blots were probed using  
589 antibodies specific for phospho-VEGFR2 (pY1175), VEGFR2, VCAM-1, ATF-  
590 2, p21, actin, cyclins A2, B or D1. (B) Quantification of relative ATF-2 levels  
591 after treatment with control (scrambled siRNA) or ATF-2 siRNA duplexes. (C)  
592 Endothelial cells subjected to treatment with control untreated, scrambled,  
593 ATF-2 and ATF-2/p53 combined siRNA duplexes were non-stimulated (-) or  
594 treated with VEGF-A<sub>165</sub> or VEGF-A<sub>121</sub> isoforms (+) followed by lysis, and  
595 immunoblotting with antibodies specific for phospho-VEGFR2, VEGFR2,  
596 VCAM-1, p53, p21, phospho-ATF-2, ATF-2, cyclin D1 or tubulin. (D)  
597 Quantification of relative levels of VCAM-1 levels in control, scrambled, ATF-2,  
598 p53 and combined ATF-2/53 siRNA treatments under normal starvation  
599 (control) or VEGF-A<sub>165</sub> isoform (165) stimulation. (E) Quantification of relative  
600 p53 levels in control, scrambled, ATF-2, p53 and combined ATF-2/53 siRNA  
601 treatments. (F) Quantification of relative levels of cyclin D1 levels after  
602 treatment with control, scrambled, ATF-2, p53 and combined ATF-2/53 siRNA  
603 duplexes. Error bars indicate  $\pm$ SEM ( $n \geq 3$ ); significance is indicated by the  
604 asterisks shown when  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*),  $p < 0.0001$  (\*\*\*\*).

605

606 **Figure 2. ATF-2 and Tpl2 regulate p53 and cyclin D1 levels in endothelial**  
607 **cells.** (A) Endothelial cells subjected to treatment with control untreated,  
608 scrambled, ATF-2 or combined ATF-2/p53 siRNA duplexes were lysed and  
609 immunoblotted with antibodies specific for VEGFR2, p53, p21, Tpl2, phospho-  
610 ATF-2, ATF-2, cyclin D1 and tubulin. (B, D, E) Quantification of relative protein  
611 levels of (B) Tpl2, (D) p53, and (E) cyclin D1 after treatment with different

612 siRNA duplexes. (C) Quantification of relative *Tpl2* RNA levels after treatment  
613 with control or ATF-2-specific siRNA duplexes. HUVECs treated with either  
614 control scrambled siRNA or ATF-2-specific siRNA duplexes, serum starved for  
615 2 h, stimulated with VEGF-A before lysis and qRT-PCR analysis (see Materials  
616 and Methods). *Tpl2* RNA levels were normalised relative to control siRNA  
617 treatment. (B-E) Error bars indicate  $\pm$ SEM ( $n \geq 3$ ); significance is indicated by  
618 the asterisks shown when  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*),  $p < 0.0001$   
619 (\*\*\*\*).

620

621 **Figure 3. The endothelial cell cycle shows regulation by ATF-2, Tpl2 and**  
622 **p53.** (A) Endothelial cells stably expressing the FUCCl reporter (see Materials  
623 and Methods) were subjected to treatment with scrambled, ATF-2, p53 and  
624 Tpl2 siRNA duplexes followed by synchronization and stimulation with 0.25 nM  
625 VEGF-A<sub>165</sub> (VEGF-A) for 8 h or 30 h before fixation and visualization using  
626 fluorescence microscopy. Quantification of endothelial cell populations after  
627 treatment with scrambled, ATF-2, p53 and Tpl2, ATF-2/p53, ATF-2/Tpl2 siRNA  
628 duplexes as shown in (B) G1/M, and (C) G0. (D) Quantification of endothelial  
629 viability in cells subjected to treatment with control, scrambled or ATF-2 siRNA  
630 duplexes. Error bars indicate  $\pm$ SEM ( $n \geq 3$ ); significance is indicated by the  
631 asterisks shown when  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*).

632

633 **Figure 4. ATF-2 levels influence endothelial cell apoptosis.** (A-D)  
634 Endothelial cells were subjected to untreated control, scrambled, ATF-2, p53,  
635 Tpl2, ATF-2/p53, ATF-2/Tpl2 siRNA duplexes before flow cytometry analysis  
636 using combined propidium iodide and Annexin V-FITC staining (see Materials  
637 and Methods). Quantification of (A) AnnexinV-labelled cell population, (B) early  
638 apoptotic cells, (C) late apoptotic cells, and (D) total apoptotic cells after  
639 treatment with different siRNA duplexes followed by flow cytometry. Error bars  
640 indicate  $\pm$ SEM ( $n \geq 3$ ); significance is indicated by the asterisks shown when

641  $p < 0.05$  (\*),  $p < 0.001$  (\*\*\*). (E) Endothelial cells subjected to treatment with  
642 scrambled or ATF-2-specific siRNA duplexes were lysed and immunoblotted  
643 with antibodies specific for ATF-2, cleaved caspase 3, caspase 3, p53, Bax  
644 and tubulin. (F) Quantification of relative levels of cleaved Caspase 3 in  
645 endothelial cells subjected to treatment with scrambled or ATF-2-specific  
646 siRNA duplexes. Error bars indicate  $\pm$ SEM ( $n \geq 3$ ); significance is indicated by  
647 the asterisks,  $p < 0.01$  (\*\*).

648

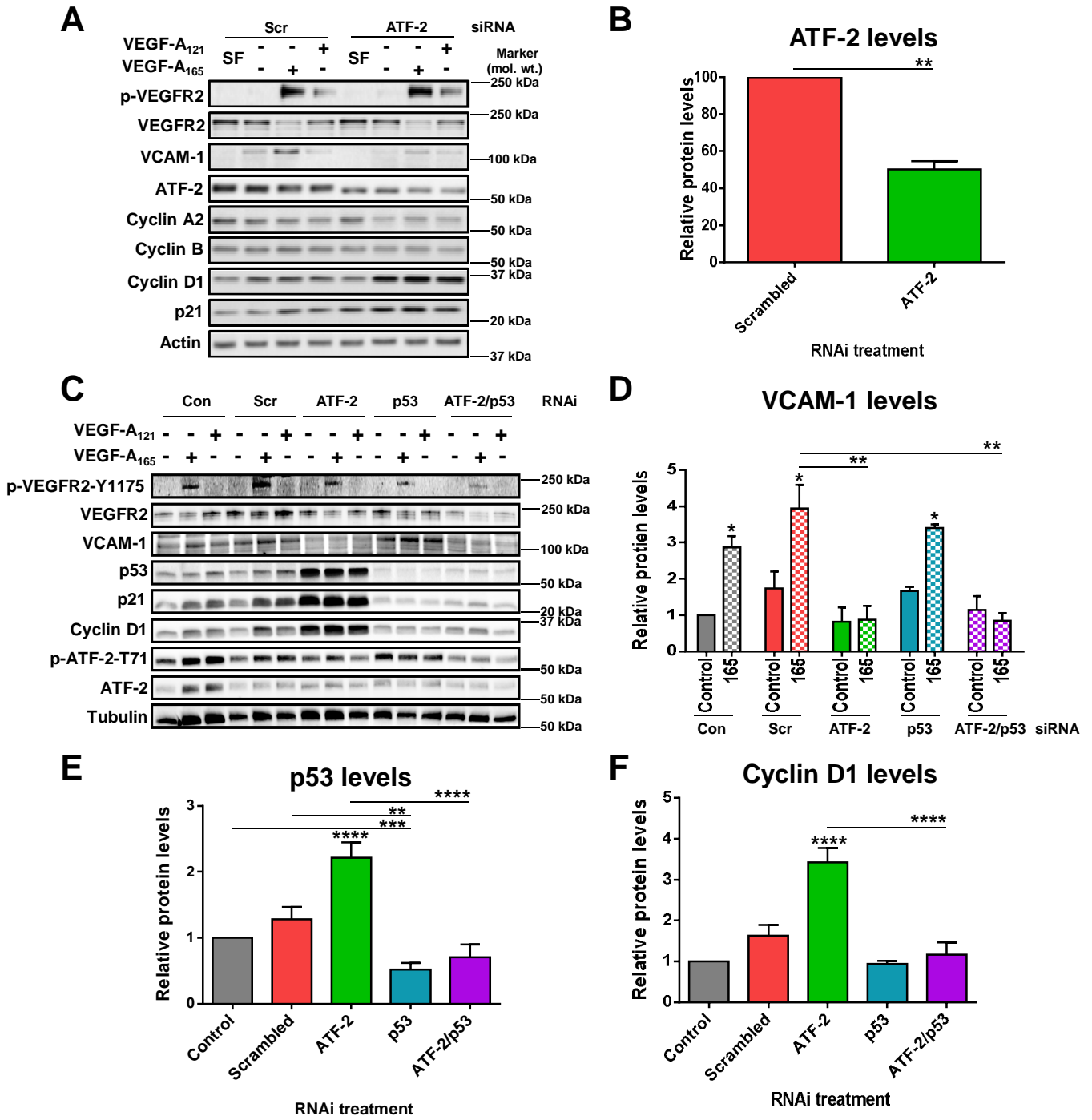
649 **Figure 5. ATF-2, Tpl2 and p53 levels modulate endothelial cell viability,**  
650 **proliferation and tubulogenesis.** Quantification of endothelial cell (A)  
651 viability, (B) proliferation, and (C) tubulogenesis after treatment with different  
652 siRNA duplexes as indicated in each panel. In panel C, endothelial cells were  
653 treated with different siRNA duplexes before assaying for tubulogenesis (see  
654 Materials and Methods) by growth in normal medium (control) or VEGF- $A_{165}$   
655 stimulated (165) tubulogenesis. In panels A-C, error bars indicate  $\pm$ SEM ( $n \geq 3$ );  
656 significance is indicated by the asterisks shown when  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  
657  $p < 0.001$  (\*\*\*),  $p < 0.0001$  (\*\*\*\*). (D) Endothelial cells were treated with different  
658 siRNA duplexes (scrambled, ATF-2, p53, Tpl2, ATF-2/p53, ATF-2/Tpl2) before  
659 assaying for tubulogenesis. PECAM-1 staining was used for detecting  
660 endothelial tubules using fluorescence microscopy (see Materials and  
661 Methods). Bar, 1000  $\mu$ m.

662

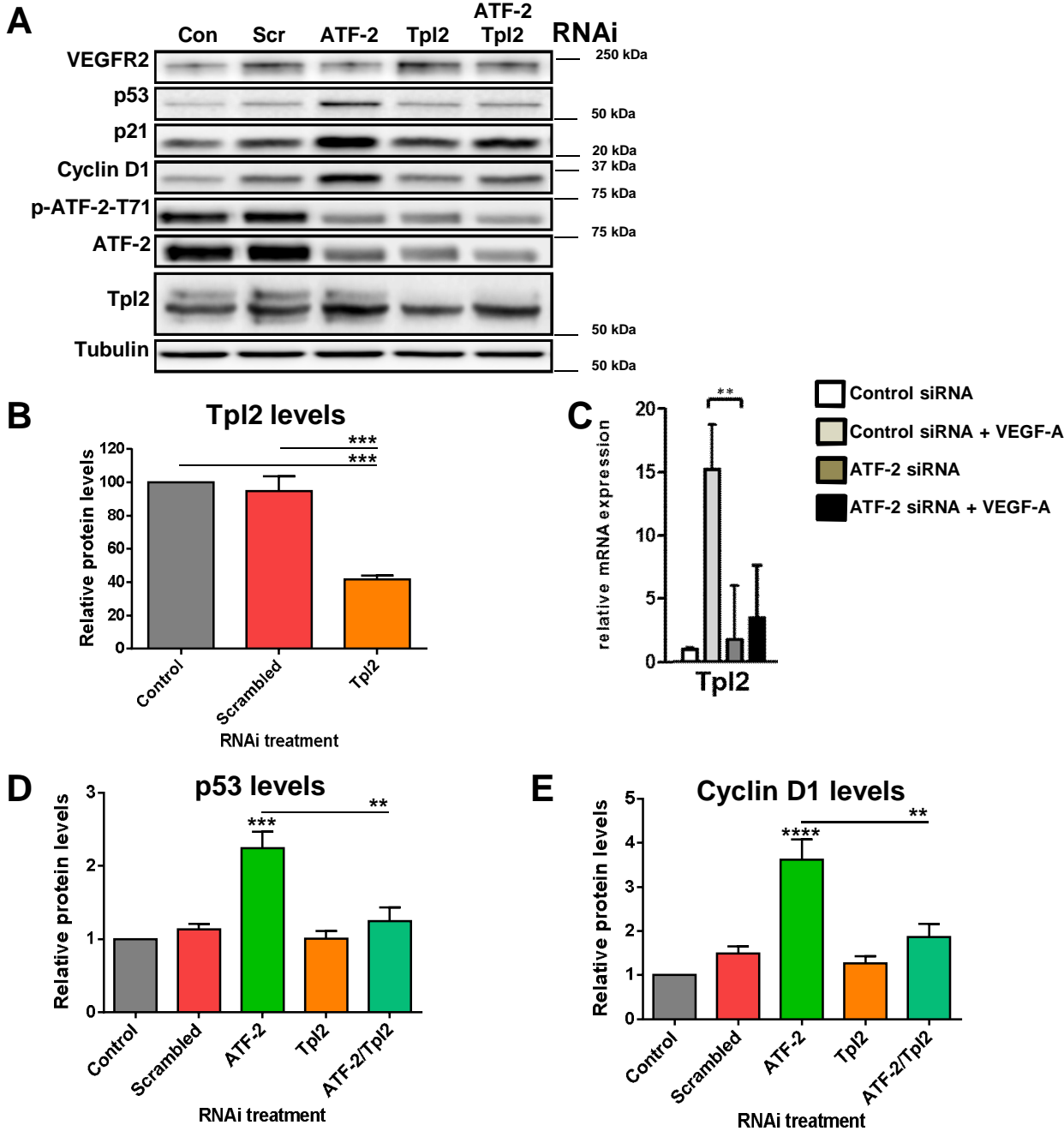
663 **Figure 6. Regulation of endothelial cell cycle progression and apoptosis**  
664 **by ATF-2, p53 and Tpl2.** (A) Under normal or steady-state conditions, the Tpl2  
665 protein kinase maintains ATF-2 phosphorylation (Step 1) which negatively  
666 regulates p53 levels, impacting on p21 and cyclin D1 expression (Step 2). ATF-  
667 2 also negatively regulates Tpl2 levels. This translates into normal endothelial  
668 cell cycle progression and function (Step 3). (B) Under conditions of reduced  
669 ATF-2 activity or levels there is a rise in Tpl2 levels (Step 4) which positively

670 regulates p53 levels, impacting on p21 and cyclin D1 expression (Step 5). This  
671 translates into cell cycle arrest and apoptosis (Step 6).

**Figure 1**



**Figure 2**



**Figure 3**

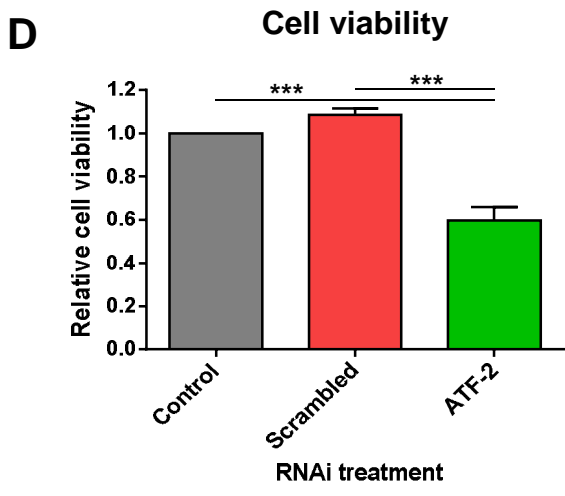
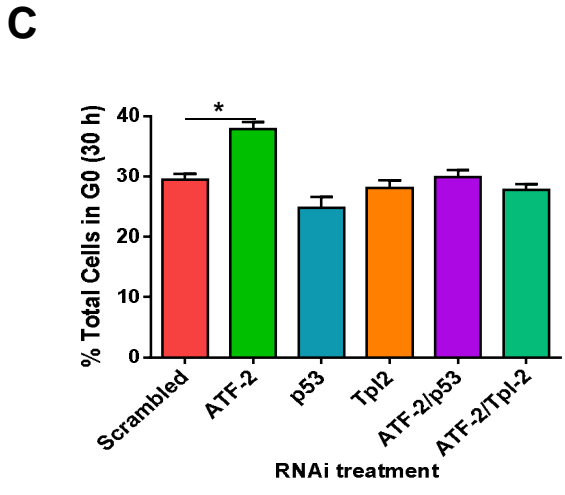
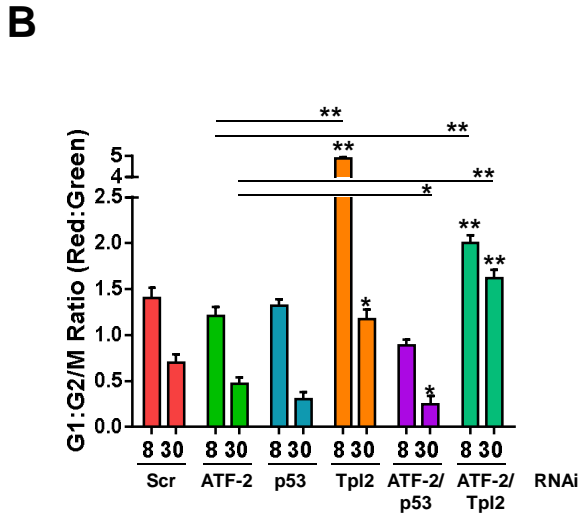
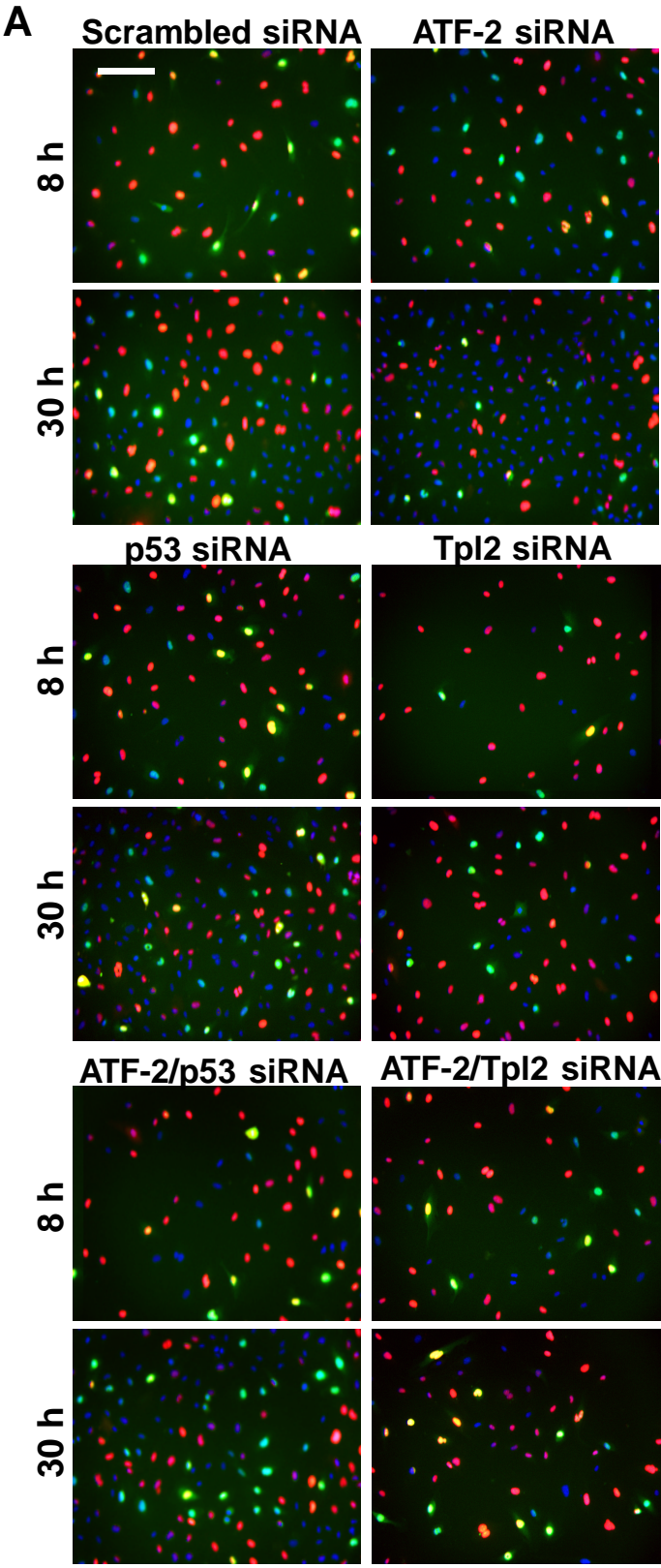


Figure 4

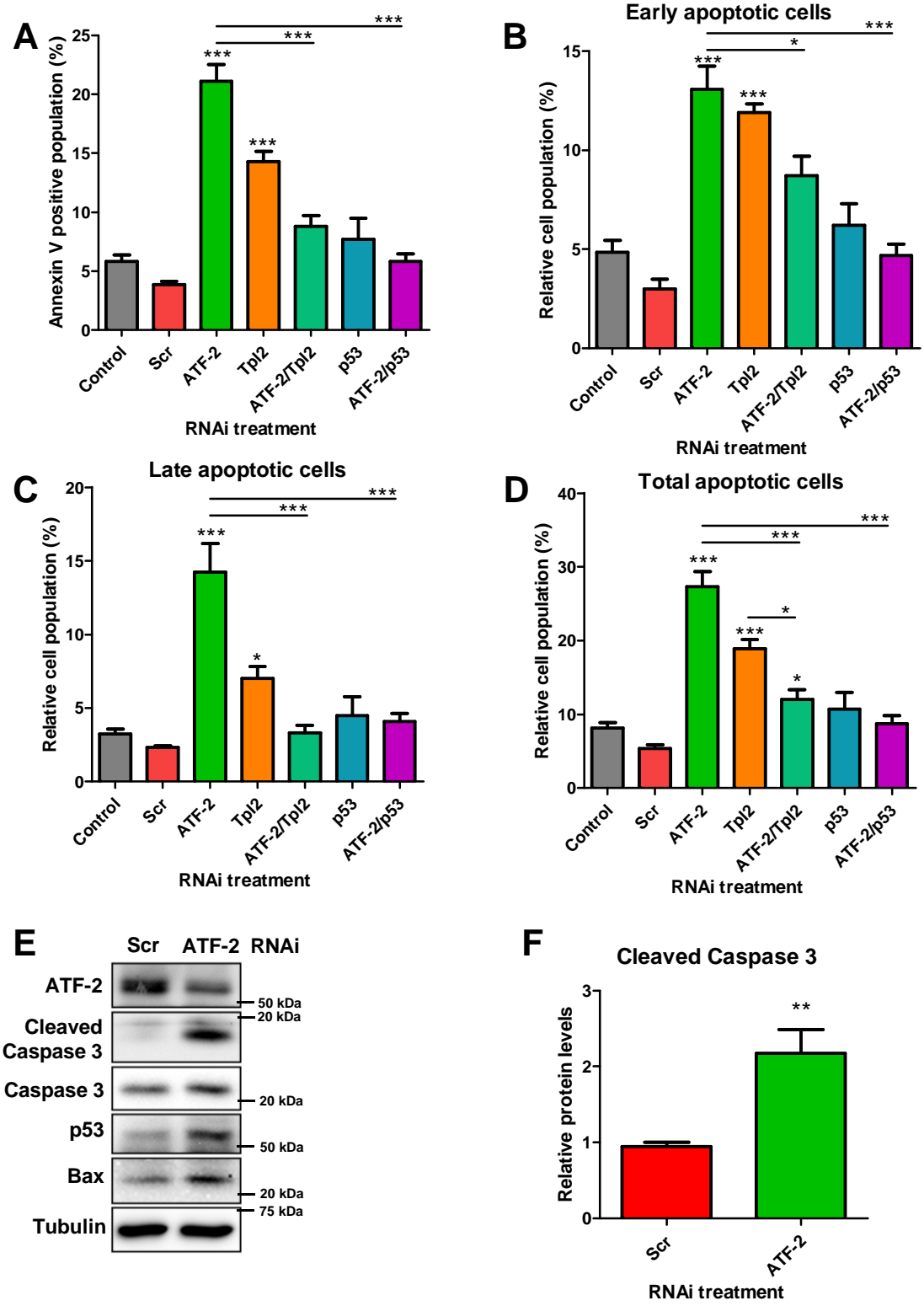


Figure 5

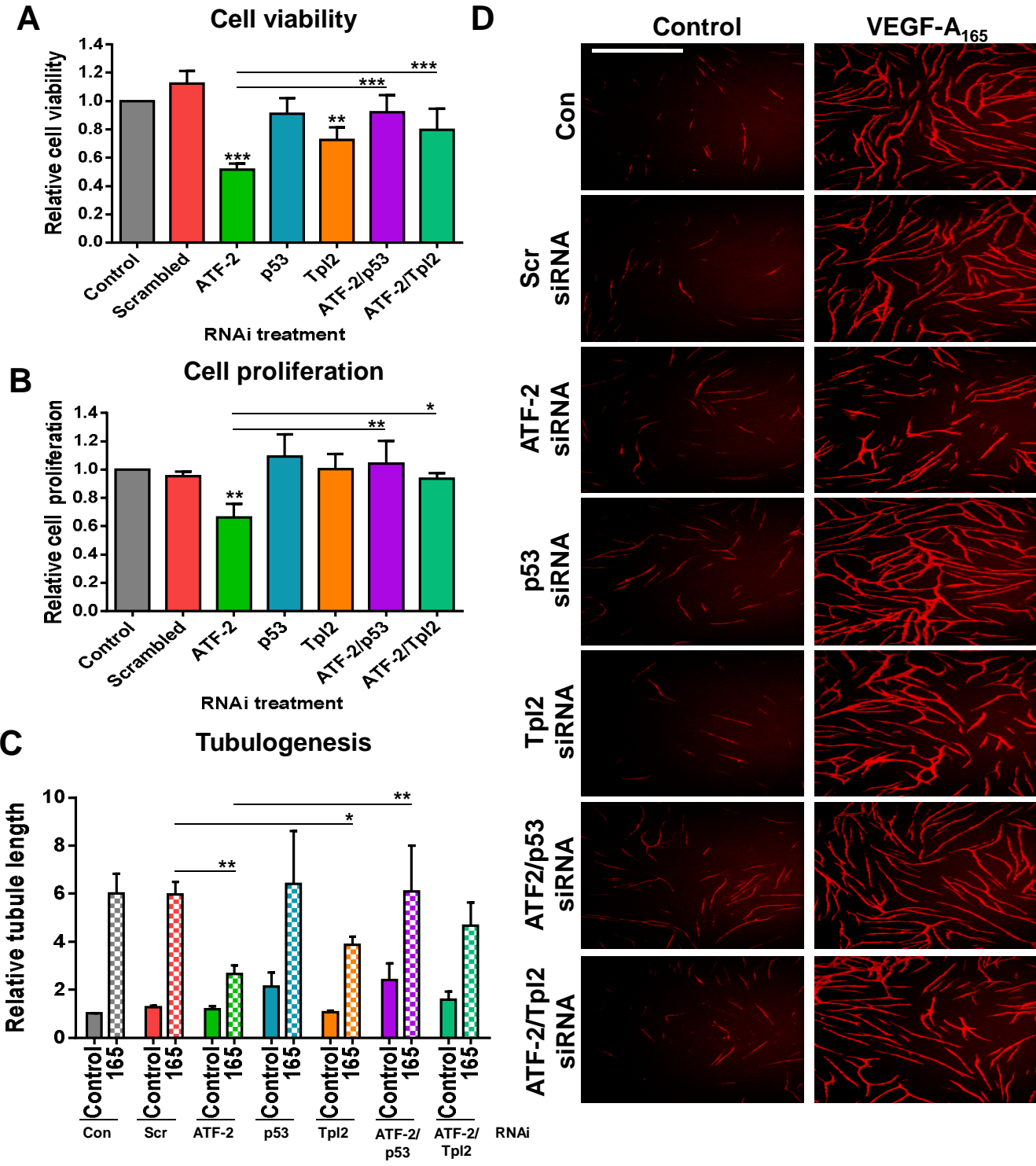
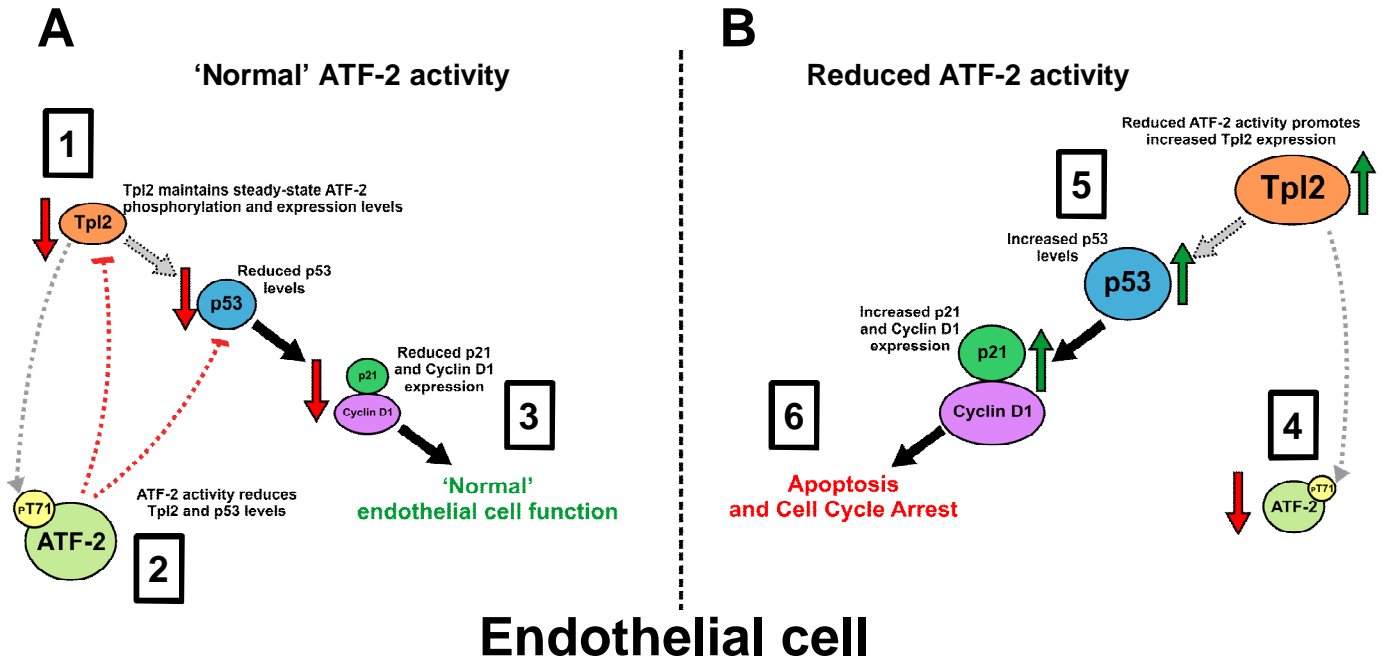
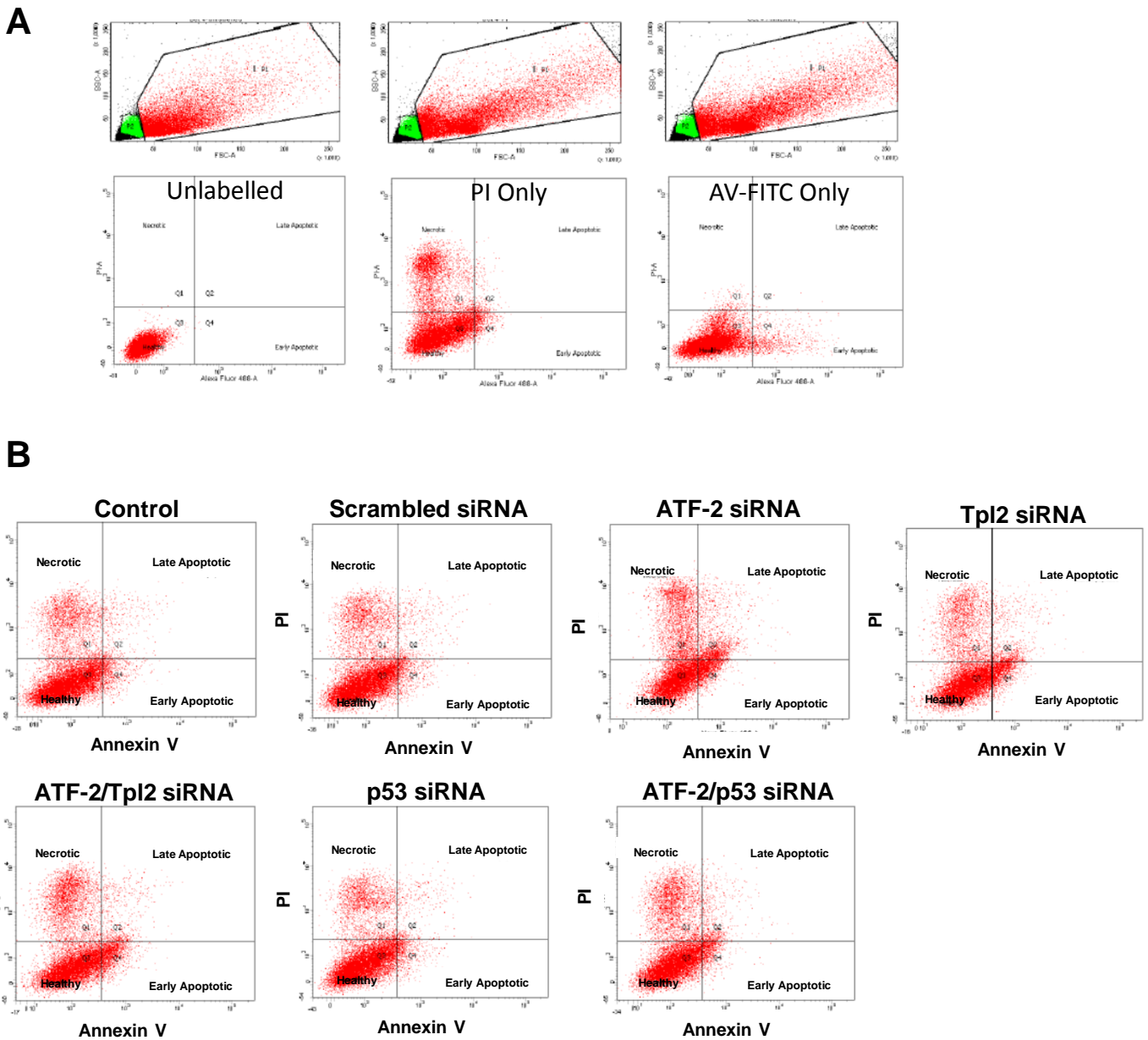


Figure 6





**Figure S1. Flow cytometry analysis of endothelial cell apoptosis.** (A) Control untreated endothelial cells were subjected to no labelling or with either propidium iodide (PI) or AnnexinV-FITC (AV-FITC). (B) Endothelial cells subjected to treatments using scrambled, ATF-2, p53, Tpl2, ATF-2/p53, ATF-2/Tpl2 siRNA duplexes before flow cytometry analysis using combined propidium iodide and Annexin V-FITC staining (see Materials and Methods). The different cell populations (healthy, necrotic, early apoptotic and late apoptotic) are indicated in each quadrant.