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

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ABSTRACT

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

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

SIGNIFICANCE

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

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

INTRODUCTION

Tissue factor (TF), a 47-kDa transmembrane cell-surface protein, is primarily known as the initiator of the coagulation cascade [1, 2] but it also has a wide variety of coagulation-independent functions: it is required for embryonic blood vessel formation [3], plays a role in tumour metastasis [4], and is involved in the maintenance of heart hemostasis and structure [5]. Fibroblasts and cardiomyocytes of the heart constitutively express high levels of TF, which is located in the sarcolemma of cardiomyocytes and the intercalated discs of myocardium, where it co-localises with cytoskeletal and adhesion proteins such as desmin and vinculin [6]. Furthermore, actin-binding protein 280 has been identified as a ligand for the TF cytoplasmic domain by two-hybrid screening [7]. These findings of co-localisation and interactions with structural cardiac proteins has led to the hypothesis that TF may also play a non-hemostatic role in the heart by maintaining the structural integrity and function of cardiac muscle [6, 7]. Furthermore, the pattern of TF expression in cardiomyocytes during human and murine heart development indicates that TF may act as a morphogenic factor in the developing heart [8].

TF is down-regulated in the myocardium of patients with dilated cardiomyopathy (DCM). Myocardial specimens from DCM hearts show that it has an altered cell localisation, being redistributed from the Z-bands to the perinuclear cytosol [9]. This further suggests that decreased TF expression and change in localisation may affect contractility and cell-to-cell contact stability, thus contributing to cardiac dysfunction in patients with DCM [9].

The *in vitro* and *in vivo* administration of lipopolysaccharide reduces TF mRNA and protein expression in cardiomyocytes, but not in lung, kidney or brain [6, 10, 11], which not only clearly demonstrates that TF is uniquely regulated in the heart, but also suggests that it has potentially unique functions in heart morphogenesis and the maintenance of cardiac muscle structure.

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

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

MATERIAL AND METHODS

Cell cultures

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

FCS (JRH Biosciences, Lenexa, KS, USA) and 100 µmol/L norepinephrine (Sigma-Aldrich, Milan, Italy) according to Prof. Claycomb's instructions [17].

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

MTT assay

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

RNA interference and cell transfection

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

Immunofluorescence analysis

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

In situ hybridisation

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

Dual-reporter cell-based splicing assay

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

Nucleosome immunoassay

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

Western blotting

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

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

RNA extraction and cDNA preparation

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

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

Real-time quantitative PCR (qRT-PCR) was carried out to detect TF mRNA, with 18S rRNA being used for sample normalisation. The primer sequences were: human TF sense: 5´-CCTTACCTGGAGACAAACCTCG-3'; human TF antisense: 5´-CCGTTCATCTTCTACGGTCACA-3'; human 18S sense: 5´-CGGCTACCACATCCAAGGAA-3´; human 18S antisense: 5´-CCTGTATTGTTATTTTTCGTCACTACCT-3´; mouse 18S sense: 5´-GTAACCCGTTGAACCCCATT-3´; mouse 18S antisense: 5´- CCATCCAATCGGTAGTAGCG-3´. The primer sequences used for mouse TF (QT00159789) came from Qiagen Inc. (Milan, Italy). A volume of 2.5 µL of cDNA was incubated in 25 µL IQ Supermix containing TF or 18S primers and

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

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

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

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

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

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

Cell proliferation assay

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

Label-free LC-MS^E analysis

The cell pellets were dissolved in 25 mmol/L NH_4HCO_3 containing 0.1% RapiGest (Waters Corporation, Milford, MA, USA) and digested as previously described [23]. The tryptic peptides were separated by means of nanoscale LC using a nanoACQUITY system coupled to a SYNAPT-MS, a hybrid Q-TOF mass spectrometer (Waters Corporation, Milford, MA, USA), for 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 (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

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

Data mining

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

Statistical analysis

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

RESULTS

Effects of TF silencing on HL-1 cardiomyocytic cells.

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

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

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

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

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

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

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

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

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

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

Effects of TF silencing on mRNA distribution and spliceosome morphology

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

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

Effects of TF silencing on pre-mRNA splicing activity

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

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

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

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

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

DISCUSSIONS

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

The dysregulation of splicing machinery caused by TF silencing is due to alterations in the expression of RNA helicases and splicing factors such as members of the hnRNP family.

We found that the expression of DEAD box UAP56 helicase was down-regulated in TF-silenced cells. UAP56 is an important factor for spliceosome assembly, mRNA export and translation, and it plays an important role in cell growth and proliferation [36-38]. DDX39, a paralogue of UAP56 that is expressed in a growth-regulated manner [39, 40], was also down-regulated after TF silencing. Another member of the helicase family that was significantly down-regulated by the silencing of TF was DEAD box RNA helicases p72 (DDX17), which plays important roles in multiple cell processes, including transcription, processing of miRNAs and pre-mRNAs and alternative splicing [41].

Five members of the hnRNP family were down-regulated by TF silencing: hnRNP C, A1, D0, L, 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.

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

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

Although our analysis is limited to few candidates, the effect of TF silencing on apoptosis-related 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 TNFα-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].

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

Our data also reveal for the first time that TF silencing up-regulates HtrA2 expression, and that blocking Akt signaling by means of the PI3K inhibitor LY294002 modulates HtrA2 in a similar manner, thus providing a further mechanism by which TF promotes cell survival through Akt. The serine protease HtrA2 leads to programmed cell death in both a caspase-dependent and caspase-independent manner. Its function closely relates to its protease activity, which is required for the cleavage of its substrates. However, the regulation of HtrA2 by a signalling molecule has not been previously documented. Yang *et al*. have demonstrated that the ectopic expression of

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

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

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

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

Nevertheless, the possibility that altered TF expression has an impact on overall splicing activity may help to explain its role in those diseases in which both TF and splicing activity are down-regulated, and opens new avenues for the investigations of splicing regulation by TF.

Although cardiomyopathies are only beginning to be explored in terms of splicing machinery, recent evidence suggests that RNA splicing is less efficient in humans with heart failure. Splicing changes have also been observed in hypertrophic myocardium with preserved systolic function, which suggests that they may occur before the onset of overt heart failure [77, 78].

It is worth noting that TF is down-regulated in the myocardium of patients with dilated cardiomyopathy [9], and that the deletion of the TF gene in mouse cardiomyocytes ($TF^{flox/flox/}$MLC2v-Cre) leads to significantly increased fibrosis [79].

The mechanisms underlying these phenomena have not been previously investigated, but our findings suggest that TF plays a role in controlling splicing machinery, although further studies are required to determine whether this mechanism is operative in cells other than cardiomyocytes.

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Conflict of interest

The authors declare that they have no competing financial interest.

REFERENCES

[1] Giesen PL, Rauch U, Bohrmann B, Kling D, Roque M, Fallon JT, et al. Blood-borne tissue factor: another view of thrombosis. Proceedings of the National Academy of Sciences of the United States of America. 1999;96:2311-5.

[2] Mackman N. The many faces of tissue factor. Journal of thrombosis and haemostasis : JTH. 2009;7 Suppl 1:136-9.

[3] Carmeliet P, Mackman N, Moons L, Luther T, Gressens P, Van Vlaenderen I, et al. Role of tissue factor in embryonic blood vessel development. Nature. 1996;383:73-5.

[4] Seto S, Onodera H, Kaido T, Yoshikawa A, Ishigami S, Arii S, et al. Tissue factor expression in human colorectal carcinoma: correlation with hepatic metastasis and impact on prognosis. Cancer. 2000;88:295-301.

[5] Eisenreich A, Rauch U. Regulation and differential role of the tissue factor isoforms in cardiovascular biology. Trends in cardiovascular medicine. 2010;20:199-203.

[6] Luther T, Dittert DD, Kotzsch M, Erlich J, Albrecht S, Mackman N, et al. Functional implications of tissue factor localization to cell-cell contacts in myocardium. The Journal of pathology. 2000;192:121-30.

[7] Ott I, Fischer EG, Miyagi Y, Mueller BM, Ruf W. A role for tissue factor in cell adhesion and migration mediated by interaction with actin-binding protein 280. The Journal of cell biology. 1998;140:1241-53.

[8] Luther T, Flossel C, Mackman N, Bierhaus A, Kasper M, Albrecht S, et al. Tissue factor expression during human and mouse development. The American journal of pathology. 1996;149:101-13.

[9] Szotowski B, Goldin-Lang P, Antoniak S, Bogdanov VY, Pathirana D, Pauschinger M, et al. Alterations in myocardial tissue factor expression and cellular localization in dilated cardiomyopathy. Journal of the American College of Cardiology. 2005;45:1081-9.

[10] Mackman N, Sawdey MS, Keeton MR, Loskutoff DJ. Murine tissue factor gene expression in vivo. Tissue and cell specificity and regulation by lipopolysaccharide. The American journal of pathology. 1993;143:76-84.

[11] Erlich J, Fearns C, Mathison J, Ulevitch RJ, Mackman N. Lipopolysaccharide induction of tissue factor expression in rabbits. Infection and immunity. 1999;67:2540-6.

[12] Bogdanov VY, Balasubramanian V, Hathcock J, Vele O, Lieb M, Nemerson Y. Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. Nature medicine. 2003;9:458-62.

[13] Boltzen U, Eisenreich A, Antoniak S, Weithaeuser A, Fechner H, Poller W, et al. Alternatively spliced tissue factor and full-length tissue factor protect cardiomyocytes against TNF-alpha-induced apoptosis. Journal of molecular and cellular cardiology. 2012;52:1056-65.

[14] Di Sanzo M, Gaspari M, Misaggi R, Romeo F, Falbo L, De Marco C, et al. H ferritin gene silencing in a human metastatic melanoma cell line: a proteomic analysis. Journal of proteome research. 2011;10:5444-53.

[15] Aguilar-Melero P, Prieto-Álamo MJ, Jurado J, Holmgren A, Pueyo C. Proteomics in HepG2 hepatocarcinoma cells with stably silenced expression of PRDX1. J Proteomics. 2013;79:161-71.

[16] Silva JC, Denny R, Dorschel C, Gorenstein MV, Li GZ, Richardson K, et al. Simultaneous qualitative and quantitative analysis of the Escherichia coli proteome: a sweet tale. Mol Cell Proteomics. 2006;5:589-607.
[17] White SM, Constantin PE, Claycomb WC. Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function. American journal of physiology Heart and circulatory physiology. 2004;286:H823-9.
[18] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods. 1983;65:55-63.
[19] Nasim MT, Eperon IC. A double-reporter splicing assay for determining splicing efficiency in mammalian cells. Nature protocols. 2006;1:1022-8.
[20] Banfi C, Brioschi M, Barbieri SS, Eligini S, Barcella S, Tremoli E, et al. Mitochondrial reactive oxygen species: a common pathway for PAR1- and PAR2-mediated tissue factor induction in human endothelial cells. Journal of thrombosis and haemostasis : JTH. 2009;7:206-16.
[21] Mahotka C, Wenzel M, Springer E, Gabbert HE, Gerharz CD. Survivin-deltaEx3 and survivin-2B: two novel splice variants of the apoptosis inhibitor survivin with different antiapoptotic properties. Cancer research. 1999;59:6097-102.
[22] Krieg A, Krieg T, Wenzel M, Schmitt M, Ramp U, Fang B, et al. TRAIL-beta and TRAIL-gamma: two novel splice variants of the human TNF-related apoptosis-inducing ligand (TRAIL) without apoptotic potential. British journal of cancer. 2003;88:918-27.
[23] Brioschi M, Lento S, Tremoli E, Banfi C. Proteomic analysis of endothelial cell secretome: A means of studying the pleiotropic effects of Hmg-CoA reductase inhibitors. Journal of proteomics. 2013;78:346-61.
[24] Brioschi M, Eligini S, Crisci M, Fiorelli S, Tremoli E, Colli S, et al. A mass spectrometry-based workflow for the proteomic analysis of in vitro cultured cell subsets isolated by means of laser capture microdissection. Analytical and bioanalytical chemistry. 2014;406:2817-25.
[25] Geromanos SJ, Vissers JP, Silva JC, Dorschel CA, Li GZ, Gorenstein MV, et al. The detection, correlation, and comparison of peptide precursor and product ions from data independent LC-MS with data dependant LC-MS/MS. Proteomics. 2009;9:1683-95.
[26] Li GZ, Vissers JP, Silva JC, Golick D, Gorenstein MV, Geromanos SJ. Database searching and accounting of multiplexed precursor and product ion spectra from the data independent analysis of simple and complex peptide mixtures. Proteomics. 2009;9:1696-719.
[27] Banfi C, Parolari A, Brioschi M, Barcella S, Loardi C, Centenaro C, et al. Proteomic analysis of plasma from patients undergoing coronary artery bypass grafting reveals a protease/antiprotease imbalance in favor of the serpin alpha1-antichymotrypsin. Journal of proteome research. 2010;9:2347-57.
[28] Colussi C, Banfi C, Brioschi M, Tremoli E, Straino S, Spallotta F, et al. Proteomic profile of differentially expressed plasma proteins from dystrophic mice and following suberoylanilide hydroxamic acid treatment. Proteomics Clinical applications. 2010;4:71-83.
[29] Maere S, Heymans K, Kuiper M. BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. Bioinformatics. 2005;21:3448-9.
[30] Lento S, Brioschi M, Barcella S, Nasim T, Ghilardi S, Tremoli E, Banfi C. Data for proteomic analysis of murine cardiomyocytic HL-1 cells treated with siRNA against Tissue Factor. Data in Brief. *submitted*
[31] Reed R, Hurt E. A conserved mRNA export machinery coupled to pre-mRNA splicing. Cell. 2002;108:523-31.

[32] Kaida D, Motoyoshi H, Tashiro E, Nojima T, Hagiwara M, Ishigami K, et al. Spliceostatin A targets SF3b and inhibits both splicing and nuclear retention of pre-mRNA. Nature chemical biology. 2007;3:576-83.
[33] O'Keefe RT, Mayeda A, Sadowski CL, Krainer AR, Spector DL. Disruption of pre-mRNA splicing in vivo results in reorganization of splicing factors. The Journal of cell biology. 1994;124:249-60.
[34] Gonzalez-Garcia M, Perez-Ballestero R, Ding L, Duan L, Boise LH, Thompson CB, et al. bcl-XL is the major bcl-x mRNA form expressed during murine development and its product localizes to mitochondria. Development. 1994;120:3033-42.
[35] Kramer A. The structure and function of proteins involved in mammalian pre-mRNA splicing. Annual review of biochemistry. 1996;65:367-409.
[36] Cordin O, Hahn D, Beggs JD. Structure, function and regulation of spliceosomal RNA helicases. Current opinion in cell biology. 2012;24:431-8.
[37] Shen H, Zheng X, Shen J, Zhang L, Zhao R, Green MR. Distinct activities of the DExD/H-box splicing factor hUAP56 facilitate stepwise assembly of the spliceosome. Genes & development. 2008;22:1796-803.
[38] Sahni A, Wang N, Alexis JD. UAP56 is an important regulator of protein synthesis and growth in cardiomyocytes. Biochemical and biophysical research communications. 2010;393:106-10.
[39] Pryor A, Tung L, Yang Z, Kapadia F, Chang TH, Johnson LF. Growth-regulated expression and G0-specific turnover of the mRNA that encodes URH49, a mammalian DExH/D box protein that is highly related to the mRNA export protein UAP56. Nucleic acids research. 2004;32:1857-65.
[40] Sugiura T, Sakurai K, Nagano Y. Intracellular characterization of DDX39, a novel growth-associated RNA helicase. Experimental cell research. 2007;313:782-90.
[41] Fuller-Pace FV, Moore HC. RNA helicases p68 and p72: multifunctional proteins with important implications for cancer development. Future Oncol. 2011;7:239-51.
[42] Dreyfuss G, Kim VN, Kataoka N. Messenger-RNA-binding proteins and the messages they carry. Nature reviews Molecular cell biology. 2002;3:195-205.
[43] Carpenter B, MacKay C, Alnabulsi A, MacKay M, Telfer C, Melvin WT, et al. The roles of heterogeneous nuclear ribonucleoproteins in tumour development and progression. Biochimica et biophysica acta. 2006;1765:85-100.
[44] Han SP, Tang YH, Smith R. Functional diversity of the hnRNPs: past, present and perspectives. The Biochemical journal. 2010;430:379-92.
[45] Gao R, Yu Y, Inoue A, Widodo N, Kaul SC, Wadhwa R. Heterogeneous nuclear ribonucleoprotein K (hnRNP-K) promotes tumor metastasis by induction of genes involved in extracellular matrix, cell movement, and angiogenesis. The Journal of biological chemistry. 2013;288:15046-56.
[46] Berson A, Barbash S, Shaltiel G, Goll Y, Hanin G, Greenberg DS, et al. Cholinergic-associated loss of hnRNP-A/B in Alzheimer's disease impairs cortical splicing and cognitive function in mice. EMBO molecular medicine. 2012;4:730-42.
[47] LaBranche H, Dupuis S, Ben-David Y, Bani MR, Wellinger RJ, Chabot B. Telomere elongation by hnRNP A1 and a derivative that interacts with telomeric repeats and telomerase. Nature genetics. 1998;19:199-202.
[48] Patry C, Bouchard L, Labrecque P, Gendron D, Lemieux B, Toutant J, et al. Small interfering RNA-mediated reduction in heterogeneous nuclear ribonucleoparticule A1/A2

proteins induces apoptosis in human cancer cells but not in normal mortal cell lines. Cancer research. 2003;63:7679-88.
[49] Fujiya M, Konishi H, Mohamed Kamel MK, Ueno N, Inaba Y, Moriichi K, et al. microRNA-18a induces apoptosis in colon cancer cells via the autophagolysosomal degradation of oncogenic heterogeneous nuclear ribonucleoprotein A1. Oncogene. 2014;33:4847-56.
[50] Salton M, Elkon R, Borodina T, Davydov A, Yaspo ML, Halperin E, et al. Matrin 3 binds and stabilizes mRNA. PloS one. 2011;6:e23882.
[51] Rymond B. Targeting the spliceosome. Nature chemical biology. 2007;3:533-5.
[52] Kotake Y, Sagane K, Owa T, Mimori-Kiyosue Y, Shimizu H, Uesugi M, et al. Splicing factor SF3b as a target of the antitumor natural product pladienolide. Nature chemical biology. 2007;3:570-5.
[53] Tanackovic G, Kramer A. Human splicing factor SF3a, but not SF1, is essential for pre-mRNA splicing in vivo. Molecular biology of the cell. 2005;16:1366-77.
[54] Wagner EJ, Garcia-Blanco MA. Polypyrimidine tract binding protein antagonizes exon definition. Molecular and cellular biology. 2001;21:3281-8.
[55] Izquierdo JM, Majos N, Bonnal S, Martinez C, Castelo R, Guigo R, et al. Regulation of Fas alternative splicing by antagonistic effects of TIA-1 and PTB on exon definition. Mol Cell. 2005;19:475-84.
[56] Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, et al. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell. 1993;74:597-608.
[57] Venables JP, Koh CS, Froehlich U, Lapointe E, Couture S, Inkel L, et al. Multiple and specific mRNA processing targets for the major human hnRNP proteins. Molecular and cellular biology. 2008;28:6033-43.
[58] Revil T, Pelletier J, Toutant J, Cloutier A, Chabot B. Heterogeneous nuclear ribonucleoprotein K represses the production of pro-apoptotic Bcl-xS splice isoform. The Journal of biological chemistry. 2009;284:21458-67.
[59] Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nature medicine. 1997;3:917-21.
[60] Jiang X, Guo YL, Bromberg ME. Formation of tissue factor-factor VIIa-factor Xa complex prevents apoptosis in human breast cancer cells. Thromb Haemost. 2006;96:196-201.
[61] Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity. 1995;3:673-82.
[62] Kocaturk B, Versteeg HH. Tissue factor-integrin interactions in cancer and thrombosis: every Jack has his Jill. Journal of thrombosis and haemostasis : JTH. 2013;11 Suppl 1:285-93.
[63] Aberg M, Siegbahn A. Tissue factor non-coagulant signaling - molecular mechanisms and biological consequences with a focus on cell migration and apoptosis. Journal of thrombosis and haemostasis : JTH. 2013;11:817-25.
[64] Zhao P, Meng Q, Liu LZ, You YP, Liu N, Jiang BH. Regulation of survivin by PI3K/Akt/p70S6K1 pathway. Biochemical and biophysical research communications. 2010;395:219-24.
[65] Bomsztyk K, Denisenko O, Ostrowski J. hnRNP K: one protein multiple processes. BioEssays : news and reviews in molecular, cellular and developmental biology. 2004;26:629-38.
[66] Naro C, Sette C. Phosphorylation-mediated regulation of alternative splicing in cancer. Int J Cell Biol. 2013;2013:151839.
[67] Lee G, Blenis J. Akt-ivation of RNA splicing. Mol Cell. 2014;53:519-20.

[68] Blaustein M, Pelisch F, Tanos T, Munoz MJ, Wengier D, Quadrana L, et al. Concerted regulation of nuclear and cytoplasmic activities of SR proteins by AKT. Nat Struct Mol Biol. 2005;12:1037-44.
[69] Vu NT, Park MA, Shultz JC, Goehe RW, Hoeferlin LA, Shultz MD, et al. hnRNP U enhances caspase-9 splicing and is modulated by AKT-dependent phosphorylation of hnRNP L. The Journal of biological chemistry. 2013;288:8575-84.
[70] Yadav AK, Vashishta V, Joshi N, Taneja P. AR-A 014418 Used against GSK3beta Downregulates Expression of hnRNPA1 and SF2/ASF Splicing Factors. J Oncol. 2014;2014:695325.
[71] Jang SW, Liu X, Fu H, Rees H, Yepes M, Levey A, et al. Interaction of Akt-phosphorylated SRPK2 with 14-3-3 mediates cell cycle and cell death in neurons. The Journal of biological chemistry. 2009;284:24512-25.
[72] Sanidas I, Polytarchou C, Hatziapostolou M, Ezell SA, Kottakis F, Hu L, et al. Phosphoproteomics screen reveals akt isoform-specific signals linking RNA processing to lung cancer. Mol Cell. 2014;53:577-90.
[73] Yang L, Sun M, Sun XM, Cheng GZ, Nicosia SV, Cheng JQ. Akt attenuation of the serine protease activity of HtrA2/Omi through phosphorylation of serine 212. The Journal of biological chemistry. 2007;282:10981-7.
[74] Sacco-Bubulya P, Spector DL. Disassembly of interchromatin granule clusters alters the coordination of transcription and pre-mRNA splicing. The Journal of cell biology. 2002;156:425-36.
[75] Chakraborty P, Satterly N, Fontoura BM. Nuclear export assays for poly(A) RNAs. Methods. 2006;39:363-9.
[76] Barabino SM, Keller W. Last but not least: regulated poly(A) tail formation. Cell. 1999;99:9-11.
[77] Kong SW, Hu YW, Ho JW, Ikeda S, Polster S, John R, et al. Heart failure-associated changes in RNA splicing of sarcomere genes. Circulation Cardiovascular genetics. 2010;3:138-46.
[78] Linke WA, Bucker S. King of hearts: a splicing factor rules cardiac proteins. Nature medicine. 2012;18:660-1.
[79] Pawlinski R, Tencati M, Holscher T, Pedersen B, Voet T, Tilley RE, et al. Role of cardiac myocyte tissue factor in heart hemostasis. Journal of thrombosis and haemostasis : JTH. 2007;5:1693-700.

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FIGURE LEGENDS

Figure 1. **TF silencing reduced cell growth and induced apoptosis in HL-1 cardiomyocytes.** **A**) TF mRNA analysed by qRT-PCR in control and TF siRNA-treated cells (n=10) *p<0.001 *vs* controls. **B**) BrdU incorporation assay of control and TF siRNA-treated cells (n=5) *p<0.01 *vs* controls. **C**) MTT assay of control and TF siRNA-treated cells (n=6) *p<0.01 *vs* controls. **D**) Apoptosis was determined by means of a nucleosome immunoassay. Mean values±SEM of seven independent experiments run in triplicate. *p<0.01 *vs* corresponding controls. Control: cells treated with a non-silencing oligonucleotide sequence; siTF: TF-silenced cells.

Figure 2. Differentially expressed proteins in TF-silenced cells identified by LC-MS^{E}. **A**) The data are expressed as the log(e) of the ratio of protein abundance in control and TF-silenced cells. The control cells' unique protein value was set at -2, and that of the TF-silenced cells at +2. The data were obtained from three independent experiments performed in triplicate. **B**) Western blot and densitometric analyses of PTB, hnRNP A1, and hnRNP L. Mean values±SEM; *p<0.05 *vs* controls (n=3). Control: cells treated with a non-silencing oligonucleotide sequence; siTF: TF-silenced cells.

Figure 3. Differentially expressed proteins in TF-silenced cells identified by 2-DE. Representative images of 2-DE gels from control **A**) and TF-silenced **B**) cells. The spots showing significant differential expression are indicated by circles (1, hnRNP K; 2, HtrA2). Representative images of 2-DE spots of hnRNP K **C**) and HtrA2 **D**) in control and TF-silenced cells, and their respective densitometric analysis. Western blot and densitometric analyses of

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

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

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

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

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

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