Phosphorylation of human Argonaute proteins affects small RNA binding

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Supplementary information

Supplementary Methods

Plasmids and mutagenesis

Plasmids encoding F/H-tagged as well as myc⁶-tagged proteins were described previously (Rudel et al., 2008). Point mutations were introduced into human Ago2 coding sequence by two PCR reactions using the following desoxy-oligonucleotides. Mutated codons are underlined: Y529A-fw: 5’-gccgcaagacgccgtgccagccaggg; Y529F-fw: 5’-gccgcaagacgccgtgccagccaggg; Y529Q-fw: 5’-gccgcaagacgccgtgccagccaggg; Y529E-fw: 5’-gccgcaagacgccgtgccagccaggg; Ago2_rev_AscI: 5’-atctggcgccttataagcagatacagtcatgctgccagagtgt. The first PCR generated mutated 3’ fragments of Ago2 that were subsequently used as reverse primers together with Ago2_fwd_FseI: 5’aaaggccggccatgtactcgggagccggcc. The resulting mutated full-length Ago2 coding sequence was digested with FseI and AscI and cloned into a modified pIRESneo vector (Meister et al., 2004).

Cell culture and transfection
In this study only HeLa and HEK 293 cells have been used and only the latter ones were transfected by calcium phosphate procedure. Cell maintenance and transfection by calcium phosphate procedure were described previously (Rudel et al., 2008).

**Total extract preparation, immunoprecipitation and Western blotting**

Total cell extracts were prepared as described before (Rudel et al., 2008), but in the presence of sodium orthovanadate (Sigma-Aldrich) that has been treated with H$_2$O$_2$ before. Immediately before lysis, cells have been incubated with phosphatase inhibitor for 10 minutes at 37°C in a final concentration of 1 mM. Vanadate was also included in the lysis buffer.

Immunoprecipitation and Western blotting were performed as reported previously (Rudel et al., 2008). For immunoprecipitation either anti-FLAG M2 agarose beads or anti-myc agarose beads (both Sigma) were used. Monoclonal anti-HA antibody was purchased from HI SS Diagnostics, Germany, monoclonal anti-myc antibody from Abcam, UK, peroxidase-conjugated secondary anti-mouse IgG antibody from Sigma and anti-rabbit IgG antibody from Jackson, West Grove, PA.

**Immunofluorescence**

Sample preparation has been described elsewhere (Rudel et al., 2008). Immunofluorescence was recorded using a TCS SP2 Confocal Laser Scanning microscope and Confocal Software softWoRx (Leica Microsystems, Germany). Monoclonal anti-LSm4 antibody was purchased from Geneway, FITC-conjugated
anti-chicken IgY antibody from Sigma and TexasRed-conjugated anti-mouse IgG antibody from Vector Laboratories.

For quantitative analysis of Ago2-Y529 mutants’ localization to P-bodies (PBs), twelve z-section images of each sample were recorded and processed to maximum projections using confocal software. PBs were identified by the presence of LSm4. For each mutant 100 PBs were analyzed for co-localization of the Ago2 mutant of interest. The ratio of PBs positive for both proteins to the total number of PBs was determined for each cell. Standard deviation was calculated using the ratios obtained for the single cells.

**Rescue experiments**

HeLa cells were reverse transfected in 6 well plates with siRNAs at 65 nM final concentration using RNAiMAX (Invitrogen) according to the manufacturer’s instructions. The following siRNAs were used (sense, antisense): Ago2-3'UTR siRNA, 5' r(CCGUCGCCAGAUUUCAACUU)dT, 5' r(AAGUUGCAGAAUCUGGACGG)dT; non-silencing control siRNA, 5' r(UUGUCUUGCAUUGCAACAU)dT, 5' r(UUAGUCGAAUGCAAGCAGA)dT. SiRNAs have been synthesized as described before (Chen et al., 2008). After 3d, cells were seeded to 48 well plates and 6 hours later transfected with 400 ng of the appropriate VP5-Ago2-Y529 plasmid and 100 ng pMIR-RNL-TK-miR21 plasmid (Hock et al., 2007) using lipofectamin 2000 (Invitrogen). 2d later, cells were lysed using passive lysis buffer (Promega, USA). Luciferase activities were measured on a Mithras LB 940 luminometer (Berthold technologies, Germany). All samples were assayed in 3 replicates and each experiment was at least repeated three times. Firefly/renilla luminescence ratios were normalized to the
ratio recorded for the control, transfected with a non-silencing siRNA but no VP5 construct. pMIR-RNL-TK-miR21 encodes firefly luciferase under the control of a HSV-Tk promoter and harbors a site perfect complementary to miR-21 in the firefly luciferase 3’UTR. In addition this plasmid encodes renilla luciferase under the control of a SV40 promoter.

**Tethering experiments**

pCI-neo-HA, pCI-neo-N-HA and pRL-5BoxB plasmids were kindly provided by the Filipowicz laboratory (Pillai et al., 2004). Protein-coding sequences were inserted into pCI-neo-HA and pCI-neo-N-HA respectively using the EcoRI and NotI restriction sites.

HeLa cells were seeded to 48 well plates and 6 hours later transfected with 300 ng N-HA or HA fusion protein coding plasmids, 120 ng pRL-5BoxB and 80 ng pMIR-REPORTER (Ambion) using lipofectamin 2000 (Invitrogen). After 2d, cells were lysed and luciferase activity measured as described for rescue experiments. Renilla/firefly luminescence ratios of the tethered N-HA fusion proteins were normalized to the ratio recorded for the not tethered HA fusion proteins.

**Mass spectrometry**

Coomassie-stained protein bands were in-gel digested by trypsin (sequencing grade, Roche, Germany) essentially as described (Shevchenko et al., 1996) and phosphorylated peptides were enriched using TiO$_2$ affinity purification (Thingholm et al., 2006). Subsequently, tryptic peptide mixtures were separated on a CAPLC (Waters, USA) or an Ultimate (Dionex, Germany) nanoHPLC system directly coupled to a QTOF (Waters, UK) or an orbitrap (Thermo, Germany)
electrospray mass spectrometer. The mass spectrometers was operated in data
dependent MS/MS mode to automatically switch between MS survey and MS/MS
fragmentation scans of the four (QTOF) or five (orbitrap) most abundant
precursor ions. Database searches were performed using the Mascot
(Matrixscience, UK) software package and identified phosphorylation sites were
further validated by visual inspection of MS/MS spectra.

References

Chen, P.Y., Weinmann, L., Gaidatzis, D., Pei, Y., Zavolan, M., Tuschl, T., and Meister,
G. (2008). Strand-specific 5'-O-methylation of siRNA duplexes controls guide
strand selection and targeting specificity. Rna 14, 263-274.

Hock, J., Weinmann, L., Ender, C., Rudel, S., Kremmer, E., Raabe, M., Urlaub, H., and
Meister, G. (2007). Proteomic and functional analysis of Argonaute-containing
mRNA-protein complexes in human cells. EMBO Rep 8, 1052-1060.

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., and Tuschl, T.
(2004). Human Argonaute2 Mediates RNA Cleavage Targeted by miRNAs and
siRNAs. Mol Cell 15, 185-197.

Pillai, R.S., Artus, C.G., and Filipowicz, W. (2004). Tethering of human Ago
proteins to mRNA mimics the miRNA-mediated repression of protein synthesis.
Rna 10, 1518-1525.

Rudel, S., Flatley, A., Weinmann, L., Kremmer, E., and Meister, G. (2008). A
multifunctional human Argonaute2-specific monoclonal antibody. Rna 14, 1244-
1253.
Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68, 850-858.

Thingholm, T.E., Jorgensen, T.J., Jensen, O.N., and Larsen, M.R. (2006). Highly selective enrichment of phosphorylated peptides using titanium dioxide. Nat Protoc 1, 1929-1935.

**Supplementary Figure legends**

Suppl. Figure 1: Multiple alignment of phosphorylated peptide sequences from different species.
| Protein | Accession Number | (1)    | (2)     | (3)       | (4)       | (5)       |
|---------|------------------|--------|---------|-----------|-----------|-----------|
| hsAgo2  | NP_036286        | 242....255 | 290....307 |
| hsAgo2  | NP_036286        |        |         |           |           |           |
| hsAgo1  | NP_036331        | NIDEQPPLTDQR | SHQTPFLOQVEST |         |           |           |
| hsAgo3  | NP_079128        | NIDEQPRLTDQR | SHQTPFLOQVEST |         |           |           |
| hsAgo4  | NP_060099        | NINEQTFPLTDQR | SHQTPFLOQVEST |         |           |           |
| Hili    | NP_00129193      | AYTQKQK---EFQ       | PDSFTEMSDGK---EIT |         |           |           |
| Hiwi2   | NP_004755        | HQTENK------FQ       | PKSTFKKADGS------EVS |         |           |           |
| mmAgo2  | NP_694818        | SIEEQQPLTDQR | SHQTPFLOQVEST |         |           |           |
| rmAgo2  | NP_067608        | SIEEQQPLTDQR | SHQTPFLOQVEST |         |           |           |
| dmAgo1  | NP_725342        | DINEQRPPLTDQR | QMQSFPLQLEQVEST |         |           |           |
| dmAgo2  | NP_648775        | AKIHINNRLDQSRH   | SSEEF---ENDGKV---T |         |           |           |
| ceAlg2  | NP_671992        | ALAX-RRASDAQR   | QTQTFPLQLETQVEST |         |           |           |
| spAgo1  | NP_587782        | NVRD------LTRFDL   | S-DSFFVRLNGEEK1S  |         |           |           |
| atAgo1  | NP_849784        | S------RPLSDAR   | RELTPVDERNTOK-----S |         |           |           |
| hsAgo2  | NP_036286        | 385....395  | 526....533  | 796....807 |
| hsAgo2  | NP_036286        |        |         |           |           |           |
| hsAgo1  | NP_036331        | NASYNL---DPYIQ  | TP-VTAE-VK   | SVSIPAPAYAH |         |           |
| hsAgo3  | NP_079128        | SAMYET---DPFQ   | TP-VTAE-VK   | SVSIPAPAYAH |         |           |
| hsAgo4  | NP_060099        | SNSNGVGPDPYLQ | TP-VTAE-VK   | SVSIPAPAYAH |         |           |
| Hili    | NP_00129193      | -- ---------    | DD-LGQ---1K   | TIRWPAPCYAH |         |           |
| Hiwi2   | NP_004755        | HKNDHV---QRELH   | KD-RYDA---IK  | VIVVAPQCYAH |         |           |
| mmAgo2  | NP_694818        | SAFNFT---DYPVR  | TP-VTAE-VK   | SVSIPAPAYAH |         |           |
| rmAgo2  | NP_067608        | SAFNFT---DYPVR  | TP-VTAE-VK   | SVSIPAPAYAH |         |           |
| dmAgo1  | NP_725342        | RADFNH---DSYIQ  | TP-VTAE-VK   | SVSIPAPAYAH |         |           |
| dmAgo2  | NP_648775        | YFQMNH---DPTIS  | RI-SYDT---IK  | SVSIPAPAYAH |         |           |
| ceAlg2  | NP_871992        | KAELESA---DPPH  | TP-VTAE-VK   | SVSIPAPAYAH |         |           |
| spAgo1  | NP_587782        | QMWDWT---DPYLT  | SPEFEGS---IK  | AVSLVPFPVYYAH |         |           |
| atAgo1  | NP_849784        | LNDYAK---DNYAQ  | NGSLYGD---IK  | SVSIVFPAYAH |         |           |