Suppression of gluconeogenic gene transcription by SIK1-induced ubiquitination and degradation of CRTC1

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ABBREVIATIONS: AMPK**,** AMP-activated protein kinase; cAMP, cyclic adenosine monophosphate; CRE, cAMP response element; CREB, CRE-binding protein; CRTC, CREB-regulated transcription coactivator; G6Pase, glucose 6-phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LTR, long terminal repeat; PEPCK phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1-α; PKA, protein kinase A; SIK, salt-inducible kinase; TORC, transducer of regulated CREB activity

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ABSTRACT

CRTCs are a group of three transcriptional coactivators required for CREB-dependent transcription. CREB and CRTCs are critically involved in the regulation of various biological processes such as cell proliferation, metabolism, learning and memory. However, whether CRTC1 efficiently induces gluconeogenic gene expression and how CRTC1 is regulated by upstream kinase SIK1 remain to be understood. In this work, we demonstrated SIK1-induced phosphorylation, ubiquitination and degradation of CRTC1 in the context of the regulation of gluconeogenesis. CRTC1 protein was destabilized by SIK1 but not SIK2 or SIK3. This effect was likely mediated by phosphorylation at S155, S167, S188 and S346 residues of CRTC1 followed by K48-linked polyubiquitination and proteasomal degradation. Expression of gluconeogenic genes such as that coding for phosphoenolpyruvate carboxykinase was stimulated by CRTC1, but suppressed by SIK1. Depletion of CRTC1 protein also blocked forskolin-induced gluconeogenic gene expression, knockdown or pharmaceutical inhibition of SIK1 had the opposite effect. Finally, SIK1-induced ubiquitination of CRTC1 was mediated by RFWD2 ubiquitin ligase at a site not equivalent to K628 in CRTC2. Taken together, our work reveals a regulatory circuit in which SIK1 suppresses gluconeogenic gene transcription by inducing ubiquitination and degradation of CRTC1. Our findings have implications in the development of new antihyperglycemic agents.

Keywords: CREB, CRTC1, SIK1, gluconeogenesis, ubiquitination, RFWD2

Highlights

- SIK1 specifically destabilizes CRTC1 protein
- S155, S167, S188 and S346 of CRTC1 are critical for destabilization
- SIK1 suppresses but CRTC1 stimulates gluconeogenic gene expression
- SIK1 induces RFWD2-mediated and K628-independent ubiquitination of CRTC1

1. Introduction

cAMP response element (CRE)-binding protein (CREB) is a multifaceted transcription factor regulating the expression of about 4000 target genes [1], which collectively exert a substantial impact on metabolism [2,3], cell proliferation [4], immune response [5], learning and memory [6], as well as other physiological and pathological processes [7]. CREB activity is regulated by two distinct but interconnected mechanisms. First, protein kinase A (PKA)-mediated phosphorylation of CREB at S133 promotes the recruitment of transcriptional coactivators in the family of histone acetyltransferases [8]. Second, CREB activation is achieved through another group of obligate transcriptional coactivators termed CREB-regulated transcriptional coactivator (CRTCs), also known as transducer of regulated CREB activities (TORCs), consisted of three isoforms CRTC1, CRTC2 and CRTC3 [9].

Although the three CRTC isoforms are structurally and functionally related, their tissue distribution patterns are distinct and some of their biological functions are non-redundant. Whereas CRTC2 and CRTC3 are more abundant in the liver, CRTC1 is more concentrated in certain areas of the brain. However, all isoforms are widely expressed but not restricted to particular types of tissue [10]. In addition, their expression can be substantially increased in response to cellular stress and other conditions. For example, CRTC1 expression is highly induced in many cancer cells [11,12] and by hepatitis B virus [13]. CRTC2 is most studied among the three isoforms. Both CRTC2 and CRTC3 are thought to be key regulators of gluconeogenesis [14], glucose uptake [15], energy homeostasis [16,17] and macrophage polarization [5,18]. A role for CRTC2 in lipid metabolism [19,20] and the requirement of CRTC3 for hormonal control of stress response [21] have also been described. In contrast to CRTC2 and CRTC3, CRTC1 has been shown to have a neuronal function. Particularly, CRTC1 is essential for long term memory [22,23], circadian

rhythm [24], dendritic growth [25] and neuronal survival after ischemia [26]. CRTC1-null mice are hyperphagic, obese and infertile [27]. They might also develop hepatic steatosis [28]. They can serve as a model for depression [29]. Disruption of CRTC2 in mice results in increased insulin sensitivity [30]. Knockout of mouse CRTC3 leads to resistance to obesity plausibly due to increased energy expenditure and increased β-adrenergic receptor signaling [31]. These phenotypes highlight the unique biological functions of the three CRTC isoforms.

The activity of CRTCs is tightly regulated by phosphorylation. Hyperphosphorylated CRTCs are bound with 14-3-3 proteins in the cytoplasm and their rapid dephosphorylation are required for activation of CREB-dependent transcription [32]. CRTC phosphorylation is catalyzed by salt-inducible kinases (SIKs), which are AMP-activated protein kinase (AMPK)-related kinases regulated by tumor suppressor kinase LKB1 [33]. SIKs comprising three isoforms were initially identified from adrenal glands of rats fed with high-salt diet [34]. They function as master regulators of sodium sensing [35], bone formation [36] and cAMP signaling [37,38]. Particularly, SIK2 directly phosphorylates CRTC2 at S171 [39] and it also phosphorylates p300 to disrupt its acetylation of CRTC2 at K628 [40]. Deacetylated and phosphorylated CRTC2 undergoes ubiquitination at K628 catalyzed by E3 ubiquitin ligase RFWD2, also known as COP1, leading to proteasomal degradation [39]. Although regulation of other CRTC isoforms by SIKs is assumed [14], it remains to be elucidated whether the modification might be isoform-specific. Particularly, whether CRTC1 is phosphorylated and regulated by SIK1 has not determined experimentally. In this regard, genetic evidence suggests that the regulation of CRTCs by SIKs is not promiscuous [41,42]. Our previous analysis of the role of LKB1 and SIKs in human T-cell leukemia virus type 1 (HTLV-1) transcription suggests that the three SIK isoforms cooperate with each other in the regulation of CRTC activity [43]. SIK1-knockout mice are viable and normoglycemic on regular

chow diet, but they exhibit increased sensitivity to insulin and their plasma insulin levels are elevated on high fat diet. They also have high arterial blood pressure [44,45]. Tissue-specific knockout reveals this specific function of SIK1 in skeletal muscle [45]. SIK2-null mice are hyperglycemic and hypertriglyceridemic [46]. Knockout of SIK3 in mice causes dwarfism. The mice are hypoglycemic and hypolipidemic, with increased insulin sensitivity and aberrant circadian rhythms [41,47-49]. Thus, the three SIK isoforms have distinct and related biological functions.

Regulatory phosphorylation of CRTC2 has also been shown to occur at several other sites including S70 [50], S275 [50,51] and S307 [52]. Additional phosphorylation sites on CRTC1 have also been suggested [53]. These findings prompted us to identify additional regulatory sites on CRTC1. Our gain-of-function and loss-of-function experiments in this study revealed SIK1-induced ubiquitination and degradation of CRTC1. The influence of this regulatory mechanism on gluconeogenic gene expression was also assessed.

2. Materials and methods

2.1. Plasmids, antibodies and reagents

Expression plasmids for CRTC1 and its mutants based on pCAGEN-V5 as well as reporter plasmids pCRE-Luc and pPEPCK-Luc have been described previously [13,43,54]. Expression plasmids pCAGEN-FLAG-SIK1/2/3 were constructed by subcloning of SIK1/2/3 cDNA from pCMV-Tag2B-SIK1/2/3 [43] to pCAGEN vector. Expression plasmid pCAGEN-RFWD2-HA was constructed by cloning of RFWD2 cDNA into pCAGEN vector. Plasmids pCMV-myc-ubiquitin and derivatives were made from constructs provided by Dr. Dirk Bohmann (University of Rochester Medical Center, Rochester, NY, USA), Dr. Ted Dawson (Johns Hopkins University School of Medicine, Baltimore, MD, USA) and Dr. Zhijian James Chen (University of Texas Southwestern Medical Center, Dallas, TX, USA) [55-57]. Plasmid pRL-SV40 was purchased from Promega (Madison, WI, USA). Expression plasmid for HTLV-1 Tax protein and reporter plasmid pLTR-Luc driven by HTLV-1 long terminal repeats (LTR) have been described [43]. The expression cassettes for FLAG, myc, V5 and HA tags are within the expression vectors and the tags cannot be found in the endogenous proteins.

Rabbit anti-SIK1 polyclonal (Y20, sc-83754), rabbit anti-myc polyclonal (A14, sc-789) and mouse anti-myc monoclonal (9E10, sc-40) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Mouse anti-V5 monoclonal antibody was bought from Thermo Fisher Scientific (Waltham, MA, USA). Mouse monoclonal antibodies against FLAG (M2 and M5), α-tubulin and β-actin were bought from Sigma-Aldrich (USA). Rabbit monoclonal antibodies against CRTC1 (C71D11) and cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C; D12F5) were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-RFWD2 monoclonal antibody (ab56400) was obtained from Abcam (Cambridge, MA, USA).

Proteasome inhibitor MG132 was purchased from Cayman Chemical (Ann Arbor, MI, USA). cAMP agonist forskolin was bought from Sigma-Aldrich (St. Louis, MO, USA). SIK inhibitors HG9-91-01 and MRT199665 were obtained from Dr. Kristopher Clark (University of Dundee, Dundee, UK) [18].

2.2. Cell culture and transfection

Human embryonic kidney cell line HEK293T and human hepatocellular carcinoma cell line HepG2 were purchased from American Type Culture Collection (Manassas, VA, USA). HEK293T and human cervical adenocarcinoma cell line HeLa were cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum. HepG2 cells were cultured in Eagle's Minimum Essential Medium. All cells were grown at 37 ^{o}C in T75 culture flask supplied with humidified atmosphere containing 5% CO_2. Plasmid DNA was transfected into cells using GeneJuice® transfection reagent (Novagen, Madison, WI, USA), while siRNA was transfected with Lipofectamine® 2000 (Thermo Fisher Scientific) according to manufacturer's protocol.

2.3. Site-directed mutagenesis

Expression plasmids for mutants of CRTC1 and SIK1/2/3 were generated by site-directed mutagenesis with a reagent kit supplied by Agilent Technologies (Santa Clara, CA, US). Mutagenic primers were designed using an online program known as QuikChange Primer Design (http://www.genomics.agilent.com/primerDesignProgram.jsp).

2.4. Dual luciferase reporter assay

Dual luciferase reporter assay was performed as described previously [43,58]. In brief, cells were transfected with reporter plasmids and target genes for 36 h. Transcriptional activity of PEPCK-C promoter and viral promoter LTR was respectively measured with reporter plasmid pPEPCK-C-Luc and pLTR-Luc. Transfection efficiencies were normalized to control plasmid pRL-SV40 expressing Renilla luciferase.

2.5. Real-time RT-PCR

Real-time reverse transcription-PCR (RT-PCR) was performed as described [43]. Normalization was made to glycerialdehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Quantitation of target mRNA was achieved with the comparative Ct method. Relative expression level of target mRNA was calculated from $2^{-\Delta\Delta Ct}$.

2.6. Western blotting

After transfection, cells were harvested and lysed with RIPA-150 buffer (50 mM Tris-Cl, pH 7.4, 250 mM NaCl, 1 mM EDTA, 1% NP-40 and 0.2% Triton X-100) supplemented with protease inhibitor cocktails (Roche, Basel, Switzerland). Protein concentration of cell lysates was determined by Bradford method (Bio-Rad, Hercules, CA, US). Protein samples were separated by SDS-PAGE, followed by electroblotting onto polyvinylidene difluoride membrane (Merck Millipore, Billerica, MA, USA). The membrane was then incubated with primary and secondary antibodies sequentially, and visualized by enhanced chemiluminescence (GE Healthcare Life Sciences, Little Chalfont, UK).

2.7. Co-immunoprecipitation

Co-immunoprecipitation (co-IP) was carried out as described [59]. Cells samples were harvested and lysed with RIPA-150 buffer supplemented with protease inhibitor cocktails. Antibodies were recovered by incubating with recombinant protein G agarose (Thermo Fisher Scientific) for 2 h, followed by overnight incubation with cell lysate at 4 °C. The protein G agarose was collected and washed for three times with lysis buffer after immunoprecipitation. The immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting.

2.8. Ubiquitination assay

For ubiquitination assay, myc-tagged ubiquitin and either CRTC1 WT or CRTC1 4A were expressed in cells for 48 h. Upstream kinase or ubiquitin ligase was also expressed. Proteasome inhibitor MG132 was added into cells 4 h prior to harvest to stabilize proteins. Cell samples were lysed with RIPA-150 buffer and immunoprecipitated with antibodies overnight at 4 °C. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-myc antibody.

2.9. Confocal microscopy

A Zeiss LSM710 confocal microscope was used for multicolor immunofluorescence imaging as described [53,60]. HepG2 cells were fixed with 4% paraformaldehyde. Cells were first blocked with 3% bovine serum albumin (BSA) for 1 h and then permeabilized with 0.2% Triton X-100 for 10 min. Cells were incubated sequentially with rabbit anti-V5 (1:400) recognizing V5-tagged CRTC1 and with rhodamine-conjugated secondary antibodies. Antibodies were diluted

with 3% BSA. Nuclei were counter-stained before mounting with 0.5 μg/ml 4′, 6-diamidino-2-phenylindole (DAPI).

3. Results

3.1. Kinase-dependent induction of CRTC1 degradation by SIK1

Phosphorylation of CRTC2 by SIK2 triggers ubiquitination and degradation [39]. To investigate whether CRTC1 might be subjected to similar mode of regulation, we expressed wild-type (WT) CRTC1 and constitutively active (CA) SIKs (SIK1 T182D, SIK2 T175D and SIK3 T163D) in HeLa cells. A significant reduction of CRTC1 protein was only observed when SIK1 T182D was expressed (Fig. 1A, lane 2 compared to 1). Neither SIK2 T175D nor SIK3 T163D had a substantial influence on the steady-state level of CRTC1 (Fig. 1A, lanes 3 and 4 compared to 1), but all three SIKs were equally competent in Tax-mediated activation of HTLV-1 LTR (Fig. 1B), which has previously been shown to require CRTC1, CRTC2 or CRTC3 [61]. SIK1 is activated by the master regulator LKB1 [33,62,63]. Although SIK1 T182D has been shown to be constitutively active [64], it would still be of interest to see how its activity compares to that of the WT. Indeed, SIK1 T182D was a more potent destabilizer of CRTC1 than SIK1 WT (Fig. 1C, lane 3 compared to 2). In keeping with this, the suppressive activity of SIK1 T182D on Tax activation of HTLV-1 transcription from LTR, as reflected in the luciferase reporter activity of pLTR-Luc, was also more potent than that of SIK1 WT (Fig. 1D). Thus, SIK1 T182D will be used throughout our study. Together, our results were compatible with the notion that CRTC1 degradation might be specifically induced by SIK1.

We next expressed CRTC1 WT with escalating amounts of SIK1 T182D in HeLa cells and observed dose-dependent effect of SIK1 on CRTC1 stability (Fig. 1E, lanes 2 to 5 compared to 1). Real-time RT-PCR results revealed that the levels of CRTC1 mRNA remained unchanged upon expression of SIK1 T182D (Fig. 1F). Thus, the possibility of SIK1 affecting CRTC1 expression at the transcriptional level was excluded.

To complement the above assays in which a CA mutant of SIK1 was used, we extended our analysis to SIK1 K56M, a catalytically inactive mutant in which the ATP binding site at K56 is disrupted [65]. CRTC1 protein was destabilized by SIK1 T182D (Fig. 1G, lanes 2 and 3 compared to 1) but unaffected by SIK1 K56M (lanes 4 and 5 compared to 1). Thus, kinase activity of SIK1 was required for its destabilizing effect on CRTC1. Importantly, the activity pattern of SIK1 T182D and SIK2 T175D on endogenous CRTC1 was the same as that on V5-tagged CRTC1. As such, SIK1 T182D was capable of destabilizing endogenous CRTC1 (Fig. 1H, lane 3 compared to 1), but neither SIK1 K56M nor SIK2 T175D had an influence (lanes 4 and 5 compared to 1). Similar results were also obtained in HEK293T and HepG2 cells (data not shown). Thus, SIK1 specifically destabilized both ectopically expressed and endogenous CRTC1 protein in a kinase-dependent manner.

3.2. SIK1-induced degradation of CRTC1 is mediated through ubiquitin-proteasome system

When we treated HeLa cells either with increasing concentrations of proteasome inhibitor MG132 for 5.5 h or with 10 μM of MG132 for 2 and 4 h, SIK1-induced destabilization of CRTC1 was reversed (Fig. 2A, lanes 4-6 compared to 3 and Fig. 2B, lanes 4 and 5 compared to 3). Notably, a stabilizing effect of MG132 on SIK1 protein was also seen. Hence, degradation of both CRTC1 and SIK1 might be executed through proteasome. Indeed, when we performed ubiquitination assay by pulling down CRTC1 with anti-V5 and probing the polyubiquitin chain with anti-myc, more robust polyubiquitination of CRTC1 was observed upon expression of SIK1 T182D (Fig. 2C, lane 4 compared to 3). To verify that polyubiquitination occurs in CRTC1 but not a CRTC1-assocaited protein, the ubiquitination assay was also conducted reciprocally by pulling down ubiquitinated proteins with anti-myc and probing CRTC1 with anti-V5. Similar results were obtained from this

experiment (Fig. 2D), lending support to the notion that CRTC1 per se was polyubiquitinated. To clarify whether K48- or K63-linked polyubiquitin chain was involved, ubiquitin mutant K48R or K63R, in which substitution at K48 or K63 prevents linkage of polyubiquitin chain, was coexpressed with SIK1 T182D and CRTC1. SIK1-induced polyubiquitination of CRTC1 was abrogated when ubiquitin K48R was expressed (Fig. 2E, lane 4 compared to 3) but unaffected upon expression of ubiquitin K63R (lane 5 compared to 3). Finally, the interaction between SIK1 and CRTC1 was probed by co-IP experiment. Detection of CRTC1 protein in the SIK1 immunoprecipitate (Fig. 2F, lane 3) suggested that they associate with each other. Our results collectively demonstrated that SIK1 induced K48-linked poly-ubiquitination of CRTC1 protein.

3.3. Requirement of S155, S188 and S346 residues of CRTC1 for SIK1-induced degradation

SIKs are known to recognize peptides with a motif of ΦX[HKR]XX[ST]XXXΦ, where Φ stands for a hydrophobic residue and X can be any residue [66]. By cross referencing with serine residues in CRTC1 that are most frequently detected to be phosphorylated based on the post-translational modification database (http://www.phosphosite.org), S155, S188 and S346 were chosen as putative SIK1 phosphorylation sites for further study. To determine whether these sites mediate SIK1-induced degradation, they were substituted with A singly or in combination in the background of CRTC1 WT and CRTC1 S167A. S167 in CRTC1 is equivalent to S171 in CRTC2, the known phosphorylation site for SIK2 [39]. Here the numbering of residues in CRTC1 is based on the longest isoform (GenBank NM_001098482.1) as in our previous studies [13,53] and some other reports [26]. However, another shorter isoform (GenBank NM_015321.2) was used in previous and some recent studies [10,67]. Thus, S155, S167, S188 and S346 of CRTC1 in our study is equivalent to S139, S151, S172 and S330 in some other papers [10,67] and in the

Phosphosite database. The CRTC1 mutants were coexpressed with SIK1 T182D in HEK293T cells and their impact on CRTC1 stability was assessed by Western blotting. Although CRTC1 S155A or CRTC1 S346A single mutant did not show increased stability in the presence of SIK1 T182D when compared to CRTC1 WT (Fig. 3A, lanes 4 and 6 compared to 2), CRTC1 S155A S346A double-mutant was stabilized to the same degree as CRTC1 S167A (lane 8 compared to 10). This suggested that S155 and S346 might jointly affect the stability of CRTC1. Moreover, when S155 and S346 in CRTC1 S167A were substituted, the resultant CRTC1 3A mutant (S155A S167A S346A) was further stabilized (Fig. 3A, lane 12 compared to 2). These results supported the model in which phosphorylation of all three sites might mediate SIK1-induced degradation cooperatively. Finally, when S188A substitution was added to CRTC1 3A, further stabilization of the resultant CRTC1 4A mutant (S155A S167A S188A S346A) was observed (Fig. 3B, lane 8 compared to 6; detection of CRTC1 protein increased from 71% to 85% as shown by densitometry). Thus, through a series of mutational analysis, we showed the requirement of S155, S188 and S346 residues for SIK1-induced CRTC1 degradation. Our results were compatible with their phosphorylation by SIK1.

CRTC1 was ambiently found in the cytoplasm and its nuclear translocation could be induced by various stimuli [53,67,68]. In this regard, whether subcellular localization of CRTC1 S167A and CRTC1 4A mutants might alter remains to be determined. We performed confocal staining of CRTC1 WT, CRTC1 S167A and CRTC1 4A and found that all these proteins localized predominantly to the cytoplasm (Fig. 3C). We next conducted ubiquitination assay to assess how ubiquitination might be affected in the unphosphorylatable CRTC1 4A mutant. Basal ubiquitination of CRTC1 WT and CRTC1 4A in the absence of SIK1 T182D was observed at similar level (Fig. 3D, lane 4 compared to 2). Notably, SIK1-induced ubiquitination of CRTC1

WT was much more robust (lane 3 compared to 2) than that of CRTC1 4A, which was not significantly increased over the basal level (lane 5 compared to 4). This result not only provided an explanation to the stabilization of CRTC1 4A, but also lent some support to the model in which SIK1-mediated phosphorylation of CRTC1 targets it to ubiquitination and degradation.

Interestingly, when co-IP assay was performed to compare CRTC1 WT and CRTC1 4A for their interaction with SIK1, CRTC1 4A was not detected in the SIK1 T182D immunoprecipitate (Fig. 3E, lane 5 compared to lane 3). These results suggested that hypophosphorylated CRTC1 might not be tightly associated with SIK1. In other words, phosphorylation of CRTC1 could facilitate its interaction with SIK1. This is consistent with the notion of the sensitizing effect for SIK1 phosphorylation of CRTC1 at certain sites.

3.4. Regulation of gluconeogenic gene transcription by SIK1-CRTC1 signaling

CRTC2 and CRTC3 have been shown to regulate the transcription of gluconeogenic genes such as PEPCK-C, glucose-6-phosphatase (G6Pase) and peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) [14,39,69].

Although we have previously found CRTC1 to be capable of activating viral transcription in liver and other cells [13,43,61], the role of CRTC1 in gluconeogenesis remains unclear. Hence, we sought to shed light on this and the regulation of CRTC1 by SIK1 in the context of gluconeogenesis. CRTC1 WT was overexpressed in HepG2 cells to assess its effect on transcription of PEPCK-C, a rate-limiting enzyme in gluconeogenesis [61]. HepG2 hepatoma cells have been widely used as a cellular model for hepatic gluconeogenesis [70]. The level of PEPCK-C mRNA in HepG2 cells was induced by CRTC1 more than 20-fold (Fig. 4A, upper panel). The induction was also observed at the level of PEPCK-C protein (Fig. 4A, lower panel). To perform

loss-of-function assay, a truncated mutant of CRTC1 termed CRTC1 M1, which has previously been shown to deplete endogenous CRTC1 activity dominantly and specifically (13), was overexpressed in HepG2 cells. The repression of PEPCK-C mRNA and protein expression by CRTC1 M1 (Fig. 4B) corroborated the notion that CRTC1 contributes to the regulation of gluconeogenesis.

We next interrogated the role of SIK1 in the regulation of PEPCK-C expression by use of constitutively active SIK1 T182D and catalytically inactive SIK1 K56M mutants. The steady-state levels of PEPCK-C mRNA and protein decreased in HepG2 cells expressing SIK1 T182D (Fig. 4C), but elevated when SIK1 K56M was expressed (Fig. 4D). These results demonstrated the negative regulatory function of SIK1 in gluconeogenesis.

We went on to knockdown endogenous CRTC1 protein expression in HepG2 cells specifically using two independent siRNAs. The knockdown effect was verified to be more than 60% by real-time RT-PCR and Western blotting (Fig. 4E). Similar to CRTC1 M1, knockdown of CRTC1 also resulted in a more than 50% drop in PEPCK-C mRNA and protein expression (Fig. 4F). Furthermore, transcription of two other gluconeogenic genes G6Pase and PGC-1α declined by 70-80% in CRTC1-knockdown cells (Fig. 4G and H). To analyze gluconeogenesis in a more physiological context, we used forskolin (FSK) to activate cAMP signaling in HepG2 cells. FSK is a cAMP agonist known to stimulate gluconeogenic gene transcription [60]. Indeed, transcription of PEPCK-C and G6Pase was highly induced by FSK and this induction was effectively blunted upon knockdown of CRTC1 (Fig. 4I). Although the induction of PGC-1α by FSK was modest, PGC-1α mRNA expression was downregulated as well when CRTC1 was compromised (Fig. 4I). Similar results were also obtained in CRTC1 M1-expressing HepG2 cells (Fig. 4J). Thus, CRTC1 was required for induction of gluconeogenic gene expression by cAMP signaling.

3.5. Influence of S155A, S167A, S188A and S346A mutations in CRTC1 on SIK1 suppression of PEPCK-C expression

We have demonstrated SIK1-induced degradation of CRTC1 and identified S155, S167, S188 and S346 of CRTC1 as four critical sites. To determine how this affects gluconeogenic gene expression, CRTC1 WT, CRTC1 S167A and CRTC1 4A were individually overexpressed with PEPCK-C-Luc reporter. CRTC1 4A activated the PEPCK-C promoter more potently than CRTC1 WT and S167A under basal condition. Upon expression of SIK1, a suppressive effect was still observed with all three forms of CRTC1, but CRTC1 4A retained the highest transcriptional activity, which was only slightly lower than in the absence of SIK1 T182D (Fig. 5A). The same pattern was observed when PEPCK-C mRNA and protein were measured. Particularly, when CRTC1 4A was expressed, PEPCK-C mRNA and protein were most abundant and minimally affected by SIK1 T182D (Fig. 5B and C). Hence, S155, S167, S188 and S346 residues in CRTC1 were influential in SIK1-induced suppression of PEKCK-C expression.

3.6. Induction of gluconeogenic gene transcription by SIK1 knockdown or SIK inhibitors

We performed a loss-of-function assay in HepG2 cells to verify the role of SIK1 in the induction of gluconeogenic gene transcription. A pre-validated siRNA against SIK1 (siSIK1) was used and its effectiveness in the depletion of SIK1 mRNA was confirmed (Fig. 6A). Knockdown of SIK1 with siSIK1 resulted in 3-5-fold increase of PEPCK-C and G6Pase mRNA expression (Fig. 6B and C), indicating a role for SIK1 in the suppression of these genes.

We also tested small-molecule inhibitors of SIK since more specific inhibitors of SIK1 are not available. A highly specific inhibitor of SIKs named HG9-91-01, which spares other AMPK1-

related kinases, has been developed and widely used [14,18]. This inhibitor is not known to inhibit all other kinases tested [18]. If SIK1 does act as a gluconeogenic suppressor, its activity should also be reversed by this pan-SIK inhibitor HG9-91-01. Another kinase inhibitor MRT199665, which inhibits SIKs and other AMPK-related kinases [18], was included as a control. To determine how HG9-91-01 might affect gluconeogenic gene expression in our experimental setting, HepG2 cells were treated with this agent for 3 h and PEPCK-C, G6Pase and PGC-1α transcripts were measured by real-time RT-PCR (Fig. 6B-D). A 5-9-fold induction of PEPCK-C, G6Pase and PGC-1α mRNA expression by HG9-91-01 indicated its gluconeogenic effect. In comparison, the pan-AMPK inhibitor MRT199665 was also capable of stimulating PEPCK-C mRNA expression about 5-fold (Fig. 6E). Although the induction was less robust than that of HG9-91-01, it plateaued in about 1 h, which was faster than the time of 3 h required for gluconeogenic action of HG9-91-01. These results are compatible with a gluconeogenic suppressor function of SIK1. The stimulatory effect of HG9-91-01 was more potent that of siSIK1 (Fig. 6B and C). Nevertheless, SIKs were gluconeogenic suppressors and SIK inhibitors can boost gluconeogenic gene expression by inhibiting the suppressive action of SIKs.

3.7. RFWD2 as a functional ubiquitin ligase for SIK1-induced CRTC1 degradation

RFWD2 is an E3 ubiquitin ligase for p53 tumor suppressor [72] and it also mediates SIK2-induced degradation of CRTC2 [39]. To investigate whether RFWD2 also targets CRTC1 for ubiquitination and degradation, we coexpressed RFWD2 with CRTC1 WT in HEK293T cells. When RFWD2 was expressed, CRTC1 protein was destabilized to the same degree as in the presence of SIK1 T182D (Fig. 7A, lane 2 compared to 1, and lane 3 compared to 2). In keeping with this, CRTC1-induced activation of PEPCK-C promoter was ablated by RFWD2 (Fig. 7B,

group 3 compared to 2). In addition, knockdown of RFWD2 with two independent siRNAs abolished the destabilizing effect of SIK1 T182D on CRTC1 (Fig. 7C, lanes 3 and 4 compared to 2). Thus, RFWD2 ubiquitin ligase is required for SIK1-induced degradation of CRTC1.

Indeed, CRTC1 ubiquitination was more robust when coexpressed with RFWD2 (Fig. 7D, lane 4 compared to 3). To assess how SIK1 might affect the recruitment of RFWD2 to CRTC1, co-IP experiment was carried out with the expression of different forms of SIK1. A weak signal of RFWD2 was detected in CRTC1 immunoprecipitate (Fig. 7E, lane 3). The RFWD2 protein band was much more pronounced when SIK1 T182D was expressed (Fig. 7E, lane 4), but it remained weak in the lysate of cells expressing SIK1 K56M (lane 5). These results were consistent with the notion that SIK1 phosphorylation promotes the recruitment of RFWD2 to CRTC1. Hence, RFWD2 served as a downstream effector in SIK1-induced ubiquitination and degradation of CRTC1.

RFWD2-induced ubiquitination of CRTC2 occurs at K628 and K628R mutant of CRTC2 cannot be destabilized by SIK2 [39]. K591 in CRTC1 is equivalent to K628 in CRTC2. However, compared to CRTC1 WT, K591R mutant of CRTC1 was equally susceptible to SIK1 T182D-induced destabilization (Fig. 7F, lane 4 compared to 3 and 2). Thus, RFWD2-mediated ubiquitination of CRTC1 might occur at another site.

4. Disscussion

In this study, we demonstrated SIK1-induced ubiquitination and degradation of the transcriptional coactivator CRTC1. In addition to S167, three other residues S155, S167 and S346 were identified to be critical in SIK1-induced degradation of CRTC1. A regulatory role of SIK1-CRTC1 signaling in gluconeogenesis was characterized. Whereas SIK1 functioned as a gluconeogenic suppressor, CRTC1 and two pharmaceutical inhibitors of SIKs exerted the opposite effect. Furthermore, RFWD2 served as an E3 ubiquitin ligase for SIK1-induced CRTC1 ubiquitination.

Several lines of evidence supported the notion that SIK1 might phosphorylate CRTC1 at S155, S188 and S346. First, they show a good match with the SIK phosphorylation motif and are commonly found to be phosphorylated in proteomic analysis as documented in the post-translational modification database. Second, disruption of these sites in the background of CRTC1 S167A stabilized the protein substantially over CRTC1 S167A alone. Third, CRTC1 4A mutant was resistant to SIK1-induced ubiquitination. Finally, CRTC1 4A mutant induced gluconeogenic gene expression more robustly and was least susceptible to inhibition by SIK1 T182D. Our findings are compatible with the model in which phosphorylation at S167 by SIK1 serves as a gatekeeper and primes or sensitizes CRTC1 to phosphorylation at other sites [50]. However, direct biochemical evidence for phosphorylation of CRTC1 by SIK1 at S155, S188 and S347 remains to be obtained in our future study. We cannot exclude the possibility that the phosphorylation at these sites might be catalyzed by other kinases including AMPK-related kinases. Further experiments are required to clarify this issue. Albeit to a lesser extent, SIK1 T182D was still capable of repressing CRTC1 4A activity. This suggested the existence of additional SIK1 phosphorylation sites on CRTC1, as suggested by others [50,52]. Although our attempt to identify additional SIK1

phosphorylation sites on CRTC1 was unsuccessful, it will be of interest to see whether residues equivalent to S70, S275 and S307 in CRTC2 might also play a role in SIK1-induced degradation of CRTC1.

Substitution of K591 of CRTC1, which is equivalent to K628 in CRTC2 known to be ubiquitinated by RFWD2 [39], did not render CRTC1 protein resistant to SIK1- and RFWD2-induced destabilization. Determination of the site for RFWD2-mediated ubiquitination of CRTC1 and comparison with CRTC2 or CRTC3 await further study. We demonstrated that SIK1 but not SIK2 or SIK3 induced degradation of CRTC1 at least in some cell types. Considered together with the previous finding that CRTC2 was targeted by SIK2 [39], we raised the possibility for specific targeting of CRTC isoforms by the three SIKs. Plausibly, the SIK isoforms might cooperate with each other to modulate the stability and activity of the CRTC isoforms in different tissues and cells. The SIK isoforms should have both unique and shared features. This also applies to the CRTC isoforms. Indeed, our loss-of-function assay provided the first evidence for the regulatory role of CRTC1 in gluconeogenesis in HepG2 liver cells. This indicates that the function of CRTC1 is not limited to the brain. We also found that the activation of PEPCK-C gene transcription by cAMP signaling required CRTC1. cAMP signaling induces not only phosphorylation of CREB [73], but also nuclear translocation of CRTC1 [68]. Exactly how CRTC1 mediates the activation of PEPCK-C gene expression by cAMP signaling merits further analysis. Our results for SIK1 regulation of CRTC1 were consistent in HeLa, HEK293T and HepG2 cells. Further analysis in other cell types and *in vivo* models will provide additional support to our general conclusion.

Our findings on SIK1 regulation of CRTC1 were obtained in the context of gluconeogenic genes in HepG2 cells. The same mechanism should also operate in other tissues in which CRTC1 expression is more abundant, including the brain and tumor cells. It might also affect other

biological processes critically controlled by CRTC1, such as learning and memory [22,23], splicing [74], AP1-dependent transcription [75], and circadian rhythm [24,76]. In this connection, the regulation of CRTC1 by SIK1 could also be implicated in human diseases in which CRTC1 plays a role, including Alzheimer's disease [77,78], Huntington's disease [79], depression [29], malignancies such as lung cancer [11,80], mucoepidermoid carcinoma [81-83] and adult T-cell leukemia [43], hepatitis B [13], as well as osteoporosis [36].

We showed the gluconeogenic effect of SIK inhibitor HG9-91-01 in HepG2 cells. This is generally consistent with previous findings obtained from mouse primary hepatocytes [14]. Notably, this agent inhibits not only SIK1, but also SIK2 and SIK3. Apparently, HG9-91-01 was a more potent gluconeogenic activator than SIK1 K56M or siSIK1, which targets SIK1 more specifically. The inhibition of SIK1 by HG9-91-01 might be more complete. Alternatively, the further induction of gluconeogenic genes by HG9-91-01 could also be attributed to its inhibition of SIK2 and SIK3. This implicates the gluconeogenic suppressor function of SIK2 and SIK3. Plausibly, SIK2 and SIK3 might contribute to the suppression of gluconeogenesis by targeting CRTC2 and CRTC3. Nevertheless, SIK inhibitors might prove useful when CRTC function is beneficial, as in the case of depression [29], neurodegeneration [22,77-79] and osteoporosis [36]. They could also be harnessed to suppress oncogenic, pro-inflammatory and pro-metastatic functions of SIKs [84-86]. On the contrary, small-molecule activators of SIKs might be developed as antihyperglycemic and antihypertensive agents [44,45,64]. The phenotypes of SIK1-, SIK2- or SIK3-null mice raise the concern that inhibition of SIKs could also be deleterious [44-48]. We therefore should be more cautious about the potential side effects of pan-SIK inhibitors. Development of SIK1-sepcific inhibitors like those targeting SIK2 [86] would certainly eliminate the side effects attributed to the inhibition of SIK2 and SIK3. However, recent model experiments

in mice indicated that metabolic and other abnormalities were not seen when mice were treated with active doses of SIK inhibitors [87]. Thus, we might have guarded optimism.

Author contributions

WWG, HMVT, Chi-PC and DYJ conceptualized and designed the study. WWG performed experiments with the help from YC and Ching-PC. All authors contributed to data analysis. WWG and DYJ wrote the paper with input from all authors.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Figure legends

Fig. 1. SIK1 induces CRTC1 degradation in a kinase-dependent manner. A. CRTC1 WT was coexpressed with SIK1/2/3 CA mutant (SIK1 T182D, SIK2 T175D or SIK3 T163D) in HeLa cells. Cell samples were harvested after 48 h, lysed and then analyzed by Western blotting. Whereas anti-FLAG recognized FLAG-tagged SIK CA (FLAG-SIK CA), anti-V5 reacted with V5-tagged CRTC1 WT (CRTC1 WT-V5). The FLAG and V5 expression cassettes were inserted in frame to the expression vectors and these tags did not appear on the endogenous proteins. B. HeLa cells were transfected with pLTR-Luc, HTLV-1 oncoprotein Tax expression plasmid and SIK1/2/3 CA mutant (SIK1 T182D, SIK2 T175D or SIK3 T163D). Fold activation is calculated from pLTR-Luc activity normalized to that of pSV-RLuc. Results are representative of three independent experiments and error bars indicate SD. The difference between groups 2 and 3 was statistically significant by Student's t test ($P < 0.01$, highlighted with $**$). C. Both WT and CA mutant of SIK1 destabilizes CRTC1. CRTC1 WT was co-expressed with SIK1 WT and SIK1 CA mutant (T182D) in HEK293T cells for 48 h. Cell lysates were then analyzed by Western blotting. D. HeLa cells were transfected with pLTR-Luc and expression plasmids for HTLV-1 Tax and either SIK1 WT or CA (T182D). Fold activation is calculated from pLTR-Luc activity normalized to that of pSV-RLuc. Results represent three independent experiments and error bars indicate SD. The differences between groups 2 and 3 as well as between groups 2 and 4 were statistically significant by Student's t test ($P < 0.01$, highlighted with $**$). E. Escalating amounts of constitutively active SIK1 T182D were coexpressed with CRTC1 WT in HeLa cells for 48 h. Cell lysates were analyzed by Western blotting. Rabbit anti-SIK1 antibodies recognized both endogenous and FLAG-tagged SIK1. F. Empty vector and SIK1 T182D was respectively overexpressed in HeLa cells for 48 h.

Cell samples were harvested and CRTC1 mRNA was analyzed by real-time RT-PCR. Results represent means from three independent experiments and error bars indicate SD. The difference was not significant (n.s.) statistically as judged by Student's t test. G. CRTC1 WT was coexpressed with increasing amount of SIK1 T182D or catalytically inactive SIK1 K56M in HeLa cells. After 48 h, cell samples were lysed and analyzed by Western blotting. H. Escalating amounts of SIK1 T182D were overexpressed in HeLa cells. SIK1 K56M and constitutively active SIK2 T175D served as controls. After 48 h, cell samples were lysed and analyzed by Western blotting for endogenous CRTC1 protein.

Fig. 2. SIK1 induces CRTC1 degradation through ubiquitin-proteasome system. A. CRTC1 WT was coexpressed with SIK1 T182D in HeLa cells for 48 h. Proteasome inhibitor MG132 was added to the indicated concentrations 5.5 h prior to harvest. DMSO was added to group 3 as a control. Cell lysates were analyzed by Western blotting. B. CRTC1 WT and SIK1 T182D were coexpressed in HeLa cells for 48 h. Cells were treated with 10 μM MG132 for the indicated lengths of time. C. CRTC1 WT, SIK1 T182D and myc-tagged ubiquitin (myc-Ub) were coexpressed in HeLa cells for 48 h. MG132 was added to 20 μM 4 h before harvest to stabilize CRTC1 protein. Anti-V5 antibody was used to pull down V5-tagged CRTC1 protein (CRTC1 WT-V5). D. CRTC1 WT, SIK1 T182D and myc-Ub were coexpressed in HeLa cells for 48 h. CRTC1 protein was stabilized by the addition of MG132 to 20 μM 4 h before harvest. Anti-myc antibody (9E10) was used to pull down ubiquitinated proteins, while anti-V5 recognized CRTC1 WT-V5. The fast-migrating protein band of about 72 kDa in the V5 blot of the input likely represents a cleavage product. E. Myc-Ub WT, K48R or K63R was individually overexpressed with CRTC1 WT and SIK1 T182D in HeLa cells for 48 h. MG132 was added to 20 μM 4 h prior to harvest. CRTC1

protein was immunoprecipitated (IP) with anti-V5. Immunoprecipitates and input samples (10%) were analyzed by Western blotting. F. CRTC1 WT was coexpressed with SIK1 T182D in HEK293T cells. After 48 h, cell samples were harvested and lysed. FLAG-tagged SIK1 protein (FLAG-SIK1 T182D) was immunoprecipitated (IP) with anti-FLAG. Immunoprecipitates and input samples (10%) were analyzed by Western blotting. The fast-migrating protein band of about 72 kDa in the V5 blot of the input likely represents a cleavage product, which is more prevalent in HEK293T cells.

Fig. 3. Serine residues of CRTC1 that are influential in SIK1-induced degradation. A and B. CRTC1 WT and different CRTC1 mutants (3A denotes S155A S167A S346A and 4A stands for S155A S167A S188A S346A) were individually overexpressed with SIK1 T182D in HEK293T cells for 48 h. Cell lysates were analyzed by Western blotting. C. HepG2 cells were transfected with V5-tagged CRTC1 WT, CRTC1 S167A and CRTC1 4A for 36 h before harvest. Cells were fixed and co-stained with DAPI (blue) and anti-V5 antibody (red). The two channels of fluorescent signal for the same field of cells were merged. Arrows indicate transfected cells. Bar, 20 μm. D. Myc-tagged ubiquitin and SIK1 T182D were coexpressed with either CRTC1 WT or CRTC1 4A in HeLa cells for 48 h. MG132 was added to 20 μM 4 h before harvest. CRTC1 protein was immunoprecipitated (IP) with anti-V5. E. SIK1 T182D was coexpressed with either CRTC1 WT or CRTC1 4A in HEK293T cells for 48 h. Cell lysates were immunoprecipitated (IP) with anti-FLAG (M2). Immunoprecipitates and input samples (10%) were analyzed by Western blotting.

Fig. 4. SIK1-CRTC1 signaling regulates gluconeogenic gene transcription. CRTC1 WT (A),

CRTC1 M1 (B), SIK1 T182D (C) and SIK1 K56M (D) were individually overexpressed in HepG2 cells. After 48 h, cell samples were harvested and subjected to both real-time RT-PCR and Western blot analysis for endogenous PEPCK-C mRNA and protein expression. Real-time PCR results represent means from three independent experiments and error bars indicate SD. The intergroup differences were statistically significant by Student's t test ($P < 0.001$, highlighted with ***; $P < 0.05$, highlighted with *). E. Two independent CRTC1-targeting siRNAs were transfected into HepG2 cells for 48 h. The knockdown effects were examined by both real-time RT-PCR and Western blotting. The differences between siCRTC1-1/2 and siNC groups were statistically significant by Student's t test ($P < 0.001$, highlighted with ***). PEPCK-C mRNA and protein (F), G6Pase mRNA (G) and PGC-1α mRNA (H) in CRTC1-compromised HepG2 cells were analyzed by real-time RT-PCR and Western blotting as appropriate. Bars represent means from three independent experiments and error bars indicate SD. The differences between the indicated groups were statistically significant by Student's t test ($P < 0.001$, highlighted with ***; $P < 0.01$, highlighted with **). I. FSK was added to 10 μM 3 h prior to harvest. PEPCK-C, G6Pase and PGC-1α transcripts were analyzed by real-time RT-PCR. Result represent means from three independent experiments and error bars indicate SD. The differences between the indicated groups were statistically significant by Student's t test ($P < 0.01$, highlighted with **). J. Empty vector or CRTC1 M1 was transfected into HepG2 cells for 48 h. FSK was added into cells to a final concentration of 10 μM 3 h before harvest. Real-time RT-PCR results represent means from three independent experiments and error bars indicate SD. The differences between the indicated groups were statistically significant by Student's t test ($P < 0.001$, highlighted with ***; $P < 0.01$, highlighted with **).

Fig. 5. S155, S167, S188 and S346 of CRTC1 are influential in SIK1-induced expression of PEPCK-C. A. Influence on PEPCK-C promoter activity. PEPCK-C-Luc reporter plasmid was transfected into HepG2 cells expressing SIK1 T182D and CRTC1 WT, CRTC1 S167A or CRTC1 4A for 36 h. Cell samples were harvested and lysed for dual-luciferase reporter assay. Results represent means from triplicate experiments and error bars indicate SD. The differences were assessed by Student's t test ($P < 0.05$, highlighted with ∗). B and C. CRTC1 WT, CRTC1 S167A or CRTC1 4A was individually overexpressed with SIK1 T182D in HepG2 cells for 48 h. PEPCK-C mRNA and protein were analyzed by real-time RT-PCR (B) and Western blotting (C). Real-time PCR results represent means from three independent experiments and error bars indicate SD. The differences between the indicated groups were statistically significant by Student's t test ($P < 0.01$, highlighted with ∗∗; $P < 0.05$, highlighted with ∗).

Fig. 6. Inhibition of SIK1 induces gluconeogenic gene transcription. A. Knockdown of SIK1 mRNA expression with a pre-validated siRNA (siSIK1). HepG2 cells were transfected with either an irrelevant scrambled siRNA as negative control (siNC) or siSIK1 purchased from Invitrogen (s45377) for 48 h. SIK1 transcript was analyzed by real-time RT-PCR. The difference between groups 1 and 2 was statistically significant by Student's t test ($P < 0.001$, highlighted with ∗∗∗). B to D. HepG2 cells were either treated with 500 nM HG9-91-01 for 3 h or transfected with the indicated siRNA for 48 h. DMSO served as a negative control. PEPCK-C, G6Pase and PGC-1α transcripts were examined by real-time RT-PCR. Results represent means from three independent experiments and error bars indicate SD. The differences between the indicated groups were

statistically significant by Student's t test ($P < 0.01$, highlighted with ∗∗ or $P < 0.05$, highlighted with ∗). E. HepG2 cells were treated with 1 mM MRT199665 for 1 h. Results represent means from three independent experiments and error bars indicate SD. The difference between the indicated groups was statistically significant by Student's t test ($P < 0.05$, highlighted with ∗).

Fig. 7. RFWD2 is a functional E3 ubiquitin ligase for SIK1-induced CRTC1 degradation. A. CRTC1 WT was coexpressed with SIK1 T182D or RFWD2 in HEK293T cells for 48 h. Cell samples were harvested and cell lysates were analyzed by Western blotting. B. PEPCK-C-Luc reporter was transfected into HepG2 cells expressing CRTC1 WT or RFWD2 for 36 h. Cell samples were lysed and analyzed by dual-luciferase reporter assay. Results represent means from three independent experiments and error bars indicate SD. The difference between groups 2 and 3 was statistically significant by Student's t test ($P < 0.01$, highlighted with ∗∗). C. Two independent RFWD2-targeting siRNAs (siRFWD2-1/2) were transfected into HEK293T cells for 36 h initially. CRTC1 WT and SIK1 T182D were then transfected into cells for an additional 36 h. Cell samples were harvested and lysed. Cell lysates were analyzed by Western blotting. D. CRTC1 WT and myc-tagged ubiquitin were coexpressed in HeLa cells expressing RFWD2. After 48 h, cell samples were harvested and lysed. CRTC1 protein was immunoprecipitated (IP) with anti-V5. Immunoprecipitates and input samples (10%) were analyzed by Western blotting. E. CRTC1 WT, RFWD2, constitutively active SIK1 T182D and catalytically inactive SIK1 K56M were overexpressed in HEK293T cells for 48 h. Cell samples were harvested and lysed. CRTC1 protein was immunoprecipitated (IP) with anti-V5. Immunoprecipitates and input samples (10%) were analyzed by Western blotting. F. CRTC1 WT or CRTC1 K591R was coexpressed with SIK1

T182D in HEK293T cells for 48 h. Cell lysates were probed by Western blotting.

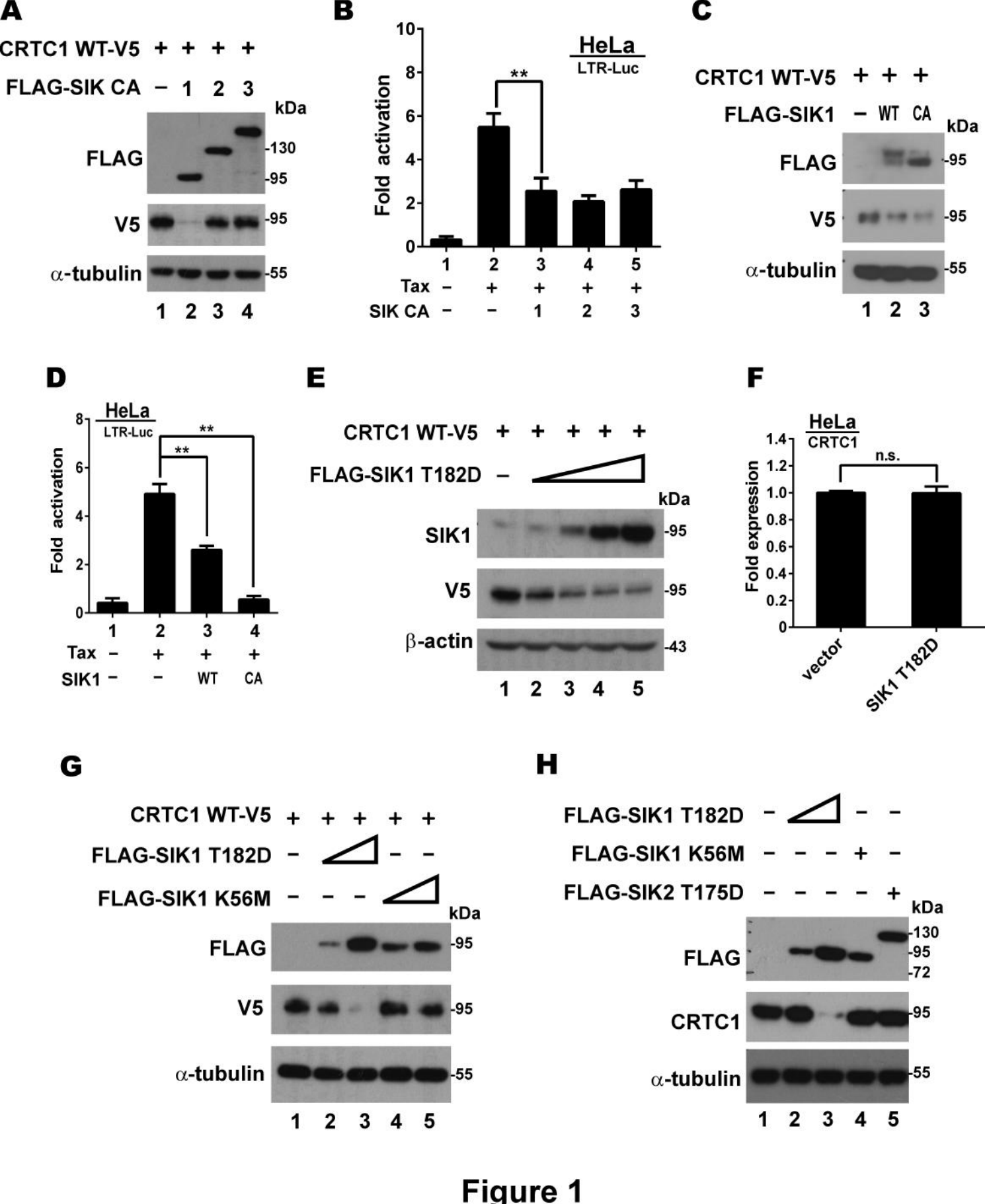
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Figure 1

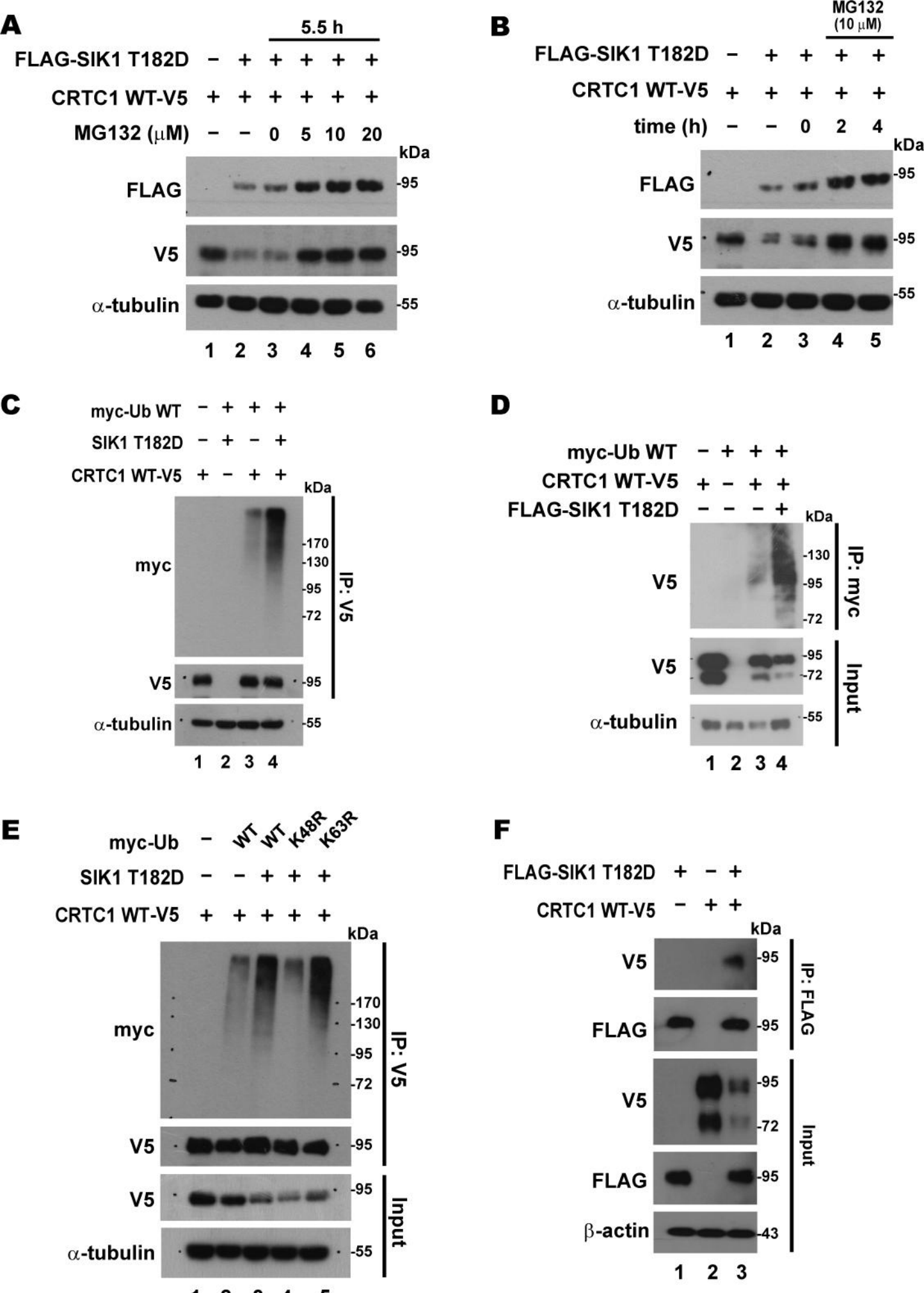
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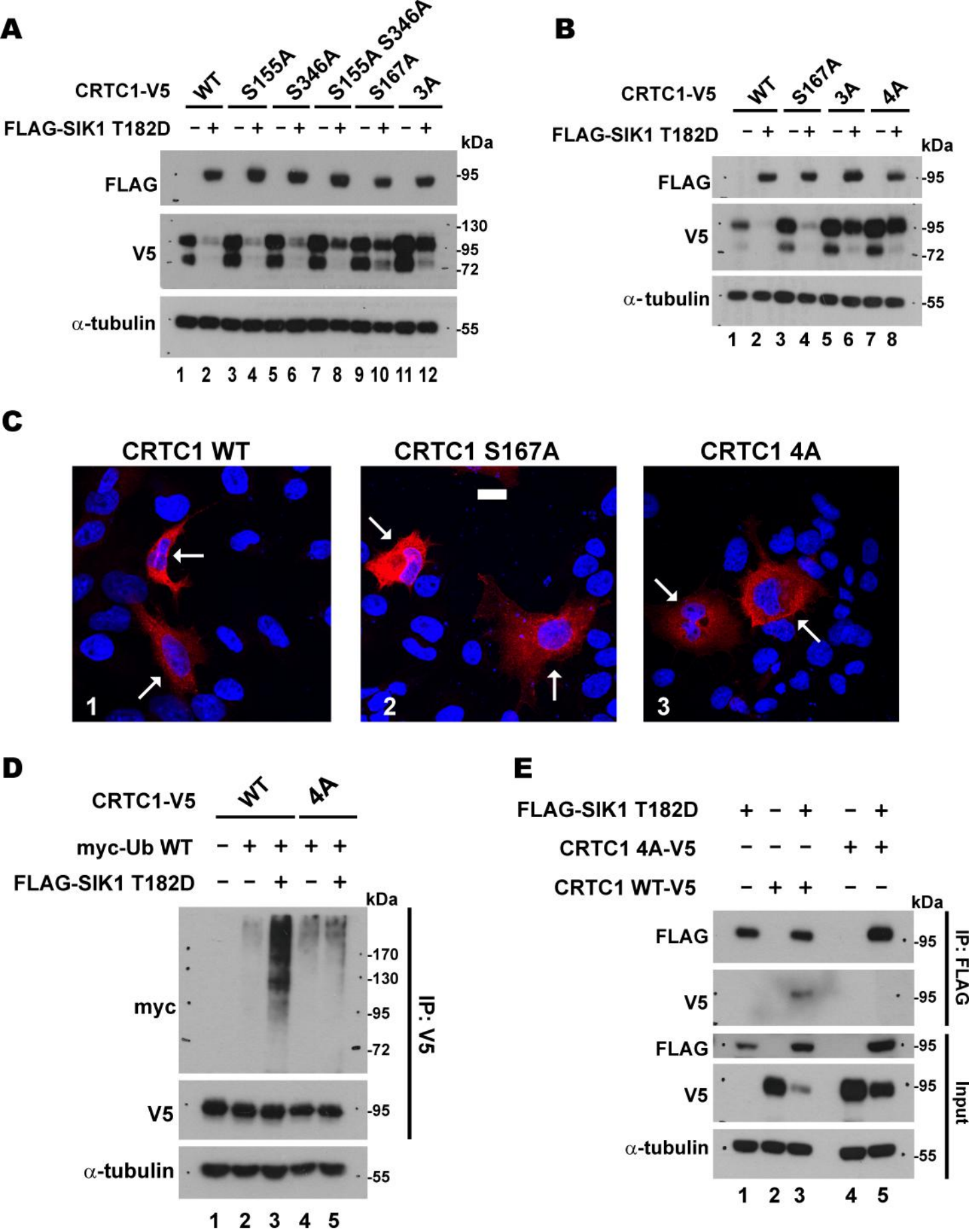
Figure 2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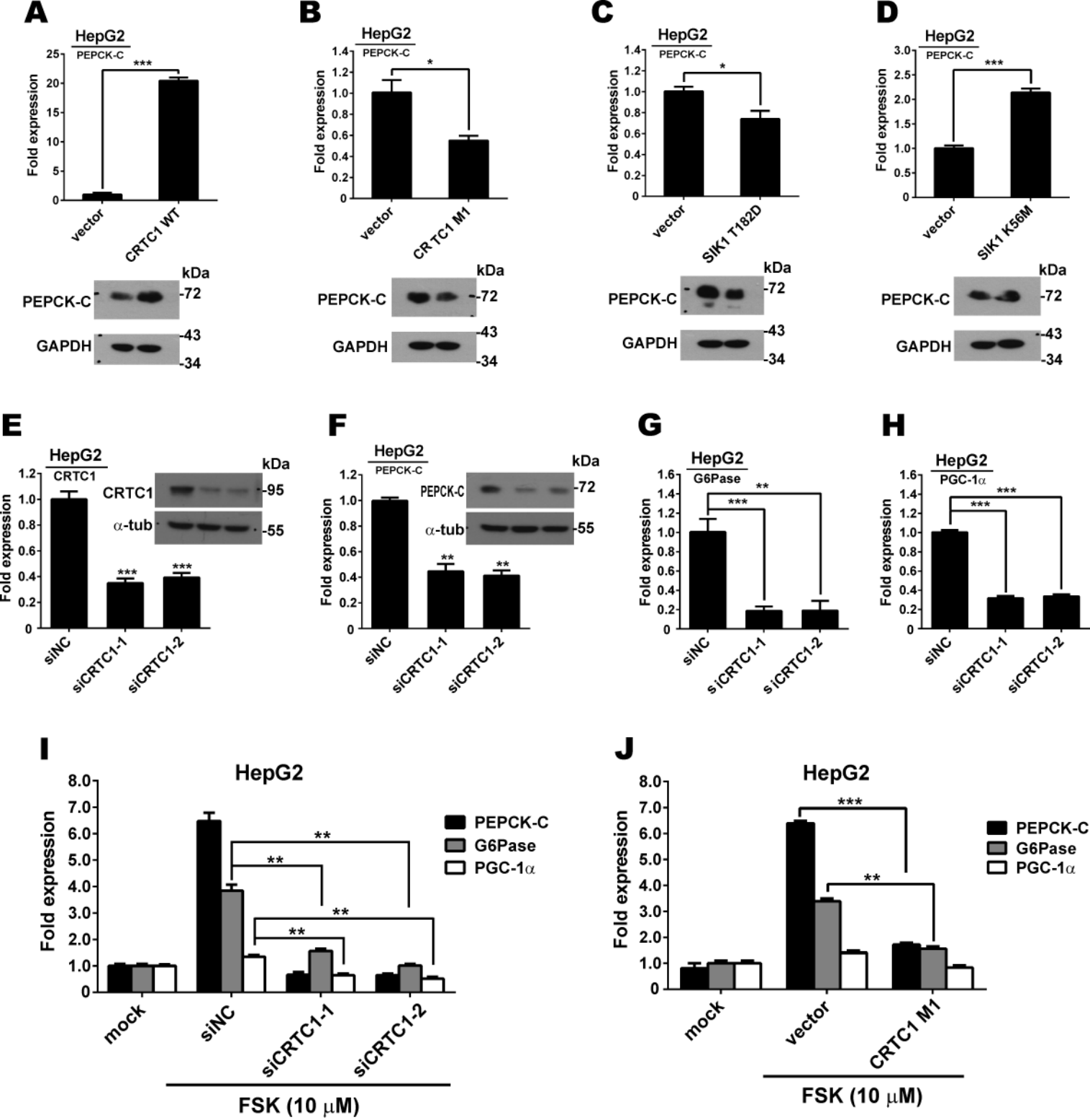
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G
HepG2
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H
HepG2
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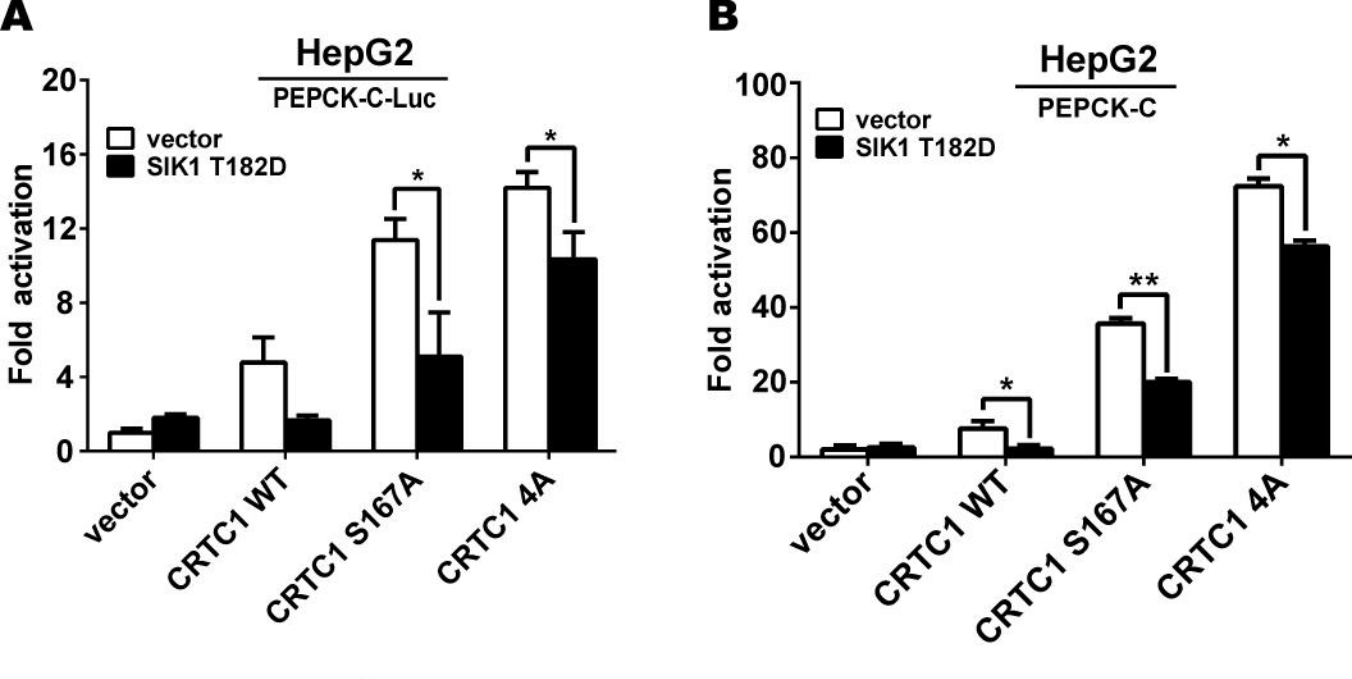
Figure 4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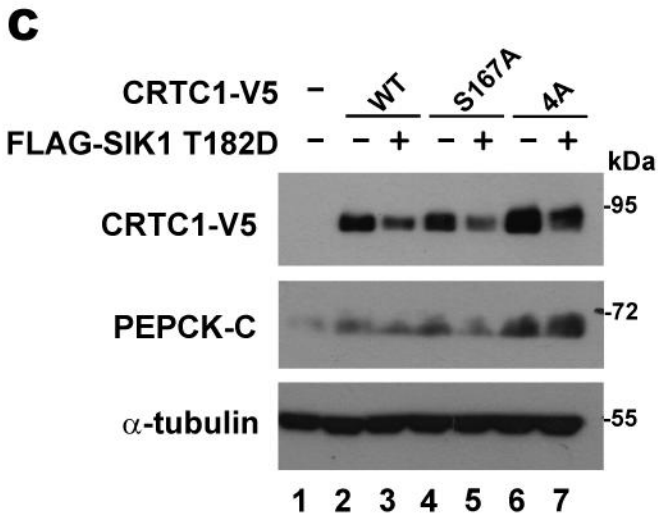
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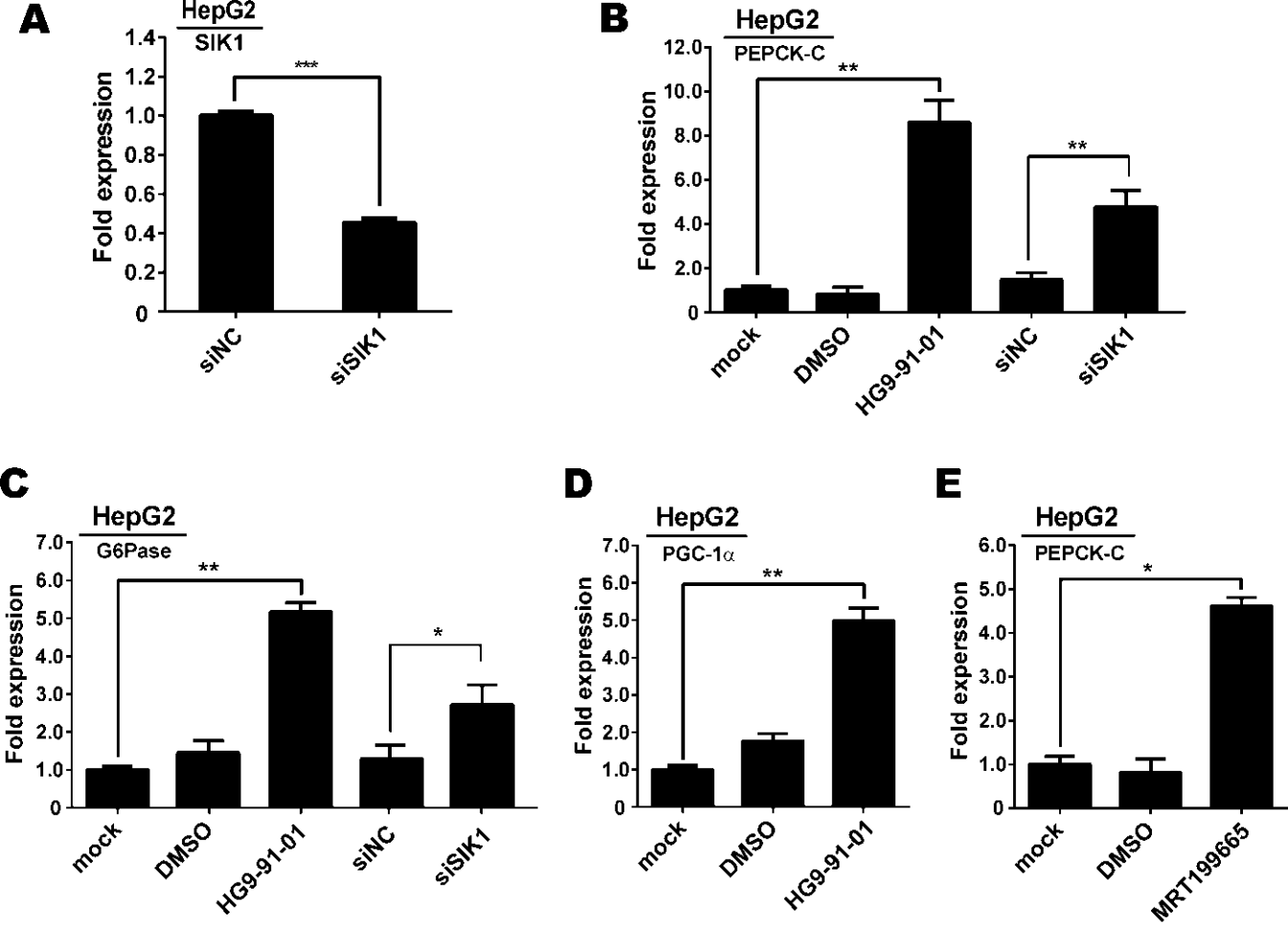
Figure 5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PEPCK-C
Fold experssion
6.0
5.0
4.0
3.0
2.0
1.0
0
*
mock
DMSO
MRT199665

Figure 6

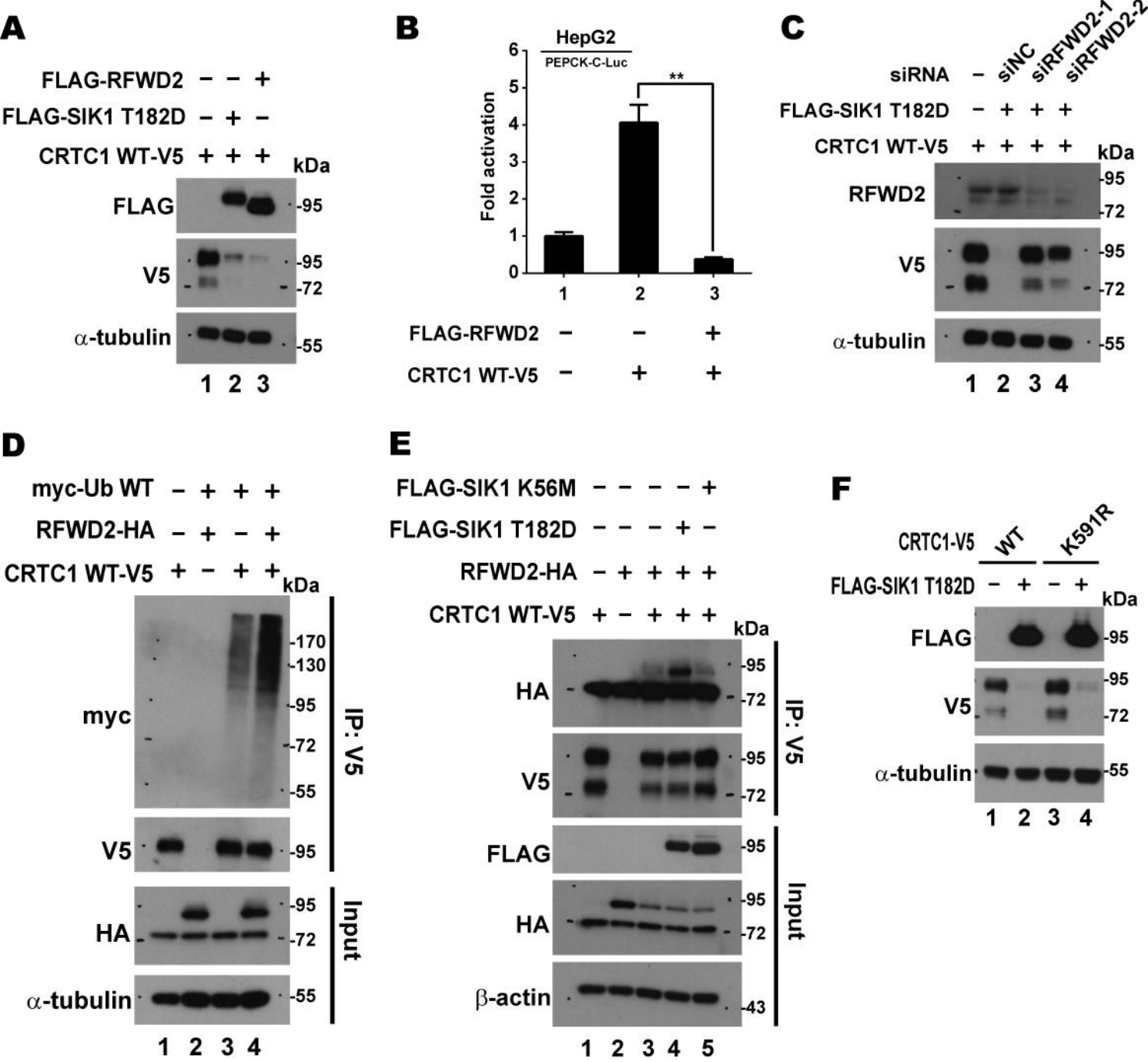
A
FLAG-RFWD2 − − +
FLAG-SIK1 T182D − + −
CRTC1 WT-V5 + + +
kDa
FLAG
V5
α-tubulin
-95
-72
-55
1 2 3
B
HepG2
PEPCK-C-Luc
Fold activation
**
FLAG-RFWD2 − − +
CRTC1 WT-V5 − + +
C
siRNA − siNC siRFWD2-1 siRFWD2-2
FLAG-SIK1 T182D − + + +
CRTC1 WT-V5 + + + +
RFWD2
V5
α-tubulin
1 2 3 4
D
myc-Ub WT − + + +
RFWD2-HA − + − +
CRTC1 WT-V5 + − + +
myc
V5
HA
α-tubulin
-170
-130
IP: V5
Input
1 2 3 4
E
FLAG-SIK1 K56M − − − − +
FLAG-SIK1 T182D − − − + −
RFWD2-HA − + + + +
CRTC1 WT-V5 + − + + +
HA
V5
FLAG
β-actin
-43
1 2 3 4 5
F
CRTC1-V5 WT K591R
FLAG-SIK1 T182D − + − +
FLAG
V5
α-tubulin
1 2 3 4

Figure 7