DR. ASHFAQUL HOQUE (Orcid ID : 0000-0003-2384-2983)
DR. JOHN W SCOTT (Orcid ID : 0000-0002-1896-9798)

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Functional analysis of an R311C variant of Ca^{2+}-calmodulin dependent protein kinase kinase-2 (CaMKK2) found as a de novo mutation in a patient with bipolar disorder

Running head: De novo CaMKK2 mutation and bipolar disorder

Naomi XY Ling[1], Christopher G Langendorf[1], Ashfaqul Hoque[1], Sandra Galic[1], Kim Loh[1], Bruce E Kemp[1,2], Andrew L Gundlach[3], Jonathan S Oakhill[1,2] and John W Scott[1,2,3*]

[1]St Vincent's Institute and Department of Medicine, The University of Melbourne, 41 Victoria Parade, Fitzroy, 3065, Australia. [2]Mary MacKillop Institute for Health Research, Australian Catholic University, 215 Spring Street, Melbourne, 3000, Australia. [3]The Florey Institute of Neuroscience and Mental Health, 30 Royal Parade, Parkville, 3052, Australia.

*To whom correspondence should be addressed. E-mail: jscott@svi.edu.au

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abstract

Objectives: Loss-of-function mutations in the gene encoding the calcium-calmodulin (Ca^{2+}-CaM) dependent protein kinase kinase-2 (CaMKK2) enzyme are linked to bipolar disorder. Recently, a *de novo* arginine to cysteine (R311C) mutation in CaMKK2 was identified from a whole exome sequencing study of bipolar patients and their unaffected parents. The aim of the present study was to determine the functional consequences of the R311C mutation on CaMKK2 activity and regulation by Ca^{2+}-CaM.

Methods: The effects of the R311C mutation on CaMKK2 activity and Ca^{2+}-CaM activation were examined using a radiolabeled adenosine triphosphate (ATP) kinase assay. We performed immunoblot analysis to determine whether the R311C mutation impacts threonine-85 (T85) autophosphorylation, an activating phosphorylation site on CaMKK2 that has also been implicated in bipolar disorder. We also expressed the R311C mutant in CaMKK2 knockout HAP1 cells and used immunoblot analysis and an MTS reduction assay to study its effects on Ca^{2+}-dependent downstream signaling and cell viability, respectively.

Results: The R311C mutation maps to the conserved HRD motif within the catalytic loop of CaMKK2 and caused a marked reduction in kinase activity and Ca^{2+}-CaM activation. The R311C mutation virtually abolished T85 autophosphorylation in response to Ca^{2+}-CaM and exerted a dominant-negative effect in cells as it impaired the ability of wild-type CaMKK2 to initiate downstream signaling and maintain cell viability.

Conclusions: The highly disruptive, loss-of-function impact of the *de novo* R311C mutation in human CaMKK2 provides a compelling functional rationale for being considered a potential rare monogenic cause of bipolar disorder.

Keywords: bipolar disorder, kinase, calcium, CaMKK2, mutation, monogenic

1 Introduction

Bipolar disorder is a recurrent chronic mood disorder that affects around 1% of the global population and is characterized by episodes of mania, with alternating periods of depression[1].

Dysregulation of neuronal calcium (Ca^{2+}) signaling networks have been implicated in the development of psychiatric conditions including bipolar disorder[2]. Ca^{2+}-signaling pathways play a major role in regulating key functions including neuronal excitability, information processing, cognition and the changes in synaptic plasticity that underpin learning and memory. Most physiological processes regulated by Ca^{2+}-signaling involve calmodulin (CaM)[3], a universal Ca^{2+}-sensing protein that binds and alters the function of numerous downstream targets in response to increased intracellular Ca^{2+}. A pivotal effector of the Ca^{2+}-CaM complex is the enzyme Ca^{2+}-CaM dependent protein kinase kinase-2 (CaMKK2), which is the central component of a Ca^{2+}-signaling pathway in neurons that regulates higher-order brain functions including memory formation[4], mood and emotional behaviour[5], and appetite[6]. CaMKK2 is activated in response to increased intracellular Ca^{2+} induced by a variety of physiological stimuli such as membrane depolarization[7], glutamate (NMDAR) and serotonin receptor (5-HT2C) activation[8], and the appetite-regulating hormone ghrelin[6]. Once activated, CaMKK2 stimulates a number of downstream processes including driving brain-derived neurotrophic factor (BDNF) expression[9], a critical regulator of neuronal function that was recently reported to be essential for the anti-mania effects of lithium[10].

Several studies indicate a potential link between loss-of-function mutations in human CaMKK2 and bipolar disorder. Although genome wide association studies did not show robust association between CaMKK2 and bipolar disorder[11], a case-control study in a modest number of subjects demonstrated a nominally significant association between bipolar disorder and an intronic polymorphism (rs1063843), where the risk allele (T) was associated with lower CaMKK2 expression in human post-mortem brains and lymphoblastoid cells[12]. Moreover, a functional magnetic resonance imaging study revealed that the carriers of the T allele of rs1063843 showed higher activation of dorsolateral prefrontal cortex during frontal lobe tasks[13]. An exonic variant (rs3817190) that encodes a threonine to serine (T85S) substitution at the regulatory T85 autophosphorylation site in human CaMKK2 is also associated with bipolar disorder[14]. T85 autophosphorylation is induced by Ca^{2+}-CaM activation, which creates a molecular memory that maintains CaMKK2 in the activated state after the Ca^{2+}-signal has dissipated[5]. Autophosphorylation of the T85S mutant is similarly triggered by the Ca^{2+}-CaM stimulus but fails to retain CaMKK2 in the activated state and instead causes a loss-of-activity. Consistent with these loss-of-function mutations in humans, mice lacking CaMKK2 display behavioral traits (anxiety and manic-like behaviors) similar to those frequently observed in bipolar disorder[5].

Whole-exome sequencing of simplex families (i.e. where there is a single, isolated case of a condition within a family) has uncovered a potential role for *de novo*, rare-variant coding mutations in the genetic architecture of neuropsychiatric conditions including bipolar disorder. Indeed, statistical analyses of non-synonymous genetic variation indicated that disorders of the central

nervous system are more likely to be caused by sporadic monogenic mutations more frequently than disorders affecting other biological systems[15]. Recently, a *de novo* mutation that encodes an arginine to cysteine (R311C) substitution in human CaMKK2 was identified from a trio family-based, whole-exome sequencing study for bipolar disorder[16]. Given the apparent relationship between loss-of-function mutations in CaMKK2 and bipolar disorder, the aim of this study was to determine the functional effects of the R311C mutation on CaMKK2, including its activity, regulation by Ca^{2+}-CaM, and signaling to downstream targets.

2 Materials and Methods

2.1 Molecular biology

Plasmid constructs for full-length, N-terminal Flag-tagged or hemagglutinin (HA)-tagged human CaMKK2 isoform-1 (NP_001257414.1) and the R311C mutant were generated by custom gene synthesis (General Biosystems, Morrisville, NC, USA), and cloned into the pcDNA3(-) mammalian expression vector using XhoI/HindIII restrictions sites. All constructs were verified by sequencing the entire open reading frame. Plasmid DNA for COS7 cell transfection was prepared using Wizard Plus SV Miniprep DNA Purification Kits (Promega, Alexandria, NSW, Australia).

2.2 CaMKK2 expression

COS7 cells and HAP1 cells were grown in Dulbecco Modified Eagle Medium (Sigma, Castle Hill, NSW, Australia) and Iscoves Modified Dulbecco Medium (Thermo Fisher Scientific, Scoresby, Victoria, Australia), respectively, supplemented with 10% fetal calf serum at 37 °C with 5% CO_2. COS7 cells were transfected at 60% confluency with 2 μg of pcDNA3(-) plasmid containing either N-terminal Flag-tagged human CaMKK2 or HA-tagged R311C mutant using FuGene HD (Roche Applied Science, Penzberg, Germany). HAP1 cells were transfected with 2 μg of plasmid at 60% confluency using Turbofectin (Life Technologies, Mulgrave Victoria, Australia). Transfected cells were harvested after 48 hr by washing with ice-cold phosphate-buffered saline (PBS) followed by rapid lysis *in situ* using 1 ml of lysis buffer (50 mM Tris.HCl [pH 7.4], 150 mM NaCl, 50 mM NaF, 1 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1% [v/v] Triton X-100) containing Complete protease inhibitor cocktail (Roche Applied Science). For the ionomycin treatments, cells were treated with either 5 μM ionomycin (Sigma-Aldrich) or a dimethylsulfoxide (DMSO) vehicle control for 10 mins prior to harvesting. Cellular debris was removed by centrifugation and total protein in the cell lysate was determined using the Bradford protein assay (Thermo Fisher Scientific)

2.3 Purification of CaMKK2

CaMKK2 was purified from transfected COS7 cell lysates using either anti-Flag M2 agarose or HA-agarose 50% [v/v] (Sigma-Aldrich) pre-equilibrated in lysis buffer, followed by successive washes in lysis buffer containing 1 M NaCl, and finally into 50 mM Hepes [pH 7.4]. The immobilized CaMKK2 was then sedimented by centrifugation and used for kinase assays or eluted overnight at 4 °C with either Flag or HA peptide (1 mg/ml; w/v) for immunoblotting (see below).

2.4 CaMKK2 kinase assay

CaMKK2 activity was measured using a synthetic peptide substrate (CaMKKtide) as previously described[17]. Briefly, CaMKK2 immobilized on either anti-Flag M2 or HA-agarose beads (50% v/v) was incubated in assay buffer (50 mM Hepes [pH 7.4], 1 mM DTT, 0.02% [v/v] Brij-35) containing 200 μM CaMKKtide (synthesized by Genscript, Piscataway, NJ, USA), 100 μM $CaCl_2$, 0-1 μM CaM (Sigma-Aldrich), 200 μM [γ-^{32}P]-ATP (Perkin Elmer, Glen Waverley, Victoria, Australia) and 5 mM $MgCl_2$ (Sigma-Aldrich) in a standard 30 μl assay for 10 min at 30 °C. Reactions were terminated by spotting 15 μl onto P81 phosphocellulose paper (GE Lifesciences, Silverwater, NSW, Australia) and washing extensively in 1% phosphoric acid. Radioactivity was quantified by liquid scintillation counting.

2.5 T85 autophosphorylation reaction

The autophosphorylation reaction was performed using 50 μl of anti-Flag agarose immobilized CaMKK2 incubated in the presence and absence of 100 μM $CaCl_2$, 1 μM CaM and 5 mM $MgCl_2$/200 μM ATP (MgATP). Reactions were incubated at 30 °C for various times, after which the autophosphorylation reaction was terminated by washing the beads successively in lysis buffer containing 1 M NaCl, then finally resuspended in 50 mM Hepes [pH 7.4] to achieve a 50% slurry. A 10 μl aliquot (50% slurry) was removed and kinase activity was measured in assay buffer containing 200 μM CaMKKtide, 200 μM [γ-^{32}P]-ATP and 5 mM $MgCl_2$, in the presence or absence of 100 μM $CaCl_2$, 1 μM CaM and 1 mM EGTA (a Ca^{2+}-chelator), using the kinase assay described above.

2.6 Immunoblotting

For the CaMKK2 phosphothreonine-85 immunoblots, a 10 μl aliquot (50% slurry) from the autophosphorylation reaction was used. For all other immunoblots, either 50 μg of transfected cell lysate or 20 ng of purified CaMKK2 was used. All samples were denatured in SDS sample buffer, resolved on a pre-cast 4-15% Mini-Protean Gradient gel (Bio-Rad, Gladesville, NSW, Australia), before transferring onto Immobilon PVDF membrane (Merck-Millipore, Bayswater, Victoria, Australia). The membrane was blocked for 30 min in PBS/1% Tween-20 (PBS-T) supplemented

with 2% non-fat milk, and then incubated for 60 mins with either mouse anti-Flag (Cell Signaling, Beverly MA, USA; Cat No 2368S; Lot No 6; 100 ng/ml), rabbit anti-phosphothreonine-85 antibodies (custom generated in-house[5,18]; 300 ng/ml), mouse anti-AMPK α subunit (Cell Signaling; Cat No 2793S; Lot No 7; 100 ng/ml), rabbit anti-phosphothreonine-172 antibodies (Cat No 2535L; Lot No 21; 100 ng/ml, respectively), rabbit anti-CaMKK2 (Cell Signaling; Cat No 16810S; Lot 1; 100 ng/ml), mouse anti-tubulin (Cell Signaling: Cat No 3873S; Lot 12; 100 ng/ml) or rabbit anti-β-actin (Cell Signaling: Cat No 4970S; Lot 11; 100 ng/ml). The membrane was then briefly washed in PBS-T, followed by incubation with goat anti-rabbit IgG IRDye680 and goat anti-mouse IgG IRDye 800 (Li-Cor, Lincoln, NE, USA) secondary antibodies for 60 min. After successive washing with PBS-T, the membrane was scanned, and the images quantified with an Odyssey CLx Infrared Imager (Li-Cor).

2.7 Cycloheximide chase assay

CaMKK2 knockout (KO) HAP1 cells were transfected with pcDNA3(-) plasmid containing either WT or R311C mutant CaMKK2 for 48 hr, after which fresh media containing 10 μM cycloheximide (Sigma Aldrich) was added to the cells. The cells were harvested post treatment at various time points by washing with ice-cold phosphate-buffered saline (PBS), followed by rapid lysis *in situ* as described above. CaMKK2 stability was then analyzed by immunoblotting. β-actin protein expression was used as a loading control due to its long half-life (2-3 days)[19,20]. CaMKK2 and β-actin expression was quantified with an Odyssey CLx Infrared Imager.

2.8 Cell viability assay

CaMKK2 knockout (KO) HAP1 cells were transfected with pcDNA3(-) plasmid containing either WT or R311C mutant CaMKK2 for 48 hr, after which fresh media containing MTS reagent (Abcam, Cambridge MA, USA) was added to the cells for 60 min at 37 °C. An aliquot of the media was removed, and absorbance measured at 490 nm using a Nanodrop Spectrophotometer (Thermo Fisher Scientific). Background absorbance was measured from plates containing culture medium and MTS reagent but no cells.

2.9 Statistical analysis

Data are presented as mean values ± standard error of the mean (SEM) for three independent experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) using Tukey's multiple comparison test (GraphPad Prism, San Diego, CA, USA). In all cases, $p<0.05$ was considered statistically significant.

3 Results

3.1 The R311C mutation is located within the HRD-motif of the CaMKK2 kinase domain

R311 constitutes part of the Histidine-Arginine-Aspartate (HRD) triad motif within the catalytic loop of the kinase domain that is invariant in CaMKK2 orthologues across a diverse range of species (Figures 1A-B), and is a highly-conserved feature across the related protein kinase superfamily[21]. The backbone of R311 traverses the intact regulatory spine (which is considered a hallmark of an active kinase) that aligns the small and large lobes of the catalytic domain (Figure 1C), and its precise positioning within the active site is required for catalytic activation. The R311 side-chain stabilizes the catalytic and activation loops by forming a network of hydrogen bonds with N336 and N347, which is critical for maintaining the optimal catalytic environment (Figure 1D). A structural model of the R311C mutant indicated that this mutation would prevent this region of the enzyme from forming these interactions and render it incapable of maintaining the activation and catalytic loops in the required conformation for efficient catalysis (Figure 1E). Furthermore, the *in silico* prediction tools, Polyphen-2 and SIFT, generated scores (1.00 and 0.00, respectively) that indicates a high probability the R311C mutation is functionally damaging[22,23].

3.2 The R311C mutation decreases CaMKK2 activity and impairs Ca^{2+}-CaM activation

In studies to determine the functional effects of the R311C mutation on CaMKK2 activity and Ca^{2+}-CaM activation, kinase activity of WT and R311C mutant CaMKK2 was measured over a range of CaM concentrations. As predicted from the Polyphen-2 and Sift scores, and the model in Figure 1E, the R311C mutant had markedly lower kinase activity than WT CaMKK2, both in the presence and absence of Ca^{2+}-CaM (Figure 2A). The R311C mutation also rendered CaMKK2 far less responsive to CaM as demonstrated by the lower degree of Ca^{2+}-CaM stimulation (6.04-fold stimulation for WT versus 4.12-fold for the R311C mutant), and the higher concentration of CaM required to achieve half-maximal activation (Table 1). Despite having a large deleterious effect on kinase activity and Ca^{2+}-CaM activation, the R311C mutation had no significant effect on CaMKK2 stability as the mutant had a similar degradation rate as WT as determined by cycloheximide chase analysis (Figures 2B-C).

3.3 The R311C mutation impairs T85 autophosphorylation

Autophosphorylation at T85 is critical for keeping human CaMKK2 in the activated state after cessation of the Ca^{2+}-signal[5]. The effect of the R311C mutation on T85 autophosphorylation was analyzed by incubating WT and R311C mutant CaMKK2 with MgATP and Ca^{2+}-CaM, after which the rate of T85 autophosphorylation was determined by immunoblot using a phosphothreonine-85 specific antibody. The R311C mutation severely impaired T85 autophosphorylation in response to

Ca^{2+}-CaM activation compared with WT (Figures 3A-B). Concordant with disabled T85 autophosphorylation, the R311C mutant was also unable to remain in the activated state after withdrawal of the Ca^{2+} signal using the Ca^{2+}-chelator, EGTA (Figure 3C).

3.4 The R311C mutation blocks CaMKK2 signaling and impairs cell viability

Since the bipolar patient carrying the R311C mutation is heterozygous for the mutant allele[16], we determined whether the R311C mutant exerts a dominant-negative effect on WT enzyme. Flag-tagged WT and HA-tagged R311C mutant were co-transfected at equimolar ratios in CaMKK2 null HAP1 cells (KO), and then purified by Flag or HA-immunoprecipitation. Figure 4A shows co-purification of the HA-R311C mutant with Flag-WT, and reciprocal co-purification of Flag-WT with the HA-R311C mutant, which indicates CaMKK2 may exist functionally as a dimer or larger oligomer. Consistent with dominant-negative inhibition, the presence of the R311C mutant impaired kinase activity of the WT enzyme (Figure 4B). We next assessed the impact of the R311C mutation on CaMKK2 signaling in cells in response to ionomycin stimulation, an ionophore that increases intracellular Ca^{2+}. Phosphorylation of the downstream substrate AMPK on the activating threonine-172 (T172) site in the α subunit (an indicator of CaMKK2 signaling) was then analyzed by immunoblot using a phosphothreonine-172 specific antibody. Ionomycin treatment increased Thr172 phosphorylation in cells expressing WT, however this effect was abolished in cells exclusively expressing the R311C mutant (Figures 4C-D). Moreover, the R311C mutant significantly impaired signaling in cells expressing WT, demonstrating that the R311C mutation exerts a dominant-negative effect on the downstream signaling capability of WT CaMKK2. There was no detectable change in basal Thr172 phosphorylation between the mock, WT and R311C transfected KO cells, which is in line with previous studies that demonstrated LKB1 is predominantly responsible for phosphorylating AMPK under basal conditions[24]. The R311C mutant also exerted a dominant-negative effect on cell viability, which was decreased ~32% in cells co-transfected with both WT and the R311C mutant, compared with cells transfected with WT alone (Figure 4E).

4 Discussion

In this study, we investigated the effects of a *de novo* R311C mutation in human CaMKK2 that was identified from a trio-family, whole-exome sequencing study of bipolar disorder. Rare *de novo* mutations are considered to play an important role in the genetic architecture of neuropsychiatric conditions including bipolar disorder, and likely account for a significant portion of the missing heritability for complex psychiatric diseases that is unobservable by genome-wide association studies[16]. *De novo* mutations in coding sequences are typically the most hazardous form of genetic

variation and are more likely to cause disease, as they tend be highly disruptive due to less stringent evolutionary pressure[25].

Consistent with the relationship between CaMKK2 loss-of-function and bipolar disorder, the R311C mutation decreased kinase activity, decreased Ca^{2+}-CaM activation and hindered autophosphorylation of the activating T85 site. At the cellular level, the R311C mutant displayed dominant-negative inhibition as it impaired downstream signaling and cell viability in the presence of functional WT CaMKK2. These detrimental effects identify the R311C variant as the most functionally disruptive among the CaMKK2 loss-of-function mutations that have been characterized so far. Notably, the large decrease (> 50%) in CaMKK2 activity displayed by the R311C mutant is similar in magnitude to the reduction in CaMKK2 observed in the dorsolateral prefrontal cortex of bipolar patients[12,26]. Of the three major effects of the R311C mutation, the impairment of T85 autophosphorylation is perhaps the most significant, as the inability to achieve T85 autophosphorylation in response to Ca^{2+}-CaM activation prevented the R311C mutant from remaining in the activated state after removal of the Ca^{2+}-signal. This is analogous to the effect of the T85S mutation in human CaMKK2, which was shown previously by two independent, candidate gene association studies to display significant links with bipolar and anxiety disorder[5,14,27].

Considering the R311C mutation is positioned within the highly conserved HRD-motif in the kinase domain, its negative impact on CaMKK2 function directly demonstrates the crucial role R311 plays in catalytic activation. The HRD-motif is a critical feature of nearly all protein kinases and is required for optimal activity and regulation[21]. Although genetic variation of the HRD motif is rare (not just for human CaMKK2 but generally across the kinome[28]), loss-of-function mutations of the cognate arginine in other protein kinases has been shown to cause a range of human diseases[29]. For example, an R274H mutation in the HRD motif of Akt2 causes a profound loss-of-function that results in severe insulin resistance and type 2 diabetes[30], whereas an equivalent R329 mutation in the activin receptor-like kinase-1 (ALK-1) causes hereditary hemorrhagic telangiectasia, an autosomal dominant disorder that leads to abnormal blood vessel formation in the skin and major organs[31]. Likewise, a sporadic R873Q mutation in the HRD motif of the receptor tyrosine kinase RET causes Hirschsprung disease[32]. This not only highlights the functional importance of the HRD motif, but also demonstrates that mutation of the conserved arginine is poorly tolerated and causes sporadic forms of human disease.

Most forms of bipolar disorder are likely to be polygenic in origin, however rare monogenic forms of the disorder might be expected to arise from single *de novo* mutations that have catastrophic, protein-altering effects. In this regard, the R311C mutation is a clear candidate as a sporadic, monogenic cause of bipolar disorder for the following reasons: [i] The R311C mutation

was detected in a bipolar patient but not their unaffected parents[16]; [ii] The R311C mutation is rare in the general population (3.97×10^{-6} minor allele frequency)[28]; [iii] The R311C mutation causes severe loss-of-function and exerts a dominant-negative effect over WT CaMKK2; [iv] CaMKK2 null mice representing a blunt loss-of-function model display behavioral traits similar to humans with bipolar disorder[5]; [v] Mutation of arginine residues functionally equivalent to R311 in the HRD motif of unrelated protein kinases cause rare, monogenic forms of various diseases[30,31,33-35]. In summary, our study reinforces the view that loss-of-function mutations in human CaMKK2 are prime candidates as underlying causes of bipolar disorder and related psychiatric conditions.

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Tables

Table 1: Effect of R311C mutation on CaM sensitivity of CaMKK2.

Kinase activity of WT and R311C mutant CaMKK2 measured over a range of CaM concentrations (0-1000 nM) in the presence of a fixed concentration of Ca^{2+} (100 μM). Data are presented as mean ± SEM; n=3 independent experiments. ***p<0.001 vs WT

CaMKK2 mutation	Kinase activity in the absence of CaM (nmol/min/mg)	Kinase activity in the presence of 1000 nM CaM (nmol/min/mg)	Concentration of CaM required for half-maximal activation (nM)
WT	0.189 ± 0.012	1.062 ± 0.052	66.37 ± 9.44
R311C	0.037 ± 0.002***	0.124 ± 0.011***	345.3 ± 55.5***

Figure Legends:

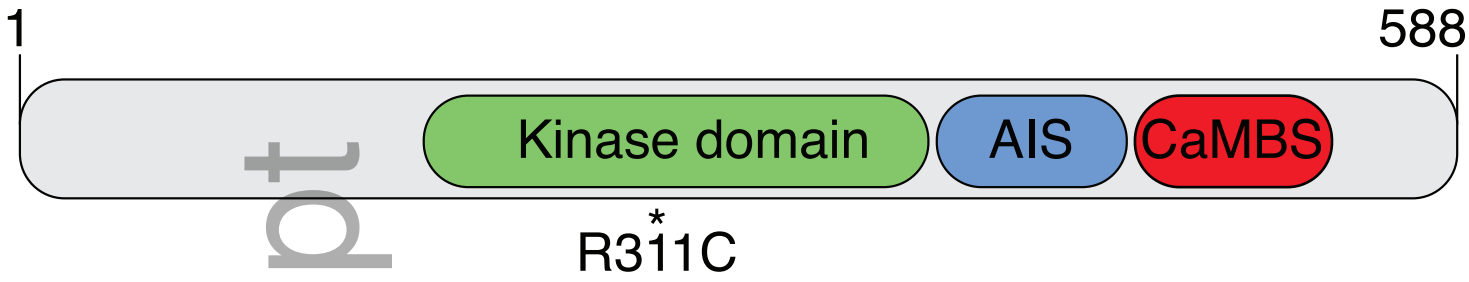
Figure 1: The R311C mutation is located within the highly conserved HRD motif of the CaMKK2 kinase domain. (A) Location of the R311C mutation in relation to known functional domains in human CaMKK2. AIS, autoinhibitory sequence; CaMBS, calmodulin-binding sequence. (B) Sequence conservation of R311 (shown in bold with an *) across CaMKK2 orthologues from diverse species. (C) Structure of the CaMKK2 kinase domain (PDB ID: 2ZV2)[36] showing the location of R311 with respect to the N- and C-terminal lobes (cyan and green, respectively), the catalytic loop (magenta), and the regulatory spine (grey shadow). (D) R311 forms a hydrogen bonding network with N336 and N347 to stabilize the catalytic and activation loops. (E) Structural model showing substitution of R311 with cysteine disrupts the hydrogen bonding interactions with N336 and N347.

Figure 2: The R311C mutation disrupts CaMKK2 activity and Ca^{2+}-CaM activation. (A) Kinase activity of WT and R311C mutant CaMKK2 measured over a range of CaM concentrations (0-1000 nM) in the presence of 100 μM Ca^{2+}. The data were fitted to the equation: Activity = Basal + ((((Fold Stimulation x Basal) - Basal) x [CaM])/($A_{0.5}$ + [CaM])), where $A_{0.5}$ is the concentration of CaM giving half-maximal stimulation. Data are presented as mean ± SEM; n=3 independent experiments. ***p<0.001 vs WT at the same CaM concentration. (B) Comparison of WT and R311C mutant CaMKK2 degradation rate as measured by cycloheximide chase analysis and immunoblot densitometry. The data were fitted to the equation: % of time zero = 100 – (% of time zero/time) x time. Data are presented as mean ± SEM; n=3 independent experiments. Representative immunoblots are shown in (C).

Figure 3: The R311C mutation impairs T85 autophosphorylation. (A) Comparison of the rate of T85 autophosphorylation between WT and R311C mutant CaMKK2 as measured immunoblot densitometry. Data are mean ± SEM, n=3 independent experiments. ####p<0.0001, vs WT at the same time point. Representative immunoblots are shown in (B). (C) Kinase activity of WT and R311C mutant CaMKK2 after autophosphorylation, measured in the presence and absence of 100 μM Ca^{2+}, 1 μM CaM and 1 mM EGTA. Data are mean ± SEM, n=3 independent experiments. **p=0.0027, ****p<0.0001, vs control within the same group; ####p<0.0001, vs WT within the same condition.

Figure 4: The R311C mutation displays dominant-negative inhibition and impairs CaMKK2 signaling and cell viability. (A) Immunoblot analysis of Flag-WT and HA-R311C mutant CaMKK2 purified by Flag or HA-immunoprecipitation from transfected KO cells. Representative immunoblots are shown. (B) Kinase activity of Flag-WT and HA-R311C mutant CaMKK2 purified by Flag or HA-immunoprecipitation from transfected KO cells, measured in the presence and absence of 100 μM Ca^{2+} and 1 μM CaM. Data are mean ± SEM, n=3 independent experiments. ####p<0.0001, vs Flag-WT within the same condition. ##p=0.004, vs HA-R311C within the same condition. (C) Thr172 phosphorylation of the AMPK α subunit quantified by immunoblot from KO cells transfected with Flag-WT or HA-R311C mutant CaMKK2, then stimulated with 5 μM ionomycin for 10 mins. Data are mean ± SEM, n=3 independent experiments. ####p<0.0001, vs Flag-WT within the same condition. Representative immunoblots are shown in (D). (E) Viability of KO cells transfected with Flag-WT or HA-R311C mutant CaMKK2, either separately or together, as measured by reduction of MTS. Data are mean ± SEM, n=3 independent experiments. ##p=0.002 and #p=0.016, vs WT.

(A)



(B)

```
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C.Elegans CaMKK2  266-YLHYQKIVHRDIKPSNLLL-284
S.Pombe CaMKK2  257-YLHYQGIIHRDIKPANLLL-275
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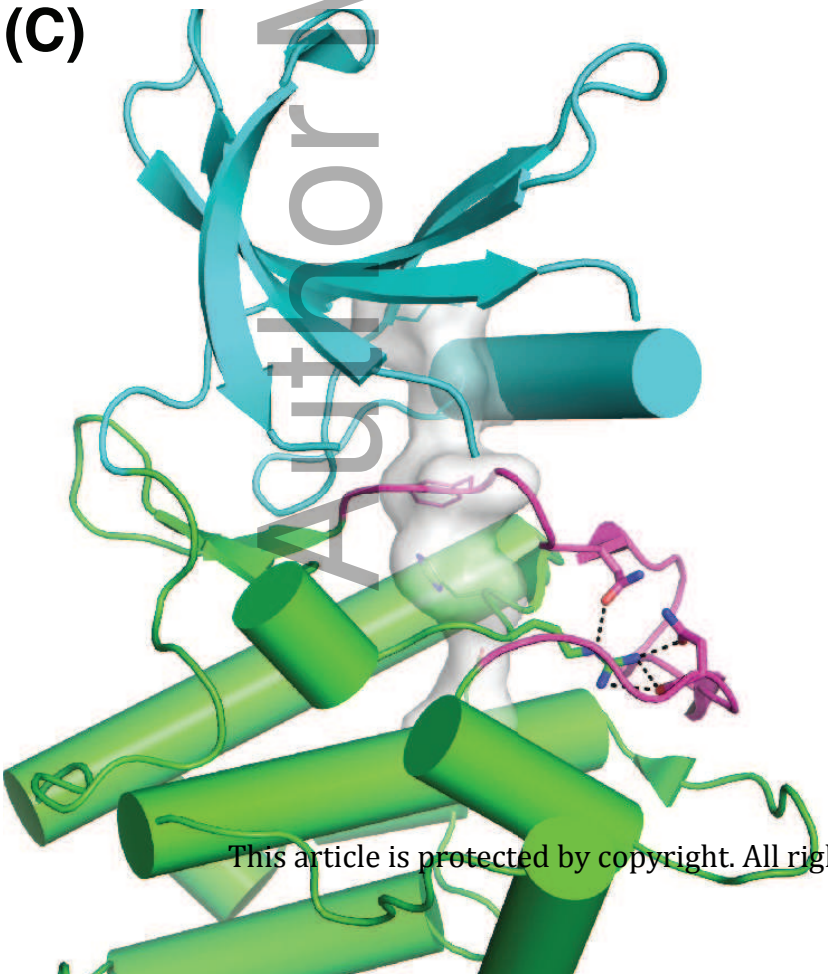
(C)



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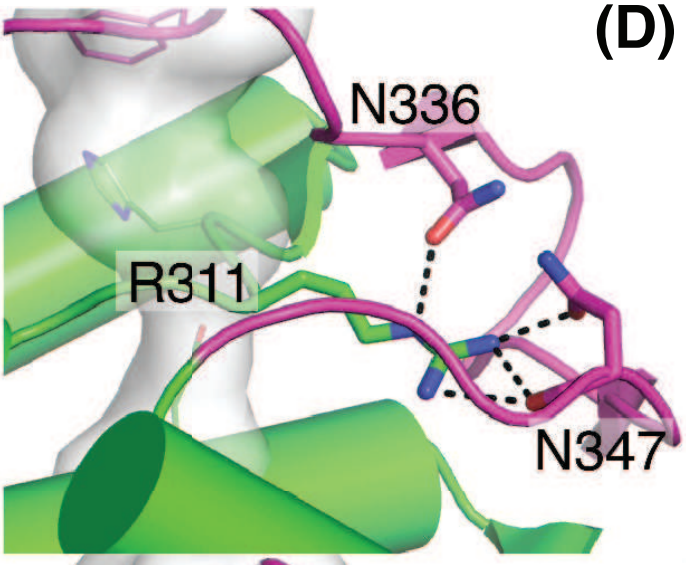

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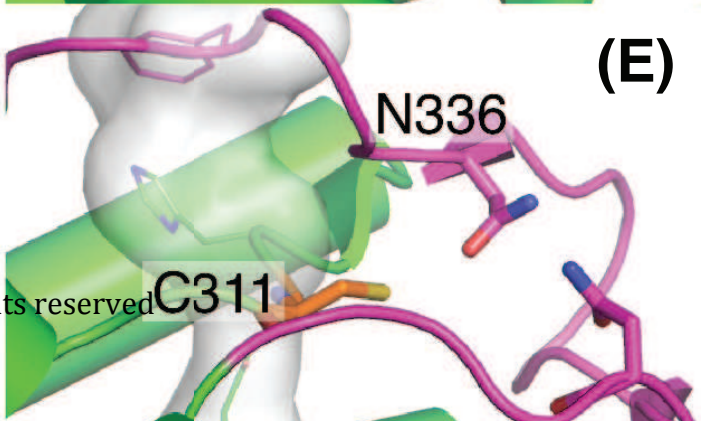

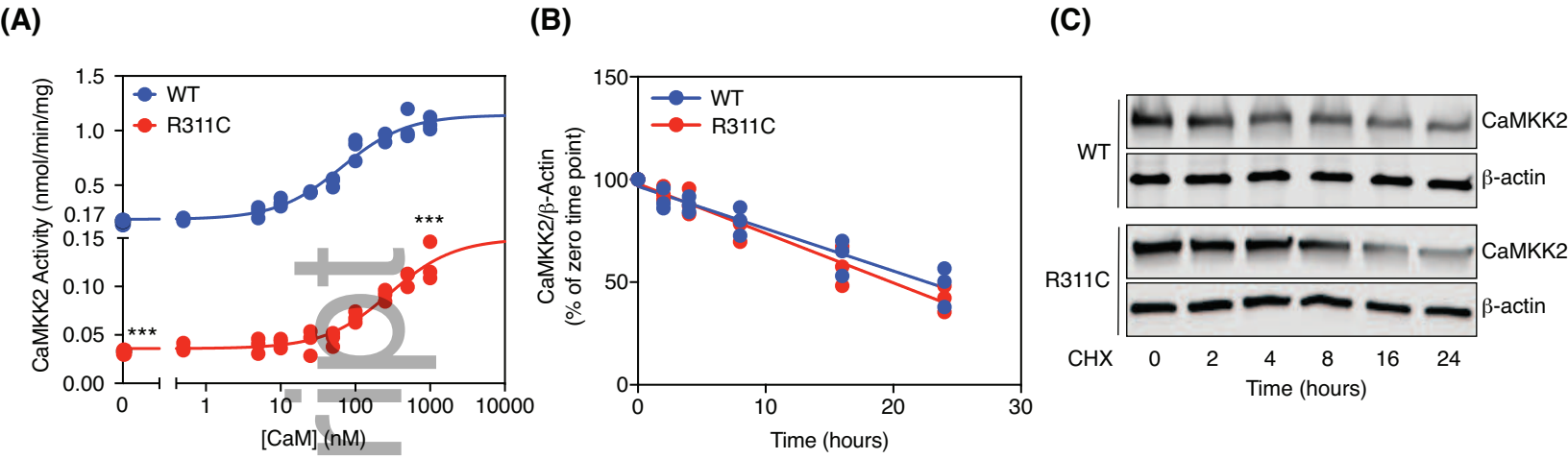

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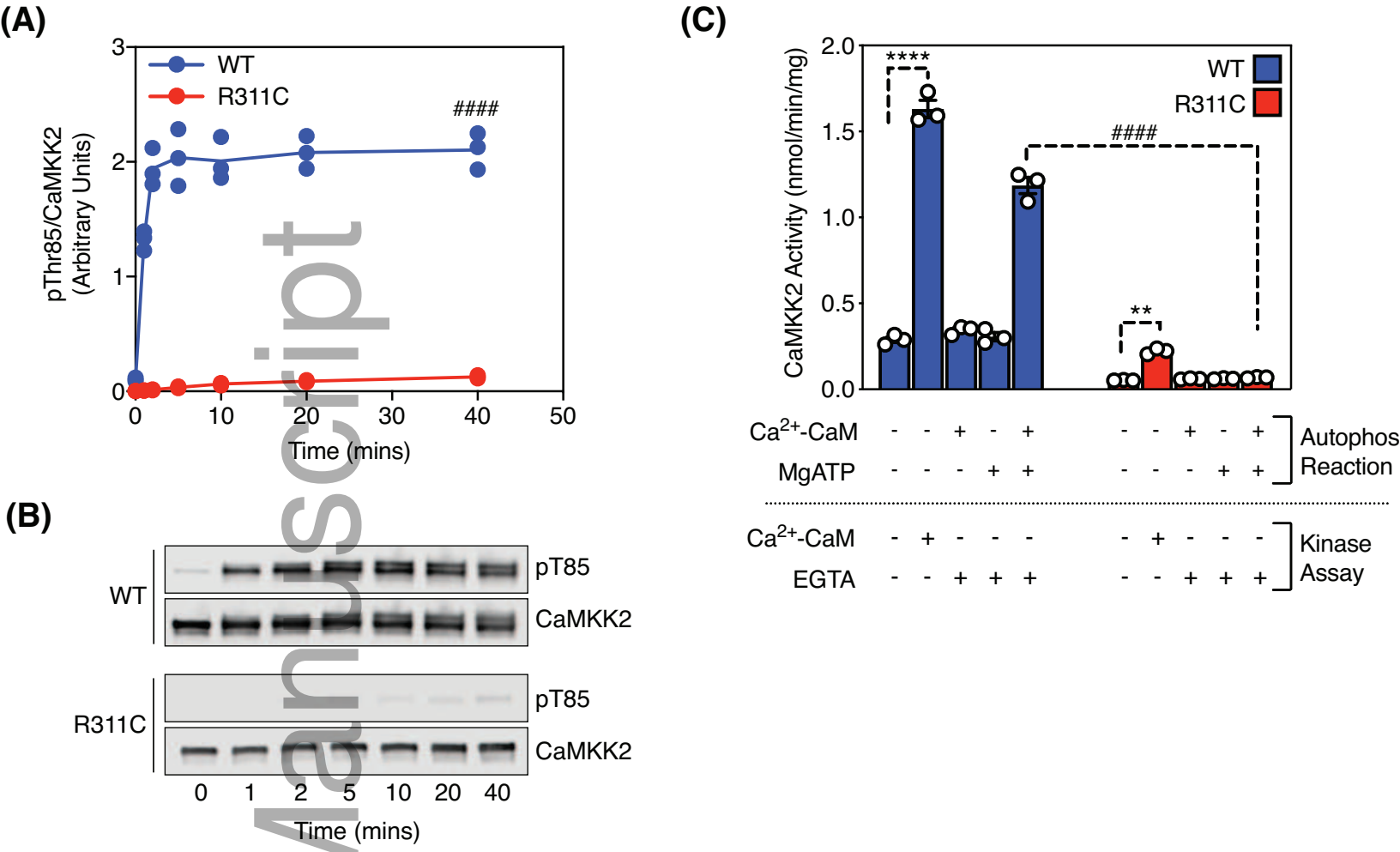

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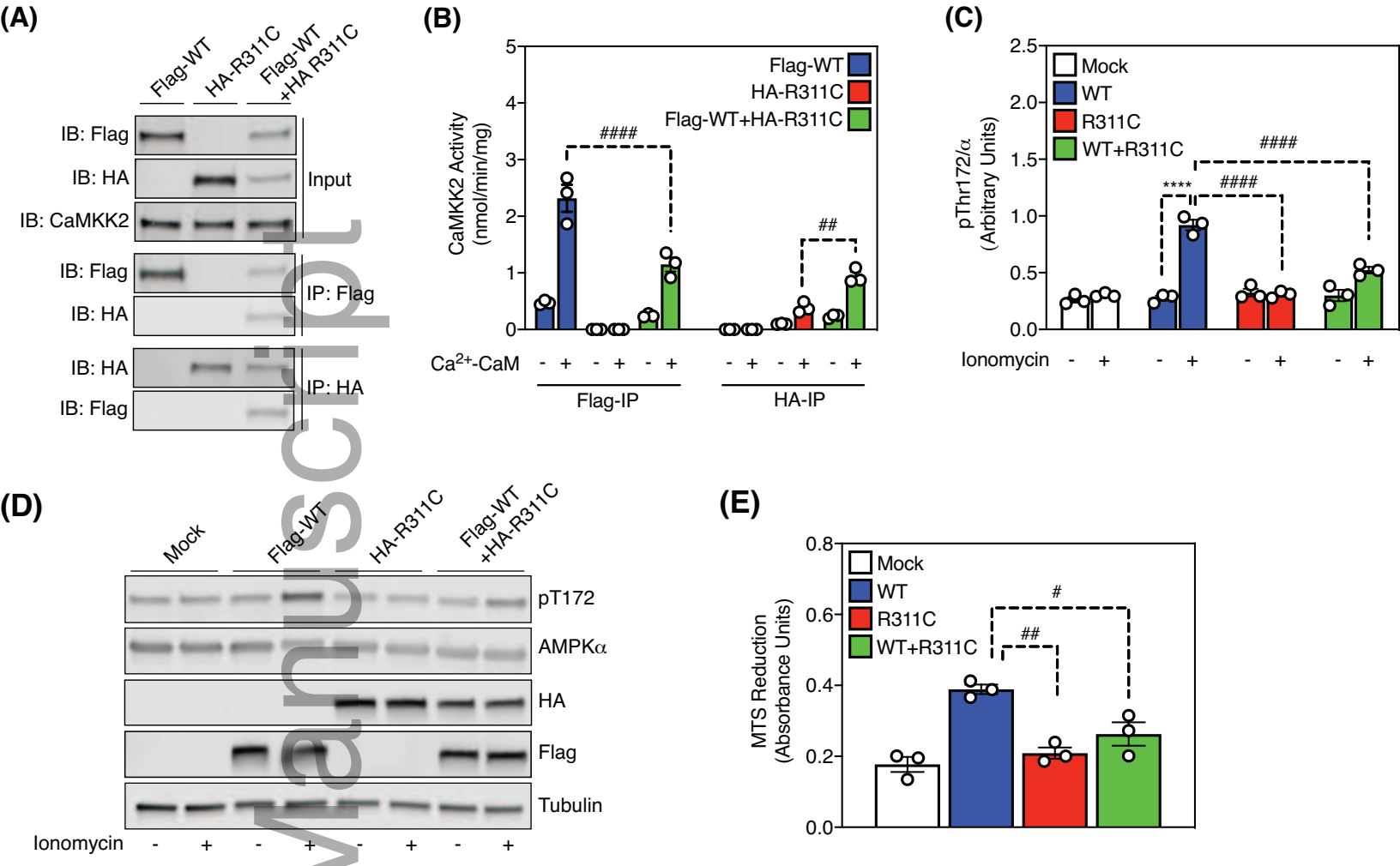

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