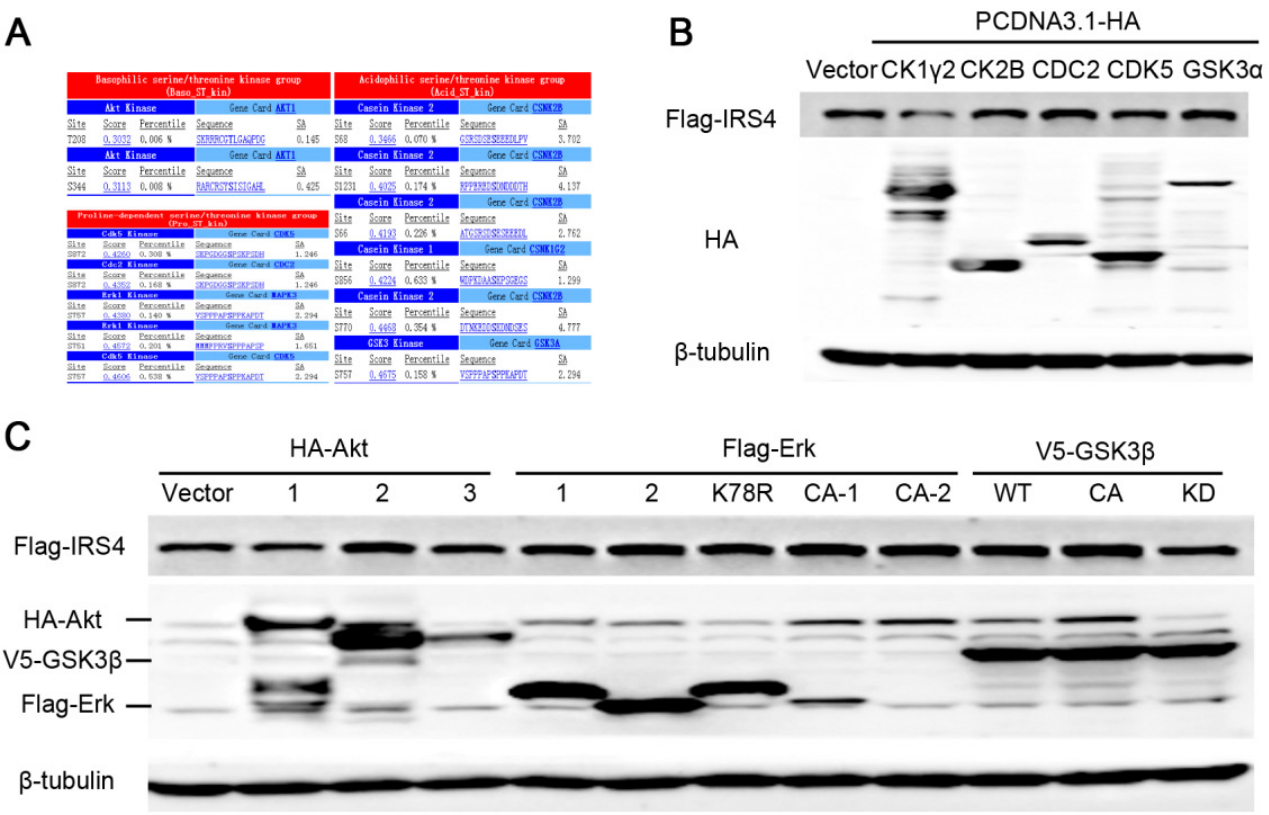
Supplementary Information

Phosphorylation of IRS4 by CK1γ2 promotes its degradation by CHIP through the ubiquitin/lysosome pathway

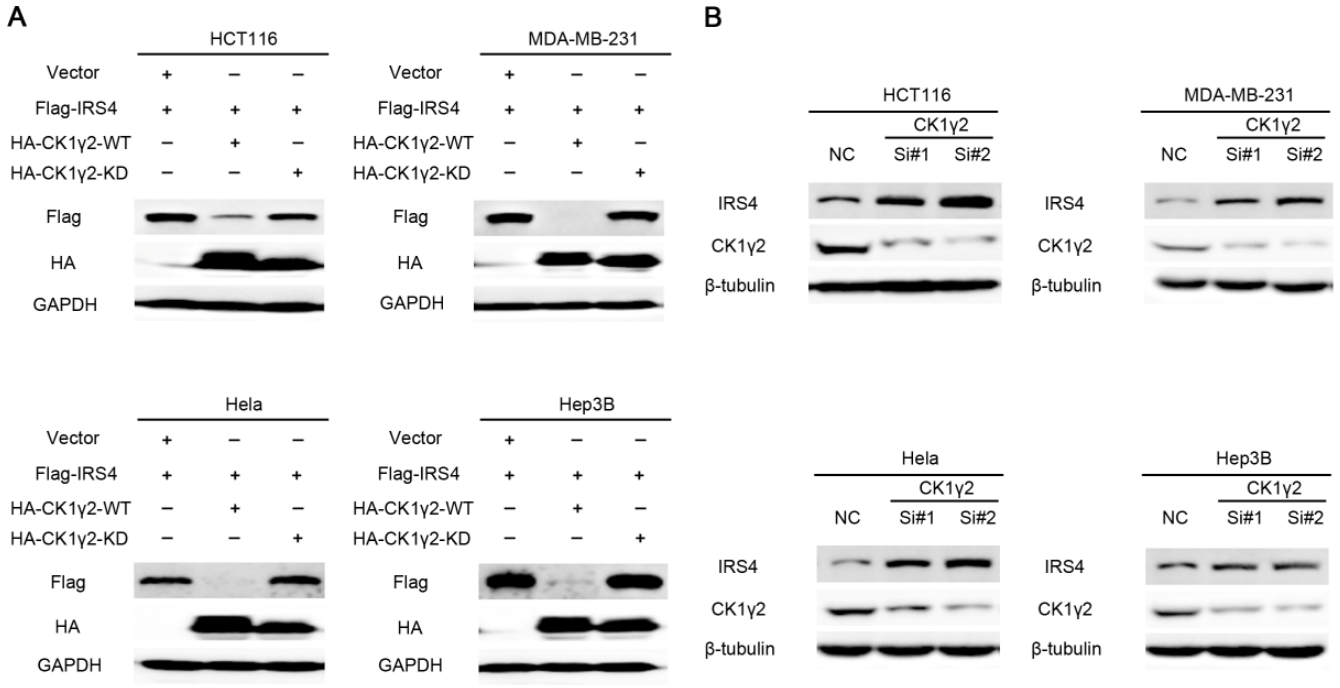
Xinchun Li[1#], Li Zhong[1#], Zhuo Wang[2#], Huiming Chen[1], Dan Liao[1], Ruhua Zhang[1], Hongyu Zhang[3*] and Tiebang Kang[1*]

Running title: Regulation of IRS4 by CK1γ2 and CHIP

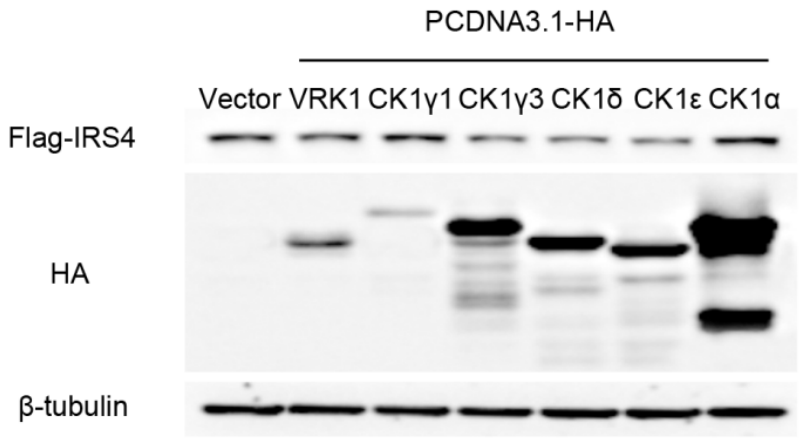
Figures S1, S2, S3, S4, S5, S6, S7 and their figure legends



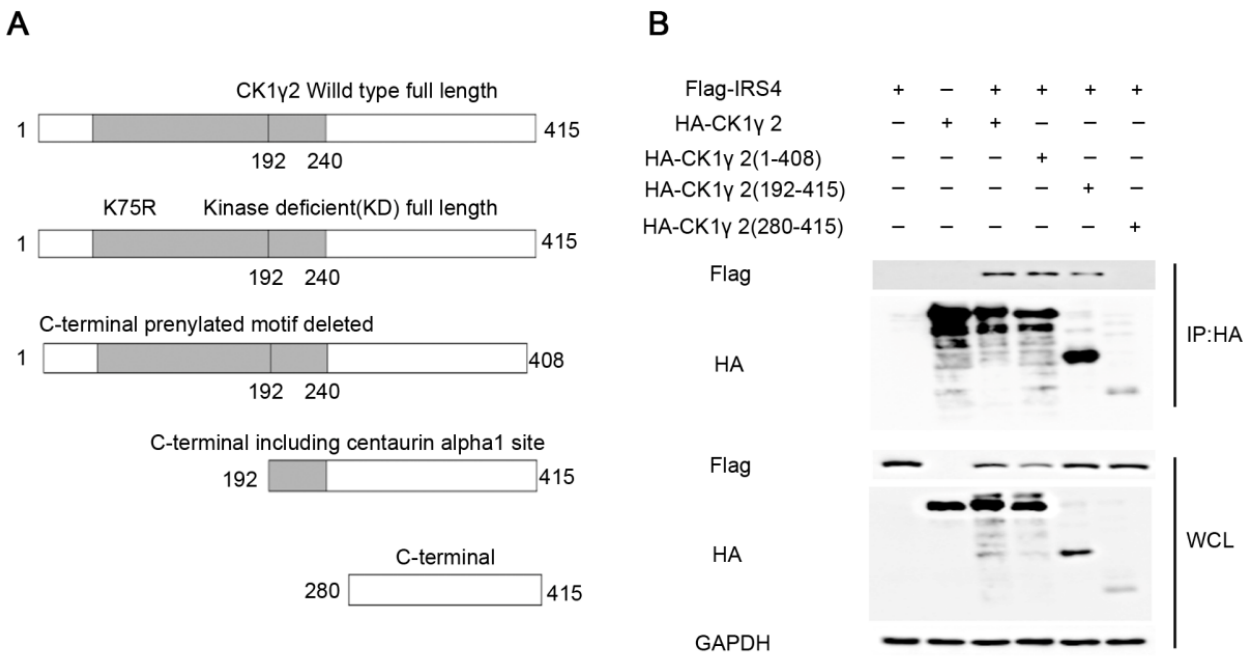
Supplementary Figure S1. CK1γ2 is the only one among the predicted kinases to down-regulate IRS4 in 293T cells. (**A**) Motif scanning result of IRS4 by Scansite. Choosing the stringency level is Medium. (**B, C**) 293T cells were transfected with the indicated plasmids for 24 h and then were analyzed by Western blotting.



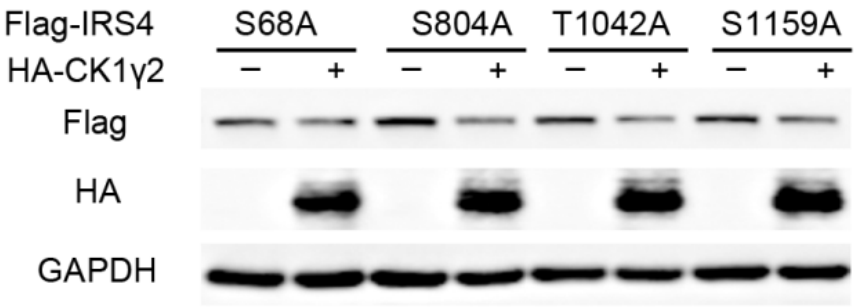
Supplementary Figure S2. CK1γ2 negatively regulates IRS4 in multiple cancer cell lines. (**A**) The indicated tumor cells were co-transfected with HA-tagged wild type (WT) or kinase-depleted (KD) CK1γ2 plasmids with Flag-IRS4 for 48 h and then were analyzed by Western blotting. (**B**) The indicated cells were transfected with CK1γ2 siRNAs and then were analyzed by Western blotting.



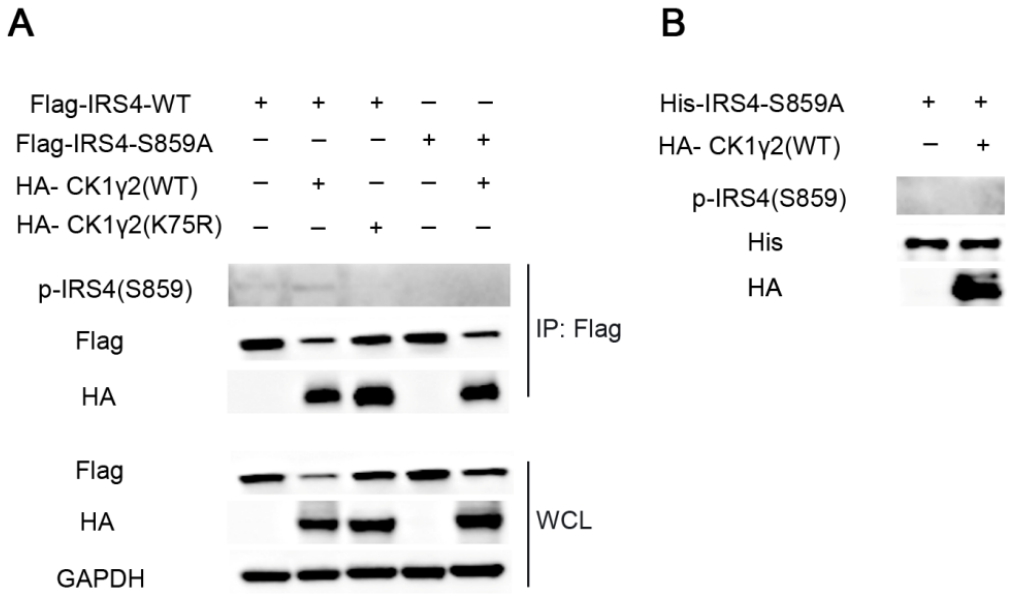
Supplementary Figure S3. Other CK1 family members such as CK1γ3, CK1δ and CK1ε, but neither CK1α, CK1γ1 nor VRK1, also down-regulate IRS4 in 293T cells. 293T cells were co-transfected with Flag-IRS4 and each of the indicated HA-tagged plasmids of CK1 family members for 48 h, and then analyzed by Western blotting.



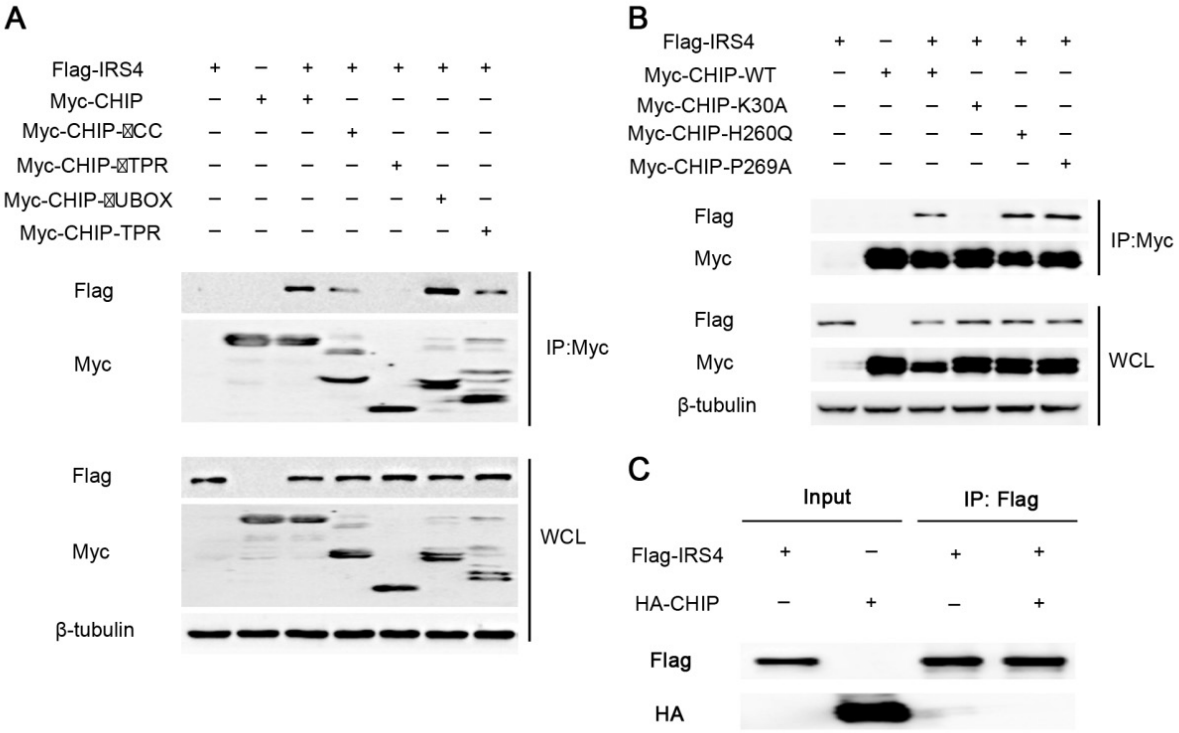
Supplementary Figure S4. Mapping of the interaction domain of CK1γ2 with IRS4.(**A**) Schematic representations of CK1γ2 constructs used in Co-IP assays. (**B**) 293T cells co-transfected with Flag-IRS4 and several HA-tagged CK1γ2 deletion mutants for 48 h were lysed and immunoprecipitated using anti-HA-agarose followed by Western blotting.



Supplementary Figure S5. Ser859 of IRS4 may be the predominant phosphorylation site by CK1γ2. 293T cells were co-transfected with HA-CK1γ2 and Flag-tagged IRS4-S868A, -S804A, -T1042A or-S1159A for 48 h, as indicated, and then analyzed by Western blotting.



Supplementary Figure S6. CK1γ2 phosphorylates IRS4 at Ser859 in vitro and in vivo. (**A**) 293T cells were co-transfected with Flag-tagged IRS4-WT or -S859A with HA-tagged WT or KD CK1γ2 for 48 h, as indicated. Then cell lysates were analyzed by Western blotting using the p-IRS4-S859 antibody we generated. Note: the phospho-specific anti-p-IRS4-S859 antibody was successfully produced. (**B**) His-tagged IRS4 S859A mutant translated in vitro was incubated with HA-CK1γ2 immunoprecipitated from 293T cells using anti-HA agarose in the presence of ATP for 2 h, as indicated. The samples were then analyzed by Western blotting.



Supplementary Figure S7. The E3 ligase CHIP is involved in the degradation of IRS4. (**A**) 293T cells co-transfected with Flag-IRS4 and Myc-tagged CHIP deletion mutants for 48 h were lysed and immunoprecipitated using anti-Myc-agarose followed by Western blotting. (**B**) 293T cells co-transfected with Myc-tagged WT or mutant CHIP plasmids with Flag-IRS4 for 48 h were lysed and immunoprecipitated using anti-Myc-agarose followed by Western blotting.(**C**)Flag-IRS4 and HA-CHIP purified from 293T cells was incubated for 4 h at 4℃, and then protein mixtures were immunoprecipitated using anti-Flag agarose overnight. The samples were then analyzed by Western blotting.