

Identification of RNA-binding proteins in macrophages by interactome capture

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Running title: Novel RNA-binding proteins in macrophages

Key words: post-transcriptional regulation/ RBPs/ LPS/ TLR4-signaling/ HCLS1/ P23

Abbreviations

| | |
|----------|---|
| ARE-BP | AU-rich element-binding protein |
| LC-MS/MS | Liquid chromatography-tandem MS |
| LPS | lipopolysaccharides |
| MAPKs | mitogen activated protein kinases |
| RBP | RNA-binding protein |
| TLR4 | Toll-like receptor 4 |
| NFκB | Nuclear factor kappa B |
| PTGES3 | Prostaglandin E synthase 3 |
| HCLS1 | Hematopoietic cell-specific LYN substrate 1 |
| GO | Gene Ontology |

Summary

Pathogen components, such as lipopolysaccharides of gram-negative bacteria that activate Toll-like receptor 4, induce mitogen activated protein kinases and NF κ B through different downstream pathways to stimulate pro- and anti-inflammatory cytokine expression. Importantly, post-transcriptional control of the expression of TLR4 downstream signaling molecules contributes to the tight regulation of inflammatory cytokine synthesis in macrophages. Emerging evidence highlights the role of RNA-binding proteins (RBPs) in the post-transcriptional control of the innate immune response. To systematically identify macrophage RBPs and their response to LPS stimulation, we employed RNA interactome capture in LPS-induced and untreated murine RAW 264.7 macrophages. This combines RBP-crosslinking to RNA, cell lysis, oligo(dT) capture of polyadenylated RNAs and mass spectrometry analysis of associated proteins. Our data revealed 402 proteins of the macrophage RNA interactome including 91 previously not annotated as RBPs. A comparison with published RNA interactomes classified 32 RBPs uniquely identified in RAW 264.7 macrophages. Of these, 19 proteins are linked to biochemical activities not directly related to RNA. From this group, we validated the HSP90 co-chaperone P23 that was demonstrated to exhibit cytosolic prostaglandin E2 synthase 3 (PTGES3) activity, and the hematopoietic cell-specific LYN substrate 1 (HCLS1 or HS1), a hematopoietic cell-specific adapter molecule, as novel macrophage RBPs. Our study expands the mammalian RBP repertoire, and identifies macrophage RBPs that respond to LPS. These RBPs are prime candidates for the post-transcriptional regulation and execution of LPS-induced signaling pathways and the innate immune response. Macrophage RBP data have been deposited to ProteomeXchange with identifier PXD002890.

Introduction

Activation of toll-like receptor 4 (TLR4) by bacterial lipopolysaccharides (LPS), results in the induction of mitogen activated protein kinases (MAPKs) and NF κ B. The related specific signaling pathways stimulate pro- and anti-inflammatory cytokine expression. Inflammatory mediators are essential to coordinate cellular responses to infection. Excessive pro-inflammatory cytokine synthesis disturbs the balance between pro- and anti-inflammatory cytokines, ultimately leading to systemic capillary leakage, tissue destruction, and lethal organ failure (1, 2). LPS induces genome-wide expression changes in alveolar macrophages and RAW 264.7 cells (3, 4). Following activation of inflammation-related genes (5, 6), post-transcriptional checkpoints are critical for the precise immune response modulation (7-9). Information about post-transcriptional mechanisms that regulate protein synthesis downstream of TLR4 to adjust the range and extent of the immune reaction is still fragmentary.

RNA-binding proteins (RBPs) that coordinate mRNA turnover and mRNA translation contribute to rapid and purposeful immune cell responses. Specific RBPs that interact with AU-rich elements (AREs) in mRNA 3' untranslated regions (3'UTR) (ARE-BPs) have been shown to directly regulate cytokine mRNA translation and/or stability. AREs were first discovered in the short-lived human and mouse tumor necrosis factor (TNF) mRNAs (10). Besides TNF, ARE-BP-mediated regulation also controls the synthesis of other pro- and anti-inflammatory factors, such as interleukins and inducible nitric oxide synthase (7, 11, 12). Several ARE-BPs have been identified (7, 12): target mRNA translation is inhibited by T-cell-restricted intracellular antigen 1-related protein, CUG-repeat binding protein 2 and Fragile-X-related protein (13-15), while target mRNA decay is initiated by tristetraprolin (TTP), butyrate response factor 1 and 2 and KH-type splicing regulatory protein (16-18) through degradation factor recruitment. In

contrast, Y-box binding protein 1 and Hu-antigen R (HUR) stabilize ARE-containing mRNAs (19-21), the latter has also been shown to regulate mRNA translation (22-24). Furthermore, AUF-1 (heterogeneous ribonucleoprotein D, HNRNP D) either inhibits or promotes target mRNA decay (25, 26). However, RBPs not only directly regulate stability and/or translation of cytokine mRNAs. Recently we could show that the synthesis of TLR4 downstream transforming growth factor- β -activated kinase 1 (TAK1), an essential signaling molecule in accurate cytokine expression control, is controlled by HNRNP K in murine macrophages (27). *In vitro* RNA-binding assays revealed that the HNRNP K homology domain 3 of HNRNP K interacts specifically with a U/CCCC_(n) motif in the TAK1 mRNA 3'UTR. HNRNP K depletion in macrophages did not affect TAK1 mRNA synthesis, but increased its translation. The resulting elevated TAK1 protein level changed the macrophage LPS response to an earlier and extended P38 phosphorylation, enhancing cytokine mRNA synthesis. This suggests that LPS-induced TLR4 activation abrogates TAK1 mRNA translational repression by HNRNP K and the newly synthesized kinase TAK1 boosts the macrophage inflammatory response (27).

To systematically identify regulatory RBPs that modulate the LPS-induced macrophage response, we employed RNA interactome capture (28) combining UV-induced protein-RNA crosslinking in LPS-activated and untreated RAW 264.7 macrophages with oligo(dT) capture of polyadenylated RNAs and bound RBPs after cell lysis, and subsequent identification of eluted proteins by mass spectrometry. Our analysis identified 402 RBPs in macrophages, referred to here as macrophage RNA interactome, including 91 proteins not previously annotated as RBPs. An comparison of the macrophage RNA interactome with the RNA interactomes of HeLa cells (29), HEK293 cells (30) and murine embryonic stems (ES) cells (31) identified 32 RAW 264.7 cell-specific RBPs. Of that group, 19 proteins that lacked RNA-related functional annotations

were classified as novel macrophage RBPs. From these RAW 264.7 cell-specific RBPs we selected two candidates: P23, which acts as a heat shock protein 90 (HSP90) co-chaperone (32) and was shown to possess cytosolic prostaglandin E2 synthase 3 (PTGES3) activity (33); and the hematopoietic cell-specific LYN substrate 1 (HCLS1, HS1), a hematopoietic cell-specific adapter molecule (34, 35). The poly(A)⁺ RNA binding activities of both proteins were validated by immunoprecipitation from cytoplasmic extracts; assaying recombinant proteins *in vitro*; and by immunofluorescence analysis combined with fluorescence *in situ* hybridization (FISH) in RAW 264.7 cells.

Experimental Procedures

Plasmids

pBSIIKS-firefly luciferase, pBSIIKS-firefly luciferase-pA (27), pBSIIKS-*Renilla* luciferase-pA (36), pGEM-CAT (37) and pET28b-PRMT1 (38) have been described. P23 (NM_019766.4) and HCLS1 (NM_008225.2) ORFs were PCR amplified from RAW 264.7 cDNA using the following primers: P23 fw: ttgtatctcgagatgcagcctgcttctg, P23 rv: gttagactcgagttactccagatctggc, HCLS1 fw: gcatgactcgagatgtggaagtctgtag, HCLS1 rv: gcatgactcgagttagaggagcttgaca and cloned into the *Xho*I site of pET16b. P23 fragments were cloned into the *Xho*I site of pET16b using the following primer pairs. P23 N (aa 1-130): P23 fw and P23 N rv: gttagactcgagttacatgtgatccatc; P23 C (aa 131-160): P23 C fw: ttgtatctcgagggtggatgatgaggatgt and P23 rv.

Cell culture and LPS treatment

RAW 264.7 cells (ATCC, TIB-71) were grown in DMEM (Thermo Fisher Scientific) supplemented with 10% heat inactivated FBS (Biochrom), penicillin and streptomycin (Thermo Fisher Scientific). For LPS treatment, 10 ng/ml *E.coli* LPS (serotype 0111:B4, Sigma-Aldrich) was added to the medium for 2 h.

UV-crosslinking of RAW 264.7 cells

Cells were kept on cell culture dishes and washed twice with ice-cold PBS. PBS was removed completely and culture dishes were placed on ice in a UV Stratalinker 2400 (Stratagene), with 15 cm distance to UV bulbs. Cells were irradiated with UV light of 254 nm (0.15 J/cm²) and ice-cold PBS was added immediately after irradiation to harvest cells by scraping.

RBP capture

In two independent biological replicate experiments the following conditions were compared: 1] LPS treatment (+LPS), UV-crosslinking (+CL), 2] no LPS (-LPS) (+CL), 3] control (ctrl.) (+LPS) non-crosslinked (no CL), 4] ctrl. (-LPS) no CL (Figure 1A). Enrichment and isolation of RBPs bound to polyadenylated RNA was performed as described (28, 29). For each sample 40 million cells were used. Crosslinked cells were collected by centrifugation for 5 min at $500 \times g$ and 4°C . The cell pellet was resuspended in 35 ml ice-cold lysis buffer (20 mM Tris/HCl pH 7.5, 500 mM LiCl, 0.5% LiDS, 1 mM EDTA, 5 mM DTT) and incubated on ice for 10 min. For homogenization the lysate was passed three times through a 26G needle. Polyadenylated *Renilla* luciferase RNA (*Renilla* poly(A)⁺) and non-polyadenylated firefly luciferase RNA (Firefly poly(A)⁻) were added as spike in-controls. 3 ml magnetic oligo(dT)₂₅-beads or magnetic non-modified beads (NEB) were activated by washing three times with 9 ml lysis buffer. Beads were incubated with the lysate for 1 h at 4°C with rotation. All following washing steps were performed at 4°C . Separation of beads and supernatant was performed in a magnetic separation rack (NEB). The supernatant was used for a second round of isolation. Beads were sequentially washed 5 min with rotation in 35 ml of the following buffers: lysis buffer, buffer I (twice) (20 mM Tris/HCl pH 7.5, 500 mM LiCl, 0.1% LiDS, 1 mM EDTA, 5 mM DTT), buffer II (twice) (20 mM Tris/HCl pH 7.5, 500 mM LiCl, 1 mM EDTA, 5 mM DTT) and buffer III (twice) (20 mM Tris/HCl pH 7.5, 200 mM LiCl, 1 mM EDTA, 5 mM DTT). RNA-protein complexes were eluted by addition of 0.5 ml elution buffer (20 mM Tris/HCl pH 7.5, 1 mM EDTA) and incubation for 3 min at 50°C with shaking at 750 rpm. Empty beads were regenerated by washing three times with 9 ml lysis buffer and then re-incubated with the

supernatant. A second isolation was performed as described above and both eluates were combined afterwards.

Isolation of proteins

For analysis of co-precipitated proteins covalently bound RNAs were digested by RNase treatment. Samples from input and eluate were incubated with 5 × RNase buffer (50 mM Tris/HCl pH 7.5, 750 mM NaCl, 0.25% NP-40, 2.5 mM DTT), 280 U RNase T1 (Thermo Fisher Scientific) and 4 µg RNase A (Thermo Fisher Scientific) for 1 h at 37°C. Reaction mixtures were concentrated with an Amicon Ultra 10000 MWCO (Merck Millipore) by centrifugation (3.220 × g, 4°C, 45 min), and the upper reservoir was refilled twice with buffer IV (10 mM Tris/HCl pH 7.5, 50 mM NaCl).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

In each of two replicate experiments, three samples were compared: (1) LPS treatment (+LPS), UV-crosslinking (+CL), (2) no LPS (-LPS) (+CL) and (3) controls (ctrl.) (+LPS, -LPS), both non-crosslinked (no CL). Controls were combined because they contain only few proteins and the background was usually fairly constant. Protein samples were processed as described before (28, 29). Briefly, proteins were digested, followed by a stable-isotope labeling step *via* reductive methylation (using differential labels producing ‘light’, ‘intermediate’ and ‘heavy’ peptides in the respective samples) (Figure 1A). Samples were combined, and peptides were fractionated by iso-electric focusing. The twelve fractions generated were analyzed by liquid chromatography (LC) coupled to an OrbitrapVelosPro mass spectrometer (Thermo Fisher Scientific) using a Proxeon nanospray source. Reverse phase chromatography was performed with a nanoAcquity

UPLC system (Waters) fitted with a trapping column (nanoAcquity Symmetry C18, 5 μ m, 180 μ m \times 20 mm) and an analytical column (nanoAcquity BEH C18, 1.7 μ m, 75 μ m \times 200 mm) directly coupled to the ion source. The mobile phases for LC separation were 0.1% (v/v) formic acid in LC-MS grade water (solvent A) and 0.1% (v/v) formic acid in can (solvent B). Peptides were separated at a constant flow rate of 300 nl/ min with a linear gradient of solvent B from 3 to 40% for 145 min. The MS1 scan was acquired in the Orbitrap from m/z 300 to 1700 at a maximum filling time of 500 ms and 106 ions. The resolution was set to 30000. Fragmentation was performed in the LTQ by collision induced dissociation, selecting up to 15 most intense ions (top15) at an isolation window of 2 Da. Target ions previously selected for fragmentation were dynamically excluded for 30s with relative mass window of 10 ppm. MS/MS selection threshold was set to 2000 ion counts. A lock mass correction was applied using a background ion (m/z 445.12003).

MS data processing

Raw files were processed with MaxQuant version 1.3.0.5 (39) and the Andromeda search engine (40). The MS/MS spectra were searched against the Mouse SwissProt database (downloaded on June 21, 2011) containing 53623 forward sequences that was appended to the same number of reverse sequences and 265 common contaminants. The precursor mass tolerance was set to 20 ppm for the first pass and 6 ppm for the 2nd pass. The fragment mass tolerance was 0.5 Da. Razor peptides, which represent non-unique peptides assigned to the protein group with the most other peptides, following Occam's razor principle (39) and unique peptides were quantified only as unmodified peptides. Cysteine carbamidomethylation and methionine oxidation were set as fixed and variable modification, respectively. The minimum peptide length was set to 6 amino

acids, the enzyme specificity was set to trypsin/P, the maximum allowed miss-cleavage was set to 2, and the false discovery rate (FDR) was set to 0.01 for both peptide and protein identifications. Re-quantification and match between runs were also performed unless stated otherwise. The protein identification was reported as a “protein group” if no unique peptide sequence to a single database entry was identified. Statistical analysis was performed using the Limma package in R/Bioconductor (41, 42) calculating FDRs from p-values using Benjamini and Hochberg's method. When comparing crosslinked cells (either untreated or after LPS-stimulation) to non-crosslinked controls, a protein was regarded as an RNA interactor, if the adjusted p-value was <0.1 and when ratios in both replicates changed in the same direction. When comparing untreated and LPS-stimulated cells to each other, a protein was considered to differentially bind to mRNA between these conditions if the adjusted p-value was <0.1 and when ratios in both replicates changed in the same direction. For RBP classification, data were collapsed to unique gene names (Supplementary table 2). The MaxQuant output text files with the raw mass spectrometry data have been deposited *via* the PRIDE partner repository in ProteomeXchange (43) and individual peptides can be viewed in the MaxQuant viewer. The ProteomeXchange accession number is PXD002890.

Statistical analyses

Enrichment analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (44, 45). A p-value was calculated, applying a modified Fisher's exact test (EASE score) for each term and corrected for multiple testing with Benjamini and Hochberg's method. The following criteria were applied: EASE score ≤ 0.1 , at least three proteins could be assigned to one term and corrected p-value ≤ 0.05 .

Experimental Design and Statistical Rationale

In each of the two replicate experiments, three samples were compared: 1] LPS treatment (+LPS), UV-crosslinking (+CL), 2] no LPS treatment (-LPS), UV-crosslinking (+CL) and 3] controls (+LPS, -LPS), both non-crosslinked (no CL). The controls (ctrl.) of the two experiments were combined because they contain only few proteins, and the background was usually fairly constant. We have digested each of these three protein samples, followed by a stable-isotope labelling step producing 'light', 'intermediate' and 'heavy' peptides in the respective samples (Figure 1A). Statistical analysis was performed with the Limma package in R/Bioconductor (41, 42) applying adjustment of p-values for multiple testing with Benjamini and Hochberg's method.

RNA preparation and quantitative real-time PCR

For analysis of precipitated RNA covalently bound proteins were removed by proteinase K. Samples from input and eluate were pre-incubated with 5× proteinase K buffer (50 mM Tris/HCl pH 7.5, 750 mM NaCl, 1% SDS, 50 mM EDTA, 2.5 mM DTT, 25 mM CaCl₂) and 40 U RiboLock (Thermo Fisher Scientific) for 30 min at 65°C. After addition of 20 µg proteinase K, samples were incubated for 1 h at 50°C. 250 pg CAT RNA (RBP capture) or firefly luciferase RNA (immunoprecipitation and poly(A)⁺ capture) were added prior to total RNA extraction using Trizol. For reverse transcription random primers and M-MLV RT (Promega) or Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) were used (46). Quantitative PCR (qPCR) was performed with Power SYBR[®]Green PCR Master Mix on a StepOnePlus (Thermo Fisher Scientific). Primers are summarized below. For analysis of spike in-control RNAs equal volumes were analyzed by qPCR. RNA levels were determined

by the $\Delta\Delta C_t$ method (47), normalized for CAT RNA. RT-PCR with primers for firefly luciferase as extraction control was performed as described (48).

Oligonucleotides for quantitative RT-PCR

| target RNA | sense | sequence |
|---------------------------|---------|-------------------------|
| firefly luciferase | forward | CCTTCCGCATAGAACTGCCT |
| | reverse | GGTTGGTACTAGCAACGCAC |
| <i>Renilla</i> luciferase | forward | GTTGTGCCACATATTGAGCC |
| | reverse | CCAAACAAGCACCCCAATCATG |
| CAT | forward | GGAATTCCGTATGGCAATGA |
| | reverse | GATTGGCTGAGACGAAAAAC |
| NDUFV1 | forward | GCCTCCAATTTGCAGGTAGCTAT |
| | reverse | CACGCACCACAAACACATCA |

Nano Chip Analysis

RNA samples from input and elute fractions were analyzed with an RNA 6000 Nano Kit on a 2100 Bioanalyzer (Agilent). The 28S/18S ratio and the RNA Integrity Number (RIN) (49) were indicated as a measure for RNA quality.

High-throughput sequencing

RNA samples from the eluate fractions were analyzed by high-throughput sequencing. RNA was chemically fragmented (NEBNext Magnesium RNA Fragmentation Module) for 4 minutes at 94°C to an average length of ~140 nt, and converted into an Illumina-compatible library using the Split Adapter method as described (50), omitting the hot SPRI bead cDNA purification step and the DSN depletion step. Amplified libraries were size-selected by polyacrylamide electrophoresis. Yield and quality were checked by running libraries on a Bioanalyzer chip.

Library sequencing was performed on an Illumina miSeq sequencer (Biomolecular Resource Facility, Australian National University). Reads were mapped to the mouse transcriptome and all non-rRNA reads were assigned to categories.

***In vitro* transcription**

RNA for spike in controls and for extraction control was transcribed with T7 MEGAscript® Kit (Thermo Fisher Scientific).

Antibodies

Antibodies were purchased from Abcam (HISTONE H3, P23), Santa Cruz (HNRNP K, HUR), Sigma-Aldrich (α -TUBULIN, VINCULIN), Cell Signaling (HCLS1) and GE Healthcare Life Sciences (HRP-conjugated secondary antibodies).

Immunoblot analysis

Western blot assays were performed as described previously (51) and analyzed on a LAS-4000 system (GE Healthcare Life Sciences).

Immunofluorescence and Fluorescence *in situ* hybridization (FISH)

Immunofluorescence staining was essentially performed as described in (48) with specific antibodies against HCLS1 and P23 and FISH with an oligo(dT) probe as in (52).

Immunoprecipitation

Crosslinked RAW 264.7 cells were collected by centrifugation for 5 min at $500 \times g$ and 4°C and lysed in 1 volume IP buffer (46) by passing ten times each through a 20G and subsequently a 26G needle. Supernatant representing cytoplasmic extract was stored at -80°C . Anti-P23 or anti-HCLS1 antibodies were incubated with 40 μl Protein G sepharose overnight at 4°C . Antibody coupled beads were incubated 2 h with 1 mg cytoplasmic extract derived from RAW 264.7 cells (untreated or 2 h LPS-induction) in IP buffer. Luciferase control IP was performed as described (27). Beads were washed twice in IP buffer and either boiled in $2 \times$ SDS sample buffer for Western blot analysis or resuspended in Trizol for RNA isolation.

Expression of recombinant proteins

His-tagged protein arginine methyltransferase 1 (His-PRMT1) was expressed as described (53). His-P23 and peptide variants as well as His-HCLS1 were expressed as described for His-HNRNP K (54).

Pulldown of His-tagged proteins

40 pmol His-P23 and peptide variants, His-HCLS1 or His-PRMT1 were immobilized on 30 μl Ni-NTA agarose in IP buffer and subsequently incubated with 50 μg of total RNA isolated from untreated or LPS-induced RAW 264.7 cells. Co-precipitated RNA was isolated using Trizol with firefly luciferase RNA as extraction control.

Poly(A)⁺ RNA detection assay

RNA isolated from immunoprecipitation or pull down assays was dot blotted on a nylon membrane, crosslinked twice (XLE-1000, Spectroline, setting Optimal Crosslink) and hybridized with Biotin-(dT)₃₀ in 4 × SSC, 1% bovine serum albumin, 2.5% dextran sulfate overnight at 4°C. Bound Biotin-(dT)₃₀ was detected by Streptavidin-HRP. Data from three independent experiments were evaluated using Student's t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).

Results

Comprehensive analysis of RNA-bound RBPs in differentially treated macrophages

Stability and translation of several cytokine mRNAs is controlled by ARE-BPs (7, 10-12). In addition, HNRNP K was identified as a specific regulator of TAK1 mRNA translation in LPS-induced macrophages (27). We aimed to systematically analyze RBPs that modulate the macrophage LPS response. To characterize the differential RNA-binding properties of RBPs in untreated and LPS-induced RAW 264.7 macrophages we applied RNA interactome capture, which had already successfully been employed to determine the RBP repertoire in other contexts (28-31, 55).

To capture early regulatory interactions, we used a 2 h LPS-treatment, based on LPS time-course experiments (27). Native RNA-protein complexes were crosslinked (CL) by UV light ($\lambda = 254$ nm) *in vivo*, cells were lysed, polyadenylated RNAs bearing the crosslinked RBPs purified on oligo(dT) beads, and treated with RNases A/T1 or proteinase K to purify the RBPs or RNAs, respectively, for further analysis (Figure 1A). Protein capture with poly(A)⁺ RNA was strongly enhanced by UV irradiation as confirmed by silver staining (Figure 1B, compare lanes 3 and 8 with 5 and 10). The protein elution pattern differs profoundly from the input (Figure 1B, lanes 1-3 and 6-8), indicating selectivity against protein abundance and specificity of RNA binding.

HNRNP K (27) and HUR (20-24), which are RBPs known to mediate post-transcriptional control of protein synthesis, were specifically enriched in oligo(dT) eluates of UV-irradiated samples from untreated and LPS-induced cells, as validated by Western blotting (Figure 1C, lanes 3-5 and 8-10). In contrast, the abundant cellular proteins VINCULIN, α -TUBULIN and HISTONE H3 were not detected in the eluates (Figure 1C, lanes 3-5 and 8-10). RNA isolation from the input and

elution from the oligo(dT)-beads yielded comparable total RNA amounts for the samples from untreated and LPS-induced RAW 264.7 cells, indicating no major changes in the overall mRNA pool, bound to oligo(dT) (Figure 1D). QPCR analysis further demonstrated that specific RNA amounts eluted from oligo(dT) were comparable independent of macrophage LPS treatment (Figure 1E). The yield of exogenously added polyadenylated *Renilla* luciferase mRNA (*Renilla* poly(A)⁺) was higher than that of non-adenylated firefly luciferase mRNA (firefly poly(A)⁻) (Figure 1E, left and middle panel). This was supported by the relative abundance of the two spike-in controls in the elution fractions as quantified by RNA-Seq (Figure 1F). In addition to the exogenously added mRNAs we investigated binding of an endogenous mRNA encoding NDUFV1 that is expressed constitutively in untreated and LPS-induced macrophages (27). 40% of NDUFV1 mRNA was recovered in the elution of the crosslinked samples compared to input (Figure 1E, right panel). The analysis of the eluted non-ribosomal RNA population by limited high-throughput sequencing revealed that mRNAs represent the major fraction of non-ribosomal RNA, independent of macrophage LPS-induction or UV-crosslinking prior to oligo(dT) capture (Figure 1G).

These data indicate that the UV-induced crosslinking between RNAs and RBPs was effectively established, while abundant proteins that lack RNA-binding activity could not be detected.

Classification of RBPs in RAW 264.7 cells

To characterize the protein pool that is bound to polyadenylated RNA, RBPs from untreated and LPS-induced macrophages were analyzed by mass spectrometry in two independent biological replicate experiments (for details see Experimental Procedures, LC-MS/MS). In total 945 proteins were identified (Supplementary table S1), 762 of them were identified in both replicates

(Supplementary table S2). Of these, 374 and 396 unique proteins were enriched compared to the non-crosslinked control in the samples of untreated and LPS-induced RAW 264.7 cells, respectively (Figure 2A, Supplementary table S2). There was a very high overlap of 368 proteins between the -LPS and +LPS samples (Figure 2B), leaving 402 proteins that we define here as the collective set of RBPs in mouse macrophages. Among them, six proteins were identified as RNA-interactors in untreated macrophages and 28 in LPS-induced macrophages, but did not pass the criteria to be considered as RNA-interactors in the other condition (Figure 2B) (for details see Experimental Procedures, MS data processing).

Within this macrophage RNA interactome we applied mouse genome-based gene set enrichment analysis to search for representative features (44, 45) (Figure 2C). As predicted, in the gene ontology (GO) domain *Molecular Function* “RNA binding” is the most significantly over-represented category of the 402 proteins with 12.5-fold enrichment compared to the mouse genome (Figure 2C, top panel). Consistently, in the GO domain *Biological Process*, RNA biology-related functions and processes are highly over-represented (Figure 2C, middle panel) and protein domain analysis (Pfam) finds RNA recognition motifs (RRM) as predominant (Figure 2C, bottom panel). Interestingly, only 188 of the 402 candidate proteins represent annotated RBPs. Examination of the remaining 214 proteins identified 80 as *RNA related* and 43 as *nucleic acid binding or related*. Notably, 91 proteins were *not related to nucleic acid binding* (Figure 2D). To further investigate protein functions we extended our GO and Pfam analysis by using Panther Protein class annotation (56, 57). This revealed that the 188 proteins categorized as RBPs bear functions from RNA synthesis to RNA processing, RNA modification and mRNA translation some of them have multiple functions (Figure 3A). For several of the 91 proteins, so

far not classified as *related to nucleic acid binding*, cellular functions linked to a broad range of processes are reported (56) (Figure 3B).

RBPs specifically identified in murine macrophages

To identify cell-specific RBPs we compared the murine macrophage RNA interactome with previously published RNA interactomes of murine ES cells (31) and two human cell lines, HeLa (29) and HEK 293 (30) (Figure 4A). This analysis classified 69 proteins only identified in murine cells (Figure 4A and B), of which 32 were exclusively detected in macrophages (Figure 4A and C). This group of 32 RBPs only identified in macrophages includes 19 proteins (Figure 4C) with activities so far not directly related to RNA (Table 1).

In addition we characterized the impact of LPS on the RNA binding activity of the 402 proteins that comprise the macrophage RNA interactome by applying the following cut-off criteria: [I] adjusted p-value 2h LPS CL *vs.* untreated CL <0.1 , [II] detectable in both replicates, [III] \log_2 ratio >1 in both replicates, [IV] detectable in both replicates from untreated and LPS-treated cells. Our analyses uncovered three proteins as differentially bound in response to LPS: TTP, transcription factor JUN-B and ribosomal RNA processing-12 homolog (RRP12). A comparison of our data with that from LPS time-course experiments in RAW 264.7 macrophages, which addressed temporal dynamics of transcription, protein synthesis and secretion (20 min, 1, 2, 3 h LPS treatment) (58) revealed differential expression levels for two of the three proteins. This study showed that TTP and JUN-B were more abundant in LPS-treated cells, suggesting that the detected increase in RNA binding may be associated with elevated expression in LPS-induced macrophages. It is noteworthy that RRP12, which remained unchanged in expression level, is involved in ribosome biogenesis and was shown to interact with poly(A) *in vitro* (59).

P23 and HCLS1 bind directly to RNA

From the 19 macrophage-specific proteins that were designated as novel RBPs (Table 1) based on our analysis, we chose two proteins to investigate their RNA-binding activity: P23, a HSP90 co-chaperone (32), also known as PTGES3 (33); and HCLS1 or HS1, an adapter molecule with transcriptional activity that is important for myelopoiesis (34, 35).

The presence of P23 and HCLS1 in eluates of oligo(dT) capture assays performed with untreated and LPS-induced macrophages was verified by Western blot (Figure 5A). Both proteins are present in the input and the eluate of UV-irradiated samples (Figure 5A, lanes 1-3 and 6-8), and absent from control eluates (Figure 5A, lanes 4, 5 and 9, 10), whereas VINCULIN, tested as negative control, was not detected in any eluate. Importantly, consistent with the mass spectrometry analysis, LPS-induction did not affect the level of P23 and HCLS1, which are specifically detected in the eluate of the crosslinked reactions (Figure 5A, lanes 3 and 8). Immunofluorescence analysis revealed that both proteins are localized in the cytoplasm, at least at steady state (Figure 5B). Therefore, we applied cytoplasmic extracts to address the interaction of endogenous P23 and HCLS1 with polyadenylated RNA in untreated macrophages and after LPS-induction. Quantification revealed that both proteins were immunoprecipitated at comparable levels independent of LPS treatment (Figure 5C and D, upper panel).

Specific co-precipitation of polyadenylated RNA was verified for both proteins, HCLS1 (Figure 5C, middle panel) and P23 (Figure 5D, middle panel) in a dot blot-based Biotin-Streptavidin assay, which utilizes biotinylated oligo(dT) hybridization to immobilized poly(A)⁺ RNA. Interestingly, a smaller proportion of poly(A)⁺ RNA was co-precipitated with P23 from cytoplasmic extracts of LPS-induced macrophages (Figure 5D, middle panel, lanes 2 and 5). This

was not due to RNA preparation differences as proven by equally detectable exogenously added firefly luciferase mRNA, shown in RT-PCR analysis (Figure 5D, lower panel).

To test whether the poly(A)⁺ RNA population binds directly to P23 and HCLS1, we purified macrophage mRNA and constructed expression vectors for both polypeptides. For initial *in vitro* interaction studies recombinant His-tagged proteins were purified from *E. coli* (Figure 6A). His-P23 and His-HCLS1 immobilized on Ni-NTA agarose precipitated specifically poly(A)⁺ RNA from total RNA isolated from untreated and LPS-induced RAW 264.7 cells, whereas His-PRMT1 did not (Figure 6B). Interestingly, P23 precipitated more poly(A)⁺ RNA isolated from untreated macrophages than from LPS-induced macrophages (Figure 6B, C), consistent with the co-immunoprecipitation of poly(A)⁺ RNA with endogenous P23 (Figure 5D). Empty Ni-NTA beads (- protein in the dot blot) (Figure 6C), which did not show poly(A)⁺ RNA binding were used for normalization (Figure 6B). Differences in co-precipitation were not due to varying extraction efficiencies shown by RT-PCR analysis with primers for firefly luciferase extraction control (Figure 6D).

To validate the interaction of HCLS1 and P23 with poly(A)⁺ RNA observed in the immunoprecipitation assay (Figure 5C and D) and with recombinant protein (Figure 6B) *in vivo* in LPS-treated RAW 264.7 cells, we applied immunofluorescence and FISH. Analysis of untreated and LPS-induced cells using a fluorescent oligo(dT) probe revealed that LPS diminishes the co-localization of P23 with poly(A)⁺ RNA in the cytoplasm (Figure 6E, left panel), but not that of HCLS1 and poly(A)⁺ RNA (Figure 6E, right panel).

Because differential poly(A)⁺ RNA-interaction could be detected *in vitro* and *in vivo* for P23, we wanted to characterize its RNA binding domains. Interestingly, P23 contains an unstructured C-terminal tail (aa 131-160) (60, 61). To specify poly(A)⁺ RNA binding domains, we constructed

and expressed P23 and deletion variants (Figure 6F, left panel), which were immobilized on Ni-NTA agarose. Consistent with Figure 6B, binding of poly(A)⁺ RNA from LPS-induced RAW 264.7 cells to His-P23 was reduced (Figure 6F, middle panel). Interestingly, the 30 aa C-terminal part (His-P23 C) confers the poly(A)⁺ RNA interaction, which declines as observed for full length P23 (Figure 6F, middle panel). This was not due to varying extraction efficiencies (Figure 6F, right panel).

These results suggest that the two proteins P23 and HCLS1, which were so far not classified as related to nucleic acid binding, exhibit RNA-binding activity.

Discussion

Employing endogenous poly(A)⁺ interactome capture we present here the first comprehensive analysis of RNA-binding proteins in murine macrophages. Our analysis identified 402 RBPs, referred to here as macrophage RNA interactome, including 91 proteins not previously annotated as RBPs. A comparison with published RNA interactomes (29-31) classified 32 RBPs as uniquely identified in RAW 264.7 macrophages (Table 1). 19 proteins of that group, which lack RNA-related functional annotations, could be ranked as novel macrophage RBPs (Table 1).

Panther protein class annotation (56, 57) of these newly identified RBPs revealed an over-representation of *cytoskeletal proteins* (Figure 4C, Table 1). Importantly, cytoskeletal proteins are involved in chemotactic and phagocytic macrophage functions, which require extensive actin cytoskeleton remodeling (62). From the seven cytoskeletal protein genes (*Capg*, *Cfl1*, *Hcls1*, *Lcp1*, *Vim*, *Tubb4b* and *Lmna*) assigned by Panther classification we chose an interesting candidate, *Hcls1*. The encoded protein not only contributes to cytoskeletal reorganization, but in complex with other factors (35) also to transcription activation in the LPS-induced monocyte to macrophage transition (63, 64). We found that the murine RAW 264.7 macrophage LPS-response does not affect HCLS1 expression (Figure 5A), consistent with mass spectrometry analyses of LPS-induced human THP-1 monocytes (64). Notably, HCLS1, which is localized to the cytoplasm (Figure 5B) showed poly(A)⁺-RNA binding activity in cytoplasmic extracts (Figure 5C) and as recombinant protein (Figure 6B) as well as in RAW 264.7 cells (Figure 6E, right panel) that was not influenced by LPS-induction. The identification of HCLS1 as an RBP now expands the view of how HCLS1 mediates the cell fate in macrophages. HCLS1 is exclusively expressed in hematopoietic cells, i.e. lymphoid, myeloid and erythroid cell lines as well as circulating lymphocytes, granulocytes and macrophages. Human HCLS1 (34) and its

murine orthologue (65) are highly homologous (87% identity). The multi-domain protein HCLS1 bears an N-terminal acidic domain (NTA) that directly binds and activates the actin-related protein (ARP) 2/3 complex, thereby promoting actin polymerization (66). The NTA is followed by a helix-turn-helix motif. Interestingly, a helix-turn-helix motif contributes to RNA binding of ROQUIN (67-69), which implicates a candidate for the RNA-interaction in HCLS1. A coiled-coil region represents the main F-actin binding site and functions synergistically with the helix-turn-helix motif (70-72). Interestingly, HCLS1 knock down in CD34⁺ cells led to an increase in F-actin expression and a resulting disturbed F-actin organization (35). Furthermore, besides a central proline-rich region HCLS1 displays a C-terminal SH3 domain (34), which initiates receptor-coupled tyrosine kinase activation (72). Tyrosine kinases SYK, LYN and LCK and the adaptor protein GRB2 phosphorylate and activate the protein (73-78). In platelets casein kinase 2 catalyzes HCLS1 threonine and serine phosphorylation (79). A comparison with the phosphoproteome of LPS-treated murine bone marrow derived macrophages (80) revealed that LPS treatment results in diminished HCLS1 phosphorylation, and it might be assumed that regulated phosphorylation causes differential interaction with individual mRNAs (27).

LPS-mediated macrophage activation not only involves cytoskeletal remodeling, but requires HSP90 activity that is essential for stabilization and maturation of protein factors involved in NFκB activation and inflammation (81-83). Among the novel RBPs we identified the HSP90 co-chaperone P23 (32) or cytoplasmic PTGES3 (33), which was therefore chosen for further analysis. LPS treatment was shown to elevate PTGES3 activity in murine peritoneal macrophages (84). Along with HSP90, P23 interacts with AGO2, stabilizes its open conformation and facilitates structural changes that promote RNA-induced silencing complex (RISC) loading (85).

During apoptosis P23 is cleaved by CASPASE-3 and 8 (86). Interestingly, we discovered that LPS-induction of RAW 264.7 macrophages leads to miR-155-mediated CASPASE-3 down-regulation and decelerated apoptosis, thereby sustaining macrophage activity (87). This is in agreement with a role of P23 in preventing ER-stress-induced apoptosis (88, 89).

Notably, after LPS-induction P23 immunoprecipitated from cytoplasmic extracts (Figure 5D) as well as recombinant P23 exhibited reduced poly(A)⁺-RNA-binding activity (Figure 6B), consistent with declining P23- poly(A)⁺-RNA co-localization in RAW 264.7 cells (Figure 6E, left panel). This is not due to altered P23 expression in LPS-induced RAW 264.7 macrophages (Fig. 5A) or changes in the pool of total RNA and poly(A)⁺ RNA captured by oligo(dT) after LPS treatment (Figure 1D).

P23 contains a compact β -strand CS domain (CHORD-containing proteins and SGT1), which provides a HSP90 binding surface. The unstructured C-terminal tail (30 aa) of P23 is necessary for optimal chaperone activity, but not for HSP90 interaction (60, 61). That C-terminal tail of P23 mediates poly(A)⁺-RNA interaction (Figure 6F, middle panel). This is in agreement with the reported function of disordered protein regions as RNA chaperones (90) and the contribution of the unstructured C-terminal tail of HIV-1 VIF to RNA binding (91). Strikingly, the identification of P23/PTGES3 as a new RBP adds that protein to the growing family of metabolic enzymes that emerged to exhibit RNA binding activity (92, 93), providing potential functions in post-transcriptional gene regulation that need to be explored.

Therefore future studies will focus on the identification and functional analysis of polyadenylated RNAs that are bound differentially in response to LPS and the potential impact of post-translational modifications on RNA-binding of P23.

In summary, our study expands the mammalian RBP repertoire, and identified specific macrophage RBPs that respond to LPS. These RBPs are prime candidates for the post-transcriptional regulation and execution of LPS-induced TLR4 signaling pathways and the innate immune response. Information about underlying molecular mechanisms of RBP functions will advance our understanding of their role in inflammatory response modulation and provide us with knowledge about their potential as therapeutic targets to prevent systemic inflammation and sepsis.

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Footnotes

Acknowledgements

We acknowledge technical support by the ACRF Biomolecular Resource Facility at JCSMR and the EMBL Proteomics Core Facility. We thank Yalin Liao for critical reading of the manuscript. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG) (OS 290/6-1) to A.O.-L.; and in part by grants from the DFG to I.S.N.-d.V. (NA 1273/1-1) and from the National Health and Medical Research Council of Australia (#1045417) to T.P. and M.W.H.

Author contributions

Biochemical experiments were performed by A.L. (Figures 1 and 5A), I.S.N.-d.V. (Figures 5 and Figure 6, except 5A and 5C-D, upper panel) and N.S. (Figure 5 C-D, upper panel). A.L. and I.S.N.-d.V. analyzed experimental data. K.E., S.F. and J.K. executed mass spectrometry experiments, analyzed the data for Figure 2A and Supplementary table 1 and 2; and deposited mass spec data in ProteomeXchange. S.K.A. and T.P. performed high-throughput sequencing experiments and data analysis presented in Figure 1F and G. A.L. analyzed the RNA interactome as shown in Figures 2B-D, 3 and 4B and C. B.U. essentially contributed to RNA interactome analysis (Figures 3 and 4) and prepared the data for Figure 4A. A.C. and M.W.H. made substantial contribution to the conceptual design of the study. G.M. contributed to the development of the project. D.H.O. and A.O.-L. designed the study, analyzed the data and wrote the manuscript.

All authors critically read the manuscript and gave their final approval of the version to be submitted.

Conflict of interest

The authors declare no conflict of interest.

Figure legends

Figure 1 RBP enrichment by *in vivo* UV-crosslinking and oligo(dT) capture.

(A) Experimental design. RAW 264.7 cells were either left untreated or induced with 10 ng/ml LPS for 2 h and subsequently UV-irradiated ($\lambda = 254$ nm) (crosslinked, CL) to stabilize RNA-protein interactions. To enrich poly(A)⁺ RNA, RNA-protein complexes were subjected to oligo(dT) capture after cell lysis and eluted, treated either with proteinase K to purify the RNA or with RNase A/T1 for Western blot analysis and mass spectrometry. **(B and C)** Following RNase A/T1 digestion, released proteins were analyzed by silver staining (B, lanes 1-10) or with antibodies specific for known RBPs (HNRNP K and HUR) or control proteins (VINCULIN, α -TUBULIN and histone H3) (C, lanes 1-10). Beads lacking oligo(dT) (beads) or preparations from non-UV-irradiated cells (no CL) served as controls. **(D)** Total amounts of isolated RNA from input and elution from oligo(dT)- and empty magnetic beads. **(E)** QPCR analysis of RNA isolated from the input and the oligo(dT)-bound fraction following proteinase K treatment of the beads, performed in triplicates. Equal proportions of the input and eluate samples were used for qPCR analysis. Primers against spike-in controls, which were added prior to oligo(dT) capture, polyadenylated *Renilla* luciferase mRNA (*Renilla* poly(A)⁺), non-adenylated firefly luciferase mRNA (firefly poly(A)⁻) or endogenous NDUFV1 mRNA were used. **(F)** Quantification of *Renilla* luciferase-poly(A)⁺ and firefly luciferase-poly(A)⁻ from high throughput sequencing of eluted RNAs. Raw read counts were normalized for total read length and the number of sequencing reads per kilobase per million mapped reads (RPKM). **(G)** High throughput sequencing of eluted RNAs. The percentage of non-rRNA reads mapped to the mouse transcriptome is displayed. Non-coding RNAs comprise miRNAs, lncRNAs and asRNAs.

Figure 2 RAW 264.7 macrophage RNA interactome.

(A) Log2 ratios of proteins identified in two biological replicates from untreated RAW 264.7 cells (-LPS CL) normalized to non-UV-irradiated cells (no CL) (left) and 2 h LPS-induced cells (+LPS CL) normalized to UV-irradiated cells (no CL) (right). Proteins represented in the RNA interactome are indicated in orange. (B) Overlap of RBPs enriched from untreated and LPS-induced cells. (C) GO domain analysis of *Molecular Function* (top) and *Biological Process* (middle), and top ten protein classification based on Pfam (bottom). Terms with lowest adjusted p-values corrected for multiple testing by Benjamini and Hochberg's method are displayed. Numbers in brackets indicate proteins identified vs. the total number of mouse genome encoded proteins according to DAVID (44, 45). (D) Classification of the identified RNA interactome as proteins implicated in RNA binding, RNA related, nucleic acid binding or related and as proteins not related to nucleic acid binding.

Figure 3 Known and novel RBPs.

(A) Classification of 188 known RBPs according to functions in RNA metabolism based on Panther (<http://www.pantherdb.org/>) (56, 57). To some proteins several functions are allocated. (B) 91 novel RBPs categorized based on Panther, several proteins were not classified.

Figure 4 Identification of RAW 264.7 macrophage-specific RBPs.

(A) Comparison of the RAW 264.7 RNA-interactome with that of HeLa cells (29), HEK293 cells (30) and murine ES cells (31). (B) Classification of 69 murine-specific RBPs in RNA- or nucleic acid binding and non-related proteins (top), assignment of 41 nucleic acid binding related RBPs to RNA metabolism (middle) and mapping of 28 novel proteins to cellular functions

(Panther protein class annotation) (bottom). **(C)** Assignment of 32 RAW 264.7 cell-specific RBPs to RNA- or nucleic acid binding and non-related proteins (top), 13 nucleic acid binding related RBPs to RNA metabolism (middle) and mapping of 19 novel proteins to cellular functions (Panther protein class annotation) (bottom).

Figure 5 Validation of RAW 264.7 macrophage-specific RBPs.

(A) Samples obtained from RAW 264.7 cells, treated as in Figure 1A and analyzed in Figure 1B were used to detect HCLS1, P23 and VINCULIN. **(B)** RAW 264.7 cells either left untreated or induced with LPS were analyzed by immunofluorescence microscopy to visualize endogenous proteins with specific antibodies as indicated, nuclei were stained with DAPI, F-actin with Phalloidin-TRITC. Scale bar: 10 μ m. Specific HCLS1 **(C)** and P23 **(D)** immunoprecipitation with respective antibodies, a Luciferase antibody served as specificity control (ctrl. ipp). Representative Western blots for HCLS1 and P23, HNRNP K served as control (top), poly(A)⁺ RNA detection with a dot blot-based Biotin-Streptavidin assay (middle) and RT-PCR with primers for firefly luciferase (Luc) extraction control (bottom). Levels of immunoprecipitated HCLS1 and P23, shown beneath the respective blots, were quantified from three independent experiments (means \pm s.d.). Co-precipitated poly(A)⁺ RNA was quantified from three independent experiments (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Figure 6 Analysis of P23 and HCLS1 poly(A)⁺ RNA binding *in vitro*

(A) Coomassie-stained SDS-PAGE of 2 μ g His-P23, His-HCLS1 and His-PRMT1. **(B)** His-P23, His-HCLS1 or His-PRMT1 were immobilized on Ni-NTA agarose and incubated with 50 μ g total RNA purified from untreated or LPS-induced RAW 264.7 cells. Co-precipitated

poly(A)⁺ RNA from three independent experiments was detected with a dot blot-based Biotin-Streptavidin assay (* = $p < 0.05$, ** = $p < 0.01$). **(C)** Representative dot blot of co-precipitated poly(A)⁺ RNA shown in (B). **(D)** RT-PCR analysis of samples described in (B) with primers for firefly luciferase (exogenous extraction control). **(E)** RAW 264.7 cells either left untreated or induced with LPS were hybridized with oligo(dT) probe (FITC, green) for *in situ* hybridization. Immunostaining of endogenous P23 (Cy3, red) (left) and HCLS1 (Cy3, red) (right) was carried out using specific antibodies, staining of nuclei with DAPI. Scale bar: 10 μ m. **(F)** Left panel: Coomassie-stained SDS-PAGE of 1 μ g His-PRMT1 and His-tagged P23 variants: P23 (aa 1-160), P23 N (aa 1-130), P23 C (aa 131-160) (left panel). Middle panel: Proteins were immobilized on Ni-NTA agarose and incubated with 50 μ g total RNA purified from untreated or LPS-induced RAW 264.7 cells. Co-precipitated poly(A)⁺ RNA from three independent experiments was detected with a dot blot-based Biotin-Streptavidin assay (* = $p < 0.05$, ** = $p < 0.01$). Right panel: RT-PCR analysis with primers for firefly luciferase.

Table 1

32 macrophage-specific RBP candidates in the RAW 264.7 cell interactome.

| gene symbol | ENSEMBL ID | gene name | Panther (protein class) | protein domains |
|--|--------------------|--|--|---|
| RNA binding | | | | |
| Phax | ENSMUSG00000008301 | Phosphorylated adapter RNA export protein | nucleic acid binding | Phosphorylated adapter RNA export protein, RNA-binding domain |
| Prkrip1 / C114 | ENSMUSG00000039737 | PRKR-interacting protein 1 | | |
| Rpl12 | ENSMUSG00000038900 | 60S ribosomal protein L12 | nucleic acid binding | |
| RNA related | | | | |
| Gm10119 | ENSMUSG00000062611 | 40S ribosomal protein S3a | nucleic acid binding | |
| Gm10154 | ENSMUSG00000066116 | 60S ribosomal protein L34 | nucleic acid binding | |
| Hic2 | ENSMUSG00000050240 | Hypermethylated in cancer 2 protein | nucleic acid binding, transcription factor | Zinc finger, C2H2-type, BTB/POZ-like |
| Lrrfip1 | ENSMUSG00000026305 | Leucine-rich repeat flightless-interacting protein 1 | nucleic acid binding, transcription factor | |
| Rplp2 | ENSMUSG00000025508 | 60S acidic ribosomal protein P2 | nucleic acid binding | WD40 repeat |
| Rps17 | ENSMUSG00000061787 | 40S ribosomal protein S17 | nucleic acid binding | |
| nucleic acid binding or related | | | | |
| Eif1 | ENSMUSG00000035530 | Eukaryotic translation initiation factor 1b | | |
| Hdgf | ENSMUSG00000004897 | Hepatoma-derived growth factor | transcription factor, signaling molecule | PWWP domain |
| Hmgb3 | ENSMUSG00000015217 | High mobility group protein B3 | nucleic acid binding, transcription factor, signaling molecule | HMG box A DNA-binding domain |
| Junb | ENSMUSG00000052837 | Transcription factor jun-B | nucleic acid binding, transcription factor | Basic-leucine zipper (bZIP) transcription factor |
| not related to nucleic acid binding | | | | |
| 1810009A15Rik | ENSMUSG00000071653 | | | |
| A230050P20Rik | | UPF0515 protein C19orf66 homolog | | |
| Arhgdia | ENSMUSG00000025132 | Rho GDP-dissociation inhibitor 1 | enzyme modulator, signaling molecule | |
| Arhgdib | ENSMUSG00000030220 | Rho GDP-dissociation inhibitor 2 | enzyme modulator, signaling molecule | |
| Capg | ENSMUSG00000056737 | Macrophage-capping protein | cytoskeletal protein, calcium-binding protein | Gelsolin |
| Cdv3 | ENSMUSG00000032803 | Protein CDV3 | | |
| Cfl1 | ENSMUSG00000056201 | Cofilin-1 | cytoskeletal protein | Actin-binding, cofilin/tropomyosin type |
| Fv4 | ENSMUSG00000075231 | MLV-related proviral Env polyprotein | Viral protein | |
| Hcls1 | ENSMUSG00000022831 | Hematopoietic lineage cell-specific protein | cytoskeletal protein, transcription factor | SH3 domain |
| Lcp1 | ENSMUSG00000021998 | lymphocyte cytosolic protein 1 | cytoskeletal protein | Calponin-like actin-binding, Calcium-binding EF-hand |
| Lmna | ENSMUSG00000028063 | Prelamin-A/C; Lamin-A/C | cytoskeletal protein, structural protein | Intermediate filament tail domain |
| Ltv1 | ENSMUSG00000019814 | Protein LTV1 homolog | | |
| Pcnp | ENSMUSG00000071533 | PEST proteolytic signal-containing nuclear protein | | |
| Ptges3 / p23 | ENSMUSG00000071072 | Prostaglandin E synthase 3 | chaperone | CS domain |
| Ranbp1 | ENSMUSG00000005732 | Ran-specific GTPase-activating protein | enzyme modulator | Pleckstrin homology-type |
| Set | ENSMUSG00000054766 | Protein SET | enzyme modulator, chaperone | |
| Tubb4b | ENSMUSG00000036752 | Tubulin beta-4B chain | cytoskeletal protein | |
| Txlna | ENSMUSG00000053841 | Alpha-taxilin | defense/ immunity protein | Myosin-like coiled-coil protein |
| Vim | ENSMUSG00000026728 | Vimentin | cytoskeletal protein, structural protein | Intermediate filament protein |

Figure 1

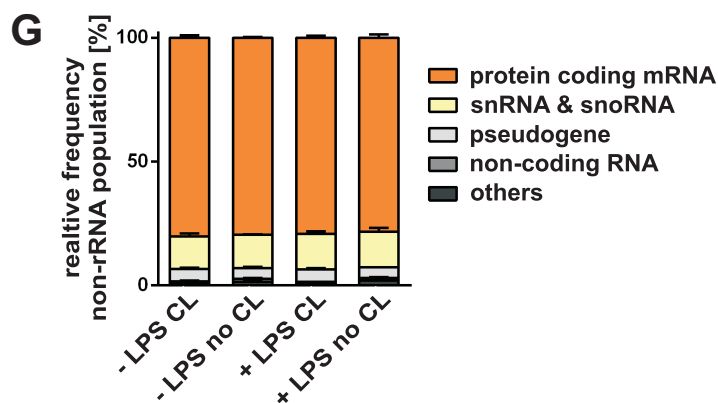
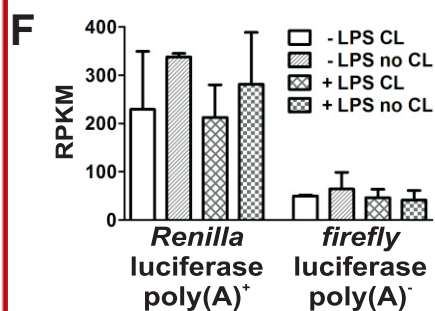
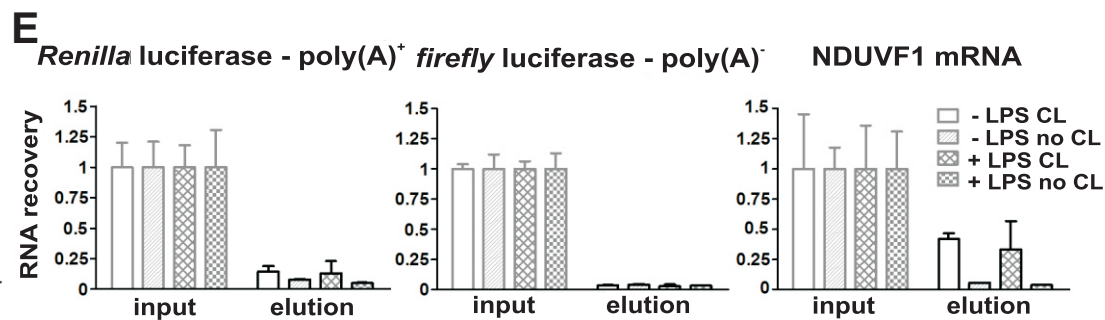
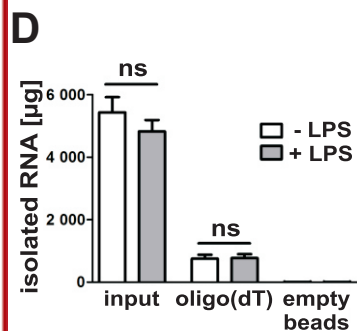
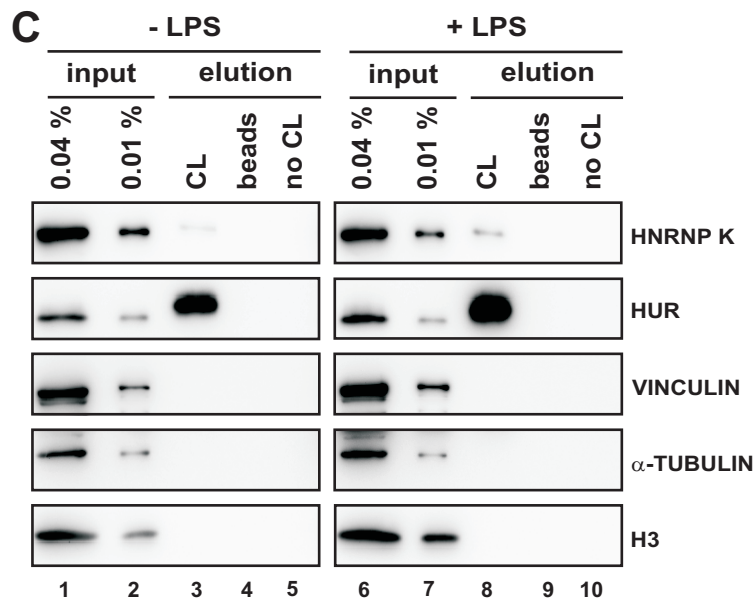
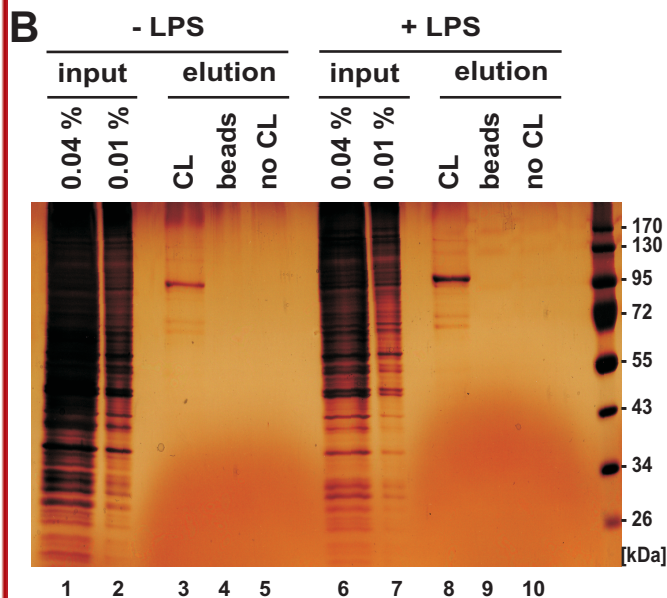
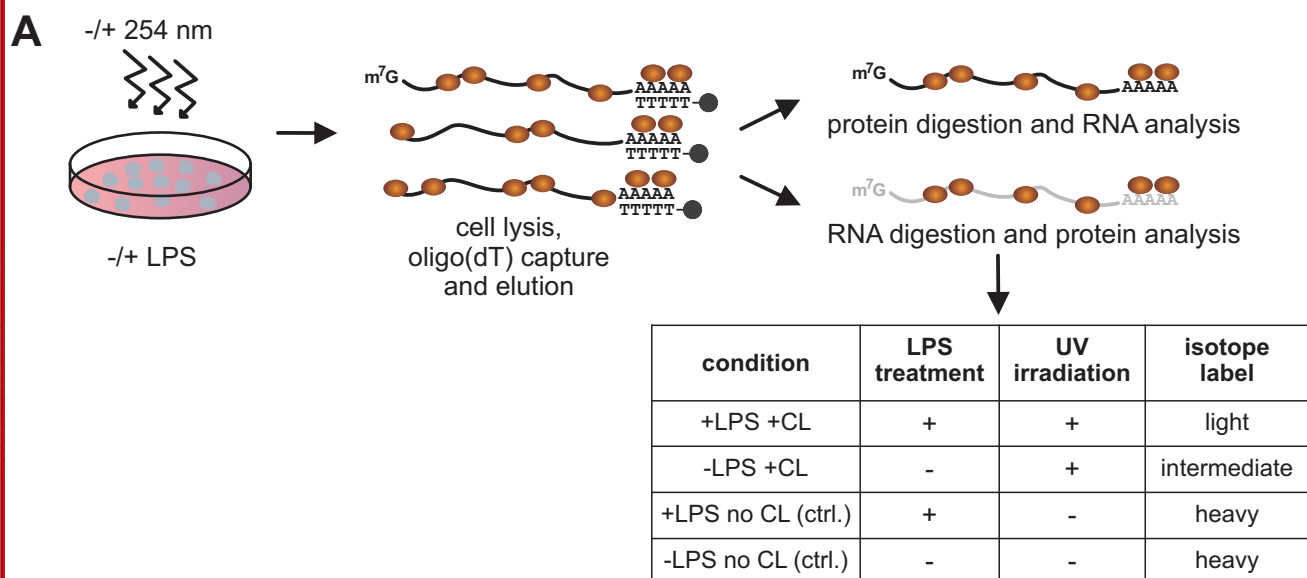


Figure 2

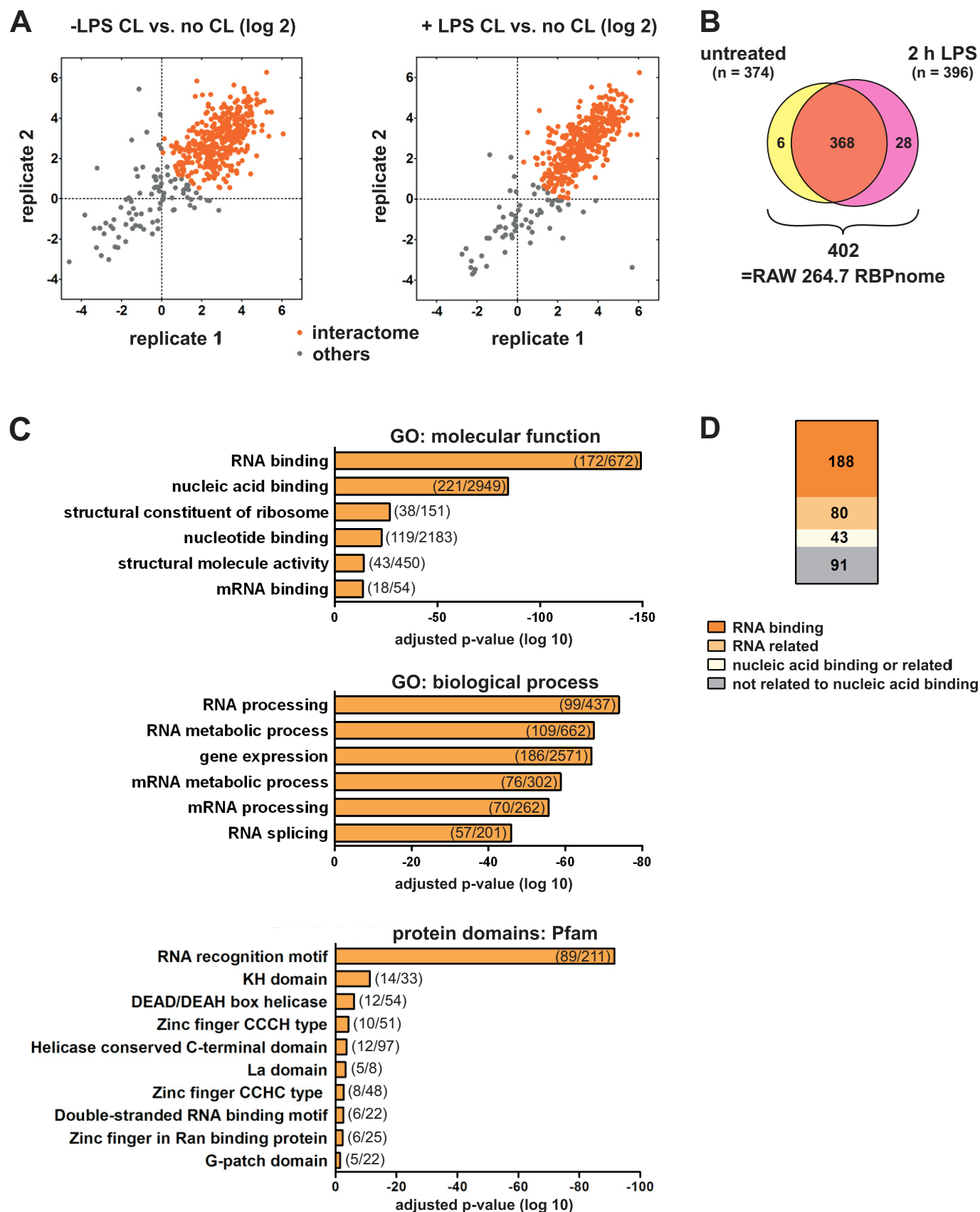
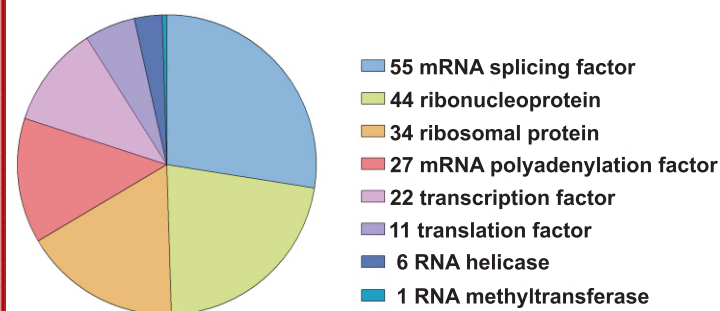


Figure 3

A known RNA binding proteins (188)



B novel RNA binding proteins (91)

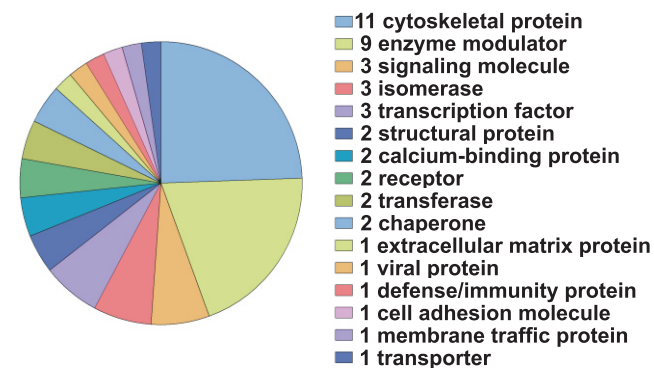
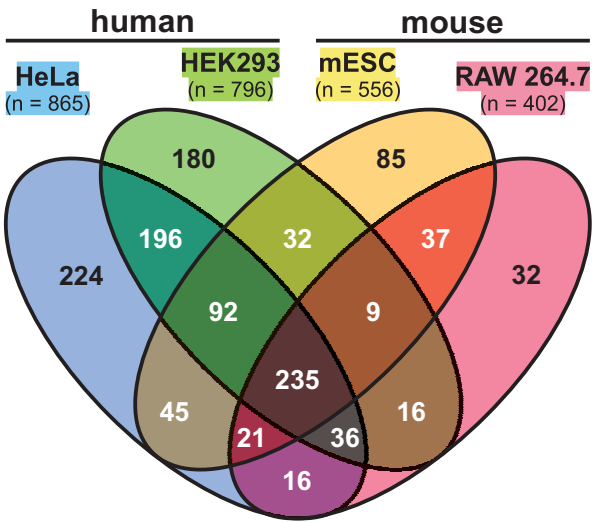
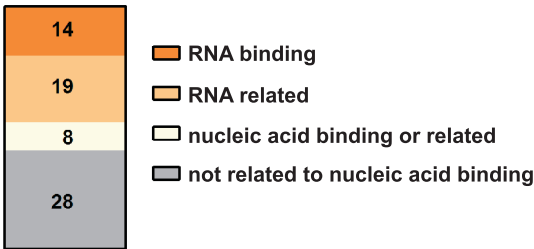


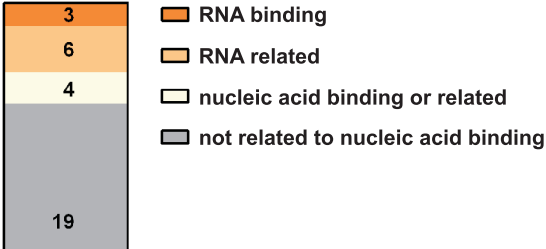
Figure 4



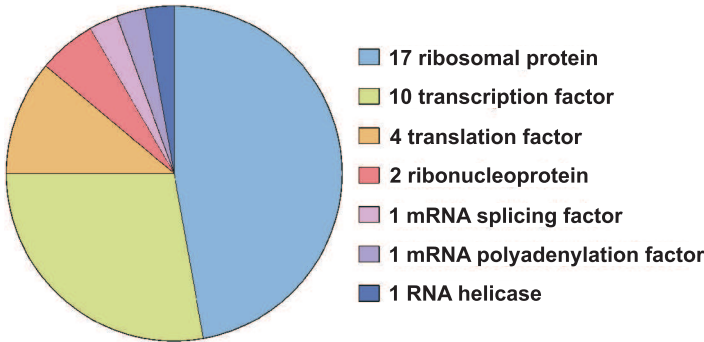
RBPs specific for murine cells (69)



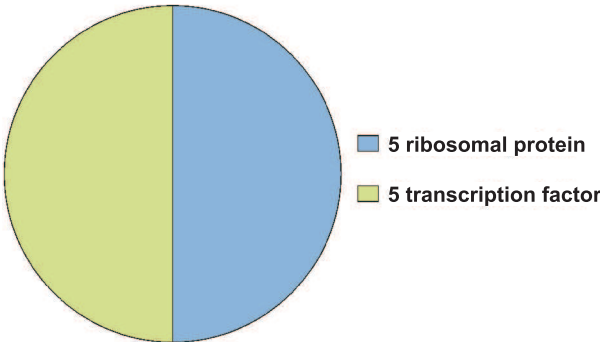
C RBPs specific for RAW 264.7 cells (32)



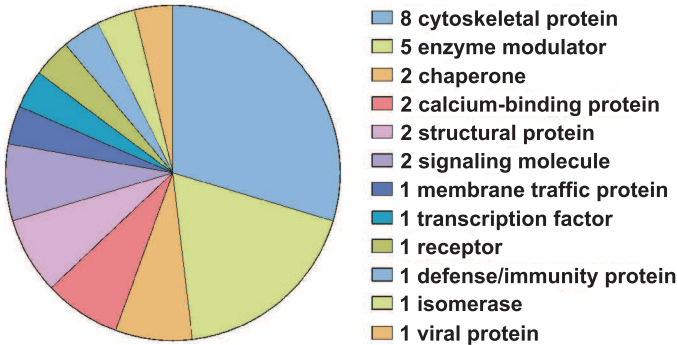
RBPs related to nucleic acid binding (41)



RBPs related to nucleic acid binding (13)



novel RBPs (28)



novel RBPs (19)

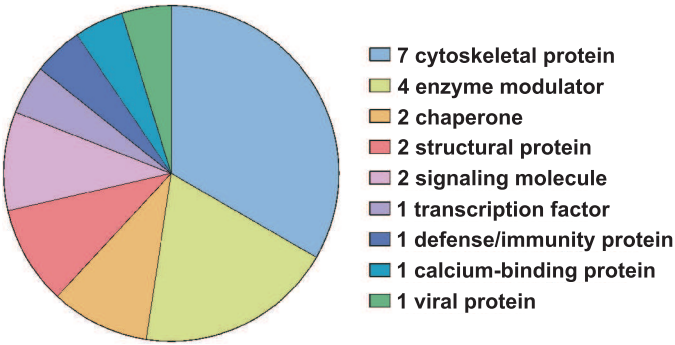
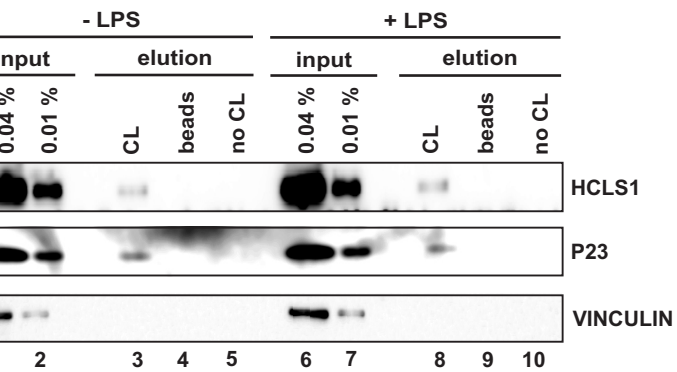
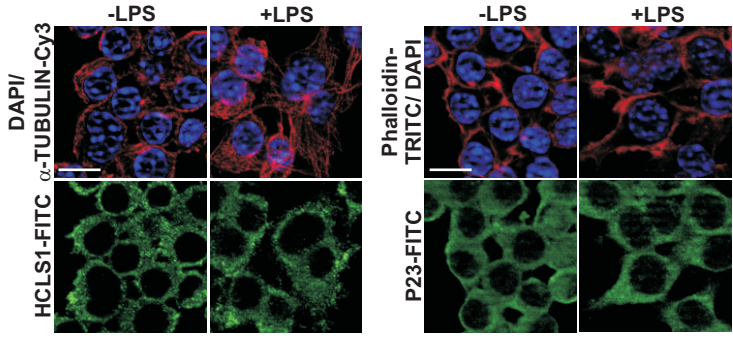


Figure 5



B



D

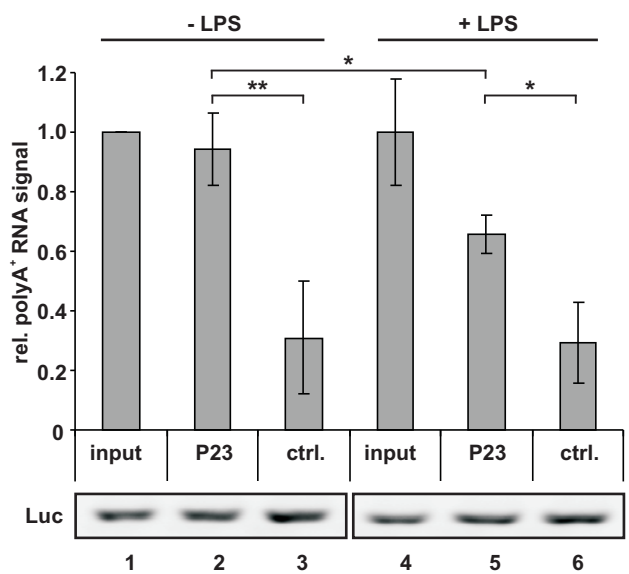
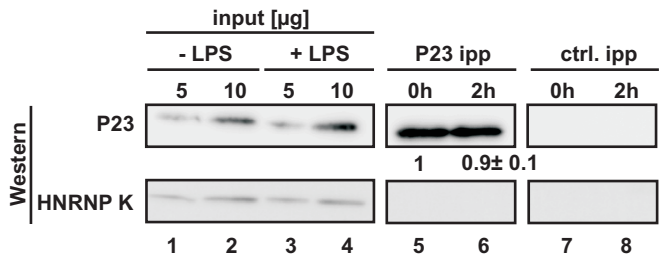
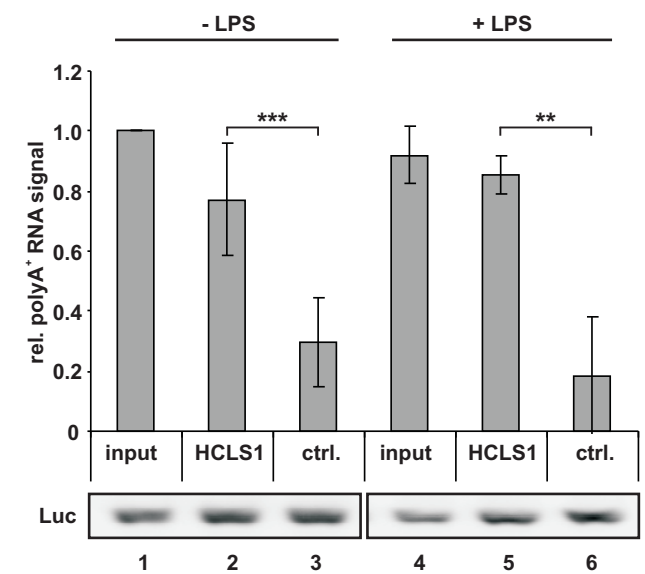
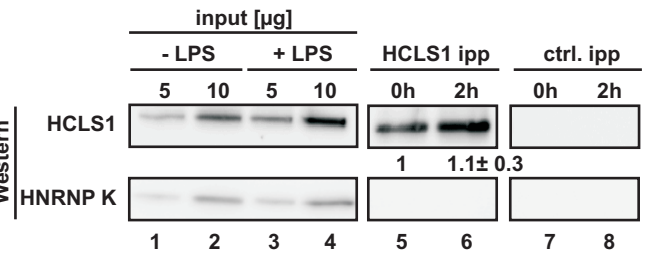


Figure 6

