Supporting Information

Gao et al. 10.1073/pnas.1019386108

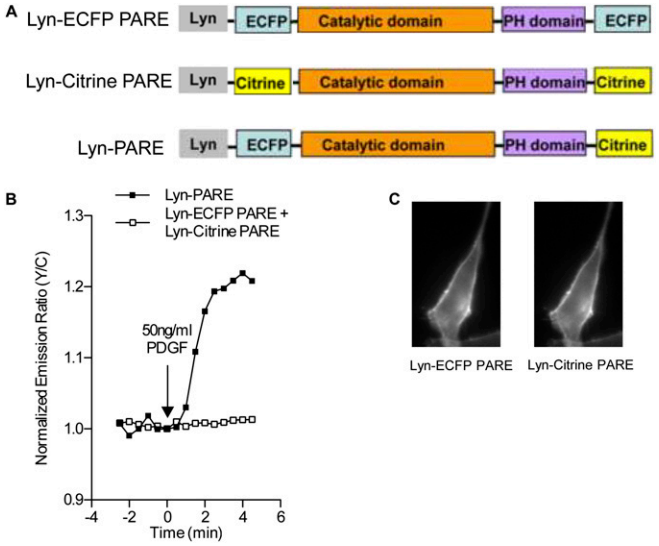

Fig. S1. Intermolecular FRET does not contribute to response of Lyn-PARE. (*A*) Lyn-ECFP PARE and Lyn-Citrine PARE were generated by sandwiching full-length PDK1 between two ECFP proteins and two Citrine proteins, respectively. The constructs were then targeted to membrane rafts by using the targeting motif from Lyn kinase, as described for Lyn-PARE. (*B*) Representative emission ratio time courses of serum-starved NIH 3T3 cells expressing Lyn-PARE ($n = 4$) or coexpressing Lyn-ECFP PARE and Lyn-Citrine PARE ($n = 4$), stimulated with 50ng/mL PDGF. No response was observed in cells expressing Lyn-ECFP PARE and Lyn-Citrine PARE. This data supports the idea that the response of Lyn-PARE results from a conformational change of PDK1 that occurs upon activation of the kinase rather than a $PI(3,4,5)P_3$-induced clustering of the kinase. (*C*) Representative images of cells coexpressing Lyn-ECFP PARE and Lyn-Citrine PARE.

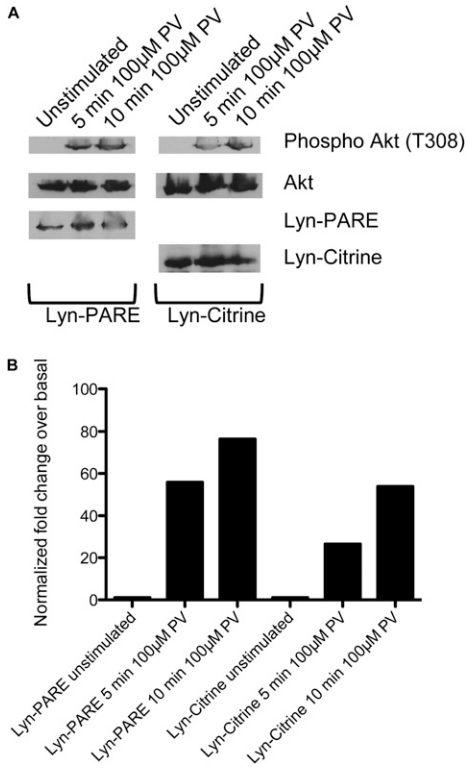

Fig. S2. Ratiometric FRET change of Lyn-PARE reflects PDK1/Lyn-PARE activation. (*A*) Upon stimulation, Lyn-PARE phosphorylates Akt T308 in a time-dependent manner. NIH 3T3 cells were transfected with Lyn-PARE or Lyn-Citrine and serum-starved for 24 h. Cells were then either left untreated or stimulated with 100 μM PV for 5 or 10 min, followed by immediate lysis. Phosphorylation of Akt at T308 is used as readout of PDK1/Lyn-PARE activation. (*B*) Quantification of Lyn-PARE and Lyn-Citrine time courses relative to unstimulated samples. Each condition was normalized to the amount of Akt in the sample.

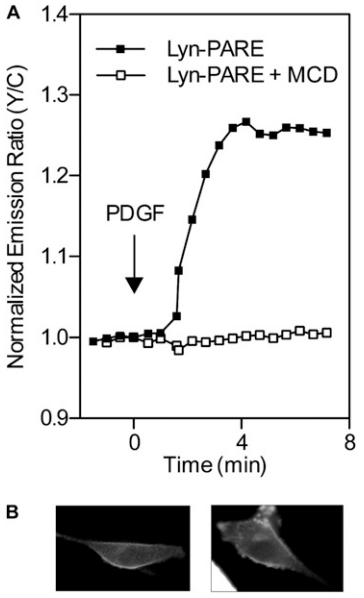

Fig. S3. Raft disruption using MCD abolishes the response of Lyn-PARE. NIH 3T3 cells were preincubated with or without 5 mM MCD for 30 min then stimulated with 50 ng/mL PDGF. (*A*) Representative emission ratio time courses of NIH 3T3 cells expressing Lyn-PARE with ($n = 3$) or without ($n = 3$) MCD treatment stimulated with PDGF. (*B*) Representative images of cells expressing Lyn-PARE with (*Right*) or without (*Left*) MCD treatment.

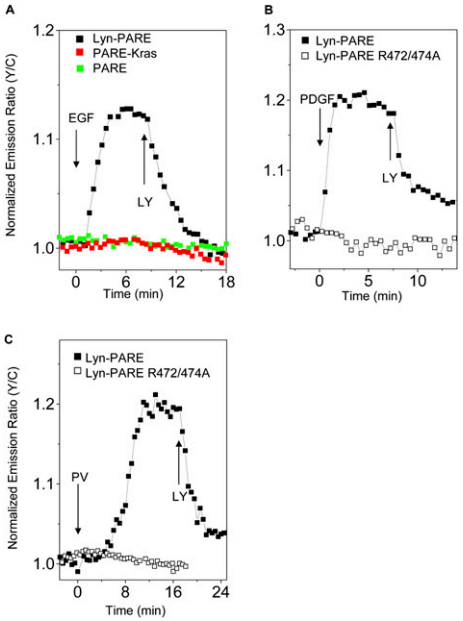

Fig. S4. The response of Lyn-PARE depends on 3′-phosphoinositides. (*A*) Stimulation of serum-starved HeLa cells with 100 ng/mL EGF induced an emission ratio change of Lyn-PARE (n = 7), but not PARE (n = 4) or PARE-Kras (n = 7). Addition of 50 μM LY294002 reversed the response of Lyn-PARE (n = 7). (*B*) A representative time course shows the response of Lyn-PARE in serum-starved NIH 3T3 cells stimulated with 50 ng/mL PDGF, followed by 50 μM LY294002 addition (n = 3). Representative response of the Lyn-PARE (R472/474A) mutant is also shown (n = 3). (*C*) The response of Lyn-PARE to PV depends on 3′-phosphoinositides. A representative time course shows the response of Lyn-PARE to PV in serum-starved NIH 3T3 cells, followed by 50 μM LY294002 treatment (n = 5). Representative response of the Lyn-PARE (R472/474A) mutant is also shown (n = 3).

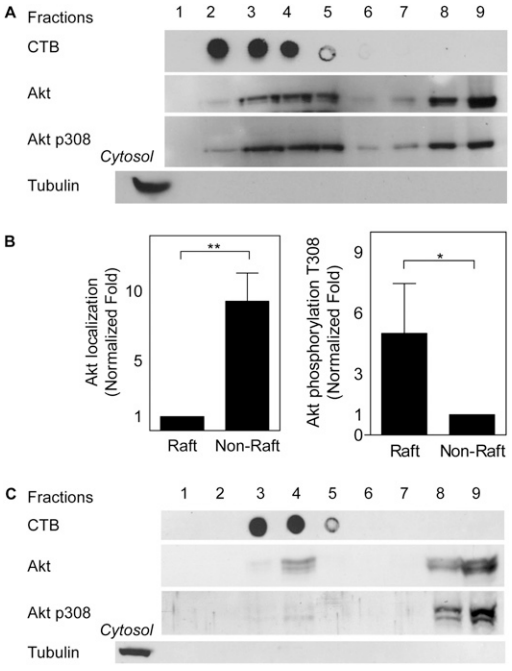

Fig. S5. Phosphorylation of Akt by active PDK1 in membrane microdomains. (*A*) Crude plasma membranes from HEK 293 cells (stimulated with 400 nM insulin) were solubilized and subjected to sucrose density gradient fractionation followed by Western blot analysis using an Akt or phospho-Akt (T308) antibody. Cholera toxin subunit B (CTB) was used as a raft marker. Anti-tubulin was used to ensure the separation of membrane proteins from cytosolic proteins. For fractions 7, 8, and 9, 10 times less volume was loaded. (*B*) Densitometric analysis of phospho-Akt normalized to total Akt (9.3 ± 2-fold stronger signal of total Akt in nonrafts compared with rafts; $P < 0.01$; $n = 3$) showing a 5.0 ± 2.5-fold ($P < 0.05$; $n = 3$) stronger signal of Akt phosphorylation (T308) in membrane rafts compared with nonrafts, suggesting that PDK1 is preferentially activated in membrane rafts (*$P < 0.05$; **$P < 0.01$). (*C*) Crude plasma membranes from serum-starved HEK 293 cells were analyzed as in *A*. Equal sample volume was loaded for each fraction. Some basal Akt phosphorylation (T308) is observed, particularly in the nonraft fractions.

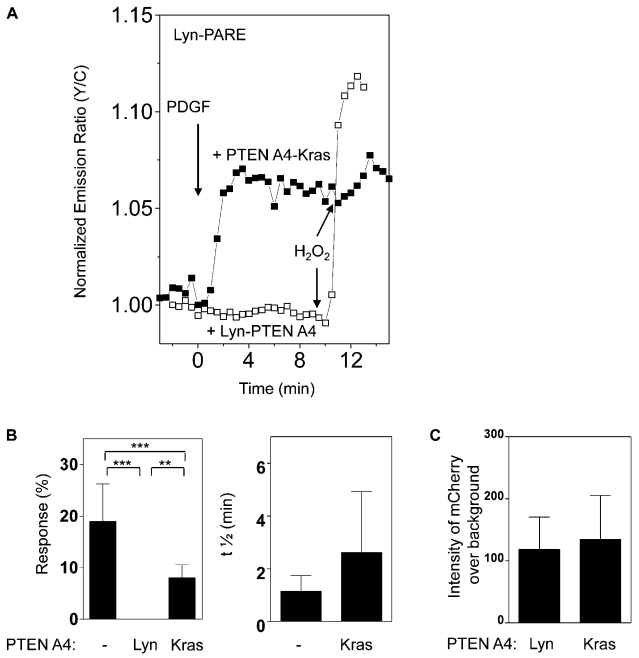

Fig. S6. Genetic targeting of PTEN A4 to rafts, but not nonrafts, abolishes PDK1 activity. (*A*) Representative time courses showing responses of Lyn-PARE in the presence of Lyn-PTEN A4 (n = 5) or PTEN A4-Kras (n = 7). Treatment with 50 mM H_2O_2 restored the response. (*B*) PDGF induced responses (amplitude and $t_{1/2}$ values) of Lyn-PARE in the presence or absence of Lyn-PTEN A4 or PTEN A4-Kras (**P < 0.01; ***P < 0.001). (*C*) Expression levels of Lyn-PTEN A4-mCherry and PTEN A4-mCherry-Kras for experiments analyzed in Fig. 2*E*.

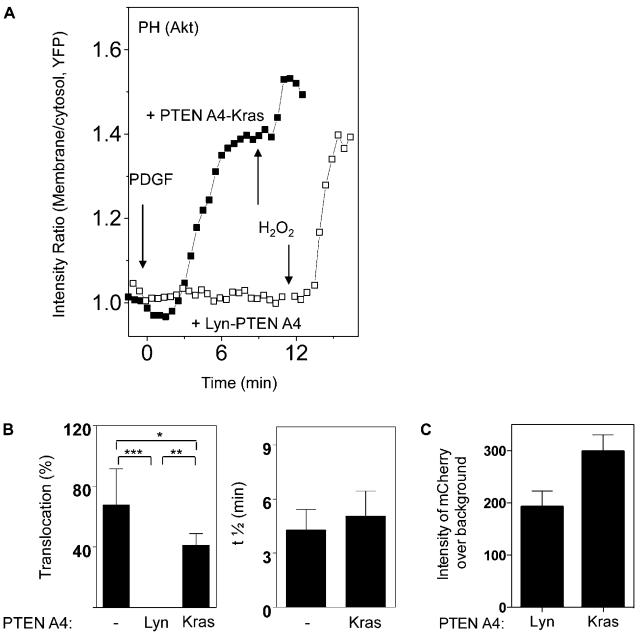

Fig. S7. Genetic targeting of PTEN A4 to rafts, but not nonrafts, abolishes membrane recruitment of Akt. (*A*) Representative time courses showing membrane translocation of Akt PH domain in the presence of Lyn-PTEN A4 (n = 4) or PTEN A4-Kras (n = 4). Treatment with 50 mM H_2O_2 restored the membrane recruitment. (*B*) PDGF induced translocation of Akt PH (response amplitude and t1/2 values) in the presence or absence of Lyn-PTEN A4 or PTEN A4-Kras (*P < 0.05; **P < 0.01; ***P < 0.001). (*C*) Expression levels of Lyn-PTEN A4-mCherry and PTEN A4-mCherry-Kras for experiments analyzed in Fig. 2*F*.

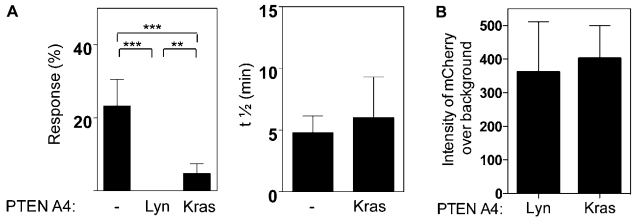

Fig. S8. Genetic targeting of PTEN A4 to rafts, but not nonrafts, abolishes Akt activity. (*A*) PDGF induced responses (amplitude and $t_{1/2}$ values) of AktAR in the presence or absence of Lyn-PTEN A4 or PTEN A4-Kras (**$P < 0.01$; ***$P < 0.001$). (*B*) Expression levels of Lyn-PTEN A4-mCherry and PTEN A4-mCherry-Kras for experiments analyzed in Fig. 2*G*.

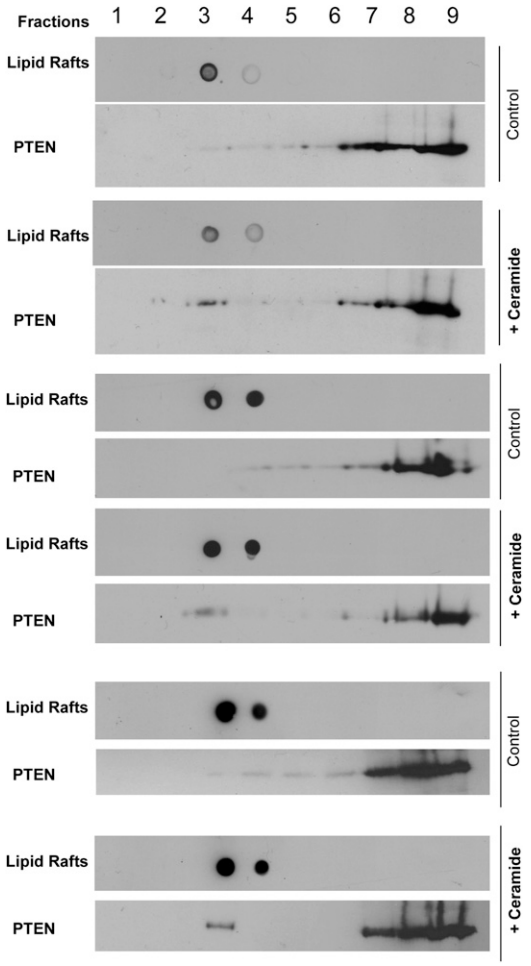

Fig. S9. Ceramide recruits PTEN in membrane rafts. Crude plasma membranes from 3T3 L1 adipocytes (in the presence or absence of 50 μM C_2-ceramide) were solubilized and subjected to sucrose density gradient fractionation, followed by Western blot analysis with PTEN antibody as shown in Fig. 4.

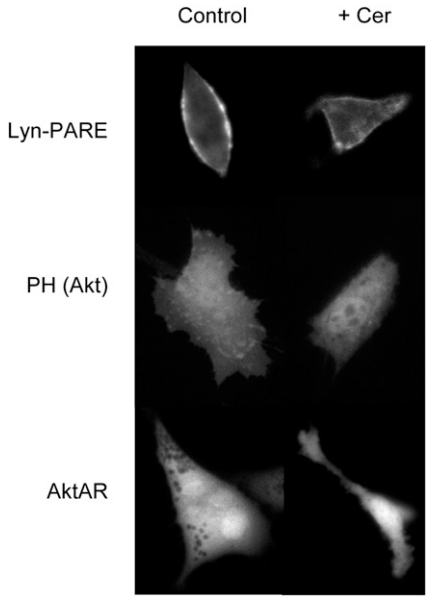

Fig. S10. Distribution of Lyn-PARE, Akt PH domain, and AktAR in the presence or absence of ceramide. Yellow fluorescence images show the distribution of Lyn-PARE, Akt PH domain, and AktAR in the presence or absence of 50 μM C_2-ceramide.

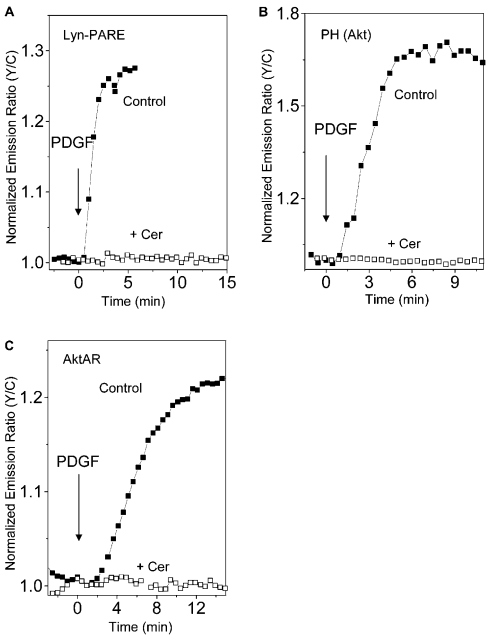

Fig. S11. Ceramide suppresses PDK1 activation, membrane recruitment of Akt, and Akt activity in NIH 3T3 cells. (*A*) Representative time courses showing the response of Lyn-PARE (n = 4) in NIH 3T3 cells was abolished with preincubation of 50 μM C_2-ceramide for 60 min (n = 9). (*B*) Representative time courses showing membrane translocation of Akt PH domain (n = 4) in NIH 3T3 cells was abolished with preincubation of 50 μM C_2-ceramide for 60 min (n = 5). (*C*) Representative time courses showing the response of AktAR (n = 4) in NIH 3T3 cells was abolished with preincubation of 50 μM C_2-ceramide for 60 min (n = 3).