Supplementary information

Figure S1. MEKK2 is specifically required for sorbitol-induced ERK activation.

(**A, B, C, D, E, F**) Requirement of MEKK2 for sorbitol-induced ERK activation. HEK293 cells were transfected with siRNAs against control, $MEKK2^{\#1}$, $MEKK2^{\#2}$, $MEKK3^{\#1}$, and $MEKK3^{\#2}$. After 48 h, cells were treated with 500 mM sorbitol for indicated time periods (A, B). The schema represents the experimental time schedules of transient stimulation with sorbitol (C). Cells were treated with 500 mM sorbitol for 1 h, and sorbitol was removed from the culture medium (D, E, F). Cell lysates were subjected to immunoblotting with indicated antibodies. Sorbitol-induced ERK phosphorylation level relative to the amount of total proteins is shown as fold increase compared with non-treated cells (0 min) (n=3, mean ± S.E., *: $p< 0.05$, **: $p< 0.01$) (C).

Figure S2. CHIP induces the ubiquitination of MEKK2.

(**A**) Kinase activity-dependent ubiquitination of MEKK2. HEK293 cells were transfected with the indicated combination of Flag-$MEKK2^{WT}$, Flag-$MEKK2^{KN}$, Myc-CHIP, and HA-Ub. After 48 h, cells were incubated with 100 nM MG132 for 18 h. Flag-MEKK2 was immunoprecipitated with anti-Flag antibody and re-immunoprecipitated with anti-Flag antibody after denaturation with 1% SDS followed by immunoblotting with indicated antibodies. The expression level of Myc-CHIP was confirmed by immunoblotting with anti-Myc antibody.

(**B**) CHIP-dependent ubiquitination of MEKK2 in response to hyperosmotic stress. $CHIP^{+/+}$ MEFs or $CHIP^{-/-}$ MEFs was cultured in medium containing 0.1% FBS for 24 h

and treated with 300 mM sorbitol or 300 mM NaCl in the presence of 100 nM MG132 for 12 h. Endogenous MEKK2 was immunoprecipitated with anti-MEKK2 antibody and re-immunoprecipitated with anti-MEKK2 antibody after denaturation with 1% SDS. Ubiquitinated MEKK2 was detected by anti-polyubiquitin antibody. The protein amount of MEKK2 and CHIP was confirmed by immunoblotting with anti-MEKK2 and CHIP antibodies.

Figure S3. CHIP interacts with dephosphorylated MEKK2.

(**A**) Phosphorylation of overexpressed MEKK2. HA-MEKK2^{WT} or HA-MEKK2^{KN} were transfected into HEK293 cells, and cell extracts were incubated with λphosphatase (λPPase) followed by immunoblotting with anti-HA antibody.

(**B**) Cell lysates-induced interaction of CHIP with dephosphorylated MEKK2 *in vitro*. Flag-MEKK2^{WT} and Flag-MEKK2^{KN} overexpressed in HEK293 cells were immunoprecipitated with anti-Flag antibody and were incubated with *CHIP*$^{-/-}$ MEF lysate in the presence or absence of PPase inhibitors (10 mM β-glycerophosphate, 10 mM NaF and 1 mM Na_3VO_4). After washing the immunocomplex beads, MEKK2 proteins were incubated with recombinant His-HA-CHIP (rec. CHIP) followed by immunoblotting with indicated antibodies. The left schema represents the experimental protocol.

Figure S4. CHIP interacts with MEKK2.

(**A**) Sorbitol-induced interaction of MEKK-CHIP in the time and dose dependent manners. HEK293 cells transfected with HA-CHIP were treated with 100, 300, or 500 mM sorbitol for 1 h, and medium was changed to new medium without sorbitol. After 1

h, cell lysates were immunoprecipitated with anti-MEKK2 antibody followed by immunoblotting with indicated antibodies. The expression level of HA-CHIP was confirmed by immunoblotting with anti-HA antibody. The bottom schema represents the experimental time schedules.

(**B**) Schematic representation of mutant forms of MEKK2.

(**C**, **D**) Interaction between CHIP and mutant forms of MEKK2-CT. HEK293 cells were transfected with the indicated combination of Flag-MEKK2-CT^{WT}, Flag-MEKK2-CT^{KN}, Flag-MEKK2-CT^{AA}, Flag-MEKK2-CT^{EE}, and HA-CHIP. Cell lysates were analyzed as described in Figure 2A.

Figure S5. CHIP is required for inhibition of the prolonged phase of ERK activity in response to sorbitol.

(**A**) No effect of CHIP siRNA on sorbitol-induced ERK activation at an early phase. HEK293 cells transfected with siRNAs against control, $CHIP^{\#1}$, or $CHIP^{\#2}$ for 48 h were treated with 500 mM sorbitol for the indicated time periods. Cell lysates were subjected to immunoblotting with indicated antibodies.

(**B**) Requirement of CHIP for the inhibition of sorbitol-induced ERK activation at the prolonged phase. $CHIP^{+/+}$ and $CHIP^{-/-}$ MEFs were treated with 300 mM sorbitol for the indicated time periods. Cell lysates were subjected to immunoblotting with indicated antibodies.

Figure S6. CHIP is required for sorbitol-induced expression of AQP1.

$CHIP^{+/+}$ and $CHIP^{-/-}$ MEFs were continuously treated with 300 mM sorbitol. At the indicated time points, mRNAs were purified from cells, and expression levels of

TonEBP, AQP1, and 18S-rRNA were measured by the real-time PCR. The values of genes expression were normalized to the value of 18S-rRNA expression and sorbitol-induced expression of TonEBP and AQP1 are shown as fold increase compared with non-treated cells. (n=3, mean ± S.E., *: $p< 0.05$, **: $p< 0.01$).

Figure S7. PP2A does not affect MEKK2-CHIP interaction and phosphorylation state of MEKK2.

Flag-MEKK2^{WT} and Myc-CHIP were transfected with the gradual amount of HA-PP2A-subunit A (HA-PP2A) into HEK293 cells. Cell lysates were immunoprecipitated with anti-Flag antibody. The expression level of Myc-CHIP and HA-PP2A- was confirmed by immunoblotting with anti-Myc and HA antibody.

Figure S8. MEKK2 is re-autophosphorylated and re-activated *in vitro.*

(**A**) The experimental protocol.

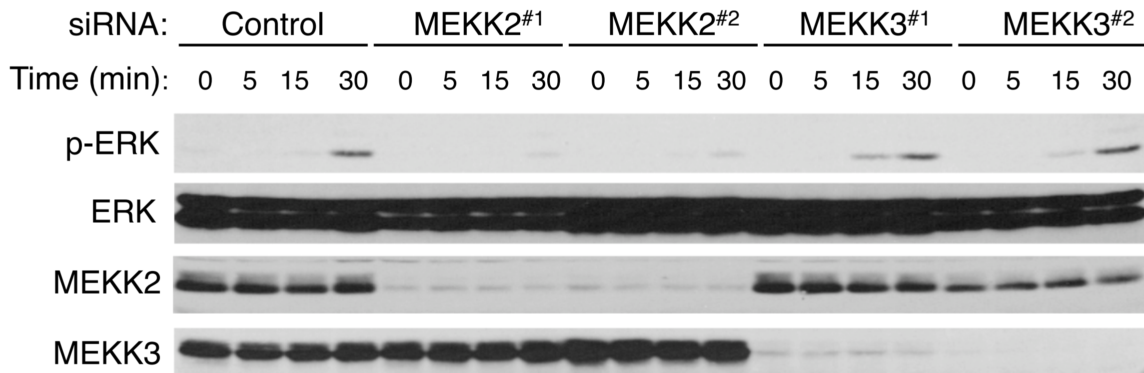
(**B**) Flag-MEKK2^{WT} transfected into HEK293 cells was immunoprecipitated with anti-Flag antibody beads. After washing the beads, they were incubated with cell lysate of *CHIP*$^{-/-}$ MEFs for 15min with or without phosphatase (PPase) inhibitor. The beads were pre-incubated with or without 100 μM ATP for 30 min After wash, Activation of MEKK2 was analyzed by *in vitro* kinase assay (IVK) using GST-SEK1 kinase inactive (SEK1KN) as a substrate for the indicated time. The amount of immunoprecipitated MEKK2 was confirmed by immunoblotting (IB) with anti-MEKK2 antibody. (MEKK2^{P}) Autophosphorylated MEKK2. (GST-SEK1KN^{P}) Phosphorylated GST-SEK1KN. Intensities of MEKK2^{P} and GST-SEK1KN^{P} relative to the amount of MEKK2 protein were calculated and shown as fold intensities.

Figure S9. Schematic representation of the regulation of MEKK2-ERK pathway by CHIP and proper gene expression in response to hyperosmotic stress.

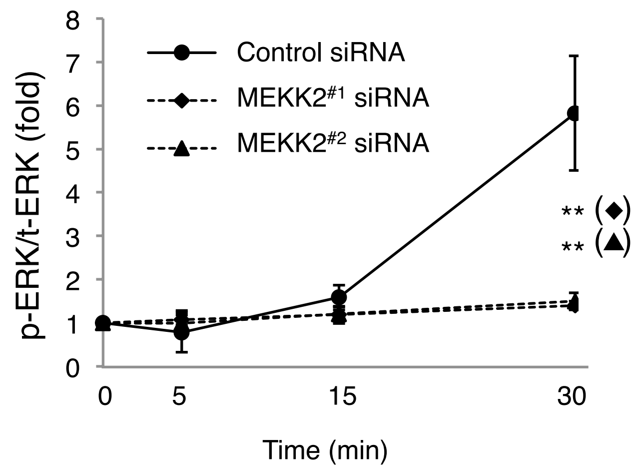
MEKK2 is phosphorylated and activated by hyperosmotic stress and then dephosphorylated by endogenous unknown phosphatase (PPase). In wild type (WT) cells, the transient ERK activation, which is mediated by MEKK2 and CHIP, culminates in proper gene expression, such as AQP1 and AQP5 (left). In the CHIP deficient cells, however, the MEKK2-ERK activation is prolonged, resulting in the aberrant gene expression (right). The sorbitol-induced expression of TonEBP, which is regulated solely by MEKK2 but not by CHIP, may be mediated by the simple ERK activation at an early phase.

Fig. S1

A



B



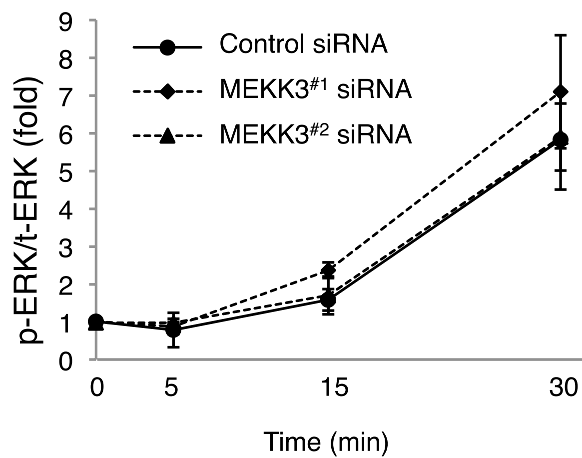
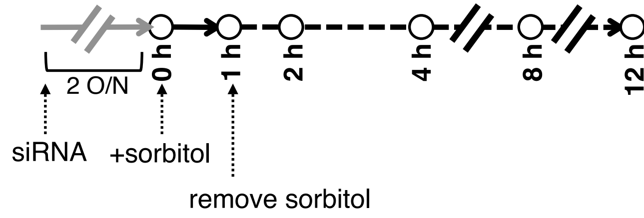
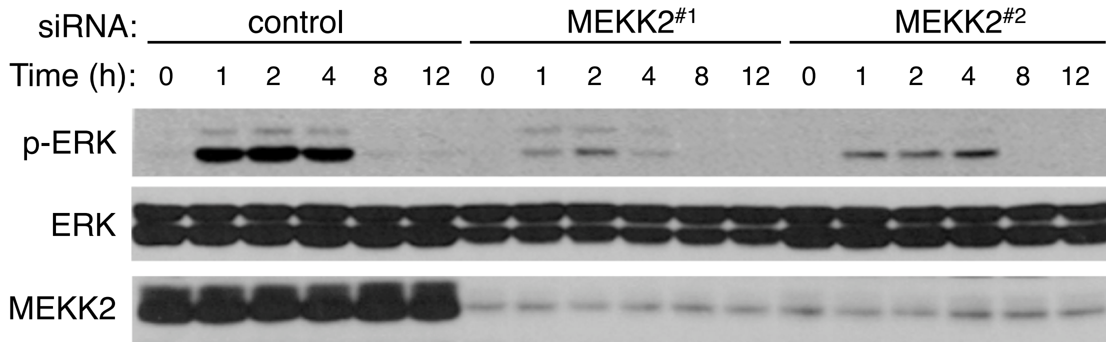


Fig. S1

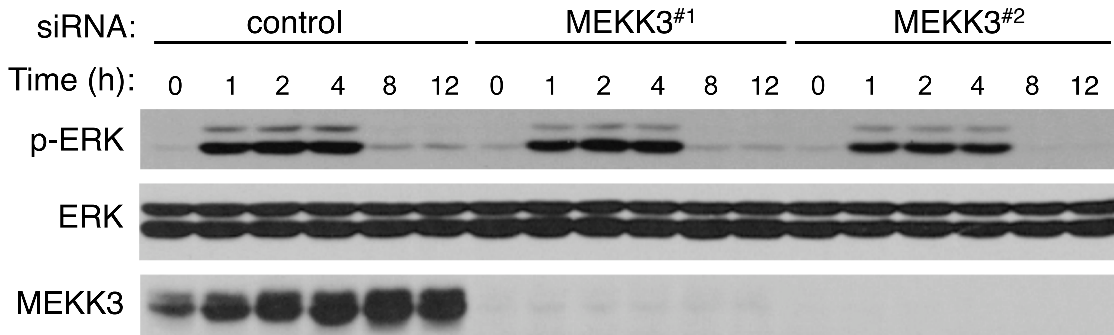
C



D



E



F

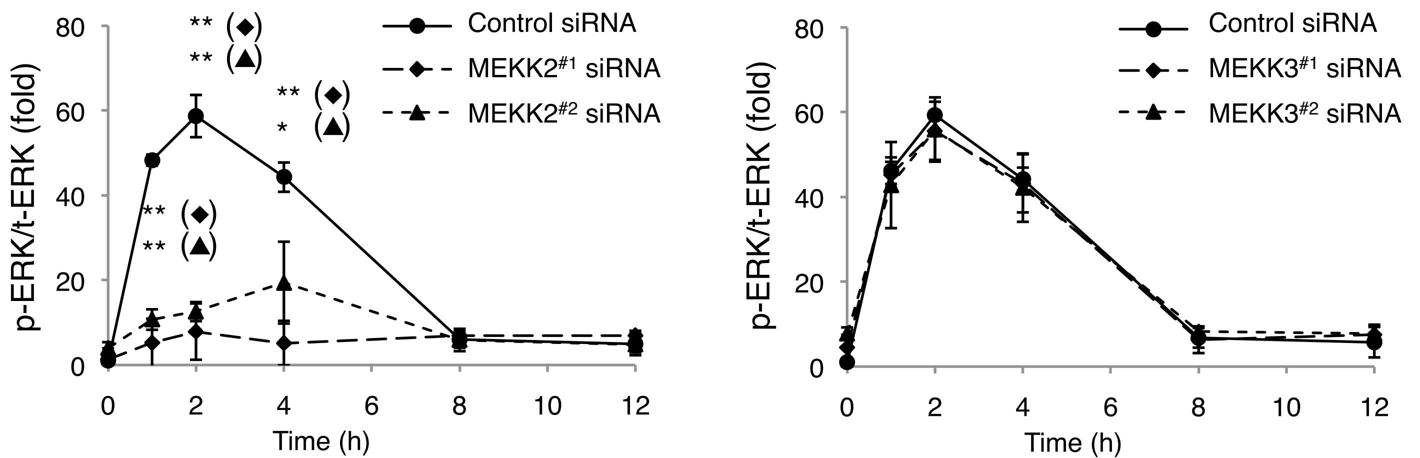
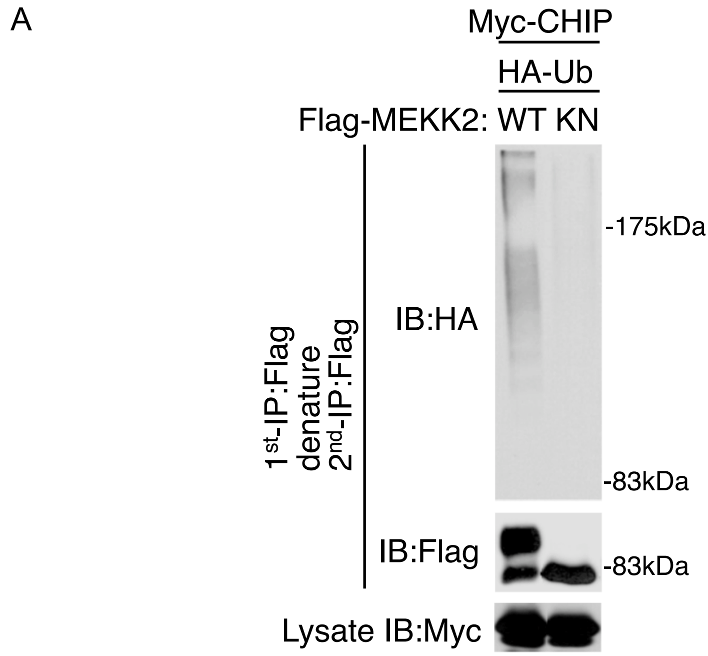


Fig. S2



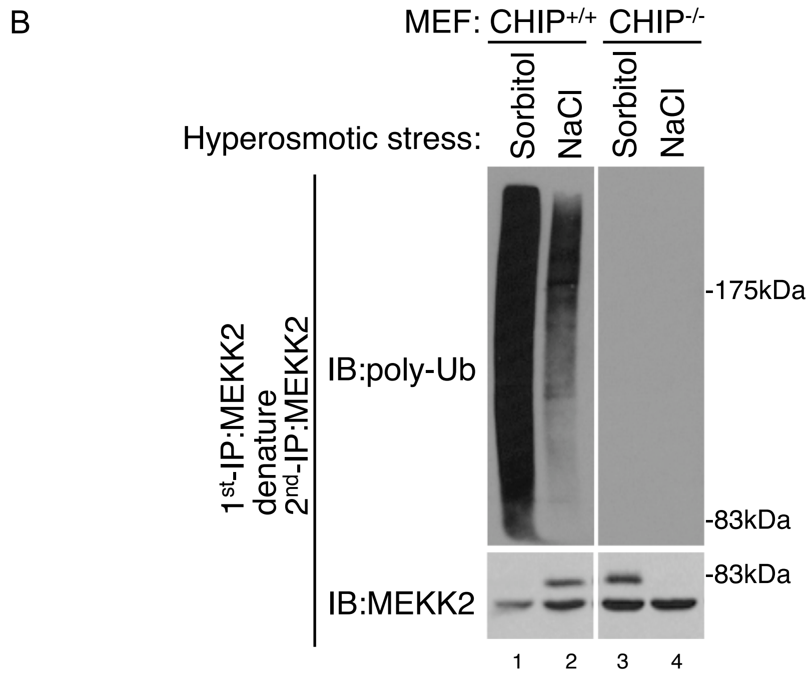
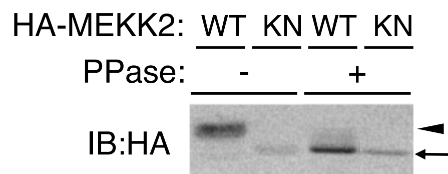
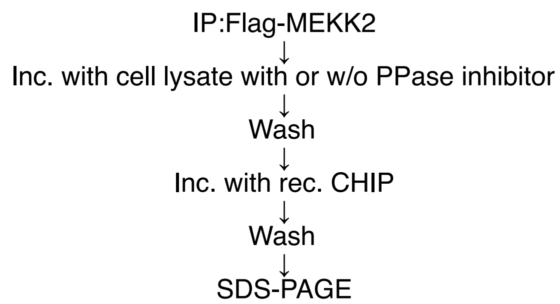


Fig. S3

A



B



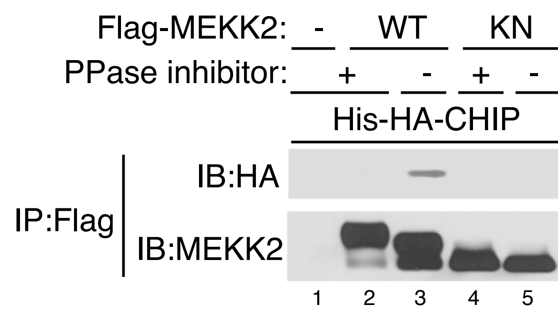
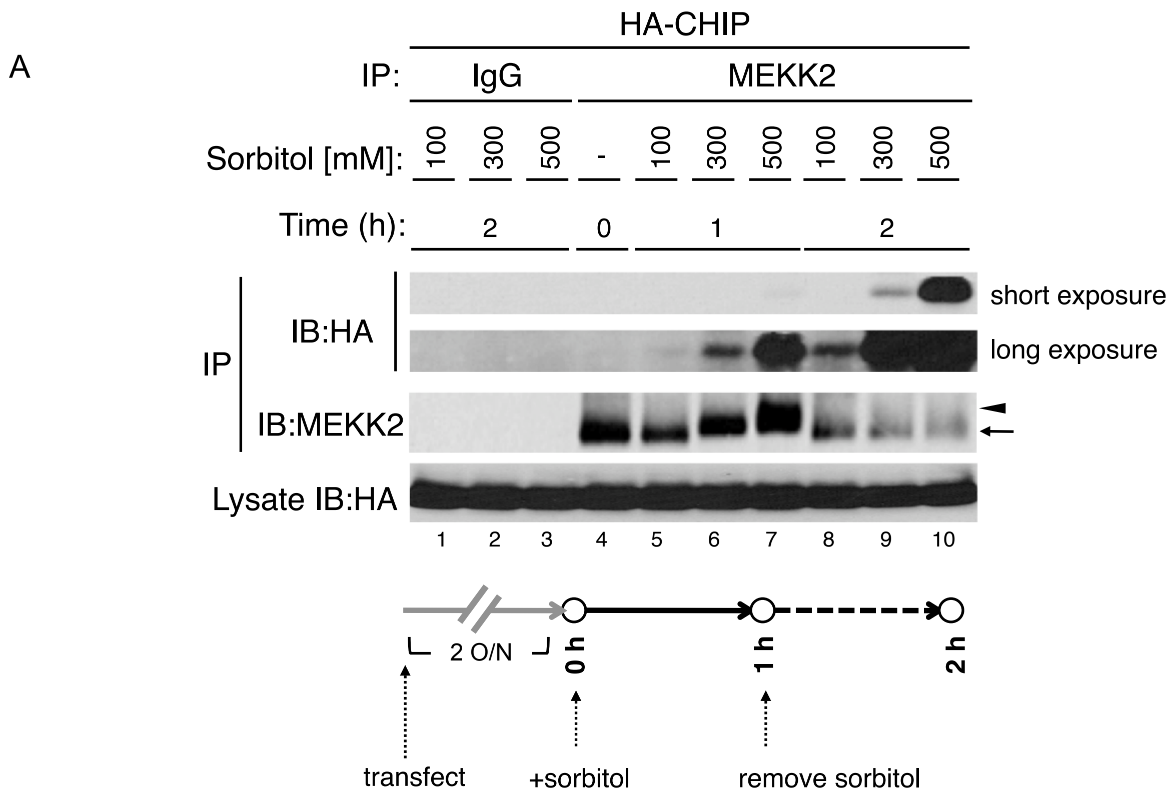
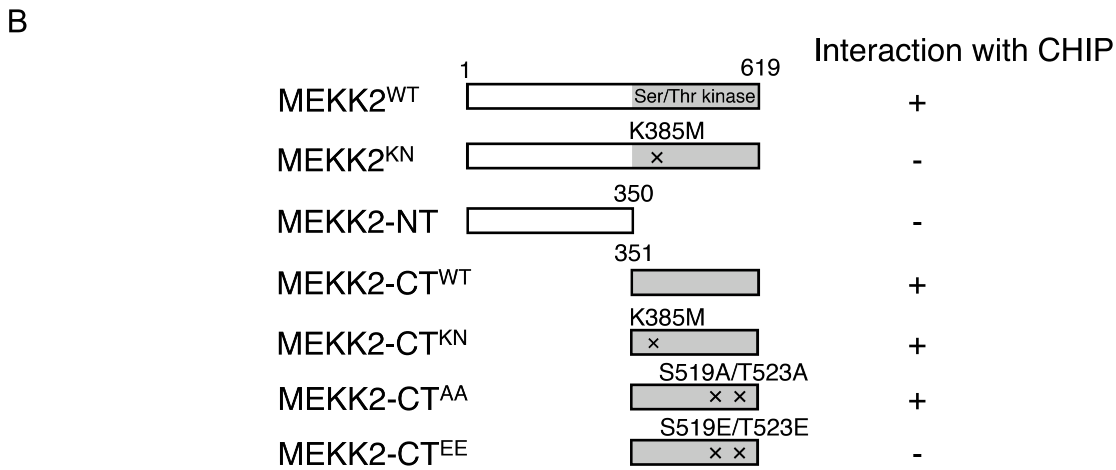


Fig. S4

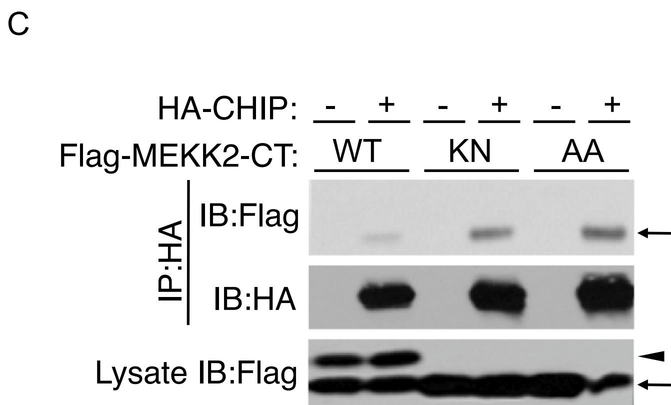
A



B



C



D

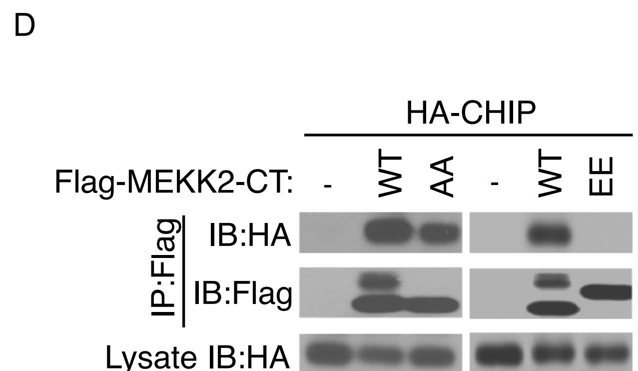
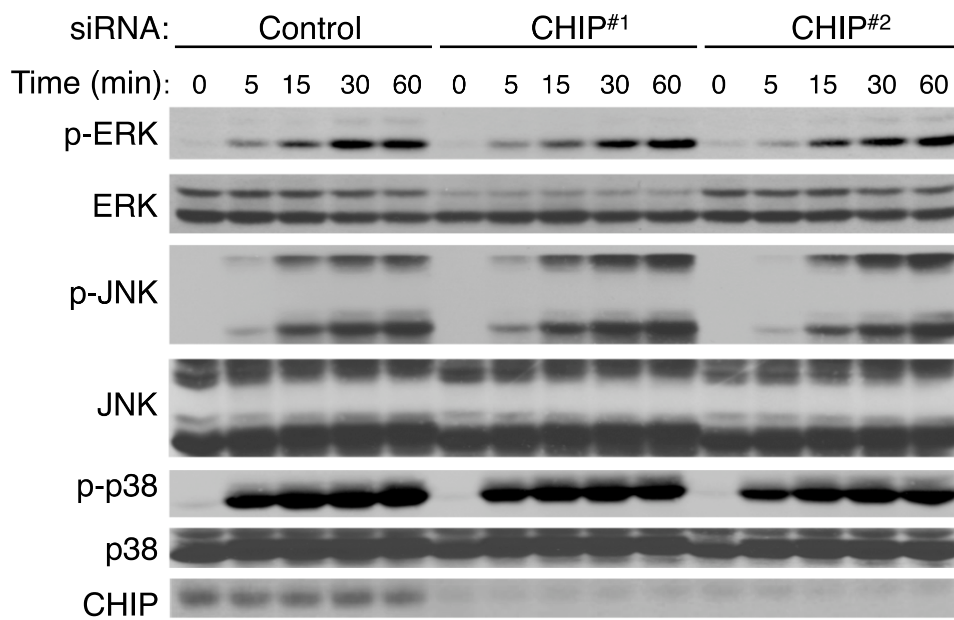


Fig. S5

A



B

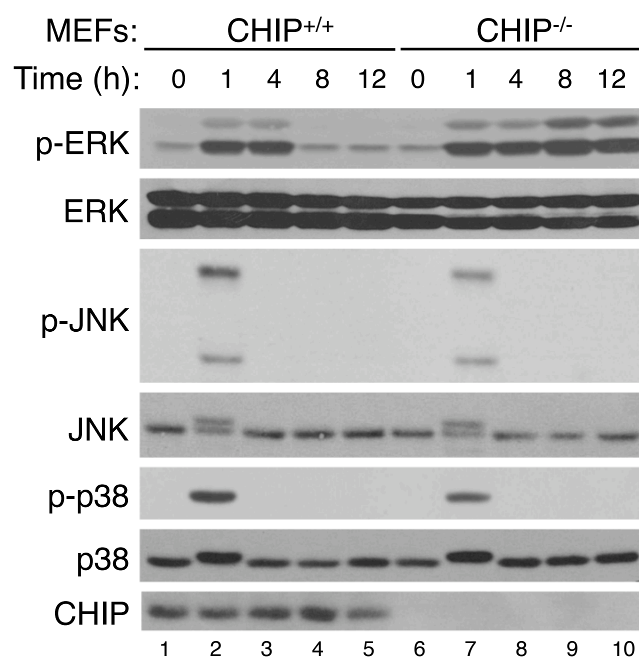


Fig. S6

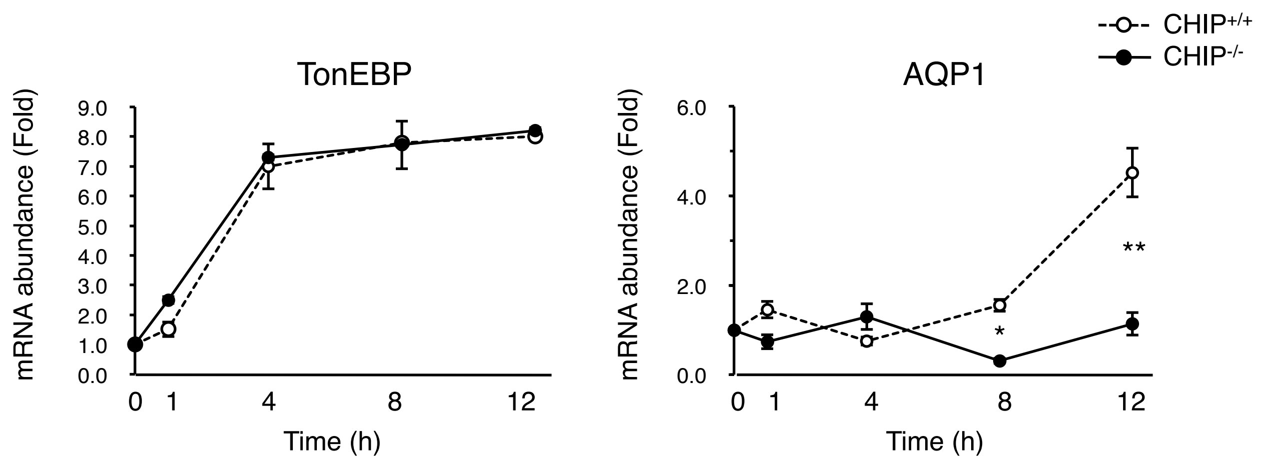


Fig. S7

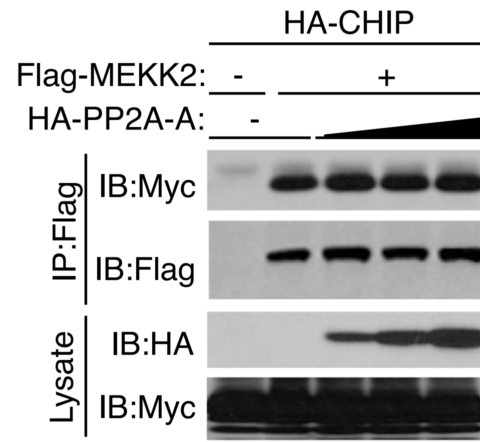
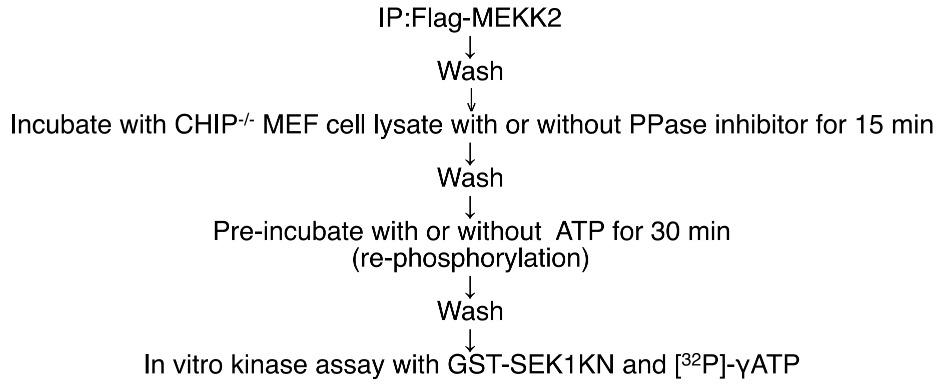


Fig. S8

A



B

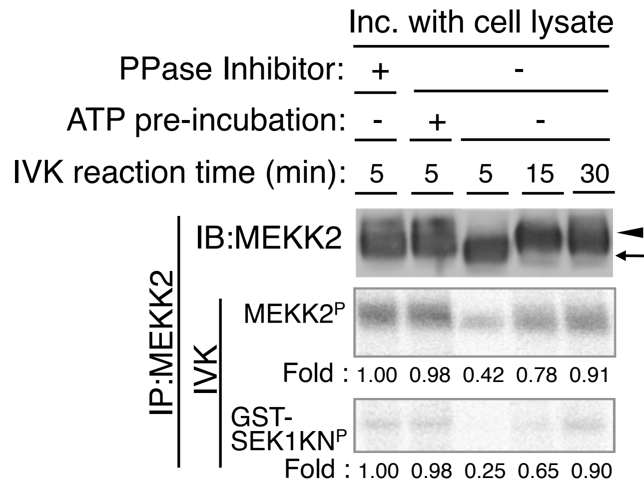


Fig. S9