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Molecules and Cells

Inhibition of Overexpressed CDC-25.1 Phosphatase Activity by Flavone in *Caenorhabditis elegans*

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We previously reported that flavone induces embryonic lethality in *Caenorhabditis elegans*, which appeared to be the result of cell cycle arrest during early embryogenesis. To test this possibility, here we examined whether flavone inhibits the activity of a key cell cycle regulator, CDC-25.1 in *C. elegans*. A gain-of-function *cdc-25.1* mutant, *rr31,* which exhibits extra cell divisions in intestinal cells, was used to test the inhibitory effects of flavone on CDC-25 activity. Flavone inhibited the extra cell divisions of intestinal cells in *rr31*, and modifications of flavone reduced the inhibitory effects. The inhibitory effects of flavone on CDC-25.1 were partly, if not completely, due to transcriptional repression.

INTRODUCTION

Several properties of *Caenorhabditis elegans*, including well-defined genetic markers and mutants, a short life cycle and large brood size, and ease of culturing and handling in the laboratory, make this nematode an ideal model organism to screen drugs with useful features. We have previously shown that flavone inhibits embryogenesis, resulting in highly frequent embryonic lethality in *C. elegans* (Lee et al., 2008). The flavone 2-phenyl chromone is abundant in plants and has been used as a structural donor in antioxidant syntheses (Ono et al., 2007). Flavone is a natural flavonoid that has been used as a food supplement to effectively exploit its antioxidative activity. However, dietary flavonoids have displayed cytotoxicity by causing cell cycle arrest (Zhang et al., 2008). Furthermore, the embryonic lethality induced by flavone in our previous study appeared to be caused by cell cycle arrest (Lee et al*.,* unpublished data). To investigate this possibility, we have developed a system for monitoring the numbers of cell divisions in *C. elegans* mutants after treatment with flavone.

CDC25 phosphatase is a key cell cycle regulator, which promotes the cell cycle by removing inhibitory phosphate residues from target cyclin-dependent kinases (Draetta and Eckstein, 1997; Hofmann et al., 1998). *Caenorhabditis elegans* has four *cdc-25* genes, all of which have highly conserved catalytic domains (Ashcroft et al., 1998). The *cdc-25.1(gf)* mutations *rr31* and *ij48* undergo extra cell divisions in intestinal cells (Clucas et al., 2002; Kostic and Roy, 2002). These extra cell divisions are caused by the hyperstabilization of mutated CDC-25.1 proteins compared to wild-type CDC-25.1 (Hebeisen and Roy, 2008). Supernumerary divisions of intestinal nuclei have been visualized by expression of an intestine-specific GFP reporter, *rrIs01,* generating an easy assay system for counting intestinal nuclei (Clucas et al., 2002; Kostic and Roy, 2002). In this study, we examined the inhibitory effects of CDC25 inhibitors and flavones on the *cdc-25.1(rr31)* gain-of-function mutant containing the *rrIs01* reporter.

MATERIALS AND METHODS

Strains and cultures

Caenorhabditis elegans strains were maintained as described (Brenner, 1974). Strain N2 was used as the wild type and strain MR142: *cdc-25.1(rr31) I; rrIs01[elt-2::GFP; unc-119(+)]* (Kostic and Roy, 2002) as the test strain. The wild-type *cdc-25.1(+)* strain containing *rrIs01* was obtained by mating MR142 hermaphrodites with N2 males and then selecting homozygous transgenic worms with the normal number of green intestinal nuclei. Strains were provided by the *Caenorhabditis* Genetics Center (University of Minnesota). Strains were maintained at 16 or 20°C on nematode growth medium (NGM) agar plates containing fresh *E. coli* OP50.

Drug treatment

The CDC25 inhibitors 2-methyl-1,4-naphthoquinone (menadione; Sigma-Aldrich Co.) and 4-dimethylamino-2-methoxy-6-((methyl-[2-(4-nitrophenyl) ethyl] amino) methyl) phenol (BN82002; Sigma-Aldrich Co.) were used as control inhibitors. 2-Phenyl chromone (flavone) and its derivatives (ICC, INC) shown in Fig. 2C were examined for their inhibitory activities on CDC-25.1. Drugs were administered as described previously (Kim and Shim, 2008). Menadione was dissolved in ddH_2O, and BN82002 and the 11 flavonoids were dissolved in dimethyl sulfoxide (Me_2SO). Worms were cultured in liquid medium, S-basal (100 mM NaCl, 44 mM KH_2PO_4, 5.7 mM K_2HPO_4) con-

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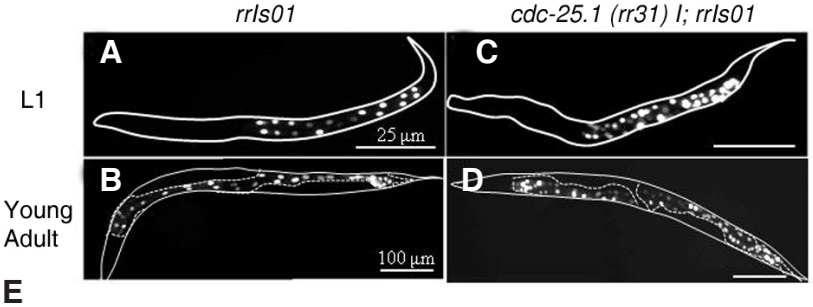

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	rrIs01		*cdc-25.1(rr31)I;rrIs01*	
Stage	Number of intestinal nuclei	N	Number of intestinal nuclei	N
L1	20.1 ± 0.2	17	38.4 ± 0.4	23
Young Adult	29.0 ± 0.5	25	54.3 ± 0.7	14

Fig. 1. Number of intestinal nuclei in *rrIs01* and *cdc-25.1(rr31) I; rrIs01* worms. The *cdc-25.1(+)* wild-type strain containing the transgene *rrIs01* displayed ~20 green intestinal nuclei at the L1 stage (A) and ~29 nuclei at the young adult stage (B). On the other hand, the *cdc-25.1* gain-of-function mutant strain *cdc-25.1(rr31) I; rrIs01* displayed ~38 intestinal nuclei at the L1 stage (C) and ~54 nuclei at the young adult stage (D). Numbers of intestinal nuclei are summarized in the table (E). Numbers are presented as means ± SD. N, numbers of worms examined. In each panel, the worms are oriented as anterior to be on the left. In panels (B) and (D), intestines are outlined with dotted lines. Scale bars, 25 μm in (A) and (C) and 100 μm in (B) and (D).

taining 5 μg/ml cholesterol and *E. coli* OP50, with or without test drugs to measure the inhibitory effects on CDC-25.1. The inhibition of CDC-25.1 is represented as the relative activity of CDC-25.1, which is the percent of intestinal nuclei in test animals relative to that in control animals. Data were evaluated by Student's *t*-test, and values of $p < 0.05$ were considered significant. Fresh *E. coli* OP50 was grown in an overnight culture, collected by centrifugation, and the supernatant removed. The pellet was then resuspended with S-basal medium. Next, 50 μl of the cell suspension were dropped onto a glass slide coated with 0.1% poly-L-lysine, and the test drug was added to the medium. Finally, synchronized L4 worms were transferred to the liquid medium and incubated for 48 h at 20°C in a wet, dark chamber to avoid evaporation. L1 or young adult progeny were observed by fluorescence microscopy.

Measurement of embryonic and larval lethality

Synchronized L4 worms were transferred to a culture plate containing *E. coli* OP50 and incubated at 20°C until the worms reached the adult stage. Four adult worms were then transferred individually to new plates containing 1-4 mM menadione, every day for 3 d. Total progeny, including hatched and unhatched embryos, were counted. Embryonic lethality was calculated as the number of unhatched embryos divided by the total progeny. Larval lethality was calculated as the number of worms that did not reach the adult stage divided by the number of hatched embryos. Embryonic lethality and larval lethality are expressed as the mean of four independent experiments with standard error of the mean (S.E.M).

Quantitative real-time PCR (qRT-PCR)

Synchronized L4 stage worms of the *rr31* mutant were treated with 0.5% Me_2SO, 0.5 mM BN82002, 5-hydroxyflavone, 2′-methoxyflavone, or 6-methoxyflavone. After reaching the gravid adult stage, they were collected into 300 μl of TRIzol® (Invitrogen) and freeze-thawed three times in liquid nitrogen. Total RNA was isolated using Phase Lock Gel (PLG; Qiagen) with 1 μl Linear Polyacrylamide (LPA; Ambion Inc.). The quality and quantity of extracted RNA were monitored by UV absorbance. The cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen) and oligo-dT primer (Bionics). qPCR was performed using Power SYBR® Green PCR Master Mix in a 96-well plate and a 7500 real-time PCR system (Applied Biosystems). Primer sequences for the internal control, *act-1,* were: forward, 5′-CCA GGA ATT GCT GAT CGT ATG CAG AA-3′, and reverse, 5′-TGG AGA GGG AAG CGA GGA TAG A-3′ (GenBank accession No. NM_073418). The primer sequences for *cdc-25.1* were: forward, 5′-ATG TTA TCA AGG TCG TCT AGT G -3′, and reverse, 5′-CTA AAT CAT TCT CCG ATT CCG T-3′ (GenBank accession No. NM_059461). The expression level of *cdc-25.1* in each treatment was normalized to that of *act-1*. Three independent experiments were performed for each treatment and are expressed as the mean ± standard error of the mean (S.E.M) for comparison. Triplicate samples were measured in each experiment. PCR conditions were as follows: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

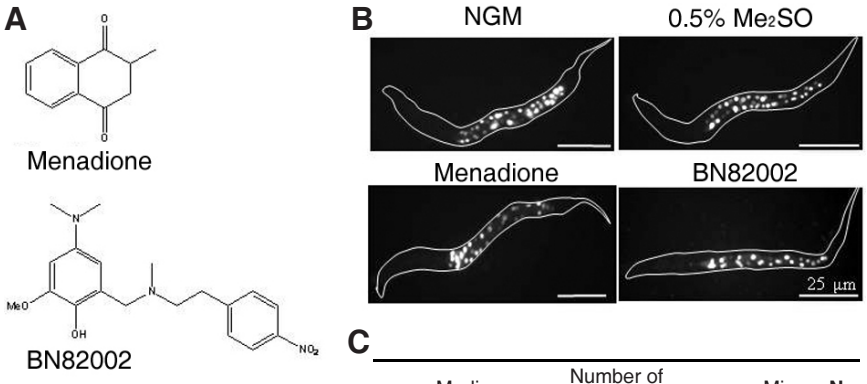
Fluorescence microscopy

Synchronized L1 or young adult stage worms containing *elt-2::GFP* transgene *rrIs01* treated with drugs were examined for their numbers of intestinal nuclei by fluorescence microscopy. Worms treated with drugs on poly-L-lysine-coated slides were harvested, washed with M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$), and mounted on a 4% agar pad, covered with a cover slip, and sealed with transparent nail polish prior to observation. The mounted specimens were observed under a fluorescence microscope (Axioskop 2 MOT; Zeiss). The images were captured using an Orca ERG digital camera (Hamamatsu) and processed with Openlab software (version 5.0; Improvision).

RESULTS

Development of a screening system for inhibitors targeting CDC-25.1 phosphatase in *C. elegans*

The *C. elegans* strain MR142 that contains the *cdc-25.1(rr31)* gain-of-function mutation, along with the transgene *rrIs01[elt-2::GFP; unc-119(+)]* (Kostic and Roy, 2002), was examined to determine whether it can be used to screen CDC25 inhibitors. In MR142 worms, the hyperstabilization of CDC-25.1 causes hyperplasia of intestinal nuclei, which can be visualized by the intestine-specific GFP reporter *elt-2::GFP* in *rrIs01* (Figs. 1C and 1D). On average, the *cdc-25.1(rr31)* mutant produced 38 intestinal nuclei in the L1 stage and 54 nuclei in the young adult stage, while the wildtype had 20 and 29 nuclei, respectively (Fig. 1E). The treatment of *cdc-25.1(RNAi)* to the *rr31* mutant restored the normal number of intestinal nuclei (Kostic and Roy, 2002). This suggests that *cdc-25.1* is required for hyperplasia of intestinal nuclei and that suppression of CDC-25.1 hyperactivity by drugs can be monitored by simply counting the number of intestinal nuclei visualized by *elt-2::GFP*. To test this possibility,



C

Medium	Number of intestinal nuclei	Min.	N
NGM	38.4 ± 0.4	35	23
0.5% Me_2SO	37.2 ± 0.3	34	35
0.5 mM Menadione	32.3 ± 0.4 (84.1%)*	28	38
0.5 mM BN82002	31.6 ± 0.5 (84.9%)*	26	31

Fig. 2. Treatment of *cdc-25.1(rr31) I; rrIs01* worms with menadione and BN82002. (A) Structures of 2-methyl-1,4-naphthoquinone (menadione) and 4-dimethylamino-2-methoxy-6-((methyl-[2-(4-nitrophenyl) ethyl] amino) methyl) phenol (BN82002). (B) Intestinal nuclei in *cdc-25.1(rr31) I; rrIs01* worms grown on NGM, NGM + 0.5% Me_2SO (0.5% Me_2SO), NGM + 0.5 mM menadione (Menadione), or NGM + 0.5 mM BN82002 (BN82002). Worms are at the L1 stage. In each panel, worms are oriented as anterior to be on the left. Scale bars, 25 μm. (C) Numbers of intestinal nuclei in worms grown in each condition are summarized. Numbers are presented as means ± SD. N, numbers of worms examined. *Relative CDC-25.1 activity is expressed as a percentage of the number of intestinal nuclei in worms treated with drugs divided by that in the control worms grown on either NGM (for menadione) or Me_2SO (for BN82002). Min, minimal number of intestinal nuclei observed.

we treated MR142 worms with two CDC25 inhibitors, menadione and BN82002 (Fig. 2A). These small molecules were identified as potent inhibitors of all three human CDC25s by their irreversible binding to the catalytic domain of CDC25 (Contour-Galcera et al., 2004). The catalytic domain of CDC25 is called the rhodanese domain, which is highly conserved from yeast to humans including *C. elegans*, suggesting that menadione and BN82002 have the potential to suppress hyperplasia of intestinal nuclei in MR142 worms by inhibiting CDC-25.1 activity. We found that both menadione and BN82002 displayed inhibitory effects on CDC-25.1 activity and reduced the number of intestinal nuclei to 84.1 and 84.9% that of their controls, respectively (MR142 worms grown on normal NGM plates were the control for menadione-treated worms, and worms treated with 0.5% Me_2SO were the control for BN82002-treated worms) (Figs. 2B and 2C). Although treatment with 0.5 mM menadione or 0.5 mM BN82002 reduced the number of intestinal nuclei in MR142, neither reduced the number to the wild-type control level, *i.e.,* to 20 in L1 worms (Fig. 1E). Higher concentrations of the drugs were tested to look for enhancement of the inhibitory effects, but these concentrations induced high embryonic and larval lethality. When 1 mM menadione was administered to L4 hermaphrodites, the embryonic lethality and larval lethality of the progeny were 26.5 ± 11.1 and 62.3 ± 15.9%, respectively. Therefore, we could not assess the effect of the drugs on hyperplasia of intestinal nuclei at concentrations above 0.5 mM.

Hyper, but not basal, CDC-25.1 activity is repressed effectively by CDC25 inhibitors

Treatment with menadione or BN82002 may also affect cell divisions in other tissues as in the intestinal cells. To examine this possibility, we administered 0.5 mM menadione and 0.5 mM BN82002 to the wild-type strain containing *rrIs01*[*elt-2::GFP; unc-119(+)*]. The worms treated with drugs exhibited no obvious defects; their morphology and movement were normal. Furthermore, neither of the CDC25 inhibitors caused a reduction in the normal numbers of intestinal nuclei in the *cdc-25.1(+)* worms at both L1 and young adult stages (data not shown). Treatment of *cdc-25.1(RNAi)* to wild-type N2 worms caused high embryonic lethality (Ashcroft et al., 1999), but the treatment of *cdc-25.1(RNAi)* to *cdc-25.1(rr31)* gain-of-function mutants did not (Kostic and Roy, 2002). Many *cdc-25.1(rr31)* worms developed normally after 24 h of RNAi treatment, but 50% of the worms contained normal numbers of intestinal nuclei (Kostic and Roy, 2002), indicating that although the hyper CDC-25.1 activity that caused hyperplasia of intestinal nuclei was repressed effectively by RNAi of *cdc-25.1*, the basal CDC-25.1 activity required for normal development was not repressed as easily by the RNAi in MR142. Similarly, although applying CDC25 inhibitors to *cdc-25.1(rr31)gf* mutants could effectively repress the hyper CDC-25.1 activity that caused hyperplasia (Figs. 2B and 2C), they could not deplete the basal CDC-25.1 activity required for normal intestinal nuclear divisions in *cdc-25.1(+)*; *rrIs01* worms. These findings suggest that at least two quantitatively different levels of repression may exist for CDC-25.1 activity. Hyper CDC-25.1 activity and the resultant phenotype, which requires high levels of CDC-25.1, is repressed by inhibitors more easily than the basal CDC-25.1 activity and its phenotype, which requires only a residual amount of CDC-25.1; the complete depletion of CDC-25.1 activity is required to achieve repression of this phenotype.

Flavone inhibits CDC-25.1 activity

Flavone and ten of its derivatives were tested for inhibition of CDC-25.1 activity. The structure of flavone and its modified positions are illustrated in Fig. 3A. The most effective inhibitor of CDC-25.1 activity was flavone itself. It repressed CDC-25.1 activity to 68.2% that of the control after treatment. However, modifications of flavone lowered the inhibitory effects (Figs. 3B and 3C). It is unclear whether CDC-25.1 is the direct target of flavones. However, the inhibition of extra nuclear divisions in *cdc-25.1(rr31)* mutants by flavones indicates that they can inhibit CDC-25.1 activity as effectively as other CDC25 inhibitors. Similar to the effects of menadione and BN82002 described above, flavone treatment to the wild-type strain containing *rrIs01*[*elt-2::GFP; unc-119(+)*] displayed no obvious defects.

Some flavones repress *cdc-25.1* mRNA levels

To investigate the possibility that flavone treatment can repress

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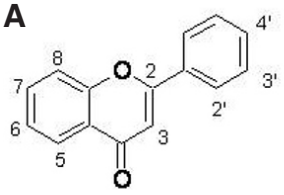

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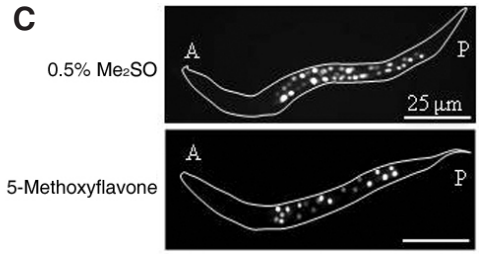

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Flavone derivatives	Name	Number of intestinal nuclei	Relative activity (%)*	Min.	N
	0.1% Me_2SO	36.3 ± 0.2	100	34	66
	0.5% Me_2SO	37.7 ± 0.3	100	34	79
1	Flavone	25.2 ± 1.3	68.2	18	13
2	5-Hydroxyflavone	28.2 ± 0.6	73.5	22	42
3	6-Hydroxyflavone	30.4 ± 0.6	80.6	21	51
4	2′-Hydroxyflavone	31.6 ± 0.7	83.9	23	30
5	5,4′-Dihydroxyflavone	31.8 ± 0.6	84.3	23	35
6	5,7-Dihydroxyflavone	32.2 ± 0.4	85.5	26	41
7	7,8-Dihydroxyflavone	31.1 ± 0.4	82.4	21	59
8	2′-Methoxyflavone	28.2 ± 0.7	74.8	23	29
9	5-Methoxyflavone	30.6 ± 0.5	84.2	22	41
10	6-Methoxyflavone	30.3 ± 0.5	80.4	22	55
11	7-Methoxyflavone	32.6 ± 0.5	86.5	24	46

Fig. 3. Treatment of *cdc-25.1(rr31) I; rrIs01* worms with flavone and its derivatives. (A) Structure of flavone (2-phenyl chromone). (B) List of flavone and its derivatives with their inhibitory effects on CDC-25.1 activity. Numbers of intestinal nuclei are presented as means ± SD. *Relative CDC-25.1 activity is expressed as a percentage of the number of intestinal nuclei in worms treated with flavones divided by that in the control worms grown on either 0.1% Me_2SO (for flavone and 5-methoxyflavone) or 0.5% Me_2SO (for the rest). Treatment concentrations were 0.1 mM for flavone, 0.1 mM (in B) or 0.5 mM (in C) for 5-methoxyflavone, and 0.5 mM for the remaining flavone derivatives. Min, minimal number of intestinal nuclei observed. N, numbers of worms examined. (C) Intestinal nuclei in *cdc-25.1(rr31) I; rrIs01* worms grown on NGM + 0.5% Me_2SO and NGM + 0.5 mM 5-methoxyflavone. Worms are at the L1 stage. In each panel, worms are oriented as anterior to be on the left. Scale bars, 25 μm.

CDC-25.1 activity at the mRNA level, we measured the expression levels of *cdc-25.1* mRNA in worms after treatment with 0.5 mM 5-hydroxyflavone, 2′-methoxyflavone, or 6-methoxyflavone, and 0.1 mM flavone. These treatments repressed the *cdc-25.1* mRNA level to 73.7, 84.4, 75, and 18.2% that of the control, respectively (Figs. 4A and 4B). The effects of flavone and 5-hydroxyflavone were significant ($p < 0.05$). Although the p values were greater than 0.05, the mRNA level repression was consistent upon treatment with the other flavones.

DISCUSSION

To develop a simple and rapid screening system for cell cycle inhibitors we tested a *C. elegans* strain as a model system with CDC-25.1 as the target. We found that strain MR142: *cdc-25.1(rr31) I; rrIs01* was very useful to screen cell cycle inhibitors because the repression of hyperplasia of intestinal nuclei by the inhibitors could be visualized easily and unambiguously using an intestine-specific GFP reporter in this strain.

The *rr31* gain-of-function mutation in *cdc-25.1* alters the first base of exon 2, causing a glycine to aspartic acid substitution at position 47 of the protein, which is located in the N-terminal destruction motif of CDC-25.1 recognized by the *C. elegans* β-TrCP ortholog LIN-23 (Hebeisen and Roy, 2008; Kostic and Roy, 2002). Consequently, the *rr31(gf)* mutation impairs efficient LIN-23-mediated degradation of the mutant CDC-25.1 protein over a precise developmental time window during embryogenesis, resulting in supernumerary cell divisions in the intestine (Hebeisen and Roy, 2008). The catalytic rhodanese domain of CDC-25.1 is in the C-terminal region of the protein, from amino acid positions 309-422 (Ashcroft et al., 1998). Menadione and BN82002 bind to the catalytic domain of CDC-25 to inhibit the phosphatase activity (Brezak et al., 2004). Therefore, they were able to repress the overexpressed mutant CDC-25.1 activity in the *rr31* mutants and consequently reduce the number of intestinal nuclei (to 84.1% for menadione and 84.9% for BN82002). The extent of repression increased with increasing concentrations of the drugs used (data not shown), but highly frequent embryonic lethality and larval lethality were observed upon drug treatment at high concentrations. Menadione and BN82002 failed to decrease the number of intestinal nuclei in the *cdc-25.1(+)* wild-type strain. This finding indicates that although the hyper CDC-25.1 activity in the *cdc-25.1(rr31)* mutant, which caused hyperplasia of intestinal nuclei, can be readily repressed, the basal CDC-25.1 activity required for normal intestinal nuclear divisions in *cdc-25.1(+)* worms cannot be depleted easily by these drugs. This finding may also suggest several aspects of the repression mechanism of CDC-25.1 activity by the drugs, as follows. (1) Hyper CDC-25.1 activity and the resultant phenotype can be more easily repressed by the cell cycle inhibitors, likely because they require higher levels of CDC-25.1. (2) Basal CDC-25.1 activity and its phenotype are more resistant to the inhibitors, likely because they require only a residual amount of CDC-25.1 activity, and the activity of CDC-25.1 remaining after drug treatment may be sufficient to undergo cell divisions. (3) Therefore, there may exist at least two quantitatively different levels of repression of CDC-25.1 activity. The treatment of *cdc-25.1(RNAi)* to the *rr31* mutants resulted in the normal number of intestinal nuclei without any other defects in the worms (Kostic and Roy, 2002), whereas the treatment of *cdc-25.1(RNAi)* to the *cdc-25(+)* wild-type strain caused highly frequent embryonic lethality due to abnormal cell divisions (Ashcroft et al., 1999). Considering the fact that the RNAi did not lead to the complete depletion of *cdc-25.1* mRNA, these results suggest that the remaining *cdc-25.1* mRNA after the RNAi might be enough to support normal numbers of intestinal nuclear division in *rr31* mutants. In addition, biochemical studies to compare the properties of mutated and

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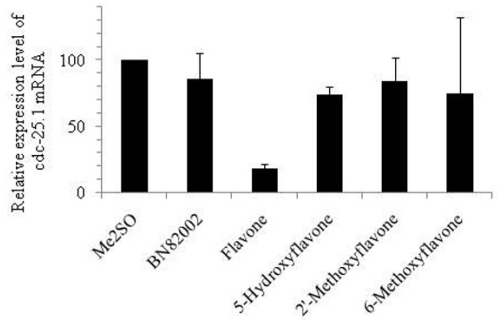

B

	Relative expression level of *cdc-25.1* mRNA (%)	*P* value
Me_2SO	100	-
BN82002	85.4 ± 20.2	0.50993
Flavone	18.2 ± 2.9	0.001287
5-Hydroxyflavone	73.7 ± 5.8	0.010587
2′-Methoxyflavone	84.4 ± 17.8	0.430313
6-Methoxyflavone	75.0 ± 57.4	0.600631

Fig. 4. Expression levels of *cdc-25.1* mRNA in the *rr31* mutant worms treated with flavones. Expression levels of *cdc-25.1* mRNA were measured by qRT-PCR using total RNA extracted from worms grown on respective drug-containing medium. Expression levels of *cdc-25.1* mRNA after each treatment were normalized to those of *act-1* mRNA, which served as the internal control, to make *cdc-25.1* gene expression levels comparable among treatments. Three independent experiments were performed for each treatment. Values are presented as means ± S.E.M. in the plot (A) and the table (B). Concentrations of 0.5 mM BN82002, 5-hydroxyflavone, 2′-methoxyflavone, and 6-methoxyflavone were administered, whereas only 0.1 mM flavone was given to worms due to highly frequent larval lethality at higher concentrations. The relative expression levels of *cdc-25.1* mRNA after each drug treatment compared to that in the Me_2SO control are summarized. The *p* values for mean differences were calculated to evaluate the significance of expression levels compared to that in Me_2SO controls using Student's *t*-test.

normal CDC-25.1 proteins may be required to distinguish whether the mutant CDC-25.1 protein is more sensitive to the drugs at the protein level. How flavones inhibit CDC-25.1 activity remains unknown. Flavone was reported to down-regulate proto-oncogenes and to inhibit cell proliferation by modulating gene expression in breast cancer cell lines (Ullmannova and Popescu, 2007). Dietary flavonoids that exhibited anti-cancer effects were reported to suppress gene expression of proto-oncogenes while activating gene expression of tumor suppressor genes (Hebeisen and Roy, 2008; Imada et al., 2008; Ullmannova and Popescu, 2007). These findings suggest that one possible way for flavone to inhibit CDC-25.1 activity is through transcriptional regulation of gene expression. Indeed, we found that some flavones reduced the mRNA level of *cdc-25.1*. It is unclear whether the reduced mRNA level is attributable to transcriptional repression, mRNA instability, or something else. Furthermore, the extent of inhibition of CDC-25.1 activity by flavones did not necessarily coincide with the reduced levels of *cdc-25.1* mRNA. In the case of flavone, it reduced the CDC-25.1 activity to 68.2% (Fig. 3B), although the *cdc-25.1* mRNA level was reduced to 18.2% that of the non-treated control (Figs. 4A and 4B). However, we consider that it is still likely that flavones can regulate or modulate the gene expression of *cdc-25.1*.

In this study we have shown that *C. elegans* strain MR142, which contains the *cdc-25.1* gain-of-function mutation *rr31* along with an intestine-specific GFP reporter *elt-2::GFP*, is an excellent animal system to screen drugs that target CDC25. This *C. elegans* system may also be useful for developing anti-cancer drugs. Mammals have three *cdc25* genes, *cdc25A*, *cdc25B*, and *cdc25C* (Sadhu et al., 1990). These are essential in cell cycle regulation, and their mutations lead to carcinogenesis (Boutros et al., 2007). Cdc25A and Cdc25B interact with Raf1 kinase in both human and mouse cells, suggesting that Cdc25s are proto-oncogenes (Galaktionov et al., 1995). This idea is supported by the observation of overexpression of CDC25A and CDC25B in a variety of human cancers. A high incidence of CDC25 overexpression was observed in prostate cancers (97%; Ngan et al., 2003), hepatocellular carcinoma (78%; Xu et al., 2003), and gastric cancers (78%; Xing et al., 2008). These results suggest the reduction of CDC25 activity as a potential cancer treatment, and the system we have developed in this study may be applicable.

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