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Caenorhabditis elegans WNK–STE20 pathway regulates tube formation by modulating ClC channel activity

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WNK kinases are a small group of unique serine/threonine protein kinases that are conserved among multicellular organisms. Mutations in WNK1–4 cause pseudohypoaldosteronism type II—a form of hypertension. WNKs have been linked to the STE20 kinases and ion carriers, but the underlying molecular mechanisms by which WNKs regulate cellular processes in whole animals are unknown. The *Caenorhabditis elegans* WNK-like kinase WNK-1 interacts with and phosphorylates *germinal centre kinase* (GCK)-3—a STE20-like kinase—which is known to inactivate CLH-3, a CIC chloride channel. The *wnk-1* or *gck-3* deletion mutation causes an Exc phenotype, a defect in the tubular extension of excretory canals. Expression of the activated form of GCK-3 or the *clh-3* deletion mutation can partly suppress *wnk-1* or *gck-3* defects, respectively. These results indicate that WNK-1 controls the tubular formation of excretory canals by activating GCK-3, resulting in downregulation of CIC channel activity.

Keywords: *C. elegans*; ClC channel; tube formation; WNK

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INTRODUCTION

WNK kinases are serine/threonine kinases that are characterized by an atypical location of its catalytic lysine (Xu *et al*, 2000). There are four mammalian WNK family members. WNK1–4 have been identified as genes mutated in families of patients with pseudohypoaldosteronism type II (PHAII) human hypertension (Wilson *et al*, 2001). Mutations of the *WNK1* gene found in PHAII subjects consist of deletions in intron 1. These mutations are reported to cause increased expression of the WNK1 protein, indicating that hypertension could result from increased expression of WNK1. The *WNK1* knockout is an embryonic lethal mutation (Zambrowicz *et al*, 2003), indicating that *WNK1* is also required for a normal development. Subsequently, WNK4 was shown to be a regulatory kinase for ion channels, transporters and tight junction proteins, indicating that WNK4 functions as a multifunctional regulator of diverse ion transport pathways (Kahle *et al*, 2003; Yamauchi *et al*, 2004; Yang *et al*, 2007a, b). Although the mechanism by which WNK4 regulates these transport pathways is unknown, recent studies have shown that WNK1–4 lie upstream of the STE20 kinases, stress protein kinase (SPAK) and oxidation stress response kinase 1 (OSR1), which are known to regulate cation/Cl co-transport (Moriguchi *et al*, 2005; Vitari *et al*, 2005; Anselmo *et al*, 2006; Gagnon *et al*, 2006). To investigate the question of WNK function in the context of the whole organism, we have taken a genetic approach using *Caenorhabditis elegans* as a model system.

RESULTS AND DISCUSSION

C. elegans contains only one gene encoding the WNK-like protein, *wnk-1* (supplementary Fig 1A online). Recent studies have shown that mammalian WNK1–4 interact with and phosphorylate the STE20 kinases, SPAK and OSR1 (Moriguchi *et al*, 2005; Vitari *et al*, 2005). As *C. elegans germinal centre kinase* (GCK)-3 is a homologue of SPAK and OSR1 (Denton *et al*, 2005; supplementary Fig 1B online), we investigated whether the biochemical interaction between WNK-1 and GCK-3 is conserved in *C. elegans*. We transiently expressed FLAG-WNK-1 together with T7-GCK-3 in human embryonic kidney (HEK) 293 cells. Cell extracts were subjected to immunoprecipitation with the T7 antibody, followed by immunoblotting assay. We found that

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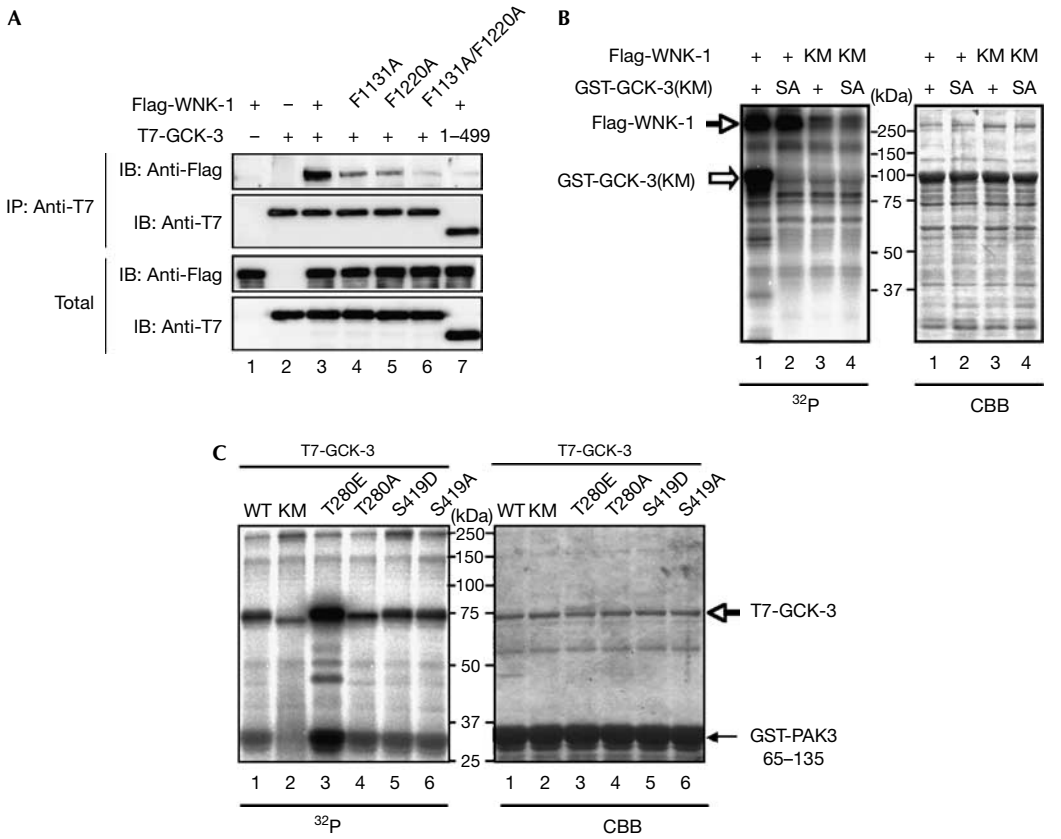

Fig 1 | Biochemical analysis of *Caenorhabditis elegans* WNK-1 and GCK-3 expressed in mammalian cells. (**A**) An association between WNK-1 and GCK-3 is shown. Human embryonic kidney (HEK) 293 cells were transfected with the indicated constructs. Cell lysates were immunoprecipitated (IP) with the T7 antibody. Association was detected by immunoblotting (IB) with the appropriate antibodies. (**B**) WNK-1 phosphorylates GCK-3 *in vitro*. HEK293 cells were transiently transfected with the FLAG-WNK-1 and FLAG-WNK-1(K344M). The proteins were immunoprecipitated with the FLAG antibody and their kinase activities assayed using bacterially expressed GST-GCK-3(K137M) or GST-GCK-3(K137M/S419A) as substrates. Right panel (CBB) shows the amounts of substrates used in this assay. (**C**) GCK-3 kinase activity. HEK293 cells were transfected with the indicated constructs. The proteins were immunoprecipitated with the T7 antibody and their kinase activities assayed toward GST-PAK3(65–135). CBB, Coomassie brilliant blue staining; GCK, *germinal centre kinase*; GST, glutathione *S*-transferase.

WNK-1 interacted with GCK-3 (Fig 1A, lane 3). Mammalian SPAK and OSR1 have conserved carboxy-terminal (CCT) domains, which recognize the RFX(V/I) motif present in WNK1–4 (Moriguchi *et al*, 2005; Vitari *et al*, 2005; Anselmo *et al*, 2006; Gagnon *et al*, 2006; Villa *et al*, 2007). Gsk-3 also contains a CCT domain (supplementary Fig 1B online) and the Gsk-3(1–499) form lacking the CCT domain failed to bind to WNK-1 (Fig 1A, lane 7). As WNK-1 contains two RFXV motifs (supplementary Fig 1A online), we investigated whether these sites are responsible for their association with GCK-3. The WNK-1(F1131A/F1220A) form, in which both RFXV motifs have been mutated, is unable to associate with GCK-3 (Fig 1A, lanes 4–6). Together, these results indicate that WNK-1 association with GCK-3 involves an interaction between the RFXV motif of WNK-1 and the CCT domain of GCK-3.

Next, we investigated GCK-3 phosphorylation by WNK-1. We produced a glutathione *S*-transferase (GST)-tagged kinase-negative form of GCK-3(K137M) in bacteria and tested its ability to be phosphorylated *in vitro*. We observed that WNK-1 phosphorylated GCK-3 in a kinase-dependent manner (Fig 1B, lanes 1,3). We carried out *in vitro* kinase assays using several deletion mutants of GCK-3 (supplementary Fig 2 online). These assays showed that the WNK-1 phosphorylation site is located in the 415–435 amino-acid region of GCK-3, within which only one Ser 419 is present (supplementary Fig 2 online). Mutation of this Ser 419 to an alanine residue abolished phosphorylation of GCK-3 by WNK-1 (Fig 1B, lane 2), which is consistent with this being the main phosphorylation site. To examine the role of Ser 419 phosphorylation in the regulation of GCK-3, we mutated Ser 419 to either alanine—to prevent phosphorylation—or aspartic acid—to mimic phosphorylation—and assayed GCK-3 kinase activity by using an amino-terminal fragment of another STE20 family member p21-activated kinase 3 (PAK3), GST-PAK3(65–135) (Okabe *et al*, 2003), as a substrate. Mutation of Ser 419 to alanine or aspartic acid had no effect on basal GCK-3 activity (Fig 1C, lanes 5,6). In mammals, WNK1–4 activate SPAK and OSR1 by phosphorylating their T-loop residues (Thr 233 of SPAK and Thr 185 of OSR1; Vitari *et al*, 2005). As this site is conserved in *C. elegans* GCK-3

(supplementary Fig 1B online), we introduced a mutation at Thr 280. Interestingly, mutation of Thr 280 to glutamic acid—to mimic phosphorylation—markedly enhanced GCK-3 activity (Fig 1C, lane 3), whereas mutation of Thr 280 to alanine slightly reduced the basal activity (lane 4). These results indicate that phosphorylation of the T-loop in GCK-3, rather than phosphorylation of Ser 419, mediates activation of GCK-3 by WNK-1. However, *in vitro* phosphorylation of recombinant GCK-3 proteins by WNK-1 or coexpression of GCK-3 with WNK-1 in mammalian cells resulted in only weak activation of GCK-3 (data not shown). Further studies will be needed to understand the mechanism of GCK-3 activation by WNK-1.

To examine the physiological role of WNK-1 in the intact organism, we isolated a presumptive null mutation in *wnk-1* (*tm487*; supplementary Fig 3A online). The *wnk-1(tm487)* homozygous mutant animals obtained from *wnk-1/+* heterozygotes did not grow beyond the early L2 larval stage (supplementary Fig 3B online). The *wnk-1(tm487)* mutation also caused an Exc (for excretory canal abnormal) phenotype. The excretory cell and its associated gland, duct and pore cells form the *C. elegans* renal system (Fig 2A; Buechner, 2002). To examine the morphology of the excretory cell, we used a *bgIs312* transgene, which expresses green fluorescent protein in the excretory cell (Berry *et al*, 2003). In wild-type L2 animals, the posterior canals terminated near the anus (Fig 2B). By contrast, in *wnk-1* L2 mutants, the posterior canals ended at or anterior to the gonad (Fig 2C). Introduction of the wild-type *wnk-1* gene driven from the heat-shock promoter (*hsp-16.2*) rescued the L2 arrest and Exc phenotypes associated with *wnk-1(tm487)*; this rescue was dependent on WNK-1 kinase activity (Fig 2D,E). Analysis using the deficiency *eDf19* indicated that *tm487* is a null allele (data not shown). Thus, WNK-1 is essential for larval development and tube formation of excretory canals.

We next constructed the deletion mutant *gck-3(tm1296)* (supplementary Fig 3C online). This deletion resulted in a truncated protein lacking the CCT domain. Although *gck-3(tm1296)* homozygous young adults obtained from *gck-3/+* heterozygotes could initially produce some fertilized eggs, they produced only unfertilized eggs 2 days after the final moult. This phenotype seemed to be associated with the loss of sperm cells from the spermatheca (supplementary Fig 3D online), indicating that *gck-3* is required for normal sperm function and fertility in hermaphrodites; in addition, they also showed an Exc phenotype. In young adult *gck-3* animals, the canals were short compared with those of wild-type animals (Fig 3A,B). Furthermore, the outer (basolateral) surface of the excretory canals grew out to most of their normal distance, whereas the luminal (apical) surface was shorter and bloated. This resulted in a wide canal with long lumen-free protrusions at the ends (Fig 3B). These long extensions indicated that canal outgrowth and guidance were largely normal, but that the lumen either did not keep pace with the canal growth, or shrank back from basal surface as the luminal surface collapsed. Although *gck-3(tm1296)* mutants did not show an early larval arrest phenotype, heterozygotes between *gck-3(tm1296)* and the *ozDf1*-deficient mutant were arrested at the early larval stage (data not shown), indicating that *tm1296* might not be a null allele. The Exc phenotype of *gck-3(tm1296)* mutants was rescued by transfection of the *gck-3* wild-type gene, but not by the kinase-negative *gck-3(K137M)*, driven from

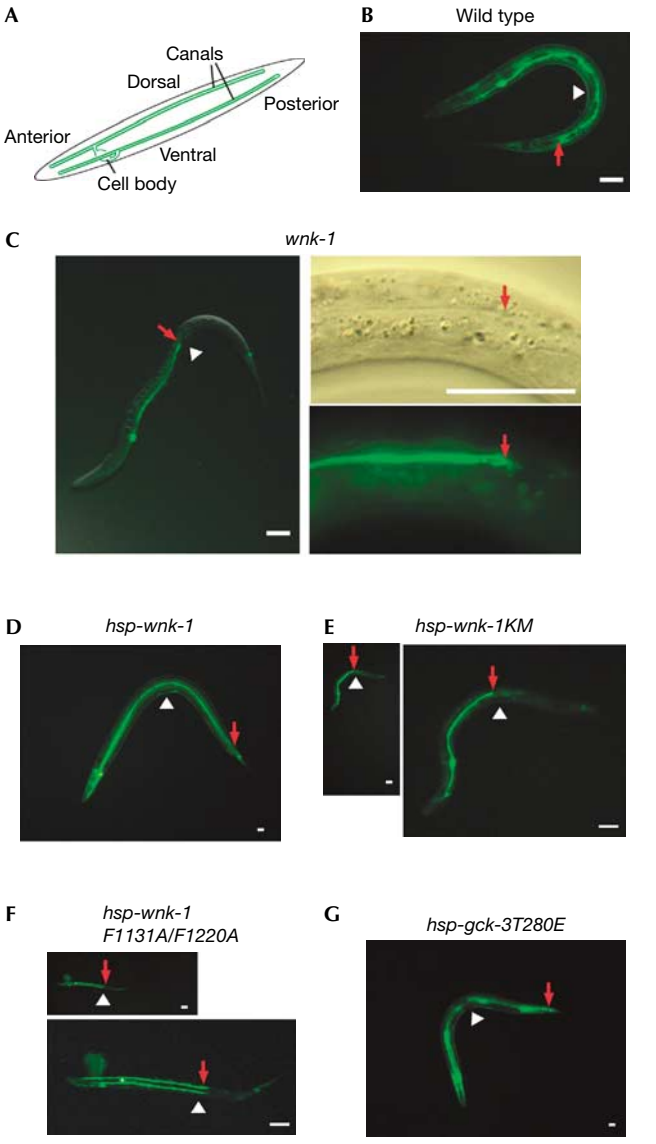

Fig 2 | Genetic analysis of *wnk-1* in *Caenorhabditis elegans*. (**A**) Diagrams showing excretory canals. The excretory cell extends tube-like canals dorsally on both sides during embryogenesis. On reaching the lateral epidermis, these canals bifurcate and grow anteriorly and posteriorly for nearly the length of the animal, creating an H-shaped canal system. (**B**,**C**) Excretory canal morphologies in (**B**) wild-type and (**C**) *wnk-1* worms at the L2 larval stage. The excretory canal is visualized using *pes-6p*::*gfp* expression. End positions of the posterior canals are indicated by red arrows; white arrowheads indicate positions of the gonads. Right panels in (**C**) show posterior canals at high magnification; anterior is to the left. Scale bars, 25 μm. (**D**–**G**) Rescue and suppression of *wnk-1* phenotypes. The *wnk-1* mutants carrying the indicated transgenes were examined for L2 arrest and Exc phenotypes. After hatching, animals were subjected to heat treatment at 37 °C for 30 min. This heat treatment was repeated three times at intervals of 24 h. Fluorescent micrographs of *wnk-1* mutants carrying the indicated transgenes are shown. Enlarged images are also shown in (**E**), right, and (**F**), bottom. Exc, excretory canal abnormal phenotype; *gck*, *germinal centre kinase*; gfp, green fluorescent protein; *hsp*, *heat-shock protein*.

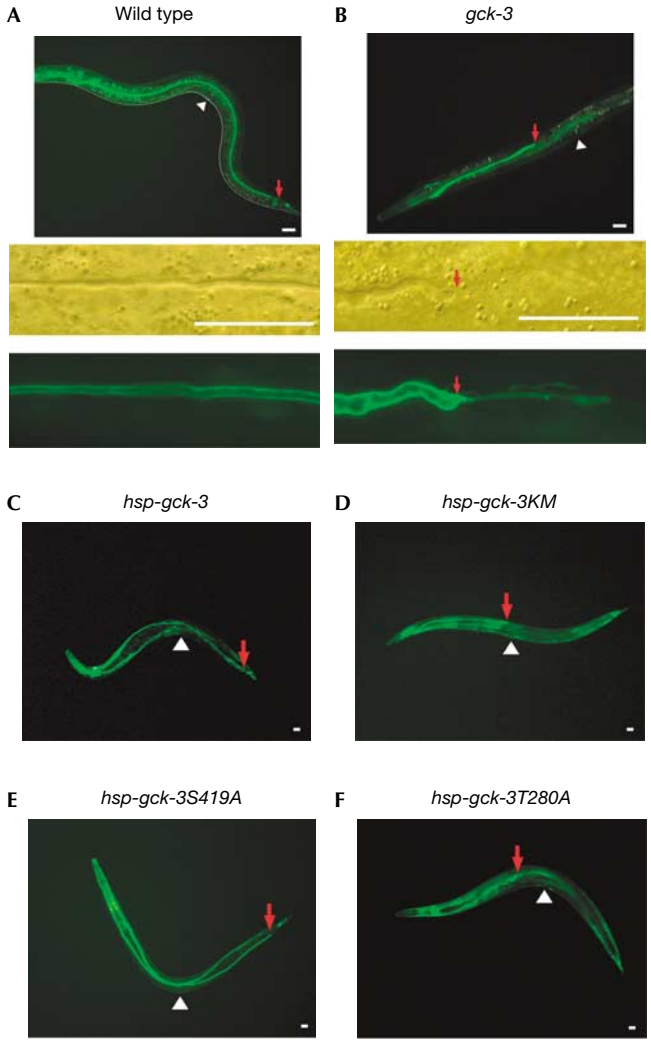

Fig 3 | Genetic analysis of *gck-3* in *Caenorhabditis elegans*. (**A**,**B**) Excretory canal morphologies in wild-type (**A**) and *gck-3* (**B**) worms at the young adult stage. The excretory canal is visualized using *pes-6p*::*gfp* expression. End positions of the posterior canals are shown by red arrows. White arrowheads show positions of the vulva. Middle and lower panels show posterior canals at high magnification. Scale bars, 25 μm. (**C**–**F**) Rescue of *gck-3* Exc phenotype. The *gck-3* mutants carrying the indicated transgenes were examined for Exc phenotype. After hatching, animals were subjected to heat treatment at 37 °C for 30 min. This heat treatment was repeated five times at intervals of 12 h. Fluorescent micrographs of *gck-3* mutants carrying the indicated transgenes are shown. Exc, excretory canal abnormal phenotype; *gck*, *germinal centre kinase*; gfp, green fluorescent proteins; *hsp*, *heat-shock protein*.

the *hsp-16.2* promoter (Fig 3C,D). Consistent with this, the *wnk-1* and *gck-3* genes are expressed in the excretory cell (Denton *et al*, 2005; Choe & Strange, 2007), indicating that both WNK-1 and GCK-3 regulate tubular formation of the excretory canals.

To determine the importance of Ser 419 and Thr 280 phosphorylation in GCK-3 function *in vivo*, we introduced a transgene carrying *gck-3(S419A)* or *gck-3(T280A)* into *gck-3* mutants. We found that the *gck-3(S419A)* mutation still rescued the *gck-3* defect (Fig 3E), whereas the *gck-3(T280A)* mutation had lost the ability to rescue the defect (Fig 3F). This result is consistent with the biochemical data, showing that phosphorylation of Thr 280 in GCK-3 is important for the activation of GCK-3. We next confirmed that WNK-1 and GCK-3 act in the same pathway *in vivo*. Expression of an activated form of GCK-3(T280E), but not of wild-type GCK-3, in *wnk-1* mutants was able to suppress both the L2 arrest and Exc phenotypes (Fig 2G; supplementary Fig 4 online), indicating that WNK-1 functions upstream of GCK-3. Furthermore, the *wnk-1(F1131A/F1220A)* mutation, defective in its interaction with GCK-3, failed to rescue the *wnk-1* defects (Fig 2F). This supports the importance of the WNK-1–GCK-3 interaction for WNK-1 function *in vivo*. In the mammalian WNK–STE20 pathway, WNK1 is activated by osmotic stress through the phosphorylation of its T-loop residue (Ser 382; Zagorska *et al*, 2007), a residue that is conserved in *C. elegans* WNK-1 at Ser 495 (supplementary Fig 1A online). To test whether Ser 495 in WNK-1 is essential for its function, we constructed the *wnk-1(S495A)* mutation, in which Ser 495 was converted to alanine. The *wnk-1(S495A)* mutation still had the ability to rescue the *wnk-1* defects (supplementary Fig 4 online).

In mammals, SPAK and OSR1 activate the Na-K-2Cl co-transporter (NKCC) by interacting with its N-terminal RFX(V/I) motif (Dowd & Forbush, 2003). *C. elegans* also has the *nkcc-1* gene encoding an NKCC-like protein that contains the RFXV motif (Fig 4A); however, the *nkcc-1(ok1621)* deletion mutation had no effect on the morphology of the excretory canal (supplementary Fig 5 online). Denton *et al* (2005) have shown that the RFXI motif mediates the interaction of GCK-3 with CLH-3, a ClC anion channel, and that knockdown of *gck-3* by RNA interference activates CLH-3. Thus, GCK-3 binds to and functions to inhibit CLH-3. This raised the possibility that downregulating CLH-3 could suppress the *gck-3* mutation. Therefore, we examined the genetic interactions of *clh-3* with *gck-3*. In *clh-3(ok763)* deletion mutants, excretory canals were formed almost normally (Fig 4B); in addition, we found that the *clh-3(ok763)* mutation was able to partly suppress the Exc phenotype of *gck-3(tm1296)* mutants (Fig 4B). These results support the possibility that the ClC anion channel negatively regulates the tube formation of excretory canals and that the WNK-1–GCK-3 cascade antagonizes the inhibitory effect of CLH-3. Furthermore, the *clh-3(ok763)* mutation partly restored fertility in *gck-3(tm1296)* mutants (Fig 4C). This is consistent with the idea that GCK-3 negatively regulates ClC channel activity *in vivo*.

The *C. elegans* excretory system functions as the worm's renal system, regulating tissue similarity and passing excess fluid along the tubules (Buechner, 2002). Here, we have shown that the WNK-1–GCK-3 pathway is important for tubular formation of the excretory canals, and that this occurs by negative regulation of CLH-3 ClC channel activity (Fig 4D). As the apical cytoskeleton is a crucial component in canal tube morphogenesis (Buechner, 2002; Berry *et al*, 2003), it is possible that CLH-3 functions to couple extension and lumen formation in a process that depends on the cytoskeleton during canal extension. Our observation that suppression of the *gck-3* Exc defect by *clh-3* was only partial raises the possibility that other ion channels or transporters are also regulated by the WNK-1–GCK-3 pathway. The redundancy indicated here might explain why the *clh-3* deletion itself has no

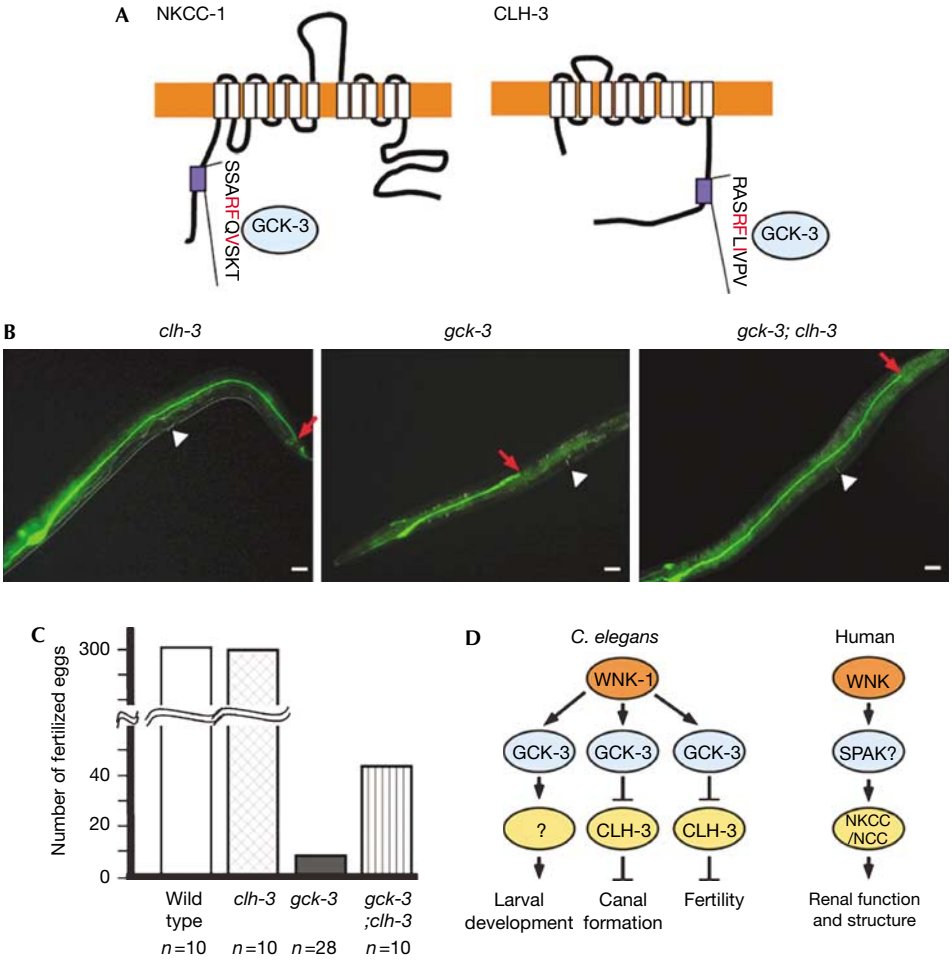

Fig 4 | Genetic interaction between *gck-3* and *clh-3* in *Caenorhabditis elegans*. (**A**) Schematic figures of NKCC-1 and CLH-3. Sequences around the putative GCK-3-binding motif are shown. (**B**) Excretory canal morphologies in worms at the young adult stage. The excretory canal is visualized using *pes-6p*::*gfp* expression. End positions of posterior canals are indicated by red arrows; white arrowheads indicate position of the vulva. Scale bars, 25 μm. (**C**) Numbers of fertilized eggs produced by wild-type and mutant animals. (**D**) Proposed model for the function of WNK-1, GCK-3 and CLH-3. CLH, CIC-type chloride channel; *gck*, *germinal centre kinase*; gfp, green fluorescent protein; NKCC, Na-K-2Cl cotransporter; SPAK, Ste20-related proline-alanine rich kinase.

effect on the tubular morphology. In addition to the Exc phenotype, *wnk-1* mutants showed an early larval arrest phenotype and *gck-3* animals showed defects in fertilization. Interestingly, *wnk-1(tm487); Ex [wnk-1]* animals were partly defective in fertilization (data not shown). This phenotype is similar to that observed in the *gck-3* mutants. Furthermore, progenies hatched from *gck-3(tm1296); clh-3(ok763)* homozygous mutants were arrested at the early larval stage (data not shown), indicating that the *clh-3* mutation is unable to suppress the early larval arrest phenotype. Thus, WNK-1 and GCK-3 are commonly involved in the regulation of tube formation of excretory canals, fertilization and larval development. Our findings suggest that the WNK-1–GCK-3 pathway regulates the former two processes by inhibiting the CLH-3 ClC channel and that CLH-3 is not implicated in the regulation of larval development (Fig 4D). Further analysis of *C. elegans* WNK-1 should provide further information on the role of WNK family kinases.

METHODS

Strains. The strains used in this work are as follows:
bgIs312.
wnk-1(tm487)/nT1[qIs48](IV, V); bgIs312.
gck-3(tm1926)/dpy-21(e428) V; bgIs312.
clh-3(ok763)II; bgIs312.
clh-3(ok763)II; gck-3(tm1926)/dpy-21(e428) V; bgIs312.
nkcc-1(ok1621)IV; bgIs312.
wnk-1(tm487)/nT1[qIs48](IV, V); bgIs312; Ex[hsp-wnk-1+rol-6d].
wnk-1(tm487)/nT1[qIs48](IV, V); bgIs312; Ex[hsp-wnk-1KM+rol-6d].
wnk-1(tm487)/nT1[qIs48](IV, V); bgIs312; Ex[hsp-wnk-1F1131A/F1220A+rol-6d].
wnk-1(tm487)/nT1[qIs48](IV, V); bgIs312; Ex[hsp-wnk-1S495A+rol-6d].
wnk-1(tm487)/nT1[qIs48](IV, V); bgIs312; Ex[hsp-gck-3+rol-6d].

wnk-1(tm487)/nT1[qIs48](IV, V); bgIs312; Ex[hsp-gck-3T280E+ rol-6d].
wnk-1(tm487)/nT1[qIs48](IV, V); bgIs312; Ex[hsp vector+ rol-6d].
wnk-1(tm487)IV; bgIs312; Ex[wnk-1+ rol-6d].
gck-3(tm1926)/dpy-21(e428) V; bgIs312; Ex[hsp-gck-3+ rol-6d].
gck-3(tm1926)/dpy-21(e428) V; bgIs312; Ex[hsp-gck-3KM+ rol-6d].
gck-3(tm1926)/dpy-21(e428) V; bgIs312; Ex[hsp-gck-3T280A+ rol-6d].
gck-3(tm1926)/dpy-21(e428) V; bgIs312; Ex[hsp-gck-3S419A+ rol-6d].
gck-3(tm1926)/dpy-21(e428) V; bgIs312; Ex[hsp vector+ rol-6d].
edf19/unc-24(e138) dpy-20(e1282) IV (CB3824).
ozDf1/sdc-3(y52y180) unc-76(e911) V (BS518).

The *tm487* and *tm1926* mutants were generated by the TMP/UV method (Gengyo-Ando & Mitani, 2000).

Plasmid constructions. The *gck-3* complementary DNA was isolated by PCR using N2 cDNA library. To create the full length of *wnk*-1 cDNA, a partial fragment of the *wnk-1* cDNA was isolated by PCR and ligated with the cDNA clone yk315d9. The mutated *wnk-1* and *gck-3* cDNA fragments were generated by PCR and inserted into the appropriate vectors. To construct *wnk-1* transgene, a 24-kb *Spe*I fragment of C46C2 was inserted into pBluescript(SK+).

Biochemical experiments. Biochemical experiments were carried out as described previously (Moriguchi *et al*, 2005).

***Caenorhabditis elegans* experiments.** Microinjections of DNA into *C. elegans* and microscopic observations were made as described previously (Kawasaki *et al*, 1999). For the fertility assay, we placed the worms individually onto 3 cm dishes with food and counted the number of fertilized eggs produced each day. To examine sperm cell nuclei, we fixed the worms in methanol for 5 min, stained with 0.1 μg/ml of the DNA-binding dye 4, 6-diamidino-2-phenyl-indole and mounted on 2% agarose slides for viewing using both fluorescence and Nomarski imaging.

Supplementary information is available at *EMBO Reports* online (http://www.emboreports.org).

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