Supplementary information

Supplementary Materials and Methods

Plasmids, mutagenesis and siRNAs

Open reading frames corresponding to database human *HORMAD2*, *MAD1*, *MAD2L1*, *PP2A-B'δ*, *SGOL1* (NM_001012410) or *SGOL2* (NM_152524) were PCR-amplified from human testis or brain cDNA libraries (Clontech). *MAD2L1BP* cDNA coding for $p31^{comet}$ was purchased from Origene. Murine *Mad2l1*, *Sgol1*, *Sgol2* and *Sgol2b* were amplified from *Mus musculus* thymus or testis cDNA libraries (kind gifts of Markus Moser). In case of *Sgol2b* the oligos 5'-AATGGCCGGCCCATGGAGTACCCAGGGATAGAA-3' and 5'-ATTGGCGCGCCCTTAACAACGCTGCCACACTTG-3' were used for amplification. *Xenopus laevis cdc20*, *mad1*, *mad2l1*, and *sgol1* were amplified from an oocyte cDNA library (kind gift of Adrian Salic). Fragments of *Xenopus cdc20*, *mad1*, *sgol1* and of human *SGOL2* were PCR-generated from plasmids containing the corresponding full length ORF. All PCR products were tagged with restriction sites of the rare 8 bp cutters FseI (5') and AscI (3') by respective extensions of the primers at their 5' ends. All PCR products were then FseI/AscI-cloned into one or several vectors of a series of FA-plasmids, all of which had their original MCSs replaced by an FseI plus AscI site. These were pCS2-Flag3-FA, pCS2-HA3-FA, pCS2-Myc_6-FA for vertebrate cell expressions (for unmodified pCS2 see: Rupp et al., 1994; Turner and Weintraub, 1994), pACT-FA or pAS2-1-FA for yeast 2-hybrid assays (unmodified vectors from Clontech), and pMAL-Tev_2-FA, pGEX-FA, pQE80-FA, or pET31-FA for bacterial expressions (original plasmids from New England Biolabs, GE Healthcare, Qiagen, or Novagen, respectively). The relative positioning of reading frames and cleavage sites were kept the same in all cases, i.e. GGC CGG CCn for FseI and N-terminal tagging and nGG CGC GCC for AscI and a C-terminal His_6-tag (pET31-FA constructs only).

siRNAs to deplete Sgo2 were "Y": 5'-GAACACAUUUCUUCGCCUAUU-3' (Huang et al, 2007; oligo #2) and "W": 5'-GCACUACCACUUUGAAUAATT-3' (Kitajima et al, 2006; Tanno et al, 2010). Mutations were introduced by PCR-, modified GeneEditor- (Promega), or modified QuikChange (Stratagene) method. To render recombinant *SGOL2* siRNA resistant the sequence 5'-cacatttcttcgccta-3' was changed to 5'-TacGtttcttcgGctT-3' - when using siRNA oligo #2 described by Huang et al (2007).

Antibodies

Antibodies used were: rabbit-anti-Myc (Santa Cruz Biotech., sc-789), rabbit-anti-Flag (Sigma, F7425), rabbit-anti-HA (Santa Cruz Biotech., sc-805), mouse-anti-Hec1 (Genetex, GTX23393), rabbit-anti-Sgo2 (Bethyl, A301-261A for I.P., A301-262A for Western blot and immunofluorescence), rabbit-anti-MCAK (gift from T.U. Mayer), mouse-anti-Mad1 (Sigma, M8069), mouse-anti-Cdc20 (Santa Cruz Biotech., sc-13162), mouse-anti-PP2A-C (Millipore, 05-421), mouse-anti-Mad2 (Western; Santa Cruz Biotech., sc-47747), rabbit-anti-Mad2 (immunofluorescence, Bethyl, A300-300A), mouse-anti-RGS-His_6 (Qiagen, 34610), mouse-anti-alpha tubulin 12G10 (Developmental Studies Hybridoma Bank) and mouse-anti-phospho Histone H3 (pSer10, Sigma, H0412); affinity matrices for I.P. were: mouse-anti-Flag (M2) agarose (Sigma, A2220), mouse-anti-Myc agarose (Upstate, 16-219) and protein G sepharose (GE Healthcare, 17-0618-01).

Two different antibodies directed against *X.l.* Sgo1 were raised by immunizing rabbits with the following recombinant *X.l.* Sgo1 fragments: (#1) a.a. 200-300, (#2) a.a. 400-500. Antibody #1 was used for Western blots at concentrations of 1 μg/ml. For immunoprecipitation experiments from meiotic tissues, 3 μg of antibody #2 per IP were bound to 7 μl (packed) protein A affiprep beads (Biorad) or 30 μl (slurry) protein A Dynabeads (Invitrogen) and subsequently crosslinked using DMP.

Yeast-2-hybrid assay and analysis of expression levels

Yeast transformation and testing for interaction on synthetic complete (SC) medium plates lacking histidine were carried out using standard procedures. The readout strain was PJ69-7A (*trp1-901, leu2-3,112, ura3-53, his3-200, gal4Δ, gal80Δ, GAL1::HIS3, GAL2-ADE2, met2::GAL7-lacZ*; Eichinger & Jentsch, 2010), a derivative of PJ69-4A (James et al, 1996). To prepare total yeast cell extract, cells from a yeast culture (equivalent to 1 ml of OD_{600nm} = 1) were harvested by centrifugation (1,000 g, 2 minutes), resuspended in 1 ml cold ddH_2O, and lysed with 150 µl 1.85 M NaOH, 7.5% β-mercaptoethanol. After incubation for 15' on ice, 150 µl of 55% trichloroacetic acid (TCA) was added and proteins were precipitated for 10 minutes before being pelleted by centrifugation for 10 minutes at 4°C and 16,000 g. The pellet was resuspended in 100 µl HU-buffer (8 M urea, 5% SDS, 200 mM Tris-HCl pH 6.8 1 mM EDTA, 1% (w/v) bromophenol blue, 100 mM DTT), heated to 65°C for 10 minutes and then resolved by SDS-PAGE on precast gradient gels (Serva, Heidelberg).

Cell culture methods

Mammalian cells (HeLa, HeLa histone-H2B-GFP, HeLa Kyoto histone H2B-mCherry / eGFP-tubulin (Steigemann et al, 2009), U2OS, HEK293T) were cultured in D-MEM at 37°C (5% CO_2). HEK293T cells were transfected with expression plasmids or siRNAs using the calcium phosphate method and cultured for at least 24 h before harvesting. In order to deplete Sgo2 or Mad2 from HeLa, cells were transfected with siRNAs targeting Sgo2 (Huang et al, (2007): siRNA #2, 60 nM or as indicated (oligo "Y"); Kitajima et al (2006): 400 nM or as indicated (oligo "W")) or Mad2 (40 nM) (Stucke et al, 2004) using Lipofectamine RNAiMAX (Invitrogen) or Hiperfect (Qiagen), respectively, and cultured for 48 hours (Sgo2) or 24 hours (Mad2) before harvesting. Either Lipofectamine 2000 (Invitrogen) or PEI (6 μg/ml) were used for transfection of HeLa cells with plasmids. To obtain robust mitotic arrest, cells were treated with 200 ng/ml nocodazole. To induce a delay in mitotic progression and chromosome misalignment, cells were treated with 10 ng/ml of nocodazole or 20 ng/ml taxol. Pre-synchronization at the G1/S-boundary was achieved by addition of 2 mM thymidine to cycling cells for 16-18 hours and subsequent release by threefold change of medium.

Immunofluorescence microscopy and live-cell imaging

For analysis by immunofluorescence microscopy, HeLa cells grown on coverslips were washed with 1 x PBS, fixed in 1 x PBS, 3.7% formaldehyde, and then permeabilized in 1 x PBS, 0.5% Triton X-100. After blocking with 1x PBS, 3% BSA, specimen were incubated with primary antibody dilutions in blocking solution for 2 hours, washed with 1x PBS, 0.1% Triton X-100, and incubated with secondary antibody dilutions (Alexa 488, Alexa 546 or Cy3 coupled, Invitrogen) in blocking solution for 1 hour. After staining with Hoechst-33342 (2 μg/ml), coverslips were mounted onto slides and analyzed using an Axio Imager A1 (Zeiss). Photos were taken using a 23.0 1.4 MP monochrome Spot Persuit camera system and Spot 4.5.9.1 software (Diagnostic Instruments).

Chromosome spreads and mitotic index determination

Chromosome spreads were prepared using Carnoy's solution as described (McGuinness et al, 2005). Hoechst-33342 (2 μg/ml) was used as DNA stain. For mitotic index determination, floating and trypsinized cells were combined, pelleted, and fixed by addition of 0.1 volume of 1 x PBS, 16% CH_2O for 15 min. Cells were washed 2 times with 1 x PBS, 100 mM glycine and permeabilized by incubation with cold methanol for 10 minutes on ice. Following a blocking step in 1 x PBS, 3% BSA (30 minutes), cells were processed essentially as for

immunofluorescence microscopy except that all steps were followed by centrifugation (400 g, 2 minutes). For cell suspensions derived from 1 well of a 6-well plate, 50 μl of 1:50 antibody dilutions were used (primary: mouse-anti-phospho-Histone H3 (pSer10, Sigma), secondary: Cy3 coupled goat-anti-mouse-IgG (Invitrogen)). Alternatively, cells were fixed with 70% ethanol, treated with RNase (0.1 mg/ml) and stained with propidium iodide (69 μM). In both cases cells were analyzed by flow cytometry using a Cytomics FC 500 system (Beckman-Coulter). For figure S4A, cells on coverslips were analyzed by anti-phospho-Histone H3 immunostaining and fluorescence microscopy.

Live cell microscopy

Time lapse live cell imaging was performed on a DMI 6000 inverted microscope equipped with digital camera (Leica Microsystems) and climate chamber to maintain 37°C and 5% CO_2 (Pecon). Images were acquired every 5 min for a total of 12 h. To measure duration of mitosis, HeLa cells were imaged using the phase contrast method in standard multi-well cell culture dishes using an HCX PL FLUOTAR L 20x/0.4 objective (Leica Microsystems). Mitosis was defined as the time from initial cell rounding until first signs of cleavage furrow ingression. For analysis of chromosome segregation, a HeLa S3 cell line stably expressing histone-H2B-GFP and cultured in 8-well slides (Ibidi) was imaged by fluorescence microscopy using an HCX PL APO 40x/0.85 objective (Leica Microsystems).

Isothermal titration calorimetry (ITC)

The peptide-protein bindings were measured by ITC using a VP-ITC MicroCalorimeter (MicroCal, Northampton, MA). All ITC experiments were carried out according to references provided by the manufacturer. Investigated peptides were used at 0.3 - 0.4 mM in PBS and titrated from a 300 µl syringe into a sample chamber holding 1.43 ml of 0.03 - 0.04 mM *X.l.* Mad2. All solutions were degassed prior to measurements. Heat generated by protein dilution was determined in separate experiments by injecting a protein solution into a PBS filled sample chamber. All data were corrected for the heat of protein dilution. Data were fitted using χ^2 minimization for a model, assuming a single set of binding sites to calculate the binding affinity K_D. All steps of the data analysis were performed using the ORIGIN (V5.0) software provided by the manufacturer.

Supplementary figure legends

Supplementary figure 1

(A) Mouse Sgo2 but not Sgo1 interacts with Mad2. HEK293T cells were transfected with an expression plasmid for HA-tagged *M.m*. Mad2 plus either a control plasmid or plasmids encoding Flag-*M.m*. Sgo1 or Flag-*M.m*. Sgo2. Anti-HA and Anti-Flag immunoprecipitates from nocodazole arrested cells were analyzed by immunoblotting and -detection of the epitope tags. **(B)** Yeast-2-hybrid assay confirming the *M.m*. Sgo2-Mad2 interaction. **(C)** A single point mutation in *X.l*. Sgo1 abolishes Mad2 binding. Amino acids 1-207 of *X.l*. Sgo1 were expressed in fusion with N-terminal AD (Gal4 transactivation domain). **(D)** Mutation of the Mad2 binding site does not affect expression levels of shugoshin. Yeast cells expressing the Gal4 transactivation domain (AD) fused to the N-terminal fragment (aa 1-250) of Sgo2-wt. or -R153A (see Fig 2E) were extracted under denaturing conditions and subjected to Western analysis using antibodies against the different Gal4 domains and phosphoglycerate kinase (PGK) as loading control.

Supplementary figure 2

(A) Mad2, PP2A and shugoshin can form a trimeric complex. Chelating agarose was loaded with His_6-tagged Mad2-wt. or -ΔC10, incubated with extract from Myc_6-PP2A-B'δ overexpressing HEK293T cells and either MBP-tagged *X.l*. $Sgo1^{1-207}$ or reference buffer. After washing, eluates were analyzed by SDS-PAGE and Coomassie staining (upper panels) or anti-Myc immunoblotting (lower panel). **(B)** ITC data for titration of Mad2 with the *H.s*. Sgo2 peptide KRISKQCKLMRLPFAR. The binding affinity of Sgo2/Mad was calculated to: K_D = 0.685 μM. **(C)** 1D NMR spectra of free Mad2 (I) titrated with the *X.l*. Sgo1 peptide SKVEEKLSSGASAILRLPIHAPISD at a molar ratio (protein:peptide) of 1:0.1 (II), 1:0.25 (III), 1:0.5 (IV), 1:1 (V), and 1:5 (VI). Examples of Trp signals that are splitting are marked by dotted lines (red: starting position, blue: final position). **(D)** Bacterially expressed MBP-TEV-tagged *X.l*. $Sgo1^{108-207}$ was combined with various forms of bacterially expressed His_6-tagged Mad2 (upper panel), affinity-purified over amylose-resin and eluted by cleavage with TEV-protease (middle panel). The eluates were affinity purified over Ni^{2+}-NTA-agarose and finally eluted with imidazole (lower panel). All panels show Coomassie stained gels. **(E)** A complex of *X.l*. $Sgo1^{99-207}$ and Mad2 was tandem affinity purified as described in (D) and analyzed for purity by Coomassie staining. **(F)** Immunoprecipitates of Flag-tagged *H.s*. Sgo2 from transfected HEK293T cells were analyzed by Western for presence of various forms of

co-expressed Myc-Mad2. In the Mad2 mutants, Thr 109 and Ser 114, 170, 178, 195 were all changed either to aspartate (Asp) or alanine (Ala). **(G)** Mutually exclusive binding of Cdc20 and *X.l.* Sgo1 to Mad2. A mixture of GST-*X.l.* $Cdc20^{114-146}$, MBP-*X.l.* $Sgo1^{108-207}$ and His_6-Mad2 (input) was passed over glutathione-, amylose- or Ni^{2+}-NTA beads. Proteins retained on the affinity resins after washing were identified by SDS-PAGE and Coomassie staining. To control for unspecific binding in each case the protein carrying the respective tag was omitted.

Supplementary figure 3

Divergent phenotypes upon depletion of Sgo2 using different siRNAs. **(A)** Western blots showing depletion of Sgo2 from HeLa Kyoto cells (stably expressing histone 2B-mCherry and eGFP-tubulin) by RNAi (48 h RNAi incl. 16 h 200 ng/ml nocodazole treatment). "G" refers to GL2 control. Sgo2 siRNAs: "W" was used at 400 nM in Tanno et al (2010) and Kitajima et al (2006); the "Y" oligo was described in Huang et al (2007), oligo #2. Antibodies used are indicated. **(B)** HeLa Kyoto cells (stably expressing histone 2B-mCherry and eGFP-tubulin) were treated with the indicated siRNAs for 56 h (incl. thymidine pre-synchronization). Phase contrast live cell microscopy was used to determine the duration of mitosis (time from first signs of cell rounding to cleavage furrow ingression; 10 min per frame). Box plot parameters are as follows. Upper box limit: 3rd quartile, lower box limit: 1st quartile, whiskers: 5th and 95th percentile, dots: values beyond 5th and 95th percentile, continuous line within the box: median, $n > 40$ **(C)** Movies obtained in (B) were analysed for signs of mitotic cell death, i.e. obvious cell lysis.

Supplementary figure 4

(A) Mad2 depletion but not Sgo2 depletion causes an override of a short-term SAC arrest. HeLa cells were transfected with control (ctrl.), Mad2, or Sgo2 siRNAs and pre-synchronized with thymidine. Six hours after release, DMSO (as control) or nocodazole (Noc, 200 ng/ml) was added. Alternatively, vinblastine (VBL) was applied at a low (lo, 6.7 nM) or a high concentration (hi, 0.5 μM) to mimic loss of tension or attachment, respectively (Skoufias et al, 2001). Another 8 hours later, the mitotic index was determined by immunofluorescence staining of Ser10-phosphorylated histone H3. The relatively low mitotic indices were most probably due to an inefficient thymidine release. **(B)** Sgo2 is dispensable for long term SAC-mediated mitotic arrests. Left panel: Sgo2 depletion efficiency by siRNA. HeLa cells were transfected with Sgo2 siRNA or control treated, pre-synchronized with thymidine and subsequently released ($t = 0$). After 2 hours (corresponding to 45 hours after siRNA transfection) nocodazole or paclitaxel were added (200 ng/ml each). At $t = 17$, 25, and 30

hours, aliquots of the cells were processed for mitotic index determination by immunostaining of Ser10-phosphorylated histone H3 and flow cytometry (middle and right panels). Cells from the 17 h time point were additionally analyzed by immunoblotting with Sgo2 and alpha-tubulin antibodies (left panel). **(C)** Sgo2 is not required for protection of centromeric cohesion in mitosis. Chromosomes from HeLa cells treated with the indicated siRNAs, synchronized by thymidine (16 h), released into nocodazole containing medium (8.5 h) and finally treated for 3 h with MG132 (60 μM). Chromosomes were spread and sister chromatid cohesion was quantified (error bars: SD). Microscopic images show exemplary spreads from Sgo1 or Sgo2 depleted cells.

Supplementary figure 5

(A) Detailed analysis of Sgo2 localization in different mitotic stages. Asynchronous HeLa cells were fixed and immunostained for Hec1 (red) and Sgo2 (green). DNA was labeled with Hoechst-33342. Mitotic stages are indicated and were determined based on chromatin morphology. Scale bars are 10 μm for the large panels and 0.5 μm for the close-up images of sister kinetochores. **(B)** Partial translocation of Sgo2 to kinetochores requires microtubule attachment but no tension. HeLa cells were treated with nocodazole or paclitaxel (200 ng/ml each) for 6 h and then processed as in (A). Scale bars as in (A).

Supplementary figure 6

(A) Sgo2 interacts with itself. HEK293T cells were transfected to express Flag-Sgo2 and/or Myc-Sgo2 as indicated. Anti-Flag immunoprecipitates from corresponding lysates were analyzed by immunoblotting using the indicated antibodies. **(B)** *H.s.* Sgo2-N58I lacks PP2A- but retains Mad2 binding capacity. Anti-Flag immunoprecipitates from transfected HEK293T cells expressing both Myc-Mad2 and Flag-tagged *H.s.* Sgo2-wt. or -N58I were analyzed by immunoblotting using the indicated antibodies. **(C)** Schematic drawing of phenotypes observed under the different experimental conditions, i.e. nocodazole alone (-MG) or in combination with MG132 (+ MG) (kinetochore: red circles, Sgo2 signals: green circles; main phenotype under control conditions: blue box; mislocalization: orange box) **(D)** Immunofluorescence signals of Sgo2 at kinetochores are split upon depletion of Mad2. HeLa cells were transfected either with control (ctrl. = GL2) or Mad2 siRNA and cultured for 24 h. Nocodazole (10 ng/ml) and MG132 (60 μM) were added and 3 hours thereafter cells were fixed and stained for the indicated markers (left panels; scale bars: 10 μm / 0.5 μm). The

predominant Sgo2 localization phenotype in metaphase cells was determined (right panel; 2 independent experiments; n > 160 each; error bars: SD).

Supplementary figure 7

(A) HeLa cells synchronized either for 24 h in G1/S phase (interphase, 2 mM thymidine) or for 16 h in G2/M phase (mitosis, 200 ng/ml nocodazole). Cells were lysed and Sgo2 was extracted from chromatin in high salt buffer (400 mM NaCl). Sgo2 was immunoprecipitated and probed for associated proteins by Western blot using the indicated antibodies. Unspecific IgG (mock) served as negative control. **(B)** Cell cycle distribution of samples from (A) determined by propidium iodide staining and flow cytometry.

Supplementary references

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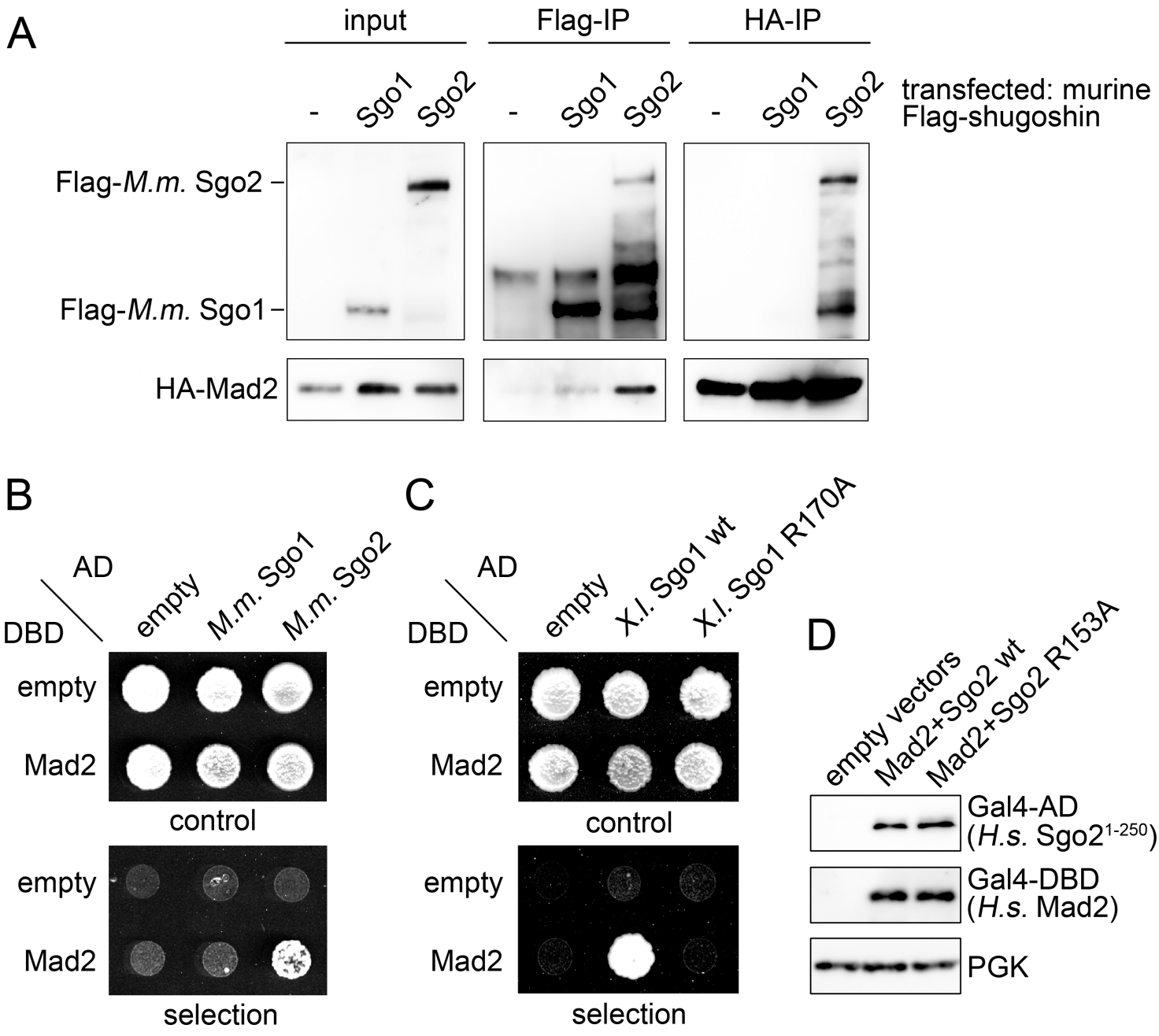

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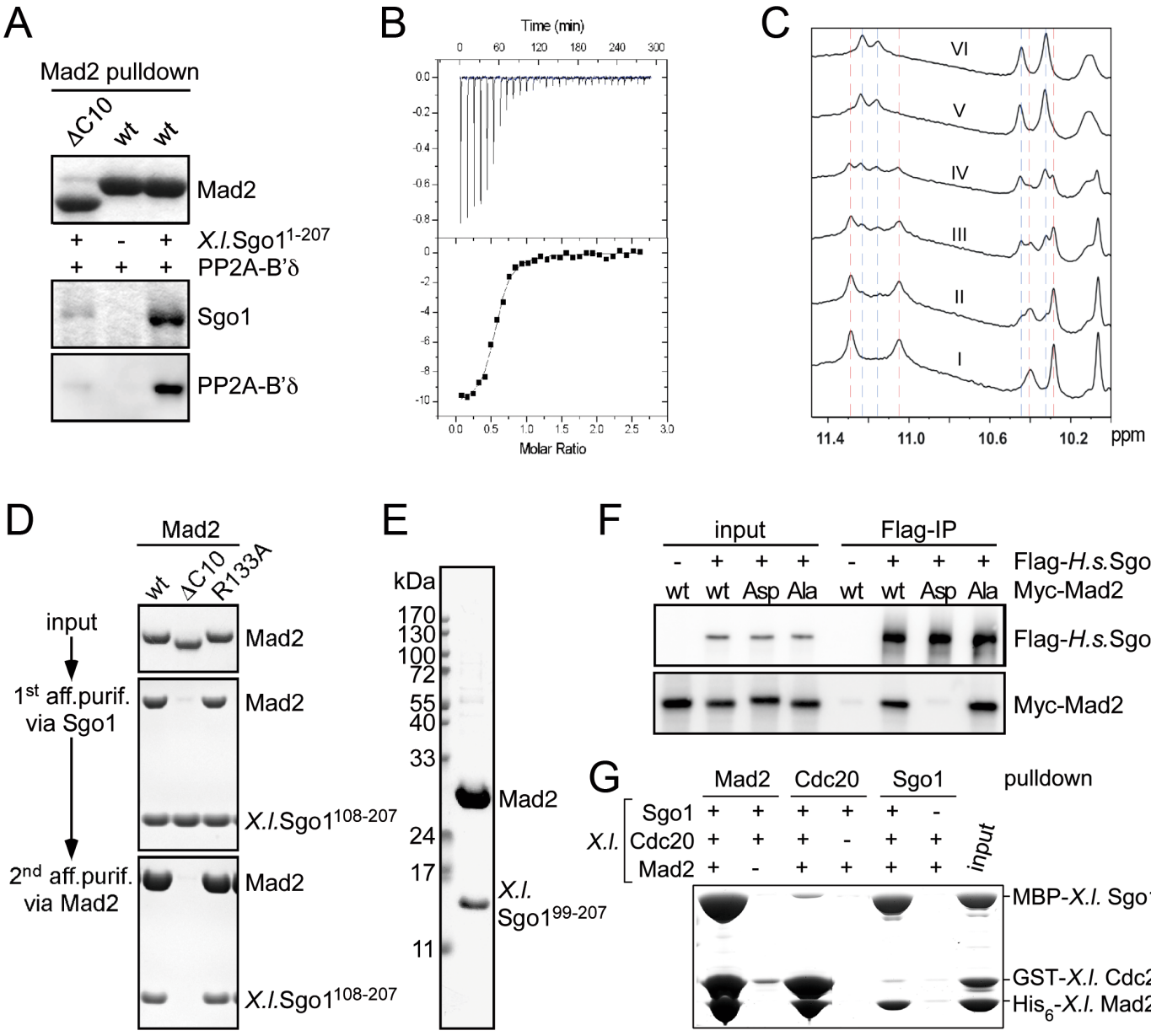

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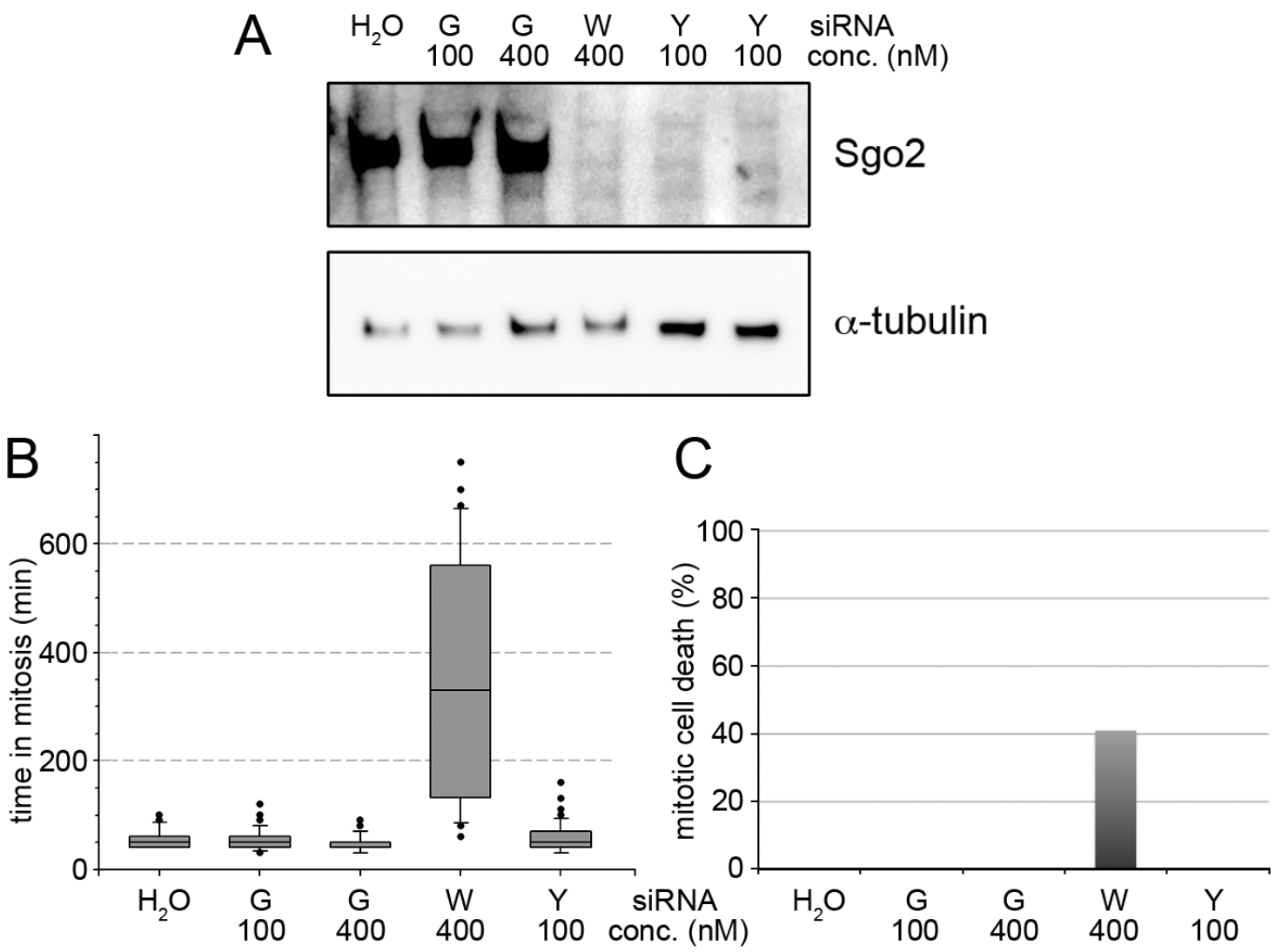

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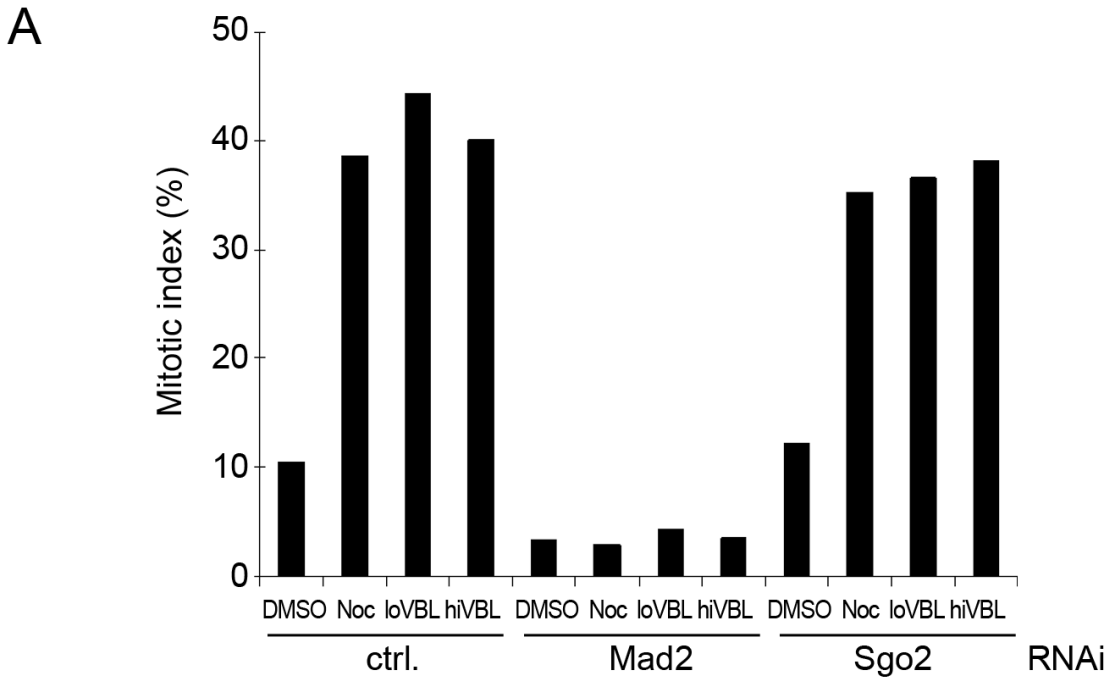

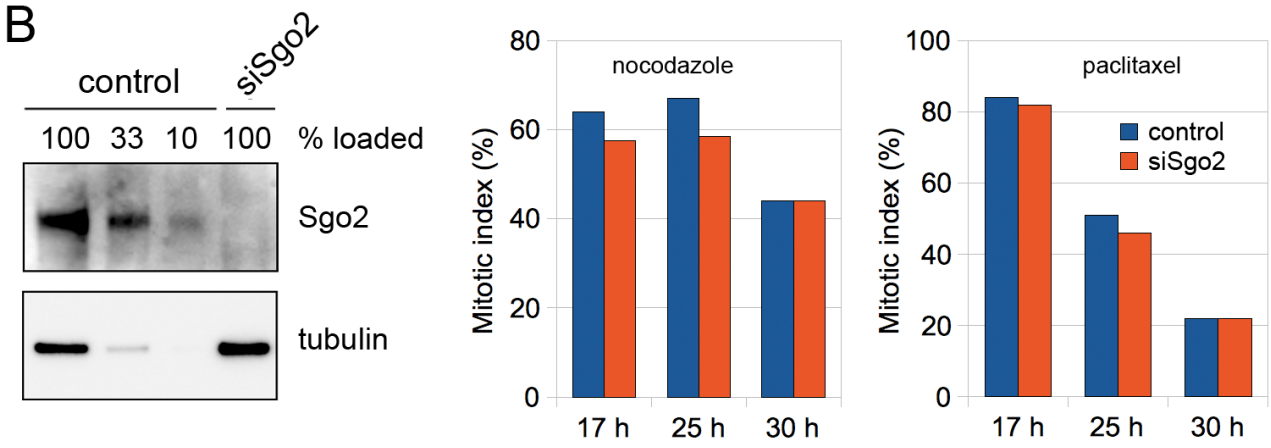

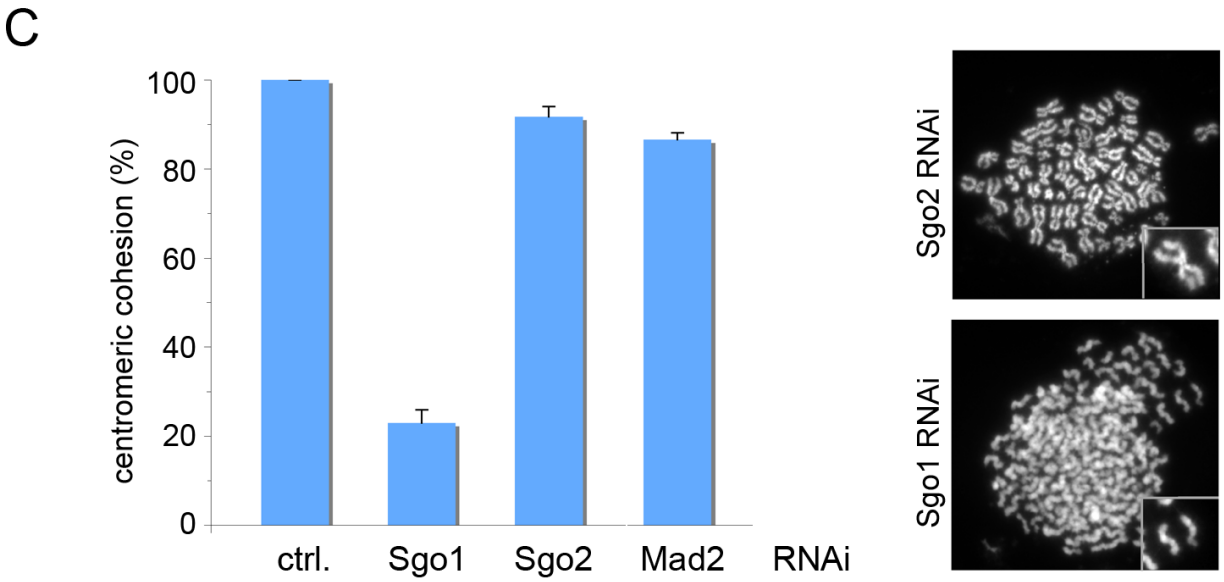

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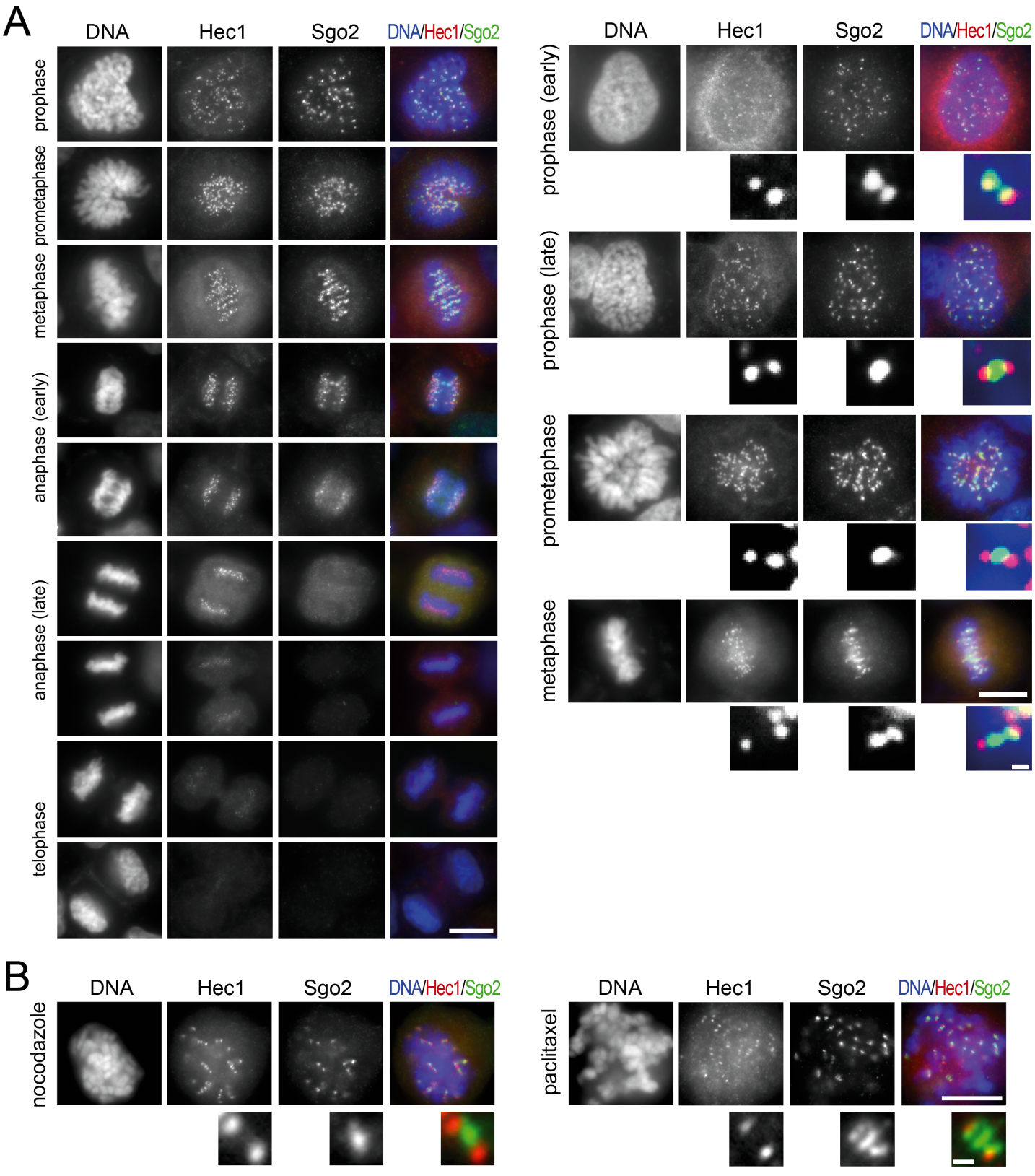

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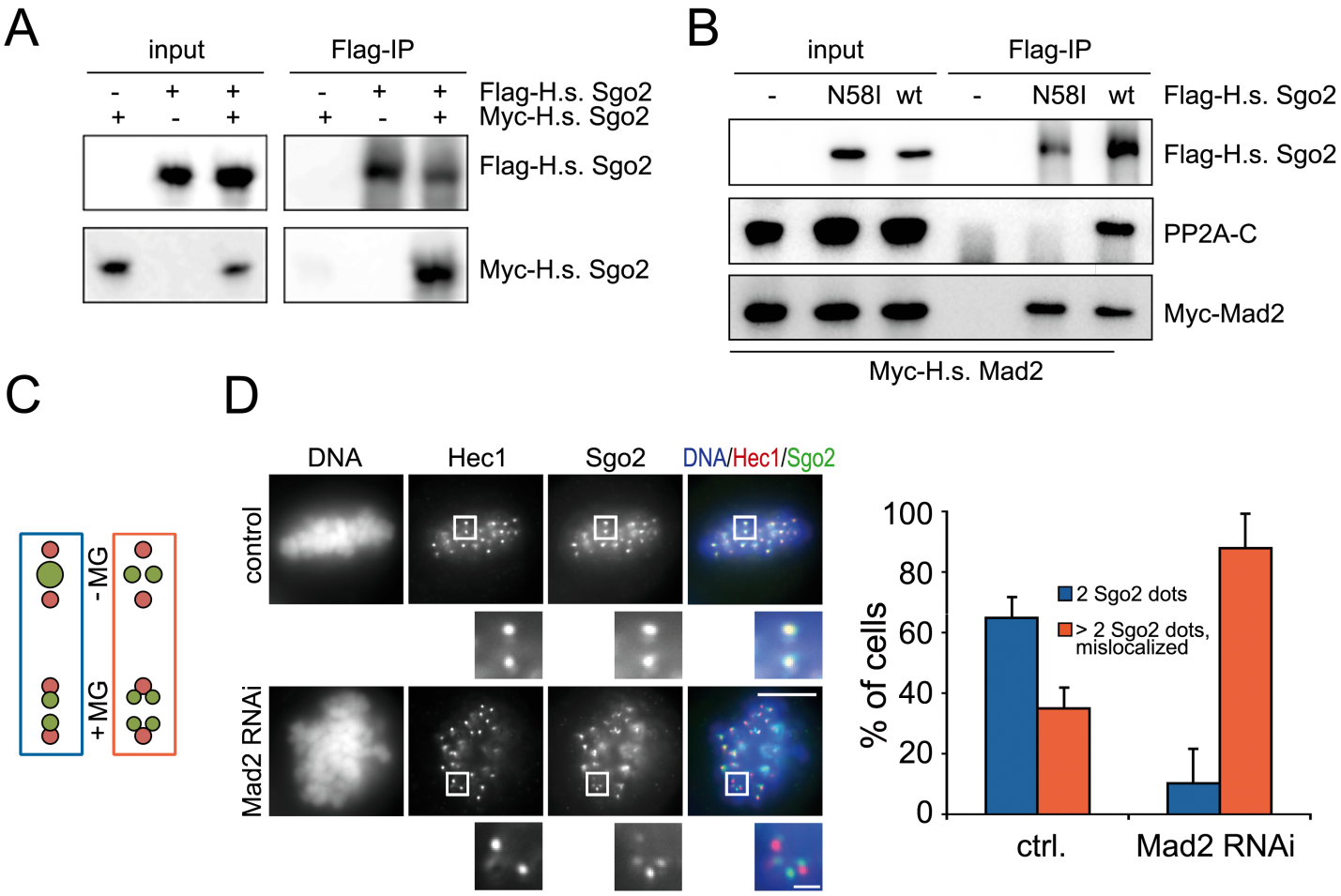

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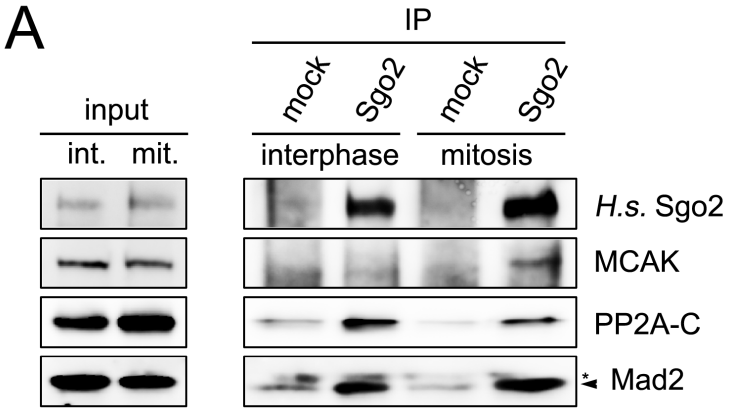

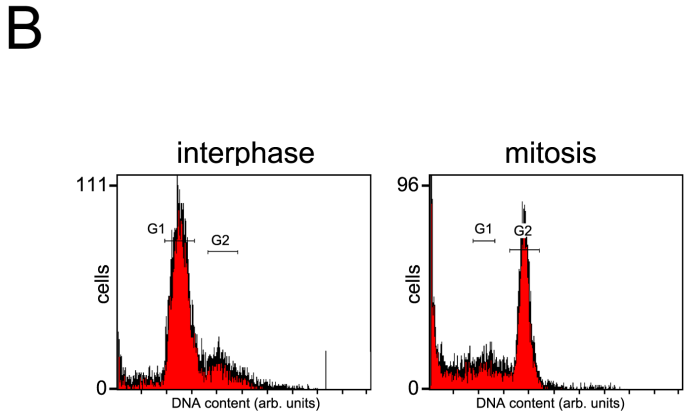

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