An approach using *Caenorhabditis elegans* screening novel targets to suppress tumour cell proliferation

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Abstract

**Objectives:** Tumour cell proliferation requires high metabolism to meet the bioenergetics and biosynthetic needs. Dauer in *Caenorhabditis elegans* is characterized by lower metabolism, and we established an approach with *C. elegans* to find potential tumour therapy targets.

**Materials and methods:** RNAi screening was used to find dauer-related genes, and these genes were further analysed in *glp-1(−)* mutants for tumour-suppressing testing. The identified tumour-related genes were verified in clinical tumour tissues.

**Results:** The lifespan of *glp-1(−)* mutants was found to be extended by classical dauer formation signalling. Then, 61 of 287 kinase-coding genes in *Caenorhabditis elegans* were identified as dauer-related genes, of which 27 were found to be homologous to human oncogenes. Furthermore, 12 dauer-related genes were randomly selected for tumour-suppressing testing, and six genes significantly extended the lifespan of *glp-1(−)* mutants. Of these six genes, *F47D12.9, W02B12.12* and *gcy-21* were newly linked to dauer formation. These three new dauer-related genes significantly suppressed tumour cell proliferation and thus extended the lifespan of *glp-1(−)* mutants in a longevity- or dauer-independent manner. The mRNA expression profiles indicated that these dauer-related genes trigged similar low metabolism pattern in *glp-1(−)* mutants. Notably, the expression of homolog gene DCAF4L2/*F47D12.9*, TSSK6/W02B12.12 and *gcy-21* was found to be higher in glioma compared with adjacent normal tissue. In addition, the high expression of TSSK6/W02B12.12 and NPR1/*gcy-21* correlated with a worse survival in glioma patients.

**Conclusions:** Dauer gene screening in combination with tumour-suppressing test in *glp-1(−)* mutants provided a useful approach to find potential targets for tumour therapy via suppressing tumour cell proliferation and rewiring tumour cell metabolism.
INTRODUCTION

Cancer remains one of the leading causes of death due to the late diagnosis, poor prognosis, metastasis and frequent occurrence of drug resistance. Usually, the treatment for tumour is surgery, chemotherapy, radiotherapy, complemented with immunotherapy and hormone therapy. These various treatments for cancer may have not been able to fully meet the clinical needs, and they frequently bring a variety of side effects to patients. Therefore, tumour still needs new treatment strategy.

The most fundamental trait of tumour cells involves their ability to sustain proliferation. In comparison with the normal tissues, high proliferating tumour cells need large amount of energy and biosynthetic precursors of macromolecules as building blocks for new cells. In clinical, patients who carry cancer cells with low proliferation can survive for relative long periods—in some cases indefinitely—without relapsing. In addition, many of us may have in situ tumours, like breast, prostate and thyroid cancer, without recognizing it. These findings suggest that these microscopic tumours are possibly dormant and need other proliferation signals to become lethal tumours.

Evidence suggests that metabolism rewiring can suppress cancer incidence, delay tumour progression and inhibit metastasis. As an example, calorie restriction impairs tumour cell proliferation, alters expression of cell cycle proteins, modifies tumour suppressor gene function and disrupts metabolic pathways. Therefore, tumour cell metabolism may be a potential target for tumour therapy. Dormancy is a special process with extremely low metabolism in nature. But there is no suitable model for screening dormancy-related targets to suppress tumour cell proliferation.

By contrast to tumour cells, dauer, a dormancy in C elegans, which is characterized by their slow growth, maintains extremely low metabolic levels. The C elegans spontaneously enter dauer during the development of L2/L3 in response to adverse environmental conditions, such as high temperature, limiting amounts of food. Several signalling cascades including insulin-like pathway, TGFβ-like pathway, steroid hormone pathway and guanylyl cyclase pathway are documented to be critical in modulating nematode dauer formation. C elegans is a fine model to study tumour. In C elegans, GLP-1 signalling promotes a proliferative germ cell state and prevents germ cells from undergoing meiosis. Thus, loss of GLP-1 signalling results in a severe proliferation defect and early meiotic entry, while constitutive activation yields a germ-line tumour with all germ cells maintaining the germ cells in the stem cell state. The expanding germ-line tumour cells eventually perforate the gonad, invade throughout the worm body and lead to early animal death. Thus, tumour mutant in combination with dauer in C elegans may present a valuable model to find the targets suppressing tumour cell proliferation.

Here, we established an approach with C elegans for screening novel targets that rewire tumour cell metabolism and suppress proliferation. We uncovered dauer formation signals could rewire metabolism and extend the lifespan of glp-1(−) mutants in a longevity- or dauer-independent manner.

RESULTS

2.1 Classical dauer formation signals significantly extended the lifespan of glp-1(−) mutants

It was reported that in glp-1(−) mutants, the germ cells in the early stage of oogenesis could re-enter into the mitotic cell cycle and overproliferate, making them resemble the overproliferation of tumour cell and shortening the lifespan of patients. In this study, we tested whether the short lifespan in glp-1(−) mutants could be extended by the classical dauer formation signals, which inhibit insulin-like pathway, guanylyl cyclase pathway, TGFβ-like pathway and steroid hormone pathway, respectively. As shown in Figure 1A, we found glp-1(−) mutants suffered a shorter lifespan compared with N2 (glp-1(+)) worms. Next, we knock down daf-2, daf-1, daf-14, daf-11 and tax-2 by RNAi, respectively, which led to the activation of dauer formation pathway. Compared to L4440 RNAi in glp-1(−) mutants, these dauer formation signals extended the mean survival rate of glp-1(−) mutants by 114.28%, 28.57%, 28.57%, 42.86% and 42.86%, respectively, for daf-2, daf-1, daf-14, daf-11 and tax-2 (Figure 1B-F, Table S1), and all the P values for each dauer formation signal in three independent trials were < .001 (Table S1), indicating a solid contribution of dauer formation signals to the lifespan extension in glp-1(−) mutants.

2.2 Classical dauer formation signals suppressed germ cell proliferation

Next, we asked whether these classical dauer formation signals extend the lifespan via suppressing the germ cell excessive proliferation in glp-1(−) mutants. We knocked down these dauer formation signals in these worms, then dissected the entire gonads at day 4 of adulthood and detected the germ cell number with DNA-intercalating dye DAPI. Our results showed that glp-1(−) mutants had more undifferentiated germ cells than glp-1(+) worms (Figure 2A, B). Furthermore, knockdown of daf-2, daf-1, daf-14, daf-11 and tax-2 resulted in reduced undifferentiated germ cells compared with the counterparts feeding with L4440 in glp-1(−) mutants (Figure 2A, B, Table S2), and all the P values were less than .001. Collectively, these data suggested that activation of classical dauer formation signals suppressed tumour cell proliferation.

2.3 Three novel dauer-related genes extended the lifespan of glp-1(−) mutants

Most of the homologues of classic dauer signals in human had anti-tumour effect, as shown in Table 1. Hence, we used the glp-1(−) mutants to screen novel genes capable of suppressing tumour cell proliferation. Protein kinases play an indispensable role in regulating cell proliferation and function, which makes them perfect targets for screening tumour-related genes.
Worms encode about 438 kinase-coding genes. To screen these genes, an RNAi library contains 287 kinase-coding genes were established (Figure 3A). We optimized conditions for RNAi library screening and performed the screening (see Figure S1). Sixty-one genes were identified to promote dauer formation after RNAi (Figures 3B, 4A), of which 27 human homologs genes were well-studied oncogenes (Table 2), demonstrating the potential value of this screening approach. Then, we randomly selected 12 genes for further evaluation. As a result, knockdown of cam-1, par-1, cdk-5, W07G4.3, Y38H6C.20 and sel-5 showed no effect on lifespan extension in glp-1(−) mutants (Figures 3B, 4B), while knockdown of genes including aak-2, akt-2, let-23, F47D12.9, W02B12.12 and gcy-21 extended the lifespan of glp-1(−) mutants (Figures 3B, 4C). Interestingly, F47D12.9, W02B12.12 and gcy-21 were first identified to be involved in dauer formation. To confirm our finding, we knock down these three genes, respectively, as a consequence, the survival of glp-1(−) mutants was significantly increased, and all the P values were <.001 (Figure 5A-C, Table S3). Furthermore, at day 4 of adulthood, these worms had less germ cell than glp-1(−) mutants feeding with L4440, (Figure 5D, Table S4), this was associated with the decreased cell proliferation (Figure 5E), and all the P values were <.001.

2.4 | The lifespan extension of dauer-related genes in glp-1(−) mutants was independent of longevity and dauer period

Most of classical dauer formation signals were reported to extend the lifespan of glp-1(+) worms; Kenyon et al. had demonstrated that longevity signals could inhibit tumour growth. The glp-1(−) mutants in our study were in the background of N2 (glp-1(+)) worms. To test whether these three dauer-related genes have inherent longevity effect, we knock down these genes in glp-1(+) worms, respectively, and no longevity effect was observed (Figure 6A-F, Table S5), which suggested the lifespan extension in glp-1(−) mutants was independent of longevity.

The dauer occurs in the L2/L3 larval stage. To determine whether the dauer period contributes to lifespan extension in glp-1(−) mutants, we knock down daf-2, gcy-21, F47D12.9 and W02B12.12 in glp-1(−) mutants at different development stages including L1, L2, L3, L4, day 1 and day 4 of adulthood (Figure 7). We found the knockdown of these genes extended the lifespan of glp-1(−) mutants at all developmental stages, and all the P values for each dauer-related gene were <.001 (Figure 7A-E, Table S6). These data showed that these dauer-related genes extend the lifespan of glp-1(−) mutants independent of dauer stage.
To investigate the molecular mechanisms underlying the antitumour effect of dauer-related genes, we compared their transcriptome profiles in worms. As shown in Figure S2A, compared to glp-1(+) worms, the glp-1(−) mutants had 5364 differentially expressed genes, of which 2514 genes were downregulated and 2850 genes were upregulated. In the glp-1(−) mutants, 86, 32, 102, 38 downregulated genes and 1022, 36, 102, 84 upregulated genes were identified after the knockdown of daf-2, gcy-21, F47D12.9 and W02B12.12, respectively (Figure S2B-E).

Next, we analysed the differentially expressed genes. RNA-related metabolism and processing were found to be significantly
increased glp-1(−) mutants when compared to glp-1(+) worms evidenced by Gene Ontology analysis, while this increment was reduced in daf-2 RNAi groups (Figures S3 and S4). As shown in Figures S3 and S5, oxidoreductase activity was upregulated in glp-1(−) mutants and it was reduced in gcy-21 RNAi group. Glp-1(−) mutants increased the structural molecule activity and transferase activity, while the knockdown of F47D12.9 or W02B12.12 reversed them (Figures S3, S6 and S7).

Furthermore, to better understand how dauer-related genes affect tumour, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed. As shown in Figure 8, compared to glp-1(+) worms, glp-1(−) mutants upregulated glycolysis, amino acid metabolism, fat acid elongation, RNA polymerase, ribosome biogenesis, peroxisome and lysosome. However, these upregulated cellular metabolisms were reversed by the RNAi of daf-2, gcy-21, F47D12.9 or W02B12.12. In addition, glp-1(−) mutants downregulated fatty acid degradation, drug metabolism, autophagy, mitophagy, longevity regulating pathway, phagosome, which were also reversed by the RNAi daf-2, gcy-21, F47D12.9 or W02B12.12. Generally, our data indicated that these dauer-related genes rewired similar metabolism pattern in glp-1(−) mutants.

2.6 | NPR1/gcy-21 and TSSK6/W02B12.12 were unfavourable prognostic indicators in glioma patients

Gcy-21, F47D12.9 and W02B12.12 are homologous to NPR1, DCAF4L2 and TSSK6 in human, respectively.40 To translate our findings from C elegans models to human individuals, we first investigated whether the antitumour effect of these three genes was parallel in human. Using the TCGA data sets, we found that NPR1, DCAF4L2 and TSSK6 were overexpressed in glioma samples compared with adjacent normal tissues in human (Figure 9A). In Figure 9B, the cluster analysis revealed that NPR1, DCAF4L2 and TSSK6 were significantly upregulated in classical and mesenchymal subtypes of glioma. Next, we evaluated the prognostic values of NPR1, DCAF4L2 and TSSK6 in human glioma patients by Kaplan–Meier analysis. Patients with higher expression of NPR1 or TSSK6 suffered shorter overall survival (Figure 9C), while DCAF4L2 had no statistical significance (Figure S8). Furthermore, we detected the NPR1 protein expression levels in glioma tissues. Consistent with the above results, NPR1 was overexpressed in these patients and the expression level was positive correlated with tumour grade (Figure 9D–F). Taken together, these results demonstrated that NPR1 and TSSK6 may be
unfavourable prognostic factors and therapeutic targets for glioma patients.

3 | DISCUSSION

The primary characteristic of tumour cells is highly proliferation and active energy metabolism. Previous studies indicated that reduced metabolism, such as calorie restriction, could inhibit tumorigenesis and progression. Dormancy is a special process with extremely low metabolism in nature. Based on these two opposite metabolism states, we proposed that the activation of dormancy could suppress tumour progression. By using C. elegans, we found that related genes significantly extended the lifespan of glioma cells and their human homolog genes had potential antitumour effect. This result suggested dauer gene screening in combination with tumour-suppressing test in glioma cells provided a useful approach for antitumour target screening.

Given that protein kinases are a large family of enzymes and control many basic bioactivity including cell proliferation and cycling. Part of protein kinase has been considered to be oncogenic, as their transforming activity can determine the survival and proliferation of
Intriguingly, our approach screened out three novel dauer-related genes, namely F47D12.9, W02B12.12 and gcy-21, which could significantly extend the lifespan of glp-1(-) mutants via suppressing tumour cell proliferation. The functions of these three genes in C. elegans were not yet clear and these genes were conserved in human beings. We found that their homologous genes NPR1, DCAF4L2 and TSSK6 were highly expressed in glioma patients and patients with high NPR1 and TSSK6 predicted a short survival.

Studies reported that NPR1/gcy-21 is the receptor for the cardiac hormone atrial natriuretic peptide, and it has been reported to be highly expressed in cancer cells such as lung cancer, prostate cancer and ovarian cancer.²⁴ The high expression and signalling of NPR1 are important for tumour growth; its deficiency protects C57BL/6 from lung, skin and ovarian cancers.²⁴ Nojiri et al.²⁵ showed that atrial natriuretic peptide prevents cancer metastasis by suppressing the inflammatory reaction. DCAF4L2/F47D12.9 is a member of WD-repeat proteins. Wang et al.²⁶ found elevated DCAF4L2 expression in colorectal cancer was significantly correlated with clinical stage and it was an independent prognosis factor for survival. TSSK6/W02B12.12 is a member of the serine/threonine protein kinase family, and it has been described as testis-specific due to effects on fertility.²⁷ Li et al.²⁶ discovered TSSK6 expression levels were positively correlated with T-cell diversity in multiple cancers and TSSK6 could be a putative vaccine target in multiple cancer types. Overall, these three new genes are worth for further evaluation in tumour patients.

Current targets for tumour therapy could induce multiple side effects, indicating that targets with more safety are needed for treating cancers. There is no successful target therapy for glioma, and chemotherapy or radiotherapy is still the major strategy for glioma clinical treatment.²⁹ P53 is a potential target to suppress tumour; however, P53 caused progeria in mouse.²⁹ Our results suggested NPR1/gcy-21 and TSSK6/W02B12.12 might be potential targets for glioma therapy via suppressing tumour cell proliferation (Figure 5). As an example, NPR1 interacts with atrial natriuretic peptide, which is an endogenous and physiological peptide, and atrial natriuretic peptide has significant antitumour effect in multiple cancers such as pancreatic cancer, breast cancer, small cell lung cancer, prostate cancer and colorectal cancer.³⁰,³¹

Notably, NPR1 and TSSK6 were highly correlated with the unfavourable prognosis of glioma patients and could be suitable prognostic indicators in glioma; these need to be confirmed by large clinical samples detection. NPR1 protein expression was increased in gastric, lung, skin, and ovarian tumours.³²,³³ However, TCGA data sets showed that the mRNA expression of NPR1 has no significant difference in multiple cancers versus normal tissues. Generally, the mRNA expression level of genes was not always consistent with protein expression level, this non-consistence of NPR1 expression at mRNA and protein level suggested that the regulation of NPR1 is complicated and the protein level modulations such as epigenetic regulation and post-translation modifications possibly play important role in these tumours. In addition, the mRNA expression of TSSK6 is increased consistently in multiple tumours; however, its protein level in tumour tissues is unknown. Overall, few molecular biological works have been performed for the
cancer cells.⁴²,⁴³ Kinase-specific inhibitors had high selectivity, efficiency and mild side effects than the cytotoxic drugs for chemotherapy.⁴⁴,⁴⁵ Using this approach, the well-known oncogenes (Tables 1 and 2), such as akt-2 and let-23, could be recognized. Akt-2 encodes a homolog of the serine/threonine kinase AKT/PKB, drugs that target the phosphoinositide 3-kinase (PI3K)/AKT pathway, which is frequently mutated in both solid tumours and haematologic malignancies.⁴⁶,⁴⁷ Let-23, an ortholog of human EGFR (epidermal growth factor receptor), is predicted to have epidermal growth factor-activated receptor activity and is involved in several processes including determination of adult lifespan, male genitalia development and nematode larval development.⁴⁸-⁵¹ EGFR is a verified therapeutic target; antibody drugs such as cetuximab and panitumumab, which target the transmembrane protein epidermal growth factor receptor (EGFR), have made a major step forward in the treatment of cancer.⁵²,⁵³

### Table 2 The homologous genes of dauer-related genes in regulating tumour cell proliferation and metabolism

| Dauer-related genes | Homologous genes | Related research in Tumour |
|---------------------|------------------|---------------------------|
| akt-2               | AKT2             | PTEN-deficient tumours⁸²   |
| C06E8.3             | PIM3             | Pancreatic cancer⁸³        |
| C01G6.8             | ROR1             | Chronic lymphocytic leukaemia⁸⁴ |
| F38E9.5             | TWF1             | Lung adenocarcinoma⁸⁵      |
| T05G5.3             | CDK1             | Melanoma⁸⁶                |
| C07G1.3             | CDK16            | Hepatocellular carcinoma⁸⁷ |
| F29C4.1             | ACVR1C           | Retinoblastoma²⁷           |
| M176.6              | AXL              | Melanoma⁸⁸                |
| C44H4.6             | GSK3B            | Pancreatic cancer⁸⁹        |
| F22E5.3             | NPR1             | Breast cancer⁹⁰            |
| T01C8.1/ aak-1      | PRKAA1           | Gastric cancer⁹¹          |
| T27E9.3             | CDK-5            | Glioma⁹²                   |
| F46F2.2             | CSNK1D           | Breast cancer⁹³            |
| C05D10.2            | MAPK15           | Chronic myeloid leukaemia⁹⁴ |
| ZK370.5             | PDK1             | Breast cancer⁹⁵            |
| ZC123.4             | CDK14            | Oesophageal cancer⁹⁶       |
| B0495.2             | CDK11B           | Cholangiocarcinoma⁹⁷       |
| H01G02.2            | CDK20            | Hepatocellular carcinoma⁹⁸ |
| K09B11.1            | Irak4            | Chronic lymphocytic leukaemia⁹⁹ |
| T06C10.3            | FER              | Melanoma¹⁰⁰                |
| R90.1               | TTBK2            | Glioma¹⁰¹                  |
| ZC404.9             | MAP4K3           | Lung cancer¹⁰²              |
| F46F6.2             | PKN3             | Prostate cancer¹⁰³         |
| ZK1067.1/ let-23    | ERBB4            | Gastric cancer¹⁰⁴          |
| Y105E8A.8           | WRAP53           | Colorectal cancer¹⁰⁵       |
| F47D12.9            | DCAF4L2          | Colorectal cancer¹⁰⁶       |
| C04G6.1             | MAPK7            | Osteosarcoma¹⁰⁶            |

<image of table>
regulation of these two genes till now; the detailed mechanisms are under investigation. These findings just provide clues for glioma target therapy, which requires a lot of work to verify its function and reveal its mechanisms in human glioma cell and mouse.

In this study, we found that dauer-related genes could extend the lifespan of glp-1(−) mutants and this extension was independent of dauer stage and longevity (Figures 6 and 7), which suggests that these genes could directly suppress the tumour cell proliferation and could be potential targets for tumour clinical therapy. The profiles of mRNA expression showed that knockdown of daf-2, gcy-21, F47D12.9 and W02B12.12 shared similar characters of low metabolism in bioenergetics and biosynthetic needs compared to controls in glp-1(−) mutants, and the rewired metabolism after the knockdown of gcy-21/NPR1 and W02B12.12/TSSK6 possibly contributes to their antitumour effect. For example, we found knockdown of daf-2, gcy-21, F47D12.9 and W02B12.12 increased the fatty acid degradation in glp-1(−) mutants. It has been reported that in cancer patients increased fatty acid degradation can suppress tumour development. Consistently, the level of fatty acid degradation was increased in low metabolic states, such as dormancy or fasting. These results suggested that dauer-related genes in glp-1(−) mutants exert antitumour effects by rewiring tumour cell metabolism.

The experimental strengths and the similarities between the cellular and molecular processes present in C elegans and other animals

**FIGURE 5** Dauer-related genes extend the lifespan of glp-1(−) mutants and reduce germ cell number in glp-1(−) mutants. (A-C) The lifespan curves of gcy-21 RNAi (A), F47D12.9 RNAi (B), W02B12.12 RNAi in glp-1(−) mutants. Adult animals were stained with the DNA-intercalating dye DAPI. Left panels, the whole gonad. Right panels, midpoints of the gonad arms. (D) glp-1(−) mutants lack oocytes and have many undifferentiated germ cells in their gonads. Knockdown of gcy-21, F47D12.9 and W02B12.12, they have far fewer undifferentiated germ cells and maintain the integrity of their gonads. Representative of n = 3 independent experiments. E, Knockdown of gcy-21, F47D12.9 and W02B12.12 reduced undifferentiated germ cells was detected and analysed using one-way ANOVA test followed by Bonferroni correction for post hoc test (*P < .05, **P < .01 for t test); data shown are mean ± SD.
across evolutionary time (metabolism, organelle structure and function, gene regulation, protein biology, etc) have made C elegans an excellent organism with which to study general metazoan biology. But no model organism can be used to answer all the research question, and working with C elegans has some limitations, for instance, C elegans lacks many genes that regulate cascades. C elegans as a
good model for research in vivo, however, there are no C. elegans cell lines exist. So our findings should be validated in higher eukaryotic model organisms, such as mammalian tumour cell lines and mice.

Conclusively, we found that dauer-related gene extends the lifespan of glp-1(−) mutants via suppressing the tumour cell proliferation and rewiring tumour cell metabolism. This work indicated that dauer gene screening in combination with tumour-suppressing test in glp-1(−) mutants provided a useful approach for antitumour target screening.

4 | METHODS

4.1 | Nematode strains and culture

We used the following strains in this study: wild-type N2, CF1370 daf-2(e1370), CF1038 daf-16(mu86), GC833 glp-1(ar202) were obtained from the Caenorhabditis Genetics Center. Worms are cultivated on standard NGM plates with Escherichia coli OP50. Nematodes were cultured at 20°C, except as noted below for temperature-sensitive.

4.2 | Worm RNAi by feeding

RNAi was performed essentially as per. Single colonies of HT115 bacteria containing L4440 plasmids with cloned fragments corresponding to target genes were from RNAi feeding libraries. Each RNAi reagent was verified by DNA sequencing except for screening. Young adult hermaphrodites were placed onto NGM plates seeded with dsRNA-expressing or empty vector control bacteria (RNAi feeding plate). After overnight incubation, worms were transferred to an identical fresh RNAi feeding plate and allowed to lay eggs for 2 hours.
4.3 | Lifespan analysis

Lifespan was measured following previous protocols. For lifespan assays, we picked about 90 animals as L4 larvae at t = 0, and we transferred worms to OP50-seeded or RNAi feeding plates (roughly 30 animals per plate) approximately every other day until the end of the reproductive period. Dead worms were counted every other day until all worms were dead. Worms that were missing or that died from extruded internal organs or from internally hatched progeny were censored and statistically incorporated into the lifespan analyses. All lifespan experiments were performed at 25°C. We generated lifespan curves using GraphPad Prism 7, and we determined P values using the Mantel-Cox log-rank test. Each lifespan experiment was repeated at least three times.

4.4 | Fluorescence microscopy

For DAPI nuclear staining, gonads were dissected, fixed and stained with DAPI as described. Intact worms were fixed in cold (−20°C) methanol for 5 minutes. Fixed worms were washed twice in modified M9 buffer (M9 without Mg2+), incubated 30 minutes in 100 ng/mL DAPI in modified M9 and washed two to three times in modified M9. To prepare dissected gonad preparations, animals of the desired age were picked onto a fresh plate containing no bacteria, immersed in 2 mL of phosphate-buffered saline (PBS) containing 0.25 mM levamisole and transferred to a circular glass dish (3 cm diam and 1.5 cm deep). Worms were decapitated by slicing with two 25-gauge syringe needles in the head region, which results in gonad extrusion. The preparations were fixed in 3 mL of 3% formaldehyde, 0.1 M K2HP04 (pH 7.2) for 2 hours Using a capillary pipette, worms with
attached extruded gonads were transferred onto a 2% agarose pad covering most of a glass slide. After removing excess liquid with a capillary, extruded gonads were manipulated for optimal positioning with a drawn capillary and then overlaid with a 25 × 50-mm coverslip. Z-stack images were acquired for the same region in entire gonad, with a 20× water objective at 2-μm intervals using a Leica Confocal Microscope. The germ cells in at least three worms for each group were detected and summarized for three independent trials. To quantify C. elegans germ cell nuclear numbers, each entire z-stack was loaded into FUJI as a single lei file. Then, we selected the Z-stack image demonstrating the DAPI-stained germ cells in the entire gonad arm for germ cell number analysis. If the two gonads were uneven size, germ cells from both gonads were measured and averaged. The average number was used to compare between groups. To distinguish undifferentiated germ cells from oocytes, only the undifferentiated proliferation germ cells, which exit pachytene, presenting nuclei fully enclosed by plasma membrane, have been detected.73

4.5 | Quantitative RT-PCR

More than 400 well-fed synchronized worms on day 1 were collected into 1.5-mL tube and washed at least three times with M9 buffer. Total RNA was extracted with Trizol reagent (Invitrogen).1 μg of total RNA was reverse-transcribed in 20 μL using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa). Real-time RT-PCR was carried out using the 7300 Real Time PCR System (Applied Biosystems) using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™). Expression level of each sample was standardized to C. elegans actin endogenous control standard.39,41 The following specific primers used were 5′-TCTTGCTCGCCTACAACCCCATC-3′ (forward) and 5′-GATCGCCTTGACTGCTACAACTCG-3′ (reverse) for W02B12.12, product size was 195 bp, 5′-CTCGTGTGGACGCTGATGTTACC-3′ (forward) and 5′-TCTTCGTCGCATCACAACCATCAC-3′ (reverse) for F47D12.9, product size was 152 bp, 5′-CAGTGGCTGGAGGCTTCAAGTG-3′ (forward) and 5′-AGATGTTGGCACCGATGTTCAGAG-3′ (reverse) for gcy-21, product size was 136 bp, 5′-CGCAATCCAAAGAGGAGTTACCTT-3′ (forward) and 5′-AGAGTGATGATCCAGATCTTCA-3′ (reverse) for act-1 as control, and product size was 120 bp. All these genes were annealed at 60°C. The folds of changes were shown as means ± SD in three independent experiments with each triplicate.

4.6 | Library preparation for Transcriptome sequencing

A total amount of 3 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext UltraTM RNA Library Prep Kit for Illumina® (NEB) following the manufacturer’s recommendations and index codes were added to attribute sequences to each sample. PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and 125/150 bp paired-end reads were generated.

4.7 | Data filtering

Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. The raw data obtained by sequencing contain a small number of reads with sequencing linker or low quality sequencing. In order to ensure the quality and reliability of data analysis, the original data need to be filtered. The content of the filtering is as follows. Remove low quality reads (Qphred ≤ 20 the number of bases in the read length face = 50% above reads). In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. At the same time, the contents of Q20, Q30 and GC in the clean data were calculated. All the downstream analyses were based on the clean data with high quality.

4.8 | Data analysis

Reference genome and gene model annotation files were downloaded from genome website directly (ftp://ftp.ensembl.org/pub/release-88/fasta/caenorhabditis_elegans/). Index of the reference genome was built using Hisat2 v2.0.5, and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5. Differential expression analysis of two conditions/groups (two biological replicates per condition) was performed using the DESeq2 R package (1.16.1). DESeq2 provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg’s approach for controlling the false discovery rate. Genes with an adjusted P-value < .05 found by DESeq2 were assigned as differentially expressed.

4.9 | GO and KEGG enrichment analysis of differentially expressed genes

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package, in which gene length bias was corrected. GO terms with corrected P value <.05 were significantly enriched by differential expressed genes.
KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput put experimental technologies (http://www.genome.jp/kegg/). We used clusterProfiler R package to test the statistical enrichment of differential expression genes in KEGG pathways.

4.10 | Sample collection

We included all available tumour samples from TCGA (https://tcgad ata. nci.nih.gov). In addition, 77 glioma tumour samples and three non-neoplastic normal brain tissues were obtained from Fudan University Minhang Hospital. All the samples were histologically graded according to the 2007 WHO Classification of Nervous System Tumors. The protocol was approved by the ethics committee of Minhang Hospital, and informed consent was signed by all participants.

4.11 | Immunohistochemistry

The immunohistochemical analysis was performed on the 4-mm-thick fraction mounted on slides and sectioned from each tumour sample. Then, each slide was deparaffinized in 60°C, followed by treatment with xylene and graded alcohol. After the antigen retrieval and being blocked with 5% bovine serum albumin, tissue slides were incubated with antibodies overnight at 4°C against NPR1 (1:50, Invitrogen, PA5-29049), DCAF4L2 (1:100, Proteintech, 21571-1-AP) and TSSK6 (1:50, H00083983-M02) respectively, followed by the addition of the appropriate biotinylated secondary antibody for 30 minutes at room temperature. Each specimen was assigned a score according to the intensity of the staining (no staining = 0; weak staining = 1; moderate staining = 2; strong staining = 3) and the extent of stained cells (0% = 0, 1-24% = 1, 25-49% = 2, 50-74% = 3, 75-100% = 4). The final immunoreactive score was determined by multiplying the intensity score with the extent of score of stained cells, ranging from 0 (the minimum score) to 12 (the maximum score).

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CONFLICT OF INTEREST

No conflict of interest was declared.

AUTHOR CONTRIBUTION

MYQ, HSF, ZSL and WLS designed the experiments; MYQ, HSF, ZSL, ZZY, KCY, CHL, LZM, CPR and HB performed experiments and statistical analysis; MYQ and ZSL wrote the manuscript; MYQ, ZSL and WLS revised manuscript. MYQ, HB and WLS provided funding support.

DATA AVAILABILITY STATEMENT

The authors declare that all the data supporting the findings of this study are available within the article and its Supplementary Information files and from the corresponding authors on reasonable request.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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