

Supplemental Figures and Tables

Supplemental Figure S1. Alignment of N-terminal kinase domain of the predicted protein sequence (September 2020) for rat MAP4K4 with mouse and human sequences. The kinase domain is in bold type.

Human MAP4K4 (NP_001371419.1)
Mouse MAP4K4 (NP_001239129.1)
Rat MAP4K4 (NP_001100374.1)

MANDSPAKSLVDIDLSSLRDPAGI**FELVEVVGNGTYGQVYKGRHVKTGQLAAIKVMDVTEDEEEEIKLEI**
MANDSPAKSLVDIDLSSLRDPAGI**FELVEVVGNGTYGQVYKGRHVKTGQLAAIKVMDVTEDEEEEIKLEI**
-----MDVTEDEEEEIKLEI

NMLKKYSHHRNIATYYGAFIKKSPPGHDDQLWLVMFEFCGAGSITDLVKNKGNLTKEDWIAYISREILRG
NMLKKYSHHRNIATYYGAFIKKSPPGHDDQLWLVMFEFCGAGSITDLVKNKGNLTKEDWIAYISREILRG
NMLKKYSHHRNIATYYGAFIKKSPPGHDDQLWLVMFEFCGAGSITDLVKNKGNLTKEDWIAYISREILRG

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LAHLHIHHVIHRDIKGQNVLLTENAEVKLVDFGVSAQLDRTVGRRNTFIGTPYWMAPEVIACDENPDATY

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DYRSDLWSCGITAIEMAEGAPPLCDMHPMRALFLIPRNPPRLKSKKWSKKFFSFIEGCLVKNYMQRPST

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EQLLKHPFIRDQPNERQVRIQLKDHDIDRTRKK-----
EQLLKHPFIRDQPNERQVRIQLKDHDIDRTRKK-----

Supplemental Figure S2. Exon structure and sequence of the rat MAP4K4 gene. The full-length sequence for the longest potential MAP4K4 isoform was determined by a combination of bioinformatics alignment of the rat gene with mouse and human genes, 5'-RACE and PCR cloning/sequencing from neonatal rat cardiomyocyte cDNA. This is aligned against the rat genomic sequence (NC_005108.4:46657454-46782545 Rattus norvegicus strain mixed chromosome 9, Rnor_6.0). Protein-coding exons are highlighted in cyan (exons identified in rat cardiomyocyte cDNA) and yellow (bioinformatics), with 5' and 3' UTRs in green. Initiation and termination codons are in red. The predicted polyadenylation signal is in bold italic type. The position of the 5' RACE primer used for cloning and sequencing is in bold type underlined. Exons M1-M9 subject to alternative splicing identified in Wright et al. [16] are indicated.

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Exon 7

Exon 8

Exon 9

Exon 10

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**Exon 19
(M5)**

Exon 20

**Exon 21
(M6)**

Exon 22

Exon 23

Exon 24

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Exon 29

Exon 30
(M8; M9)

Exon 31

Exon 32

Exon 33

Supplemental Figure S3. Alignment of MAP4K4 cloned from neonatal rat cardiomyocyte cDNA (blue type) with the predicted sequence for transcript variant X22 for rat Map4k4 (XM_008767015.2; red type). Start and stop codons are highlighted in yellow.

GGGCGACGCCCGGGGGTGGGTGGGGCCGAGGGAGGGCCGCCGCGCCATGGTGTGAGCCCCGTGGCCCCGCGCTC
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GAGCTGGTAG-----

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Supplemental Figure S4. Protein sequence of potential full-length rat Map4k4 containing all possible exons (upper rows with individual exons in yellow and green alternating) compared with the 1233 residue Map4k4 cloned from neonatal rat cardiomyocytes (lower rows, bold type). Map4k4 1233 is missing exons 16 and 24, plus 5' sequence from exon 15 and 3' sequence from exons 20 and 30 (missing extensions in italics). Residues highlighted in red are coded across an exon boundary. Exon numbers are provided above the potential full-length sequence.

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1          2          3          4
MANDSPAKSLVDIDLSSLRDPAGIFELVEVVGNGTYGQVYGRHVKTGQLAAIKVMDVTEDEEEEIKLEINMLKKYSHHR
MANDSPAKSLVDIDLSSLRDPAGIFELVEVVGNGTYGQVYGRHVKTGQLAAIKVMDVTEDEEEEIKLEINMLKKYSHHR

          5          6
NIATYYGAFIKKSPPGHDDQLWLVMEFCGAGSITDLVKNTKGNTLKEDWIAIYISREILRGLAHLHIHHVIHRDIKGQNVL
NIATYYGAFIKKSPPGHDDQLWLVMEFCGAGSITDLVKNTKGNTLKEDWIAIYISREILRGLAHLHIHHVIHRDIKGQNVL

          7          8          9
LTENA EVKL VDFGVSAQLDRTVGRRNTFIGTPYWM APEVIACDENPDATYDYRS DLWSCGITAIEMAEGAPPLCDMHPMR
LTENA EVKL VDFGVSAQLDRTVGRRNTFIGTPYWM APEVIACDENPDATYDYRS DLWSCGITAIEMAEGAPPLCDMHPMR

          10         11
ALFLIPRNPPRLKSKKSKKFFSFIEGCLVKNYMQRPSTEQLLKHPFIRDQPNERQVRIQLKDHIDRTRKKRGEKDETE
ALFLIPRNPPRLKSKKSKKFFSFIEGCLVKNYMQRPSTEQLLKHPFIRDQPNERQVRIQLKDHIDRTRKKRGEKDETE

          12
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YEYSGSEEEEEEVPEQEGEPSSIVNVPGESTLRRDFLRLQENKERSEALRRQQLLQEQQLREQEYKRQLLAERQKRIE

          13         14
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QQKEQRRRLEEQQRREREARRQQEREQRRREQEEKRRLLEELERRRKEEEERRRAEDEKRRVEREQEYIRRQLEEEQRHLE

          15
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          16         17
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          18
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QQNSQAGQRNSTSSIEPRLWERVEKLVPRPGSGSSSGSSNSGSQPGSHPGSQSGSGERFVRVSSSKSEGPSQRLENAA

          21 22
KKPEDKKEVFRPLKPAVRI GEV DLTALAKELRAVEDVRPPHKVTDYSSSSEESGTTDEEEEDVEQEGADDSTSGPEDTRA
KKPEDKKEVFRPLKPA---GEV DLTALAKELRAVEDVRPPHKVTDYSSSSEESGTTDEEEEDVEQEGADDSTSGPEDTRA

          23         24
ASSLNLNNGETESVKTMIVHDDVESE PAMTPSKEGTLIVRQSTVDQKRASHHESNGFAGRIHLLPDL LQQSHSSSTSSTS
ASSLNLNNGETESVKTMIVHDDVESE PAMTPSKEGTLIVRQ-----

          25         26
SSPSSSQPTPTMSPQTPQDKLTANE TQSASSTLQKHKSSSSFTPFIDPRL LQISPSSGTTVTVSVVGFSCDGLRPEAIRQD
-----TQSASSTLQKHKSSSSFTPFIDPRL LQISPSSGTTVTVSVVGFSCDGLRPEAIRQD

          27
PTRKGSVVNVNPTNTRPOSDTPEIRKYKKRFNSEILCAALWGVNLLVGTESGLMLLDRSGQGKVYPLISRRRFQQMDVLE
PTRKGSVVNVNPTNTRPOSDTPEIRKYKKRFNSEILCAALWGVNLLVGTESGLMLLDRSGQGKVYPLISRRRFQQMDVLE

          28         29
GLNVLVTISGKKDKLRVYYLSWLRNKILHNDPEVEKKQGWTTVGDLEGCVHYKVV KYERIKFLVIALKSSVEVYAWAPKP
GLNVLVTISGKKDKLRVYYLSWLRNKILHNDPEVEKKQGWTTVGDLEGCVHYKVV KYERIKFLVIALKSSVEVYAWAPKP

          30         31
YHKFMAFKSFGELVHKPLLVDLTV EEGQRLKVIYGSCAGFHAVDVDSGSVYDIYLPTHVRKNPHSMIQCTIKPHAI IILP
YHKFMAFKSFGELVHKPLLVDLTV EEGQRLKVIYGSCAGFHAVDVDSGSVYDIYLPTH-----IQCTIKPHAI IILP

          32
NTDGMELLVCYEDEGVYVNTYGRITKDVVLQW GEMPTSVAYIRSNQTMGWGEKAIEIRSVETGHL DGVFMHKRAQRLKFL
NTDGMELLVCYEDEGVYVNTYGRITKDVVLQW GEMPTSVAYIRSNQTMGWGEKAIEIRSVETGHL DGVFMHKRAQRLKFLH

          33
CERNDKVFFASVRS GGSSQVYFMTLGRTSLLSW
CERNDKVFFASVRS GGSSQVYFMTLGRTSLLSW

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Supplemental Table S1. Primers used for MAP4K4 cloning and mutation.

Primer ID	Sequence (5' to 3')	Notes
F1	AACTCAGAGATCCTGTGTGC	
F2	CTGT <u>CTCGAG</u> ATCCCATTCC	Includes an endogenous <u>Xho1</u> site (underlined)
F3	AGAGAGGCGAGAAAGATGAG	
F4	GACGACAAG <u>GGTACC</u> ATGGCGAACGACTCTCCGGC	Includes a <u>KpnI</u> site adjacent to the ATG start codon
F5	GGTCTATATAAGCAGAGCTG	
F6	AGCTGGCGGCCATCAGGGTTATGGATGTCAC	mutant base to generate K54R underlined
F7	GTTGGACGGAGAAATGCATTCATAGGCACAC	mutant base to generate T187A underlined
F8	ATCCAGCTTAAGGAGGAGGAGGAGGAGGAAGTGC	AflII site underlined
F9	TCTGGAGGAACTGGAAAGGC	
F10	CGCGGAGGTGAACTTGTTG	
F11	CCATGCAATCATTATCCTC	
F12	GAGCT <u>TCGAG</u> GGAGGTTCTGTGAGAACGACATCTCG	Includes an additional four bases (TCGA, emboldened) to generate the Xho1 site (underlined)
F13	GTTGGACGGAGAAAT <u>GAC</u> TTTCATAGGCACAC	mutant bases to generate T187D underlined
F14	CCCTGTCTCGAG <u>A</u> TGTGCTGCCTTATGGGGAGTGA	The additional A is needed to keep the correct reading frame.
F15	CGAGAAAGATGAGGCGGAGTACGAGTACGCTGGCGCT GAGGAGGAGGAGG	
F16	GATCACATAGACCGGGCCAGGAAGAAGAGAGGC	
R1	CTTATCTAGA <u>AAGCTT</u> CTACCAGCTCAGAAGGGAAG	Includes a <u>HindIII</u> site adjacent to the stop codon TAG (CTA)
R2	TCACTCCCCATAAGGCAGC	
R3	TCACAGGAACCCTTGGAG	
R4	CTCGTACTCCGTCTCATCTTTC	
R5	GTGACATCCATAACC <u>C</u> TGATGGCCGCCAGCT	mutant base to generate K54R underlined
R6	CTCAGGAAATCACGTCGCAG	
R7	GTGTGCCTATGAATG <u>C</u> ATTTCTCCGTCCAAC	mutant base to generate T187A underlined
R8	CACACCAGAAGCTCCATTCC	
R9	CAGCTGCTGCTGCAGCATCT	
R10	CTCGACT <u>TCGAG</u> AGCTCTGTGTCAGCAGGGTCGTAG	Includes an additional four bases (TCGA, emboldened) to generate the Xho1 site (underlined)
R11	GTGGTATGGCTGATTATGATCAG	
R12	GTGTGCCTATGAA <u>GTC</u> ATTTCTCCGTCCAAC	mutant bases to generate T187D underlined
R13	GGATCGGATATCCTACAGGATCTCTGAGTTAAATCTC	
R14	CCTCCTCCTCCTCAGCGCCAGCGTACTCGTACTCCGCC TCATCTTTCTCG	
R15	GCCTCTCTTCTTCTGGCCCGGTCTATGTGATC	

Supplemental Table S2. Mass-spectrometry of MAP4K4 expressed in cardiomyocytes. Rat neonatal cardiomyocytes were infected with adenoviruses for expression of FLAG-tagged MAP4K4 and treated without or with calyculin A (CalA; 100 nM, 10 min). MAP4K4 was immunoprecipitated using EZ-FLAG and proteins separated by SDS-PAGE. FLAG-MAP4K4 was extracted from gels stained with Coomassie blue (see **Figure 2A**) and digested with trypsin. Peptide fragments and post-translational modifications (PTMs) were identified by mass spectrometry. Full details of peptides and PTMs are shown in Supplemental Spreadsheet S1.

Treatment	No. of peptides	% coverage	No. of PTMs
Minus CalA	124	78.4	820
Plus CalA	121	82.4	227

Supplemental Table S3. Identification of proteins in Band 3. Rat neonatal cardiomyocytes were infected with adenoviruses for expression of FLAG-tagged MAP4K4 and treated without or with calyculin A (CalA; 100 nM, 10 min). The expressed proteins were immunoprecipitated using EZ-FLAG and separated by SDS-PAGE. Band 3 was extracted from gels stained with Coomassie blue (see **Figure 2A**) and analysed by mass spectrometry following digestion with trypsin for protein identification. Proteins identified with >20 peptides and >20% coverage are shown.

Accession	Gene symbol	Gene name	% Coverage	No. of peptides	Score
IPI00189809.2	Myh6	Myosin heavy chain 6	72.00	218	38996.92
IPI00189811.1	Myh7	Myosin heavy chain 7	69.87	214	37413.99
IPI00209113.3	Myh9	Myosin heavy chain 9	69.40	193	22081.04
IPI00391300.3	Myo10	Myosin X	60.31	140	11747.71
IPI00764167.1	Myh11	Myosin heavy chain 11	38.61	71	6025.76
IPI00957592.1	Myh11	Myosin heavy chain 11	38.39	71	5964.10
IPI01007580.1	Myh11	Myosin heavy chain 11	37.44	68	5724.38
IPI00366370.3	Eif5b	Eukaryotic translation initiation factor 5B	30.10	31	1264.12
IPI00367479.5	Myh14	Myosin 14	29.70	47	3273.00
IPI00764433.3	MAP4K4	Mitogen-activated protein kinase kinase kinase 4	24.70	24	982.55
IPI00568786.4	MAP4K4	Mitogen-activated protein kinase kinase kinase 4	23.53	24	983.65

Supplemental Table S4. MAP4K4 peptide fragments associated with Band 3.

Residues	Region	Peptides	% coverage
1-289	Kinase domain	SPPGHDDQLWLVMFcGAGSITDLVK GQNVLLTENAEVK LVDFGVSAQLDR ALFLIPR FFSFIEGCLVK	14.1
290-492	Coiled-coil	RQQLLQEQQLR RLEEQQR	8.9
493-919	Unstructured linker	SKPSYHAPEPK EVQWSHLASLK SEGLSQSSDSKSEVPEPTQK RDSPLQGSGQQNSQAGQR NSTSSIEPR LVPRPGSGSSSGSSNSGSQPGSHPGsQSGSGERFR AASSLNLSNGETESVK QTQSASSTLQK	30.2
920-1233	Citron homology domain	FQQMDVLEGLNVLVTISGK VYYLSWLR ILHNDPEVEKK FLVIALK SSVEVYAWAPK DVVLQWGEMPTSVAYIR	23.2