

Supplementary Materials for

**Signaling by p38 MAPK Stimulates Nuclear Localization of the
Microprocessor Component p68 for Processing of Selected Primary
MicroRNAs**

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The PDF file includes:

Materials and Methods

Fig. S1. Western blotting analysis of MK2 abundance.

Fig. S2. Microarray analysis comparing the relative expression of 345 miRNAs in wild-type and MK2^{-/-} MEFs.

Fig. S3. Mass spectrometry analysis of the MK2-dependent phosphorylation of p68.

Fig. S4. The p72 protein physically interacts with and is a substrate of MK2.

Fig. S5. Assessment of the knockdown of p68 in wild-type MEFs.

Table S1. The binding partners of MK2 as determined by yeast two-hybrid screening.

Reference (50)

Materials and Methods

Antibodies

The following antibodies were used for Western blotting analysis (all antibodies were used at a working dilution of 1:1000): p68 monoclonal antibody (Millipore, Cat # PAb204), Drosha polyclonal antibody (Millipore, Cat # 07-717), MK2 polyclonal antibody (Cell Signalling Technology, Cat # 3042), HA monoclonal antibody (Santa Cruz Biotechnology, Cat # sc-7392), Paxillin polyclonal antibody (Santa Cruz Biotechnology, Cat # sc-5574), LaminA/C monoclonal antibody (Chemicon, Cat # JoL2), c-Myc polyclonal antibody (Santa Cruz Biotechnology, Cat # sc-788), β -Actin monoclonal antibody (Santa Cruz Biotechnology, Cat # sc-47778). The following antibodies were used for immunohistochemical analysis (all antibodies were used at working dilution of 1:100): p68 monoclonal antibody (Millipore, Cat # PAb204), MK2 polyclonal antibody (Cell Signalling Technology, Cat # 3042). The following antibodies were used for immunoprecipitation (in all cases, 2 μ g of antibody was used in each immunoprecipitation reaction): p68 monoclonal antibody (Millipore, Cat # PAb204), MK2 polyclonal antibody (Cell Signalling Technology, Cat # 3042), HA monoclonal antibody (Santa Cruz Biotechnology, Cat # sc-7392), c-Myc polyclonal antibody (Santa Cruz Biotechnology, Cat # sc-788).

Reagents

U0126, SP600125 and SB203580 were purchased from the LC Laboratory and were dissolved in dimethylsulfoxide (DMSO). The c-Myc siRNA pool was purchased from Dharmacon. Recombinant active MK2 was obtained from Millipore.

Murine MK2 and p68 shRNA target sequences

The following target sequences were used to generate lentiviruses expressing murine MK2- and p68-specific shRNAs. For murine MK2: GCACATCGTGGATGTCTATGA. For murine p68: GCTGAATATTGTCGAGCTTGT.

qRT-PCR analysis

Amount of pri-miR-145, pri-miR-181a1 and pri-miR-1991 were measured by qRT-PCR using the primers in the following. For pri-miR-145: forward primer: TCAGGGCAATTGAAGTTCCGGTCAC; reverse primer: ACCAGCTGGAGTTCTTCTGCATC. For pri-miR-181a: forward primer: CCTCTGCCTCCCTCCTGCTCC; reverse primer: GATGTGCTGATTTAAACAG. For pri-miR-199a: forward primer: AAGATTCTGGGAGGAGGGTGGAAA; reverse primer: CGTTGGAAATAAGGAGAAAGGCC. β -actin was used as the internal control for standardization: forward primer: CCAGCTCACCATGGATGATG; reverse primer: GACAACGGCTCCGGCAT. The amounts of pre-miR-145, pre-miR-181a1, and pre-miR-181a were measured with pre-miRNA-specific miScript pre-miRNA assay kits (Qiagen).

Primers used for generating pri-miRNA transcripts

For pri-miR-145: forward primer: TTCAGGGATCCAAAAGCTATACAAAGGTCAGAAGAG; reverse primer: AACAGAATTCTTCCAGAGCAGGACGCTGCATCCC. For pri-miR-181a1: forward primer: TTCAGGGATCCATGTGTGACAGGTTTGGTTAAAGG; reverse primer: AACAAAGCGGCCGCCCTTTGTAAACATAGTCTCTGAAG. For pri-miR-199a: forward

primer: TTCAGGGATCC CCTACTTTTTCCACCCTCCTCCC; reverse primer:
ACAAGCGGCCGCAGAGTTCAGGGTGCCTGGATGAAC.

Primers used to generate p68 mutants and individual p68 regions

To generate the DEAD→DQAD mutant: forward primer:
CCTACCTTGTCTTGATCAAGCAGATAGAATGCTTG; reverse primer:
CAAGCATTCTATCTGCTTGATCAAGGACAAGGTAGG. To generate the SAT→AAA mutant: forward primer:GCAAACCTAATGTGGGCTGCGGCTTGGCCAAAAGAAGTAAG; reverse primer: CTTACTTCTTTTGGCCAAGCCGCAGCCACATTAGAGTTTGC. To amplify the sequence encoding the N-terminal region of p68: forward primer: GCGGATCCCATGTCTGGGTTATTCGAGTGACCG; reverse primer: CGAAGCTTTTAATCCAATCCACTTAGAGCAACTG. To amplify the core region of p68: forward primer: GCGGATCCCATGGTTGGAGTGGCACAGACTGG; reverse primer: CGAAGCTTTTATCCAATTCGATGAATATAATCCTC. To amplify the C-terminal region of p68: forward primer: GCGGATCCCAGAACTGCTCGCAGTACCAAAC; reverse primer: CGAAGCTTTTATTGGGAATATCCTGTTGGC.

MicroRNA microarray

Microarray experimental procedures were performed as previously described (50). Briefly, total RNA was isolated from MEF cell using the *mirVana* RNA Isolation kit (Ambion) according to the manufacture's protocol. The *mirVana* miRNA isolation kit (Ambion) was further used to isolate miRNA from total RNA, and sent to Genome Exploration (Knoxville, TN) for analysis.

Mass Spectrometry analysis

Recombinant GST-p68 was incubated in a kinase buffer (20mM Tris-HCl pH 7.5, 5mM MnCl₂, 100μM ATP) in the absence or presence of 1 μg active MK2 at 30°C for 30 min and then resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The spot containing p68 was excised from the gel and sent to MS Bioworks (Ann Arbor, MI) for the assessment of p68 phosphorylation.

Preparation of polyclonal antibody specific for p68 phosphorylated at Ser¹⁹⁷

This antibody was commercially prepared by EZ Biolab with synthesized RLK(pS)TCIYGGAPKG-C peptide. A titer of 1:1,000 was used for Western blotting analysis.



Fig. S1. Western blotting analysis of MK2 abundance. WT MEFs were transduced with lentivirus expressing MK2-specific shRNA or luciferase-specific shRNA (Luc shRNA), whereas MK2^{-/-} MEFs were transduced with lentivirus expressing MK2. After overnight culture, cells were lysed, and cell lysates were subjected to Western blotting analysis with polyclonal antibody against MK2. Membranes were then stripped and incubated with antibody against b-actin to determine protein loading. As a control, MK2^{-/-} MEFs were transduced with lentivirus expressing empty vector. Data are representative of three individual experiments.

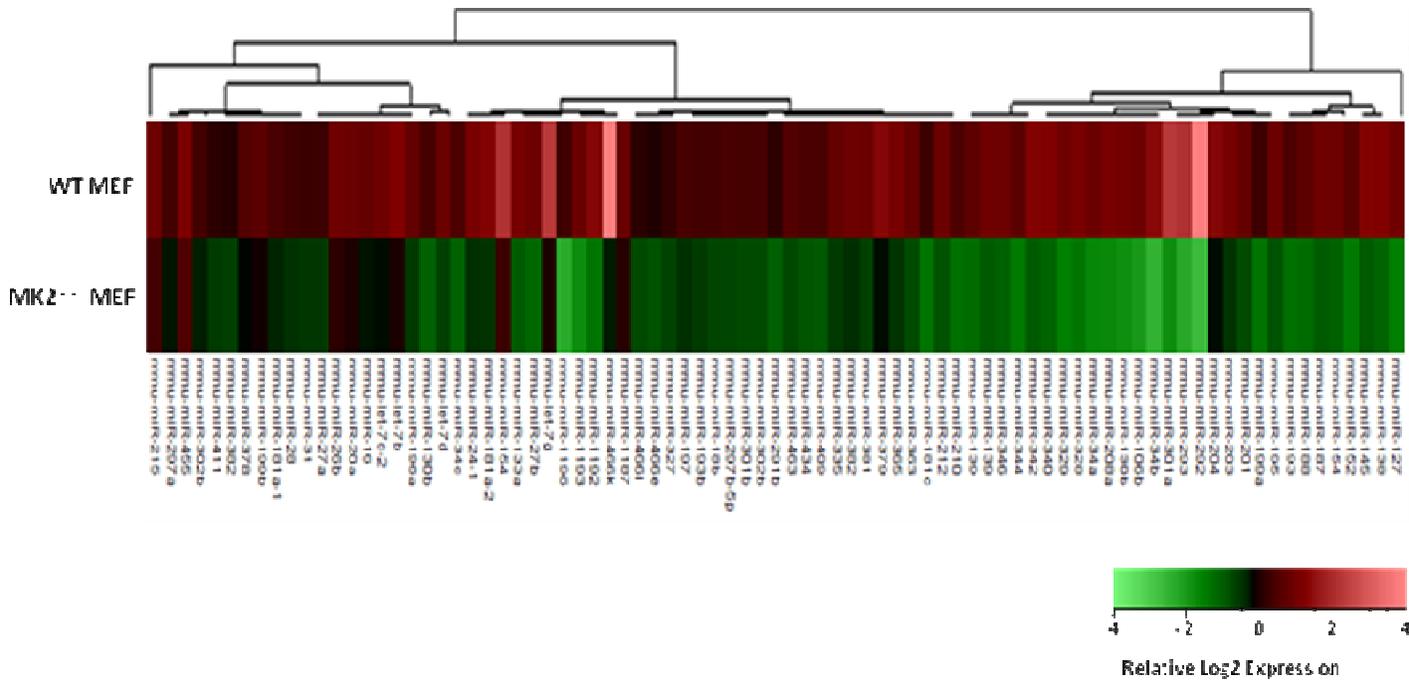


Fig. S2. Microarray analysis comparing the relative expression of 345 miRNAs in wild-type and MK2^{-/-} MEFs. Of the 345 miRNAs tested, the relative expression of 96 miRNAs were more than two-fold different between WT and MK2^{-/-} MEFs from three experiments. $P < 0.05$ by one-way ANOVA.

A

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MSGYSSDRDRGRDRGFGAPRFGGSRAGPLSGKKFGNPGEKLVKKKWNLDELPKFEKNFY
QEHPDLARRTAQEVEITYRRSKEITYRQHNCPPVNLNFEANFPANVMDVIARQNFTEPTAIQ
AQQWVPVALSGLDMYGVAAQTGSGKTLSELLPAIVHINHQPFLERGDGPICLVLAPTRELQQV
QQVAAEYCRACRLKSTCIYGGAPKGPQIRDLERGVETCIATPGRLLIDFLECGKTNLRRTTYLV
LDEADRMLDMGFEPQIRKIYDQIRPDRQTLMWSATWPKEVRQLAEDFLKDYIHINIGALELSA
NHNILQIVDYCHDVEKDEKLIRLMEEIMSEKENKTIVFVETKRRCDDELTRKMRRDGWPAMGI
HGDKSQQERDWWVLNEFKHGKAPILIATDVASRGLDVEDYKFINYDYPNSSEDIHRIGRTA
RSTKTGTAYFFTPNNIKQVSDLISVLRANQAINPKLLQLVEDRGSGRSRGRGGMKDDRRD
RYSAGKRGGFNTFRDRENYDRGYSSLLKRDVFGAKTQNGVYSAANYTNGSFGSNFVSAGIQT
SFRTGNPTOTYQNGYDSTQQYGSNVPNMHNMGMNQQAYAYPATAAAPMIGYPMPTGYSQ

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B

Site	Sequence	Max of Ion Score	Modifications	Observed	Actual Mass	Charge	Delta PPM	Start	Stop
S197	KLSTCIYGGAPKGPQIR	49.27	Phospho (+80)	0.13415	1412.42	2	1.1415	197	211

Fig. S3. Mass spectrometry analysis of the MK2-dependent phosphorylation of p68. (A) Amino acid sequence of p68. Mass spectrometry analysis of gel-recovered recombinant p68 protein included 72 unique peptides, 136 unique spectra, 233 total spectra, and 443 of 614 amino acid residues (72% coverage). There was one putative phosphorylation site: Ser¹⁹⁷ (marked in red). (B) The phosphorylated spectra.

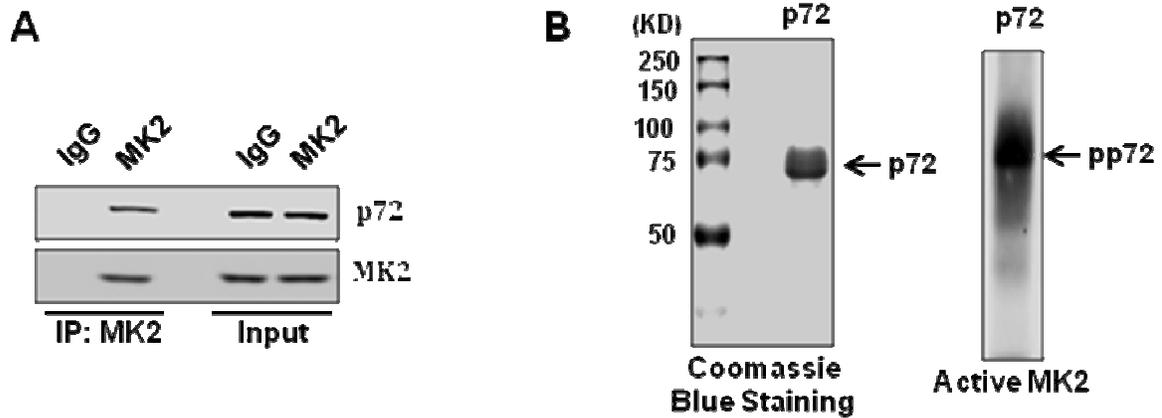


Fig. S4. The p72 protein physically interacts with and is a substrate of MK2. **(A)** WT MEFs were subjected to immunoprecipitation with either rabbit IgG or a polyclonal antibody against MK2. Immunoprecipitates were analyzed by Western blotting with antibody specific for p72. **(B)** Left panel: Coomassie blue staining of recombinant p72. Right panel: In vitro kinase assay with recombinant active MK2 to determine the ability of MK2 to phosphorylate p72. Data are representative of two individual experiments.

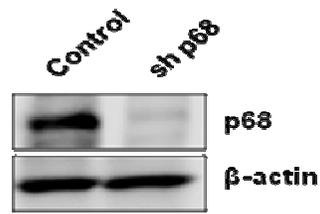


Fig. S5. Assessment of the knockdown of p68 in wild-type MEFs. WT MEFs were transduced with lentiviruses expressing p68-specific shRNA (sh p68) or luciferase-specific shRNA (Control). Four days later, cells were lysed, and lysates were analyzed by Western blotting with an antibody against p68. Membranes were stripped and incubated with an antibody against β -actin to determine protein loading. Data are representative of three individual experiments.

Table S1. The binding partners of MK2 as determined by yeast two-hybrid screening. cDNA encoding murine MK2 was subcloned into pGBKT7 (Clontech) and the generated plasmid was used to screen a mouse seven-day Embryo Matchmaker cDNA Library (Clontech). A total of 10^7 transformants were screened, and identified clones were further verified by the X-Gal colony filter assay. “Hits” indicates the obtained clones that represent the same gene. “Expect” is a parameter that describes the number of hits one can “expect” to see by chance when searching a database of a particular size. The lower the value, the more statistically significant the match is.

	<u>Name</u>	<u>Hits</u>	<u>Expect</u>
1	Human putative tumor suppressor(LUCA15) mRNA	2	1.00E-180
2	H.sapiens mitochondrial genome	1	2.00E-27
3	Human Jun activation domain binding protein	1	3.00E-107
4	Human butyrophilin(BTF5), subfamily 3	1	3.00E-17
5	protein BEX2 isoform 1	1	3.00E-15
6	aryl hydrocarbon receptor nuclear translocator	1	3.00E-12
7	Human polyhomeotic 1 homolog	2	2.00E-04
8	Early development regulator2	1	2.00E-04
9	Sentrin specific protease3	2	9.00E-97
10	CAEEL hypothetical protein	1	3.00E-12
11	Scribble	1	4.00E-50
12	Human presynaptic protein, SAP97	1	4.00E-08
13	Channel associated protein of synapse-110	1	1.00E-07
14	KIAA1016 protein	1	2.00E-16
15	RPA interacting protein	1	3.00E-31
16	Microphin1	1	2.00E-18
17	Solute carrier family9(Isoform A3)	1	3.00E-63
18	SLC9A3R1 protein	1	3.00E-101
19	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	1	9.00E-77
20	Chromosome7 ORF1 1	1	1.00E-66
21	Fibronectin type3 domain containing protein	1	7.00E-04
22	Human mRNA for p68 protein	3	5.00E-74