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Comparative Proteomic Profiling of Unannotated Microproteins and Alternative Proteins in Human Cell Lines

Xiongwen Cao[1,2,4], **Alexandra Khitun**[1,2,4], **Zhenkun Na**[1,2], **Daniel G. Dumitrescu**[1], **Marcelina Kubica**[2,5], **Elizabeth Olatunji**[2,5], **Sarah A. Slavoff**[1,2,3,*]

[1]Department of Chemistry, Yale University, New Haven, Connecticut 06520, United States

[2]Chemical Biology Institute, Yale University, West Haven, Connecticut 06516, United

[3]Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06529, United States

[4]These authors contributed equally

[5]These authors contributed equally

Abstract

Ribosome profiling and mass spectrometry have revealed thousands of small and alternative open reading frames (sm/alt-ORFs) that are translated into polypeptides variously termed microproteins and alt-proteins in mammalian cells. Some micro-/alt-proteins exhibit stress-, cell type- and/or tissue-specific expression, and understanding this regulated expression will be critical to elucidating their functions. While differential translation has been inferred by ribosome profiling, quantitative mass spectrometry-based proteomics is needed for direct measurement of microprotein and alt-protein expression between samples and conditions. However, while label-free quantitative proteomics has been applied to detect stress-dependent expression of bacterial microproteins, this approach has not yet been demonstrated for analysis of differential expression of unannotated ORFs in the more complex human proteome. Here, we present global micro-/alt-protein quantitation in two human leukemia cell lines, K562 and MOLT4. We identify 12 unannotated proteins that are differentially expressed in these cell lines. The expression of six micro/alt-proteins was validated biochemically, and two were found to localize to the nucleus. Thus, we demonstrate that label-free comparative proteomics enables quantitation of micro-/alt-protein expression between human cell lines. We anticipate that this workflow will enable discovery of regulated sm/alt-ORF products across many biological conditions in human cells.

Graphical Abstract

*Correspondence: sarah.slavoff@yale.edu.

SUPPORTING INFORMATION

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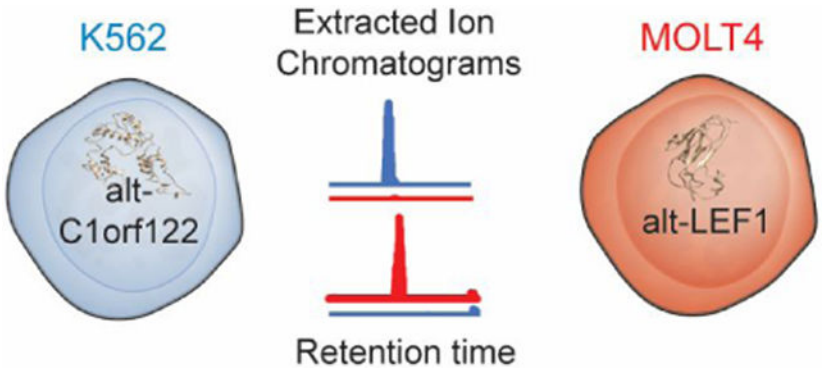

Keywords

comparative proteomics; microprotein; alternative protein; unannotated protein; proteogenomics

INTRODUCTION

Recent advances in genomic and proteomic technologies have revealed that mammalian genomes harbor thousands of previously unannotated small and alternative open reading frames (sm/alt-ORFs) which are <100 codons and >100 codons in length, respectively[1-6]. Sm/alt-ORFs encode polypeptide products that have been referred to as smORF-encoded polypeptides (SEPs), micropeptides, or microproteins (<100 amino acids), and alt-proteins (>100 amino acids); we will collectively refer to all unannotated polypeptides, regardless of length, as alt-proteins in this study to comport with recommended nomenclature[7]. These proteins were previously excluded from genome annotation due to their short size, initiation at non-AUG start codons, and overlap with other coding regions. A rapidly increasing number of sm/alt-ORFs have been shown to play important roles in mammalian biology[8]. For example, a recent genome-scale Cas9-based knockout screen revealed that 570 out of 2353 smORFs regulate growth of human induced pluripotent stem cells[9]. A number of sm/alt-ORF-encoded proteins have been characterized at the molecular level, recently including PIGBOS, which regulates the endoplasmic reticulum (ER) stress response[10], and BRAWNIN, which is essential for mitochondrial function[11]. These findings demonstrate that identification and characterization of sm/alt-ORFs can provide new biological insights.

Translation of thousands of mammalian sm/alt-ORFs has been predicted via ribosome profiling[12,13]. Mass spectrometry offers lower coverage, but provides more direct evidence for the presence of translated alt-proteins in cells[2]. Differential expression of some functional alt-proteins has been reported to occur under stress conditions, in disease, and during cell division[14,15]. For example, in humans, CASIMO1 is upregulated in breast tumors, and regulates proliferation of cancer cell lines[16]. In mouse, AW112010 is induced upon lipopolysaccharide (LPS) treatment, and is required for the innate immune response[17]. In bacteria, YmcF and YnfQ are small, cold shock-induced alt-proteins[18], while GndA is a heat shock-induced membrane alt-protein[19]. These bacterial stress-response microproteins were identified using a label-free quantitative proteomic approach. Notably, Saghatelian and colleagues have shown that mammalian sm/alt-ORFs exhibit cell- and tissue-specific

expression patterns[4]. Quantitative proteomic profiling of differential sm/alt-ORF expression is therefore needed to further identify stress-induced, and disease- and tissue-specific, functional mammalian alt-proteins. While quantitative proteomics has been applied to a human alt-protein during oxidative stress, differential expression was not observed[20], leaving open the question of whether this approach can quantify differences in human alt-proteins between samples.

In this report, we identify 16 and 15 sm/alt-ORF encoded alt-proteins, including microproteins, larger alt-proteins, N-terminal extensions, and an unannotated transcript variant, in K562 and MOLT4 cells, respectively. 28 exhibit differential expression, three are shared by these two cell lines and 12 are reported in this study for the first time. We confirmed differential expression of >30% of these genes with mRNA-seq, performed bioinformatic analysis and tested expression of six alt-proteins from overexpressed, epitope tagged cDNA constructs. Overall, we extend label-free quantitative proteomics to identify differentially expressed, unannotated alt-proteins in human cell lines.

MATERIALS AND METHODS

Cloning

For alt-CCNA2, alt-LEF1, alt-SLC1A5 and alt-SCRIB, a construct comprising the full 5'UTR of the annotated mRNA through the stop codon of each smORF was synthesized by Genscript with a FLAG epitope tag appended to the 3' end of each smORF coding sequence, and was then subcloned into pcDNA3. For ERVK3-1 and alt-C1orf122, a construct comprising the full annotated mRNA sequence was synthesized by Genscript with a FLAG epitope tag appended to the 3' end of each alt-ORF, and was then subcloned into pcDNA3.

Cell Lysis and Protein Size Selection

Cell pellets (2.5 x 10^6 K562 or MOLT4 cells) were resuspended with 200 μL standard SDS loading buffer, followed by heating for 10 min at 100°C until homogenous. 40 μL of the lysates were loaded into one Tricine gel well, and separated according to established protocol[21], followed by Coomassie blue staining and destaining. The gel bands corresponding to 2-5 kDa, 5-10 kDa and 10-15 kDa were excised for further LC-MS/MS analysis. Two biological replicates were performed for MOLT4, and one replicate for K562, which has been extensively profiled in the past[3,4,20,22].

Proteomics and Database Searches

Protein-containing gel slices were digested with 13.3 μg/mL trypsin (Promega) at 37°C for 14-16 h. The resulting peptide mixtures were extracted from the gel, dried, extracted with ethyl acetate to remove residual detergent, and then re-suspended in 15 μl of 3:8 70% formic acid:0.1% TFA. A 5 μL aliquot of each sample was injected onto a pre-packed column attached to a nanoAcquity UPLC (Waters) in-line with an LTQ Orbitrap Q Exactive (Thermo Scientific) and a 130-min gradient was used to further separate the peptide mixtures as follows (solvent A: 0.1% formic acid; solvent B: acetonitrile with 0.1% formic acid): Single pump trapping was turned on for 6 min at a flow rate of 2.5 μL/min at 99% A. Isocratic flow was maintained at 0.25 μL/min at 1% B for 40 min, followed by linear gradients from 1% B

to 6% B over 2 min, 6% B to 24% B over 58 min, 24% B to 48% B over 5 min, 48% B to 80% B over 5 min. Isocratic flow at 80% B was maintained for 5 min, followed by a gradient from 80% B to 1% B over 5 min, then maintained for 10 min. The full MS was collected over the mass range of 298-1,750 m/z with a resolution of 30,000. MS/MS data was collected using a top 10 high-collisional energy dissociation method in data-dependent mode with a normalized collision energy of 33.0 eV and a 2.0 m/z isolation window. The first mass was 100 m/z in fixed mode. MS/MS resolution was 7,500 and dynamic exclusion was 60 seconds.

For identification of alt- and microproteins, ProteoWizard MS Convert was used for peak picking and files were analyzed using Mascot (version 2.5.1). Oxidation of methionine and N-terminal acetylation were set as variable modifications. A mass deviation of 20 p.p.m. was set for MS1 peaks, with a peptide tolerance of 0.6 Da. A maximum of two missed cleavages were allowed. The false discovery rate (FDR) was set to 1% both on peptide and protein levels. The minimum required peptide length was five amino acids. Protein quantitation was accomplished via spectral counting, followed by comparing the MS1 extracted ion chromatograph (EIC) peak intensity in both cell lines using Xcalibur 4.0 (Thermo). As previously reported[3,23], peptide spectra were matched against a 3-frame translation of mRNA-seq from the corresponding cell line, permitting identification of both known and unannotated peptides. Annotated peptides were excluded with a string-matching algorithm via comparison to the human proteome. Because many sm/alt-ORFs are only identified by a single peptide-spectral match, putative unannotated peptide-spectral matches were manually examined for score and sequence coverage, as previously reported[18].

PepQuery (version 1.4.1)[24] was used to further filter candidate unannotated peptide-spectral matches identified by Mascot. Each spectrum was compared to a database of human proteins (UniProt) and common contaminants using unrestricted post-translational modification searching to exclude the possibility that any annotated, modified peptide would produce a higher-scoring spectrum match. Only peptides which were identified as the top-ranking match by PepQuery were retained.

mRNA-seq and Data Analysis

Whole RNA was isolated from 2.5 x 10^6 K562 or MOLT4 (n = 3) cells using Qiagen RNeasy Mini Kit spin columns, then treated in solution with DNase I prior to Qiagen column clean-up according to the manufacturer's protocol. Whole RNA was submitted to the Yale Center for Genomic Analysis for preparation according to the standard Illumina protocol for paired-end sequencing with enrichment of poly-A RNA. Samples were multiplexed and 75 bp fragments were sequenced on the Hiseq2500 sequencer. The reads were mapped to the human genome (hg38) using TopHat (v2.1.1). To identify the genes differentially expressed between these two cell lines, we counted the RNA reads in exons and calculated the reads per kilobase per million reads (RPKM) for each gene as a measure of expression using Cufflinks (v.2.2.2) with the Cuffdiff tool using default parameters and a $\log_2$ (fold change) cutoff of 0.5 with p value ≤ 0.05.

Cell Culture and Transfection

All cell lines were purchased from ATCC and early-passage stocks were established in order to ensure cell line identity. Cells were maintained up to only 10 passages. K562 and MOLT4 cells were maintained at a density of 1-10 × 10^5 cells/mL in RPMI 1640 medium (Gibco, 11875101) with 10% FBS (Sigma, F0392) and 1% penicillin-streptomycin (VWR, 97063-708). HEK 293T cells were cultured in DMEM (Corning, 10-013-CV) with 10% FBS (Sigma, F0392) and 1% penicillin-streptomycin (VWR, 97063-708) in a 5% CO_2 atmosphere at 37°C. Plasmid transfection was performed with Lipofectamine 2000 and Opti-MEM (GIBCO, 31985-070) according to the manufacturer's instructions, or polyethyleneimine (PEI, Polysciences, 23966-1) according to established protocol[25].

Data Availability.—The mRNA-seq data have been deposited in the NCBI Gene Expression Omnibus under accession GSE148451.

The mass spectrometry proteomics data have been deposited to the PRIDE Archive (http://www.ebi.ac.uk/pride/archive/) via the PRIDE partner repository with the data set identifier PXD018565 and 10.6019/PXD018565.

RESULTS

Comparative Proteomics Reveals Differential Expression of Unannotated Alt-Protein in Two Human Cell Lines

Previously, we reported a label-free quantitation protocol for comparative profiling of unannotated polypeptides between two conditions in bacteria[18,19]. As shown in Figure 1A, we aimed to extend this quantitative proteomic workflow to human polypeptides using two human leukemia cell lines, K562 and MOLT4, as a model. We hypothesized that, since both K562 and MOLT4 are leukemia-derived cell lines, they would exhibit similar expression levels of some alt-proteins; however, since they arise from different types of leukemia (chronic myelogenous leukemia and acute lymphoblastic leukemia, respectively) and different progenitor cells (bone marrow and T lymphoblast, respectively)[26,27], we reasoned that a subset of alt-proteins would be differentially expressed. Our approach is based on a previously reported method to qualitatively enrich and identify unannotated peptides[23]. Briefly, after gel-based protein size selection, tryptic digest and LC-MS/MS analysis, all MS/MS spectra are matched to a 3-frame translated RNA-seq database to identify both annotated and unannotated peptides and proteins (Table S1). Subsequently, unannotated peptides are filtered using a string-matching algorithm, and MS/MS spectra are inspected using stringent criteria to remove false positives, as previously reported[23]. In this work, we additionally utilized PepQuery[24] to further analyze any putative smORFs identified by only one peptide-spectral match (Table S2), a common challenge in peptidomics[3]. The final list of unannotated peptides that passed all filters is provided in Table S3. Finally, differential expression of unannotated peptides identified in only one sample (or non-differential expression of unannotated peptides detected in both) was validated by comparing the MS_1 extracted ion chromatograph (EIC) peak intensity in both samples, as previously reported[18-20].

Prior to analysis of unannotated sequences, we first validated our workflow and proteomic data quality by analyzing the numbers and sizes of annotated proteins identified from searching the raw spectral data against the UniProt human database. 2,285 and 2,579 annotated proteins were identified in the 2-15 kDa size range from K562 and MOLT4 cells respectively, and a clear enrichment of small proteins was observed (Figure 1B), similar to the number of identifications and size distributions in reported LC-MS/MS proteomics studies of smORFs[4,23]. Detections of proteins larger than the excised gel band have been previously reported, and may be due to proteolysis during cell lysis[20,23].

Subsequently analyzing unannotated sequences, we identified peptides mapping to 16 and 15 alt- proteins in K562 and MOLT4 cell lines, respectively. Among alt-proteins identified in this study, 3 are detected in both cell lines, while the rest are detected in only K562 or MOLT4, suggesting differential expression of these sm/alt-ORF candidates (Figure 1C and Tables S1, S2, and S3). Considered together, the 28 sm/alt-ORFs that encode these proteins fall into six categories (Figure 1D): 1) upstream ORFs (uORFs) located in the 5' UTR; 2) downstream ORFs in the 3' UTR; 3) N-terminal extensions of known protein coding sequences from alternative translation initiation sites[12]; 4) ORFs within “noncoding” RNAs (ncRNAs); 5) ORFs that overlap annotated protein coding sequences in a different reading frame (CDS) and 6) one peptide mapping to an unannotated protein isoform derived from a novel transcript variant, which is supported by reads in our mRNA-seq data (Figure S1). 68% of the 28 alt-proteins presumptively initiate with a non-AUG start codon (Figure 1E). These features are consistent with prior smORF proteomics studies[3,4,28], further validating our workflow. Of the 28 detected alt-proteins, 12 (42.9%) are unannotated and reported in this study for the first time; 5 (17.8%) are predicted in the UniProt database without protein-level evidence; and 11 (39.3%) have been detected and/or recently reported[3,4,20,28-30] (Figure 1F and Table S3). While only a single replicate of K562 cells was analyzed, the alt-protein identifications in this work were compared to previous studies which have extensively analyzed K562 alt-proteins; 6 out of the 16 alt-proteins detected in this study in K562 cells were reported before in this cell line (Table S3)[3,4,29].

Confirmation of Alt-Protein Differential Expression

We validated label-free quantitation of six selected alt-proteins detected only in K562, only in MOLT4, or in both cell lines, by comparing the area under the MS_1 peak in the extracted ion chromatogram (EIC) for each of these peptides. As shown in Figure 2 and Figure S2, the EIC comparisons revealed that peptides derived from alt-C1orf122 (Figure 2A) were present in K562 cells only, and peptides derived from alt-LEF1 (Figure 2B), alt-CCNA2 (Figure S2A) and alt-SLC1A5 (Figure S2B) were present in MOLT4 cells only, consistent with spectral counting results (Tables S1 and S3). In contrast, peptides derived from ERVK3-1 (Figure 2C) and alt-SCRIB (Figure S2C) were present in both cell lines, again consistent with the spectral counting results (Tables S1 and S3).

To orthogonally confirm alt-protein differential expression, we compared the levels of transcripts encoding them in these two cell lines. As shown in Figure 3A, the results of mRNA-seq revealed that, globally, 4,135 genes are upregulated ($\log_2$(Fold change) ≥0.5, *p-value* ≤ 0.05), and 3,664 genes are downregulated ($\log_2$(Fold change) ≤ −0.5, *p-value* ≤ 0.05)

in K562 cells compared with MOLT4 cells. We then focused on transcript encoding the alt-proteins of interest. Figure 3B illustrates enrichment of sm/alt-ORF-encoding transcripts in either K562 or MOLT4 cells. For 42% of detected alt-proteins that map to annotated genes, RNA-level quantitation is consistent with the quantitative proteomics analysis, similar to moderate RNA-protein correlation observed in prior studies[31]. This analysis is therefore partially consistent with our proteomics results, suggesting our proteomics workflow can quantitate differential alt-protein expression.

Overexpression Analysis of Alt-Proteins

The six alt-proteins for which MS_1 quantitation was performed above represent a mix of K562-specific, MOLT4-specific, and ubiquitous expression. While alt-CCNA2, alt-SCRIB, ERVK3-1 and alt-C1orf122 have been previously reported in proteomics studies[3,20,29], alt-LEF1 and alt-SLC1A5 are reported for the first time in this work, and none, except alt-CCNA2[3], have been previously validated to express at the molecular level. Therefore, we generated constructs containing the full-length cDNA (or cDNA sequence from the 5'UTR through the stop codon of the alt-ORF) with a FLAG tag appended to the C-terminus of each putative alt-ORF. These constructs were overexpressed in HEK 293T cells and visualized with immunofluorescence. As shown in Figure 4, immunostaining revealed that all six constructs produce FLAG signal, indicating all six alt-ORFs can be translated from cDNA in cells. Alt-C1orf122 and alt-LEF1 are primarily nuclear. We note that confirmation that the endogenous alt-ORFs are expressed requires genomic tagging or specific antibodies, so these identifications must be regarded as putative.

To confirm the immunostaining results, we performed Western blotting. As shown in Figure 5, ERVK3-1, alt-LEF1, alt-C1orf122 and alt-SCRIB were robustly detected. We did not see clear Western blot bands for alt-CCNA2 and alt-SLC1A5, although they were detected via immunostaining, suggesting their expression levels may be low. ERVK3-1 produces two bands, which may be caused by phosphorylation[32], proteolytic processing or other post-translational modifications.

Alt-ORF Start Codon Prediction

Next, we identified the start codon used by the six alt-ORFs by searching the upstream, in-frame DNA sequences relative to the detected tryptic peptides for ATG or near-cognate start codons in strong Kozak consensus sequences. As shown in Figure S3, we found that ERVK3-1 and alt-SCRIB contain an upstream, in-frame ATG start codon, $A_{205}TG$ and $A_{326}TG$, respectively (numbered relative to the first nucleotide of the cDNA), and are predicted to encode 109 amino acid (aa) and 120 aa proteins, respectively. Alt-C1orf122, alt-LEF1, alt-CCNA2 and alt-SLC1A5 contain near-cognate start codons in a strong Kozak sequence context: $A_{138}CG$, $A_{40}GG$, $C_{135}TG$ and $A_{301}AG$, respectively, and may respectively encode 317 aa, 141 aa, 115 aa and 61 aa proteins. The predicted protein sizes are generally consistent with the observed molecular weights (MW) in Western blot analysis (Figure 5), supporting these start codon assignments. The observed MW of alt-LEF1 is 20 kDa, while the predicted MW is 15 kDa, which we hypothesize may be explained by the basic amino acid composition of alt-LEF1 (PI = 10.61); other similarly basic alt-proteins such as NBDY (PI = 9.51) and MRI-2 (PI = 9.30) also show aberrantly low mobility in SDS-

PAGE gels[33,34]. Deletion analysis of the alt-LEF1 predicted start codon will be required to confirm its assignment.

Alt-Protein Bioinformatic Analysis

Previous studies have suggested that many sm/alt-ORF-encoded proteins contain signal peptides and/or localize to organelles[3,11]. To determine whether any of our 28 alt-proteins contain signal peptides, mitochondrial transfer peptides, or subcellular localization motifs like nuclear localization sequences (NLS), we performed bioinformatics analyses with TargetP 2.0[35] and cNLS-mapper[36,37]. These analyses revealed that CHTOP (N-terminal extension) is predicted to contain a mitochondrial transfer peptide (Figure S4A), which we hypothesize may change the localization of the annotated CHTOP protein from nuclear to mitochondrial. Previous reports of localization signal-encoding N-terminal extensions, for example FGF2 initiation at either an upstream CUG start codon or a downstream AUG to produce two protein isoforms with distinct localizations and interactions[38,39], provide precedent for this observation. Additionally, SF3A1 N-terminal extension is predicted to contain a signal peptide (Figures S4B and Table S4). Furthermore, three alt-proteins (alt-C1orf122, alt-LEF1 and A0A499FIZ0) are predicted to encode NLS sequences (Figure S5), consistent with nuclear immunofluorescence for two of these candidates upon overexpression (Figures 4C and 4D). These results suggest that differentially expressed alt-proteins may be imported into the secretory pathway or organelles.

Establishing conservation of small open reading frames throughout evolution remains a challenge due to poor annotation of orthologs across species and limited sequence lengths[40]. To determine whether any of the six selected alt-proteins are conserved, *CCNA2, LEF1, SLC1A5, C1ORF122* and *SCRIB* mRNA sequences from different species were obtained from NCBI nucleotide database, then translated in the +1, +2 and +3 reading frames using the ExPASy translate tool. ATG or near-cognate start codons in strong Kozak consensus sequences in frame with sequences homologous to human alt-CCNA2, alt-LEF1, alt-SLC1A5, alt-C1orf122 or alt-SCRIB were identified in the 5'UTR of each transcript in order to predict the full-length sequence of hypothetical homologs. ClustalW alignment of these hypothetical homologs against human alt-CCNA2 (Figure S6A), alt-LEF1 (Figure S6B), alt-SLC1A5 (Figure S6C), alt-C1orf122 (Figure S6D), and alt-SCRIB (Figure S6E), revealed significant sequence similarity, though protein-level existence for these hypothetical homologs does not currently exist. Finally, both alt-C1orf122 and alt-LEF1 are predicted to be structured using I-TASSER[41] (Figure S7). The observations that alt-proteins contain localization signals, exhibit conservation and may be structured permit hypothesis generation about their possible functions.

DISCUSSION

In this study, we applied a workflow for comparative proteomics to quantify expression of 28 unannotated alt-proteins in two human leukemia cell lines. The observed differential expression can be partially confirmed by mRNA-seq. It is worth noting that (1) mRNA levels are poorly correlated with protein levels in general due to post-transcriptional regulation[31], and (2) mRNA-seq quantifies the annotated genes taking all transcript variants

into account, while alt-proteins may be encoded by a specific transcript isoform[34], so mRNA-seq is not expected to perfectly correlate with alt-protein expression levels. For six selected alt-proteins, we confirmed via MS_1 quantitation that two are expressed in both cell lines, while the other four are specifically expressed in either K562 or MOLT4 cell lines, suggesting that leukemia-derived cells can have substantially different alt-protein expression profiles. Taken together, these results show that this workflow enables comparison of the expression levels of alt-proteins in human cell lines.

Several of the alt-proteins examined in this study are conserved in mammals or primates. While this degree of conservation is limited, similar clade-specific alt-proteins have previously been shown to be functional. For example, alt-proteins specific to mammals, such as NBDY and Minion, have been shown to impact biological processes such as RNA decapping and cellular fusion[33,42], and Storz and colleagues have noted similarly limited conservation of functional smORFs in bacteria[40].

Interestingly, alt-C1orf122, a K562-specific alt-protein, and alt-LEF1, a MOLT4-specific alt-protein, both localize primarily to the nucleus and may fold into tertiary structures. Therefore, the differentially expressed proteins identified in this study, especially those exhibiting specific subcellular localization, may be candidates for follow-up functional characterization. In the future, we anticipate that this method can be extended to profiling of unannotated human alt-proteins expressed under different conditions, including stress or disease states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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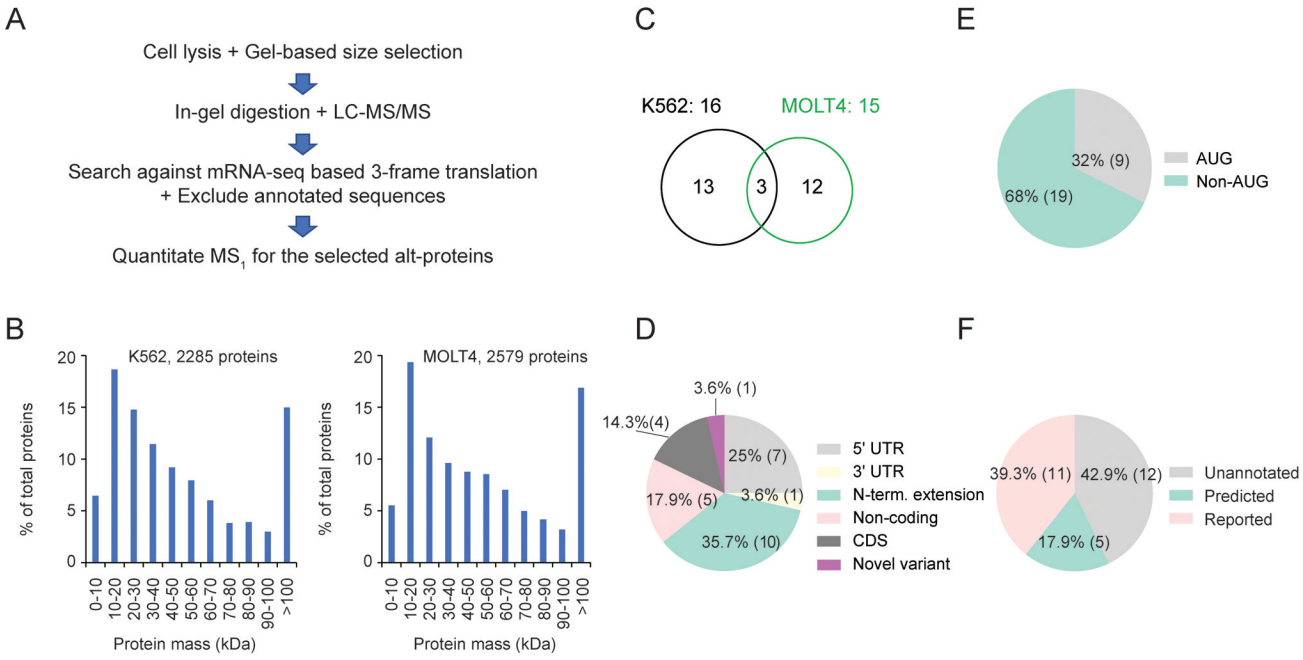

Figure 1.

Comparative proteomics reveals differential expression of unannotated alt-proteins in two human cell lines. (A) Schematic overview of the quantitative proteomics protocol. (B) Size distribution of annotated proteins identified in K562 (left) and MOLT4 (right) cells. (C) Venn diagram of alt-proteins identified in K562 (left) or MOLT4 (right) cells. (D) Distribution of locations of the detected alt-proteins relative to the annotated coding sequence (CDS). (E) Frequency of cognate (AUG) vs near-cognate (non-AUG) predicted start codons used by the detected alt-ORFs. (F) Annotation status of the detected alt-proteins: unannotated, predicted in UniProt database without protein level evidence (predicted), and recently published (reported).

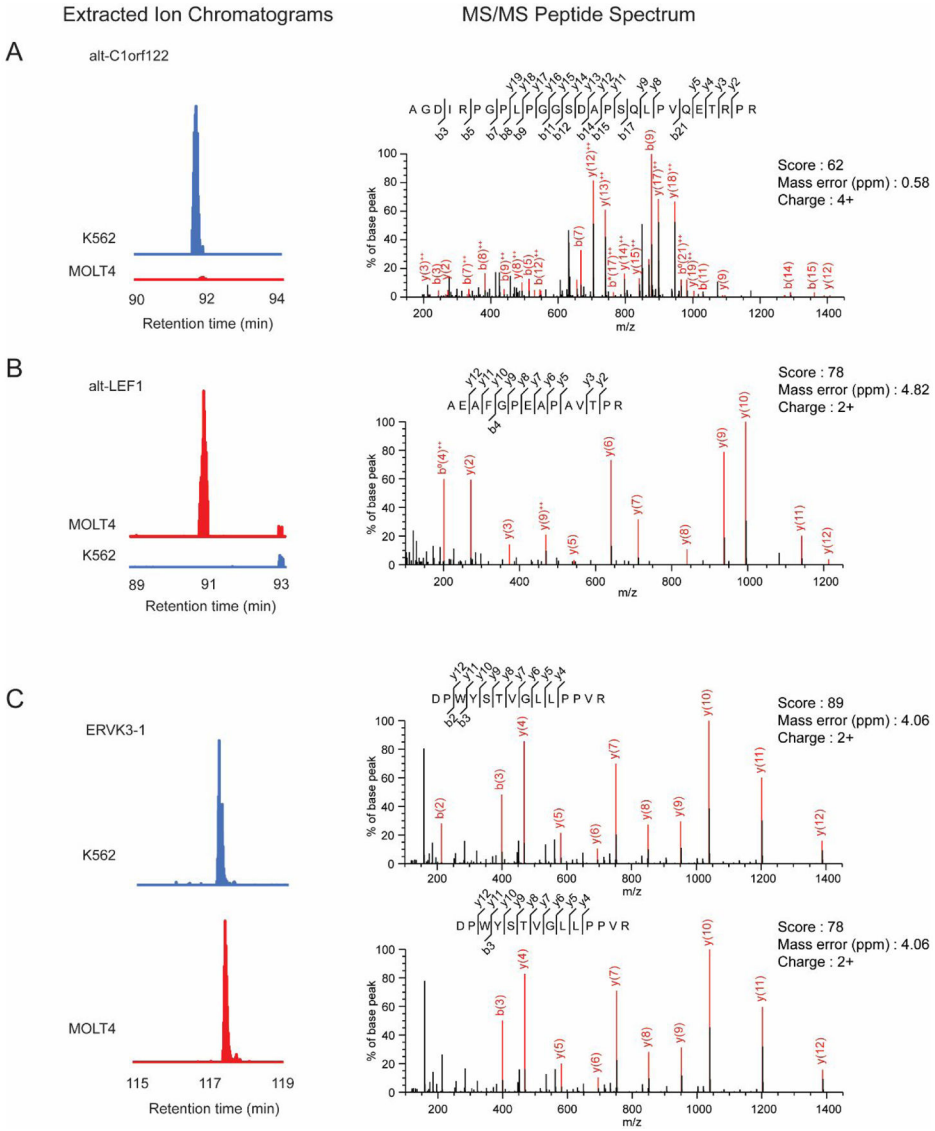

Figure 2.

Confirmation of differential expression of three detected alt-proteins with extracted ion chromatograms (EICs). Shown are EICs (left) from MS_1 spectra corresponding to MS/MS spectra (right) of three tryptic peptides identified in K562 cells only (A), MOLT4 cells only (B) or both cells (C). The same y-axis scale is used for each matched EIC pair. The EIC intensity at the same retention time was compared for the paired samples.

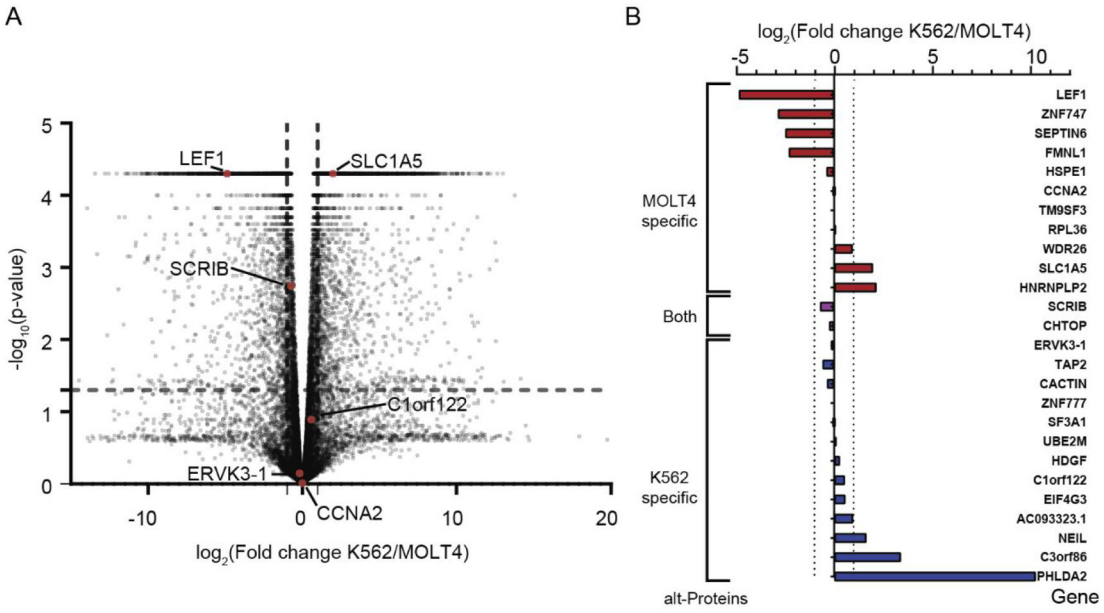

Figure 3.

Differential expression of the detected alt-ORFs at the mRNA level. (A) Global volcano plot of mRNA-seq from K562 and MOLT4 cells. Transcripts corresponding to the six alt-ORFs selected for further confirmation are indicated in red and gene names are labeled. (B) Differential expression of transcripts encoding the detected alt-ORFs in K562 and MOLT4 cells. Genes corresponding to MOLT4- and K562-specific alt-proteins are represented in red, and blue, respectively. Genes corresponding to alt-proteins detected in both cell lines are shown in purple. Gene names are indicated to the right.

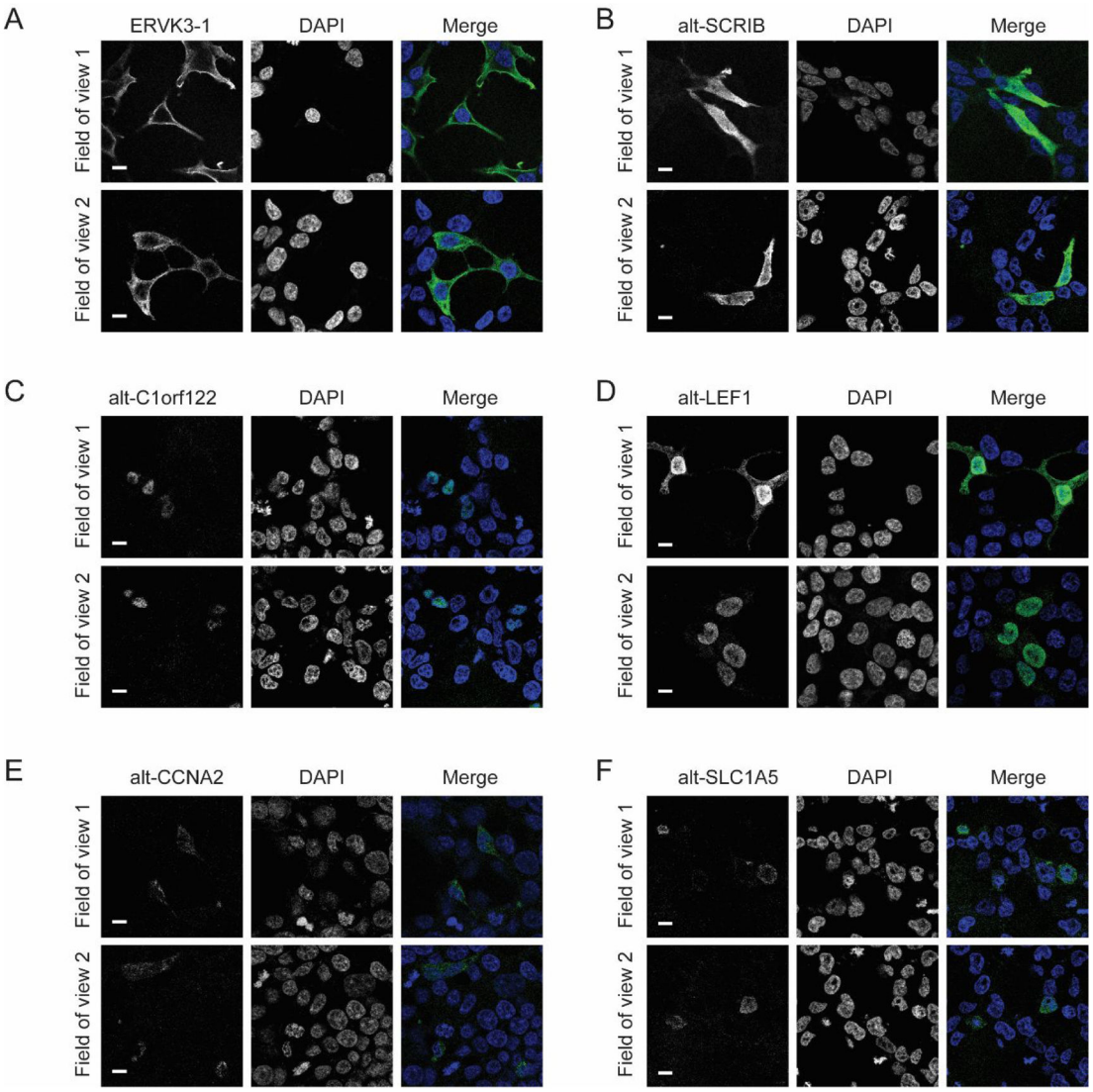

Figure 4.

Confirmation of the expression of six detected alt-proteins with immunostaining. HEK 293T cells were transfected with a construct containing the full 5'UTR or transcript and the indicated alt-protein coding sequence with a FLAG tag appended to the C-terminus, followed by immunostaining with anti-FLAG (left) and DAPI (center). Scale bar 10 μm. (A-B) show two proteins, ERVK3-1 and alt-SCRIB, detected in both K562 and MOLT4 cells, (C) alt-C1orf122, detected in K562 only, and (D-F) three alt-proteins, alt-LEF1, alt-CCNA2, and alt-SLC1A5, which were detected in MOLT4 only.

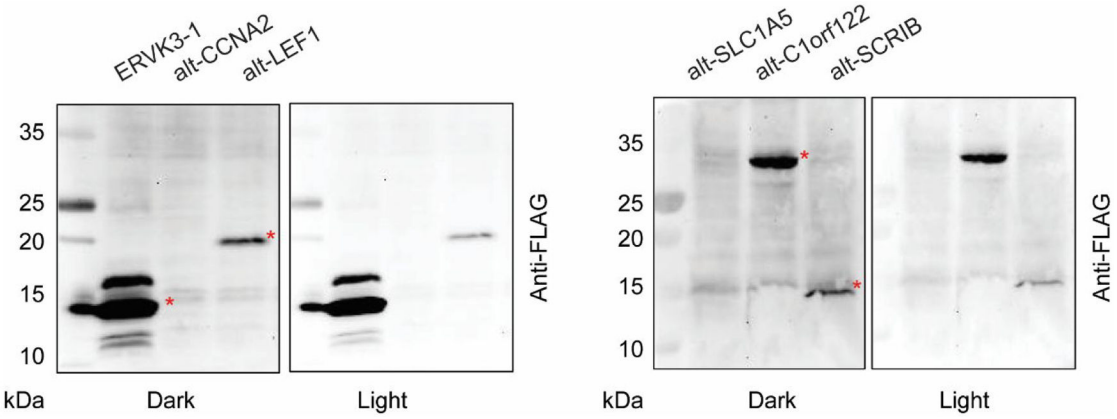

Figure 5.

Confirmation of alt-protein expression with Western blotting. HEK 293T cells were transfected with a construct containing the full 5'UTR or transcript and the indicated alt-protein coding sequence with a FLAG tag appended to the C-terminus, followed by Western blotting with anti-FLAG antibody. The bands indicated by a red asterisk correspond to ERVK3-1, alt-LEF1, alt-C1orf122 and alt-SCRIB.