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Twitchin kinase interacts with MAPKAP kinase 2 in Caenorhabditis elegans striated muscle

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ABSTRACT In Caenorhabditis elegans, twitchin is a giant polypeptide located in muscle A-bands. The protein kinase of twitchin is autoinhibited by 45 residues upstream (NL) and 60 residues downstream (CRD) of the kinase catalytic core. Molecular dynamics simulation on a twitchin fragment revealed that the NL is released by pulling force. However, it is unclear how the CRD is removed. To identify proteins that may remove the CRD, we performed a yeast two-hybrid screen using twitchin kinase as bait. One interactor is MAK-1, C. elegans orthologue of MAPKAP kinase 2. MAPKAP kinase 2 is phosphorylated and activated by p38 MAP kinase. We demonstrate that the CRD of twitchin is important for binding to MAK-1. mak-1 is expressed in nematode body wall muscle, and antibodies to MAK-1 localize between and around Z-disk analogues and to the edge of A-bands. Whereas unc-22 mutants are completely resistant, mak-1 mutants are partially resistant to nicotine. MAK-1 can phosphorylate twitchin NL-Kin-CRD in vitro. Genetic data suggest the involvement of two other mak-1 paralogues and two orthologues of p38 MAP kinase. These results suggest that MAK-1 is an activator of twitchin kinase and that the p38 MAP kinase pathway may be involved in the regulation of twitchin.

INTRODUCTION
Muscle sarcomeres contain giant polypeptides (>700,000 Da) of the titin-like family, consisting primarily of multiple copies of immunoglobulin (Ig) and fibronectin type 3 (Fn3) domains and one or two protein kinase domains at their C-termini. These giant proteins are involved in sarcomere assembly and in sensing and transducing mechanical signals in the sarcomere (Kontrogianni-Konstantopoulos et al., 2009; Krüger and Linke, 2011). These mechanical signals drive the development and regulation of muscle in adaptation to physical demands. Members of this family include titin and obscurin in mammals; UNC-22 (twitchin), UNC-89 (obscurin), and TTN-1 in Caenorhabditis elegans; twitchin in molluscs; and projectin (quite similar to twitchin), UNC-89, and stretchin in insects (Bullard et al., 2002; Kontrogianni-Konstantopoulos et al., 2009).

Bacterially expressed C. elegans twitchin kinase has protein kinase activity in vitro, both in autophosphorylation and toward peptides derived from vertebrate myosin light chains (Lei et al., 1994; Heierhorst et al., 1996). Twitchin kinase is autoinhibited by a 60-residue-long regulatory tail that lies just C-terminal of the kinase catalytic core (called the C-terminal regulatory domain [CRD]; Lei et al., 1994). Crystal structures of twitchin kinase from C. elegans (Hu et al., 1994) and Aplysia (Kobe et al., 1996) and from human titin kinase (Mayans et al., 1998) show that the CRD wedges itself between the two subdomains of the catalytic core, blocking binding sites for ATP and protein substrates. Activation of these kinases requires removal of the CRD from the active site, but how this occurs is unknown. Steered molecular dynamics simulations on human titin kinase indicate that small pulling forces occurring during muscle activity are sufficient to remove the CRD from the catalytic pocket and permit access to substrates and, thus, catalytic activity (Grater et al., 2005). Although this model has not been proven by experiments, atomic force microscopy (AFM) experiments on titin kinase demonstrate that pulling force permits ATP to bind to the kinase (Puchner et al., 2008; Puchner and Gaub, 2010). In addition, there is some evidence that stretching force activates twitchin kinase in Mytilus: using permeabilized smooth muscles, Butler and Siegman (2011) demonstrated that with a 10% stretch there is a twofold increase in phosphorylation of a model peptide substrate that previously had been
shown to be a substrate for molluscan twitchin kinase in vitro. Moreover, the titin kinase CRD binds as a complex to the autophagosome receptors nbr1 and p62 and the E3 ubiquitin ligase MuRF2 (Lange et al., 2005). Force-dependent regulation of this complex was suggested by finding that in cultured cardiac muscle cells, when induced to stop beating, there was disassembly of this complex and nuclear translocation of MuRF2. Correlated nuclear export of serum response factor resulted in decreased transcription of mRNAs for sarcromeric proteins (Lange et al., 2005). Force was initially believed to induce phosphorylation by titin kinase of nbr1 and/or p62, and this might explain decreased binding affinity, and, at least in vitro, nbr1 and p62 could be weakly phosphorylated by titin kinase (Lange et al., 2005). However, more recently, titin kinase has been demonstrated to be an inactive pseudokinase (Bogomolovas et al., 2014).

The crystal structure of the largest segment of any giant kinase, the Fn–N-terminal linker (NL)-kinase-CRD-Ig segment from C. elegans twitchin, has been reported (von Castelmur et al., 2012). Molecular dynamic simulations indicate that, unexpectedly, the mechanically sensitive portion is an NL of 45 residues lying between the Fn3 domain and the kinase catalytic core, not the CRD. In fact, the CRD remains attached to the large lobe of the kinase even after the small lobe has been unwound. In mammalian titin kinases, the NL sequence is shorter, ~26 residues long (von Castelmur et al., 2012), and was not present in the original crystal structure (Mayans et al., 1998) or the molecular dynamics simulations (Grater et al., 2005). However, in AFM pulling experiments on a larger segment of human titin including Ig25-Fn3-NL-Kin-CRD-Ig, the first unfolding peak was attributed to the unfolding of a “23-residue” NL (Puchner et al., 2008). Therefore the NL of mammalian titin kinase may also be mechanically sensitive.

For nematode twitchin kinase, in vitro kinase assays using a model peptide substrate show that NL and CRD each inhibits kinase activity by one-half (von Castelmur et al., 2012). One possibility is that the purpose of this CRD interaction is to stabilize the kinase catalytic core and protect its active site from being destroyed. Another possibility is that under some physiological conditions, twitchin kinase needs to be fully activated by pulling force removing the NL and additional, unknown factors removing the CRD from the catalytic pocket. These unknown factors could be binding to other protein activators or phosphorylation by another kinase. Twitchin kinase is 52% identical in sequence to chicken smooth muscle myosin light chain kinase (MLCK; Benian et al., 1989). MLCKs are also autoinhibited by an ~60-residue tail C-terminal of their catalytic core (Kemp et al., 1987) and are activated upon binding of this autoinhibitory sequence to calmodulin (Stull et al., 1986). Although calmodulin binds to the CRD of twitchin kinase in vitro, it does not lead to significant activation (Lei et al., 1994).

In C. elegans, unc-22 is one of ~40 genes that, when mutant, result in slow or paralyzed adult nematodes and disorganized sarcomeric structure (“unc” for “uncoordinated”; Waterston et al., 1980; Zengel and Epstein, 1980). The unc-22 gene encodes twitchin, a ~754-kDa polypeptide consisting primarily of 31 Fn3 domains, 30 Ig domains, and a single protein kinase domain near its C-terminus (Moerman et al., 1986, 1988; Benian et al., 1989, 1993). The sequence of C. elegans twitchin revealed that it was the first intracellular protein to join the Ig superfamily and helped define the intracellular, mostly muscle, branch of this superfamily. unc-22 mutants display a characteristic “twitching” of the animal’s surface, ~1–2 times/s, which originates from the underlying muscle, and also show variably disorganized sarcomeres (Waterston et al., 1980; Moerman et al., 1988). Whether twitching represents unregulated contraction or relaxation is unknown, but twitching is enhanced by choline agonists such as nicotine (Moerman and Baillie, 1979). Indeed, whereas exposure to nicotine causes wild-type nematodes to stop moving, unc-22 mutants continue to move and twitch violently. Moreover, although under typical culture conditions, unc-22 heterozygotes move normally and have normal muscle structure, in nicotine solutions, they twitch; that is, unc-22 is “conditionally dominant” (Moerman and Baillie, 1979). Because this dominance occurs even with null alleles, this suggests that twitchin is required stoichiometrically. Genetic data strongly suggest interaction of twitchin with myosin: an unc-22-null allele shows disorganization of thick filaments (Moerman et al., 1988), and rare missense alleles of unc-54 residing in the head domain of myosin heavy chain B suppress the twitching of unc-22 mutants and also improve their locomotion and muscle structure (Moerman et al., 1982).

To determine what might remove the CRD and permit full activation, we used a region of C. elegans twitchin containing the kinase catalytic core and the CRD to screen a yeast two-hybrid library. Our bait included the N-terminal 45 residues of the 60-residue CRD, based on results of titin kinase yeast two-hybrid screening: titin kinase including a truncated but not full-length CRD was found to interact with Nbr1 (Lange et al., 2005). We found that twitchin kinase interacts with MAK-1, an orthologue of mammalian mitogen-activated protein (MAP) kinase--activated protein (MAPKAP) kinase 2, known to be phosphorylated and thereby activated by p38 MAP kinase.

**RESULTS**

**Identification of MAK-1 as a binding partner for twitchin kinase**

To identify proteins that might regulate the activity of the protein kinase domain of twitchin, we used Ig25-Fn3-NL-Kin-CRD (45 amino acids [aa]) as bait to screen a yeast two-hybrid library of C. elegans cDNAs (Figure 1A). A total of 1.7 × 10^6 colonies were screened, and we obtained 82 prey clones that were positive upon retransformation, identifying 26 genes (Supplemental Table S1). We then queried Serial Analysis of Gene Expression (SAGE) data available in Meissner et al. (2009) to identify which of these genes were primarily expressed in body wall muscle. Many of the genes are expressed in multiple tissues (muscle, neurons, and gut), and several others are expressed primarily in neurons. Two genes are primarily expressed in muscle, deb-1 and K08F8.1. deb-1 encodes vinculin, a component of the base of the dense body (Z-disk analogue; Barstead and Waterston, 1989), but this interaction does not seem biologically relevant, as twitchin is located in a different portion of the sarcomere, the A-band (Moerman et al., 1988). K08F8.1, which we renamed mak-1 (see later discussion), caught our attention because the encoded protein is an orthologue of a protein that was first identified and purified from rabbit skeletal muscle called MAPKAP kinase 2 (Stokoe et al., 1992a). As shown in Figure 1B, the protein MAK-1 is 521 residues in length and contains a protein kinase domain. The kinase domain of human MAPKAP kinase 2 is 53% identical to the kinase domain of nematode MAK-1 (Figure 1B). Mammalian MAPKAP kinase 2 requires phosphorylation by p38 MAP kinase for activity; the sites have been identified (Ben-Levy et al., 1995), and these are conserved in MAK-1. Our analysis of the C. elegans proteome reveals that there are two additional MAPKAP kinase 2 paralogues, MAK-2 and MNK-1, which have kinase domains that are 52 and 31% identical to the kinase domain of MAK-1, respectively (Figure 1B).

The Y2H screen identified two independent prey clones for MAK-1 encoding residues 28–521 and 81–521. To further determine a minimal region of MAK-1 required for interaction of twitchin kinase,
Similarly, by Y2H assays, we determined that the Ig-Fn31 region upstream of twitchin kinase is not required for interaction with MAK-1; interaction was found when the NL-Kin-CRD (45) of twitchin was tested (top three rows of Figure 1D). We next explored the specificity of the interaction of twitchin kinase with MAK-1. In C. elegans, there are two additional giant polypeptides with protein kinase domains near their C-termini, TTN-1 and UNC-89. Moreover, the we created deletion derivatives of full-length MAK-1 and used Y2H assays to assess interaction with Ig25-Fn31-Kin-CRD (45 aa). As indicated in Figure 1C, the smallest portion of MAK-1 that interacts with twitchin kinase is MAK-1 (aa 81–405), essentially the kinase catalytic core plus 61 residues of N-terminal sequence. When tested for interaction with twitchin kinase, the MAK-1 paralogues MAK-2 and MNK-1 fail to interact (bottom portions of Figure 1C).

Similarly, by Y2H assays, we determined that the Ig-Fn31 region upstream of twitchin kinase is not required for interaction with MAK-1; interaction was found when the NL-Kin-CRD (45) of twitchin was tested (top three rows of Figure 1D). We next explored the specificity of the interaction of twitchin kinase with MAK-1. In C. elegans, there are two additional giant polypeptides with protein kinase domains near their C-termini, TTN-1 and UNC-89. Moreover, the
domain organization upstream of the kinase domains is also conserved, such that all three proteins are organized as Ig-Fn-Kinase. When tested by Y2H assays for interaction with MAK-1, the homologous regions of TTN-1 or UNC-89 PK2 failed to interact (bottom two rows of Figure 1D).

**Twitchin CRD is important for binding to MAK-1**

Taking advantage of the lack of interaction of TTN-1 with MAK-1 (Figure 1D), we created TTN-1 ("T")/twitchin ("W") chimeras and tested them by Y2H to determine which portion of twitchin NL-Kin-CRD (45 aa) is critical for interaction with MAK-1. Analysis of these many chimeras (Figure 1E) suggests that the C-terminal ~140 residues of twitchin, including the end of the kinase catalytic core and most of the CRD, is crucial for this interaction. However, we cannot rule out the possibility that the chimeras that show no interaction are not folded correctly.

To verify the interaction of twitchin kinase with MAK-1 and obtain additional evidence that the C-terminal region of NL-Kin-CRD (60 aa) is important for this interaction, we conducted two types of in vitro binding experiments using purified recombinant proteins. We produced histidine (His)-tagged fragments of twitchin kinase—NL-Kin-CRD (60 aa), Kin-CRD (60 aa), NL-Kin, and Kin—and a maltose-binding protein (MBP) fusion of MAK-1 (81–405). A far-Western assay was conducted in which the twitchin fragments were present on a membrane and incubated with either MBP or MBP-MAK-1 in solution. (A Coomassie-stained gel of 2 μg of each protein used in this experiment is shown in Supplemental Figure S1.) As shown in Figure 2A, the apparent strength of binding depended on the presence of the twitchin CRD (60 aa) region: stronger binding to NL-Kin-CRD (60 aa) and Kin-CRD (60 aa) and weaker binding to NL-Kin and Kin. A pull-down assay was conducted in which the His-tagged fragments of twitchin kinase were incubated in solution with either MBP or MBP-MAK-1 and the proteins pulled down using anti-His antibody conjugated beads; after separation on a gel and transfer to a membrane, MBP-MAK-1 or MBP was detected using anti-MBP. As shown in Figure 2B, only MBP-MAK-1 and not MBP was detected, and the amount of MBP-MAK-1 pulled down was reduced when CRD was missing from the twitchin fragments.

**The mak-1 gene is expressed in body wall muscle, intestine, and hypodermis**

Although SAGE data indicate that mak-1 is expressed in body wall muscle (Meissner et al., 2009; Supplemental Table S1), we sought additional evidence for this expression pattern. A 6.4-kb genomic fragment that includes the putative promoter sequence upstream of mak-1, a putative 5′-untranslated region, initiator ATG, and the first 12 nucleotides of coding sequence of mak-1 was fused to green fluorescent protein (GFP), and transgenic worms carrying this segment were generated. This mak-1 promoter reporter is expressed in body wall muscle and intestine (Figure 3A), as well as in hypodermis (unpublished data).

**mak-1 loss-of-function mutants and generation of specific anti-MAK-1 antibodies**

Two mak-1 intragenic deletion strains were obtained from the Cae
norhabditis Genetics Center (University of Minnesota, Minneapolis, MN) and the National Bioresource Project (Tokyo, Japan). As depicted on WormBase (www.wormbase.org/species/c_elegans), mak-1(ok2987) is a 754–base pair deletion, removing all of exon 3 (Figure 3B). We determined that mak-1(tm3455) has both a 7–base pair insertion and a 267–base pair deletion extending from the 3′ end of exon 3 until the 5′ end of intron 4.
FIGURE 3: mak-1 expression pattern, mutations, and MAK-1 antibodies. (A) The mak-1 gene is expressed in the intestine and body wall muscle. To detect mak-1–expressing cells, we created transgenic worms expressing GFP from a 6.4-kb segment of genomic DNA upstream of the mak-1 predicted translational start. GFP was detected in the intestine (left) and body wall muscle cells (right) at the upstream of the worm expressing GFP from a 6.4-kb segment of genomic DNA. (B) Schematic representation of the gene, with boxes denoting exons, and introns denoting introns, tm3455 (insertion/deletion), ...ttggtctgtctgact...wild type...ttggtctgtctgact[754 bp deletion]ttggtctgtctgact...ok2987...taaatagttgctgttaagc...wild type...taaatagttgctgttaagc[267 bp deletion]taaatagttgctgttaagc...tm3455 [insertion]. (C) Location of immunogens used to generate antibodies to MAK-1 and Western blot analysis of wild-type and mak-1 mutants. Antibodies raised to either an N-terminal or a C-terminal region detect an ∼60-kDa protein, the size expected for MAK-1. For the anti–N-terminal antibody, MAK-1 cannot be detected from the two mutant alleles. On longer exposure, however, two novel bands are detected from tm3455, likely representing truncated MAK-1 polypeptides. On longer exposure, no protein is detected from ok2987, other than bands found from all three strains likely arising from cross-reactivity to bacterial products. For the anti–C-terminal antibody, MAK-1 proteins cannot be detected even after long exposure time. On the basis of these results, we conclude that ok2987 is a likely null allele for mak-1.

MAK-1 localizes between and around dense bodies and partially colocalizes with MHC B (and, by inference, twitchin) at the edge of the A band

The anti-C-terminal MAK-1 antibody was used to determine whether MAK-1 is localized in the sarcomere and, if so, where it is localized. In immunofluorescence experiments of wild-type body wall muscle, anti-MAK-1 was costained with anti-PAT-6 (α-parvin) to mark the M-lines and dense bodies. As shown in Figure 4A (top row), anti-MAK-1 localizes between and around dense bodies. No signal was obtained with anti-MAK-1 upon staining either mak-1 mutant animal (Figure 4A, rows 2 and 3), indicating that the localization pattern in wild-type is specific.

Both anti-MAK-1 and all available anti-twitchin antibodies (Moerman et al., 1988; Benian et al., 1996) have been generated in rabbits. Thus we could not costain anti–MAK-1 with anti-twitchin. However, previous studies demonstrate that twitchin colocalizes with MHC B (Moerman et al., 1988), and a mouse monoclonal to MHC B is available (monoclonal S-8; Miller et al., 1983). Therefore we costained anti-MAK-1 with anti-MHC B. As shown in Figure 4B, some MAK-1 colocalizes with MHC B at the outer edges of the A-band (seen as white dots in the merged image). This result shows that some MAK-1 colocalizes with twitchin, consistent with their interaction in vivo.

We next investigated whether loss of function of twitchin would affect the localization of MAK-1. Using two independent unc-22 alleles, e66 and e105, we observed no obvious difference in the localization of MAK-1 as compared with wild type (Figure 4C).

mak-1 mutants have slightly reduced motility but normal muscle structure

The locomotion of adult C. elegans can be assessed by measuring the number of times a worm moves back and forth for a given time in a droplet of liquid (Epstein and Thomson, 1974), often referred to as a "swimming assay." Figure 5A presents swimming assay results for wild type, the two mak-1 mutants, and three unc-22 mutants. Although mak-1(ok2987) worms show almost the same motility as wild-type worms, mak-1(ok2987) worms show slightly reduced motility that is statistically significant. The fact that ok2987 shows slightly reduced motility but tm3455 shows normal motility is expected for MAK-1 (∼60 kDa) from a wild-type extract on Western blot. For the anti–N-terminal antibody, MAK-1 cannot be detected from the two mutant alleles. On longer exposure, however, two novel bands are detected from tm3455, likely representing truncated MAK-1 polypeptides. On longer exposure, no protein is detected from ok2987, other than bands found from all three strains likely arising from cross-reactivity to bacterial products. For the anti–C-terminal antibody, MAK-1 proteins cannot be detected even after long exposure time.
are much slower than wild type. Of interest, unc-22(e105) shows slightly increased locomotion (Figure 5A).

Given that mak-1(ok2987) has a slightly reduced motility, we expected that there might be some defect in the organization of its sarcomeres. To examine this possibility, we conducted immunofluorescence localization of various sarcomere proteins in wild type and mak-1(ok2987). In addition to twitchin, we assessed the localization of an ECM component, UNC-52(perlecan), the M-line and dense body proteins UNC-112 (kindlin) and UNC-95, and the dense body protein ATN-1 (α-actinin), the M-line protein UNC-89 (obscurin), and MHC A (a myosin heavy chain isoform that lies in the middle of thick filaments). Antibodies to all these proteins showed normal localization in mak-1(ok2987) mutant muscle (Figure 5B). Thus we can attribute the slightly reduced motility of mak-1(ok2987) to a possible defect in regulation of muscle contraction rather than a defect in sarcomere organization.

Sarcomeres are disorganized in unc-22(e66) but normal in unc-22(e105)

There are many available mutant alleles for unc-22. Although some alleles, such as the null ct37 or the amber allele s32, are very slow moving, other alleles, such as e105, show near-normal motility (Moerman et al., 1988). Despite this variation in locomotion defects, all alleles “twitch”—that is, shallow bends occur in multiple places along the surface of the animal one to several times per second, whether the animal is stationary or moving in its usual snake-like locomotion. Given that our swimming assays showed reduced motility for e66 and increased motility for e105, we wondered how sarcomere organization would be affected. Figure 6 presents immunostaining results using antibodies to a battery of sarcomeric proteins. In unc-22(e66) muscle, all of these proteins display abnormal localization. In contrast, in unc-22(e105) muscle, all of these proteins show normal localization. Therefore a mild allele of unc-22, e105, and a null allele of mak-1, ok2987, each displays normal sarcomere organization. Our interpretation is that at least one function of UNC-22 (twitchin), and the main function of MAK-1, is in regulating muscle contraction. However, it is also possible that MAK-1 does have a role in sarcomere organization, but our immunostaining did not detect subtle defects in mak-1(ok2987).

mak-1 mutants are partially resistant to nicotine

Nicotine is an acetylcholine agonist, and when wild-type animals are exposed to a solution of nicotine, they become paralyzed (i.e., they consistent with the Western blot results (Figure 3C) indicating that ok2987 is a likely null allele. For comparison, the unc-22 null allele, ct37 (Moerman et al., 1988), and the unc-22 canonical allele, e66,
antibodies. UNC-52 (perlecan), UNC-112, and UNC-95 all localize to wild type or structure. Images show part of a body wall muscle cell from either locomotion in this assay. (B) type. In contrast, ct37 reduced motility that is statistically significant. The motility as wild-type animals, nematodes of the indicated genotypes. Data are means and SEs with slightly reduced locomotion. Results of swimming assays on adult FIGURE 5: Phenotype of mak-1 mutants. (A) A mak-1 mutant has (ok2987) shows normal or even increased mak-1(ok2987) shows normal sarcomeric mak-1(ok2987) shows normal dense bodies. UNC-89 (obscurin) is located at M-lines full depth, mutant has the dense bodies. UNC-89 (obscurin) is located at M-lines full depth, MHC A localizes to the middle of A-bands. For all these proteins, localization is no different in mak-1; mnk-1 complete resistance, just like unc-22(RNAi). That is, unc-22 is epistatic to mak-1.

The mak-1 paralogues mk-1 and mak-2 are also expressed in body wall muscle
We wondered whether loss of function of either or both of the mak-1 paralogues mk-1 or mak-2 might also show a phenotype similar to mak-1, that is, partial resistance to nicotine. Before examining this question, we wanted to know whether mk-1 and mak-2 are even expressed in adult body wall muscle. Thus we created transgenic animals that express GFP from genomic segments upstream of the putative start sites for mk-1 or mak-2. WormBase indicates two predicted isoforms of mk-1, mk-1a and mk-1b, which differ in their 5′-most exons, suggesting the existence of two different promoters. We examined the expression patterns of transgenic worms expressing GFP driven by sequences upstream of each of these alternative 5′-most exons. As shown in Figure 8A, mk-1a and mk-1b are expressed in the same tissues/organs: the pharynx, the intestine, the vulval muscles, and body wall muscle. The promoter for mak-2 is expressed in the pharynx, the intestine, and body wall muscle (Figure 8B).

mk-1, mak-2, pmk-1, and pmk-3 are all partially resistant to nicotine
Using the same WMicrotracker assays, we found that mk-1 and mak-2 are partially resistant to nicotine (Figure 9, A and B), and their resistance is in the order mak-1 > mak-2 > mk-1. We also examined the nicotine response of the double mutants mak-1; mak-2 and mak-1; mk-1. The mak-1; mak-2 mutant showed more resistance than mak-1 alone (Figure 9, C and D) but less resistance than unc-22 (e105) (Figure 9, E and F). These results suggest that mak-1 and mak-2 contribute to nicotine resistance in the same pathway, but other unidentified players are probably also involved. As noted, mammalian MAPKAP kinase 2 is phosphorylated, and activated, by p38 MAP kinase. C. elegans has three p38 MAP kinases, encoded M-lines and dense bodies; UNC-52 in the extracellular matrix; and UNC-112 and UNC-95 in the muscle cytoplasm close to the cell membrane. ATN-1 (α-actinin) localizes to the main, deeper portion of the dense bodies. UNC-89 (obscurin) is located at M-lines full depth, from muscle cell membrane, up through the deepest part of the myofilament lattice. Twitchin localizes to the outer portions of A-bands, and MHC A localizes to the middle of A-bands. For all these proteins, localization is no different in mak-1(ok2987) muscle than in wild-type muscle. Scale bar, 10 μm.
by separate genes that are organized into a single operon in the order pmk-3, pmk-2, and pmk-1 (Berman et al., 2001). SAGE data suggest that pmk-3, -2, and -1, like mak-1, are also expressed in body wall muscle (Meissner et al., 2009). Although an intragenic deletion of pmk-2 is lethal at the L1 stage, this phenotype is not related to the disruption of the pmk-2 gene (Berman et al., 2001). This lethality prevented our analysis using the WMicrotracker. However, as shown in Figure 9, G and H, loss of function of either pmk-1 or pmk-3 results in partial nicotine resistance, each fairly similar to mak-1. This result is at least consistent with pmk-1 and pmk-3 being part of the same genetic pathway as mak-1.

**MAK-1 can phosphorylate twitchin kinase in vitro**

Given that our results indicate that MAK-1 and twitchin interact both physically and genetically and have overlapping mutant phenotypes, we explored the enzyme/substrate relationship of these two protein kinases. First, we used as source of kinase the minimum portion of MAK-1, residues 81–405, which interacts with twitchin and is predicted to contain an intact protein kinase domain. As substrate, we used twitchin NL-Kin-CRD, in which the highly conserved lysine (lysine 185 in von Castelmur et al., 2012; lysine 6290 in UNC-22b [WormBase]) was converted to alanine. This lysine is known in other kinases to coordinate ATP, and when mutated to alanine or several other amino acids, it will inactivate the kinase.

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We discovered that the twitchin kinase domain interacts with another protein kinase, the nematode orthologue of MAPKAP kinase 2, MAK-1. For each protein, the minimal necessary sequence includes the kinase catalytic core plus some flanking sequence; for MAK-1, it is the catalytic core plus 62 residues N-terminal (Figure 1C); for twitchin, it is NL plus catalytic core plus 45 of 60 residues of the CRD (Figure 1D). Both yeast two-hybrid assays using twitchin/TTN-1 chimeras (Figure 1E) and in vitro binding assays using purified recombinant deletion derivatives of twitchin NL-Kin-CRD (Figure 2) indicate that the CRD is crucial for this interaction. Of interest, a sequence in the second protein kinase domain of UNC-89 (PK2) in the analogous position of the CRD of twitchin is also required for interaction with SCPL-1, a CTD-type protein phosphatase (Qadota et al., 2008).

We further demonstrate that the mak-1 promoter is expressed in body wall muscle (Figure 3A) and that antibodies to MAK-1 detect a protein of appropriate size from nematode extracts (Figure 3C) that localizes in the sarcomere around and between dense bodies with some overlap with the A-band localization of twitchin (Figure 4, A and B). We characterized the phenotype of two loss-of-function mak-1 mutant alleles (Figure 3B). mak-1(ok2987) shows a slight decrease in worm locomotion (Figure 5A) and no effect on sarcomere organization (Figure 5B). We also found that unc-22(e105), although showing the expected “twitching phenotype,” shows no defects in sarcomere organization (Figure 6). Both unc-22 and mak-1 mutants have an abnormal response to nicotine; unc-22 mutants, regardless of the strength of the allele, showed complete resistance to nicotine, whereas mak-1 mutants showed partial resistance to nicotine (Figure 7). An unc-22; mak-1 double mutant showed complete...
wild-type animals stop moving, whereas mak-1 after 60 min. (B) Response of wild-type, nicotine, wild-type animals nearly stop moving by 30 min. In contrast, (A) Response of wild-type and three over time during exposure to a solution of 0.1 or 0.05% nicotine. A device called a WMicrotracker (DesignPlus) was used to monitor the locomotion of multiple worms per well in a microtiter dish to nicotine. A device called a WMicrotracker (DesignPlus) was used to monitor the locomotion of multiple worms per well in a microtiter dish to nicotine. FIGURE 7: Response of wild-type, unc-22, and mak-1 mutant strains to nicotine. A device called a WMicrotracker (DesignPlus) was used to monitor the locomotion of multiple worms per well in a microtiter dish over time during exposure to a solution of 0.1 or 0.05% nicotine. (A) Response of wild-type and three unc-22-mutant alleles. In 0.1% nicotine, wild-type animals nearly stop moving by 30 min. In contrast, unc-22-mutant animals, regardless of allele, continue moving even after 60 min. (B) Response of wild-type, unc-22(e105), and two mak-1–mutant alleles. In 0.1% nicotine, after 60 min of exposure, wild-type animals stop moving, whereas unc-22(e105) animals continue to move. mak-1(ok2987), the putative null allele of mak-1, but not mak-1(tm3445), shows an intermediate response; that is, ok2987 is partially resistant to nicotine. (C) At a slightly lower concentration of nicotine (0.05%), even mak-1(tm3445) shows partial resistance to nicotine. (D) In 0.1% nicotine, wild-type animals fed bacteria with an empty RNAi plasmid stop moving, but wild-type animals fed bacteria with an unc-22(RNAi) plasmid continue to move after 60 min exposure. Whereas mak-1(ok2987) animals fed bacteria with an empty RNAi plasmid show an intermediate response, mak-1(ok2987) animals fed bacteria with an unc-22 RNAi plasmid continue to move. Therefore unc-22(e105) is epistatic to or genetically downstream of mak-1. Two mak-1 paralogues, mak-2 and mnk-1, also are expressed in body wall muscle (Figure 8), and each showed partial but less resistance to nicotine than mak-1 (Figure 9, A–F). Loss of function of each of two of the three p38 MAP kinases in C. elegans showed partial resistance to nicotine (Figure 9, G and H). These data suggest that pmk-1, pmk-3, mak-2, and mnk-1 are part of the same pathway as mak-1 and twitchin (Figure 11).

unc-22(e105) has a fascinating phenotype. Although, like all known unc-22–mutant alleles, it twitches and is resistant to nicotine (Figure 7), it shows normal (Figure 6) rather than abnormal muscle structure and increased rather than decreased motility in swimming assays (Figure 5A). unc-22(e105) is a missense mutation in the seventh Ig domain of twitchin, changing a highly conserved glycine to an arginine, and results in normally localized twitchin by immunostaining (unpublished data). Studies in Aplysia and Mytilus suggest that the normal function of twitchin is to inhibit the rate of relaxation (Probst et al., 1994; Siegman et al., 1998; Funabara et al., 2007). If this same physiological function for twitchin pertains to C. elegans muscle, then in unc-22(e105), reduced inhibition of relaxation (i.e., faster relaxation time) might result in an overall faster contraction/relaxation cycle and thus faster worm locomotion.

Our results suggest that MAK-1 is an activator of twitchin kinase. This is suggested by the finding that the CRD of twitchin kinase is crucial for this interaction and because the CRD is one of two flanking sequences (the other being NL) that independently inhibit twitchin kinase activity in vitro (von Castelmur et al., 2012). Although molecular dynamics simulations of the twitchin Fn-NL-kinase-CRD-Ig segment show that pulling force removes the NL inhibitor, the CRD inhibitor remains attached to the kinase catalytic core. We suggest that pulling force removes the NL and activates the kinase to half-maximal activity and that full activation occurs when MAK-1 interacts with or possibly phosphorylates the CRD (Figure 11). Alternatively, partial activation by mechanical force and partial activation by MAK-1 occur independently according to different physiological demands. One interpretation of our genetic epistasis experiment is that twitchin kinase is downstream of MAK-1, and one possibility is that MAK-1 phosphorylates twitchin kinase in or near the CRD. Consistent with this hypothesis, in in vitro protein kinase assays, we can show that MAK-1 phosphorylates twitchin NL-Kin-CRD (Figure 10A), but the reciprocal experiment failed to show that twitchin kinase can phosphorylate MAK-1 (unpublished data). To roughly map the phosphorylation site(s), we tested Kin-CRD, Kin, and NL-Kin, but we could only unambiguously demonstrate that the intact NL-Kin-CRD was phosphorylated (the twitchin fragments ran too closely to the position of MAK-1 on the gel). Further experiments are needed to address the questions of phosphorylation sites, whether phosphorylation by MAK-1 activates twitchin kinase catalytic activity, and whether this phosphorylation and/or activation occurs in vivo.
Although our data suggest that MAK-1 phosphorylates twitchin kinase, there could be additional substrates. Quite different substrates have been putatively identified for mammalian MAPKAP kinase 2. In vitro, MAPKAP kinase 2 phosphorylates mouse heat shock protein 25 (hsp25) and human heat shock protein 27 (hsp27); fractionation of rabbit skeletal muscle proteins reveals that hsp25 kinase activity copurifies with MAPKAP kinase 2 activity (Stokoe et al., 1992b). Of interest, in C. elegans, one of the small heat shock proteins, hsp25, localizes to sarcomeric dense bodies and M-lines (near the localization of MAK-1) and has been shown to exist in a complex with the dense body components vinculin and α-actinin (Ding and Candido, 2000), so there is a possibility that these proteins could also be substrates for MAK-1 in vivo.

We postulate that MAK-1 is part of a cascade that involves p38 and twitchin kinase to regulate muscle contractile activity. Because mammalian p38 and MAPKAP kinase 2 are activated by and respond to intracellular stress, perhaps the model we propose, at least in C. elegans, provides a mechanism by which muscle activity is adjusted in response to stress. MAPKAP kinase 2 is involved in stress-activated signaling in heart muscle: MAPKAP kinase 2 is highly expressed in the human heart; in cultured rat cardiac muscle cells, there is increased MAPKAP kinase 2 activity and phosphorylation of hsp25 in response to experimental stressors—heat shock, hydrogen peroxide, or phorbol ester exposure (Zu et al., 1997). A “stress” that could be encountered in vivo is ischemia/hypoxia: MAPKAP kinase 2 activity is increased during ischemia in “preconditioned” rabbit hearts (Nakano et al., 2000). A MAPKAP kinase 2–mediated stress response may not be beneficial: Shiroto et al. (2005) report that ischemia/reperfusion of the mouse heart leads to activation of both p38 and MAPKAP kinase 2; a mouse that carries a knockout of MAPKAP kinase 2 is resistant to injury after ischemia/reperfusion, including a decreased number of apoptotic cardiomyocytes.

We also postulate that our putative p38/MAK-1/twitchin kinase cascade (Figure 11) results in inhibition of muscle relaxation. Support for this idea comes from recent studies of mouse skeletal muscle: Scharf et al. (2013) report that a MAPKAP kinase 2 and MAPKAP kinase 3 double-knockout mouse shows enhanced expression of the slow oxidative skeletal muscle gene program; use of reporter gene and electrophoretic mobility shift assays demonstrates that MAPKAP kinase 2 catalytic activity directly regulates the promoters of fast fiber myosin heavy chain and slow fiber SERCA2; moreover, there is accelerated relaxation and enhanced contractility of cardiomyocytes and improved force parameters of skeletal muscle.

The abnormal response of mak-1 mutants to nicotine may be mechanistically related to a finding about MAPKAP kinase 2 in mammalian smooth muscle. Carbachol is a muscarinic acetylcholine agonist. When canine airway smooth muscle is exposed to carbachol, increased phosphorylation of recombinant hsp27 results (Larsen, 1997).

### FIGURE 8: Adult expression patterns of mnk-1 and mak-2 promoters.

(A) On WormBase there are two predicted mnk-1 splicing patterns, mnk-1a and mnk-1b. To detect mnk-1a–expressing cells, transgenic worms were created that express GFP from a 3.7-kb segment of genomic DNA upstream of the mnk-1a predicted translational start. GFP was detected in the pharynx, intestine, vulva muscle, and body wall muscle at the adult stage. To detect mnk-1b–expressing cells, transgenic worms were made that expressed GFP from a 4.8-kb segment of DNA upstream of the mnk-1b translational start. GFP was detected in the pharynx, including pharyngeal muscle, intestine, vulva muscle, and body wall muscle. (B) To detect mak-2–expressing cells, transgenic worms were made that expressed GFP from a 3.9-kb genomic segment upstream of the mak-2 predicted translational start. Expression was detected in pharynx, intestine, and body wall muscle. Scale bar, 25 μm.
FIGURE 9: Response of mnk-1, mak-2, pmk-1, and pmk-3 mutants to nicotine. The WMicrotracker was used monitor
the locomotion of the indicated strains to the indicated concentrations of nicotine. (A, B) Response of wild type,
mnk-1(ok2987), mk-1(tm4266), and mnk-2(gk1110) to 0.05 and 0.1% nicotine, respectively. Note that mnk-1 and mak-2
are partially resistant to nicotine, although to a lesser extent than mak-1. (C,D) Response of wild type and mak-1, mak-1;
mak-2 and mak-1; mnk-1 double mutants to 0.05 and 0.1% nicotine, respectively. Note that the mak-1; mak-2 double
mutant shows enhanced resistance compared with either mak-1 alone or the mak-1; mnk-1 double mutant.
(E, F) Response of wild type, mak-1, the mak-1; mak-2 double, and unc-22(e105) to 0.05 and 0.1% nicotine, respectively.
Although the mak-1; mak-2 double is more resistant than mak-1, it is less resistant than unc-22(e105). (G, H) Response
of wild type, mak-1, pmk-1(km25), and pmk-3(ok169) to 0.05 and 0.1% nicotine, respectively. pmk-1 and pmk-3 are also
partially resistant but consistently less resistant than mak-1.
impaired p38/MAPKAP kinase 2 signaling leads to apoptosis of intestinal epithelial cells and subsequent loss of hindgut epithelial integrity (Seisenbacher et al., 2011).

MATERIALS AND METHODS
Screening of yeast two-hybrid library
A twitchin bait plasmid containing Ig25-Fn32-Kinase-CRD (45 aa) (Qadota et al., 2008) was used for screening a C. elegans yeast two-hybrid cDNA library (RB2, a gift from Robert Barstead, Oklahoma Medical Research Foundation, Oklahoma City, OK). The screening procedure was described previously (Miller et al., 2006). Yeast two-hybrid assays were conducted as described in Mackinnon et al. (2002).

Deletion derivatives of MAK-1 and twitchin
cDNA fragments from MAK-1 and twitchin were amplified by PCR (primers: YHM1–YHM5, YHM13–YHM15, and TWI-F; see Supplemental Table S2) and cloned into pBluescript and then confirmed by DNA sequencing. Error-free fragments were transferred into yeast two-hybrid vectors (MAK-1 fragments into pGAD, prey; twitchin fragments into pGBDU, bait).

Cloning of MAK-2 and MNK-1
cDNA fragments of MAK-2 and MNK-1 were amplified by PCR (primers: YHM6–YHM12) from the RB2 library. Amplified fragments were first cloned into pBluescript. After confirmation of DNA sequences, error-free fragments were cloned into pGAD, the yeast two-hybrid prey vector.

Construction of twitchin/TTN-1 chimeras
Chimeric fragments were created by PCR (primers: YHM15–YHM21) using chimeric primers and twitchin and TTN-1 cDNAs (Qadota et al., 2008) according to a protocol described previously (Qadota et al., 1994). Error-free fragments were placed into pGAD, the yeast two-hybrid prey vector.

In vitro binding assays with purified recombinant proteins
The expression of His-tagged twitchin kinase fragments containing the kinase catalytic core were described previously (von Castelmur et al., 2012). For expression of MBP-MAK-1 (81–405), the corresponding MAK-1 cDNA fragment (PCR amplified with primers YHM4 and YHMS) was cloned into pMAL-KK-1 (a gift from Kozo Kaibuchi, Nagoya University, Nagoya, Japan). His-tagged twitchin kinase fragments and MBP-MAK-1 (81–405) were purified as described previously (Mercer et al., 2006). A far-Western assay was conducted as follows: 1 μg of each

Finally, we found that the promoters for mak-1 and its paralogues mnk-1 and mak-2 are expressed not just in muscle; they are also expressed in the intestine. Similarly, the p38 kinases are expressed in body wall muscle and the intestine, based on SAGE analysis (Meissner et al., 2009), and in the intestine based on promoter analysis (Berman et al., 2001). The function of intestinal expression is unknown, but may involve stress response: in Drosophila, a complex of p38 and MAPKAP kinase 2 is required to prevent stress-dependent damage to intestinal epithelial cells;
His-tagged twitchin fragment (His-Kin, His-NL-Kin, His-Kin-CRD, His-NL-Kin-CRD) was resolved by SDS–PAGE and transferred to nitrocellulose membranes. The blots were incubated with 1 µg/ml MBP or MBP-MAK-1 (81–405), washed, and then reacted with anti-MBP conjugated to horseradish peroxidase (anti-MBP–horseradish peroxidase [HRP]; New England BioLabs, Ipswich, MA) at 1:5000 dilution and visualized by enhanced chemiluminescence (ECL; Pierce, Thermo Fisher Scientific, Waltham, MA) using horseradish peroxidase [HRP] (New England BioLabs, Ipswich, MA) as a substrate.

Transgenic animals for determining tissue expression of mak-1, mak-2, and mnk-1

The following plasmids were constructed: mak-1p::gfp has a 6.4-kb genomic fragment that includes the putative promoter sequence upstream of mak-1, a putative 5′-untranslated region, initiator ATG, and the first 12 nucleotides of the coding sequence of mak-1, amplified by PCR with primers YHM22 and YHM23 and cloned into the pPD95.75 vector (kindly provided by Andrew Fire, Stanford University, Stanford, CA) using SphI and BamHI sites. mak-2p::gfp has a 3.0-kb genomic fragment that includes the putative promoter sequence upstream of mak-1, 5′-untranslated region, initiator ATG, and the first 24 nucleotides of the coding region of mak-2, amplified by PCR with primers YHM24 and YHM25 and cloned into pPD95.75 vector using SphI and BamHI sites. mnk-1p (a)::gfp has a 3.7-kb genomic fragment that includes the putative promoter sequence upstream of mak-1a, 5′-untranslated region, initiator ATG, and the first 12 nucleotides of the coding region of mnk-1a, amplified by PCR with primers YHM26 and YHM27 and cloned into pPD95.75 vector using SphI and SalI sites. mnk-1p (b)::gfp has a 4.8-kb genomic fragment that includes putative promoter sequence upstream of mnk-1b, 5′-untranslated region, initiator ATG, and the first 12 nucleotides of the coding region of mnk-1b, amplified by PCR with primers YHM28–YHM29 and cloned into pPD95.75 vector using SphI and BamHI sites. The constructed plasmids were injected at 20 ng/µl into the gonads of wild-type animals along with the pRF4 [rol-6(su1006)] plasmid as coinjection marker (80 ng/µl; Mello and Fire, 1995). At least three independent stable transgenic lines were generated for each plasmid.

Nematode strains

N2 (Bristol) is the primary wild-type strain, and standard growth conditions were used (Brenner, 1974). The following alleles were used in this study: lev-1(e211) IV, mak-1(ok2987) II, mak-1(tm3455) II, mak-2(gk1110) IV, mnk-1(tm4266) II, pmk-1(km25) I, pmk-3(ok169) I, unc-22(e66) IV, unc-22(e105) IV, unc-22(ct37) IV, unc-29(e1072) I, and unc-63(x37) I. The double-mutant animals mak-1(ok2987); mak-2(gk1110) and mak-1(ok2987); mnk-1(tm4266) were generated by crossing the respective single-mutant animals. To check for deletions of mutant animals, we carried out PCR with primers YHM31–YHM47: mak-1(ok2987) and mak-1(tm3455) were each outcrossed three times to wild type.

Mutation site in mak-1(tm3455)

Genomic DNA of the mak-1 locus was amplified from mak-1(tm3455) mutant worms using nested PCR with designed primers (YHM48–YHM51). The deletion junction was determined by DNA sequencing.
**Generation of antibodies to MAK-1 and immunoblot analysis**

Residues 81–142 and 416–468 of MAK-1 were expressed and purified in *Escherichia coli* as glutathione S-transferase (GST) and MBP fusion proteins, respectively. To do this, each cDNA fragment was amplified by PCR (primers for aa 81–142: YHMS and YHMS2; primers for aa 416–468: YHMS3 and YHMS4) from mak-1 cDNA clone (MAK-1 aa 81–521 fragment). cDNA fragments were cloned into pGEX-KK1 and pMAL-KK1 using BamHI and XhoI sites. After finding an error-free clone, we expressed GST-fusion proteins as described in Mercer et al. (2006). The resulting GST-MAK-1(81-142) and GST-MAK-1(416-468) were shipped to Spring Valley Laboratories (Woodbine, MD) for generation of rabbit polyclonal antibodies. Anti–MAK-1 (81–142) and anti–MAK-1 (416–468) antibodies were affinity purified using Affigel conjugated to MBP-MAK-1 (81–142) or MBP-MAK-1 (416–468), as described previously (Mercer et al., 2003).

**Immunofluorescence localization**

Adult nematodes were fixed using the method described previously (Nonet et al., 1993; Wilson et al., 2012). Primary antibodies were used at the following dilutions: anti–ATN-1 (α-actinin; MH35; Francis and Waterston, 1991) at 1:200, anti–MAK-1 (416-468) at 1:100, anti–myosin heavy chain A (MHC A; 5–6; Miller et al., 1983) at 1:200, anti–myosin heavy chain B (MHC B; 5–8; Miller et al., 1983) at 1:200, anti–PAT-6 (Warner et al., 2013) at 1:200, anti–twitchin (I I II; Benian et al., 1996) at 1:200, anti–UNC-52 (MH2; Rogalski et al., 1993) at 1:200, anti–UNC-89 (MH42; Benian et al., 1996) at 1:200, anti–UNC-95 at 1:200 (Gadota et al., 2007), and anti–UNC-112 (Hikita et al., 2005) at 1:200. For anti–MAK-1, anti–twitchin, anti–UNC-95, and anti–UNC-112, the secondary antibody was anti-rabbit Alexa 488 (Invitrogen). For anti–ATN-1, anti–MHC A, anti–MHC B, anti–UNC-52, and anti–UNC-89, the secondary antibody was anti-mouse Alexa 594 (Invitrogen, Grand Island, NY). For anti–PAT-6, the secondary antibody was anti-rat Alexa 594 (Invitrogen). Each secondary antibody was used at 1:200 dilution. Stained samples were mounted on a glass slide with a coverslip containing mounting solution (20 mM Tris, pH 8.0, 0.2 M Dabcyo, and 90% glycerol). Images were captured at room temperature with a Zeiss confocal system (LSM510) equipped with an Axiovert 100M microscope and an Apochromat 63×/1.4 numerical aperture objective in 2.5× zoom mode. The color balances of the images were adjusted by using Photoshop (Adobe, San Jose, CA).

**Swimming assays**

L4 animals of each genotype were picked to fresh OP50 plates and allowed to mature at 20°C for 24 h. The swimming assays were carried out on individual young adult animals in M9 buffer. For locomotion (swimming), body bends of individual young adult animals were counted for 1 min. A body bend was scored as a complete deflection of the anterior portion of the nematode from the midline. Approximately 20 animals of each genotype were analyzed.

**Nicotine sensitivity assays using a WMicrotracker device**

Synchronized young adult animals of each genotype were collected in M9 buffer. After a final wash with M9 buffer containing 0.01% Triton X-100 (M9T), a worm slurry (wet pellet:M9T = 1.5) was prepared. For the assays, 96-well plates were used. To each well was added 40 μl of M9T followed by 10 μl of slurry, delivering ~50–100 worms/well. The plate was placed in the dark at room temperature for 1 h. Then locomotive activities under no-nicotine conditions were measured for 1 h using a WMicrotracker (Phylumtech, Santé Fe, Argentina) with the program C. elegans (>L4) supplied by the manufacturer. Supplemental Figure S2 shows the locomotive activities in 0% nicotine of all the worm strains used in these experiments. Supplemental Figure S2B also shows the behavior of several acetylcholine receptor mutants (unc-29, unc-63, and lev-1) in 0.05% nicotine as “positive controls,” since these are known to be resistant to nicotine (Gottschalk et al., 2005). After the measurement, 50 μl of M9T containing 0.2 or 0.1% nicotine solution was added to each well, and locomotive activities were measured using the WMicrotracker for each strain and nicotine concentration, eight independent wells were assayed. On the graphs of locomotive activity versus time, each point represents the mean and SE. RNA interference (RNAi) of unc-22 was performed by using an Ahringer library clone and the protocol described in Kamath et al. (2003).

**Protein kinase assays with purified recombinant proteins**

cDNA fragments containing the twitchin kinase domain (Fn31-Kinase-ig26) harboring the K185A mutation, expected to abolish kinase activity, were amplified by PCR using primers twcF-Kinlg-F, twcF-Kinlg-R, YHMS5, and YHMS6 (see Supplemental Table S2). Derivatives of twitchin kinase fragments containing the K185A mutation were amplified by PCR using twitchin kinase with K-to-A cDNA as a template and the following primer combinations: TwiK-1 and TwiK-2 for NL-Kin-CRD, TwiK-3 and TwiK-2 for Kin-CRD, TwiK-1 and TwiK-4 for NL-Kin, and TwiK-3 and TwiK-4 for Kin only. BsmBl-Xhol fragments of these twitchin kinase derivatives were cloned into the Ncol and Xhol sites of pETM11. To construct the His-tagged MAK-1 (aa 81–405) expression plasmid, cDNA was amplified by PCR using YHMS5 and YHMS4. The Ncol-Xhol fragment of this PCR product was cloned into Ncol/Xhol-cut pETM11. All of these plasmids were transformed into Rosetta 2 (DE3)–competent E. coli (EMD Millipore, Billerica, MA), and protein expression was induced with addition of 680 μM isopropyl-β-D-thiogalactoside and grown for 5 h at 20°C for twitchin fragments and 15°C for MAK-1. Bacteria from a 500-ml culture were suspended in 30 ml of lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 40 mM imidazole, 10 mM β-mercaptoethanol, 10% glycerol, pH 8.0, plus cComplete Mini EDTA-free protease inhibitor cocktail [Roche]), and broken in a French pressure cell at 10,000 psi; after addition of 80 μl of 20% Triton X-100 and 20 μl of Benzonase nuclease (purity >90%; EMD Millipore) the lysate was centrifuged at 39,000 × g for 45 min. To the cleared lysate was added 2 ml of a 50% suspension of Ni-agarose beads (Qiagen, Valencia, CA), which was mixed at 4°C for 30 min. The beads were pelleted and washed 5× in wash buffer (same as lysis buffer, but having 60 mM imidazole) and applied to a small column, and the protein was eluted with elution buffer (same as lysis buffer, but having 200 mM imidazole). Peak fractions were pooled and dialyzed against 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, and 2 mM β-mercaptoethanol. Protein concentrations were determined by Bradford assays. Kinase assays were performed in the following manner. We first preincubated 1 μg of each twitchin kinase fragment (K to A) with an equal molar amount of MAK-1 (81–405) in a volume of 15 μl containing 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, and 0.05% Tween-20 at 25°C for 1 h with shaking. To this mixture was added 15 μl of 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, and 0.05% Tween-20 at 25°C for 1 h with shaking. After mixing, the solution was incubated at 30°C for 1 h, followed by addition of 7.5 μl of 5x Laemmli sample buffer, heated at 95°C for 5 min, separated on a 10% polyacrylamide SDS gel, stained with Coomassie, destained, dried, and exposed to film overnight. SDS–PAGE showing the proteins used in this assay is shown in Figure 10C. For kinase assays in the reverse direction (twitchin kinase as enzyme, MAK-1 as substrate), the source of twitchin kinase was His-tagged twitchin kinase.
domain, only which has been reported to be active (von Castelmur et al., 2012). As a substrate, full-length MAK-1 with the K-to-A mutation, also expected to abolish kinase activity, was amplified by PCR using YHM1, YHM2, YHM58, and YHM59 and cloned into pMAL-KK-1. MBP-MAK-1 (K to A) was purified as described previously (Mercer et al., 2006). The kinase assay was performed similarly to that described.

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