ARTICLE

Supplementary Materials

Placental mammals acquired functional sequences in NRK for regulating the CK2-PTEN-AKT pathway and placental cell proliferation

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These Supplementary Materials contain the following

- Supplementary Materials and Methods
- Supplementary Table S1 and S2
- Supplementary Figures S1 through S6

Supplementary Materials and Methods

Antibodies and reagents

The following primary antibodies were used in this study: anti-FLAG M2 antibody (clone M2, Sigma-Aldrich, St Louis, MO, USA), anti-myc antibody (clone 9E10, Santa Cruz Biotechnology, Dallas, TX, USA), HRP-conjugated anti-GST antibody (#RPN1236, Cytiva, Marlborough, MA, USA), anti-NRK rabbit serum (Denda et al. 2011), anti-PARP antibody (clone C-2-10, Calbiochem, San Diego, CA, USA), anti-AKT antibody (clone C67E7, Cell Signalling Technology (CST), Danvers, MA, USA), anti-phospho AKT (T308) antibody (clone D25E6, CST), anti-phospho AKT(S473) antibody (clone D9E, CST), anti-p42/44 MAPK antibody (#9102, CST), anti-phospho p42/44 MAPK (T202/T204) antibody (clone E10, CST), anti-CK2α antibody (clone 1AD9, Santa Cruz Biotechnology), anti-CK2α' antibody (A300-199A, Bethyl Laboratories, Montgomery, TX, USA), anti-CK2β antibody (A301-984A, Bethyl Laboratories), anti-phospho CK2 substrate antibody (#8738, CST), anti-PTEN antibody (clone 138G6, CST), anti-phospho PTEN (S380) antibody (clone H-3, Santa Cruz Biotechnology), anti-α-Tubulin antibody (#013-25033, Wako Pure Chemical, Osaka, Japan), anti-phosphatidylinositol 4,5-bisphosphate (PIP_2) antibody (clone 2C11, Santa Cruz Biotechnology), and anti-phosphatidylinositol 3,4,5-triphosphate PIP_3 (#Z-P345, Echelon Biosciences, Salt Lake City, UT, USA). For immunoprecipitation, anti-FLAG M2 affinity gel (Sigma-Aldrich) was used. Peroxidase-labelled anti-mouse IgG antibody and anti-rabbit IgG antibody (GE Healthcare, Chicago, IL, USA) were used as secondary antibodies for immunoblotting. Alexa Fluor 488- or 594-conjugated anti-mouse IgG antibody, anti-mouse IgM antibody, and anti-rabbit IgG antibody (Thermo Fisher Scientific, Waltham, MA, USA) were used as secondary antibodies for immunostaining. Cells were treated with the following reagents: doxycycline (dox; Sigma-Aldrich), epidermal growth factor (EGF; PeproTech, Rocky Hill, NJ, USA), cycloheximide (Nacalai Tesque, Kyoto, Japan), tumour necrosis factor-α (TNFα; Suntory, Tokyo, Japan), and a PTEN inhibitor VO-OHpic (BioVision, Milpitas, CA, USA).

Plasmids

Mouse *Nrk* cDNA was pre-prepared by our group (Nakano et al. 2000). Chicken *Nrk* cDNA (NM_001031126.2) was amplified using polymerase chain reaction (PCR) from the chicken embryo cDNA library kindly donated by Dr. Mikiko Tanaka (Tokyo Institute of Technology, Yokohama, Japan). Mouse *Nik* (XM_006496042.3), *Tnik* (NM_026910.1), *Mink1* (NM_016713.2), *CK2α'* (see below), *CK2β* (NM_001303445.1), and *PTEN* (NM_008960.2) cDNAs were amplified by using PCR from mouse embryo and brain cDNA libraries. The mouse *CK2α'* cDNA sequence is identical to NM_009974.3, except for the 84 bp deletion in the predicted coding region, which is thought to be an isoform because other rodent *CK2α'* cDNA sequences also lack this 84 bp sequence. The following vectors were used in this study:

mammalian expression vectors, pFLAG-CMV2 (Sigma-Aldrich) and pCMV5 (kindly donated by Dr. Jun Nakae, Keio University, Tokyo, Japan); dox-induced mammalian expression vector, pcDNA5/FRT/TO (Invitrogen, Carlsbad, CA, USA); bacterial expression vectors, pGEX-4T1 and pGEX-6P2 (GE Healthcare). N-terminal FLAG-tagged mouse NRK (mNRK), mNRK CNH (aa 1133-1455), mNRK ΔCNH (aa 1-1137), mNRK ΔCNH-CaaX (mNRK aa 1-1137 fused with HRAS CaaX motif (GCMSCKCVLS) at the C-terminus), other mNRK truncated mutants, mouse NIK (mNIK), mNIK CNH (aa 1011-1330), mouse TNIK (mTNIK), mTNIK CNH (1042-1361), mouse MINK1 (mMINK1), mMINK1 CNH (982-1301), chicken NRK (cNRK), and cNRK CNH (aa 918-1237) were expressed using pFLAG-CMV2 or pcDNA5/FRT/TO. N-terminal myc-tagged mouse CK2α' and N-terminal myc-tagged mouse CK2β were expressed using pCMV5. GST-tagged mNRK (aa 565-868) was expressed using pGEX-6P2 (Cytiva). GST-tagged NRK (aa 565-831), GST-tagged PTEN (aa 190-403), GST-tagged mNRK CNH (aa 1133-1455), and GST-tagged cNRK CNH (aa 918-1237) were expressed using pGEX-4T1 (Cytiva).

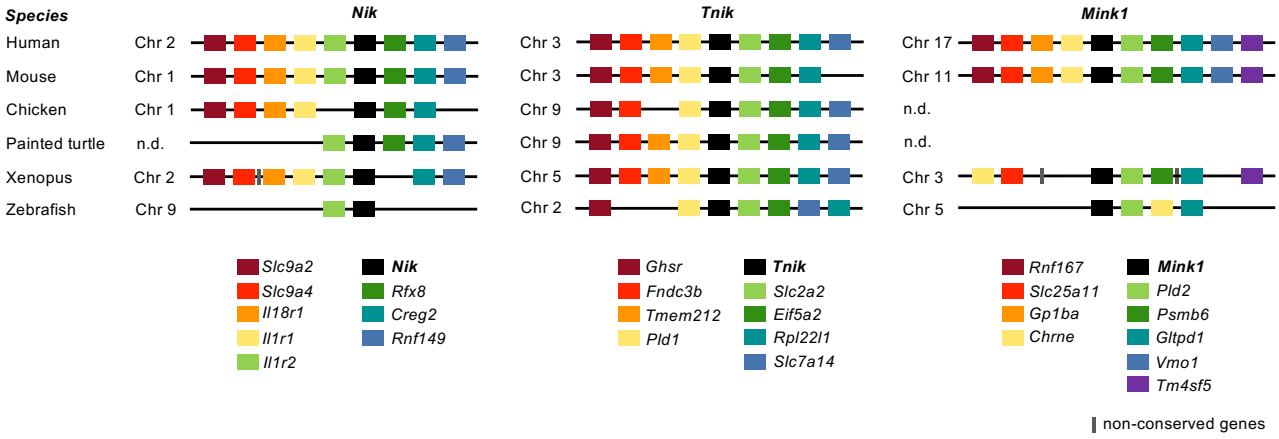
Supplementary Table 1. List of accession numbers of *Nrk* orthologs of selected species used in evolutionary analysis

Species (binomial name)	Species (common name)	NCBI Accession Number	UCSC Genome Data	Ensembl ID
Homo sapiens	Human	NM_198465.4		
Pongo abelii	Orangutan	NM_198465		
Carlito syrichta	Tarsier		KE939067V1:141656-280484	
Mus musculus	Mouse	NM_013724.2		
Canis lupus familiaris	Dog		chrX:79336562-79493046	
Equus caballus	Horse		chrX:87765874-87891413	
Bos taurus	Cow		chrX:32465316-32599498	
Sorex araneus	Shrew		JH798166:7825193-7975312	
Dasypus novemcinctus	Armadillo	NW_004462463.1		
Loxodonta africana	Elephant			scaffold_32:25936048-26099519
Notamacropus eugenii	Wallaby		GL154379:1-69272	
Phascolarctos cinereus	Koala		MSTS01000267.1	
Sarcophilus harrisii	Tasmanian devil		chrX:990069-1335659	
Monodelphis domestica	Opossum		chrX:21189130-21556422	
Ornithorhynchus anatinus	Platypus	XM_029067556.1		
Gallus gallus	Chicken	NC_006091.5		
Chrysemys picta	Painted turtle			ENSCPBT00000034663.1
Xenopus tropicalis	Xenopus	XM_031891524.1		
Latimeria chalumnae	Coelacanth			ENSLACT00000019211.1
Oryzias latipes	Medaka			ENSORLT00000044323.1
Salmo salar	Salmon	NW_012336144.1		
Danio rerio	Zebrafish			ENSDARG00000098680
Lepisosteus oculatus	Spotted gar	XM_006632839.2		
Erpetoichthys calabaricus	Reedfish	XM_028815688.1		

Supplementary Table 2. List of accession numbers of *Nik, Tnik, Mink1* of selected species used in synteny analysis

Species (binomial name)	Species (common name)	Accession Number		
		Nik	***Tnik***	***Mink1***
Homo sapiens	Human	ENST00000302217.9	NM_015028.4	NC_000017.11
Mus musculus	Mouse	ENSMUST00000163854.8	NM_026910.1	ENSMUSG00000020827
Gallus gallus	Chicken	ENSGALG00000016781	NC_052540.1	n.d.
Chrysemys picta	Painted turtle	NW_024885785.1	ENSCPBG00000015935	n.d.
Xenopus tropicalis	Xenopus	NC_030678.2	ENSXETG00000001826	ENSXETG00000001083
Danio rerio	Zebrafish	ENSDARG00000098670	ENSDARG00000056218	ENSDARG00000035360

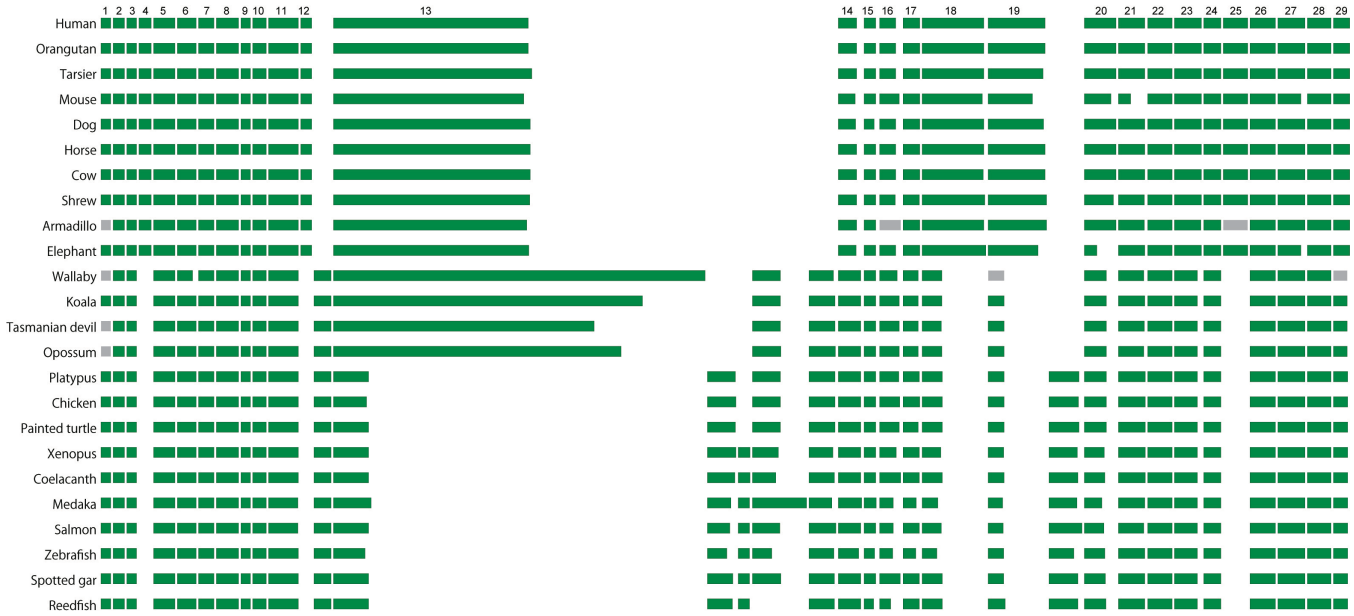
Supplementary Figure S1 (related to Figure 1)



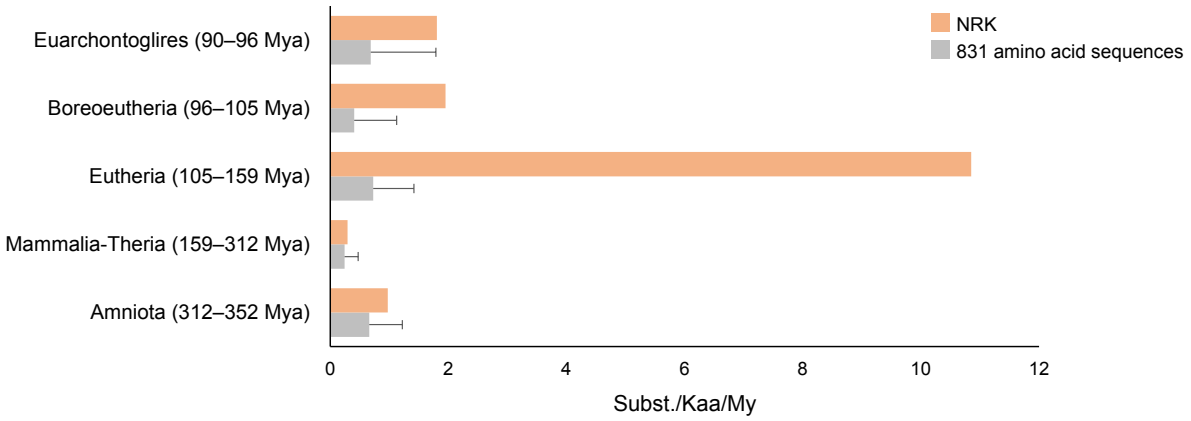
Supplementary Figure S1. Synteny analysis of the *Nik, Tnik, and Mink1* genes (Related to Fig. 1).
Synsteny conservation of the region surrounding the *Nik, Tnik, and Mink1* genes. Gray boxes indicate non-conserved genes.

Supplementary Figure S2 (related to Figure 2)

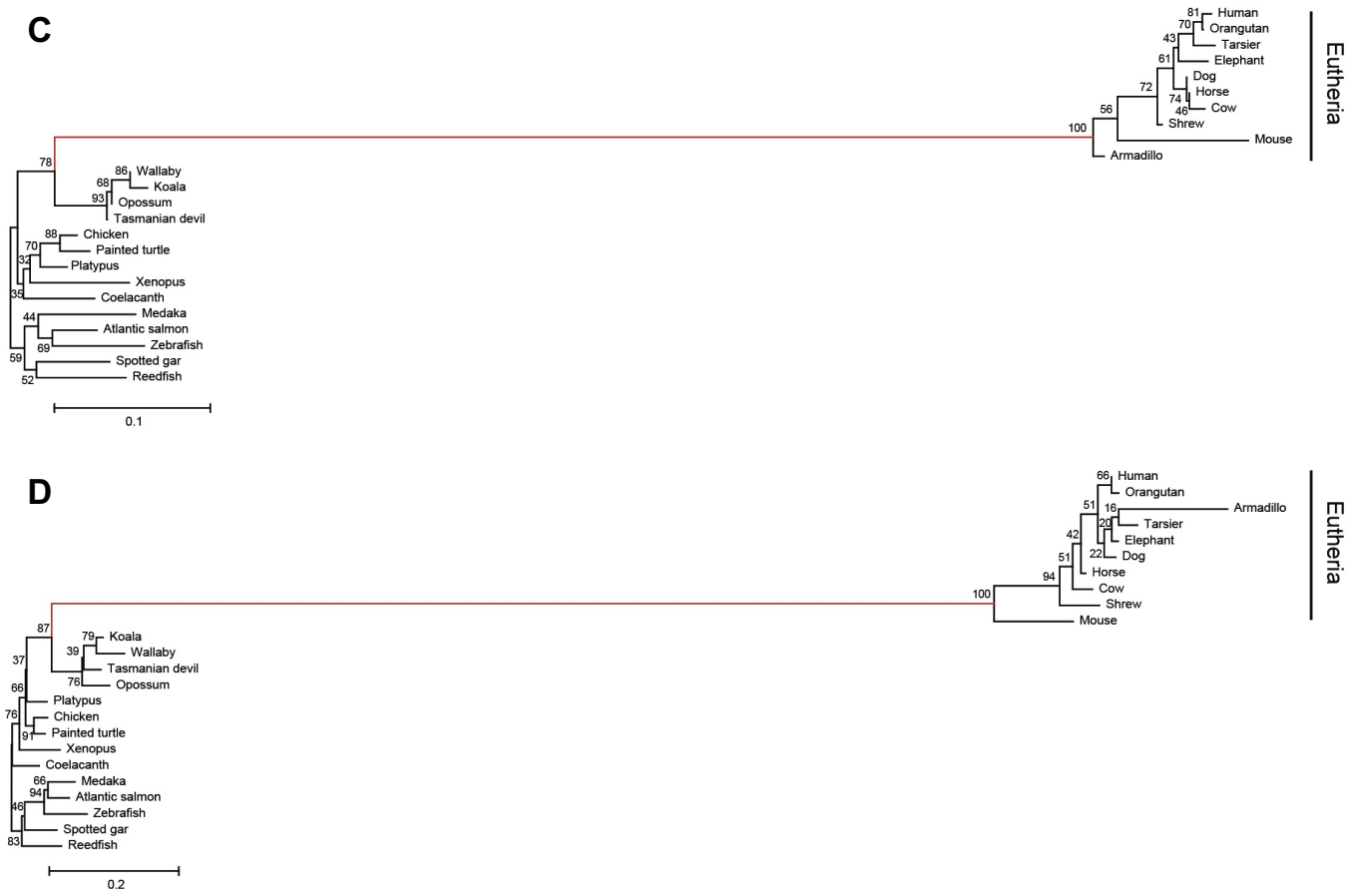
A



B



Supplementary Figure S2 (continued, related to Figure 2)



Supplementary Figure S2. Exon structures of the *Nrk* gene and neighbour-joining tree for the amino acid sequences of the NRK domains (Related to Fig. 2)

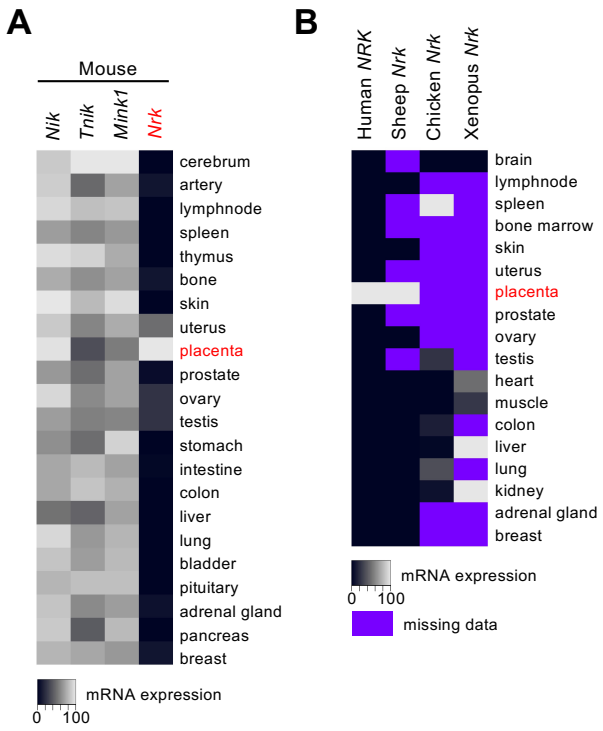
A) Exon structures of vertebrate *Nrk*. Green boxes correspond to the coding exons, while grey boxes indicate undetermined exons. All boxes are arranged left for each exon. The numbers above represent exon numbers in eutherians.

B) Substitution rates in the ancestral lineages of the five taxonomic groups compared between NRK and the control (831 amino acid sequences). The ages of the ancestral lineages are based on TimeTree. Subst./Kaa/My; the number of substitutions per 1,000 amino acid residues per million years.

C) Neighbour-joining (NJ) tree of the kinase domain corresponding to exons 1–11 of *Nrk*, excluding the eutherian-specific exon 4. Numbers on branches are node support based on 1,000 bootstrap replicates.

D) NJ tree of the CNH domain corresponding to exons 21–29 of *Nrk*, excluding the eutherian-specific exon 25.

Supplementary Figure S3

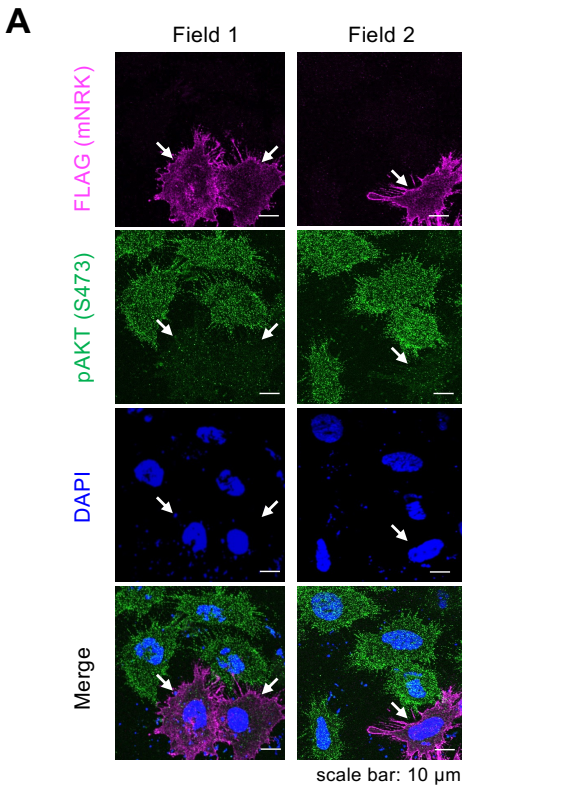


Supplementary Figure S3. Tissue expression patterns of vertebrate *Nrk*

A) Heatmap of *Nrk* gene expression in different mouse tissues in comparison to the expression patterns of the other GCK IV family members (*Nik, Tnik, Mink1*). The colour intensity reflects the mRNA expression levels from the lowest (black) to the highest (white).

B) Heatmap of *Nrk* gene expression in human, sheep, chicken, and *Xenopus* tissues. The deep purple panels represent the missing data.

Supplementary Figure S4 (related to Figure 3)

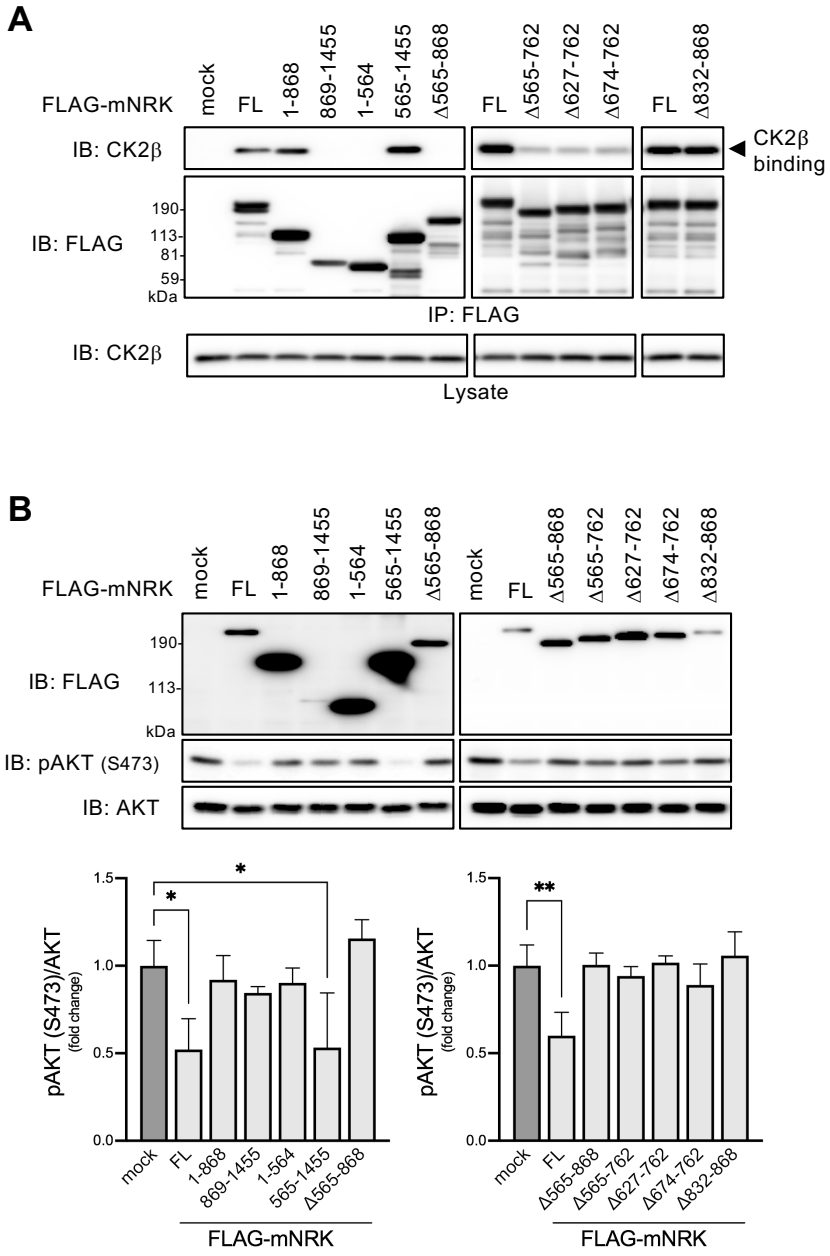


Supplementary Figure S4. Effects of mNRK on AKT signalling and cell apoptosis (Related to Fig. 3)

A) Effects of mNRK on AKT signalling. Different field images in the experiment contributing to Fig. 3C are shown.

B) HEK293T cells were transfected with plasmid encoding FLAG-mNRK. As a positive control, HeLa cells were stimulated with TNF (10 ng/mL for 0.5 or 4 h) and CHX (10 μg/mL for 4 h). The lysates were subjected to immunoblotting using an anti-PARP1 antibody.

Supplementary Figure S5 (related to Figure 5)

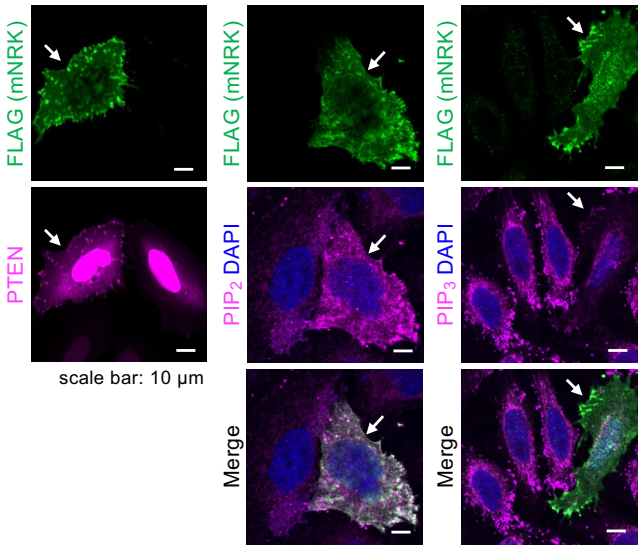


Supplementary Figure S5. Binding of mNRK truncated mutant to CK2 and its effects on AKT signalling (Related to Fig. 5)

A) Regions in mNRK responsible for binding to CK2β. HEK293T cells expressing indicated proteins were subjected to immunoprecipitation and immunoblotting.

B) Regions in mNRK responsible for the suppression of AKT phosphorylation. HEK293 cells expressing indicated proteins were subjected to immunoblotting, followed by densitometric analyses. The graph shows the mean ± standard deviation (SD) of three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$ (one-way analysis of variance [ANOVA], followed by Dunnett's multiple comparisons test).

Supplementary Figure S6 (related to Figure 7)



Supplementary Figure S6. Effects of mNRK on PTEN localisation and the levels of PIP_2 and PIP_3 (Related to Fig. 7)
Different field images in the experiment contributing to Fig. 7B are shown.