SUPPLEMENTARY METHODS AND FIGURES

Enzymes: Asp-N endoproteinase was purchased from Roche Applied Science (Mannheim, Germany), Lys-C endopeptidase by Wako Chemicals USA (Richmond, VA) and mass spectrometry-grade trypsin by Promega (Madison, WI). Phusion high-fidelity polymerase was purchased from New England Biolabs (Ipswich, MA). Protein phosphatase 2A C subunit was obtained from Cayman Chemical (Ann Arbor, MI).

***Cells and cell culture reagents*:** HEK-293 cells were purchased from Cell Culture Services at the Cleveland Clinic (Cleveland, OH) and Phoenix Ampho cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Media and fetal bovine serum for culture of mammalian cells were purchased from HyClone (Logan, UT). Sf9 insect cells were purchased from ATCC and High-Five insect cells were a kind gift of Drs. Valerie and John Tesmer (University of Michigan, Ann Arbor, Michigan). The Sf-900 III and Express Five media as well as all other reagents required for culture of insect cells and generation of baculovirus were purchased from Invitrogen (Carlsbad, CA).

***Antibodies*:** IS4 monoclonal and UW28 rabbit polyclonal anti-GC antibodies were generated in our laboratory (1-4). Goat-anti-mouse secondary antibody labeled with Alexa Fluor 647 was purchased from Invitrogen (Carlsbad, CA).

***Plasmid vectors*:** pGEM-T-Easy was purchased from Promega (Madison, WI). A retroviral vector (pMXs-IG3) was modified from pMXs-IG (5), which was kindly provided by Toshio Kitamura (University of Tokyo, Tokyo, Japan)(6).

***Chemicals and disposables*:** ANAPOE-NID-P40 was purchased from Anatrace (Maumee, OH). Protein A Sepharose was obtained from GE Healthcare (Uppsala, Sweden). Pro-Q Diamond and SYPRO Ruby gel stains were bought from Invitrogen - Molecular Probes (Eugene, OR). MonoTip TiO_2, pipette tips for phosphopeptide enrichment, were supplied by GL Sciences (Saitama, Japan). Protoblot II immunoblotting systems were purchased from Promega (Madison, WI). OPTI-FLUOR scintillation liquid was obtained from PerkinElmer (Shelton, CT). Alumina was purchased from MP Biomedicals (Solon, OH). EDTA-free protease inhibitors were purchased from Roche Applied Science (Mannheim, DE). Microcystin-LR was obtained from Alexis Biochemicals (Lausen, CH). Bio-Rad Protein Assay kits were obtained from Bio-Rad (Hercules, CA). Organic solvents were supplied by Fisher Scientific (Fair Lawn, NJ) and other chemicals were purchased either from Sigma-Aldrich (St. Louis, MO) or USB Corp. (Cleveland, OH).

***Radiochemicals*:** [α-^{33}P]GTP was purchased from Perkin Elmer (Boston, MA) and 8-N_3-[α-^{32}P]ATP was obtained from ALT Bioscience (Lexington, KY).

Instrumentation

The FACSAria cell sorter was supplied by BD Biosciences (San Jose, CA). The Molecular Imager FX with Quantity One software was purchased from Bio-Rad (Hercules, CA) and the Typhoon 9410 with ImageQuant TL software was supplied by GE Healthcare (Uppsala, Sweden). The scintillation counter model LS 6500 was furnished by Beckman Coulter (Fullerton, CA). A sonifier Model 150D was purchased from Branson (Danbury, CT). A UV crosslinker model XL-1000 was purchased from Spectronics Corp. (Westbury, NY). The Leica TCS SP2 confocal microscope was supplied by Leica (Heidelberg, Germany).

Genotyping

Knock-out genotypes of *Gnat1*$^{-/-}$ and *Lrat*$^{-/-}$ mice were confirmed by the absence of PCR products with primers Gnat1-F 5'- GGCTATGACCACGCTCAACATTC -3', Gnat1-R 5'-

CTGGAGTCACCAGACGCTCTAGG -3' and LRAT-F 5'-ATGAAGAACCCAATGCTGGAAGC-3', LRAT-R 5'-CTTCTCAGCCTGTGGACTGATCC-3', respectively. Genotyping of *Gcap1/2*$^{-/-}$ mice was performed as previously described (7). The knock-out genotype was confirmed by the presence of the ~200 base pair PCR product obtained with primers GCAP2-F GAAGCAGCCAGGCCAAGCCACACCCATCACA and Neo-R CGGTGGATGTGGAATGTGTGCGAGGCCAGA. Absence of the WT allele was confirmed by lack of the 863 base pair product with primers GCAP2-F and GCAP2-R GGCAGGGTTTCTTCAGCTTGTAAATCGCCTGCCAGA. Positive controls were processed simultaneously with all tested samples.

Cloning, expression and purification of mouse GCAP1

The full-length mouse GCAP1 cDNA clone was obtained from mouse retinal cDNA by PCR amplification with primers: mGCAP1-F CAGGGGACATTAGAAAATAAGATCTACCTG, and mGCAP1-R GTTAAGACAAGAACAGAACAACCACACC followed by ligation into a pGEM-T-Easy vector to generate plasmid pGTE-mGCAP1. The GCAP1 coding sequence was then cloned into pFastBac HT vector to generate pFBmGCAP1 with GCAP1 flanked by the following sequences:

...[ATG]TCGTACTAC[CATCACCATCACCATCAC]GATTACGATATCCCAACGACC[GAAAACCTGTATTTTCAG**GGC]AACATCATG~GAGGCTGCGGGT**[TGA][AAGCTT]...

where sequential brackets denote: the initiation codon, the 6xHis-tag coding sequence, the tobacco etch virus (TEV) recognition site coding sequence, the stop codon, and the *Hind*III recognition site, respectively, with the GCAP1 coding sequence shown in bold. This DNA sequence corresponds to the following protein sequence: MSYY[HHHHHH]DYDIPTT[ENLYFQ/**G]NIMEGKSVEEL~AGTGDLAAEAAG** with brackets denoting the 6xHis-tag and TEV recognition site, respectively, the slash indicating the TEV cleavage site and GCAP1 shown in bold. The pFBmGCAP1 was used to generate bacmid and then baculovirus by using Sf9 cells and following the producer's manual. For protein expression suspended High-Five insect cells were cultured in 1-2 L glass baffled flasks at 27 °C in a shaking incubator set at 120 rotations per min in Express Five SFM media supplemented with 90 ml/L of 200 mM L-glutamine. Just prior to addition of baculovirus, cells were diluted about twofold with fresh media to a density of 2 x 10^6 cells/ml. To initiate expression, 1 ml of baculovirus (passage 2) was added per 100 ml of culture and cells were collected 50 h later by a 10 min centrifugation in 50 ml conical tubes at 300*g*. Cell pellets were resuspended in 3 ml of 50 mM HEPES, pH 7.0, 40 mM NaCl, and 360 mM KCl with one tablet of EDTA-free protease inhibitors per 50 ml of buffer, snap frozen in liquid nitrogen and stored at -80 °C. To prepare cell clarified lysates suitable for column purification, cell pellets from 250 ml of culture were thawed and sonicated three times for 20 s with 1 min incubations on ice with a Sonifier Model 150D set to power-level 5. To remove crude cell debris, a sample was first centrifuged at 25,000*g* for 30 min at 4 °C and the supernatant then was ultracentrifuged at 100,000*g* for 30 min and then again for 1 h at 4 °C to remove cell membranes. After sample loading, a column (7.5 mm diameter), packed with 2.5 ml of TALON metal affinity resin (Clontech, Mountain View, CA) and equilibrated with 10 resin volumes of 50 mM HEPES, pH 7.0, 40 mM NaCl, 360 mM KCl, and 10 mM imidazole buffer, was washed with 20 resin volumes of the same buffer. Purified GCAP1 was eluted with 50 mM HEPES, pH 7.4, 360 mM KCl, 40 mM NaCl, and 200 mM imidazole in 1 ml fractions. Fractions containing protein were identified with the Bio-Rad protein assay, combined, and diluted 4x with 50 mM HEPES, pH 7.4, which resulted in a final GCAP1

concentration of ~0.5 mg/ml. To cleave the 6xHis-tag, TEV protease was added at a molar ratio of 1:50 (TEV:GCAP1), incubation was performed for 24-48 h at 4 °C; and complete removal of the tag was confirmed by SDS-PAGE. GCAP1 was aliquoted, frozen in liquid nitrogen and stored at -80 °C. A total of 7 mg of >95% pure GCAP1 was obtained from 250 ml of insect cell culture.

Photolabeling of GC with 8-N_3-[α-^{32}P]ATP

Washed cell membranes, obtained exactly as for the GC assay, were resuspended in 45 μL of labeling buffer (50 mM HEPES, pH 7.4, containing 90 mM KCl, 10 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EGTA, and 8 μM (2 Ci/mmol) 8-N_3-[α-^{32}P]ATP) to obtain a final reaction mixtures. Some samples also contained 4 mM GTP or 4 mM ATP to compete out the 8-N_3-[α-^{32}P]ATP label. Resulting samples were incubated for 3 min at 22 °C and than irradiated in a UV crosslinker with 254 nm UV light (3,500 μW/cm^2) for 3 min with mixing and cooling on ice every 1 min. Immediately afterwards, the samples were resuspended in 1 ml of HEPES-RIPA buffer (10 mM HEPES, pH 7.4, 10 mM NaCl, 1% ANAPOE-NID-P40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 μM leupeptin) and GC was purified by immunoprecipitation and resolved by SDS-PAGE as described above. Each gel was fixed by two 30 min washes in 100 ml of fixative solution (50% methanol, 10 % acetic acid), which was then removed by three 10 min incubations in water. Finally, gels were placed in a clear polypropylene cover and exposed to a phosphor storage screen for 24 h followed by image acquisition with a Typhoon imager and quantification with ImageQuant TL software.

Immunocytochemistry and confocal imaging

HEK-293 cells expressing GC were seeded at 2.5 x 10^5 cells / 9.6 cm^2 glass bottom dish (thickness 0.17 mm) and cultured for 36 h at 37 °C. The fixative (136 mM NaH_2PO_4, pH 7.4, containing 2.5 mM $MgCl_2$, and 2% paraformaldehyde) was freshly prepared by first dissolving paraformaldehyde in 30 ml of 130 mM NaOH at 60 °C followed by addition of the remaining components. Cells were rinsed once with 2.5 ml of warm (37 °C) DPBS (8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4, 137 mM NaCl, and 2.7 mM KCl) and then incubated for 20 min at 21 °C in fixative, which was subsequently removed by three more rinses with 2.5 ml of DPBS. Next, cells were permeabilized by incubation with 2.5 ml of DPBS-T (0.1% Triton X-100 in DPBS) for 15 min at 21 °C and rinsed three times with 2.5 ml of DPBS. Subsequently, the samples were incubated in 1.5 ml of blocking solution (10% normal goat serum in DPBS) for 1 h at 21 °C, which was then replaced with 1.5 ml of IS4 monoclonal anti-GC antibody at 50 ng/ml diluted in blocking solution, and the incubation was continued for 2 h at 21 °C. After three 10 min washes with 2.5 ml of wash solution (1% normal goat serum in DPBS) samples were incubated for 1 h at 21 °C with Alexa Fluor 647 labeled goat anti-mouse secondary antibody diluted with blocking solution to 2 μg/ml. After three more 10 min washes with 2.5 ml of wash solution, samples were mounted in ProLong Gold according to the producer's instructions. Finally, samples were imaged with a Leica TCS SP2 confocal microscope.

Dephosphorylation of GC1 in ROS by the catalytic subunit of protein phosphatase (PP2Ac)

Four to eight week old C57BL/6J mice were dark adapted overnight and ROS were isolated as described under "Isolation of mouse ROS," but using a different buffer (10 mM HEPES, pH 7.4, 100 mM NaCl) that does not interfere with the GC assay. Isolation of ROS, PP2Ac treatment and

subsequent GC assay were carried out in the darkroom under red light to prevent the activation of phosphodiesterase 6. For the composition of buffers mentioned below, see “GC activity assay”. ROS purified from 75 retinas were resuspended in 350 µL of protein buffer and added to solution composed of 350 µL of assay buffer concentrate, 350 µL of 5 mM 3-isobutyl-1-methylxanthine in water and 175 µL of water. The resulting mixture was sonicated (5 s, sonifier model 150D, power-level 3) and 70 µL aliquots were transferred to microtubes. Next, either 16 µL of 0.5 mg/ml stock solution of GCAP1 in protein buffer or buffer alone was added to the samples. Finally, 4 µL of 0.13 mg/ml stock solution of PP2Ac supplied in PP2A buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, and 25% v/v glycerol) or this buffer alone was added followed by incubation at 30°C for 30 min. To measure the activity and phosphorylation levels of GCs, samples were chilled on ice, 10 µL of nucleotide premix was added (see “GC activity assay”) and the temperature was returned to 30°C. The cGMP synthesis was stopped after 10 min by addition of 30 µL of 0.4 M HCl, brief vortexing and a 7 min centrifugation at 16,000*g*. Forty µL of the supernatant was assayed for GC activity as described under “GC activity assay” and the excess supernatant was discarded. Pellets of ROS membranes frozen at -80 °C were later used for purification of GC as described under “Purification of GC from ROS membranes by immunoprecipitation” but with less UW28 antibody (7 µg per sample). Finally, phosphorylation levels of GC were examined as described under “Phosphoprotein staining, visualization and quantification” in the ”Materials and Methods”.

SUPPLEMENTARY FIGURES

Figure S1. Modulators of GC1 activity.
A. Effect of Ca^{2+} concentration on GC1 activity. Assays were carried out with HEK-GC1 cell sonicates in the presence or absence of 4 μM GCAP1 as described under "Materials and Methods". The results demonstrate robust activation of GC1 by GCAP1 in the presence of low Ca^{2+} concentrations.
B. Effect of nucleotides on the activity of GC1. Activities of HEK-GC1washed cell membranes were assayed in the presence of 4 μM GCAP1, 45 nM (low) free Ca^{2+} and increasing concentrations of ATP or AMP-PNP. Concentrations at which the maximal stimulatory effect (EC_{max}) and 50% inhibition (IC_{50}) were observed were determined from the plot. All measurements were done in triplicate and the standard deviations of the mean were lower than 10% for all data points shown.

Figure S2. Phosphorylation sites in the KH domain of membrane GCs.
White letters on a black background indicate phosphorylation sites. Multiple Ser residues of the GC1 kinase homology domain are phosphorylated *in vivo*. Likewise, membrane GC NPRs were reported to be multiply phosphorylated within this domain (8,9). Residues are numbered from the initiating Met, and thus their numbers might differ from reports where mature protein lacking the signal peptide is used as reference.

Figure S3. Localization and expression of GC1mutants in HEK-293 cells.
A. Immunocytochemical localization of GC1 mutants stably overexpressed in HEK-293 cells. GC1 (red signal) has a similar localization in all cell lines. All cells also express EGFP (green signal) as a selection marker. A cell line labeled EGFP that expresses EGFP but not GC1 was used as a negative control. Images were collected with a Leica TCS SP2 Confocal Scanner.
B. Colocalization of GC1 with calreticulin, an endoplasmic reticulum marker.
This analysis demonstrates that GC1 is localized predominantly in the endoplasmic reticulum. Images were collected with a Leica TCS SP2 Confocal Scanner. **C.** Immunoblot with anti-GC1 IS4 monoclonal antibody demonstrating similar expression of GC1 mutants in HEK-293 cells per equal number of cells.

Figure 4. Effect of phosphorylation on GC1 regulation by Ca^{2+} and ATP.
Activities of WT and mutant GC1 washed cell membranes were determined in the presence of 4 μM GCAP1 and increasing concentrations of Ca^{2+} **(A)** or ATP **(B)**. The results demonstrate that phosphorylation did not affect the sensitivity of GC1 to Ca^{2+} or ATP. Samples were assayed in triplicate and the vertical lines represent the standard deviations of the mean.

Figure 5. Effect of dephosphorylation of GC1 by the catalytic subunit of protein phosphatase 2A (PP2Ac) on GC1 activity.
The GC activity in sonicates of mouse ROS treated with the catalytic subunit of PP2Ac was compared with that in untreated controls **(A)**. To evaluate the extent of dephosphorylation of GCs in the assayed samples, GCs were purified by immunoprecipitation, resolved by SDS-PAGE and stained with Pro-Q-Diamond **(B, top)**. Immunoblot probed with IS4 anti-GC1 antibody is also presented to show that the amount of GC1 was similar in all samples **(B, bottom)**. This experiment was performed twice with triplicate samples. The result demonstrates that GC1 is a

substrate for PP2Ac and that dephosphorylated GC1 does not display altered enzymatic activity.

Figure 6. Contribution of residues N674 and D687 to binding of Mg^{2+} within the KH domain.

A. The model of the Mg^{2+} binding site within the KH domain was generated based on homology to the insulin receptor tyrosine kinase (IR-TK, PDB ID: 1ir3). Mg^{2+} ions and water molecules are shown as green and red spheres, respectively. Residues coordinating ATP and Mg^{2+} are shown as sticks. Hydrogen bonds are indicated by dashed lines. The overall architecture of the GC1 KH domain was modeled based on the structure of leukocyte-specific protein tyrosine kinase; LCK (PDB:ID 1qpca) using the Phyre webserver (10). Subsequently, ATP, Mg^{2+} and surrounding water molecules were modeled into the nucleotide binding pocket based on the structure of insulin receptor tyrosine kinase (IR-TK, PDB:ID 1IR3) using Coot (11). The final figure was generated with PyMOL ((12) and *http://www.pymol.org*).

B. Alignment of the protein sequence fragments of GC1 and IR-TK encompassing predicted Mg^{2+} binding residues i.e. N674 and D687. Substantial identity of these sequence fragments indicates that residues N674 and D687 were modeled with high accuracy.

Figure 7. GC1 mutants deficient in Mg^{2+} binding retain the ability to bind ATP.

An autoradiograph of WT and mutant GC1 photolabeled with 10 μM 8-N_3-[α-^{32}P]ATP in the absence or presence of competing nucleotides 4 mM ATP or GTP as described under "Materials and Methods". The efficiency of 8-N_3-[α-^{32}P]ATP incorporation and the extent to which such labeling was diminished by competing ATP or GTP were similar in WT and mutant GC1s, implying that Mg^{2+} is not required for binding of ATP to the KH domain of GC1. This result is representative of two replicated experiments.

Figure 8. GC1 Ser residues found phosphorylated in the current study are highly conserved among species.

Protein sequences of a GC1 KH domain fragment containing the phosphorylation sites were aligned by using Clustal W (13-15). Residues matching the consensus sequence are shaded black. Conserved residues (positive BLOSUM62 score) are shaded gray and non-conserved (negative BLOSUM62 score) are shown in white. Phospho-Ser residues identified in this study are colored red. Mm - *Mus musculus* (mouse), Bt - *Bos taurus* (cow), Hs - *Homo sapiens* (human), Rn - *Rattus norvegicus* (rat), Cf - *Canis familiaris* (dog), Md - *Monodelphis domestica* (opossum), La - *Loxodonta africana* (elephant), Gg - *Gallus gallus* (chicken), Xt - *Xenopus tropicalis* (frog).

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Figure S1

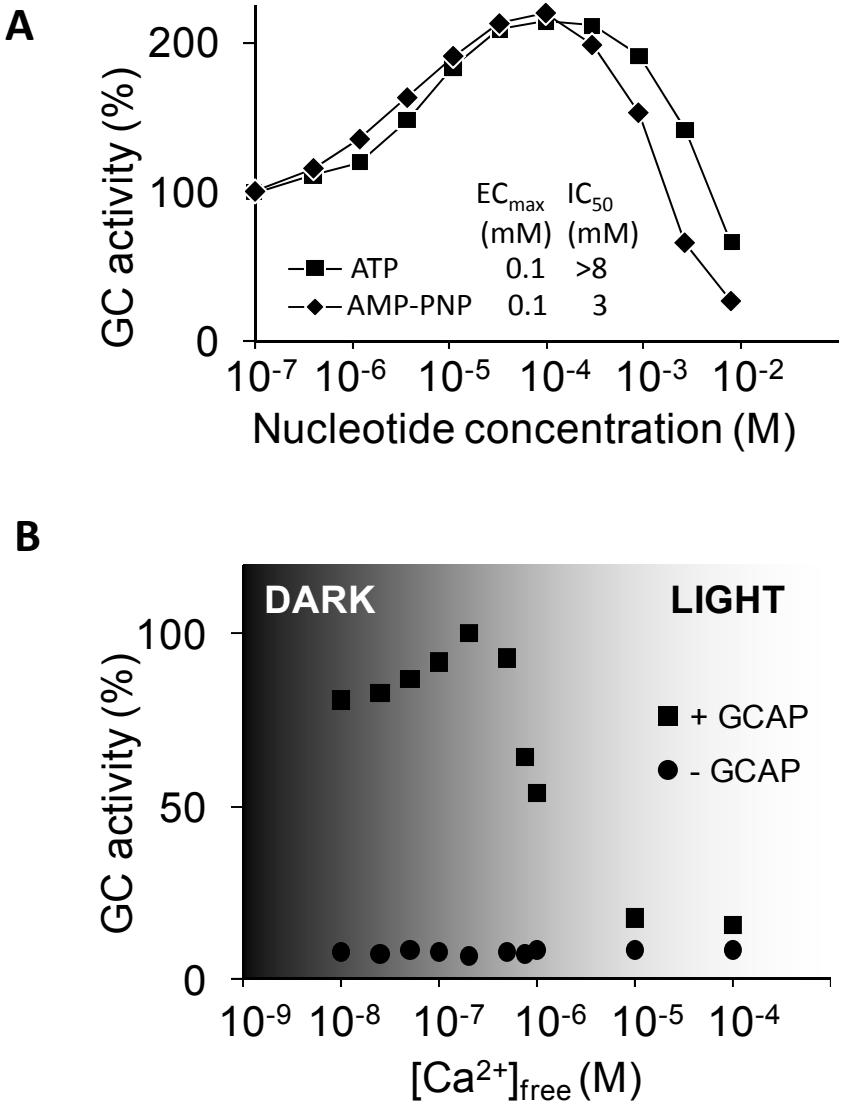
A
200
100
0
GC activity (%)
EC$_{max}$ (mM)
IC$_{50}$ (mM)
ATP 0.1 >8
AMP-PNP 0.1 3
10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2}
Nucleotide concentration (M)
B
DARK
LIGHT
100
50
0
GC activity (%)
+ GCAP
- GCAP
10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
$[Ca^{2+}]_{free}$ (M)

Figure S2

PROTEIN	SPECIES	SOURCE	KH DOMAIN PHOSPHORYLATION SITES
GC1	MOUSE	RETINA	^{528}QG**S**R**SS**LATR**S**ASDIRSVPS
NPR-A	RAT	HEK-293	^{523}AG**S**RL**T**L**S**GRG**S**NYG**S**LL**T**T
NPR-B	RAT	HEK-293	^{511}AG**S**RL**T**L**S**LRGS**S**YG**S**LMTA

Figure S3

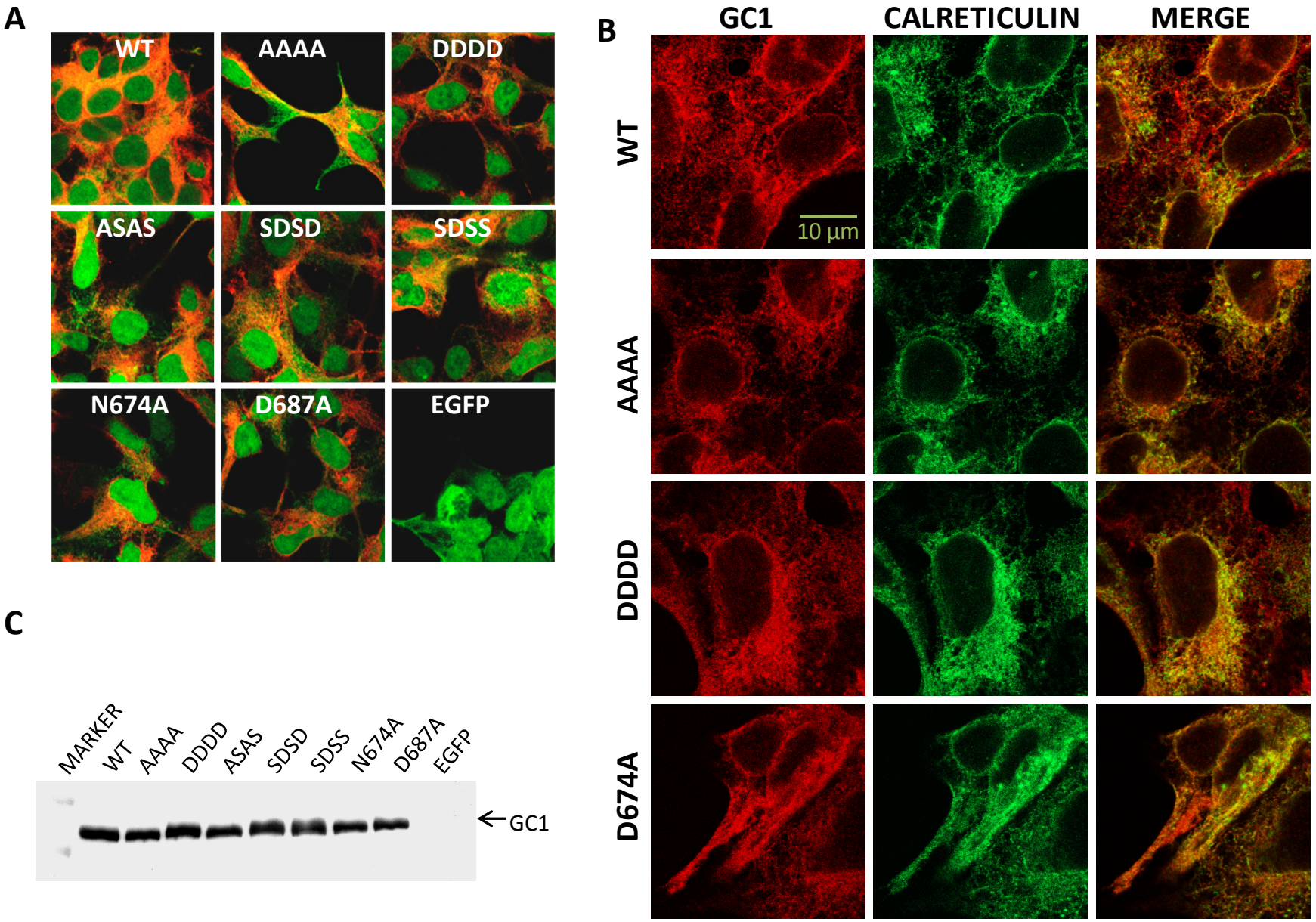
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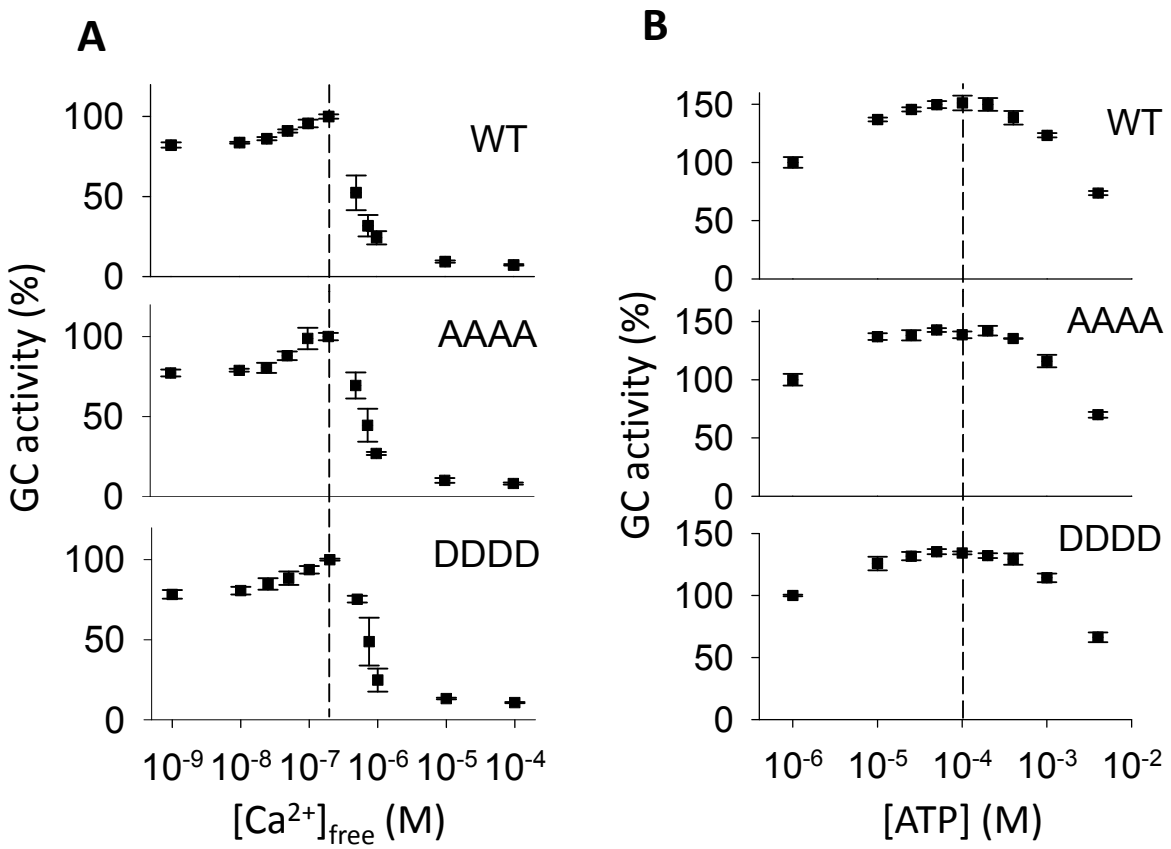
Figure S4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B
GC activity (%)
WT
AAAA
DDDD
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50
0
150
10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
$[Ca^{2+}]_{free}$ (M)
10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2}
[ATP] (M)

Figure S5

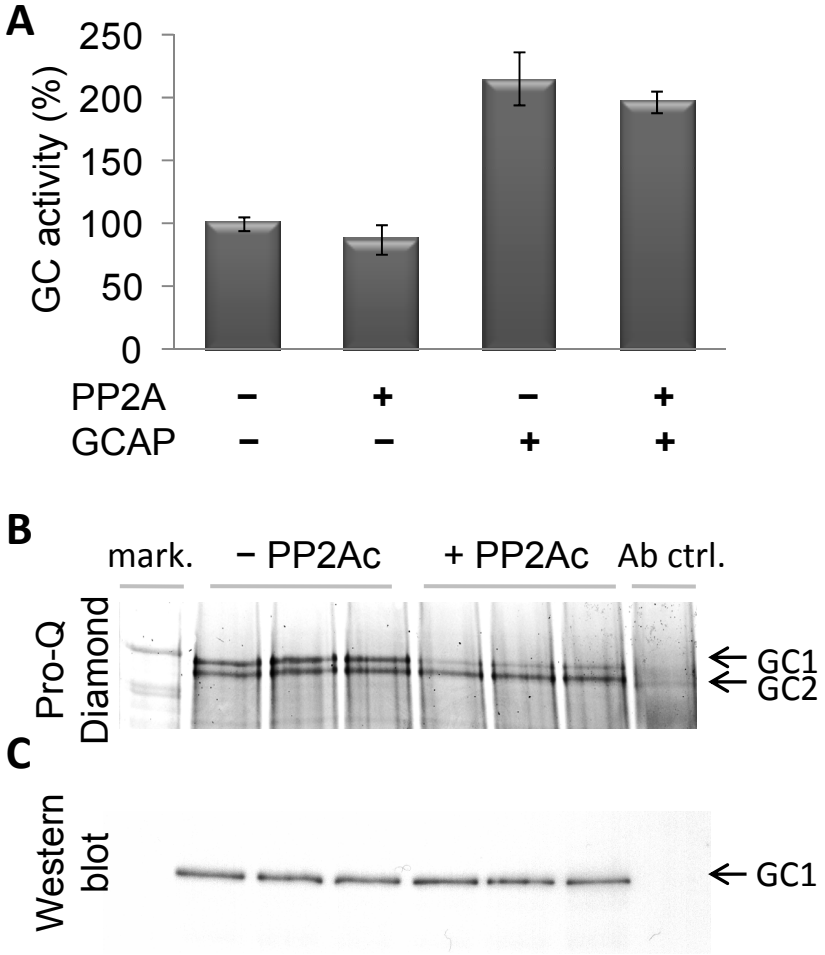
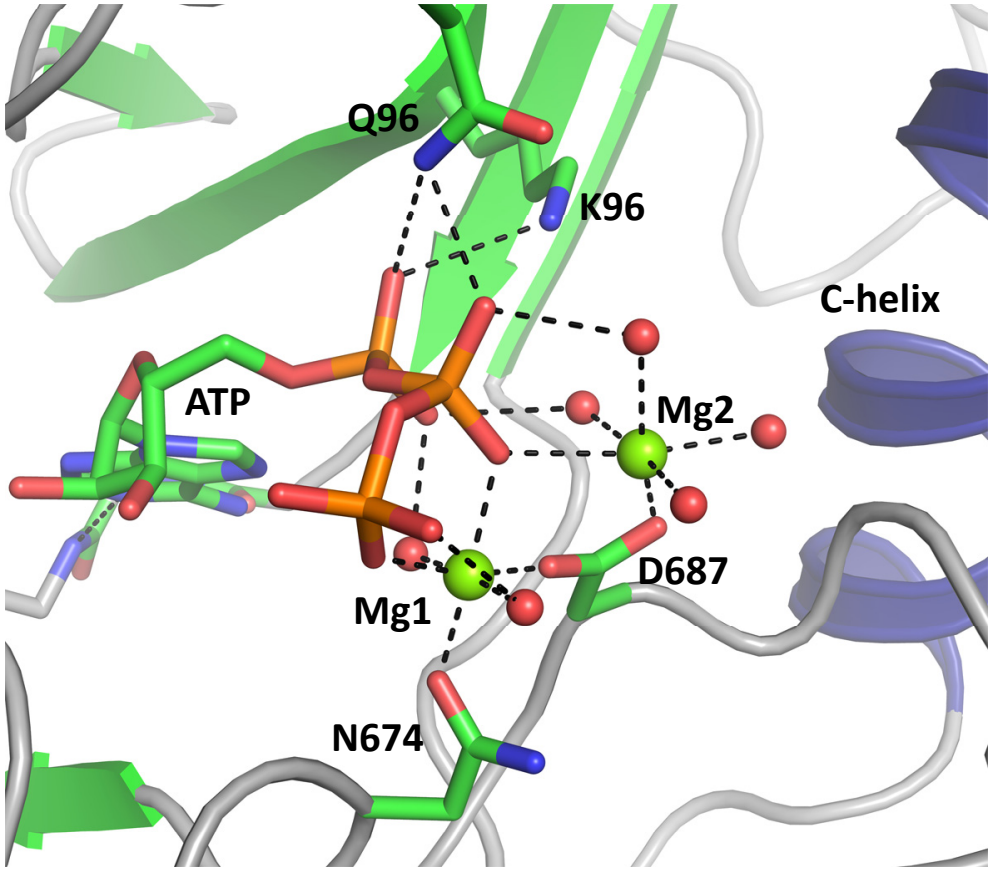

A
GC activity (%)
250
200
150
100
50
0
PP2A – + – +
GCAP – – + +
B
mark.
– PP2Ac
+ PP2Ac
Ab ctrl.
Pro-Q Diamond
GC1
GC2
C
Western blot
GC1

Figure S6

A



B

GC1 ^{667}HGRLKSR**N**CVVDGRFVLKVT**D**HGHGR

IR-TK ^{1157}HRDLAAR**N**CMVAHDFTVKIG**D**FGMTR

N674 D687

Figure S7

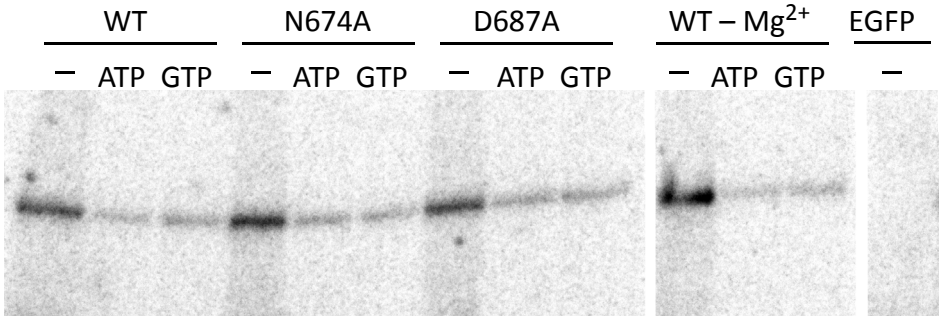


Figure S8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