Supplementary Results

Ubiquitination of AGO2 by TRIM71 and TRIM32

Previous work on TRIM71 had shown that TRIM71 ubiquitinates AGO2 thereby targeting it for proteasomal degradation (1). The mammalian TRIM71 paralog TRIM32 also interacts with AGO proteins and has ubiquitin ligase activity but was reported not to mediate AGO ubiquitination (2). In fact, TRIM32 was described as a positive regulator of the pathway enhancing miRNA mediated repression (2), while TRIM71 was proposed to antagonize miRNA mediated repression (1). Our findings that identify TRIM71 as a repressor of mRNAs co-operating with miRNAs to repress certain mRNA targets resemble the situation described for TRIM32. With the aim to get some understanding what determines the differences between TRIM71 and TRIM32, we first tested the activity of TRIM71 and TRIM32 to ubiquitinate AGO2. We adapted the *in vivo* ubiquitination protocol established by Rybak et al. (1), which examines ubiquitination of overexpressed tagged AGO2 upon co-expression of TRIM-NHL proteins in HEK293 cells. FLAG-tagged AGO2 was immunopurified from whole cell lysates and analyzed by western blotting using an antibody against either AGO2 or ubiquitin. AGO2 ubiquitination was induced upon expression of either TRIM71 or TRIM32, as indicated by a high molecular weight smear visible with both the AGO2 and the ubiquitin antibodies (Supplementary Fig. S8a, lane 2 and 3). To show that TRIM32 mediated ubiquitination of AGO2 is dependent on TRIM32's ubiquitin ligase activity, we performed the assay with a TRIM32 mutant that lacks catalytic activity due to the mutation of two cysteine and one histidine residues in the RING domain (TRIM32 mut)(3). As expected, mutation of the catalytic domain abolished the ability of TRIM32 to ubiquitinate AGO2 (Supplementary Fig. S8b). A similar experiment demonstrating that the TRIM71 ability to ubiquitinate AGO2 is dependent on its ligase activity has already been described (1). Collectively, these data indicate that TRIM71 and TRIM32 are both able to ubiquitinate AGO2.

TRIM71 and TRIM32 do not affect AGO2 stability

Although we observed AGO2 ubiquitination by TRIM71 and TRIM32, we did not detect a decrease in AGO2 proteins levels upon TRIM-NHL protein overexpression (see Supplementary Fig. S8a and b). We thus tested the effect of TRIM-NHL proteins on AGO2 stability. First, we analyzed the stability of endogenous AGO2 in HEK293 cells stably expressing either FLAG-HA-TRIM71 or FLAG-HA-TRIM32 in a time-course experiment following addition of cycloheximide (CHX) (Supplementary Fig. S8c). In control HEK293 cells, AGO2 was very stable and its levels did not decrease after inhibition of protein synthesis with CHX even for 8 h, a time-point at which the levels of the unstable c-Myc protein had clearly diminished. Analysis of the TRIM71 and TRIM32 cell lines revealed that expression of neither TRIM71 nor TRIM32 had an effect on AGO2 stability.

As overexpressed AGO2 had been reported to be less stable than the endogenous protein (4), we also monitored the stability of overexpressed AGO2. Indeed, overexpressed AGO2 showed a shorter half-life than the endogenous protein but again we observed no effect of TRIM32 or TRIM71 expression on AGO2 turnover (Supplementary Fig. S8d). We also checked AGO2 protein levels in mES cells after knock-down of TRIM71. Although TRIM71 levels were strongly down-regulated, we did not observe an increase in AGO2 protein level (Fig. 3c).

Collectively, these data indicate that although TRIM71 and TRIM32 can induce AGO2 ubiquitination, they do not accelerate AGO2 decay, making it unlikely that ubiquitination of AGO2 by TRIM71 or TRIM32 marks the protein for proteasomal degradation. These results are consistent with a recent report that similarly did not find evidence for a destabilizing effect of TRIM71 on AGO2 (5).

Supplementary Methods

Ms analysis

The eluates of the FLAG-IP's were processed by reduction and alkylation of the cysteines followed by digestion with trypsin. The peptides were separated by nano-HPLC (Agilent 1100 nanoLC system, Agilent Technologies) coupled to an LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Scientific) using a Top10 method. Proteins were identified searching SwissProt database (version 2011-08) using Mascot 2.3.0 (Matrix Science). Data were compiled and evaluated with Scaffold 3.4.3 (Proteome Software) applying a peptide and protein threshold of 80% with at least two peptides per protein, resulting in the identification of 154 proteins with an FDR of less than 0.1%.

In vivo ubiquitination assay

The *in vivo* ubiquitination assay was adapted from Rybak et al. (1). Briefly, HEK293 cells were transfected with plasmids encoding either 3xFLAG-AGO2 and GFP-TRIM32 or GFP-TRIM71 or with FLAG-HA-AGO2 and His-myc-TRIM32 or His-myc-TRIM32 mut. 48 h post-transfection cells were lysed in preboiled 25 mM HEPES, pH 7.7 containing 150 mM NaCl, 0.5 mM EDTA, 1% SDS, 0.1% NP-40, 50 mM chloracetamide, 1 mM DTT (denaturing buffer) and incubated for 5 min at 95ºC. Lysates were clarified by centrifugation for 15 min at 13,000xg and diluted 1/10 with NP-40 buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 0.2% NP-40, 10% glycerol, 10 mM NaF, 8 mM glycerophosphate, 1 mM NaVanadate, 50 mM chloracetamide, 1mM DTT, and Complete protease inhibitor cocktail). FLAG-M2 beads were added to the lysate and incubated at 4ºC oN. Beads were washed 3x with NP-40 buffer and bound proteins were eluted by boiling in Laemmli buffer and analyzed by western blotting.

Immunofluorescene

HEK293 Flp-In cells constitutively expressing FLAG-HA-TRIM71 were plated on acid-washed cover slips and grown overnight. Cells were washed with PBS, fixed with 4% PFA in PBS, permeablised with 0.1% Triton-X in PBS, blocked with 10% horse serum (HS) in PBS containing 0.05% TWEEN (PBS-T) and incubated with primary antibodies diluted in 2% HS in PBS-T for 1h at RT. Following primary antibodies were used anti-HA (9F10), 1:200 (Roche); anti-FLAG M2, 1:500 (Sigma); anti-AGO2 (11A9), 1:100 (Ascenion); anti-DDX6, 1:500 (Bethyl, A300-461A). Incubation with Alexa-488- or Alexa-647- coupled secondary antibodies (Molecular probes) was for 30 min at RT. Nuclei were stained with DAPI and cells analyzed with the Axioimger Z1 (Zeiss) equipped with a AxioCamMRCc using the axiovison software.

Real-time quantitative PCR (RT-qPCR)

RNA from whole cells or, in case of RIP experiments, from whole cell lysates or immune-precipitates was extracted using Trizol, treated with RNase-free DNaseI and reverse transcribed using SuperScript III (Invitrogen). Briefly, 1 µg total RNA (in 10 µl volume) was mixed with 1 µl of 50 µM random hexamers (Invitrogen) and 1 µl of 10 mM dNTP-Mix, incubated at 65ºC for 5 min and cooled down on ice. 4 µl 5x buffer, 1 µl 0.1 mM DTT, 1 µl RNaseOUT (40 U/µl) and 1 µl SuperScript III (200 U/ µl) were added and incubated at 42ºC for 60 min. Reactions were inactivated for 15 min at 70ºC. Control reactions lacked SuperScript III. The resulting cDNA was used as template for qPCR with the ABI 7500 Real Time PCR system and SYBR green PCR Master mix (Applied Biosystems). Each reaction was performed in triplicate and the data was normalized to *GAPDH* or *Tubulin*. For each RT-qPCR experiment shown, values represent means of two independent experiments, each performed in triplicate, and error bars show s.e.m. For the analysis of mRNA decay rates (shown in Fig. 2f) cytoplasmic RNA was isolated as described previously (6). Briefly, cells were lysed in RNA lysis buffer (50 mM TrisHCl pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0,5% NP-40) and RNA was isolated using Nucleo Spin RNA II columns (Machery-Nagel). cDNA synthesis was performed with the iScript cDNA synthesis kit (Biorad) according to manufacturer's instructions. The resulting cDNA was used as a template for qPCR with the CFX Real Time PCR system (Biorad) and SSO Fast Eva Green Supermix (Biorad). Values are the mean of three independent experiments, each performed in duplicates and error bars show s.e.m.

Primer sequences are listed in Supplementary Table S3.

Microarray analyses

100 ng of extracted total RNA was amplified using the Ambion WT Expression kit (Ambion) and the resulting sense-strand cDNA was fragmented and labeled using the Affymetrix GeneChip WT Terminal Labeling kit (Affymetrix). GeneChip Mouse Gene 1.0 ST arrays were hybridized following the "GeneChip Whole Transcript (WT) Sense Target Labeling Assay Manual" (Affymetrix, Santa Clara, CA) with a hybridization time of 16 h. The Affymetrix Fluidics protocol FS450_0007 was used for washing. Scanning was performed with Affymetrix GCC Scan Control v. 3.0.1 on a GeneChip® Scanner 3000 with autoloader (Affymetrix). Raw data were loaded and and probeset-level values were calculated and normalized with justRMA function from R (version 2.14.0) / Bioconductor (version 2.9) package "affy" using the CDF environment MoGene-1_0-st-v1.r3.cdf (as provided by Bioconductor) and annotation from Netaffx (www.netaffx.com).

Differentially expressed genes were identified using the empirical Bayes method (F test) implemented in the LIMMA package and adjusted with the false discovery rate method(7). Hierarchical clustering and visualization were done in R. Probe sets with a log2 average contrast signal of at least 4, an adjusted P value of <0.01 or of <0.05 for

HEK293 (Supplementary Table S2) or mES cells (Supplementary Table S3), respectively, and an absolute log2 fold-change of >0.585 (1.5-fold in linear space). The complete microarray data are available in the Gene Expression Omnibus (GSE37714).

PUM motif analysis

Using a PUM weight matrix (8) and the software mast (9), we scanned the 3'UTRs of RefSeq mRNA transcripts for potential binding sites. High scoring PUM sites were selected based on a minimum meme score of 1100 and weak binding sites (serving as controls) based on a score between 500 and 550.

Supplementary references:

1. Rybak, A., Fuchs, H., Hadian, K., Smirnova, L., Wulczyn, E.A., Michel, G., Nitsch, R., Krappmann, D. and Wulczyn, F.G. (2009) The let-7 target gene mouse lin-41 is a stem cell specific E3 ubiquitin ligase for the miRNA pathway protein Ago2. *Nat Cell Biol*, **11**, 1411-1420.
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4. Johnston, M., Geoffroy, M.C., Sobala, A., Hay, R. and Hutvagner, G. (2010) HSP90 protein stabilizes unloaded argonaute complexes and microscopic P-bodies in human cells. *Mol Biol Cell*, **21**, 1462-1469.
5. Chen, J., Lai, F. and Niswander, L. (2012) The ubiquitin ligase mLin41 temporally promotes neural progenitor cell maintenance through FGF signaling. *Genes Dev*, **26**, 803-815.
6. Bethune, J., Artus-Revel, C.G. and Filipowicz, W. (2012) Kinetic analysis reveals successive steps leading to miRNA-mediated silencing in mammalian cells. *EMBO Rep*, **13**, 716-723.
7. Smyth, G.K. (2005) *Limma: linear models for microarray data.* Springer, New York.
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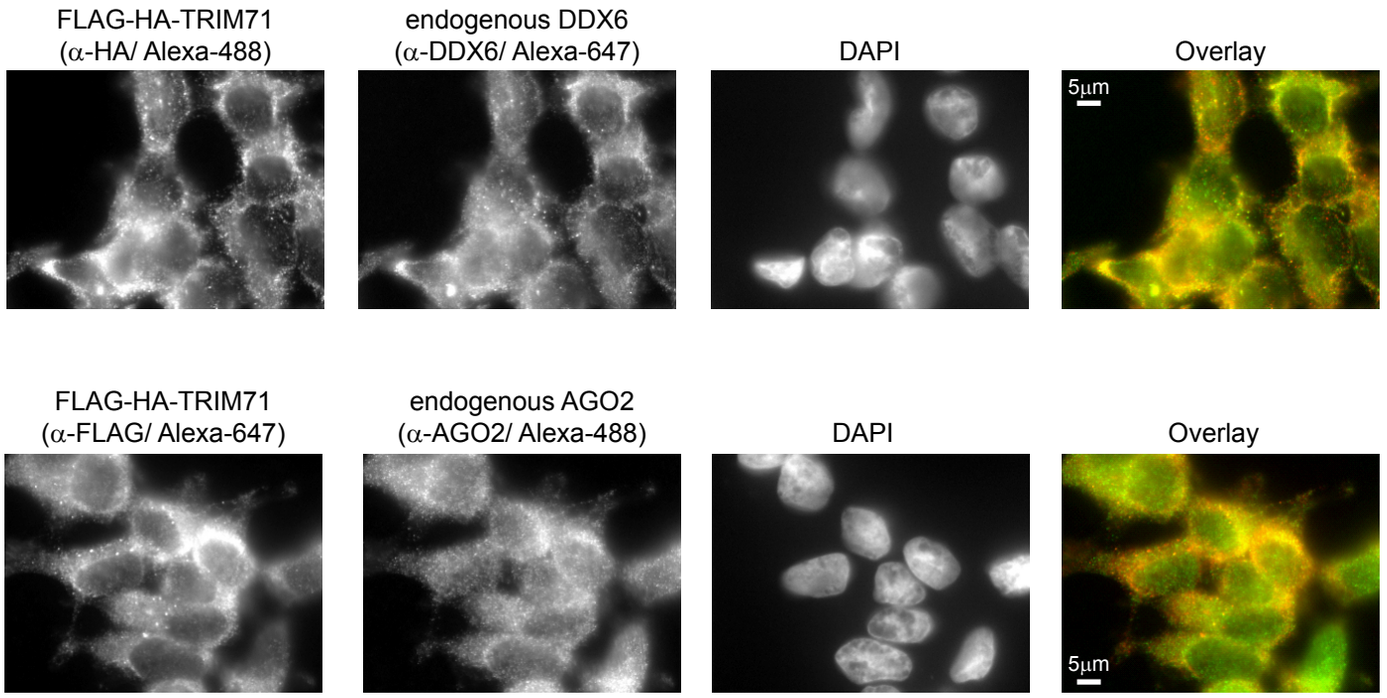
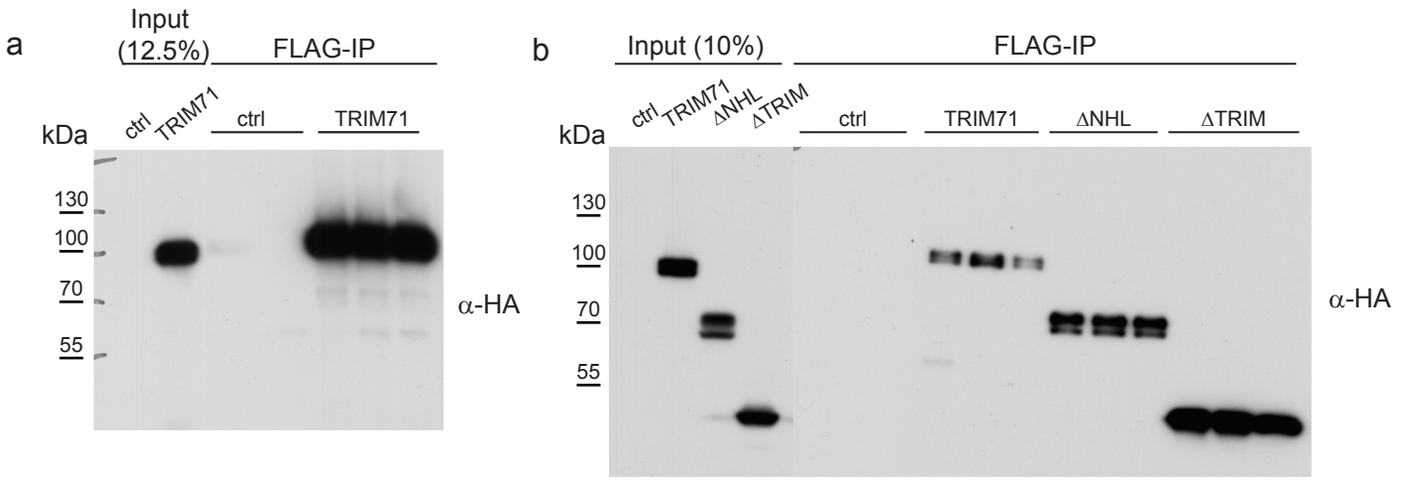
9. Bailey, T.L., Boden, M., Buske, F.A., Frith, M., Grant, C.E., Clementi, L., Ren, J., Li, W.W. and Noble, W.S. (2009) MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res*, **37**, W202-208.

Supplementary Figure S1



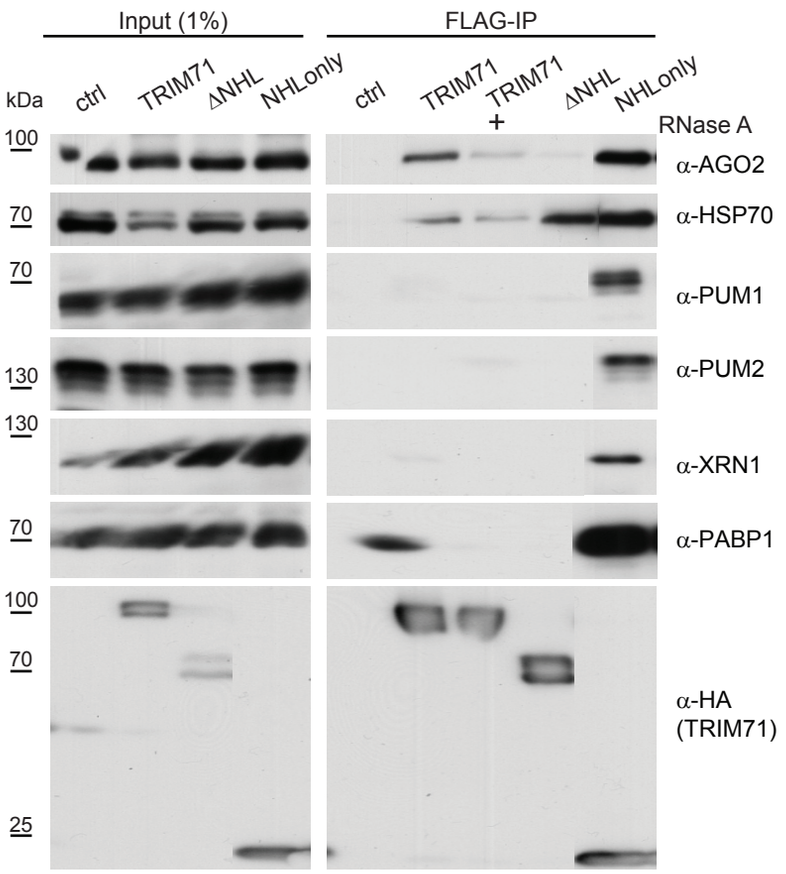
Supplementary Figure S1 Imunofluorescence analyses of HEK293 cells constitutively expressing FLAG-HA-TRIM71. HEK293 cells stably expressing FLAG-HA-tagged TRIM71 were stained with antibodies against FLAG or HA as indicated and antibodies against either endogenous AGO2 or endogenous DDX6.

Supplementary Figure S2



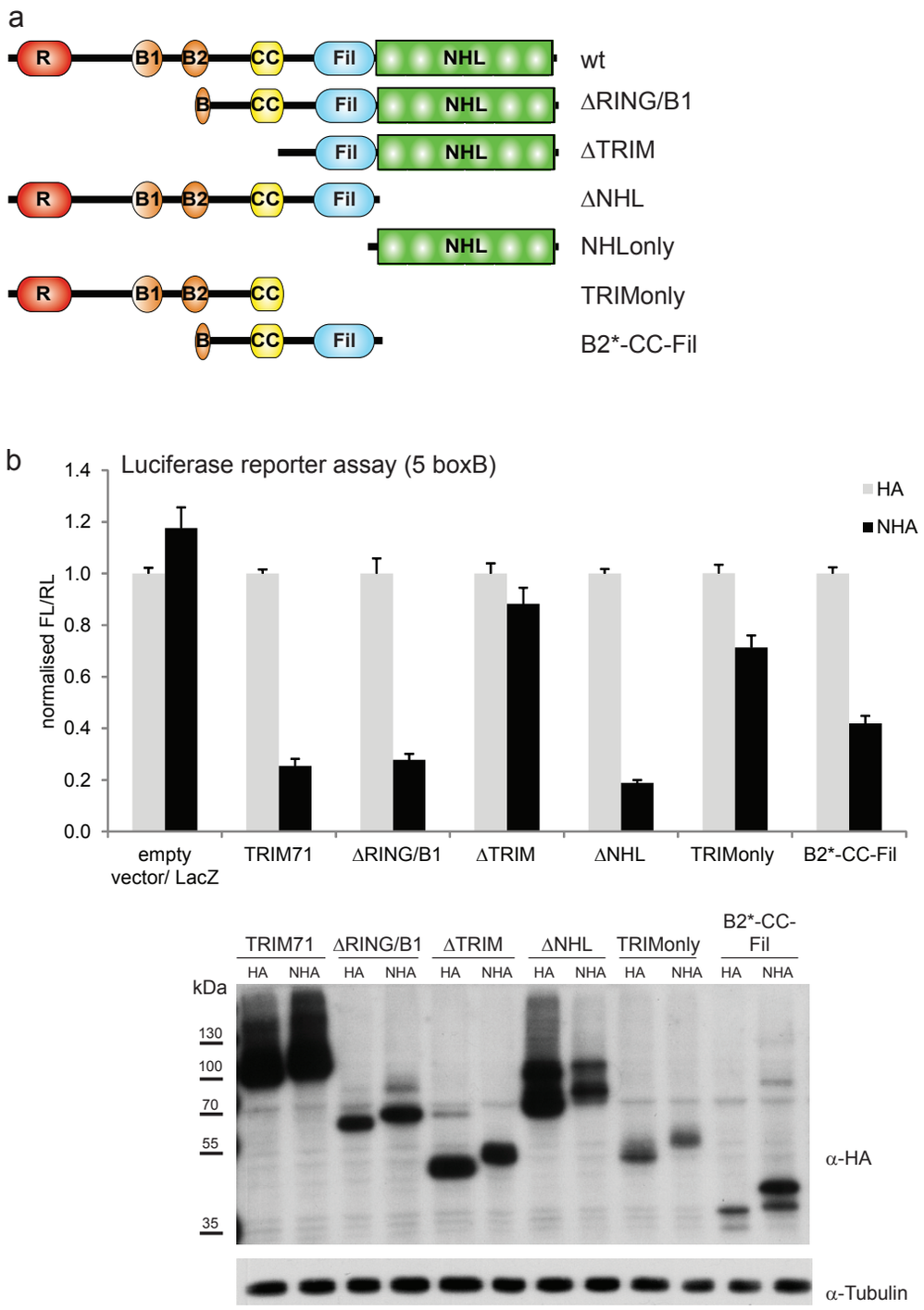
Supplementary Figure S2 Western blots for RNA-Immunoprecipitation (RIP) experiments shown in Figures 2d (**a**) and 4d (**b**). (**a**, **b**) Immunoprecipitation experiments with anti-FLAG M2 affinity gel (FLAG-IP) were performed with extracts from HEK293 cells stably expressing either FLAG-HA-tagged TRIM71 (TRIM71) (**a**) or indicated FLAG-HA tagged TRIM71 deletion mutants (ΔNHL and ΔTRIM) (**b**). Parental cell line not expressing any FLAG-HA-tagged protein was used as control (ctrl). The experiments were performed in triplicates and repeated twice. Shown are single representative experiments. Western blotting was performed with anti-HA antibody.

Suplementary Figure S3



Supplementary Figure S3 The NHL domain mediates association of TRIM71 with mRNA binding proteins. Immunoprecipitation experiment with anti-FLAG M2 affinity gel (FLAG-IP), performed with extracts from HEK293 cells stably expressing FLAG-HA-tagged TRIM71, FLAG-HA-tagged TRIM71 ΔNHL, FLAG-HA-tagged TRIM71 NHLonly, or the parental cell line not expressing any FLAG-HA-tagged protein (ctrl). Where indicated (+) RNase A was added to the reaction. Western blots were probed with antibodies against the indicated endogenous proteins or against HA. Notably, HSP70 associated with all tested TRIM71 mutants, indicating that it interacts with multiple regions of the protein.

Suplementary Figure S4



Supplementary Figure S4 TRIM71 deletion analysis to define the minimal repressive domain. (**a**) Schematic representation of human TRIM71 and TRIM71 deletion mutants. See Fig. 4a for details. (**b**) (**top**) Repression of RL-5boxB by NHA-TRIM71 and indicated deletion mutants in HEK293 cells. Note that TRIM71 deletion mutants already tested and described in Fig. 4b were included also in this assay for comparison. HEK293 cells were co-transfected with plasmids expressing RL-5boxB, the indicated NHA- or HA-fusion proteins, and a FL control plasmid. RL was normalized to FL and values of normalized RL produced in the presence of the proteins without N-peptide (HA) were set to one. Tethering of LacZ served as an additional control. (**bottom**) Expression of TRIM71 deletion mutants was estimated by western blotting with antibodies against HA. α-Tubulin was measured as a loading control.

Suplementary Figure S5

a

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                                                  D                                                  L
sp|Q8MQJ9|BRAT_DROME  767 HCKFGEFGVMEGQFTEPSGVAVNAQNDIIVADTNNHRIQIFDKEGRFKFQ
sp|Q2Q1W2|LIN41_HUMAN 640 HHKFGTLGSRPGQFDRPAGVACDASRRIVVADKDNHRIQIFTFEGQFLLK
sp|Q13049|TRI32_HUMAN 358 LKKMGAKGSTPGMFNLPVSLYVTSQGEVLVADRGNYRIQVFTRKGFLKEI
                            *:*  *   * *  * .:   :.  ::*** .*:***:*  :* :

                                                  A
sp|Q8MQJ9|BRAT_DROME      F-GECGKR---------DSQLLYPNRVAVVRNSGDIIVTERSPTHQIQIY
sp|Q2Q1W2|LIN41_HUMAN     F-GEKGTK---------NGQFNYPWDVAV-NSEGKILVSD-TRNHRIQLF
sp|Q13049|TRI32_HUMAN     RRSPSGIDSFVLSFLGADLPNLTPLSVAM-NCQGLIGVTD-SYDNSLKVY
                            .  *           :     *  **: . .* * *:: :  : ::::

                                                  A
sp|Q8MQJ9|BRAT_DROME      NQYGQFVRKFGA-----TILQHPRGVTVDNKGRIIVVECKVMRVIIFD-- 899
sp|Q2Q1W2|LIN41_HUMAN     GPDGVFLNKYGFEGALWKHFDSPRGVAFNHEGHLVVTDFNNHRLLVIHP- 777
sp|Q13049|TRI32_HUMAN     TLDGHCVACHR------SQLSKPWGITALPSGQFVVTDVEGGKLWCFTVD 488
                            *  :  .       . :. * *::   .*:::*.: :  ::  :
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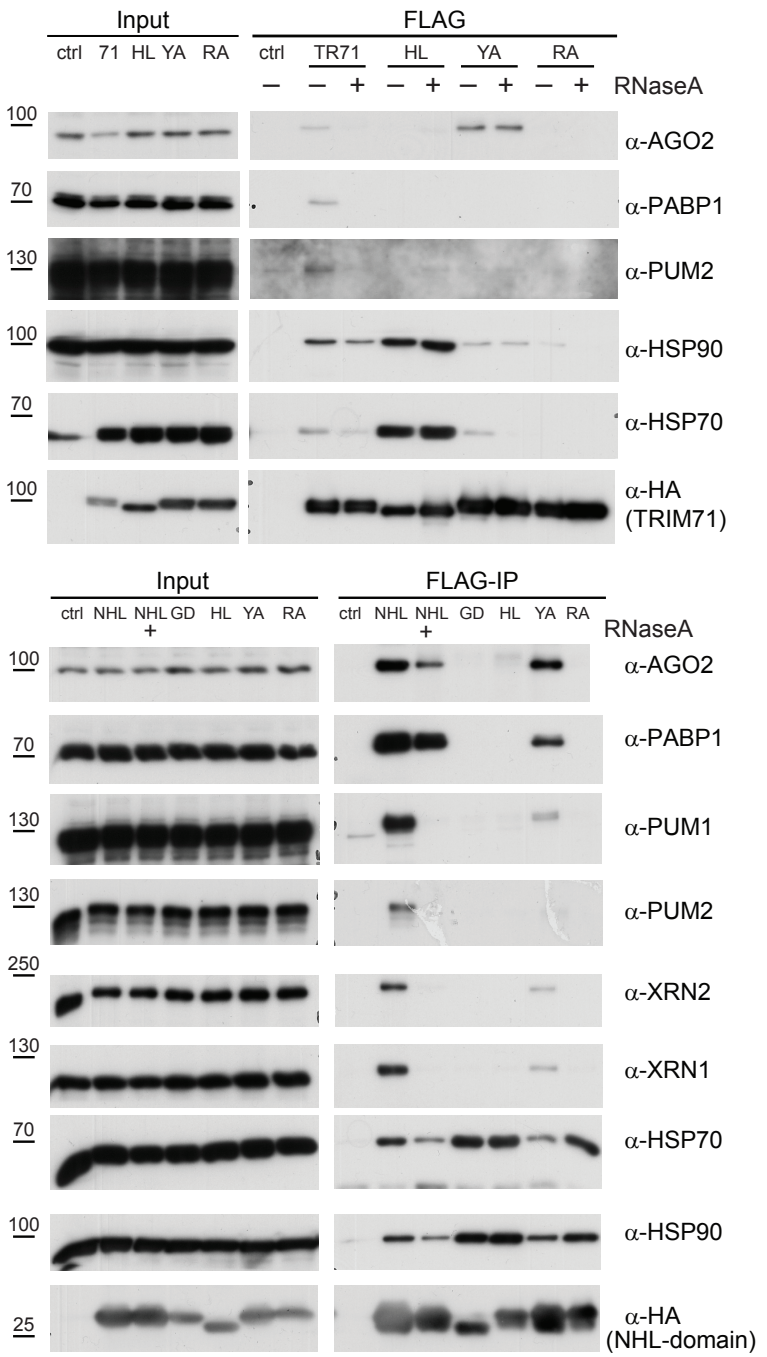
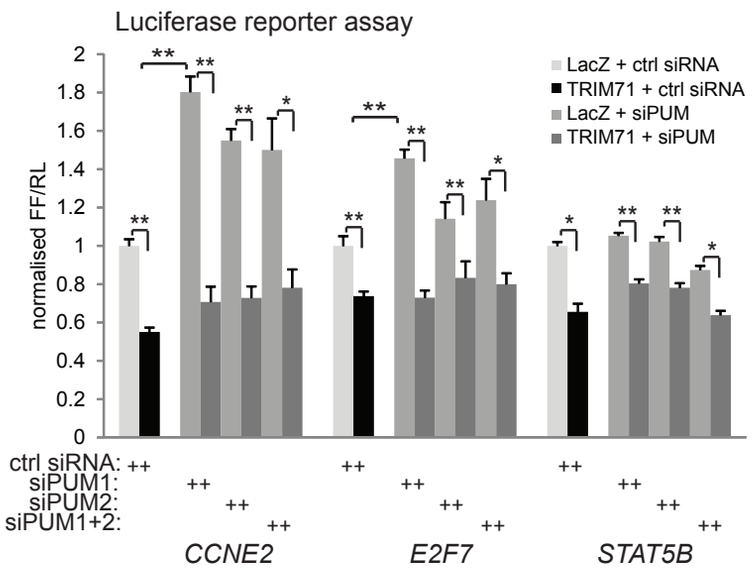
b



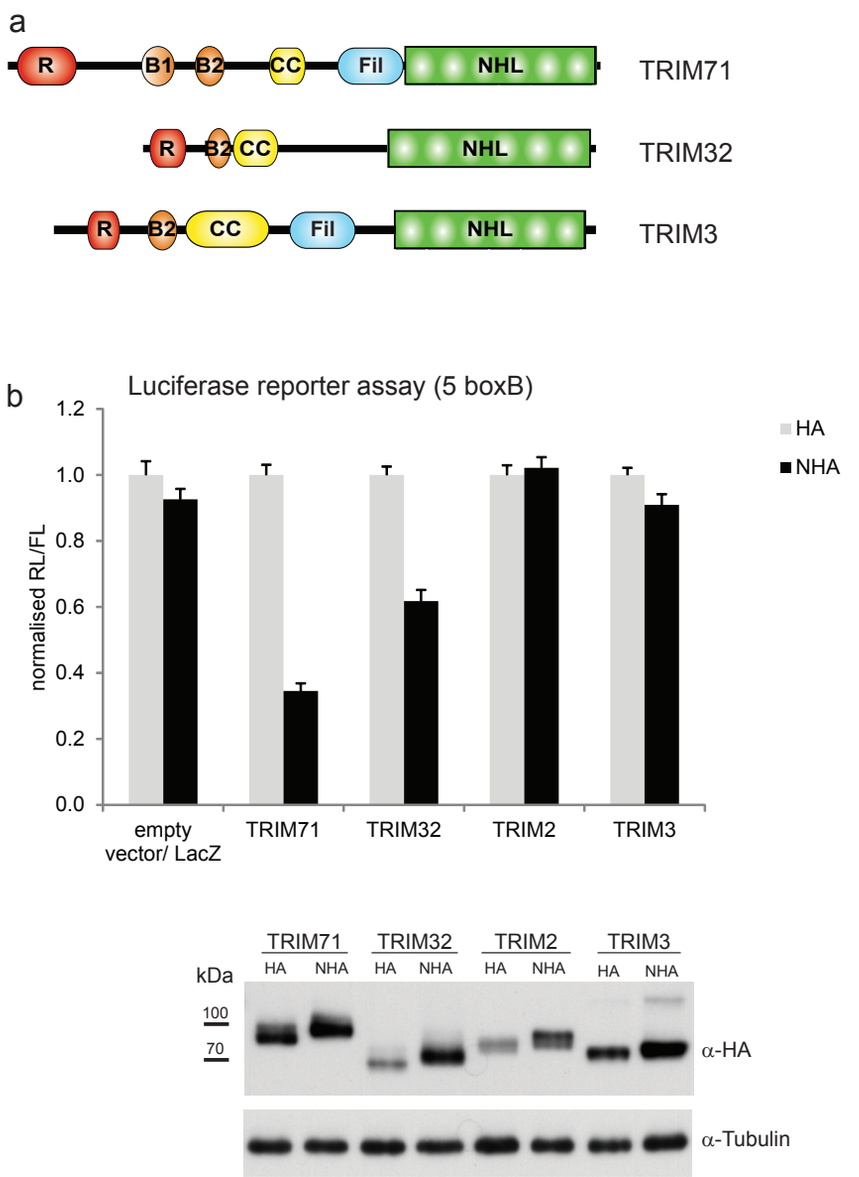
Supplementary Figure S5 (**a**) Alignment of a fragment of the NHL domain of *Drosophila* BRAT and human TRIM71 (LIN41) and TRIM32 (TRI32). Alignment was created using TCoffee. Identical residues are denoted with an asterisk (*), conserved substitutions with two dots (:) and semi-conserved substitutions with one dot (.). The alignment encompasses NHL repeats 2-4 and numbers of amino acid positions are indicated. Residues (within the BRAT NHL domain) that line up the electropositive top surface of the ß-propeller structure and that are important for the interaction between BRAT and PUM are shown in red. Conserved residues (within the TRIM71 NHL domain) that mutated by us are shown in bold black. (**b**) Immunoprecipitation experiment with anti-FLAG M2 affinity gel (FLAG-IP) from HEK293 cells stably expressing either FLAG-HA-tagged TRIM71 (wt) and indicated FLAG-HA-tagged point mutants thereof (HL, YA, RA) (**top**) or FLAG-HA-tagged TRIM71 NHLonly (wt) and indicated FLAG-HA-tagged point mutants thereof (GD, HL, YA, RA) (**bottom**). The parental cell line not expressing any FLAG-HA-tagged protein served as negative control (ctrl). Western blots of cell lysate (Input) and FLAG-IP were probed with antibodies against the indicated endogenous proteins or against HA.

Supplementary Figure S6



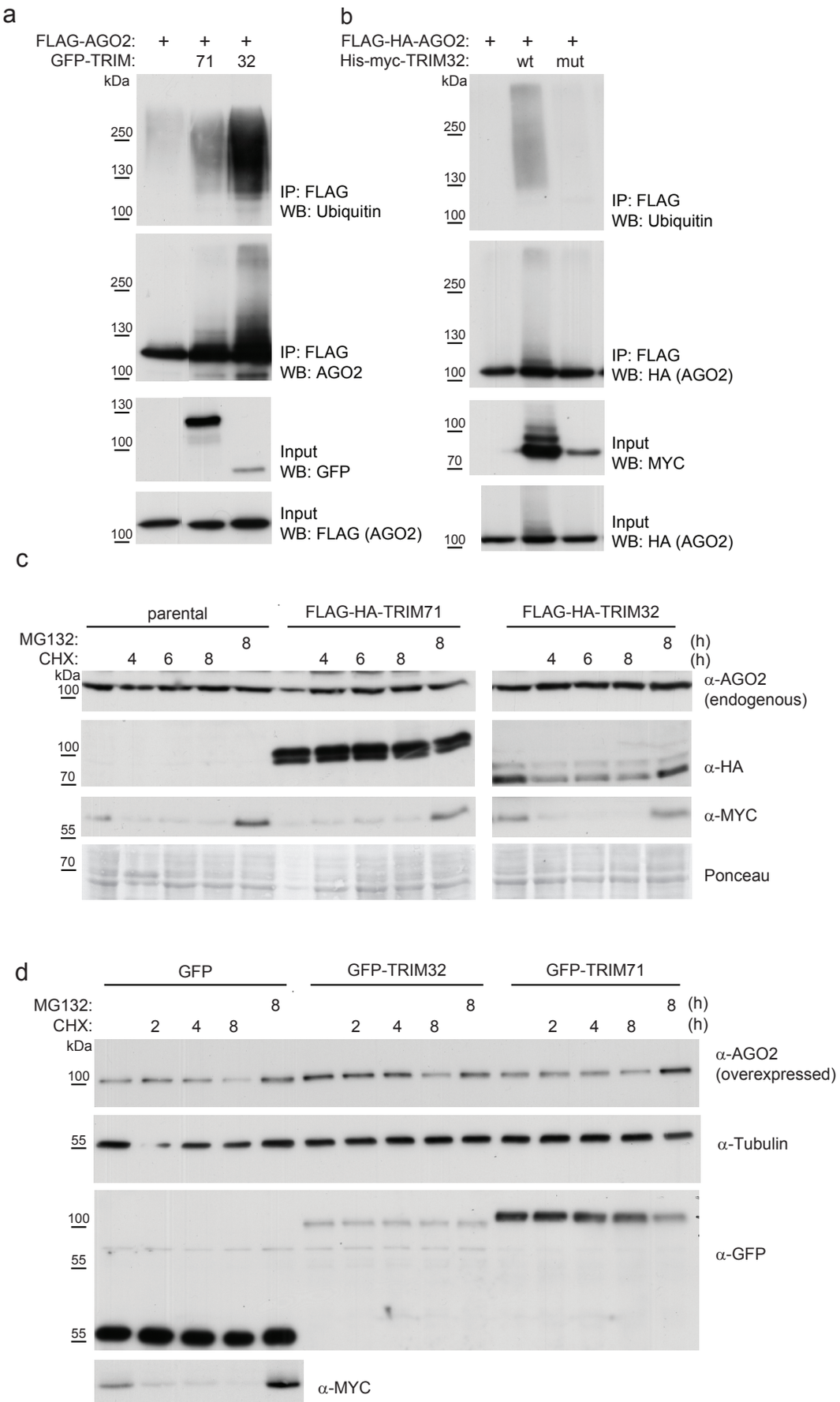
Supplementy Figure S6 Knock-down of PUM1 and PUM2 does not affect TRIM71-mediated repression. HEK293 cells were co-transfected with indicated FL-3'UTR reporter constructs, plasmids expressing TRIM71 or LacZ and siRNAs against PUM1 and PUM2, either individual or in combination, or a non-targeting control siRNA as indicated. FL was normalized to RL and values of normalized FL produced from samples expressing LacZ and the non-targeting control siRNA were set to one. In addition to Fig. 5b, this figure also shows normalised FF values for experiments with knock-down of individual PUM proteins. * p-value < 0.05; ** p-value < 0.01

Supplementary Figure S7



Supplementary Figure S7 Tethering reporter assay with human TRIM-NHL proteins. (**a**) Schematic representation of human TRIM71, TRIM32, and TRIM3. As TRIM2 and TRIM3 are highly similar only TRIM3 is depicted. See Fig. 4a for more details. (**b**) (**top**) Repression of RL-5boxB by human TRIM-NHL proteins. HEK293 cells were co-transfected with plasmids expressing RL-5boxB, the indicated NHA- or HA-fusion proteins and a FL control plasmid. RL was normalized to FL and values of normalized RL produced in the presence of the proteins without N-peptide (HA) were set to one. (**bottom**) Protein expression was confirmed by western blotting with antibody against HA. Tubulin alpha was measured as a loading control.

Suplementary Figure S8



Supplementary Figure S8 Effect of TRIM71 and TRIM32 on AGO2 ubiquitination and stability. (**a, b**) *In vivo* ubiquitination assay. HEK293 cells were transient transfected with FLAG-tagged AGO2 and either GFP-TRIM71, GFP-TRIM32, or empty vector control, as indicated (**a**), or with FLAG-HA-tagged AGO2 and either His-MYC-tagged TRIM32 (wt) or its catalytic inactive mutant (mut) (**b**). 48 h post-transfection cells were lysed in denaturing buffer, the lysate was diluted 1/10 with immunoprecipitation buffer and subjected to immunoprecipitation with FLAG-M2 affinity gel. Western blots of input and IP samples were probed with indicated antibodies. Ubiquitination of AGO2 is manifested by the presence of a high-molecular-weight smear visible with both the AGO2 and ubiquitin antibodies in the IP samples. (**c**) Stability of endogenous AGO2 upon TRIM71 or TRIM32 overexpression. HEK293 cells lines stably expressing FLAG-HA-tagged TRIM71 or FLAG-HA-tagged TRIM32 or the parental control cell line not expressing any FLAG-HA-tagged protein (parental) were treated with cycloheximide (CHX) or with MG132 for indicated times. Western blots of cell lysates were probed with indicated antibodies. Drug effectiveness is monitored by western blotting against the unstable protein c-Myc. Ponceau staining of the membranes served as loading control. (**d**) Stability of overexpressed AGO2. HEK293 cells were transiently transfected with FLAG-HA-tagged AGO2 and either GFP, GFP-TRIM32 or GFP-TRIM71, as indicated. 48 h post-transfection cells were treated with CHX or MG132 for times indicated and western blots of cell lysates were probed with indicated antibodies. Western blot with anti-c-Myc antibody reveals effectiveness of treatments with CHX and MG132. alpha-Tubulin was quantified as a loading control.

Supplementary Table S1
TRIM71 associated proteins identified by mass spectrometry

Supplementary Table S2
Microarray data for transcripts both up- and down-regulated by 1.5 fold in HEK293 cells constitutively expressing FLAG-HA-TRIM71
Microarray data for transcripts both up- and down-regulated by 1.5 fold in mES cells upon TRIM71 knock-down

Supplementary Table S3
siRNA sequences
Primers for PCR amplification and cloning of 3'UTRs into pMIR
qPCR primers