Identification of a protein that interacts with *Caenorhadbitis elegans* CLK-2 in a yeast two-hybrid assay

Ying Wang
Department of Biology
McGill University, Montreal

June 2003
A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the Master degree of Science

ABSTRACT

The gene clk-2 of C. elegans is required in both the germline and the soma, for subsequent embryonic viability, and for developmental and behavioural rates, respectively. clk-2 encodes a protein that is homologous to Tel2p in yeast, which is required for the telomere length regulation. It has been demonstrated that clk-2 affects telomere length also in worms and human cells. By now the exact biochemical function of CLK-2 is unknown. In order to shed light onto the function of the gene clk-2, a two-hybrid screen was carried out to identify the interactors of the protein CLK-2. A potential interactor of CLK-2, Y105C5B.19, was identified in the screen. Y105C5B.19 is a novel gene and does not have homologues in other species. Y105C5B.19 contains an MSP (major sperm protein) domain, therefore it is possible that it could be involved in the processes that regulate oocyte maturation, gonadal sheath contractions, or sperm mobility. Interestingly, given that *clk-2* is required for subsequent embryogenesis at some point during a narrow time between the end of oocyte maturation and the 2-cell stage, it is tempting to speculate that the interaction between CLK-2 and Y105C5B.19 might be functionally relevant. The lethality of *clk-2(qm37)* mutants might result from delayed consequences of defects in ovulation and/or fertilization, and perhaps such defects could result from the disruption of the interaction between CLK-2 and Y105C5B.19. The amino acid substitution C772Y resulting from the *clk-2(qm37)* mutation was found to disrupt the interaction between CLK-2 and Y105C5B.19 in a two-hybrid assay, lending support to the idea that the interaction between CLK-2 and Y105C5B.19 takes place *in vivo*.

RÉSUMÉ

La fonction du gène *clk-2* du nématode *C. elegans* est requise dans le soma pour la régulation des rythmes du développement et du comportement, ainsi que dans la lignée germinale pour le développement embryonnaire ultérieur. *clk-2* code pour une protéine qui est similaire à la protéine Tel2p de la levure. *clk-2*, comme *tel2* chez la levure, participe au maintien d'une longeur normale des télomères chez le ver et dans les cellules humaines. Cependant, la fonction biochimique de la protéine CLK-2 reste inconnue. Afin de dévoiler sa fonction, un criblage 'double hybride' a été effectué pour identifier des protéines qui interagissent avec CLK-2. Une protéine qui interagit avec CLK-2 dans le crible 'double hybride' a été ainsi identifiée, Y105C5B.19. Cette protéine est nouvelle et n'a pas d'homologues dans d'autres espèces. Elle contient cependant un domaine MSP (pour 'major sperm protein'), et donc il est possible qu'elle participe à la régulation de la maturation des oocytes, aux contractions de la gonade, et/ou à la mobilité du sperme. Étant donné que la fonction de *clk-2* est requise entre la fin de la maturation des oocytes et le stade embryonnaire de deux cellules pour le développement embryonaire, il semble possible que l'interaction entre CLK-2 et Y105C5B.19 soit importante pour la fonction de CLK-2. Ainsi, la létalité des mutants *clk-2(qm37)* pourraient être la conséquence de défauts lors de l'ovulation et/ou fertilization, due à l'absence d'interaction entre Y105C5B.19 et CLK-2. La substitution d'une cystéine par une tyrosine au résidu 772 de la protéine CLK-2, qui correspond à la mutation *clk-2(qm37)*, brise l'interaction entre CLK-2 et Y105C5B.19 et donc suggère qu'eu ces deux protéines interagissent *in vivo*.

ACKNOWLEDGMENTS

I would like to express my gratitude to all those who gave me the possibility to complete this thesis. The first person I would like to thank is my supervisor **Siegfried Hekimi**, whose help, stimulating suggestions and encouragement helped me in all the time of research. Under his guidance, I successfully overcame many difficulties and learn a lot about research work. His overly enthusiasm and integral view on research has made a deep impression on me.

Second, my great appreciation goes to **Claire Bénard**, who taught me genetics, worm manipulations and molecular biology. She kept an eye on the progress of my work and was always available when I needed her help. Whenever I have questions, she always was there to help me and explained in detail. I would also express my thanks for her much precious instruction at very step of this thesis and correcting and offering suggestions for improvement. I own thanks to her also for her kind help and consideration about my personal life.

I am grateful to lab members in Dr Hekimi lab. Thanks to **Ning Jiang, Liesbeth de Jong** and **Madjid Hihi** for their helpful advice throughout this work. I would also like to acknowledge **Hania Kéhir** for her kind guidance about western blots. I own thanks to **Robyn Branicky** for providing me *dsc-1 ds*RNA and other kind help. I also would like to thank **Melissa Carroll, Irene Oviedo Landaverde** for sharing happiness with me, as well as other past and present lab members.

I owe thanks to **Huijuan Li**, a former post-Doctor in **Dr Bussey's** lab. She helped me a lot in my experiments with two-hybrid screening. I would like to thank **Anne-Marie Sdicu**, the senior technician, and Ph.D student **Federico Carlo Angioni** in Dr Bussey's lab for all their kind and generous help with yeast manipulation.

I am also thankful to Professor **Christian Hardtke** and Professor **Monique Zetka** for being my supervisory committee meeting members and taking effort in reading and providing me with valuable comments on my progress report.

I would like to acknowledge Yuji Kohara for cDNA clones and Andrew Fire for the RNAi vector.

Finally, special thanks to my family, particular to my sister. Without their endless love for me, I would have never achieved my current position. Heartfelt thanks to my husband Zhan for his love, with that I have never felt alone.

CONTENTS

LISTS OF TABLES

INTRODUCTION

MATERIALS AND METHODS

RESULTS

LIST OF FIGURES

INTRODUCTION

RESULTS

DISCUSSION

INTRODUCTION

C. elegans as a model organism

C. elegans is a good model for studying numerous biological processes, including development, behaviour, nerve function and the genetic basis of life span, due to their particular biological characteristics. Firstly, *C. elegans* is a predominantly self-fertilizing hermaphrodite and hence homozygosity can be easily created and is well tolerated. Although *C. elegans* males are produced at very low frequency, they make it possible to perform genetic crosses. Secondly, *C. elegans* is a multi-cellular organism. They have a high degree of cell differentiation and specialization, and distinct somatic and germ-line cell lineages (Gershon and Gershon, 2002). Third, the worm body is transparent, so its cells are easily visualized by microscopy. It has a constant cell number and stereotyped cell positions. Furthermore, *C. elegans* can be easily raised and maintained under simple laboratory conditions. Generally, the worms are grown on petri plates seeded with bacteria. Mutants can be obtained easily by chemical mutagenesis or exposure to ionizing radiation. A large number of *C. elegans* strains can be stored for very long period of time, since they can be frozen in liquid nitrogen indefinitely.

clk genes

clk stands for 'abnormal function of biological clocks'. By now 11 *clk* genes have been isolated, including *clk-1, clk-2, clk-3, clk-4, clk-5, clk-6, clk-7, clk-8, clk-9, clk-10* as well as *gro-1*(Hekimi et al., 1995; Hodgkin and Doniach, 1997; unpublished data). The *clk* mutants share a number of pleiotropic phenotypes known as "the clock phenotype". They develop slowly on average at both the embryonic and postembryonic stages. Rhythmic behavioural

rates, including swimming, pharyngeal pumping and defecation, are also slowed down. In addition, their life span is extended (Hekimi et al., 1998).

Except for *gro-1,* all other *clk* genes are identified in a screen for viable maternal-effect mutations. A maternal effect can be detected when homozygous mutants issued from heterozygous hermaphrodites are wild type, and only the homozygous mutants issued from homozygous mothers display mutant phenotypes (Hekimi et al., 1995). The mechanism of action of the *clk* genes is being extensively studied. Evidence shows that increased life span of the *clk* mutants is likely a consequence of reduced physiological rates. However, *clk* mutations seem to slow physiological rates by different mechanisms, because various combinations of *clk* double mutants, like *clk-1clk-2, clk-3clk-2 and clk-3clk-1* can live much longer than single *clk* mutants (Hekimi et al., 2001). So far, it is unclear how the mutations in *clk* genes slow the worm's physiological rates. The *clk* mutants, when combined with *daf-2,* which is involved in the dauer formation pathway and whose mutations lead to highly resistant worms that have extended life-span, live much longer than the single mutants, suggesting that *clk* mutations increase the life span in different ways from that of the dauer pathway (Hekimi et al., 1998).

clk-1 is the *clock* gene which has been best characterized. It encodes a mitochondrial hydroxylase, which is involved in ubiquinone biosynthesis, where it converts demethoxyubiquinone (DMQ) to 5-hydroxyubiquinone (UQ) (Stenmark et al., 2001). The function and subcellular localization of CLK-1 appear to be highly conserved from yeast to mammals (Ewbank et al., 1997). It has been demonstrated that ubiquinone is an essential electron carrier in the mitochondrial respiratory chain and it is required not only in mitochondria, but also at non-mitochondrial sites, playing numerous roles in cells (Hihi et al.,

2002). *clk-1* mutants do not synthesize UQ_9 (the subscript refers to the length of the isoprenoid side chain), instead its precursor DMQ_9 is accumulated. DMQ_9 can replace in part the function of UQ_9 at the mitochondrial respiratory chain, but is not capable to carry out functions of UQ_9 at non-mitochondrial sites (Hihi et al., 2002). Although it is still unclear how the defects of ubiquinone contribute to the *clk-1* phenotypes, evidence indicates that the life span extension of *clk-1* mutants may result from decreased ROS level. In *clk-1* mutants the accumulation level of the by-products of oxidative damage is likely lower than in wild-type worms (Hekimi and Guarente, 2003). The most important organelles for ROS production are the mitochondria, where ROS is produced during the electron transport process in the respiration chain. Cytoplasm is another source of ROS production. Given that the mitochondrial respiration level is normal in *clk-1* mutants and ubiquonone is also used at non-mitochondrial sites in cells, ROS reduction in *clk-1* mutants may result from decreased production of extramitochondrial ROS. ROS are short-lived toxic molecules that react with macromolecules, including nucleic acids, proteins and lipids, and inhibit or damage their function. The theory that ROS damage is the major cause of aging is supported by the observations that many age-related pathologies result from the damage of ROS to macromolecules (Hekimi and Guarente, 2003).

The gene *clk-2*

The gene *clk-2*, which is defined by the recessive temperature-sensitive mutant allele *qm37,* belongs to the *clk* gene family (Hekimi et al., 1995; Benard et al., 2001). *clk-2(qm37)* mutants display Clk phenotypes: compared with wild-type (N2) worms, they develop more

slowly and their behavioural rates, including defecation, pumping and egg-laying, are dramatically slowed down. Also, their life span is extended. In addition, the mutation is temperature sensitive. At the permissive temperature (15-20°C), the *clk-2(qm37)* mutants are viable, their embryos all develop and grow up to become long-lived adults. But at the restrictive temperature (25°C), the *qm37* mutants display a more severe phenotype, that is embryonic developmental arrest. At 25°C all *clk-2(qm37)* embryos arrest development at various developmental stages, which appears to be the null phenotype of *clk-2*, since this is also produced by RNA interference treatment with *clk-2 ds*RNA at all temperatures. The *clk-2(qm37)* mutants exhibit the temperature sensitivity through their whole life cycle. Shifting the mutants at any stage (for larval to adulthood) from 20°C to 25°C results in sterility and unhealthy appearance (Benard et al., 2001), suggesting that *clk-2* is required throughout the entire life cycle of the worm.

At both temperatures 20°C and 25°C, developmental and behavioural phenotypes of *clk-2(qm37)* mutants can be fully maternally rescued (Benard et al., 2001). All progeny from heterozygous *clk-2(qm37)/clk-2*(+) mothers develop and grow up to become wild-type adults. However, the reduction of brood size at the permissive temperature (20°C) can only be partially maternally rescued. At the restrictive temperature (25°C), the maternal-rescue effect fails to rescue the fertility: homozygous progeny from heterozygous mothers can develop into wild-type adults, but they produce only a few dead eggs. In addition, the maternal effect is strict at 25°C: homozygous *clk-2(qm37)* mutants mated with wild-type males produce only dead progeny, and only the presence of a wild-type copy of *clk-2* in mothers can rescue normal embryonic development (Benard et al., 2001).

Another allele of *clk-2*, *rad-5(mn159)*, was isolated as a *C. elegans* UV-radiation-hypersensitive mutant. The mutation is defective for ionizing-radiation damage-induced cell cycle arrest. In wild-type worms, mitotic cells in the germline transiently arrest cell proliferation following radiation, but in *mn159,* the mitotic cells continue to proliferate after radiation. In addition, unlike wild-type worms, the *mn159* germline meiotic cells do not undergo apoptosis following irradiation (Ahmed et al., 2001). *clk-2(qm37)* mutants display the same abnormal responses to radiation. Furthermore, *mn159* mutants display similar Clk phenotypes as *clk-2(qm37)* mutants, such as slow growth, extended life span, lethality at 25°C and a maternal effect (Ahmed et al., 2001). *clk-2* is the only *clock* gene which has been shown to be required for the normal response to ionizing radiation.

Since *clk-2* mutants are defective for ionizing-radiation-damage-induced cell cycle arrest and apoptosis, it has been suggested that CLK-2 is a DNA damage checkpoint protein. However, as irradiation damages biological macromolecules through the production of reactive oxygen species (ROS), it might be possible that in *clk-2* mutants the failure to respond properly to irradiation does not result from their defects in a DNA damage checkpoint, but is due to decreased sensitivity to oxidative stress or abnormal response to oxidative damage. In fact, overexpression of human CLK-2 (hCLK2) does not show hypersensitivity to the agents or treatments that can damage DNA directly, but leads to an increased apoptotic response to oxidative stress (Jiang et al., 2003). In addition, it has been demonstrated that the human cells with overexpressed human CLK-2 are also hypersensitive to HU (hydroxyurea), a drug that blocks DNA replication. However, HU is also an oxidizing agent (Jiang et al., 2003). Thus, CLK-2 might not be involved in a DNA damage checkpoint, but involved in the cellular reaction to oxidative stress or damage (Jiang et al., 2003).

The function of CLK-2 is conserved from yeast to human

CLK-2 protein is homologous to *Saccharomyces cerevisiae* Tel2p and has a unique homologue in every eukaryotic genome examined. The similarities between the *C. elegans* CLK-2 and yeast Tel2p are throughout the whole CLK-2 protein, but similarity is low and can only be picked up convincingly by Psi-BLAST (Benard et al., 2001).

S. cerevisiae TEL2 is an essential gene which functions in the regulation of telomere length. *C. elegans* CLK-2 and human CLK-2 also show effects on telomere. However, the effects are different. In yeast, a partial loss-of-function mutation *tel2-1* results in telomere shortening, in *C. elegans* the *clk-2(qm37)* mutation lengthens telomeres (Benard et al., 2001), while in the human cells, overexpressing human CLK-2 increases telomere length (Jiang et al., 2003).

In addition, the *tel2* knockout yeast cells cannot divide more than 3 times and arrest development with abnormal morphology. It has been reported that the *tel2-1* mutation, like the mutations in the gene *clk-2* in *C. elegans*, is also temperature-sensitive and leads to a slow growth rate (Benard et al., 2001). Overexpression of hCLK2 decreases the population doubling time of human cells and growth arrest is detected in the human cells treated with *si*RNA of hCLK2 to knock down the hCLK-2 expression (Jiang et al., 2003).

The biochemical function of CLK-2 is unknown

The *C. elegans* CLK-2 is 877 amino acids long. The *clk-2(qm37)* mutation results in a cysteine to tyrosine substitution at residue 772 (Benard et al., 2001). The *mn159* mutation is in the N-terminal of CLK-2 protein, resulting in a glycine to cysteine substitution at residue

135 (Ahmed et al., 2001). No recognizable functional motifs or domains can be found in the protein CLK-2.

A full-length *C. elegans* CLK-2::GFP fusion protein localizes to the cytoplasm (Benard et al., 2001). The subcellular distribution of hCLK2 is ubiquitous. By immunocytochemistry, it has been shown that hCLK2 is present in all cellular compartments, including cytosol, nuclei, heavy and light membranes and both as a soluble and as a membrane-associated form (Jiang et al., 2003). Also, in worms, the CLK-2 protein is detected at all developmental stages by western blots, consistent with the requirement for *clk-2* throughout the whole life cycle of the worm.

CLK-2 protein may play important roles in many processes in cells. A way to further study CLK-2 is to identify its interactors, which will provide insights into the function of CLK-2. A screen for *clk-2(qm37)* suppressors was carried out to identify CLK-2 interactors by a genetic approach. Unfortunately, all 8 *clk-2(qm37)* suppressors that have been isolated so far are intragenic (unpublished data). Therefore, it is worth taking another approach to identify CLK-2 interactors. The research described in this thesis has been focused on the identification of CLK-2 interactors by a yeast two-hybrid approach.

The Yeast Two-Hybrid System

Yeast two-hybrid systems pioneered the field of identification of protein interactions. The basic principle of the yeast two-hybrid systems is the reconstitution of functional transcriptional activators, such as the yeast GAL4 protein. Genes encoding two-hybrid proteins are generated, by fusing the GAL4 DNA binding domain to a protein of interest

referred to as a bait (DB-X) and fusing the GAL4 activating domain to other proteins referred to as preys (AD-Y) (Colas and Brent, 1998). Interactions between baits and preys bring together the DNA binding domain and transcription activation domain thus reconstitute a functional transcriptional activator, leading to the transcription of the reporter genes downstream of GAL4 binding sites. The expression of the reporter genes can be easily detected by observing cell growth on selective media or by a quick enzyme assay (Colas and Brent, 1998; White, 1996), hence the interactions can be identified.

Over the past ten years, yeast two-hybrid systems have been proven to be extremely effective methods for identification of partners of given proteins, mapping the regions of interactions between known proteins, and more recently in deciphering the network of associations between all putative proteins encoded by a given genome (Drees, 1999). In comparison with the traditional methods for studying protein-protein interactions, yeast two-hybrid systems present several advantages. First, in yeast two-hybrid systems interactions happen *in vivo*, thus there is no need for complicated manipulation of proteins. Secondly, they are more sensitive than co-immunoprecipitation in detecting weak interactions. Lastly, yeast two-hybrid systems readily lend themselves to the design of genetic experiments, such as testing the existence of the interactions between mutant variants of proteins that are known to interact when wild-type (Luban and Goff, 1995).

For the commonly encountered difficulties of yeast two-hybrid systems, the major limitation of these systems is that they rely on transcriptional activations. DB-X and AD-Y must enter nucleus to trigger transcriptional activations. A standard two-hybrid assay gives rise to a certain proportion of false positives and false negatives. In addition, yeast two-hybrid assays cannot take into account the specificity of protein-protein interactions, so

interactions identified by two-hybrid screens should be viewed as hypotheses until they are validated by other biochemical assays. Furthermore, the yeast does not have some types of post-transcriptional modifications that could be required for interactions between higher eukaryotic proteins (Legrain and Selig, 2000).

The *Proquest* Two-Hybrid System

The *Proquest* two-hybrid system was used to carry out a yeast two-hybrid screen to identify *C. elegans* CLK-2 interactors. An overview of the system is shown in Figure 1. In this system, baits and a cDNA library are fused to the DNA binding domain and the activation domain of GAL4, respectively. This system contains modifications that further reduce false positives and false negatives, which are the major shortcomings of yeast two-hybrid systems. First, the use of low-copy number vectors, rather than high-copy vectors, provides decreased and more constant protein level, reducing false positives due to non-specific interactions and reducing false negatives resulting from toxicity of overexpressed fusion proteins. Secondly, the use of 3 independent promoters (*HIS3, GAL4, and SPAL10*) reduces false positives resulting from the interaction of the AD fusion proteins with the promoter regions of the reporter genes. Last, this system provides three reporter genes (*HIS3, lacZ,* and *URA3*) and four phenotypes to assess whether candidate yeast colonies contain interacting proteins (http://www.invitrogen.com/content/sfs/manuals/10835023.pdf *Proquest*^{TM} Two-Hybrid System Manual).

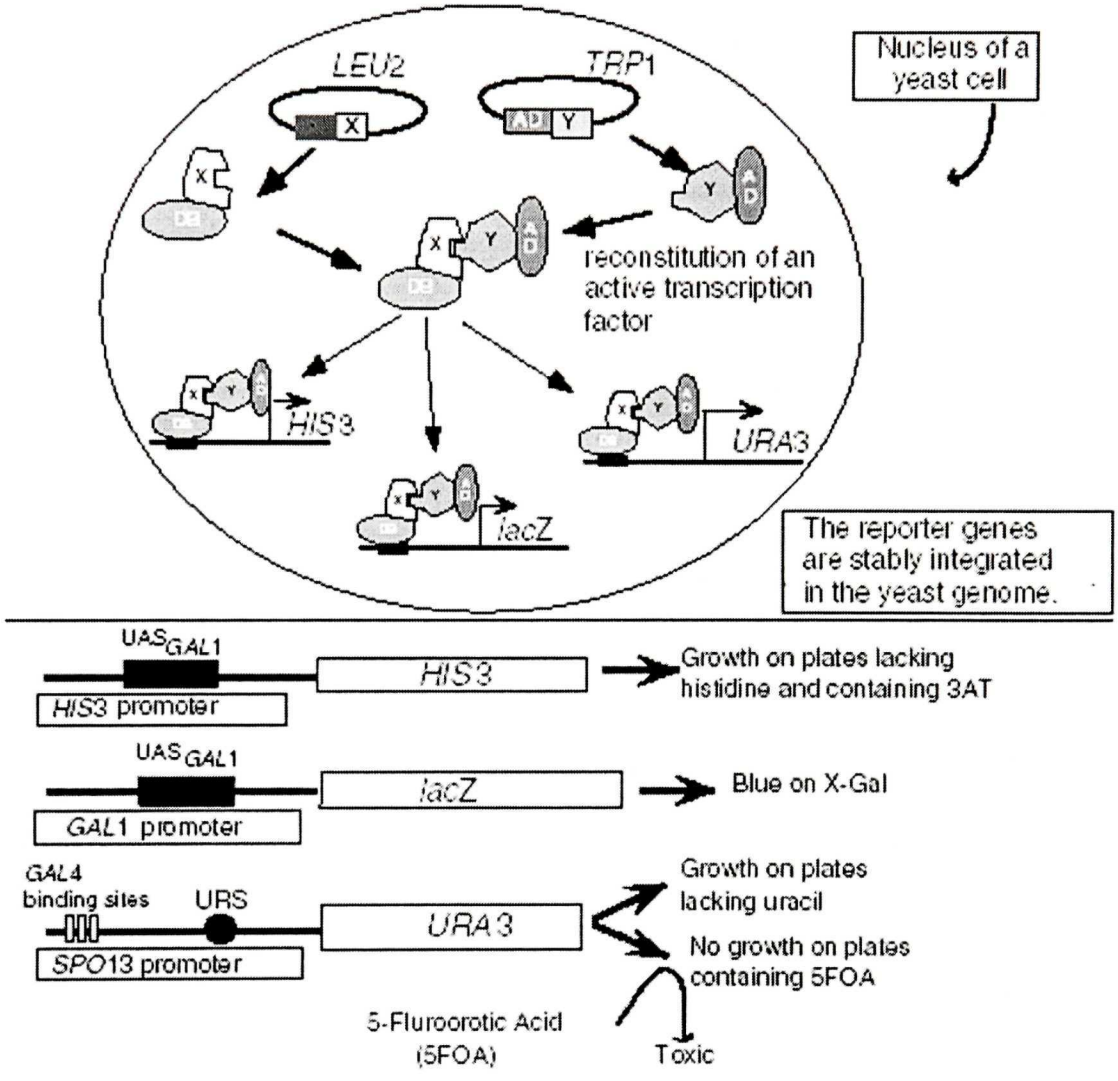
The yeast strain MaV203 used in the *Proquest* two-hybrid system contains the following features. First, a set of non-reverting auxotrophic mutations: *Leu-2* and *Trp-1* to allow

selection for DB fusions (pDBLeu-X) and AD fusions (pPC86-Y), respectively; second, a mutation of *HIS3* for growth dependence upon induction of the report gene *GAL1::HIS3;* third, the deletions of the *GAL4* and *GAL80* genes encoding GAL4 and its repressor GAL80. In the absence of GAL80, galactose is not required for activation of the GAL4 inducible promoters. The two-hybrid system contains three stably integrated low-copy GAL4-inducible reporter genes: *SPAL10::URA3* integrated at *URA3*; $HIS3_{UAS\ GAL1}$*::HIS3* integrated at *LYS2*; and *GAL1:: lacZ* integrated at an unknown locus. Furthermore, this system provides 5 yeast control strains for indicating the interaction strength between two proteins (Table 1).

This system seems to be a good two-hybrid system to identify protein-protein interactions. *Proquest* also provides *C. elegans* cDNA library, which can be used with the two-hybrid system. Dr Vidal's laboratory uses the same system and the same initial cDNA library to build the *C. elegans* protein-protein interaction map. By now, they have reported *C. elegans* interaction networks in vulva development (Walhout et al., 2000), 26S proteasome (Davy et al., 2001) and DNA damage repair (Boulton et al., 2002).

To identify *C. elegans* CLK-2 interactors, a yeast two-hybrid screen was carried out using the *Proquest* two-hybrid system and *Proquest C. elegans* cDNA library. The whole procedure followed during my screen is shown in Figure 2. In the thesis, methods and materials used during the screening, the results obtained so far, and a discussion about the results will be presented.

Figure 1: The *Proquest* two-hybrid system. *Top*: MaV203 cells containing pDB-Leu-X and pPC86-Y encode fusion proteins DB-X and AD-Y, respectively. The interaction of X::Y reconstitutes an active transcription factor that binds to the *GAL4* DNA binding sequences present in the promoter regions of the 3 chromosomally-integrated reporter genes and activates transcription. *Centre*: Structure of the promoter regions expressing each of the reporter genes and the expected growth/color results. *Bottom*: Expected growth or color results when tested for induction of the reporter genes for interacting and non-interacting DB-X and AD-Y (Derived from *Proquest* Two-Hybrid System Manual, http://www.invitrogen.com/content/sfs/manuals/10835023.pdf).



	His$^+$ (3AT^R)	β-Gal	Ura$^+$	5FOA
X:Y do not interact	−	White	−	+
X:Y do interact	+	Blue	+	−

Figure 2. An overview of the entire procedure involved in the yeast two-hybrid screen to identify interactors of *C. elegans* CLK-2.

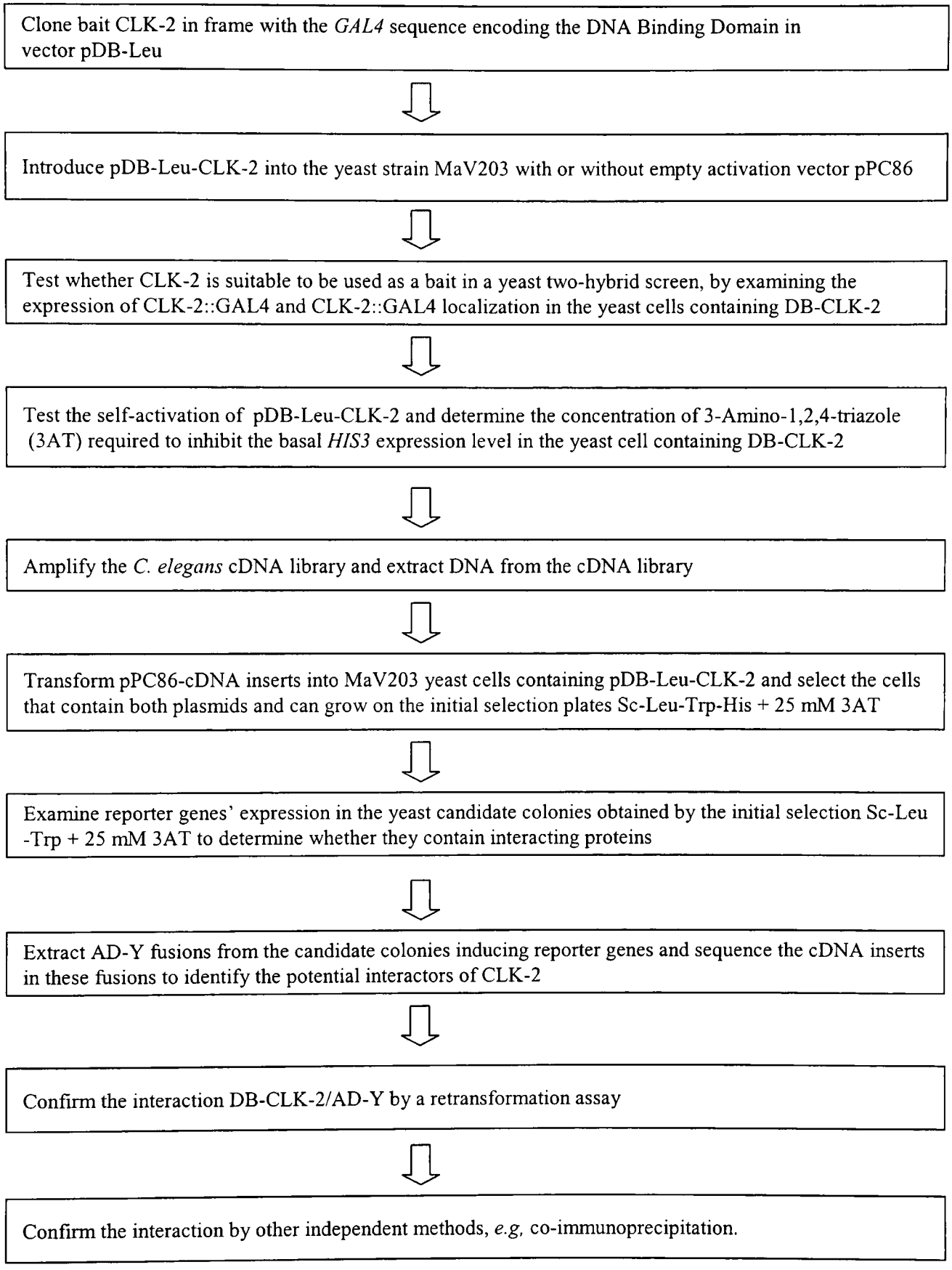

Clone bait CLK-2 in frame with the *GAL4* sequence encoding the DNA Binding Domain in vector pDB-Leu
Introduce pDB-Leu-CLK-2 into the yeast strain MaV203 with or without empty activation vector pPC86
Test whether CLK-2 is suitable to be used as a bait in a yeast two-hybrid screen, by examining the expression of CLK-2::GAL4 and CLK-2::GAL4 localization in the yeast cells containing DB-CLK-2
Test the self-activation of pDB-Leu-CLK-2 and determine the concentration of 3-Amino-1,2,4-triazole (3AT) required to inhibit the basal *HIS3* expression level in the yeast cell containing DB-CLK-2
Amplify the *C. elegans* cDNA library and extract DNA from the cDNA library
Transform pPC86-cDNA inserts into MaV203 yeast cells containing pDB-Leu-CLK-2 and select the cells that contain both plasmids and can grow on the initial selection plates Sc-Leu-Trp-His + 25 mM 3AT
Examine reporter genes' expression in the yeast candidate colonies obtained by the initial selection Sc-Leu -Trp + 25 mM 3AT to determine whether they contain interacting proteins
Extract AD-Y fusions from the candidate colonies inducing reporter genes and sequence the cDNA inserts in these fusions to identify the potential interactors of CLK-2
Confirm the interaction DB-CLK-2/AD-Y by a retransformation assay
Confirm the interaction by other independent methods, *e.g,* co-immunoprecipitation.

Table 1. The control yeast strains from the *Proquest* two-hybrid system used to help to decide which candidate colonies likely represent true interactors, as well as to estimate the strength of interaction (Derived from *Proquest* Two-Hybrid System Manual).

Control Strains	Resident Plasmids	cDNA Insert	Interaction Strength
Control A	pPC97	no insert	none
	pPC86	no insert	
Control B	pPC97-RB	human RB	weak
	pPC86-E2F1	human E2F1	
Control C	pPC97-*CYH2*^{s}-dDP	*Drosophila* DP	moderately strong
	pPC86-dE2F	*Drosophila* E2F	
Control D	pPC97-Fos	rat cFos	strong
	pPC86-Jun	mouse cJun	
Control E	pCL1	GAL4	very strong
	pPC86	No insert	

MATERIALS AND METHODS

Construction of the DB fusions with baits CLK-2

To identify *C. elegans* CLK-2 interactors by two-hybrid screening, three DB fusions were constructed, with full-length CLK-2 (1-877th aa) (CDB 555), the first half of CLK-2 (1-464th aa) (CDB 556), and the second half of CLK-2 (458th-877th aa) (CDB557), respectively. CLK-2 cDNAs with compatible in-frame restriction sites *Spe*I and *Nhe*I at the two ends respectively were generated using polymerase chain reaction (PCR) with oligonucleotide primers containing the restriction sites. Using full-length CLK-2 cDNA as template and primers SHP2084 and SHP2085 (Table 4), full-length CLK-2 cDNA was amplified by PCR. Primers SHP2084 and SHP2087 (Table 4) were used to generate the first half of CLK-2, and the second half of CLK-2 was generated by PCR using primers SHP2085 and SHP2086. PCR amplifications were done by *Pfu* polymerase (Stratagene). Following digestion of the CLK-2 cDNA segments and the empty DB vector pDB-Leu (*Proquest* Two-Hybrid System), the CLK-2 cDNA segments had been cloned into the DB vector pDB-Leu. Sequencing results confirm that the clonings have been done properly and all inserts are free of mutations.

Construction of the DB fusion with CLK-2 containing the substitution C772Y resulting from the *qm37* mutation (CDB 607)

By site-directed mutagenesis (*Quickchange^{TM} Site-Directed Mutagenesis Kit*, Stratagene) using primers SHP2357 and SHP2358 (Table 4) and *Pfu Turbo* polymerase (Stratagene), a mutated CLK-2 cDNA, which contains the *clk-2(qm37)* mutation, was generated. Then the PCR product of the CLK-2 cDNA containing the *clk-2(qm37)* mutation with the compatible

in-frame restriction sites *Spe*I and *Nhe*I at ends was generated by PCR using primers SHP2084 and SHP2085. Following digestion of the PCR product and the DB vector pDB-Leu, the CLK-2 cDNA with the *qm37* mutation has been cloned into the DB vector pDB-Leu. Sequencing results confirm that the cloning has been done properly. All clone constructs are shown in Table 3.

Construction of the AD fusion with C34G6.5 (CDB 609)

Full-length C34G6.5 cDNA with the compatible in-frame restriction sites *Sal*I and *Not*I at two ends respectively was generated using the polymerase chain reaction (PCR) by oligonucleotide primers SHP2385 and SHP2386, with full-length C34G6.5 cDNA clone yk712c12 (from Dr. Yuji Kohara lab) as template. Following digestion of the C34G6.5 cDNA segment and the AD vector pPC86 (*Proquest* two-hybrid system), the C34G6.5 cDNA segment encoding the entire C34G6.5 has been cloned into the AD vector pPC86. Sequencing results confirm that the cloning has been done properly.

Y105C5B.19 RNAi construct (CDB 608)

The segment covering bases 118367 to 118124 of Y105C5B.19, which shows no homology to any other gene in *C. elegans* genome, was amplified by PCR from the fusion pPC86-Y105C5B.19 using primers SHP2371 and SHP2372 containing compatible in-frame restriction sites *Spe*I and *Nhe*I. Following digestion the PCR segment has been cloned into the RNAi vector L1440 (from Fire lab). Sequencing results confirm that the clone has been done properly.

Immunoblot analysis

The yeast colonies containing the DB fusion with full-length CLK-2, or the first half of CLK-2 or the second half of CLK-2 respectively, were suspended in 50 µl autoclaved water and spread onto the centre of an Sc-Leu-Trp plate. Another Sc-Leu-Trp plate was prepared by the same way. After incubation of both plates for 18-24 hr at 30°C, the yeast cells were suspended in 100 ml Sc-Leu-Trp liquid medium in a 500 ml flask. Following incubating the medium for 12 hr with shaking (250 rpm), the cells were harvested by centrifugation at 1800 rpm for 5 min at room temperature. Cell pellets were suspended in 500 µl breaking buffer (100 mM Tris, 20% glycerol, 1 mM EDTA, 0.1% Triton X-100, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM phenylmethysulfonylfluoride (PMSF), 1 µg/ml leupeptin, 1 µg/ml pepstain), and 400 µl 0.5 mm glass beads (Comeau) were added. The debris was pelleted by centrifugation at 14000 *g* for 15 min at 4°C after vortex at full speed for 10 min at 4°C. The supernatants were transferred into new tubes and the protein concentration in the supernatants was estimated by Bradford assay (Bio-Rad).

The protein samples extracted from the yeast cells were denatured by boiling at 85°C for 5 min. The protein samples were then run on an acrylamide gel. Following the separation on an acrylamide gel, the proteins were then transferred onto a nitrocellulose membrane and blocked for 1 hr at room temperature in TBS-T (0.2M Tris, 1.5M NaCl, 0.05% Tween 20) buffer with 5% milk. The nitrocellulose membrane was incubated overnight at 4°C with the rabbit anti-CLK-2 polyclonal antibody (Hekimi lab antibody # MG19), which is directed against amino acids of CLK-2, at a 1:1000 dilution. The membrane was then rinsed 3 times with TBS-T buffer. A second block was performed using TBS-T buffer with 5% milk powder

and 1% normal donkey serum for 1 hr at room temperature. The membrane was then incubated for 2 hr at room temperature with the HRP-conjugated goat anti-rabbit antibody (Sigma) at a 1:2000 dilution. After the incubation, the membrane was washed 3 times with TBS-T and visualized using the ECL kit (Amersham).

Immunofluorescence microscopy

The yeast cells containing the DB fusion with full-length CLK-2, or the first half of CLK-2, or the second half of CLK-2 were grown to logarithmic phase, fixed with 3.7% formaldehyde in TBS-T (100 mM Tris, pH 8.0; 150 mM NaCl; 0.05% Tween) for 30 min, washed twice with TBS-T, and digested with zymolyase 100T (Seikagaku) for 25 min at room temperature. Cell pellets were then washed with TBS-T twice. After incubation with the rabbit anti-CLK-2 polyclonal antibody (Hekimi lab antibody # MG19) at 1:1000 overnight at 4°C, cells were washed with TBS-T 3 times. Biotinylated goat anti-rabbit IgG (7.5 μg/ml) in TBS-T containing 1% BSA were used as second antibody. Incubation time for the second antibody was 2 hr at room temperature. The yeast cells were then incubated for 30 min at room temperature with 10 μg/ml fluorescein streptavidin in TBS-T containing 1% BSA. After the cells were washed with TBS-T 3 times, dihydrochloride hydrate (at 1 μg/ml, in TBS-T containing 1% BSA) was added and the cells were then incubated at room temperature for 30 min. Cells were visualized under a Leitz fluorescence microscope.

Amplification of the *C. elegans* cDNA library and preparation of DNA from the amplified cDNA library

500 µl of the bacterial stock of *C. elegans* cDNA library (catalogue number: 11288-016, *Proquest*) was incubated in 100 ml terrific both (1L: 12g bacto-tryptone, 24g bacto-yeast, 4 ml glycerol) with 10% potassium phosphate (1L: 23g KH_2PQ_4, 125g K_2HPO_4) containing 100 µg/ml of ampicillin for 16 hr at 30°C with shaking. When A_{590} of the cDNA library bacterial culture reached to 2-8, bacterial cell pellets were collected by centrifugation. The cells pellets were then suspended in 10 ml of buffer I [15 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 µg/ml RNase A, 1200 U/ml RNase T1], and 10 ml of buffer II (0.2 M NaOH, 1% SDS) were added to the suspended cells. After mixing by inverting, they were incubated for 5 min at room temperature. Ten ml of cold 7.5 M NH_4OAc were then added to the cell mixture, and the cell mixture was incubated for 10 min on ice. After centrifugation, supernatants were poured through cheesecloth to get rid of white flocculent material. An equal volume of cold isopropanol was then added, and the DNA pellets were collected by centrifugation. After that, the DNA pellets were suspended in 1 ml of buffer I, and were then transferred to a microcentrifuge tube. The tube was incubated for 10 min at 37°C. In the following step, the tube was incubated at 65°C for 5 min and the sample in the tube was split into two equal parts. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was then added to each sample. After centrifugation, 400 µl of upper and aqueous phase were transferred into new tubes. An equal volume of isopropanol was then added to each tube and DNA pellets were collected by centrifugation again. After this, the DNA pellets were washed with 70% ethanol and finally were suspended in TE buffer. The concentration of the extracted DNA

from cDNA library was determined by A_{260}. They were then aliquot into tubes; each tube contains 12 μg DNA at 1.75 μg/μl. These tubes were stored at -20°C.

Preparation of the working plates from the stocks of the yeast strain MaV203 and the control strains A-E

Samples of the glycerol stocks of the yeast control strains A-E, provided by *Proquest*, were streaked onto plates Sc-Leu-Trp using autoclaved loops. A sample of the glycerol stock of the yeast strain MaV203 from *Proquest* was streaked onto an YPAD plate. The plates were then incubated for 48 hr for isolated colonies at 30°C. After this, the plates were stored at 4°C for later use.

Small-scale transformation of MaV203 with pDB-Leu-CLK-2 (and pPC86)

Several isolated colonies from a working plate of the yeast strain MaV203 were suspended in 50 μl autoclaved distilled water and spread onto the centre of a 10 cm YPAD (1L: 10g bacto-yeast extract, 20g bacto-poptone, 20g Dextrose, 100mg adenine sulfate) plate using autoclaved loops. The plate was then incubated overnight at 30°C. The next day the MaV203 cells were scraped from the YPAD plate and completely suspended in 5 ml autoclaved water. A sufficient amount of the cell suspension was then added to 100 ml YPAD medium to produce a final OD_{600} of 0.1. The YPAD medium containing MaV203 cells were then shaken at 30°C. When the OD_{600} of the cell culture reached ~0.4, the 100 ml YPAD medium was split into 4 of 25 ml aliquots and the cell pellets were collected by centrifugation at room temperature at 3000 *g* for 5 min. Each cell pellet was suspended in 10

ml autoclaved distilled water and then collected by centrifugation again. Each centrifuged cell was then suspended in 5 ml 1 X TE/LiAC and centrifuged again. After this, all cells pellets were suspended in 1 X TE/LiAC with the final volume 350 µl. The cell suspension was then aliquot into 7 microcentrifugation tubes for transformation. For each transformation, 5 µl of freshly boiled salmon sperm DNA (10 mg/ml, Invitrogen) and 100 ng of each plasmid were combined with 50 µl cell suspension. After mixing gently, 300 µl PEG/LiAC (1XTE, 1X LiAC in 50% PEG-3500) was added and mixed very gently. The tubes were incubated for 30 min at 30°C, and were then heat shocked for 15 min at 42°C. Cells were then collected by centrifugation at 6000*g* for 30 sec. After this, each cell pellet was suspended in 400 µl of autoclaved water. Each 100 µl of transformation cell suspension was spread onto a selective plate. All plates with the transformation cell suspension were then incubated at 30°C for 48-72 hr. A small-scale transformation, that was carried out to transform pDB-Leu-full-length CLK-2 (and pPC86) into the yeast cell MaV203, is shown in Table 2.

Replica plating and Replica cleaning

Replica plating is performed by pressing a master plate onto autoclaved velvet to transfer colonies on the master plate to selection plates. After incubation of master plates, each master plate was gently pressed onto autoclaved velvet. Prewarmed selection plates of interest are then gently pressed onto this "inoculated" velvet to have the colonies or patches from master plates. Immediately following replica plating or following incubation, new autoclaved velvets were pressed onto the surface of selection plates to remove excessive cell material

transferred to the selection plates. Replica cleaning was repeated several times if necessary, until all visible cell material on the surface of selection plates had been removed.

Determination of the 3AT concentration necessary to inhibit the basal expression of the *HIS3* gene in the yeast strain containing pDBLeu-CLK2

Four different colonies of the strains transformed with pDBLeu-CLK-2 and pPC86 were patched on a Sc-Leu-Trp plate along with two colonies of each yeast control strain provided by *Proquest*. After incubation at 30°C for 18 hr, these colonies were replica plated from the Sc-Leu-Trp master plate onto Sc-Leu-Trp-His plates containing 3-Amino-1.2.4-Triazole (3AT) at different concentrations (10 mM, 25 mM, 50 mM, 75 mM, and 100 mM) and replica cleaned immediately. After incubation for 24 hr at 30°C, these plates were replica cleaned again and were then incubated for 2 days at 30°C. The lowest concentration of 3AT inhibiting the growth of the strains transformed with pDB-Leu-CLK-2 and pPC86 was determined as the basal amount of 3AT to be added to all plates lacking histidine in the following two-hybrid screen.

Sequential transformation with pPC86-cDNA library of *C. elegans*

Several colonies of MaV203 (pDBLeu-CLK-2) were suspended in ~50 µl of autoclaved distilled water and spread onto the centre of a Sc-Leu plate. The second Sc-Leu plate was prepared in the same way. Both plates were incubated at 30°C for 18-24 hr. On the next day, the cells were scraped and completely suspended in 10 ml autoclaved water. A sufficient

amount of the cell suspension was then added to 500 ml Sc-Leu medium to give an OD_{600} of ~0.1. The Sc-Leu medium was shaking at 30°C. When the OD_{600} reached to ~0.4, the 500 ml Sc-Leu medium was split into two 250 ml aliquots and the cell pellets were collected by centrifugation at room temperature at 3000 *g* for 5 min. After this, each cell pellet was suspended in 100 ml autoclaved distilled water and collected by centrifugation. Each centrifuged cell pellet was then suspended in 50 ml 1X TE/LiAC and centrifuged again at 3000 *g* for 5 min. After this, all cell pellets were suspended in 1X TE/LiAC with a final volume of 2.5 ml. To transform AD-cDNAs of *C. elegans* into the yeast strain containing DB-CLK-2, 125 µl of freshly boiled salmon sperm DNA (10 mg/ml) and 12.5 µg DNA extracted from *C. elegans* cDNA library were combined with 2.5 ml of the cell suspension. After mixing gently, 15 ml PEG/LiAC (1XTE, 1X LiAC in 50% PEG-3500) was added and then mixed very gently and completely. In the following step, they were aliquot into 25 tubes of 700 µl each and the tubes were incubated for 30 min at 30°C. The tubes were then heat shocked for 15 min at 42°C. Cells were collected by centrifugation at 6000 *g* for 30 sec after heat shock. Each cell pellet was suspended in 400 µl of autoclaved water. Each 200 µl of the transformed cell suspension was spread on a selective plate Sc - Leu - Trp - His + 25 mM 3-AT. In the meantime, to estimate the number of cDNA clones screened, diluted cell suspensions were made (1:10, 1:100, and 1:1000) and 100 µl of each diluted cell suspension were spread on plates Sc-Leu-Trp. All plates were then incubated at 30°C for 60 hr. The number of cDNA clones screened was calculated by multiplying 'the number of colonies on a plate spread with diluted cell suspension with dilution factor and with the number of tubes of transformation performed'. All plates Sc – Leu – Trp - His + 25 mM 3AT where yeast

colonies had grown were replica cleaned and were then incubated for 2 - 3 days at 30°C. The transformants, which keeps growing after that, are referred to as "candidate colonies" and are further analyzed (see section below) to determine whether they contain interacting proteins.

Characterization of candidate colonies for interacting proteins

The transformants that can grow on the initial selective plates Sc-Leu-Trp-His + 25 mM 3AT, which are referred to as candidate colonies below, were streaked on Sc-Leu-Trp plates to get isolated colonies. These plates were then incubated for 48 hr at 30°C, along with the plates containing yeast control strains A-E for interacting strength.

Four isolated colonies derived from each candidate colony were then patched onto Sc-Leu-Trp plates (referred to as master plates below). In addition, two isolated colonies of each yeast control strain (A-E), as well as two isolated colonies containing the fusion DB-CLK-2 and the empty AD vector (pPC86) were patched onto the same plates (Figure 5). The plates were incubated for 18 hr at 30°C, then the colonies on these master plates were replica plated onto the following selection plates, in the order listed: 1) YPAD containing a nitrocellulose membrane for X-Gal assay, 2) Sc-Leu-Trp-Ura, 3) Sc-Leu-Trp-His + 25 mM 3-AT, and 4) Sc-Leu-Trp + 0.2% 5FOA (5 - fluoroorotic acid). The plates Sc-Leu-Trp-His + 25 mM 3-AT and Sc-Leu-Trp + 0.2% 5FOA were replica cleaned after replica plating. Following 18 - 24 hr incubation, an X-Gal assay (described below) was performed on the membrane on the YPAD plates to determine the phenotype of the reporter gene *lacZ* (see section below). All other selection plates were replica cleaned after incubation for 24 hr at 30°C. After these

plates were incubated for an additional 2 days at 30°C, growth of these candidate colonies on selection plates was examined.

Growth of yeast candidate colonies on plates Sc-Leu-Trp-Ura and their absence of growth on plates Sc-Leu-Trp + 0.2% 5FOA indicate the expression of reporter gene *URA3*. Growth of yeast candidate colonies on the plates Sc-Leu-Trp-His + 25 mM 3AT indicates the activation of expression of the reporter gene *HIS3* in these colonies. But to conclude, all control strains must behave properly on these selection plates. One example of candidate colonies on the selection plates is shown in Figure 4. In addition, if there is expression of reporter gene *LacZ*, candidate colonies appear to be blue during the X-Gal assay (shown below). Only the candidate colonies that induce the reporter genes, referred to as candidate colonies for interaction are further analyzed.

X-Gal [5-bromo-5-chloro-3-indolyl-C-(-D-galactoside)] assay

Two round 7 mm filter papers (Whatman) were staked in a 10 cm plate and saturated with ~2 ml of Z buffer (16.1 g $Na_2HPO_4 \cdot 7H_2O$, 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 0.246 g $MgSO_4 \cdot 7H_2O$ in 1 L autoclaved H_2O, pH 7.0) containing X-Gal solution [10 mg X-Gal (5-bromo-5-chloro-3-indolyl-D-galactoside) in 100 µl DMF (N,N-dimethyl formamide)] and 60 µl 2-mercaptoethanol. The membranes, which have been replica plated with candidate colonies along with yeast control strains were removed carefully from YPAD plates and immersed in liquid nitrogen for 30 sec. The frozen membranes were then placed on top of the two soaked filter papers with colonies side up. Excessive solution was then removed. The plates were incubated with the colonies facing up at 37°C for 24 hr and the appearance time

of blue colors was recorded. An appearance time of the blue color of the candidate colonies of less than 1 hr indicates a strong interaction. Weak interactions show blue color within 24 hr. All control strains should behave properly: control strain E should appear to be blue within 1 hr and control B should appear to be blue within 24 hr. Control strain A, which does not contain interacting proteins, should never appear to be blue during the assay.

Plasmid DNA extraction from yeast cells

A single candidate colony for interaction, where the reporter genes had been induced, was suspended in 1 ml Sc-Trp-Leu media and the media was incubated at 30°C for 24 hr with shaking. The cell pellet was collected by centrifugation at 14000 *g* for 30 sec at room temperature. The cell pellet was then suspended in 100 µl freshly prepared 3% sodium dodecyl sulfate with 0.2 N NaOH. After this, they were incubated at room temperature for 15 min with occasional mixing by inversion. 500µl TE (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) was then added and mixed completely by rapid inversion. 60 µl 3 M sodium acetate was then added and mixed completely. After this, 600µl phenol: chloroform: isomyl alcohol (25:24:1) was added and they were vortex for 2 min at full speed. The tubes were then centrifuged at 14000 *g* for 2 min and the upper aqueous phase was transferred to new microcentrifuge tubes. The extraction with phenol: chloroform: isomyl alcohol was repeated twice. In the following step, 650 µl ice cold isopropanol was added and the tubes were then incubated at -20°C for 20 min. DNA pellets were collected by centrifugation. Then the DNA pellets were washed with 70% ethanol once and finally suspended in 10 µl TE.

Retransformation assay

The plasmids DNA extracted from the candidate colonies for interaction were introduced into *E. coli* ElectroMaX DH10B cells with selection for ampicillin to selectively isolate the AD fusions. After extracting the AD fusions containing potential CLK-2 interactors from the *E. coli* colonies growing on plates LB + 100 μg/ml ampicillin, each AD fusion and pDB-Leu-CLK2 were reintroduced into fresh yeast cells MaV203 along with control transformations (pDB-Leu-CLK-2 + pPC86, pDB-Leu + the AD fusion extracted from the candidate colonies for interaction). These transformants were then characterized as described before. If the transformants freshly introduced with pDB-Leu-CLK-2 and an AD fusion extracted from yeast candidate colonies for interaction reproduce the phenotypes due to expression of reporter genes, the AD fusion likely contains a CLK-2 interactor.

Amplification of Y105C5B.19 from candidate yeast colonies

To determine whether the yeast colonies, that grew on the initial selection plate Sc-Leu-Trp-His + 25 mM 3AT, contain an AD fusion with Y105C5B.19, amplification of Y105C5B.19 from the yeast colonies was performed by PCR, using primers specific to Y105C5B.19 (SHP2341 and SHP2342). Successful amplification of Y105C5B.19 indicates that the yeast colonies contain Y105C5B.19 fused to the AD vector pPC86.

Y105C5B.19 RNAi by feeding

Single colonies of the HT115 bacteria containing the Y105C5B.19 RNAi construct [or empty RNAi vector L1440 (from Fire's lab)], were picked and grown in culture of LB with

50 μg/ml ampicillin overnight. On the next day, the overnight culture was re-grown in LB with 50 μg/ml for 6 hr, the re-grown culture was then seeded directly onto NGM plates with 1 mM IPTG and 50 μg/ml ampicillin. The seeded plates were allowed to dry at room temperature for ~24 hr before use. Five L4-stage hermaphrodites (N2 or *qm37)* were then placed onto each of the seeded plates. After incubation at 20°C for every 24 hr, worms were transferred onto new seeded plates in the same way. The brood size of the P0 worms and the defecation cycle (Wong et al., 1995) of F1s were scored. For F1 worms, the duration of their development (from eggs to young adult) was also measured.

Y105C5B.19 *ds*RNA preparation

Linear Y105C5B.19 DNA template for *in vitro* transcription was prepared by amplification of Y105C5B.19 from the Y105C5B.19 RNAi construct (construction of this construct was described before) using the T7 primer. *In vitro* transcription reaction was set up by *Ribomax*^{TM} Large-Scale RNA Production System (Proquest), which contains rNTP, T7 transcription buffer, T7 enzyme mix and linear Y105C5B.19 DNA template. The reaction was incubated at 37C° for 4 hr, and then the DNA template was removed by digestion with DNAase RQ1 then phenol-chloroform extraction. The concentration of RNA suspended in nuclease-free water was determined approximately by electrophoresis.

Y105C5B.19 RNAi by injection

Y105C5B.19 *ds*RNA prepared by *Ribomax*^{TM} Large-Scale RNA Production System (described above) was injected into the gonads of N2 and *qm37* adult worms at concentration

~ 1 μg/μl by standard injection protocol. Injected worms were singled and transferred onto new plates 8 hr after injection, and then they were transferred into new plates twice a day. The progeny of the injected worms and percentage of embryonic viability after injection were scored. In addition, the defecation cycle, post-embryonic developmental rate and brood size of F1s reproduced by the injected worms during 8-24 hr after the injection were scored.

Generation of the transgenic worms with the Y105C5B.19 transgene

The full-length gene Y105C5B.19 along with its upstream regulatory region of the operon was obtained by PCR (*Tag* Polymerase, Invitrogen), using Y105C5B as template, primers SHP2645 and SHP 2646. The accession number of YAC Y105C5B is AL110479. The PCR product was microinjected into *clk-2(qm37)* and N2 worms at a concentration of 20 ng/μl along with pRF4 (200 ng/μl). F1 and F2 transgenic worms were examined.

Table 2. A small-scale transformation of MaV203. Transformation **1** and **2** were used as transformation controls. Transformation **3** was then used to test whether the bait CLK-2 can self-activate the reporter genes in the present of the empty activation domain vector (pPC86). In transformation **4**, only pDB-Leu-CLK-2 was transformed into MaV203, which was then used in a sequential transformation during the following yeast two-hybrid screening.

Transformation	Plasmid 1	Plasmid 2	Selection	Purpose
1	pDB-Leu	None	Sc-Leu	transformation control
2	none	none	Sc-Leu and Sc-Leu-Trp	transformation control
3	pDB-Leu-CLK-2	pPC86	Sc-Leu-Trp	test self-activation of bait CLK-2
4	pDB-Leu-CLK-2	None	Sc-Leu	sequential transformation in the following screen

Table 3. The clone fusions constructed for the two-hybrid screening.

Name	Vector backbone	Insert	Restriction sites used	Purpose
CDB 555	pDB-Leu	*C. elegans* CLK-2 cDNA encoding entire CLK-2 (aa 1-877)	*Spe*I and *Nhe*I	Bait construct
CDB 556	pDB-Leu	*C. elegans* CLK-2 cDNA encoding the first half of CLK-2 (aa 1-464)	*Spe*I and *Nhe*I	Bait construct
CDB 557	pDB-Leu	*C. elegans* CLK-2 cDNA encoding the second half of CLK-2 (aa 458-877)	*Spe*I and *Nhe*I	Bait construct
CDB 607	pDB-Leu	*C. elegans* CLK-2 cDNA encoding entire CLK-2 with the C772Y substitution	*Spe*I and *Nhe*I	To test whether *qm37* disrupts the interaction between Y105C5B.19 and CLK-2
CDB 608	pPD129.36	A segment from bases 118367 to bases 118124 of Y105C5B.19	*Spe*I and *Nhe*I	Y105C5B.19 RNAi construct
CDB 609	pPC86	*C. elegans* C34G6.5 cDNA encoding entire C43G6.5 (aa 1-411)	*Sal*I and *Not*I	To test the possible interaction between C34G6.5 and CLK-2

Table 4. The sequences of the primers used for the construction of the clones used in the two-hybrid screen.

SHP 2084
CCTCTA GCTAGCATGAATTTACGAAGTCGCCTGG

SHP 2085
GGCTAG ACTAGTTTAAACGTCTTGGTGTTGCAGTAC

SHP 2086
CCTCTA GCTAGCTCTGAGCGAGTTCGAGAAACC

SHP 2087
GGCTAGACTAGTTTAGGTTTCTCGAACTCGCTCAGA

SHP 2341
GGCTCTTCGTATTGCTTATGG

SHP 2342
AGCTCTCCATCCTCCTTGCG

SHP 2357
CGATGGTTTATGTGAGATATGGCGTATGTCCTCAAATTC

SHP 2358
GAATTTGAGGACATACGCCATATCTCACATAAACCATCG

SHP 2371
GGCTAGACTAGTA GAGTACATACTCGGTGGGTTC

SHP 2372
CCTCTAGCTAGCTTTGAGCTCTCCATCCTCCTTG

SHP 2385
CTACGCGTCGACCATGTCCATAAAATCGTCACGG

SHP 2386
ATAAGAATGCGGCCGCTTAGAATGGATCTTGATTCGAC

SHP 2645
CTAAACTGTT GCTAGATTGATCG

SHP 2646
GCGACTCTAC AAATTGTGGC

RESULTS

In order to help understand the cellular function of CLK-2, I have carried out a yeast two-hybrid screen to identify proteins that interact with *C. elegans* CLK-2. The interactions may provide insight into the function of CLK-2. Constructs coding for binding domain fusions with the entire CLK-2 protein, as well as the parts of the protein CLK-2 were constructed. These fusions were analyzed for their suitability as baits and subsequently were used in two-hybrid screening. Below, I present my bait constructs and their characterization as suitable baits to be used in two-hybrid screening, as well as the results of the two-hybrid screen.

Full-length *C. elegans* CLK-2 is a suitable bait for a yeast two-hybrid screen

Since *C. elegans* CLK-2 protein is 877 amino acids long, it is possible that it is too big to be expressed at a stable level in yeast cells and to enter the yeast nucleus. Thus, three bait fusions, which contain different length of CLK-2 have been constructed: pDBLeu-full-length CLK-2; pDBLeu-first half of CLK-2 (amino acid residues 1-464); pDBLeu-second half of CLK-2 (amino acid residues 458-877).

To determine whether the CLK-2 fusions could be used as baits in two-hybrid screening, the expression of CLK-2 in the yeast cells containing each of the three bait fusions was analyzed by western blots using the rabbit polyclonal anti-CLK-2 antibody. Western blot results show that the full-length CLK-2 and the 2nd half of CLK-2 can be expressed in the yeast cells at a stable and reasonably high level (Figure 3). The band corresponding to the 1st

half of CLK-2 has not been detected, but the anti-CLK-2 antibody we have should only recognize the second half of CLK-2. Thus we do not know whether in the yeast cells the 1st half of CLK-2 can be expressed or not. To know this, an anti-GAL4 antibody (ClonTech) was used in a western blot analysis. Unfortunately, the anti-GAL4 antibody did not work even after numerous trials. In fact, the anti-GAL4 antibody did not work at all as it did not even recognize other GAL4 fusions which we know are expressed, *e.g.* it did not recognize the full length CLK-2 fusion with GAL4 which we could detect with the anti-CLK-2 antibody.

Second, I examined whether the baits CLK-2 can enter yeast nucleus, since the detection of protein-protein interactions by a yeast two-hybrid screen relies on transcriptional activation. Results of a immunofluorescence staining with the anti-CLK-2 antibody show that approximately 10% of the yeast cells containing the binding domain vector fused with the full-length CLK-2 have stained nuclei (data not shown), indicating that the bait DB-full-length *C. elegans* CLK-2 can enter the yeast nucleus.

Third, the undesirable possibility that the CLK-2 baits could self-activate the reporter genes was examined before carrying out the two-hybrid screen, since if the CLK-2 baits can self-activate, they cannot be used as baits to fish their interactors. In the *Proquest* two-hybrid system, the initial selection for interactions is the induction of the *HIS3* reporter gene. Thus, firstly I tested whether the construct pDBLeu-full-length CLK-2 could self-activate the reporter gene *HIS3* expression. To maximize the sensitivity of the *HIS3* reporter gene, the yeast strain MaV203 in the *Proquest* two-hybrid system expresses a basal level of *HIS3*. *HIS3* encodes imidazole glycerol phosphate dehydratase, an enzyme involved in the histidine

biosynthesis. This enzyme can be specifically inhibited in a dose-dependent manner by 3-Amino-1.2.4-Triazole (3AT). Therefore, before performing two-hybrid screens, the basal expression level of the *HIS3* reporter gene in the yeast cells containing the DB fusion with full-length CLK-2 and the concentration of 3AT necessary to inhibit the basal expression of the *HIS3* reporter gene, was determined. Since it is possible that the CLK-2 bait fusion and the AD vector could form a complex and thus activate the transcription of reporter genes, I tested the CLK-2 bait fusion in presence of the empty AD vector to determine the 3AT concentration required to inhibit the basal expression of *HIS3* gene, which does not result from an interaction.

The results show that on Sc-Leu-Trp-His plates added with 10 mM 3AT, the yeast cells containing pDBLeu-full length CLK-2 and the empty AD vector pPC86 can grow slightly, but on the Sc-Leu-Trp-His plates added with 25 mM 3AT, the yeast strains do not grow (Figure 4). On the Sc-Leu-Trp-His plates added with 25 mM 3AT, the yeast control strain B which contains control vectors expressing proteins that weakly interact can grow well. Therefore, 25 mM 3AT have been added to Sc-Leu-Trp-His plates to inhibit the basal expression of the gene *HIS3* during the following two-hybrid screens.

Since no motifs or domains are recognizable in *C. elegans* CLK-2, we preferred to use full-length CLK-2 as a bait to carry out two-hybrid screening. All the previous results show that *C. elegans* full-length CLK-2 is suitable to serve as a bait. Therefore, to identify proteins that interact with *C. elegans* CLK-2, I performed yeast two-hybrid screens with the full-length CLK-2 as a bait. The cDNA library used in the screening is a *C. elegans* cDNA library

(Proquest), prepared from a mixture of developmentally staged worms. The library is enriched for inserts of approximately 1.4 kb and therefore contains a lot of full-length cDNAs. Below I will present the screening results obtained so far.

Y105C5B.19 is a potential interactor of *C. elegans* CLK-2

Using the *Proquest* two-hybrid system and the full-length CLK-2 as a bait, I screened the *C. elegans* cDNA library approximately 2 times in total and only one CLK-2 interactor was found, namely the protein encoded by the gene Y105C5B.19. Henceforth, Y105C5B.19 will be used to refer either to the gene or to the encoded protein product. Below some screening results will be presented to show that Y105C5B.19 might be a real interactor of *C. elegans* protein CLK-2.

The first series of my two-hybrid screening (using full-length CLK-2 as bait) covered 75% of the *C. elegans* cDNA library and yielded a total of 33 candidate colonies from the initial selection (lacking histidine with 25 mM 3AT). All the candidate colonies have been characterized following the procedure shown in Figure 6. An example of the candidate colonies on the selection plates is shown in Figure 5. The results show that all these candidate colonies grow on plates Sc-Leu-Trp-Ura, do not grow on plates Sc-Leu-Trp + 0.2% 5FOA (5-fluoroorotic acid), do grow on plates Sc-Leu-Trp-His + 25 mM 3AT and appear to be blue during the X-Gal [5-bromo-5-chloro-3-indolyl-C-(-D-galactoside)] assay, indicating that all three reporter genes *URA3*, *HIS3* and *LacZ* have been induced in these colonies, which is indicative of interaction. The yeast strain containing pDBLeu-full length CLK-2 and the

empty AD vector pPC86 do not grow on Sc-Leu-Trp-Ura plates, grow on Sc-Leu-Trp + 0.2% 5FOA plates, do not grow on plates Sc-Leu-Trp-His + 25 mM 3AT and do not appear to be blue after incubation in X-Gal solution. Furthermore, all yeast control strains, except for the control D, behave properly on the selection plates. The control D is not reliable as this strain generates both large and small colonies and also varies for growth on all selection plates. However, according to the phenotypes of the colonies on the selection plates, the candidate colonies seem to contain interacting proteins.

To identify the interactors of CLK-2 in these candidate colonies, the plasmids containing the activation domain fusions were extracted from these 33 candidate colonies and sent to sequence the cDNA inserts of these fusions. In all these colonies, the cDNA inserts correspond to the same predicted gene, Y105C5B.19. It is worth noting that the 33 cDNA inserts are not identical at DNA level. They can be divided into 11 groups according to their DNA sequences (some have small 5' deletions, other have small 3' deletions, etc.).

To exclude the possibility that the expression of reporter genes in the candidate yeast colonies identified above is a result from a mutation in the bait or in the yeast cells that allowed activation of the reporter genes, a retransformation assay was carried out. The activation domain fusion with Y105C5B.19 (pPC86-Y105C5B.19) extracted from these candidate colonies that induce all reporter genes and the original bait construct pDB-Leu-full-length CLK-2 were introduced into a freshly prepared yeast cell MaV203. Results show that the reporter genes phenotypes have been reproduced in the yeast cells with freshly introduced pDBLeu-full length CLK-2 and pPC86-Y105C5B.19 (Figure 7). In the yeast strains that contain pPC86-Y105C5B.19 and empty DB vector pDBLeu, the reporter genes

have not been induced. All these results show that only when the yeast cells contain the binding domain fusion with full-length CLK-2 and Y105C5B.19 fused to the activation domain of GAL4, the reporter genes are activated in the cells. Thus, the interaction between CLK-2 and Y105C5B.19 is authentic.

The second series of my two-hybrid screening using full-length CLK-2 as a bait covered the *C. elegans* cDNA library 1.5 times, and roughly 300 yeast colonies able to grow on the initial selection plates (Sc-Leu-Trp-His + 25 mM 3AT) were obtained. Knowing that Y105C5B.19 would likely be picked again, I carried out PCR amplification using the primers specific to Y105C5B.19 (SHP 2341 and SHP 2342) to directly amplify Y105C5B.19 from these candidate colonies in case they again contain the same interactor. DNA extracted directly from the yeast colonies was used as the template for the PCR. 180 of the 300 candidate colonies were analyzed by PCR. A PCR product with the expected size of 1 kb was obtained in all 180 colonies. A picture of a gel where the PCR products were run is shown in Figure 8. Therefore, again all these cDNA inserts correspond to the same predicted gene, Y105C5B.19.

In summary, the two-hybrid screen using *C. elegans* full-length CLK-2 as a bait has covered the *C. elegans* cDNA library 2 times, and in total about 330 candidate colonies were obtained. 213 out of 330 colonies were analyzed and the results show that all of them contain activation domain fusions with the cDNAs corresponding to the same gene, Y105C5B.19. The interaction between CLK-2 and Y105C5B.19 can induce the expression of all reporter genes. In addition, a retransformation assay confirmed the interaction is authentic. Therefore, Y105C5B.19 is a potential interactor of CLK-2 in worms.

The CLK-2 interactor Y105C5B.19 is a novel gene

The potential CLK-2 interactor Y105C5B.19 is a novel gene and does not have homologues in other species. It is expressed in all somatic embryonic stages until adulthood (http://www.wormbase.org/db/gene/gene?name=y105c5b.19). The predicted protein product of this gene is 484 amino acids long. The only recognizable domain in the protein product of this gene is an MSP domain (from residues 45 to 178 of the protein), which is 66% identical to the MSP proteins. The structure of the protein product of this gene is shown in Figure 9. In worms, MSP proteins are encoded by a multigene family, which consists of 40 genes. The MSP proteins are 99% identical to each other and only differ by one to four amino acids (Miller et al., 2001). MSP is the most abundant protein present in sperm, and it is well known to be a cytoskeletal protein, responsible for sperm mobility. In addition, it has been demonstrated that MSP is a signal for oocyte maturation and gonadal sheath contraction for ovulation in the worm (Miller et al., 2001).

X-ray crystallography reveals that MSP proteins fold into an immunoglobulin-like seven-stranded β sandwich, which is called an MSP domain. Although MSP orthologs have not been identified outside of nematodes, proteins containing MSP-like domains are found in yeast, plants and animals. For example, the transmembrane proteins, named as VAMP-associated proteins and seem to function in somatic cells in numerous ways including neurosecretion, contain NH_2-terminal MSP-like domains that are also found in fungi, plants and animals (Miller et al., 2001).

Identification of the interacting region between *C. elegans* CLK-2 and Y105C5B.19

Direct two-hybrid assays were carried out to determine which region of CLK-2 interacts with Y105C5B.19 using the binding domain fusions encoding the two portions of CLK-2 namely, the first half of CLK-2 (amino acid residues 1-464) and the second half of CLK-2 (amino acid residues 458-877). The DB fusions with the first half of CLK-2 and the second half of CLK-2 respectively were transformed into the yeast strain MaV203 with AD-Y105C5B.19. The reporter genes in these transformants were then characterized. The results show that the cotransformation of the DB-1st half of CLK-2 and AD-Y105C5B.19 cannot induce the expression of reporter genes (Figure 10). But the expression of all reporter genes was activated in the transformants containing the DB-2nd half of CLK-2 and the AD-Y105C5B.19. Therefore, the 2nd half of CLK-2 seems to interact with Y105C5B.19 by the two-hybrid assay, but the first half of CLK-2 does not appear to interact with Y105C5B.19. However, we do not exclude that the interaction between the 1st half of CLK-2 and Y105C5B.19 cannot be detected by the two-hybrid assay, as a result of the 1st half of CLK-2 possibly not being expressed in the yeast cells at a stable and reasonably high level, or not entering the yeast nucleus.

Since the 1st half of CLK-2 does not seem to interact with Y105C5B.19, I tried to fish other CLK-2 interactors using the 1st half of CLK-2 as a bait by a yeast two-hybrid screen. Firstly, the 3AT concentration necessary to inhibit the basal expression of *HIS3* in the yeast cells containing the DB fusion with the 1st half of CLK-2 was determined. Our results indicate that when the 3AT concentration is 10 mM or more than 10 mM, the yeast cells

containing DB-1st half of CLK-2 and pPC86 do not grow, but on the Sc-Leu-Trp-His + 10mM 3AT plates, the control A, which does not have interacting proteins, can still grow (Figure 11). Therefore, 25 mM is the necessary concentration of 3AT to inhibit the basal expression of *HIS3*, which does not result from an interaction. Then, a yeast two-hybrid screen was carried out using the 1st half of CLK-2 as a bait. However, no colonies able to grow on the initial selection plates were obtained after screening 80% of clones of the *C. elegans* cDNA library. Thus, no additional interactors of CLK-2 could be identified using this strategy.

The *clk-2(qm37)* mutation disrupts the interaction between CLK-2 and Y105C5B.19

clk-2(qm37) is a temperature-sensitive partial loss-of-function mutation, which results in a C772Y amino acid substitution and leads to a severely reduced level of CLK-2 protein at all temperatures (Benard et al., 2001). The *clk-2(qm37)* mutants display slow growth and behavioural rate, lifespan extension at 20°C, as well as embryonic developmental arrest at the restrictive temperature 25°C. To investigate whether the substitution C772Y resulting from the *clk-2(qm37)* mutation could disrupt the interaction between CLK-2 and Y105C5B.19, the DB fusion with CLK-2(C772Y) and the AD fusion with Y105C5B.19 were cotransformed into the yeast cells MaV203. The yeast colonies containing both fusions were then characterized to determine whether the reporter genes in these colonies were induced or not. The interaction between CLK-2(C772Y) and Y105C5B.19 was not detected by the two-

hybrid assay, indicating that it is likely that the *clk-2(qm37)* mutation affects the ability of the CLK-2 protein to interact with Y105C5B.19 (Figure 12). However, it is possible that CLK-2 (C772Y) is not stable in the yeast cells, even though it is detected in the *clk-2(qm37)* mutant worms.

RNA interference (RNAi) phenotypes of Y105C5B.19

To investigate the function of Y105C5B.19, an RNAi experiment was carried out to determine the consequence of the loss of function of this gene. First, RNAi by feeding approach was used. No morphological defects were observed in P0 or F1 wild-type N2 and *clk-2(qm37)* mutant worms fed with Y105C5B.19 *ds*RNA. Quantitatively, the brood size, the defecation cycle and the post-embryonic developmental rate of the first generation of the fed worms were scored. No difference between the worms fed with Y105C5B.19 *ds*RNA and the worms fed with the bacteria containing the empty RNAi vector was detected (Table 5).

Secondly, I did RNAi interference of Y105C5B.19 by injecting Y105C5B.19 *ds*RNA into the gonads of adult worms (P0s). In the F1s of the wild-type N2 worms injected with Y105C5B.19 *ds*RNA morphological defects were not observed. In the F1s of the injected *clk-2(qm37)* mutant worms no other morphological defects, except for the *clk-2* phenotypes, were detected. In addition, the defecation, the post-embryonic developmental rates and the brood size of the F1s are not different from the uninjected worms (data not shown). However, the injected worms (P0s) show reduced brood size and lethality, and in the *clk-2(qm37)* background the effect is more severe.

To determine, whether the effect on the brood size and embryonic lethality is specific to the injection of Y105C5B.19 *ds*RNA, the brood size and the number of dead embryos produced by the wild-type N2 and *clk-2(qm37)* mutant worms injected with water alone, as well as with *ds*RNA of an unrelated gene (*dsc-1*) that should not be functionally related to *clk-2,* were scored. The results show that the worms injected with water or *dsc-1 ds*RNA also have reduced brood size, and they also lay some dead embryos (Table 6). In N2 background no difference of the brood size reduction and percentage of dead embryos were detected between the worms injected with Y105C5B.19 *ds*RNA, water, or *dsc-1 ds*RNA. However, in the *clk-2(qm37)* background, the worms injected with Y105C5B.19 *ds*RNA show more severe reduction of brood size, and they lay more dead embryos (26% dead embryos) than the *qm37* mutant worms injected with water or *dsc-1 ds*RNA. But, the worms that were injected are adults and the injection treatment may mechanically affect the function of gonad/germline. Thus, in order to confirm this result, more experiments to control the experimental variations and bias should be carried out.

In summary, no specific phenotypes have been observed after silencing the expression of Y105C5B.19 by RNAi treatment, except the possibly for an increased embryonic lethality in the *clk-2(qm37)* mutants. Since there is a homologue of this gene in the *C. elegans* genome, namely M199.2, the absence of more obvious phenotypes in the worms treated with Y105C5B.19 *ds*RNA might due to a possible functional complementation brought about by M199.2. The simultaneous knockdown of both genes may be required to reveal the functional requirements for these genes.

Overexpression of Y105C5B.19 cannot rescue *clk-2* mutants

In order to get more evidence for the functional relationship between CLK-2 and Y105C5B.19, I determined whether the overexpression of Y105C5B.19 carried on a transgene could rescue *clk-2(qm37)* mutants, including the slow growth and behavioural rate, the reduction of brood size at 20°C and the lethality at 25°C. High expression level of the protein product of Y105C5B.19 in the *clk-2(qm37)* mutants may allow more interaction between CLK-2 and Y105C5B.19, and thus rescue some of the *clk-2* phenotypes. A PCR product containing the entire Y105C5B.19, including its upstream promoter region, along with the rolling marker pRF4 was injected into the gonads of the *clk-2(qm37)* mutant worms. The defecation cycle and the brood size of F2s/F3s transgenic worms carrying the transgene Y105C5B.19 were scored at 20°C. No difference was detected between the transgenic worms and *qm37* mutants (data not shown). In addition, some F2s/F3s (at L4 or young adult stage) of the transgenic *qm37* mutant worms were shifted to 25°C, and their embryos were examined. The transgenic *qm37* mutant worms at 25°C lay only dead eggs (data not shown), indicating that the lethality of the *clk-2(qm37)* mutation cannot be rescued with the Y105C5B.19 transgene.

C. elegans CLK-2 does not interact with C34G6.5, the *C. elegans* homologue of Cdc7p of *S. cerevisiae*

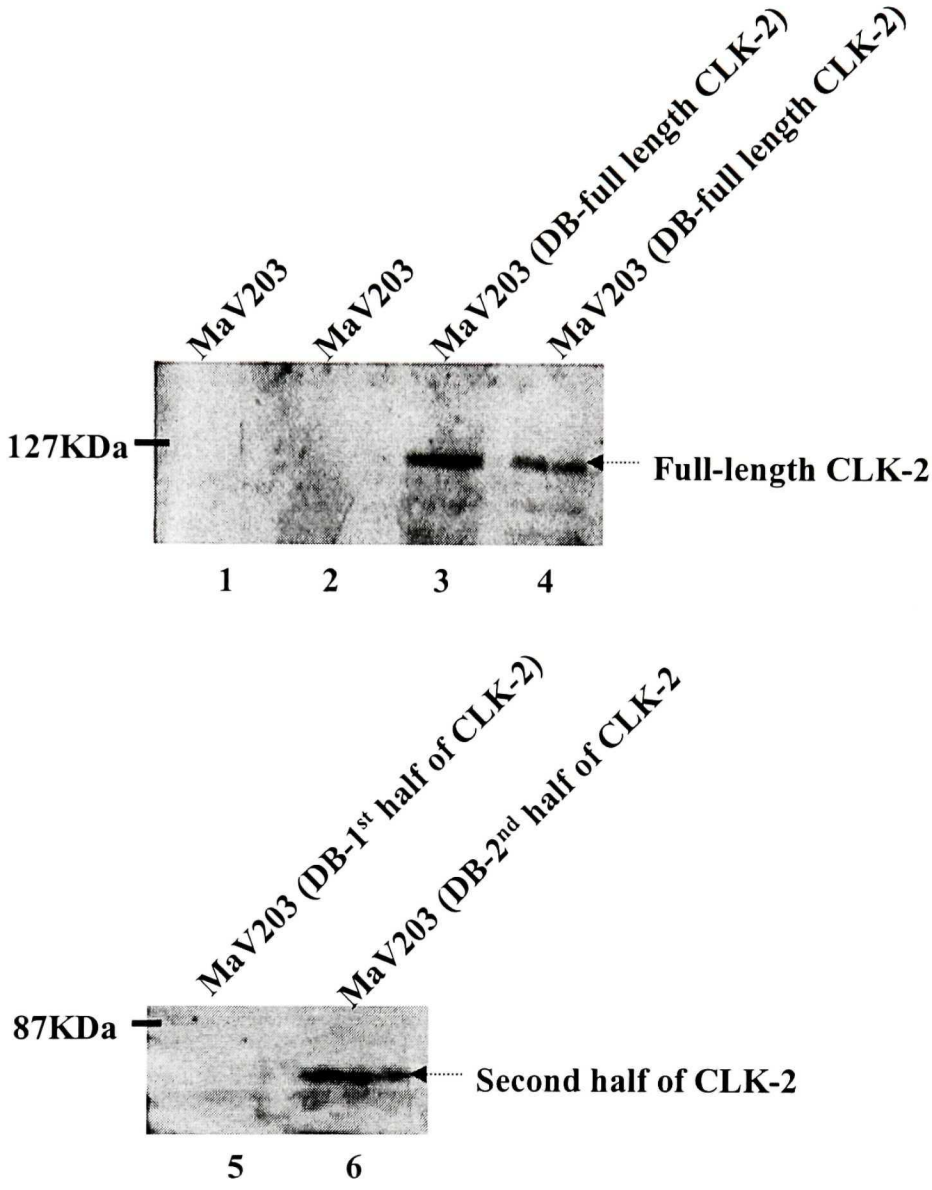
During a large-scale two-hybrid screening in *Saccharomyces cerevisiae,* Tel2p (the homologue of *C. elegans* CLK-2) has been picked up twice by Cdc7p (Uetz et al., 2000). It will be interesting to examine whether CLK-2 and the *C. elegans* Cdc7p homologue also

interact. Therefore, a direct two-hybrid assay was carried out to test the possibility. The results are presented below.

The *C. elegans* Cdc7p homologue was identified by BLAST. Using the Cdc7p sequence in budding yeast, and the sequences of the Cdc7p homologues in frog, mouse, fly and human to do sequence alignments, the same *C. elegans* protein (C34G6.5) was picked up, indicating that this protein is the closest homologue of Cdc7p, despite a poor conservation of the proteins (the identity between Cdc7p and C34G6.5 is 32%).

To test whether *C. elegans* CLK-2 and C34G6.5 interact, a two-hybrid assay was carried out using the binding domain fusion with full-length CLK-2 and the activation domain fusion with full-length C34G6.5. The expression of the reporter genes in the yeast cells, containing these two fusions, was characterized to determine whether *C. elegans* CLK-2 and C34G6.5 could interact. The results obtained indicate that CLK-2 and C34G6.5 do not interact by the two-hybrid assay (Figure 13). In the yeast colonies containing the two fusions pDB-Leu-full-length CLK-2 and pPC86-C34G6.5 the reporter genes have not been induced, since they do not grow on plates Sc-Leu-Trp-Ura, grow on plates Sc-Leu-Trp + 0.2% 5FOA, do not grow on plates Sc-Leu-Trp-His + 25 mM 3AT and do not appear to be blue during X-Gal assay. Therefore, the interaction between the *C. elegans* CLK-2 and the *C. elegans* homologue of Cdc7p was not detected by the two-hybrid assay.

Figure 3. Immunoblot analysis of the expression of CLK-2 in the yeast cells containing CLK-2 bait constructs. Lane **1, 2:** protein extracts were prepared from the yeast cells MaV203 and reacted with the anti-CLK-2 polyclonal antibody. Lane **3, 4:** protein extracts were prepared from the yeast cells MaV203 containing pDB-Leu-full-length *C. elegans* CLK-2 and reacted with anti-CLK-2 polyclonal antibody. Lane **5:** protein extracts were prepared from the yeast cells MaV203 containing pDB-Leu-first half of *C. elegans* CLK-2 and reacted with the anti-CLK-2 polyclonal antibody. Lane **6:** protein extracts were prepared from the yeast cells MaV203 containing pDB-Leu-second half of *C. elegans* CLK-2 and reacted with the anti-CLK-2 polyclonal antibody.

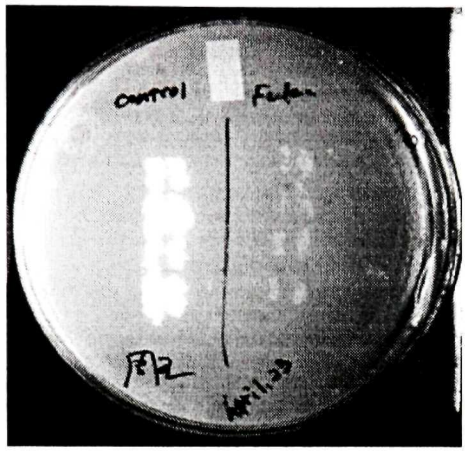


Full-length CLK-2: amino acid residues 1-877

1st half of CLK-2: amino acid residues 1-464

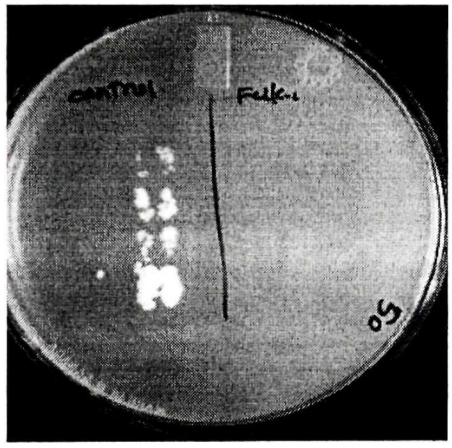
2nd half of CLK-2: amino acid residues 458-877

Figure 4. Determination of the 3AT concentration required to inhibit the basal expression of *HIS3*, which does not result from interactions. Growth of the yeast cells containing **pDB-Leu-full length CLK-2** and empty AD vector **pPC86** along with the control yeast strains on the Sc-Leu-Trp-His plates added with 3AT at different concentration is shown. The colonies marked as **1** were patched from the isolated colonies derived from the yeast cells transformed with **pDB-Leu-full length CLK-2** and **pPC86.** The yeast colonies marked as **A, B, C, D, E** on the plates were patched from two isolated colonies derived from yeast control strains provided by *Proquest* two-hybrid system. The interaction strength in these control strains is shown in Table 1. All these colonies have been replica plated on these plates from same master plate.

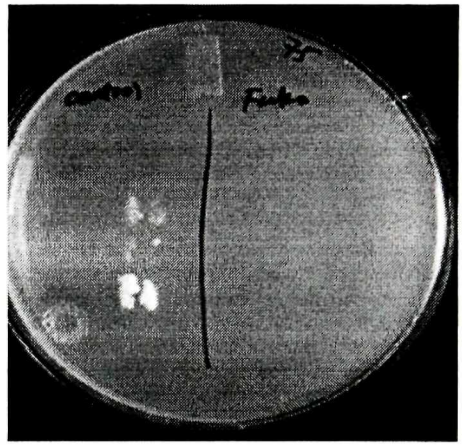


Sc-leu-Trp-His + 10 mM 3-AT

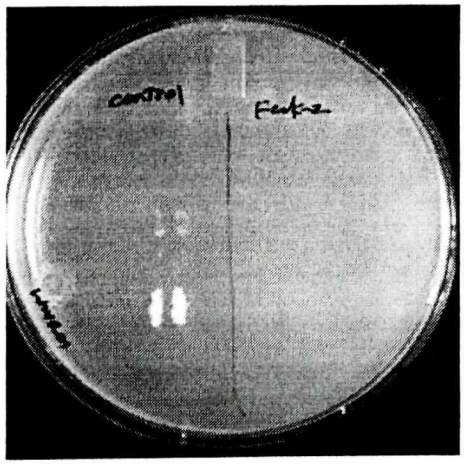
Sc-leu-Trp-His + 25 mM 3-AT



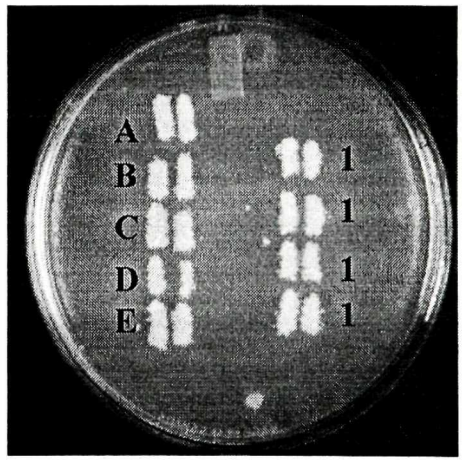
Sc-leu-Trp-His + 50 mM 3-AT



Sc-leu-Trp-His + 75 mM 3-AT



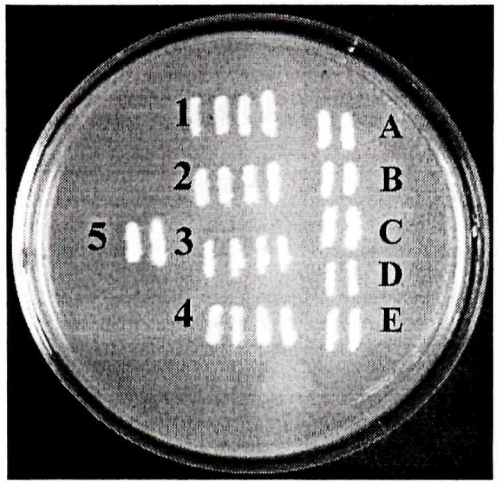
Sc-leu-Trp-His + 100 mM 3-AT



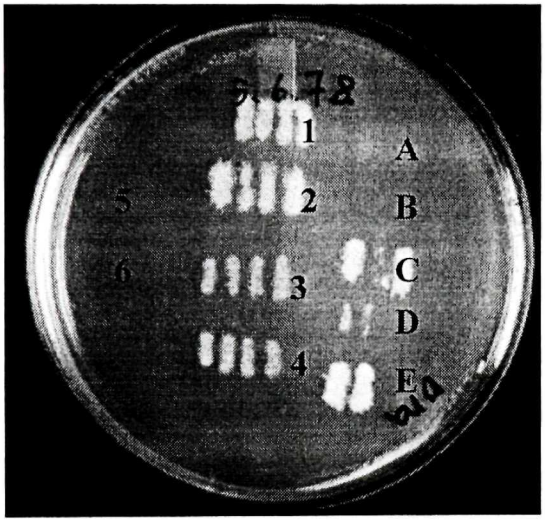
Sc-leu-Trp-His + 0 mM 3-AT

Figure 5. Characterization of the candidate colonies obtained from the initial selection plates (Sc-Leu-Trp-His + 25 mM 3AT). **A.** Candidate colonies with the control strains on a master plate (Sc-Leu-Trp). Four short lines marked as **1, 2, 3, 4** were patched onto the centre of the plate from four isolated colonies derived from each candidate colony, identified by initial selection 'lacking histidine with 25 mM 3AT'. Two short lines marked as **5** were patched onto the left side of the plate from two isolated colonies derived from the yeast strain containing pDB-Leu-full-length CLK-2 and pPC86. Two short lines marked as **A, B, C, D, E** were patched onto the right side of the plate from two isolated colonies derived from the yeast control strains provided by the *Proquest* two-hybrid system. The interaction strength in these control strains is listed in Table 1. **B.** Candidate yeast colonies along with the yeast control strains on the selection plates (Sc-Leu-Trp-Ura; Sc-Leu-Trp + 0.2% 5FOA; Sc-Leu-Trp-His + 25 mM 3AT; membrane saturated with X-Gal solution). The colonies marked as **1, 2, 3, 4** on the centre of the selection plates and membrane saturated with X-Gal solution were patched from four isolated colonies derived from each candidate colony, which can grow on the initial selection plates Sc-Leu-Trp-His + 25 mM 3AT. The colonies marked as **5** on the selection plates and membrane saturated with X-Gal solution were patched from 4 isolated colonies derived from the yeast strain containing pDB-Leu-full length CLK-2 and pPC86. The yeast colonies marked as **A, B, C, D, E** on the selection plates and the membrane saturated with X-Gal solution were patched from two isolated colonies derived from the yeast control strains provided by *Proquest* two-hybrid system. All these colonies have been replica plated onto these selection plates from same master plate.

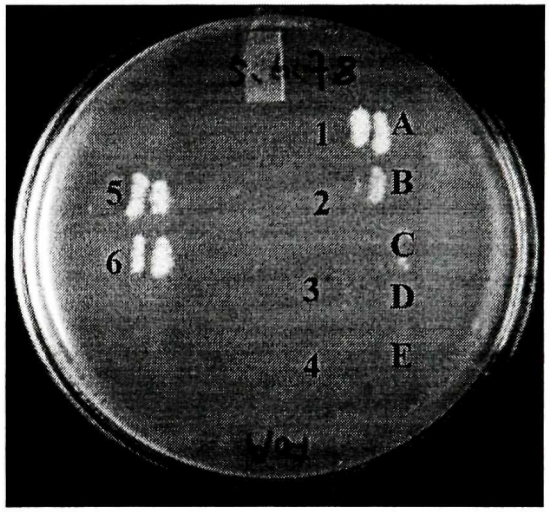
A



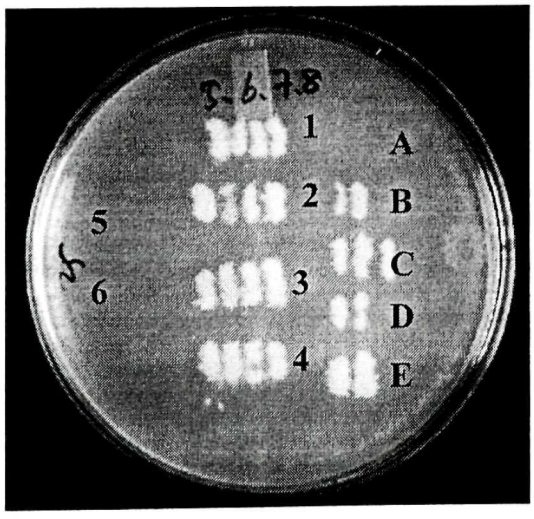
B



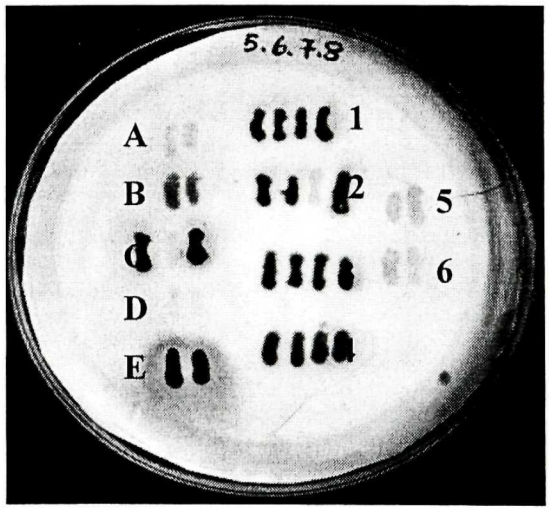
Sc-Leu-Trp-Ura



Sc-Leu-Trp + 0.2% 5FOA

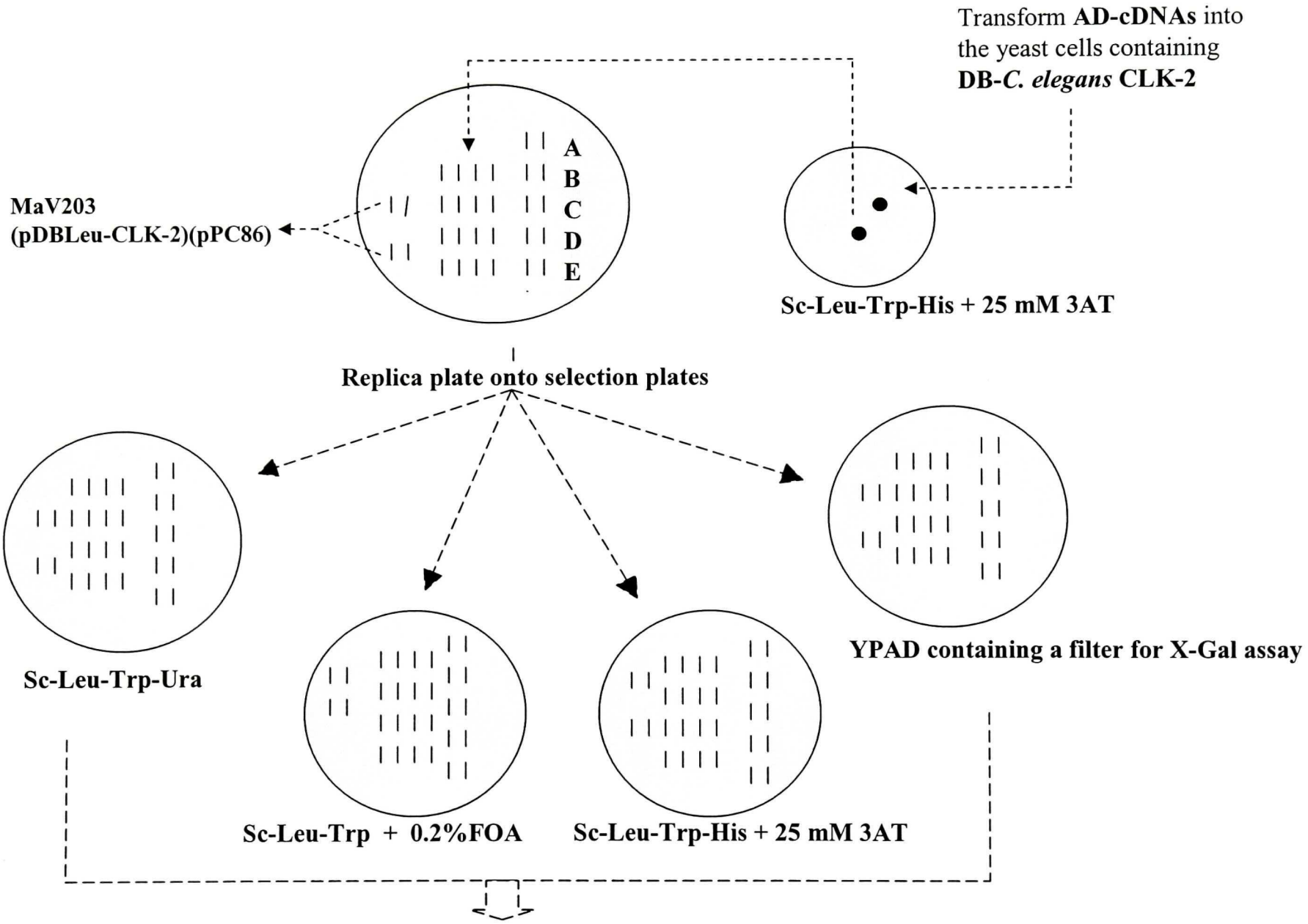


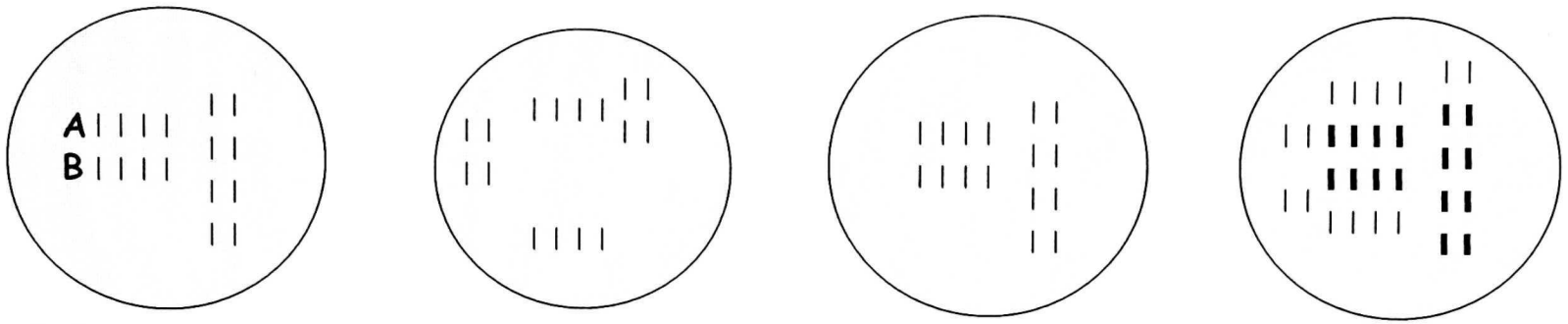
Sc-Leu-Trp-His + 25 mM 3AT



X-Gal assay

Figure 6. The procedure followed to characterize the candidate colonies obtained during the initial selection (lacking histidine). The circles represent yeast plates. The dark dots are candidate colonies that can grow on the initial selection plates (Sc-Leu-Trp-His + 25mM 3AT). The short lines on the plates are yeast cells patched from the isolated colonies derived from the yeast candidate colonies or the control strains.


Transform **AD-cDNAs** into the yeast cells containing **DB-*C. elegans* CLK-2**
A
B
C
D
E
MaV203
(pDBLeu-CLK-2)(pPC86)
Sc-Leu-Trp-His + 25 mM 3AT
Replica plate onto selection plates
Sc-Leu-Trp-Ura
YPAD containing a filter for X-Gal assay
Sc-Leu-Trp + 0.2%FOA
Sc-Leu-Trp-His + 25 mM 3AT
Determine the phenotypes of the candidate colonies on the selection plates



Sc-Leu-Trp-Ura **Sc-Leu-Trp + 0.2%FOA** **Sc-Leu-Trp-His + 25 mM 3AT** **YPAD containing a filter for X-Gal assay**

Extract AD fusions from the candidate colonies where the reporter genes have been induced (e.g. colony A and B)

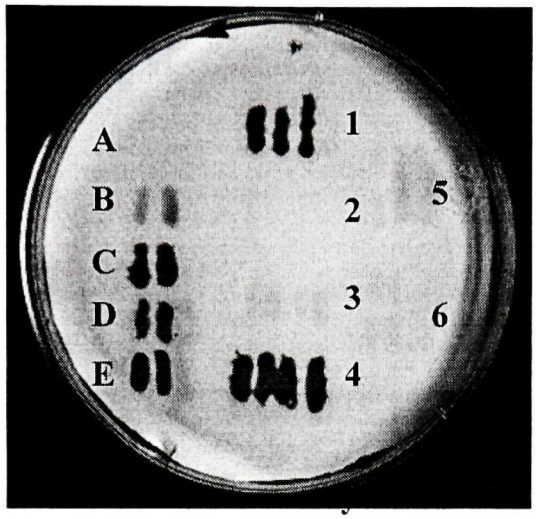
Transform the AD fusions extracted from the candidate colonies and DB-*C. elegans* CLK-2 into freshly prepared yeast cells MaV203 (retransformation assay)

Examine expression of the reporter genes in the yeast cells from the retransformation assay.

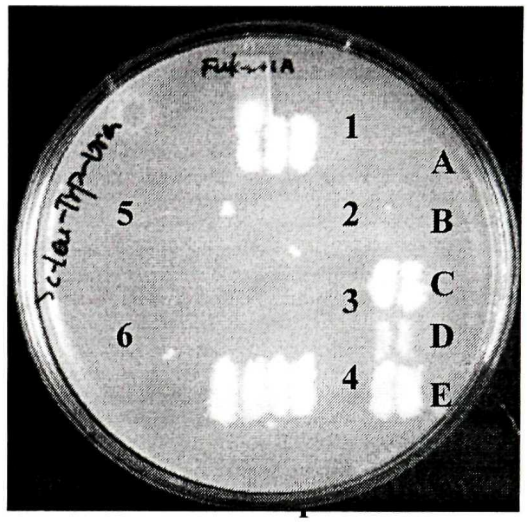
If the reporter genes' phenotypes have been reproducted in the retransformation assay, the AD-fusions likely contain CLK-2 interactors

Sequence the cDNA inserts in the AD fusions, identify the CLK-2 interactors

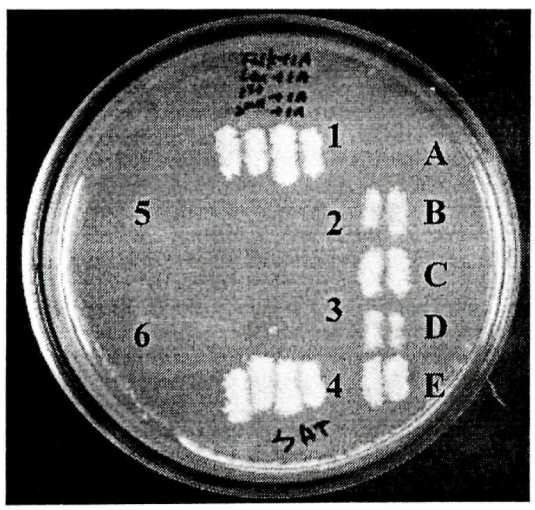
Figure 7. Retransformation assay to verify the candidate interaction between CLK-2 and Y105C5B.19. The yeast colonies from the retransformation assay with the yeast control strains on the selection plates (Sc-Leu-Trp-Ura; Sc-Leu-Trp + 0.2% 5FOA; Sc-Leu-Trp-His + 25 mM 3AT; membrane saturated with X-Gal solution) are shown. The colonies marked as **1** were patched from four isolated colonies derived from the yeast cells transformed with **pDB-Leu-full length CLK-2** and **pPC86-Y105C5B.19.** The colonies marked as **2** were patched from four isolated colonies derived from the yeast cells transformed with **pDB-Leu** and **pPC86-Y105C5B.19.** The colonies marked as **3** were patched from four isolated colonies derived from the yeast cells transformed with **pDB-Leu-1st half CLK-2** and **pPC86-Y105C5B.19.** The colonies marked as **4** were patched from four isolated colonies derived from the yeast cells transformed with **pDB-Leu-2nd half CLK-2** and **pPC86-Y105C5B.19.** The colonies marked as **5** were patched from the isolated colonies derived from the yeast cells containing **pDB-Leu-1st half of CLK-2** and **pPC86**. The colonies marked as **6** were patched from the isolated colonies derived from the yeast cells containing **pDB-Leu-2nd half of CLK-2** and **pPC86**. The yeast colonies marked as **A, B, C, D, E** on the plates were patched from two isolated colonies derived from the yeast control strains provided by *Proquest* two-hybrid system. The interaction strength in these yeast control strains is listed in Table 1. These colonies have been replica plated onto these selection plates from same master plate.



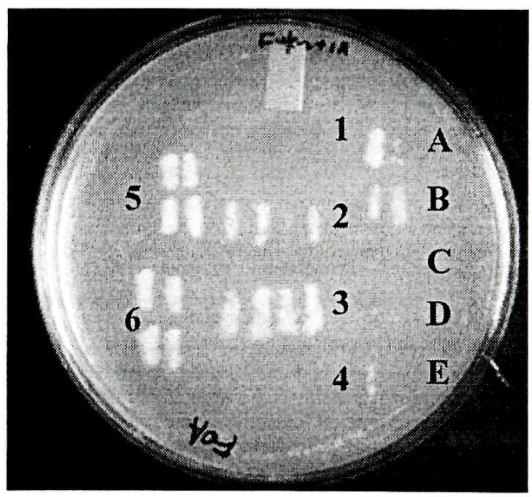
X-Gal



Sc-Leu-Trp-Ura



Sc-Leu-Trp-His + 25 mM 3AT



Sc-Leu-Trp + 0.2% 5FOA

Figure 8. An example of a PCR amplification of **Y105C5B.19** DNA from the candidate colonies for CLK-2 interactors, that can grow on the initial selection plates Sc-Leu-Trp-His + 25 mM 3AT, with the primers (SHP 2341 and SHP 2342) specific to Y105C5B.19. The PCR template is the DNA extracted from the candidate colonies. A PCR product, which size is 1 kb, was successfully amplified in the samples, indicating all cDNA inserts in these candidate colonies correspond to the same predicted gene, Y105C5B.19.

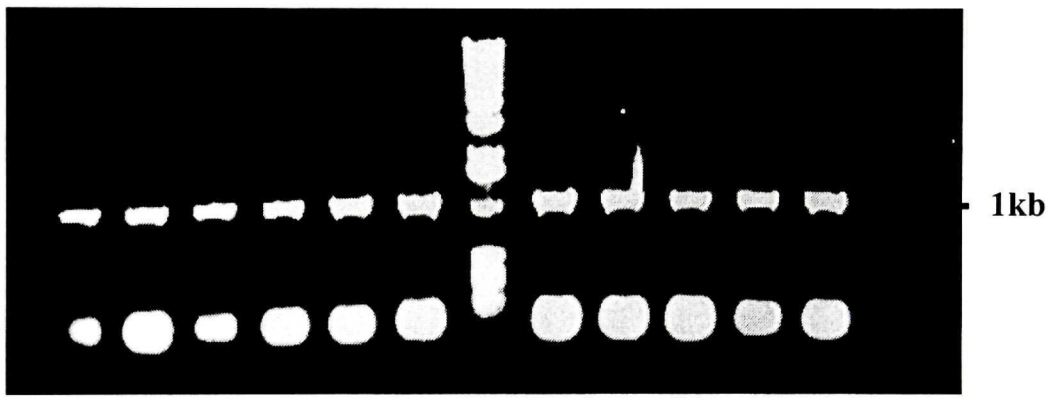
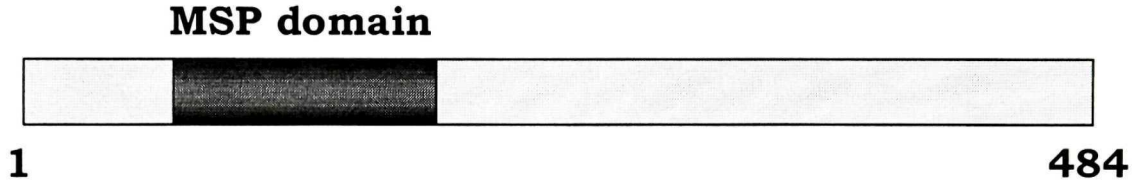
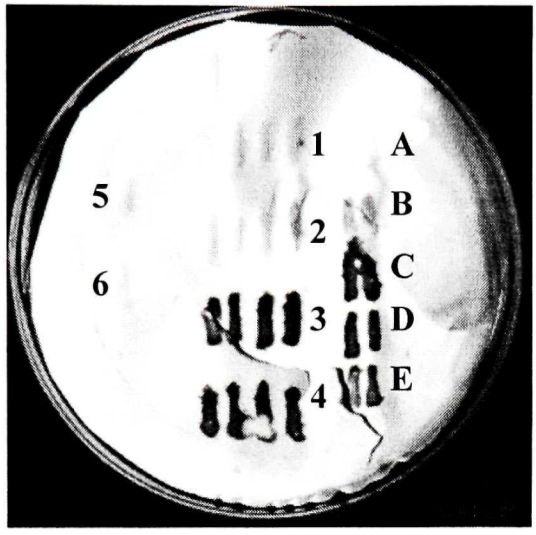

1kb

Figure 9. The structure of the protein product of Y105C5B.19, the potential CLK-2 interactor identified in the two-hybrid screen. The predicted protein product of this gene is 484 amino acids long. The only recognizable domain in the protein product of this gene is an MSP domain (from residues 45 to 178 of the protein), which is 66% identical to the MSP proteins.

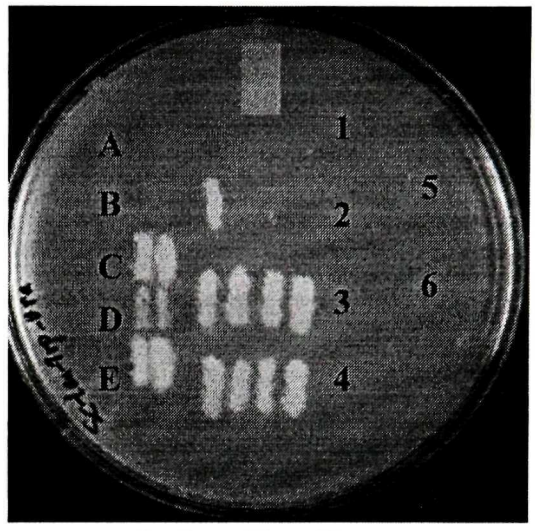


Y105C5B.19

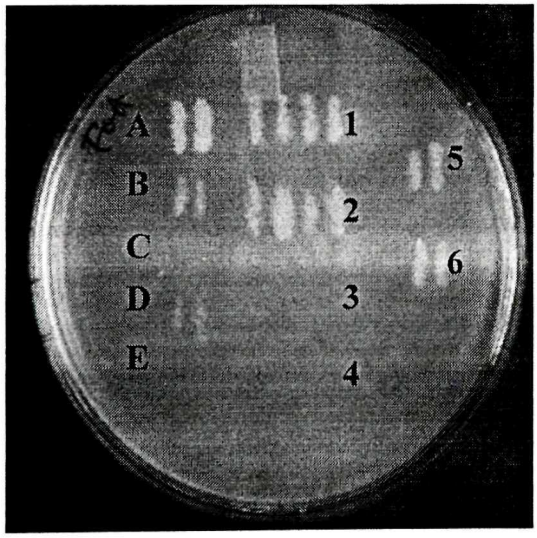
Figure 10. A two-hybrid assay for the interactions between the two portions of the protein CLK-2 and Y105C5B.19. The yeast colonies transformed with **DB-1st half of CLK-2**(amino acid residue from 1 to 464) or **DB-2nd half of CLK-2** (amino acid residue from 458 to 877) and **AD-Y105C5B.19** along with the yeast control strains on the selection plates (Sc-Leu-Trp-Ura; Sc-Leu-Trp + 0.2% 5FOA; Sc-Leu-Trp-His + 25 mM 3AT; membrane saturated with X-Gal solution) are shown. The colonies marked as **1, 2** were patched from the isolated colonies derived from the yeast cells containing **pDB-Leu-1st half of CLK-2** and **pPC86–Y105C5B.19.** The colonies marked as **3, 4** were patched from the isolated colonies derived from the yeast cells containing **pDB-Leu-2nd half of CLK-2** and **pPC86-Y105C5B.19.** The colonies marked as 5 and 6 were used as controls. The colonies marked as **5** were patched from the isolated colonies derived from the yeast cells containing **pDB-Leu-1st half of CLK-2** and **pPC86**. The colonies marked as **6** were patched from the isolated colonies derived from the yeast cells containing **pDB-Leu-2nd half of CLK-2** and **pPC86**. The yeast colonies marked as **A, B, C, D, E** on the plates were patched from the isolated colonies derived from each yeast control strain provided by *Proquest* two-hybrid system. The interaction strength in the yeast control strains is shown in Table 1. All these colonies have been replica plated onto these selection plates from same master plate.



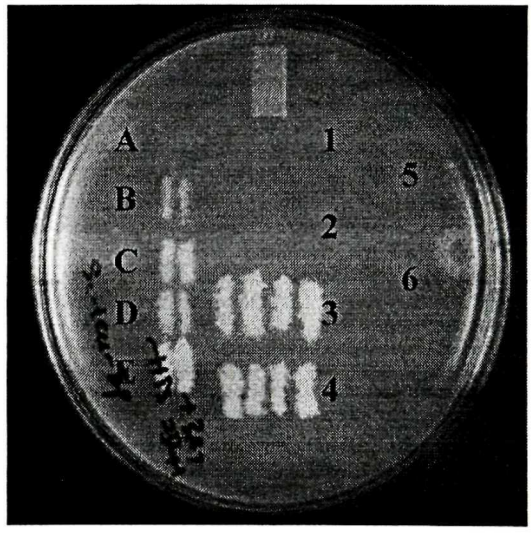
X-Gal Assay



Sc-Leu-Trp-Ura

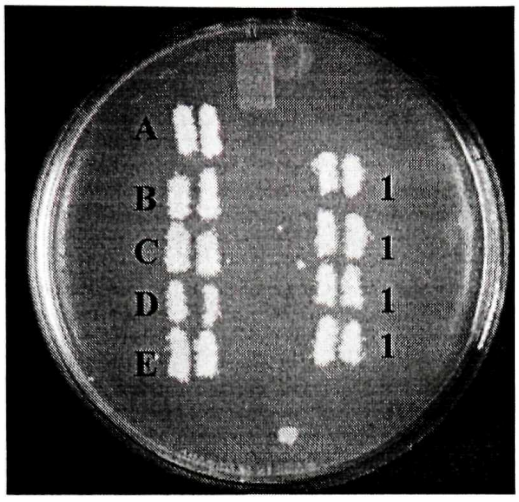


Sc-Leu-Trp + 0.2% 5FOA

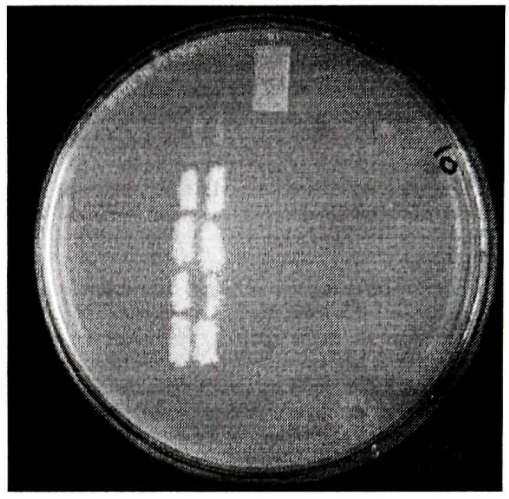


Sc-Leu-Trp-His + 25 mM 3AT

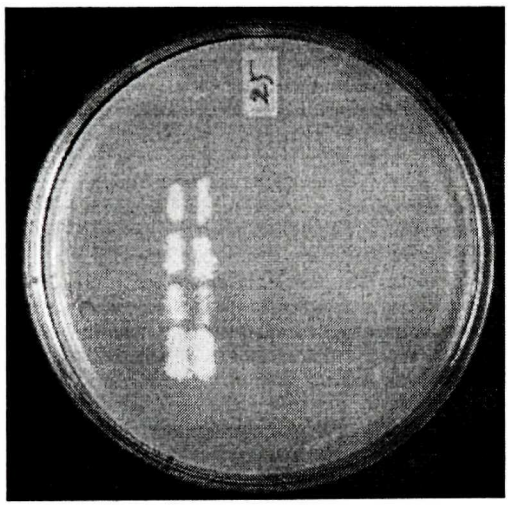
Figure 11. Determination of the 3AT concentration necessary to inhibit the basal expression of *HIS3* for the bait first half of CLK-2. Growth of the yeast cells containing **pDB-Leu-1st half of CLK-2** and **pPC86** along with the control yeast strains on Sc-Leu-Trp-His plates added with 3AT at different concentration is shown. The colonies marked as **1** were patched from the isolated colonies derived from the yeast cells transformed with **pDB-Leu-1st half of CLK-2** and **pPC86.** The yeast colonies marked as **A, B, C, D, E** on the plates were patched from the isolated colonies derived from the yeast control strains provided by *Proquest* two-hybrid system. All these colonies have been replica plated onto these plates from same master plate.



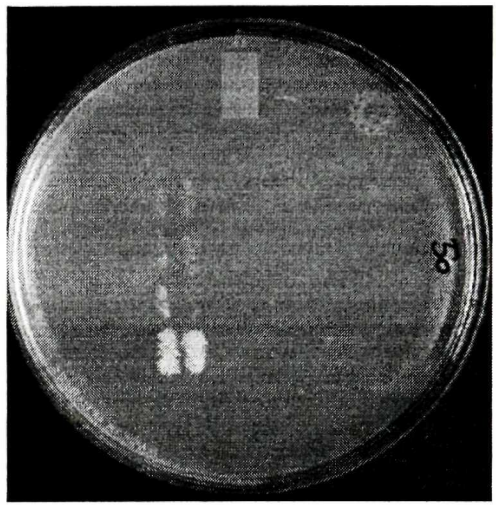
Sc-leu-Trp-His + 0 mM 3AT



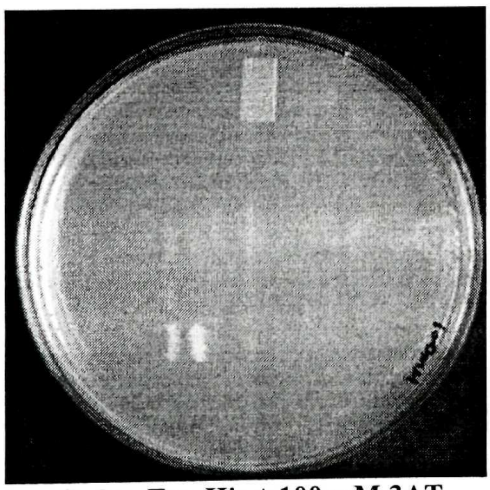
Sc-leu-Trp-His + 10 mM 3AT



Sc-leu-Trp-His + 25 mM 3AT

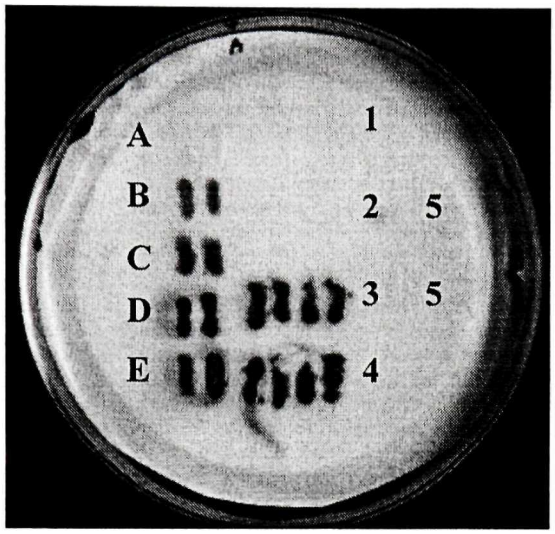


Sc-leu-Trp-His + 50 mM 3AT

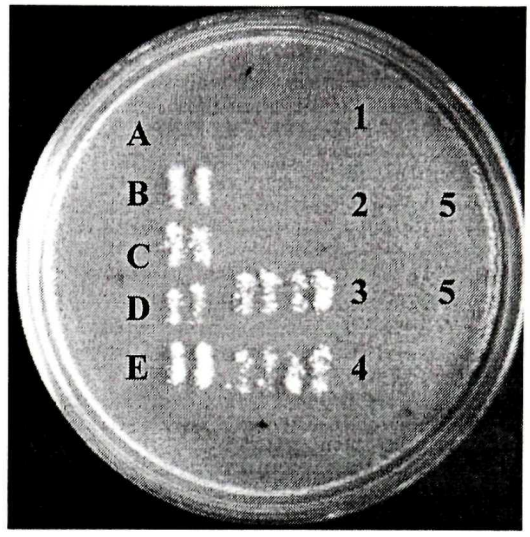


Sc-leu-Trp-His + 100 mM 3AT

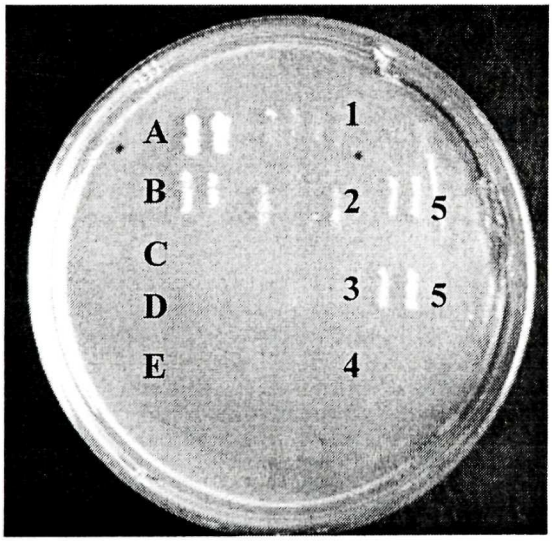
Figure 12. A two-hybrid assay to test whether the *clk-2(qm37)* mutation could disrupt the interaction between CLK-2 and Y105C5B.19. The yeast colonies containing **DB-wild-type CLK-2** or **DB-CLK-2 (C772Y)** and **AD-Y105C5B.19** along with the yeast control strains on the selection plates (Sc-Leu-Trp-Ura; Sc-Leu-Trp + 0.2% 5FOA; Sc-Leu-Trp-His + 25 mM 3AT; membrane saturated with X-Gal solution) are shown. The C772Y substitution corresponds to the *clk-2(qm37)* mutation. The colonies marked as **1** and **2** were patched from the isolated colonies derived from the yeast cells containing **pDB-Leu-CLK-2 (C772Y)** and **pPC86-Y105C5B.19.** The colonies marked as **3** and **4** were patched from the isolated colonies derived from the yeast cells containing **pDB-Leu-wild-type CLK-2** and **pPC86-Y105C5B.19.** The colonies marked as **5** were used as controls. The colonies marked as **5** were patched from isolated colonies derived from the yeast cells containing **pDB-Leu-CLK-2 (C772Y)** and **pPC86**. The yeast colonies marked as **A, B, C, D, E** on the plates were patched from two isolated colonies derived from each yeast control strain provided by *Proquest* two-hybrid system. The interaction strength in the yeast control strains is listed in Table 1. All these colonies have been replica plated onto these selection plates from same master plate.



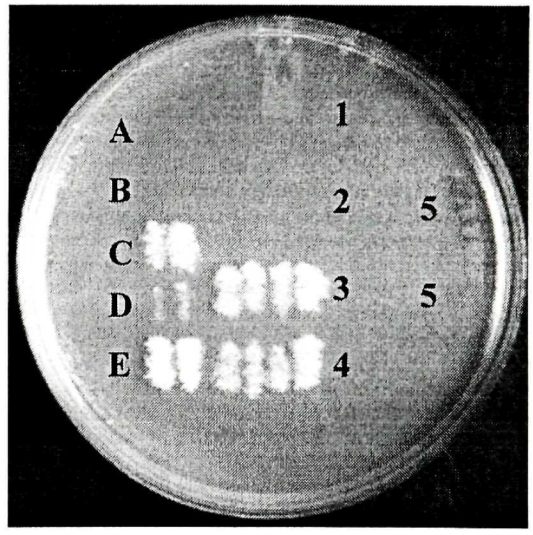
X - Gal assay



Sc-Leu-Trp-His + 25 mM 3AT



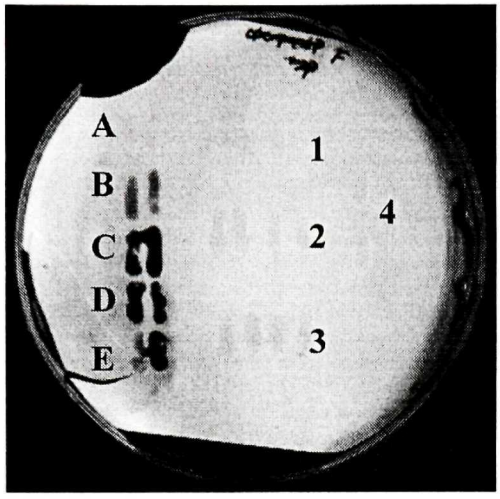
Sc-Leu-Trp + 0.2%FOA



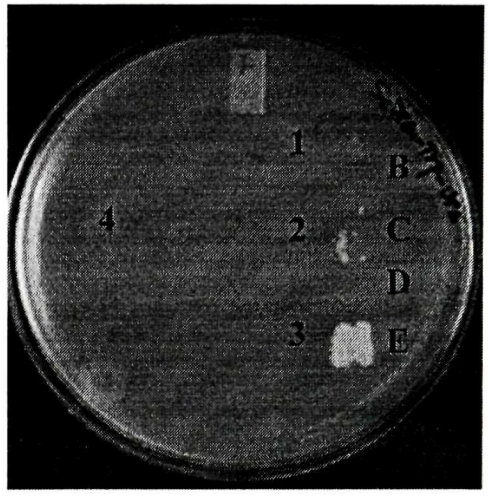
Sc-Leu-Trp-Ura

Figure 13. A yeast two-hybrid assay of the possible interaction between *C. elegans* CLK-2 and C34G6.5. The yeast colonies transformed with **DB-CLK-2** and **AD-C34G6.5** along with the yeast control strains on the selection plates (Sc-Leu-Trp-Ura; Sc-Leu-Trp + 0.2% 5FOA; Sc-Leu-Trp-His + 25 mM 3AT; membrane saturated with X-Gal solution) are shown. The colonies marked as **1** were patched from four isolated colonies derived from the yeast cells containing **pDB-Leu-full length CLK-2** and **pPC86-C34G6.5.** The colonies marked as **2** were patched from four isolated colonies derived from the yeast cells containing **pDB-Leu** and **pPC86-C34G6.5.** The colonies marked as **3** were patched from four isolated colonies derived from the yeast cells containing **pDB-Leu-Rb*** and **pPC86-Y105C5B.19.** The colonies marked as **4** were patched from the isolated colonies derived from the yeast cells transformed with **pDB-Leu-full length CLK-2** and **pPC86.** The yeast colonies marked as **A, B, C, D, E** on the plates were patched from two isolated colonies derived from each yeast control strain provided by *Proquest* two-hybrid system. The interaction strength in these yeast control strains is listed in Table 1. All these colonies have been replica plated onto these selection plates from same master plate.

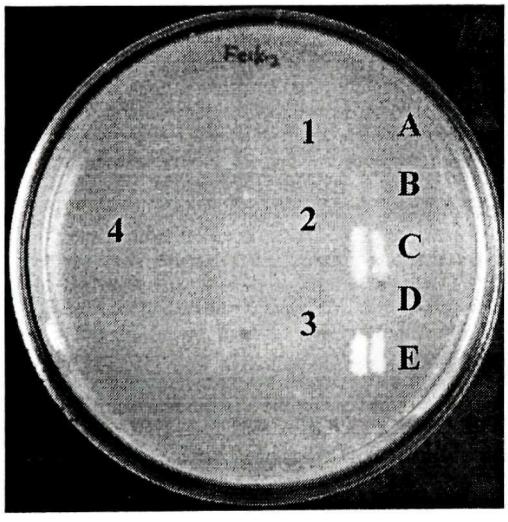
Rb is human retinoblastoma (amino acids 342-437). The fusion pDB-Leu-Rb was extracted from the yeast control strain B. Since, on occasion, a false positive AD-Y will not induce the reporter genes when tested with DB vector alone, but will when an unrelated protein is fused to DB, AD-Y105C5B.19 was tested against an unrelated protein (Human Rb). The colonies containing **pPC86-Y105C5B.19** and **pDB-Leu-Rb** marked as **3** do not grow on plates Sc-Leu-Trp-Ura, grow on plates Sc-Leu-Trp + 0.2%5FOA, do not grow on plates Sc-Leu-Trp-His + 25 mM 3AT and do not appear to be blue during X-Gal assay, indicating that AD-Y105C5B.19 not induce the reporter genes when with a unrelated protein human Rb. Thus, Y105C5B.19 might be a real interactor of CLK-2, not a self-activating false positive.



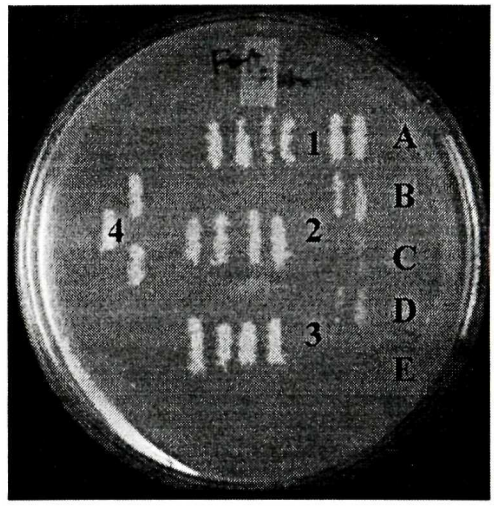
X-Gal membrane



Sc-Leu-Trp-Ura



Sc-Leu-Trp-His + 25 mM 3AT



Sc-Leu-Trp + 0.2% 5FOA

Table 5. The phenotypes of the wild-type **N2** and ***clk-2(qm37)*** mutant worms fed with **Y105C5B.19 *ds*RNA**. The brood size, the post-embryonic developmental and defecation rates of F1s of the wild-type N2 and *clk-2(qm37)* mutant worms fed with Y105C5B.19 *ds*RNA, produced by the bacteria containing the Y105C5B.19 RNAi construct, are shown. The wild-type N2 and *clk-2(qm37)* mutant worms fed with the bacteria containing the empty RNAi vector L1440 were used as control.

	N2 fed with *ds*RNA	N2 fed with no *ds*RNA	*qm37* fed with *ds*RNA	*qm37* fed with no *ds*RNA
Brood size	197 (n =15)	193 (n = 10)	96 (n =10)	100 (n = 5)
Post -embryonic Developmental rate (hours)	62 (n = 10)	62 (n = 10)	86 (n = 10)	86 (n = 10)
Defecation (seconds)	50 (n = 8)	52 (n = 7)	100 (n = 4)	100 (n = 4)

Table 6. The brood size and percentage of dead embryos of the worms injected with Y105C5B.19 *ds*RNA, *dsc-1* *ds*RNA or water. All *ds*RNAs and water were injected into the gonads of adult worms. After injection, worms have been singled and the number of F1s and the number of dead embryos produced by the injected worms were scored. The mean and standard deviation of the number of F1s produced by the injected worms are indicated at the bottom of left columns of each group. The percentage of dead embryos produced by the injected worms is shown in the right columns of each group. The brood size of wild-type N2 worms is 302.4±30.7. The brood size of *clk-2(qm37)* mutant worms is 83.4 (from Benard, et al., 2001).

Injection treatment	N2 worms			*qm37* mutants		
	P0	Number of F1s	Percentage of dead embryos	P0	Number of F1s	Percentage of dead embryos
Y105C5B .19 *ds*RNA	A	66	1%			26%
	B	31		B	10	
	C	44		C	1	
	D	39		D	2	
	E	134		E	1	
	F	24		F	1	
	G	51		G	4	
	H	2		H	3	
	49±39			3±3		
***dsc-1* dsRNA**	A	99	1%	A	12	12%
	B	139		B	2	
	C	106		C	15	
	D	52		D	1	
	E	75		E	2	
	F	76		F	6	
	G	104		G	3	
	93±28			H	7	
				7±6		
water	A	103	0.6%	A	11	2%
	B	70		B	6	
	C	85		C	5	
	D	46		D	5	
	E	67		E	13	
	F	83		F	8	
	G	84		G	4	
	77±18			8±4		

DISCUSSION

C. elegans clk-2 is an essential gene that is required for multiple aspects of somatic and germline function (Benard et al., 2001). A partial loss-of-function mutation in *clk-2*, *qm37,* results in slow developmental and behavioural rates, a reduced brood size, and an extended life span at 20°C. At the restrictive temperature (25°C), the *clk-2(qm37)* mutation leads to embryonic arrest at different times during development, which is likely the null phenotype of *clk-2* (Benard et al., 2001). In addition, *clk-2* mutants display an abnormal response to ionizing radiation (Ahmed et al., 2001).

The CLK-2 protein is 877 amino acids long and its expression level is similar at all developmental stages. Functional CLK-2::GFP accumulates in the cytoplasm of cells in *C. elegans* (Benard et al., 2001). In the yeast *Saccharomyces cerevisiae,* Tel2p is the homologue of *C. elegans* CLK-2 and it is known to function in the regulation of telomere length (Benard et al., 2001; Runge and Zakian, 1996). *C. elegans* CLK-2 and hCLK2 (the human protein homologous to *C. elegans* CLK-2) also have effects on telomere length (Benard et al., 2001; Jiang et al., 2003). Furthermore, both a partial loss-of-function mutation in the yeast gene *tel2* and mutations in *C. elegans clk-2* lead to slow growth (Benard et al., 2001). In human cells the overexpression of hCLK2 decreases the cell population doubling time and growth arrest was detected after treatment with *si*RNA to knock down the expression of hCLK2 in the cells (Jiang et al., 2003). Therefore, although the protein similarity is low, the function of CLK-2 is conserved from yeast to human.

In order to shed light onto the function of the gene *clk-2*, I have carried out a two-hybrid screen for interactors of the *C. elegans* protein CLK-2. A novel protein, encoded by the gene

Y105C5B.19, was identified as a *C. elegans* CLK-2 interactor during the screen. In the thesis, Y105C5B.19 is used to refer either to the gene or to the encoded protein product.

Identification of the interaction between CLK-2 and Y105C5B.19 by a two-hybrid screen

The protein encoded by the gene Y105C5B.19 was identified as an interactor of *C. elegans* CLK-2, after characterizing 213 candidate colonies obtained by screening the *C. elegans* cDNA library approximately 2 times. Since Y105C5B.19 has been picked up numerous times, it seems to be a strong potential interactor of the *C. elegans* CLK-2 by the two-hybrid assay.

Y105C5B.19 is a novel gene and does not have homologues in other species. The predicted protein product of this gene is 484 amino acids long. An MSP domain is the only recognizable domain in the protein product of this gene. The MSP domain present in Y105C5B.19 is 66% identical to the MSP proteins. In worms, MSP proteins are 99% identical to each other and only differ by one to four amino acids (Miller et al., 2001). MSP is the most abundant protein present in sperm, and it is well known to be a cytoskeletal protein, responsible for sperm mobility. Furthermore, it has been demonstrated that MSP is a signal for oocyte maturation and gonadal sheath contraction for ovulation in the worm (Miller et al., 2001). During *C. elegans* reproduction, oocytes arrest in cell cycle progression at the first meiotic prophase until fertilization. Sperm are stored in the storage compartment named spermatheca. When sperm are present in the spermatheca, they signal oocytes to resume their meiosis and promote the gonadal sheath cell contraction, resulting in ovulation (oocytes enter

into spermatheca). Then the oocytes undergo fertilization in spermatheca (Kuwabara, 2003). Injection of sperm-conditioned medium or purified MSP isoform into *fog-2* mutants, which show very slow oocyte maturation and sheath contraction rate resulting from a lack of sperm, produces a robust increase in oocyte maturation and sheath contraction rate. Deleting MSP by microinjecting anti-MSP antiserum resulted in suppression of ovulation through reducing the oocyte maturation rate (Miller et al., 2001). The VAB-1, which is an ephrin receptor protein-tyrosine kinase, is a receptor of MSP as well as for ephrines. Evidence indicates that MSP antagonizes Eph/ephrine signalling to promote oocytes maturation and MAPK activation, which is involved in the signalling process (Kuwabara, 2003).

Y105C5B.19 contains an MSP domain, so it is possible that it could be involved in the signalling for oocyte maturation and gonadal sheath contraction and/or sperm mobility (defects in sperm mobility lead to loss of sperm), that are essential for the ovulation and fertilization processes, as are the MSP proteins. *clk-2(qm37)* mutants display lethality at the restrictive temperature 25°C. The phenotype does not result from a failure of the gonad and germline to develop properly, since it has been found that after shifting down *clk-2(qm37)* mutants that grow at 25°C and become sterile to 20°C, they will produce numerous live embryos, indicating that their germline are functional and they have functional sperm and female gametes (Benard et al., 2001). Therefore, the lethality of *clk-2(qm37)* mutants at 25°C might result from delayed consequences of defects in ovulation and/or fertilization processes, and perhaps such defects could result from the disruption of the interaction between CLK-2 and Y105C5B.19. In *clk-2(qm37)* mutants the CLK-2 protein expression level is lower than in the wild type (Benard et al., 2001), and the aminoacid substitution C772Y corresponding

to the *clk-2((qm37)* mutation disrupts the interaction between CLK-2 and Y105C5B.19 in a two-hybrid assay. Hence, if CLK-2 and Y105C5B.19 indeed interact *in vivo*, it is likely that in the *clk-2(qm37)* mutants the interaction between these proteins is disrupted. Given that *clk-2* is required at some point during a narrow time between the end of oocyte maturation and the 2-cell stage for subsequent embryogenesis (Benard et al., 2001), it is tempting to speculate that the interaction between CLK-2 and Y105C5B.19 might be functionally relevant.

Since this two-hybrid interaction is potentially biologically relevant, it is worth to carry out additional experiments to further characterize this interaction. For example, co-immunoprecipitation of these two proteins from worm protein extracts could be performed to confirm that the interaction occurs *in vivo*.

Y105C5B.19 interacts with the second half of *C. elegans* CLK-2

The two-hybrid assays using the 1st half or 2nd half of CLK-2 as a bait and Y105C5B.19 as a prey showed that the second half of CLK-2 (aminoacid residues 458-877) interacts with Y105C5B.19, but the 1st half of CLK-2 (aminoacid residues 1-464 aa), however, does not, perhaps due to a problem of expression. This result indicates that the presence of the 2nd half of CLK-2 is sufficient for the interaction with Y105C5B.19 likely because the site of interaction between CLK-2 and Y105C5B.9 is entirely contained within the second half of CLK-2. It remains to be determined which part of Y105C5B.19 is required for its interaction with CLK-2. It is possible that it is not through the MSP domain as no MSP protein was found as an interactor of CLK-2 during the two-hybrid screen.

A yeast two-hybrid screen was carried out using the 1st half of CLK-2 as bait, to fish other CLK-2 interactors, since the 1st half of CLK-2 does not seem to interact with Y105C5B.19 by a two-hybrid assay. However, no colonies able to grow on the initial selection plates were obtained after screening 80% of the *C. elegans* cDNA library. Two possibilities may contribute to the failure of identifying CLK-2 interactors through the use of the bait 1st half of CLK-2. One possibility is that Y105C5B.19 is the only interactor of CLK-2 that is detectable in the two-hybrid system. Since the 1st half of CLK-2 does not seem to interact with Y105C5B.19, no candidate colonies could be obtained by screening with the 1st half of CLK-2 as bait. Another possibility is that the 1st half of CLK-2 is not a suitable bait. It is likely that it is not stably expressed in yeast cells or/and that it cannot enter the yeast nucleus.

The *clk-2(qm37)* mutation disrupts the interaction between CLK-2 and Y105C5B.19

The mutant protein CLK-2(C772Y) containing the aminoacid substitution resulting from the *clk-2(qm37)* mutation does not interact with Y105C5B.19 by a two-hybrid assay. This result indicates that the *clk-2(qm37)* mutation probably disrupts the interaction between CLK-2 and Y105C5B.19. It is possible that the aminoacid substitution C772Y alters the protein conformation in such a way that it disrupts the interaction with Y105C5B.19, which could suggest that the site of interaction between CLK-2 and Y105C5B.19 is towards the carboxyl terminus of CLK-2. Alternatively, a failure to detect the interaction between CLK-2(C772Y) and Y105C5B.19 could result from the instability of the protein CLK-2(C772Y).

To exclude this possibility, a western immunoblot analysis should be performed to determine the expression and stability of CLK-2(C772Y) in the yeast cells containing the fusion DB-CLK-2(C772Y).

CLK-2 is possibly a part of an interaction complex

Y105C5B.19 identified as a CLK-2 interactor in the screen, does not have homologues in other species. However, CLK-2 is conserved through evolution. It is possible that CLK-2 is a component of a complex, that carries out a conserved cellular role and contains not only conserved components but also species-specific components. An example of a complex in which some components are evolutionarily well conserved and other components are not is that of the spindle pole body in the budding yeast. The yeast spindle pole body consists of multiple components. Apart from Cdc31p, Ndc1p and the components of the Tup4p complex, homologues of other SPB components have not been found in other species (Adams and Kilmartin, 2000). Evidence suggests that the microtubule-organizing centers of animals and yeast (the spindle pole body) share a common molecular mechanism for microtubule nucleation, in spite of the great morphological differences between them (Murphy et al., 1998). Similarly, CLK-2 might be a conserved component in a complex which also contains species-specific proteins (such as Y105C5B.19 in *C. elegans*) but whose function is conserved through evolution. The interaction complex in which CLK-2 might be involved, could be found through identification of interactions with the CLK-2 interactor (Y105C5B.19). The identification of the complex would shed light onto the function of the gene *clk-2*. Therefore, it would be worth to perform a two-hybrid screen to identify the

interactors of Y105C5B.19 if the interaction between CLK-2 and Y105C5B.19 is confirmed to be real *in vivo*.

Overexpression of Y105C5B.19 cannot rescue *clk-2* mutants

Overexpression of Y105C5B.19 in *clk-2(qm37)* mutants does not rescue the embryonic lethality at 25°C, the slow defecation and the reduction of brood size at 20°C of the *clk-2* mutants. It is possibly because of that the Y105C5B.19 transgene cannot be well expressed in the germline as most transgenes in *C. elegans* (Kelly et al., 1997), where the gene Y105C5B.19 likely functions if it is indeed required for ovulation and fertilization. Another possibility is that even if the interaction between CLK-2 and Y105C5B.19 is real *in vivo*, since the *qm37* mutation disrupts the interaction or/and the CLK-2 level decreases in *clk-2(qm37)* mutants, a high level of expression of Y105C5B.19 cannot restore a functional deficiency of *clk-2*. In the *clk-2(qm37)* mutants carrying transgenic copies of Y105C5B.19, CLK-2 still cannot interact with Y105C5B.19 or the interaction has been slightly restored but not to the functional levels.

Y105C5B.19 is the only interactor identified in the screening

Surprisingly, Y105C5B.19 is the only interactor identified after screening the cDNA library approximately 2 times and it was picked out 213 times. It is possible that the gene Y105C5B.19 is abnormally highly represented in the cDNA library, as a result of bias during amplification of the cDNA library used in the two-hybrid screen. However, firstly, some genes that are not particularly abundant in the *C. elegans* cDNA library, including C34G6.5 and *clk-1*, have been successfully amplified from the cDNA library. The quality control of

the Proquest company consists of detecting 20 out of 23 different inserts of any newly produced library. Also, the number of candidate colonies obtained during the screening suggests a normal representation of Y105C5B.19 in the library. In fact, only about 30 candidate colonies, which can grow on the initial selection plates, were obtained after screening ~10^6 cDNA clones. Furthermore, this library has been successfully used by other researchers, for example, Dr Vidal's lab has reported *C. elegans* interaction networks in vulva development (Walhout et al., 2000), 26S proteasome (Davy et al., 2001) and DNA damage repair (Boulton et al., 2002) using the same initial cDNA library and the same two-hybrid system. For all these reasons, we believe that the isolation of a unique CLK-2 interactor does not result from it being highly or overrepresented in the library used during this screen.

In addition, the Y105C5B.19 homologue M199.2 in the *C. elegans* genome was never picked during the screening, although the protein encoded by the gene M199.2 displays 70% identity to Y105C5B.19 at the amino acid level (the alignment between Y105C5B.19 and M199.2 shown in Figure 14). It is possible that M199.2 can also interact with CLK-2, but that M199.2 is not represented in the cDNA library. Alternatively, M199.2 may not interact with CLK-2, making the interaction between CLK-2 and Y105C5B.19 very specific.

Independent assays need to be carried out to confirm the interaction between CLK-2 and Y105C5B.19

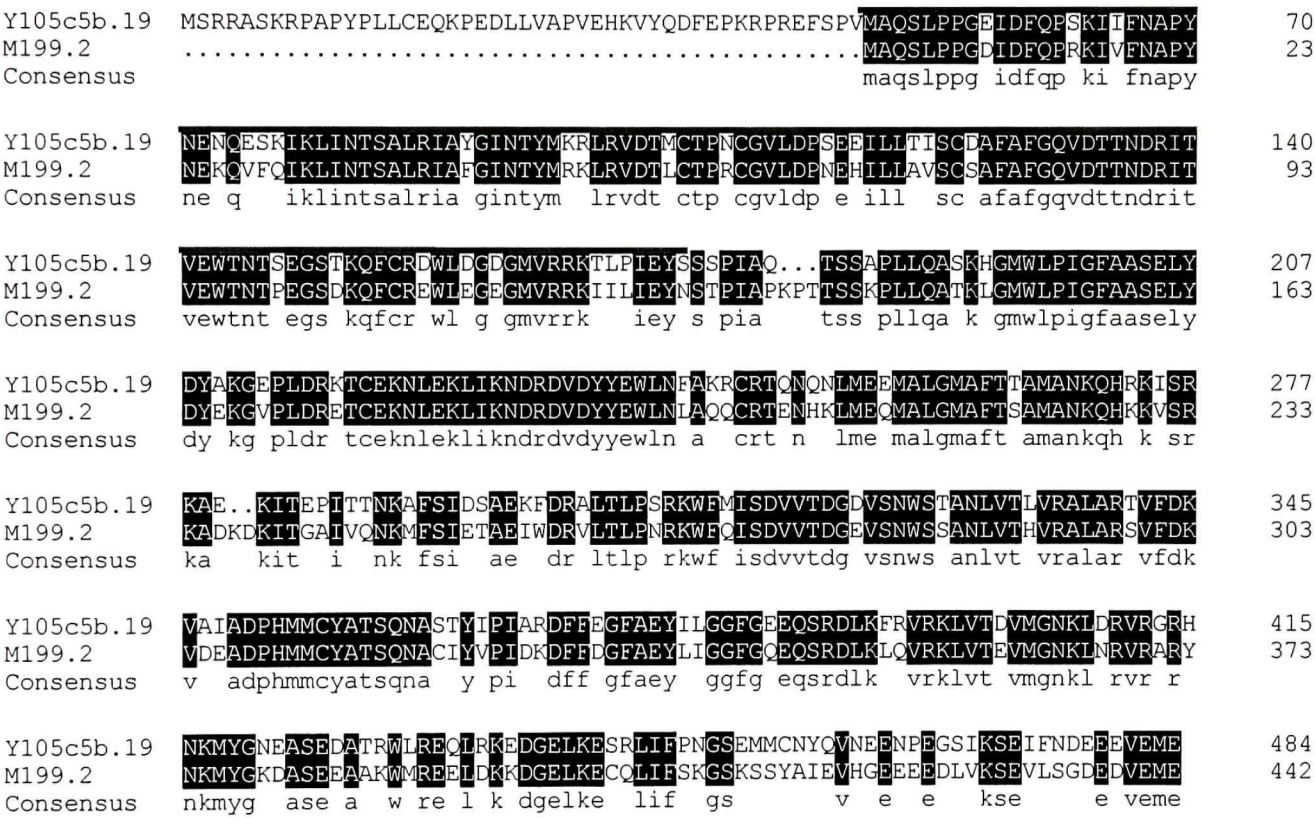
Although the interaction between CLK-2 and Y105C5B.19 seems to be real, we cannot entirely exclude the possibility that this interaction is a false positive of the two-hybrid

system. To verify the interaction, independent assays such as coimmunoprecipitation, pull-down assays, and quantitative analysis on the *lac*Z reporter gene induction in liquid culture, must be performed. In addition, it is also possible that the interaction is real, but CLK-2 also interacts with other proteins as well. Other CLK-2 interactors may not have been identified in the screening possibly because of some undetected problems in the cDNA library, for instance, some rare proteins are underrepresented in the amplified cDNA library used in the screen. In addition, the nature of the interactions between CLK-2 and other proteins, and/or the localization of these interactions within the cell, could prevent the detection of such interactions through a two-hybrid screen.

Other approaches to elucidate the function of CLK-2

Y105C5B.19 is the only interactor identified during the two-hybrid screen using the *C. elegans* CLK-2 as a bait. Y105C5B.19 is a novel gene, thus the interaction provides limited insights into the function of CLK-2. Hence, to further investigate the function of CLK-2 through the identification of proteins that physically interact with CLK-2, a two-hybrid screen using the human protein homologous to the *C. elegans* CLK-2 (hCLK2) as a bait might be useful. Furthermore, biochemical approaches, such as co-immunoprecipitation, might identify additional interactors of CLK-2.

Figure 14. The amino acid sequence alignment between Y105C5B.19 and its homologue M199.2. The region in red represents the MSP domain, which is 66% identical to the MSP proteins.


Y105c5b.19 MSRRASKRPAPYPLLCEQKPEDLLVAPVEHKVYQDFEPKRPREFSPVMAQSLPPGEIDFQPSKIIFNAPY 70
M199.2 ..... MAQSLPPGDIDFQPRKIVFNAPY 23
Consensus maqslppg idfqp ki fnapy
Y105c5b.19 NENQESKIKLINTSALRIAYGINTYMKRLRVDTMCTPNCGVLDPSEEILLTISCDAFAFGQVDTTNDRIT 140
M199.2 NEKQVFQIKLINTSALRIAFGINTYMRKLRVDTLCTPRCGVLDPNEHILLAVSCSAFAFGQVDTTNDRIT 93
Consensus ne q iklintsalria gintym lrvdt ctp cgvldp e ill sc afafgqvdttndrit
Y105c5b.19 VEWTNTSEGSTKQFCRDWLDGDGMVRRKTLPIEYSSSPIAQ...TSSAPLLQASKHGMWLPIGFAASELY 207
M199.2 VEWTNTPEGSDKQFCREWLEGEGMVRRKIILIEYNSTPIAPKPTTSSKPLLQATKLGMWLPIGFAASELY 163
Consensus vewtnt egs kqfcr wl g gmvrrk iey s pia tss pllqa k gmwlpigfaasely
Y105c5b.19 DYAKGEPLDRKTCEKNLEKLIKNDRDVDYYEWLNEAKRCRTQNQNLMEEMALGMAFTTAMANKQHRKISR 277
M199.2 DYEKGVPLDRETCEKNLEKLIKNDRDVDYYEWLNLAQQCRTENHKLMEQMALGMAFTSAMANKQHKKVSR 233
Consensus dy kg pldr tceknleklikndrdvdyyewln a crt n lme malgmaft amankqh k sr
Y105c5b.19 KAE..KITEPITTNKAFSIDSAEKFDRALTLPSRKWFMISDVVTDGDVSNWSTANLVTLVRALARTVFDK 345
M199.2 KADKDKITGAIVQNKMFSIETAEIWDRVLTLPNRKWFQISDVVTDGEVSNWSSANLVTHVRALARSVFDK 303
Consensus ka kit i nk fsi ae dr ltlp rkwf isdvvtdg vsnws anlvt vralar vfdk
Y105c5b.19 VAIADPHMMCYATSQNASTYIPIARDFFEGFAEYILGGFGEEQSRDLKFRVRKLVTDVMGNKLDRVRGRH 415
M199.2 VDEADPHMMCYATSQNACIYVPIDKDFFDGFAEYLIGGFGQEQSRDLKLQVRKLVTEVMGNKLNRVRARY 373
Consensus v adphmmcyatsqna y pi dff gfaey ggfg eqsrdlk vrklvt vmgnkl rvr r
Y105c5b.19 NKMYGNEASEDATRWLREQLRKEDGELKESRLIFPNGSEMMCNYQVNEENPEGSIKSEIFNDEEEVEME 484
M199.2 NKMYGKDASEEAAKWMREELDKKDGELKECQLIFSKGSKSSYAIEVHGEEEEDLVKSEVLSGDEDVEME 442
Consensus nkmyg ase a w re l k dgelke lif gs v e e kse e veme

REFERENCES

Adams, I. R. and Kilmartin, J. V. (2000). Spindle pole body duplication: a model for centrosome duplication? *Trends Cell Biol* **10**, 329-35.

Ahmed, S., Alpi, A., Hengartner, M. O. and Gartner, A. (2001). *C. elegans* RAD-5/CLK-2 defines a new DNA damage checkpoint protein. *Curr Biol* **11**, 1934-44.

Benard, C., McCright, B., Zhang, Y., Felkai, S., Lakowski, B. and Hekimi, S. (2001). The *C. elegans* maternal-effect gene *clk-2* is essential for embryonic development, encodes a protein homologous to yeast Tel2p and affects telomere length. *Development* **128**, 4045-55.

Boulton, S. J., Gartner, A., Reboul, J., Vaglio, P., Dyson, N., Hill, D. E. and Vidal, M. (2002). Combined functional genomic maps of the *C. elegans* DNA damage response. *Science* **295**, 127-31.

Colas, P. and Brent, R. (1998). The impact of two-hybrid and related methods on biotechnology. *Trends Biotechnol* **16**, 355-63.

Davy, A., Bello, P., Thierry-Mieg, N., Vaglio, P., Hitti, J., Doucette-Stamm, L., Thierry-Mieg, D., Reboul, J., Boulton, S., Walhout, A. J. et al. (2001). A protein-protein interaction map of the *Caenorhabditis elegans* 26S proteasome. *EMBO Rep* **2**, 821-8.

Ewbank, J. J., Barnes, T. M., Lakowski, B., Lussier, M., Bussey, H. and Hekimi, S. (1997). Structural and functional conservation of the *Caenorhabditis elegans* timing gene clk-1. *Science* **275**, 980-3.

Gershon, H. and Gershon, D. (2002). *Caenorhabditis elegans*--a paradigm for aging research: advantages and limitations. *Mech Ageing Dev* **123**, 261-74.

Hekimi, S., Benard, C., Branicky, R., Burgess, J., Hihi, A. K. and Rea, S. (2001). Why only time will tell. *Mech Ageing Dev* **122**, 571-94.

Hekimi, S., Boutis, P. and Lakowski, B. (1995). Viable maternal-effect mutations that affect the development of the *nematode Caenorhabditis elegans*. *Genetics* **141**, 1351-64.

Hekimi, S. and Guarente, L. (2003). Genetics and the specificity of the aging process. *Science* **299**, 1351-4.

Hekimi, S., Lakowski, B., Barnes, T. M. and Ewbank, J. J. (1998). Molecular genetics of life span in *C. elegans*: how much does it teach us? *Trends Genet* **14**, 14-20.

Hihi, A. K., Gao, Y. and Hekimi, S. (2002). Ubiquinone is necessary for *Caenorhabditis elegans* development at mitochondrial and non-mitochondrial sites. *J Biol Chem* **277**, 2202-6.

Jiang, N., Benard, C. Y., Kebir, H., Shoubridge, E. A. and Hekimi, S. (2003). Human CLK2 Links Cell Cycle Progression, Apoptosis, and Telomere Length Regulation. *J Biol Chem* **278**, 21678-84.

Kelly, W. G., Xu, S., Montgomery, M. K. and Fire, A. (1997). Distinct requirements for somatic and germline expression of a generally expressed *Caernorhabditis elegans gene*. *Genetics* **146**, 227-38.

Kuwabara, P. E. (2003). The multifaceted *C. elegans* major sperm protein: an ephrin signaling antagonist in oocyte maturation. *Genes Dev* **17**, 155-61.

Luban, J. and Goff, S. P. (1995). The yeast two-hybrid system for studying protein-protein interactions. *Curr Opin Biotechnol* **6**, 59-64.

Miller, M. A., Nguyen, V. Q., Lee, M. H., Kosinski, M., Schedl, T., Caprioli, R. M. and Greenstein, D. (2001). A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation. *Science* **291**, 2144-7.

Murphy, S. M., Urbani, L. and Stearns, T. (1998). The mammalian gamma-tubulin complex contains homologues of the yeast spindle pole body components spc97p and spc98p. *J Cell Biol* **141**, 663-74.

Stenmark, P., Grunler, J., Mattsson, J., Sindelar, P. J., Nordlund, P. and Berthold, D. A. (2001). A new member of the family of di-iron carboxylate proteins. Coq7 (clk-1), a membrane-bound hydroxylase involved in ubiquinone biosynthesis. *J Biol Chem* **276**, 33297-300.

Uetz, P., Giot, L., Cagney, G., Mansfield, T. A., Judson, R. S., Knight, J. R., Lockshon, D., Narayan, V., Srinivasan, M., Pochart, P. et al. (2000). A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. *Nature* **403**, 623-7.

Walhout, A. J., Sordella, R., Lu, X., Hartley, J. L., Temple, G. F., Brasch, M. A., Thierry-Mieg, N. and Vidal, M. (2000). Protein interaction mapping in *C. elegans* using proteins involved in vulval development. *Science* **287**, 116-22.

Wong A., Boutis P. and Hekimi S. (1995). Mutation in the *clk-1* gene of *Carnorhabditis elegans* affect developmental and behavioral timing. *Genetics* **139**, 1247-1259.