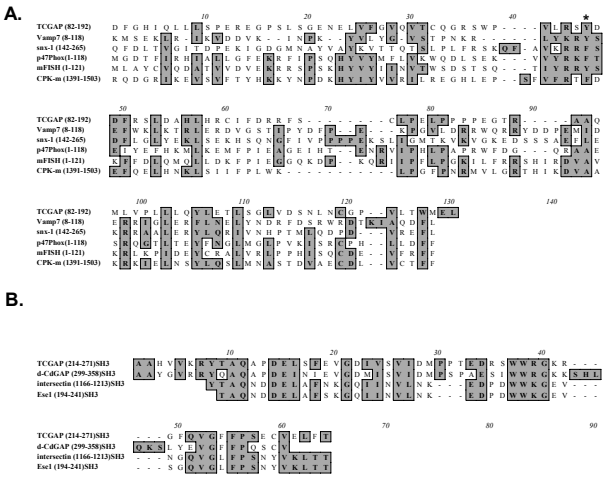
SUPPLEMENTARY DATA

1. Characterization of TCGAP

To search for the human ortholog of TCGAP, we performed a BLAST search of the non-redundant and human EST (dbEST) databanks with the mouse TCGAP cDNA sequence. A human cosmid F25965 (AC002398) located on chromosome 19q13.1 between D19S208 and COX7A1 was predicted to encode a hypothetical protein with strong similarity to mouse TCGAP. Moreover, a human full-length TCGAP cDNA, sorting nexin 26 (snx 26), was identified from a databank search for PX domain-containing proteins (Teasdale et al., 2001). Alignment of the human snx 26 and mouse TCGAP proteins showed an overall 89% identity. The two sequences are almost identical in the PX, SH3 and GAP domains and the C-terminal proline-rich regions.

The PX domain (residues 84-193) was originally identified as a conserved region in the NADPH oxidase family of proteins. Recently, a series of non-phox family proteins were also found to contain this domain (Ago et al., 2001; Bravo et al., 2001; Hiroaki et al., 2001; Ponting, 1996; Sato et al., 1998). Sequence alignments of PX domains showed that TCGAP contains all of the consensus amino acids common to other PX domain-containing proteins (supplementary data 1A). Amino acid residues 213-271 encompassing the SH3 domain shared the highest homology with the SH3 domains of Drosophila CdGAPr, and the endocytotic proteins intersectin and Ese1 (Okamoto et al., 1999; Sengar et al., 1999) (Supplementary data 1B).

Supplementary data 1

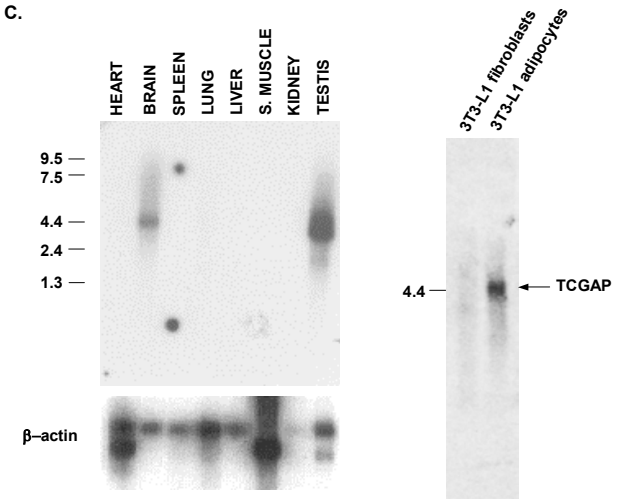


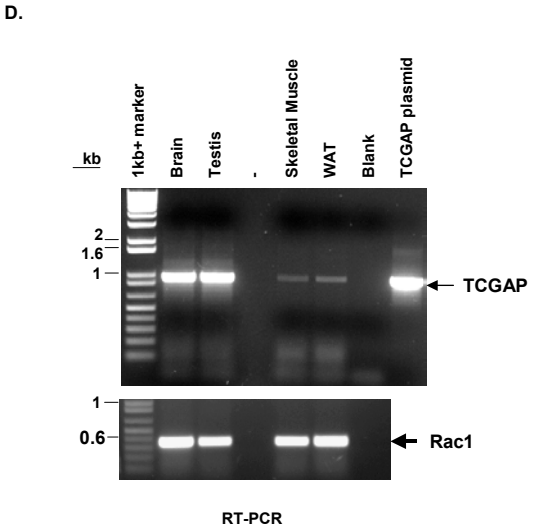
Supplementary data 1 (A) Alignment of TCGAP PX domain with other identified PX domains. (B) Alignment of the predicted TCGAP SH3 domain with other similar and identified SH3 domains. Identical residues are boxed in black; dashes indicate where space was added in the sequence to achieve optimal alignments.

The tissue distribution of TCGAP mRNA was determined using a mouse multiple-tissue Northern blot hybridized with a radiolabeled probe containing the 3' 1.5kb of the TCGAP cDNA. A major 4.5kb transcript of TCGAP was found predominantly in brain and testis (Supplementary data 1C). A Northern blot with identical amounts of total RNA from 3T3-L1 fibroblasts and fully differentiated adipocytes revealed that TCGAP mRNA levels were significantly increased in differentiated adipocytes. RT-PCR was also performed with tissue RNA from skeletal muscle and white adipose tissue, and compared to brain and testis as control. Consistent with the results from the multiple tissue

Northern blots; TCGAP was highly expressed in brain and testis, and also expressed in white adipose tissue and muscle at a lower level (supplement 1D).

Supplementary data 1.

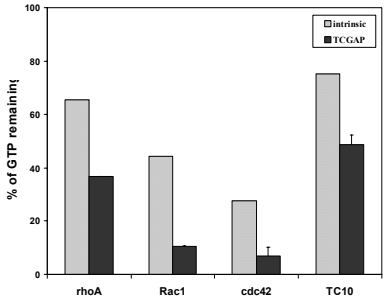




Supplementary data 1 (C) Mouse multiple tissue Northern blot (Clontech) and a blot with same amount of total RNA from 3T3-L1 fibroblasts and day 10 adipocytes hybridized with a ^{32}P-labelled 3' end 1.5 kb TCGAP cDNA probe. (D) Total cellular RNA was purified from brain, testis, skeletal muscle and white adipose tissue (WAT) using the TRIzol reagent (Invitrogen). To examine TCGAP expression, cDNA were amplified with PCR, with the gene specific primers. Rac1 was amplified as control. PCR products were visualized by agarose gel electrophoresis. It showed that TCGAP highly expressed in brain and testis, and also expressed in WAT and muscle in a low level.

2. GAP activity of a GST-TCGAP GAP domain fusion protein *in vitro.*

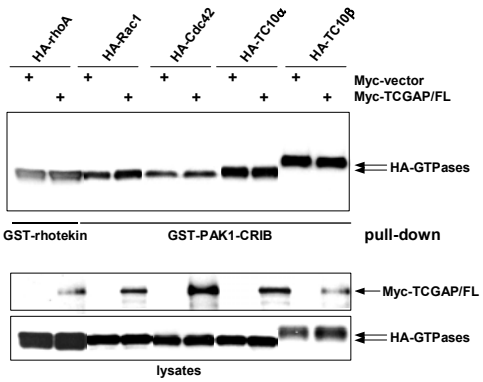
Supplementary data 2



Supplementary data 2. Identical amounts of purified GST-TCGAP GAP domain were incubated for 20 minutes with Rho family GTPases preloaded with [γ-^{32}P]GTP. The remaining GTPase-bound radioactive GTP was determined. Remaining GTP in control without addition of TCGAP at time 0 was set at 100%. It showed the TCGAP/GAP domain stimulated the intrinsic GTPase activity of Cdc42 and Rac1, and was less active on RhoA and TC10.

3. Lack of GAP activity of Myc-TCGAP *in vivo*.

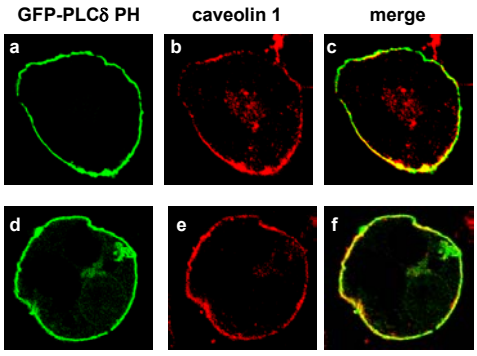
Supplementary data 3



Supplementary data 3. 0.1 μg of HA-tagged wild-type RhoA, Rac1, cdc42, TC10α or TC10β in a combination with 2 μg of Myc-tagged vector control or full-length TCGAP were transfected in Cos-1 cells in the presence of serum. 24 hrs after transfection, cells were collected and lysed in NP40 lysis buffer. About 20μg of cell lysates were incubated with 15 μg of GST-rhotekin conjugated beads (Upstate Biotech.) for HA-RhoA transfected samples or 5 μg of GST-PAK1-CRIB conjugated beads (Cytoskeleton Inc.) for the rest of the transfected samples. Bound lysates were subject to electrophoresis and immunoblotting with anti-HA monoclonal antibody. Lysates were immunoblotted with anti-HA and anti-Myc monoclonal antibody to determine the expression levels. The overexpression of full-length TCGAP did not influence the GTP-bound fraction of any GTPases, compared to vector control.

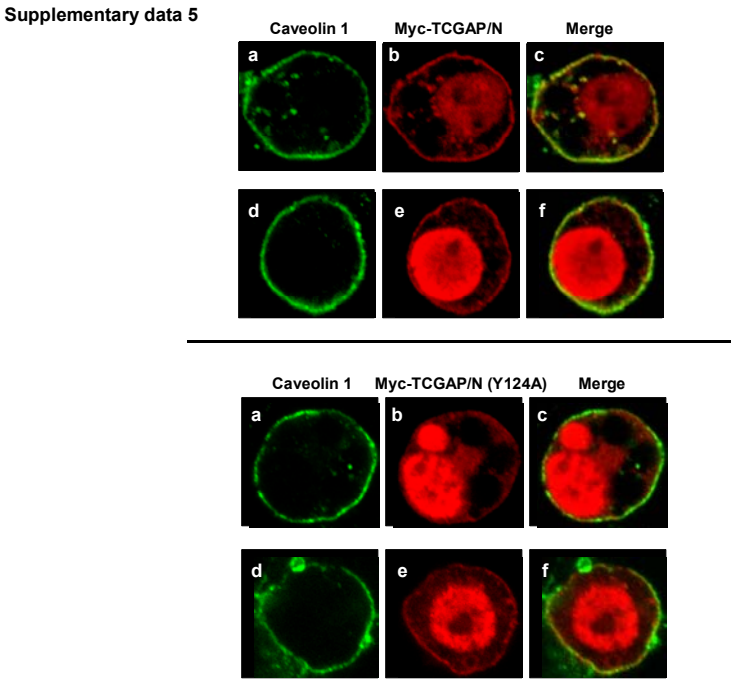
4. Localization PtdIns(4,5)P_2 at the plasma membrane in adipocytes.

Supplementary data 4



Supplementary data 4. 3T3L1 adipocytes were electroporated with GFP-PLCδ/PH domain. After 48 hrs, cells were starved and treated without (a,b,c) or with 100nM insulin (d,e,f) for 5 min, followed by co-immunostaining with anti-caveolin 1 polyclonal antibody. Confocal microscopy was used to detect GFP-PLCδ/PH domain (green) (a and d) and endogenous caveolin 1 (red) (b and e). Overlapping images are shown in c and f. GFP-PLCδPH domain exclusively localized at the plasma membrane, indicating the membrane localization of PtdIns(4,5)P_2 in adipocytes. In addition, the localization of this phospholipid was affected by exposure of the cells to insulin.

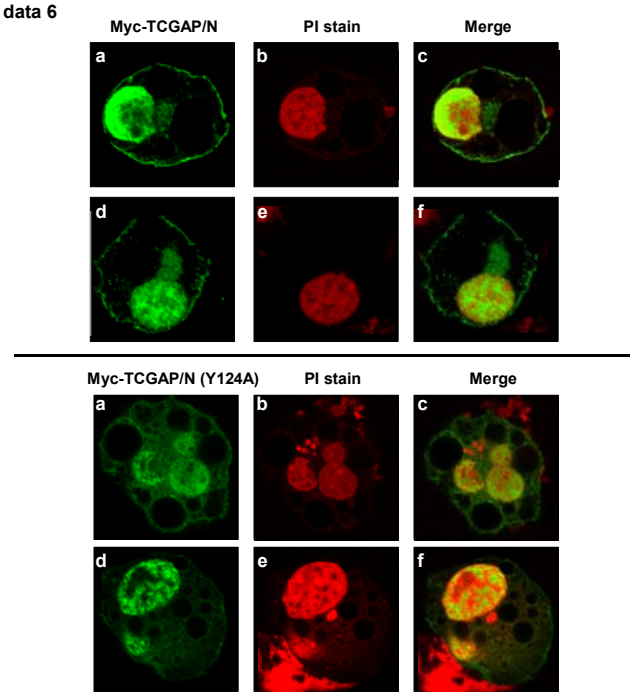
5. TCGAP N-PX mutant $Y^{124}A$ abolished the plasma membrane localization.



Supplementary data 5. 3T3L1 adipocytes were electroporated with Myc-TCGAP/N wild type (top panel) or $Y^{124}A$ mutant (lower panel). After 48 hrs, cells were starved and treated without (a,b,c) or with 100nM insulin (d,e,f) for 5 min, followed by co-immunostaining with anti-caveolin polyclonal antibody and anti-Myc monoclonal antibody. Confocal microscopy was used to detect endogenous caveolin 1 (green) (a and d) and transfected Myc-TCGAP (red) (b and e). Overlapping images are shown in c and f. It showed TCGAP wild type N-terminal fragment costained with caveolin 1, at the plasma membrane. The plasma membrane staining of TCGAP N-terminal fragment is not changed in response to insulin. Y124A mutant significantly lost the plasma membrane staining compared to wild type.

6. TCGAP N-PX mutant $Y^{124}A$ still localized in nucleus.

Supplementary data 6



Supplementary data 6. 3T3L1 adipocytes were electroporated with Myc-TCGAP/N wild type (top panel) or $Y^{124}A$ mutant (lower panel). After 48 hrs, cells were starved and treated without (a,b,c) or with 100nM insulin (d,e,f) for 5 min, followed by co-immunostaining anti-Myc monoclonal antibody and propidium iodide. Confocal microscopy was used to detect Myc-TCGAP/N (green) (a and d) and nucleus by propidium iodide stain (red) (b and e). Overlapping images are shown in c and f. It showed the nucleus staining of TCGAP/N was not affected by this $Y^{124}A$ mutation, suggesting the existence of a nuclear targeting signal other than PX domain in the N-terminal fragment.

References

Ago, T., Takeya, R., Hiroaki, H., Kuribayashi, F., Ito, T., Kohda, D. and Sumimoto, H. (2001) The PX domain as a novel phosphoinositide- binding module. *Biochem Biophys Res Commun*, **287**, 733-738.

Bravo, J., Karathanassis, D., Pacold, C.M., Pacold, M.E., Ellson, C.D., Anderson, K.E., Butler, P.J., Lavenir, I., Perisic, O., Hawkins, P.T., Stephens, L. and Williams, R.L. (2001) The Crystal Structure of the PX Domain from p40(phox) Bound to Phosphatidylinositol 3-Phosphate. *Mol Cell*, **8**, 829-839.

Hiroaki, H., Ago, T., Ito, T., Sumimoto, H. and Kohda, D. (2001) Solution structure of the PX domain, a target of the SH3 domain. *Nat Struct Biol*, **8**, 526-530.

Okamoto, M., Schoch, S. and Sudhof, T.C. (1999) EHSH1/intersectin, a protein that contains EH and SH3 domains and binds to dynamin and SNAP-25. A protein connection between exocytosis and endocytosis? *J Biol Chem*, **274**, 18446-18454.

Ponting, C.P. (1996) Novel domains in NADPH oxidase subunits, sorting nexins, and PtdIns 3-kinases: binding partners of SH3 domains? *Protein Sci*, **5**, 2353-2357.

Sato, T.K., Darsow, T. and Emr, S.D. (1998) Vam7p, a SNAP-25-like molecule, and Vam3p, a syntaxin homolog, function together in yeast vacuolar protein trafficking. *Mol Cell Biol*, **18**, 5308-5319.

Sengar, A.S., Wang, W., Bishay, J., Cohen, S. and Egan, S.E. (1999) The EH and SH3 domain Ese proteins regulate endocytosis by linking to dynamin and Eps15. *Embo J*, **18**, 1159-1171.

Teasdale, R.D., Loci, D., Houghton, F., Karlsson, L. and Gleeson, P.A. (2001) A large family of endosome-localized proteins related to sorting nexin 1. *Biochem J*, **358**, 7-16.