

1 **PROTEOMICS OF TISSUE FACTOR SILENCING IN CARDIOMYOCYTIC CELLS**
2 **REVEALS A NEW ROLE FOR THIS COAGULATION FACTOR IN SPLICING**
3 **MACHINERY CONTROL.**

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22 **ABSTRACT**

23 It has long been known that Tissue Factor (TF) plays a role in blood coagulation and has a direct
24 thrombotic action that is closely related to cardiovascular risk, but it is becoming increasingly
25 clear that it has a much wider range of biological functions that range from inflammation to
26 immunity. It is also involved in maintaining heart hemostasis and structure, and the observation
27 that it is down-regulated in the myocardium of patients with dilated cardiomyopathy suggests that
28 it influences cell-to-cell contact stability and contractility, and thus contributes to cardiac
29 dysfunction. However, the molecular mechanisms underlying these coagulation-independent
30 functions have not yet been fully elucidated.

31 In order to analyse the influence of TF on the cardiomyocytic proteome, we used functional
32 biochemical approaches incorporating label-free quantitative proteomics and gene silencing, and
33 found that this provided a powerful means of identifying a new role for TF in regulating splicing
34 machinery together with the expression of several proteins of the spliceosome, and mRNA
35 metabolism with a considerable impact on cell viability.

36

37 **SIGNIFICANCE**

38 In this study, using quantitative proteomics and functional biochemical approaches, we define for
39 the first time that, in addition to its primary role in blood coagulation, Tissue Factor also plays a
40 novel role in regulating cell splicing machinery, with a relevant impact on cell survival.

41 This new function may help to explain the wide range of biological activities of TF, and thus
42 provide fruitful clues for developing new strategies for treating human diseases in which TF is
43 dysregulated.

44

45 INTRODUCTION

46 Tissue factor (TF), a 47-kDa transmembrane cell-surface protein, is primarily known as the
47 initiator of the coagulation cascade [1, 2] but it also has a wide variety of coagulation-
48 independent functions: it is required for embryonic blood vessel formation [3], plays a role in
49 tumour metastasis [4], and is involved in the maintenance of heart hemostasis and structure [5].
50 Fibroblasts and cardiomyocytes of the heart constitutively express high levels of TF, which is
51 located in the sarcolemma of cardiomyocytes and the intercalated discs of myocardium, where it
52 co-localises with cytoskeletal and adhesion proteins such as desmin and vinculin [6].
53 Furthermore, actin-binding protein 280 has been identified as a ligand for the TF cytoplasmic
54 domain by two-hybrid screening [7]. These findings of co-localisation and interactions with
55 structural cardiac proteins has led to the hypothesis that TF may also play a non-hemostatic role
56 in the heart by maintaining the structural integrity and function of cardiac muscle [6, 7].
57 Furthermore, the pattern of TF expression in cardiomyocytes during human and murine heart
58 development indicates that TF may act as a morphogenic factor in the developing heart [8].
59 TF is down-regulated in the myocardium of patients with dilated cardiomyopathy (DCM).
60 Myocardial specimens from DCM hearts show that it has an altered cell localisation, being
61 redistributed from the Z-bands to the perinuclear cytosol [9]. This further suggests that decreased
62 TF expression and change in localisation may affect contractility and cell-to-cell contact stability,
63 thus contributing to cardiac dysfunction in patients with DCM [9].
64 The *in vitro* and *in vivo* administration of lipopolysaccharide reduces TF mRNA and protein
65 expression in cardiomyocytes, but not in lung, kidney or brain [6, 10, 11], which not only clearly
66 demonstrates that TF is uniquely regulated in the heart, but also suggests that it has potentially
67 unique functions in heart morphogenesis and the maintenance of cardiac muscle structure.

68 Recent studies of murine cardiomyocytes have demonstrated that the stably transfected full-
69 length and alternatively spliced form of TF lacking exon 5 (asTF) [12] have anti-apoptotic
70 activity in cells exposed to TNF- α [13] as a result of increased Akt expression and
71 phosphorylation and the up-regulation of the anti-apoptotic protein Bcl-x_L. Finally, a study by
72 Eisenreich *et al.* has shown that the overexpression of asTF in murine cardiomyocytic cells also
73 increases the expression of the pro-angiogenic and pro-migratory factors, such as VEGF, FGF2,
74 and Cyr61 [5].

75 The mechanisms underlying this plethora of effects have not yet been fully elucidated. In order to
76 analyse the influence of TF on the protein expression in cells, it is essential to use a global
77 approach such as that provided by proteomic analyses, which allow protein clusters to be
78 identified in a single experiment. We analysed for the first time the influence of TF knock-down
79 on the proteome of cardiomyocytic cells by identifying, quantifying, and classifying the proteins
80 directly or indirectly regulated by endogenously expressed TF, and found that combining gene
81 silencing technology with proteomic analysis [14, 15] is a very promising means of identifying
82 the molecular patterns in which the silenced gene might be involved. We therefore used the data-
83 independent analysis (DIA) mass spectrometry-based method, named LC/MS^E [16], for label-free
84 quantitative proteomics in order to detect the changes in the expression profile of cardiomyocytes
85 caused by the siRNA-mediated gene silencing of TF.

86

87 **MATERIAL AND METHODS**

88 *Cell cultures*

89 The HL-1 cardiomyocytes, a kind gift of Prof. W.C. Claycomb, (LSU Health Sciences Center,
90 New Orleans, LA, USA), were cultured in complete Claycomb medium supplemented with 10%

91 FCS (JRH Biosciences, Lenexa, KS, USA) and 100 $\mu\text{mol/L}$ norepinephrine (Sigma-Aldrich,
92 Milan, Italy) according to Prof. Claycomb's instructions [17].

93 The MDA-MB-231 human breast adenocarcinoma cells were purchased from ATCC (LGC
94 Standards S.r.l., Milan, Italy), and cultured in complete DMEM supplemented with 10% FCS
95 (Euroclone S.p.A., Milan, Italy).

96

97 ***MTT assay***

98 The MTT assay was based on the protocol first described by Mosmann [18]. Briefly, after being
99 treated with small interfering RNAs or LY 294002 (Sigma-Aldrich, Milan, Italy), the cells were
100 incubated for 4 h at 37°C with 0.1 mg/mL of MTT, dissolved in serum-free medium. Washing
101 with PBS was followed by the addition of DMSO (1 mL in a well of a six wells/plate) and gentle
102 shaking for 5 min in order to ensure complete dissolution. Absorbance was recorded at 550 nm
103 using the microplate spectrophotometer system (Mithras LB940, Berthold Technologies, Bad
104 Wildbad, Germany).

105

106 ***RNA interference and cell transfection***

107 Validated high-performance purity grade small interfering RNAs (siRNA) against TF were
108 synthesised by Qiagen Inc. (Milan, Italy) using the HiPerformance siRNA design algorithm and a
109 proprietary homology analysis tool. Control siRNA, with a nonsilencing oligonucleotide
110 sequence that does not recognise any known homology to mammalian genes, was also generated
111 as a negative control. The siRNAs were dissolved in an appropriate volume of sterile water (20
112 $\mu\text{mol/L}$). The cells were transfected with siRNA at 70-80% confluence using the HiPerFect
113 Transfection Reagent (Qiagen Inc, Milan, Italy). After 24 h, the transfection procedure was
114 repeated before analysis.

115

116 ***Immunofluorescence analysis***

117 The cells were fixed with 2% w/v paraformaldehyde containing 0.2% v/v Triton X-100 for 10
118 min at room temperature and, after washing with PBS, were treated with acetone at -20°C for 5
119 min, and rinsed three times with PBS. The samples were first incubated with the anti-SRSF2
120 antibody (Sigma-Aldrich, Milan, Italy), and then with the Alexa Fluor 488 goat anti-mouse IgG
121 antibody (Molecular Probes, Life Technologies Italia, Monza, Italy); the nuclei were stained with
122 DAPI (Sigma-Aldrich, Milan, Italy). The images were acquired using an LSM710 Zeiss confocal
123 inverted microscope equipped with Ar/Kr and UV lasers, and Zeiss Zen 2008 software.

124

125 ***In situ hybridisation***

126 The cells were prepared for fluorescence *in situ* hybridisation by means of fixation with 4% w/v
127 paraformaldehyde at room temperature for 8 min. After washing with PBS, the cells were
128 permeabilised with 0.5% v/v Triton X-100 on ice for 5 min, and rinsed with PBS. Hybridisation
129 was carried out at 42°C in a solution containing 2X saline sodium citrate buffer (SSC), 25% v/v
130 formamide, 1 mg/mL of tRNA, 10% w/v dextran sulfate, and 50 µg/mL oligo-dT probe labelled
131 with Alexa 488 at the 3' end. After hybridisation, the cells were washed twice for 15 min in 2X
132 SSC, and once with 0.5X SSC at 42°C, and then fixed with 4% w/v paraformaldehyde at room
133 temperature for 8 min. After three washes in PBS, the images were acquired using an LSM710
134 Zeiss confocal inverted microscope equipped with Ar/Kr and UV lasers, and Zeiss Zen 2008
135 software.

136

137 ***Dual-reporter cell-based splicing assay***

138 HL-1 cells were transiently transfected with the pTN24 dual-reporter splicing construct, with or
139 without the human pTra2- α construct, for 24 h using DreamFect™ Gold (OZ Biosciences,
140 Marseille, France) as described by the manufacturer. The sequence of both plasmids have been
141 previously described in detail [19]. The cells were subsequently treated with control siRNA and
142 TF siRNA (Qiagen Inc., Milan, Italy) for 48 h. After silencing, the cells were lysed, and equal
143 amounts of proteins for each condition were assayed for luciferase and β -galactosidase activity
144 using the Dual-Light® Assay System (Applied Biosystems, Foster City, CA, USA) according to
145 the manufacturer's instructions. The assay data are expressed as the relative ratio of luciferase
146 and β -galactosidase activity.

147

148 ***Nucleosome immunoassay***

149 The histone-complexed DNA fragments (mono- and oligonucleosomes) were quantified using a
150 one-step sandwich immunoassay (Roche Diagnostics, Mannheim, Germany), according to the
151 manufacturer's instructions. The data are expressed as absorbance/mg of cell proteins as
152 determined by the Bradford protein assay.

153

154 ***Western blotting***

155 Cell monolayers were harvested in Laemmli buffer containing 1 mmol/L PMSF, 10 μ g/mL aprotinin
156 and 5 mmol/L benzamidine, and equal amounts of proteins were separated on 12% SDS-
157 polyacrylamide gel and transferred to nitrocellulose membranes as previously described [20]. The
158 membranes were incubated with primary antibodies against phosphorylated and total ERK1/2 (Cell
159 Signalling Technologies, Danvers, MA, USA), phosphorylated and total Akt (Cell Signalling
160 Technologies, Danvers, MA, USA), HtrA2 (Cell Signalling Technologies, Danvers, MA, USA),

161 hnRNP L and hnRNP K (Abcam, Cambridge, UK), polypyrimidine tract-binding protein-associated-
162 splicing factor, PTB (Antibodies-online, Aachen, Germany), heterogeneous nuclear
163 ribonucleoprotein A1 protein, hnRNP A1 (Cell Signalling Technologies, Danvers, MA, USA). The
164 blots were subsequently incubated with goat anti-rabbit or goat anti-mouse horseradish peroxidase-
165 conjugated secondary antibody (Bio-Rad Laboratories, Milan, Italy), and the bands were visualised
166 by means of enhanced chemiluminescence (GE Healthcare, Milan, Italy). The densitometric analysis
167 was carried out using QuantityOne software (Bio-Rad Laboratories, Milan, Italy).

168

169 ***RNA extraction and cDNA preparation***

170 Total cellular RNA was extracted using TRIzol Reagent (Invitrogen Life Technologies, Milan,
171 Italy) and reverse transcribed at 42°C for 50 min, and at 70°C for 15 min (Bio-Rad Laboratories,
172 Milan, Italy). For 1 µg of total cellular RNA, we used 200 units of reverse transcriptase (RT;
173 SuperScript II, Invitrogen, Life Technologies, Milan, Italy), 3 µg random hexamer primers, 1
174 mmol/L dNTPs, and 40 units Rnase inhibitor.

175

176 ***Real-time reverse transcription polymerase chain reaction (RT-PCR)***

177 Real-time quantitative PCR (qRT-PCR) was carried out to detect TF mRNA, with 18S rRNA being
178 used for sample normalisation. The primer sequences were: human TF sense: 5'-
179 CCTTACCTGGAGACAAACCTCG-3'; human TF antisense: 5'-
180 CCGTTCATCTTCTACGGTCACA-3'; human 18S sense: 5'-CGGCTACCACATCCAAGGAA-3';
181 human 18S antisense: 5'-CCTGTATTGTTATTTTTTCGTCACCT-3'; mouse 18S sense: 5'-
182 GTAACCCGTTGAACCCATT-3'; mouse 18S antisense: 5'-CCATCCAATCGGTAGTAGCG-3'.

183 The primer sequences used for mouse TF (QT00159789) came from Qiagen Inc. (Milan, Italy). A
184 volume of 2.5 µL of cDNA was incubated in 25 µL IQ Supermix containing TF or 18S primers and

185 fluorescence dye SYBRGreen (Bio-Rad Laboratories, Milan, Italy). Real-time RT-PCR was carried
186 out in triplicate for each sample using an iCycler optical system (Bio-Rad Laboratories, Milan, Italy)
187

188 ***PCR assay for the analysis of full-length and alternatively spliced TF (asTF)***

189 After denaturation at 94°C for 2 min, 1 µg of total cellular RNA was amplified using the one-step
190 Invitrogen SuperScript III kit and a GeneAmp 9700 thermal cycler (Applied Biosystems, Life
191 Technologies, Monza, Italy) for 40 cycles (94°C for 15 s, 30 s at an annealing temperature of 66°C,
192 and then 68°C for 30 s), with a final extension at 68°C for 5 min using the following primers: mouse
193 TF sense: 5'-ACGAGATCGTGAAGGATGT-3'; mouse TF sense: 5'-
194 GAGATATGGACAGGAGGATG-3' (Invitrogen Life Technologies, Milan, Italy). The amplified
195 products were separated by means of electrophoresis on a 2% agarose gel.

196

197 ***PCR assay for the analysis of apoptotic genes affected by alternative splicing***

198 The Bcl-x_L/Bcl-x_S, Survivin, and TNF-related apoptosis inducing ligand (TRAIL) isoforms were
199 analysed by means of semi-quantitative RT-PCR. The RNA was treated with DNase I (Invitrogen,
200 Life Technologies, Milan, Italy) and then underwent RT-PCR as described above. The primer
201 sequences were: human Bcl-x sense: 5'-GAGGCAGGCGACGAGTTTGAA-3'; human Bcl-x
202 antisense: 5'-TGGGAGGGTAGAGTGGATGGT-3'; human survivin sense 5'-GCA TGG GTG
203 CCC CGA CGT TG-3'; human survivin antisense: 5'-GCT CCG GCC AGA GGC CTC AA-3' [21];
204 human TRAIL sense: 5'-GAA TCC CAT GGC TAT GAT GGA GGT CCA G-3'; human TRAIL
205 antisense: 5'-GGA TTC GAG GAC CTC TTT CTC TCA CTA-3' [22]; human Glyceraldehyde-3-
206 phosphate dehydrogenase (GAPDH) sense: 5'-ACG GAT TTG GTC GTA TTG GGC G-3'; human
207 GAPDH antisense: 5'-CTC CTG GAA GAT GGT GAT GG-3'.

208 The amplification products were analysed by means of agarose gel electrophoresis using 2% w/v
209 agarose gels and Gel Red (Società Italiana Chimici, Rome, Italy), and the bands were captured
210 under UV illumination. QuantityOne software (version 4.5.2, Bio-Rad Laboratories, Milan, Italy)
211 was used for image acquisition and densitometric analysis.

212

213 *Cell proliferation assay*

214 Cell proliferation was evaluated using the Cell proliferation ELISA, BrdU (Roche Diagnostic,
215 Monza, Italy), in accordance with the manufacturer's instructions. Briefly, the cells were grown
216 in 96-well tissue-culture microplates, and labelled by the addition of BrdU for 4 h. During the
217 labelling period, BrdU is incorporated in place of thymidine in the DNA of the cycling cells.
218 After removing the labelling medium, the cells were fixed, and the DNA was denatured in one
219 step by adding FixDenat buffer. After removing the buffer, the anti-BrdU-POD antibody was
220 added (which binds to the BrdU incorporated into the newly synthesised cellular DNA). The
221 immune complexes were detected by means of the subsequent substrate reaction. The reaction
222 product was quantified by measuring light emission using a scanning multi-well luminometer
223 (Mithras LB940, Berthold Technologies, Bad Wildbad, Germany).

224

225 *Label-free LC-MS^E analysis*

226 The cell pellets were dissolved in 25 mmol/L NH₄HCO₃ containing 0.1% RapiGest (Waters
227 Corporation, Milford, MA, USA) and digested as previously described [23]. The tryptic peptides
228 were separated by means of nanoscale LC using a nanoACQUITY system coupled to a SYNAPT-
229 MS, a hybrid Q-TOF mass spectrometer (Waters Corporation, Milford, MA, USA), for
230 qualitative and quantitative multiplexed LC-MS^E analysis. The control siRNA- and TF siRNA-

231 treated samples (0.5 μg) were mixed with 50 fmol yeast alcohol dehydrogenase (ADH) digest as
232 an internal standard for molar amount estimation [16], and analysed as previously described [24].
233 The LC-MS^E data were processed and searched using ProteinLynx Global Server (PLGS) v 2.3
234 (Waters Corporation, Milford, MA, USA). Ion detection, data clustering, and the normalisation of
235 the data-independent LC-MS^E data have been previously explained in detail [23, 25, 26].
236 Quantification of differentially expressed proteins identified by LC-MS^E was performed by
237 quantifying the peptides identified for each protein using the Expression^E algorithm in PLGS v
238 2.3. The regulation likelihood uses the confidence of identification of each peptide as a
239 quantification weighting mechanism, and is specifically designed for independently acquired LC-
240 MS data. The overall likelihood of regulation is calculated using the Bayesian statistics-based
241 Quantify algorithm in PLGS v 2.3, and is expressed by the probability of up-regulation ($P > 1$): if
242 this is < 0.05 (i.e. between 0 and 0.05), the likelihood of down-regulation is $> 95\%$; if it is > 0.95
243 (i.e., between 0.95 and 1) the likelihood of up-regulation is $> 95\%$ [23].
244 The significance of the regulation level was determined at a 20% fold change, but only proteins
245 identified with at least 2 peptides with similar intensities in the technical replicates ($\text{CV} < 10\%$)
246 were considered. The entire data set of differentially expressed proteins was further filtered by
247 considering only the identifications from data with identified peptides that replicated at least two
248 out of three technical instrument replicates and in two out of three biological replicates.
249

250 *2-dimensional electrophoresis (2-DE) and protein identification*

251 The cell pellets were dissolved in urea buffer (8 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 0.8%
252 carrier ampholytes, pH 3-10, 20 mmol/L Tris, 55 mmol/L DTT, and bromophenol blue), and 2-
253 DE was carried out in accordance with the manufacturer's protocol (Protean IEF cell, Bio-Rad
254 Laboratories, Milan, Italy) as previously described [27]. 2-DE was performed with three

255 independent biological experiments of control and TF-silenced cells, analysing each sample in
256 triplicate. Progenesis SameSpot software (v 4.0, NonLinear Dynamics, Newcastle upon Tyne,
257 UK) was used for gel alignment, spot detection, spot quantification, and normalisation for total
258 spot volume in each gel, and the data were statistically analysed using the incorporated statistical
259 package [27]. Significant between-group differences for each protein were computed using
260 Student's t test, considering a p value of <0.05 as statistically significant with a fold change cutoff
261 of 1.5. The protein spots selected for mass spectrometry analysis by LC-MS^E were in-gel digested
262 with trypsin as previously described [28].

263

264 *Data mining*

265 Cytoscape (v 2.7) and BiNGO plugin (v 2.3) were used to make gene ontology (GO) assignments
266 and determine the cell component and biological process GO categories that were significantly
267 under- and over-represented, as previously described [23]. Statistical significance was determined
268 by means of hypergeometric analysis followed by Benjamini and Hochberg's false discovery rate
269 correction (p<0.001) [29].

270

271 *Statistical analysis*

272 The data are expressed as mean values \pm SEM. The control and treated groups were statistically
273 compared using Student's t-test or ANOVA for repeated measures followed by Tukey's test. The
274 accepted level of significance was p<0.05; n=the number of individual experiments performed in
275 duplicate.

276

277 **RESULTS**

278 *Effects of TF silencing on HL-1 cardiomyocytic cells.*

279 The transfection of HL-1 cells with TF siRNA led to a $83.7\pm 5.6\%$ reduction in TF mRNA levels
280 as evaluated by qRT-PCR (Figure 1A), and induced a greater decrease in cell viability in
281 comparison with control cells. Specifically, the BrdU incorporation assay performed after TF
282 silencing showed a significant decrease in the relative number of BrdU-positive cells (Figure 1B)
283 accompanied by a remarkable inhibition of cell growth assessed by means of the MTT assay
284 (Figure 1C). A nucleosome immunoassay used in order to investigate whether these effects were
285 followed by an increase in apoptosis showed an increase in the apoptotic index after 16 h of up to
286 2.2 ± 0.3 times the control value (Figure 1D).

287

288 *Effects of TF silencing on the proteome of HL-1 cardiomyocytic cells.*

289 To analyse the effect of TF on the cardiomyocytic proteome further, we quantitatively compared
290 the proteome of control and TF-silenced cells using a label-free LC-MS^E analysis of three
291 independent biological replicates analysed in triplicate. Quality controls of the analysis showed a
292 high mass precision, a retention time CV of $<1\%$, and an intensity CV of $<4.5\%$, which allowed
293 an accurate comparison of the 370 proteins present in at least two out of three technical and
294 biological replicates. The molecular weights, pI, PLGS scores, the mean number of unique
295 identified peptides, sequence coverage, precursor RMS mass errors, and product RMS mass
296 errors are reported in Supplementary Table 1 in [30].

297 After auto-normalisation, the intensities of accurate mass-retention time component clusters from
298 control and TF-silenced cells were compared using PLGS v 2.3, as described in the Material and
299 Methods section, thus providing a table of the relative fold change in the level of each identified
300 protein.

301 There were 33 non-redundant proteins that were differentially expressed under the two
302 experimental conditions (Figure 2A); five were identified only in the control cell proteome, five

303 only in the TF-silenced cells, and 23 in both (nine down-regulated and 14 up-regulated in the TF-
304 silenced cells).

305 Table 1 shows the proteins significantly up- or down-regulated in the control and TF-silenced
306 cells, their scores, fold changes (expressed as $\log(e)$ of the ratio between the control and TF-
307 silenced cells), standard deviations, probabilities, and biological variations.

308 The Biological Networks Gene Ontology (BiNGO) plugin was employed to search for the GO
309 terms statistically over-represented among the differentially expressed proteins.[29] As shown in
310 Supplementary Figure 1A, among the proteins that were down-regulated after TF-silencing or
311 were unique in the control cells, the most over-represented GO terms in the biological process
312 category were mRNA metabolic process and RNA splicing (for additional details see also
313 Supplementary Table 2 in [30]). Furthermore, as shown in Supplementary Figure 1B, in the cell
314 compartment category, the most over-represented GO term was the spliceosome, thus suggesting
315 that TF silencing may principally alter the expression of proteins belonging to the spliceosome
316 that are involved in mRNA processing and RNA splicing (for additional details see also
317 Supplementary Table 2 in [30]). The down-regulation of polypyrimidine tract-binding protein-
318 associated-splicing factor (PTB), heterogeneous nuclear ribonucleoprotein A1 protein (hnRNP
319 A1), and heterogeneous nuclear ribonucleoprotein L protein (hnRNP L) was further validated by
320 immunoblotting (Figure 2B).

321 We also used 2-DE analysis as a complementary approach to analyse the effect of TF silencing on
322 the HL-1 proteome. Comparison of the 2-DE maps of the control and TF-silenced cells (Figure
323 3A-B) revealed a statistically significant decrease in the expression of the heterogeneous nuclear
324 ribonucleoprotein K protein, hnRNP K (Figure 3C), and a significant increase in the expression
325 of the pro-apoptotic serine protease HtrA2 (Figure 3D), both of which were confirmed by means
326 of immunoblotting (Figure 3E-F). Supplementary Table 1 shows the protein name, Uniprot

327 accession code, fold increase in the TF-silenced cells, p value, molecular weight, pI, protein
328 score, percentage of coverage, precursors RMS error, and peptide sequences and scores of the
329 differentially expressed proteins in the 2-DE gels.

330

331 ***Effects of TF silencing on mRNA distribution and spliceosome morphology***

332 As splicing activity is required for efficient mRNA transport [31], we next examined whether TF
333 silencing affects the subcellular distribution of mRNA. The *in situ* hybridisation of poly(A) RNA
334 showed that large populations of mRNA species were distributed in the cytoplasm of the control
335 cells, whereas, after cell treatment with TF siRNA, a lower cytoplasmic RNA signal was
336 observed (Figure 4).

337 We also investigated whether TF silencing affects the distribution of splicing factors such as
338 SRSF2, which is known to be organised in a specific “speckled” distribution pattern in the
339 nucleus. The sub-nuclear organisation of SRSF2-containing speckles was also greatly affected by
340 TF silencing, with an increase in both size and content as measured by immunofluorescence
341 (Figure 5). A similar effect was observed in cells in which SF3a (another U2 snRNP component)
342 has been knocked down [32], or in cells treated with oligonucleotides that inhibit pre-mRNA
343 splicing [33].

344

345 ***Effects of TF silencing on pre-mRNA splicing activity***

346 In order to examine whether TF plays a role in pre-mRNA splicing *in vivo*, we used the dual-
347 reporter system described by Nasim *et al.* [19]. The reporter construct (pTN24) contains genes
348 encoding β -galactosidase and luciferase proteins. The assay was constructed in such that in the
349 absence of splicing, it gives rise to β -galactosidase, whereas an efficient splicing reaction
350 generates the fusion of β -galactosidase and luciferase (Figure 6A). The splicing efficiency was

351 therefore measured as a relative ratio of luciferase and β -galactosidase activities. TF silencing
352 significantly decreased splicing activity (Figure 6B). The co-transfection of pTN24 and pTra2- α ,
353 an established splicing activator [19], increased the luciferase/ β -galactosidase ratio 1.6 ± 0.2 times
354 in comparison with the cells transfected with pTN24 alone ($p < 0.05$) and, also under this
355 condition, TF silencing significantly decreased splicing activity (Figure 6B). Overall, these
356 findings indicate that TF acts as a spliceosome modulator, and that TF silencing inhibits mRNA
357 splicing activity.

358

359 ***TF silencing decreases cardiomyocytic cell viability by influencing the regulators of apoptosis***

360 The levels of the phosphorylated forms of Akt and Erk1/2 were examined in order to investigate
361 which of the pathways that ultimately lead to cell apoptosis is affected by TF silencing. The level
362 of phosphorylated Akt was lower in the TF-silenced cells than in the control cells (Figure 7A),
363 whereas Erk1/2 levels were not affected (data not shown). Interestingly, Akt inhibition by LY
364 294002 up-regulated the pro-apoptotic serine protease HtrA2, and concomitantly reduced cell
365 growth (Figure 7B and 7C).

366 Finally, semi-quantitative RT-PCR revealed an increase in the mRNA of the pro-apoptotic Bcl-x_S
367 isoform over the anti-apoptotic Bcl-x_L transcripts following TF silencing in human MDA cells
368 which, unlike murine cells, express detectable levels of Bcl-x_S [34] (Figure 7D). The anti-
369 apoptotic Survivin and its splice variant, Survivin-DEx-3 were both downregulated in TF-
370 silenced cells (Figure 7E). The splice variant Survivin-2B, which shows a markedly reduced anti-
371 apoptotic potential, was not detectable in our experimental conditions. TNF-related apoptosis
372 inducing ligand (TRAIL), detectable only as regularly spliced TRAIL α , was not affected by TF-
373 silencing (Figure 7F).

374

375 **DISCUSSIONS**

376 Our proteomic analysis revealed for the first time that TF silencing affects various proteins
377 belonging to the spliceosomal complex [35] and, by modulating spliceosome proteins, inhibits
378 splicing activity, as assessed by means of a dual-reporter mRNA splicing assay, and changes the
379 normal speckled pattern of SRSF2 together with a decreased poly(A) RNA signal in the
380 cytoplasm. The impaired splicing efficiency due to TF silencing was accompanied by a loss of
381 cell survival, which was likely attributable to a decreased level of phosphorylated Akt, an
382 increase in the Bcl-x_S spliced isoform, a decrease of the anti-apoptotic wild-type Survivin and
383 Survivin DEx-3 isoforms, and the induction of pro-apoptotic HtrA2 expression. Our results
384 further confirm and extend previous findings by Boltzen *et al.* [13] showing that TF
385 overexpression protects cardiomyocytes from apoptosis induced by TNF- α by activation of Akt
386 and up-regulation of anti-apoptotic Bcl-x_L.

387 The dysregulation of splicing machinery caused by TF silencing is due to alterations in the
388 expression of RNA helicases and splicing factors such as members of the hnRNP family.
389 We found that the expression of DEAD box UAP56 helicase was down-regulated in TF-silenced
390 cells. UAP56 is an important factor for spliceosome assembly, mRNA export and translation, and
391 it plays an important role in cell growth and proliferation [36-38]. DDX39, a paralogue of
392 UAP56 that is expressed in a growth-regulated manner [39, 40], was also down-regulated after
393 TF silencing. Another member of the helicase family that was significantly down-regulated by
394 the silencing of TF was DEAD box RNA helicases p72 (DDX17), which plays important roles in
395 multiple cell processes, including transcription, processing of miRNAs and pre-mRNAs and
396 alternative splicing [41].

397 Five members of the hnRNP family were down-regulated by TF silencing: hnRNP C, A1, D0, L,
398 and K. HnRNP proteins are among the most abundant nuclear proteins (rivalling histones),

399 participate in pre-mRNA processing activities such as splicing and are important determinants of
400 mRNA export, localisation, translation and stability [42]. Given their central role in regulating
401 gene expression, it is not surprising that hnRNPs have been linked to numerous diseases [43][44],
402 ranging from cancer to neurodegenerative diseases [45, 46].

403 Interestingly, hnRNP A1, which was significantly down-regulated in TF-silenced cells, plays a
404 key role in the regulation of cell survival, promoting telomere length extension [47, 48]. More
405 recently, Fujiya *et al.* demonstrated that either siRNA targeting of hnRNP A1 or
406 autophagolysosomal degradation of hnRNP A1 induced by miR-18a lead to cell apoptosis [49].

407 One new finding is that TF silencing profoundly down-regulated the expression of matrin 3, a
408 highly conserved inner nuclear matrix protein which, through a direct interaction with hnRNP
409 proteins and with the DNA and RNA helicase DHX9, might control RNA metabolism and
410 stabilisation [50].

411 The proteomic analysis of TF-silenced cells also revealed that other spliceosome proteins were
412 also significantly down-regulated, including SF3b (SAP130) and PTB. It is interesting to note
413 that two natural products, pladienolide B and spliceostatin A, targeting the SF3b subunit of the
414 spliceosome [51], provoke the accumulation of incompletely processed pre-mRNA, inhibit cell
415 growth [52] and alter the size and number of snRNP-enriched nuclear speckles [32], similarly to
416 the effects observed by Tanackovic *et al.*, when specific splicing factors are inactivated [53].

417 PTB binds to uridine-rich sequences and regulates alternative splicing of a variety of pre-mRNAs
418 [54], acting as a general repressor of exon inclusion. For example, PTB, binding to an exonic
419 splicing silencer, promotes Fas exon 6 skipping and generates a soluble isoform that prevents
420 apoptosis [55]. The finding that TF silencing down-regulates PTB protein level suggests that this
421 splicing modulator is no longer operating.

422 TF silencing also increased the expression of the spliced isoform of the pro-apoptotic Bcl-x_S.
423 This is in line with the finding of Boltzen *et al.*, who showed that Bcl-x_L is an important factor
424 mediating anti-apoptotic effects in HL-1 cells stably transfected with TF [13]. Bcl-X is closely
425 related to Bcl-2 and exists in several isoforms generated by alternative splicing [56]. The large
426 isoform, Bcl-x_L, protects cells against apoptosis, whereas the short isoform Bcl-x_S, antagonises
427 the inhibition of cell death by interacting with Bcl-x_L and Bcl-2 [56]. It is worth noting that
428 alternative splicing can be regulated by hnRNP proteins binding exonic splicing silencer
429 elements, thus excluding exons from mature mRNA. One study has found that hnRNP K may
430 play a prominent role in alternative splicing control because nearly half of 56 alternative splicing
431 events in apoptotic genes were affected upon hnRNP K depletion, either enhancing or
432 suppressing exon inclusion [57]. In support of our finding, it has been shown that hnRNP K
433 represses the production of the pro-apoptotic Bcl-x_S spliced isoform, whereas its down-regulation
434 enhances the splicing of Bcl-x_L to Bcl-x_S [58].

435 We also found that the anti-apoptotic Survivin and its splice variant, Survivin-DEx-3, were both
436 down-regulated by TF silencing. Survivin is a member of the inhibitor of apoptosis protein (IAP)
437 family and plays an important role in inhibiting cell apoptosis [59]. In addition to wild-type
438 Survivin, different isoforms characterised by distinct localisation and functions have been
439 identified. For example, Survivin 2B has a pro-apoptotic function, whereas the nuclear Survivin
440 DEx-3 isoform has typically been shown to prevent apoptosis [21]. Our finding is in line with
441 previous results showing that TF-FVIIa-FXa complex prevents apoptosis by a thrombin-
442 independent pathway mediated in part by an increase in cell Survivin levels [60].

443 Although our analysis is limited to few candidates, the effect of TF silencing on apoptosis-related
444 genes does not appear to be widespread because, for example, the TRAIL gene is not affected in

445 TF-silenced cells. TRAIL- α is a member of the TNF family [61] which induces programmed cell
446 death in a wide range of neoplastic cells.

447 The inability to detect, in our experimental system, the pro-apoptotic splice variant of Survivin,
448 Survivin 2B, and the two alternative TRAIL-splice variants, TRAIL- β and TRAIL- γ , which are
449 characterised by the loss of their pro-apoptotic properties [22], does not allow us to establish a
450 clear relationship between TF-mediated deregulation of splicing factors and these proteins
451 involved in the apoptotic process.

452 As stated above, in addition to being the main initiator of the blood coagulation cascade, TF is
453 also a true signalling receptor that acts by means of various mechanisms, including the activation
454 of protease-activated receptors 1 and 2, via the TF cytoplasmic domain and the transactivation of
455 receptor tyrosine kinases. The activation of signalling cascades such as the mitogen-activated
456 protein kinase and phosphoinositide 3-kinase (PI3K)/Akt pathways relates TF to a multitude of
457 cell functions, such as cell survival, migration, and proliferation [62, 63].

458 It is worth noting that TF silencing significantly reduced phosphorylated Akt levels, whereas the
459 levels of phosphorylated Erk1/2 were unaffected. Furthermore, Akt inhibition led to the induction
460 of apoptosis because Akt is a serine/threonine kinase that, once activated by phosphorylation,
461 plays an important role in regulating a number of cell processes that deliver an anti-apoptotic
462 signal. Supporting the association between TF level and cell survival, cardiomyocytes stably
463 transfected with TF show less TNF α -induced apoptosis and a higher degree of activation of the
464 Akt pathway [13]. Moreover, anti-apoptotic Bcl-x_L expression was increased in HL-1 cells
465 overexpressing TF, thus underlining the positive association between TF, the Akt pathway and
466 anti-apoptotic Bcl-x_L. Interestingly, PI3K/Akt signaling pathway has been implicated to play an
467 important role in the regulation of Survivin [64].

468 The nature of the interaction between signalling pathway and splicing machinery in TF-silenced
469 cells was not elucidated in this study, but we can hypothesise that splicing factors may represent
470 targets of the signaling molecules. A wide variety of interactions have been described between
471 components of the RNA processing machinery and traditional signalling molecules: for example,
472 hnRNP K protein contains multiple modules that bind kinases while recruiting chromatin,
473 transcription, splicing and translation factors [65]. We focused our analysis to kinases such as the
474 mitogen-activated protein kinase and PI3-kinase/Akt pathways because they are related TF
475 signalling, and we have not analysed the full spectrum of kinases and phosphatases (reviewed in
476 [66]) involved in the post-traslational modifications of the spliceosomal components and
477 accessory splicing factors which significantly contribute to their activity. Regulation of splicing
478 factors by Akt is not heretical [67]; activated Akt has been involved in directly modulating
479 serine/arginine-rich proteins (SR proteins) [68]; more recently, hnRNP L has been recently
480 identified as a direct substrate of Akt, thus influencing the alternative splicing of caspase-9 [69],
481 and down-regulation of splicing factors has been also observed in U373 glioma cells after Akt
482 silencing [70]. Furthermore, SR-specific protein kinases (SRPKs) are also directly regulated by
483 Akt [71]; lastly, a phosphoproteomic screen of Akt isoforms identified 25 RNA processing
484 proteins including splicing factors as Akt isoform-dependent targets [72].

485 Our data also reveal for the first time that TF silencing up-regulates HtrA2 expression, and that
486 blocking Akt signaling by means of the PI3K inhibitor LY294002 modulates HtrA2 in a similar
487 manner, thus providing a further mechanism by which TF promotes cell survival through Akt.

488 The serine protease HtrA2 leads to programmed cell death in both a caspase-dependent and
489 caspase-independent manner. Its function closely relates to its protease activity, which is required
490 for the cleavage of its substrates. However, the regulation of HtrA2 by a signalling molecule has
491 not been previously documented. Yang *et al.* have demonstrated that the ectopic expression of

492 HtrA2 considerably induces programmed cell death, whereas apoptosis is largely reduced by
493 constitutively active Akt [73].

494 We recognise some limitations in our study. One limitation is the fact that we could not
495 discriminate the relative contribution of full-length and alternatively spliced TF, which are both
496 expressed in the cardiomyocytic cells although to different extents (full-length TF is the main
497 isoform and accounts for $85\pm 6\%$ of expressed TF) (Supplementary Figure 2) and, although they
498 have functionally different features, appear to be involved in similar biological processes [62].

499 We evidenced a reorganization of SRSF2 in TF-silenced cells in which “speckles became larger,
500 rounded and decreased in number with a concurrent increase in their fluorescent intensity”
501 attributable to the “return of splicing factor to their site of storage and/or assembly, until splicing
502 is resumed”, similarly to what observed by O’Keefe *et al.* [33]. However, a precise picture of the
503 SR proteins involved in this phenomenon has not been depicted as the antibody used does not
504 discriminate between SRSF2 and SRSF2-related non-snRNP factor SF2/ASF. As a detailed
505 analysis of the phosphorylation state of SR proteins has not been addressed, we can only
506 speculate that, similarly to what observed by Sacco-Bubulya P. *et al* [74], a reduced
507 phosphorylation causes retention of SR proteins in nuclear speckles.

508 Finally, the decreased level of poly(A) signal in the cytoplasm of TF-silenced cells does not
509 necessarily imply that poly(A) transcripts are improperly spliced and retained in the nucleus, as
510 we could not see any visible increase of the nuclear signal. Thus the effect of TF silencing on
511 mRNA biogenesis or on the mRNA export machinery [75] deserves further detailed
512 investigations, however, it is not unlikely that splicing factors might alter the polyadenylation
513 process as observed for PTB (reviewed in [76])

514 Nevertheless, the possibility that altered TF expression has an impact on overall splicing activity
515 may help to explain its role in those diseases in which both TF and splicing activity are down-
516 regulated, and opens new avenues for the investigations of splicing regulation by TF.
517 Although cardiomyopathies are only beginning to be explored in terms of splicing machinery,
518 recent evidence suggests that RNA splicing is less efficient in humans with heart failure. Splicing
519 changes have also been observed in hypertrophic myocardium with preserved systolic function,
520 which suggests that they may occur before the onset of overt heart failure [77, 78].
521 It is worth noting that TF is down-regulated in the myocardium of patients with dilated
522 cardiomyopathy [9], and that the deletion of the TF gene in mouse cardiomyocytes
523 (TF^{flox/flox}/MLC2v-Cre) leads to significantly increased fibrosis [79].
524 The mechanisms underlying these phenomena have not been previously investigated, but our
525 findings suggest that TF plays a role in controlling splicing machinery, although further studies
526 are required to determine whether this mechanism is operative in cells other than cardiomyocytes.

527

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533

534 **Conflict of interest**

535 The authors declare that they have no competing financial interest.

536

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542 **FIGURE LEGENDS**

543

544 **Figure 1. TF silencing reduced cell growth and induced apoptosis in HL-1 cardiomyocytes.**

545 **A)** TF mRNA analysed by qRT-PCR in control and TF siRNA-treated cells (n=10) *p<0.001 vs

546 controls. **B)** BrdU incorporation assay of control and TF siRNA-treated cells (n=5) *p<0.01 vs

547 controls. **C)** MTT assay of control and TF siRNA-treated cells (n=6) *p<0.01 vs controls. **D)**

548 Apoptosis was determined by means of a nucleosome immunoassay. Mean values±SEM of seven

549 independent experiments run in triplicate. *p<0.01 vs corresponding controls. Control: cells

550 treated with a non-silencing oligonucleotide sequence; siTF: TF-silenced cells.

551

552 **Figure 2. Differentially expressed proteins in TF-silenced cells identified by LC-MS^E.** **A)** The

553 data are expressed as the log(e) of the ratio of protein abundance in control and TF-silenced cells.

554 The control cells' unique protein value was set at -2, and that of the TF-silenced cells at +2. The

555 data were obtained from three independent experiments performed in triplicate. **B)** Western blot

556 and densitometric analyses of PTB, hnRNP A1, and hnRNP L. Mean values±SEM; *p<0.05 vs

557 controls (n=3). Control: cells treated with a non-silencing oligonucleotide sequence; siTF: TF-

558 silenced cells.

559

560 **Figure 3. Differentially expressed proteins in TF-silenced cells identified by 2-DE.**

561 Representative images of 2-DE gels from control **A)** and TF-silenced **B)** cells. The spots showing

562 significant differential expression are indicated by circles (1, hnRNP K; 2, HtrA2).

563 Representative images of 2-DE spots of hnRNP K **C)** and HtrA2 **D)** in control and TF-silenced

564 cells, and their respective densitometric analysis. Western blot and densitometric analyses of

565 hnRNP K **E**), and HtrA2 **F**). Mean values \pm SEM; * $p < 0.05$ vs controls (n=3). Control: cells treated
566 with a non-silencing oligonucleotide sequence; siTF: TF-silenced cells.

567

568 **Figure 4. Subcellular localisation of poly(A) RNA in TF-silenced cells.** Poly(A) RNA
569 localisation was visualised using a digoxigenin-labelled oligo-dT probe. The nucleus was stained
570 with DAPI (shown in blue). The image is representative of four independent experiments (scale
571 bar, 20 μ m). Control: cells treated with a non-silencing oligonucleotide sequence; siTF: TF-
572 silenced cells.

573

574 **Figure 5. Subcellular localisation of SRSF2 in TF-silenced cells.** SRSF2 was visualised using
575 an anti-SRSF2 antibody. The nucleus was stained with DAPI (shown in blue). The image is
576 representative of four independent experiments (scale bar, 10 μ m). Control: cells treated with a
577 non-silencing oligonucleotide sequence; siTF, TF-silenced cells.

578

579 **Figure 6. Pre-mRNA splicing assay in TF-silenced cells.** The test system **A**) for *in vivo* splicing
580 based on the reporter genes encoding β -galactosidase and luciferase, which were fused in-frame
581 via a recombinant fragment of adenovirus and human α s-tropomyosin genes. The recombinant
582 fragment contains three in-frame translation stop signals (XXX) in the intronic region. β -
583 galactosidase is generated in the absence of splicing, whereas an active fusion of β -galactosidase
584 and luciferase is generated after the splicing of the primary transcript (adapted from [19]). **B**)
585 The cells were transiently transfected with the reporter construct (pTN24) and the splicing
586 activator Tra2- α plasmid. The graph shows the percentage stimulation of splicing derived from
587 the luciferase/ β -galactosidase ratio. Mean values \pm SEM (n=7). * $p < 0.05$ vs controls; # $p < 0.05$ vs

588 controls transfected with pTN24 alone. Control: cells treated with a non-silencing oligonucleotide
589 sequence; siTF: TF-silenced cells.

590

591 **Figure 7. Effect of TF silencing on regulators of apoptosis.** **A)** Immunoblotting of
592 phosphorylated Akt in TF-silenced and control cardiomyocytic cells. **B)** Immunoblotting of
593 HtrA2 expression in cardiomyocytic cells treated with LY 294002 for four hours. **C)** MTT assay
594 in cardiomyocytic cells treated with LY 294002 for four hours. **D-F)** RT-PCR analysis of Bcl-x,
595 Survivin, and TRAIL in TF-silenced MDA-MB-231 cells. The densitometric analysis was made
596 using QuantityOne software (v 4.5.2, Bio-Rad Laboratories). GAPDH mRNA was used for
597 sample normalisation. The images are representative of three individual experiments. * $p < 0.01$ vs
598 controls (n=3). Control: cells treated with a non-silencing oligonucleotide sequence; siTF: TF-
599 silenced cells; - : vehicle-treated cells.

600 **Table 1.** Differentially expressed proteins in TF-silenced and control cells identified by LC-MS^E.

601 The data were obtained from three independent experiments performed in triplicate.

602

Accession ^a	Description	PLGS Score	Si-:SiTF ^b Log(e)Ratio	Si-:SiTF Log(e)StdDev	P value	CV ^c
Q8K310	Matrin-3	158.1	0.52	0.16	1	14.8%
Q64012	RNA-binding protein Raly	99	0.39	0.17	1	17.7%
Q8R081	Heterogeneous nuclear ribonucleoprotein L	225.5	0.27	0.06	1	9.1%
Q60668	Heterogeneous nuclear ribonucleoprotein D0	204	0.24	0.1	1	1.2%
Q501J6	Probable ATP- dependent RNA helicase DDX17	337.5	0.24	0.09	1	2.3%
Q8VIJ6	Splicing factor, proline- and glutamine-rich, PTB	227.2	0.23	0.11	1	5.5%
Q9Z1N5	Spliceosome RNA helicase Bat1, UAP56	218.4	0.21	0.08	1	10.3%
P62631	Elongation factor 1- alpha 2	309.6	0.19	0.08	1	4.1%
Q8VDW0	ATP-dependent RNA helicase DDX39	167.7	0.19	0.1	1	6.6%
Q9DBG6	Dolichyl-diphospho- oligosaccharide--protein glycosyltransferase 63 kDa subunit precursor	252.1	-0.21	0.11	0.02	7.0%
P99024	Tubulin beta-5 chain	450.3	-0.23	0.07	0	17.1%
Q01853	Transitional endoplasmic reticulum ATPase (TER ATPase)	432.0	-0.23	0.1	0	11.4%
Q922F4	Tubulin beta-6 chain	254.5	-0.23	0.14	0	9.5%
P68372	Tubulin beta-2C chain	352.5	-0.24	0.11	0	16.9%
P58771	Tropomyosin-1 alpha chain	277.1	-0.25	0.09	0	5.6%
P47738	Aldehyde dehydrogenase, mitochondrial precursor	321.5	-0.27	0.1	0	9.5%
P17182	Alpha-enolase	210.5	-0.28	0.13	0	20.4%
P05213	Tubulin alpha-2 chain	345	-0.34	0.09	0	20.2%
P05214	Tubulin alpha-3/alpha-7 chain	230.1	-0.34	0.09	0	15.4%
P68373	Tubulin alpha-6 chain	396.3	-0.34	0.1	0	18.3%
P68369	Tubulin alpha-1 chain	419.4	-0.35	0.12	0	16.5%

P68368	Tubulin alpha-4 chain	131.3	-0.43	0.1	0	26.2%
Q9JJZ2	Tubulin alpha-8 chain	182.6	-0.44	0.12	0	19.3%
<i>Control cells unique</i>						
P49312	Heterogeneous nuclear ribonucleoprotein A1	180.2	Control unique			
P63011	Ras-related protein Rab-3A	132.4	Control unique			
Q05920	Pyruvate carboxylase, mitochondrial precursor	333.1	Control unique			
Q921M3	Splicing factor 3B subunit 3	245.5	Control unique			
Q9QXY	Tight junction protein ZO-3	205.4	Control unique			
<i>TF-silenced cells unique</i>						
P17183	Gamma-enolase	127.3	TF-silenced unique			
P21550	Beta-enolase	116.7	TF-silenced unique			
Q9QVP4	Myosin regulatory light chain 2, atrial isoform	64	TF-silenced unique			
P06151	L-lactate dehydrogenase A chain	154.6	TF-silenced unique			
Q9ES82	Popeye domain-containing protein 2	85.8	TF-silenced unique			

^a Accession code in UniProt

^b Si- refers to control cells treated with a non-silencing oligonucleotide sequence. SiTF refers to TF-silenced cells.

603 ^c CV , coefficient of variation of the ratios in the three biological replicates