Supplementary Information

Supplementary Methods

Reagents – All Chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

Cell Culture – Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's medium (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). For transient transfections, cells were grown to approximately 60% confluence and transfections were performed according to the calcium phosphate method. GFP-transfected cells were observed by fluorescent microscopy (Olympus BX41) and the percentage blebbing/round cells was scored. For inhibition of PP2A, 24 h post transfection, cells were treated with 100% ethanol as negative control or with 100 nM okadaic acid (Sigma) for 50 minutes, 2 or 3h. Where indicated, 10μM ionomycin (Sigma) was added 10 min after addition of okadaic acid for the remaining time of treatment**.**

Plasmids - Plasmids encoding N-terminal Flag and hemagglutinin (HA)-tagged human full-length DAPk (Flag-DAPk and HA-DAPk), HA-tagged DAPk lacking the cytoskeletal interacting region (ΔCyto - amino acids 641–835), C-terminal GFP-tagged DAPk (GFP-DAPk) and C-terminal Flag-tagged DAP1 have been described previously (Bialik et al, 2004; Cohen et al, 1997; Koren et al, 2010). Additionally, pcDNA3 encoding the non-relevant protein luciferase and pEGFP-C1 encoding GFP were used as controls. The plasmids encoding DAPk mutants, HA-ΔROC (deletion between amino acids 667-954), HA-ΔKD (lacking amino acids 1-285), and Flag-ΔP-Loop (deletion between amino acids 695-702) were generated by site directed

mutagenesis using HA-DAPk or Flag-DAPk as templates. N-terminal Flag and HA-tagged DAPk ROC fragment (amino acids 667-954) were generated by PCR and ligation into the expression vector pcDNA3. Flag-tagged ROC fragment ΔP-Loop (lacking amino acids 695-702) was generated by site directed mutagenesis using Flag-ROC as template. The plasmid encoding GDP-bound state mimicking construct (DAPk T701N) was generated by site directed mutagenesis. The plasmid pET3d-hMLC for expression of hMLC in bacteria was a kind gift of Mathias Gautel. Finally, DAPk ROC-COR (amino acids 667-1288) domains was cloned into the pETMBPH vector, fused to N-MBP-His and separated by a Tobacco Etch virus (TEV) cleavage site (Peleg & Unger, 2008) in the Israel Structural Proteomics Center (ISPC) using standard molecular cloning techniques.

Western Blot Analysis - Total cell lysates or protein immunoprecipitates were resolved on 10% polyacrylamide gels, transferred to nitrocellulose membranes, and incubated with monoclonal antibodies to Flag (Sigma), His (Qiagen), actin (Sigma), DAPk (Sigma), HA (BAbCO), DAPk phospho-Ser308 (Sigma) or PP2A-B subunit (Cell Signaling). Secondary antibodies consisted of horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch Laboratories), which were detected by SuperSignal enhanced chemiluminescence (Pierce).

Analytic size exclusion chromatography – Immunopurified Flag-DAPk was loaded onto an analytical Superdex™ 200 10/300 (GE Healthcare) gel-filtration column equilibrated with TBS. The eluting protein was detected by UV absorbance at 280 nm. Fractions were collected using the AKTA Prime fraction collector and analyzedby Western blot with anti-Flag antibodies. The migration position of the

fractions containing Flag-DAPk was assigned relative to the migration position of Gel Filtration Calibration HMW standards (GE Healthcare) on the same column.

Protein Purification and Immunoprecipitation – Recombinant human Ras (Ras WT) was a kind gift from Joel Hirsch. MBP fusions of DAPK ROC and DAPk ROC-COR were expressed in *E. coli* BL21(DE3) over night at 15°C following induction with 200 μM IPTG. The cells were lysed by sonication in buffer A (30 mM Tris pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 3 mM β-mercaptoethanol). Amylose beads (E8021L New England Biolabs, Ipswich, MA) were added to the cleared lysate and the slurry was stirred for 1h at 4°C. The proteins were eluted from the washed beads with buffer A containing 10 mM maltose. Recombinant human MLC was expressed in BL21 (DE3) (Novagen) and purifications were carried out using the standard HiTrap Nickel affinity column (GE Healthcare). MBP-AcPS was produced by a similar procedure in the Israel Protein Structural Proteomics Center (ISPC) and used as a negative control for GTP hydrolysis experiments.

Different constructs were transfected in HEK 293T cells, which were lysed in G buffer (100 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM DTT, 1 mM EDTA, 1% Triton X-100) supplemented with protease and phosphatase inhibitor cocktails (Sigma). In order to immunopurify sufficient amounts of Flag-DAPk for size exclusion chromatography experiments, cells were first treated with the actin-depolymerizing agent latrunculin B (Sigma) at 20 μM for 45 min to release all of the kinase from the actin cytoskeleton, and then immediately lysed in G buffer. Extracts were immunoprecipitated with anti-Flag M2 monoclonal antibody conjugated to protein G beads (Sigma), and proteins were eluted with excess Flag peptide (Sigma) at 150 nM in TBS.

GTP-binding assays - GTP-agarose beads (Sigma) were blocked with G buffer containing 100 μg/mL BSA (Sigma) for 1 h at 4^0C. Next, 100 μg of total cell lysates were incubated with the pre-treated GTP-agarose beads for 1 h at 4^0C on rotator. Where indicated, nucleotides were added followed by continuous incubation for additional 2 h. Then, the beads were washed three times with G buffer and the binding proteins eluted by addition of 40 μL 2X SDS sample buffer and boiling for 5 minutes. Eluted proteins were subjected to western blot analysis.

GTP hydrolysis assays – The indicated recombinant proteins (3 μM) were pre-incubated for 10 min at room temperature with GTPase buffer (50mM Tris-HCL, pH 7.9, 20mM NaCl, 200 nM [α-32P]GTP (3000 Ci/mmol)). The reactions were then started by addition of 10 mM $MgCl_2$ and incubation at 37^0C. At the indicated time points, 5 μL aliquots of each reaction were removed and immediately mixed with 5 μL of reaction stop buffer (0.2% (v/v) SDS, 2mM EDTA, 1mM GDP and 1mM GTP) followed by boiling for 5 minutes to completely inactivate the enzymes and stop the reaction. Next, the samples were analyzed by thin layer chromatography (TLC) on PEI cellulose (Sigma) by spotting 1 μL of each sample on membranes, which was resolved in running buffer (0.75M KH_2PO_4, pH 3.4). Finally, the membrane was dried and exposed to X-ray film.

Cell Detachment Assays - Cell detachment was assessed as described (Widau et al, 2010) by counting adherent cells left on the culture plate 24h post-transfection with either DAPk WT or ΔP-loop.

Supplementary Figure Legends

S1. A, expression and purification of MBP-ROC-COR – SDS-PAGE stained with GelCode® Blue showing total soluble cell lysate, flow-through from the amylose resin and eluted MBP-ROC-COR used in GTP binding and hydrolysis assays. **B, MBP-ROC-COR binds GTP** - Purified MBP-ROC-COR was incubated with GTP-agarose beads. GTP (2 mM) was added or not as indicated. After washing, bound proteins were subjected to western blot with anti-His antibody. **C, Controls for GTP hydrolysis assay –** TLC of reactions in which either the active G-protein Ras or MBP fused to the non-relevant protein AcPS were incubated with [α-32P]GTP. Note that whereas Ras produced a remarkable shift from GTP to GDP along the reaction time course, MBP-AcPS did not induce detectable GTP hydrolysis during the 2 h reaction.

Fig. S2. Schematic representation of hypothetical mechanistic models for GTP dependent regulation of DAPk. The models are based on comparisons between WT and the GTP-binding deficient mutants and are extrapolated to GTP/GDP exchange. A, GTP-binding blocks the docking of PP2A, consequently inhibiting Ser 308 dephosphorylation and DAPk activation. Upon cycling to GDP, the PP2A holoenzyme (composed of catalytic (C), regulatory (B) and structural (A) subunits) is recruited and DAPk is activated by dephosphorylation. Note that the docking site of PP2A, although not precisely mapped, was determined to range between aa 628-1215 (Widau et al, 2010), and was further narrowed down here to the ROC domain. **B**, PP2A is constitutively bound to the ROC-COR domain, yet GTP hydrolysis effects a conformational change that enables access of PP2A to Ser308 within the CaM domain, leading to dephosphorylation. **C,** Conformational change imposed by GTP-binding favors DAPk activity towards Ser 308 autophosphorylation, bringing about

catalytic inactivation independently of PP2A. Upon GTP hydrolysis, the CaM regulatory domain is removed from the active site and the kinase domain preferentially phosphorylates DAPk substrates such as MLC, instead of its own Ser308. PP2A constitutively removes the pre-existing Ser308 phosphorylation during GTP/GDP exchange.

Supplementary References

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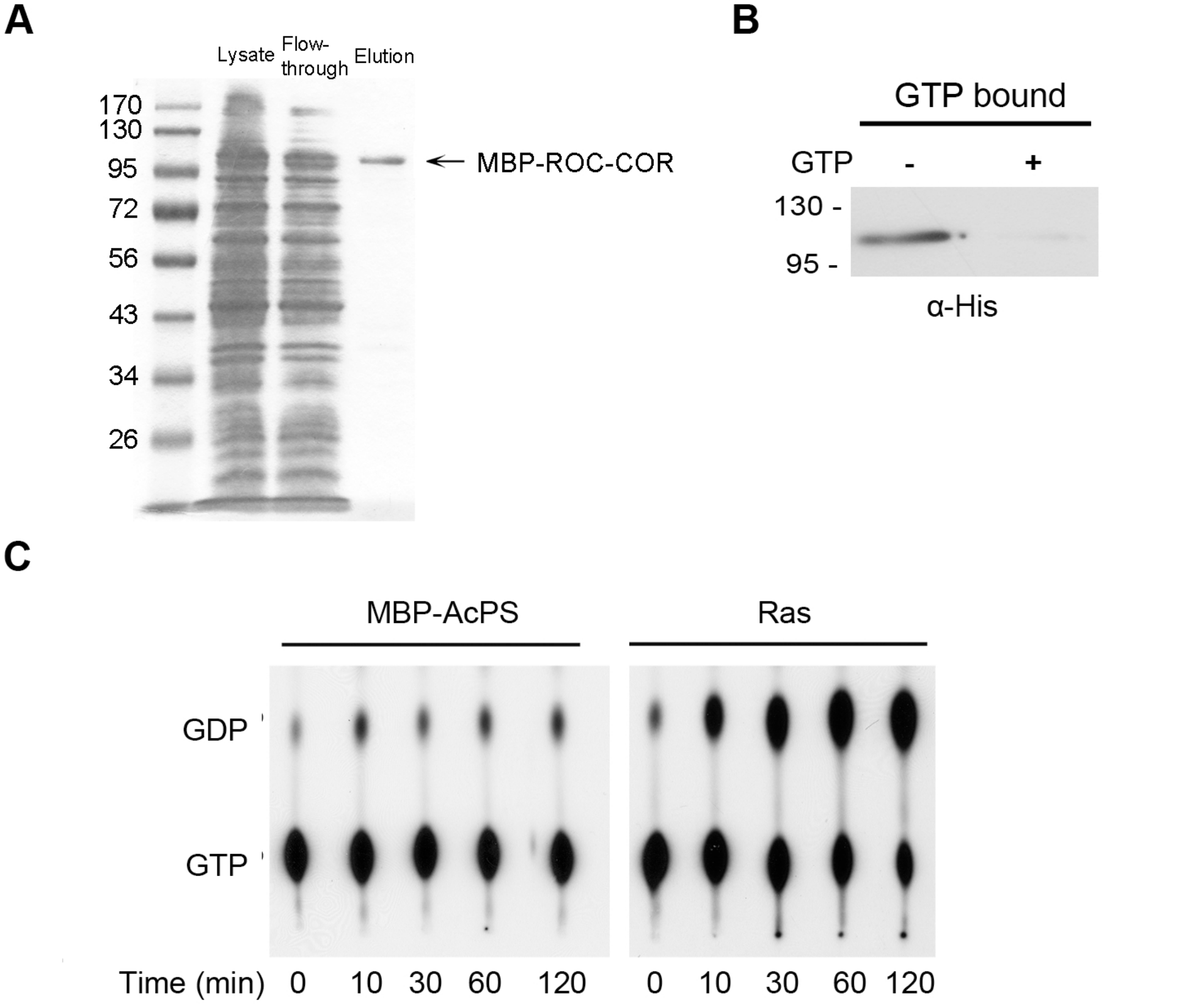
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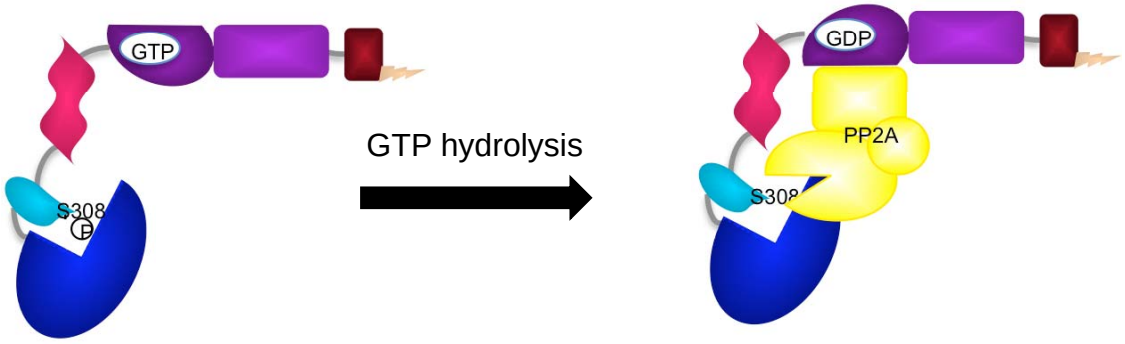
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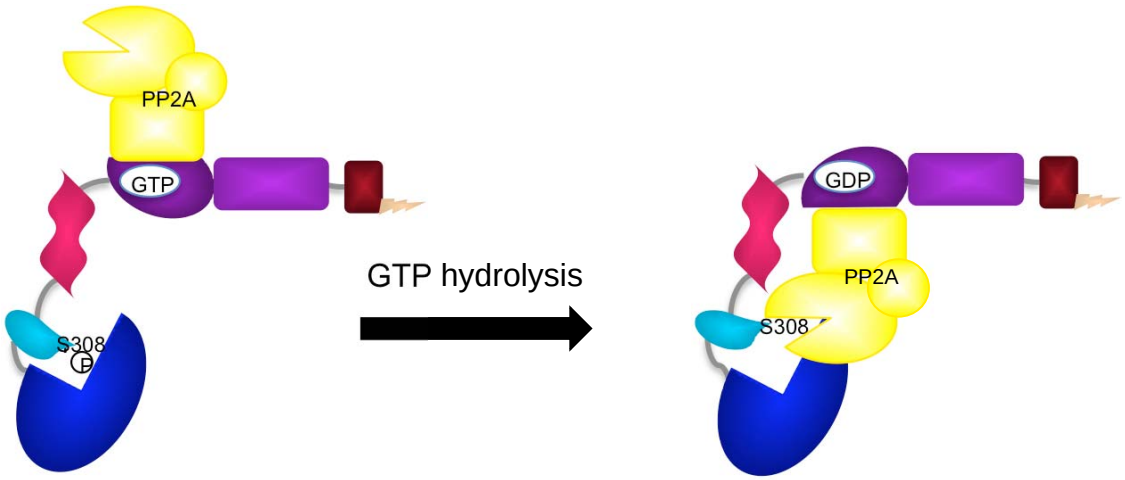
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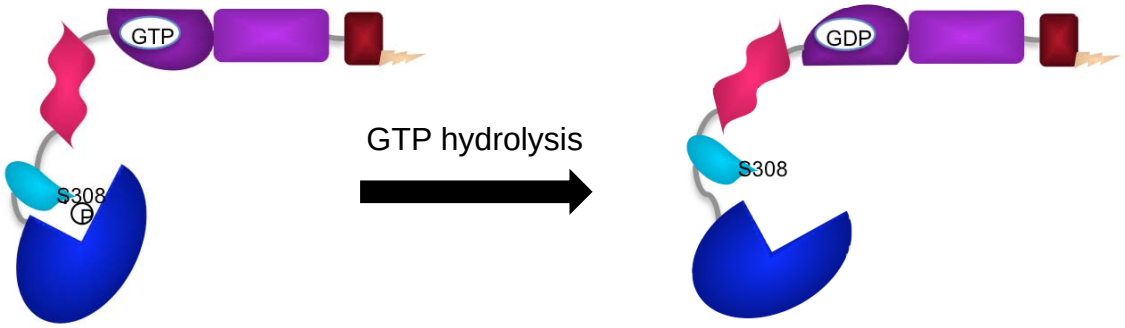
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