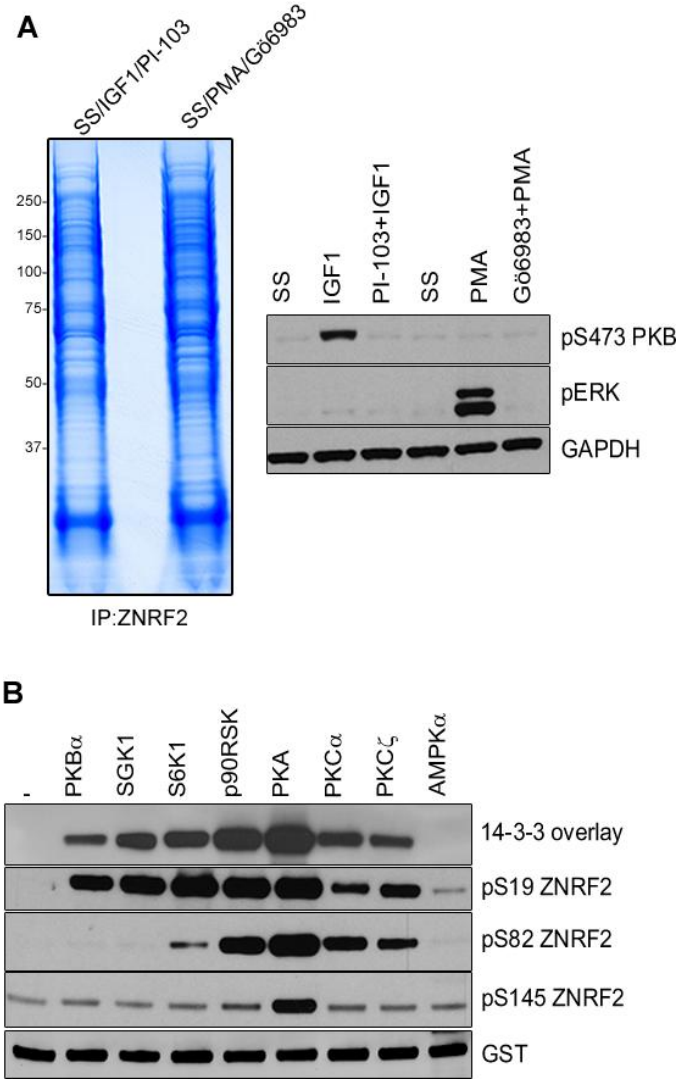
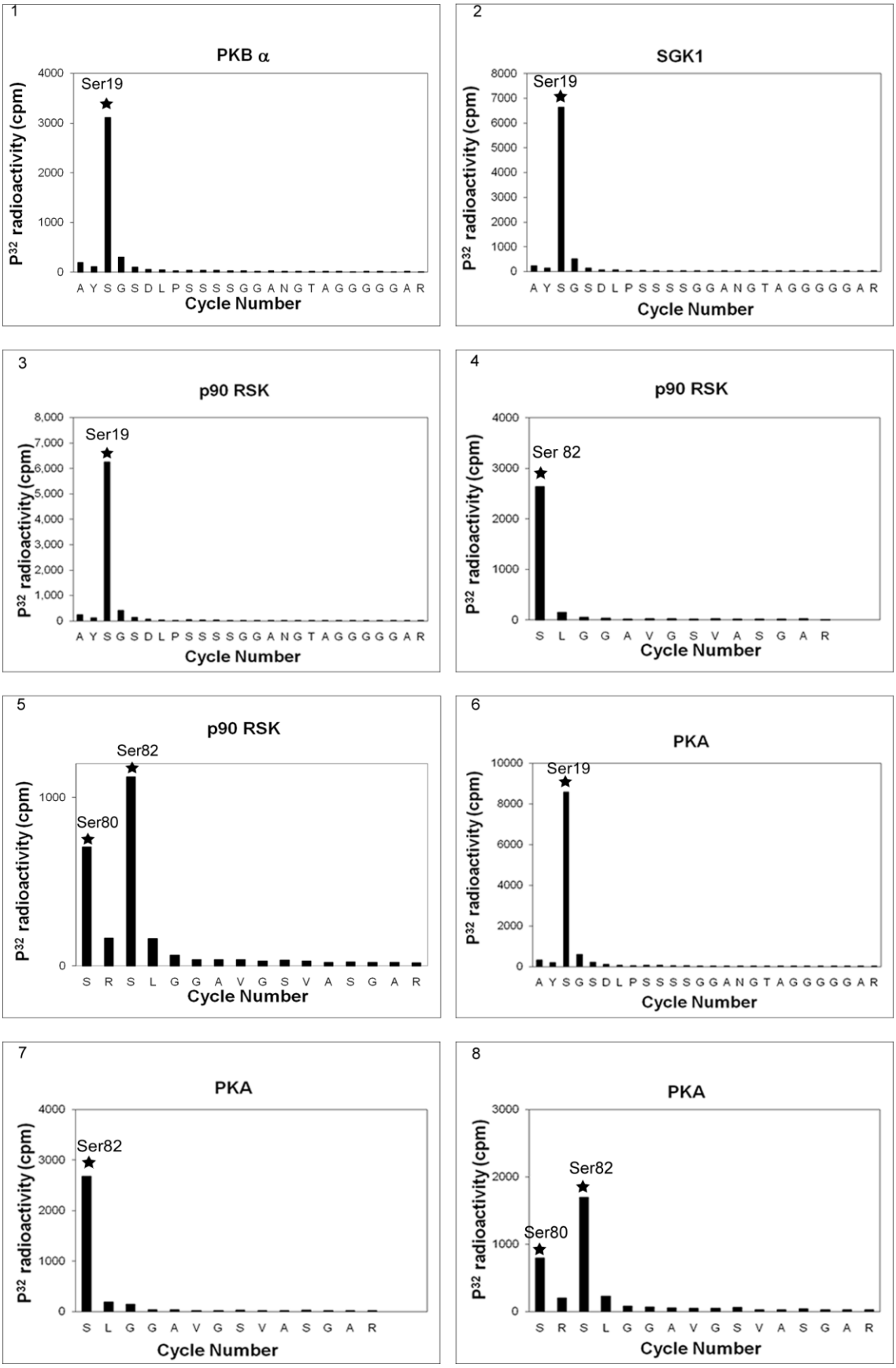


Fig. S1. Identification of residues on ZNRF2 that are phosphorylated *in vivo* and *in vitro.* (**A**) HEK293 cells were cultured for multiple passages in R10K8 (heavy), R6K4 (medium) or R0K0 (light) SILAC medium. Cells were serum starved overnight (12 h) and treated with PI-103/IGF1 or Gö6983/PMA (R10K8-grown cells), IGF1 or PMA (R6K4), and cells in R0K0 were serum starved only (SS). Cells were lysed and 9 mg of lysate from (SS/IGF1/PI-103) or (SS/PMA/Gö6983) were mixed. Endogenous ZNRF2 was immunoprecipitated, reduced, alkylated, and resolved on 4 to 12% Novex SDS-polyacrylamide gels, which were stained with Colloidal Blue. Each entire lane was excised, digested with trypsin as described previously (Dissanayake et al (2011) Biochem. J. 433, 515-525), processed for analysis by Orbitrap MS and phosphopeptides quantified using MaxQuant (version 13.13.10) as in Materials and Methods. The results are presented in Table 1 in the main text as fold ratio. (**B**) Wild-type GST-ZNRF2, expressed and purified from *E. coli*, was phosphorylated *in vitro* using purified constitutively-active PKBα, SGK1, S6K1, RSK1, PKA, PKC isoforms and AMPK. The phosphorylated proteins (100 ng per lane) were analysed by Western blotting using phosphospecific ZNRF2 antibodies (pSer19, pSer82 and pSer145) and for their ability to bind directly to 14-3-3 in a Far-Western overlay assay.

A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PKB α
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P32 radioactivity (cpm)
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Cycle Number
2
SGK1
Ser19
P32 radioactivity (cpm)
A Y S G S D L P S S S S S G G A N G T A G G G G G A R
Cycle Number
3
p90 RSK
Ser19
P32 radioactivity (cpm)
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Cycle Number
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Ser 82
P32 radioactivity (cpm)
S L G G A V G S V A S G A R
Cycle Number
5
p90 RSK
Ser82
Ser80
P32 radioactivity (cpm)
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Cycle Number
6
PKA
Ser19
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Cycle Number
7
PKA
Ser82
P32 radioactivity (cpm)
S L G G A V G S V A S G A R
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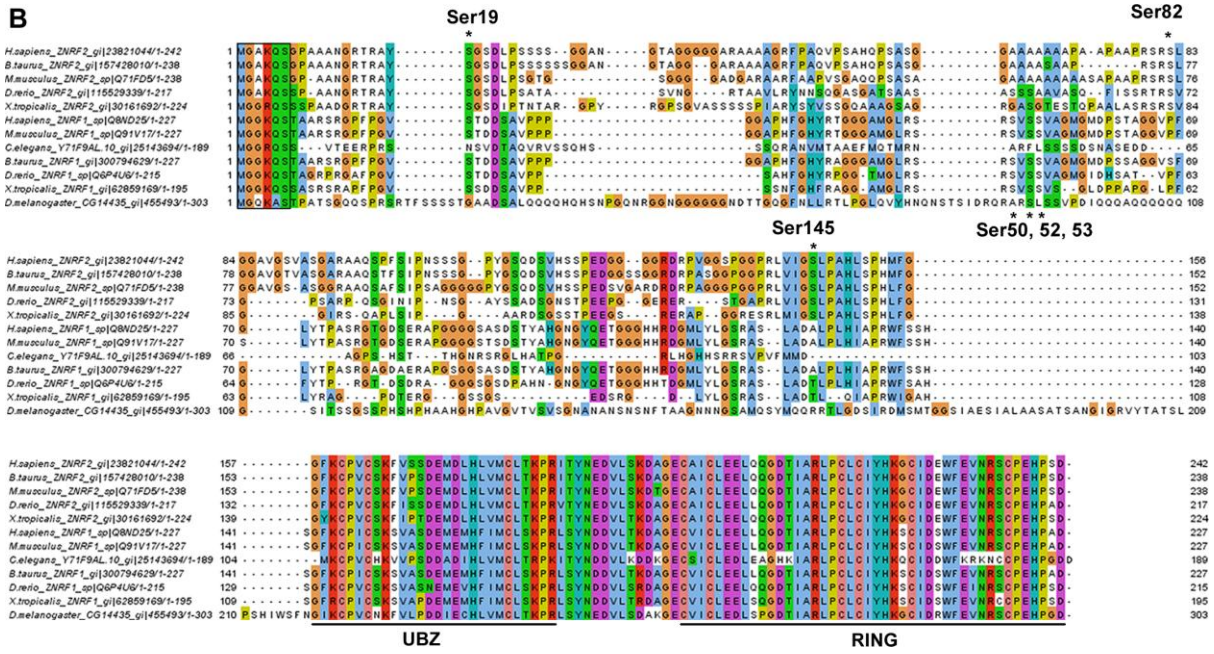


C *In vivo* phosphorylation site mapping for ZNRF2-GFP (Qtrap data)

Treatment	Phosphopeptides	Potential phosphorylated residues
Serum starved	No phosphopeptides	-
IGF1	sLGGAVGSVASGAR SRsLGGAVGSVASGAR LVIGsLPAHLSPHMFGGFK AYSGsDLPSSSSGGANGTAGGGGGAR	Ser82 Ser82 Ser145 Ser19
IGF1 $^{+}$ PI-103	No phosphopeptides	-

D *In vivo* phosphorylation site mapping for ZNRF2-GFP (Orbitrap data)

Treatment	Phosphopeptides	Potential phosphorylated residues
Serum starved	DRPVGGsPGGPR SRSLGGAVGSVASGAR AYSGSDLPSSSSGGANGTAGGGGGAR AAQSPFSIPNSSSGPYGSQDSVHSSPEDGGGGR	Ser135 Ser80/82 Ser19/21 Ser108/Y111
IGF1	DRPVGGsPGGPR sLGGAVGSVASGAR SRSLGGAVGSVASGAR LVIGSLPAHLSPHMFGGFK AYSGSDLPSSSSGGANGTAGGGGGAR AAQSPFSIPNSSSGPYGSQDSVHSSPEDGGGGR	Ser135 Ser82 Ser80/82 Ser145 Ser19/21 Ser108/Y111
IGF1 $^{+}$ PI-103	DRPVGGsPGGPR SRSLGGAVGSVASGAR AYSGSDLPSSSSGGANGTAGGGGGAR AAQSPFSIPNSSSGPYGSQDSVHSSPEDGGGGR ITYNEDVLSKDAGECAICLEELQQGDTIAR	Ser135 Ser80/82 Ser19/21 Ser108/Y111 T186
PMA	DRPVGGsPGGPR sLGGAVGSVASGAR SRSLGGAVGSVASGAR AYSGSDLPSSSSGGANGTAGGGGGAR	Ser135 Ser82 Ser80/82 Ser19/21

	AAQSPFSIPNSSSGPYGSQDSVHSSPEDGGGGR ITYNEDVLSKDAGECAICLEELQQGDTIAR	Ser108/Y111 T186
PMA + Gö6983	DRPVGGsPGGPR SRSLGGAVGSVASGAR AYSGSDLPSSSSGGANGTAGGGGGAR AAQSPFSIPNSSSGPYGSQDSVHSSPEDGGGGR	Ser135 Ser80/82 Ser19/21 Ser108/Y111
forskolin	DRPVGGsPGGPR sLGGAVGSVASGAR SRSLGGAVGSVASGAR AYSGSDLPSSSSGGANGTAGGGGGAR AAQSPFSIPNSSSGPYGSQDSVHSSPEDGGGGR	Ser135 Ser82 Ser80/82 Ser19/21 Ser108/Y111
forskolin + H-89	DRPVGGsPGGPR ITYNEDVLSK sLGGAVGSVASGAR SRSLGGAVGSVASGAR AYSGSDLPSSSSGGANGTAGGGGGAR AAQSPFSIPNSSSGPYGSQDSVHSSPEDGGGGR	Ser135 T186 Ser82 Ser80/82 Ser19/21 Ser108/Y111

E *In vitro* phosphorylation site mapping for ZNRF1-GST (Orbitrap data)

Treatment	Phosphopeptides	Potential Residues
No kinase	No phosphopeptides	-
PKBα	R.SVSSVAGMGMDPSTAGGVPFGLYTPASR.G R.SRSVSSVAGMGMDPSTAGGVPFGLYTPASR.G	Ser50 Ser52 Ser53
PKBβ	R.SVSSVAGMGMDPSTAGGVPFGLYTPASR.G R.SRSVSSVAGMGMDPSTAGGVPFGLYTPASR.G	Ser50 Ser52 Ser53

Fig. S2. HPLC-Edman degradation analysis of *in vitro* phosphorylated of ZNRF2 by PKB*α*, SGK1, p90RSK and PKA; amino acid sequences of ZNRF1, ZNRF2 and related proteins; and phosphorylation sites identified by mass spectrometric analyses.

(A) GST-tagged ZNRF2 was expressed in *E. coli*, purified and subject to *in vitro* phosphorylation by PKBα (A 1), SGK1 (A 2), p90RSK (A 3, A 4 and A 5) and PKA (A 6, A 7 and A 8) in the presence of γ-^{32}P ATP. Phosphorylated ZNRF2 was digested by trypsin, resolved on HPLC and fractions with radioactive label were analyzed by Edman degradation. Ser19 was identified to be phosphorylated by PKBα (A 1), SGK1 (A 2), p90RSK (A 3) and PKA (A 6), while Ser82 was identified to be phosphorylated by RSK1 and PKA (A 4 and A 7). Ser80 might also be phosphorylated by p90RSK and PKA (A 5 and A 8).

(B) The UBZ and RING domains, and N-terminal myristoylation consensus sequences, are conserved in the ZNRF1 and ZNRF2 proteins of the vertebrates, and related singleton proteins of the Ecdysozoa (*Drosophila melanogaster* and *Caenorhabditis elegans*). However, the N-terminal halves of the proteins vary: Ser19, Ser82 and Ser145 are conserved in ZNRF2 of vertebrates, but not the Ecdysozoal proteins. The phosphorylatable Ser50, Ser52 and Ser53 of ZNRF1 are also indicated. Unfortunately, the evolutionary origin of the regulatory sites on ZNRF1 and ZNRF2 cannot be deduced because the only sequence available for the related protein from a basal invertebrate of the chordate phylum is incomplete (F6YWJ3 for *Ciona intestinalis*). (The superphylum classification for these species is outlined in Telford, M.J. and Copley, R.R. (2011). Improving animal phylogenies with genomic data. *Trends Genetics*

27, 186–195). (**C, D, E**) *In vivo* phosphorylation site mapping data for ZNRF2-GFP using Qtrap **(C)** and Orbitrap mass spectrometry **(D)** and *in vitro* phosphorylation site mapping for ZNRF1-GST using Orbitrap mass spectrometry **(E)**. Lower case '**s**' indicates phosphorylated residues that could be assigned definitively, and underlines are used where there is uncertainty about which are the phosphorylated residues.

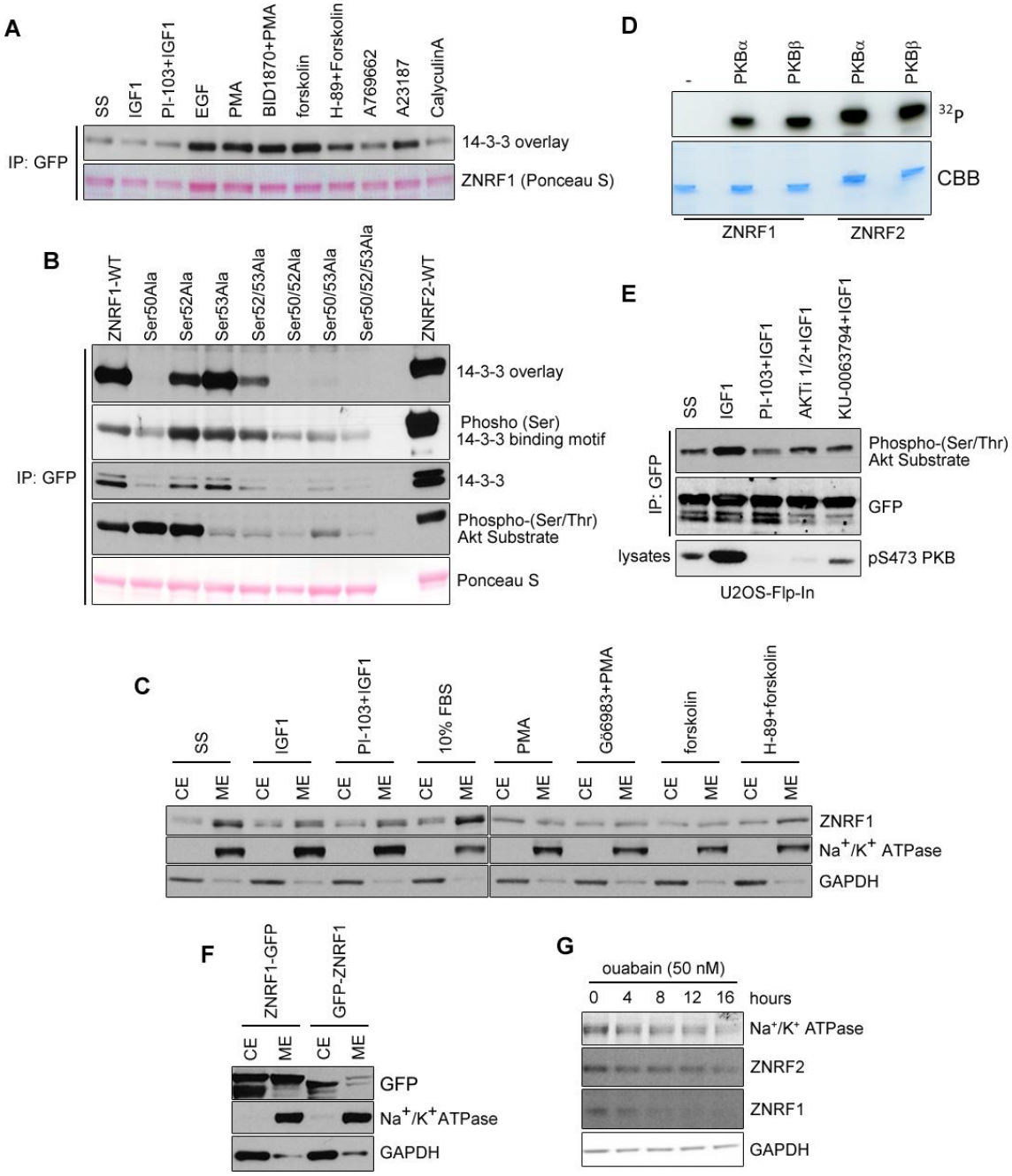
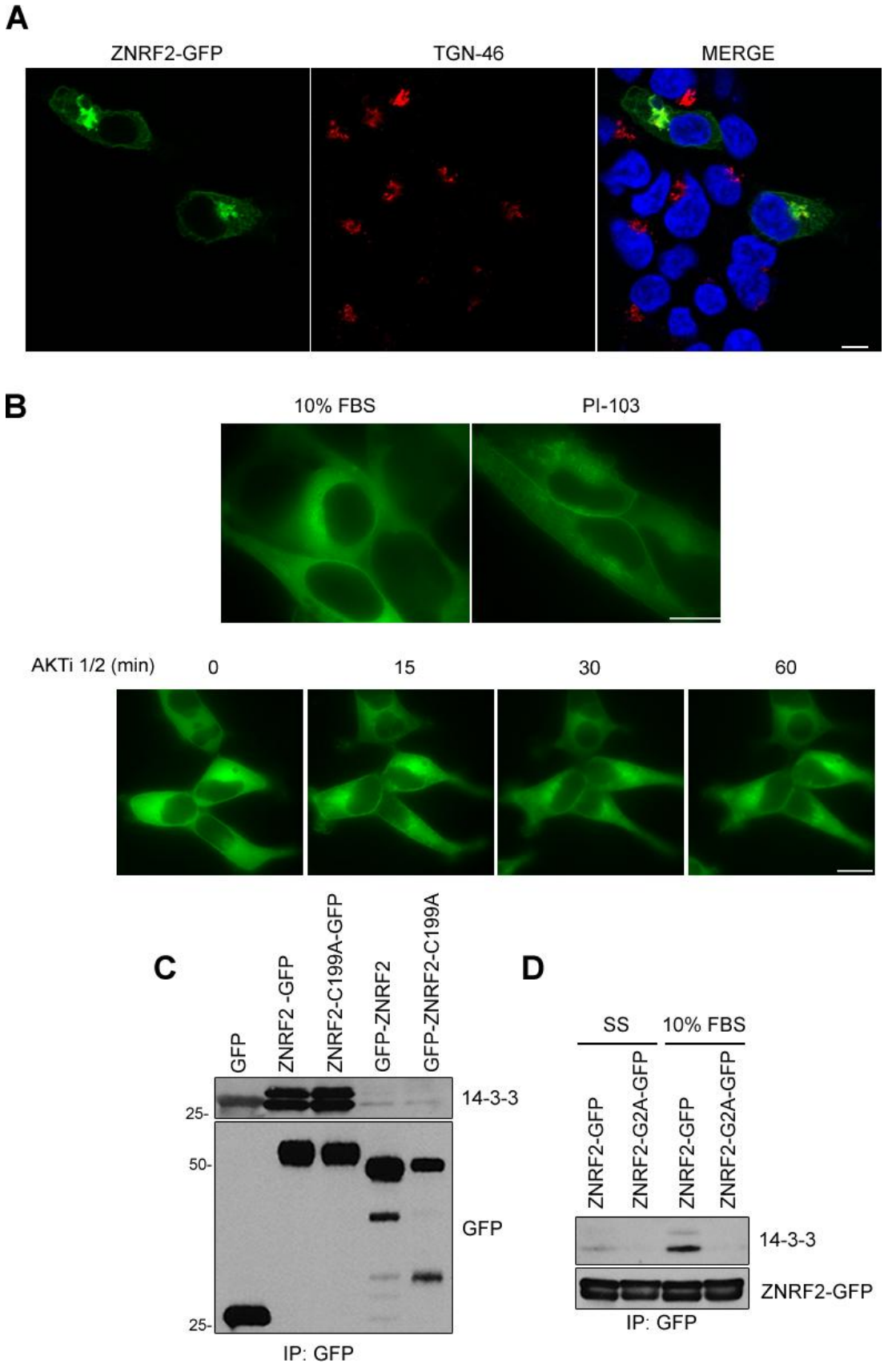


Fig. S3. Phosphorylation, 14-3-3 binding and membranal localization of ZNRF1.
(A) ZNRF1-GFP isolated from transfected HEK293 cells exposed to various stimuli/inhibitor combinations was tested for binding to 14-3-3 in a Far-Western overlay.

(B) Wild-type ZNRF1-GFP or single/double/triple serine to alanine mutants were isolated from transfected HEK293 cells grown in 10% FBS. ZNRF2-GFP was used as positive control. The proteins were tested for binding to 14-3-3 in a 14-3-3 Far-Western, phospho-(Ser) 14-3-3 binding motif antibody (Cell Signaling 9601), and for co-immunoprecipitating endogenous 14-3-3s (K19, Santa Cruz). Given the proximity of the three potential phosphorylated sites (Ser50, 52 and 53) the phospho(Ser/Thr)-Akt substrate (PAS) antibody (Cell Signaling 9611) was used to test if the mutated sites resembled Akt/PKB substrate sites. The data indicate that the 14-3-3 and PAS antibody have different phosphorylated determinants on ZNRF1.
(C) HEK293 cells exposed to various stimuli/inhibitor combinations were fractionated into cytosol extracts and membrane extracts by ultracentrifugation as described in Materials and Methods. Cell lysates were subjected to Western blotting with the indicated antibodies.
(D) GST-tagged ZNRF1 and ZNRF2 (positive control) were expressed and purified from *E. coli* and subjected to *in vitro* phosphorylation by PKBα and PKBβ in the presence of 100 μM γ-^{32}P ATP in a 50 μl reaction at 30^{o}C for 30 min. Samples were subjected to SDS-PAGE followed by colloidal Coomassie Brilliant Blue (CBB) staining and autoradiography. Phosphorylated residues were identified in a parallel set of non-radioactive proteins using mass spectrometric analyses (Supplementary S1E).
(E) ZNRF1-GFP isolated from U2OS Flp-In cells (stably expressing ZNRF1) stimulated with IGF1 in the presence or absence of the PI 3-kinase inhibitor (PI-103), PKB inhibitor (AKTi1/2) and mTOR kinase inhibitor (Ku-0063794) was tested for regulation of phosphorylation by Akt using PAS antibody. Phospho-Ser473-PKB was used as a control for the stimulation used. These data indicate the IGF1 stimulates phosphorylation of ZNRF1 at site(s) (most likely Ser53) that does not lead to 14-3-3 binding (compare with (A)), though the regulation of each residue will have to be tracked individually.
(F) HEK293-Flp-In-Trex cells stably expressing ZNRF1-GFP (N-myristoylated) and GFP-ZNRF1 (not N-myristoylated) were subjected to subcellular fractionation to generate membrane (ME) and soluble (CE) fractions as described in Materials and Methods. Cell fractions were subjected to Western blotting for GFP, Na^+/K^+ATPase α1 and GAPDH.
(G) HeLa cells were treated with 50 nM ouabain for the indicated times. Cell lysates were prepared in modified RIPA buffer (see Materials and Methods). The levels of Na^+/K^+ATPase α 1, ZNRF2, ZNRF1 and control GAPDH were analysed by Western blotting.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ZNRF2-GFP
TGN-46
MERGE
B
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PI-103
AKTi 1/2 (min)
0
15
30
60
C
GFP
ZNRF2 -GFP
ZNRF2-C199A-GFP
GFP-ZNRF2
GFP-ZNRF2-C199A
14-3-3
25-
50-
GFP
25-
IP: GFP
D
SS
10% FBS
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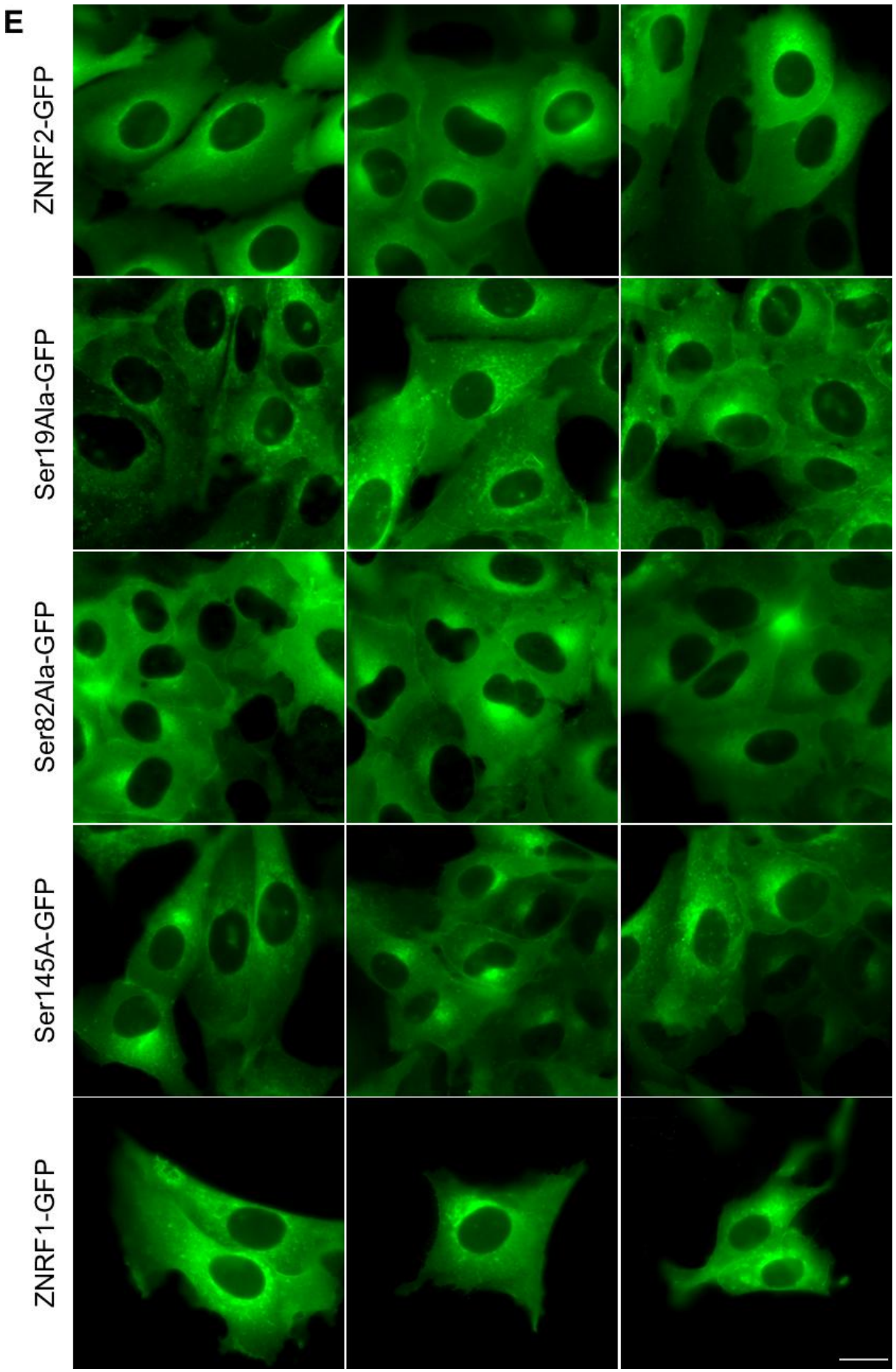
E
ZNRF2-GFP
Ser19Ala-GFP
Ser82Ala-GFP
Ser145A-GFP
ZNRF1-GFP

Fig. S4. Reversible membrane-to-cytosol translocation of ZNRF2.
(A) ZNRF2-GFP (green) was expressed in HEK293 cells. Cells were fixed and permeabilized as described in Materials and Methods, and co-stained with TGN46 antibody (Abcam ab16052) (Golgi apparatus marker) and DAPI (4,6-diamidino-2-phenylindole, Invitrogen) (nuclear stain). Bar, 10 µm.
(B) HEK293-Flp-In-TRex cells stably expressing ZNRF2-GFP were used for live cell imaging. Cells were treated with 1 µM PI-103 or 10 µM (AKTi-1/2) for 60 min. Bar, 10 µm.
(C) Extracts of cells stably expressing GFP alone, ZNRF2-GFP and ligase dead ZNRF2-C199A-GFP (C-terminal tag), and GFP-ZNRF2 and ligase dead GFP-ZNRF2-C199A-GFP (N-terminal tag and no N-myristoylation) were subjected to immunoprecipitation with GFP-Trap® beads and precipitates were subject to SDS-PAGE and Western blotting to detect GFP and co-precipitating 14-3-3s (K19 antibody). Molecular masses of markers are indicated (kDa).
(D) Wild-type and G2A mutated (non-myristoylated) ZNRF2-GFP (C-terminal tag) were isolated from stably transfected HEK293 cells, which had been serum starved or grown in 10% FBS. The ZNRF2-GFP proteins were tested for co-precipitation of cellular 14-3-3s using the K19 antibody. [This experiment reaches similar conclusions to those reached in Fig. 8 D, though the latter shows data for transiently transfected cells for which the G2A-ZNRF2 mutant is generally expressed at higher levels than the wild-type protein].
(E) U2OS-Flp-In cells stably expressing ZNRF1-GFP, ZNRF2-GFP or the indicated mutants were mounted in a temperature-controlled chamber (37^{o}C) for live-cell imaging. Bar, 10 µm.

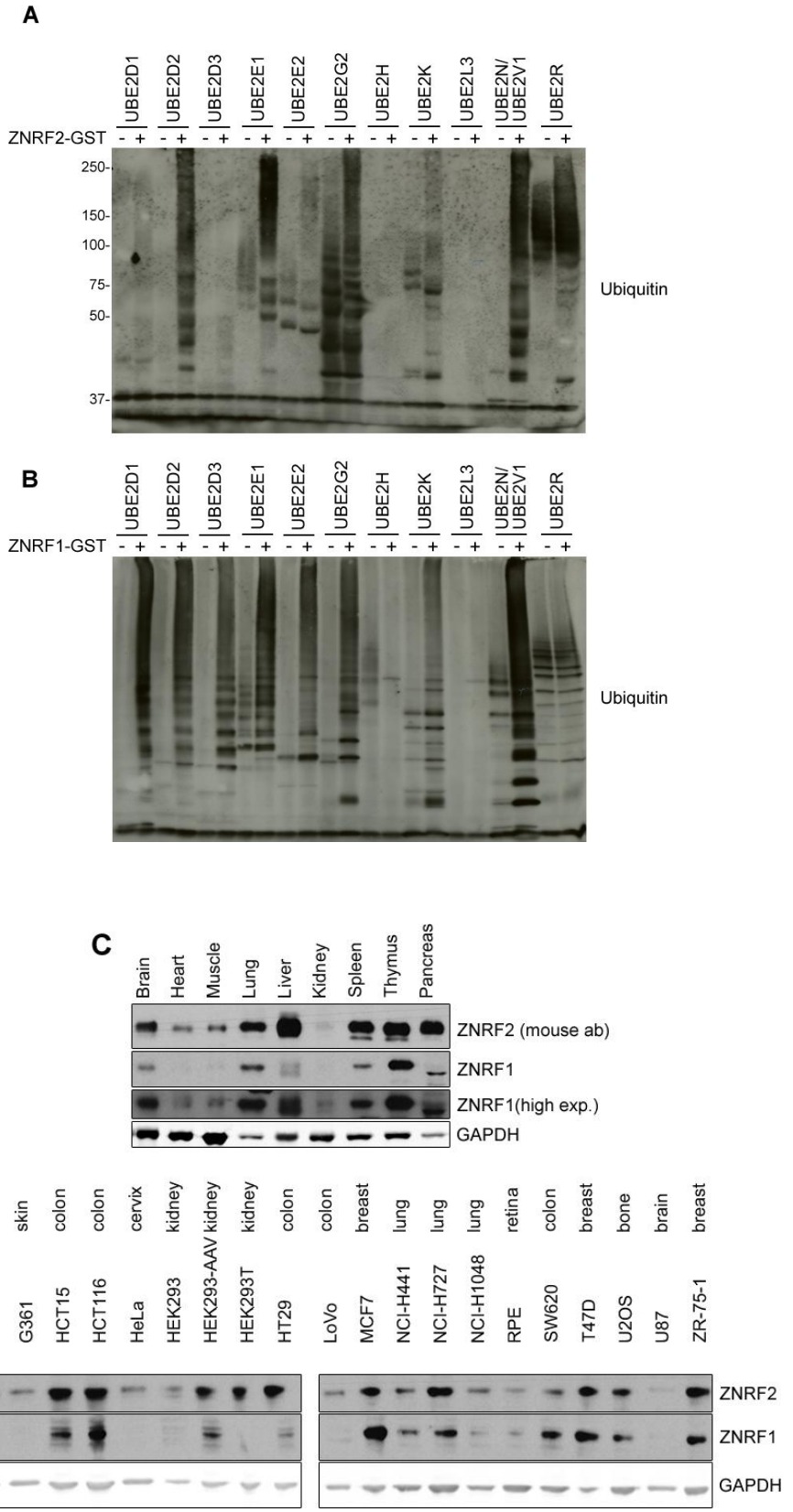

Fig. S5. Activities of ZNRF2 and ZNRF1 with a variety of E2 enzymes. and their expression of in tissue lysates and in various cell lines. (A) GST-ZNRF2 (0.5 µg) and **(B)** GST-ZNRF1 (0.5 µg) were incubated with 2.5 µg ubiquitin, 0.1 µg E1, 0.3 µg E2 for 2 h at 37°C. Reaction products were analyzed by Western blotting using an ubiquitin antibody. **(C and D)** Expression of ZNRF1 and ZNRF2 in tissue lysates and in various cell lines was

detected with a ZNRF2 antibody raised against bacterially-expressed GST-ZNRF2 (mouse) in sheep S098D or bacterially-expressed GST-ZNRF2 (human) in sheep S617C. ZNRF1 antibody was raised against bacterially-expressed GST-ZNRF1 (human) in sheep S219D.

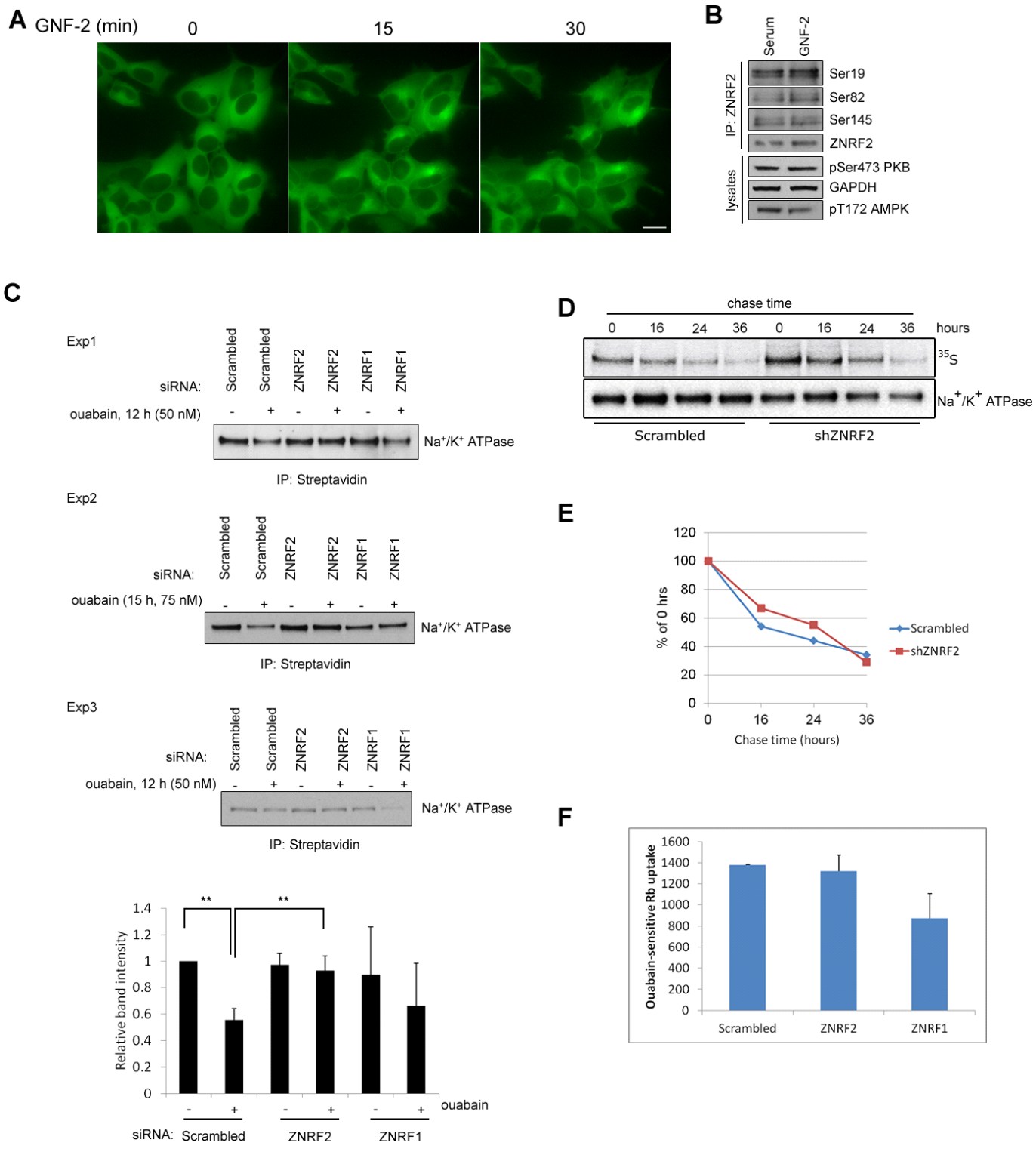

Fig. S6. ZNRF2 is targeted to membranes upon treatment of cells with myristoyl-binding pocket-interacting drug GNF-2, and effects of knockdowns of ZNRF1 and ZNRF2.

(A) HEK293-Flp-In-TRex cells stably expressing ZNRF2-GFP in a tetracycline inducible manner were mounted in a heated chamber for live-cell fluorescent imaging. Before treatment with 10 µM GNF-2, cells were visualized in complete media (10% FBS). **(B)** Phosphorylation status of ZNRF2 isolated from cells with and without 10 µM GNF-2 for 1 h. Bar, 10 µm. **(C)** Signals from Figure 8B and two other similar experiments with ouabain concentrations from 50 to 70 nM for 12 to 15 h, were quantitated using the Image J program and used for the graph that is also shown as Figure 8C. A student's t-test showed that the differences indicated are statistically significant, $p<0.005$. (**D**) HeLa cells were infected with control (Scrambled) or ZNRF2 lentiviral shRNA particles for 12 h and selected for 3 days in media containing pyromycin. Cells plated in 10-cm dishes were depleted from methionine/cysteine for 60 min at 37°C and pulse-labeled for 120 min with 0.2 mCi/ml of Expre^{35}S^{35}S Protein labeling mix and chased in DMEM containing 5 mmol/l unlabeled methionine and 5 mmol/l unlabeled cysteine for the indicated times. Cells were lysed and endogenous Na^+/K^+ATPase α1 was immunoprecipitated. The bound [*S35*]-labeled Na^+/K^+ATPase α1 was detected using a *phosphorimager* screen for 24 h. A small amount of the same immunoprecipitates were also analyzed by blotting against Na^+/K^+ATPase α1. (**E**) Graphical representation of data in (D). The 100% values in the experiment shown are 6.67 (arbitrary 35S/protein units; scrambled) and 13.1 (ZNRF2) knockdown. (**F**) HeLa cells transfected with control (scrambled) or ZNRF2 siRNA were plated in 12-well plates. ^{86}Rb uptake assay was performed on the cells 56 h post-transfection. Culture media was removed from the wells and replaced with media in the presence or absence of 1 mM ouabain for 15 min. Cells were then incubated with identical uptake medium containing 1 µCi/ml ^{86}Rb for 15 min. After this period, cells were rapidly washed thrice with ice-cold PBS, lysed in 700 µl ice-cold RIPA buffer and ^{86}Rb-uptake quantified by Cherenkov counting. The values presented are the counts per minute normalized to protein concentration. *P-values* were >0.05.