SUPPLEMENTARY INFORMATION

MATERIAL AND METHODS

General reagents: PC, PS and PImix were purchased from Sigma. PA, PI4P and PI(4,5)P2 were purchased from Avanti Polar Lipids, Inc. (Alabama, USA). Ni-NTA agarose beads were from Qiagen. U73122 was purchased from Tocris Bioscience (Bristol, UK). EGF, Hoechst 33342, 1-butanol, Glutaraldehyde and other chemicals were purchased from Sigma.

Antibodies: Monoclonal antibodies against p115 and VSV-G as well as rabbit polyclonal antibodies against Nir2 and GRASP65 were described previously [1, 2]. Monoclonal and rabbit polyclonal anti-Myc antibodies, as well as anti-ERK1/2 and pERK1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-α-tubulin and anti-pP38 antibodies were purchased from Sigma. Antibodies against pAKT (pS473 and pT308) were purchased from Cell Signaling Technologies (Beverly, MA). Anti-PI(4,5)P2 antibody was purchase from Echelon Biosciences Inc. (UT, USA). Alexa-488 donkey anti-mouse and anti-rabbit immunoglobulin Gs (IgGs) were purchased from Invitrogen (Carlsbad, CA). Cyanine (Cy)3-conjugated goat anti-rabbit and goat anti-mouse IgGs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

DNA Constructs, Transfections and Lentivirus Infections

The C-terminal region of Nir2 (aa 911-1244) as well as the PITD (aa 1-277) of the wild-type or the Nir2(D1128A) and Nir2(S164A) mutants were cloned into pET21a bacteria expression vector (Novagen) and expressed as His-tagged recombinant proteins in bacteria. Myc tag was fused in frame to the C-terminal end of the wild type (WT) Nir2, its ΔPI (aa 278-1244), ΔCter (aa 1-910), PITD (aa 1-277) and the C-terminal region (aa 911-1244) by PCR. Additional constructs expressing the C-terminal region (aa 911-1244) fused to a Myc-tag at its N-terminal end or the PITP fused to GFP at its C-terminal end were also constructed. Point mutations (S164A, D1128A and S1026/D1028/D1128A) were introduced by site directed mutagenesis. All these Nir2 constructs were cloned downstream of CMV promoter using the pCDNA3 expression vector (Invitrogen, CA), its modified version pCAN-Myc, or the pEGFPN1 (Clontech). Four different shRNA sequences of Nir2 cloned in the pLKO.1-puro lentiviral vector were obtained from Sigma and validated. The following shRNA clones were used: #1 (TRCN0000029763), #2 (TRCN0000029760), #3 (TRCN0000029761), #4 (TRCN0000029762). TRCN0000029763 was most potent. Lentivirus packaging was performed in HEK293T cells by co-transfecting the pLKO.1-puro-Nir2-shRNA constructs with pMDL-g/p-RRE (HIV *gag/pol*) packaging plasmid, the *rev*

expression plasmid pRSV-REV, and the pCMV VSV-G envelope plasmid. Twelve hours later, the medium was changed and viruses were collected 24 and 48 h later. Viral supernatants were filtered through 0.45-μm pore size filters and stored at -80 °C [3]. Cells were infected with the viruses in the presence of 8 μg/ml polybrene for 16 h and were then subjected to selection in 0.5 μg/ml puromycin for 72 h. The cDNA of Nir2-Myc containing silent mutations within the target sequences of shRNA #1, #2, and #3, was subcloned into pHAGE PGK-IRES-Hygro-W lentivirus vector, a variant of pHAGE PGK-GFP-IRES-Luciferase-W vector [4] that was kindly provided by Darell Kotton (Boston University). Replacing the GFP and the Luciferase genes from the original vector with the cDNA of Nir2-Myc or its mutants and Hygromycin, respectively, produced the pHAGE PGK-Nir2-Myc-IRES-Hygro-W construct. Lentivirus packaging was performed in a five plasmid system by co-transfecting HEK293T cells with Nir2 lentivirus constructs together with vectors encoding the Gag-Pol (pHDM-Hgpm2), Tat (pHDM-tat1b), Rev (pRC-CMV rev1B) and VSV-G (pHDM VSV-G). Lentiviruses were collected as above, and infected cells were selected in 200 μg/ml hygromycin for 72 h. All transfections were performed by the calcium phosphate method. Stable HeLa cell lines expression control shRNA or Nir2 shRNA#1 were established following DNA transfection and colony selections. At least 5 independent clones were examined to exclude clonal variation.

Liposome sedimentation assays

The C-terminal region of Nir2 was expressed as His-tagged protein in bacteria (BL21) and purified on Ni-NTA agarose beads (Qiagen) according to the manufacturer's instructions. Liposome sedimentation assays were performed essentially as described previously [5]. In brief, purified recombinant C-terminal of Nir2 was dialyzed against PIPES buffer (20 mM PIPES, 137 mM NaCl, 3 mM KCl pH 6.8) and centrifuged at 213,000 x g for 15 min at 4˚C to remove aggregated protein. Multilamellar liposomes were prepared from PC alone or from a mixture of PC with PS, PA or PImix at a molar ratio of PC:PA (2:1), PC:PS (2:1) or PC:PImix (5:1) unless indicated otherwise. The mixtures were dried under nitrogen, and resuspended at final concentration of 300 μM total lipids in buffer containing 20 mM Tris pH 7.5, 100 mM NaCl. The resuspended lipids were incubated at room temperature for 1 hour to hydrate lipid film and then vortexed to detach hydrated lipids from the walls of tube. Liposomes (16.5 μl) were mixed with the recombinant C-terminal protein (5 μg in 50 μl in the same buffer) and incubated for 10 min at room temperature. Proteins bound to the liposomes were sedimented by centrifugation at 16,000 × *g* for 30 min. Proteins present in the pellet

and supernatant were resolved on SDS-PAGE and detected by Coomassie brilliant blue staining.

Cells Culture and Immunofluorescence Microscopy

HEK293 and HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin. For immunoflourescence analysis of Nir2 localization, HeLa cells grown on coverslips were fixed in 1% paraformaldehyde (PFA) in KM buffer (10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.2, 10 mM NaCl, 1.5 mM $MgCl_2$ and 2.5% glycerol) for 20 min at room temperature. The cells were washed with PBS containing 0.1M glycine, permeabilized with 0.5% Triton-X 100 in PBS, and then immunostained and analyzed by confocal microscopy essentially as we previously described [6]. When indicated, the cells were serum starved for 16-24 hr, and then stimulated with EGF or PA as described. Immunostaining with anti-PI(4,5)P2 antibody was performed as described by Komaba et al., [7] with some modifications. All steps were performed at 4°C. The cells were washed with iced cold PBS and then fixed for 1 hr in 3.7% PFA and 0.1% glutaraldehyde in PBS. The cells were incubated for 15 min in PBS containing 0.1M glycine, and then incubated in blocking buffer containing 0.5% saponin, 5% goat serum, and 2.5% BSA in PBS for 30 min, followed by 1 h incubation with the primary mouse anti-PI(4,5)P2 antibody (Echelon Biosciences Inc. Salt Lake City, UT) in 0.1% saponin, 5% goat serum, and 2.5% BSA in PBS. The samples were washed in PBS, incubated with secondary antibody in the same buffer for 1 hr, washed again with PBS, mounted on microscopic slides, and processed by confocal microscope (LSM510, Zeiss).

Cell extracts and Western Blotting

HeLa cells were serum starved for 16-24 hr, and then stimulated with EGF as indicated. Cells were lysed in lysis buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM $MgCl_2$, 1 mM EGTA, 50 mM NaF, 20 mM β-glycerophosphate, 0.5 mM $NaVO_3$, 0.4% saponin, and protease inhibitors). Protein content was measured using Bradford reagent (Bio-Rad), and samples were normalized accordingly. Western blot was performed by standard procedures using blocking buffer containing 5% nonfat dry milk in TBS-Tween (0.05%). The intensity of protein bands was measured using the Image J software (NIH, USA).

Measurment of PIP3 content

The PIP3 content of control and Nir2-depleted HeLa cells was determined by the PIP_3 Mass Elisa Kit (Echelon Biosciences. Inc. Catalog No. K-2500s) according the manufacturer's instructions. In brief, 10^7 cells were serum-starved for 18 hr and then stimulated with EGF

(100ng/ml) for the indicated time. The cells were incubated with ice cold 0.5M TCA for 5 min, centrifuged, and cell pellet was washed twice with 5% TCA containing 1mM EDTA. Neutral lipids were extracted with methanol:chloroform (2:1) at RT. Acidic lipids were extracted with 2.25 ml methanol:chloroform:12M HCl (80:40:1) for 15 min at RT. The samples were centrifuged for 5 min and the supernatant recovered. The supernatant was then treated with 0.75 ml chloroform and 1.35 ml 0.1M HCl and centrifuged to separate organic and aqueous phases. The organic phase was collected and dried. PIP3 levels were then determined using a competitive ELISA assay providing by the kit. Standard curve was used to measure the content of PIP3 in pmols.

Measurment of PIP2 production

For measuring PIP2 production, control and Nir2-depleted HeLa cells (4×10^6) were metabolically labeled for 1 hr with 100 µCi [^{32}P]Orthophosphate/ml (PerkinElmer) in phosphate-free media. Acidic lipids were extracted with chloroform/methanol as previously described [8]. In brief, metabolically labeled HeLa cells in 10 cm plates were rinsed with cold PBS, and then scraped into 0.8 ml mixture of 1 N HCl and MeOH (1:1 v/v). The cells were collected into glass tubes and 0.4 ml chloroform was added. The samples were vortexed for 1 min and centrifuged (400 x *g* for 10 min) to separate the organic phase. The organic phase (lower) was collected and re-extracted with 0.4 ml of a mixture of MeOH:EDTA (0.1M, pH 8.0) (1:0.9 v/v) and centrifuged at 400 x *g* for 10 min. The organic layer was dried under N_2 stream, and separated by thin layer chromatography (TLC) on siliga gel 60 (Merck). The TLC plates were impregnated with 1.2% potassium oxalate and 1.2 mM EDTA in MeOH: H_2O (2:3) solution and then air-dried and activated at 110˚C for 20 min. The dried lipids were resuspended in chloroform:MeOH (2:1) and spotted on to the pretreated TLC plate. TLC was developed in a solvent containing: chloroform:acetone:methanol:acetic-acid:H_2O (40:15:13:12:7) [9]. PI, PIP and PIP_2 standards (Sigma, Avanti) were identified by iodine vapor and used as marks for the radiolabeled phosphoinositides. PI(4,5)P2 synthesis was quantified by phosphorimager and densitometry analysis (Image J). The experiments were performed in duplicates, and were repeated at least 7 times. The intensity of the radiolabeled lipids was normalized according total radiolabeled lipids.

Accession Numbers: Nir1 (Accession; AAK01446), Nir2 (Accession; AAK01444) and Nir3 (Accession; AAK01445) [10] and the three human lipins; Lipin1 (Accession; AAH30537), Lipin2 (Accession; NP_055461) and Lipin3 (Accession; NP_075047)

FIGURE LEGENDS

Figure S1: PA and PI(4,5)P2 turnover in response to growth factor stimulation. Key lipid intermediates are shown along with the enzymes (labeled in red) that regulate their production or turnover. The positive feedback loop between PA and PI(4,5)P2 production at the PM is marked by a dashed blue line. The bulk synthesis of PA takes place at the ER, where PA functions as a precursor for PI and other phospholipids. PA is converted into CDP-DAG by CDP-DAG synthase (CDS), which is then utilized by PI synthase (PIS) to produce PI; the precursor of higher phosphoinositide species [11]. The potential transport of PA and PC from the ER to the PM could be mediated by vesicular transport or potentially by lipid-transfer protein as previously was proposed [12-15]. CK: Choline kinase, CCT: cholinephosphate cytidyltransferase, CPT: choline phosphotransferase. Other metabolic enzymes are mentioned in the text. For simplicity we omitted the PI3K pathway and other lipid kinases and phosphatases that can regulate PI(4,5)P2 levels.

Figure S2: Nir2 influences downstream signals of EGFR and phosphoinositides levels in MCF7 cells. (A) Endogenous Nir2 translocates to the plasma membrane in response to EGF treatment. The localization of Nir2 (red) and the Golgi marker p115 (green) in serum-starved and EGF-induced MCF7 cells are shown by the representative confocal images. Scale bar, 10 μm. **(B)** Nir2 influences downstream signals of EGFR in MCF7 cells. MCF7 cells were infected with the indicated lentiviruses. Three days later the cells were serum starved for 18 hr and then stimulated with EGF (100ng/ml) for the indicated time periods. The effect of Nir2 overexpression (right panels) or its downregulation by shRNA (left panels) on the indicated downstream pathways was assessed by Western blotting using the indicated antibodies. As shown, overexpression of Nir2 enhances AKT phosphorylation, whereas depletion of Nir2 inhibits AKT and ERK1/2 phosphorylation, similar to the results obtained in HeLa cells. **(C)** Nir2 depletion affects PI(3,4,5)P3 production in MCF7 cells in response to EGF. MCF7 cells were infected with either control or Nir2 shRNA and then transfected with an expression vector encoding the PH-AKT-GFP; a PI(3,4,5)P3 reporter. The localization of PH-AKT-GFP was examined in serum starved or EGF-induced infected MCF7 cells. Shown are representative confocal images. Scale bar, 10 μm. **(D)** Nir2 depletion affects PI(4,5)P2 levels in MCF7 cells. MCF7 cells were infected with either control or Nir2 shRNA and then transfected with expression vector encoding PH-PLCδ-GFP; a PI(4,5)P2 reporter. The distribution of PH-PLCδ-

GFP was examined in live cells, and representative confocal images are shown. Scale bar, 10 μm.

Figure S3: Effects of Nir2 shRNAs on EGF-induced ERK1/2 activation, Golgi morphology and membrane trafficking. (A) The different shRNAs of Nir2 efficiently downregulate its expression in HeLa cells. HeLa cells were infected with lentiviruses carrying the indicated shRNAs of Nir2. The expression of Nir2 was examined 5 days later either by Western blotting (left panel) or by IF analysis. As shown, the different shRNAs reduced the expression level of Nir2 by at least 60%. shRNA #1 appears to be the most potent. Yet, it had no influence on Golgi morphology, or the steady state distribution of different Golgi markers (C). Similar results were obtained for the other shRNAs. **(B)** Similar to Nir2 shRNA#1 (Fig. 3B) Nir2 shRNA#2 and shRNA#3 also reduced the phosphorylation of ERK1/2 in response to EGF. **(D)** shRNA#1 of Nir2 has no effect on transport along the secretory pathway as determined by VSV-G transport assay [2]. Control and Nir2-depleted HeLa cells were infected with ts045 strain of VSV at 37°C for 30 min, washed, and incubated at 40°C for 3 hrs. The cells were then shifted to 32°C in the presence of cyclohexamide (100 μg/ml) for the indicated time periods, fixed, and double-immunostained with anti-VSV-G and anti-Grasp65 antibodies. Shown are representative confocal images of tsO45 VSV transport from the ER to the PM at the indicated time points. Scale bar, 10 μm.

Figure S4: The PI-transfer activity of Nir2 and its influence on AKT phosphorylation in HeLa cells. (A) The PLCγ inhibitor U73122 fails to restore the PI(4,5)P2 levels of Synaptojanin-expressing cells. HeLa cells were transfected with Myc-tagged Synaptojanin (kindly provided by Y. Yarden, Weizmann Institute). Twenty-four hours later the cells were either treated with DMSO or with 10 μM U73122 for 30 min, and then fixed, double immunostained with anti-PI(4,5)P2 and anti-Myc antibodies and analyzed by confocal microscope. As seen, synaptojanin-expressing cells were apparently depleted of PI(4,5)P2, and U73122 could not rescue this effect. **(B)** The S164A mutation within the PITD of Nir2 abolishes its PI/PC-transfer activity *in vitro*. His tagged PITPα (kindly provided by Karel Wirtz, Utrecht University), as well as the PITD of wild type Nir2 and its S164A mutant were purified from bacteria on Ni-NTA column and assessed for PI and PC transfer activity *in vitro*. PI transfer activity was measured from [^{3}H]PI-labeled microsomes to unlabeled liposomes, whereas PC transfer activity from [^{14}C]PC-labeled liposomes to unlabeled mitochondria, essentially as previously described [16, 17]. Transfer activities were analyzed in the presence of the indicated purified recombinant proteins at a concentration of 100 μg/ml. Transfer activity is represented as a percentage of

total radiolabeled PI or PC in the assay. Each recombinant protein was analyzed in duplicates in three independent experiments. Shown are mean values ± S.E. As shown, the PI/PC-transfer activity of wild-type Nir2 was very similar to recombinant PITPα. However, the S164A mutation apparently abolished this activity, consistent with the critical role of S166 in PITPα and PITPβ [15, 18]. **(C)** The wild-type Nir2, but neither its ΔPI nor its S164A mutant enhanced AKT phosphorylation in response to EGF. HeLa cells were infected with lentiviruses encoding GFP, Myc-tagged wild-type Nir2, or its ΔPI or S164A mutant. Three days later the cells were serum starved for 18 hr and then stimulated with EGF for the indicated time periods, lysed and immunoblotted with antibodies against the indicated proteins. Shown are representative results of at least four independent experiments. Densitometric analysis of the pAKT(T308) signals obtained from 4 experiments was performed and the mean values ± S.D. are shown in the graph. Note that we often compare the effects of the different proteins on pAKT from similar nitrocellulose membranes in order to reduce the effects of the proteins transfer, exposure etc. We therefore show the influence of the wild type Nir2 once with the S164A mutant and once with the ΔPI mutant.

References

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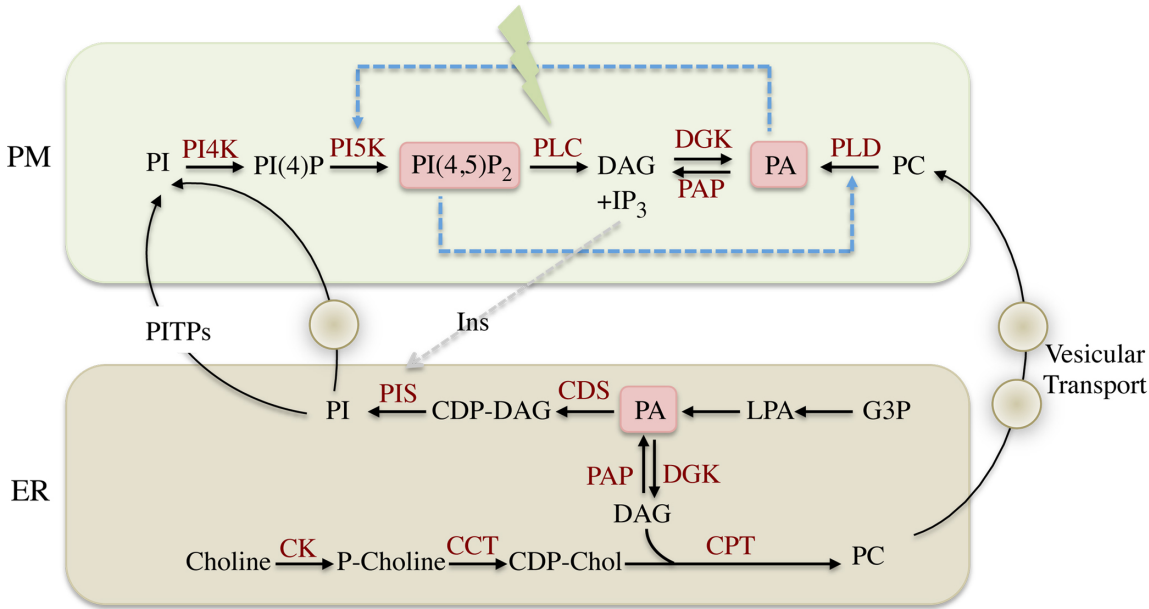
Figure S1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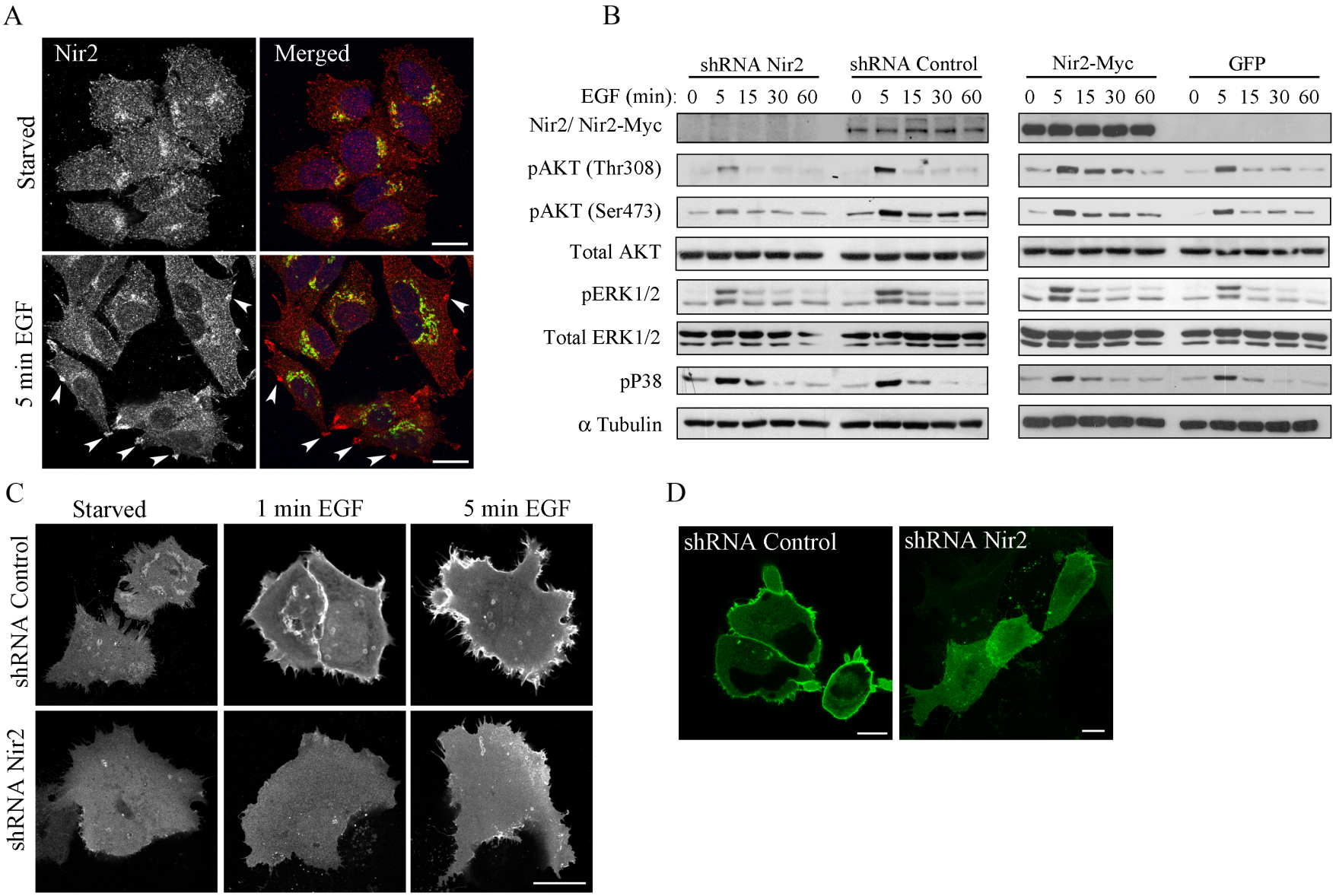
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pERK1/2
Total ERK1/2
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Figure S3

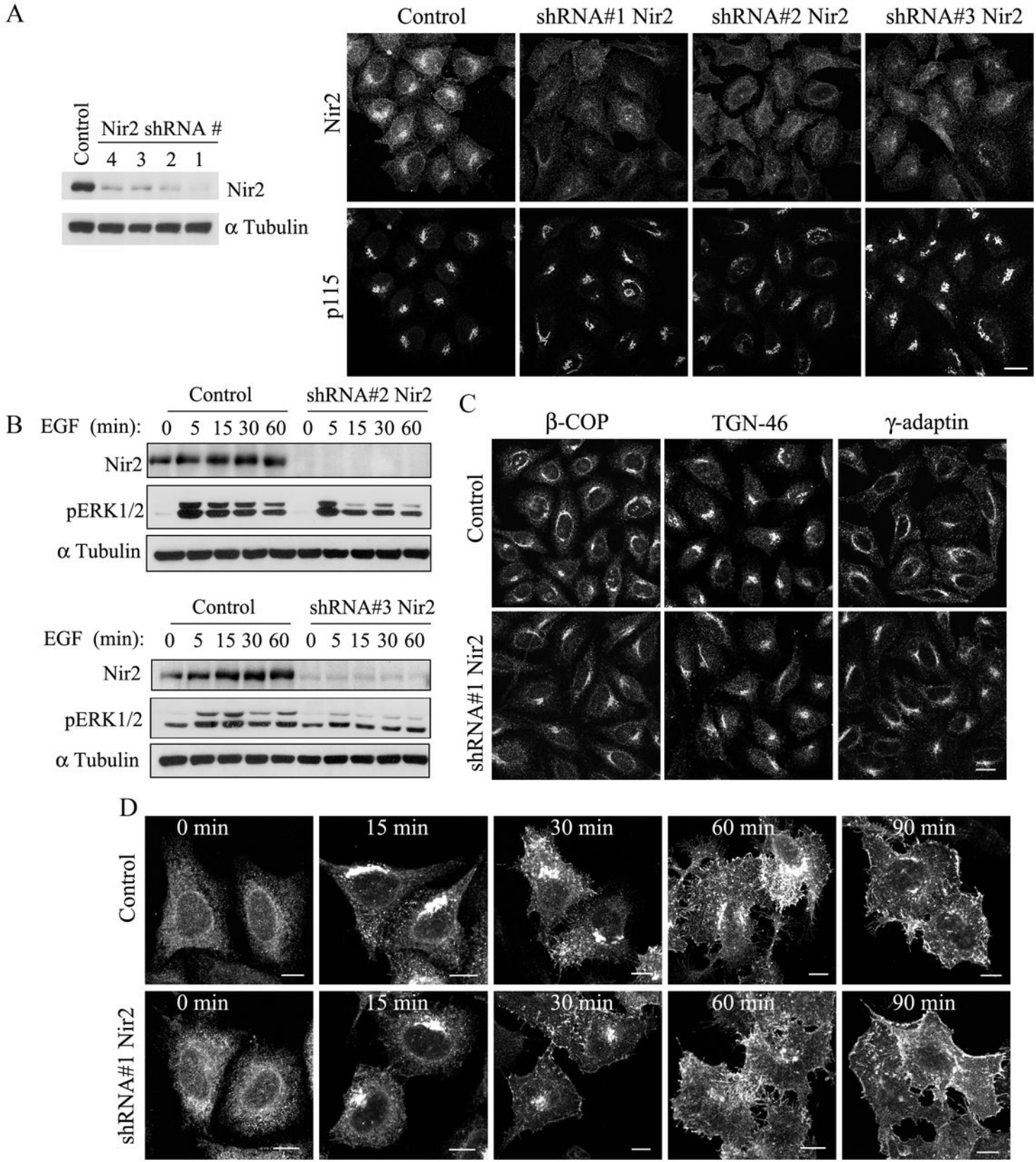
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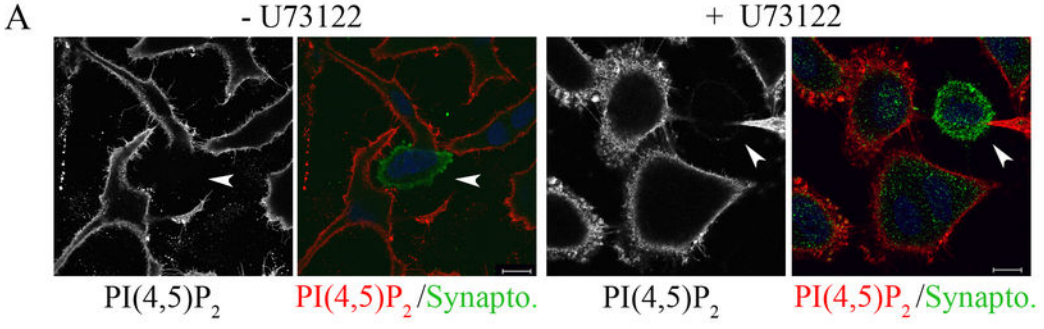
Figure S4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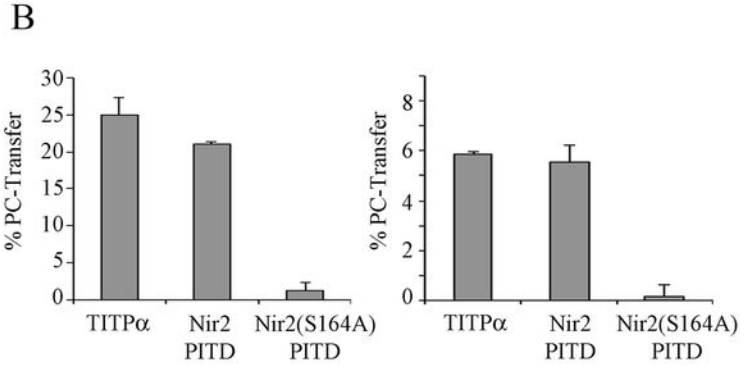
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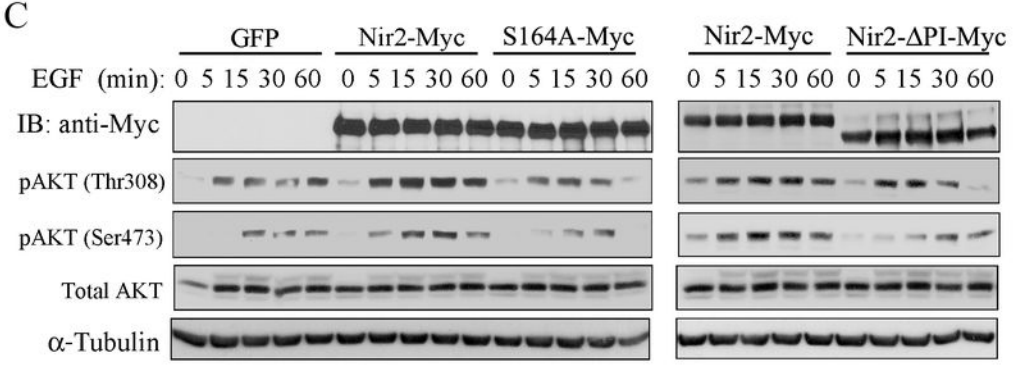
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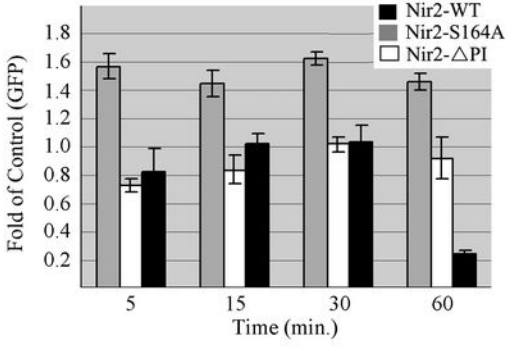
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