Quantitative proteomics reveals key roles for post-transcriptional gene regulation in the molecular pathology of FSHD.

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Abstract

DUX4 is a transcription factor whose misexpression in skeletal muscle causes facioscapulohumeral muscular dystrophy (FSHD). While DUX4's transcriptional activity has been extensively characterized, the DUX4-induced proteome remains undescribed. Here, we report concurrent measurement of RNA and protein levels in DUX4-expressing cells via RNA-seq and quantitative mass spectrometry. DUX4 transcriptional targets were robustly translated, confirming the likely clinical relevance of proposed FSHD biomarkers. However, a multitude of mRNAs and proteins exhibited discordant expression changes upon DUX4 expression. Our dataset revealed unexpected proteomic, but not transcriptomic, dysregulation of diverse molecular pathways, including Golgi apparatus fragmentation, as well as extensive post-transcriptional buffering of stress response genes. Key components of RNA degradation machinery, including UPF1, UPF3B, and XRN1, exhibited suppressed protein, but not mRNA, levels, explaining the build-up of aberrant RNAs that characterizes DUX4-expressing cells. Our results provide a resource for the FSHD community and illustrate the importance of post-transcriptional process to DUX4-induced pathology.

Introduction

Facioscapulohumeral muscular dystrophy is caused by the inappropriate expression of an early embryonic transcriptional activator, DUX4, in adult muscle, leading to cell death (1, 2). Decades of work have generated a detailed parts-list of the genes and pathways affected by DUX4 that may underlie FSHD pathophysiology (3-10). Yet, an integrated model for how those DUX4-induced changes lead to disease has remained elusive (11-13). Considering that transient and pulsatile expression of DUX4 is sufficient induce pathology and cell death (14), it is particularly important that we understand the cellular events and pathways set in motion by DUX4 that lead to eventual cell death in order to develop effective therapeutics for FSHD.

DUX4 induces changes in the expression of hundreds of genes that impact dozens of highly interconnected pathways (3-10), making a cause-effect relationship between the measured gene expression and the observed pathology difficult to discern. Given that DUX4 is a strong transcription factor, most studies on DUX4 activity have focused on measuring gene expression at the transcript-level (3, 14, 15), making the assumption that the transcriptome is an accurate representation of the cellular proteome in DUX4-expressing cells. While this is a reasonable assumption, it is well known that RNA and protein levels are not always concordant and post-transcriptional regulation can cause them to significantly diverge (16). The few early proteomics studies that exist were conducted on FSHD muscle biopsies and lack the depth necessary to draw meaningful comparisons with the DUX4 transcriptome (17-19). Given our recent discovery that DUX4 induces proteolysis of a key RNA binding protein, UPF1 (7), we hypothesized that post-transcriptional gene regulation could be an important factor to consider in understanding DUX4 biology. Hence, we set out to generate reliable protein-level measurements of DUX4-induced gene expression and thereby elucidate the extent of post-transcriptional gene regulation in DUX4-expressing cells.

64 Using SILAC-based quantitative mass spectrometry (20), we measured the fold-change
65 at the protein level for ~4000 genes upon DUX4 expression, with high confidence. Comparing the
66 transcript fold-change to the protein fold-change shows three distinct patterns of expression for
67 different subsets of genes: 1) Concordant change in expression at the RNA and protein level for
68 a number of direct DUX4 transcriptional targets; 2) Post-transcriptional buffering of the expression
69 of a large number of genes, especially of those involved in stress response; and 3) Discordant
70 gene expression change at the RNA versus protein level that included genes involved in RNA
71 surveillance. Together, these findings highlight the importance of considering the expressed
72 proteome to fully understand DUX4 biology and the FSHD disease process.

Results

Determining protein fold-change in DUX4-expressing cells via quantitative mass spectrometry.

In order to measure DUX4-induced changes to the cellular proteome, we conducted SILAC-based mass spectrometry in two independent DUX4 expression systems (**Figure 1A).** We have previously shown that DUX4 expressed via a lentiviral vector versus an inducible transgene integrated into the genome of a myoblast cell line both yield comparable gene expression profiles (21). Here, we use both of the expression systems to provide a stringent biological replicate for our proteomic analysis.

In a pilot-scale proteomics experiment, human muscle cells that were adapted to light or heavy SILAC media for 3 weeks were transduced with lentivirus carrying DUX4 (vDUX4) or GFP (vGFP) expression constructs and samples were collected 24 and 36 hours post-transduction. In an independent experiment, cells carrying a doxycycline-inducible DUX4 transgene (iDUX4; (21)) were adapted to SILAC media for 3 weeks and DUX4 expression was induced with 1µg/ml of doxycycline for 14 hours in two replicates carrying heavy and light SILAC labels. Paired controls with no treatment were also collected with both heavy and light labels. Total protein from cells expressing DUX4 were mixed with an equal amount of total protein from cells without DUX4 expression containing the opposite SILAC label to generate samples that were then subjected to mass spectrometry.

Peptide-spectrum matches (PSMs) with quantified heavy to light ratios were subject to thorough screening for quality (e.g., filtering out single-peak spectra and spectra without unique mapping; see *Materials & Methods* for further details; Supplemental Data Set 1). Peptides mapping to a DUX4 target gene, ZSCAN4, from both vDUX4 and iDUX4 datasets showed highly skewed $\log_2$ (DUX4/Control) ratio that showed significant upregulation of the proteins upon DUX4

97 expression (**Figure 1B-C**). In contrast, plotting the $\log_2$ (DUX4/Control) ratio of all individual
98 peptides mapping to a housekeeping gene RPL15 showed that the ratio is centered around zero
99 (**Figure 1B-C**), as would be expected for a gene with no differential expression upon DUX4
100 induction. These example plots illustrate the strong agreement between the expected and
101 observed protein fold-change values determined by SILAC mass spectrometry. Moreover, out of
102 the 65 genes identified by Yao *et al* (22) as potential FSHD biomarkers based on transcriptome
103 analysis of FSHD patient samples, 8 were quantified in the vDUX4 proteomics study and 25 were
104 quantified in the iDUX4 proteomics study and both show high induction at the protein level (**Figure**
105 **1D, E**). Note that the lower number of peptides (and hence quantified proteins) from the vDUX4
106 sample indicates the lower depth of this dataset, and yet yields fold-changes that are highly
107 consistent with the iDUX4 dataset with a higher depth.

108

109 **Assessing concordance of RNA and protein expression fold-change.**

110 Next, using the iDUX4 dataset, we performed peptide to protein summarization via median
111 heavy/light ratios of all the peptides mapping to a certain protein in both replicates to obtain gene-
112 level $\log_2$ (DUX4/Control) ratios (**Figure S1**; Supplemental Data Set 2). After filtering out genes
113 that were only observed in one of the two label-swap replicates, we obtained quantitative
114 proteomics information for 4005 genes, 3961 of which also had a corresponding RNA-seq
115 measurement (**Figure 2A;** RNA-seq data previously reported in (21)). The lower number of genes
116 quantified via proteomics compared to RNA-seq is expected as proteomics is known to have lower
117 sensitivity compared to RNA-seq.

118 To qualitatively compare the RNA and protein expression level changes upon DUX4
119 expression, we assessed the overlap of genes with an expression change of 4-fold or above.
120 Among genes that are upregulated (> 2 log2 fold change), the concordance between RNA and
121 protein was roughly 40-50%, whereas similarly downregulated genes show very little concordance

122 (**Figure 2A**). To obtain a more quantitative measure of concordance, we generated a scatter plot
123 of the RNA versus protein fold-change for the 3961 genes (**Figure 2B**). We found a reasonable
124 level of correlation between these values with a Pearson's correlation coefficient, r, of 0.51 (p-
125 value < 2.2e-16).

126 To assess whether similar pathways were affected at the RNA versus protein level, we
127 conducted gene ontology analysis for genes up- or down-regulated at the RNA and protein
128 (**Figure 2C**). Surprisingly, we observed that the pathways that are affected at the RNA versus
129 protein levels are quite distinct. While the transcript level changes occur in genes involved in
130 transcription and mRNA processing, protein-level changes impact pathways including humoral
131 immune response, proteolysis and exocytosis. As the exocytosis pathway has not been implicated
132 in any of the previous DUX4 gene expression studies, we sought to examine this phenomenon
133 further by imaging the Golgi apparatus, which is the source of exocytotic vesicles in the cell (23).
134 We found that DUX4 expressing cells showed severe fragmentation of Golgi apparatus, which
135 could be an indicator of a perturbation in the cellular secretory pathways (24) (**Figure 2D;**
136 quantified in **Figure 2E**).

137 Taken together, these results demonstrate that analyzing the protein measurements may
138 give us insights that are not discernable in the transcriptome fold-change analysis performed in
139 earlier studies.

140

141 **Post-transcriptional buffering of stress response genes may exacerbate DUX4 toxicity.**

142 Though many of the genes induced at the transcript level are largely also induced at the
143 protein-level, a subset of genes showed no change in their protein level despite their transcripts
144 were up- or down-regulated to a significant degree (678 genes, shaded blue in **Figure 3A**),
145 indicating post-transcriptional buffering of the protein levels. Most notably, several housekeeping
146 genes that respond to protein folding stress or dsRNA-induced stress showed transcriptional
147 upregulation with minimal protein-level upregulation (**Figure 3B**).

148 Given that both unfolded protein and dsRNA induced stresses converge in
149 phosphorylation of eIF2α and lead to translation inhibition (25), we asked if the timing of
150 transcription of various stress-response genes coincide with translational downregulation. We
151 found that HSPA5, a prominent marker of the unfolded protein response pathway (26), shows
152 transcriptional upregulation at a time period that temporally coincides with eIF2α phosphorylation
153 and reduced incorporation of S35-labeled methionine, a proxy for bulk translation efficiency
154 (**Figure 3 C-E**). These data demonstrate that translation inhibition caused by various cellular
155 stresses and the resulting post-transcriptional buffering prevents DUX4-expressing cells from
156 mounting a robust stress response.

157

158 **Post-transcriptional modulation of RNA quality control pathway by DUX4.**

159 Next, we focused our analysis on the subset of genes that showed significant changes at
160 the protein level with either no change in their transcript abundance or a change in the opposite
161 direction (198 genes shown as 'gold' circles in **Figure 4A**). Pathway analysis did not reveal any
162 significant trends among these genes. So instead, we decided to focus on one of the pathways
163 that we have previously shown to be post-transcriptionally modulated – namely, the nonsense-
164 mediated RNA decay (NMD) pathway (7).

165 A diagram showing RNA- vs protein-level changes to various components of this pathway
166 demonstrates substantial post-transcriptional regulation in this pathway (**Figure 4B**). Many of
167 these genes, including UPF1, UPF2, UPF3B and XRN1, showed downregulation at the protein
168 level. The downregulation of XRN1 is of particular interest as it is the 5'-3' exonuclease that
169 degrades NMD targets upon cleavage by the endonuclease, SMG6 (27). Moreover, SMG6 too is
170 downregulated to a log2 fold change of -4.7, though it is only detected via a single peptide and
171 hence was filtered out of our analysis. Thus, DUX4-induced NMD inhibition appears to be a result

172 of post-transcriptional downregulation of multiple key players of the NMD pathway, which explains
173 the severity of NMD inhibition in DUX4-expressing cells.

174 Post-transcriptional downregulation of a gene can be achieved via two means: reduced
175 translation or increased protein degradation. We have previously shown that DUX4 induces
176 proteasome-mediated degradation of UPF1 (7). Hence, we asked if DUX4 affects known
177 components and regulators of the ubiquitin proteasome. A scatterplot of all ubiquitin proteasome
178 regulators shows a change in the expression of several such genes, one or more of which may
179 underlie the rapid degradation of UPF1 (**Figure 4C**). Further studies are needed to understand
180 the precise molecular mechanism behind this regulatory pathway and its downstream
181 consequences. In summary, we propose that the post-transcriptional gene regulation plays a
182 critical role in inhibiting NMD and perturbing the proteostasis in DUX4-expressing cells and may
183 thus underlie key aspects of FSHD pathology (**Figure 4D**).

184 Finally, in order to enable researchers and patients in the FSHD community to access the
185 data generated in this study, we developed a web tool for easy visualization of these data
186 (Screenshot shown in **Figure S2**). This tool can be freely accessed at the following URL:
187 https://dynamicrna.shinyapps.io/dataviz/.
188
189
190

191 **Discussion**

192 Most of the highly induced DUX4 transcriptional targets are germline and early embryonic
193 genes that are normally never expressed in adult muscle (3). So, it is possible that despite being
194 expressed at the transcript level, such genes may be translated poorly and/or be degraded rapidly
195 upon translation due to the lack of cell-type chaperones or other factors. Here, we show using
196 quantitative mass spectrometry that DUX4-induced transcripts are efficiently translated to
197 produce stable proteins in the muscle cell. This is an important confirmation for the altered identity
198 of DUX4-expressing cells, and also provides validation to pursue some of these proteins as
199 potential FSHD biomarkers (22).

200 Next, we asked if the changes in the DUX4 proteome are largely reflective of the changes
201 to the transcriptome. We find that this is not the case. While there is a positive correlation between
202 these measurements (Pearson's correlation coefficient 0.51), hundreds of genes deviate from this
203 trend. GO analysis of the most differentially expressed genes at the transcript versus protein level
204 shows splicing and RNA processing as being the prominent categories impacted at the transcript
205 level while protein-level changes impact an entirely different set of pathways. We take these
206 results as indication that the transcriptome analysis does not paint a complete picture of DUX4
207 biology and needs to be complemented with proteome analysis to develop a more thorough
208 understanding of how DUX4 misexpression causes FSHD.

209 We next pursued the various mechanisms by which protein-level changes deviate from
210 the corresponding transcriptomic changes. We found that genes induced by dsRNA and unfolded
211 protein stress are transcriptionally induced, but translationally buffered as a result of the
212 translation repression that accompanies these stress-response pathways. As a result, we
213 postulate that the DUX4-expressing cells are unable to mount a robust stress response, despite
214 inducing the transcripts necessary to alleviate stress. We also show that multiple proteins in the
215 RNA surveillance pathway, including UPF1 and XRN1, are downregulated at the protein level,

216 which may explain the drastic reduction in RNA quality control capacity in DUX4-expressing cells.
217 From the proteomics data, we have identified a number of genes involved in the ubiquitin
218 proteasome pathway that could impact the stability of proteins, which should serve as a starting
219 point for further investigation into this novel regulatory mechanism of RNA surveillance in DUX4-
220 expressing cells.

221 **Materials and Methods**

222 **Accession codes.** Mass spectrometry proteomics data have been deposited to the
223 ProteomeXchange Consortium via the PRIDE partner repository (Vizcaino et al., 2013) with the
224 dataset identifier PXD010221 (Reviewer account details: Username: reviewer37110@ebi.ac.uk;
225 Password: aMrE4BW0). The RNA-seq data are available through the NCBI SRA database under
226 accession number GSE85461 (21).

227

228 **Cell culture and SILAC labeling.** Proliferating human myoblasts were cultured in F10 medium
229 (Gibco/Life Technologies) supplemented with 20% fetal bovine serum (Thermo Scientific), 10ng
230 bFGF (Life Technologies), 1μM dexamethasone (Sigma) and 50U/50μg penicillin/streptomycin
231 (Life Technologies). To induce DUX4 expression, 1μg/ml of doxycycline was added for 8 or 14
232 hours, as indicated. Cells were labeled in SILAC media containing heavy lysine (Lys8) and
233 arginine (Arg10) for 3 weeks before DUX4 induction experiments.

234

235 **Gel slice digestion.** Total RNA and protein were extracted from whole cells using TRIzol^{RT}
236 reagent (Ambion) following the manufacturer's instructions. 50 μg of total protein was subjected
237 to SDS PAGE using a 4-15% bis-TRIS gel. The gel was stained using GelCode blue (Pierce) as
238 per manufacturer's instructions, destained overnight in ultrapure water and the entire lane
239 containing protein was cut into 16 fractions using a GelCutter (Gel company Inc.). Individual gel
240 slices in 1.5 mL tubes (Eppendorf) were consecutively washed with water and incubated with
241 25mM ammonium bicarbonate, 50% acetonitrile for 2 hrs. The gel pieces were dehydrated with
242 acetonitrile. And the dried gel slices were reduced by covering them with 10 mM dithiothreitol in
243 100 mM ammonium bicarbonate and heating them at 56°C for 45 min. The solution was removed
244 and discarded. The gel slices were alkylated by covering them with a solution of 50 mM
245 iodoacetamide in 100 mM ammonium bicarbonate and incubating in the dark at ambient

246 temperature for 30 min. The solution was removed and discarded. The gel slices were dehydrated
247 with acetonitrile, then washed with 100 mM ammonium bicarbonate 100 mM for 10 min. The
248 solution was removed, discarded and the gel slices were dehydrated once again with acetonitrile.
249 After removing acetonitrile, the gel slices were then hydrated with 5 ng/uL sequencing grade
250 trypsin (Promega) in 50 mM ammonium bicarbonate and digested overnight at 37°C on an orbital
251 shaker. Following digestion, the supernatants were collected, and the gel slices were washed with
252 0.1% trifluoroacetic acid, and after 30 min an equal volume of acetonitrile was added followed by
253 washing for an additional 1 hour. The original digestion supernatant and the wash for a single
254 sample were combined into a single tube and dried by vacuum centrifugation. The digestion
255 products were desalted using Ziptips (Millipore) per the manufacturer's instructions, eluted with
256 70% acetonitrile/0.1% trifluoroacetic acid, and dried by vacuum centrifugation.

257

258 **Mass spectrometry.** The desalted material was resuspended in 20 µL of 2% acetonitrile in 0.1%
259 formic acid, and 18 µL was analyzed using one of two LC/ESI MS/MS configurations. The first
260 configuration consisted of an Easy-nLC II (Thermo Scientific) coupled to a Orbitrap Elite ETD
261 (Thermo Scientific) mass spectrometer using a trap-column configuration as described (28). A
262 trap of 100 µm × 20 mm packed with Magic C_{18}AQ (5-µm, 200 Å resin; Michrom Bioresources)
263 packing material was used for in-line desalting and a column of 75 µm × 250 mm packed with
264 C_{18}AQ (5-µm, 100 Å resin; Michrom Bioresources) was used for analytical peptide separations.
265 Chromatographic separations were carried out using a 60-minute gradient from 5% to 35%
266 solvent B (solvent A: 0.1% formic acid, solvent B: 0.1% formic acid in acetonitrile) at a flowrate of
267 300 nL/min. The analytical column temperature was maintained at 40℃. The Orbitrap Elite
268 instrument was operated in the data-dependent mode, switching automatically between MS
269 survey scans in the Orbitrap (AGC target value 1E6, resolution 240,000, and maximum injection
270 time 250 ms) and collision induced dissociation (CID) MS/MS spectra acquisition in the linear ion

271 trap (AGC target value of 10,000 and injection time 100 ms). The twenty most intense precursor
272 ions from the OrbiTrap full scan were each consecutively selected for fragmentation by CID in the
273 linear ion trap using a normalized collision energy of 35%. Ions of +2 and +3 charge states were
274 selected for MS/MS and selected ions were dynamically excluded for 30 seconds. The second
275 configuration consisted of an Easy nanoLC 1000 (Thermo Scientific) HPLC connected to an
276 OrbiTrap Fusion (Thermo Scientific) mass spectrometer. In-line chromatographic separations (no
277 trap column) were carried out using a 75 µm × 400 mm column packed with Magic C_{18}AQ (5-µm,
278 100 Å resin; Michrom Bioresources) packing material at a flowrate of 300 nL/min.
279 Chromatographic elution consisted of a 90-minute gradient from 3% to 27% B and the column
280 temperature was maintained at 40℃. The OrbiTrap Fusion was operated in the 2 second "top
281 speed" data dependent acquisition mode with MS survey scans in the OrbiTrap at least every 2
282 seconds (AGC target value 4E5, resolution 120,000, and maximum injection time of 50 ms).
283 Quadrupole isolation was set to 1.6 FWHM and higher energy collision dissociation (HCD) was
284 used for fragmentation at a collision energy of 28% and MS/MS detection was carried out in the
285 linear ion trap set at rapid scan speed (injection time of 250 ms and AGC target of 10E2).
286 Positively charged ions from 2 to 6 were selected for MS/MS and selected ions were dynamically
287 excluded for 30 seconds.

288

289 **Data analysis and statistical methods.** Qualitative and quantitative data analysis were
290 performed using Proteome Discoverer 2.1 (Thermo Scientific). The data were searched against
291 a human UniProt database (downloaded 11-04-16) that was appended with protein sequences
292 from the common repository of adventitious proteins (cRAP; www.thegpm.org/crap/) an *in silico*
293 translation products of noncanonical transcript isoforms stabilized due to NMD inhibition. In
294 downstream analyses, peptides that only mapped to NMD targets were not considered any further
295 in the current study and will be pursued in a future investigation. Searches were conducted with

296 the trypsin enzyme specificity. The precursor ion tolerance was set to 10 ppm and the fragment
297 ion tolerance was set to 0.6 Da. Variable modifications were set for oxidation on methionine
298 (+15.995 Da), carbamidomethyl (+57.021 Da) on cysteine, and acetylation (+42.010 Da) on the
299 N-terminus of proteins. Heavy SILAC amino acids for lysine (+8.014 Da) and arginine (10.008)
300 were also accounted for in the analysis as variable modifications. All search results were
301 evaluated by Percolator (29) for false discovery rate (FDR) evaluation of the identified peptides.
302 Peptide identifications were filtered to a peptide FDR of 1%. All downstream data analysis was
303 conducted using the R statistical programming language. The complete code to reproduce the
304 analyses and figures in this manuscript is deposited in github, available at the following URL:
305 https://github.com/sjaganna/2018-jagannathan_et_al.
306

307 **Gene Ontology analysis.** GO analysis was performed via the Overrepresentation Enrichment
308 Analysis method using WebGestalt server (pmid: 15980575; www.webgestalt.org). One of the
309 GO categories identified as enriched in this analysis was skin development, which we
310 subsequently removed from Figure 2C as many of the genes that contributed to this GO category
311 were extracellular proteins including keratins that could be environmental contaminants.
312

313 **Immunofluorescence microscopy.** Cells were permeabilized with PBS containing 0.1% Triton
314 X-100 for 5 minutes at room temperature and rinsed thrice in PBS. Primary antibody against
315 GM130 (Bethyl Laboratories, Cat # A303-402A-T) was diluted 1:200 in PBS and incubated for 1
316 hour at room temperature. After three washes in PBS, secondary anti-Rabbit TRITC (Jackson
317 ImmunoResearch, cat # 711-025-152) diluted 1:400 was added and incubated for 45 minutes at
318 room temperature. Cells were washed thrice in PBS with the nuclear counterstain DAPI included
319 in the final wash. Images were collected on a Cytation 5 multimode reader (BioTek) and analyzed
320 using GenPrime software (BioTek).

321 **Figure legends**

322

323 **Figure 1. Quantitative mass spectrometry of DUX4-expressing cells.**

324 A) Schematic of experimental set up to measure protein fold-change in cells expressing vDUX4
325 or iDUX4.

326 B-C) Histogram shows the DUX4/Control ratios (log2) for all peptides for the DUX4 target gene,
327 ZSCAN4 and the housekeeping gene, RPL15 in the vDUX4 (B) and iDUX4 (C) datasets.

328 D-E) FSHD biomarker transcripts identified by Yao et al (2015) in vDUX4 (D) and iDUX4 datasets
329 (E).

330

331 **Figure 2. Concordant changes in RNA and protein abundance of several DUX4**
332 **transcriptional targets.**

333 A) Table showing overlap of genes with a quantified RNA and protein level change in expression.

334 B) Scatter plot of RNA versus protein log2 fold-change (DUX4/control) of all genes.

335 C) GO analysis of genes that are up- or down-regulated > 4-fold at the RNA level or protein level.
336 Multiple testing correction done via Benjamini-Hochberg procedure.

337 D) Fluorescence micrographs of cells with and without DUX4 induction for 14h, stained with DAPI,
338 anti-GM130 (Golgi) and phalloidin-555 (actin).

339 E) Quantification of percent of cells with DUX4 positive nuclei and fragmented Golgi apparatus.

340

341 **Figure 3. Extensive post-transcriptional buffering of stress response genes in DUX4-**
342 **expressing cells.**

343 A) Scatter plot of RNA versus protein log2 fold-change (DUX4/control).

344 B) Representative genes that show post-transcriptional buffering.

345 C) qRT-PCR for a chaperone HSPA5 over a time course of vDUX4 expression.

346 D) Western blot showing phosphorylation of eIF2a over DUX4 expression time course.

347 E) S35-methionine incorporation over DUX4 time course.

348

349 **Figure 4. DUX4 induces post-transcriptional gene regulation.**

350 A) Scatter plot of RNA versus protein log2 fold-change (DUX4/control), highlighting genes that
351 are post-transcriptionally regulated.

352 B) Schematic representation of RNA- and protein-level changes for the genes involved in mRNA
353 surveillance. Colors represent a heat map of actual fold-change values. Proteins outlined by
354 dotted lines represent those lacking high-quality data.

355 C) Scatter plot of RNA versus protein log2 fold-change (DUX4/control) of genes in the ubiquitin
356 proteasome pathway.

357 D) Model for DUX4-induced post-transcriptional gene regulation.

358

359 **Supplementary Figures.**
360
361
362 **Figure S1. Demonstration of peptide to protein summarization for two candidate genes.**

363 A) Histogram shows the DUX4/Control ratios (log2) for all peptides

364 B-C) Histogram shows the DUX4/control ratios (log2) for all peptides mapping to RPL15 (B) or

365 ZSCAN4 gene (C)

366 D) Box and whisker plot showing the median log2 DUX4/control ratio for RPL15 and ZSCAN4.

367

368 **Figure S2. Tool for easy access of the data generated in this study**

369 Screenshot of the Shiny web server showing a sample analysis of the RNA and protein-level data

370 for a DUX4 transcriptional target, ZSCAN4.

371

372

373 **Supplementary Data**

374 Supplementary data 1: PSM-level data (deposited to Dryad; Link:

375 https://datadryad.org/review?doi=doi:10.5061/dryad.ck06k75)

376 Supplementary data 2: Gene level data for RNA and protein fold-change

377 Supplementary file 3: R markdown document with the code needed to process mass spectrometry

378 data and generate figures.

379

380

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Competing interests

No competing interests declared.

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Figure 1

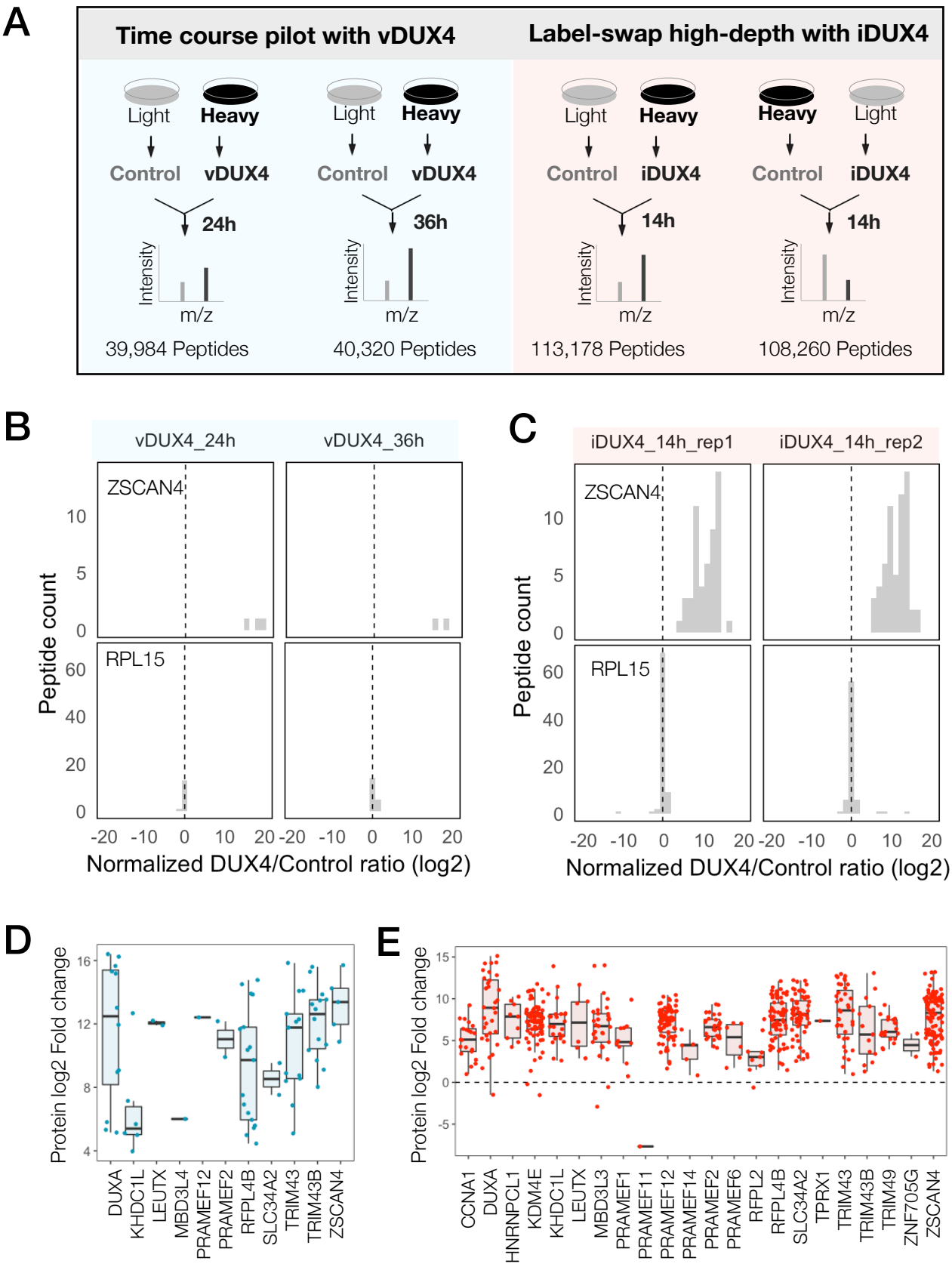
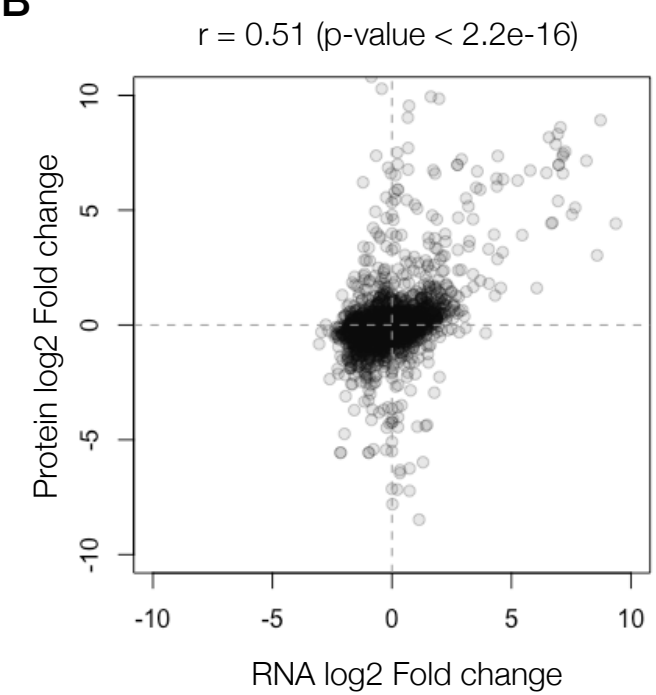

A
Time course pilot with vDUX4
Label-swap high-depth with iDUX4
Light
Heavy
Control
vDUX4
24h
36h
iDUX4
14h
Intensity
m/z
39,984 Peptides
40,320 Peptides
113,178 Peptides
108,260 Peptides
B
vDUX4_24h
vDUX4_36h
ZSCAN4
RPL15
Peptide count
Normalized DUX4/Control ratio (log2)
C
iDUX4_14h_rep1
iDUX4_14h_rep2
D
Protein log2 Fold change
DUXA
KHDC1L
LEUTX
MBD3L4
PRAMEF12
PRAMEF2
RFPL4B
SLC34A2
TRIM43
TRIM43B
ZSCAN4
E
CCNA1
HNRNPCL1
KDM4E
PRAMEF1
PRAMEF11
PRAMEF14
PRAMEF6
RFPL2
TPRX1
TRIM49
ZNF705G

Figure 2

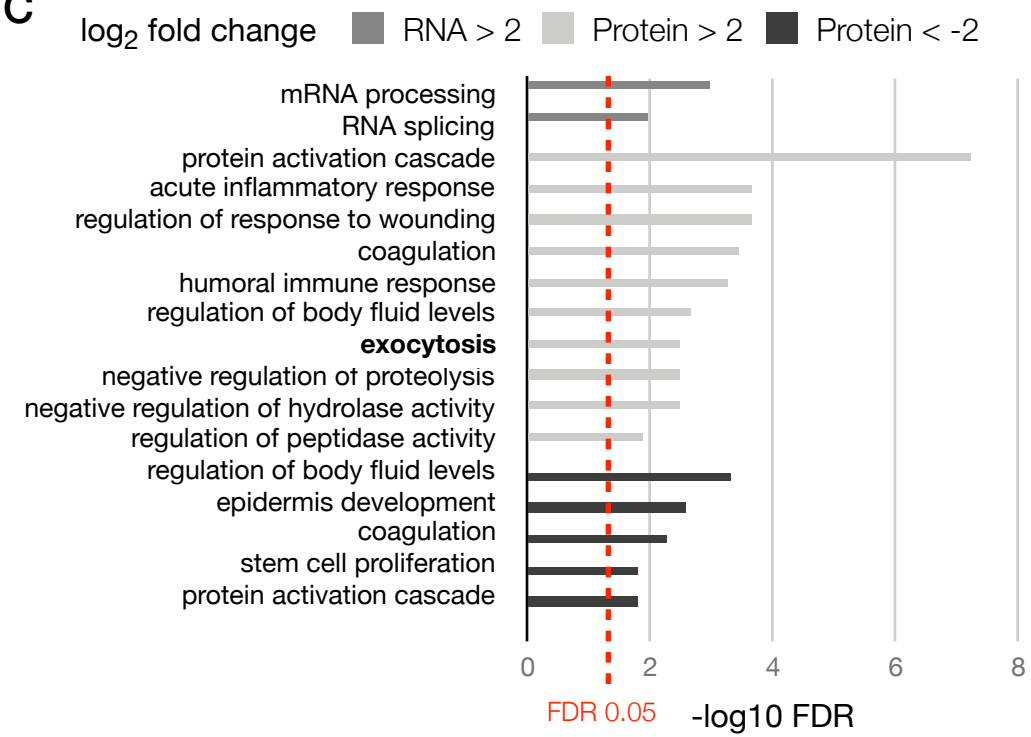
A

	Gene number		
Criteria	**RNA**	**Protein**	**Both**
Measured	21184	4005	3961
log2 fold change > 2	107	132	54
log2 fold change < -2	36	76	3

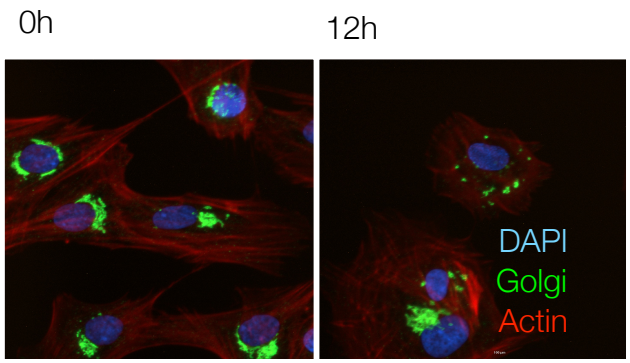
B



C



D



E

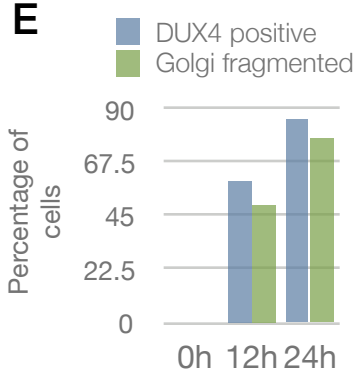


Figure 3

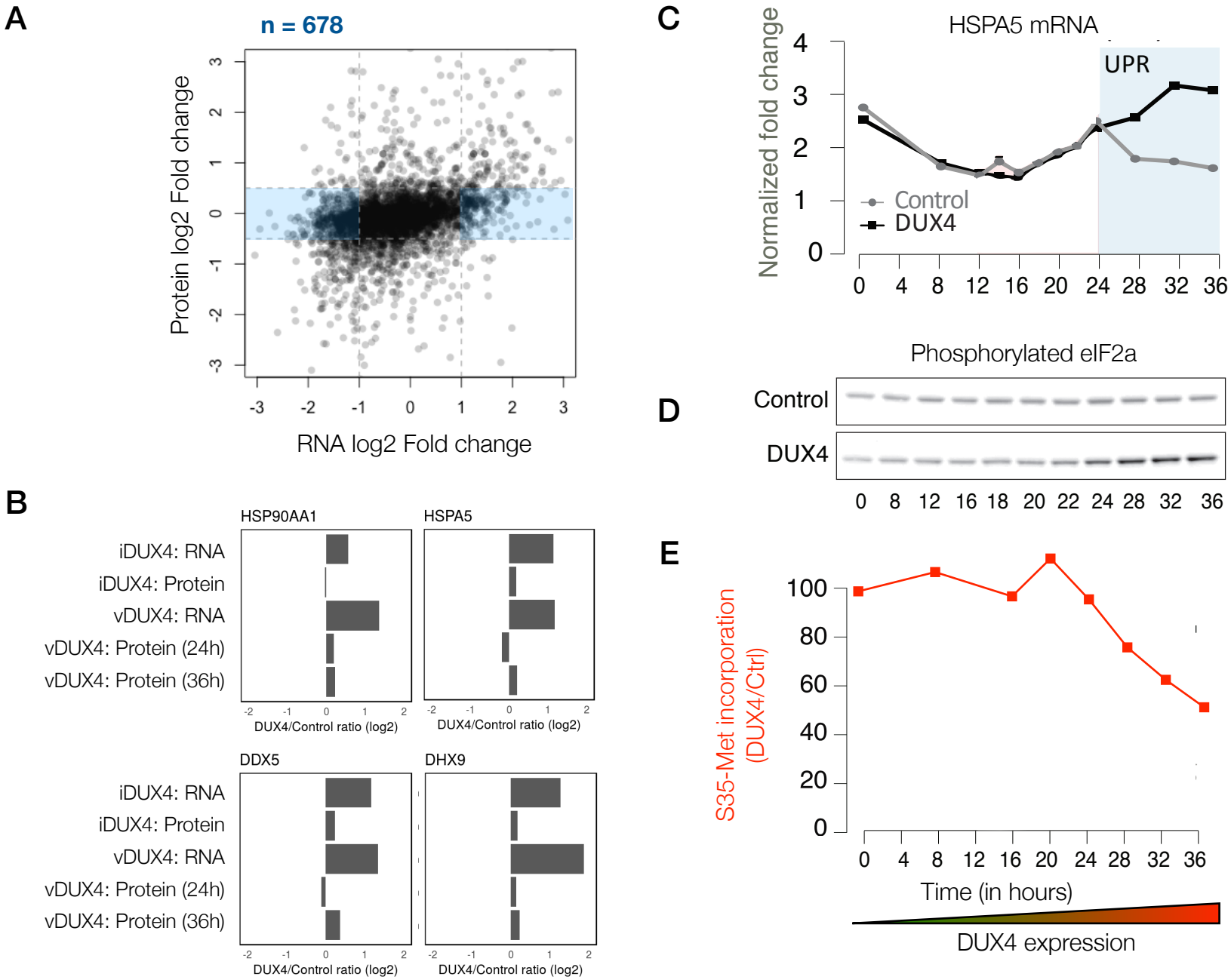

A
n = 678
Protein log2 Fold change
RNA log2 Fold change
B
HSP90AA1
HSPA5
DDX5
DHX9
iDUX4: RNA
iDUX4: Protein
vDUX4: RNA
vDUX4: Protein (24h)
vDUX4: Protein (36h)
DUX4/Control ratio (log2)
C
HSPA5 mRNA
UPR
Normalized fold change
Control
DUX4
D
Phosphorylated eIF2a
Control
DUX4
E
S35-Met incorporation (DUX4/Ctrl)
Time (in hours)
DUX4 expression

Figure 4

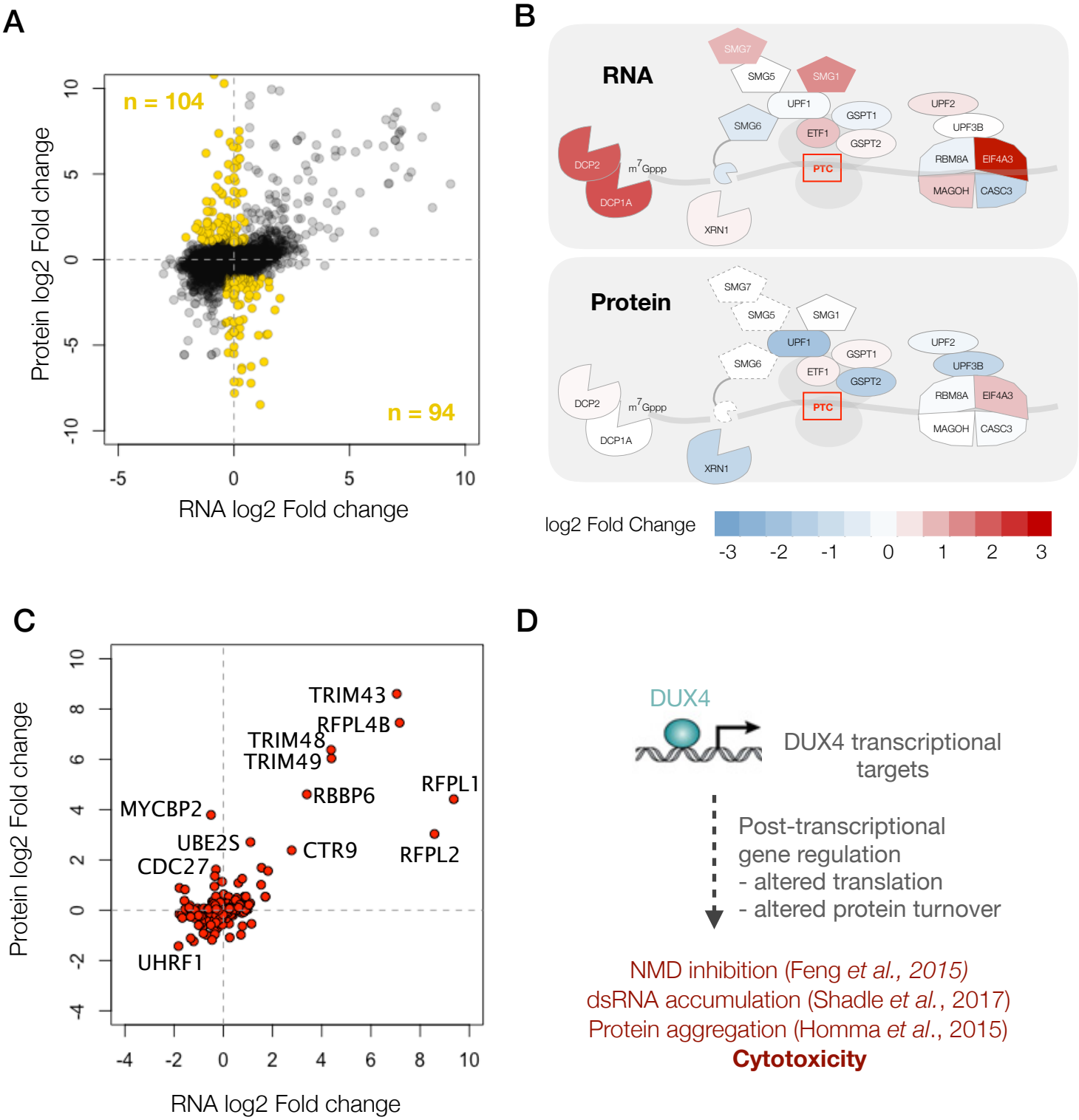

A
n = 104
n = 94
Protein log2 Fold change
RNA log2 Fold change
B
RNA
Protein
SMG7
SMG5
SMG1
UPF1
SMG6
GSPT1
ETF1
GSPT2
PTC
UPF2
UPF3B
RBM8A
EIF4A3
MAGOH
CASC3
DCP2
DCP1A
m7Gppp
XRN1
log2 Fold Change
-3 -2 -1 0 1 2 3
C
TRIM43
RFPL4B
TRIM48
TRIM49
RFPL1
RBBP6
MYCBP2
UBE2S
CTR9
RFPL2
CDC27
UHRF1
Protein log2 Fold change
RNA log2 Fold change
D
DUX4
DUX4 transcriptional targets
Post-transcriptional gene regulation
- altered translation
- altered protein turnover
NMD inhibition (Feng et al., 2015)
dsRNA accumulation (Shadle et al., 2017)
Protein aggregation (Homma et al., 2015)
Cytotoxicity

Figure S1

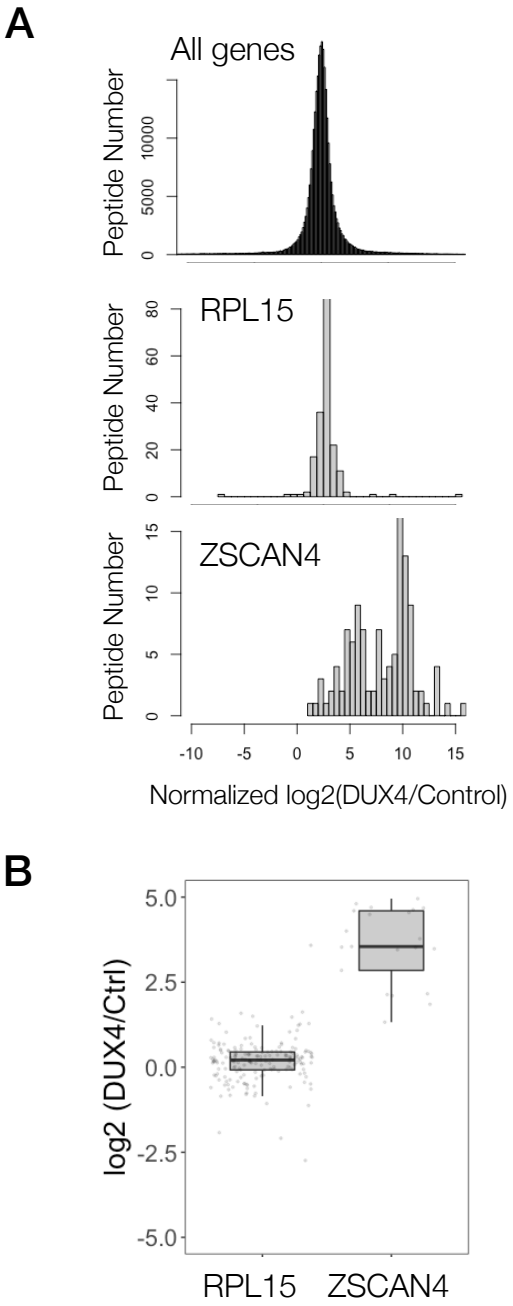
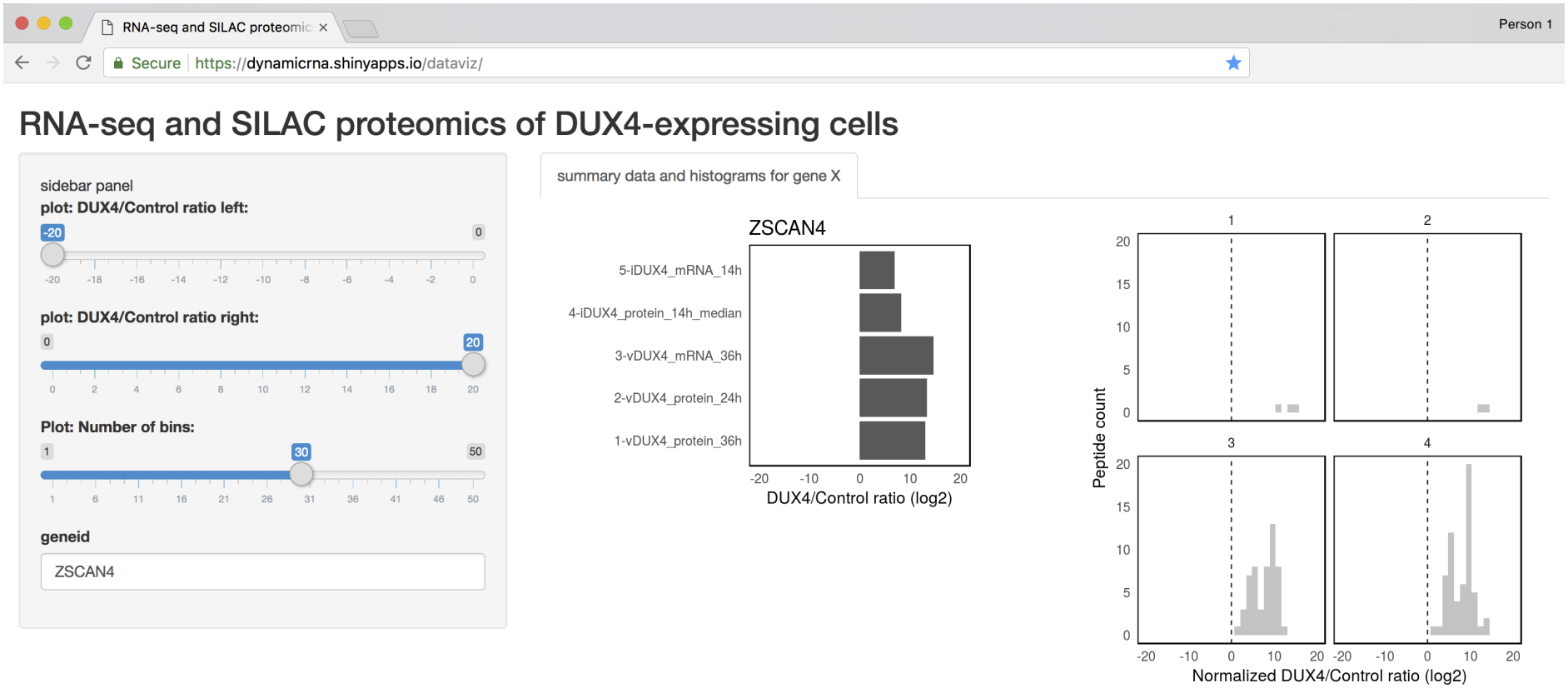

A
All genes
Peptide Number
0
5000
10000
RPL15
Peptide Number
0
20
40
60
80
ZSCAN4
Peptide Number
0
5
10
15
-10
-5
0
5
10
15
Normalized log2(DUX4/Control)
B
log2 (DUX4/Ctrl)
5.0
2.5
0.0
-2.5
-5.0
RPL15
ZSCAN4

Figure S2


RNA-seq and SILAC proteomic
Person 1
Secure
https://dynamicrna.shinyapps.io/dataviz/
RNA-seq and SILAC proteomics of DUX4-expressing cells
sidebar panel
plot: DUX4/Control ratio left:
-20
0
plot: DUX4/Control ratio right:
0
20
Plot: Number of bins:
1
30
50
geneid
ZSCAN4
summary data and histograms for gene X
ZSCAN4
5-iDUX4_mRNA_14h
4-iDUX4_protein_14h_median
3-vDUX4_mRNA_36h
2-vDUX4_protein_24h
1-vDUX4_protein_36h
DUX4/Control ratio (log2)
Peptide count
Normalized DUX4/Control ratio (log2)