TNIP2 is a Hub Protein in the NF-κB Network with Both Protein and RNA Mediated Interactions*

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The NF-κB family of transcription factors is pivotal in controlling cellular responses to environmental stresses; abnormal nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling features in many autoimmune diseases and cancers. Several components of the NF-κB signaling pathway have been reported to interact with the protein TNIP2 (also known as ABIN2), and TNIP2 can both positively and negatively regulate NF-κB-dependent transcription of target genes. However, the function of TNIP2 remains elusive and the cellular machinery associating with TNIP2 has not been systematically defined. Here we first used a broad MudPIT/Halo Affinity Purification Mass Spectrometry (AP-MS) approach to map the network of proteins associated with the NF-κB transcription factors, and establish TNIP2 as an NF-κB network hub protein. We then combined AP-MS with biochemical approaches in a more focused study of truncated and mutated forms of TNIP2 to map protein associations with distinct regions of TNIP2. NF-κB interacted with the N-terminal region of TNIP2. A central region of TNIP2 interacted with the endosomal sorting complex ESCRT-I via its TSG101 subunit, a protein essential for HIV-1 budding, and a single point mutant in TNIP2 disrupted this interaction. The major gene ontology category for TNIP2 associated proteins was mRNA metabolism, and several of these associations, like KHDRBS1, were lost upon depletion of RNA. Given the major association of TNIP2 with mRNA metabolism proteins, we analyzed the RNA content of affinity purified TNIP2 using RNA-Seq. Surprisingly, a specific limited number of mRNAs was associated with TNIP2. These RNAs were enriched for transcription factor binding, transcription factor cofactor activity, and transcription regulator activity. They included mRNAs of genes in the Sin3A complex, the Mediator complex, JUN, HOXC6, and GATA2. Taken together, our findings suggest an expanded role for TNIP2, establishing a link between TNIP2, cellular transport machinery, and RNA transcript processing. Molecular & Cellular Proteomics 15: 10.1074/mcp.M116.060509, 3435–3449, 2016.

The Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway is used to orchestrate cellular responses to environmental changes. Signals originating from a variety of sources control the localization of a family of transcription factors (NFκB1, NFκB2, REL, RELA, and RELB), and hence control the transcription of their target genes. One NFκB1 (p105) interacting protein, TNIP2 (also known as ABIN2), was initially identified in a yeast two hybrid screen for binding partners of a negative regulator of NF-κB signaling, A20 (also known as TNFAIP3) (1). As well as binding to A20, TNIP2 can inhibit NF-κB signaling (1). Curiously, TNIP2 may also play a role in activation; the C-terminal half of TNIP2 fused to a DNA binding domain (2) can translocate to the nucleus (2) and activate expression of a reporter gene in human cells. One model has proposed that N-terminal half of

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The abbreviations used are: NF-κB, Nuclear Factor kappa-light-chain-enhancer of activated B cells; AHD, ABIN homology domain; AP-MS, Affinity Purification Mass Spectrometry; CAM, Chloroacetamide-N-methylol; CMV, Cytomegalovirus; DAPI, 4',6-diamidino-2-phenylindole; DNA/cDNA, Deoxyribonucleic acid/Complementary DNA; dNSAF, Distributed Normalized Spectral Abundance Factor; EABR, ESCRT and ALIX binding region; ESCRT, Endosomal sorting complexes required for transport; FDR, False Discovery Rate; FPLC, Fast protein liquid chromatography; HEK293T, Human Embryonic Kidney 293 cells expressing the SV40 T antigen; IκB, Inhibitor of NF-κB; KH domain, Heterogeneous nuclear ribonucleoprotein K homology domain; MudPIT, Multidimensional Protein Identification Technology; PLGEM, Power Law Global Error Model; qPCR, Quantitative polymerase chain reaction; RNA/mRNA, Ribonucleic acid/messenger RNA; RNase, Ribonuclease; RP, Reversed Phase; SCX, Strong Cation Exchange; Si21, Spodoptera frugiperda 21 cells; TCEP, Tris(2-carboxyethyl)phosphine; TEV, Tobacco etch virus; Tris, Tris(hydroxymethyl)aminomethane.
the endogenous TNIP2 might normally interact with a cytoplasmic protein, preventing such nuclear localization, hence controlling TNIP2’s activation activity (2), although a signaling mechanism controlling this has not been established. Recent work has also shown that when the NF-κB pathway is stimulated by overexpression of the regulatory kinase CHUK (also known as IKKα), TNIP2 can act as a positive regulator of NF-κB target genes by activating CHUK (3).

Some physical associations between TNIP2 and other proteins have previously been investigated. Importantly, N-terminal TNIP2 sequences are required for an interaction between TNIP2 and the NFKB1 protein p105 (p105 functions both as a precursor to the NFKB1 transcription factor p50, and also as an inhibitor of NF-κB signaling) (4). Together, TNIP2 and p105 can form a ternary complex with MAP3K8 (also known as Tp2) (4, 5). There is also evidence that TNIP2 may interact with several other proteins: STK11, SMARCD1, and TEK (6); and TNIP2 binds to ubiquitin via a ubiquitin binding domain (UBD) (7). A comprehensive affinity purification-based proteomics approach to explore interactions between FNK12 and other cellular proteins.

screens (2, 6, 7); exogenously coexpressed TNIP2 and IKBKG copurify (8); and TNIP2 binds to ubiquitin via a ubiquitin binding domain (UBD) (9). A comprehensive affinity purification-based proteomics approach to explore interactions between TNIP2 and other cellular proteins.

Having established TNIP2 as an NF-κB network hub protein, we found that in addition to NFKB1 (p105), our TNIP2 preparations contained: components of the ESCRT-I complex, the 220 kDa protein YLPM1, and a number of RNA binding proteins that associate separately with YLPM1. In order to define the parts of TNIP2 needed for association with these factors more precisely, we used TNIP2 mutants to determine the approximate regions of TNIP2 required for the associations, and thus extended and refined the TNIP2 protein interaction network. Our results suggest that TNIP2 interacts with ESCRT-I complex via the TSG101 subunit— the ESCRT-I/TSG101 machinery is involved in a number of cellular processes including vacuolar protein sorting and HIV-1 viral budding (10). Lastly, we determined that the short region of TNIP2 required for its association with both the ESCRT-I complex and YLPM1 (and associated proteins), also copurifies mRNA transcripts that code for proteins involved in regulating transcription. Taken together these findings suggest an expanded role for the NF-κB network hub protein TNIP2, and lay the groundwork for future investigations into TNIP2 function.

EXPERIMENTAL PROCEDURES

Materials— Anti-CP56 rabbit polyclonal (SAB4200258), anti-FLAG® M2 mouse monoclonal (F3165), and anti-α-Tubulin mouse monoclonal (T9026) antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Anti-p105 (ab7971), anti-TJIP2 (ab130663), anti-TSG101 (ab30871), and anti-TNIP2 (ab13683) rabbit polyclonal antibodies, and anti-GAPDH (ab9484) mouse monoclonal antibodies were from Abcam (Cambridge, UK). Anti-TNIP2 (NPB1-32689) and anti-YLPM1 (NBPM2-22326) rabbit polyclonal antibodies were from Novus Biologicals (Minneapolis, MN). Anti-Halo rabbit polyclonal antibody (G9281) and Magne™ HaloTag® magnetic affinity beads were purchased from Promega (Madison, WI). The following clones from the Kazusa DNA Research Institute (Kisarazu, Chiba, Japan) were used: NFκB1 (AB489154), NFκB2 (AB334801), REL (AB463450), RELA (AB464351), Halo-TNIP2 (FHC21846), and Halo-TSG101 (FHC07226). In addition, we used Halo-REL (EX-G0029-M49) and Halo-YLPM1 (EX-E2833-M49) from GeneCopoeia™ (Rockville, MD).

Construction of Vectors for Expression of Affinity Tagged Proteins in Human Cells—Construction of pcDNA5-FLAG, pcDNA-Halo, and CMV-δ pcDNA-Halo has been previously described (11, 12). The vector pCLIP-FLAG was constructed by inserting a PCR product (containing a flag-tag and SgfI and PmeI restriction sites) into the multiple cloning site of the pCLIP-FLAG vector (New England Biolabs, Ipswich, MA), using the primers listed in the supplemental Data S1. For the experiments described in Fig. 1, ORFs coding for NFKB1, NFKB2, REL, RELA, and RELB were subcloned into pcDNA5-Halo, essentially as described previously (11). Vectors expressing Halo-TNIP2 1–195 and Halo-TNIP2 1–346 (described in Fig. 2A) were constructed by using site directed mutagenesis of Halo-TNIP2 (FHC21846) to insert premature stop codons. Vectors expressing the Halo tag in tandem with other regions of the TNIP2 ORF (described in Fig. 2A and Fig. 3) were constructed using the primers described in supplemental Data S1 to generate PCR products, which were subcloned into either pFN21K (Halo-TNIP2 253–429 and Halo-TNIP2 196–346) or Halo-pcDNA5 (Halo-TNIP2 196–429 and TNIP2 Y230A). CLIP-TSG101 (Fig. 4) and FLAG-TNIP2 196–429 (supplemental Fig. S4) were constructed by subcloning sequences coding for either TSG101 (FHC07226) or TNIP2 (FHC21846) into pCLIP-FLAG or pcDNA5-FLAG respectively.

Construction of Stable Cell Lines— The construction of the Flp-In™-293 cell line stably expressing Halo-TNIP2 has been previously described (12). A Flp-In™-293 cell line stably expressing Halo-TNIP2 (196–429 was constructed using the Flp-In™ system (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions (manual 25–0306). In brief, a DNA sequence coding for TNIP2 196–429 was first subcloned into CMV-δpcDNA-Halo, which contains both a hygromycin resistance gene and two Flp recombination target (FRT) sites. The resulting vector was cotransfected into Flp-In™-293 cells (also containing FRT sites) with a vector expressing Flp recombinase (pOG44), and the cells were grown in media containing hygromycin B. Expression of Halo-TNIP2 196–429 in the resulting colonies of hygromycin-resistant cells was confirmed by Western blotting.

Preparation of Whole Cell Extracts from Human Cells—Approximately 2 × 107 HEK293T or HeLa cells were transfected with DNA constructs encoding Halo or FLAG tagged proteins indicated in the figures using Lipofectamine LTX (Thermo Fisher Scientific). Cells were harvested ~48 h after transfection. Alternatively, 1 × 108 Flp-In™-293 cells stably expressing Halo-tagged proteins were cultured for 72 h and harvested. Cells were washed twice in ice-cold PBS, and the cell pellets frozen at −80 °C and thawed. Cells were then resuspended in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton® X-100, 0.1% sodium deoxycholate, 0.1 mM benzamidine HCl, 55 μM phenanthroline, 10 μM bestatin, 20 μM leupeptin, 5 μM pepstatin A, and 1 mM PMSF. Cells were then passed through a 26-gauge needle 5 times and insoluble material was removed by centrifuging the lysates at 21,000 × g for 30 min at 4 °C. The resulting 300 μl whole cell extracts from transiently transfected cells were then diluted with 700 μl of PBS (50 mM Tris-HCl pH 7.4, 157 mM NaCl, 2.7 mM KCl);
1000 µl whole cell extracts from stable cell lines were used without dilution. Extracts were centrifuged at 21,000 × g for 10 min at 4 °C to remove insoluble material.

Expression of Recombinant Proteins in Insect Cells—Sequences coding for either Halo-TNIP2, FLAG-TSG101, or FLAG-mCherry (control) were first cloned into pBacPAK8 (Takara Biotechnology, Mountain View, CA). The Baculovirus Expression System (Takara Biotechnology) was used to generate recombinant baculoviruses according to the manufacturer’s instructions. SI21 cells were cultured in SF9-900 III serum free medium (Thermo Fisher Scientific) at 27°C. Flasks containing ~1 × 10^6 SI21 cells were infected with the viruses indicated in Fig. 4B and harvested 48 h after infection. Cells were lysed in 10 ml of ice-cold buffer containing 50 mM Tris, 0.15 M NaCl, 5 mM MgCl₂, 0.2% Triton X-100, 20% (v/v) glycerol, 0.1 mM benzamidine HCl, 55 µM phenanthroline, 10 µM bestatin, 20 µM leupeptin, 5 µM pepstatin A, and 1 mM PMSF. Lysates were centrifuged at 20,000 × g for 30 min at 4 °C to remove insoluble material.

Halo Affinity Purification of Protein Complexes—Whole cell extracts containing the Halo tagged bait protein were incubated with beads prepared from 100 µl Magne® T1 HaloTag® bead slurry according to the manufacturer’s instructions for 2 h at 4 °C. The beads were washed four times with buffer containing 50 mM Tris-HCl pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 0.05% Nonidet® P40. Bound proteins were eluted by incubating the beads with buffer containing 50 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.005 mM DTT, and 2 Units AcTEV™ Protease (Thermo Fisher Scientific) for 2 h at 25 °C. Residual traces of beads were removed by centrifuging the eluates through Micro Bio-Spin columns (Bio-Rad, Hercules, CA) prior to further analysis.

FLAG Immunoprecipitation of Protein Complexes—Whole cell extracts containing the FLAG tagged bait protein were incubated with 50 µl anti-FLAG (M2) agarose beads for 2 h at 4 °C. The beads were washed four times with buffer containing 50 mM Tris-HCl pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 0.05% Nonidet® P40. Bound proteins were eluted by incubating the beads overnight at 4°C with 100 µl TBS containing 0.3 mg/ml 3× FLAG peptide. Residual traces of beads were removed by centrifuging the eluates through Micro Bio-Spin columns (Bio-Rad) prior to further analysis.

MudPIT Analysis of Protein Complexes—Halo purified proteins were precipitated with trichloroacetic acid, the resulting pellet was washed twice with acetone, and proteins were resuspended in buffer containing 100 mM Tris-HCl pH 8.5 and 8 µl urea(13). Samples were incubated with 0.5 mg/ml tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) to reduce disulfide bonds and then with chloroacetamide (CAM) to prevent disulfide bond reformation. Proteins were then digested with endoproteinase Lys-C; the urea concentration was reduced to 2 M by addition of a suitable volume of 100 mM Tris-HCl pH 8.5, and proteins were further digested with trypsin overnight. Samples were then loaded onto three phase fused silica microcapillary columns (reversed phase (RP); strong cation exchange (SCX); reversed phase (RP)). Peptides were gradually eluted from the column using a quaternary FPLC pump (Agilent, Santa Clara, CA) in 10 – 2 h MudPIT steps as described previously(13). Peptides were analyzed using a linear ion trap (LTQ) mass spectrometer (Thermo Fisher Scientific) in positive ion mode. An in-house software package, RAWD-istiller v. 1.0, was used to convert the resulting .raw files to .ms2 files. MS/MS spectra were then matched to 29,375 human protein sequences (Uniprot) were first reported is included in the mass spectrometry data sets used here and details of where they were first reported is included in supplemental Table S2. Only fully tryptic peptides were considered. Using these selection criteria, the average spectral FDR for the 92 MudPIT runs was 0.28% ± 0.31% (standard deviation), and the average protein FDR was 2.62% ± 1.83% (standard deviation). Proteins that were subsets of others were removed using the parsimony option in Contrast. Peptides that were identified by the same set of peptides (including at least one peptide unique to the set to distinguish between isoforms) were grouped together; one representative accession number was used to describe the set. We have reported some of the mass spectrometry data used in this study previously (11, 12); a list of all the mass spectrometry data sets used here and details of where they were first reported is included in supplemental Table S2. For experiments using transiently transfected cells, the numbers of biological replicates used for each bait was as follows: 11 (Halo-control); 5 (Halo-REL); 6 (Halo-RELA); 5 (Halo-RELB); 5 (Halo-NFKB1); 5 (Halo-NFKB2); 5 (Halo-TNIP2); 4 (Halo-TNIP2 196C); 5 (Halo-TSG101); 3 (Halo-YLPM1); 3 (Halo-YLPM1 Y230A); 3 (Halo-TNIP2 1–195 #1); MudPIT run failure occurred either when the microcapillary column became clogged (3 samples) or when the end of the column became misaligned with the mass spectrometer during the run (1 sample).

Fluorescence Microscopy—For experiments using transiently transfected cells, HEK293T cells were seeded onto glass bottom culture dishes (MatTek, Ashland, MA) and transfected with the Halo-tagged or CLIP-tagged constructs indicated (Fig. 2C and Fig. 4C). Affinity tagged proteins were fluorescently labeled during growth, with either Halo-Tag TM Direc ligand (Promega), with CLIP-Cell505 ligand (New England Biolabs), or with both ligands according to the manufacturer’s instructions. For the experiment of Fig. 2C row 3, cells were fixed prior to imaging in a solution containing 4% paraformaldehyde, 200 mM sucrose, and 1× PBS for 10 min at room temperature; cells were then washed twice with 1× PBS. DNA was stained with either DAPI (fixed cells) or Hoechst dye (live cells). Images were
collected using an UltraVIEW spinning disk confocal microscope (Perkin Elmer, Waltham, MA).

NanoBRET Assay—Transfection master mixes were prepared by mixing NanoLUC-TNIP2 donor plasmid with TSG101-HaloTag acceptor plasmid (1:2 ratio), NanoBRET Control Vector, or control pSPORT6 at a total of 50 ng with 0.1 μl X-tremeGENE HP (Roche, Indianapolis, IN) lipid in phenol red and serum free DMEM (Lonza, Basel, Switzerland) with a final volume of 20 μl. Transfection mix was dispensed into a 384w white opaque tissue culture treated microwell plate (Corning, Corning, NY) and incubated at room temperature for 20 min. 293T (ATCC, Manassas, VA) cells were suspended at a concentration of 250,000 cells/ml in phenol red and serum free DMEM containing 20% charcoal-stripped FBS (Corning) and 20 μl was dispensed into each well. Cells were incubated at 37°C and 5% CO₂ for at least 24 h.

NanoBRET 618 ligand (Promega) was diluted to 200 nM in phenol red and serum free DMEM just prior to adding 2 μl of NanoBRET 618 ligand or DMSO control to each well. Cells were incubated at 37°C and 5% CO₂ for 4 h. A 30 μM NanoBRET NanoGlo stock solution (Promega) was prepared in phenol red and serum free DMEM prior to adding 20 μl to each well. Plates were plated on a plate shaker with gentle agitation for 5 min at room temperature. Plates were then incubated for 5 min at room temperature to allow to equilibrate. Plates were read on an Envision Multimodal reader using the time-resolved fluorescence intensity mode with the 460 nm emission filter (donor signal) (PerkinElmer), Chroma ET610lp emission filter (acceptor signal) and luminescence dual mirror (PerkinElmer).

RNA Pull-down Assays—Approximately 1 × 10⁸ transiently transfected HEK293T cells were lysed in 300 μl of ice cold buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton® X-100, 0.1% sodium deoxycholate, 0.1 mM benzamidine HCl, 55 μM phenanthroline, 10 μM bestatin, 20 μM leupeptin, 5 μM pepstatin A, 1 mM PMSF, 1 mM diithiothreitol, and 0.4 μl RNaseOUT™ (Thermo Fisher Scientific). The lysates were then passed through a 26-gauge needle 5 times, centrifuged at 21,000 × g at 4°C for 30 min, and the resulting supernatant was diluted with 700 μl of TBS (50 mM Tris-HCl pH 7.4, 137 mM NaCl, 2.7 mM KCl). Protein complexes were purified from 900 μl of whole cell extract using HaloTag® magnetic affinity beads essentially as described above; complexes were eluted with 2 Units AcTEV™ Protease (Thermo Fisher Scientific) in 100 μl buffer containing 50 mM Tris-HCl pH 8.0, 0.5 mM EDTA, and 0.005 mM DTT and 40 Units of RNaseOUT™. Total RNA was prepared from either 100 μl of the whole cell extract or from 90 μl of the Halo purified samples using RNeasy® Mini kits (Qiagen, Germantown, MD) according to the manufacturer’s instructions. Residual traces of DNA were removed during RNA purification with Dnase I. RNA was reverse transcribed using iScript™ Reverse Transcription Supermix (Bio-Rad) and the resulting cDNA was analyzed either by PCR followed by agarose gel electrophoresis (Fig. 5B), or by qPCR using a MyiQ™ real-time detection system (Bio-Rad) (Fig. 5C). The PCR primers used for analyzing the cDNA are listed in supplemental Data S1. RNA sequencing was performed essentially as described previously (11). The sequenced libraries were aligned to the UCSC hg19 reference genome with Tophat (19). RNA-seq data sets have been deposited to the NCBI GEO (Gene Expression Omnibus) repository under the accession number: GSE79656.

RESULTS

Halo/MudPIT Analysis of NF-κB Family Transcription Factors—Previously, we developed a workflow which we had used to investigate RELA associated proteins using Halo/MudPIT AP-MS (affinity purification mass spectrometry) analysis (11) (this workflow is outlined in Fig. 1A—modified version of Fig. 1A in our previous work (11) shown here for clarity). To develop the network of protein interactions between all of the NF-κB transcription factors, we further expressed Halo-tagged versions of the other NF-κB transcription factors (NFκB1, NFκB2, REL, and RELB) in HEK293T cells. Protein complexes purified from cells using Halo affinity chromatography were analyzed by MudPIT mass spectrometry as described above (a list of the proteins identified in the NF-κB Halo/MudPIT analyses, as well as a description of which of our purifications were first reported previously (11), are listed in supplemental Table S2). The NF-κB protein interaction network that we constructed using all five bait proteins is shown in Fig. 1B, and reveals several proteins that associate with more than one of the NF-κB transcription factors. Among the network hub proteins, we identified the IκB proteins (NFKBIA, NFKBIB, and NFKBIE), which have been reported to bind to and inhibit NF-κB dimers by sequestering them in the cytoplasm (20). Consistent with a previous study by Bouwmeester et al. (5), we also identified a network hub protein TNIP2 (also known as ABIN2 or FLIP1) in our preparations of Halo-NFκB1, Halo-REL, and Halo-RELA (Fig. 1B). Although TNIP2 has previously been shown to interact with unprocessed NFκB1 (p105) (4) and can regulate NFκB activity (7), interactions between TNIP2 and other cellular components have not been fully characterized.

TNIP2 Associates with the ESCRT-I complex and mRNA Processing Factors—Having identified TNIP2 as a hub protein in our NF-κB network, we wanted to investigate the association between TNIP2 and other groups of proteins. We had recently identified 98 proteins (FDR < 0.05) that associated with Halo-TNIP2 purified from transiently transfected HEK293T cells (12) (reproduced for clarity in supplemental Table S3). We now analyzed the gene ontology terms associated with these proteins to identify groups of proteins with a common biological function, and found that many TNIP2 associated proteins were involved in protein transport and mRNA metabolism (Fig. 2A). Specifically, in addition to NFKB1, TNIP2 purifications contained: subunits of the endosomal sorting complex ESCRT-I (10, 21–25) and its associated proteins PDCD6IP (26) (also known as ALIX) and TBK1 (27); the nuclear YLPM1 protein (also known as ZAP3) and its associated RNA binding protein KHDRBS1 (also known as Sam68) (28); the RNA splicing factor KHSRP (also known as KSRP) (29); the nuclear matrix protein MATR3 (which also binds to RNA) (30); and proteins involved in polyadenylating RNA transcripts (including CPSF6 and CPSF7) (31) (Fig. 2B).

Although transient overexpression of affinity tagged bait proteins is commonly used to investigate protein interactions, Gibson and coworkers have suggested that problems caused by overexpressing baits (for example improper protein localization) can be offset by engineering cell lines that stably express bait proteins at levels similar to the endogenous protein (32). We had previously constructed a cell line stably expressing Halo-TNIP2 in HEK293 cells under the control of the weaker CMVδ2 promoter at close to endogenous levels
Mapping Interactions for the TNIP2 Hub Protein

Fig. 1. Halo/MudPIT analysis of proteins associated with NF-κB family transcription factors. A, AP-MS workflow for the MudPIT analysis of proteins copurifying with a Halo tagged bait protein. Proteins purified from HEK293T cells expressing a Halo-tagged bait protein or the Halo tag alone (controls) are digested with trypsin and identified by MudPIT mass spectrometry. Only proteins detected in the majority of experimental samples are considered. The statistical algorithm PLGEM is used for quantitative comparison of experimental and control replicates and prey proteins likely enriched in experimental samples, but not controls, and are categorized according to their adjusted p values (FDRs). B, Endogenous TNIP2 copurifies with several NF-κB family transcription factors. Halo tagged versions of the five NF-κB family transcription factors (NFKB1, NFKB2, REL, RELA, and RELB, green circles) were expressed as baits for AP-MS experiments (supplemental Table S2). TNIP2 (pink circle) copurified with NFKB1, REL, and RELA (FDR < 0.01). Components of the IκB complex are shown in blue. The network layout was constructed using relative average dNSAF values for each interaction as an input for the "edge weighted spring embedded" model in Cytoscape (47).

(supplemental Fig. S1), and had also identified proteins present in complexes affinity purified from these cells (12) (we have reproduced this previous analysis in supplemental Table S3 for clarity). As with the transiently transfected cells, in these stably expressing Halo-TNIP2, we detected NFKB1, subunits of the ESCRT-I complex, and PDCD6IP, but we detected lower levels of MATR3 and failed to detect the other RNA processing proteins and YLPM1 that had copurified with transiently overexpressed TNIP2 (Fig. 2B).

These differences between the proteins copurifying with transiently overexpressed Halo-TNIP2 and stably expressed Halo-TNIP2 are consistent with a model for TNIP2 localization, proposed by Chien and coworkers (2, 7). They proposed that the localization of TNIP2 is regulated: TNIP2 is usually in the cytoplasm (they suggest that TNIP2 might normally be sequestered in the cytoplasm through an interaction between its N terminus and FLNA (2)); they found that removing the N terminus of TNIP2 caused partial relocalization to the nucleus (2, 7). Consistent with this we found that, whereas our full length stably expressed Halo-TNIP2 is detected primarily in the cytoplasm (Fig. 2C 1), a TNIP2 mutant that we constructed, which lacks the N terminus (Halo-TNIP2 196–429), was partially localized to the nucleus (Fig. 2C 2). In addition, we noticed that the overexpressed (full length) Halo-TNIP2 in our transiently transfected cells had a small amount of nuclear Halo-TNIP2 (Fig. 2C 3) in some of the cells that appeared to express the protein at particularly high levels. These observations are consistent with the model proposed by Chien et al. (2), with TNIP2 relocalizing to the nucleus as conditions change, and might explain why some TNIP2 associated proteins were only identified under conditions where Halo-TNIP2 was partially nuclear. To further test this idea, we analyzed the cells stably expressing a version of TNIP2 missing the N terminus (Halo-TNIP2 196–429) to test whether YLPM1 once again copurified with the bait (Fig. 2D, Supplemental Table S3). In contrast to full length Halo-TNIP2, Halo-TNIP2 196–429 stably expressed using the CMvd2 promoter associates not only with the ESCRT-I complex, but also with: YLPM1; the YLPM1 associated proteins KHSRP, MATR3, SFPQ, NONO, and PSCP1; and CPSF6/7 (Fig. 2D). This association between the partially nuclear TNIP2 196–429 and YLPM1/YLPM1 associated proteins is also consistent with the reported nuclear localization of YLPM1 (and associated proteins) in human cells (www.proteinatlas.org (33)).

NFKB1 and ESCRT-I Associate with Distinct Regions of TNIP2—TNIP2 is one of a family of three homologous proteins (also called ABINs) that was originally identified following a yeast two-hybrid screen for binding partners of TNFAIP3 (A20)
Fig. 2. **TNIP2 associated proteins.** A, TNIP2 associated proteins are enriched for mRNA processing factors and subunits of the ESCRT-I complex. Protein complexes were purified from HEK293T cells transiently transfected with a plasmid expressing Halo-TNIP2 (supplemental Table S3). TNIP2 associated proteins (FDR < 0.05) were used as an input for the bioinformatics discovery tool David version 6.7 (48). Enriched gene ontology terms (biological process domain) with an adjusted p value < 0.05 were visualized using “TreeMap” view of REVIGO (49). The size of each rectangle reflects the associated adjusted p value (larger areas for smaller p values). **B, D,** Copurification of ESCRT-I complex and YLPM1 associated proteins with TNIP2. TNIP2 associated proteins (FDR < 0.05, supplemental Table S3) were purified from cells expressing Halo-TNIP2 (full length) or Halo-TNIP2 196–429, either transiently transfected with a plasmid with a CMV promoter (high expression), or stably transfected with expression controlled by the CMVd2 promoter (low expression). Relative average dNSAF values have been calculated from five biological replicates (transient) or three biological replicates (stable); error bars are standard deviation. C, Subcellular localization of Halo-TNIP2 in human cells. Cells were stably or transiently transfected with either: 1 & 3 Halo-TNIP2 1–429 (full length) or 2 Halo-TNIP2 196–429. Halo-tagged proteins have been labeled with Halo-Tag TMRDirect fluorescent ligand (red) and DNA (nuclei) stained with either Hoechst dye or DAPI (blue).

(34, 35). The three proteins share domains of homology termed ABIN homology domains (AHD). The 429 amino acid TNIP2 protein contains three AHD domains, as well as a ubiquitin binding domain or UBD (Fig. 3A). TNIP2 also contains a region with homology to the ESCRT- and ALIX-binding region (EABR) within the protein CEP55. In CEP55, this EABR region mediates an interaction between CEP55 and the TSG101 subunit of the endosomal sorting complex ESCRT-I (36, 37). Nonetheless, interactions between TNIP2 and the ESCRT-I complex have not previously been empirically characterized. Having identified a number of factors copurifying with the full length TNIP2 protein, we sought to define the regions of TNIP2 that might be important for the association between these factors and TNIP2 more precisely. To do this we first subcloned Halo-tagged versions of the regions of the TNIP2 ORF described in Fig. 3A into mammalian expression vectors for expression in HEK293T cells (the Halo-TNIP2 1–429 and Halo-TNIP2 253–429 constructs were also used in a previous study (12)). Of the proteins previously highlighted as interacting with TNIP2 (Fig. 2), those which consistently purified with these various TNIP2 mutants (FDR < 0.05) are described in Fig. 3B. The complete lists of TNIP2 associated factors for each TNIP2 mutant analysis are listed in supplemental Table S4 (again we have included our previously reported analysis of proteins associated with Halo TNIP2 1–429 and Halo-TNIP2 253–429 in this table for clarity (12)).
sistent with a previously characterized interaction between NFKB1 and TNIP2 (4), endogenous NFKB1 did not copurify with TNIP2 mutants that lacked the N-terminal 195 amino acids (Fig. 3B, lanes 5–7) but did copurify with mutants which included these amino acids (Fig. 3B, lanes 1–4). To test whether the annotated EABR region within TNIP2 was important for the association between TNIP2 and the ESCRT-I complex, we constructed a point mutant, Halo-TNIP2 Tyr230Ala. Lee and coworkers (37) had identified Tyr187 within the EABR of CEP55 as a key residue for the interaction between CEP55 and the ESCRT machinery. We identified Tyr230 as the equivalent residue within the EABR of TNIP2 (outlined in supplemental Data S1). Halo-TNIP2 Tyr230Ala failed to associate with the ESCRT-I complex (Fig. 3B, lane 2). This is consistent with a physical interaction between the EABR region of TNIP2 and the ESCRT-I complex. In addition we observed a decrease, but not a complete loss, of the amounts of YLPM1/associated proteins copurifying with Halo-TNIP2 Tyr230Ala compared with Halo-TNIP2 (compare Fig. 3B, lanes 1 and 2). A 151 amino acid region within TNIP2 that includes the EABR (Halo-TNIP2 196–346) associated with both the ESCRT-I complex and YLPM1 and its associated RNA binding proteins (Fig. 3B, lane 5), and an overlapping region that did not contain the EABR but did include two ABIN homology domains (Halo-TNIP2 253–429), bound neither the ESCRT-I complex nor YLPM1. This suggests that regions within TNIP2 196–252 are necessary for the associations between TNIP2 and both ESCRT-I and YLPM1 (Fig. 3B, lane 6).

To provide additional evidence that distinct regions of TNIP2 were important for its association with NFKB1 and the ESCRT-I complex, we purified proteins associated with our various TNIP2 baits again, and this time fractionated the eluates using SDS-PAGE and visualized proteins either by

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**Fig. 3.** Distinct regions of TNIP2 are used for interactions with either NFKB1 or the ESCRT-I complex. A, Features of TNIP2 (NP_077285.3, also known as ABIN2) include three ABIN homology domains, a putative ESCRT/ALIX binding region (EABR), and a ubiquitin binding domain (UBD). Halo-tagged versions of the seven TNIP2 regions indicated were constructed for transient expression in AP-MS experiments. B, Average relative dNSAF values (see supplemental Table S4) for the indicated prey proteins (FDR < 0.05) copurifying with the TNIP2 bait proteins described in A. Results are from 7, 3, 3, 5, 7, 5, or 4 biological replicates of the seven bait proteins shown. C, The recombinant Halo-tagged proteins indicated were expressed in HEK293T cells and purified, together with endogenous associated proteins, using Halo affinity chromatography. Eluates were analyzed using SDS-PAGE, and proteins visualized by silver staining to confirm the presence of the bait. SDS-PAGE fractionated eluates were also analyzed by Western blotting using antibodies specific to TSG101, YLPM1, or CPSF6 as indicated.
silver staining or by Western blotting (Fig. 3C). As before, we detected NFKB1 in TNIP2 purifications that included the region TNIP2 1–195 (Fig. 3C lanes 1–4), and we detected TSG101 (an ESCRT-I subunit), YLPM1, and CPSF6 in purifications that included the region TNIP2 196–346 (Fig. 3C lanes 1, 3, 5, and 7). A control experiment using antibodies to a protein not detected in TNIP2 complexes by mass spectrometry (TJP2) indicated that we were not detecting proteins nonspecifically in our Western blot analysis (supplemental Fig. S2).

To further validate our results, we performed additional similar experiments using either a different cell type or a different purification method. First, we expressed either Halo-TNIP2 or Halo–TNIP2 196–429 in HeLa cells and performed Halo pulldown experiments (supplemental Fig. S3). As with HEK293T cell extracts, we found that in HeLa cell extracts, endogenous YLPM1 copurified with both the full length and the truncated versions of TNIP2. Also consistent with our HEK293T cell observations, we detected NFKB1 (p105) co-purifying with full length TNIP2, but not with TNIP2 196–429. Second, we expressed FLAG-TNIP2 196–429 in HEK293T cells and performed FLAG coimmunoprecipitation experiments (supplemental Fig. S4). As they had with Halo-TNIP2 196–429, YLPM1, and TSG101 copurified with FLAG-TNIP2 196–429.

TNIP2 Interacts with ESCRT-I Subunit TSG101—As we had observed that TNIP2 association with the ESCRT-I complex was disrupted by mutating a single amino acid within the EABR, we wanted to investigate whether TNIP2 was physically interacting with an ESCRT-I subunit. Lee and coworkers had previously demonstrated a direct interaction between the EABR domain of CEP55, and the ESCRT-I subunit TSG101 (37), so we decided to examine whether TNIP2 similarly associates with the ESCRT-I subunits via TSG101. We first asked whether recombinant TSG101 could associate with other components of the ESCRT-I complex in human cells. Using recombinant Halo-TSG101 expressed in HEK293T cells, we were able to pull down the other ESCRT-I subunits that we had observed copurifying with TNIP2 (Fig. 4A, purple bars). None of the other TNIP2 associated proteins that we had focused on copurified with Halo-TSG101. In addition, when we used Halo–YLPM1 as bait for AP-MS studies, we found that endogenous ESCRT-I did not copurify with YLPM1. Interestingly, we did identify a number of the other TNIP2 associated proteins in Halo–YLPM1 purifications, which we now refer to as YLPM1 associated proteins (Fig. 4A, red bars). These include: the KH domain containing, RNA binding proteins KHSRP and KHDRBS1 (38, 39), and the nuclear paraspeckle components PSPC1, NONO, and SFPQ (40), together with MATR3 (MATR3 has also previously been found in a complex with SFPQ/NONO (41)). Having observed an association between Halo–TSG101 and components of the ESCRT-I complex in human cells, we decided to examine whether recombinant TSG101 and TNIP2 proteins could interact in the absence of other human proteins that might mediate an interaction. We infected Sf21 insect cells with recombinant baculoviruses coding for FLAG-TSG101 and Halo–TNIP2, or with FLAG-TSG101 alone, and subjected the resulting whole cell extracts to Halo affinity purification (Fig. 4B). FLAG-TSG101 copurified with Halo–TNIP2 (lane 3); the interaction between TSG101 and TNIP2 appeared to be direct, as only TEV protease and very small amounts of other proteins were detected in our TNIP2/TSG101 preparation (lane 5). In a control experiment, Sf21 cell expressed FLAG–mCherry did not spuriously copurify specifically with Halo–TNIP2 (supplemental Fig. S5). In addition to using a biochemical approach to ask whether TNIP2 and TSG101 could interact in insect cell extracts, we used the NanoBRET™ protein-protein interaction system (42) to interrogate the interaction between TNIP2 and TSG101 in living cells. First, we confirmed that coexpressed TNIP2 and TSG101 both localized to the cytoplasm in HEK293T cells (Fig. 4C). Second, we expressed NanoLuc® tagged TSG101 (or TSG101 Tyr230Ala as a control), together with fluorescently labeled TSG101-Halo (or unlabeled TSG101-Halo as a control), and tested for bioluminescence resonance energy transfer (NanoBRET) in HEK293T cells (42). We detected similar levels of donor luminescence for NanoLuc®–TNIP2 and NanoLuc®–TSG101 Tyr230Ala samples (Fig. 4D). We detected a decrease in energy transfer to the TSG101-Halo fluorophore (BRET ratio) between the TNIP2 and TSG101 Tyr230Ala samples. This is consistent with an interaction between TNIP2 and TSG101 in living cells with reduced affinity between TNIP2 Tyr230Ala and TSG101. Both of these BRET ratios were larger than those detected in control samples with unlabeled TSG101 (Fig. 4D). We repeated the NanoBRET experiment using RPE-1 cells and obtained a similar result (supplemental Fig. S6). Taken together, the copurification of insect cell expressed proteins and the resonance energy transfer between proteins in cells suggest a direct interaction between TNIP2 and TSG101.

TNIP2 Associates with mRNA Transcripts—A number of the TNIP2 associated proteins had previously been reported to bind RNA, and so we investigated whether some of these proteins might be present in our TNIP2 preparations as a result of their interactions with RNA. To test this, we used the TNIP2 196–346 truncation mutant that we had earlier observed associating with ESCRT-I, the YLPM1 associated proteins, and CPSF6/7. When we purified Halo–TNIP2 196–346 associated proteins in the presence of Benzonase®, an endonuclease that digests both DNA and RNA, we detected the bait proteins shown in Fig. 5A (light gray bars). There was no significant change in the relative amounts of the subunits of the ESCRT-I complex detected after performing purifications with Benzonase (Fig. 5A). In contrast, although YLPM1 itself still copurified with Benzonase® treated Halo–TNIP2 complexes, we detected reduced amounts of some of the YLPM1 associated proteins (notably KHDRBS1) in the Benzonase® treated Halo–TNIP2 purifications. This would be consistent
**Fig. 4.** TNIP2 interacts with ESCRT-I complex via the TSG101 subunit.

A, Distinct sets of TNIP2 associated proteins copurify with TSG101 or YLPM1. HEK293T cells were transiently transfected with the indicated bait proteins. Relative average dNSAF values are shown for the indicated prey proteins (FDR < 0.05), calculated from 6 (TSG101) or 3 (YLPM1) biological replicates. Error bars represent standard deviation.

B, Interaction of TNIP2 and TSG101 in vitro. Lysates from Sf21 cells coinfected with baculoviruses encoding Halo-TNIP2 and/or FLAG-TSG101 were used for Halo affinity chromatography. Eluates were analyzed by SDS-PAGE and proteins detected either by Western blotting or staining with Coomassie Blue R-250. TNIP2 was detected with anti-TNIP2 polyclonal antibodies and IRDye® 800CW labeled anti-Rabbit secondary antibodies (green); FLAG-tagged proteins were detected with anti-FLAG (M2) monoclonal antibodies and IRDye® 680LT labeled goat anti-Mouse secondary antibodies (green). Note, the 33 kDa Halo tag is cleaved from the 49 kDa TNIP2 protein during purification.

C, Subcellular localization of ectopically expressed Halo-TNIP2 and CLIP-TSG101 in HEK293T cells. Cells were cotransfected with Halo-TNIP2 and CLIP-TSG101. Halo-tagged proteins have been labeled with Halo-Tag TMRDirect fluorescent ligand (red), CLIP-tagged proteins with CLIP-Cell505 fluorescent ligand (green) and DNA (nuclei) stained with Hoechst dye (blue).

D, Physical interaction between TNIP2 and TSG101 in living cells. NanoBRET™ protein interaction assays (42) were performed using HEK293T cells coexpressing TNIP2 (or TNIP2 Tyr230Ala) tagged with NanoLuc® (N terminus), and TSG101 tagged with a Halo tag (C terminus). The NanoLuc® tag (energy donor) can transfer energy to the fluorescently labeled Halo tag (acceptor) when they are brought into close proximity (42). Both raw donor luminescence values (in relative light units (RLU)), and BRET ratios are calculated from four biological replicates.
with, for example, KHDRBS1 associating with TNIP2 via interactions with RNA. Having observed a reduction in the amount of KHDRBS1 associated with TNIP2 196–346 purified in the presence of Benzonase®, we decided to test preparations of TNIP2 for the presence of known KHDRBS1-associated RNA transcripts. KHDRBS1 had previously been found to associate with mRNAs encoding for regulators of apoptosis (43), so we first tested the samples for the presence of Bcl-2 family mRNA transcripts reported to associate with KHDRBS1 (43). We purified Halo–TNIP2 196–429 in the presence of RNase inhibitors, and analyzed RNA extracted from the affinity purified samples by reverse transcription followed by PCR.

Fig. 5. **Copurification of mRNA with TNIP2 complexes.** A, Purification of TNIP2 complexes in the presence of nuclease. Lysates from HEK293T cells transiently transfected with Halo-TNIP2 196–346 were subjected to Halo affinity chromatography with (three biological replicates) or without (seven biological replicates) the endonuclease Benzonase®. Eluates were analyzed by MudPIT and relative average dNSAF values plotted for the indicated prey proteins (supplemental Table S5). Error bars are standard deviation. B, TNIP2 complexes contain KHDRBS1 associated mRNAs. Lysates from HEK293T cells transfected with Halo-TNIP2 196–429 or Halo-control (tag alone) were subjected to Halo affinity chromatography in the presence of RNase inhibitors. Total RNA was purified from the eluates and reverse transcribed. The resulting cDNA was analyzed for the presence of KHDRBS1 associated mRNAs by PCR followed by agarose gel electrophoresis essentially as described (43) (KHDRBS1 is also known as Sam68). Details of the primers used are in supplemental Data S1. C, mRNA copurifies specifically with the TNIP2 196–346 region. Halo purified protein complexes were purified from HEK293T cells transfected with the indicated proteins (three biological replicates). RNA extracted from these purifications was reverse transcribed and analyzed by quantitative PCR using the primers for the two transcript variants of BCL2L1 and GAPDH described in supplemental Data S1. The relative amounts of the proteins NFKB1, TSG101, YLPM1, and CPSF6 detected in similar purifications (see also Fig. 3B and supplemental Table S4) are shown for comparison.
(RT-PCR) (Fig. 5B); we detected PCR products consistent with BCL2L1, BCL2L11, and BCL2 mRNA transcripts (Fig. 5B). Having detected mRNA transcripts copurifying with TNIP2 196–420, we sought to further define which region of TNIP2 might be sufficient for its association with RNA. We isolated RNA from affinity purified Halo-TNIP2 1–429, Halo-TNIP2 1–195, Halo-TNIP2 196–346, and Halo-TNIP2 253–429, as well from controls expressing only the Halo tag. As we had previously detected BCL2L1 transcripts in the TNIP2 196–429 samples, we designed qPCR primers specific to the two BCL2L1 transcript variants (described in the supplemental Data S1), as well as for the housekeeping gene GAPDH. When we analyzed the samples by qPCR, we found very low levels or no RNA in Halo-TNIP21–195, Halo-TNIP2 253–429, or Halo-control purified samples (Fig. 5C). In contrast, we detected products corresponding to modest levels of mRNA in samples purified via full-length Halo-TNIP2, and to larger quantities of mRNA in Halo-TNIP2 196–346 purified samples (Fig. 5C). The region of TNIP2 196–346, which is sufficient for purifying mRNA transcripts, is also sufficient for purifying the ESCRT-I complex (including TSG101), YLPM1 and associated proteins, and the RNA processing factors CPSF6 and CPSF7, but not NFKB1 (Fig. 5C).

TNIP2 Associated Transcripts Code for Factors Controlling Transcription—As we had detected GAPDH transcripts in the TNIP2 purifications, we reasoned that TNIP2 associated RNA might not be limited to transcripts of BCL2 family proteins. One possibility was that TNIP2 associated non-specifically with all transcribed RNA species; alternatively, TNIP2 might be associating with a specific subset of these RNAs. To address this, we sequenced samples of RNA copurifying with Halo-TNIP2 196–346 (pulldown RNA), and RNA samples prepared from a portion of the whole cell extract prior to protein purification (input RNA). We identified 314 RNA species enriched in Halo-TNIP2 196–346 purified samples compared with input RNA \( p_{adj} < 0.01, \log_2 \text{fold change (pulldown/input)} > 2, \text{ mean FPKM (pulldown) > 20, supplemental Table S6}. \) This set of RNAs was significantly enriched for GO terms related to transcription factor binding (Fig. 6A, 6B), and also contained BCL2L11, which we had initially identified by qPCR. To corroborate these findings, we repeated our analysis but this time we compared Halo-TNIP2 196–346 purified RNA with Halo-control pulldown samples, rather than with input RNA samples (supplemental Table S6). We identified a similar set of 368 RNA species again enriched for GO terms related to transcription factor binding, with 290 of the RNA species identified by both analysis methods (supplemental Table S6). A list of the mRNA species associated with the gene ontology term “transcription factor binding” is provided in Fig. 6B. Included in this list are mRNAs coding for the Sin3A complex subunits SAP18 and SAP30, the Mediator complex subunits MED13 and MED30, the homeobox protein HOXC6, the transcription factor coded by the JUN proto-oncogene, and the transcription factor GATA2. We confirmed the presence of SAP30 and GATA2 transcripts in Halo-TNIP2 196–346 purified samples by qPCR (supplemental Fig. S7).

We had previously shown that common protein contaminants in protein purifications tend to be proteins that are highly expressed in cells (11). This might also be true for nonspecific RNA contaminants not adequately removed during washes. To assess whether our TNIP2 associated RNAs were simply a population of more abundant RNA species copurifying nonspecifically, we compared the distribution of RNA abundance for all RNAs in our whole cell extracts (Fig. 6C gray bars) with the distribution of RNA abundances for the set of TNIP2 196–346 associated RNAs (Fig. 6C green bars). The TNIP2 associated RNAs were not predominantly RNAs that are more abundant in cells and so are likely not simply RNA contaminants with high cellular abundance. In addition to identifying the transcripts associated with TNIP2, we looked at the distribution of the sequence reads across several TNIP2 associated transcripts to assess whether these transcripts were different in character to those in the whole cell extract. Depth graphs are shown for one of these, GATA2, in Fig. 6D (GATA2 has the largest value for \( \log_2 \text{fold change in the TNIP2 196–346 pulldown/control pulldown analysis}. \) The most significant TNIP2 196–346 enriched GATA2 sequences appear to correspond to GATA2 transcript exons (and specifically the exons present in transcript variant 3). This is consistent with an association between TNIP2 and spliced GATA2 transcripts (Fig. 6D lower graph).

DISCUSSION

This study identifies subunits of the ESCRT-I complex, together with YLPM1 associated factors, as proteins associated with TNIP2, a hub protein in an NF-κB transcription factor interaction network (Fig. 7). We have also shown that the ESCRT-I complex interacts with TNIP2 via the subunit TSG101 and that both the ESCRT-I complex and YLPM1 associated factors associate with amino acid sequences in a central region of TNIP2 (Fig. 7). This region does not include the N terminus of TNIP2, which is required for the TNIP2/ NFKB1 interaction (4). This central TNIP2 region also copurifies mRNAs that code for proteins that function as transcription factors, such as GATA2, or as transcriptional cofactors, such as subunits of the mediator complex. Consistent with this, many of the YLPM1 associated factors found in TNIP2 preparations are RNA processing proteins. Some of these proteins might copurify with TNIP2 because of interactions mediated by RNA (Fig. 7). Taken together, these findings establish a link between the NF-κB network hub protein TNIP2, the ESCRT-I complex, and a distinct set of mRNAs coding for transcriptional regulators (Fig. 7).

Previously, we had used an affinity purification mass spectrometry (AP-MS) approach, which used the Halo tag coupled with MudPIT mass spectrometry, to investigate proteins associating with two NF-κB factors, RELA and NFKB1 (11). Although AP-MS studies have been widely used for mapping
The networks of interactions between cellular proteins (44), mapping interaction networks centered on transcription factors has been particularly challenging (45), and so we first used a broad Halo/MudPIT approach to expand the NF-κB transcription factor interaction network. Having generated this expanded network, which included all five NF-κB family proteins, we selected TNIP2 as a network hub protein for more focused follow up studies to map TNIP2 associations at sub-protein resolution. Giambruno and coworkers have suggested using multiple complementary AP-MS strategies as a way of

Fig. 6. Sequencing analysis of RNA copurifying with Halo-TNIP2 196–346. A, Following RNA sequencing, 314 RNAs were identified as enriched in Halo-TNIP2 196–346 purifications compared with input RNA in R using the DESeq2 library (50) (padj < 0.01, log2 fold change (pulldown/input) > 2, mean FPKM (pulldown) > 20). Gene ontology terms associated with these RNAs were analyzed using David version 6.7 (48). Enriched gene ontology terms (molecular function domain) with an adjusted p value < 0.05 were visualized using “TreeMap” view of REVIGO (49), B, TNIP2 associated mRNA species with associated gene ontology term “transcription factor binding” (GO:0008134). C, Distribution of the cellular abundances of TNIP2 associated mRNAs. RNA purified from whole cell extracts of three biological replicates of cells transfected with Halo TNIP2 196–346 (input RNA) was sequenced and mean FPKM values calculated to assess the cellular abundance of the different RNA species. RNAs were binned according to FPKM values (x axis) with the number of RNA species in each bin on the y axis. Data for the set of all RNA species (gray bars) or the subset of the 314 TNIP2 196–346 associated RNAs (green bars) is shown. D, Analysis GATA2 RNA sequences from Halo-TNIP2 196–346 pulldowns. Mean depth graph profiles for GATA2 RNA sequences were generated using the Integrated Genome Browser (51). The y axis of these profiles represents the mean number of annotations aligned at each location from three biological replicates of Halo control (gray bars) or Halo TNIP2 196–346 samples (green bars). Results both for RNA purified from whole cell extracts (input) and for RNA from Halo purified samples (Halo pulldown) are shown. The pulldown/input depth graph ratio for the Halo-TNIP2 196–346 samples is also shown (red/brown bars).
increasing both the efficiency and the reliability of detecting interactions (45). Therefore, in order to help understand the role of TNIP2 in cells more clearly, we examined TNIP2 associated proteins in several ways. First, we used cells transiently transfected with Halo-TNIP2 expressed at relatively high levels; second, we used cells stably expressing Halo-TNIP2 expressed at near endogenous levels; and third, we used a series of TNIP2 truncation mutants for mapping interaction domains.

When we analyzed TNIP2 associated proteins purified from transiently transfected populations of cells heterogeneously expressing Halo-TNIP2 at relatively high levels, we found these samples were enriched both for subunits of the complex ESCRT-I, for the protein YLPM1, and for proteins involved in RNA processing (Fig. 2A and 2B). An association between TNIP2 and YLPM1 or between TNIP2 and RNA processing proteins has not been previously reported. In addition, although TNIP2 contains a conserved EABR domain (ESCRT and ALIX binding region), which is homologous to a region in a protein CEP55 that does bind to ESCRT-I (37), empirical evidence for an association between TNIP2 and the ESCRT-I complex has also not been previously reported.

To determine whether the level of Halo-TNIP2 expression affected the complement of proteins associating with TNIP2, we repeated the experiments with Halo-TNIP2 stably expressed at levels similar to the endogenous TNIP2 protein (transiently transfected cells can receive multiple copies of DNA coding for the bait protein resulting in high bait expression). At these lower levels of Halo-TNIP2 expression, we still found both NFkB1 and subunits of the ESCRT-I complex in TNIP2 purifications, but we could no longer detect YLPM1 or the YLPM1 associated RNA binding proteins associated with TNIP2. We hypothesized that this could be because of differences in Halo-TNIP2 localization. Previously, Liu and cowork-
ers had proposed a model in which TNIP2 was normally sequestered in the cytoplasm, but might, under certain physiological conditions, enter the nucleus and interact with nuclear proteins (7). Consistent with this idea, although we detected stably expressed Halo-TNIP2 primarily in the cytoplasm, we observed that some of the transiently transfected cells expressing Halo-TNIP2 at relatively high levels appeared to contain some Halo-TNIP2 in the nucleus (Fig. 2C). This pool of nuclear Halo-TNIP2 in these transiently transfected cells could be associating with nuclear YLPM1. As previous studies had established that TNIP2 mutants without the N terminus could localize to the nucleus (2), we next stably expressed Halo-TNIP2 196–421 at close to endogenous levels, reasoning that this TNIP2 mutant might once again associate with YLPM1. Indeed when we analyzed proteins associated with stably expressed TNIP2 196–421, we once again detected YLPM1 and other YLPM1 associated proteins (Fig. 2D).

Intriguingly, both our Halo-TNIP2 and Halo-YLPM1 purifications contain the core paraspeckle proteins NONO, SFPQ, and PSPC1 (Fig. 7). Paraspeckles are nuclear RNP structures that influence gene expression by controlling the nuclear retention of RNA transcripts (40). In addition to these paraspeckle proteins, TNIP2 mutants that included a central region containing amino acids 196–346 also associated with mRNA transcripts. The association with such transcripts appeared to be specific, for example with the set of TNIP2 associated mRNAs enriched for species with the GO term “transcription factor binding.” These include mRNAs coding for DNA binding transcription factors such as GATA2, JUN and HOXC6, as well as mRNAs coding for subunits of complexes that function as transcriptional coactivators like Mediator, or as corepressors like the SIN3A complex. It is possible that TNIP2 has a role in coordinating post-transcriptional processing of these mRNAs.

In summary, our work reveals an association between TNIP2 and YLPM1, together with many RNA processing proteins and a distinct set of mRNAs. We also established a physical interaction between TNIP2 and the ESCRT-I complex, a component of the cellular transport machinery, via its TSG101 subunit (Fig. 7). ESCRT-I is and its TSG101 subunit in particular has been shown to be essential for releasing HIV-1 viral RNA from late endosomes to the cytosol (10, 46). This suggests some potential new avenues of research into TNIP2 function, which could help to determine whether the NF-κB signaling pathway uses TNIP2 to influence intracellular transport, or the processing, and possibly localization, of RNA transcripts.

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