Supplementary Materials and methods

Cell culture and transfection

HeLa cells, HeLa cells stably expressing ss-HRP, HeLa cells stably expressing ManII-GFP, and HEK 293T cells were grown in complete medium consisting of DMEM (PAA laboratories) containing 10% FCS, 100U/ml penicillin, and 100µg/ml streptomycin at 37°C with 7 % CO_2. To synchronize cells in S-phase or pre-metaphase, the cells were incubated for 18 h with 2 mM thymidine or 1.5 µM nocodazole, respectively. For the transfection of siRNAs, HeLa cells were transfected in suspension with siRNA duplexes and then seeded onto culture plates, and retransfected a second time the following day with the same siRNA duplexes. Transfection was performed with Lipofectamine2000 (Invitrogen) following the manufacturer's recommendations. Myt1, MEK1 and SCFD1 mRNA were silenced by transfecting cells with respectively, Myt1 siRNA duplexes (5'-GCCUGGGCCAUGGCUCCUACGdTdT-3' and 5'-AGCCGCCUGGGCCAUGGCUCCdTdT-3' and their antisense sequences), MEK1 siRNA duplexes (5'-GAGGGAGAAGCACAAGAUCdTdT-3' and 5'-AAGAUUCUACUCUUGUCAUUUdTdT-3' and their antisense sequences), and SCFD1 siRNA duplex (5'-AGACUUAUUGAUCUCCAUAdTdT-3' and its antisense sequence) at the final concentration of 25nM. Control include a siRNA duplex targeting the luciferase mRNA (5'-CGUACGCGGAAUACUUCGAdTdT-3' and its antisense sequence). All siRNA duplexes were purchased from Eurofins MWG. For the transfection of DNA plasmids, HeLa cells and HEK 293T cells were transfected with FuGENE 6 (Roche) and TransIT®-293 Transfection Reagent (Mirus) respectively, following the manufacturer's recommendations.

Cell synchronization in S-phase and G2, and Golgi membrane fragmentation in intact cells.

HeLa cells were synchronized in S-phase with double thymidine block. Briefly, cells were plated at 60% confluence in 24-wells plates on 12-mm coverslip, maintained in growth medium for 6 h, and incubated for 18 h with 2mM thymidine. Then cells were washed 3 times with PBS and maintained in growth medium for 10 h. Cells were then incubated in 2mM thymidine for additional 18 h before final release. After release, at the indicated times, cells were stained with DAPI and an anti-phospho-histone H3 antibody and analyzed by immunofluorescence microscopy to calculate mitotic index. Mitotic cells are positive for the phospho-histone H3 staining and exhibit condensed DNA.

To monitor cells in G2, after the double thymidine block, the cells were washed, and stained with DAPI and an anti-phospho-histone H3 antibody, and visualized by immunoflorescence microscopy. The cells in G2 were positive for phospho-histone H3 labeling as reported previously (Colanzi et al, 2007) and contained uncondensed DNA. In order to analyze the kinetics of G2-M transition, for each time point, we calculated the number of cells in G2-phase over total cells in G2/M-phase.

To monitor Golgi membrane fragmentation in intact cells, HeLa cells expressing ManII-GFP were arrested in S-phase with a double thymidine block, the cells were then washed and cultured in normal medium for 8 h. The cells were stained with an anti-phospho-histone H3 antibody and visualized by immunoflorescence microscopy. The Golgi membrane organization was monitored only in G2 cells (uncondensed DNA and phospho-histone H3 positive).

When double thymidine block was performed along with siRNA transfection, the first transfection was performed in suspension cells prior to seeding cells onto culture plates. The second transfection was performed after the first incubation with thymidine. Where indicated, DMSO and PD98059 were added 4 h after release.

Expression and purification of recombinant proteins

Recombinant Myt1 proteins: GST-tagged Myt1 wild type (WT) and GST-tagged Myt1 kinase dead (KD) were expressed in bacteria, the recombinant proteins adsorbed to glutathione Sepharose beads (GE Healthcare) and eluted from the beads with 30mM glutathione. The eluate was dialyzed against kinase buffer (50mM Tris, pH 7.2, 10mM $MgCl_2$). Recombinant MEK1 proteins: His-tagged MEK1 constitutively active (CA) and MEK1 KD were expressed in bacteria, the recombinant proteins adsorbed to Ni-agarose beads (Qiagen) and eluted from the beads with 150mM imidazole. The eluate was dialyzed against kinase buffer (50mM Tris, pH 7.2, 10mM $MgCl_2$). Recombinant MEK1 proteins: FLAG-tagged MEK1 from S-phase and pre-metaphase cytosol were obtained by expression in HEK 293T cells synchronized in S-phase by incubation with thymidine for 18 h, or in pre-metaphase by incubation with nocodazole for 18 h. FLAG-tagged MEK1 was purified with FLAG-beads and eluted from the beads with 3 X FLAG peptide. The eluate was dialyzed against kinase buffer (50mM Tris, pH 7.2, 10mM $MgCl_2$).

In vitro kinase assay

Recombinant proteins MEK1, ERK2, Myt1 or GST were incubated in kinase buffer (50mM Tris, pH 7.2, 10mM $MgCl_2$), 1mM DTT, 50μM ATP and γ-(^{32}P) ATP

10μCi/20μl for 30 min at 30°C. The reactions were stopped by the addition of SDS sample buffer and analyzed by SDS-PAGE followed by autoradiography.

ss-HRP secretion

HeLa cells stably expressing ss-HRP were transfected with control, Myt1, and SCFD1 specific siRNA oligos. Seventy-two hours after the transfection, cells were washed with PBS and incubated for 4 h in complete medium. Fifty microliters of the medium was collected and mixed with ECL reagent (Thermo Fisher Scientific), and luminescence was measured with a multi-label counter (WALLAC1420; Perkin Elmer). For normalization, cells were lysed with RIPA buffer, and internal HRP activity was measured.

Immunofluorescence microscopy

Cells were fixed with 3.7% paraformaldehyde in PBS and blocked with blocking buffer (2.5% FCS, 0.1% triton X-100 in PBS). Cells were then incubated with primary antibody followed by a PBS wash and incubation with secondary antibody (Donkey anti-mouse or anti-rabbit Alexa Fluor-488 or 594). DAPI was used to stain the DNA. Samples were analyzed with a Leica SPE confocal microscope using a 63X plan Apo NA1.3 objective and Leica Microsystem LAS AF software.

Fluorescence recovery after photobleaching

Seventy-two hours after the siRNA transfection of HeLa cells stably expressing ManII-GFP, cells were imaged using a Leica SP5 confocal laser-scanning microscope equipped with an environmental control system set at 37°C and 5% CO_2 atmosphere. Cells were imaged on Opti-MEM (Invitrogen) without phenol red containing 10%

FCS by using the argon laser line at 488 nm and using a 63X oil immersion objective. Part of the Golgi was bleached using a single laser pulse, and recovery of the fluorescence was followed every 5 s for a total of 400 s. The recovery of fluorescence was calculated as the ratio of the intensity in the bleached area to the intensity of the total Golgi complex. Normalization was set between the pre bleach and immediate post bleach values.

Immunodepletion of Plk from mitotic cytosol

A mouse monoclonal anti-Plk antibody was coupled to protein G-sepharose beads (GE Healthcare) in PBS for 2 h at 4°C. The antibody-protein G complex was washed three times with KHM buffer and incubated with mitotic cytosol. After 30 min, the beads were pelleted and the supernatant used as Plk-depleted mitotic cytosol. Mock incubation was performed in the same conditions with a mouse IgG2a isotype control.

GRASP55 immunoprecipitation

HeLa cells synchronized in S-phase (thymidine treated cells) and in mitosis (nocodazole treated cells) were incubated in the presence or absence of PD and lysed in lysis buffer (50mM Tris, pH 7.4, 150mM NaCl, 1% NP-40 and 0.5% sodium deoxycholate) supplemented with protease inhibitors, 1mM Na_3VO_4 and 25mM sodium fluoride, and centrifuged at 20,000 X g for 15 min. One mg of total cell lysate was precleared for 30 min at 4°C with 50µl of protein A-Sepharose beads (GE Healthcare) Proteins that nonspecifically bound the protein A-Sepharose were removed by centrifugation to generate the precleared lysates. Then 2 µg of rabbit IgG and rabbit polyclonal anti-GRASP55 were added to the precleared lysates overnight at 4°C, and 50µl of protein A-Sepharose beads were added with further incubation for

45 min at 4°C. Beads were washed 5 times in lysis buffer and the bound proteins were eluted by boiling in SDS-PAGE loading buffer without DTT or β-mercaptoethanol. GRASP55 was resolved by SDS-PAGE and analyzed by immunoblotting with anti MPM2-antibody.

Western blotting

Cells were lysed in lysis buffer (50mM Tris, pH 7.4, 150mM NaCl, 0,1% SDS, 1% NP-40 and 0.5% sodium deoxycholate) supplemented with protease inhibitors, 1mM Na_3VO_4 and 25mM sodium fluoride and centrifuged at 20,000 X g for 15 min. Cells extracts were subjected to western blotting with the primary antibodies mentioned above. Proteins were detected using the specific secondary antibody and fluorescent bands visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Phosphatase treatment

Cells were lysed in phosphatase buffer (50mM Hepes, pH 7.5, 100 mM NaCl, 0.1 mM EGTA, 0.5% NP-40, 2 mM DTT, 1 mM PMSF, 2 mM $MnCl_2$, supplemented with protease inhibitors) and centrifuged at 20,000 X g for 15 min. Cell extracts were incubated with 300 U of purified λ protein phosphatase for 45 min at 30°C. The phosphatase reaction was stopped by the addition of SDS sample buffer and the samples analyzed by western blotting.

Golgi membrane preparation and incubation with S-phase and mitotic cytosol

Golgi membranes were purified as described previously (Balch et al, 1984) from S-phase synchronized HeLa cells after incubation for 18 h with 2mM thymidine. Briefly, cells were washed three times with PBS, resuspended in breaking buffer

(10mM Tris, pH 7.4, 250mM sucrose) and centrifugated for 10 min at 600 x g. The cells were resuspended in breaking buffer and homogenized with an EMBL cell cracker. Two milliliters of homogenate was adjusted to 1.4 M sucrose by the addition of 2ml ice-cold 2.3M sucrose containing 10mM Tris, pH 7.4. Then, 1mM EDTA was added from a 100mM stock solution, and the homogenate was loaded into ultracentrifuge tube and overlaid with 4.5ml of 1.2M sucrose, 10mM Tris, pH 7.4, and then with 3.5ml of 0.8M sucrose, 10mM Tris, pH 7.4. The samples were centrifuged for 2.5 h at 4°C at 30,000 rpm in SW40Ti rotor (Beckman-Coulter). The turbid band at the 0.8/1.2M sucrose interface was harvested in a minimum volume by syringe. This fraction was used for the experiment, reported as a Golgi isolated membranes. 10μg of isolated Golgi membranes were incubated for 1 h at 37°C with 10mg/ml of S-phase or mitotic cytosol preincubated with either DMSO, 75 μM PD, 20 μM SL0101 or 20 μM olomoucine, and an ATP-regenerating system. The reaction was terminated by the addition of an excess cold KHM buffer supplemented with phosphatase inhibitors. The Golgi membranes were collected after ultracentrifugation for 1 h at 4°C at 48,000 rpm in a TLS55 rotor and resuspended in the SDS sample buffer.

Supplementary References

Balch WE, Dunphy WG, Braell WA, Rothman JE (1984) Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. *Cell* **39**(2 Pt 1)**:** 405-416

Colanzi A, Hidalgo Carcedo C, Persico A, Cericola C, Turacchio G, Bonazzi M, Luini A, Corda D (2007) The Golgi mitotic checkpoint is controlled by BARS-dependent fission of the Golgi ribbon into separate stacks in G2. *EMBO J* **26**(10)**:** 2465-2476

Supplementary Figure Legends

Figure S1: Effectiveness of the chemical inhibitors used.

HeLa cells deprived of serum for 16 h were incubated in the presence or absence of 75μM PD (A), 10μM U0126 (B), 20μM SL0101 (C) and 10μM BI-D1870 (D) for the indicated times, then stimulated with 400 nM PMA for 20 min. The expression of the indicated proteins were analysed by western blotting the total cell lysate with specific antibodies as described in the materials and methods section. (E and F) HeLa cells were arrested in S-phase with a double thymidine block, the cells were washed to remove thymidine, and incubated in a thymidine free medium during 6 h (release) in the presence or absence of 20μM olomoucine (E) and 5μM RO-3306 (F) for the indicated times. Histone H3 phosphorylation was analysed by western blotting the total cell lysate. Western blotting with an anti-β-actin antibody was used as a loading control.

Figure S2: MEK1 regulates mitotic Golgi membrane fragmentation in permeabilized cells.

(A) Left panel. After incubation with thymidine for 12 h, permeabilized and salt-washed cells stably expressing ManII-GFP were incubated with mitotic cytosol preincubated with either DMSO, U0126, BI-D1870 or RO-3306 and an ATP-regenerating system. The organization of the Golgi membranes was visualized by fluorescence microscopy. Scale bar is 10 μm. Right panel. Percentage of cells with fragmented Golgi in the experimental conditions describe above. For each condition, 200 cells were counted on 2 different coverslips (mean ± SD, n=3, *$p<0.05$).

Figure S3: MEK1 is not directly phosphorylated by MEK1 or ERK2.

100 ng of FLAG-tagged MEK1 protein purified from mitotic cells were incubated with 2 μg of recombinant ERK2 protein and 1 μg of kinase dead (KD) form of Myt1 in the presence of γ-(^{32}P)ATP for 30 min at 30°C. The reactions were stopped by the addition of SDS sample buffer and analyzed by SDS/PAGE. Coomassie blue staining and the autoradiogram of the gels are shown.

Figure S4: MEK1-mediated GRASP55 phosphorylation during the mitosis does not require Myt1.

(A) HeLa cells synchronized in S-phase (thymidine treated cells) and in mitosis (nocodazole treated cells) and incubated with DMSO or PD were subjected to immunoprecipitation with the anti-GRASP55 antibody and a corresponding control IgG. The resulting immunoprecipitates were analysed by western blotting with an anti-MPM2 and anti-GRASP55 antibodies. (B) Control and Myt1 siRNA transfected cells synchronized in mitosis and incubated with DMSO or PD were subjected to immunoprecipitation with the anti-GRASP55 antibody and an corresponding control IgG . The resulting immunoprecipitates were analysed by western blotting with an anti-MPM2 and anti-GRASP55 antibodies.

Figure S1

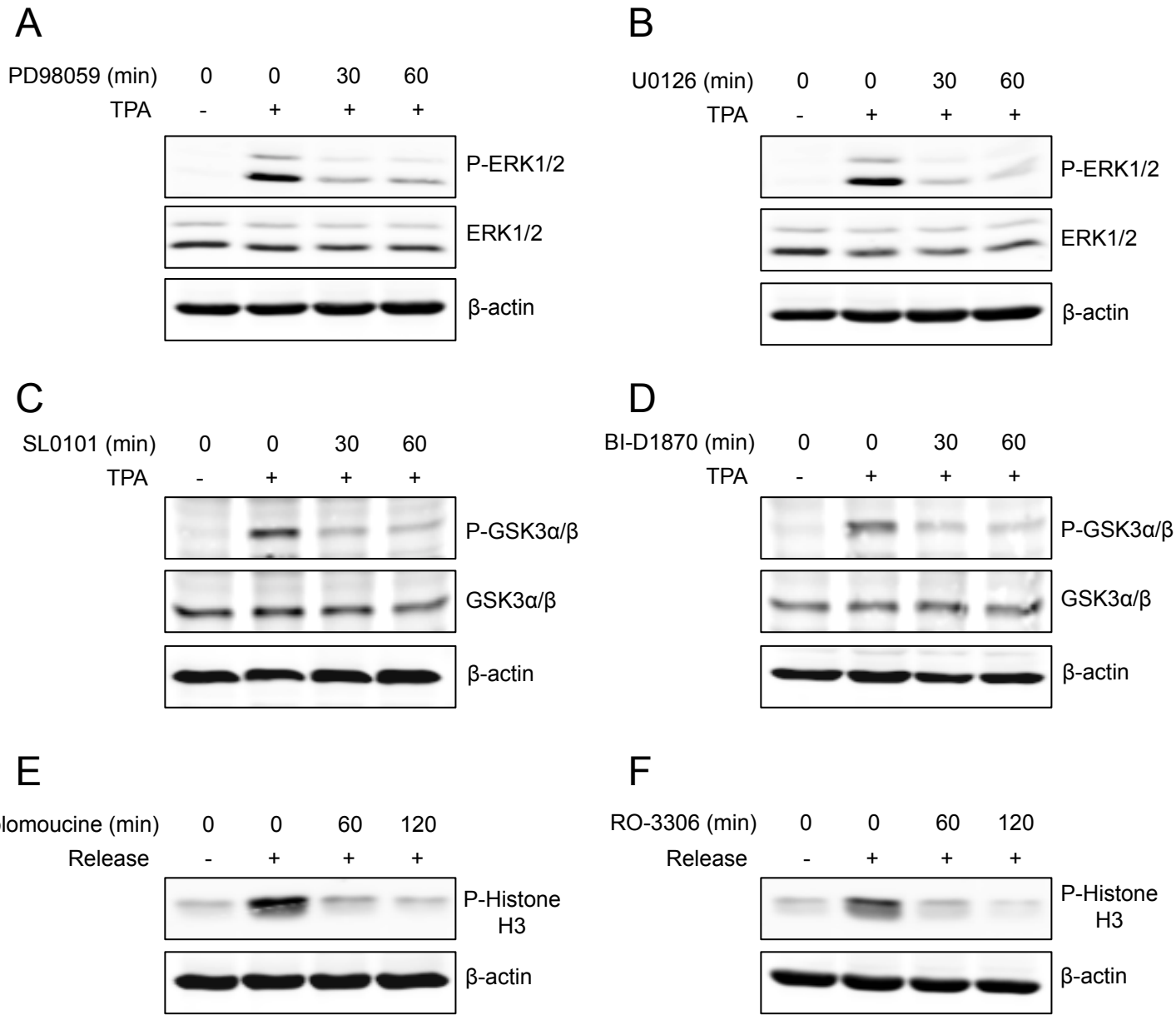
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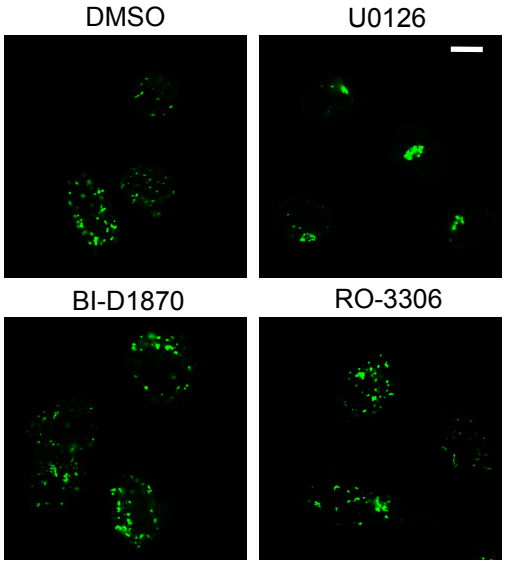
Figure S2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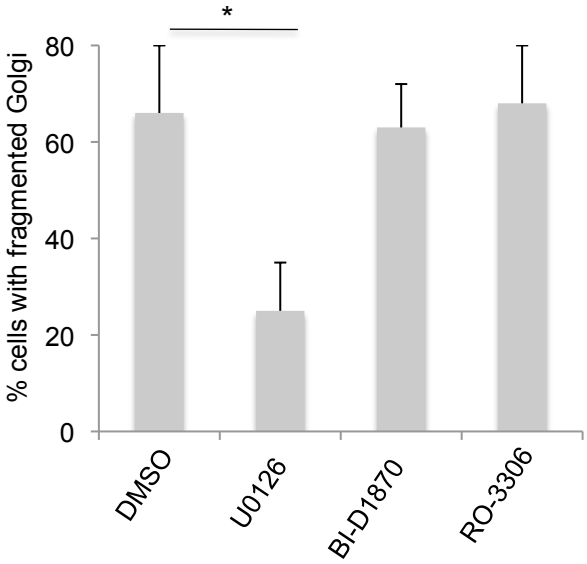
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20
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DMSO
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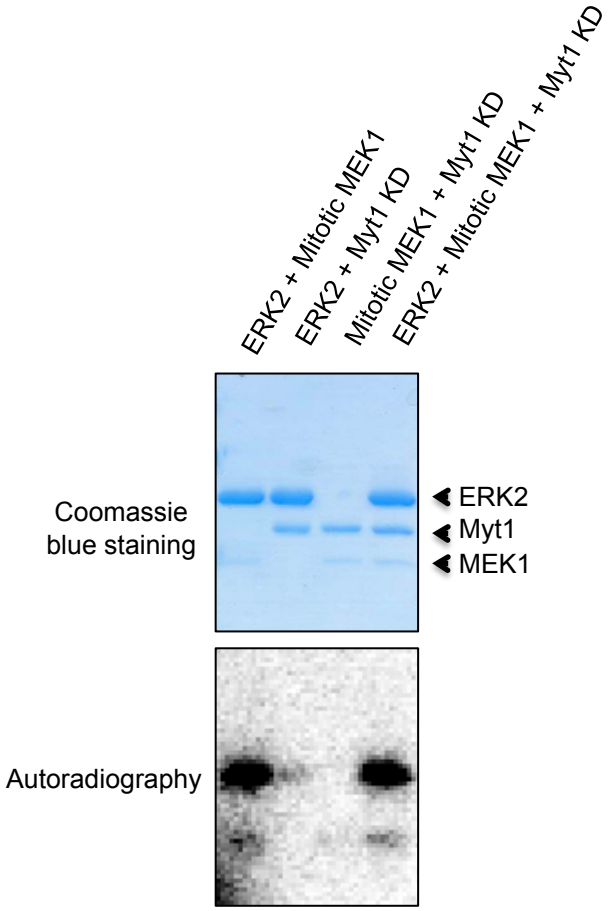
BI-D1870
RO-3306

Figure S3


ERK2 + Mitotic MEK1
ERK2 + Myt1 KD
Mitotic MEK1 + Myt1 KD
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Coomassie blue staining
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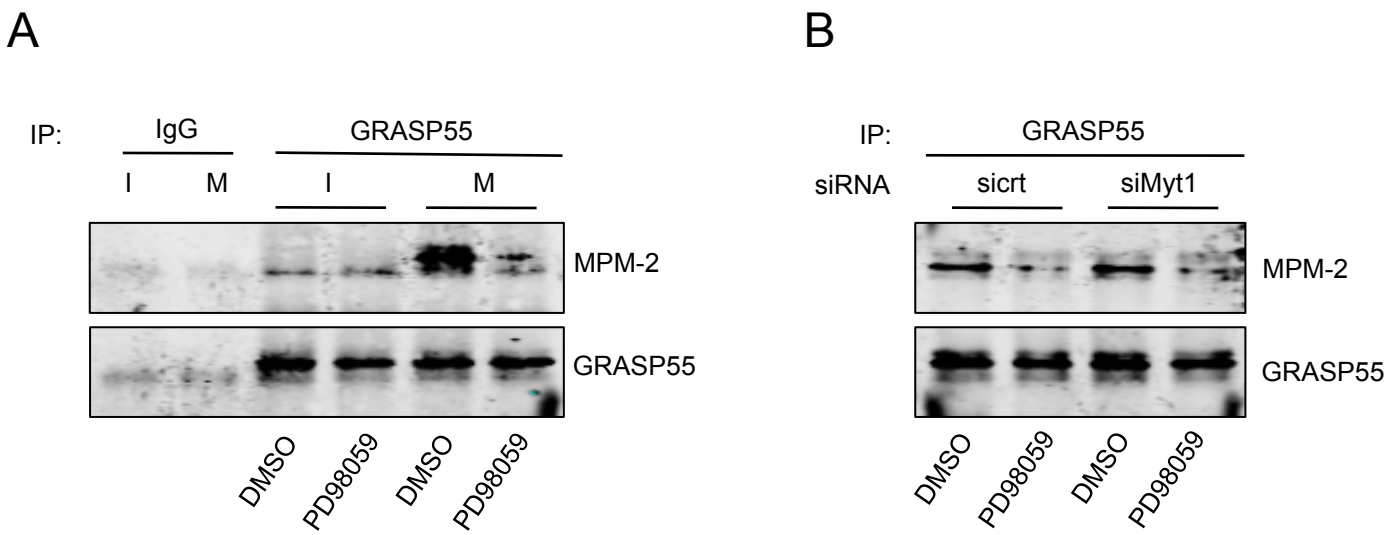
Myt1
MEK1
Autoradiography

Figure S4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