NDK-1, the Homolog of NM23-H1/H2 Regulates Cell Migration and Apoptotic Engulfment in C. elegans

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Abstract

Abnormal regulation of cell migration and altered rearrangement of cytoskeleton are characteristic of metastatic cells. The first described suppressor of metastatic processes is NM23-H1, which displays NDPK (nucleoside-diphosphate kinase) activity. To better understand the role of nm23 genes in cell migration, we investigated the function of NDK-1, the sole Caenorhabditis elegans homolog of group I NDPKs in distal tip cell (DTC) migration. Dorsal phase of DTC migration is regulated by integrin mediated signaling. We find that ndk-1 loss of function mutants show defects in this phase. Epistasis analysis using mutants of the α-integrin ina-1 and the downstream functioning motility-promoting signaling module (referred to as CED-10 pathway) placed NDK-1 downstream of CED-10/Rac. As DTC migration and engulfment of apoptotic corpses are analogous processes, both partially regulated by the CED-10 pathway, we investigated defects of apoptosis in ndk-1 mutants. Embryos and germ cells defective for NDK-1 showed an accumulation of apoptotic cell corpses. Furthermore, NDK-1::GFP is expressed in gonadal sheath cells, specialized cells for engulfment and clearance of apoptotic corpses in germ line, which indicates a role for NDK-1 in apoptotic corpse removal. In addition to the CED-10 pathway, engulfment in the worm is also mediated by the CED-1 pathway. abl-1/Abi and abi-1/Abi, which function in parallel to both CED-10/CED-1 pathways, also regulate engulfment and DTC migration. ndk-1(-)abi-1(-) double mutant embryos display an additive phenotype (e.g. enhanced number of apoptotic corpses) which suggests that ndk-1 acts in parallel to abi-1. Corpse number in ndk-1(-);ced-10(-) double mutants, however, is similar to ced-10(-) single mutants, suggesting that ndk-1 acts downstream of ced-10 during engulfment. In addition, NDK-1 shows a genetic interaction with DYNS-1/dynamin, a downstream component of the CED-1 pathway. In summary, we propose that NDK-1/NDKP might represent a converging point of CED-10 and CED-1 pathways in the process of cytoskeleton rearrangement.

Introduction

The human nm23 (nm) gene family consists of ten members named after the first identified metastasis suppressor nm23-H1 (non metastatic clone 22). The metastasis suppressor function has been extensively corroborated using metastatic cell lines (melanoma, breast-, colon-, hepato- and oral squamous cell carcinoma) where, for the most part, overexpression of NM23-H1 was associated with reduced cell motility (reviewed in [1]). Proteins encoded by the nm23 family are classified into two groups. Isoforms of group I (NM23-H1–NM23-H4) possess nucleoside diphosphate kinase activity and are highly conserved in eukaryotes from yeast to mammals [2]. Beyond their nucleoside diphosphate kinase activity, additional molecular functions are associated with NDPKs such as histidine-dependent protein kinase activity [3–4], 3′-5′ exonuclease action [5–6], DNase activity in caspase-independent apoptosis [7] and transcriptional regulation [8]. Together, group I members display essential functions; both up- and down-regulation can disrupt growth and/or differentiation [9;10].

The most extensive set of studies analyzing group I members’ role in cell motility and migration have utilized Drosophila. aced, the fly orthologue of nm23-H1/H2 is a negative regulator of migrating tracheal and border cells via modulating endocytosis of different receptors, such as platelet-derived growth factor receptor (PDGFR)/vascular endothelial growth factor receptor (VEGFR) [11] and fibroblast growth factor receptor (FGFR) [12]. In the process which affects the level of FGFRs Awd functions together with the dynamin/Shibire in endocytosis as a putative GTP

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supplier for the GTPase [9]. Although no physical association of Abw and Shibire could be demonstrated in Drosophila, in rat a direct interaction was detected between NDPK and dynamin I by in vitro pulldown and coimmunoprecipitation [13]. Independent studies using Dictyostelium also confirm links to light-dependent, vectorial cell migration and cell nutrition through different forms of endocytosis [10].

**C. elegans** serves as a particularly amenable model to investigate the process of cell migration. The nematodes are transparent and have simple anatomy making it possible to follow the migration of individual cells in the living animal throughout development. Well studied migrating cell types of *C. elegans* include sex myoblasts (SM), two Q neuroblast (QL and QR) and their descendants, and distal tip cells (DTCs) or the gonadal leader cells [14–16]. In *C. elegans*, we identified and described a single group I NDPK ortholog, NDK-1, which shows 85% and 86% similarity to NM23-H1 and H2, respectively [17]. In the current study we examine the role of NDK-1 in DTC migration and apoptosis.

The tightly regulated migratory path of DTCs provides an elegant system to explore how cell migration can be guided within the spatial and temporal context of the organism. Distal tip cells are specialized leader cells and are responsible for gonad morphogenesis via their migration in C. elegans [16]. During the four stages of larval development (L1, L2, L3, and L4), DTCs migrate in response to attractive and repulsive cues to properly form two mirror image U-shaped gonad arms (Figure 1C). During development, somatic cells dying by apoptosis are engulfed by the neighboring cells as there are no specialized engulfing cells. However, during germ cell death, which occurs as a part of the oogenesis program, at least half of all oogenic germ cells [17] are eliminated by apoptosis and are engulfed by gonadal sheath cells which surround the germ cells [18].

DTC migration and engulfment of apoptotic corpses are analogous processes in *C. elegans* (Figure 1). Both require cytoskeletal rearrangements and membrane trafficking/recruitment (Figure 1A, B) sharing genes regulating both processes (Figure 1D) [22–23]. Two major partially redundant pathways regulate engulfment. The CED-10/Rac pathway consists of unc-73/Trio, ced-10/DOK1, 80, 12/ELMO and ced-10/Rac genes acting downstream of the alpha integrin receptor ino-1, while the CED-1 pathway includes ced-7/ABC1, the ced-1/MEG10 receptor, ced-6/GULP, and dyn-1/Dynamin [22–24]. The CED-10/Rac pathway controls both engulfment and the movement of DTCs by rearranging the cytoskeleton of the engulfing and migrating cells. The CED-1 pathway is involved only in engulfment where it recruits membranes to extend the surface of the engulfing cell. It has been suggested that a third, distinct pathway consisting of abl-1 and abl-1 also influences both DTC migration and engulfment in parallel to the CED-10 Rac and CED-1 pathways (Figure 1D) [22].

In this study we demonstrate that NDK-1 is required for normal DTC migration and engulfment of apoptotic corpses. We show that ndk-1 influences both processes via common genes, acts downstream of ced-10 (cell death abnormality)/Rac and in parallel to abl-1 (Abl interaction)/ABL, and additionally shows a genetic interaction with dyn-1/dynamin. Thus, NDK-1 affects the rearrangement of cytoskeleton in both DTC migration and apoptotic engulfment. We also show that NDK-1 functions similar to its human counterparts in cell migration, as it inhibits the migratory potential of invasive breast adenocarcinoma cells. Our results might help to better understand the function of nm23 genes in metastasis.

Results

**C. elegans** FLAG::NDK-1 reduced the motility of MDA-MB-231T cells

Our group is investigating the function of nucleoside diphosphate kinases (NDPKs) in the model organism *C. elegans*. NDK-1 is the single group I NDPK homolog of the worm and shows high sequence similarity to NM23-H1 and H2 [25]. It is known that nm23 genes regulate cell migration [26]. For example overexpression of NM23-H1 and its sponge ortholog both reduced the migratory and invasive potential of CAL27 (oral squamous carcinoma of the tongue) cells [27]. Based on the high sequence similarity one might expect that the *C. elegans* homolog of NM23-H1/H2 is also able to act likewise. Therefore we investigated the effect of NDK-1 overexpression on the cell migration capacity of the breast adenocarcinoma MDA-MB-231T cell line. MDA-MB-231T cells are far more migratory than CAL27 cells, and the influence of NM23-H1 is much more obvious in these cells. Stably transfected MDA-MB-231T cells overexpressing FLAG::NDK-1, FLAG::NM23-H1 and MYC-NM23-H2 (Figure 2A, B, C) were used for migration assay. Three independent experiments demonstrated that NDK-1 clones CE1 and CE2 both diminished the migratory potential of MDA-MB-231T cells in a similar manner to overexpressing NM23-H1 (clones HA1 and HA2) or NM23-H2 (HB1 and HB2) clones (Figure 2D). The suppression of migratory potential reached or exceeded 50% in almost all clones overexpressing either the worm or a human NM23 homolog compared to MDA-MB-231T control clones (clones K1 and K2).

**ndk-1(0f)** mutants show incomplete migration of DTCs

We recently characterized the worm ortholog of group I NDPKs, *ndk-1*, and identified the strong loss of function allele *ok314*. *ok314* is a 1157 bp-long deletion, which removes the entire *ndk-1* ORF, as well as upstream and downstream regulatory sequences [25].

**ndk-1(ok314)** mutants show a Pvl (protruding vulva) phenotype and are sterile due to germ cell arrest in mitotic phase [25]. Morphological studies using Nomarski optics revealed that *ndk-1(ok314)* animals have insufficiently elongated gonad arms suggesting defects in DTC migration. Detailed analysis (Figure 3G, H) of 318 gonad arms showed that in the majority of *ok314* mutants (60.3%) DTCs turned to the proper direction and side however their migration is stopped prior to reaching the vulva. In 14.5% of the cases DTCs turned back to the vulva in the ventral [instead of the dorsal] side or initiated ventral migration but subsequently vectored to the dorsal side before the turn (7.2%). Other defects manifested low penetrance (wrong direction, lack of the turn, wandering, bizarre twists) and we observed normal DTC migration in only 4.7% of the animals. Altogether we conclude that the prominent phenotype of *ok314* mutants is incomplete migration of DTCs.

In order to determine whether NDK-1 is expressed in DTCs, we studied NDK-1 expression in transgenic strains TTV2 and TTV3. Both were generated using the same translational construct but by distinct methods. In case of TTV2, the strain was created by ballistic bombardment, which resulted in extrachromosomal arrays of the transgene and therefore genetic mosaicism. For TTV3 the MosSCI method was applied to integrate a single copy of the transgene and therefore genetic mosaicism. For TTV3 the MosSCI method was applied to integrate a single copy of the transgene into the genome. The latter method allowed the rescue of Ndk-1 mutant phenotypes. Among TTV2 and TTV3 animals we observed expression in DTCs from L3 (Figure 3A, B) to L4 (Figure 3C, D) stage while in the isolated gonads of TTV3 animals DTC expression was also detected in adults (Figure 3E, F).
As ndk-1 is expressed in the distal tip cells in different developmental stages and knockout of ndk-1 results in different DTC migration defects (Figure 3G,H), we hypothesized that ndk-1 functions in the process of DTC migration.

ndk-1(-) reduces the extra turn phenotype of CED-10 pathway mutants while abi-1 restores the Ndk-1 DTC migration phenotype

Integrins are heterodimeric receptors consisting one α and one β subunit. They provide connections between the extracellular matrix and the actin cytoskeleton regulating cellular shape, motility and cell cycle. Integrin mediated signaling is well known to be involved in the process of DTC migration [21]. Interestingly, both NM23-H1 and H2 have been linked to integrins [28–30].

The C. elegans genome encodes two α (ina-1, integrin alpha) and two β (pat-3) integrin subunits [21]. All these genes are expressed and function in DTCs [21,31–32]. Genes encoding C. elegans integrins are essential, null mutations in any of them cause embryonic lethality [21,31]. Since hypomorph integrin mutants are viable, we applied these mutant alleles for DTC analysis. Hypomorph ina-1 mutants show an abnormal migration (i.e. the migratory path of DTCs is often longer compared to wild-type and extra turns occur due to pathfinding defects, see also definition in methods) phenotype because DTCs do not stop prior to reproduction [21]. Silencing of pat-2 by RNAi resulted in dorsal pathfinding defects [21].
incomplete migration with an enlarged and blunt end of the gonad arm can be observed with DTC-specific expression of a dominant-negative pat-3 transgene or by pat-3 (RNAi) [21,31–32].

vab-3 (variable abnormal morphology) encodes a homeodomain protein, the C. elegans orthologue of Pax6. vab-3/Pax6 transcriptionally regulates both α-integrins in different manners: it downregulates ina-1 (Figure 1D) to cause the cessation of DTC migration and activates pat-2 expression at L3 stage which is necessary for normal dorsal pathfinding [21]. vab-3 reduction of function mutants show an overmigration phenotype similar to ina-1(rf) mutants.

The Rac GTPases CED-10 and MIG-2 (abnormal cell migration) have been shown to act downstream of INA-1 during the migration of DTCs [21]. Additionally, ced-10 was found to function together with ced-2 and ced-3 in DTC migration (reviewed in [16]). Moreover, in the last decade, an entire pathway (often called as CED-10 pathway) was built using also the above mentioned genes (Figure 1D). [19,21–22]. Mutations in CED-10 pathway components manifest characteristically extra turns due to pathfinding defects. Furthermore, these genes – similar to integrin signaling components – function during the dorsal (or third) phase of DTC migration [16].

Figure 2. Western blot analysis and migration assay of transfected MDA-MB-231T cells. MDA-MB-231T cells were stably transfected with, pcDNA3 (K1 and K2), pcDNA3/FLAG-nm23-H1 (HA1 and HA2), pcDNA3/FLAG-ndk-1 (CE1 and CE2) and pcDNA3/MYC-nm23-H2 (HB1 and HB2). A: Western blot with anti-α-tubulin antibodies (loading control). B: Western blot with anti-FLAG- antibodies, visible band in HA1, HA2, CE1 and CE2 proves stable overexpression of introduced transgenes. C: Western blot with anti-MYC- antibodies, visible band in HB1 and HB2 (overexpression of NM23-H2). D: Migration assay. MDA-MB-231T cells stably transfected with one of the following constructs: pcDNA3 (K1 and K2), pcDNA3/FLAG/nm23-H1 (HA1 and HA2), pcDNA3/FLAG/ndk-1 (CE1 and CE2) and pcDNA3/MYC-nm23-H2 (HB1 and HB2) were tested for migration potential. The cells were stained with crystal violet and counted (the number of migrated cells were counted in four representative microscopic fields per each clone). The CE1 and CE2 clones as well as HA1, HA2, HB1 and HB2 exhibited significantly diminished migration potential compared to control (K1 and K2) clones (Student’s t-test, p<0.05). The results are presented as an absolute number of migrated cells in 4 representative fields for every clone (±SD).

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ABL-1 and ABI-1 function in a distinct, recently discovered cascade, acting parallel to the CED-10 pathway in DTC migration (Figure 1D, [22]). abl-1(ok171) and abl-1(n1963) alleles manifest no obvious gonadal phenotype [22] but the DTC migration defects of ced-2, ced-5, ced-12, and ced-10 mutants were suppressed in abl-1 mutants. ABL-1 inhibits ABI-1 in C. elegans, abi-1(tm494) mutation itself or abi-1(RNAi) caused only weak defects in DTC migration [22], however abi-1(RNAi) enhanced the extra turns of ced-5(n1812) and ced-12(n3261) [22]. We analyzed DTC migration in abi-1(ok640) mutants and observed 26% of abnormal gonad arms in which the overshoot phenotype was the most frequent (13%) (Figure 4 A, B).

NDK-1 expression was detected in DTCs from L3 larval stage onwards, and lack of NDK-1 activity resulted in early cessation of migration in the dorsal phase in most of the cases (Figure 3G). Based on these facts we investigated the connection between ndk-1 and integrin signaling genes during the process of DTC migration.

**Figure 3. NDK-1 functions in DTCs.** NDK-1::GFP shows expression in distal tip cells (DTCs) in TTV2 (B, D) and TTV3 (F) translational reporter lines in L3 (A, B), L4 (C, D) larvae and adults (E, F and I). A, C, and E are the corresponding DIC images of B, D, and F, respectively. DTC locations are indicated with arrows. We note that on panel D GFP expression in DTC (marked by an arrow) is overshadowed by intense expression of autofluorescent granules in the intestine. E, F and I: gonads were isolated from adult animals. H: DIC image of an adult wild-type (N2) gonad arm. G: Variation and distribution of DTC migration phenotypes observed in ndk-1(ok314) mutants (see details in the main text). The migratory path of DTCs is marked by dashed lines. I: In TTV3 lines carrying the integrated transgene, NDK-1::GFP expression is also observed in gonadal sheath cells. DIC, fluorescent and merged images derive from the gonadal loop region of a transgenic animal. On the DIC panel white arrows show dying cells, which are surrounded by sheath cells strongly expressing NDK-1::GFP (white arrows on the fluorescent and merged panels).

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We performed an epistasis analysis (a genetic tool suitable for ordering genes into pathways) using the ok314 mutation or RNAi mediated silencing of ndk-1 and the following loss of function or reduction of function mutants: vab-3(e1796), ina-1(gm39), unc-73(e936), mig-2(lq13), ced-5(n1812), ced-12(n3261), abl-1(ok171), abi-1(ok640). Except abl-1(ok171) and abi-1(ok640), all these mutants display large gonads with extra turns (Figure 4A).

To examine the effect of the absence of NDK-1 in the above mentioned mutant backgrounds, we either depleted the function of NDK-1 by RNAi or generated double mutants. In the cases of vab-3(e1796), ina-1(gm39), unc-73(e936), ced-5(n1812) and ced-12(n3261) RNAi was applied to knockdown NDK-1 function in the corresponding mutant background. In the cases of mig-2(lq13), ced-10(n1993), abl-1(ok171) and abi-1(ok640) double mutants were created by introducing the ok314 mutant allele of ndk-1 into the appropriate single mutants.

Loss of ndk-1 suppressed the extra turns of vab-3, ina-1, unc-73, mig-2, ced-5, ced-12 and ced-10 mutants and mostly resulted in Ndk-1-like gonad [reduced gonad with fewer extra turns] (Figure 4A).
B. Although, as mentioned above, abl-1(ok171) was able to suppress the phenotype of the cdg mutants [22], it did not influence the reduced migration of ndk-1 mutants. Unlike abl-1(ok171); abi-1(ok943) mutation was able to restore the DTC migration defects of ndk-1(ok314) animals in 37% of the cases (Figure 4A, B), suggesting that ndk-1 might either act upstream of abl-1 or loss of ABI-1 compensates for the absence of NDK-1, indicating parallel functions of the two genes.

Based on these results we suggest that ndk-1 might act downstream of or in parallel to cdg-10/Rac and upstream of or in parallel to abi-1/Abi (Figure 1D) in the process of DTC migration. Our data raise the possibility that ndk-1 acts downstream of cdg-10 and in parallel to abi-1. Although this tendency could be hypothesized on the basis of the distribution of migration phenotypes, it could not be confirmed by statistical analyses (Figure 4A). Thus, the epistatic relationship of NDK-1 and the two parallel pathways cannot be deduced unambiguously, as DTC migration phenotypes are difficult to quantify. To specify NDK-1’s site of action more precisely, we decided to analyze the role of NDK-1 in apoptosis, where phenotypes, e.g. number of apoptotic corpses, so called Ced phenotype can be quantified with precision.

ndk-1(-); abi-1(-) double mutants show an additive Ced phenotype, suggesting that NDK-1 acts downstream of CDG-10, in parallel to ABI-1

Since CDG-10/Rac signaling controls both DTC migration and engulfment, and our data derived from the DTC analysis suggested that NDK-1 acts downstream of or in parallel to CDG-10, we hypothesized that NDK-1 also functions in the engulfment phase of apoptosis. In somatic tissues of the worm, elimination of apoptotic corpses occurs by the non-specialized engulfment, and our data derived from the DTC analysis (Figure 4A). Thus, the epistatic relationship of NDK-1 and the two parallel pathways cannot be deduced unambiguously, as DTC migration phenotypes are difficult to quantify. To specify NDK-1’s site of action more precisely, we decided to analyze the role of NDK-1 in apoptosis, where phenotypes, e.g. number of apoptotic corpses, so called Ced phenotype can be quantified with precision.

ndk-1(ok314) embryos show a phenotype characteristic for dyn-1(-) mutants: late embryonic lethality with persistent cell corpses

NDPK/AWD is known as a potential GTP supplier of the large GTPase, Dynamin/Shibire in several model systems [11–13,37]. The C. elegans ortholog of dynamin/shibire, dyn-1 acts in the CDG-10 engulfment pathway downstream of cdg-6 [36]; Figure 1D). DYN-1 is proposed to organize vesicle transport: 1. to the phagocytic cups for extending pseudopods during engulfment and 2. to phagosomes for apoptotic cell degradation [36].

dyn-1 loss of function mutants are embryonic lethal [36,38] due to endocytosis defects. Beyond this main phenotype, defective DYN-1 causes failure in engulfment [36]. Many dyn-1 mutant alleles bearing missense mutations in the GTPase domain result in embryonic lethality at later embryonic stages and a strong Ced phenotype. These embryos do not move inside the eggshell in contrast to the other same-aged embryos. The combination of persistent cell corpses and late-embryonic lethality was defined as a new phenotypical class [24,36].

Previously we characterized Ndk-1 mutant phenotypes and showed that in the progeny of ndk-1(ok314)/+;abi-1(tm1704) double mutants displayed 36.7 cell corpses on average (Figure 5 F, G, H, L, M). This additive phenotype suggests that ndk-1 acts in parallel to abi-1. Namely, if two engulfment genes act in the same linear pathway, the phenotype of the double mutants (number of corpses) should not be more severe than that of the stronger single (null) mutant. If the two genes function in parallel pathways a (null) mutation in one of them should enhance significantly the phenotype caused by the other, resulted in an additive phenotype [24,36]. Therefore our results suggest that in the process of engulfment ndk-1 acts in parallel to abi-1 and downstream of cdg-10 (Figure 1D).
Figure 5. *ndk-1(ok314);abi-1(ok640)* double mutants show additive Ced phenotype. *Plim::ced-1::gfp* transgenic worms treated by *ndk-1(RNAi)* (D) show an excess of apoptotic corpses in the germline compared to worms carrying the same transgene treated by control RNAi (B). A, C are corresponding DIC images of B, D respectively. Arrows indicate apoptotic germ cells. E: Increase of apoptotic germ cell death in *ndk-1(RNAi)* animals compared to the control, where *Plim::ced-1::gfp* transgenic worms were grown on control RNAi (e.g. *E. coli* HT115(DE3) carrying an empty vector). F–K: Monitoring apoptotic corpses in wild-type embryos (F), *ndk-1* (G), *abi-1* (H), *ced-10* (J) single mutants and *ndk-1(–);abi-1(–)* (I), *ndk-1(–);ced-10(–)* (K) double mutants using DIC optics. Embryos slightly before or at the comma stage were scored. Each panel shows two focal planes (F–K). Arrowheads indicate apoptotic corpses. Panel L shows a summary of apoptotic corpses scored in *ndk-1(ok314), abi-1(ok640), ced-10(n1993)* single...
crossing we used ndk-1(ok314) and the viable ky51 allele of dyn-1, wherein we took the advantage of thermosensitivity.

We performed this experiment at the restrictive temperature for ky51 (25°C) (Figure 6A). 15.8% of ndk-1(ok314) homozygotes died as embryos and ndk-1/+ heterozygotes laid on the average 122.7 eggs. We measured approximately 50% lethality of dyn-1(ky51) single mutants and 51% lethality in the F1 progeny of dyn-1(ky51);ndk-1(ok314)/+ animals, but the broodsize was decreased (43.9) in the latter category compared to dyn-1 single mutants (63).

At the restrictive temperature we did not notice any Pvl, Ste (double mutants) in the F1 progeny. Putting the F1 animals to the permissive temperature (15°C) the Pvl, Ste phenotype appears in F2 generation. Therefore these observations suggest that the double mutant is not viable indicating that ndk-1 and dyn-1 interact genetically in the worm as well.

**Discussion**

Group I NDPKs are negative regulators of cell migration and motility. To investigate the function of the sole worm group I homolog, NDK-1 in cell migration, we overexpressed NDK-1 and human NM23-H1 and H2 in MDA-MB231T, an invasive breast carcinoma cell line. We found that NDK-1, similar to its human counterparts, dramatically suppressed the migratory potential of MDA-MB231T cells. This result shows that NDK-1 bears an evolutionary conserved function in cell migration and that *C. elegans* serves as a tractable model system to monitor the effects of the NDPK gene family.

Next, we studied the role of ndk-1 in the process of the migration of distal tip cells (DTCs) which are responsible for leading gonad morphogenesis. During larval development distal tip cells guide the migrating gonad arms which finally form two U-shaped tubes [16]. Studying different transgenic strains, we detected NDK-1::GFP expression in DTCs. Detailed analysis of ndk-1 loss of function mutants showed different DTC migration defects, but predominantly, incompletely elongated gonad arms were the consistent feature. The majority of ok314 mutants showed a third (dorsal) phase migratory defect, as a consequence of a premature stop of DTCs, after the turn, that was not present in wild-type worms.

Thus, overexpression of NDK-1 inhibited cell migration and reduced motility of metastatic breast adenocarcinoma cells, but loss of NDK-1 function in the worm predominantly caused incomplete gonadal migration and not excess migration. These data suggest that NDK-1 might have opposing functions depending on the cellular environment, e.g. NDK-1 might promote cell migration in the worm, but inhibit it in human cell lines. In support of this idea, recent work showed that NDPK affects *Dictyostelium* growth in opposing ways in different cellular environment [10]. To further investigate this issue, it will be important to overexpress NDK-1 in the worm and analyze the phenotypes in response to overexpression.
Previously *nm23* genes were linked to integrin signaling in human studies [28–30]. In *C. elegans*, integrins act in the dorsal/third phase of DTC migration [16,21], where the majority of defects conferred by *ndk-1(ok314)* mutants are also observed. Consistently, NDK-1::GFP expression was detected in the third phase of migration (from L3 to adulthood). Based on these findings we performed an epistasis analysis with genes involved in CED-10/Rac signalling (*ok314;par-6, unc-11/alpha-integrin, unc-73/Trio, mig-21/RhoG, ced-5/Dock180, ced-12/Erho, ced-10/Rac*) and with *abl-1/ABL* and *abi-1/ABI* which were shown to act in parallel to the CED-10 pathway. All mutants of CED-10 pathway genes have large gonads with extra turn(s). Putative null mutant alleles of *abl-1* (*ok171* and *n1963*) display no obvious gonad phenotype [22]. We observed 30% of abnormal gonad arms in *abi-1(ok460)* mutants in which the overshoot phenotype was the most frequent (14%). This result is consistent with previous data, e.g. *abi-1* mutations or *abi-1(RX4)* results in overextensioned DTC migration [22]. Loss of *ndk-1* reduced the extra turn phenotype of *ok314, ima-1, unc-73, mig-2, ced-5, ced-12* and *cd-10* mutants and mostly resulted in *ndk-1* like gonad (similar to that of *ndk-1(-) mut*ants). These results lead to the hypothesis that NDK-1 might act downstream of or in parallel to *ced-10*. Although *abl-1(ok171)* was able to suppress the extra turn phenotype of the *cd-10* mutants [22], it did not influence the reduced migration of *ndk-1* mutants. Unlike *abl-1(ok171)*, *abi-1(ok460)* mutation was able to restore partially the DTC migration defects of *ndk-1(ok314)* animals, showing a genetic interaction between *ndk-1* and *abi-1*.

Both distal tip cell migration and engulfment of apoptotic cell corpses require precise regulation of cytoskeleton to extend cell surfaces [19–20,22]. Although *nm23* has been implicated in apoptosis [7,39–43] in these studies NM23’s function was detected in the dying, not in the engulfing cells. In *C. elegans*, during somatic cell death neighboring cells engulf the nascent apoptotic corpses; however germ cell corpses are eliminated by specialized engulfing cells, the sheath cells [17]. We noticed increased numbers of apoptotic corpses in the germline of *ndk-1(RX4)* animals and in *ndk-1(ok314)* embryos, furthermore, NDK-1 is expressed in sheath cells (this study) as well as in embryos [25]. These results suggested that NDK-1 plays a role in engulfment of apoptotic cells. We note that subsequent experiments are necessary to further confirm the role of NDK-1 in engulfment, such as investigating the persistence of apoptotic corpses in *ndk-1(-)* single mutants.

Starting from the observation that *ndk-1* genetically interacts with *abi-1* in DTC migration and functions likely downstream of *ced-10*, we hypothesized that *ndk-1* regulates engulfment in the same manner. We analyzed *ndk-1(-), abi-1(-) and ced-10(-)* single mutant embryos and *ndk-1(-);abi-1(-) and ndk-1(-);ced-10(-)* double mutants. *ced-10(-)*, *ndk-1(-) and *abi-1(-)* mutants contain more apoptotic corpses than wild type animals. Moreover, we observed an increased number of corpses, an additive phenotype in *ndk-1(-);abi-1(-) double mutant embryos, whereas *ndk-1(-);ced-10(-) double mutants displayed apoptotic corpses reminiscent to *ced-10(-) single mutants*. These results suggest that *ndk-1* acts in parallel to *abi-1* and downstream of *ced-10* during apoptotic engulfment.

We also analyzed the putative interaction of *ndk-1* and *dyn-1*/dynamamin, which is a downstream factor of the CED-1 pathway involved in engulfment. The CED-1 pathway is thought to recruit membranes to extend the surface of the engulfing cell, and DYN-1 is proposed to organize vesicle transport during this process. Interaction between NDPKs and Dynamins in endocytosis is well known in other model systems [9,12–13,36,44]. *ndk-1(-) and dyn-1(-)* single mutants display a phenotypic similarity in the worm, the late-embryonic lethality with persistent cell corpses. Double mutant analysis revealed that *ndk-1(ok314);dyn-1(ky51)* animals are not viable indicating a genetic interaction between the two genes in *C. elegans* as well.

Together, we propose that NDK-1 acts downstream of CED-10 and in parallel to ABI-1 in the process of engulfment and DTC migration. In addition, *ndk-1* shows a genetic interaction with *dyn-1*/dynamamin, which raises the possibility that NDK-1 might represent a converging point of the CED-1 and CED-10 pathways. Interestingly, in 10% of *ndk-1(-);ced-10(-) double mutant embryos we observed an increased number of cell corpses (note that in 90% of the double mutants the corpse number is similar to *ced-10(-) single mutants*). The convergence of the two pathways would explain this partial additivity observed in *ndk-1(-);ced-10(-) double mutants*. There are also data showing that silencing of *dyn-1* results in a DTC migration phenotype [38], although the CED-1 pathway is not considered to influence DTC migration. Earlier it was suggested that the CED-10 and CED-1 engulfment pathways converge at *ced-10* [45], but later studies dealing with DYN-1’s function presume that the two engulfment pathways do not converge [36,46]. Recently, it was found that the CED-1 pathway not only regulates membrane recruitment during engulfment, but is also connected through CHC-1/clathrin heavy chain to F-actin assembly [46]. Thus, the CED-1 pathway – besides the CED-10 pathway – is also linked to cytoskeleton remodeling. Earlier studies confirmed NDPKs’ role in cell migration and endocytosis [12,26], processes, which are both related also to CED-1 and CED-10 pathways.

In summary, our genetic analysis shows that *ndk-1* acts downstream of *ced-10* in the regulation of engulfment and DTC migration and genetically interacts with *dyn-1*/dynamamin. These data might contribute to our understanding of how NDK-1 exerts its function in the rearrangement of the cytoskeleton. Although NDPKs were already related to phagocytosis in different systems [10,47], this is the first time that we link *ndk-1*/NDPK to apoptotic engulfment. We show that impaired NDK-1 function causes inefficient engulfment. In mammals it is known that failure of engulfment leads to inflammation [48], moreover inflammation favors tumour progression in certain circumstances [49]. In further studies it would be interesting to overexpress or silence NDK-1 in specialized engulfing cells, such as the macrophage-monocyte system, to further investigate NDPKs’ function in apoptotic engulfment.

**Materials and Methods**

**Nematode strains and alleles**

* C. elegans* strains were cultivated at 20°C on NGM plates seeded with OP50 bacteria [50]. The N2 Bristol strain was used as the wild-type strain. The following transgenic or mutant strains were used:

- TTV1 *ndk-1(ok314) I/hT2[ bli-4(e937) let-2(q782) QIs8][I;III];
- TTV2 *eluEx1[NDK-1::gfp; unc-119(e1395)]III; TTV3 *eluSi1[NDK-1::GFP+unc-119(e1395)]III; L1: MT11068 ced-12(n3261), CB3203 ced-1(e1735), CB3203 unc-73(e936), LGII: RB829 B036.6(ok640) corresponds to *abi-1(ok640)*, NG39 ina-1(d235), CB13203 ced-1(e1735), CB3203 unc-73(e936), LGII: TTV1 *ndk-1(ok314) I/hT2[ bli-4(e937) let-2(q782) QIs8][I;III];
- RNAi

- For RNAi through ingested dsRNAs, an XhoI digested fragment of the full-length cDNA corresponding to *P25H2.5* *(yk1105e04)* was cloned into *pPD129.36*, and the obtained

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**RNA interference**

For RNAi through ingested dsRNAs, an XhoI digested fragment of the full-length cDNA corresponding to *P25H2.5*(yk1105e04) was cloned into pPD129.36, and the obtained
construct was transformed into HT115 (DE3) bacteria. RNAi experiments were performed at 25 °C essentially as described [51].

Quantitation of DTC defects. Adult animals were anaesthetized and examined visually. Only completely visible gonads were analyzed. Gonadal length was defined as reduced when the gonad tip was before the vulva and as overshoot when the gonad was elongated past vulva. Extra turn category means that we observed more than one complete turn. DTC migration was scored as ‘other’ when the gonad was morphologically abnormal (wrong direction, lack of the turn, wondering, extra arm, bizarre twists). As animals affected by ndk-1(RNAi), sterile adults possessing a protruding vulva were picked. For statistical analysis DTC migration phenotypes were grouped into three classes: reduced migration, migration positioned to vulva and excess migration (in the last class overshoot and extra turn categories were merged, see Figure 3A). Animals belonging to the ‘other’ category were not included in the statistical analysis, as they display a broad phenotypic variance, cannot be grouped in any category, and their ratio does not reach 10% in any mutant population used. p-values of pairwise comparisons of DTC defect rates were calculated by Fisher’s exact probability test.

Quantitation of apoptotic cell corpses. Apoptotic corpses (as refractile discs) were scored in comma stage embryos directly by microscopy [33], using Nomarski/DIC (differential interference contrast) optics on Olympus BX51. p-values for pairwise comparisons of apoptotic corpse numbers were calculated using the Student’s t-test.

Cloning of the FLAG::NDK-1 (pcDNA3FLAG-nm23-NDK) construct

The insert of the construct was generated from the cDNA yk1105e04 by PCR using the following primers: 5’-cag aat gct atc tat gga cta cca gga cga cga taa gat gag caa cac tga gag aac c-3’ (which contains the flag sequence) and 5’-ata gtt tag cgg ccc ctg tat tgc tag acc cat gag tgc-3’. The insert was ligated into pCDNA3.1 using BamHI and NotI sites.

Preparation of stably transfected MDA-MB-231T clones

MDA-MB-231T cells were transfected as follows: 4 × 10⁶ cells in DMEM supplemented with 10% FBS were subjected to electrotransformation (250 V, 950 m). After 15 minutes at room temperature. The cells were stained with 0.1% crystal violet, cut out from the inserts, mounted in (DAKO) on slide, analyzed by light microscopy, and photographed. The cells from four representative images of every clone were assembled in Adobe Photoshop and counted. The experiments were performed three times.

Supporting Information

Figure S1 Monitoring apoptotic corpses in embryos slightly after the comma stage in different mutant backgrounds. A–F. Monitoring apoptotic corpses in wild-type embryos (A), ndk-1(–) (D), abi-1(–) (B), cod-10(–) (C) single mutants and ndk-1(–);abi-1(–) (E), ndk-1(–);cod-10(–) (F) double mutants using DIC optics. Embryos slightly after the comma stage were scored. Each panel shows two focal planes (A–F). Arrowheads indicate apoptotic corpses. (TIF)

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Author Contributions

Performed the experiments: LF EM ZF EP NM MD MHB BH KTV. Analyzed the data: LF MHB AM TV BH AZ KTV. Contributed reagents/materials/analysis tools: MHB BH TV AM AZ KTV. Wrote the paper: MHB AM LF KTV. Designed research: LF MHB AM TV KTV.

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