**Supplementary Material for:**

# RITA a novel modulator of Notch signaling acts via nuclear export of RBP-J

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**Files in this Data supplement:**



**Supplementary Figure and Movie Legends**

**Figure S1: Genomic organization, mRNA- and protein sequences of human RITA.** (A) The human RITA gene is located on chromosome 12, spans approximately 6.5 kb and consists of four exons (E1 to E4). The coding sequences (yellow) are located on E3 and E4. (B) The human RITA mRNA encodes a 269 amino acid protein. Exon/intron boundaries are marked in red. A putative polyadenylation signal is underlined.

**Figure S2: RITA interacts with the BTD of RBP-J.** (A) Cell free synthesized $^{35}$S-labeled full length RBP2N (1-487, lane 1) and fragments 1-315 (lane 2), 166-487 (lane3), 166-334 (lane 4) were incubated with GST alone (B), GST-mNICD (C) and GST-RITA (D). (E) Schematic representation of the RBP-J fragments, used in the experiment. RBP-J (166-334) represents the BTD (black). (F) GST (lane 1) and GST fusion proteins (lanes 2 and 3) used as baits.

**Figure S3: RITA is a tubulin binding protein.** (A) Inhibition of tubulin polymerization leads to a decrease of RITA-GFP fibrillar structures within 2 hours. A HEK293 cell, stably expressing GFP-RITA was photographed before and 116 minutes after addition of 10 µM nocodazole (see also supplementary movie S1). (B) Single frames of a movie from dividing HEK293 cells, stably expressing GFP-RITA (see also supplementary movie S2). During mitosis, tubulin depolarization, breakdown of the nuclear membrane, centriole movements, spindle formation and tubulin dynamics during cytokinesis are clearly visible (arrows). (C) RITA shows tubulin like structures in *Xenopus laevis* embryonic cells. Stages of cell division of a dissociated late gastrula stage cell. Photographs were made in 30-second intervals (a). A dissociated mesendodermal late gastrula stage cell before spreading on the fibronectin substrate (b). A dissociated mesendodermal late gastrula stage cell spread on fibronectin. The meshwork of microtubules is found immediately above the level of substrate (c). A ciliated cell from the epidermis of a tailbud stage embryo (d). (D) RITA(wt) and RITA(156-269) but not RITA(1-257, ΔTub) colocalize with α-tubulin. HeLa cells were transiently transfected with the indicated for GFP-RITA expression plasmids. 24 h after transfection, cells were fixed and immunostained using an anti-tubulin antibody. The subcellular localization of GFP-RITA(wt) (left panel), GFP-RITA(1-257, ΔTub) (middle panel), GFP-RITA(156-269) (right panel) compared to α-tubulin was determined by fluorescence microscopy; scale bar; 5 µm.

**Figure S4: RITA does not colocalize with NICD and protects RBP-J from NICD loading.** (A) NICD shows nuclear localization when RITA is coexpressed (upper panel). A RBP-J binding defective NICD (ΔRBP/ΔEP) served as a control (lower panel). HeLa cells were transiently transfected with the indicated expression plasmids for RITA(wt) fused to GFP together with mNICD-mRuby. 24 after transfection subcellular localization was assayed by fluorescence microscopy, scale bar; 5 µm (B) In a cell-free system, GST-RITA bound RBP-J can not be co-immunoprecipitated together with NICD. Cell-free synthesized RBP-J alone (lanes 2 and 5) or together with cell-free synthesized NICD (lanes 3, 6, 7) were incubated with GST (lanes1 to 3), GST-RITA (lanes 4 to 6) or beads alone (lane 7). Only when not occupied by GST-RITA, RBP-J can be co-immunoprecipitated together with NICD (lanes 3 and 7). The asterisk denote the heavy chain of the antibody used for immunoprecipitation

**Figure S5: RITA is an evolutionary conserved protein.** (A) Protein alignments were performed using the CLC free Workbench 2.5.1 software, Abbreviations: HU, human, accession: AAH22092, MA, *Macaca mullata*, accession: XP_001111386, BO, *Bos taurus*, accession: NP_001039809, MO, *Mus musculus*, accession: NP_083372, RA, *Rattus norvegicus*, accession: NP_001037691, XL, *Xenopus laevis*, accession: BC070862 (EST), TA, *Trichoplax adhaerens*, accession: XP_002114203. (B and C) RITA-RBP-J interaction and subcellular localization is conserved in *Trichoplax adhaerens*. (B) Cell free synthesized RBP-J from *T. adhaerens* interact with GST-RITA(ta) (lane 2) and with GST-RITA(hs) (lane 3) but not with GST alone (lane 1). (C) taRITA is tubulin associated. HeLa cells were transiently transfected with pcDNA3-GFP-taRITA. 24h after transfection, cells were analyzed by fluorescence microscopy. (D) Human RITA binds to Su(H), the *Drosophila* homologue of RBP-J. Cell free synthesized Su(H) from D. melanogaster interacts with GST-RITA(hs) (lanes 2 and 3) but not with GST alone (lanes 1 and 4). Notch-IC (GST-NICD) served as a positive control for Su(H) interaction (lane 5).

**Figure S6: Expression of RITA mRNA.** (A) Expression in mouse embryos. Sagittal sections through whole body embedded embryos of stage embryonic day (ED) 12.5 and 17.5, enlargements from the area of the inner ear (above) and the area of the pharynx (below) and a cross section at the height of the forelimbs of a postnatal day 2 (PND2) mouse are shown. Weak but easily discernible enhanced expression is seen in several ganglia (trigeminal ganglion, ventral and dorsal root ganglia of the spinal cord) in the layer of the neocortex and also in the sensory epithelia of the inner ear. Signals are also present in some epithelial organs as the salivary glands, thymus and the developing kidney (malpighian tubules at ED 12.5, kidney cortex at ED 17.5) and in striated muscle (muscle cords of the trunk and heart). Preparation of mouse embryos and RNA in situ hybridization was carried out as described previously (Wilda et al., 2000). Abbreviations: 5g, trigeminal ganglion; Cki, cortex of kidney; cl, cochlea; DG, dorsal root ganglia; ED, embryonic day; he, heart; mc, muscle cords; MP, Malpighian tubules; NCx, neocortex; PND, postnatal day; R, ventral root ganglia; sg, submandibular gland, glandula parotis, thymus. (B) Expression in adult mouse tissues and embryonic stem cells. RITA-mRNA is expressed in all tissues tested as well as in ES cells. The highest level of expression was measured in brain and heart. Real-time PCR analysis with oligonucleotides derived from the murine RITA cDNA sequences were performed. The RITA mRNA expression levels were normalized to the endogenous HPRT mRNA levels for each tissue. The highest level of expression was set to 1. (C) Expression during embryonic development of *Xenopus laevis*. RITA(xl) mRNA is detected maternally (stage 1 to 6). Zygotic expression levels during gastrula stages (stages 8 to 13) are low and then increase during neurula (stage 16). RITA(xl) mRNA was detected by classical RT-PCR (left) or real-time PCR (right) using cDNA from the indicated embryonic stages and oligonucleotides shown in supplementary table 1. Histone H4 mRNA was used as internal control. Maximal expression of RITA(xl) was set to 1 (left). (D) Spatial expression at the neurula stage 17 of Xenopus laevis was analyzed using real-time PCR with cDNA from embryonic halves and

oligonucleotides as listed in supplementary table 1. mRNA levels (related to the expression of the housekeeping gene H4 $x10^{-2}$) are shown for RITA, the anterior marker Otx-2, the posterior marker HoxB9, the dorsal marker N-Tub and the ventral marker Hex. Minor changes in RITA levels indicate an equal distribution. (E) Whole mount in situ hybridization using full-length sense and antisense Xenopus sequences as probes. A neurula stage (S17, dorsal view, head up) and a tadpole stage (S35, lateral view, head up) are shown. A broad, perhaps ubiquitous, signal was detected at these stages.

**Figure S7: RITA/RBP-J interaction is conserved in *Xenopus laevis*.** (A) Cell free synthesized RBP2N and its Xenopus homologue xlSu(H) interact with GST-RITA(hs) (lanes 2 and 5) and with GST-RITA(xl) (lanes 10 and 13) but not with GST alone (lanes 1, 4, 9 and 12) or beads alone (lanes 3, 6, 11 and 14). (B) Endogenous RBP-J protein is coimmunoprecipitated with an anti-GFP antibody in HEK293 cell lysates after transient transfection with plasmids expressing mNotch-1-IC-GFP (lanes 2 and 3) or GFP-RITA(xl) (lanes 4 and 5). RBP-J protein is not immunoprecipitated in cellular lysates from HEK293 cells transfected with plasmids expressing EGFP alone (lane 1) or Flag-tagged mNotch-1-IC (lanes 6 and 7). Cellular lysates from HEK293 cells stably expressing human GFP-RITA(wt) (lane 8) and purified RBP-J protein (lane 9) served as positive controls. (C) A Flag-tagged RITA(xl) protein is not downregulated by morpholino. (upper) schematic representation of binding site for the RITA(xl) morpholino, MOa. (lower) Specificity of the MOa morpholino in downregulation of RITA(xl) translation. In an in vitro transcription/translation coupled protein synthesis assay, only the RITA(xl) protein without Flag-Tag, starting from the second start codon, is efficiently downregulated by MOa (lane 6). Translation of the Flag-Tagged RITA(xl), (detected by western blotting in lanes 2 and 3) is not affected by the morpholino (lanes 4, 5 and 6).

**Supplementary movie S1: Disappearance of fibrillar structures after inhibition of tubulin polymerization.** A HEK293 cell stably expressing GFP-RITA(wt) was photographed in minute intervals before and after addition of 10 µM Nocodazole. Magnification 630x.

**Supplementary movie S2: GFP-RITA(wt) localization in living cells is not distinguishable from tubulin.** HEK293 cells stably expressing GFP-RITA were photographed in minute intervals. Magnification: 200x.

**Supplementary movie S3: Localization of GFP-RITA and pericentrin at centrosomes.** HeLa cells were transiently transfected with a plasmid expressing GFP-RITA(wt). Pericentrin was labeled using an anti pericentrin antibody as described in materials and methods. From the centrosomal region a Z-stack was photographed, using a confocal microscope. The 3D reconstruction was made using the ImageJ software (http://rsb.info.nih.gov/ij/).

**Supplementary movies S4, S5 and S6: Nuclear accumulation of GFP-RITA** (wt) after treatment with the nuclear export inhibitor Leptomycin B (LMB), (S4). Nuclear import is independent of tubulin binding, (S5). An amino-terminally truncated RITA protein shows increased nuclear localization even without LMB treatment, (S6). HEK293 cells stably expressing GFP-RITA (wt), (S4), GFP-RITA (1-257), (S5) or GFP-RITA (25-269), (S6) were photographed every minute after treatment with 2.5 ng/ml Leptomycin B. left, DIC images, right, fluorescence images.

**Supplementary Materials and Methods**

**Plasmids**

PCR aided cloning was performed using cDNA-libraries from human peripheral blood mononuclear cells (PBMC), *Xenopus laevis* (stage 6) and *Trichoplax adhaerens* [kindly

provided by Bernd Schierwater (ITZ, Hannover)]. All oligonucleotides used in this study are shown in Table S1. PCR products were digested with appropriate restriction enzymes and inserted into the corresponding sites of pcDNA3-Flag1 (Oswald et al., 2002) or pcDNA3 (Invitrogen) (Table S2). To generate pcDNA3-Flag1-hsRITA-ΔRBP, the expression plasmid pcDNA3-Flag1-hsRITA(wt) was digested with SacII and XbaI to delete the carboxy-terminal part of RITA. A fragment was amplified (hsRITA_ΔRBP_UP, hsRITA_DO). After SacII/XbaI digestion, the PCR product was inserted into the opened vector. Amino acid substitution in hsRITA(NESmut), xlRITA(NESmut) and xlRITA(NLSmut) expression plasmids were introduced by PCR based site directed mutagenesis. To generate expression plasmids for RITA-GFP fusion proteins [pcDNA3-GFP-hsRITA_1-269 (wt), pcDNA3-GFP-hsRITA_1-257 (ΔTub), pcDNA3-GFP-hsRITA_85-269, pcDNA3-GFP-hsRITA_105-269, pcDNA3-GFP-hsRITA_156-269, pcDNA3-GFP-hsRITA_25-155, pcDNA3-GFP-hsRITA_ΔRBP (Δ128-156), pcDNA3-GFP-hsRITA(NESmut), pcDNA3-GFP-RITA(xl) pcDNA3-GFP-xlRITA(NESmut), pcDNA3-GFP-xlRITA(NLSmut), pcDNA3-GFP-xlRITA(ΔTub), pcDNA3-GFP-RITA(ta)], the EGFP cDNA was amplified from pEGFPC1 (Clontech) using the oligonucleotides EGFP_UP and EGFP_DO. The PCR product was digested with Acc65I/EcoRI and inserted into the corresponding sites of the Flag-tagged constructs, thereby exchanging the EGFP-cDNA with the Flag-tag. The expression plasmid for GFP fused to a tandem of the hsRITA-NLS, pcDNA3-GFP-(LTPRKKNKY)2, was made as follows: pcDNA3-GFP-hsRITA (wt) was digested with EcoRI and XbaI to delete RITA sequences. The 5-prime phosphorylated oligonucleotides NLS_UP and NLS_DO were annealed and ligated into the opened vector. The RBP2N specific expression plasmids for mapping the RBP-RITA interaction, pcDNA3-Flag1-RBP-J (aa 1-315), pcDNA3-Flag1-RBP-J (aa 166-487) and pcDNA3-Flag1-RBP-J (aa 166-334), as well as the expression plasmids for *T. adhaerens* RBP-J [pcDNA3-Flag1-RBP(ta)] and *D. melanogaster* Su(H) [pcDNA3-Flag1-dmSu(H)] were made by PCR. Products were digested with the indicated restriction enzymes and inserted into the corresponding sites of pcDNA3-Flag1 (Table S2). Plasmids for bacterial expression of GST-

fusion-proteins from various species were made as follows: The human RITA cDNA were isolated from pcDNA3-Flag1-hsRITA(wt) after XbaI/Klenow/EcoRI treatment and ligated into pGEX6P1 (GE healthcare) after digestion with XhoI/Klenow/EcoRI resulting in pGEX6P1-hsRITA. The expression plasmid pcDNA3-F1-RITA(xl) was incubated with ApaI/Klenow/EcoRI. The isolated RITA(xl) cDNA was ligated into the EcoRI/SmaI opened pGEX6P1 resulting in pGEX6P1-RITA(xl). The same strategy was used for construction of pGEX6P1-RITA(ta) starting with pcDNA3-Flag1-RBP(ta).

Table S1: Oligonucleotides used in the study for plasmid construction and real time PCR as well as morpholinos and siRNAs used for specific knock-down in *Xenopus laevis* and HEK293 cells.

| Constructs | |
|---|---|
| hsRITA_UP | 5'-GCGAATTCAAGACCCCCGTGGAGCTGGCC-3' |
| hsRITA_DO | 5'-GCTCTAGATGAAAGAGTATCATTTCCAAGGG-3' |
| hsRITA_25_UP | 5'-GCGAATTCTACCGGGTCAAGGCCAGG-3' |
| hsRITA_65_UP | 5'-GCGAATTCGGCGTGGGCAAGGAGGCAT-3' |
| hsRITA_155_DO | 5´-ATGATATCGCGGGGCGAGTGGCTACCC-3 |
| hsRITA_190_DO | 5´-ATGATATCTTAAGAGTGTAACCCACCCATAGATAAC-3 |
| hsRITA_216_DO | 5'-CGGATATCTTAACTGGTGGCTGGATGACC-3' |
| hsRITA_156_UP | 5'-CGGAATTCCCCAGGGAGGCACCACTG-3' |
| hsRITA_85_UP | 5'-CGGAATTCTCAAGGGGCAGCACCCCC-3' |
| hsRITA_269_DO | 5'-GCGATATCATTTCCAAGGGGGCTTTGG-3' |
| hsRITA_105_UP | 5'-CGGAATTCCACACCCCGTCTTACTGT-3' |
| hsRITA_257_DO | 5'-GCTCTAGATTAGCTAACTGAGCGAGCCCTGGAAG-3' |
| hsRITA_ΔRBP_UP | 5´-ACCCGCGGGAGGCACCAC-3´ |
| EGFP_UP | 5´-ATGGTACCATGGTGAGCAAGGGCG-3´ |
| EGFP_DO | 5´-ATGAATTCCTTGTACAGCTCGTCCAT-3´ |
| NLS_UP | 5´-AATTCCTCACACCAAGGAAGAAGAACAAATACCT CACACCAAGGAAGAAGAACAAATACTAAT-3´ |
| NLS_DO | 5´-CTAGATTAGTATTTGTTCTTCTTCCTTGGTGTGA GGTATTTGTTCTTCTTCCTTGGTGTGAGG-3´ |
| xlRITA_UP | 5´-CGGAATTCATGCCTGATAATCTGTATGCTACCAAC-3´ |
| FLAG_xlRITA_UP | 5´-CGGAATTCCCTGATAATCTGTATGCTACCAAC-3´ |
| xlRITA_DO | 5´-GCTCTAGATCATTTCCATGGAGGTCTCTCC-3´ |
| hsRITA_NESmut_UP | 5´-GTCAGTGGGGCGCAGACCGCCGGCGCTCAGCACCGC-3´ |
| hsRITA_NESmut_DO | 5´-GCGGTGCTGAGCGCCGGCGGTCTGCGCCCCACTGAC-3´ |
| xlRITA_NESmut_UP | 5´-CCAACATGTCTGCGGATGCCTCTGCAACTGGGCATAG-3´ |
| xlRITA_NESmut_DO | 5´-CTATGCCCAGTTGCAGAGGCATCCGCAGACATGTTGG-3´ |
| xlRITA_NLSmut_UP | 5´-CAGCAGGGACTCCCGCGGCAGCGATTCAATACAGG-3´ |
| xlRITA_NLSmut_DO | 5´-CCTGTATTGAATCGCTGCCGCGGGAGTCCCTGCTG-3´ |
| xlRITA_DTub_DO | 5´-TATCTAGATTACTCCTGCATTTTAACAGAGCCTC-3´ |
| RBP_1-315_UP | 5´-AACTCGAGATGGCGCCTGTTG-3´ |
| RBP_1-315_DO | 5´-GGTCTAGATTACCAGGAAGCGCCA-3´ |
| RBP_166-478_UP | 5´-AACTCGAGTTATGCATTGCCTCAGG-3´ |
| RBP_166-334_DO | 5´-GGTCTAGATTAGGCAAGGACAGGGCC-3´ |
| RBP_166-487_DO | 5´-GGTCTAGATTATACCACTGTGGCTGTAG-3´ |
| RITA_TA_UP | 5´-ATGAATTCTCCGATTCTATTGCTAGAAGAAGC-3´ |

| | |
|---|---|
| RITA_TA_DO | 5´-ATTCTAGATTATCTCCATGGTGGTCGAACAG-3´ |
| FlagtaRBP_UP | 5´-ATGAATTCAGAAAATACCTTGCTGGAAGATGTTATC-3´ |
| FlagtaRBP_DO | 5´-TATCTCGAGTTAAATTATAGGTAATAGTGAACAGTCAG-3´ |
| dmSUH_UP | 5´-ATGAATTCAAGAGCTACAGCCAATTTAATTTAAACG-3´ |
| dmSUH_DO | 5´-ATCTCGAGTCAGGATAAGCCGCTACCATG-3´ |
| | |
| real-time PCR | |
| | *mus musculus* |
| mRITA_F | 5´-CAAACCTCACACCAAGGAAGAAA-3´ |
| mRITA_R | 5´-ACAGTGACTCATCGCAGTAGGATG-3´ |
| mCyclophilin-F | 5´-ATGGTCAACCCCACCGTGT-3´ |
| mCyclophilin-R | 5´-TTCTTGCTGTCTTTGGAACTTTGTC-3´ |
| | *homo sapiens* |
| hsRITA_F | 5´-CCCTCACACCAAGGAAGAAG-3´ |
| hsRITA_R | 5´-CCAAACAGCGACTCATCACA-3´ |
| hsGAPDH_F | 5-GCTCTCTGCTCCTCCTGTTC-3´ |
| hsGAPDH_R | 5´-ACGACCAAATCCGTTGACTC-3´ |
| | *xenopus laevis* |
| xRITA.1_F | 5´-CACGCCTTCATATTGTGATGA-3´ |
| xRITA.1_R | 5´-ATCCCACGTGCATTCCTC-3´ |
| xDelta1_F | 5´-GCAGTAATGCGGACCCCAC-3´ |
| xDelta1_R | 5´-CCAGGCCATGTGAATCCAA-3´ |
| xESR1_F | 5´-GGAAAAGATGCGCAGAGACAG-3´ |
| xESR1_R | 5´-GGGTTCCTGCTTGTGAAATTCT-3´ |
| xESR2_F | 5´-AGATGCGCAGGGACCGTAT-3´ |
| xESR2_R | 5´-GGCTGGAGGAGCTGGAATTC-3´ |
| xESR7_F | 5´-CAAAAGCAGCCAGAAACCGA-3´ |
| xESR7_R | 5´-ATGATGAAATGCAGCTGGCA-3´ |
| xESR10_F | 5´-AGCAGCACATGACAACCAAA-3´ |
| xESR10_R | 5´-AGCAGCACATGACAACCAAA-3´ |
| xHESR1_F | 5´-AGCAGCACATGACAACCAAA-3´ |
| xHESR1_R | 5´-TGCAGAGAGTCCTGGAGACA-3´ |
| xN-Tub_F | 5´-AGCAGCACATGACAACCAAA-3´ |
| xN-Tub_R | 5´-TGCAGAGAGTCCTGGAGACA-3´ |
| xOtx-2 F | 5´-AAGTGAGCTCGGAGAGTGGG-3´ |
| xOtx-2 R | 5´-GAGATGACTGGACCAGAGGTGC-3´ |
| xHex F | 5´-CTATGGAGCCAGCACCTATGC-3´ |
| xHex R | 5´-CCAGAGAGTCGTGTCGGATGA-3´ |
| xHoxB9 F | 5´-TGCAGTTTCAGCGCCTCC-3´ |
| xHoxB9 R | 5´-GTTGGATATAGGGCTGATAGACTGG-3´ |
| xH4_F | 5´-GGCAAAGGAGGAAAAGGACTG-3´ |
| xH4_R | 5´-GGTGATGCCCTGGATGTTGT-3´ |
| | |
| morpholinos | |
| MOa | 5´-TGGTAGCATACAGATTATCAGGCAT-3´ |
| MOb | 5´-TGGCAGTACACGGATTATCAAGCAT-3´ |
| Ctrl | 5’-CCTCTTACCTCAGTTACAATTTATA-3’ |
| | |
| siRNAs | |
| hsRITA_siRNA1 | 5´-GGCUAACAGAACCAGAGGCtt-3´ (sense)<br>5´-GCCUCUGGUUCUGUUAGCCtt-3´ (antisense) |
| hsRITA_siRNA2 | 5´-GGAAGAAGAACAAAUACAGtt-3´ (sense)<br>5´-CUGUAUUUGUUCUUCUUCCtt-3 (antisense) |

Table S2: Expression constructs for flag-tagged as well as untagged RITA and RBP-J proteins from various species were made by PCR. The table gives plasmid names, used oligonucleotides (see table 1) and restriction enzyme sites for cloning into pcDNA3-Flag1 or pcDNA3.

| constructs RITA | oligonucleotides | restriction enzymes |
|---|---|---|
| pcDNA3-Flag1-hsRITA(wt)_1-269 | hsRITA_UP/hsRITA_DO | EcoRI/XbaI |
| pcDNA3-Flag1-hsRITA_25-269 | hsRITA_25_UP hsRITA_DO | EcoRI/XbaI |
| pcDNA3-Flag1-hsRITA_65-155 | hsRITA_65_UP/hsRITA_155_DO | EcoRI//EcoRV |
| pcDNA3-Flag1-hsRITA_65-190 | hsRITA_65_UP/hsRITA_190_DO | EcoRI//EcoRV |
| pcDNA3-Flag1-hsRITA_65-216 | hsRITA_65_UP/hsRITA_216_DO | EcoRI//EcoRV |
| pcDNA3-Flag1-hsRITA_156-269 | hsRITA_156_UP/hsRITA_269_DO | EcoRI//EcoRV |
| pcDNA3-Flag1-hsRITA_85-269 | hsRITA_85_UP/hsRITA_269_DO | EcoRI//EcoRV |
| pcDNA3-Flag1-hsRITA_105-269 | hsRITA_105_UP/hsRITA_269_DO | EcoRI//EcoRV |
| pcDNA3-Flag1-hsRITA_25-155 | hsRITA_25_UP/hsRITA_155_DO | EcoRI//EcoRV |
| pcDNA3-Flag1-hsRITA_1-257 | hsRITA_UP/hsRITA_257_DO | EcoRI/XbaI |
| pcDNA3-Flag1-hsRITA_ΔRBP | hsRITA_ΔRBP_UP/hsRITA_DO | SacII/XbaI |
| pcDNA3-Flag-1hsRITA(NESmut) | hsRITA_NESmut_UP/DO | EcoRI/Xba |
| pcDNA3-Flag-1-RITA(xl) | FLAG_xlRITA_UP/xlRITA_DO | EcoRI/XbaI |
| pcDNA3-RITA(xl) | xlRITA_UP/xlRITA_DO | EcoRI/XbaI |
| pcDNA3-Flag-1xlRITA(NESmut) | xlRITA_NESmut_UP/DO | EcoRI/Xba |
| pcDNA3-Flag-1xlRITA(NLSmut) | xlRITA_NLSmut_UP/Do | EcoRI/XbaI |
| pcDNA3-Flag-1xlRITA(ΔTub) | FLAG_xlRITA_UP/xlRITA_DTub_DO | EcoRI/XbaI |
| pcDNA3-Flag-1-RITA(ta) | RITA_TA_UP/RITA_TA_DO | EcoRI/XbaI |
| | | |
| constructs RBP/Su(H) | | |
| pcDNA3-Flag1-RBP-J (1-315) | RBP_1-315_UP/RBP_1-315_DO | XhoI/XbaI |
| pcDNA3-Flag1-RBP-J (166-478) | RBP_166-478_UP/RBP_166-487_DO | XhoI/XbaI |
| pcDNA3-Flag1-RBP-J (166-334) | RBP_166-478_UP/RBP_166-334_DO | XhoI/XbaI |
| pcDNA3-Flag1-RBP(ta) | FlagtaRBP_UP/FlagtaRBP_DO | EcoRI/XbaI |
| pcDNA3-Flag1-dmSu(H) | dmSUH_UP/dmSUH_DO | EcoRI/XhoI |

**Cell fractionation and coimmunoprecipitation experiments**

Nuclear and cytoplasmic fractions were prepared using standard procedures. Briefly, cell lysis (approx. 3 x $10^6$ cells) was performed with 100 µl sucrose buffer (320 mM sucrose, 3 mM $CaCl_2$, 2 mM MgAc, 0.1 mM EDTA, 10 mM Tris/HCl pH 8.0, 1 mM Dithiothreitol (DTT), 0.5 mM phenylmethanesulfonylfluoride (PMSF)) containing 0.5% Nonidet P-40 (NP-40, Fluka). After centrifugation step 1 (720 g, 5 min, 4 °C) the supernatant containing the cytoplasmic fraction was collected, diluted with 5 fold extraction buffer (150 mM HEPES, pH 7.9, 700 mM KCl, 15 mM $MgCl_2$), and centrifuged again (20.000 g, 15 min, 4 °C). After collection and addition of 25 % glycerol, the supernatant was used for western blotting. The pellet from centrifugation step 1 was resuspended in low salt buffer (20 mM HEPES, pH 7.9,

25 % glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF). Finally the nuclear proteins were extracted with high salt buffer (20 mM HEPES, pH 7.9, 25 % glycerin, 1.5 mM $MgCl_2$, 800 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1% NP-40) for 20 min on ice. After dilution with 1,5 fold volume of dilution buffer (25 mM HEPES, pH 7.9, 25 % glycerol, 0.1 mM EDTA, 0,5 mM DTT, 0.5 mM PMSF) the samples were centrifuged (20.000 g, 15 min, 4°C). Supernatants containing the nuclear fraction were used for further analysis. For IP experiments, cells were lysed with 700 µl CHAPS lysis buffer. The extracts were incubated with 40 µl agarose-conjugated anti-Flag antibody (M2, Sigma) at 4 °C overnight. Extracts from HEK293 cells stably expressing GFP or GFP-RITA fusion proteins were incubated with 4.0 µg anti-GFP antibody (Roche) at 4 °C overnight. After pre-clearing, 40 µl protein-G sepharose beads (4-fastflow, GE healthcare) were added and incubated for 2 hours at 4 °C. The beads were washed 6 to 8 times with CHAPS lysis buffer and resuspended in SDS-polyacrylamide gel loading buffer. For Western blotting the proteins resolved in SDS-polyacrylamide gels (10%) were transferred electrophoretically at room temperature to PVDF membranes (Millipore) for 1 h at 50 mA using a Tris-glycine buffer system. The membranes were pre-blocked for 1 h in a solution of 2% or 3% milk powder in PBS-T (0.1% Tween 20 in PBS) before adding antibodies. The following antibodies were used: anti GFP (7.1/13.1, mouse monoclonal IgG, secondary antibody peroxidase-conjugated sheep anti-mouse IgG, NA931V, GE healthcare), anti-Flag (M5, Sigma; secondary antibody, NA931V, GE healthcare), anti-RBP-J (rat monoclonal IgG2a, T6709, Institute of Immunology Co., Ltd.; secondary antibody peroxidase-conjugated goat anti-rat IgG, Dianova), anti-αTubulin (mouse monoclonal IgG, T9026, Sigma; secondary, NA931V, GE healthcare). For densitometric analysis scanned Western blots from three experiments were measured with the ROI manager tool of the ImageJ software ((http://rsb.info.nih.gov/ij/). After background subtraction, the signal intensities for tubulin served as loading control.

**Microinjections**

In all injections, a volume of 10 nl per blastomere was injected at stage 2. For loss of function experiments two anti-sense morpholino oligonucleotides (MOs, Gene-Tools Inc.) were designed. MOa exactly fits the start of translation of RITA, MOb is mutated in 5 positions to reduce the blocking of translation. The function and specificity of the MOs was tested *in vitro* using the T7-coupled transcription and translation system (Promega, Figure S7C). Concentrations of 1 to 3 ng/nl of MOs were injected resulting in doses of 20 to 60 ng per embryo. Best results were obtained with 2.5 ng/nl (50ng per embryo). The Gene tools' standard control morpholino (ctrl MO) was used as additional negative control. Two different capped mRNAs for *X. laevis* RITA [RITA(xl) and Flag1-RITA(xl)] were synthesized in vitro as previously described (Oswald et al., 2002) and used for injections. Concentrations from 1 pg/nl to 30 pg/nl (i.e. 20 pg per embryo to 600 pg per embryo) were tested. Doses below 200 pg per embryo resulted in weak phenotypes at low frequencies, doses below 100pg showed no significant effects at all. The mRNA encoding *mNotch1ΔE* was injected at concentrations of 100 to 200 pg per embryo. The numbers of injected embryos and resulting amounts of phenotypes are listed in table S3.

Table S3. Numbers of injected Xenopus embryos and distribution of phenotypes as shown in figure 8. The amounts of injected MO per embryo (in ng) and of different injected mRNAs per embryo (in pg) are given. Labels of injection are as in figure 8. MOa RITA specific MO, MOb mutated MO, ctrl MO unspecific control MO, S15 ISH stage 15 in situ hybridization, S45 phenotyp. phenotypical analysis at stage 45, N cumulated number of analyzed embryos.

| **S15 ISH** | | | normal develop. | | reduced N-tub | | other phenotype | |
|---|---|---|---|---|---|---|---|---|
| figure | Injection | N | abs. | % | abs. | % | abs. | % |
| 8A | uninjected | 24 | 24 | **100** | 0 | **0** | 0 | **0** |
| | MOa 50ng | 24 | 3 | **13** | 21 | **88** | 0 | **0** |
| | MOb 50ng | 24 | 22 | **92** | 1 | **4** | 1 [1] | **4** |
| | ctrl MO 50ng | 24 | 24 | **100** | 0 | **0** | 0 | **0** |
| **S15 ISH** | | | normal develop. | | **increased N-tub** | | other phenotype | |
| figure | Injection | N | abs. | % | abs. | % | abs. | % |
| 8B | uninjected | 32 | 32 | **100** | 0 | **0** | 0 | **0** |
| | RITA(wt) 100pg | 32 | 27 | **84** | 2 | **6** | 3 [2] | **9** |
| | RITA(wt) 200pg | 24 | 0 | **0** | 24 | **100** | 0 | **0** |
| | RITA(wt) 300pg | 24 | 0 | **0** | 24 | **100** | 0 | **0** |
| **S15 ISH** | | | normal develop. | | reduced N-tub | | other phenotype | |
| figure | Injection | N | abs. | % | abs. | % | abs. | % |
| 8C | uninjected | 24 | 24 | **100** | 0 | **0** | 0 | **0** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MOa 50ng | 24 | 3 | **13** | 20 | **83** | 1 [1] | **4** |
| | MOa 50ng+Flag1-RITA(wt) 40pg | 24 | 19 | **79** | 5 | **21** | 0 | **0** |
| | Flag1-RITA(wt) 40pg | 24 | 23 | **96** | 0 | **0** | 1 [1] | **4** |
| **S15 ISH** | | | normal develop. | | reduced N-tub | | other phenotype | |
| figure | Injection | N | abs. | % | abs. | % | abs. | % |
| 8D | uninjected | 68 | 67 | **100** | 0 | **0** | 1 [1] | **0** |
| | N1ΔE 200pg | 68 | 15 | **22** | 53 | **78** | 0 | **0** |
| | N1ΔE 200pg+Flag1-RITA(wt) 100pg | 24 | 20 | **83** | 4 | **17** | 0 | **0** |
| | Flag1-RITA(wt) 100pg | 24 | 21 | **88** | 1 | **4** | 2 [1] | **8** |
| **S15 ISH** | | | normal develop. | | reduced N-tub | | other phenotype | |
| figure | Injection | N | abs. | % | abs. | % | abs. | % |
| 8E | MOa 50ng+Flag1-RITA(wt) 100pg | 57 | 39 | **68** | 18 | **32** | 0 | **0** |
| | MOa 50ng+Flag1-RITA(ΔRBP) 100pg | 30 | 11 | **37** | 19 | **63** | 0 | **0** |
| | MOa 50ng+Flag1-RITA(NLSmut) 100pg | 25 | 10 | **40** | 13 | **52** | 2 [3] | **8** |
| | MOa 50ng+Flag1-RITA(ΔTub) 100pg | 32 | 22 | **69** | 10 | **31** | 0 | **0** |
| **S15 ISH** | | | normal develop. | | reduced N-tub | | other phenotype | |
| figure | Injection | N | abs. | % | abs. | % | abs. | % |
| 8F | N1ΔE 200pg+Flag1-RITA(wt) 100pg | 24 | 15 | **63** | 9 | **38** | 0 | **0** |
| | N1ΔE 200pg+Flag1-RITA(ΔRBP) 100pg | 24 | 7 | **29** | 17 | **71** | 0 | **0** |
| | N1ΔE 200pg+Flag1-RITA(NLSmut) 100pg | 24 | 6 | **25** | 18 | **75** | 0 | **0** |
| | N1ΔE 200pg+Flag1-RITA(ΔTub) 100pg | 24 | 14 | **58** | 10 | **42** | 0 | **0** |

| **S45 phenotyp.** | | | normal development | | described MOa phenotype | | other phenotype | | not developed | |
|---|---|---|---|---|---|---|---|---|---|---|
| figure | injection | N | abs. | % | abs. | % | abs. | % | abs. | % |
| 8A | uninjected | 58 | 56 | **97** | 0 | **0** | 1 [1] | **2** | 1 | **2** |
| | MOa 50ng | 58 | 12 | **21** | 41 | **71** | 3 [1] | **5** | 2 | **3** |
| | MOb 50ng | 48 | 40 | **83** | 4 | **8** | 4 [1] | **8** | 0 | **0** |
| | ctrl MO 50ng | 22 | 22 | **100** | 0 | **0** | 0 | **0** | 0 | **0** |
| **S45 phenotyp.** | | | normal development | | described RITA phenot. | | other phenotype | | not developed | |
| figure | injection | N | abs. | % | abs. | % | abs. | % | abs. | % |
| 8B | uninjected | 24 | 24 | **100** | 0 | **0** | 0 | **0** | 0 | **0** |
| | RITA(wt) 100pg | 24 | 18 | **75** | 5 | **21** | 1 [1] | **4** | 0 | **0** |
| | RITA(wt) 200pg | 24 | 5 | **21** | 18 | **75** | 0 | **0** | 1 | **4** |
| | RITA(wt) 300pg | 24 | 0 | **0** | 22 | **92** | 2 [1] | **8** | 0 | **0** |
| **S45 phenotyp.** | | | normal development | | described MOa phenot. | | other phenotype | | not developed | |
| figure | injection | N | abs. | % | abs. | % | abs. | % | abs. | % |
| 8C | uninjected | 28 | 27 | **96** | 0 | **0** | 0 | **0** | 1 | **4** |
| | MOa 50 ng | 28 | 1 | **4** | 20 | **71** | 0 | **0** | 7 | **25** |
| | MOa 50ng+Flag1-RITA(wt) 40pg | 28 | 19 | **68** | 5 | **18** | 0 | **0** | 4 | **14** |
| | Flag1-RITA(wt) 40pg | 30 | 21 | **70** | 3 | **10** | 0 | **0** | 6 | **20** |
| **S45 phenotyp.** | | | normal development | | described N1ΔE phenot. | | other phenotype | | not developed | |
| figure | injection | N | abs. | % | abs. | % | abs. | % | abs. | % |
| 8D | uninjected | 61 | 61 | **100** | 0 | **0** | 0 | **0** | 0 | **0** |
| | N1ΔE 200pg | 55 | 8 | **15** | 47 | **85** | 0 | **0** | 0 | **0** |
| | N1ΔE 200pg+Flag1-RITA(wt) 100pg | 34 | 26 | **76** | 7 | **21** | 0 | **0** | 1 | **2** |
| | Flag1-RITA(wt) 100pg | 34 | 34 | **100** | 0 | **0** | 0 | **0** | 0 | **0** |

[1] embryos with oedema, but no other defects, [2] reduced expression of N-tubulin, [3] changed pattern due to axial deformation

**Production of antiserum directed against NEK2**

The NEK2-specific peptide C Q E L E K R L E A T G G P I was coupled to Keyhole Limpet Hemocyanin (KLH) for the first, to bovine serum albumin (BSA) for the second, and to ovalbumin for the third immunization. For the immunization of rabbits, the coupled peptide emulsified in an equal volume of complete (first immunization) or incomplete (subsequent immunizations) Freund´s adjuvant (Gibco Laboratories) was used for subsequent subcutaneous injections in rabbits. Test bleed of each animal was taken 10 days after each boost, and after three to four boosts the animals were bled out. The specificity of the antiserum was confirmed by competition experiments.

**Immunofluorescence imaging**

HeLa cells were cultured on glass cover slips in a 25-well plate (Bibby Sterilin Ltd) at a density of $10^5$ cells per $cm^2$. After 16 hours, cells were transfected with 500 ng of expression plasmids. Cells were rinsed with PBS 24 hours after transfection, fixed and permeabilized with 0.1 % Triton X-100. Nonspecific immunostaining was blocked by incubating the cells in 3 % BSA in PBS with 0.1 % TWEEN-20. The following antibodies and antisera were used: anti α-Tubulin, mouse monoclonal IgG, (T9026, Sigma), secondary antibody, Cy3™ coupled sheep anti mouse IgG (C2181, Sigma), anti Pericentrin (Martin-Subero et al., 2003), secondary antibody, Alexa-Fluor-568 coupled goat anti rabbit IgG, (A11011, Invitrogen). When required, specimens were embedded in ProLong© Gold antifade reagent (Invitrogen) supplemented with 2-(4-carbamimidoylphenyl)-1H-indol-6-carboximidamide (DAPI) and stored at 4 °C overnight.

Imaging was performed using a fluorescence microscope (IX71, Olympus) equipped with a digital camera (C4742, Hamamatsu), and a 100-W mercury lamp (HBO 103W/2, Osram). The following filter sets were used: Green, (EGFP), ex: HQ470/40, em: HQ525/50; Red (Alexa-Flour-568), ex: HQ545/30, em: HQ610/75; Blue (DAPI), ex: D360/50, em: D460/50.

Confocal imaging was performed essentially as described previously (Kredel et al., 2009). Briefly, images were collected on a Leica TCS 4Pi scanning confocal laser microscope (Leica Microsystems) equipped with a 100×, NA 1.35, glycerol immersion objective and 488-nm excitation for EGFP, whereas Alexa-Fluor-568 was excited at 561 nm. Emitted photons were registered with two avalanche photodiodes (APDs; SPCM-AQR-14, Perkin-Elmer). For life-cell imaging, HeLa and HEK293 cells were grown on chambered cover glasses (Nunc). When required, Nocodazole (10 µM, Sigma) or Leptomycin B (2.5 ng/ml, Sigma) was added to the medium prior to analysis.

**Imaging of living cells from *Xenopus laevis***

Embryos were injected with 500pg of RITA-GFP mRNA at stage 2 and cultivated at 16°C. Coverglass chambers (Nunc Lab-Tek) were coated with 200 µg/ml of bovine plasma fibronectin (Sigma-Aldrich) for 30 minutes and then saturated with 5% BSA for 30 minutes. Mesendoderm from late gastrula embryos was explanted and then dissociated into single cells in $Ca^{2+}$ and $Mg^{2+}$ free MBS. Chambers were filled with MBS and cells seeded on the coated cover glass. Details about standard methods of explantation and cultivation of Xenopus cells were published before (Wacker et al., 1998; Wacker et al., 2000; Winklbauer, 1990). Cells were photographed using the Keyence BZ8000K fluorescent microscope with a Nikon Plan-Apo VC 60x/1.40 oil lens and a GFP filter set (GFP-B, ex: HQ470/40, em: HQ525/50).

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Wacker_Figure S1

A


B

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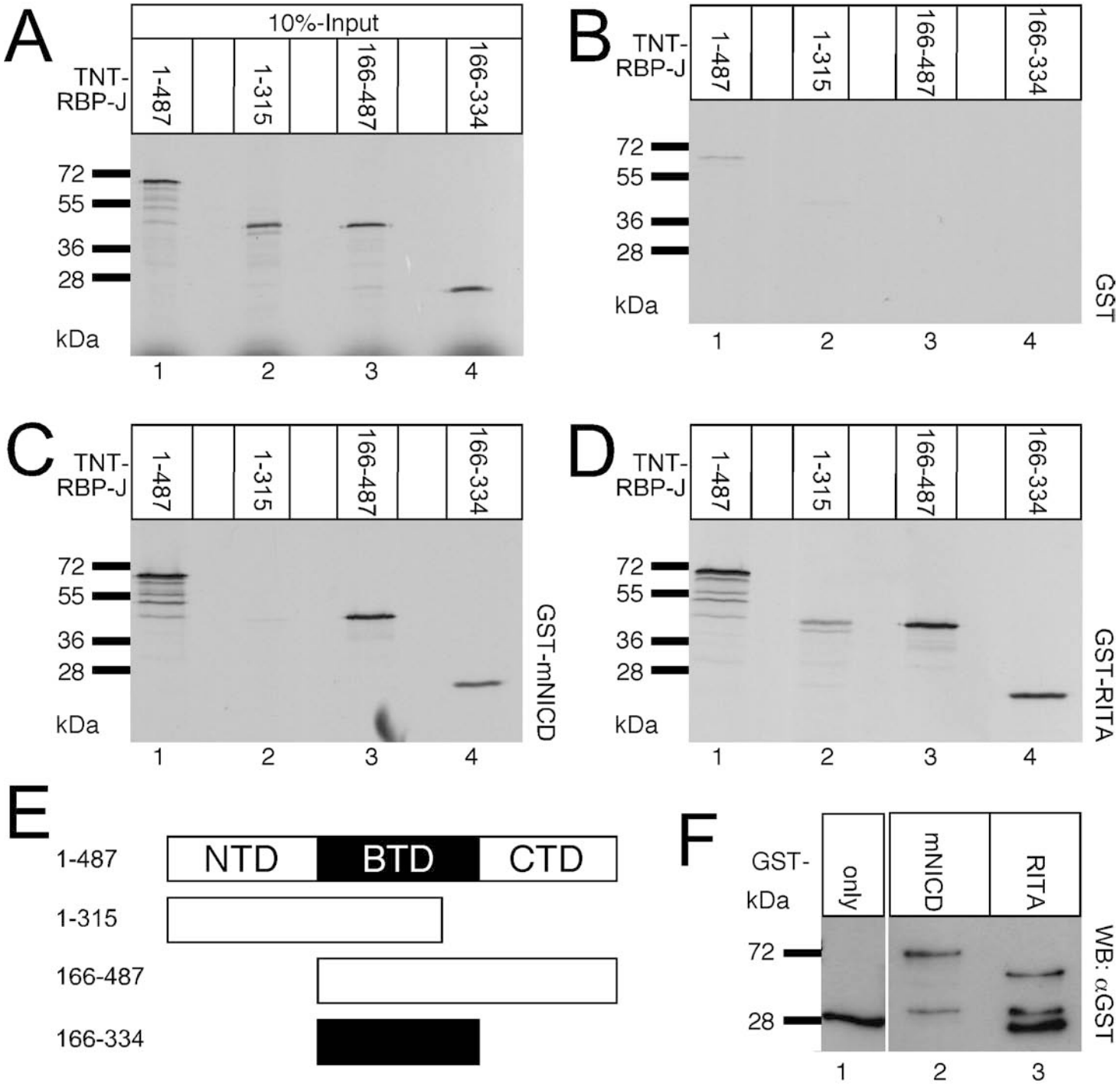

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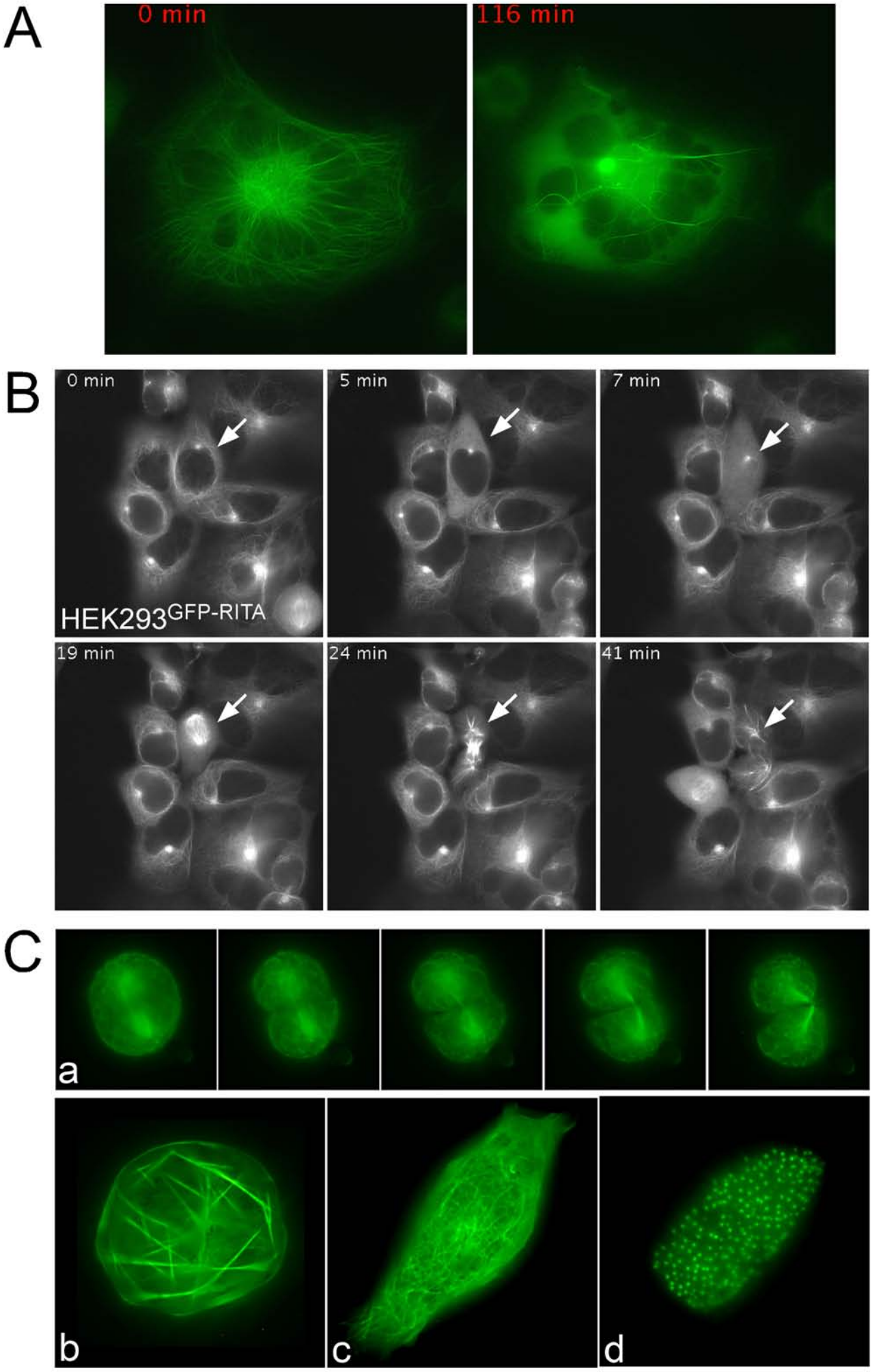

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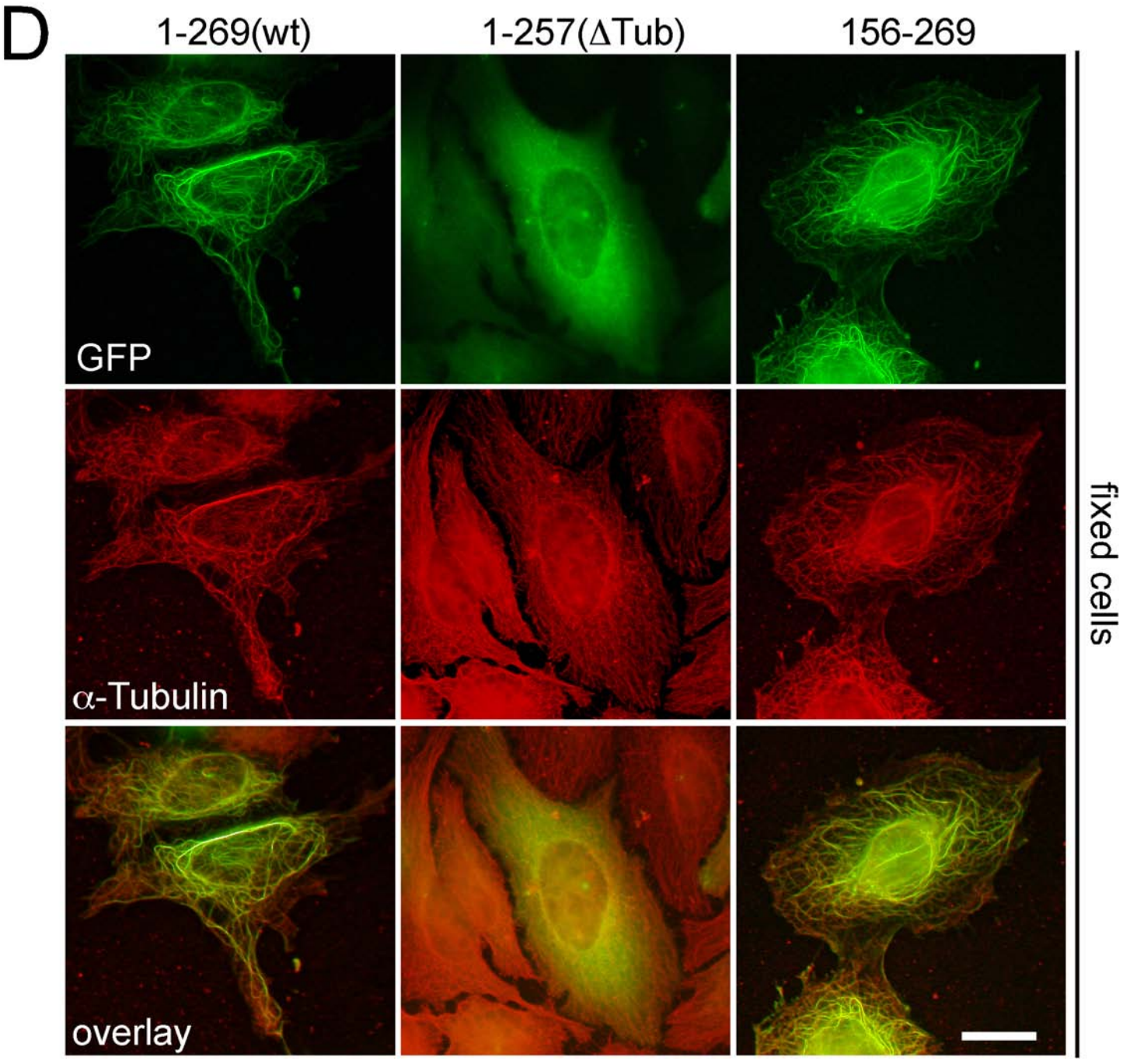

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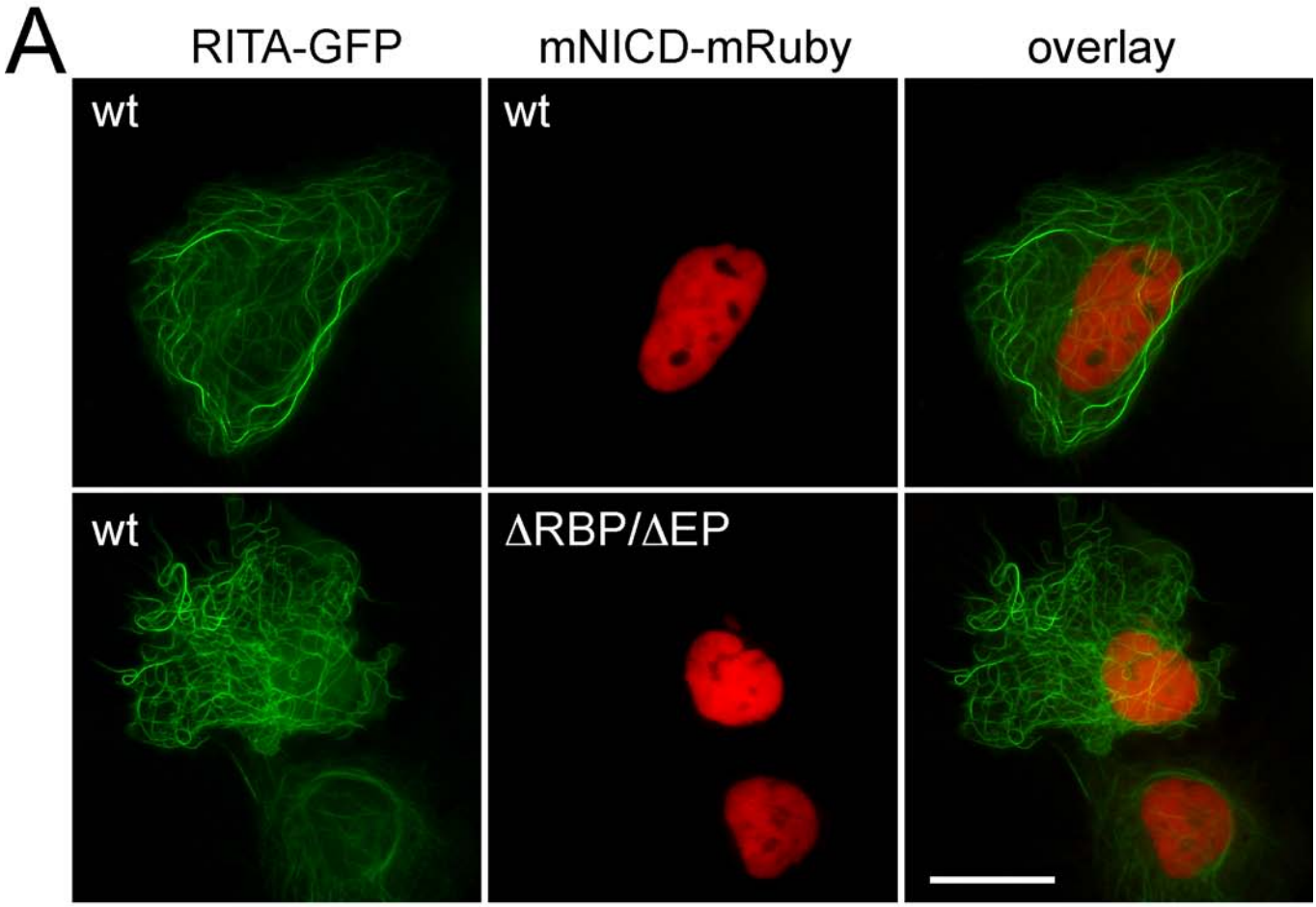


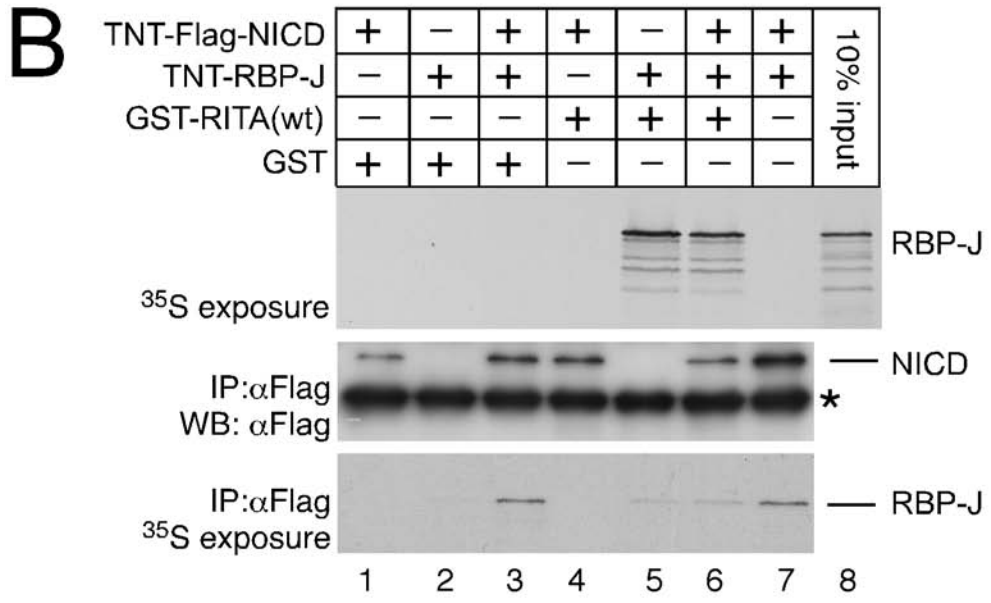

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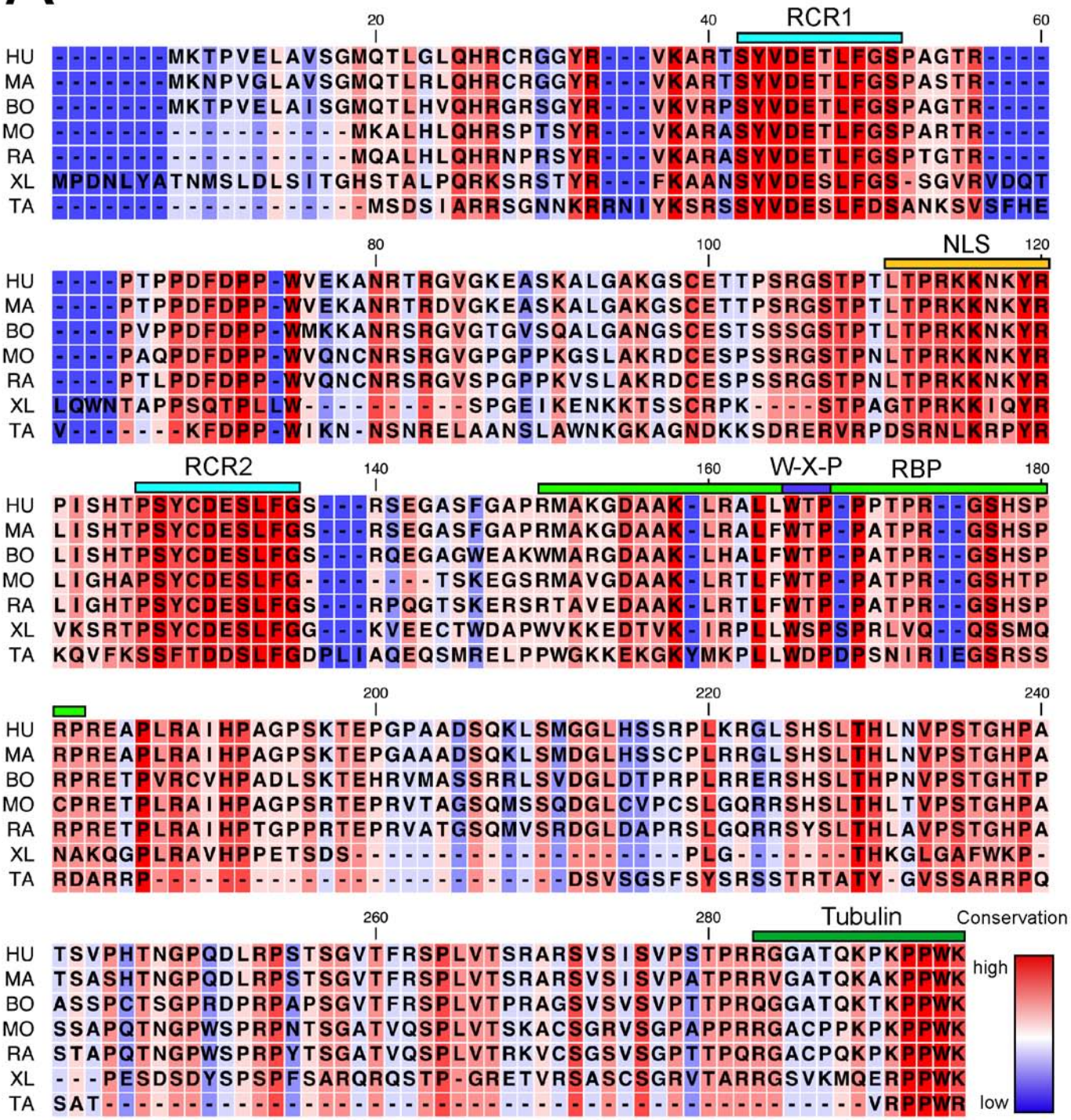

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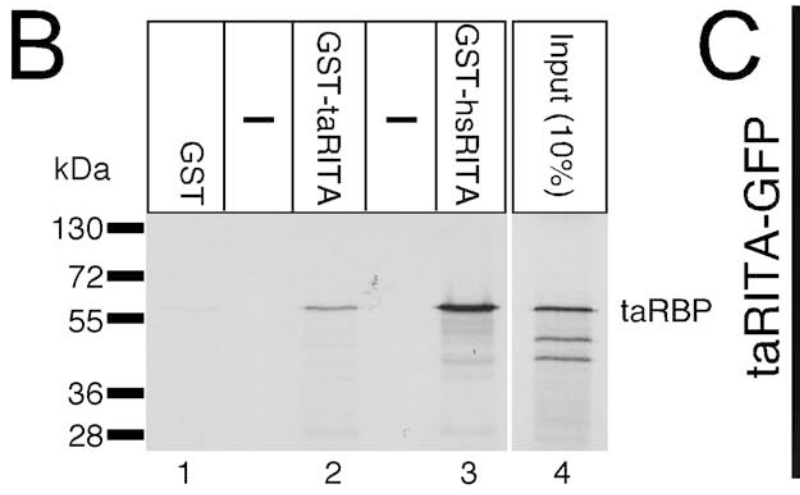
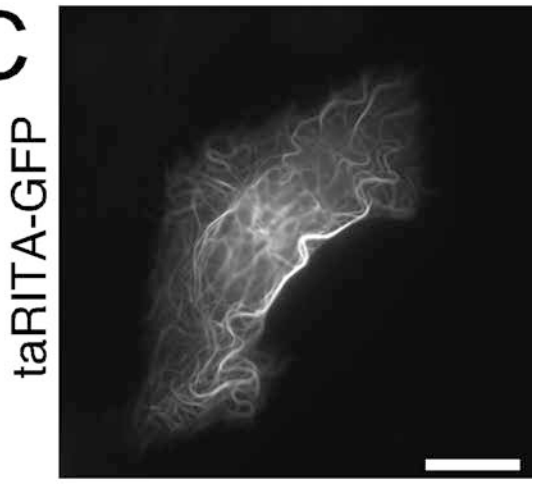
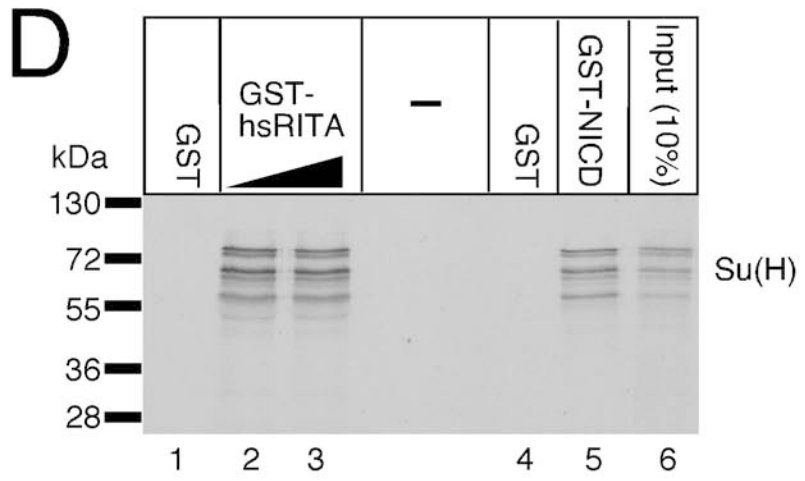
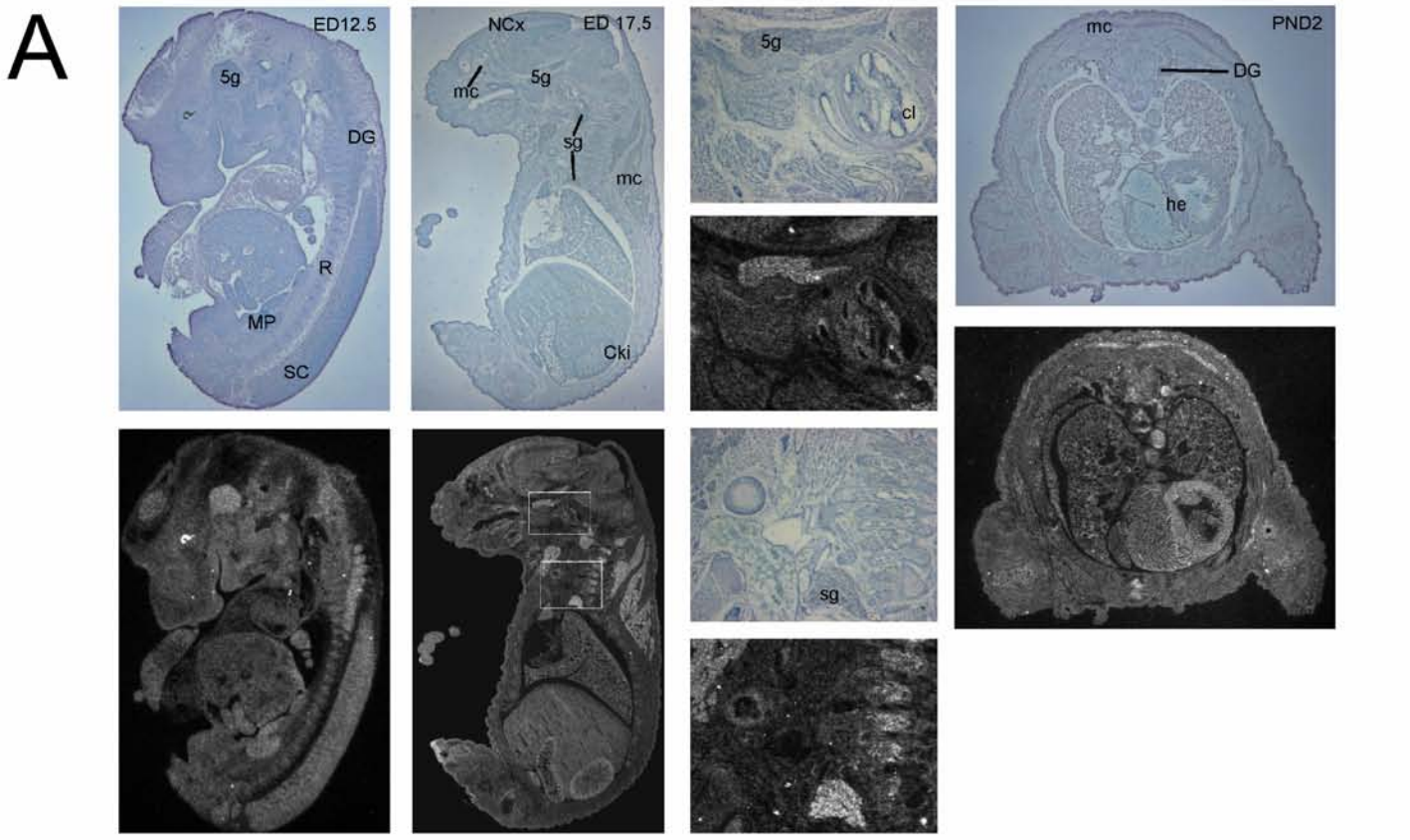
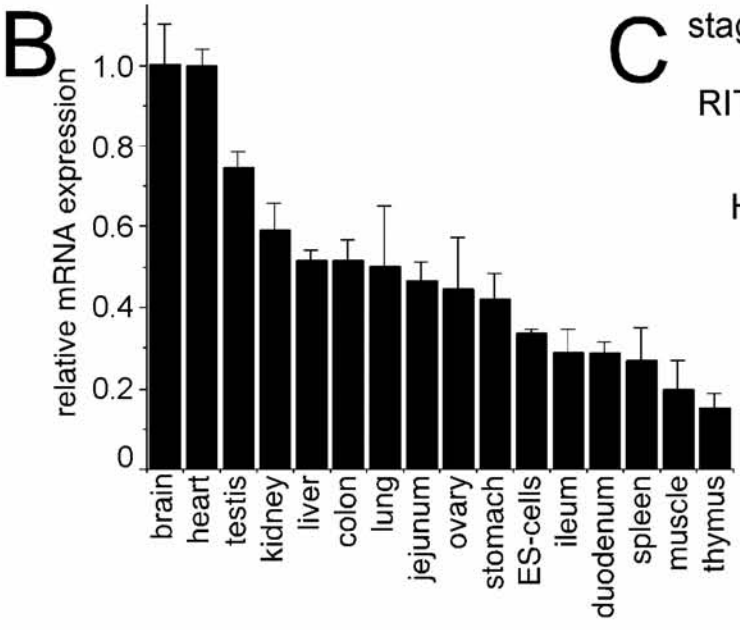


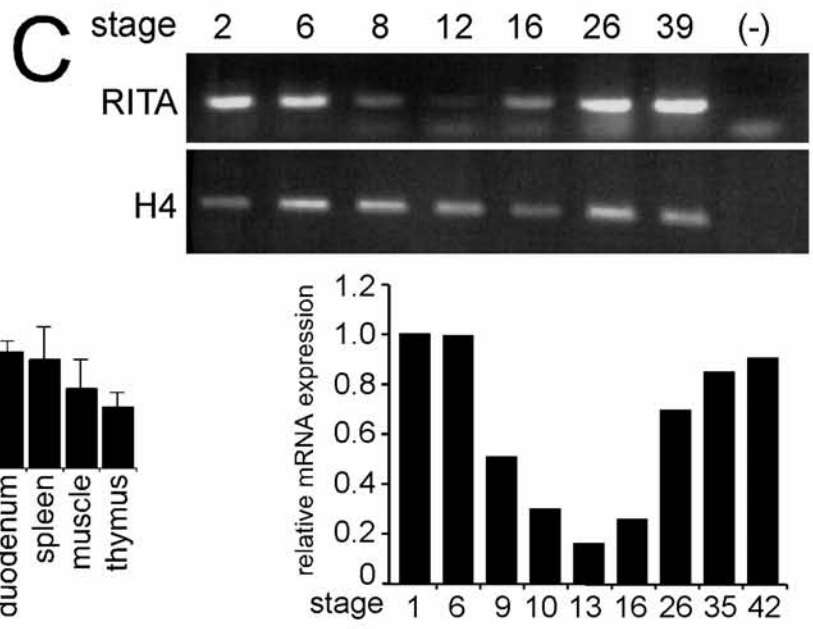



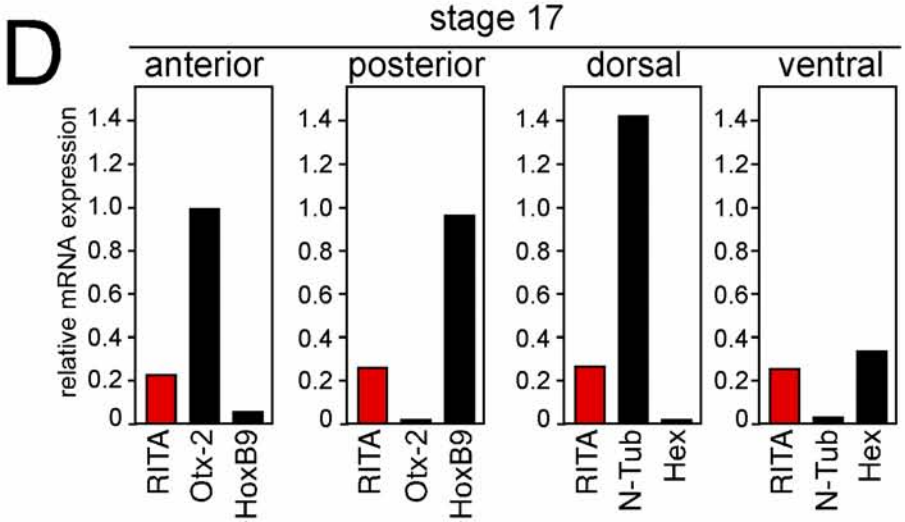



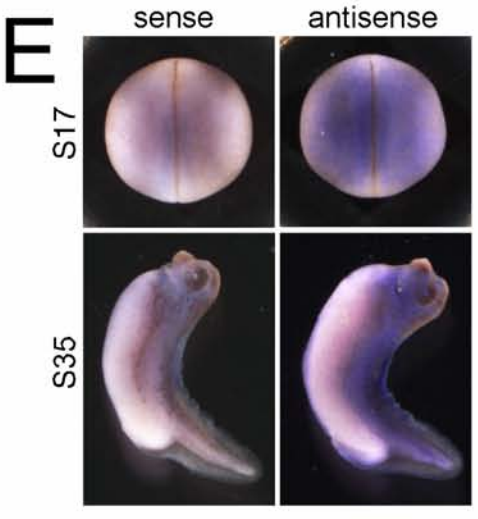
Wacker_Figure S6



Wacker_Figure S7