SUPPLEMENTARY FIGURE 1. **The intracellular staining pattern of Dab2 is altered in mitosis in HeLa cells.** A-B Unsynchronized HeLa cells were plated onto glass coverslips. 16 h post-plating cells were fixed and stained against microtubules, Dab2 and DNA. Cells were imaged by confocal microscopy and assigned to different stages of the cell cycle according to the distribution pattern of DNA and microtubules. For the sake of simplicity, only pictures depicting interphase and anaphase cells are shown. Bar, 10 μm. C- Graph depicts the quantification of the ratio of the variance-of-fluorescence-signal to the mean-of fluorescence-signal (VtM) of Dab2 in the bottom plane of cells imaged as in A and B. Values were calculated with Slidebook™. Each bar represents the average of 10-17 cells imaged in each condition. Significance was calculated by the Student's t-test. ***, p<1E-6, when comparing the average VtM of interphase cells to the average VtM values obtained in all other cell cycle stages; §, p< 0.001, when comparing the VtM values of metaphase and anaphase cells to the value obtained with cells in cytokinesis.

SUPPLEMENTARY FIGURE 2. **Cell fractionation of 2ME2-treated cells reveals a displacement of Dab2 to the cytosol.** A- ES-2 cells, treated with 2ME2 (16 h ,4.4 μM) or vehicle were resuspended in hypotonic buffer (400 μl, 50 mM Tris pH 7.4, supplemented with protease and phosphatase inhibitors), submitted to repeated freeze-thaw cycles and sonicated. Cell lysates were ultra-centrifuged (100000g, 30 min, 4 °C) generating the S100 (soluble) and the P100 (particulate) fractions. The P100 fraction was either dissolved in SDS-PAGE sample buffer or treated with a mild detergent solution (40 μl, [150 mM NaCl, 1% Triton, 0.5% Igepal CA-630, 10 mM Hepes ph 7.4, protease and phosphatase inhibitors], 30 min, 4 °C) prior to the clearance of the nuclear fraction by centrifugation. The nuclear fraction was re-suspended in SDS-PAGE sample buffer. Note that 1/10 of the cytosolic fraction and the entirety of all other fractions was separated by SDS-PAGE prior to immunoblotting with a polyclonal α-Dab2 antibody. B- ES-2-based cell lines (expressing p82-wt-*h* or p82-5A) were re-suspended in hypotonic buffer (400 μl, [20 mM Hepes pH 7.4, supplemented with protease and phosphatase inhibitors]) submitted to repeated freeze-thaw cycles and sonicated. The nuclear fraction was cleared by centrifugation (5 min, 1000 g, 4 °C). The cleared lysate was then ultra-centrifuged (100000g, 30 min, 4 °C) generating the S100 (soluble) and the "P100-Nuclear" (particulate) fractions. 40 μl of the S100 fraction and the entirety of the "P100-Nuclear" fraction were separated by SDS-PAGE and immunoblotted with α-myc, α-Epidermal Growth Factor Receptor (EGFR, employed as a membrane marker), and α-ERK (yielded similar signals in all fractions, indicative of a 10 fold enrichment in the S100 fraction, data not shown).

SUPPLEMENTARY FIGURE 3. **Roscovitine inhibits cell cycle progression and leads to the maintenance of clathrin/Dab2 interactions in the presence of 2ME2.** A- ES-2 cells, grown on coverslips, were treated with 2ME2 (4.4 μM, 16 h), roscovitine (80 μM, 16 h), a combination of both drugs or the same concentration of vehicles. Cells were fixed, permeabilized and stained as in Figure 2. Panels depict the bottom plane of representative cells in each condition. B- Graph depicts the quantification of the maximum Pearson's correlation coefficient (CCF) of the fluorescence signals of Dab2 and clathrin in the bottom planes of cells imaged as in (A). Values were calculated with ImageJ software. Each bar represents the average of 10 cells imaged in each condition. Significance was calculated by the Student's t-test; $p < 0.001$. C- Cells treated as in (A) were analyzed by FACS as in Fig. 3. D- ES-2 cells, grown in 10 cm dishes were treated as in (A), re-suspended and lysed. Lysates were separated by SDS-PAGE and immunobloted with a monoclonal anti-Dab2 antibody.

SUPPLEMENTARY FIGURE 4. **Mass spectrometry analysis reveals unique phosphorylation sites in p82-wt-*h*.** α-myc immunoprecipitates, from ES-2 cells stably expressing either p82-wt-*h* (A to F) or p82-5A (G and H), treated with 2ME2 (C,D,F,H) or vehicle (A,B,E,G), were processed for mass spectrometry as described in Supplementary Experimental Procedures. [A-D] Chromatograms of the 392-K.SSPNPFVGSPPK.G-405 peptide in its phosphorylated form (at S401; B, D) or unphosphorylated form (A, C). Note that the phosphorylated form was prominent in the vehicle treated sample (B). (E-F) Representative chromatograms of the 321-K.ANPGSLSTPQSK.G-334 peptide in its phosphorylated form (at S326 or S328; F) or unphosphorylated form (E, G and H). Note that the phosphorylated form of 321-K.ANPGSLSTPQSK.G-334 was apparent exclusively in the p82-wt-*h* sample treated with 2ME2 (F). In vehicle-treated cells expressing p82-wt-*h* (E), and in cells expressing p82-5A (either treated with vehicle,

G; or treated with 2ME2, H) this phosphopeptide was not identified. m/z is the mass to charge ratio. NL is the frequency of occurrences. The retention time is written immediately above the peaks.

SUPPLEMENTARY FIGURE 5. **2ME2 induces the phosphorylation of p82-wt-*h* on threonine/ serine residues adjacent to prolines (pT-P/pS-P).** ES-2 cells, stably expressing p82-wt-*h*, p82-5A or p59-wt-*r*, were treated with 2ME2 (4.4 μM, 18 h) or vehicle and lysed (with [150 mM NaCl, 1% Triton, 0.5% Igepal CA-630, 10 mM Hepes pH 7.4, protease and phosphatase inhibitors], 30 min, 4 °C)**.** Lysates were immunoprecipitated with α-myc antibodies. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with [monoclonal α-myc/ goat-anti-mouse-peroxidase antibodies], lower panel; striped and re-probed with [polyclonal α-(pT-P/pS-P)/biotinilated-goat-anti-rabbit/streptavidin-peroxidase], upper panel.

SUPPLEMENTARY FIGURE 6. **In mitotic/cycling cells p82-wt-*h* is displaced from the membrane while p82-5A retains its membrane localization.** ES-2-based cell lines expressing p82-wt-*h* or p82-5A were plated on glass coverslips, cultured for 16 h, fixed, permeabilized and stained with [α-myc/Alexa-555-G-α-M] and DAPI. Cells at different stages of mitosis were identified by their DNA staining pattern. The entire cell volume was acquired by spinning disk confocal microscopy. Panels depict the bottom and middle plane of cells expressing p82-wt-*h* or p82-5A at different stages of mitosis. Note the mitosis-dependent alteration to the staining pattern of p82-wt-*h* and the maintenance of the punctuate/membrane-bound staining pattern of p82-5A throughout mitosis.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES.

Generation of constructs and mutants.

p82-w.t. *h* was generated by mutating asparagine 227 (of Rat p82) to serine by overlapping PCR employing the following primers:

5`-CTCCTGACCTAAATTCGCCAACAGAAAGCAGAGATATCCTG-`3;

5`-TCTGCTTTCTGTTGGCGAATTTAGGTCAGGAGGTGTAGACATGTCC-`3. p82-3A in which serines 393, 394 and 401 were mutated to alanines, was generated through sequential reactions employing the following primers:

5`-TTCCATATCAAAGCGGCTCCGAATCCTTTTGTGGGAAGC-`3;

5`-CACAAAAGGATTCGGAGCCGCTTTGATATGGAACCCGTTCTG-`3;

5`-AACCCATTTGTCGGAGCGCCTCCCAAAGGACTATCGGTACC-`3;

5`TAGTCCTTTGGGAGGCGCTCCGACAAATGGGTTAGGGGAAG-`3.

p82-5A was generated through the mutation of serine 423 to alanine, employing the following primers:

5`-AGCTCTGTCCAGTCCGCAGCGCATGACTCCATAGCCATTATCC-`3;

5`-ATGGAGTCATGCGCTGCGGACTGGACA GAGCTTTCC-`3; and by mutating threonine 221 to alanine, employing the following primers:

5`-TTGGGGACATGTCTGCGCCTCCTGACCTAAATAATCCAACAG-`3

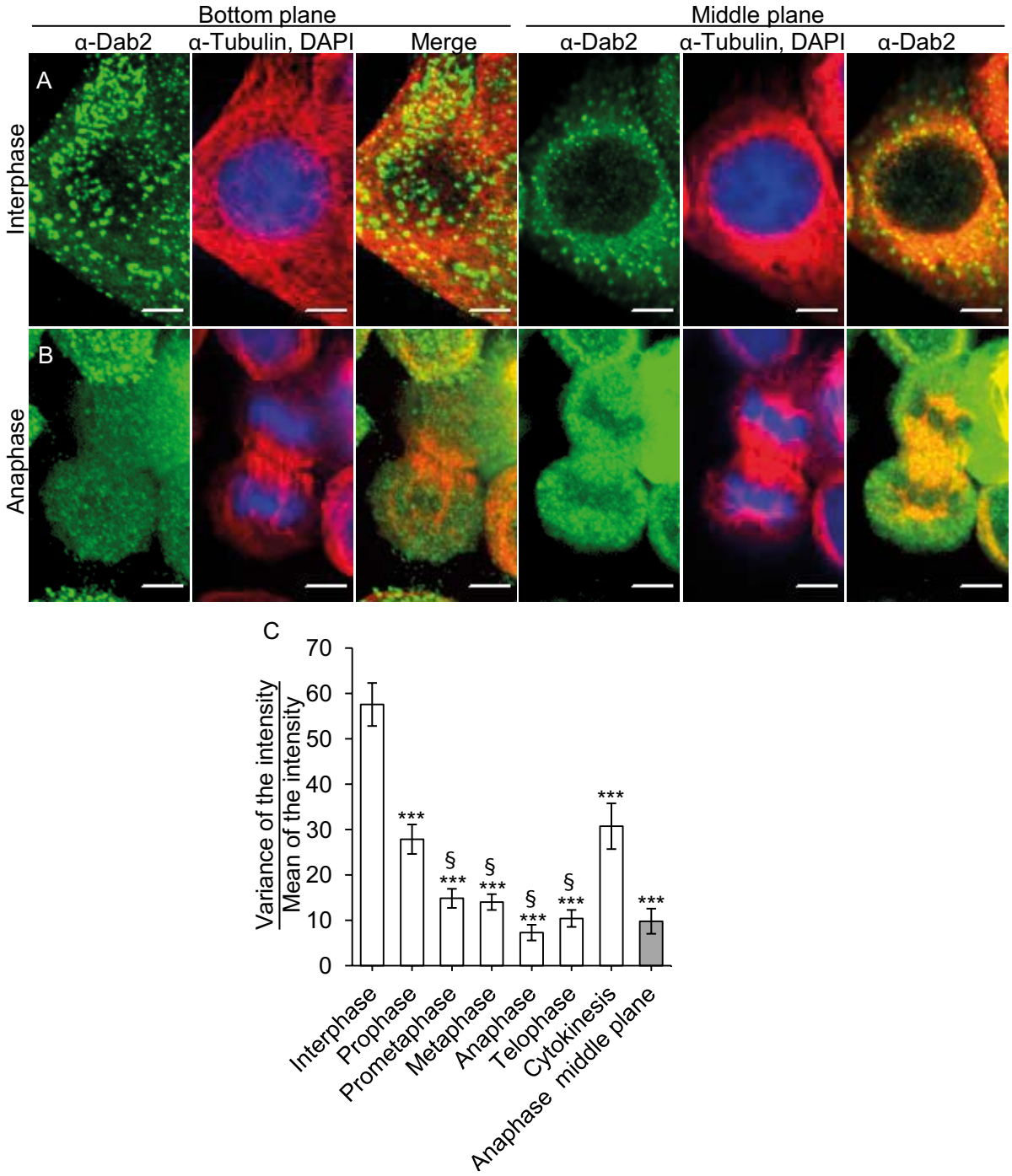
5`- TTTAGGTCAGGAGGCGCAGACATGTCCCCAAACAAATCCATC-`3, using p82-3A as a template.

In gel proteolysis and mass spectrometry analysis.

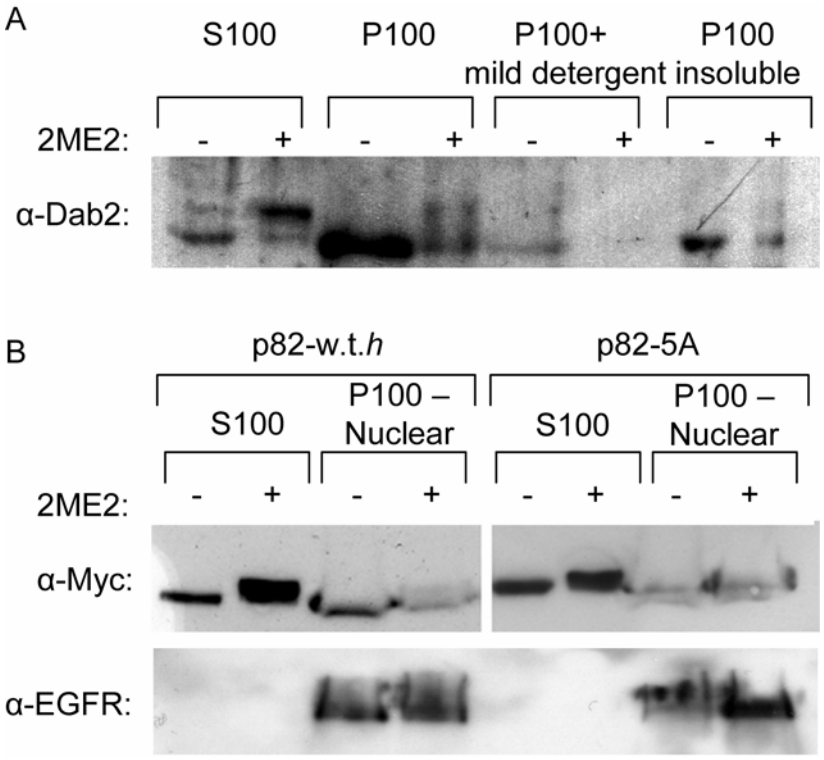
Immunoprecipitates of the myc-p82 constructs, in the different treatment/transfection conditions, were separated by SDS-PAGE. Proteins in the gel were reduced (3mM DTT), modified with 12mM iodoacetamide and trypsinized (modified trypsin (Promega)) at a 1:10 enzyme-to-substrate ratio. The resulting tryptic peptides were resolved by reverse-phase chromatography on 0.075 X 200-mm fused silica capillaries (J&W) packed with Reprosil reversed phase material (Dr Maisch GmbH, Germany). The peptides were eluted with linear 65 minutes gradients of 5 to 45% and 15 minutes at 95% acetonitrile with 0.1% formic acid in water at flow rates of 0.25 μl/min. Mass spectrometry was performed by an ion-trap mass spectrometer (OrbitrapXP, Thermo) in a positive mode using repetitively full MS scan followed by multistage activation collision induces dissociation (CID) of the 7 most dominant ions selected from the first MS scan. Alternatively fragmentations were done on selected ions from a defined list. The mass spectrometry data was analyzed using the Sequest 3.31 software (J. Eng and J.Yates, University of Washington and Finnigan, San Jose) searching against the uniprot database.

LEGEND TO SUPPLEMENTARY MOVIES

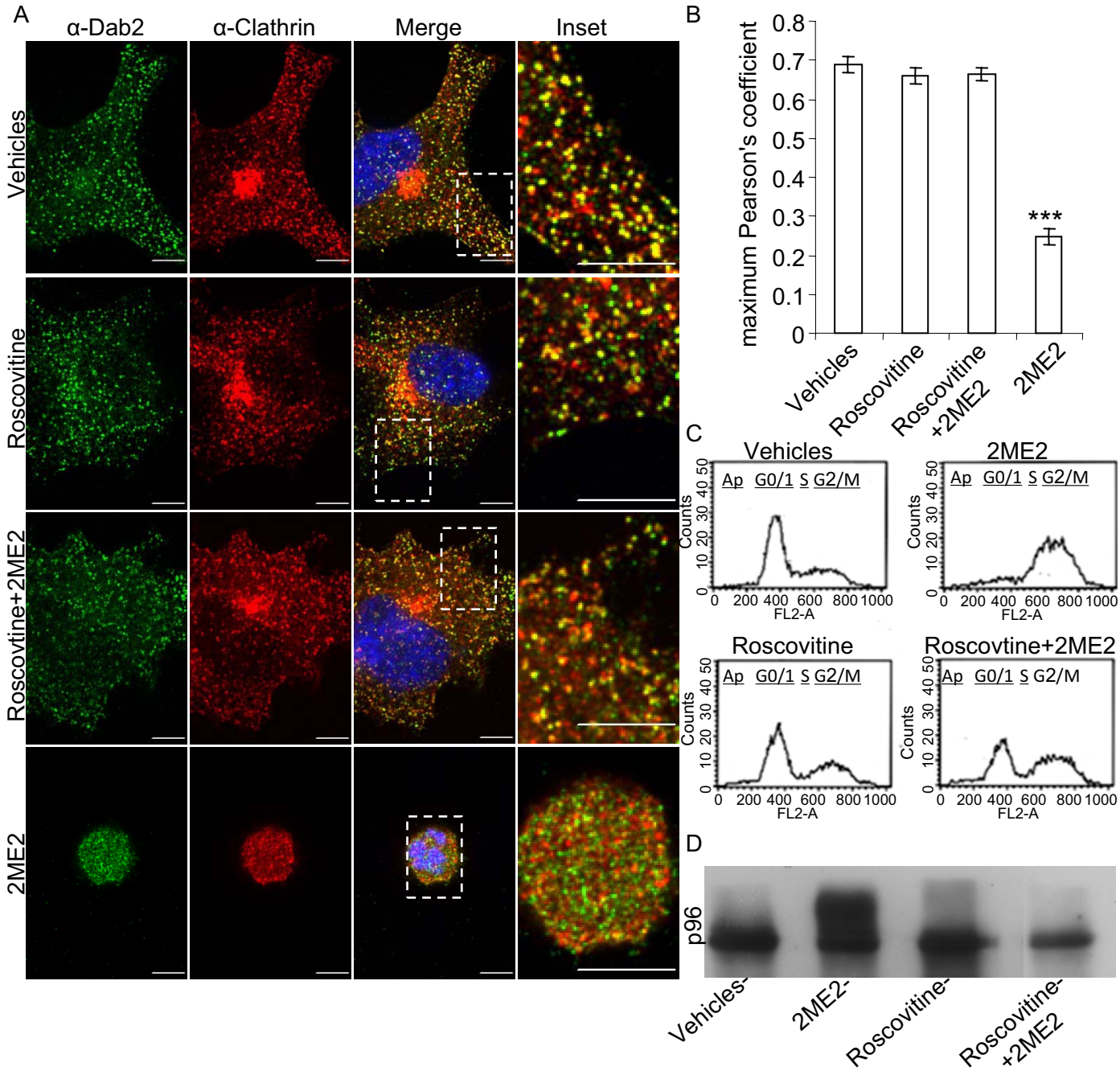
ES-2 cells were "pulsed" with Alexa-555-labeled transferrin (50 μg/ml in Hepes-buffered serum free medium, 2 min, 37 °C), after which they were imaged by spinning disk confocal microscopy (1s acquisition, no interval between frames). Supplementary Movie 1 depicts an untreated cell, while Supplementary Movie 2 depicts a cell treated with 2ME2 (4.4 μM, 16 h).



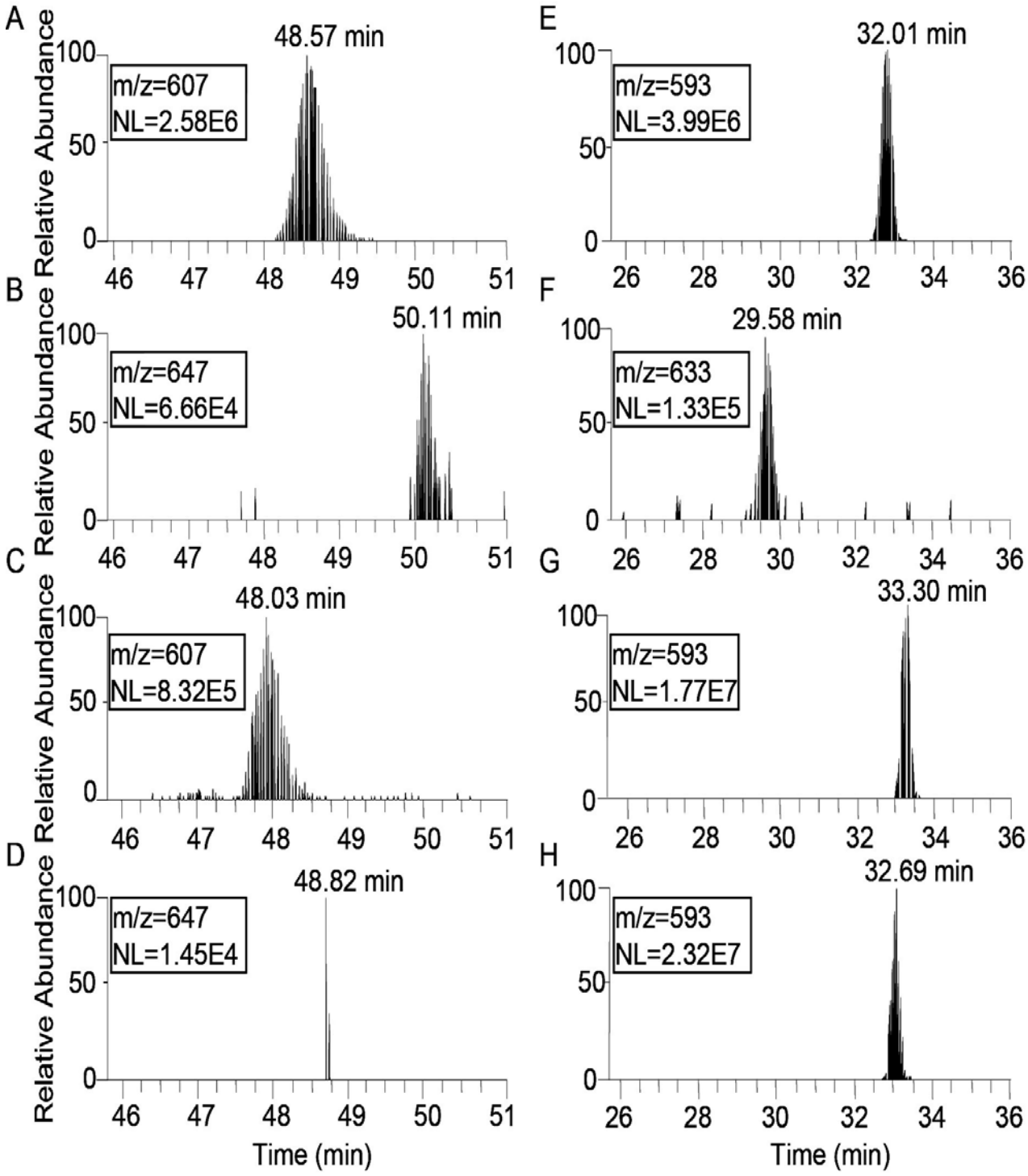
Chetrit et al., Supplementary Fig. 1



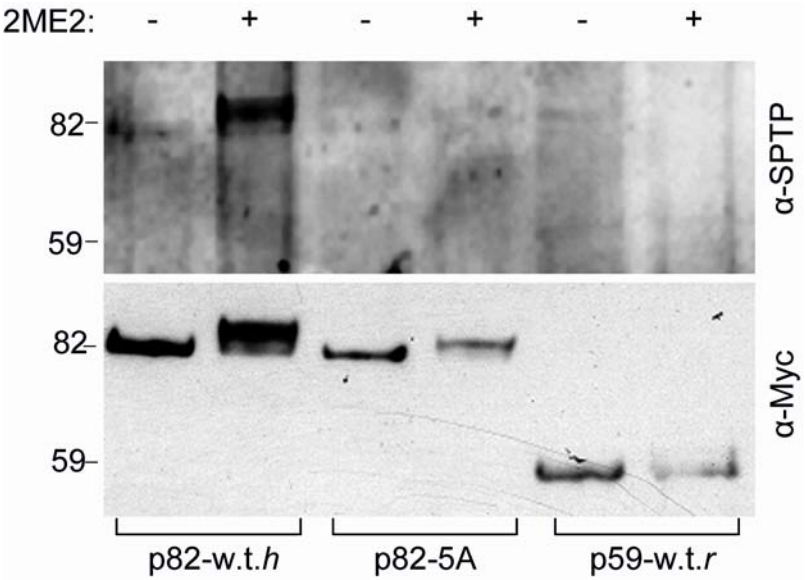
Chetrit et al., Supplementary Fig. 2



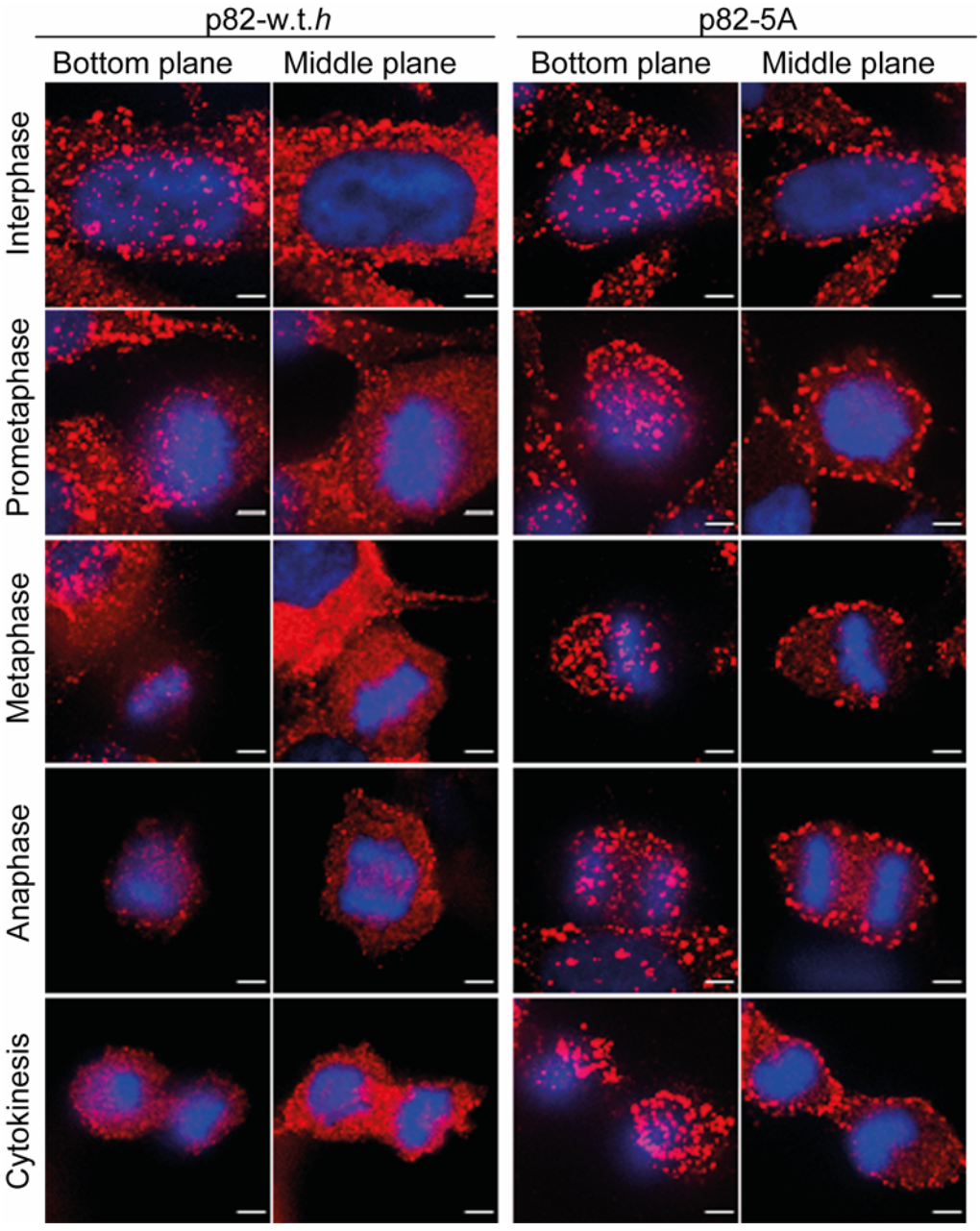
Chetrit et al., Supplementary Fig. 3



Chetrit et al., Supplementary Fig. 4



Chetrit et al., Supplementary Fig. 5



Chetrit et al., Supplementary Fig. 6