The Germinal Center Kinase GCK-1 Is a Negative Regulator of MAP Kinase Activation and Apoptosis in the C. elegans Germline

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Abstract

The germinal center kinases (GCK) constitute a large, highly conserved family of proteins that has been implicated in a wide variety of cellular processes including cell growth and proliferation, polarity, migration, and stress responses. Although diverse, these functions have been attributed to an evolutionarily conserved role for GCKs in the activation of ERK, JNK, and p38 MAP kinase pathways. In addition, multiple GCKs from different species promote apoptotic cell death. In contrast to these paradigms, we found that a C. elegans GCK, GCK-1, functions to inhibit MAP kinase activation and apoptosis in the C. elegans germline. In the absence of GCK-1, a specific MAP kinase isoform is ectopically activated and oocytes undergo abnormal development. Moreover, GCK-1- deficient animals display a significant increase in germ cell death. Our results suggest that individual germinal center kinases act in mechanistically distinct ways and that these functions are likely to depend on organ- and developmental-specific contexts.

Introduction

The Ste20-related germinal center kinases comprise a large protein family that has been implicated in cellular processes ranging from cytoskeletal dynamics and stress responses, to cell growth, proliferation, and death [1–4]. The founding member, S. cerevisiae Ste20, activates MAP kinase signaling in response to mating pheromone as a MAP kinase kinase kinase kinase (MAP4K) upstream of the MAP3K Ste11p [5,6]. Dozens of proteins with kinase domains highly similar to Ste20 were subsequently found in invertebrates and vertebrates [1]. Based on the location of this conserved kinase domain and the presence or absence of a p21-activated kinase (PAK) domain, these proteins have been divided into two large families, PAKs and germinal center kinases (GCKs) [7]. Based on slightly different kinase signature sequences and divergence outside the conserved kinase domain, the GCKs have been further grouped into eight distinct subfamilies (GCK I – GCK VIII) that (with the exception of subfamily VII) consist of one C. elegans, one Drosophila, and multiple vertebrate/mammalian proteins [7].

Like the founding kinase Ste20, members of the GCK I, II, III, IV, V, VII, and VIII subfamilies have been reported to activate MAP kinase signaling, including JNK, p38, and ERK pathways [7–13]. Likewise, members of multiple subfamilies are potent inducers of apoptosis [2,7,16,17]. However, there are exceptions to these observations. Although two human members of the GCK VII family, TAO1 and TAO2 activate p38 [14,15], a third human GCK VII kinase, JIK, appears to inhibit JNK signaling [16]. Interestingly, while the human GCK V kinase SLK induces apoptosis when overexpressed [17], expression of the highly related Drosophila SLIK kinase inhibits cell death [19]. Though some of these conflicting results could be due to species-specific differences, caveats remain since the majority of the existing data has been gleaned from over-expression studies in tissue culture coupled with in vitro kinase assays [2,7]. Clearly, addressing the physiological role of these kinases in the context of a whole organism will be essential for parsing the functions of each family member.

In addition to intracellular events, MAP kinase signaling pathways regulate a remarkable number of developmental processes [20,21]. For instance, a conserved ERK signaling cascade is essential for oocyte maturation in both vertebrates and invertebrates [22,23]. In C. elegans, ERK is activated at two distinct times during oocyte development: first, as germ cells progress through pachytene, and second, in maturing diakinetic oocytes residing proximal to the spermatheca [24,25]. Mutation or depletion via RNA mediated interference (RNAi) of the C. elegans ERK ortholog MPK-1 and other components of the ERK cascade results in sterility characterized by the failure of germ cells to progress through pachytene [24,26]. The affected nuclei clump...
GCK-1 Inhibits MAP Kinase

together and eventually disintegrate [26]. MAP kinase activation in pachytene is also required for a developmentally programmed germ cell death switch that reduces the number of maturing oocytes by one half [27].

It has been recognized for a number of years that C. elegans oocyte maturation is regulated by sperm [20]. Recently, elegant biochemical analyses yielded the surprising result that the Major Sperm Protein (MSP), a highly abundant, sperm-specific cytoskeletal protein, is released from intact sperm and is required for MAP kinase activation, oocyte maturation, and ovulation [25,29]. In the absence of MSP, Ephrin binding of VAB-1, an Eph receptor protein-tyrosine kinase, and a parallel pathway regulated by CEH-18, a POU-class homeoprotein, inhibit MAP kinase signaling [30]. Together, these inhibitory pathways ensure that MAP kinase activation and oocyte maturation are tightly linked to the presence of sperm. The upstream signal required for MAP kinase activation during pachytene is not known.

Here we report that MAP kinase activation in pachytene is inhibited by GCK-1, the sole C. elegans member of the GCK III subfamily. Loss of GCK-1 results in the hyper-activation of a specific MAP ERK kinase isoform, abnormal oocyte development, and increased germ cell death. Altogether, this study has uncovered a novel role for a germinal center kinase in germ cell development and reveals that GCK kinases can have opposing roles in the regulation of MAP kinase activation and apoptosis.

Results

T19A5.2 encodes a germinal center kinase required for oogenesis

The C. elegans genome encodes 8 recognizable members of the germinal center kinase family [7,31]. One these, T19A5.2/GCK-1, can be grouped with the GCK III subfamily (Figure S1) [7]. To determine the functional role of GCK-1 during C. elegans development, dsRNA corresponding to the full-length gck-1a isoform was microinjected or fed to wild-type (wt) young adult or L4 larval stage hermaphrodites. gck-1(RNAi) by either method resulted in progressive sterility. The animals were completely sterile within 24 hours of RNAi induction. gck-1(RNAi) hermaphrodites produced broods of <70 progeny as compared to >250 progeny from untreated wt age-matched controls. The F1 progeny of gck-1(RNAi) animals were completely sterile, and displayed a phenotype that was indistinguishable from their sterile mothers (below and data not shown). Consistent with these results, hermaphrodites homozygous for a gck-1 deletion allele that lacks a significant portion of the kinase domain (gck-1(km15)) (Figure S1) had a similar sterile phenotype. Since animals depleted of GCK-1 activity by any of the above means display a similar phenotype (described in detail below), they will be collectively referred to as gck-1(ːf), except as noted.

The C. elegans hermaphrodite gonad consists of two U-shaped arms that share a common uterus (Figure S2). Beginning in the fourth larval stage (L4), developing germ cells within the gonad undergo spermatogenesis, while at the L4 to adult molt, a genetic switch occurs and the germ cells develop as oocytes [32]. The region of the gonad most distal to the uterus contains proliferating germ nuclei which then transition into meiosis with crescent shapes characteristic of the leptotene and zygote stages of meiotic prophase I [33]. As the nuclei pass through the gonad, they enter into pachytene, where pairing of thread-like chromosomes becomes evident. Pachytene nuclei are arranged in an orderly fashion on the gonad surface, forming a tube surrounding a common amnueate cytoplasm, the rachis [34]. The rachis terminates at the exit from pachytene, usually near the bend in the gonad arm, although small connections between the oocytes and the syncytial cytoplasm remain [35]. Upon pachytene exit, approximately 50% of the developing oocytes undergo apoptosis [27]. The surviving nuclei become cellularized and organized into a single row as they grow and progress through diplotene into diakinesis. These oocytes remain in diapause with six highly condensed bivalent chromosomes until a maturation signal is received from sperm [25,28].

To determine the cause of sterility in gck-1(ːf) animals, gonads dissected from wt and gck-1(RNAi) hermaphrodites were examined by differential interference contrast (DIC) microscopy (Figure 1A,B). Each wt gonad displayed a highly ordered progression of germ cells through meiotic prophase with a clearly visible rachis and a single row of growing oocytes in the proximal gonad (Figure 1A, arrows). In contrast, gonads from gck-1(ːf) animals had little to no discernable rachis, and the proximal ends were filled with dozens of small germ cells (Figure 1B).

To characterize gck-1(ːf) germline defects, gonads dissected from wt, gck-1(RNAi), gck-1(km15) heterozygotes, and gck-1(km15) homozygous animals were fixed, stained with DAPI, and examined by deconvolution microscopy (Figure 1C–F). This analysis revealed that gck-1(km15)/nT1, gck-1(RNAi), and gck-1(km15) gonads had progressively fewer germ cells than wt (Figure S3). Differences were most pronounced in pachytene, where gck-1(km15)/nT1 heterozygotes, gck-1(RNAi), and gck-1(km15) gonads had 35, 60, and 81% fewer pachytene nuclei respectively than wt (Supplemental Table S1). Although there was not a statistically significant difference in the number of mitotic metaphase figures in all four genotypes/conditions (Figure S3), the number of nuclei at all other stages of oogenesis was decreased in gck-1(RNAi) and gck-1(km15) gonads (Figure S3 and Table S1). gck-1(ːf) gonads also contained a large number of nuclei whose stage in meiotic prophase could not be readily determined by morphology or position (Figures 1E,F and S3; Table S1).

Although the pachytene region of gck-1(km15)/nT1 heterozygotes contained fewer germ cells, all other aspects of oogenesis were grossly normal and these animals were fertile (Figure 1D). However, in gck-1(RNAi) and gck-1(km15) animals, striking morphological differences from wt became apparent during pachytene (Figure 1E,F). Unlike wt, gck-1(ːf) nuclei with paired, thread-like chromosomes were not arranged in an orderly pattern on the gonad periphery, and there was no clear transition from pachytene into diplotene (usually just prior to the bend in wt gonad arms) (Figure 1C–F). Moreover, unlike the single row of growing oocytes found in wt and gck-1(km15)/nT1 heterozygotes (Figure 1A,C,D, arrows), the proximal gonads of gck-1(ːf) hermaphrodites contained a large number of small cells/oocytes (Figure 1B,E,WF). While some of these cells had diakinet-like chromosomes (Figure 1E,F, arrows), the exact stage of many of these cells could not be determined, and there was no clear progression from diplotene into diakinesis, as diakinet-like cells were scattered throughout the proximal half of gck-1(ːf) gonads (Figure 1E,F).

To determine whether GCK-1 acts in the soma or germline to affect germ cell development, wt and rrf-1(pk1417) hermaphrodites were fed control and gck-1 dsRNAs. RRF-1 encodes an RNA directed RNA polymerase that is required for RNAi in somatic cells but not the germline [36]. Hence RNAi inhibition does not affect somatically expressed genes in rrf-1(pk1417) mutant animals, but is still potent in the germline. DAPI staining revealed that the gck-1(RNAi) phenotype does not differ between wt and rrf-1(pk1417) gonads (Figure 2), indicating that GCK-1 functions in the germline.
To ascertain if the abnormal diakinetic-like oocytes in \textit{gck-1(lf)} gonads display other characteristics of diakinetic oocytes, wt and \textit{gck-1(RNAi)} gonads were immunostained for two markers that delineate diakinetic oocytes from earlier stages of meiotic prophase. Staining of wt hermaphrodite gonads with antibodies that recognize a phosphorylated residue on the C-terminal domain (CTD) of RNA polymerase II (H14), and histone H3 phosphorylated at serine 10 (pH 3) results in a specific pattern during \textit{C. elegans} oogenesis \cite{37,38}. H14 stains germ cell chromosomes from the distal mitotic region through diplotene \cite{37}, and recognizes a cytoplasmic antigen in diakinetic oocytes. Although this cytoplasmic antigen is unlikely to be RNA polymerase, H14 staining clearly delineates diakinesis from earlier stages of meiotic prophase (Figure 3C, arrowheads). pH 3 immunostains mitotic nuclei in the distal gonad and the diakinetic chromosomes of maturing oocytes residing close to the spermatheca (\cite{38} and Figure 3E, small arrowheads). The appearance of pH 3 on diakinetic chromosomes is sperm dependent \cite{39–41}. In late diakinetic oocytes, H14 and pH 3 immunostaining patterns are complementary (Figure 3G,I). H14 staining is always cytoplasmic in oocytes with chromosomal pH 3 (Figure 3G,I).

As in wt animals, H14 immunostaining in \textit{gck-1(lf)} gonads was associated with all germ cell nuclei from the distal mitotic region...
GCK-1 functions in the germline. Gonads dissected from wt and rrf-1(pk1417) hermaphrodites fed control or gck-1 dsRNA were stained with DAPI. Deconvolved, flattened image stacks are shown. Scale bar, 20 μm.

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Meiotic prophase progression is disrupted in gck-1(0) hermaphrodites. Control(RNAi) (A,C,E,G,I) and gck-1(RNAi) (B,D,F,H,J) dissected gonads were stained with DAPI (blue: A,B,G,H,I,J) and H14 (green: C,D,G,H,I,J) and pH 3 antibodies (red: E,F,G,H,I,J). Merged images are shown in (G,H,I,J). (I,J) Enlarged images of boxed regions in (G,H). Spermatheca is to the right in all images. *, distal tip (A,B); }pach, pachytene (A,B); l, line, H14 nuclear staining (C,D); large arrowheads, cytoplasmic H14 staining (C,D,H); small arrowheads, pH 3 positive cells (E,F,I,J); arrows, diakinetic and diakinetic-like nuclei (A,B); S, spermatheca; Scale bars, 20 μm.

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through pachytene. However, in the proximal gonad, some of the cells displayed chromosomal H14 staining while in others, it was clearly cytoplasmic (Figure 3D, H, J, arrowheads). Importantly, germ cells with cytoplasmic H14 staining nearly always had chromosomes with diakinetic-like morphology, suggesting that H14 immunostaining remains a useful marker for delineating different germ cell stages in the disorganized gk-1(+/+) germine.

As in wt, the pH 3 antibody stained the chromosomes of gk-1(+/+) mitotic germ cell nuclei (Figure 3E, F). However, in the gk-1(+/+) proximal gonad, pH 3 immunostaining was not limited to oocytes residing next to the spermatheca, but was associated with the diakinetic-like chromosomes of cells scattered throughout the proximal half of gk-1(+/+) gonads (Figure 3F, arrowheads). These scattered nuclei had the chromosome morphology and patterns of H14 and pH 3 immunostaining that are consistent with the diakinetic stage of oogenesis (Figure 3H, J).

GCK-1 inhibits the activation of a specific MAP kinase isoform

A canonical MAP kinase pathway regulates proliferation, development, and apoptosis in the C. elegans germine [25,27, 42,43]. MAP kinase is activated by an unknown signal as germ cells transit through pachytene and again in the proximal gonad by MSP signaling in maturing oocytes [25]. Since GCK-1 affects germ cell development, the pattern and extent of MAP kinase activation was assessed in gk-1(+/+) gonads.

Immunostaining of wt and gk-1(+/km15)/nt1 gonads with an antibody specific for activated MAP kinase (MAPK-YT) resulted in the expected pattern of MAP kinase activation during pachytene (Figure 4A, white line) and increasing amounts in oocytes as they progressed closer to the spermatheca, consistent with activation by a diffusible signal from sperm (Figure 4A, arrows) [25,29]. In gk-1(+/km15) animals there was a statistically significant increase in activated MAP kinase in pachytene compared to wt (Figures 4B (white line) and 4C). In addition to pachytene, MAPK-YT immunostaining was also present in a few random germ cells in the gk-1(+/km15) proximal gonad (Figure 4B, arrowheads).

Two isoforms of C. elegans MAP kinase/ERK are encoded by the mpk-1 locus (MPK-1a, 43 KD, and MPK-1b, 50 KD) and differ only by the presence of an additional 5’ exon in the larger isoform [44]. Immunostaining with the MAPK-YT antibody does not distinguish between activated MPK-1a and MPK-1b since the phosphorylated epitope is found in both isoforms. However, since the two isoforms encode proteins of different sizes, they are easily discernable by western analysis with MAPK-YT antibody (Figure 4D) [44].

Western analysis of protein extracts from wt and gk-1(+/km15) young adult hermaphrodites (4A+24 hours) revealed a significant decrease in MPK-1b activation concomitant with a striking increase in MPK-1a activation in gk-1(+/km15) animals (Figure 4D, lanes 1, 2). The increased levels of MPK-1a activation in the absence of MPK-1b activation, and the increased intensity of MAPK-YT immunostaining during pachytene in gk-1(+/km15) gonads suggest that loss of GCK-1 leads to germline activation of MPK-1a specifically at pachytene. However, it was previously proposed that the MPK-1b isoform is specific for germ cell development since it is only activated in animals with a functional germine, and there were no discernible germine dependent changes in the activation of the MPK-1a isoform [44] and Figure 4D, lanes 1, 7, 8. The simplest interpretation of these results is that the MPK-1a and MPK-1b isoforms are activated at distinct stages of oocyte development, with MPK-1a activation occurring in mid-pachytene by an unknown signal, while MPK-1b is activated by MSP in oocytes most proximal to the spermatheca.

To test this model, we asked whether MAPK-YT staining of maturing oocytes in the proximal gonad is due to specific activation of the MPK-1b isoform by MSP. The F-box protein FOG-2 is required for hermaphrodite spermatogenesis [45]; hence, a fog-2(q71) mutant strain produces fertile males and females, but no hermaphrodites [32]. Consistent with our model, western analysis revealed that MPK-1b was specifically activated in mated fog-2(q71) females but not unmated fog-2(q71) females (Figure 4D, lanes 3, 5). MPK-1a activation was not affected by the presence or absence of sperm (Figure 4D, lanes 3, 5).

We predicted that increased MPK-1a activation in pachytene in gk-1(+/+) gonads should be independent of sperm and MSP signaling. To test this, MAP kinase activation was examined in unmated fog-2(q71) females fed gk-1 or control dsRNA. DAPI staining confirmed that the gk-1(RNAi)/fog-2(q71) gonads retained the gk-1(+/+) phenotype, with multiple rows of small oocytes in the proximal gonad (Figure 4E, F). As expected MAPK-YT staining was low in the proximal gonads of both control and gk-1(RNAi) females, whereas there was a clear increase in pachytene MAP kinase inactivation in gk-1(RNAi)/fog-2(q71) animals (Figure 4F). Western blotting revealed a corresponding specific increase in MPK-1a activation (Figure 4D, lane 6). A similar activation pattern was seen in mated gk-1(RNAi)/fog-2(q71) animals (Figure 4D, lane 4, and data not shown). Altogether, these results are consistent with the hypothesis that the MPK-1a and MPK-1b isoforms are differentially activated at distinct times during oocyte development. We posit that MPK-1a activation occurs during pachytene and is inhibited by the GCK-1 kinase, while MPK-1b activation occurs in proximal oocytes and is dependent on MSP signaling.

Oocyte development in gck-1(lf) gonads requires the MAP kinase pathway

A second facet of our model predicts that the gk-1(+/+) phenotype should be dependent on MAP kinase, mpk-1 mutations and mpk-1 (RNAi) lead to sterility characterized by defects in pachytene progression, where the “stuck” germ cell nuclei clump together and degenerate, leaving the proximal gonad devoid of nuclei [24,26]. Hence, MPK-1 was depleted from wt and gk-1(+/km15)/nt1 heterozygous animals by feeding mpk-1 dsRNA and their progeny were examined. All of the wt, gk-1(+/km15)/nt1, and gk-1(+/km15) hermaphrodite progeny of mpk-1(RNAi) treated mothers exhibited a strong pachytene arrest phenotype as evidenced by clumping of pachytene nuclei and proximal gonads devoid of germ cells (Figure 5 and data not shown). Similar results were found in gk-1(+/km15) animals treated with, Ksr/ksr-2, Mek/mek-2 or Raf/lin-45(RNAi) (Figure 5). These results indicate that the gk-1(+/+) phenotype is dependent on the MAP kinase signaling pathway. Hence, GCK-1 is likely to act upstream of Raf or in a parallel pathway.

GCK-1 directly interacts with MPK-1 in vitro

The GCK-1 N-terminal harbors a predicted ERK docking site [46]. To determine whether MPK-1 binds directly to GCK-1 via this site, various GCK-1 protein fragments were expressed as maltose-binding protein (MBP) fusion proteins, purified from bacteria, and mixed with GST- or GST-MPK-1a-coated glutathione- beads (Figure 6A, B). Of the six GCK-1 fragments tested, only the N-terminal fragment bound specifically to MPK-1a (Figure 6B). To determine whether the ERK docking site was necessary for this interaction, two separate docking site mutations were introduced into the GCK-1 N-terminal fragment (R55G and I64N). Both mutations resulted in a significant loss of GCK-1 binding (Figure 6C). These data indicate that GCK-1 and MPK-1 can directly interact through an ERK docking site in the GCK-1 N-terminal, suggesting that GCK-1-mediated inhibition of MPK-1a
activation may occur via direct binding/sequestration of the MPK-1a isoform. GCK-1 loss results in increased germ cell death. Under normal growth conditions, approximately 50% of developing C. elegans oocytes undergo physiological cell death [27]. This apoptosis requires progression through pachytene and MAP kinase activation [27]. Since GCK-1 is a negative regulator of MAP kinase signaling in the germline, we examined the frequency of germ cell apoptosis in wt and \( gck-1\) (\( km15 \)) animals by staining live animals with Syto12, a dye that is specific for apoptotic cells [27]. At 24 hours past the L4 stage (L4 + 24 hours), \( gck-1(km15) \) gonads had a statistically significant increase in apoptotic germ cells over wt controls (Figure 7). This germ cell death was dependent on the apoptotic machinery, as no dying cells were detected in \( cdg-3 \) or \( cdg-4 \) mutant animals (Figure 7) [27].

We reasoned that the increase in cell death in \( gck-1\) (\( km15 \)) gonads could be due to an upregulation of the physiological germ cell death pathway or to introduction of cell damage that triggered a checkpoint-dependent apoptotic response. The C. elegans checkpoint genes \( him-7 \) and \( rad-5/clk-2 \) mediate apoptosis in response to DNA damage. Mutations in these genes decrease DNA damage-induced but not physiological germ cell death [47]. The frequency of germ cell death in \( him-7(e1480) \) and \( clk-2(mu259) \) hermaphrodites depleted of GCK-1 was significantly higher than control treated animals (Figure 7), indicating that the increase in germ cell death in \( gck-1\) (\( km15 \)) animals is not mediated by the DNA checkpoint pathway. Altogether, these results suggest that GCK-1 is an inhibitor of physiological apoptosis in the C. elegans germline.
Discussion

Here we demonstrate that the *C. elegans* germinal center kinase GCK-1 is required to inhibit abnormal oocyte development and germ cell apoptosis. Furthermore, loss of GCK-1 activity results in ectopic activation of a specific MPK-1 isoform that has not previously been shown to have a significant role in the germline. An ERK docking site dependent in vitro interaction between GCK-1 and MPK-1 suggests that GCK-1 may physically sequester MPK-1 from activation. These results are in striking agreement with a recent study demonstrating that GCK-1 is required to prevent germ cell apoptosis in a regulatory role for the MAP kinase pathway in the germline of *Drosophila* ([21]).

Figure 5. The *gck-1(lf)* phenotype requires the MAP kinase pathway. (A–D) DAPI stained gonads from (A,C) wt and (B,D) *gck-1(km15)* animals fed control (A,B) or *mpk-1* dsRNA (C,D). TZ, transition zone; bracket, pachytene; arrows, diakinetic oocytes; arrowheads, diakinetic-like nuclei; S, spermatheca. Scale Bar, 20 μm.
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Figure 6. GCK-1 binds to MPK-1 in vitro. A) Schematic of the MBP-GCK-1 fragments used in the GST-MPK-1 binding assay (B). The putative ERK docking site in the GCK-1 N-terminus is shown. B) MBP-GCK-1 protein fragments (A) were incubated with GST or GST-MPK-1 coated glutathione beads. Top panel: α-MBP western analysis of washed beads. Lower panel: α-MBP and α-GST western analysis of protein loading in each binding reaction (0.5% of load). Molecular weights are indicated. C) The wt MBP-GCK-1A protein fragment and two different ERK docking motif mutant GCK-1A proteins were incubated with GST and GST-MPK-1 coated beads as in (B). Top panel: α-MBP western analysis of washed beads. Lower panel: α-MBP and α-GST western analysis of protein loading in each binding reaction (1.0% of load). Molecular weights are indicated.
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contrast to the reported functions of other germinal center kinase family members, the majority of which have been shown to induce MAP kinase activation and apoptosis [2,7], raising the question as to whether different germinal center kinases have disparate functions in different organisms or developmental contexts. This view is supported by the recent discovery that the Drosophila GCK-III kinase family member is a negative regulator of EGFR/ERK activation in fly neurons [48]. Loss of GCK-III/Happyhour leads to increased ERK activation and reduced sensitivity to alcohol sedation. Although the mechanism of Happyhour mediated inhibition of ERK activation is unknown, these findings are consistent with our data and reveal a conserved role for GCK-III family members in the inhibition of ERK signaling pathways.

MAP kinase activation is an essential component of oogenesis in many organisms [49–53]. Although strongly correlated with oocyte maturation in C. elegans, the requirement for MAP kinase activation in this process has been difficult to assess due to the essential role of MPK-1 in pachytene progression [24–26]. Moreover, the minor difference between MPK-1a and MPK-1b has made it difficult to study the specific function and localization of each isoform. Previous studies revealed that MPK-1b activation is specific to the germ line. However, there was no discernable difference in MPK-1a activation in the presence or absence of germ cells, suggesting that MPK-1a functions may be restricted to the soma [44]. Whilst there is little doubt that MPK-1b plays a key role in the germline, we conclude that MPK-1a is also critical for germ cell development. While other models are formally possible, the simplest interpretation of our data is that the MPK-1a and MPK-1b isoforms are activated at distinct stages of oocyte development. MPK-1a is activated in mid-pachytene by an unknown signal, while MPK-1b is activated by MSP in maturing oocytes residing close to the spermatheca. We hypothesize that inappropriately high MPK-1a activation at mid-pachytene in the absence of GCK-1 leads to abnormal oocyte development and increased apoptosis.

Significantly fewer germ cells populate gck-1(lf) gonads as compared to wild type C. elegans. Interestingly, LET-60/Ras gf alleles or loss of a MAP kinase inhibitor, LIP-1, also result in fewer germ cells [43,54]. Hence, the decreased germ cell population in gck-1(lf) gonads might also be attributed to inappropriately high MAP kinase activity. Conversely, GCK-1 could be affecting cell division and/or cell survival via a MAP kinase independent pathway. Indeed, other germinal center kinases, including Drosophila Slik and Hippo, play critical roles in cell cycle progression and growth control through novel signaling pathways [19,55]. Interestingly, both the Slik and Hippo kinases are regulated in part by Raf, although independently of Raf’s role as an activator of the MAP kinase pathway [16,19,56]. However, unlike GCK-1, both of these kinases act to limit cell proliferation, and loss-of-function mutants display gross tissue overgrowth [19,56].

Deciphering the exact mechanism through which GCK-1 affects germ cell development and apoptosis will require the identification of GCK-1 binding partners and the physiological substrates of this interesting kinase. This study contributes to a growing body of evidence suggesting that the in vivo functions of the Germinal Center kinases are likely to vary in different developmental and cellular contexts. Hence the analysis of each member of this large kinase family in a variety of model organisms will be necessary to elucidate these functions and their contribution to human development and disease.

Materials and Methods

**cDNAs**

The C. elegans genome database predicts four alternatively spliced gck-1 isoforms (http://wormbase.org Release WS203, June 29, 2009). The gck-1a isoform was PCR amplified from a C. elegans cDNA library and DNA sequencing confirmed that it was
identical to the predicted gck-1a cDNA (Genbank: AAC69038.1). All of the relevant experiments described here were performed with this cDNA.

Nematode strains and culture methods

Standard techniques were used to culture and genetically manipulate all strains [57]. All mutant strains are derivatives of the wt strain C. elegans var. Bristol. The following strains were maintained at 20°C: N2 (wild-type), NL2090 (mt-1(pk1477)), MT1522 (ed-3(n471)/IV), MT2547 (ed-4(n162)/I), CB1400 (him-7(t1409)), SP506 (rad-5/ed-2::bn-15/I), CB4001 (sg-1(q71)/V), JS947 (gck-1(bm15)/ms100U) and JS10: gfy-21::GFP expressing Chr. V balancer that results in GFP positive muscle cells [58], JS945 (gck-1(bm15)/nT1[gl51]/IV), nT1[gl53] was used as a dominant green fluorescent balancer chromosome, allowing gck-1a-containing animals to be scored beginning at the four-cell stage of embryogenesis [59,60]. SS104 (gpf-4(bm20)/IV) is germine-less at 25°C and was therefore maintained at 15°C. The gck-1(bm15) deletion mutant was generated by a TnP/UV method and isolated using a sieb-selection protocol [61]. The sequences flanking the breakpoint are: GAA-TACTTTTGTG and TATTACACTTTT, corresponding to a 5′-CGATATCGAGAATGCACCAGCATCAG-3′ and 5′-GGCTAAGGCTTTCACCCGGGTTGTCAGTC-3′, respectively. For MAPK-YT immunostaining, dissections were performed as previously described [64], with the exception that dissected gonads were dissected at 4°C methanol for 5 minutes as previously described [43]. After washing, specimens were fixed with the appropriate primary antibody overnight at room temperature (RT). The following day, the gonads were washed and incubated with secondary antibodies for one hour at RT. Animals were then mounted in Vectashield mounting media containing 4′,6-Diamidino-2-phenylindole (DAPI; Vector Laboratories) on a microscope slide and gently covered with a coverslip.

For MAPK-YT immunostaining, sections were performed as above, while fixation and MAPK-YT staining was performed as previously described [64], with the exception that dissected gonads were directly fixed in 4% paraformaldehyde and 0.1 M potassium phosphate overnight at 4°C. The following day, gonads were washed, incubated with primary antibody for 4 hours at RT, washed again, and incubated with secondary antibody overnight at 4°C as previously described [64]. Gonads were then treated with RNase, stained with To-Pro-3 (Invitrogen), washed in water, and mounted on a microscope slide in Slow Fade Gold mounting media (Invitrogen). Confocal images were captured and MAPK-YT immunostaining intensity was quantified using Imaris X64 software (Bitplane Inc, St. Paul, MN) as follows: an isosurface of pachytene MAPK-YT immunostaining was created for each image (threshold = 29) and the mean intensity for each gonad was recorded and plotted.

Nuclei Counts

Animals were dissected at 14-24 hours, fixed in 3% paraformaldehyde and 0.1 M potassium phosphate for one hour and stained with DAPI. Image stacks were acquired and deconvolved as described above. Nuclei were counted using the manual count option in Metamorph. Nuclei counts for each region were compared to wt using Dunnett’s Statistical Test using a 99% family confidence interval [65].
In wt gonads, condensed nuclei in the distal region that had a metaphase chromosome arrangement were counted as mitotic metaphase nuclei. The mitotic region as a whole was defined as nuclei residing from the distal tip up to the transition zone [66]. The transition zone was defined as the region containing crescent-shaped nuclei [33,67]. Pachytene nuclei were counted based on chromosome morphology [68]. Nuclei spanning from the end of the pachytene region to the spermatheca were counted as diplotene and diakineti nuclei.

The gck-1 loss-of-function [lf] germline nuclei were counted using the same methods, except that nuclear morphology was the only criteria used for distinguishing different stages since the temporal and spatial order of developing germ cell nuclei was severely disrupted from pachytene to diakinesis in gck-1[lf] gonads. Some nuclei did not have distinct chromosome morphology and were therefore grouped as “other.”

**Western Analysis**

100 adult (L4+24 hours) animals were individually picked into M9 solution in a microfuge tube, allowed to purge for three to five hours, and washed three times in M9. Animals were then resuspended in SDS-PAGE loading buffer [69], boiled for five minutes, and centrifuged before loading the supernatant on an SDS/PAGE gel. One tube of 100 worms was used per lane. Separated proteins were transferred to an Immobilon-NC membrane (Millipore, Billerica, MA), and blocked in 1x western membrane (Millipore, Billerica, MA), and blocked in 1x western

**In vitro binding assays**

DNA sequences corresponding to the GCK-1 protein fragments in Figure 6A were PCR amplified from a full-length GCK-1 cDNA and subcloned into a pMAL-Maltose Binding Protein (MBP) E. coli expression vector (New England Biolabs (NEB), Beverly, MA). The MBP-GCK-1 fusion proteins were expressed and purified on amylose beads following manufacturer’s instructions (NEB). A Glutathione-S-Transferase (GST)-MPK-1a fusion protein was created by PCR amplification of the MPK-1a open-reading frame from a C. elegans cDNA library. The MPK-1a cDNA was subcloned into the pGEX-6P-1 E. coli expression vector (GE LifeSciences). GST and GST-MPK-1a proteins were expressed and purified from E. coli using glutathione beads (GE LifeSciences) following manufacturer’s instructions, except that the proteins were not eluted from the beads. The GST and GST-MPK-1a coated beads were incubated with approximately 2 µg eluted MBP-GCK-1 fusion protein in 100 µl binding buffer (200 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA and 0.5% Triton X-100, 100 µg/ml BSA) for more than 2 hours at 4°C. The beads were washed four times in binding buffer, resuspended and boiled in SDS/PAGE buffer, and the bound proteins separated on a 10% Tris-glycine polyacrylamide gel. A fraction of the protein in each binding reaction was also loaded on the gel. Separated proteins were transferred to nitrocellulose and subjected to western analysis with α-MBP (NEB) and α-GST [70] antibodies. To create the ERK docking site mutations, the MBP-GCK-1A construct was subjected to PCR-based site-directed mutagenesis (Stratagene/Agilent Technologies, Santa Clara, CA). Binding reactions with these proteins were carried out as described above.

**Cell Death Assays**

Cell death assays were performed by staining hermaphrodite animals (L4+24 hours) with Syto12 (Invitrogen) as previously described [27]. Data were analyzed using both the Kruskal-Wallis Test and Two-Way Analysis of Variance (ANOVA) at a 99% confidence level. Results from both statistical tests were consistent.

**Supporting Information**

| Table S1 Germ nuclei counts |
|-----------------------------|
| Found at: do1:10.1371/journal.pone.0007450.s001 (0.03 MB DOC) |

**Figure S1** An alignment of GCK-1 and other GCK-III subfamily protein sequences. The GCK-III subfamily was defined in (Dan et al., 2001). The aligned sequences are human MASK (GenBank: BAA92785.2); human MST3 (Swiss-Prot: Q9Y6E0.1); human SOK1/YSK1 (Swiss-Prot: O00506.1); Drosophila GCKIII (GenBank: AAF55388.1); and C. elegans GCK-1a (GenBank: AAC90381.1). The sequences were aligned using the ClustalW2 EMBL-EBI server: [http://www.ebi.ac.uk/Tools/clustalw2/index.html] and BOX SHADE 3.21 ([http://www.ch.embnet.org/software/BOX_form.html]). Identical amino acids are in solid boxes and similar residues are shaded. The kinase domain is underlined and the GCK-III subfamily signature sequence is overlined. The breakpoints in the gck-1(km15) allele are indicated (*).

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**Figure S2** Schematic of the C. elegans gonad. The C. elegans hermaphrodite gonad consists of two mirror image U-shaped arms. Each arm consists of a distal mitotic region (*) with proliferating germ cells. These nuclei transition into meiosis with a crescent shape characteristic of leptotene and zygotene. Pachytene nuclei are arranged on the surface of the gonad and surround a common anucleate cytoplasm, the rachis. Germ cells remain in the pachytene stage for an extended period before passing through diplotene (condensing chromosomes enclosed in a nuclear membrane) and arresting in diakinesis (six bivalent chromosomes in a nuclear membrane). In response to MSP, the oocyte migrates distally and nuclear envelope breakdown occurs. The mature oocyte is then ovulated through the spermatheca (S) where it is fertilized and passed into the uterus where the embryo (E) develops before being extruded into the environment.

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**Figure S3** Germ cell numbers are reduced in gck-1[lf] hermaphrodites. (A) The average total number of germline nuclei in a single gonad of the indicated genotype as grouped by nuclear stage (see Materials and methods). (B) The average number of mitotic metaphase nuclei per gonad arm. (n = 4 for each genotype; **p<0.001; error bars represent standard error of the means.)

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**Figure S4** The gck-1[lf] phenotype requires the MAP kinase pathway. (A–H) DAPI stained gonads from (A,C,E,G) wt and (B,D,F,H) gck-1[km15] animals fed control (A,B), ksr-2 (C,D), mek-2 (E,F), or lin-45 (G,H) dsRNA. Scale Bar, 20 µm.

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Concepted and designed the experiments: JS. Performed the experiments: KRS YK TF. Analyzed the data: KRS YK JS. Contributed reagents/materials/analysis tools: NH KM. Wrote the paper: KRS JS.
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