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Calcium/calmodulin-dependent protein kinase kinase 2 mediates pleiotropic effects of epidermal growth factor in cancer cells

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ABSTRACT

Aims: Engagement of epidermal growth factor (EGF) with its receptor (EGFR) produces a broad range of cancer phenotypes. The overriding aim of this study was to understand EGFR signaling and its regulation by the Ca^{2+}/calmodulin (CaM) dependent protein kinase kinase 2 (CaMKK2) in cancer cells.
Results: In ovarian cancer cells and other cancer cell types, EGF-induced activation of oncogenic Akt is mediated by both the canonical PI3K-PDK1 pathway and by CaMKK2. Akt activation induced by EGF occurs by both calcium-dependent and calcium-independent mechanisms. In contrast to the canonical pathway, CaMKK2 neither binds to, nor is regulated by phosphoinositides but is activated by Ca^{2+}/CaM. Akt activation at its primary activation site, T308 occurs by direct phosphorylation by CaMKK2, but activation at its secondary site (S473), is through an indirect mechanism requiring mTORC2. In cells in which another CaMKK2 target, 5'AMP-dependent protein kinase (AMPK) was deleted, Akt activation and calcium-dependency of activation were still observed. CaMKK2 accumulates in the nucleus in response to EGF and regulates transcription of phosphofructokinase platelet (PFKP) a glycolytic regulator. CaMKK2 is required for optimal PFK activity. CaMKK2 regulates transcription of plasminogen activator, urokinase (PLAU) a metastasis regulator. The EGFR inhibitor gefitinib synergizes with CaMKK2 inhibition in the regulation of cell survival and increases the dose-reduction index. CRISPR/Cas9 knockout of CaMKK2 leads to compensatory PTEN downregulation and upregulation of Akt activation.
Conclusions: CaMKK2-mediation of EGFR action may enable cancer cells to use intracellular calcium elevation as a signal for growth and survival.

1. Introduction

The epidermal growth factor (EGF) receptor (EGFR) is a receptor tyrosine kinase and prototypical member of the ErbB (HER) family of proteins. The connection of the EGFR (ErbB1) to cancer was revealed when it was found that the ErbB oncogene of the avian erythroblastosis virus was a mutated homologue of the EGFR [1,2]. EGFR mutations and overexpression are drivers of a large number of malignancies including non-small cell lung cancer, glioblastoma, colon cancer, skin, and breast cancers and the focus of this study, ovarian cancer (OVCa) [3]. The significance of EGFR-mediated signaling was recently highlighted as a key outcome of the Human Genome Project based on the finding that EGFR mutations (e.g. EGFRvIII, L858R, T790M) are predictive of responses to EGFR inhibitors [4]. EGFR alterations hyper-activate downstream signaling pathways, most prominently the RAS-RAF-MEK-ERK cascade in the MAPK pathway and the phosphoinositide 3-kinase (PI3K)-phosphoinositide-dependent kinase-1 (PDK1)-Akt pathway. Activation of these pathways result in cell cycle progression, inhibition of apoptosis, angiogenesis, transcriptional regulation and other cancer phenotypes [5–7].

Regulation of cellular function by increased intracellular calcium (Ca^{2+}_i) is often mediated by the ubiquitous calcium receptor calmodulin (CaM). An important class of effectors of Ca^{2+}/CaM are the multifunctional Ca^{2+}/CaM-dependent protein kinases, CaMK I, II and IV [8].

Activated CaMKs perform essential cellular functions including growth, cell cycle progression, gene transcription, mRNA translation and cell survival. We found CaMKs I and IV to be activated not only allosterically by Ca^{2+}-liganded CaM but also by phosphorylation catalyzed by two upstream kinase kinases CaMKKα and CaMKKβ (gene names, *CaMKK1* and *CaMKK2* respectively) [9]. CaMKK2 was subsequently reported to function as a kinase kinase for the regulator of energy homeostasis 5′AMP-dependent protein kinase (AMPK) [10–12]. Due to its comprehensive role in promoting cell growth, CaMKK2 have been associated with progression of multiple cancers such as prostate, and hepatic, cancers [13–17].

In a previous report we found that CaMKK2 directly phosphorylates and activates Akt at its primary activation site, T308 in vitro and in OVCa cells and is necessary for optimal activation of Akt at its secondary activation site, S473 [18]. However this regulation was observed under normal growth (steady-state) conditions. Thus, the critical participation of EGFR signaling was not examined. Although, EGFR mutations and overexpression are drivers of many cancers, EGFR signaling is of particular importance for OVCa since about 70% of ovarian carcinomas express activated EGFR [5]. And there is evidence that EGFR mutation or amplification may develop with drug treatment consistent with a role in chemoresistance [7]. However there are currently no EGFR inhibitors approved for OVCa suggesting a pressing need to better understand signaling downstream of the EGFR in OVCa.

Given the pleiotropy of the EGFR in cellular physiology, the overriding aim of this study was to discern the participation of calcium, CaM and CaMKK2 in a broad range of EGFR-regulated events in OVCa, and other cancer cells. Regulation of the c-proto-oncogene Akt by the EGFR was studied since Akt is one of the most heavily mutated and hyperactivated effectors among all cancers and plays an important role in chemotherapy resistance [6,19]. Other objectives of the study were to examine whether CaMKK2 and PDK1 share mechanistic similarities in Akt activation, regulation of p-Akt (S473) and the role of mTORC2, possible participation of the CaMKK2 target AMPK in Akt activation, and CaMKK2 nuclear translocation and its role in transcriptional regulation. Another, and potentially clinically relevant objective of the study was to examine the potential for synergy between inhibitors of the activities of the EGFR and CaMKK2. The final objective of the study was to assess whether a CRISPR/Cas9 knockout of CaMKK2 could serve as a model for its therapeutic targeting in OVCa.

2. Materials and methods

2.1. Materials

EGF (#E9644), Rapamycin (#553211-1MG), W7 (#681629), CaM (#208690) and Gefitinib (#SML1657) were purchased from Sigma-Aldrich. STO-609 was purchased from Tocris (#1551). BAPTA-AM (#B6769), ionomycin (#I24222), baculovirus expressed PDK1 (#P3001) and CaMKK2 (#PV4206) were purchased from ThermoFisher Scientific. CaMKK2-GFP (#RG222692) was obtained from Origene. Recombinant Akt1 was provided by Thomas Franke. A PIP Array-Lipid Protein Interaction Assay (#P-6100) was purchased from Echelon Biosciences. Water soluble PIP3 (C18) (#64930) was purchased from Cayman Chemical. AZD2014 (#A11303) was purchased from AdooQ. myc-Rictor was from Dr. David Sabatini, obtained from Addgene (plasmid #11367). p-Akt (T308) (#13038), p-Akt (S473) (#4060), pan-Akt (#4691), PDK1 (#13037), p-AMPK (T172) (#2525) and AMPK antibodies (#2603) were purchased from Cell Signaling Technology. CaMKK2 antibody for western blotting (#H00010645) was purchased from Novus and CaMKK2 antibody for immunofluorescence microscopy (#11549-1-AP) was purchased from Proteintech group. GAPDH (sc-47724) and Rictor (sc-271081) antibodies were purchased from Santa Cruz Biotechnology.

2.2. Cell culture

OVCAR-3, A431, A549, N87, LNCaP and M059J cells were obtained from American Type Culture Collection (ATCC). WT HeLa and AMPK α1/α2 double knockout (DKO) HeLa cells were cultured as previously described [20]. OVCAR-3 cells were cultured in RPMI-1640 with 20% fetal bovine serum (FBS) and 0.01 mg/mL insulin. A431, A549 cells were cultured in F-12 K with 10% FBS. N87 and LNCaP cells were cultured in RPMI-1640 with 10% FBS. M059J cells were cultured in DMEM/F12K with 10% FBS.

2.3. RNA interference

Cells were transfected with 40 nM of non-specific (NS), PDK1 or CaMKK2 siRNAs (Life Technologies) using Lipofectamine 2000 for 2 d. CaMKK2 siRNA Silencer Select (sense, 5′-GCAUCGAGUACUUACA-CUAtt-3′) and PDK1 siRNA Silencer Select (sense, 5′-GGAACA GCGCA-GUACGUUUtt-3′) siRNAs were used in RNAi experiments as previously described [18]. A second CaMKK2 siRNA was used as control, CaMKK2 siRNA #2 (sense, 5′-GGAUCUGAUCAAAGGCAUCtt-3′).

2.4. EGF and drug treatments

EGF stimulation was performed by treating cells with 100 nM of EGF for 5 min after 30 min of serum starvation. Cells were treated with 1 μM ionomycin for 15 min after 30 min serum starvation. For BAPTA and W7 experiments, cells were pre-treated with 10 μM BAPTA-AM or 10 μM W7 during the 30 min serum starvation period. AZD2014 and Rapamycin experiments were conducted by pre-treating cells with AZD2014 (500 nM) or Rapamycin (500 nM) for 1 h.

2.5. PIP array assay

The PIP array assay was conducted as described in the instruction manual. Briefly, the PIP array membrane was blocked with 3% bovine serum albumin (BSA) for 1 h at room temperature. The membrane was then incubated with 1 μg/mL of PDK1 or CaMKK2 in the presence or absence of 1 mM $CaCl_2$ and 1 μM CaM for 1 h at room temperature. To detect protein binding, the membrane was incubated with PDK1 or CaMKK2 antibodies and secondary antibodies. Binding was detected by enhanced chemiluminescence (ECL).

2.6. CaMKK2 activity assays

Baculovirus-expressed CaMKK2 (187.5 nM) was incubated with a peptide derived from the Akt sequence flanking T308 (KTFCG**T**PEYLA-PEVRR) (360 μM) for 20 min at 30 °C in kinase reaction buffer (50 mM HEPES, 2 mM DTT, 10 mM $MgCl_2$, 0.5 mg/mL BSA, ATP (200 μM) with [γ-32P] ATP (PerkinElmer). Peptide phosphorylation was quantified as described previously [18] in the presence and absence of PIP3 (20 μM) and/or Ca^{2+} (1 mM)/CaM (1 μM). Recombinant baculovirus-expressed Akt1 was incubated with CaMKK2 in the same reaction buffer for 20 min at 30 °C. Akt phosphorylation at T308 was detected by western blot using p-Akt (T308) antibody.

2.7. Rictor overexpression

CaMKK2 siRNA (40 nM) and myc-Rictor (3 μg) were co-transfected in OVCAR-3 cells for 2 d. Cells were then stimulated with 100 nM EGF for 5 min or 1 μM ionomycin for 15 min after 30 min of serum starvation.

2.8. Imaging of nuclear CaMKK2

OVCAR-3 cells transfected with CaMKK2-GFP for 2 d were plated on 12 mm cover slips. After EGF stimulation for 30 min, cells were fixed with 4% paraformaldehyde (PFA) and stained with DAPI. Cells with

nuclear or cytoplasmic CaMKK2 were counted (blindly as to condition) under a fluorescence microscope. Nuclear/Cytosol CaMKK2 = the number of cells with nuclear CaMKK2 divided by the number of cells with cytosolic staining (n = 3, 1009 cells counted). For endogenous CaMKK2 imaging, OVCAR-3 cells were plated on 12 mm cover slips overnight then simulated by EGF for 30 min. Cells were fixed with 4% PFA and permeabilized with 0.2% Triton X-100. After blocking with 5% BSA, cells were incubated with CaMKK2 primary antibody, Alexa Fluor 488-conjugated secondary antibody and DAPI. Confocal images were taken to visualize CaMKK2 localization.

2.9. Subcellular fractionation

OVCAR-3 cells were transfected with CaMKK2-GFP for 2 d then stimulated with EGF for 10 min after 30 min of serum starvation. The subcellular fractionation protocol was modified from the method of Schreiber et al. [21]. Cells were harvested in 1 mL cold TBS with scrapers and pelleted at 1500 $\times g$ for 30 s at 4 °C. The cell pellet was re-suspended in 100 μL cold buffer A (10 mM HEPES pH 7.9, 1 mM DTT, 10 mM KCL, 0.1 mM EGTA, 0.1 mM EDTA, 0.5 mM PMSF) by gentle tapping. After sitting on ice for 15 min, 6.25 μL of a 10% solution of NP-40 alternative (Sigma-Aldrich) was added followed by vigorous vortexing for 10 s. The mixture was centrifuged at 1500 $\times g$ for 30 s to produce a crude nuclear pellet. The supernatant was re-centrifuged at 100,000 $\times g$ for 5 min. The supernatant was transferred to a fresh tube with buffer B (10 mM Tris pH 7.5, 20 mM EDTA, 7 M urea, 0.3 M NaAc, 1% SDS) and saved as the cytosolic fraction. The nuclear pellet was re-suspended in 400 μL cold buffer A, swelled on ice for 15 min followed by the addition of 25 μL of a 10% solution of NP-40 alternative. The tube was vigorously vortexed for 10 s and re-centrifuged at 1500 $\times g$ for 30 s. The supernatant was removed and the nuclear pellet was re-suspended in 50 μL ice-cold buffer C (20 mM HEPES pH 7.9, 1 mM DTT, 1 mM EGTA, 1 mM EDTA 0.4 M NaCl, 1 mM PMSF) with vigorous vortexing for 15 min at 4 °C. After centrifugation at 1500 $\times g$ for 5 min at 4 °C, the supernatant was stored as the nuclear fraction.

2.10. qRT-PCR

OVCAR-3 cells were transfected with CaMKK2 siRNA for 2 d, following which they were serum starved for 24 h, then treated with EGF (100 nM) for 3 h. RNA was extracted using an RNeasy kit (Qiagen). qRT-PCR was performed as described previously [18]. Quantification was performed by the ΔΔCt method with GAPDH as the reference gene. Primer sequences for quantification of the expression of PFKP, PLAU and GAPDH are respectively: (sequences are 5′ → 3′): PFKP, forward: CAGCTATGACGTGTCGGACT, reverse: ATGGGCACTCGCCGATTAG; PLAU, forward: CCGCTTTCTTGCTGGTTGTC, reverse: AGGCC-TATGCCTGAGGGTAA; GAPDH, forward: TGGCATTGCCCTCAACGAC-CACTTTG, reverse: TCCTCTTGTGCTCTTGCT GGGGCTG.

2.11. Dose-response and drug combination assay

OVCAR-3 cells were plated overnight then cells were treated with varying concentrations of Gefitinib and STO-609 individually or in combination. The two-drug combination was conducted by mixing the drugs at a constant concentration ratio of 1:1. Live and dead cells were counted after 3 days by Trypan blue (#T8154, Sigma-Aldrich) staining using a TC20 cell counter (Bio-Rad). IC-50, combination index (CI) and dose-reduction index (DRI) parameters were calculated using Compu-Syn software.

2.12. Phosphofructokinase (PFK) activity assay

PFK activity was measured using a colorimetric kit (# K776, Bio-Vision). In brief, OVCAR-3 were transfected with 40 nM NS or CaMKK2 siRNAs for two days. Cells were harvested in ice-cold assay buffer. The

reaction mix was prepared according to manufacturer's instructions. 0.5 μg of protein/well were used for each sample and the background control. 50 μl of the reaction mixture was added to each well and mixed thoroughly. Absorbance was taken at 450 nm on a microplate reader in kinetics mode at 37 °C for 20 min.

2.13. CaMKK2 knockout using CRISPR/Cas9 genome editing

Guide RNA was designed based on the following. Exon 10 of the CaMKK2 gene is shared by all 17 of its transcript variants. Thus to avoid splice products with residual activity this exon was targeted. Moreover D312 within the DFG motif in exon 10 of CaMKK2 is important for Mg^{2+}-ATP binding within the catalytic cleft and therefore for catalysis. Therefore R311 5′ of D312 was targeted [22,23]. The gRNA (TGGAAGGTTTGATGTCACGGTGG) was designed and verified by the Genome Institute of Washington University, St. Louis. The gRNA was then inserted into the plasmid expressing Cas 9 and GFP (#48138, Addgene) and transfected into OVCAR-3 cells for 2 days. A wild type control was generated by transfecting an empty vector plasmid without gRNA. GFP positive cells were FACS sorted into 96 well plates as single cell clones. Surviving cells were gradually expanded into clonal cell lines in about two months. CaMKK2 knockout in the clonal cell lines was verified by sequencing of each allele with TA cloning (#450245, Thermo Fisher). Western blotting was also used to verify CaMKK2 knockout. The two clonal CaMKK2 KO lines are described in more detail in Supplemental Fig. 5. As shown, no residual fragments that could behave as dominant negative inhibitors were detected, due presumably to nonsense-mediated decay.

2.14. RNA-seq and analysis

Total RNA was extracted from CaMKK2 WT and CaMKK2 KO cells using the RNeasy kit (#74104, Qiagen) with three biological replicates for each cell line. The RNA-seq libraries were constructed by Illumina Truseq Stranded Total RNA kit with ribosomal RNA removal beads. Sequencing was carried out with the NextSeq 500 platform at the Genomics and Bioinformatics Core of the University at Buffalo, State University of New York. RNA-seq reads were aligned with HISAT2 version 2.1.0 using default parameters. UCSC hg38 was used for the reference genome and gene annotation set. Sequence alignments were compressed and sorted into binary alignment map (BAM) files using Samtools version 1.3. Counting of mapped reads for genomic features was performed using Subread featureCounts version 1.6.2. Differentially expressed genes were detected using the Bioconductor package DESeq2 version 1.20.0 with alpha of 0.05. Pathway enrichment analysis was done by Metascape using the Reactome Gene Sets.

2.15. Western blotting

Western blotting was performed as described previously [18]. Protein levels were quantified with local background subtraction using Bio-Rad Image Lab software.

2.16. Statistical analysis

Statistical significance was determined by unpaired Student's *t*-test for experiments with two conditions and one-way ANOVA followed by Sidak's post hoc test for multiple comparisons.

3. Results and discussion

3.1. CaMKK2 mediates EGF-induced Akt activation

We previously documented requirements of calcium, CaM and CaMKK2 for optimal activation of Akt by its phosphorylation at T308 and S473 during steady-state cell growth [18]. Since Akt is a crucial
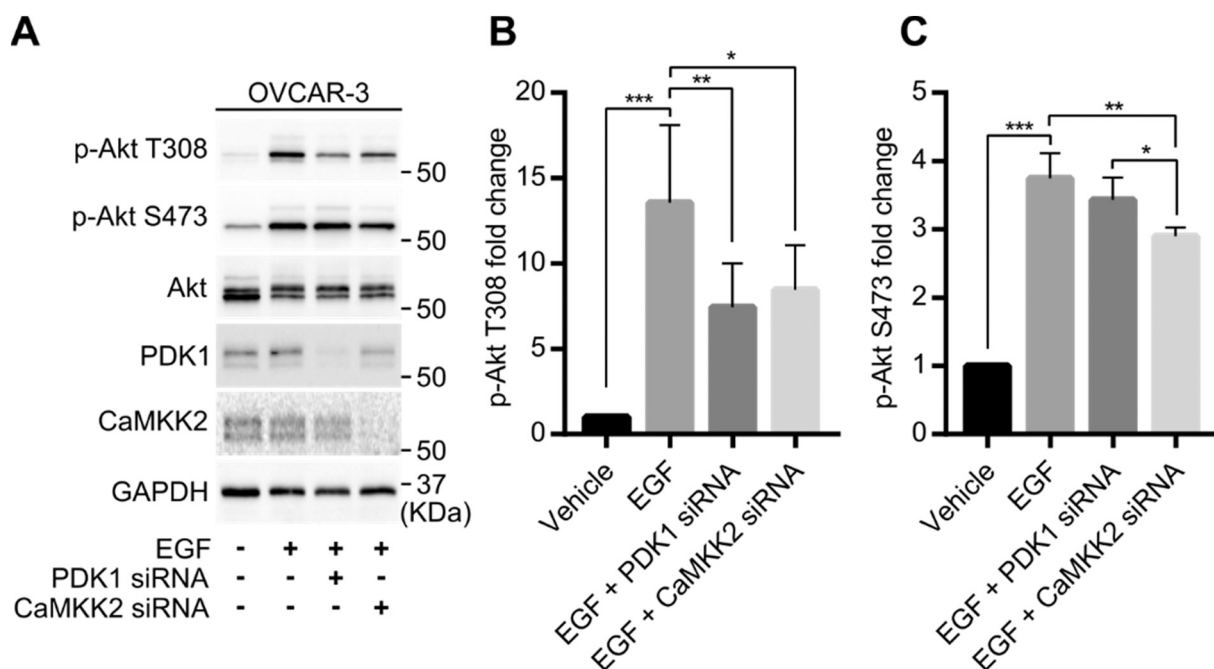
Fig. 1. CaMKK2 and PDK1 mediate Akt phosphorylation stimulated by EGF. (A) OVCAR-3 cells were transfected with non-specific (NS), PDK1 or CaMKK2 siRNAs for 2 days as shown. Cells were then serum-starved for 30 min, followed by EGF treatment (100 nM) for 5 min. Samples were analyzed by Western Blotting. Quantification of Akt phosphorylation at T308 and S473 is shown in (B) and (C) respectively (n = 4). Results are normalized to vehicle control and shown as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

mediator of EGFR signaling, an important objective of the present research was to examine Akt activation under acute stimulation by EGF. As shown, OVCAR-3 cells responded to EGF with Akt phosphorylation at its primary activation site, T308 (Fig. 1A, B). RNAi mediated knockdowns of PDK1 and CaMKK2 produced similar levels of reductions in p-Akt (T308). At S473 of Akt, EGF stimulation produced, in general, less dependence on CaMKK2 and PDK1 phosphorylation than at T308. At this site regulation by CaMKK2 was still observed while PDK1 knockdown was ineffective at reducing EGF-induced p-Akt (S473) (Fig. 1A, C).

Pinilla-Macua1 et al. had reported that EGF concentrations in *in vivo* tumor xenografts are in the ng/mL range [24]. Therefore a concentration of EGF at 10 ng/mL was tested. Consistent with the results of Fig. 1, CaMKK2 regulated Akt phosphorylation in this range (Fig. S1B).

To determine whether regulation of Akt phosphorylation by both CaMKK2 and PDK1 is restricted to OVCa cells, CaMKK2 and PDK1-mediation of EGF-induced Akt phosphorylation at both sites was studied in A431 (epidermoid carcinoma cells), A549 (non-small cell lung cancer cells), NCI-N87 (epithelial gastric cancer cells) and LNCaP (prostate cancer cells) (Fig. S1A). CaMKK2 and PDK1 knockdowns both reduced Akt phosphorylation. Considering both ovarian and non-ovarian cancer cells, these results indicate that the role CaMKK2 plays in Akt activation is not restricted to OVCa cells and is of comparable importance to PDK1 for EGF-induced Akt activation at its primary phosphorylation site T308. CaMKK2 is possibly of greater importance for phosphorylation at S473 since all cell lines showed prominent reductions in p-Akt (S473) after CaMKK2 knockdown. Ultimately, large scale gene expression profiling studies or tissue microarrays may yield a more complete accounting of those cancers in which EGF-dependent CaMKK2 mediation of Akt activation is of particular significance.

3.2. Akt phosphorylation demonstrates Ca²⁺/CaM dependence in response to EGF stimulation

CaMKK2 is an enzyme dependent on the presence of adequate levels of calcium for its activity [9]. We therefore investigated the role of intracellular calcium (Ca^{2+}_i) in EGF-induced Akt activation and its

potential mediation by CaMKK2 and/or PDK1. The respective Ca^{2+}_i requirements for CaMKK2 and/or PDK1 were evaluated in gain- and loss-of-function experiments in which the calcium ionophore, ionomycin, was used to increase Ca^{2+}_i, and conversely, BAPTA-AM to chelate and reduce its concentration. Both PDK1 and CaMKK2 mediated Akt phosphorylation at T308 in response to ionomycin treatment (Fig. 2A, B). And similarly to responsiveness to EGF (Fig. 1), depletion of CaMKK2, but not that of PDK1, reduced p-Akt (S473) elevation in response to ionomycin (Fig. 2A, C). Similar effects of CaMKK2 knockdown on Akt phosphorylation was observed using a second CaMKK2 siRNA (Fig. S3).

In addition to its activation of PI3K, EGF induces Ca^{2+}_i elevation through the generation of inositol trisphosphate (IP3) via activation of phospholipase C γ (PLCγ) [25]. We asked whether EGF stimulation of p-Akt (T308) and/or p-Akt (S473) are Ca^{2+}_i-dependent events. As shown, Akt phosphorylation at both activating sites is Ca^{2+}_i regulated (Fig. 2D–F). In addition there is a non-Ca^{2+}_i requiring component(s) which contributes to the effects of EGF stimulation. Since CaMKK2 depends for its activity on allosteric activation by Ca^{2+}/CaM we examined effects of the CaM inhibitor W-7 on Akt activation. At both sites, CaM inhibition reduced Akt phosphorylation with effects very similar to those observed for Ca^{2+}_i-dependence (Fig. 2G–I). Ca^{2+}/CaM dependence of Akt phosphorylation was also observed in A431, A549 and N87 cells and in OVCAR-3 cells with CaMKK2 siRNA #2 (Figs. S2, 3). Overall, these results indicate that EGF stimulation of Akt activating phosphorylation is a Ca^{2+}_i/CaM-regulated process.

The inability of BAPTA-AM and W7 to fully block EGF-induced Akt phosphorylation at the two sites as well as the lower efficacy of ionomycin relative to that of EGF, suggest that unlike cells growing under steady-state conditions in which BAPTA-AM reduces Akt phosphorylation ~10-fold [18], the data shown here are consistent with EGF signaling involving both Ca^{2+}/CaM-dependent, and -independent mechanisms of Akt activation [18]. It will be of interest in future studies to identify such EGF-, but non-Ca^{2+}/CaM-dependent mechanisms. In addition to kinases not directly activated by Ca^{2+}/CaM such as PDK1, the ability of CaMKK2 to auto-phosphorylate and regulate the degree to
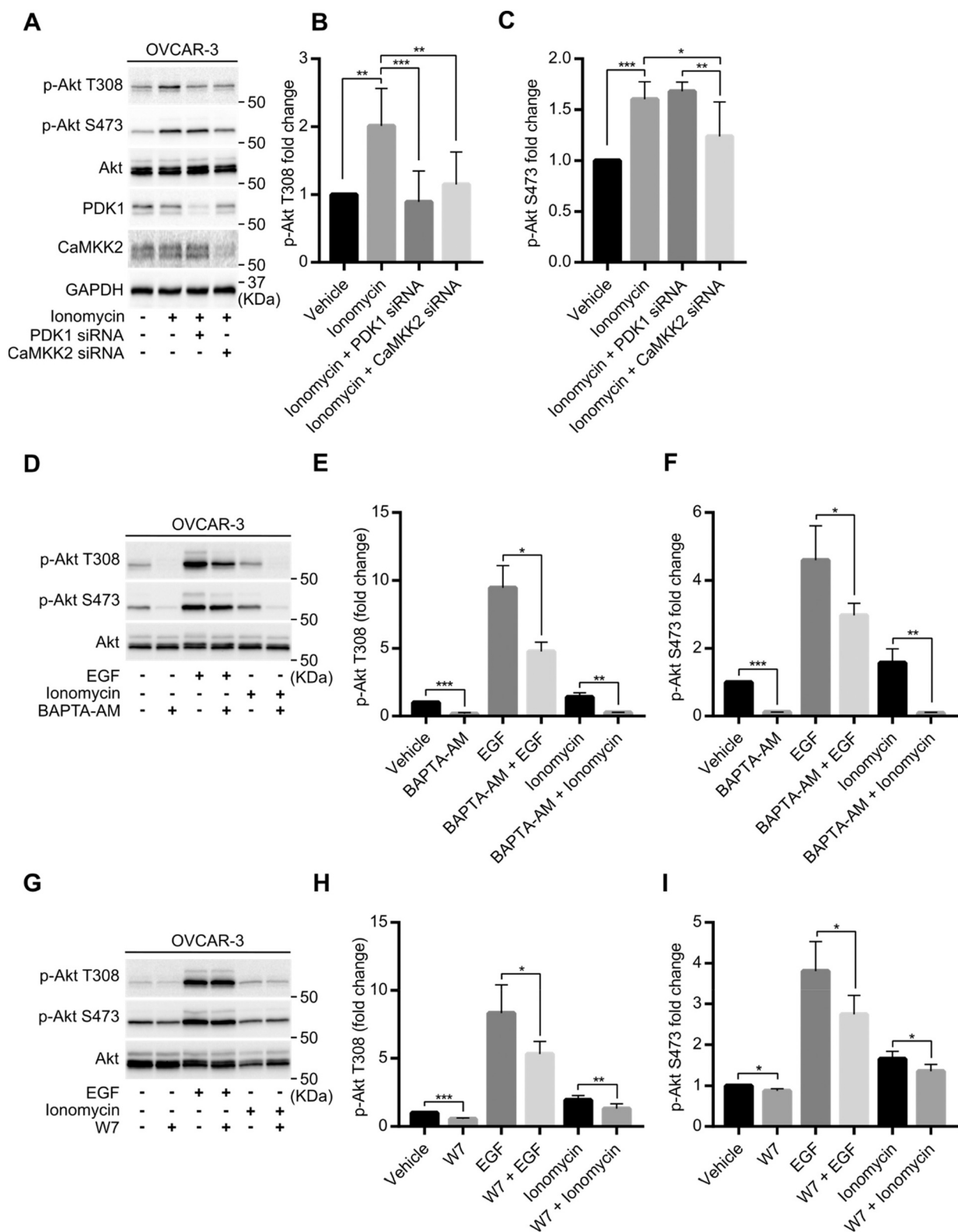
Fig. 2. Calcium and calmodulin regulation of Akt phosphorylation in response to EGF or the calcium ionophore, ionomycin. (A) OVCAR-3 cells were transfected with NS, PDK1 or CaMKK2 siRNAs for 2 days as shown. Cells were then serum starved for 30 min, followed by ionomycin treatment (1 μM) for 15 min. Samples were analyzed by Western Blotting. Quantification of Akt phosphorylation at T308 and S473 is shown in (B) and (C), respectively (n = 6). (D) OVCAR-3 cells were pre-treated with BAPTA-AM (10 μM) as shown during the 30 min serum starvation. Cells were then treated with EGF (100 nM) for 5 min or ionomycin (1 μM) for 15 min. Samples were analyzed by Western Blotting. Quantification of Akt phosphorylation at T308 and S473 is shown in (E) and (F), respectively (n = 3). (G) OVCAR-3 cells were pre-treated with W7 (10 μM) as shown during the 30 min serum starvation. Cells were then treated with EGF (100 nM) for 5 min or ionomycin (1 μM) for 15 min. Samples were analyzed by Western Blotting. Quantification of Akt phosphorylation at T308 and S473 is shown in (H) and (I), respectively (n = 6). (B, C, E, F, H, I) Results are normalized to control and shown as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
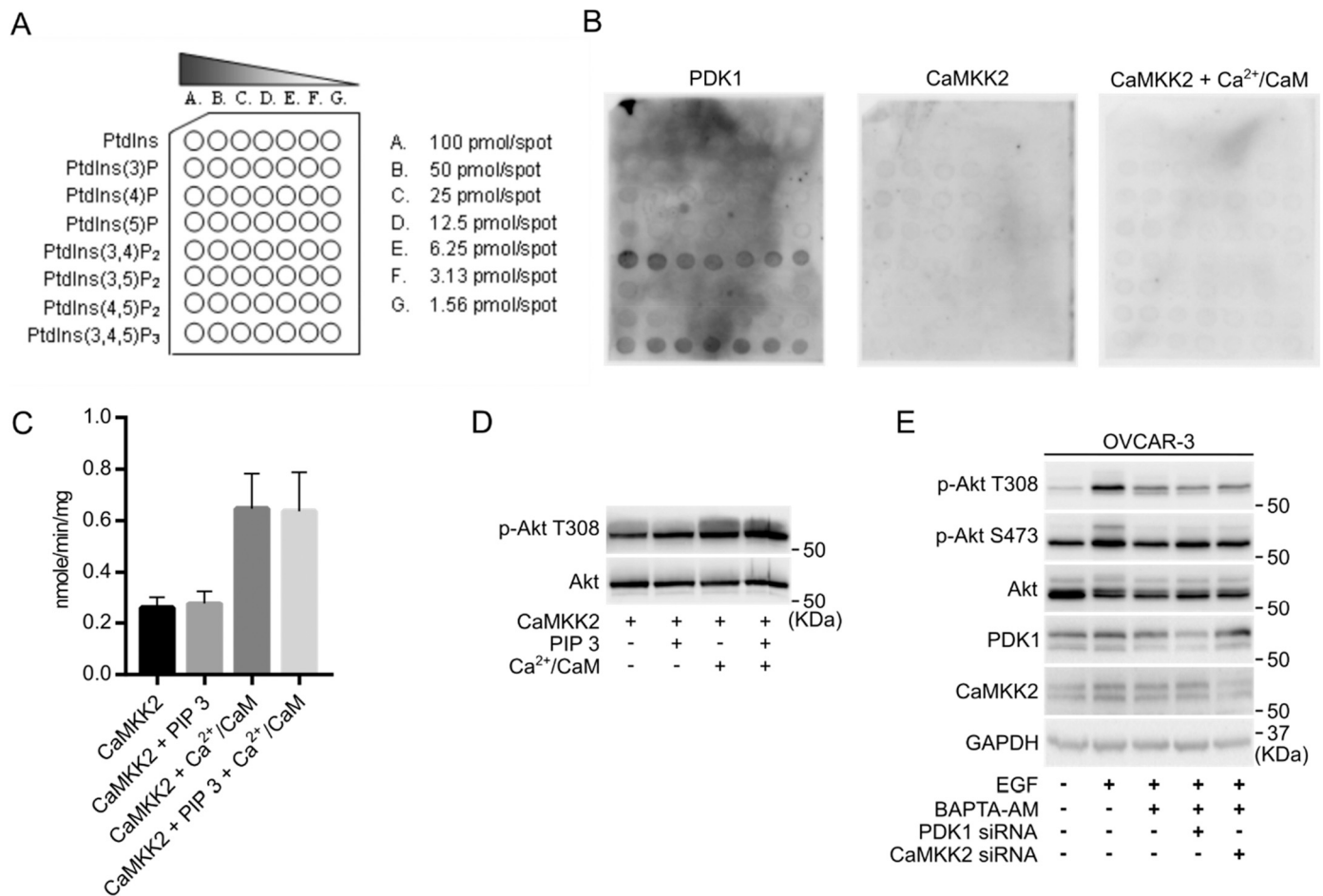
Fig. 3. CaMKK2 neither binds to, nor is regulated by, PIP3 but is activated by Ca^{2+}/CaM. (A) Schematic diagram of the phosphoinositide (PIP) array (Echelon Biosciences) used in these experiments. (B) The PIP array was incubated with baculovirus-expressed-PDK1 or CaMKK2 in the presence or absence of Ca^{2+}/CaM. Proteins binding on the array were detected by blotting with PDK1 and CaMKK2 antibodies. (C) CaMKK2 kinase activity was measured using a peptide substrate derived from the Akt sequence flanking T308 and [γ-32P] ATP in the presence or absence of PIP3 and/or Ca^{2+}/CaM (n = 3) (D) Baculovirus-expressed CaMKK2 and Akt were incubated under phosphorylating conditions in the presence or absence of PIP3 and/or Ca^{2+}/CaM. Akt phosphorylation at T308 was analyzed by Western Blotting. (E) OVCAR-3 cells were transfected with PDK1 or CaMKK2 siRNAs for 2 days. Cells were then pre-treated with BAPTA-AM (10 μM) as shown during the serum starvation for 30 min, followed by EGF treatment (100 nM) for 5 min. Samples were analyzed by Western Blotting.

which it is Ca^{2+}/CaM-dependent could be at least partially responsible for such Ca^{2+}/CaM autonomy [26].

Differences between steady state and non-steady state systems are starting to be examined in more detail. For example, Ponsioen and colleagues recently demonstrated that oscillations in the MAPK pathway are amplified by EGFR signaling [27], a process which could also play a role in the extent to which EGFR signaling requires Ca^{2+}/CaM.

3.3. CaMKK2 neither binds to, nor is regulated by, PIP3 but is activated by Ca^{2+}/CaM

Although CaMKK2 and PDK1 are structurally dissimilar, since both enzymes regulate p-Akt (T308) in response to both EGF and ionomycin, we asked whether their individual activation mechanisms were in any way also similar. As the canonical PI3K/PDK1 pathway relies on the generation of PIP3, we investigated whether CaMKK2 binds to PIP3 using PDK1 as a positive control. A PIP array with phosphatidylinositol (PtdIns) and seven species of phosphatidylinositol phosphates over a 64-fold concentration range was used to compare binding of PDK1 with that of CaMKK2 (Fig. 3A). PDK1 demonstrated binding to PtdIns (3, 4, 5) P3 (PIP3) and to PtdIns (3, 4) P2 whereas CaMKK2 binding to any of the phosphoinositides in either the presence or absence of Ca^{2+}/CaM was not detected (Fig. 3B).

To determine if CaMKK2 activity is regulated by PIP3, we performed *in vitro* kinase assays using a synthetic peptide encompassing Akt T308 or WT recombinant Akt1 as substrates for CaMKK2 with or without Ca^{2+}/CaM and/or PIP3 in the assays. CaMKK2 activity was enhanced by Ca^{2+}/CaM equally in the presence and absence of PIP3 with both the synthetic peptide and WT Akt1 indicating a lack of effect of PIP3 on CaMKK2 activity (Fig. 3C, D). In OVCAR-3 cells pre-treated with BAPTA-AM, the effect of CaMKK2 knockdown to reduce Akt phosphorylation was abolished supporting the conclusion of the kinase assays that CaMKK2 activity is Ca^{2+}/CaM dependent (Fig. 3E). Ca^{2+} chelation also reduced the ability of PDK1 knockdown to reduce Akt phosphorylation which may be due to an indirect effect in which Ca^{2+}_{i}/CaM aids Akt translocation to the plasma membrane for phosphorylation by PDK1 [28].

3.4. Evidence that CaMKK2 regulates mTORC2 activity to induce Akt S473 phosphorylation

We previously reported that CaMKK2 phosphorylates and activates, baculovirus-expressed and purified Akt at T308 but not at S473 and yet in cells, RNAi mediated knockdown of CaMKK2 significantly reduced p-Akt (S473) indicating that CaMKK2 regulates p-Akt (S473) through an indirect mechanism [18]. Two upstream regulators of p-Akt (S473) have
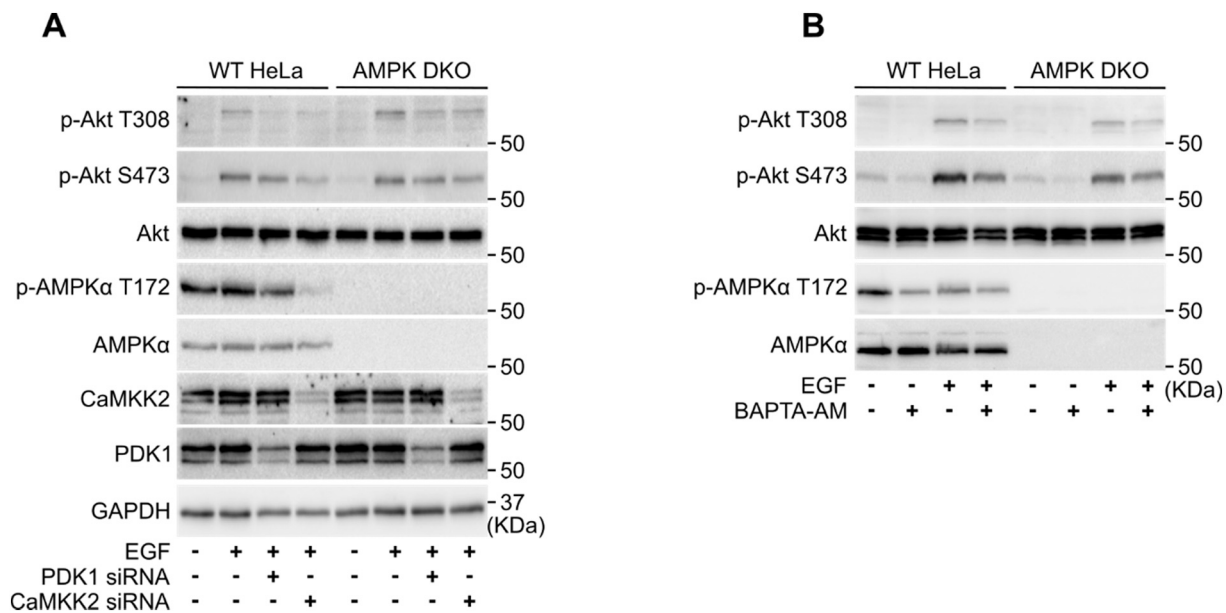
Fig. 4. AMPK is not required for regulation by calcium and CaMKK2 of Akt phosphorylation in HeLa cells. (A) WT and AMPK DKO HeLa cells were transfected with NS, PDK1 or CaMKK2 siRNAs for 2 days then serum starved for 30 min, followed by EGF treatment (100 nM) for 5 min or ionomycin treatment (1 μM) for 15 min. Samples were analyzed by Western Blotting. (B) WT and AMPK DKO HeLa cells were pre-treated with BAPTA-AM (10 μM) as shown during the 30 min serum starvation. Cells were then treated with EGF (100 nM) for 5 min. Samples were analyzed by Western Blotting.

gained significant experimental support, mTOR Complex 2 (mTORC2) and DNA-dependent protein kinase (DNA-PKcs) [29–31]. However, the mechanism by which Akt is phosphorylated at S473 in response to growth factor stimulation remains to be established. To gain insight into the participation of CaMKK2 in Akt S473 phosphorylation we used a number of different approaches.

The M059J human glioma cell line is DNA-PKcs$^{-/-}$ due to a spontaneous mutation resulting in a premature stop codon [32]. However, as shown in Fig. S4A–C, CaMKK2 depletion in M059J cells remained effective in reducing p-Akt (S473) when stimulated by EGF or ionomycin indicating that DNA-PKcs is dispensable for regulation by CaMKK2 of p-Akt (S473).

Using OVCAR-3 cells, we then addressed the possibility that in response to EGF, CaMKK2 regulates mTORC2 which in turn induces p-Akt (S473). At the present time there is no inhibitor that is mTORC2-specific. AZD2014 is a dual inhibitor of both mTORC1 and mTORC2 and rapamycin is an mTORC1-specific inhibitor. We reasoned that a differential effect between the two inhibitors would be due to mTORC2 inhibition. As shown in Fig. S4D, AZD2014 abolished p-Akt (S473) whereas rapamycin produced no decrease and instead, a modest increase in Akt phosphorylation. The latter effect was likely due to relief of inhibition by the negative feedback loop mediated by P70-S6 Kinase 1 (S6K1) and Insulin receptor substrate 1 (IRS-1) [33,34]. There was no effect of CaMKK2 knockdown on p-Akt (S473) after EGF stimulation in AZD2014 treated cells. Similarly, there was no effect of CaMKK2 knockdown in ionomycin stimulated cells treated with AZD2014. Conversely, in rapamycin treated cells in which mTORC2 is not inhibited, p-Akt (S473) was significantly reduced by CaMKK2 knockdown in cells stimulated by either EGF or ionomycin. These data are consistent with CaMKK2 regulation of mTORC2. Similar results were obtained under steady-state conditions under which, AZD2014 but not rapamycin deprived CaMKK2 of the ability to regulate p-Akt (S473) (Fig. S4E).

To further explore the mechanism by which CaMKK2 regulates p-Akt (S473) via mTORC2, we focused on a critical component of the complex, rapamycin-insensitive companion of mTOR (Rictor). We expressed a myc-Rictor transgene in OVCAR-3 cells to upregulate mTOCR2 activity. Rictor expression rescued the decease of p-Akt (S473) caused by CaMKK2 siRNA when cells were stimulated with EGF or ionomycin

(Fig. S4F). This gain of function approach supports the data from the loss of function approach using AZD2014 that mTORC2 is necessary for the regulation by CaMKK2 of p-Akt (S473). In sum, these data indicate that CaMKK2 may activate mTORC2 to provide optimal activation of Akt via its phosphorylation at S473. Since these results are not definitive with respect to the mechanism of control of p-Akt (S473), future investigation into the mechanism by which CaMKK2 regulates mTORC2 is an important goal. As a kinase kinase, CaMKK2 activates its kinase substrates by phosphorylation within their respective activation loops [9]. However, as an atypical kinase in the Phosphatidylinositol 3-kinase-related kinases (PIKK) family, the activity of mTORC2 is not regulated by activation loop phosphorylation. Thus, it is unlikely that CaMKK2 directly phosphorylates mTOR to regulate mTORC2 activity. Discerning the target(s) of phosphorylation by CaMKK2 which regulate mTORC2 may ultimately require phospho-proteomic analysis of CaMKK2 knockdown-sensitive mTORC2 components. It may also be speculated that more indirect mechanisms could be involved. For example, since, mTORC2 is activated by association with ribosomes, CaMKK2 could indirectly regulate mTOC2 complex integrity or the association of mTORC2 with ribosomes [35].

3.5. AMPK is not an obligate intermediate in CaMKK2 regulation of Akt

Several recent studies have proposed models in which Akt activation requires, in certain settings, AMPK [36,37]. Han et al. [36] proposed a signaling pathway in which CaMKK2-dependent regulation of Akt in response to EGF requires AMPK as an intermediate kinase under conditions of stress and tumorigenesis. Since Akt and AMPK are downstream targets of CaMKK2, we investigated a potential role for AMPK as an intermediate in Akt activation. We knocked down PDK1 and CaMKK2 in AMPK WT and AMPK α1/α2 double knockout (AMPK DKO) HeLa cells, then stimulated cells with EGF. In both WT and AMPK DKO cells, p-Akt (T308) was reduced by PDK1 or CaMKK2 knockdown and CaMKK2 knockdown also reduced p-Akt (S473) (Fig. 4A). Moreover, the Ca^{2+}_i dependence of Akt phosphorylation at both sites was retained in AMPK DKO cells (Fig. 4B). These data support a model in which CaMKK2 acts in an AMPK-independent manner to phosphorylate Akt at T308 and regulates Akt at S473 via mTORC2.
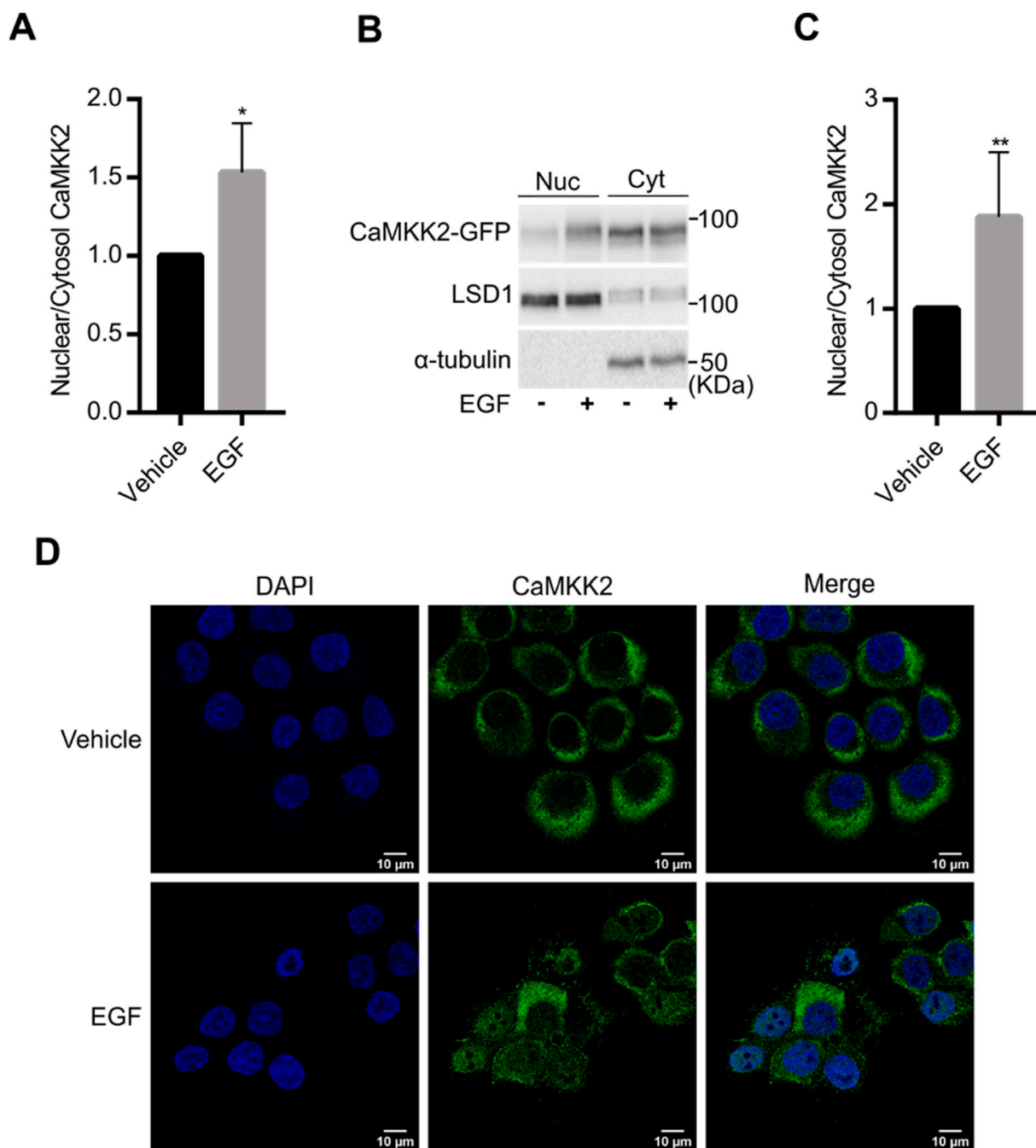
Fig. 5. CaMKK2 translocates to the nucleus in response to EGF. (A) OVCAR-3 cells were transfected with CaMKK2-GFP for 2 days then serum starved for 30 min, followed by EGF treatment (100 nM) for 30 min. Cells were imaged by fluorescence microscopy and cells with or without nuclear CaMKK2 were quantified by blinded counting. The nuclear to cytosolic ratio of CaMKK2 was calculated by the number of cells with nuclear CaMKK2 divided by the number of cells with only cytosolic CaMKK2 (n = 3, 1009 cells counted). (B) OVCAR-3 cells were transfected with CaMKK2-GFP for 2 days then serum starved for 30 min, followed by EGF treatment (100 nM) for 10 min. Nuclear (Nuc) and cytosolic (Cyt) fractions were isolated by subcellular fractionation as described under Materials and Methods and were analyzed by Western Blotting. Lysine-specific histone demethylase 1A (LSD1) and α-tubulin served as Nuc and Cyt markers respectively. (C) CaMKK2-GFP in subcellular fractions as shown in (B) was quantified as the Nuc/Cyt ratio normalized to control (n = 6). (D) Confocal microscopic images of endogenous CaMKK2 as detected by immunocytochemistry in OVCAR-3 cells after EGF treatment (100 nM) for 30 min as compared to vehicle control are shown. (A and C) Results are normalized to control and shown as mean ± SD. *$p < 0.05$, **$p < 0.01$.

Our observations that AMPK is not required for Akt activation in HeLa cells indicates that AMPK is not required for Akt activation in all cell types i.e. that it is not an "obligate" intermediary which is always required for Akt activation. The factors that may determine whether AMPK participates in Akt activation could include different cancer environments or differential responsiveness to different stimuli. For example, in this study we used AMPK DKO HeLa cells while previous studies used AMPK DKO mouse embryonic fibroblasts (MEFs). In addition, previous studies used a much longer serum starvation time of 16–60 h, while we studied EGF stimulation with only 30 min serum starvations. An extended time of serum starvation could result in metabolic stress activating AMPK, serving as a cellular energy sensor resulting in AMPK involvement in Akt activation to support cell survival.
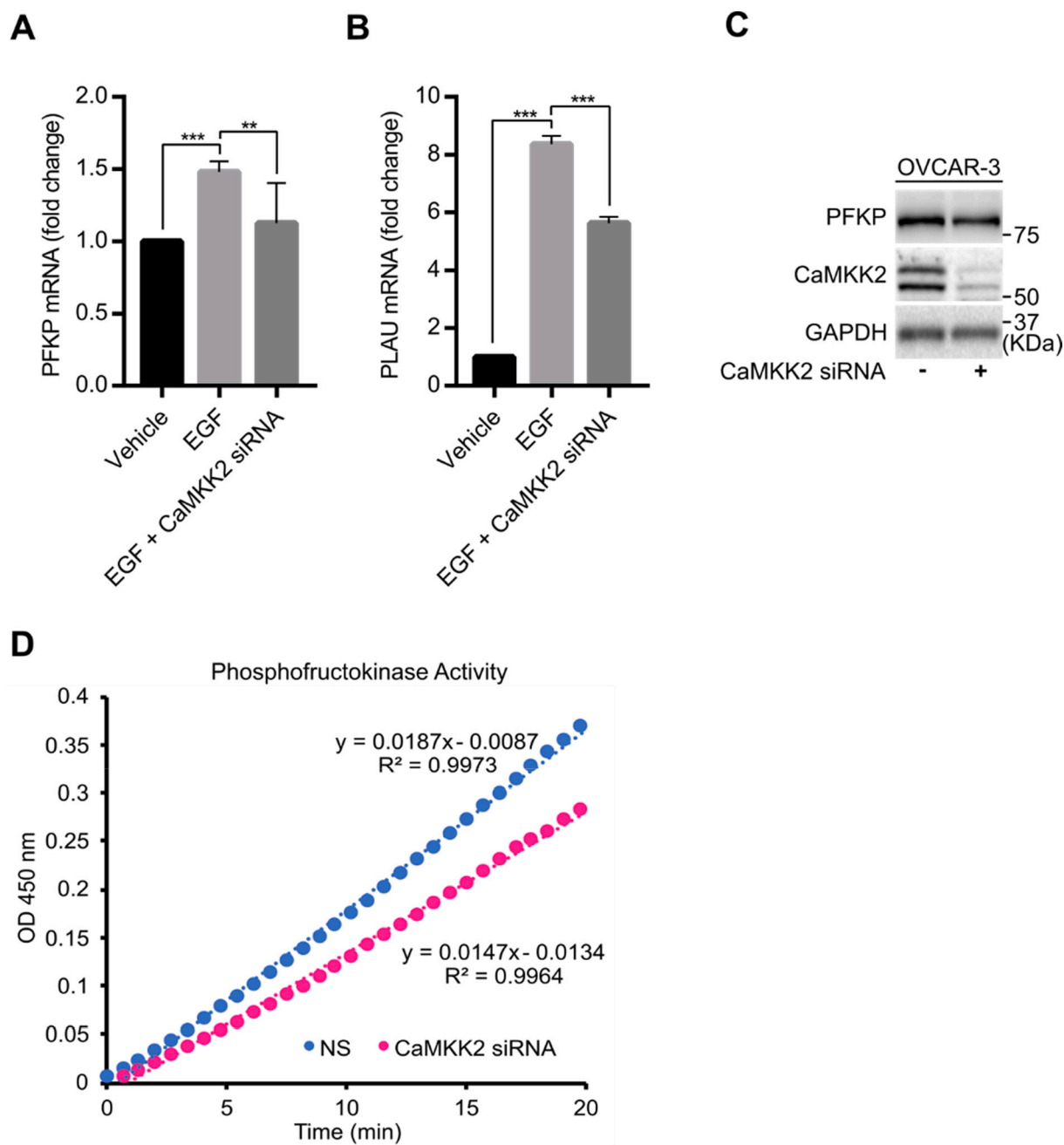
Fig. 6. CaMKK2 regulates EGF-stimulated gene transcription. (A, B) OVCAR-3 cells were transfected with CaMKK2 siRNA for 2 days, following which they were serum starved for 24 h, then treated with EGF (100 nM) for 3 h. mRNA levels of PFKP and PLAU were measured by rt-qPCR (n = 3). Results are normalized to control and shown as mean ± SD. **$p < 0.01$, ***$p < 0.001$. (C) OVCAR-3 cells were transfected with CaMKK2 siRNA for four days then PFKP was analyzed by Western Blotting. (D) OVCAR-3 cells were transfected with CaMKK2 siRNA for 2 days. PFK activity was assayed in cell lysates as described in Materials and Methods. The plot shown was repeated an additional two times with similar results.

3.6. CaMKK2 translocates to the nucleus in response to EGF stimulation-potential role in transcriptional regulation

EGF is a regulator of the Ras-Raf-Mek-Erk and PI3K/Akt pathways that can ultimately result in transcriptional regulation of multiple genes mediating proliferation and survival. In addition, the EGFR has been identified as a transcriptional co-activator with the ability to translocate to the nucleus in response to EGF [38]. High levels of nuclear EGFR have been observed in different cancer types and have been linked to pro-tumorigenic phenotypes [39].

In Karacosta et al. [16,17] we observed that in prostate cancer cells, in response to an agonist (dihydrotestosterone) CaMKK2 translocated to

the nucleus and played a role in transcriptional regulation at the Prostate-Specific Antigen (PSA) enhancer. Thus we investigated whether, similarly, CaMKK2 translocates to the nucleus in response to the agonist EGF, and is involved in transcriptional regulation.

CaMKK2-GFP-expressing OVCAR-3 cells were stimulated with EGF and the percentage of cells with nuclear CaMKK2 was quantified by blinded counting of fluorescent images. Nuclear CaMKK2 increased significantly in response to EGF stimulation (Fig. 5A). To confirm these findings, we used subcellular fractionation to quantify nuclear-to-cytosolic ratios of CaMKK2-GFP in EGF stimulated cells. As shown, EGF induced an increase in the nucleus to cytosol distribution of CaMKK2 (Fig. 5B, C). Expression of CaMKK2-GFP could potentially skew
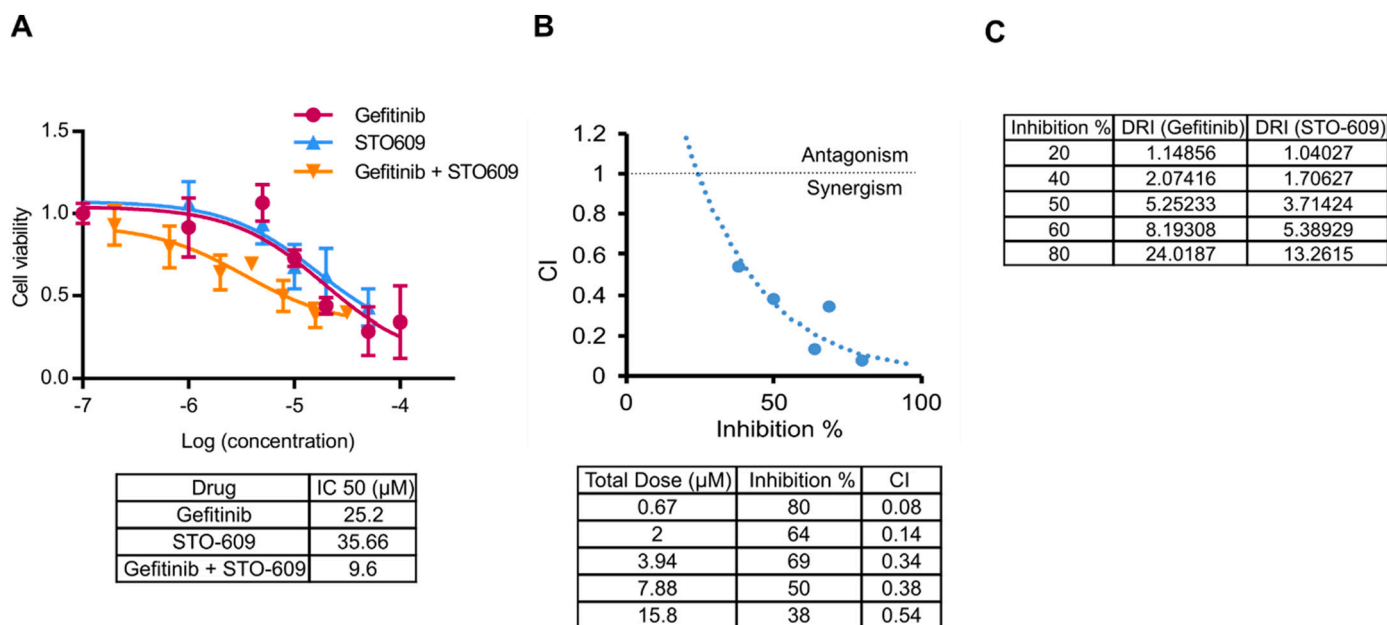
Fig. 7. The CaMKK2 inhibitor STO-609 synergizes with the EGFR inhibitor Gefitinib in inducing cell death. (A) OVCAR-3 cells were treated with Gefitinib, STO-609 or their combination at different concentrations for 3 days following which the number of viable cells were quantified. (B) The Combination index (CI) was plotted as a function of inhibition percentages at different doses. (C) The Dose-Reduction Index (DRI) was calculated for each drug at different inhibition percentages.

the distribution of CaMKK2, however we also show by immunofluorescence that endogenous CaMKK2 accumulates in the nucleus in response to EGF stimulation (Fig. 5D).

EGF-induction of CaMKK2 nuclear translocation raised the possibility of a role for CaMKK2 in the expression of EGFR target genes. We show here by rt-qPCR that phosphofructokinase, platelet (PFKP) and plasminogen activator, urokinase (PLAU) mRNAs are increased by EGF in a CaMKK2-dependent manner (Fig. 6A, B). Both proteins play important roles in malignancy. PLAU-mediated conversion of plasminogen to plasmin leads to extracellular matrix degradation facilitating metastasis. Cancer cells have a high rate of glycolysis with increased glucose uptake followed by lactic acid fermentation (the Warburg effect) [40]. Phosphofructokinase (PFK) as a rate-limiting step in glycolysis has been shown to be overexpressed in many cancers [41]. PFKP protein downregulation after CaMKK2 knockdown is consistent with the decrease of PFKP mRNA (Fig. 6C). Besides PFKP, there are two other isoforms PFK, liver (PFKL) and PFK, muscle (PFKM). A PFK activity assay was performed in CaMKK2 knockdown OVCAR-3 cells. Although the PFK activity assay measures the activity of all PFK isoforms CaMKK2 knockdown still impaired the activity of PFK (Fig. 6D).

Future studies will be necessary to fully evaluate the impact of CaMKK2 nuclear translocation. Such studies could involve addressing the following questions. Is the presence of CaMKK2 required for regulation of other nuclear events such as DNA-repair, counteracting replication stress etc.? Does nuclear CaMKK2 regulate other transcriptional targets of the EGFR with roles in tumorigenesis? Does the involvement of CaMKK2 in transcriptional regulation require nuclear targets of CaMKK2 (CaMKI, CaMKIV, AMPK, Akt) or of EGFR signaling (e.g. ERK) or does CaMKK2 directly interact with nuclear EGFR?

3.7. The CaMKK2 inhibitor STO-609 synergizes with the EGFR inhibitor Gefitinib to induce cell death

Given the ability of CaMKK2 to mediate EGFR signaling we asked whether the CaMKK2 inhibitor STO-609 would produce a synergistic effect in inducing cell death when combined with the EGFR inhibitor Gefitinib. As shown in Fig. 7A, in OVCAR-3 cells, the individual IC50s of Gefitinib and STO-609 were 25.2 μM and 35.66 μM, respectively.

However, the combination of Gefitinib with STO-609 at a 1:1 ratio produced a leftward shift of the cell kill curve with an IC50 of 9.6 μM indicating a synergistic effect. To assess the degree of synergism, we employed Chou-Talalay methodology based on the median effect principle. The Combination Index (CI) was calculated using CompuSyn software following the authors' instructions [42,43]. The CI of Gefitinib and STO-609 varied from 0.08 to 0.54 at different concentrations indicating strong- to- moderate synergism (Fig. 7B). The Dose-reduction index (DRI) was also calculated. The DRI is highly relevant to the use of these agents therapeutically since it represents the reduction in dosages based on synergy of the combination that will achieve the same efficacy and thus reduce toxicity. In this case to induce 50% cell killing, only 19% and 27% of the original concentrations of Gefitinib and STO-609 respectively were needed to produce the same efficacy when combined (Fig. 7C). Overall, these results showed a promising synergistic interaction between EGFR inhibition and CaMKK2 inhibition consistent with our findings that CaMKK2 is an important mediator of EGF signaling.

Even though many ovarian cancer patients overexpress EGFR, clinical trials of EGFR inhibitors in ovarian cancer have been disappointing due to acquired and innate resistance [44]. The resistance can be developed by a variety of mechanisms and the hyperactivation of Akt has been well documented in this setting [45–47].

Besides Gefitinib, a first generation EGFR inhibitor, multiple EGFR inhibitors have recently been developed [48]. The second generation is represented by Afatinib which irreversibly inhibits ATP-binding to EGFR. Osimertinib and Olmutinib are third generation EGF inhibitors. They covalently bind to and inhibit the T970M mutant of EGFR which causes resistance to the early generations of EGFR inhibitors in about 50% of patients [49,50]. Based on our findings, the combination of the STO-609 with other EGFR inhibitors may be worth investigating in multiple cancers with EGFR amplification or mutation.

3.8. CaMKK2 knockout triggers compensation by Akt activation

CaMKK2 regulation of EGFR/Akt signaling suggested that CRISPR/Cas9 CaMKK2 knockout (KO) cell lines could provide models of long term inhibition of CaMKK2 at maximally tolerated dosages. To this aim,
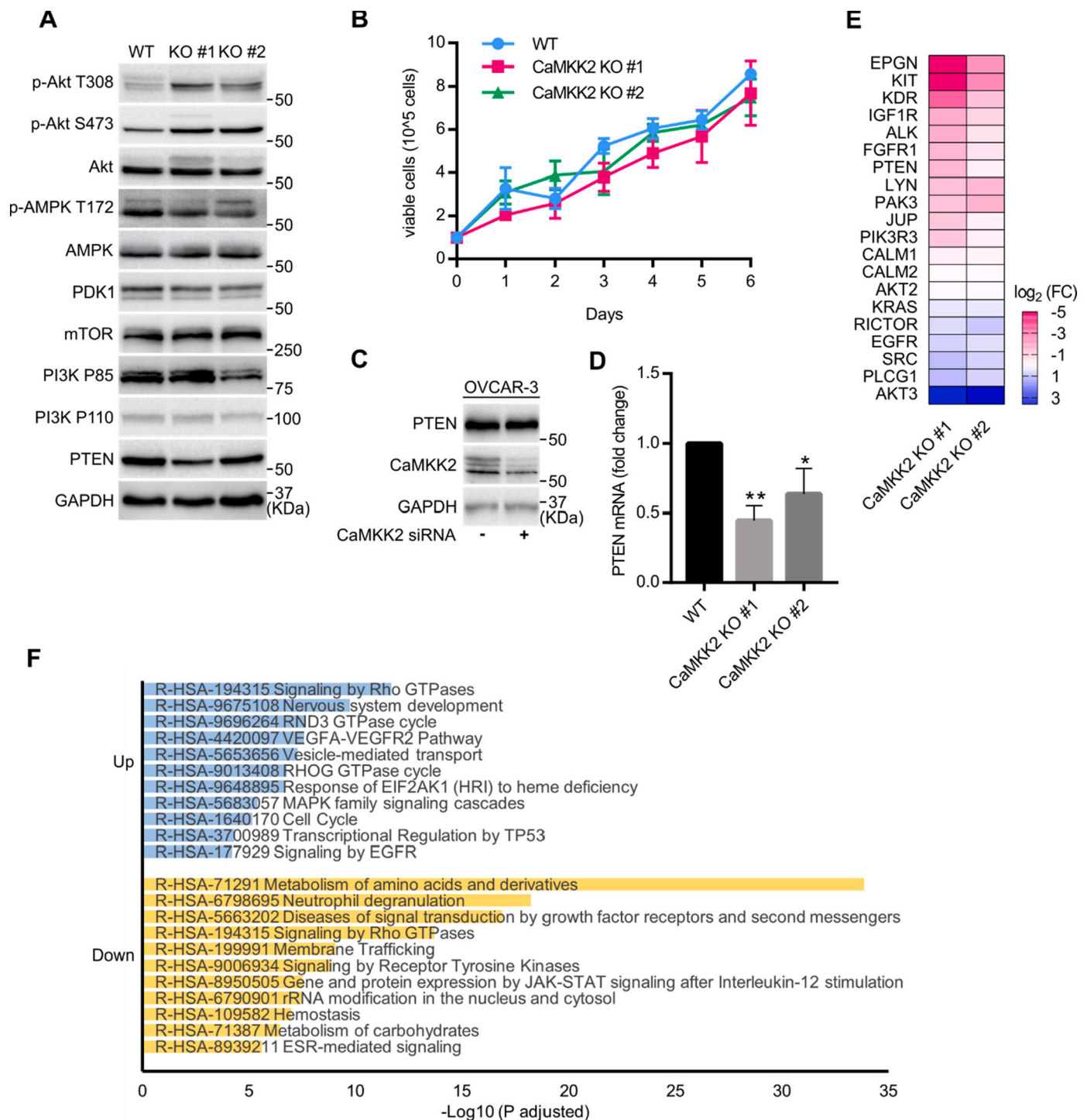
Fig. 8. Compensation of Akt phosphorylation in CaMKK2 KO cells. (A) Expression of Akt, AMPK and proteins involved in Akt regulation were analyzed in CaMKK2 KO cells by Western Blotting. (B) CaMKK2 WT and KO cells were seeded overnight then viable cell growth was quantified for six days. (n = 3) (C) OVCAR-3 cells were transfected with CaMKK2 siRNA for two days then PTEN was analyzed by Western Blotting. (D) Downregulation of PTEN mRNA in CaMKK2 KO cells was verified by rt-qPCR (n = 3). Results are normalized to WT and shown as mean ± SD. *p < 0.05, **p < 0.01. (E) The Heatmap shown represents the $\log_2$ fold change (FC) of genes in the RTK pathway. (F) Pathway enrichment of up- and down- regulated genes that are shared by both CaMKK2 KO cell lines are listed.

a control WT cell line and two CaMKK2 KO clonal OVCa cell lines were developed (Fig. S5).

RNAi of CaMKK2 results in decreases of ~50–80% in Akt activating phosphorylation (Figs. 1, 2) [18]. We therefore expected the knockouts to yield at least this level, if not somewhat more, reduction of Akt activation. Surprisingly, the knockouts produced prominent increases of Akt phosphorylation. These occurred at both T308 and S473 in CaMKK2

KO #1 and #2 cells (Fig. 8A). This suggests that a compensatory mechanism is involved to maintain Akt activation in the face of a long-term loss of functional CaMKK2. During the ~2 month period of outgrowth in which knockout cells were expanded, there was presumably selective pressure to promote Akt phosphorylation and thereby continue to drive growth and survival. Given the difference in time frames, such compensation would not be expected to occur in the 2–7
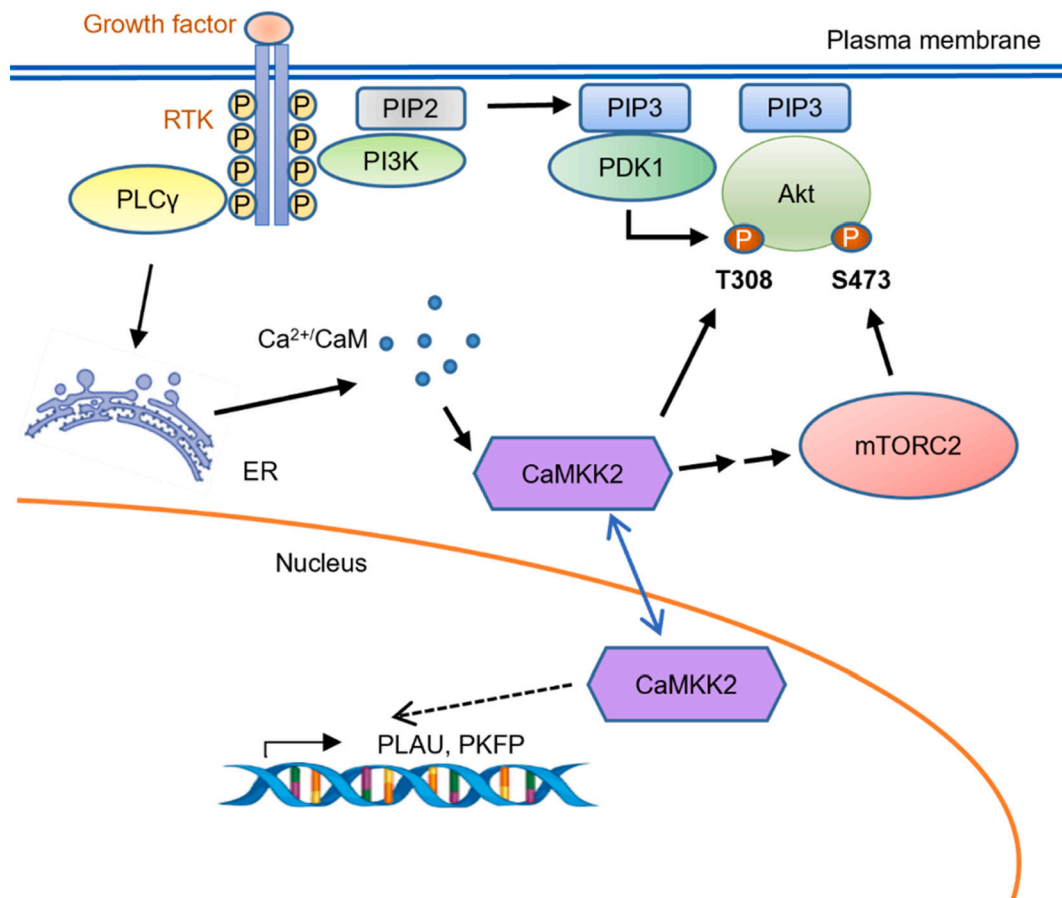
Fig. 9. Hypothetical model for the roles of Ca^{2+}, CaM and CaMKK2 in the activation of Akt and regulation of gene transcription.

day periods in which RNAi was conducted in this study.

It should be noted that this compensation only occurred in the Akt pathway since the phosphorylation of another CaMKK2 substrate, AMPK at T172, decreased in both CaMKK2 KO #1 and #2 cells as predicted (Fig. 8A). We hypothesize that the reason for this difference is the critical role of Akt in proliferation and survival during normal growth in the absence of significant cellular stress. The fact that this presumed selection occurred due to loss of CaMKK2 is consistent with its important role in Akt activation. Our hypothesis also predicts that the selective pressure during outgrowth does not function to accelerate growth. Rather, it exists simply to compensate for the loss of an important regulator, in this case, CaMKK2. Consistent with this prediction is the data of Fig. 8B showing similar time courses of cell growth in the two KO lines and WT cells.

To understand this compensation mechanism, the levels of proteins that are important for regulating Akt phosphorylation were examined, such as PDK1 mTOR, PI3K (P85 and P110) and PTEN. As shown in Fig. 8A, there was no dramatic and consistent change of all targets in KO #1 and #2 cells except the decrease of PTEN. The significant decrease of PTEN mRNA level supported the finding of PTEN loss in both CaMKK2 KO #1 and #2 cells (Fig. 8D). However, as hypothesized, the short-term loss of CaMKK2 with CaMKK2 siRNA did not downregulate PTEN level consistent with the time dependence of the compensation effect (Fig. 8C).

A similar compensation of Akt activation was recently reported by Wang and colleagues who observed that the allosteric Akt inhibitor MK2206 initially downregulated Akt phosphorylation at both T308 and S473, however inhibition was subsequently compensated for by hyperactivation of PI3K and mTOR [51]. In order to avoid compensatory resistance, PI3K and mTOR single or dual inhibitors such as NVP-

BEZ235 have been used in combination with Akt inhibitors however the inhibition was transient and Akt hyperactivation reoccurred [52]. Together, evidence points to EGFR/Akt activation as regulated in a complex fashion with backup systems requiring a better understanding of the various mechanisms causing resistance and the need for a multi-drug strategy for targeting this important oncogenic mechanism.

From a different perspective, the complete depletion of CaMKK2 in cancer cells over an extended period of time and the subsequent compensation of Akt activation may be analogous to drug resistance observed in patients with a time frame of months to years. Indeed, knockout may bear some similarity in its extent of inhibition to maximally tolerated doses (MTDs) used clinically. This in turn suggests that further studies to understand the mechanism(s) underlying compensation such as PTEN down regulation may be worth pursuing.

3.9. RNA-seq of CaMKK2 KO cells identifies potential physiological functions of CaMKK2

In addition to identification of potential resistance mechanisms such as Akt compensation, we sought to gain a more comprehensive understanding of the physiological effects of CaMKK2 depletion in cancer cells. RNA-seq was performed on CaMKK2 WT and both KO cell lines. Three biological replicates were used for each cell line and differentially expressed genes (DEGs) were identified between CaMKK2 WT vs KO #1 and CaMKK2 WT vs KO #2. Approximately 5300 genes were significantly up- or down-regulated in CaMKK2 KO #1 cells and about 3500 genes in #2 cells (Fig. S6A). Among those genes, 1174 genes were upregulated in both KO #1 and KO #2 cells and 1175 genes were downregulated in both cell lines. The PCA plot in Fig. S6B indicates good consistency among biological replicates within each cell line. However,

KO #1 and #2 did not cluster into a single group indicating that although they shared some changes in their transcriptomes, they underwent different evolutionary paths during the two-month expansion from single cells. Volcano plots of DEGs in CaMKK2 KO #1 and #2 cells with the most prominently changed genes marked are shown in Fig. S6C and D.

DEGs related to growth factor pathways are shown in the Fig. 8E. EGFR, RICTOR, SRC, KRAS, PLCγ1 and Akt3 were upregulated in both KO cells, all of which may have roles in the compensation effect together with the downregulation of PTEN. However, growth factors like Insulin-like growth factor 1 receptor (IGF1R) and Fibroblast growth factor receptor 1 (FGFR1) were downregulated in the KO cells suggesting that not all growth factor signaling mechanisms are required for optimal cellular growth. Pathway enchainment analysis was performed with DEGs that are shared by both KO cell lines. Upregulated pathways such as EGFR, VEGFA-VEGFR2 and MAPK signaling suggested a complex compensatory mechanism that may act at the level of Akt activation but also by initiating signaling crosstalk among intersecting pathways (Fig. 8F).

Although Akt phosphorylation was compensated, some pathways involving growth factor receptors and second messengers were downregulated supporting the comprehensive role of CaMKK2 in regulating pathways mediating proliferation and survival. Downregulated pathways also suggest unrecognized functions of CaMKK2 such as regulation of the metabolism of amino acids and carbohydrates. Combined with the ability of CaMKK2 to mediate the transcription of PFKP, our data and further study may reveal CaMKK2 to have a far reaching regulatory role in cell metabolism through the regulation of gene transcription.

3.10. Hypothetical model for CaMKK2 function

Fig. 9 represents a working model for how CaMKK2 may regulate both cytoplasmic and nuclear events producing a pro-oncogenic phenotype in response to EGFR signaling.

4. Conclusions

The results of this research have two main conclusions and taken together lead to an overriding hypothesis. First, CaMKK2 is an effector of EGFR signaling, some of which, although not all of which, occurs through the Akt pathway and relies for optimal effects on intracellular calcium and the calcium-binding protein calmodulin. Second, CaMKK2 is an important effector in maintaining Akt signaling since its complete depletion induces a compensatory up-regulation of Akt signaling. This appears to be specific since it was not observed in another prominent CaMKK2 substrate, AMPK. We hypothesize that the evolutionary rationale for these findings is that intracellular calcium elevation may represent a mechanism for cell survival over an extended period of time. Future experiments exploring these issues may aid our understanding of the capacity of an EGFR/CaMKK2 axis to regulate a broad spectrum of cellular processes related to tumorigenesis such as aerobic glycolysis and metastasis.

CRediT authorship contribution statement

Shuhang Dai: Conceptualization, Validation, Investigation, Writing – original draft, Writing – review & editing. **Elisa Venturini:** Conceptualization, Validation, Investigation, Methodology. **Saveg Yadav:** Conceptualization, Validation, Investigation, Methodology. **Xiaoxuan Lin:** Conceptualization, Validation, Investigation, Methodology. **Dylan Clapp:** Conceptualization, Validation, Investigation, Methodology. **Martin Steckiewicz:** Conceptualization, Validation, Investigation, Methodology. **Angela M. Gocher-Demske:** Conceptualization. **D. Grahame Hardie:** Resources, Conceptualization, Methodology. **Arthur M. Edelman:** Conceptualization, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2022.119252.

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