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Control of AMPK-related kinases by USP9X and atypical Lys29/Lys33-linked polyubiquitin chains.

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Running Title: USP9X deubiquitinates NUAK1 and MARK4

Key words: Ubiquitin specific protease, protein kinase, ubiquitination, deubiquitination, LKB1.

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Abstract AMPK-related kinases regulate cell polarity as well as proliferation and are activated by the LKB1-tumour suppressor kinase. We demonstrate that the AMPK-related kinases, NUAK1 and MARK4, are polyubiquitinated in vivo and interact with the deubiquitinating enzyme USP9X. Knockdown of USP9X increased polyubiquitination of NUAK1 and MARK4, whilst overexpression of USP9X inhibited ubiquitination. USP9X catalysed the removal of polyubiquitin chains from wild type NUAK1, but not a non-USP9X-binding mutant. Topological analysis revealed that ubiquitin monomers attached to NUAK1 and MARK4 are linked by Lys29 and/or Lys33 rather than the more common Lys48/Lys63. We find that AMPK and other AMPK-related kinases are also polyubiquitinated in cells. We identified non-USP9X binding mutants of NUAK1 and MARK4 and find that these are hyper-ubiquitinated and not phosphorylated at their T-loop residue targetted by LKB1 when expressed in cells, suggesting that polyubiquitination may inhibit these enzymes. Our results demonstrate that NUAK1 and MARK4 are substrates of USP9X and provide the first evidence that AMPK family kinases are regulated by unusual Lys29/Lys33-linked polyubiquitin chains.

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Introduction.

Mutation of the LKB1 protein kinase in humans causes Peutz Jeghers Syndrome (PJS), a disease in which subjects are predisposed to develop benign and malignant tumours [1]. LKB1 forms a complex with the regulatory proteins termed STRAD and MO25 and phosphorylates and activates at least 14 protein kinases that are closely related to AMPK (reviewed [2]). The enzymes activated by LKB1 are AMPKγ 1, AMPKγ 2, QSK, SIK, QIK, MARK1, MARK2, MARK3/Par-1A/C-TAK1, MARK4, NUAK1/ARK5, NUAK2/SNARK, BRSK1/SAD-A, BRSK2/SAD-B and SNRK [2]. LKB1 activates these enzymes by phosphorylating a conserved Thr residue located within the T-loop of the kinase domain. AMPKγ 1 and AMPKγ 2 have been extensively analysed and are activated by LKB1 in response to conditions that lower cellular energy and increase intracellular levels of 5'-AMP. Activation of AMPK enables cells to control their energy resources under situations of stresses [3]. Less is understood about the cellular roles of other LKB1-activated kinases, that are collectively termed AMPK-related kinases [4]. Genetic analysis suggests that MARK isoforms regulate cell polarity [5-7]. The BRSK/SAD enzymes have recently been shown to control axon initiation during neuronal polarisation [8-10]. The functions of the other AMPK-related kinases including isoforms of NUAK are largely uncharacterised.

Several AMPK-related kinases including QSK and MARK isoforms interacted with the 14-3-3 adaptor proteins which was shown to regulate their localisation as well as activity [7, 11-14]. In addition to 14-3-3 isoforms, other proteins interacted with overexpressed TAP-tagged AMPK-related kinases in 293 cells, however, their physiological roles were not explored further [11, 13]. One of these was the deubiquitinating enzyme termed ubiquitin specific protease-9 (USP9X), the orthologue of the *Drosophila* fat facets (dFAF) [11, 13]. USP9X is one of the ~15% of genes encoded on the X chromosome that escapes X-inactivation, a phenomena that ensures most genes located on this chromosome are expressed at the same levels in male and female cells. The Y chromosome encodes a gene possessing 91% identity in sequence to USP9X, termed USP9Y that is reportedly expressed in male cells [15].

USP9X is a large 2547-residue enzyme which is widely expressed in all tissues [15]. Apart from a deubiquitinating catalytic domain located between residues 1531 and 1971 belonging to the USP class of deubiquitinating enzymes, and a putative ubiquitin like domain (residues 873-963) no other obvious domains are present [16, 17]. Genetic analysis in *Drosophila*, suggests that dFAF regulates the polarity and fate of particular cells in the eye and also plays roles in ovary development as well as embryo viability [18, 19]. Overexpression of dFAF in neuronal cells of *Drosophila* led to profound synaptic overgrowth and disruption of synaptic function [20]. Biochemical as well as genetic data suggest that USP9X interacts with and deubiquitinates the endocytic adaptor protein Epsin-1 [21-23]. This may play a role in the receptor-induced endocytosis regulated by the Notch pathway [24]. Others have reported that USP9X deubiquitinates β-catenin [25], the ras-target AF-6 [26], Survivin [27] and more recently the E3 ubiquitin ligase Itch [28].

Posttranslational modification of proteins by the small ubiquitin molecule is emerging as a key regulatory mechanism that is beginning to rival phosphorylation in its global importance [29]. It is also becoming clear that ubiquitination is controlling almost all aspects of protein function not only stability [29]. The regulation of AMPK or AMPK-related kinases by ubiquitination has not previously been investigated. In this study we provide the first evidence that the NUAK1 and MARK4 kinases are ubiquitinated in vivo and that these enzymes are deubiquitinated by USP9X. Our results suggest that ubiquitination of NUAK1 and MARK4 is not controlling their stability, but rather may be inhibiting their phosphorylation and activation by LKB1. Moreover, our data suggest that polyubiquitination of NUAK1 and MARK4 is mediated by an unusual Lys29 and Lys33 linkage, rather than the more common Lys48 or Lys63 linkages.

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Materials and Methods.

Materials. Protein G–Sepharose, Calmodulin-Sepharose 4B, glutathione-Sepharose, streptavidin-Sepharose, ^{32}P -ATP and enhanced chemiluminescence reagent were purchased from Amersham Bioscience; protease-inhibitor cocktail tablets, and precast SDS polyacrylamide Bis-Tris gels were from Invitrogen; Tween-20, rabbit IgG-Agarose, HA-agarose resin, dimethyl pimelimidate, N-ethylmaleimide and polybrene were from Sigma; MG-132 was from Calbiochem; Protein Desalting Spin Columns were from PIERCE; Lys48-linked tetra-ubiquitin chains and Lys63-linked tetra-ubiquitin chains were from BostonBiochem; NP-40 was from Fluka; and phosphocellulose P81 paper was from Whatman. The hexahistidine tagged TEV protease was expressed in E.coli by Elton Zeqiraj and purified using nickel agarose affinity chromatography and gel filtration. All peptides were synthesised by Dr Graham Bloomberg at the University of Bristol.

Antibodies. The following antibodies were raised in sheep and affinity purified on the appropriate antigen: anti-USP9X (residues 2311-2547 of human USP9, used for immunoblotting (IB) and immunoprecipitation (IP)), anti-NUAK1 (residues 1-661 of human NUAK1, used for IB and IP), Phospho-anti-T-loop MARK (residues 204-218 of human MARK3 phosphorylated at Thr211, TVGGKLDT(P)FCGSPPY, used for IB), Phospho-anti-T-loop NUAK (residues 305-319 of human NUAK2, HQGKFLQT(P)FCGSPLY, used for IB), anti-AMPKγ 1 (residues 344-358 of rat AMPKγ 1, CTSPPDSFLDDHHLTR) and the anti-GST (raised against the glutathione S-transferase protein, used for IB). Mouse monoclonal antibodies recognizing HA epitope tag was purchased from Roche (#1666606) and monoclonal antibodies recognizing FLAG epitope tag was purchased from Roche (#F3165). Rabbit polyclonal anti-ubiquitin was purchased from DakoCytomation (#Z0458) and secondary antibodies coupled to horseradish peroxidase used for immunoblotting were obtained from Pierce.

General methods. Tissue culture, transfection, immunoblotting, restriction enzyme digests, DNA ligations, and other recombinant DNA procedures were performed using standard protocols. All mutagenesis was carried out by the QuickChange site-directed mutagenesis method (Stratagene) using KOD polymerase (Novagen). DNA constructs used for transfection were purified from *E. coli* DH5γ using Qiagen plasmid Mega or Maxi kit according to manufacturer's protocol. All DNA constructs were verified by DNA sequencing, which was performed by The Sequencing Service, School of Life Sciences, University of Dundee, Scotland, UK, using DYEnamic ET terminator chemistry (Amersham Biosciences) on Applied Biosystems automated DNA sequencers.

Buffers. Lysis Buffer contained: 50 mM Tris/HCl pH 7.5, 1 mM EGTA, 1mM EDTA, 1 % (w/v) NP-40, 1 mM sodium orthovanadate, 10 mM sodium-β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM dithiothreitol (DTT) and complete proteinase inhibitor cocktail (one tablet/50 ml). To detect ubiquitination in lysates, 5 mM N-ethylmaleimide (NEM) (SIGMA no E1271) was added to lysis buffer lacking DTT just prior to use. Buffer A contained: 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.27 M sucrose and 1 mM DTT. Buffer B contained: 50 mM Tris/HCl pH 7.5, 0.15 M NaCl, 0.27 M sucrose, 1% (w/v) NP-40 and 1 mM DTT. Buffer C contained: 50mM Tris pH 7.5, 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM Imidazole, 2 mM CaCl2, 0.27 M sucrose and 1 mM DTT. Buffer D contained: 50 mM Tris pH 7.5, 20 mM EGTA, 150 mM NaCl and 5 mM DTT. TBS-Tween Buffer contained: 50 mM Tris/HCl pH 7.5, 0.15 M NaCl and 0.2% (v/v) Tween-20. Sample Buffer was 1μ NuPAGE® LDS (lithium dodecyl sulphate) sample buffer (Invitrogen) containing 0.14 M Tris 2% (w/v) **LDS**, 10% (v/v) glycerol, final pH 8.5.

Plasmids. The cloning of NUAK1, MARK4 and other AMPK related kinases have been described earlier [4]. In order to clone USP9X (transcript variant 4, accession number EAW59412) an EST was ordered from Geneservice ltd. (IMAGE CLONE 30422528, NCBI acc. CD657533), which covered the coding region of residues 540-2554 (end). The missing N-terminal 2.2 kb of the USP9X cDNA was obtained by RT-PCR from HeLa mRNA (Stratagene) using SuperScript III PCR amplification kit

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(Invitrogen) and the following oligonucleotides: 5-atgacagccacgactcgtggctct-3 and 5'-caagtcatccatcatataggctct-3'. The resulting PCR product was subcloned into pSC-A intermediate vector (Stratagene). To create the full length USP9X cDNA in several mammalian expression vectors a 5'-BamH1 site and a 3'-Not1 site was incorporated by PCR into the intermediate constructs, then 3 way ligations were carried out using BamH1-Spe1 5' 2.2. kb 5' end and Spe1-Not1 5.4 kb 3' end fragments. Full length USP5 (NCBI Acc. P45974) was PCR amplified from IMAGE clone 3506801(NCBI Acc. BC005139) and subcloned as a BamH1-Not1 fragment into several expression vectors. Full length USP7 (NCBI Acc. EAW85194 was PCR amplified from EST dkfzp434f179q2/NCBI Acc. AL046721 (Imagenes) and subcloned as a BamH1-Not1 fragment into several expression vectors. CYLD (NP_056062) was PCR amplified from IMAGE clone 4552767 using the GC Rich PCR System (Roche). The resulting fragment was ligated into pCR2.1 (Invitrogen), sequenced to completion and subcloned into the BamH1 site of pFBHTb (Invitrogen) to form pFNHTb CYLD. Ubiquitin (NCBI P62988) was RT-PCR amplified from total RNA (human peripheral blood) using the One Step RTPCR Superscript III kit (Invitrogen), then cloned into pSC−a (Stratagene) and sequenced to completion. It was subcloned into the BamH1−Not1 sites of pCMVFLAG−1 to form pCMV-FLAG ubiquitin.

Generation of USP9X stable cell lines. HEK-293 cells were cultured in 10-cm diameter dishes to 50-70% confluence and transfected with 2 μg of pEGFP-C2-TAP construct [13], encoding human full length USP9X wild type or human full length mutant USP9X [C1559A] using Fugene 6 reagent (Roche) according to the manufacturer's instructions. After 24 h, G418 was added to the medium to a final concentration of 3 mg/ml, and the medium was changed every 24 h maintaining G418. After 14-20 days, individual surviving colonies expressing low levels of GFP-fluorescence were selected and expanded. FACS analysis was also performed to ensure uniform expression of GFP in the selected cell lines and anti-USP9X immunoblotting analysis of lysed cells was also undertaken to ensure that the expressed proteins migrated as a single molecular weight species at the expected apparent molecular mass (the isolated GFP-TAP tag adds 50 kDa to the molecular mass of a protein).

Purification of full length USP9X protein. The purification method was adapted from the previously described TAP-purification protocol [30]. For each purification, twenty-five 15 cm dishes of confluent 293 cell lines stably expressing wild type USP9X or catalytically inactive USP9X[C1559A] were employed. Cells were washed twice with ice-cold Phosphate Buffered Saline and lysed in 1.0 ml of ice-cold Lysis Buffer. The combined lysates were centrifuged at 26,000 x g for 30 min at 4°C and the supernatant incubated with 0.2 ml of rabbit IgG-agarose beads for 1 h at 4°C. The IgG-agarose was washed extensively with Lysis Buffer containing 0.15 M NaCl, then with several washes in Buffer B prior to incubation with 0.250 ml of Buffer B containing 0.1 mg of TEV protease. After 3 h at 4°C ~70-90% of the TAP-tagged protein had been cleaved from the IgG-agarose and the eluted protein was incubated with 0.1 ml of rabbit calmodulin-Sepharose equilibrated in Buffer C. After 1 h at 4°C, the calmodulin-Sepharose was washed with Buffer C. To elute the protein, the calmodulin-Sepharose was then incubated with 0.1 ml of Buffer D for 10 min at 4°C. The eluate was removed from the beads and the elution repeated 2-3 times. To remove the NaCl present in the buffer containing the eluate protein, the eluates were centrifuged for 1 min at 4°C in protein desalting spin columns.

Deubiquitination assays. Ubiquitinated NUAK1 was employed as a substrate that was obtained by transecting 293 cells with HA-NUAK1 and FLAG-ubiquitin. 36 h post-transfections cell lysates were generated in the presence of 5 mM NEM and for each assay NUAK1 was immunoprecipitated from 1 mg of cell extracts. Assays were set-up in a total volume of 25 μl containing 50 mM Tris-HCl pH 8.3, 25 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 0.5 μg of purified USP9X or other deubiquitination enzyme and immunoprecipitated ubiquitinated HA-NUAK1 conjugated to 5 μl of HA-agarose. The same conditions were employed when tetraubiquitin was used as the substrate except that HA-NUAK1 was replaced with 100 ng of Lys48 or Lys63-linked

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tetraubiquitin. Reactions were initiated by the addition of the deubiquitinating enzyme and performed on a vibrating platform at 30°C. For reactions containing ubiquitinated HA-NUAK1, reactions were terminated by brief centrifugation to pellet the HA-NUAK1-agarose conjugate and the supernatant was removed and added to LDS-Sample Buffer. The agarose resin was washed twice in Buffer A containing 0.15M NaCl and twice with Buffer A containing no added NaCl then added to an equal volume of 2X LDS-Sample Buffer. The reactions employing tetraubiquitin as substrates were terminated by addition of LDS-Sample Buffer. Deubiquitination was assessed by subjecting the samples in LDS-Sample Buffer to immunoblot analysis with anti-ubiquitin antibodies.

Immunoblotting. Total cell lysate (10-50 μg) or immunoprecipitated samples were heated at 70°C for 5 min in SDS sample buffer, subjected to polyacrylamide gel electrophoresis and electrotransfer to nitrocellulose membrane. To improve detection of ubiquitinated proteins nitrocellulose membranes were heated at 100 °C for 5 min in Milli-Q water prior to the blocking step [31]. Membranes were blocked in TBS-Tween Buffer containing 10% (w/v) skimmed milk. The membranes were probed with 1 μg/ml of indicated antibodies sheep antibodies or a 1:1000 dilution of commercial antibodies in TBS- Tween, 5% (w/v) skimmed milk for 16 h at 4°C. Detection was performed using horseradish peroxidase conjugated secondary antibodies and the enhanced chemiluminescence reagent.

Expression and purification of USP5, USP7 and CYLD. The pGEX expression constructs encoding human full-length wild type USP5 or USP7 [214-522] were transformed into *E.coli* BL21 cells. One-litre cultures were grown at 37°C in Luria broth containing 100 mg/ml ampicillin until the absorbance at 600 nm was 0.8. Induction of protein expression was carried out by adding 100 μM isopropyl-B-D-galactoside and the cells were cultured for a further 16 hr at 26°C. Cells were isolated by centrifugation, resuspended in 15 ml of ice-cold lysis buffer and lysed in one round of freeze/thawing, followed by sonication to fragment DNA. The lysates were centrifuged at 4°C for 30 min at 26,000 x g, and the recombinant proteins were affinity purified on glutathione-Sepharose and eluted in Buffer A containing 20 mM glutathione. Full length human His-tagged CYLD was expressed in insect cells using baculovirus expression system and purified on nickel NTA-Sepharose as described previously for PDK1 [32].

Expression and purification GST-AMPK-related kinases in HEK-293 cells. Typically ten 10-cm diameter dishes of HEK-293 cells were cultured and each dish transfected with 10 μg of the pEBG-2T construct encoding wild-type AMPK-related kinase using the polyethylenimine method [33]. The cells were cultured for a further 36 h and lysed in 0.5 ml of ice-cold lysis buffer, the lysates pooled and centrifuged at 4°C for 10 min at 26,000 x g. The GST-fusion proteins were purified by affinity chromatography on glutathione-Sepharose and eluted in Buffer A containing LDS sample buffer.

Transfection and generation of lysates. Typically ten 10-cm diameter dishes of HEK-293 cells were cultured and each dish transfected with a total of 10 μg indicated plasmids using the polyethylenimine method [33]. The cells were cultured for a further 36 h and lysed in 0.5 ml of ice-cold lysis buffer either in the absence or presence of NEM. Cell lysates were clarified by centrifugation at 4°C for 10 min at 26,000 x g. Rat brain extracts were generated by homogenising 3 rat brains in a 10X volume of Lysis Buffer not containing NEM and clarified as above.

Immunoprecipitation. 0.1-1 mg of clarified cell lysates were subjected to immunoprecipitation employing 5 μg the indicated antibody conjugated to 5 μl Protein G-Sepharose or in the case of HA-epitope tagged proteins 5 μg of HA antibody covalently conjugated to 5 μl of agarose resin (purchased from SIGMA). Immunoprecipitation was undertaken for 1 hr at 4°C on a vibrating platform, the immunoprecipitates washed twice with 1 ml of Lysis Buffer containing 0.15 M NaCl and twice with 1 ml of Buffer A lacking DTT. The immunoprecipitates were assayed for kinase activity or analysed by immunoblotting.

In vitro kinases assays employing the AMARA peptide. The activity of the immunoprecipitated AMPK-related kinases was quantified by measurement of

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phosphorylation of the AMARA (AMARAASAAALRRR) peptide substrate [4]. The immunoprecipitated HA-NUAK1 or HA-MARK4 were incubated in a 50 μl mixture containing 50 mM Tris-HCl pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM [γ-32P]-ATP (300 cpm/pmol) and 200 μM AMARA peptide for 20 min at 30°C. Incorporation of ^{32}P-phosphate into the peptide substrate was determined by applying 40 μl of the reaction mixture onto P81 phosphocellulose paper and scintillation counting after washing the papers in phosphoric acid as described previously. One Unit (U) of activity was defined as that which catalysed the incorporation of 1 nmol of ^{32}P into the substrate. In assays requiring LKB1 activation, the immunoprecipitated NUAK or MARK4 proteins were pre-incubated with 5 μg of LKB1:STRAD:MO25 complex kindly provided by Elton Zeqiraj in Buffer A containing 10mM $MgCl_2$ and 0.1 mM ATP in a final volume of 20 ml. Following the LKB1 activation the kinases were either assayed using the AMARA peptide substrate or subjected to immunoblot analysis.

Lentivirus production and infection. The pLK0.1 lentiviral-based shRNA constructs were employed to knock-down USP9X protein levels. We obtained 4 independent short-hairpin constructs specific for human USP9X from the MISSION™ TRC-HS 1.0 library (SIGMA reference no. NM_004652). Only one of the four constructs tested significantly reduced USP9X protein expression and was employed in our studies (no. TRC shRNA, TRCN0000007361 5'CCGGGAGAGTTTATTCACTGTCTTACTCGAGTAAGACAGTGAATAAACT CTCTTTTT-3'). We also utilised control shRNA construct in the same vector (5'CCTAA GGTTA AGTCG CCCTC GCTCT AGCGA GGGCG ACTTA ACCTT AGG3'). To generate lentivirus, HEK-293 cells grown on 10 cm diameter dishes were transfected with a plasmid mix containing 3.5 μg of the shRNA-encoding plasmid, 3.5 μg of pCMV delta R8.2 (packaging plasmid) and 3.5 μg of pCMV-VSV-G (envelope plasmid) using the polyethylenimine method [33]. 60 hr post-transfection, the virus-containing medium of cells was collected and filtered through a 0.2 μm filter. Typically 6 to 7 ml of viral supernatant was used to infect HEK-293 cells cultured in 10-cm diameter dishes in the presence of 5 μg/ml polybrene. After 16 hours the virus-containing medium was replaced with fresh medium not containing virus.

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Results.

Interaction of USP9X with NUAK1 and MARK4. To corroborate findings of previous proteomic screens that indicated USP9X interacted with certain AMPK-related kinases [11, 13], we overexpressed thirteen AMPK-related kinases and tested whether these interacted with endogenous USP9X in 293 cells. Under these conditions, USP9X interacted with NUAK1 and MARK4, but not with the other AMPK-related kinases (Fig 1A). We also found that endogenous NUAK1 and MARK4 were co-immunoprecipitated with endogenous USP9X from rat brain or 293 cells (Fig 1B).

Evidence that NUAK1 and MARK4 are ubiquitinated and constitute substrates for USP9X in vivo. To study whether NUAK1 or MARK4 were ubiquitinated in vivo, HEK 293 cells overexpressing NUAK1 or MARK4 were lysed in the absence or presence of N-ethylmaleimide (NEM) in order to inhibit deubiquitinating enzymes and thus prevent deubiquitination of proteins in cell extracts [16, 34]. Ubiquitination was assessed following immunoprecipitation and electrophoresis of NUAK1 and MARK4 on a polyacrylamide gel and immunoblot analysis with an anti-ubiquitin antibody. In the presence of NEM, a higher molecular weight ubiquitinated species of immunoprecipitated NUAK1 and MARK4 was readily detected that ran as a smear on a polyacrylamide gel, typical of a polyubiquitinated protein (Fig 2A). When cells were lysed in the absence of NEM, much reduced ubiquitination of NUAK1 or MARK4 was observed. Endogenously expressed NUAK1 from 293 cells was also polyubiquitinated (Fig 2B).

To explore the role of USP9X in controlling polyubiquitination of NUAK1 and MARK4, we generated 293 cells stably overexpressing either full length wild type USP9X or catalytically-inactive USP9X[Cys1559Ala] and investigated how the overexpression of these forms of USP9X affected the ubiquitination of MARK4 and NUAK1. In cells stably overexpressing wild type USP9X, no significant ubiquitination of NUAK1 or MARK4 was observed (Fig 2C). In cells stably expressing inactive USP9X[Cys1559Ala], NUAK1 and MARK4 were ubiquitinated to a higher level than observed in the control 293 cells not overexpressing USP9X (Fig 2C), suggesting that this mutant acts in a dominant negative manner preventing deubiquitination of NUAK1/MARK4 by endogenous USP9X.

We also investigated how reduction of USP9X expression in 293 cells influenced ubiquitination of NUAK1 and MARK4. For these studies we utilised a USP9X shRNA encoding lentivirus, derived from the MISSION™ TRC-HS 1.0 library (SIGMA), which reproducibly reduced endogenous USP9X protein expression by 80-90%. Under these conditions, both NUAK1 and MARK4 were ubiquitinated to a markedly greater extent than observed in control cells (Fig 2D).

Full-length USP9X deubiquitinates NUAK1 in vitro. To verify whether USP9X can directly catalyse the deubiquitination of NUAK1, we purified either full-length wild type USP9X or catalytically inactive USP9X[Cys1559Ala] from stable 293 cells expressing these enzymes as described in the Materials and Methods. The large 2547-residue USP9X protein isolated in this manner was not degraded and was the major Comassie-staining protein on a polyacrylamide gel (Fig 3A). We incubated the purified USP9X with immunoprecipitated ubiquitinated NUAK1 conjugated to an agarose resin. Incubation of immunoprecipitated ubiquitinated wild type NUAK1 with USP9X, resulted in a significant release to the supernatant of a polyubiquitinated species migrating within a range of ~20-250 kDa (Fig 3B). There was no evidence of USP9X catalysing the release of mono-ubiquitin from NUAK1 (Fig 3B). In contrast, catalytically inactive USP9X[C1559A], did not induce detectable deubiquitination of immunoprecipitated NUAK1 in parallel experiments (Fig 3B). We also found that other deubiquitinating enzymes tested, namely USP5, USP7 and CYLD failed to deubiquitinate NUAK1 (Supplementary Fig 1) under conditions where they hydrolysed Lys48- or Lys63-linked tetraubiquitin (Fig 3C). We also found that full-length USP9X under conditions in which it deubiquitinated NUAK1, failed to hydrolyse Lys48 or Ly63 linked tetraubiquitin (Fig 3C).

Evidence that polyubiquitination of NUAK1 and MARK4 involves atypical Lys29 and Lys33 linkages. Polyubiquitin chains normally result from one of the

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seven Lys residues on ubiquitin (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) forming an isopeptide bond with the C-terminal Gly residue of another ubiquitin molecule [29]. To investigate which of the Lys residue(s) on ubiquitin were required for formation of polyubiquitin chains on NUAK1 and MARK4, these enzymes were co-expressed in 293 cells with FLAG-ubiquitin mutants in which all Lys residues except one were changed to Arg (Fig 4A) or in which individual Lys residues were mutated to Arg (Fig 4B). NUAK1 and MARK4 were then immunoprecipitated and immunoblotted with a FLAG antibody to determine whether these were polyubiquitinated with the mutant forms of the FLAG-ubiquitin. Strikingly, these studies revealed that only ubiquitin molecules containing Lys residues at positions 29 and/or 33 were capable of ubiquitinating NUAK1 or MARK4 (Fig 4A). Consistent with this, ubiquitin molecules lacking either or both of these Lys residues were significantly less able to ubiquitinate NUAK1 or MARK4, whereas mutation of the other Lys residues in ubiquitin did not affect ubiquitination (Fig 4B). Lys29/Lys33 linked polyubiquitin chains were directly linked to NUAK1 rather than co-immunoprecipitated associated proteins, as NUAK1 when co-transfected with either wild type ubiquitin or Lys29/Lys33-only ubiquitin mutants, was still polyubiquitinated after its immunoprecipitation from a buffer containing 1% (by mass) SDS (Supplementary Fig 2). In parallel experiments, TRAF6 a protein regulated by Lys48 and Lys63 polyubiquitination [35], was only polyubiquitinated in the presence of ubiquitin chains containing Lys48 or Lys63 (Fig 4A) and mutation of both Lys48 and Lys63 were required to prevent its ubiquitination (Fig 4B).

Evidence that AMPK and other AMPK-related kinases are polyubiquitinated in vivo. Using a similar approach we found that several other AMPK-related kinases including isoforms of MARK and BRSK were ubiquitinated in cells to various degrees when co-expressed with either wild type ubiquitin or mutant ubiquitin containing only Lys29 and Lys33 (Fig 5A). MARK3 was ubiquitinated to the same extent as MARK4, however MARK2 was not significantly ubiquitinated (Fig 5A). Eight other kinases not related to AMPK were not ubiquitinated when co-transfected with ubiquitin mutant possessing only Lys29 and Lys33 (Fig 5A). We also found that endogenous AMPK immunoprecipitated from fibroblast cells lysed in the presence of NEM was significantly polyubiquitinated (Fig 5B). In the absence of NEM, conditions where AMPK is normally analysed, it was not significantly polyubiquitinated (Fig 5B).

Identification of non-USP9X interacting forms of NUAK1 and MARK4. To investigate the functional significance of the NUAK1/MARK4 and USP9X interaction we attempted to generate mutants of NUAK1/MARK4 that were unable to bind USP9X. Fragments of NUAK1 were expressed in 293 cells and their ability to bind to endogenous USP9X analysed (Fig 6A). This revealed that the removal of a region encompassing the C-terminal boundary of the NUAK1 kinase domain (residues 300-310), abolished binding to USP9X. Removal of the equivalent residues on MARK4 also prevented interaction with USP9X (Fig 6B). We next mutated residues within the kinase C-terminal boundary region and studied the effect that this had on USP9X-binding. This revealed that mutation of Trp305 to Ala (Fig 6C) or Phe, Val or Ile (Fig 6D) prevented binding of NUAK1 to USP9X. Sequence alignments indicate that the Trp305 residue is the only residue within the kinase C-terminal boundary region that is conserved in all AMPK family protein kinases (Fig 6E). Mutation of the equivalent Trp residue in MARK4 (Trp309) also prevented USP9X interaction (Fig 6F). Residues surrounding the conserved Trp residue are less well conserved between AMPK-related kinases (Fig 6E) and mutations of these residues had variable effects on USP9X binding (Fig 6C and 6F). The C-terminal boundary of NUAK1 may not interact directly with USP9X as GST-fusions encompassing this region when expressed in 293 cells failed to interact with endogenous USP9X (Supplementary Fig 3). Significantly, wild type purified USP9X protein was unable to catalyse the deubiquitination of the non-USP9X-binding NUAK1[W305F] mutant, indicating that USP9X needs to interact with NUAK1 in order to deubiquitinate it (Fig 3B).

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Evidence that ubiquitination of NUAK1 and MARK4 may inhibit activity and phosphorylation by LKB1. Modulating the degree of ubiquitination of NUAK1 or MARK4 by overexpression or knock-down of USP9X in 293 cells did not markedly affect stability of these enzymes (Fig 2). Moreover, treatment of cells with the proteasome inhibitor MG132 or lysosome inhibitor leupeptin/NH_4Cl in the presence of the protein synthesis inhibitor cycloheximide, did not significantly alter the cellular levels of NUAK1 or MARK4 (Supplementary Fig 4). We next investigated whether ubiquitination of NUAK1 and MARK4 could be correlated with catalytic activity as well as T-loop phosphorylation. As NEM potently inhibited the catalytic activity of NUAK1 and MARK4 (AZ data not shown), cells were lysed in the absence of NEM for experiments in which protein kinase activity was analysed. Strikingly, in contrast to wild type NUAK1 or MARK4, the non-USP binding NUAK1[W305A] and MARK4[W309A] mutants, were devoid of catalytic activity and were also not phosphorylated at their T-loop residues when expressed in 293 cells (Fig 7A & 7B, upper panel). Consistent with the NUAK1[W305A] and MARK4[W309A] mutants being unable to interact with USP9X, these were ubiquitinated to a greater extent than wild type enzymes when expressed in 293 cells (Fig 7A & 7B, lower panel). Analysis of other NUAK1 and MARK4 mutants revealed that ubiquitination, activation and T-loop phosphorylation of these enzymes could also be correlated with their ability to bind USP9X (compare Fig 6C with Fig 7A or Fig 6F with Fig 7B). For example, the NUAK1[H303A], MARK4[I310A], MARK4[N311A], MARK4[G313A] and MARK4[Y314A] mutants, which either fail to interact with USP9X or bind weakly (Fig 6), were hyper-ubiquitinated and displayed low activity and markedly reduced T-loop phosphorylation (Fig 7A & 7B). The other mutants of NUAK1 and MARK4 that interact with USP9X to a greater extent were significantly more active and phosphorylated at their T-loop residues (Fig 7A & 7B). In vitro studies demonstrated that the LKB1:STRAD:MO25 complex phosphorylated and activated the NUAK1[W305A] and MARK4[W309A] non-USP9X binding mutants, showing that the mutations did not affect intrinsic structure and catalytic properties of these enzymes. The four other non-USP9X-binding mutants of MARK4 (Ile310, Asn311, Gly313 and Tyr314), were also phosphorylated and activated by the LKB1:STRAD:MO25 complex. Only the NUAK1[H303A] mutant was not phosphorylated or activated by LKB1, indicating that this mutation disrupted the structure of the enzyme. However, it should be noted that our data does not rule out the possibility that mutation of Trp305/Trp309 residues destabilises the structure of NUAK1 and MARK4, in a manner that prevents in vivo phosphorylation by LKB1 independently from ubiquitination and/or its ability to bind USP9X.

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Discussion

To our knowledge regulation of AMPK or AMPK-related kinases by ubiquitination has not previously been investigated. It is likely that ubiquitination has been missed in previous studies, as these enzymes are normally analysed after their isolation from cell extracts generated in the absence of inhibitors of deubiquitinating enzymes such as NEM. Our results also indicate that NUAK1 and MARK4 are likely to be deubiquitinated in vivo by USP9X, as overexpression of USP9X inhibited NUAK1 and MARK4 ubiquitination, whilst knock-down of USP9X expression enhanced ubiquitination. Interaction of USP9X with NUAK1 and MARK4 is required for deubiquitination, as the non-USP9X binding mutants are not substrates for the purified USP9X protein in vitro and these mutants are also hyper-ubiquitinated when expressed in cells.

In vitro we were able to demonstrate that full length USP9X protein catalysed the release of polyubiquitin species from ubiquitinated NUAK1 immunoprecipitated from 293 cells. Under the same conditions, other deubiquitinating enzymes including USP5, USP7 and CYLD failed to deubiquitinate NUAK1. Interaction of USP9X with NUAK1 was required for the deubiquitination, as the non-binding NUAK1[W305A] mutant could not be deubiquitinated by USP9X. We also found that USP9X catalysed the release of polyubiquitinated products from NUAK1 rather than free ubiquitin monomers (Fig 3). This suggests that USP9X interacts with NUAK1 and catalyses release of polyubiquitin chains rather than ubiquitin monomers from the end of the polyubiquitin chain. Overexpression of USP9X in cells prevented detectable ubiquitination of NUAK1 (Fig 2C), indicating that USP9X plays a rate-limiting role in the removal of the entire polyubiquitin chains from NUAK1. Consistent with the notion that USP9X needs to interact with its substrate prior to catalysing deubiquitination, we found that full length USP9X was unable to hydrolyse Lys48 or Lys63-linked tetraubiquitin, under conditions in which USP5, USP7 and CYLD acted upon these substrates. Other proposed substrates for USP9X including epsin-1, [21-23] and Itch [28] have also been reported to bind USP9X. It would be important to define the region on USP9X that interacts with NUAK1 and MARK4 and determine whether this was also involved in enabling USP9X to associate with its other substrates.

Our results indicate that the polyubiquitin chains attached to NUAK1 and MARK4 are linked through Lys29 and Lys33. Global mass spectrometry analysis of ubiquitinated proteins has revealed that all Lys residues in ubiquitin including Lys29 and Lys33 are employed to form branch points [36, 37]. Another USP9X substrate, the Itch ligase was reported to auto-ubiquitinate itself through Lys29 conjugated chains [38]. As three USP9X substrates namely NUAK1, MARK4 and Itch contain Lys29-linked ubiquitin chains, it is tempting to speculate that USP9X may have evolved specificity and/or preference for deubiquitinating proteins conjugated to polyubiquitin chains possessing Lys29 and/or Lys33 linkages. However, it should be noted in the case of the Survivin substrate, USP9X reportedly deubiquitinates Lys63-linked polyubiquitin chains [27]. Similar to NUAK1/MARK4, USP9X is not affecting stability of Survivin, but instead regulates chromosome alignment and segregation by controlling association of Survivin with centromeres as well as co-localising Survivin and Aurora B to centromeres.

Deubiquitination of Itch by USP9X reportedly protected it from degradation by the lysosome. Similarly USP9X has been proposed to stabilise the expression of its other substrates epsin-1 [22], β-catenin [25, 39] and AF-6 [40]. In the case of NUAK1 and MARK4, our results indicate that polyubiquitination of these enzymes is not controlling their stability. Instead the data suggest that interaction of NUAK1 and MARK4 with USP9X is regulating their phosphorylation and activation by the LKB1 tumour suppressor (Fig 7). In future work it would be important to identify the ubiquitin ligases that introduce the Lys29/Lys33 linked polyubiquitin chains on to NUAK1 and MARK4. We have also found that several other AMPK-related kinases become ubiquitinated when co-expressed with ubiquitin mutants containing only Lys29 and Lys33 (Fig 5), suggesting that the ubiquitin ligases acting upon NUAK1 and MARK4 might also ubiquitinate other members of the AMPK family. This

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modification appears specific to AMPK family kinases, as other kinases tested were not ubiquitinated with mutant ubiquitin containing only Lys29 and Lys33 (Fig 5). It will also be important to establish the Lys residues in NUAK1 and MARK4 that the polyubiquitin chains are attached to as well as the role(s) that Lys29 and Lys33 linked polyubiquitination plays and whether these types of chains are capable of interacting with a specific subset of proteins. Endogenous AMPK was also observed to be polyubiquitinated when immunoprecipitated from cells lysed in the presence of NEM. Stimulation of cells with agonists that activate AMPK (AICAR, phenformin and A769662) increased phosphorylation of AMPK at Thr172 (the site of LKB1 phosphorylation), but did not markedly alter ubiquitination status (data not shown). Further work is required to analyse which AMPK subunits are ubiquitinated, what types of ubiquitin chains are attached and how this affects AMPK function.

MARK4, possesses a ubiquitin-associated (UBA) domain located between residues 324-368, in close proximity to the region required for interaction with USP9X (residues 300-310). The role of the UBA domain on MARK4 and several other AMPK-related kinases is poorly understood. Mutations of conserved residues within the UBA domains of several AMPK-related kinases prevented phosphorylation and activation by LKB1, suggesting that the UBA domain might regulate the conformation of the kinase domain and its accessibility to LKB1 [41]. Recently, an elegant study demonstrated that the UBA domain of MARK3 possessed significant intrinsic conformational instability and had a tendency to unfold explaining the very low affinity of this domain for ubiquitin [42]. This study also established that at the expense of its capacity to engage ubiquitin, the UBA domain of MARK3 preferentially interacted with the N-terminal lobe of the MARK3 kinase domain, thereby stabilising it in an open active conformation [42]. An intriguing possibility is that when MARK isoforms are polyubiquitinated, the ubiquitin chains might compete with the kinase domain for the UBA domain binding. Polyubiquitination of MARK isoforms would therefore be acting to disrupt the kinase-UBA domain interaction. This would lead to destabilisation of the kinase domain similarly to the UBA domain mutations that have previously been investigated [41], and in so doing inhibit LKB1 T-loop phosphorylation. A model of how polyubiquitination might regulate the activity of AMPK-related kinases in presented in Figure 8. Although domain identification programmes fail to recognize a UBA domain in NUAK1, sequence alignments imply some similarity between the UBA domain on other AMPK-related kinases and the equivalent region on NUAK1, suggesting that NUAK1 may possess a UBA-like motif [41]. The catalytic subunit of AMPK may also contain a UBA-like motif in the equivalent region [41]. Other possibilities of how polyubiquitination of MARK4/NUAK1 might regulate their activity is if polyubiquitin chains sterically shield the T-loop Thr residue and/or a site on these enzymes that interacts to LKB1. Ubiquitination of NUAK1 and MARK4 could also induce conformational changes promoting their dephosphorylation by a protein phosphatase.

A major challenge in this area of research is to develop a cell lysis condition that would preserve NUAK1 and MARK4 ubiquitination as well as kinase catalytic activity. In this study, cells were lysed in the presence of 5 mM NEM in order to maintain ubiquitination, a condition that completely inactivates NUAK1 and MARK4 protein kinase activity. We found that other USP-inhibiting alkylating agents such as iodoacetimide also inactivated NUAK1 and MARK4 (AZ data not shown). We also attempted to lyse cells in the presence of the cysteine protease inhibitor E64 as well as ubiquitin aldehyde that do not inhibit kinase activity of NUAK1 and MARK4, but found that these compounds were much less effective than NEM in preserving polyubiquitination of NUAK1/MARK4 (AZ data not shown). Further work is required to develop a potent USP inhibitor that works in cell extracts, but does not inhibit protein kinases.

In conclusion, the results of this study provide the first indication that the family of AMPK kinases is regulated by ubiquitination. They also demonstrate that ubiquitinated forms of NUAK1 and MARK4 are substrates for the USP9X deubiquitinating enzyme. Our data indicate that ubiquitination of NUAK1 and MARK4 may regulate their phosphorylation and activation by LKB1, but further

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work is required to validate this conclusion. More work is also required to define the mechanism by which ubiquitination of NUAK1 and MARK4 regulates these enzymes. It will also be important to define how USP9X is regulated and the significance of the atypical Lys29/Lys33-linked ubiquitin chains attached to NUAK1 and MARK4. It will also be interesting to determine the identity of the ubiquitin ligases that introduce the Lys29 and Lys33-conjugated ubiquitin chains to NUAK1 and MARK4.

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Figure Legends.

Figure 1. **USP9X interacts specifically with NUAK1 and MARK4**. (**A**) HEK-293 cells were transfected with constructs encoding the indicated GST-AMPK-related kinases. 36h post-transfection, the AMPK-related kinases were affinity purified from the cell lysates using glutathione-Sepharose and subjected to immunoblot analysis with GST antibody (bottom panel) or USP9X antibody (top panel). (**B**) Endogenously expressed USP9X was immunoprecipitated from rat brain extracts or from HEK-293 cell lysates. As a control, immunoprecipitation from rat brain extracts and HEK-293 lysates were also performed in parallel with a pre-immune antibody. The immunoprecipitates were subjected to immunoblot analysis with the indicated antibodies. The results presented are representative of two or three independent experiments.

Figure 2. **USP9X deubiquitinates NUAK1 and MARK4 in vivo**. (**A**) HEK-293 cells were transfected with constructs encoding either the empty pCMV-HA vector or HA-NUAK1 or HA-MARK4. 36 h post transfection, cells were lysed in the absence (-) or in the presence (+) of 5 mM NEM. The kinases were then immunoprecipitated with the HA antibody and subjected to immunoblot analysis with NUAK1, MARK4 or ubiquitin antibodies. (**B**) Endogenous NUAK1 was immunoprecipitated from HEK-293 cell lysates prepared with 5 mM NEM. Immunoprecipitates were subjected to immunoblot analysis with anti-NUAK1 or ubiquitin antibody. As a control, lysates were precipitated with Protein G-Sepharose not coupled to NUAK1 antibody (-). (**C**) Constructs encoding HA-NUAK1 or HA-MARK4 were transfected in either normal HEK-293 cells or in HEK-293 cells stably expressing the catalytically inactive USP9X[C1559A] or wild type (WT) USP9X. 36h post transfection, cells were lysed, NUAK1 and MARK4 immunoprecipitated with the HA antibody and subjected to immunoblot analysis with the HA or ubiquitin antibodies (lower panel). Lysates were also immunoblotted with the indicated antibodies (upper panel) (**D**) HEK-293 cells were transfected with constructs encoding HA-NUAK1 or HA-MARK4. 16 h post-transfection, cells were left non-infected (-) or infected with a control shRNA or USP9-directed shRNA vectors packaged within lentiviral particles as described in the materials and methods. At 60 h post-infection, cells were lysed in the presence of 5 mM NEM and analysed as in (**C**). The results presented are representative of two or three independent experiments.

Figure 3. **Evidence that USP9X deubiquitinates NUAK1**. (**A**) Full length wild type USP9X or catalytically inactive USP9X[C1559A] protein was purified as described in materials and methods and 2 g were subjected to a electrophoresis on a polyacrylamide gel which was stained with Colloidal Blue. 0.5 g of each preparation of USP9X was also analysed by immunoblot analysis. (**B**) HEK-293 cells were co-transfected with constructs encoding FLAG-ubiquitin with either wild-type HA-NUAK1 or the non-USP9X-binding HA-NUAK1[W305A] mutant. 36 h post-transfection, cells were lysed in the presence of 5 mM NEM and wild type and mutant NUAK1 immunoprecipitated from 1.0 mg of cell extract. The immunoprecipitates were incubated in the absence (-) or presence of ~0.5 g of wild type (WT) USP9X or catalytically inactive USP9X[C1559A]. After incubation for 60 min at 30°C the reaction was centrifuged and the supernatant and pellet fractions analysed by immunoblot analysis with the FLAG antibody to detect ubiquitin. (**C**) The indicated deubiquitinating enzymes were incubated for 60 min at 30°C with either 100 ng of Lys-48 (upper panel) or Lys-63 (lower panel) tetraubiquitin. The reaction mixtures were analysed by immunoblot analysis with a ubiquitin antibody. The results presented are representative of two or three independent experiments.

Figure 4. **Lys29 and Lys33 linkages are required for ubiquitin of NUAK1 and MARK4**. (**A** & **B**) HEK-293 cells were co-transfected with constructs encoding for HA-NAUK1, HA-MARK4 or HA-TRAF6 together with either wild type or the indicated mutant forms of FLAG-ubiquitin. In (**A**) ubiquitin mutants are employed in which all Lys residues, except the one(s) indicated are mutated to Arg. In (**B**)

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ubiquitin mutants are utilised in which only the indicated Lys residue(s) are mutated to Arg. 36h post-transfection, cells were lysed in the presence of 5 mM NEM and NUAK1, MARK4 and TRAF6 immunoprecipitated with a HA-antibody and subjected to immunoblot analysis with either HA antibody or the FLAG antibody in order to detect ubiquitinated forms of these proteins. Similar results were obtained in 3 independent experiments.

Figure 5. **Lys29 and Lys33 linkages are required for ubiquitination of AMPK and several AMPK-related kinases**. (**A**) HEK-293 cells were co-transfected with constructs encoding for the indicated GST tagged kinases together with either wild type (WT) or ubiquitin mutants in which all Lys residues, except Lys29 and Lys33 are mutated to Arg. 36 h post-transfection, the GST-tagged kinases were affinity purified using glutathione-Sepharose and subjected to immunoblot analysis with GST antibody or FLAG antibody to detect ubiquitination. Dashed line indicates that immunoblots were undertaken on separate gels. (**B**) Endogenous AMPKα1 was immunoprecipitated from 1 mg of cell lysate derived from mouse embryonic fibroblast cells prepared in the absence (-) or presence (+) of 5 mM NEM. Immunoprecipitates were subjected for immunoblot analysis with anti-AMPKα1 or ubiquitin antibody. As a control lysates were immunoprecipitated with pre-immune IgG antibody. Similar results were obtained in 2-3 independent experiments.

Figure 6. **Identification of non-USP9X binding forms of NUAK1 and MARK4**. (**A** & **B**) Upper panels: schematic representation of the domain structure of NUAK1 (**A**) and MARK4 (**B**) numbering based on the human sequence. Abbreviations: UBA, ubiquitin-associated domain; KA, kinase associated domain. Lower panels: HEK-293 cells were transfected with constructs encoding the indicated forms of HA-NUAK1 and HA-MARK4. 36h post-transfection, endogenously expressed USP9X was immunoprecipitated from HEK-293 cells and subjected to immunoblot analysis with either the USP9X antibody or the HA antibody to detect NUAK1 or MARK4. Cell lysates (10 μg) were also subjected to immunoblot analysis with the USP9X or HA antibodies (panel labelled lysate). (**C**, **D** and **F**) Constructs encoding the indicated mutants of HA-tagged NUAK1 and MARK4 were transfected into HEK-293 cells. 36 h post-transfection, endogenous USP9X was immunoprecipitated and immunoblotted as above. Lanes with control immunoprecipitates from non-transfected cells were labelled (-). The results presented are representative of two or three independent experiments. (**E**) Sequence alignment of the C-terminal boundary of AMPK and AMPK-related kinases overlapping the region of NUAK1/MARK4 required for binding of USP9X. Asterisks indicate the identical Trp residue found in all sequences, which is also shaded in black. Residues conserved in seven or more of the aligned kinases are shaded in grey.

Figure 7. **Evidence that ubiquitination of NUAK1 and MARK4 inhibits activity and phosphorylation of these enzymes by LKB1**. (**A** & **B**) HEK-293 cells were transfected with constructs encoding wild-type (WT) or the indicated mutants of HA-NUAK1 (**A**) or HA-MARK4 (**B**). 36 h post-transfection, the cells were lysed in the absence (Upper panel) or presence (Lower panel) of 5 mM NEM. (Upper panel) NUAK1 and MARK4 were immunoprecipitated with the HA-antibody and their catalytic activity assayed using the AMARA peptide. The HA-NUAK1 and HA-MARK4 activity is the mean ±s.d. for each sample assayed in duplicate. Aliquots of each of the immunoprecipitates were subjected to immunoblot analysis with the indicated antibodies. Thr211 of NUAK1 and Thr213 of MARK4 represents the T-loop LKB1 site of phosphorylation on these enzymes [4]. (Lower panel) NUAK1 and MARK4 kinases were immunoprecipitated and subjected to immunoblot analysis with ubiquitin antibody. (**C** & **D**) The wild type and indicated mutants of HA-NUAK1 and HA-MARK4 were immunoprecipitated from 293 cell extracts generated in the absence of NEM as described above. The kinases were then preincubated in the absence (-) or presence (+) of recombinant LKB1:STRAD:MO25 complex in the presence of MgATP. After incubation for 40 min at 30 °C activity of NUAK1 and

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MARK4 was assessed employing the AMARA peptide, which LKB1 does not phosphorylate. Results are presented as the mean ± s.d. for a duplicate experiment. Aliquots of the reaction mixture were also analysed by immunoblotting with the indicated antibodies. The results presented are representative of two or three independent experiments.

Figure 8 Model of how USP9X and ubiquitination controls AMPK family kinases.

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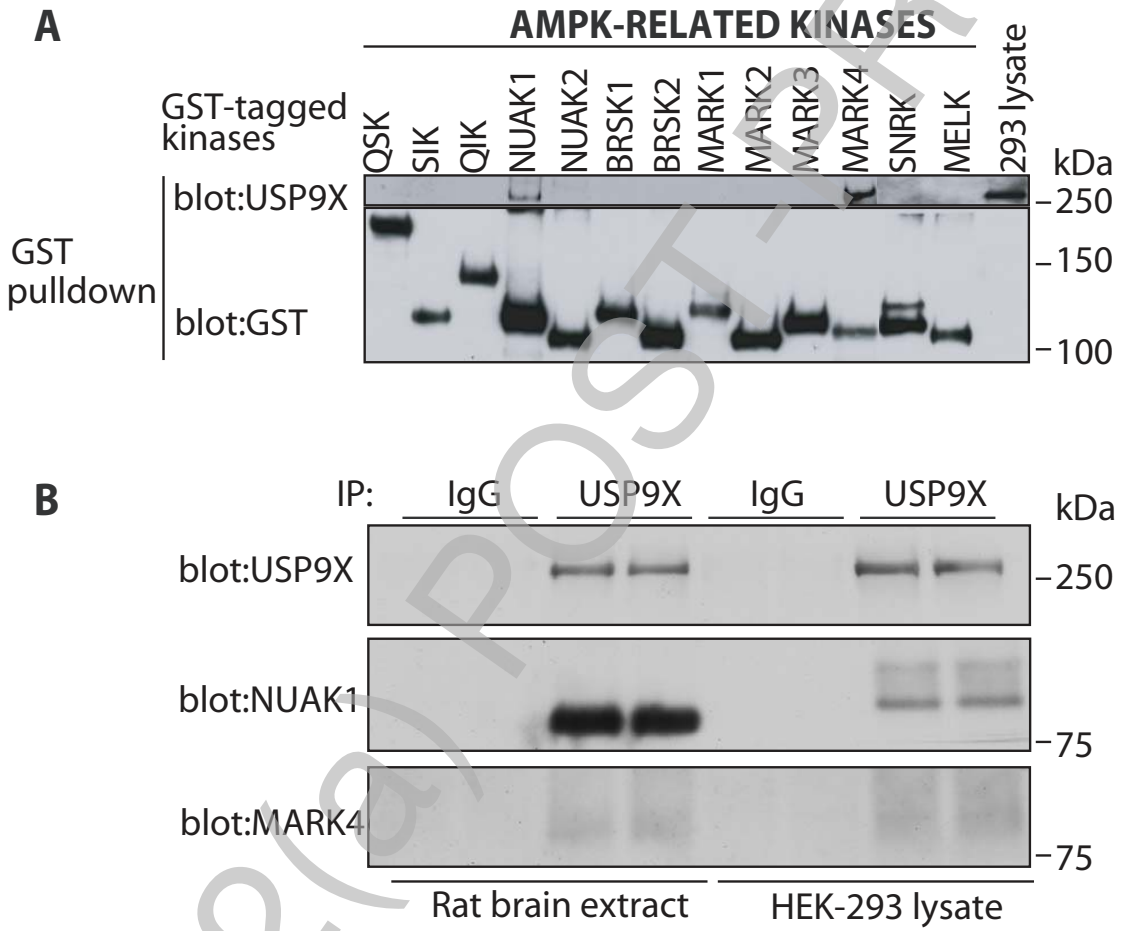

FIGURE 1

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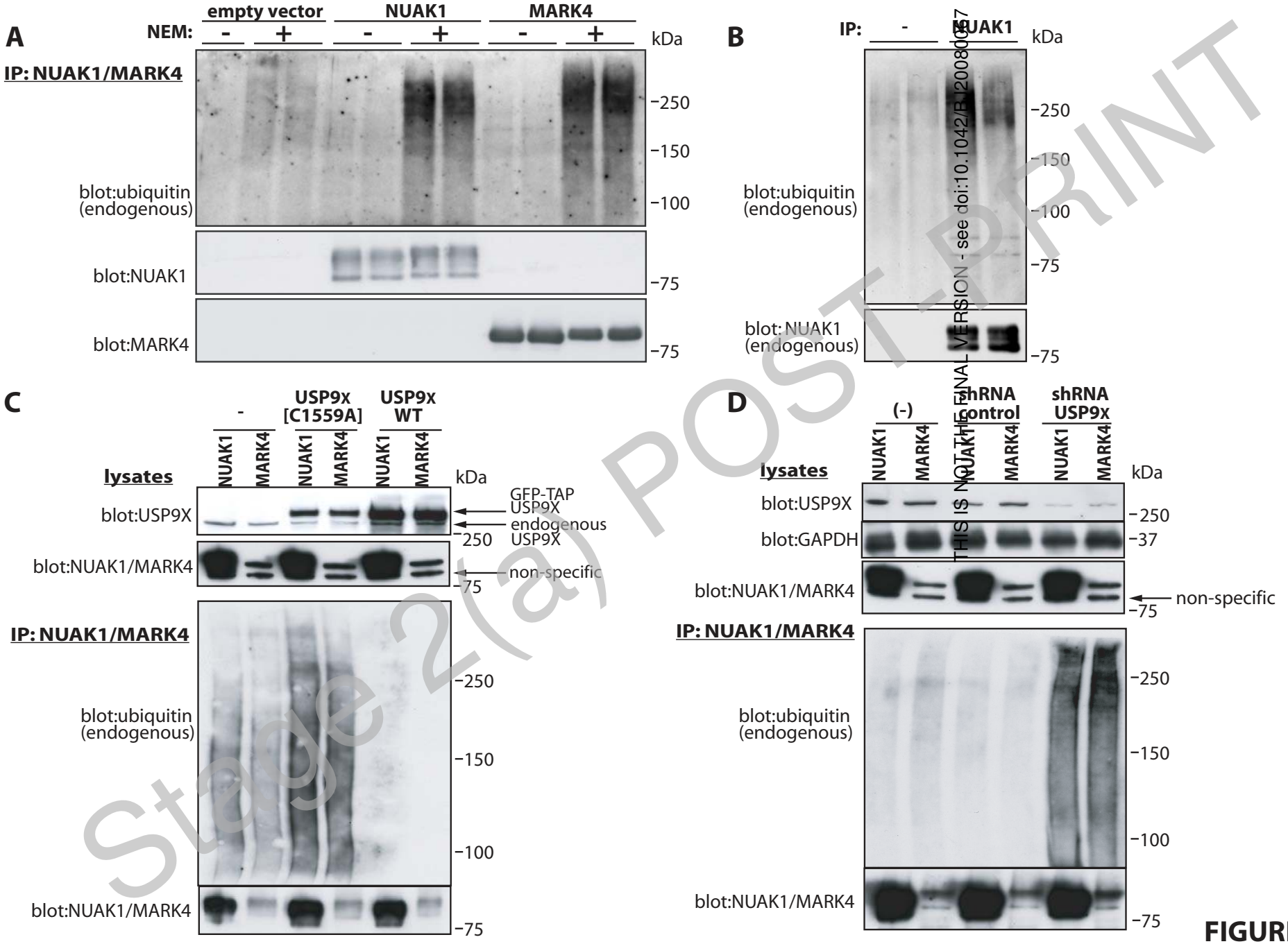

FIGURE 2

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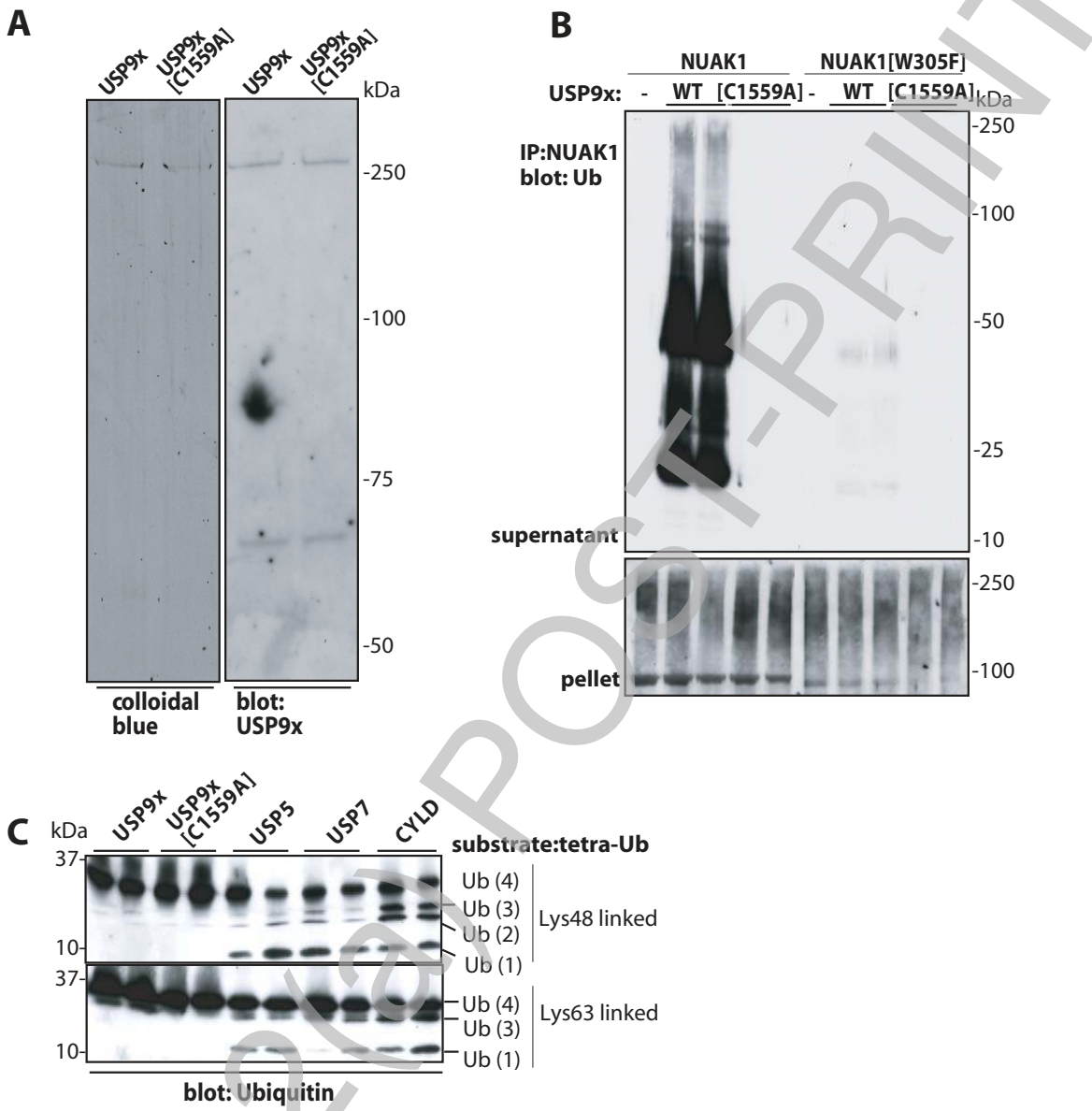

FIGURE 3

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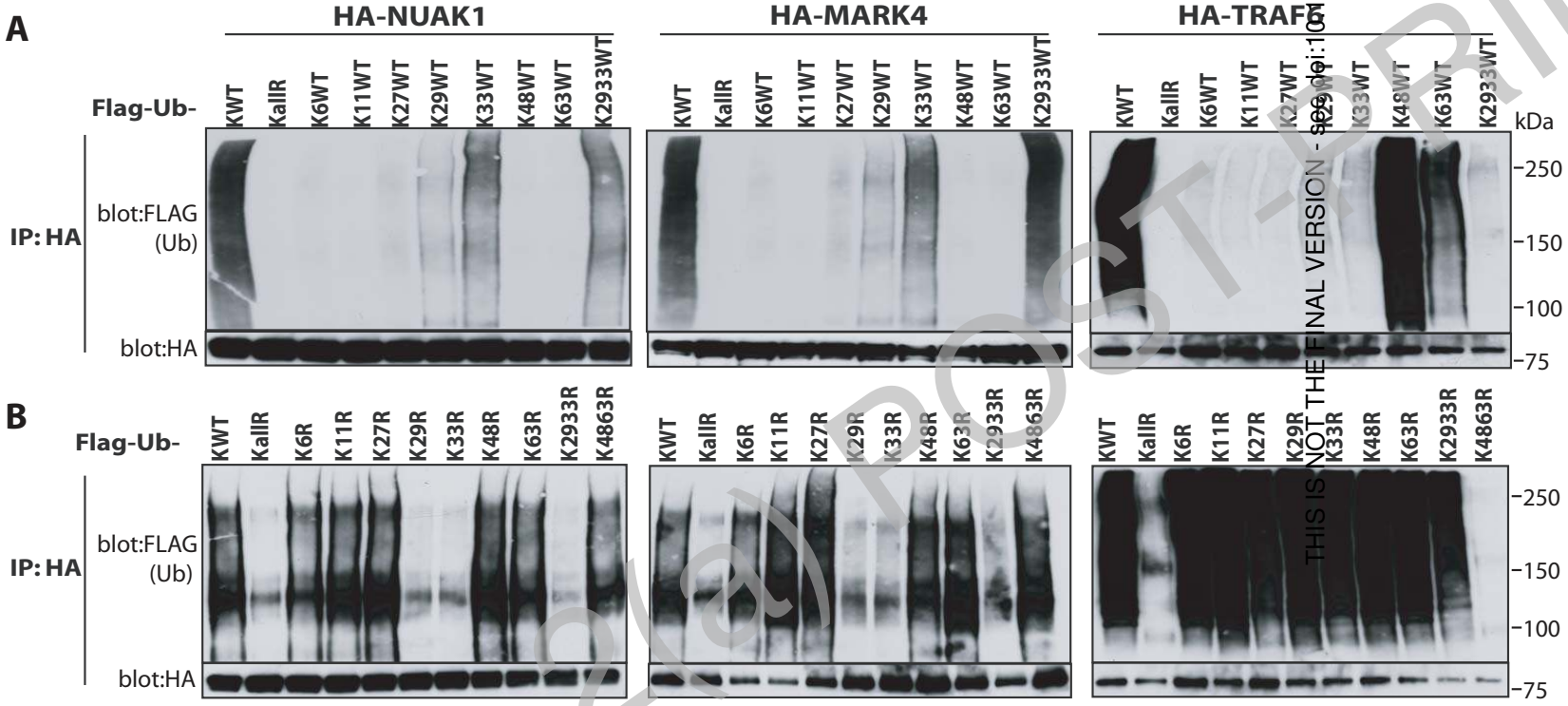

FIGURE 4

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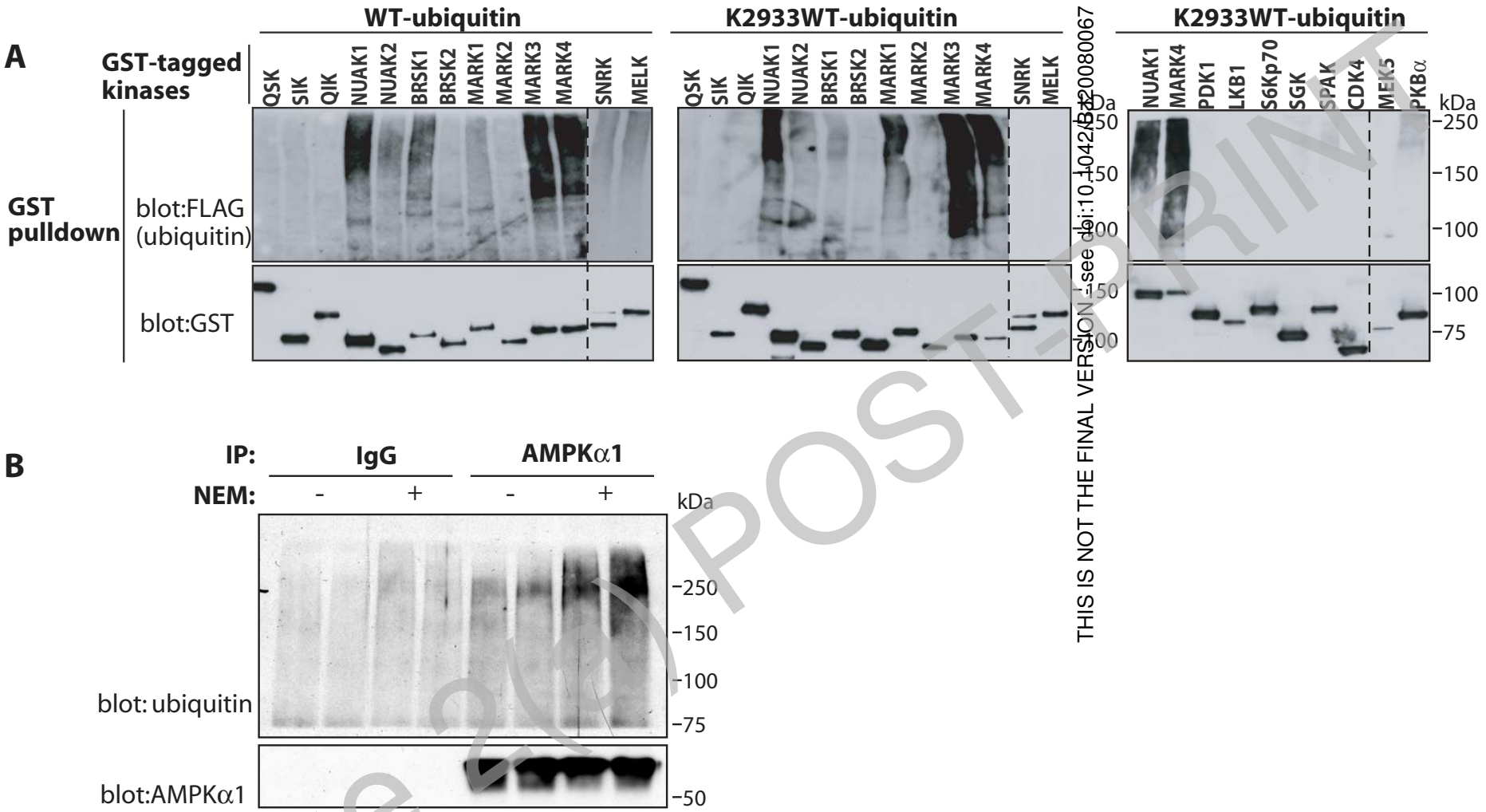

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FIGURE 5

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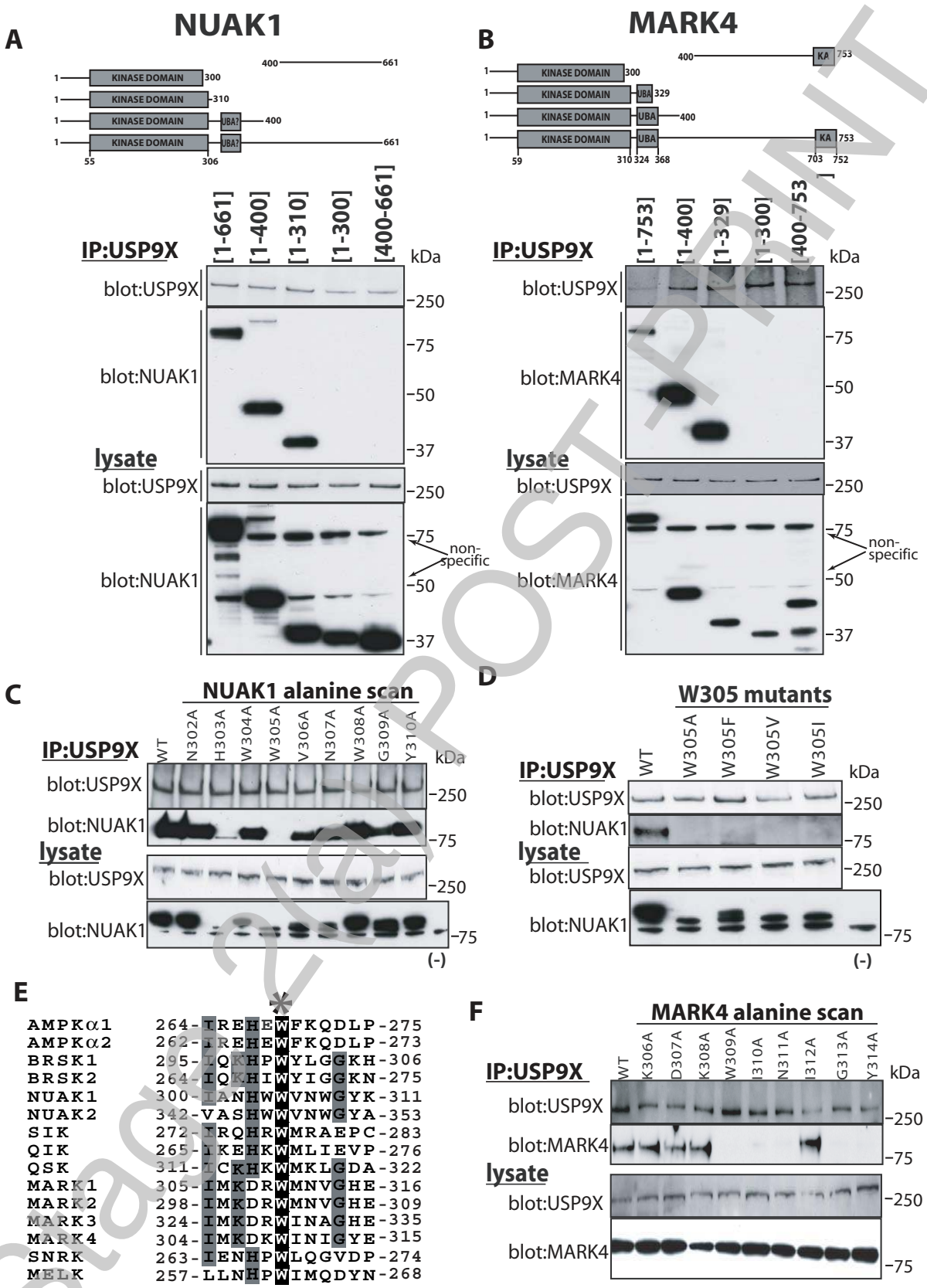

FIGURE 6

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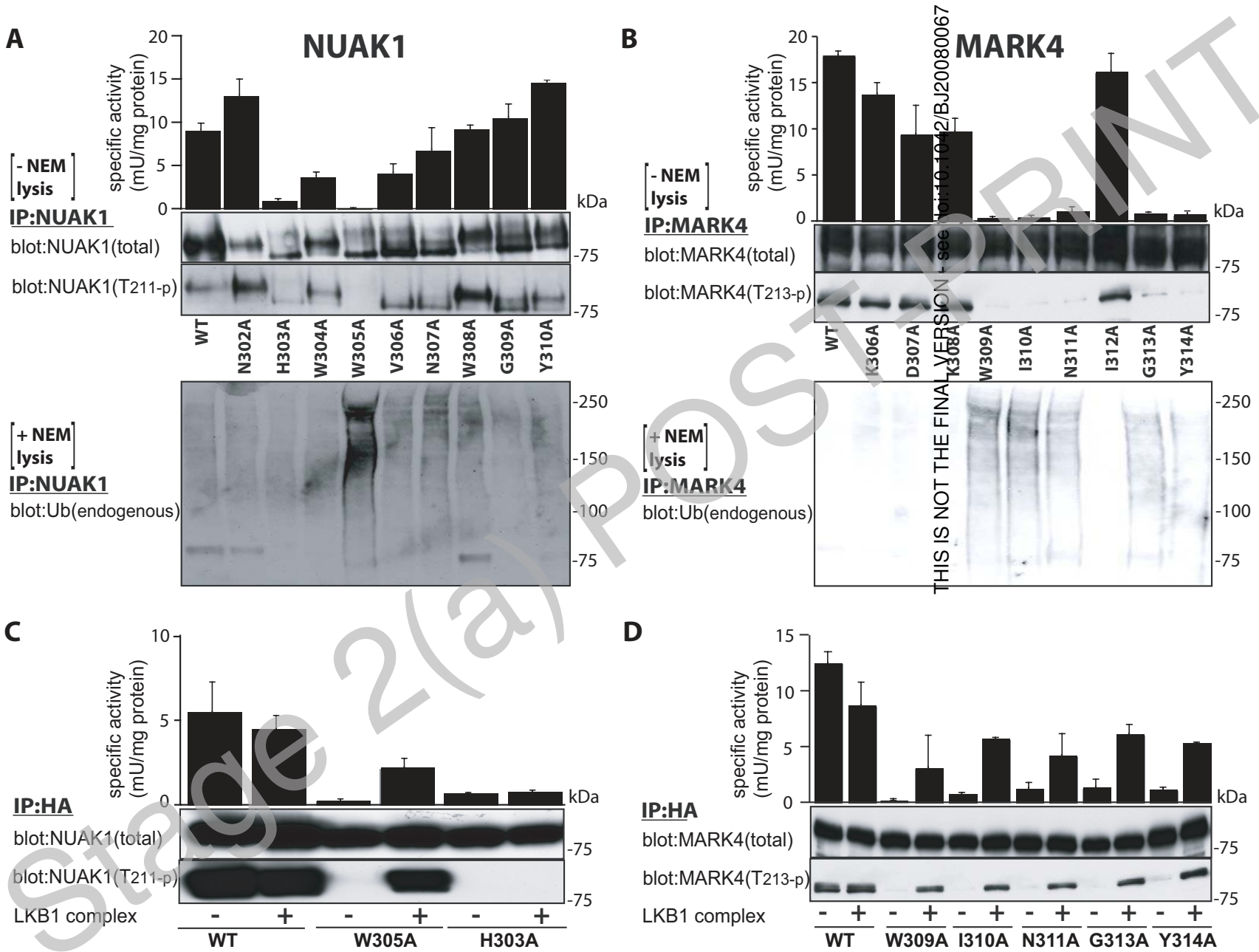

FIGURE 7

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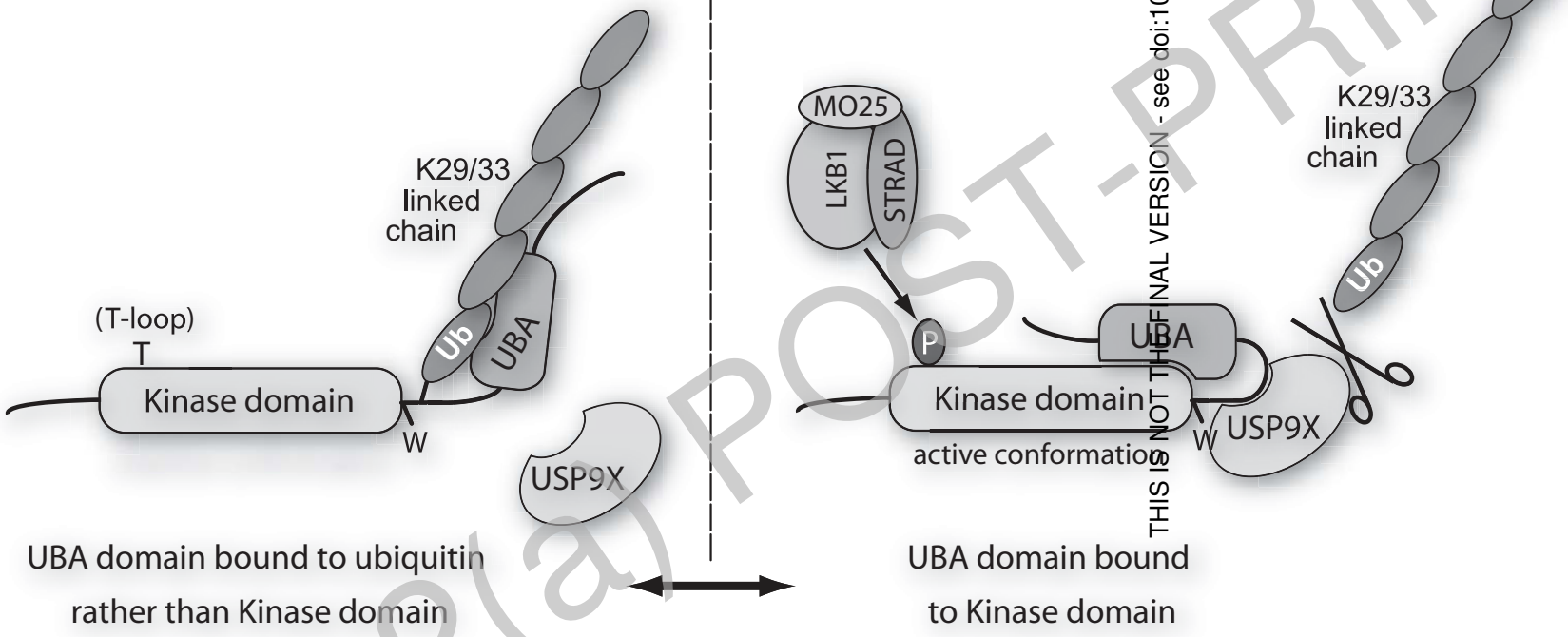

FIGURE 8

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