Legends for supplemental figures

fig. S1. Contribution of N-linked glycosylation to the subcellular distribution of NCEH

Wild-type or mutant NCEH containing all of N270Q, N367Q and N389Q was expressed as a GFP fusion protein in Hela cells. NCEH-myc was expressed and immunostained as in Fig. 5. The intercellular localization of these GFP fusion proteins was compared with that of NCEH-myc. Images were acquired with a confocal microscope (Leica) using an argon laser and a 100× objective. Data are representative of three independent experiments.

fig. S2. Site-directed mutagenesis of catalytic sites in NCEH.

Whole cell lysate was prepared from HEK293 cells transfected with wild-type or mutant NCEH containing G114A or S191A. The lysate was subjected to measurements of enzymatic activity using PNPB as a substrate (**B**) and a Western blot analysis with anti-NCEH antibody (**A**). Data are expressed as means±S.E. for three independent measurements. (*, $P < 0.01$, compared with WT NCEH).

fig. S3. Subcellular distribution of GFP fusion proteins

HEK 293 cells transfected with GFP, NCEH-GFP or $NCEH_{1-33}$-GFP were fractionated as described in Materials and Methods and analyzed by Westtern blotting with anti-GFP antibody.

fig. S4. Regulation of HSL and NCEH by phosphorylation

HEK293 cells transfected with FLAG-NCEH or FLAG-HSL were preincubated in Krebs-Ringer buffer for 6 h and subsequently incubated in 1 ml of Krebs-Ringer buffer containing 0.5 mCi [^{32}P] orthophosphate for 1 h, and then in the presence or absence of a PKA activator, forskolin (20 μM) for 30 min. Cells were suspended in buffer A containing 1% (v/v) TritonX-100 and centrifuged. Next, 0, 30 or 60 μg of the supernatant was immunoprecipitated with 5 μg of mouse anti-FLAG monoclonal antibody. After SDS-PAGE, the supernatant was immunoblotted by mouse anti-FLAG monoclonal antibody (**A**). Alternatively, the gels were dried, and autoradiographed with a Phosphoimager Screen (**B**)**.** Data are representative of two independent experiments.

(**C**) Whole cell lysate of HEK293 cells overexpressing NCEH or HSL was incubated with the indicated concentration of cAMP in 250 mM sucrose, 0.5 mM ATP, 1.25 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.0, for 30 min at 37 °C. Activation was stopped by the addition of an equal volume of substrate mixture containing excess

EDTA (6.25 mM), and NCEH activity was assayed for 1 h at 37 °C. Data are expressed as means ± S.E. of three measurements and are representative of three independent experiments. (*, $P < 0.001$, compared with 0 μM cAMP)

fig.S1

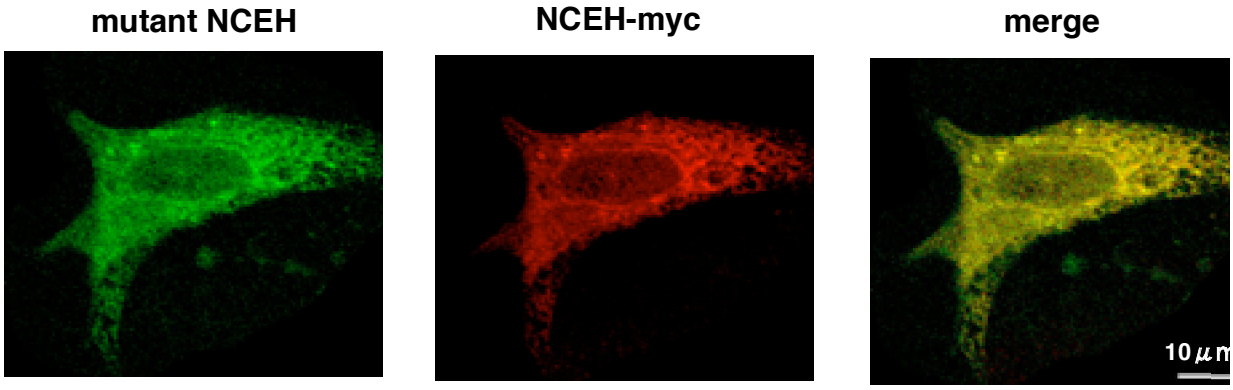
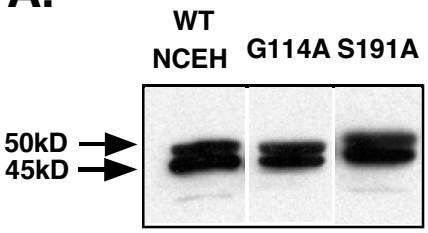


fig.S2

A.


WT
NCEH
G114A S191A
50kD
45kD

B.

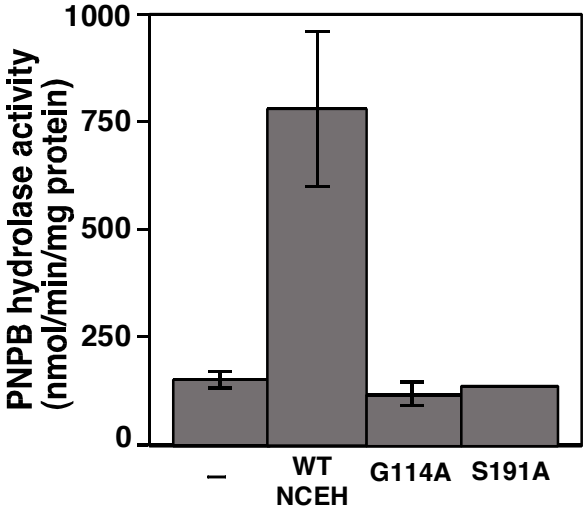

PNPB hydrolase activity
(nmol/min/mg protein)
1000
750
500
250
0
–
WT
NCEH
G114A
S191A

fig.S3

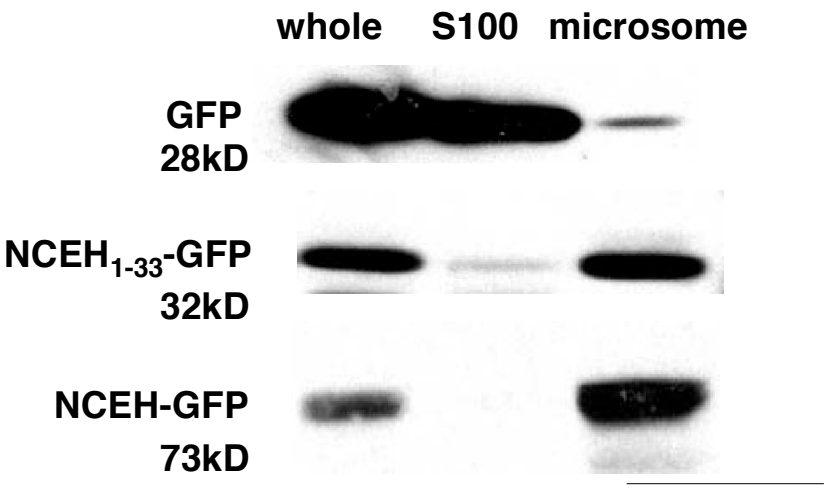
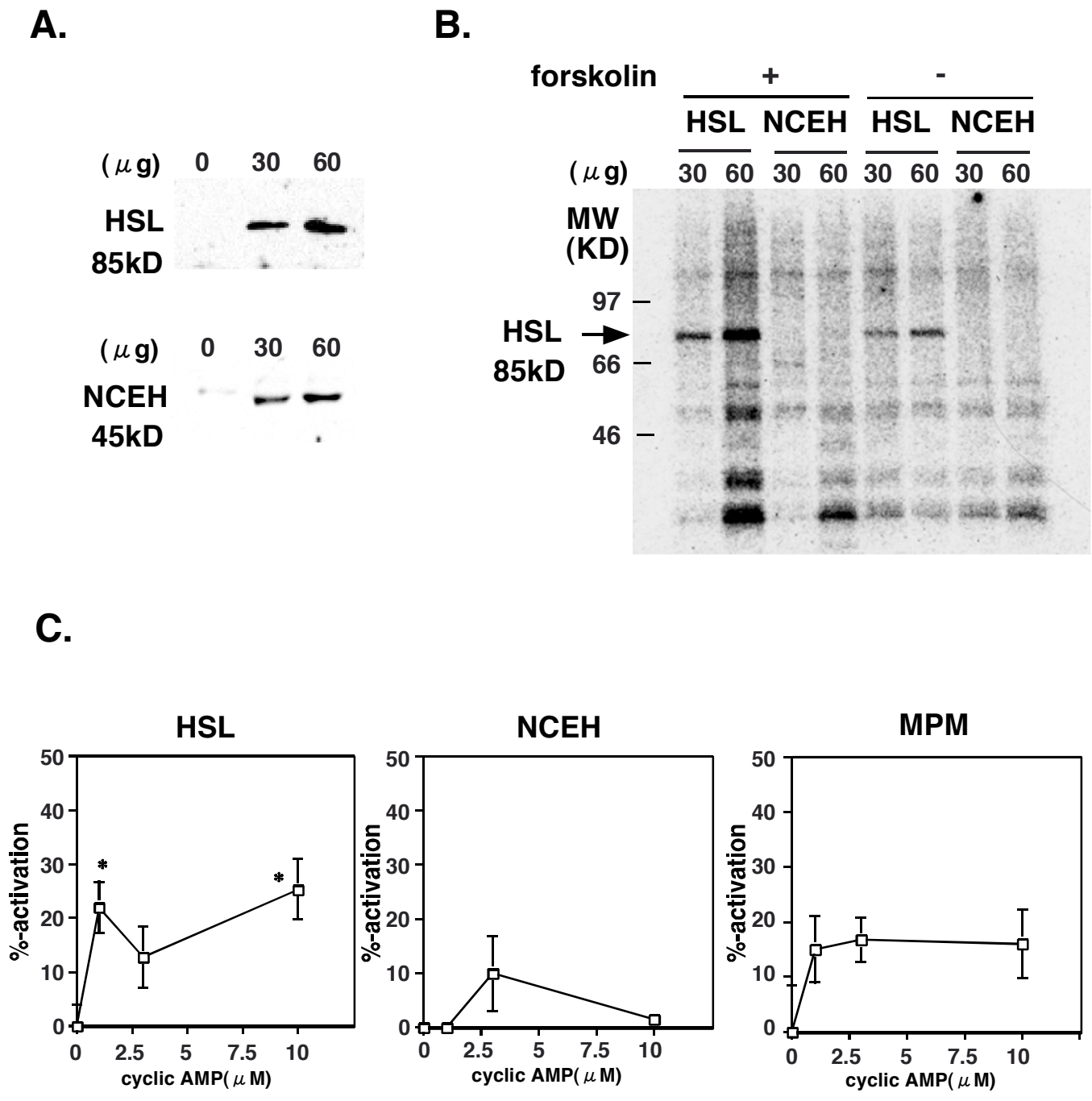

whole
S100
microsome
GFP
28kD
NCEH1-33-GFP
32kD
NCEH-GFP
73kD

Fig.S4


A.
(μg) 0 30 60
HSL
85kD
(μg) 0 30 60
NCEH
45kD
B.
forskolin + -
HSL NCEH HSL NCEH
(μg) 30 60 30 60 30 60 30 60
MW
(KD)
97
HSL
85kD
66
46
C.
HSL
NCEH
MPM
%-activation
cyclic AMP(μM)
0 2.5 5 7.5 10
0 10 20 30 40 50