Proteomic Profiling of Ewing Sarcoma Reveals a Role for TRAF6 in Proliferation and Ribonucleoproteins/RNA Processing

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Abstract
Notwithstanding advances over the last decade in the comprehension of the molecular biology of Ewing sarcoma (ES), we still lack an understanding of critical issues, especially those regarding its genesis. In particular, little effort has been devoted to characterization of the proteic component of the mechanisms and pathways deployed by the activation of the fusion protein resulting from chromosomal translocation. We decided to investigate the proteic alterations of an ES cell line bearing a representative fusion protein. The combination of RNA interference of EWS-FLI1 in the ES cell line TC-71, a proteomic analysis by 2-D electrophoresis and subsequent mass-spectrometry identification, and a global overrepresentation study detected changes in more than 500 spots. Forty-three proteins were identified as being significantly differentially abundant. As expected, we found and validated changes in proteins linked to nucleotide processing, transcription regulation, ribonucleoproteins, helicases, cell-cycle control and proliferation, and metabolic processes. Additionally, TNF receptor-associated factor 6 was revealed as a hub node. Our strategy showed the potential to reveal the protein interplay associated with the known functions of the fusion protein: binding to DNA and RNA in order to act as aberrant transcription factors or potent repressors, or by altering RNA processing.

Keywords: Comparative proteomics; Ewing sarcoma; Molecular pathogenesis; Two-dimensional gel electrophoresis

Introduction
Ewing sarcoma (ES) is a high-grade malignancy in which approximately 75% of cases are localized at diagnosis, and 25% are initially metastatic [1,2]. Refractory and/or recurrent ES remains a clinical challenge because the disease’s resistance to therapy makes it difficult to achieve long-lasting results. There is an urgent need to improve cure rates for localized, metastatic, and recurrent disease while decreasing treatment-related morbidity. The general treatment paradigm for localized ES is relies on a multidisciplinary approach, coupling chemotherapy with cyclic combinations of locoregional management consisting of surgery with or without radiation treatment [3]. Different combinations of drugs involving doxorubicin, vincristine, cyclophosphamide, ifosfamide, etoposide, and dacarbazine have been employed in consecutive first-line trials, with the aim of tailoring therapies to the individual molecular profile of the patients. However, the current overall disease-free survival rate for metastatic disease is 25%. For long-term survivors of childhood ES (defined as patients that survived ≥ 5 years as of diagnosis) with localized disease the overall reported mortality is 25%. Disease recurrence/progression accounts for 60% of deaths. These data indicate the limitations of conventional cytotoxic chemotherapy and underscore the need for novel targeted therapies in ES, reflecting the current knowledge into the biology of the EWS-FLI1 protein in the initiation and progression of ES.

ES is characterized by the presence of specific balanced chromosomal translocations, the most frequent being t(11;22)(q24;q12), which invariably fuses the 5’ region of the Ewing sarcoma breakpoint region 1 locus (EWSR1) with the 3’ region of one of several members of the ETS family of transcription factors. The resulting fusion is translated into a chimeric protein linking the NH2-terminal domain of the RNA-binding protein EWS with the DNA-binding portion of a given ETS transcription factor, FLI1, in 90% of cases [5-8]. EWS-FLI1 acts as an aberrant transcription factor [9] both as a transcriptional activator and repressor of similarly sized sets of target proteins, including transcription, apoptotic, or signal transduction factors [10-12]. There is strong evidence for the oncogenic potential of EWS-ETS fusions [7]. In addition to the chimeric protein itself, the cellular context also contributes to the phenotype, as it has been extensively reported that the introduction of EWS-ETS fusions into different cell models resulted in diverse outcomes, ranging from the induction of cell-cycle arrest, or apoptosis, to dedifferentiation [13-17].

Keywords: Comparative proteomics; Ewing sarcoma; Molecular pathogenesis; Two-dimensional gel electrophoresis

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Although substantial evidence suggests that genetic abnormalities play a primary function in the development of tumors, there is also ample proof that aberrations in cancer can occur through changes in the phenotype that arise in the absence of alterations in the DNA sequence, and that many such phenotypic changes are directly related to proteins, some examples of which include splice variation, time-dependent changes in abundance levels, cellular location, turnover number, alterations in signaling pathways, interactions, post-translational modifications such as phosphorylation, glycosylation, acetylation, etc., and degradation processes; these processes are aberrantly regulated in many types of cancers and cannot be predicted by DNA sequencing or measurement of mRNA expression. In fact, it has become evident that there is considerable discrepancy between mRNA and protein translation [18]. Assuming the classical paradigm that proteins are the cell's ultimate tools, its de facto effectors, and given that the majority of treatments with antitumor drugs are directed against specific proteins, we considered the potential advantages of monitoring protein abundance in a global manner in an effort to gain a better understanding of the underlying biology of ES.

We developed a stable RNA interference model by knocking down EWS-FLI1 in the ES cell line TC-71 [19], so we can analyze in depth, using proteomic approaches, the alterations in the landscape of proteins influenced by the chimeric protein. By inquiring on the differences between specific experimental conditions and using highly sensitive techniques as the final readout, we were able to typify the differences in protein abundance that were associated with the expression/repression of EWS-FLI1. Using two-dimensional gel electrophoresis (2D-PAGE) and mass spectrometry (MS), we performed a comparative global protein abundance study of the TC-71 with shEWS-FLI1. Our shEWS-FLI1 model unveiled a primary dynamic play of proteins involved in different aspects of nucleotide processing, from changes in histone levels wrapping the chromatin in the nucleosome, DNA unwinding through helicases, RNA binding and splicing, RNA elongation from polymerases and aminoacyl tRNA ligases, to transcription initiation from transcription factors. All these modulations are suggestive of an internal cellular program of transcriptional misregulation in cancer. In addition, other changes in protein expression point to alterations in diverse metabolic pathways, which might be indicative of disruption of energetic fueling and consumption.

Materials and Methods

Cell lines and shRNAi interference

A-4537, A-673, CADO-ES, RD-ES, RM82, SK-ES-1, SK-N-MC, STA-ET-1, STA-ET-2.1, STA-ET-10, TC-32, TC-71, TTC-466, VH-64, and WE-68 ES cell lines, as well as MCF7 (breast) and PC-3 (prostate) cell lines were obtained and maintained as previously described [20]. Briefly, shRNAi sequences were designed to target the fusion point between EWS and FLI1, and the resulting insert was sequenced and then transfected into TC-71 cells, which were finally selected for positive clones. RNA interference was monitored by analyzing EWS-FLI1 protein and mRNA levels using Western blotting (WB) and qRT-PCR, respectively. Up to 40 clones were analyzed at both early and late stages (8 cellular passages later).

Two-dimensional gel electrophoresis

Cell culture plates were washed 3 times with 10 ml of ice-cold PBS. Next, lysates were obtained by scraping cells into 200 μl of ice-cold lysis buffer containing 8 M urea, 2 M thiourea, 4% (w/v) CHAPS and protease inhibitor cocktail (Roche). Proteins were precipitated with the 2-D Clean-Up Kit (GE Healthcare) to remove salts and impurities and were resuspended in lysis buffer. Total protein (150 μg, estimated using the 2-D Quant Kit (GE Healthcare)) was prepared in 350 μl of rehydration solution (8 M urea, 2% (w/v) CHAPS, 0.5% (w/v) DTT, 0.75% (v/v) IPG buffer and 0.002% bromophenol blue), and subsequently, IPG strips (18 cm, pH 3-11 non-linear Immobiline DryStrip gels, GE Healthcare) were rehydrated overnight with the sample/rehydration buffer mixture. Isoelectrofocusing for the first-dimensional separation was performed in an Etan IPGPhor 3 apparatus (GE Healthcare) for 50,000 Vh at high voltages and 20°C. Reduction and alkylation were accomplished by equilibration of the strips in buffer I containing 6 M urea, 2% SDS, 375 mM Tris-HCL (pH 8.8), 20% glycerol, and 2% (w/v) DTT, and buffer II containing 6 M urea, 2% SDS, 375 mM Tris-HCL (pH 8.8), 20% glycerol and 2.5% (w/v) iodoacetamide. Next, the gel strips were electrophoresed in vertical 12% SDS-PAGE gels for the second dimension, using a Protean II XL Cell (Bio-Rad), under a constant power of 17 W/gel.

Image analysis of 2-D protein spots

Silver staining (Silver Staining Kit, GE Healthcare) according to a modified protocol (without glutaraldehyde fixation solution) was used to detect protein spots and perform image analysis. Subsequently, synthetic gels were constructed, and the resultant protein patterns were qualitatively and quantitatively analyzed by Melanie 5 software (GeneBio). For subsequent MS analysis, preparative 2-D gels were run in parallel and stained with Sypro Ruby (Sigma-Aldrich), which does not interfere with the technique. All samples were run in sextuplicate.

MS analysis

All proteins were processed and analyzed for their identification in the Genomics and Proteomics Unit of the Centro de Investigación del Cáncer of Salamanca. Differentially abundant protein spots were manually excised from the gels, digested with trypsin, and processed for MALDI-ToF MS analysis in an AutoFlex system (Bruker Daltonics) in a positive ion reflector mode. The ion acceleration voltage was 20 kV. Each spectrum was internally calibrated with the masses of two trypsin autolysis products. For protein identification by matching peptide-mass fingerprinting, the tryptic peptide mass maps were transferred through MS BioTools’ program (Bruker Daltonics) as inputs to search the Swiss-Prot non-redundant human database (minimal score change cut-off was set to 54; p<0.05) using MASCOT software (Matrix Science). Fully-tryptic digestion with up to one missed tryptic cleavage were considered, a mass accuracy of 100 ppm, and 0.8 Da mass tolerances for precursor and product ions were used for all the tryptic-mass searches. Carbamidomethylation of cysteines, variable oxidation of methionine and N-terminal acetylation were considered. Peptides having MASCOT scores below a significant threshold (5%) were not considered for analysis. One percent false discovery rate using the Percolator was used for peptide validation [21]. Only proteins with at least two significant peptides were considered for analysis.

Bioinformatic analyses

We performed functional profiling of the proteomic data using the FatiGO tool from the Babelomics server [22]. Functional enrichment analysis of overrepresented ontology terms allowed us to categorize by Gene Ontology (GO) the molecular function, biological process, and cellular localization of the unique proteins identified in this study. Likewise, overrepresentation analysis of pathways was launched against the Biocarta, KEGG, and Reactome databases, in order to find the

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pathways of relevance in which the annotated proteins on our list were strongly enriched. Finally, we searched for enrichment in regulatory elements within our list of protein IDs, thus analyzing both miRNA databases in search of miRNA targets, and transcription factor binding-site databases (Jasper TFBS). In all the cases, only those terms showing FDR <0.05 were considered statistically significant.

**Gene expression analysis by quantitative real-time PCR**

Total RNA from cell lines was isolated using the RNasy FFPE kit (Qiagen). cDNA was produced using the Universal Transcriptor cDNA synthesis kit (Roche Diagnostics, Spain) according to the manufacturer’s recommendations for random hexamer primer. The levels of PA2G4, PP1B, PRDX6, and TRAF6 gene expression were determined using a quantitative real-time PCR assay using ATSP5e as a housekeeping gene. Primers were designed based on the sequences from GeneBank listed in Table S1. qPCR assays were performed using the LightCycler480 II system (Roche Applied Science, Switzerland) for 45 cycles with the sets of specific primers described in Table S1 (Figure S1). PA2G4, PP1B, PRDX6, and TRAF6 expression levels were normalized to the calibrator levels (IMR90 fibroblast human cell line) and compared to hMSC (mesenchymal stem cells) from healthy donors recruited from the Salamanca University Hospital.

**Immunohistochemistry (IHC)**

Sections (5 μm) from formalin-fixed, paraffin-embedded (FFPE) ES tissue samples were stained with hematoxylin and eosin. Representative malignant areas were carefully selected from the stained sections of each tumor, and 21-mm diameter tissue cores were obtained from each specimen. IHC was carried out on sequential tissue microarray sections by the Envision method (Dako, Denmark) using the following primary antibodies: PA2G4, PRDX6 and TRAF6. Three tissue microarrays, including 42 ES samples available from the HUVR Biobank, were used to validate biomarker abundance. Only those tumor cells evaluated as clearly stained were considered to be positive. The IHC results were evaluated by 2 pathologists (E.A. and D.M.), who scored the average abundance of markers in duplicate samples.

**WB analysis**

WB assays were performed as described earlier [20]. Protein abundance was determined using the following antibodies: anti-PA2G4 (Sigma-Aldrich HPA016484); anti-PRDX6 (Sigma-Aldrich HPA006983); anti-TRAF6 (Sigma-Aldrich HPA020599); anti-β-tubulin (Sigma-Aldrich T5293); anti-rabbit IgG, HRP, and anti-mouse IgG, HRP (Cell Signaling). Protein bands were visualized using the chemiluminescence detection kit Clarity Western ECL Substrate (Bio-Rad).

**Results and Discussion**

**Proteomic profiling of EWS-FLI1 knockdown cells**

EWS-ETS gene fusions are generally accepted as the major drivers of ES pathogenesis [23]. Given the function of the chimera protein as an aberrant transcription factor [9], the subsequent identification of EWS-FLI1 targets by proteomic approaches could be a key aspect in understanding the molecular behavior of ES. The alternative approach of inducing the ectopic expression of EWS-FLI1 in heterologous tumor cells did not always produce the desired effect, but rather in a wide variety of adverse effects, such as dedifferentiation or growth arrest [23]. So far, various proteomic approaches have been used to address biological issues in ES, including the study of disease mechanisms, molecular target identification, and biomarker development, though we are now only beginning to recognize their benefits [24-26].

In order to identify proteins associated with changes induced by EWS-FLI1 we compared the TC-71 cell line with a downregulated shEWS-FLI1 clone showing the most pronounced decrease in EWS-FLI1 expression [19]. Two-dimensional gel electrophoresis (2D-PAGE) analyses of 6 shEWS-FLI1 extracts and 6 control (pSUPER empty vector) from independent replicates were performed over a pH range of 3-11. Overall, images of 2-D gels showed similar distribution of proteins (Figure 1A). The digital analysis of the images detected changes in protein abundance levels for more than 500 spots in a virtual gel master. A pattern of more than 400 spots with identical positions was found on every gel replicate of each group. Forty-six spots were analyzed as being differentially abundant with significant changes of more than ± 1.8 folds (p<0.05). Of these spots, 43 proteins were successfully identified by MS, as summarized in Table 1. Among them, 25 proteins were overabundant in the shEWS-FLI1 model, whereas 14 proteins were highly abundant in the control cells.

**Dependency on DNA/RNA processing proteins is revealed by GO analysis**

The molecular functions and cellular localization of the proteins identified in the analysis was performed using GO as shown in Figure 2A. Interestingly, many of the identified proteins with the highest scores were molecules directly involved in DNA/RNA functioning and regulation, which makes sense in the context of the role played by the chimeric protein EWS-FLI1 in ES as an aberrant transcription factor. Next, we displayed the relative distribution of proteins in selected GO terms by their molecular function (Figure 2B). The first striking observation was the high number of proteins implicated in DNA and RNA processing. More than 15 molecular functions related to RNA processing were described for the proteins overrepresented in the shEWS-FLI1 dataset. In particular, there were at least 7 proteins bearing significant annotations in ribonucleoprotein complexes (nucleophosmin, different helicases, ribosomal proteins), and several proteins with helicase activity were described as altered in the shEWS-FLI1 model (DDX5, DDX39B, RUVBL2). The analysis also suggested that another 3 proteins were involved in DNA replication-associated processes (like proteins of proliferation, and proteasome components).

RUVBL2 is a component of both the RNA polymerase II holoenzyme complex (and therefore is involved in DNA recombination, DNA repair, and protein folding) and of the Nua4 histone acetyltransferase complex (involved in transcriptional activation of specific genes) [27]. When modified, it promote interaction of the histones H4 and H2A with transcription regulators, which will activate transcriptional programs associated with oncogene and proto-oncogene mediated growth induction, tumor suppressor mediated growth arrest and replicative senescence, apoptosis, and DNA repair. In addition, several histones (histone H2B type-1B, histone H2B type-1C, histone H2B type-2F, and histone H3.3) were differentially overabundant in the interfered model. This suggests that EWS-FLI1 may play a role in DNA accessibility and that ES cells exhibit an activated cell-dividing activity, according to its tumorigenic capability. Recent reports have furnished evidence of the role of the chimera in the regulation of epigenome and transcriptome rewiring [28].

Similarly to RNA helicase A, which has been already described in ES [29], we found helicases of the DEAD box protein family, as well as helicases DDX5 and DDX39B, all of which may stimulate the transcriptional activity of EWS-FLI1 target gene promoters by directly
Figure 1: A) Representative 2-DE map of the proteome obtained from TC-71 cell line with shRNAi interference for EWS-FLI1. Differentially abundant spots that were subsequently analyzed by MS are marked with numbered circles. B) 2-DE images for PA2G4, PPIB, PRDX6, and TRAF6 proteins. Arrowheads indicate spots that were excised for MS analysis. Numbers indicate spot numbers as in panel A. C) MALDI-ToF mass spectra of tryptic digests of PA2G4, PPIB, PRDX6, and TRAF6.

| Protein                                | UniProtKB/Swiss-Prot accession | Accession | Gene name | Spot no. | Abundance level variation | P value | Mass (kDa) | pI | MASCOT score |
|----------------------------------------|--------------------------------|-----------|-----------|----------|----------------------------|---------|------------|----|--------------|
| Translationally-controlled tumor protein | P13693                         | TCTP_HUMAN| TPT1      | 6        | ↑ 6.42                     | 0.040   | 19.7       | 4.8 | 81           |
| FSD1-like protein                      | Q98XM9                         | FSD1L_HUMAN| FSD1L     | 8        | ↑ 6.61                     | 0.009   | 17.0       | 5.9 | 89           |
| Creatine kinase B-type                 | P12277                         | KCRB_HUMAN| CKB       | 9        | ↑ 4.73                     | 0.005   | 42.9       | 5.4 | 115          |
| RuvB-like 2                           | Q9Y230                         | RUVB2_HUMAN| RUVBL2    | 11       | ↑ 2.01                     | 0.042   | 51.2       | 5.5 | 134          |
| Spliceosome RNA helicase DDX39B        | Q13838                         | DX39B_HUMAN| BAT1      | 11       | ↑ 2.01                     | 0.042   | 49.4       | 5.4 | 125          |
| Inorganic pyrophosphatase              | Q15181                         | IPYR_HUMAN | PPA1      | 14       | ↑ 7.12                     | 0.019   | 33.1       | 5.5 | 118          |
| TNF receptor-associated factor 6       | Q9Y4K3                         | TRAF6_HUMAN| TRAF6     | 17       | ↑ 5.70                     | 0.023   | 61.2       | 6.0 | 48           |
| Histone H2B type 1-B                  | P33778                         | H2B1B_HUMAN| HIST1H2BB | 18       | ↑ 5.22                     | 0.018   | 13.8       | 10.3| 132          |
| Tryptophan--tRNA ligase. cytoplasmic   | P23381                         | SYWC_HUMAN| WARS      | 19       | ↑ 2.30                     | 0.021   | 53.5       | 5.8 | 203          |
| Rab GDP dissociation inhibitor beta    | P50395                         | GDI2_HUMAN| GDI2      | 21       | ↑ 1.88                     | 0.029   | 51.1       | 6.1 | 89           |
| Dimethylamine monoxygenase 2           | Q99518                         | FMO2_HUMAN| FMO2      | 24       | ↑ 2.23                     | 0.044   | 61.4       | 6.9 | 49           |
| T-complex protein 1 subunit zeta       | P40227                         | TCPZ_HUMAN| CCT6A     | 26       | ↑ 6.34                     | 0.002   | 58.3       | 6.3 | 114          |
interacting with EWS-FLI1. In fact, a recent study has confirmed the relevance of EWS-FLI1 as a network hub that modulates systemic alternative splicing as an oncogenic process [30]. Similarly, hnRNP L, PCNA, and TAF70 are components of several nuclear complexes that play a central role in mediating promoter responses to various activators and repressors [31]. These factors show changes in their expression levels, and it is suspected that some of them bear activating or deactivating signals in their posttranslational modification patterns, as a result of EWS-FLI1 induction in ES cells [25]. Proteins related to the translational programme were also altered. This is the

Table 1: List of 43 unique proteins identified as differentially abundant in ES TC-71 cells, as a result of the inhibition of EWS-FLI1 fusion by shRNAi. They included proteins with at least 1.8-fold increased abundance levels in shEWS-FLI1 model and proteins with at least 1.8-fold decreased abundance levels. P values measure the significance of the difference between proteins from shEWS-FLI1 gels and proteins from control gels, calculated by an independent-samples T-test for each spot. Spot numbers correspond to those in Figure 1.

| Protein Name | Accession | Fold Change | Spot Number | Description |
|--------------|-----------|-------------|-------------|-------------|
| 3.5-Delta(2.4)-diensol-CoA isomerase mitochondrial | P17644 | DDX5_HUMAN | DDX5 | Probable ATP-dependent RNA helicase DDX5 |
| Peroxiredoxin-6 | P30041 | PRDX6_HUMAN | PRDX6 | Transcription initiation factor TFIIId subunit 6 |
| Histone H2B type 1-C/E/F/G/I | P62607 | H2B1C_HUMAN | HIST1H2BC | Fructose-bisphosphate aldolase A |
| Histone H3.3 | P84243 | H33_HUMAN | H3F3A | Prostate-specific antigen |
| Histone H2B type 2-F | Q50NW6 | H2B2F_HUMAN | HIST2H2BF | V-type proton ATPase isomerase A3 |
| Citrate synthase mitochondrial | Q75390 | CISY_HUMAN | CS | Peptidyl-prolyl cis-trans isomerase B |
| Probable ATP-dependent RNA helicase DDX5 | P23284 | PPIB_HUMAN | PPIB | Nucleophosmin |
| Proliferating cell nuclear antigen | P12004 | PCNA_HUMAN | PCNA | Proliferating cell nuclear antigen |
| Proteasome subunit alpha type-3 | P25788 | PSA3_HUMAN | PSMA3 | V-type protein ATPase subunit B. brain isoform |
| Protein disulfide-isomerase A3 | P30101 | PDA3_HUMAN | PDA3 | Protein disulfide-isomerase A3 |
| V-type proton ATPase subunit B. brain isoform | P21261 | VATB2_HUMAN | ATP6V1B2 | GOS acidic ribosomal protein P0 |
| 60S acidic ribosomal protein P0 | P05388 | RLA0_HUMAN | RPLP0 | T-complex protein 1 subunit beta |
| T-complex protein 1 subunit beta | P78371 | TCPB_HUMAN | CCT2 | Proliferation-associated protein 2G4 |
| Proliferation-associated protein 2G4 | Q9UQ80 | PA2G4_HUMAN | PA2G4 | Serine/threonine-protein kinase 2G4 |
| Proliferation-associated protein 2G4 | Q9UQ80 | PA2G4_HUMAN | PA2G4 | Serine/threonine-protein kinase 2G4 |
| Serine/threonine-protein phosphatase PP1-beta catalytic subunit | P62140 | PP1B_HUMAN | PPP1CB | Alpha-enolase |
| Alpha-enolase | P06733 | ENOA_HUMAN | ENO1 | Sepsin-5 |
| Sepsin-5 | Q97919 | SEPTS_HUMAN | SEPTS | S-formylglutathione hydrolase |
| Heterogeneous nuclear ribonucleoprotein L | P14866 | HNRPL_HUMAN | HNRPL | Heterogeneous nuclear ribonucleoprotein L |
| Phosphoserine aminotransferase | Q9Y617 | SERC_HUMAN | PSAT1 | Sorbitol dehydrogenase |
| Sorbitol dehydrogenase | Q0796 | DHSO_HUMAN | SORD | Voltage-dependent anion-selective channel protein 2 |
| Voltage-dependent anion-selective channel protein 2 | P45880 | VDAC2_HUMAN | VDAC2 | Fibroblast growth factor 2 |
| Fibroblast growth factor 2 | P09038 | FGFR2_HUMAN | FGFR2 | Fibroblast growth factor 2 |
A protein binding                    10%
ATP binding                          7%
nucleotide binding                 7%
helicase activity                     7%
ydrolase activity                  7%
DNA binding                          7%
identical protein binding        4%
isomerase activity                 4%
RNA binding                          3%
unfolded protein binding        3%
other molecular functions    42%

Figure 2: GO annotation of the proteins altered in our shEWS-FLI1 TC-71 cell line model, as listed in Table 1. A) Graphic network of the significantly enriched GO categories (FDR<0.05) for cellular components and molecular function. The gray-colored gradient is directly proportional to the significance, from white (non-significant) to black (most). B) Relative distribution of proteins in selected GO terms by their molecular function. C) Pathway analysis report showing the interactions between the proteins altered in TC-71 cells by shEWS-FLI1. A GeneMANIA-generated network seeded with the proteins specific to EWS-FLI1 knocking-down (Table 1), pointing out TRAF6 as a hub node. The predictions of GeneMANIA were based on co-expression, co-localization, genetic interactions, pathways, physical interactions, predicted interactions, and shared protein domains. Protein queries—selected from our analysis—are black-filled circles. Other network proteins are gray-filled circles. Black lines correspond to physical interactions, while grey lines represent pathways.
case of nucleophosmin, which has already been proposed as a candidate independent prognostic marker for ES patients, as identified by another proteomic approach [26]. Taken together, all these alterations reflect direct consequences of an aberrant transcriptional activity of the chimera protein.

Cell-cycle proteins involved in the growth of tumors are overabundant in ES cells

Another group of proteins altered in TC-71 cells are associated with cell cycle and cell division activities. One of these is PA2G4, whose abundance level is reduced in the shEWS-FLI1 silenced cells (Figure 1B). This result is consistent with the data from the gene expression microarray study of this clone (PA2G4 score value = -6.62) [19]. PA2G4 forms part of a ribonucleoprotein complex that acts as a negative regulator of transcription, and may be involved in regulation of rRNA processing, either directly or through an E2F switch. Additionally, PA2G4 may also play a role in erbB-3-regulated signal transduction pathway. Interestingly, erbB-3-expression levels have been reported to be typically upregulated in pediatric alveolar rhabdomyosarcoma [32]. Since ES proteins share the same N-terminal EWS domain, PA2G4 overabundance may reflect changes in erbB-3 levels and in its downstream transcriptional activation pathways. This point merits further confirmation.

Another protein involved in cell-cycle is PP1B, which is essential for cell division as it participates in the regulation of glycogen metabolism and protein synthesis. PP1B decreased its protein abundance levels in the EWS-FLI1 knockdown cells (Figure 1B), in agreement with previous reports that elucidate its role in malignant bone and soft tissue tumors [33]. From IHC analysis, it was proven that the percentage of cells stained positively with antiserum against PP1B was significantly higher in ES than in benign tumors. Furthermore, the malignant tumor lesions showed a markedly high number of cells in the S-phase fraction of the cell cycle, as compared to benign tumors. Taken together, these results suggest that PP1B is involved in the accelerated growth of malignant tumor cells.

Finally, PSA3, a component of the proteasome, and septin-5 are more abundant in TC-71 tumor cells. Differential levels of both proteins have been associated with several tumor types.

Changes in protein abundance related to metabolism reprogramming

The analysis of overrepresentation confirmed the previous findings and revealed a pattern of metabolic changes in the shEWS-FLI1 model. Since the mid-1950s, it has been well-known that cancer cells adjust their energy metabolism in order to fuel cell growth and division [34], and many genes have been related to this switch during recent decades. In our study, TC-71 cells exhibited abundant levels of alpha-enolase (ENO1). This is a multifunctional enzyme that is upregulated in cancers of the cartilage or bone marrow (among others), and that has been proposed as being of diagnostic and prognostic value [35]. Although some attention has been devoted to the role of metabolism in other pediatric sarcomas (rhabdomyosarcoma for instance) [36], this is one of the few studies reporting about metabolism in ES.

Other proteins in the EWS-FLI1-downregulated cells are engaged in protein binding, folding, rearranging of polytopic structures, or even modulation of p53-dependent functions (PP1B, PDA13, TCPB). High levels of these proteins may provide some clues about the molecular changes accompanying the redifferentiation program of cells after impairment of the fusion protein expression.

TRAF6 is a hub connecting multiple targets

Notably, many of the proteins listed in Table 1 are described in the overrepresentation analysis as showing direct physical interactions among each other. Among them, TRAF6 (TNF receptor-associated factor 6) appears as a hub node, highly connected to several other proteins on the list (Figure 2C). Given that TRAF-family proteins are associated with, and mediate, signal transduction from various signaling pathways, the high number and functional heterogeneity of the TRAF6 counterparts found in our analysis (isomerases, proteasome subunits, Rab GDP dissociation inhibitor, citrate synthase, etc.) are unsurprising. It also provides a link between the different signaling pathways, as it interacts with various protein kinases (IRAK1/IRAK, SRC, and PKCzeta). Similarly, it has been linked to the incorrect regulation of NF-kappaB as well as to alterations in JNK and p38 signaling pathways. In consequence, TRAF6 upregulation in the absence of EWS-FLI1 would fit in the context of regulatory changes in the abundance levels of signaling proteins leading to tumorigenic processes.

Finally, the search for enrichment in miRNA targets uncovered miR-363 as a potential regulatory candidate for 7 proteins within our list (FDR<0.05): 4 targets were upregulated in the control cells, whereas 3 proteins were increased in the shEWS-FLI1 cells. It is generally accepted that miR-363 functions in many cancer types as an oncimR. Recent studies have identified upregulation of miR-106a–363 cluster in ES, and provide support for a pro-oncogenic role of this microRNA cluster [37]. In numerous ES cell lines, however, and according to our own miRNA profiling research, miR-363 basal expression levels are usually low (including TC-71 cell line), and they barely change when the EWS-FLI1 protein fusion is silenced (unpublished data). In our proteomic study, the enrichment for miR-363 targets was significant (7/44 proteins), with 3 of the target proteins upregulated in the silenced cells and 4 in the TC-71 cells. It is clear that some other miRNA must be playing a role in the regulation of these protein alterations following EWS-FLI1 knocking-down. In consequence, from the integration of this whole body of data, we cannot provide conclusive information on the nature of the modulation of miR-363 in response to EWS-FLI1 knockdown in the TC-71 ES cell line.

Validation of abundance levels of selected candidates in ES cell lines

We selected 4 proteins from Table 1 for which abundance levels were analyzed in several ES cell lines as well as in samples from patients: TRAF6 (as a hub node connected to other proteins on the list); PRDX6 (participating in metabolic pathways); PP1B (accelerates the folding of proteins by binding to peptides and RNA polymerase, ultimately contributing to bone development); and PA2G4 (a RNA processing protein that interacts with erbB3). PA2G4 is more abundant in the TC-71 cell line, whereas PP1B, PRDX6, and TRAF6 increase their levels when the chimera protein is silenced.

We initially characterized the gene expression levels of these proteins by quantitatively analyzing their corresponding mRNA levels across a panel of 15 ES cell lines (Figure 3A). Data were normalized with respect to the levels of the 1MI90 fibroblast line, and hMSC (human mesenchymal stem cells) were used as controls for comparison of levels. qPCR results showed different trends in the patterns of expression for the assayed genes, i.e., more or less expressed in the ES cells than in the controls, although the results were homogeneous in the 4 scenarios: 85% of the cell lines showed similar mRNA levels for a given gene. PA2G4 levels were consistently elevated (FC=3.0) in the tumor cells as well as in the hMSC. On the other hand, PP1B levels were decreased in all the
Figure 3: Assessment of PA2G4, PPIB, PRDX6, and TRAF6 levels. A) mRNA expression levels for selected target genes were determined by qPCR analysis in 15 ES cell lines and in hMSC control line. Experiments were repeated three times. *: P<0.05. B) Total protein extract levels were assayed by WB for PA2G4, PRDX6, and TRAF6 abundance measurement in 11 ES cell lines. Two additional cell lines (MCF7, breast; PC-3, prostate) were included as controls. C) Representative images of ES cases and non-ES controls obtained by IHC with PA2G4, PRDX6, and TRAF6 antibodies in tissue microarray sections.
cell lines with respect to both the fibroblasts and the hMSC. Meanwhile, most of the cell lines displayed TRAF6 and PRDX6 expression levels similar to the controls. For PA2G4, PRDX6, and TRAF6, mRNA levels were 2-3 times higher in hMSC than in IMR90.

In general, protein abundance levels, as estimated by WB analysis, revealed a similar pattern. PA2G4 levels were homogeneous in a panel of 11 ES cell lines (plus 2 additional prostate and breast cancer lines, used as controls, Figure 3B), with moderately superior abundance levels as compared to the controls. PRDX6 protein levels varied from cell line to line with 2-3 fold differences, and all cases were between levels for prostate and breast cancer cells. TRAF6 was abundant in most ES cell lines, but apparently not in the controls. If we take a closer look at the TC-71 cell line, from which the shRNA model was constructed, we can observe that TRAF6 levels were low, in accordance with 2-D PAGE results (Figure 1B). Most of the other cell lines exhibited higher abundance levels for TRAF6.

ES tumor samples show low levels of selected proteins by IHC

PA2G4, PRDX6, and TRAF6 antibodies gave consistent signals in 3 different ES TMAs, for a total number of 42 ES samples (Figure 3C). A pattern of sample heterogeneity was visualized in the IHC analysis of the PA2G4 protein. The signal for PA2G4 was ubiquitously cytoplasmic, but the extent of abundance in tumor samples was unexpectedly low, with 2% of positive cases. On the contrary, tumor cells were not supposed to express either PRDX6 or TRAF6, and the IHC results confirmed that issue (5% of cases staining for PRDX6 and 0% for TRAF6). In both cases, staining was also cytoplasmic, as seen in the control samples (pancreas, epididymis, prostate, prostate carcinoma, breast, breast carcinoma). The signal for TRAF6 was also visualized in the surrounding inflammatory cells but not in the tumor cells.

Concluding Remarks

In spite of the significant progress made over recent years in describing the molecular biology of ES, it remains a largely unknown disease in many key points of its pathogenesis. A combination of EWS-FLI1 chimera silencing, 2-DE/mass spectrometry analysis, and ontology modeling have been employed to address this pathology. In summary, it has revealed that EWS-FLI1 modulates RNA processing and transcription regulation, and it might be involved in cell-cycle control and metabolism functioning. This study confirmed the value of proteomic approaches to uncover complex regulatory mechanisms unleashed by the aberrant behavior of a key transcription factor. Under this assumption, our work is a valuable example for gaining knowledge to the role of EWS-FLI1 as an epitome of fusions governing the pathogenesis of sarcomas.

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Author Contributions

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