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***Trypanosoma brucei*: Two mitogen activated protein kinase kinases are dispensable for growth and virulence of the bloodstream form**

Bryan C. Jensen¹, Charles T. Kifer¹, and Marilyn Parsons^{1,2}

¹ Seattle Biomedical Research Institute, 307 Westlake Ave. N., Seattle, WA 98109 USA

² Dept. of Global Health, University of Washington, Seattle, WA 98195 USA

Abstract

Mitogen activated protein kinase cascades function in eukaryotic responses to the environment and stress. Trypanosomatid parasites possess protein kinases with sequences characteristic of kinases in such cascades. In this report we use gene knockouts to demonstrate that two mitogen activated kinase kinase genes, *MKK1* (Tb927.3.4860) and *MKK5* (Tb927.10.5270), are not essential in the pathogenic bloodstream stage of *Trypanosoma brucei*, either *in vitro* or *in vivo*. Bloodstream forms lacking *MKK1* showed decreased growth at 39°C as compared to the parental line. However, unlike its *Leishmania* orthologue, *T. brucei* *MKK1* does not appear to play a significant role in flagellar biogenesis.

1. Introduction

The protozoan pathogen, *Trypanosoma brucei brucei*, possesses many protein kinases which show high homology to the catalytic domains of mitogen activated protein kinases (MAPKs) and their upstream regulators. In other eukaryotes, the MAP kinase cascades include the upstream activators of the MAPKs, the MAPK kinases (MKK) and, in turn, the activators of these kinases, the MKK kinases (MKKKs). In other organisms, MAPK cascades promote cell survival and proliferation and integrate responses to a variety of stimuli such as mitogenic signals, mating pheromone, or osmotic stress (Fremin and Meloche, 2010; Qi and Elion, 2005; Saito, 2010). The cascades are kept functionally aligned by a combination of kinase specificities, spatial segregation within the cell, and inhibitory cross-talk (Saito, 2010). Human MAPK pathways are major drug targets in a variety of chronic diseases, primarily in cancer (Fremin and Meloche, 2010; Montagut and Settleman, 2009), but also in inflammatory and other diseases (Kaminska, 2005). Trypanosomatids possess numerous kinases which cluster by catalytic domain sequence similarity into the MAPK and MKKK families, as well as a smaller number of MKKs (Parsons et al., 2005). However, the close relationship of MKK and MKKK kinase domains combined with the phylogenetic distance between trypanosomatids and model organisms makes direct experimentation required to delineate functional attributes of the parasite kinases.

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*Corresponding Author. marilyn.parsons@seattlebiomed.org; Tel: 206-256-7315, FAX: 206-256-7229.

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Genetic approaches have revealed the functions of some of the MAPK proteins and their activators in *T. brucei* and the related parasites of the genus *Leishmania*. Most of the *Leishmania* predicted MAPKs, MKKs and MKKKs have orthologues in *T. brucei* (although the nomenclature is not always parallel). Some of the genes appear to be essential or play more significant roles in a particular stage of parasite development. For example, *Leishmania mexicana* *LmxMPK3* knockouts show a dramatically reduced flagellum (Erdmann et al., 2006), whereas *LmxMPK9* knockouts (Bengs et al., 2005) possess an elongated flagellum; these phenotypes are manifested in the flagellated promastigote stage. Conversely *LmxMPK* (orthologues of *L. major* *MPK1*) knockouts show no phenotype in cultured promastigote but do not proliferate as amastigotes (Wiese, 1998). Five *T. brucei* MAPKs have been studied to date, identifying roles in growth (ECK1, (Ellis et al., 2004)), response to interferon (KFR1, (Hua and Wang, 1997)), and virulence (MAPK5, (Domenicali et al., 2006)). MAPK2 and MAPK4 appear not to be important in bloodstream forms (BF). Instead MAPK2 is required for differentiation to insect midgut procyclic forms (PF) (Muller et al., 2002) while MAPK4 conveys tolerance to heat stress in PF (Guttinger et al., 2007).

In contrast to MAPKs, which phosphorylate a variety of effector proteins, MKKs are thought to be specific for one or more MAPKs, on which they typically phosphorylate both threonine and tyrosine within the activation loop. In *L. mexicana* two cognate pairs of MAPKs and MKKs have been defined. The first pair is the MAPK MPK3 and its activator MKK (MKK1 in *L. major*) (Erdmann et al., 2006). As expected from the role of MPK3 in regulating flagellar length, *MKK1* knockouts also have a shortened flagellum. The second pair, as demonstrated by in vitro studies, is comprised of *LmxMPK4* and its activator MKK5 (von Freyend et al., 2010). *LmxMPK4* has been proposed as a potential drug target. While these specific relationships are clearly important, given that there are about twice as many MAPKs as predicted MKKs in the trypanosomatids, it is possible that MKK1 and MKK5 have additional targets. The work reported here shows that neither of the *T. brucei* orthologues of these MKKs is essential in BF.

2. Methods

2.1. Parasites

Strain 29.13 (which is a derivative of strain Lister 427) (Wirtz et al., 1999) was used for experiments employing PF whereas strain Lister 427 was used for experiments employing BF. Our 427 strain, like many others (Peacock et al., 2008), has lost the ability to differentiate efficiently into PF. PF were grown in SDM79 with 15% fetal calf serum and BF cell were grown in HMI-9 with 10% fetal calf serum media. G418, hygromycin, phleomycin, and puromycin were added as necessary at 15 µg/ml, 50 µg/ml, 2.75 µg/ml and 1 µg/ml respectively for PF and 2.75 µg/ml, 5 µg/ml, 2.75 µg/ml and 1 µg/ml respectively for BF. Growth tests were performed by inoculating 10⁴ BF parasites into 1 ml in a 24 well plate. The cell number was measured daily using a Coulter Counter, starting 2 days after inoculation. Cultures were diluted as needed, to avoid cell numbers surpassing 2×10⁶/ml. Stress conditions tested included: temperature (39°C) and osmotic stress (NaCl 10–100 mM; glycerol 2–500 mM), and oxidative stress (10–900 µM H₂O₂). Each condition was tested in triplicate wells and in at least two experiments.

2.2. Plasmids

Gene knockout constructs were made by cloning approximately 500 bp from the genomic regions flanking *T. brucei* *MKK1* (Tb927.3.4860) and *MKK5* (Tb927.10.5270) into plasmid pLEW13 (Wirtz et al., 1999) (see Table 1 for primers) and were transfected into BF. These flanking regions facilitated homologous recombination into the locus of interest. First allele knockouts utilized *NEO* as the selectable marker and brought in T7 RNA polymerase.

Second allele knockouts utilized *HYG* as the selectable marker and brought in the Tet repressor derived from pLEW90 (Wirtz et al., 1999). For expression of epitope-tagged proteins in *T. brucei*, the coding regions were amplified using primers MKK1 AvrII-ATG and MKK1 3'-HindIII or MKK5 AvrII-ATG and MKK5 3'-HindIII and the products cloned into pGEM. The sequences were verified and subcloned into pLew79-3V5(PAC) (Flaspohler et al., 2010) such that three V5 epitopes were appended to the C-terminus, allowing tetracycline (Tet)-regulated expression of the fusion protein. Following transfection into *T. brucei* BF and PF and selection of stable transfectants, expression was induced the addition of Tet (2 µg/ml).

2.3. Antisera, immunofluorescence analysis, and immunoblots

Both *MKK1* and *MKK5* coding regions were subcloned from pLew79-3V5(PAC) into a modified version of pRSETA, in which the multiple cloning site had been replaced with a different polylinker which includes contains appropriately placed stop codons. Plasmids were transformed into *E. coli* BL21. Few transformants were obtained with the constructs encoding either wild-type gene, and these transformants did not yield expression of the protein of interest. Mutant versions of each kinase, in which the active site lysine in subdomain II was mutated to methionine, were readily expressed in *E. coli* after induction with IPTG. Both MKK1 and MKK5 remained in the insoluble fraction. The inclusion bodies were solubilized in 8 M urea and His-tagged MKK1 and MKK5 protein purified by affinity chromatography on nickel resin. Proteins were separated by SDS-PAGE and stained in a water-based Coomassie blue to visualize the protein. The bands were excised and used to raise polyclonal antisera in rabbits (R&R Rabbitry, Washington). Only MKK5 immunizations yielded useful antisera.

Immunofluorescence was carried out using anti-V5 monoclonal antibodies (Invitrogen) as previously described (Das et al., 1998; Jensen et al., 2003). BF were fixed in 4% paraformaldehyde for 10 min and then applied to poly-L-lysine coated cover slips, whereas PF were fixed directly on uncoated coverslips. Bound antibodies were revealed with fluorescein isothiocyanate-conjugated goat anti-mouse Ig (Southern Biotechnology). Cells were analyzed on a Deltavision microscope (Applied Precision), with images deconvolved using the conservative ratio option. Western blots using 0.5 µg/ml anti-V5, anti-CK2α (Jensen et al., 2007) 1:4000, anti-TbMKK5 1:4000, E7 monoclonal anti-β-tubulin 1:100,000 (Developmental Studies Hybridoma Bank, University of Iowa), anti-NOG1 1:4000 (Park et al., 2001) employed IRDye coupled secondary antibodies and blots were scanned on the LiCor Odyssey. For Triton X-100 extractions, washed PF were resuspended in 1% Triton X-100 in 25 mM HEPES pH 7.8, 50 mM potassium acetate, 250 mM sucrose, 1 mM EDTA and incubated for 10 min on ice. The cytoskeletal fraction was pelleted by centrifugation at maximum speed for 10 min in a microfuge. Flagella were isolated, along with the kinetoplast DNA (kDNA), as described (Robinson and Gull, 1991), except that 3 mM CaCl₂ was utilized following extraction with Triton X-100.

2.4. Mouse virulence studies

All procedures were performed under IACUC approved protocols. Mice were housed in the Seattle BioMed rodent facility, under controlled light, temperature, and humidity with adequate food, water, and bedding. Animals were under the care of vivarium staff and an attending veterinarian. Seattle BioMed has a PHS Assurance issued by OLAW and has a USDA Research Registration. BALB/c mice were infected with 10⁴ parasites by intraperitoneal injection (day 0). Starting on day 2, parasitemias were monitored daily by microscopic analysis of blood obtained by tail prick. Blood was diluted 1:100 in 1X SSC containing 4% formaldehyde, and counted on a hemocytometer. Once parasites reached 10⁸/

ml the animals were euthanized by CO₂ asphyxiation, since the parasitemia would reach lethal levels within a day.

Results

MKK1 and *MKK5* are two *T. brucei* MKKs which cluster by phylogenetic inference with MKKs of yeast and human (Parsons et al., 2005). *MKK1* and *MKK5* transcripts are present in similar, albeit low, levels in BF and PF according to our previous microarray studies (Jensen et al., 2009), although a more recent RNASeq analysis suggested up-regulation of *MKK5* in BF (Siegel et al., 2010). Estimates suggest that the genes are expressed at the level of 2–3 mRNAs per PF cell (Kolev et al., 2010). These genes are syntenic with their *Leishmania* orthologues, and share similar features except that the *Leishmania* *MKK5* has a considerably longer C-terminal extension following the protein kinase domain (197 aa for *LmxMKK5* versus 58 for *TbMKK5*).

Recent results have suggested that some trypanosomatid MAPK pathways play a role in flagellar biogenesis or function, including that mediated by *L. mexicana* MKK, the orthologue of *MKK1* (Wiese et al., 2003). We therefore examined the localization of *T. brucei* *MKK1* and *MKK5* in PF and BF following isolation of stable transfectants expressing epitope tagged versions of the proteins. In both cases, in PF the proteins were expressed and appeared to be predominantly present in the cytoplasm (Fig. 1A). Expression in of the tagged protein BF was low, which precluded immunofluorescence analysis. Western analysis (Fig. 1B) demonstrated the expression of the V5-tagged kinases at the appropriate molecular mass (42.1 kDa for *MKK1*; 41.6 for *MKK5*) considering the size of the three V5 tags (5.4 kDa). Extraction with Triton-X 100, which leaves the cytoskeleton intact, fully extracted both of the tagged kinases, similarly to the soluble protein CK2 α and unlike the cytoskeletal protein β -tubulin. These data indicate that the kinases were not strongly associated with the cytoskeleton (Fig. 1B). We also raised antibody to *MKK5*, which detected a 43 kDa protein within parasite lysates of both BF and PF stages, although faint background bands were also observed (Fig 1C).

We generated knockouts for each gene in BF parasites by homologous recombination, utilizing *NEO* as the selectable marker for the first knockout and *HYG* for the second. Parasite clones arose, even in the absence of a complementing copy of the gene. PCR experiments (Fig. 2A) as well as either Southern analyses (*MKK1*, Fig. 2B) or Western analysis (*MKK5*, Fig. 2C) verified that the target gene was deleted. The *mkk5* knockouts did not show any anomalies on PCR analysis of integration events. However, PCR analysis suggested and Southern blots confirmed that (unbeknownst to us at the time of transfection of the second knockout construct), the two *mkk1::NEO* transfectants had become homozygous for the *NEO* deletion allele. Upon introduction of the second knockout construct, Southern analysis suggested that the *HYG* gene was inserted 3' to the *NEO* gene, likely via a 115 bp region of identity that is present both upstream and downstream of the drug markers as derived from pLEW13 and pLEW90 (Wirtz et al., 1999) (see Fig. 2B, black boxes). PCR analysis using a sense *NEO* primer and antisense *HYG* primer confirmed the presence of such a *NEO-HYG* allele in these $\Delta mkk1$ clones as shown by the presence of the predicted ~580 bp product (Fig. 2A). Subsequently, one clone (N1H) became homozygous for the $\Delta mkk1::NEO-HYG$ allele and the other (N2H) showed two allelic forms, ($\Delta mkk1::NEO/\Delta mkk1::NEO-HYG$). The relative signal between the two forms in the latter suggests that the *NEO* allele may be present in higher copy number than the *NEO-HYG* allele. Digestion with three other restriction enzymes confirmed this scenario (data not shown).

To analyze growth of the *Δmkk1* and *Δmkk5* parasites *in vitro*, we inoculated BF at low density and monitored the increase in the population over time (Fig. 3A). Growth of the knockout clones was indistinguishable from the parental wild type lines under these conditions.

Because some MAP kinase pathways function in stress responses we tested the growth of the knockout parasites under conditions of elevated temperature (39°C), hyperosmotic stress (NaCl and glycerol) or elevated temperature. The two *mkk1* knockout clones were moderately more sensitive to temperature stress (Fig. 3B, $p < .0001$). To determine whether this resistance was due to clonal variation independent of the gene knockout, we examined whether expression of a Tet-regulated copy of *MKK1* would rescue this phenotype. Without Tet induction, the growth resembled that of the null mutant. The addition of Tet restored wild-type levels of growth at 39°C. The two independent *mkk5* knockouts were slightly more resistant to high temperature ($p < .002$). When exposed to hyperosmotic stress, no obvious differences were noted in the growth of the knockout parasites as compared to the control parental lines (Fig. 3C, D). Interestingly, although the *mkk1* knockout in *L. mexicana* leads to a dramatically shortened flagellum (Wiese et al., 2003), the flagellum appeared normal upon Giemsa staining of *Δmkk1 T. brucei* BF (not shown). Analysis of isolated flagella confirmed that there was no significant difference in their length (Fig. 3E).

Although deletion of either *MKK1* or *MKK5* had no effect on growth *in vitro*, we wanted to know if there was any effect in an *in vivo* animal model. Precedence for such a case was seen in the RNAi mediated depletion of cathepsin L (brucipain) (Abdulla et al., 2008) as well as calflagin (Emmer et al., 2010). BALB/c mice were infected with 10^4 parasites by intraperitoneal injection. Mice infected with the wild-type parental line had a steady increase in parasitemia that exceeded 10^8 cells/ml four days post infection, at which time they were euthanized (Fig. 3F). We tested the virulence of two clones of *Δmkk1* and 5 clones of *Δmkk5* (Fig. 3F). Survival of mice infected with either of the *Δmkk1* clones was nearly indistinguishable from those infected with wild type parasites, with a mean survival of 4.2 days compared to 4 days for mice infected with wild type parasites. Five of the six *Δmkk5* clones showed virulence similar to wild type, albeit with a slightly increased mean survival time of 5.2 days. One *Δmkk5* clone repeatedly showed dramatically reduced virulence (with survival of infected mice for more than two weeks) despite normal growth *in vitro*. The reason for this decrease in virulence is unknown, but presumably reflects genetic or epigenetic changes specific to that clone.

4. Discussion and conclusions

The data presented here demonstrate that neither *MKK1* nor *MKK5* is required for growth of BF *in vitro* or virulence *in vivo*. The *Δmkk1* parasites showed modest but statistically significant increase in sensitivity to increased temperature whereas *Δmkk5* parasites were slightly more resistant. However, the response of the knockout lines to osmotic stresses did not differ from the parental line. Preliminary studies revealed no compelling differences in survival upon exposure to H_2O_2 or the DNA damaging agent phleomycin, or survival upon reaching stationary phase (data not shown). Thus the data indicate that *MKK1* and *MKK5* are unlikely to be effective drug targets.

Interestingly, BF *MKK1* single knockouts (*MKK1/mkk1::NEO*) repeatedly underwent loss of heterozygosity to a null phenotype under our standard drug selection regimens, suggesting that under those conditions increased levels of the resistance protein (NEO) may be more beneficial than having a copy of the *MKK1* gene. Additionally it could be that *MKK1* resides in a particularly recombinogenic region of the genome. Interestingly it lies less than 5 kb from a divergent strand switch region, and as such is close to a region of the chromosome

with distinctive histone modifications (Siegel et al., 2009). Work in other organisms has demonstrated that increased histone acetylation stimulates homologous recombination (Cummings et al., 2008). That MKK1 and MKK5 play apparently minor roles in BF is all the more striking given the major role of MKK1 in flagellar biogenesis or function in *L. mexicana* (Wiese et al., 2003) and the essentiality of the target of MKK5 in the same species (von Freyend et al., 2010; Wang et al., 2005). However, we should note that TbMAPK2, the orthologue of the *L. mexicana* MKK5 substrate LmxMPK4, is not essential in *T. brucei* BF (Muller et al., 2002). In contrast, null mutants of *LmxMPK4* could not be obtained in *L. mexicana*, where the gene is thought to be required for lesion formation (Wang et al., 2005). These data point out that the specific functions of protein kinases with strong reciprocal blast relationships within trypanosomatids are not always parallel. Although the studies did not reveal an important role for *MKK1* and *MKK5* in *T. brucei* BF, our data do not preclude important roles in another developmental stage. Alternatively, potential functions of the kinases could be revealed by analysis of pleiomorphic strains which, unlike our commonly used monomorphic strain, retain the capacity to develop into non-dividing forms, and thus may be more sensitive to environmental perturbations.

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Figure 1. Localization of MKK1 and MKK5

A) Stable PF transfectants bearing a Tet-regulated gene encoding a V5-tagged copy of MKK1 or MKK5 were stained with anti-V5. Parasite DNA was stained with DAPI. +Tet, induced for expression; –Tet, not induced.

B) MKK1 and MKK5 are extracted by non-ionic detergents. PF expressing the V5-tagged proteins were extracted with Triton X-100 and centrifuged to pellet the insoluble material. The pellet was washed and the samples were loaded in cell equivalents for western blot analysis with anti-V5, anti-CK2 α , and anti- β -tubulin. T, total lysate; S, Triton X-100 soluble fraction; W, wash; P, Triton X-100 pellet fraction. Salt concentrations in the three fractions differ, which likely accounts for the small differences in migration between fractions. Hash marks at left indicate the migration of the listed molecular mass markers (kDa).

C) Western blot showing expression of MKK5 in both PF and BF. The blot was additionally probed with anti-NOG1 to normalize the loading of the gel.

Figure 2. Generation and verification of *Amkk1* and *Amkk5* knockout strains

A) PCR analysis showing gene loss. DNA from the 427, wild type parental line and two independent deletions of *MKK1* (*mkk1::NH*) and *MKK5* (*mkk5::NH*) was amplified with primers located in the UTRs for either *MKK1* or *MKK5*. PCR extension times were optimized for the intact gene, as opposed to longer products from knockout alleles. As a control for the PCR the DNAs were amplified with primers directed against the MAPK, Tb927.8.3350. The integration of the *HYG* gene 3' to the *NEO* gene in the *Δmkk1* strains was verified using a primer in unique region just 3' to the *NEO* gene (NEO-S) and primer located within the hygromycin resistance gene (*HYG*-AS).

B) Southern analysis of *Δmkk1* strains. Genomic DNA from various clones was digested with *Eco47*. The probe was a randomly primed PCR fragment which included the ORF plus approximately 400 bp from both 5' and 3' UTRs. Two independent first round knockouts bearing the *NEO* marker (*mkk1::N*) are shown. Earlier passages of these clones were transfected with the second knockout construct containing the *HYG* gene to generate *Amkk1* clones 1 and 2 designated here as *mkk1::NH*. The cartoon shows the alleles found (arrowheads), with the gray and white boxes representing *MKK1* 5' and 3' UTRs and the black box representing a segment of the actin 5' UTR which is present both upstream and downstream of the drug resistance markers. The predicted sizes of these fragments upon cleavage with *Eco47* is listed. Migration of DNA markers (kDa) is indicated.

C) Western blot of MKK5 strains. The blot was probed with anti-MKK5 and anti-β-tubulin. The strains examined are: wild type parental line (427), single knockout clone 1 (*mkk5::+/-NI*) and double knockout clone 1 (*mkk5::NH1*).

Figure 3. Phenotypic analysis of *Amkk1* and *Amkk5* strains

A) *In vitro* growth.

B) Effect of high temperature stress. The number of cells following growth for two days at 39°C were compared to parallel cultures of the same clone grown at 37°C within the same experiment. The means and standard deviations across multiple experiments are graphed; at least two parasite clones were examined. P values from t-tests comparing different cell lines are indicated.

C) Effect of osmotic stress with glycerol. Population sizes at two days for each concentration of glycerol are compared to the no glycerol control of the same clone. Shown are the means and standard deviations across multiple experiments; at least two clones were examined.

D) Effect of osmotic stress with NaCl. Details as in Fig. 3C.

E) Flagellar length is not altered in *mkk* knockout parasites. For each of the indicated parasite lines, the length of 25 isolated flagella was measured by tracing each flagellum from the associated kDNA to the tip using Metamorph software. The mean length and standard deviation for each line are shown. The inset shows a typical image of a detergent-extracted flagellum with the DAPI signal from the kDNA in white. 427, single marker clone of strain 427 (Wirtz et al., 1999). Bar, 5 µm.

F) Survival of mice infected with knockout strains. Mice were infected intraperitoneally with cultured BF and sacrificed when parasitemias rose above 10^8 /ml. The number of mice surviving on each day is plotted. Data is pooled from two experiments, showing infections with wild type parasites (10 mice), *Amkk1* parasites (10 mice, 2 parasite clones), and *Amkk5* (15 mice, 5 parasite clones). The single *Amkk5* clone which showed dramatically decreased virulence was excluded from this analysis.

Table 1**Primers**

primer	purpose	sequence
MKK1 5' UTR-S	<i>MKK1</i> knockout	5'-ATAGCGGCCGCGGGTTGGACGGTGGTTACAG-3'
MKK1 5'UTR-AS	<i>MKK1</i> knockout	5'-ATAACGCGTCTCGAGTTGGATTGGAATCCGTTTCAGAGAC-3'
MKK1 3' UTR-S	<i>MKK1</i> knockout	5'-ATATCTAGAATTTAAATATTAAAGGGTGTGCTGTCCTG-3'
MKK1 3'UTR-AS	<i>MKK1</i> knockout	5'-ATAAGGCCTGCGGCCGCACCTCTAACCTGATACGCTG-3'
MKK5 5' UTR-S	<i>MKK5</i> knockout	5'-ATAGCGGCCGCCATGCCGATTGCTAATGCAG-3'
MKK5 5'UTR-AS	<i>MKK5</i> knockout	5'-ATAACGCGTCTCGAGGGTTGACTCAACACACAGTGAGGCAG-3'
MKK5 3' UTR-S	<i>MKK5</i> knockout	5'-ATATCTAGAATTTAAATAAGTGTGGAGTGCCGAAACATGGTG-3'
MKK5 3'UTR-AS	<i>MKK5</i> knockout	5'-ATAAGGCCTGCGGCCGCTAGCACCATATCGCGTCCTC-3'
MKK1 AvrII-ATG	Clone <i>MKK1</i> for V5 tag	5'-CGGCCTAGGATGAAAAGGAAGGGGATTGCGTTAG-3'
MKK1 3'-HindIII	Clone <i>MKK1</i> for V5 tag	5'-CGGAAGCTTCAGAACACCTAGCATTTCCAATTTTATGTTC AC-3'
MKK5 AvrII-ATG	Clone <i>MKK5</i> for V5 tag	5'-CGGCCTAGGATGACCGGGTTGAAGATTGACATC-3'
MKK5 3'-HindIII	Clone <i>MKK5</i> for V5 tag	5'-CGGAAGCTTCTGCACCAACTTATTTAGTTCCTCGTC-3'
NEO-S	Verify <i>NEO-HYG</i> allele	5'-CGAGTTCTTCTGAGCGGGAC-3'
HYG-AS	Verify <i>NEO-HYG</i> allele	5'-TATCCACGCCCTCCTACATC-3'