Unité de Biologie cellulaire et de développement
Département de Biologie
Université de Fribourg (Suisse)

Identification of differentially expressed target genes of human nucleosome remodelling Mi-2 orthologue LET-418 in *C. elegans*

THESE

Présentée à la Faculté des Sciences de l'Université de Fribourg (Suisse) pour l'obtention du grade de *Doctor rerum naturalium*

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Thèse No.1532

Imprimerie St-Paul, Fribourg
2006

Acceptée par la Faculté des Sciences de l'Université de Fribourg (Suisse) sur la proposition de:

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Fribourg, le 05 Octobre 2006

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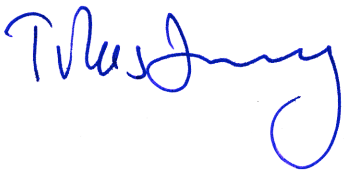

To my wife Yinghua and my newborn son Yuhao

ACKNOWLEDGMENTS

In the wake of footsteps of the new Millennium, I came here to Switzerland, this world-famous home of brave, diligent and hospitable people. I would like to thank here all those who helped providing me with the valuable information of Swiss culture, politics and science.

I want to express my deepest gratitude to my supervisor, Professor Fritz Muller (and Professor Heinz Tobler) for giving me the opportunity to undertake this fascinating and challenging project in his laboratory. I strongly appreciated the freedom and the confidence he granted me during the last five years.

My sincere thanks to Dr. Frederic Guerry for his passionate involvement of LET-418/Mi-2 on LIN-12/Notch pathway and the confirmation of *lag-2* overexpresssion in *let-418* RNAi animals and his initial finding of *lin-39* overexpresssion in *let-418* mutant and other contributions. I owe a great deal to Claude-Olivier Marti for his assistance of Chromatin Immunoprecipitation and other efforts, and Paolo Moroni for some collaboration.

Dr. Thomas von Zelewsky is acknowledged for introducing me to *C.elegans* at the beginning. Particularly I appreciated Dr. Tibor Vellai for inviting me to his original project: ceTOR on life span

I am greatly indebted to Dr. Chantal Wicky, Dr. Karin Brunschwig, Dr. Adrian Strait, Dr. Alex Puoti, Dr. Kristina Takacs-Vellai, Dr. Myriam Passannante and Dr.Thomas Berset for their kind helps, fruitful discussions, advices and encouragements.

Particular thanks to Verena Zimmerman, Yolande Molleyres, Laurence Buillard, Jean-Claude Dougoud, Hélène Panchaud, Kitty von Overbeck and Bertrand Clement for excellent technical support.

I would like to thank all members of the Muller lab and Puoti lab for their technical help, interesting discussions and suggestions. I could have never forgotten all members of the Department of Biology/Zoology, University of Fribourg for the pleasant atmosphere.

I am very grateful to Professor Bruno Betschart and Dr. Alex Puoti for accepting to be experts on the thesis committee and their important suggestions concerning the writing of this manuscript and for carefully reviewing English language of this thesis.

Finally, many thanks go to my Chinese friends (Professor Zhihong Yang, Professor Yicheng Zhang, Dr. Huanfa Liu, Dr. Yonghua Zhu, *etc*.) in Fribourg for sharing both good and bad days of research and life, playing football and basketball, etc; warmest thanks to my family for their support, and especially to my wife Yinghua for her understanding, sequencing support to my work and her love.

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Résumé

Les complexes multi-protéiques remodelant la chromatine ont été démontrés comme étant impliqués dans différents procédés tels la transcription, la réplication, l'assemblage de la chromatine et la condensation des chromosomes. Des mutations dans les composants de ces complexes multi-protéiques causent diverses maladies chez l'être humain, comme la maladie d'Alzheimer ou différents types de cancer.

Parmi ces complexes, nous trouvons le complexe Mi-2/NuRD (nucleosome remodelling and histone deacetylase) qui a d'abord été montré comme étant impliqué dans la régulation ou la modulation de nombreux procédés cellulaires et développementaux en réprimant l'expression des gènes par la désacétylation des histones. De récentes études ont montré que le complexe Mi-2/NuRD pouvait également être impliqué dans l'activation de l'expression génique. C'est pourquoi, les fonctions biologiques du complexe Mi-2/NuRD ne pourraient être totalement comprises que lorsque les conséquences de son association dans les différentes niches biochimiques ne seraient définies.

Le génome de *C. elegans* comprend des orthologues de tous les composants du complexe Mi-2/NuRD des vertébrés dont les deux homologues de Mi-2, LET-418 et CHD-3. On a montré que ces derniers jouaient non seulement des rôles essentiels mais aussi partiellement redondants durant le développement. Par des approches ciblées sur des candidats potentiels et des criblages systématiques, nous avons utilisé des techniques génétiques et biochimiques pour comprendre les fonctions de LET-418. Ces techniques incluaient le développement de protocoles de précipitation de la chromatine (ChIP), de SSH (suppression subtractive hybridization) et de RNA interference (RNAi) afin d'identifier les cibles de LET-418 et de caractériser leurs fonctions. Nous avons ainsi montré que LET-418 se liait directement au ligand *lag-2*/Delta de Notch en contrôlant le sort de la cellule « ancre » (anchor cell fate) et influençait le destin de plusieurs autres cellules potentiellement impliquées dans la cascade du Notch. De plus, nous avons aussi démontré que LET-418 avec l'aide de LIN-1/ETS se liait sur le promoteur du gène *lin-39*/Hox afin de réprimer son expression. Pour comprendre les rôles de LET-418 dans d'autres cascades de signalisation, nous avons généré un criblage SSH afin d'identifier de nouvelles cibles de LET-418. Nous avons confirmé que le gène F59A2.4 (*clp-1*) qui est impliqué dans la formation des teminaisons 3' et dans la machinerie transcriptionelle de poly-adénylation, est surexprimé dans les animaux mutants *let-418ts*. De plus, la dérégulation confirmée des gènes *sqt-2* and *sqt-3* établit un lien potentiel entre LET-418/Mi-2 et la cascade de signalisation du TGF-beta. Finalement, les animaux mutants *chd-3*(*eh4*) montrent un phénotype létal après un traitement à la chaleur. Cependant ce dernier phénotype inattendu reste à être élucidé.

Summary

Chromatin-remodeling multiprotein complexes are critically involved in processes that include transcription, replication, chromatin assembly, and chromosome condensation. Furthermore, multiple human diseases, including Alzheimer's disease and several types of cancer, are caused by mutations in chromatin-remodeling complexes.

One of these complexes is the NuRD complex (nucleosome remodelling and histone deacetylase). In the regulation or modulation of many cellular and developmental processes, the multiprotein Mi-2/NuRDcomplex was first proposed through histone deacetylation to repress gene repression, but the recent study of Mi-2/NuRD complex showed that it is to be involved in gene expression activation as well. Thus, the biological functions of Mi-2/NuRD complex can be fully understood only when the consequences of its association in biochemical niche are defined.

The *C. elegans* genome encodes orthologues of all components of the vertebrate Mi-2/NuRD complex, among them the two Mi-2 homologues LET-418 and CHD-3. LET-418 and CHD-3 were shown to play essential and partially redundant roles during development. Using both a candidate gene approach and a systematic screen, we have applied genetic and biochemical techniques to elucidate the functions of LET-418, including the development of a chromatin immuno-precipitation protocol and suppression subtractive hybridization (SSH) and RNA interference screens to assay its targets and characterize their functions. Here we show that LET-418 binds directly *lag-2*/Notch ligand Delta thereby controlling the anchor cell fate and influence fates of other cells putatively influenced by the Notch pathway. LET-418 together with LIN-1/ETS directly binds promoter of the *lin-39* /Hox gene to repress its expression. To further understand the roles of LET-418 in other pathways, we have performed a SSH screen to identify some novel targets of LET-418. We confirmed that the gene F59A2.4 (*clp-1*) is over-expressed in *let-418(ts)* animals that is involved in 3'end formation and polyadenylation transcriptional machinery. The confirmed disregulations of *sqt-2* and *sqt-3* make a putative link between LET-418/Mi-2 and the TGF beta pathway. Unexpectedly, the *chd-3(eh4)* animals show a heat shock lethal phenotype but its mechanism remains unclear.

General Introduction

During the development of an organism, a wide variety of cell fate decisions are taken based on specific inheritance or cell interactions. Over the past decade, it has been shown that alterations of gene expression can occur by the mean of chromatin modifications (Ahringer, 2000; Ng and Bird, 2000; Struhl, 1998).

One of the most extensively studied cases so far is correlated with the acetylation state of histones. Enzymes that carry out either histone acetylation (histone acetyltransferase, HATs) or deacetylation (histone deacetylases, HDACs) have been shown to function in large multiprotein complexes, that can be targeted via transcription factors to specific sites of DNA, leading to local chromatin modification (Knoepfler and Eisenman, 1999; Ng and Bird, 2000).

Recently, multiprotein complexes (such as the Mi-2/NuRD or SIN3 complex) have been shown to be implicated in the regulation or modulation of many cellular processes (Ahringer, 2000). Often, one protein can be discovered in different complexes, with each complex performing its unique function (Duchaine et al 2006). Thus, the biological functions of a given protein can be understood only when the consequences of its association in complexes are defined. The biochemical approach to define targeted complexes is often performed by using highly efficient immunopurification protocols to isolate the endogenous complexes from nuclear extracts in highly purified form. Furthermore, one protein could be involved in many distinct pathways and the global transcription remodeling and regulation of its nuclear regulatory complexes could be quite complex such that both systematic assay and candidate approach has been intensively explored for downstream targets of one protein.

Regulation of the eukaryotic chromatin structure plays an important role in the control of gene expression. In the eucaryotic nucleus, the chromatin structures that allow efficient storage of genetic information also tend to render the DNA inaccessible to metabolizing enzymes. The repressive chromatin structure must be remodeled to allow transcription and other metabolic reactions to occur. Chromatin-remodeling multiprotein complexes are critically involved in processes that include transcription,

replication, chromatin assembly, and chromosome condensation. Furthermore, multiple human diseases, including Alzheimer's disease and several types of cancer, are caused by mutations in remodeling complexes (Jarriault et al., 2002, von Zelewsky et al., 2000). Aging in several lower species (and in several human disorders with features of premature aging) can be modulated by alterations in remodeling enzymes as well.

One of these multiprotein complexes is the Mi-2/NuRD complex (*nu*cleosome *remodeling* and histone *d*eacetylase complex, Fig.1). Mi-2 protein has been shown to be a central component of the NuRD complex isolated from human cell lines and *Xenopus* egg extracts (Bowen et al., 2004; Marhold et al., 2004; Tong et al., 1998; Wade et al., 1999; Wade et al., 1998; Xue et al., 1998; Zhang et al., 1998). It has been originally identified as an autoantigen in human dermatomyositis (Ge et al., 1995; Seelig et al., 1995), and belongs to the highly conserved CHD family of proteins which, in addition to an ATPase/helicase domain of the SWI/SNF2 class, also contains two PHD zinc-finger motifs, two chromo domains, and a truncated helixturn-helix DNA-binding motif with limited similarity to the telobox DNA-binding domain (Woodage et al., 1997). Homologs of Mi-2 proteins have been identified in vertebrates, *Drosophila*, plants and also in *C.elegans* (Bowen et al., 2004; Delmas et al., 1993; Eshed et al., 1999; Kehle et al., 1998; Marhold et al., 2004; Woodage et al., 1997). In mammals, two Mi-2 proteins exist.

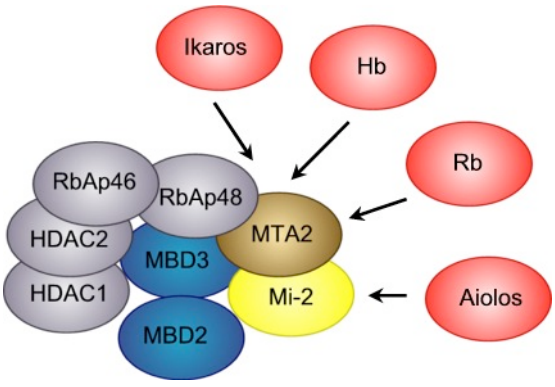

Figure 1: Scheme of the Mi-2/NuRD complex.

The Mi-2 a protein (also called CHD-4), is a component of the NuRD complex. Its paralog, CHD-3, however, is presently suggested to exist in a complex different from

NuRD (Xue et al., 1998). Besides Mi-2, the NuRD complex contains at least seven other proteins, including the class I histone deacetylases HDAC1 and HDAC2 and the histone-binding proteins RbAp46 and RbAp48. Therefore, the complex was proposed to mediate transcriptional repression of target genes by establishing or maintaining hypoacetylated chromatin domains at their promoters (Xue et al., 1998; Zhang et al., 1999). Moreover, findings that in *Drosophila* and mouse, Mi-2 proteins are targeted through sequence-specific DNA-binding proteins suggest that they might generally link remodelling complexes to transcription factors (Cortes et al., 1999; Kehle et al., 1998; Kim et al., 1999).

The eukaryotic Mi-2/NuRD multiprotein complexes are also involved in both DNA expression and genome stability. It was found that the Mi-2/NuRD components Mi-2β and HDAC2 physically interact with ATR (*A*taxia *t*elangiectasia mutated and *R*ad3-related protein), a phosphatidylinositol-kinase-related kinase that has been implicated in the response of human cells to multiple forms of DNA damage and may play a role in DNA replication checkpoint (Schmidt and Schreiber, 1999). The defects in ATR-X syndrome may result from inappropriate expression of genes controlled by this complex. The Mi-2 protein was also shown to be part of the SNF2h/cohesin complex (Hakimi et al., 2002). This suggests a function of the Mi-2/NuRD complex in the sister chromatid segregation.

Two *C. elegans* Mi-2 orthologs, LET-418 and CHD-3 have been cloned and characterized (von Zelewsky et al., 2000, Fig.2). *let-418* has revealed to be an essential gene expressed in most if not all nuclei of the worm. Mutations in *let-418* have pleiotropic phenotypes including vulval defects and sterility and, without maternal contribution, result in L1 larval arrest (von Zelewsky et al., 2000). Furthermore, *let-418* is required for the maintenance of somatic differentiation in *C. elegans* (Unhavaithaya et al., 2002). On the contrary, mutations in *chd-3* do not display any obvious phenotype. However, a requirement for *chd-3* becomes apparent in *let-418(lf);chd-3(lf)* double mutants, which display strong vulval defects and L4 arrest or, without any *let-418* maternal contribution, embryonic arrest. These results suggest that *let-418* and *chd-3* have essential and partially redundant functions during development (von Zelewsky et al., 2000,Fig.2).

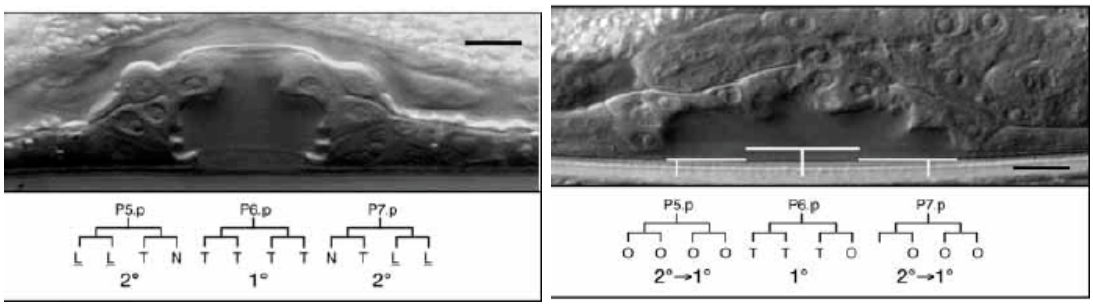

Figure 2. Nomarski images of vulval phenotypes of wild type (left) and *let-418; chd-3* (right) hermaphrodites.

To further investigate the functions of the *C. elegans* Mi-2 proteins and their relationship with putative remodelling complexes, we first searched for possible target genes with candidate approach and investigated how they may be brought to their sites of action. Formation of the hermaphrodite vulva of *C. elegans* exemplifies the fundamental mechanisms of pattern formation during organogenesis. Its well-defined and fixed lineage, amenability to analyses at the single-cell level, and availability of genetic, cellular and molecular tools (Horvitz and Sternberg, 1991), make it an important model system. Vulva formation is a microcosmos of events important in the development of all animals and these events utilize molecules that appear to be conserved in all animals. Indeed, some of these molecules or their involvement in development were first identified through studies of vulval development in *Caenorhabditis elegans.* Since *let-418* and *chd-3* mutants display striking phenotypes in the vulva and gonad (von Zelewsky et al., 2000), we decided to focus our investigations on these organs.

The Mi-2/NURD complex could function as a master on/off switch to determine whether particular genes are active or inactive. Our study also aims to discover novel targets of Mi-2/NuRD chromatin-remodeling molecules and to investigate their composition and mechanism of action. We have taken genetic and biochemical approaches to elucidate the functions of *C.elegans* Mi-2 complexes, including chromatin immunoprecipitation, SSH (suppression subtractive hybridization) screening and RNA interference technique to isolate the targets and characterize their functions: (1). In-detail analysis of their roles in Ras and Notch pathways, mainly focus on either directly regulated target genes (*lin-39* and *lag-2*) or indirectly regulated genes (*lin-39*, *lag-2*, *lin-12*) through LIN-39; (2). Identification of binding

sites of transcription factor LIN-1/ETS that recruits Mi-2 /NuRD complex; (3). Isolation of some novel putative target genes, which are involved in RNA interference, the TGF beta pathway, the Insulin/IGF-1 pathway, *etc*.

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Development of a *C.elegans* ChIP procedure

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Summary

ChIP (Chromatin Immunoprecipitation) offers an attractive solution to transcription analysis by combining the specificity of immunoprecipitation, the sensitivity of PCR and the genome-screening power of array profiling. ChIP has been classically performed to study histone modifications. The technique of ChIP involves antibodies specific to transcription factor or chromatin remodeling complex components of interest. The method allows monitoring of the interactions between DNA and transcription factors and/or components of chromatin remodeling complexes, but it is technically challenging due to the low abundance and/or only temporary interactions of these proteins. Here, we describe the development of a worm ChIP protocol upon modification on commonly used chemical cross-linking protocols developed for cultured cell lines. The feasibility of worm ChIP is expected to accelerate the further understanding of transcription mechanisms in *C.elegans.*

Keywords: Chromatin immunoprecipitation; Formaldehyde; cross-linking; transcription factor; Mi-2; *C.elegans*

Introduction

To unravel the transcription mechanisms, transcription factor and gene promoter activity has been analyzed *in vitro* using reporter gene assays, gel shift assays, South-western blotting (Bowen B *et al*, 1980) and DNA microarrays. However, these approaches can not demonstrate that a particular protein is bound to a specific DNA sequence in living cells. The determination of sites of interaction of nuclear proteins

with genomic DNA has become increasingly interesting. The ChIP (chromatin immunoprecipitation) represents a powerful tool for the spatial and temporal mapping of chromatin bound factors *in vivo* (Orlando V *et al*, 1997; Kuo M and Allis CD, 1999). This technique allows to determine whether, where and when a protein is bound and to see if the interaction with DNA is direct or indirect (see Figure 1 for the general principle of the experiment). Widely used protocols rely either on chemical reagents or UV mediated physical cross-linking to preserve native nuclear structures for subsequent biochemical and molecular analysis.

Formaldehyde is an attractive and widely used cross-linking reagent in mapping protein–DNA interactions. It penetrates membranes readily and allows cross linking in intact cells, thereby reducing the risk of redistribution or reassociation of chromosomal proteins during the preparation of cellular or nuclear extracts. The chemical targets for formaldehyde are primary amino groups (lysine amino groups and side chains of adenine, guanine and cytosine) leading to cross links between both proteins or proteins and DNA. Both types of cross-linkings can be reversed by heating (65°C for protein-DNA, boiling for protein-protein interactions) (Solomon MJ and Varshavsky A, 1985; Orlando V *et al*, 1997). After cross-linking, the cells are lysed and crude extracts are sonicated to shear the crosslinked chromatin DNA. Short chromatin DNA fragments provide higher mapping resolution and allow to determine the precise chromosomal location of chromatin associated proteins. Extensive sonication is a way to generate fairly uniformly sized pieces of chromatin, although the PCR amplification efficiency will decrease with the size of the DNA molecules. After preparation of the cellular extracts and chromatin fragmentation, proteins together with cross-linked DNA are immunoprecipitated. Protein-DNA cross-links in the immunoprecipitated material are then reversed and the DNA fragments are purified. If the protein under investigation is associated with a specific genomic region *in vivo*, DNA fragments of this region should be enriched in the immunoprecipitate (IP) as compared to other portions of the genome. The presence of the relevant genomic regions in the IP is determined by PCR amplification using specific primers from the region of interest. Comparison of the ratio of the PCR products from the region in question and from the control regions obtained in the IP relative to the input (non immunoprecipitated whole cell extract) allows to quantify the enrichment of the region of interest. Although ChIP has been used for cultured

cells for some years, a few people recently began to apply it on the tissue or whole animal (Liu L *et al*, 2005). Here we report on the development of a worm ChIP protocol modified from commonly used chemical cross-linking protocol in mapping the genomic occupancy of the Hox transcription factor LIN-39, Mi-2 chromatin remodeling protein LET-418 and acetylated histone 3 in mixed stage worms (see also attached manuscript).

Chromatin Immuno-Precipitation (ChIP)

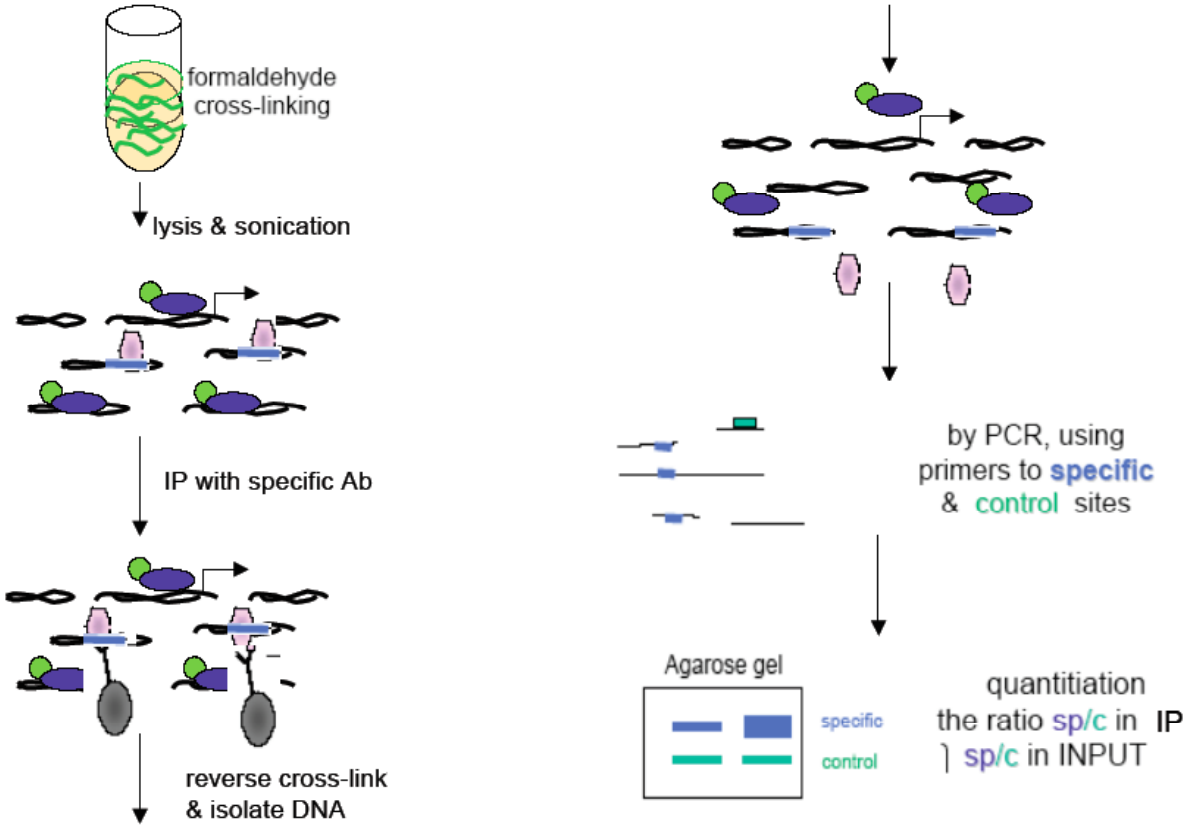


Figure 1 The principle of the worm ChIP

Living worms are treated with formaldehyde to fix the chromatin structures and lyzed . The chromatin is isolated, sheared by sonication and cross-linked protein-DNA and protein-protein complexes are solubilized. A selective precipitation of the protein of interest (pink) by a specific antibody (black Y) co-immunoprecipitate the associated DNA (IP). Cross-linking is reversed and the DNA is purified from both the INPUT (WCE) and IP samples. The precipitated DNAs are subjected to PCR amplification and gel electrophoresis to identify the associated sequences (blue). Nonassociated DNA sequences are shown in green. Associated factors are shown as a green circlee and a blue ellipse .

Method:

Cross-linking

Formaldehyde can cross-link both protein and DNAand protein and protein, so that ChIP-coIP or ChIP-re-IP is feasible too. Generally, chromatin remodeling complexes have many different components, either in direct or indirect contact with DNA. In the latter case, an adjunct cross-linking reagent (e.g. DTBP, Bis (1,1-dimethylethyl) peroxide tert-Butyl peroxide) can be helpful to facilitate retention of the intergrity of the chromatin remodeling complexes thereby improving the cross–linking (Fujita N et al., 2003).

Chromatin shearing

This is a critical step because the extent of chromatin fragmentation will influence the resolution capacity of the mapping experiment. To establish the conditions for sonication, a cellular lysate is prepared and subjected to various number of sonication cycles. Small aliquots are removed after each cycle and finally DNA is isolated and analyzed by agarose gel electrophoresis and ethidium bromide staining to determine how many cycles of sonication are needed to shear the chromatin to the desired size range. When choosing a certain size range for the chromatin, one should also keep in mind that the amplification efficiency by PCR may decrease as the average DNA fragment size approaches the distance between the PCR primers used.

Immunoprecipitation

The outcome of the experiment depends critically on the quality of the antibodies used (check for cross reactions). The antibodies may need to be affinity purified for a better specificity. The amount of antibodies necessary for the quantitative immunoprecipitation of the protein of interest has to be determined. This can be done in pilot experiments in which increasing amounts of antibodies are combined with the cell lysate. A control sample with no antibody should be included. The precipitated proteins and aliquots of the supernatant after IP are analyzed by western blotting to monitor the condition of the protein of interest in the precipitate. It is necessary to control the efficiency of the IP of each experiment by analyzing the protein content of the WCE (whole cellular extracts, input), the supernatant and the IP by Western blot. The salt and detergent concentrations in the buffers used for lysis, IP and washing

determine the stringency of the analysis. Increasing the salt concentrations or the presence of 0.1%SDS will increase the stringency and reduce non-specific IP. These conditions have to be optimised for each protein analyzed.

For each experiment one might check the specificity of the ChIP by performing IP with no antibody or with a control antibody. This allows to evaluate the background due to non-specific IP by the antibody support used for the pull down (Sepharose, magnetic beads…). For DNA binding proteins one should also perform a non cross-linked control to check that no rebinding of the protein occurs during the precipitation steps.

PCR analysis

To deduce whether a protein is associated with a particular genomic region, one compares the relative abundance of PCR products from the region in question relative to a reference. Therefore, it is important that the PCR is quantitative by either conventional PCR or real time PCR.

Detailed protocol for Chromation Immunoprecipitation in *C.elegans*

2. Cross-linking of *C.elegans* worms

Here, we present a simple protocol for the application of the formaldehyde cross-linking protocol to *C.elegans* mixed stage worms. A similar technique has been recently developed for *C.elegans* embryos (Chu S, et al 2002).

2.1. Materials required

• Mixed stage worms, grown on 6 cm standard worm NGM plates.
• M 9 (3 g KH2PO4, 6 g Na2HPO4, 5 g NaCl, 1 ml 1 M MgSO4, H2O to 1 liter).
• Formaldehyde.

2.2. Cross-linking protocol

1. Mixed stage worms, grown on 6 cm standard worm NGM plates with ice-cold M9 (pH 8.0).

2. 10 ml of ice-cold M9 (pH 8.0) containing 5 mM DTBP (Bis (1,1-dimethylethyl) peroxide tert-Butyl peroxide) is added and the worms are incubated on ice for 30 min. DTBP is relatively unstable and should be freshly prepared. DTBP concentration, pH, and cross-linking duration should be optimized.

3. Worms are washed twice with 10 ml ice- cold M9 (pH 8.0) using 15 ml Falcon tubes.

4. The remaining DTBP is quenched by adding 10 ml of ice-cold DTBP quenching buffer (100 mM Tris–HCl, pH 8.0, 150 mM NaCl) and incubation. Incubate on ice for 10 min.

Note: step 2-4 are optional. They are recommended particularly for the analysis of transcription factors or components of low abundant chromatin remodeling complexes, only temporary interaction with DNA).

5. Worms are washed three times with ice-cold M9 (pH 8.0).

6. For cross-linking with formaldehyde, worms can be treated in M9 or PBS. Formaldehyde is added to M9 (or PBS) to a final concentration of 2% (540 μl of 37% formaldehyde are added to 10 ml M9 (or PBS)). Worms are cross-linked at room temperature for 30 min by shaking gently. Note: The final concentration of formaldehyde can be rised to 4% to ensure an optimal cross-linking.

7. Excess formaldehyde is removed by three washes with ice-cold M9 or PBS. Finally, add 500 μl of 2.5 M glycine are added to 10 ml M9 or PBS to stop further cross-linking.

3. Preparation of soluble chromatin

3.1. Materials required

• Lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0, and 1 mM PMSF).

• Dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.0, and 167 mM NaCl).

• Sonicator (Misonix Sonicator 3000).

3.2. Protocol

1. Add 200 μl lysis buffer to about 200μg packed worms (corresponding to about 10^4 -10^5 individual worms), and incubate for 10 min on ice.

2. Collect lysates in a microfuge tube.

3. Sonicate on ice. We use a Misonix 3000 Sonicator with microtip and sonicate for 20 s (output Control =2, 15% duty cycle) for 4–8 cycles with cooling periods of 1

minute between each sonication step. Sonication is critical for the ChIP protocol and proper sonication conditions must be empirically determined. After the sonication, the aliquot of 5- 10% fragmented chromatins should be visualized by EtBr on agarose gel electrophoresis. We generally obtain soluble chromatin fragments with an average size of 200–1000 bp. Note: for the quantitation, add 20 μl of 5 M NaCl to the aliquot chromatin samples and 500 μl elution buffer and incubate at 65 °C for 4 h, then add 10 μl of 0.5 M EDTA, 10 μl of 1 M Tris–HCl, pH 6.5, and 2 μl Proteinase K. Incubate at 45 °C for 1 h. Purify the DNA with a QIAquick PCR product purification kit (QIAGEN) and measure the O.D. value. For one Immunoprecipiation to ensure > 40 μg chromatin DNA in the lysate.

4. Clear lysate by centrifugation (13,000 x*g*, 4 °C, 10 min).

4. Immunoprecipitation

4.1. Materials required

• Dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.0, 167 mM NaCl, and 1 mM PMSF).

• Protein A–agarose/salmon sperm DNA (Upstate Biotech, Catalog: 16-157c).

• Washing buffer 1 (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, and 0.1% SDS).

• Washing buffer 2 (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 1% Triton X-100, 2 mM EDTA, and 0.1% SDS).

• Washing buffer 3 (10 mM Tris–HCl, pH 8.0, 250 mM LiCl, 1% NP-40, 1% sodium deoxycholate, and 1 mM EDTA).

• TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA).

• Elution buffer (1% SDS, 0.1 M NaHCO3).

• 5 M NaCl.

• 0.5 M EDTA, pH 8.0.

• 1 M Tris–HCl, pH 6.5.

• Proteinase K (10 mg/ml).

• QIAquick PCR product purification kit (QIAGEN)

4.2. Protocol

Immunoprecipitation

1. To 200 μl soluble chromatin, add 1800 μl of ice cold dilution buffer.
2. Add 80 μl of protein A/salmon sperm DNA slurry (Upstate Biotech). Rotate at 4 °C for 30 min.
3. Spin for 20 s, 3000 rpm at 4 °C. Preserve supernatant and discard pellet.
4. To the supernatant, add precipitating antibodies. Note that the optimal concentration of the primary antibody for ChIP must be determined empirically. Incubate at 4 °C overnight.
5. Add 60μl of protein A / salmon sperm DNA slurry (Upstate Biotech). Rotate at 4 °C for 1 h. Spin for 20 s, 3000 rpm at 4 °C. Save the supernatant (containing the unbound fraction).
6. Add 1 ml of washing buffer 1 to the pellet, rotate for 5 min at 4 °C, and spin for 20s (3000 rpm at 4 °C). Discard the supernatant.
7. Add 1 ml of washing buffer 2 to the pellet, rotate for 5 min at 4 °C, and spin for 20s (3000 rpm at 4 °C). Discard the supernatant.
8. Add 1 ml of washing buffer 3 to the pellet, rotate for 5 min at 4 °C, and spin for 20s (3000 rpm at 4 °C). Discard the supernatant.
9. Add 1 ml of TE buffer to the pellet, rotate for 5 min at 4 °C, and spin for 20s (3000 rpm at 4 °C). Discard the supernatant.
10. Add 250 μl of elution buffer to pellet. Vortex briefly, rotate at room temperature for 15 min. Spin for 20s (3000 rpm at 4 °C). Preserve the supernatant.
11. Repeat elution step 10. Pool the supernatants from the elutions steps 10 and 11.

Reverse the cross-linking fixation

12. Add 20 μl of 5 M NaCl and incubate at 65 °C for 4 h.

The immunoprecipitated DNA purification

13. Add 10 μl of 0.5 M EDTA, 10 μl of 1 M Tris–HCl, pH 6.5, and 2 μl Proteinase K. Incubate at 45 °C for 1 h.
14. Purify the DNA with a QIAquick PCR product purification kit (QIAGEN).

4.3. Analysis of the immunoprecipitated DNA

The immunoprecipitated DNA is now analyzed by PCR amplification using appropriate primer pairs for the region of interest and the control region. The careful use of both positive and negative controls is crucial for the interpretation of the ChIP data.

Results

Application of the worm ChIP protocol—the acetylated Histone 3 on promoter of gene *act-4*.

Introduction

In order to optimize the parameters of the worm ChIP protocol, we chose the putative house-keeping and abundantly expressed gene *act-4*. We used antibodies against acetylated histone 3 (Upstate, 06-599), which were produced in rabbits immunized with a synthetic di-acetylated histone 3 peptide wth acetylated lysine 9 and lysine 14 and the acetylation correlates with active transcription (Strahl, B.D., and C.D. Allis, 2000). As acetylated histone 3 is abundant in chromatin, we expected a better yield of pull-downed DNAs by anti-acetylated histone 3 than for anti-LET-418 or No antibody after chromatin immunoprecipitation. If this would be the case, anti-acetylated histone 3 could be used as a positive control for ChIP assays. It is important to determine the efficiency of the ChIP protocol, since result validation and interpretation are difficult without appropriate controls.

Sonication

After cross-linking (see the above method), about 1g of packed mixed stage N2 worms were lysed in 1 ml of lysis buffer. Then, sonication was performed as follows: samples were sonicated on ice using a Misonix 3000 Sonicator with a microtip. Each sonication step (20 s, output control =2) was followed by a cooling period of 1 minute. Totally, 8 sonication cycles were performed, and we obtained sonicated DNA fragments with a length of about 500bp (Figure 2).

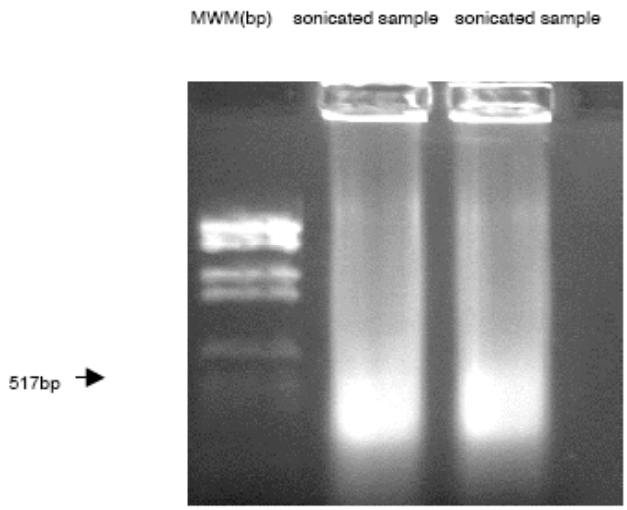


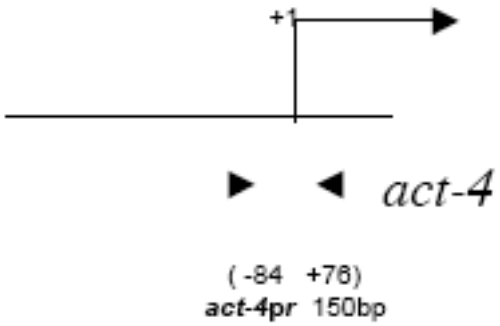
Figure2 Fragmentation of chromatin using sonication

Agarose gel electrophoresis of the sonicated samples. The estimated size of chromatin fragments is around 500bp. MWM: Molecular weight marker (pBR328/*Hind* III +pBR328/*Bgl* I)

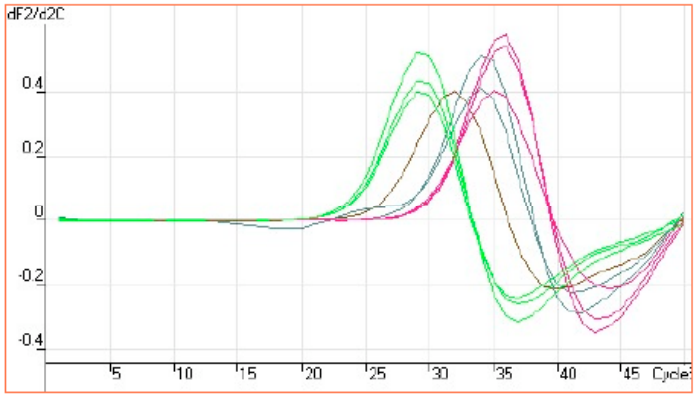
Quantitation

ChIP assays were done as described (see the above method). For a quantitation, the coimmunprecipitated DNAs were amplified by PCR using *act-4* promoter specific primers (Table 1). Quantitative real-time PCR with SYBR green was performed on a Rotorgene 3000, using the primers at a concontration of 500 nM according to the manufacturer's recommendations. A threshold cycle (C_t) value was obtained from each amplification curve using the software provided by the manufacturer. The comparative quantitation was essentially as described previously by Wang H et al (2004). A ΔC_t value was calculated by subtracting the C_t value for the 0.25% input sample from the C_t value for the immunoprecipated (IP) sample, i.e. $\Delta C_t = C_{t\,(Input)} - C_{t\,(IP)}$). The percentage of the total input amount for the IP sample was then calculated by raising 0.25 to the Ct power, i.e. the total percentage of IP sample sample $=2^{\Delta Ct} \times 0.25$. The following primers, corresponding to the *C.elegans act-4* promoter sequence (Table 1), were used for real time PCR. PCR cycle parameters: denaturation at 94°C for 15s, annealing at 57°C for 20s, and extension at 72°c for 20s, with a total of 50 cycles. The melting curve analysis (Figure3a) and agarose gel-run (not shown) were performed to rule out primer-dimers for the primer specificity.

(a)



(b)



(c)

No.	Colour	Name	Takeoff	Amplification
13		INPUT.act4pr	27.4	1.79
14		NoAb-act4pr	29.8	1.79
15		NoAb-act4pr	30.1	1.76
17		Mi2-act4pr	31.5	1.80
18		Mi2-act4pr	31.5	1.83
19		Mi2-act4pr	30.8	1.80
20		H3-act4pr	25.2	1.79
21		H3-act4pr	25.1	1.80
22		H3-act4pr	25.1	1.80

Figure3a Real time quantitation of ChIPed DNA

Each reaction was run in duplicate or triplicate and the melting curves were monitored using Rotor-gene software to ensure that only a single product was amplified. The left upper panel (a) shows schematically the *act-4* promoter/ATG region (-84,+75). The right middle panel (b) shows results of a real-time comparative quantitation report. The table at the bottom right (c) shows the Ct values and the amplification efficiency. To do this, DNA samples obtained in the assay (Input, LET-418/Mi-2, H3 /anti-acetylated histone 3 and No Ab) were used.

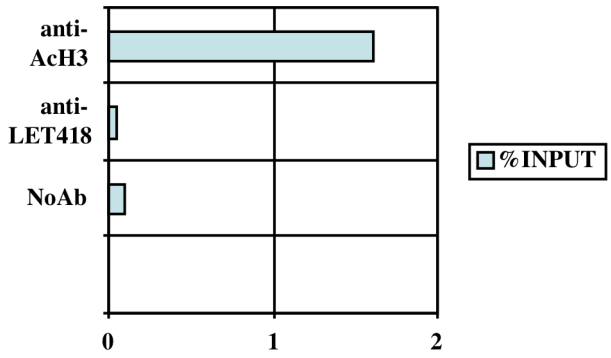


Figure 3b Comparative quantitation of ChIPed DNAs pulled down by anti LET-418 antibody, anti acetylated histone 3 antibody or no antibody from the *act-4* promoter

The immunoprecipited DNAs pulled downn by anti LET-418 antibody, anti acetylated histone 3 antibody or no antibody were quantified by real time PCR and represented by the percentage of the total input (Figure3a and also see the text for details).

Table 1 The sequences of primers for the promoter of gene *act-4*

Name	Forward/reverse	sequences
act-4pr	F	5'GCG TGC AGC AGT ATA AAT AGG C3'
	R	5'ATC AAC TCA CCT TGC ACA TTC C3'

Conclusion

The results show a significant enrichment of coimmunoprecipitated DNA with acetylated histone 3 antibodies, but not with the LET-418/Mi-2 antibodies or in the mock–IP (no antibody). This demonstrates that the enrichment of *act-4* promoter DNA was due to the acetylated anti-histone 3 antibodies, and was not simply a result of non-specific sticking to IgG or Protein G beads. Under this condition, acetylated histone 3 binds specifically to the tested region of the *act-4* promoter but LET-418 does not. The fact that the gene *act-4* is constitutively and abundantly expressed, is consistent with our data: the tested region of promoter of *act-4* is aceylated on histone 3. Aceylation of histone 3 is expected to correlate with the absence of LET-418 in the tested promoter region of *act-4*, since the Mi-2/NuRD complex is supposed to be a transcriptional repressor. Background levels can vary, but a typical background level for DNA fragments that do not associate with the protein of interest is 0.025% to 0.05% of total input (this study, not shown). However, this anti-acetylated histone 3 antibody pulls downed about 1.6 % of total INPUT. If a specific antibody gives some

immunoprecipitation efficiency like 0.02%-0.1% level, which is close to background(Wang H et al 2004), thus such antibody could be meaningful as a positive technical antibody control. It can demonstrate if ChIP assay works.

Application of the worm ChIP protocol—the Hox transcription factor LIN-39 on the promoter of *egl-17* (Figure 4)

Introduction

Based on reporter gene assays and bioinformatics, the *C.elegans* fibroblast growth factor gene *egl-17* has been recently proposed to be one direct target gene of LIN-39 (Cui M and Han M 2003). To test this hypothesis, we have used chromatin immunoprecipitation. Furthermore, we have also tested, whether LET-418 binds to the *egl-17* promoter.

Sonication

Lysis and sonication was done as described in experiment 1: about 1g packed mixed stage N2 worms were lysed in 1 ml lysis buffer. The chromatin sonicated to an average length of 500bp (not shown), and the soluble chromatin were divided equally into 5 tubes for further processing.

Quantitation

For the input DNA control, the DNA was extracted from 2% of the diluted starting lysates as detailed in step 1. Quantitative conventional PCR amplifications (30-33 cycles at 94°C for 30 sec, 57 °C annealing for 40 sec and 72°C extension for 90 sec in 50 µl of reaction volume) were carried out on 2 µl of precipitated DNA. PCR primer pairs are listed in Table 2. For the analysis on agarose gels, the number of PCR amplification cycles was adjusted so that no or only a very weak signal could be detected for the mock-IP DNA (no antibody). Multiplex PCR was performed using QIAGEN multiplex kit according to the manufacturer's description. The PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining.

Results

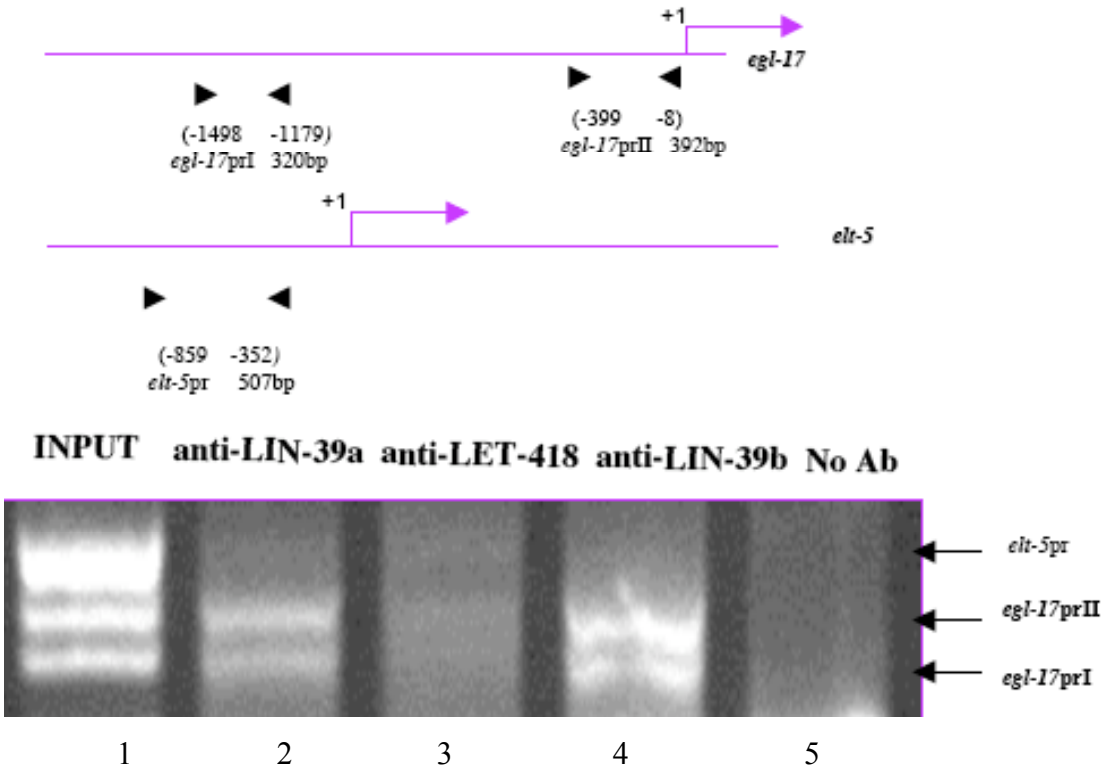


Figure 4 ChIP assay of the LIN-39 to the *egl-17* promoter and *elt-5* promoter

Agarose gel electrophoresis of the PCR products of immuoprecipitated DNA samples from different antibodies and the primers specific either to *egl-17* promoter or *elt-5* promoter (as negative control).

Lane 1: PCR amplification using 2% INPUT and specific primers for the promoters of the genes of *egl-17* and *elt-5*.

Lane 2: PCR amplification using IP samples with LIN-39 antibody (Santa-cruz Biotech Inc.) and specific primers for the promoters of the genes of *egl-17* and *elt-5*.

Lane 3: PCR amplification using IP samples with LET-418 antibody (LET-418Ab) and specific primers for the promoters of the genes of *egl-17* and *elt-5*.

Lane 4: PCR amplification using IP samples with LIN-39 antibody (Dr.C.Kenyon lab) and specific primers for the promoters of the genes of *egl-17* and *elt-5*.

Lane 5: PCR amplification using IP samples with no antibody (NoAb) and specific primers for the promoters of the genes of *egl-17* and *elt-5*.

Table 2 The sequences of the primers specific for the promoter of the genes *egl-17* and *elt-5*

Name	Forward/reverse	sequences
egl-17prI (promoter site I)	F	5'CAAAACCAATAGCGCTCAAA3'
	R	5'AAATTGGCGTACGCTTTCTG3'
egl-17prII (promoter site II)	F	5'CGGTGTTCGTTGGAAGAAAT3'
	R	5'ACTCCAGAAGGGGATTTTTG3'
elt-5pr (promoter site)	F	5'TTGATATATTTCGAGTTTCCTTGACA3'
	R	5'AAACATAGGGATCTACTCAAAATTAGC3'

This ChIP assay clearly demonstrates a strong enrichment of IP (immunoprecipitated) DNA from *egl-17* promoter with LIN-39Ab in comparison with LET-418 antibody (LET-418Ab) or No antibody (NoAb). But there are no obvious enrichments from *C.elegans* GATA transcription factor *elt-5* promoter with LIN-39Ab.

Conclusion: LIN-39 is specifically associated with the *egl-17* promoter but not with *elt-5* promoter. Hence, LIN-39 may directly regulate the transcriptional expression of the gene *egl-17*. The fact that no obvious enrichments of the IP DNAs with LET-418 Ab was seen, suggested that LET-418 probably does not localize these examined regions and therefore does not directly regulate the transcriptional expression of the gene *egl-17*.

General conclusion and perspectives

This worm ChIP protocol has been optimized by using acetylated histone 3 antibody and was successfully applied for the determination of the distribution of the *C.elegans* Hox transcription factor LIN-39 on the promoter of the gene *egl-17* using mixed stage worms. The application of bifunctional cross-linking reagents in mapping protein–DNA interactions in native chromatin promises to be helpful. Once a positive ChIP data is obtained for specific transcription factors or chromatin remodeling complex using mixed stage worms, the detailed stage–specific ChIP analysis can be performed. For further investigation, artificial constructs can be made and further ChIP assays should be performed. The powerful ChIP in combination with tiling arrays, mass spectrometry or direct new sequencing technology will dramatically speed up our understanding of direct downstream targets.

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The Mi-2 nucleosome-remodelling protein LET-418 is targeted via LIN-1/ETS to the promoter of *lin-39*/Hox to regulate vulval development in *C. elegans*

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Short title: LET-418 binds to the *lin-39*/Hox promoter

Key Words : *C. elegans*; vulva; Mi-2/NuRD ; RTK/Ras; *lin-39*/Hox; LIN-1/ETS

Summary

During vulval development in *C. elegans*, an inductive RTK/Ras signalling pathway is antagonized by the inhibitory action of redundantly acting classes of synMuv genes. Some class B synMuv genes encode orthologs of NuRD (nucleosome remodelling and histone deacetylase) complex components, including LET-418/Mi-2, suggesting that regulation of chromatin structure might be important for vulval cell-fate specification in *C. elegans*. Here we show that *let-418* antagonizes the RTK/Ras pathway by negatively interfering with the transcription of the Hox gene *lin-39*, a key regulator for vulval development. LET-418 controls *lin-39* transcription by directly associating with its promoter. Targeting of LET-418 to the promoter of *lin-39* depends on the transcription factor LIN-1/ETS, a direct downstream target of the inductive RTK/Ras signalling pathway. Our findings suggest that a *C. elegans* NuRD-like complex acts as co-repressor of LIN-1/ETS to negatively regulate the expression of *lin-39* Thus, LIN-1/ETS provides a direct link between RTK/Ras signalling and chromatin structure.

Introduction

The vulva of *Caenorhabditis elegans* is made from the descendants of the three hypodermal blast cells, P(5-7).p. These cells are members of the vulval equivalence group P(3-8).p, a set of six cells with the potential to adopt either a vulval or a nonvulval fate. They are referred to as the vulval precursor cells (VPCs). The process of vulval cell specification can be divided into two major sequential steps (for review see Sternberg, 2005). During the first and second larval stages (L1 and L2), the six VPCs are rendered competent to acquire a vulval fate by remaining unfused with the surrounding hypodermal syncytium (hyp7). The other Pn.p cells (VPCs) fuse to the hypodermis and can no longer become vulval cells (Beitel et al., 1995; Sulston and Horvitz, 1977). The second step of vulval fate specification, termed vulval induction, occurs during the third larval stage (L3). During this process three out of the six unfused VPCs adopt either a 1° (P6.p) or a 2° (P5.p and P7.p) vulval cell fate and undergo series of cell divisions, whereas the remaining three VPCs (P3, 4, 8.p) adopt a non-vulval 3° cell fate (Sternberg and Horvitz, 1986).

The fates of the VPCs are specified, at least partially, through a highly conserved Ras mediated signalling cascade (Han et al., 1990; Hill and Sternberg, 1992). During L3, this RTK/Ras signal transduction pathway is activated in P6.p by a LIN-3/EGF signal from the neighbouring gonadal anchor cell through the receptor tyrosine kinase (RTK) LET-23, and leads to the induction of the 1° vulval fate (Sternberg and Han, 1998). The RTK/Ras pathway negatively regulates the activity of the transcription factor LIN-1/ETS, an important inhibitor of vulval induction (Beitel et al., 1995). A *lin-1* loss-of-function (lf) mutation causes all six Pn.p cells to adopt vulval cell fates,

resulting in a multivulva (Muv) phenotype (Beitel et al., 1995; Ferguson et al., 1987; Lackner et al., 1994). Both, lf mutations in genes involved in the inductive RTK/Ras signal transduction, and gain-of-function (gf) mutations in the *lin-1* gene reduce the output of the anchor cell-signalling pathway and result in a partial to complete vulvaless (Vul) phenotype (Aroian et al., 1994; Clark et al., 1992; Jacobs et al., 1998; Lackner and Kim, 1998). The same RTK/Ras signalling cascade is also required to prevent the fusion of the VPCs with hyp7 during the L1/L2 larval stages (Aroian et al., 1990; Beitel et al., 1990; Chen and Han, 2001; Han et al., 1990). An important player during vulval development is *lin-39*. The Hox gene *lin-39* functions downstream of both, the RTK/Ras and the Wnt signalling pathways, as well as the gene *lin-1*, and plays a pivotal role in controlling vulval cell competence and induction (Chen and Han, 2001; Eisenmann et al., 1998; Maloof and Kenyon, 1998; Shemer and Podbilewicz, 2002). At the L1/L2 larval stages its activity prevents fusion of the P(3-8).p cells to the hypodermal syncytium hyp7. During vulval induction, *lin-39* expression is upregulated by RTK/Ras signalling in the vulval precursor cells (Wagmeister et al., 2005). If *lin-39* function is absent at this time, no vulval induction takes place (Maloof and Kenyon, 1998; Shemer and Podbilewicz, 2002).

SynMuv(Synthetic Multivulva) genes are believed to antagonize the RTK/Ras signalling during vulval induction (Ferguson et al., 1987; von Zelewsky et al., 2000). They encode the components of at least three functionally redundant pathways (synMuvA, synMuvB and synMuvC) that repress the vulval fate in all VPCs (Ceol and Horvitz, 2004). Single lf mutations in either class do not cause any obvious vulval phenotype, whereas the combination between two mutations from different classes (e.g. a class A and a class B mutation) results in an increase of vulval specification of

the Pn.p cells and leads to a Muv phenotype (Ceol and Horvitz, 2004; Fay and Han, 2000; Ferguson and Horvitz, 1989). Induction of the vulval cell fates in absence of the inhibitory synMuv gene activity requires basal levels of the RTK/Ras signalling transduction cascade (Sternberg and Horvitz, 1989; Thomas and Horvitz, 1999). The molecular mechanisms by which synMuv genes antagonize processes stimulated by RTK/Ras signalling are poorly understood and may be complex.

Recently it has been shown that the gene *hda-1*/HDAC and the class B synMuv gene *lin-53*/RbAp48 antagonize RTK/Ras activity during vulval cell specification by negatively interfering with the expression of *lin-39* (Chen and Han, 2001). Since these genes encode homologs of components of the human chromatin remodelling complexes NuRD and Sin3A (reviewed in Ahringer, 2000), it has been speculated that chromatin remodelling may be important for the regulation of *lin-39* gene activity during vulval cell-fate specification in *C. elegans* (Chen and Han, 2001).

To investigate the molecular mechanisms by which the synMuv genes regulate the activity of the key gene *lin-39* during vulval specification, we have focussed on *let-418*. The class B synMuv gene *let-418* encodes an ortholog of the mammalian Mi-2 protein, a SNF2-like ATP-dependent chromatin remodeler, which has been identified as specific component of the nucleosome remodelling and histone deacetylase (NuRD) complex from human cell lines and *Xenopus* egg extracts (Tong et al., 1998; Wade et al., 1999; Wade et al., 1998; Xue et al., 1998; Zhang et al., 1998). *let-418* is an essential gene expressed in most nuclei of the worm. Mutations in *let-418* cause a pleiotropic phenotype, including vulval defects, sterility and, without the maternal contribution, L1 larval arrest (von Zelewsky et al., 2000). Moreover, *let-418* is

required for the maintenance of somatic differentiation in *C. elegans* (Unhavaithaya et al., 2002).

Here we show that *let-418* antagonizes RTK/Ras activity during vulval cell specification by negatively interfering with the expression of *lin-39*. LET-418 interacts directly with the promoter of *lin-39* suggesting that it represses its transcription through chromatin remodelling. Furthermore, LET-418 interacts physically with the transcription factor LIN-1/ETS, and its association with the *lin-39* promoter depends on the activity of LIN-1. Based on these results we propose a model in which LIN-1 recruits LET-418 (and probably other components of a *C. elegans* NuRD-like complex) to the promoter of *lin-39*. RTK/Ras signalling relieves the inhibitory activity of LIN-1, resulting in the release of the LET-418 containing complex from the promoter of *lin-39*. This allows up-regulation of *lin-39* transcription, which is required for vulval induction.

Materials and methods

Nematode strains and culture conditions

Nematodes were grown at 20°C under standard conditions, unless otherwise indicated. Wild-type strain and parent of all mutant strains was *C. elegans* Bristol N2. Mutations and balancer chromosomes used in this study: LGII *lin-31(n1053)* ; LGIII *lin-39(n709ts), lin-39(n1760), dpy-18(e364*), eT1(III;V) ; LGIV *lin-1(e1275ts), lin-1(sy254);* LGV *unc-46(e177), let-418(s1617), let-418(n3536ts)* ; LGX *lin-15A(n767)*; *lin-1(e1275ts); ex[lin-1::gfp].*

RNAi by feeding

Primers used for PCR amplification of the cDNAs were 5'-GTATCCATGGGCTGCACACGCCAATCGTCA-3' and 5'-AGTTCCATGGTCCATTTTCAGACATGAATT for *let-418*, 5'-CATCATCCGTCGACTCAATC-3' and 5' ACGATGGGAACTTGAACGAG-3' for *lin-1*, 5' TGTTGGCCTACTGGGGTTA 3' and 5' TTTGCGCTCGTACAAATCCT 3' for *lin-15B* and 5' ATTATGGTCAAGGGCACGTC 3' and 5' TTTTCATTTGCTGCATTCGAC 3' for *hda-1*. The PCR amplified cDNA sequences of *let-418* (440 bp) and *lin-1* (1206 bp) were inserted into the vector pPD129.36 (gift from A. Fire; Timmons L and Fire A, 1998). The cDNA of *hda-1* (993bp) was cloned into the *Xcm*I site of pYZT. The vector pYZT was constructed by ligating the *Xcm*I site containing adapter sequences 5' gatccaccaagcttcccttggttacccaggtagct 3' and 5' cacctgggtaaccaagggaagcttggtg 3' into *Bam*HI/*Sac*I digested LITMUS 28i. *let-418 RNAi* strongly phenocopied the *let-418* mutant phenotype, giving rise to 100% L1 larval arrest when from young treated mothers (this study).

Construction of pEXPR/LIN-1::GFP

The vector pEXPR/LIN-1::GFP was constructed based on a previous report by Beitel et al., 1995. For cloning we used the Invitrogen Gateway Technology. A 15 kb long *lin-1* genomic DNA fragment was amplified with the Expand Long template PCR System from Roche, using the primers lin-1attB1 left and lin-1attB2 right. These primers contained a B1 or B2 Att site for the generation of a pENTRlin-1 entry vector by BP recombination of the PCR product and pDONR201 donor vector. We then generated a pDEST-GFP destination vector using the Gateway Vector Conversion System by inserting a RfA cassette in the *Sma*I site of the pPD95.77 gfp reporter plasmid from the Fire Lab Vector Kit. Finally the pEXPR/LIN-1::GFP vector was obtained by LR recombination of the pENTRlin-1 and the pDEST-GFP vectors.

Primers: lin-1attB1left: 5' ggggacaagtttgtacaaaaaagcaggctagttttcttgtttcgggaag 3'

lin-1attB2right: 5' ggggaccactttgtacaagaaagctgggtccaaagttggcatttttatgg 3'

Generation of LET-418 antibodies

Rat and rabbit anti-LET-418 antibodies were generated using LET-418 specific peptides. The antibodies recognized a protein of the expected size in extracts from wild-type worms, that was absent in extracts from *let-418(s1617)* animals (data not shown).

Protein co-immunoprecipitation

Co-immunoprecipitation experiments were adapted from (Chuang et al., 1996; Rocheleau et al., 1999). Wild-type, *lin-1(e1275ts), lin-1(e1275ts); ex[lin-1::gfp]*, or *lin-31(n1053)* mixed stage worms were homogenized in lysis buffer (25 mM HEPES NaOH (pH 7.4), 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100, 1 mM EDTA NaOH

and protease inhibitor cocktail (Roche)) using a 5mm POLYTRON stainless steel homogenizer (Kinematica AG). After centrifugation of the worm carcasses for 15 min at 4°C, approximately 1 mg of protein extract from the supernatant was used for immunoprecipitation in combination with 1 µg of antibodies or serum and 10 % protein G sepharose beads (Zymed). Following overnight incubation at 4°C on a rotating wheel, several washes in PBS were performed. The proteins in the incubated lysates (beads) were separated by gel electrophoresis, immunoblotted as described (Sambrook J., 1989) and revealed using either rat or rabbit anti-LET-418 antibodies at a dilution of 1:500, or rabbit anti-HDA-1 antibodies (Santa Cruz Ce-87) at a dilution of 1:1000. Co-immunoprecipitation of LIN-1::GFP was done using mouse anti-GFP native antibodies (Quantum Biotechnology).

Realtime RT-PCR analysis

The quantitative analysis of *lin-39* expression was performed by real time RT-PCR. For each experiment, total RNA was isolated from 30 wild-type, *let-418 (s1617)* or *lin-1(sy254)* tightly synchronized late L2 stage larvae. Each RNA sample was split into 3 identical RT-PCR reactions. The *lin-39* mRNA levels were determined using the Qiagen SybrGreen RT-PCR system on Rotorgene 2000 (adapted after Menzel et al., 2004). For each genotype, the mRNA levels of the reference gene *gpd-1* were determined as an internal standard by using the same protocol. The mean values from two independent experiments were calculated and normalized using *gpd-1* as internal standard. Primers: 5'-cctggaaggagacgatgatg-3' and 5'-cgcgtgaacctcctgtagtt-3' for *lin-39* and 5'-aaaggacacggttcaagtgg-3' and 5'-acaacgaaatcggctttgac-3' for *gpd-1*.

Chromatin immunoprecipitation (ChIP) and quantitative PCR

ChIP experiments were adapted from Chu et al., 2002. Wild-type, *let-418*(*n3536ts*) or *lin-1(n1275ts)* mixed stage worms were fixed in M9 buffer containing 2% formaldehyde at room temperature for 30 min. Excess formaldehyde was quenched and removed with a 0.1 M Tris-HCl (pH 7.5) wash and two M9 washes. Worm lysates were prepared by sonication in ChIP lysis buffer and protease inhibitor cocktail (Roche). Cellular debris were cleared by centrifugation. The average sonicated chromatin size of the fragments tested each time was around 500 bp (data not shown). The sonified chromatin was centrifuged at 14,000 g for 20 min and the supernatants, containing soluble chromatin fragments were saved. The chromatin fractions were precleared twice against Protein G Sepharose (ZyMed) and kept for 4 h at 4°C on a rotating plate. The suspensions were then centrifuged at 14,000 g for 30 s to discard nonspecifically-bound chromatin fragments. Aliquots from the supernatant (equivalent to 50 μg DNA) were taken in equal portions for each ChIP reaction and an additional 1% volume of such a portion was saved as input control. Each reaction was incubated 4 hrs/overnight with either 4 μg of the corresponding affinity-purified antibody except 2 μg of acetylated histone 3 antibody or with 4 μg of normal rabbit serum or without antibody as control. After clearing non-specific aggregates by centrifugation at 14,000 rpm, the immunocomplexes were captured with Protein G Sepharose, subjected to two 1 ml ChIP low salt buffer washes, two 1 ml ChIP high salt buffer washes and two 1 ml TE buffer washes and finally eluted with 1% SDS, 0.01 M Tris-HCl (pH8.0). For ChIP analysis, formaldehyde crosslinks were reversed by incubation at 65°C overnight in 0.2 M NaCl. Proteins were removed by proteinase K digestion and the DNA was purified with QIAquick PCR products purification kit (QIAGEN). For input DNA control, the total DNA was extracted from 1% of starting lysates as described above. PCR cycle number was adjusted to be 2-4 cycles beyond

the cycle in which a band was first seen for the input DNA, and generally adjusted until no or only a very weak signal was detected for the mock-IP DNA (no antibody). PCR products resolved on a 2% agarose gel were visualized by ethidium bromide staining and ranged from 30-35 cycles for different primer pairs. ChIP performed with normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, California; 4mg/ml) as serum control, consistently gave negative results and with the anti-acetylated histone 3 antibody (Upstate, 06-599, 2 mg/ml) as for ChIP- procedure positive control, gave positive results. ChIP performed on the unrelated gene *ntl-1* gave no DNA signals. ChIP experiments and PCR amplifications were performed at least twice for each sample.

Promoter analysis and constructs

The alignment of the *lin-39* promoter sequences from *C. elegans* and *C. briggsae* were performed using the online softwares LALIGN (Huang X., 1991) (http://www.ch.embnet.org/software/LALIGN_form.html) and VISTA (Mayor et al., 2000) (http://www-gsd.lbl.gov/vista/index.shtml). Sequences conserved between the two species were identified with the Matinspector software (Quandt et al., 1995) (http://www.gene-regulation.com/; http://www-gsd.lbl.gov/vista/index.shtml) using the Transfac database (Wingender et al., 2000).

Results

LET-418 promotes Pn.p cell fusion and represses vulval induction by negatively controlling *lin-39* activity

The *C. elegans* Hox gene *lin-39* plays at least two important roles in vulval formation. During the L1/L2 larval stage, it prevents fusion of the VPCs P(3-8).p with the hypodermal syncytium hyp7 (Clark et al., 1993; Shemer and Podbilewicz, 2002; Sternberg and Horvitz, 1986; Wang et al., 1993). In wild-type animals, all VPCs P(4-8).p and about 50% of the P3.p cells remain unfused (Table 1, Sternberg and Horvitz, 1986). In *let-418(RNAi)* animals, however, we observed a significant increase of the number of unfused P3.p cells. Whereas in wild-type animals 54% of the P3.p cells remained unfused (n=24), *let-418(RNAi)* depleted animals showed 85% (n=20) and *let-418(n3536ts)* mutants 90% of unfused P3.p cells (n=50) (von Zelewsky et al., 2000, this study). This suggested that *let-418* might negatively regulate the activity of the Hox gene *lin-39*. To further investigate this issue, we used *lin-39* mutants carrying the weak temperature-sensitive allele *n709ts*. At the restrictive temperature of 25ºC, *lin-39* activity was reduced, resulting in an increased level of P(3-8).p fusion (Table 1). We observed a significant rescue of the fusion phenotype in all VPCs P(3-8).p in *lin-39(n709ts);let-418(RNAi)* worms (Table 1). Remarkably, *let-418(RNAi)* was not able to rescue the fusion phenotype of *lin-39(n1760)* null mutants (Maloof and Kenyon, 1998; Shemer and Podbilewicz, 2002) (Table 1), indicating that minimal levels of *lin-39* activity are required. *lin-1(RNAi)* resulted in a similar rescue of the fusion phenotype of *lin-39(n709ts)* mutant animals (Table 1, Chen and Han, 2001), suggesting that *lin-1*, like *let-418*, negatively controls *lin-39* activity.

Table 1

Depletion of *let-418* rescues the fusion phenotype of *lin-39(rf)* mutants

Genotype	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	Number
wild-type	54	100	100	100	100	100	24
RNAi control	52.5	100	100	100	100	100	40
lin-39 (n709ts)	30	75	92	98	84	57	63
lin-39 (n709ts) ; RNAi control	34	74	91	97	77	54	35
lin-39 (n709ts) ; let-418 (RNAi)	56[+]	90[+]	100[+]	100	93	76[+]	70
lin-39 (n709ts) ; lin-1 (RNAi)	59[+]	91[+]	100	100	95	77[+]	44
lin-39 (n1760)	0	9	11	11	11	7	46
lin-39 (n1760) ; let-418 (RNAi)	0	10	10	10	10	7	20

L2 larvae, grown at 25°C, were stained with anti-MH27 antibodies that specifically mark the adherens junction of unfused cells. Numbers indicate the percentage of unfused Pn.p cells. [+] $p < 0.05$. P values were derived from comparing data from *lin-39(n709)* animals to those of *lin-39(n709);let-418(RNAi)* or *lin-39(n709);lin-1(RNAi)* animals using Fischer's exact test. As RNAi controls, we used HT115 E. Coli strains transformed with pPD129.39 vector alone (a gift from A. Fire)

During the L3 larval stage of wild-type animals, *lin-39* activity is upregulated in P(5-7).p by RTK/Ras signalling from the gonadal anchor cell (Maloof and Kenyon, 1998; Wagmeister et al., 2006). High levels of LIN-39 are required for the induction of the vulval fates in these cells. At restrictive temperature, the reduced activity of the weak *lin-39* allele *n709ts* results in an incomplete induction of the P(5-7).p cells (Table 2 and Clandinin et al., 1997). We found that depletion of *let-418* by RNAi partially restored the induction defects of the P5-7.p descendants in *lin-39(n709ts)* mutants (Table 2). In summary, our data suggested that the rescue of the *lin-39(n709ts)* phenotype through loss of *let-418* function is due to an increase of *lin-39* activity or to a deregulation of downstream target genes or both, indicating that *let-418* may act as a negative regulator of *lin-39* activity in the VPCs.

Table 2

Depletion of *let-418* partially rescues *lin-39 rf* vulval induction defects

Genotype	Fate	P5.p	P6.p	P7.p	n
Wild-type					
	1°	0.0	100.0	0.0	
	2°	100.0	0.0	100.0	
	3°/4°	0.0	0.0	0.0	
					120
lin-39 (n709)					
	1°	0.0	93.2	0.0	
	2°	69.9°	0.0^{+}	65.0°	
	3°/4°	30.1°	6.8^{+}	35.0°	
					103
lin-39 (n709) ; RNAi control					
	1°	0.0	91.7	0.0	
	2°	71.7	0.0	65.0	
	3°/4°	28.3	8.3	35.0	
					60
lin-39 (n709); let-418 (RNAi)					
	1°	0.0	97.6	0.0	
	2°	90.6°	0.0	81.2^{+}	
	3°/4°	9.4°	2.4	18.8^{+}	
					85

Animals were grown at 25°C. The percentage of the different cell fates in the descendants of the P5.p, P6.p and P7.p cells of mid-L4 larvae is indicated in percent.

$^{+}$ $p < 0.05$
° $p < 0.001$

lin-39(n709) p values was derived from comparing data from *lin-39(n709)* to those from wild-type animals, the others were derived from comparing data from *RNAi* depleted animals to those from *lin-39(n709)* animals in a Fisher's exact test.

LET-418 regulates *lin-39* expression

To test an influence of *let-418* on *lin-39* expression in the VPCs, we have analyzed the vulval specific expression pattern of an integrated, partially rescuing *lin-39::gfp* reporter transgene. The construct contained the entire *lin-39* coding sequence and about 5 kb of the upstream promoter region. It was frequently expressed in P3.p to P6.p and at lower levels in P7.p and P8.p during the late L2 stage (see Table 3). We

determined the frequency of visible *lin-39::gfp* expression in each VPC of wild-type and *let-418* depleted late L2 larvae. The percentage of *lin-39::gfp* expressing VPCs was significantly higher in *let-418(s1617)* and *let-418(RNAi)* animals than in wild-type worms (Table 3). The *lin-39::gfp* expression levels in the synMuv background *lin-15A(n767);let-418(RNAi)* were elevated to the same extent as in *let-418* single mutant worms (Table 3). Mutations in the synMuv A gene *lin-15A(n767)*, however, caused no significant change in *lin-39::gfp* expression as compared to wild-type animals (Table 3). Moreover, *lin-1(RNAi)* animals had a similar increase in the *lin-39::gfp* expression frequency in the VPCs (Table 3). Finally, we have also tested the influence of *hda-1* (that encodes an ortholog of the vertebrate NuRD component HDAC1), since it co-immunoprecipitated with LET-418 (result not shown). We observed that RNAi depletion of *hda-1* resulted in a similar increase of the percentage of *lin-39* expressing VPCs as *let-418(RNAi)* (Table 3), suggesting that the two proteins may act in the same complex to repress *lin-39*.

Table 3

***let-418* negatively regulates *lin-39* expression**

	***lin-39 ::gfp* expression (% expressing cells)**						
Genotype	**P3.p**	**P4.p**	**p5.p**	**p6.p**	**p7.p**	**P8.p**	**n**
Wild-type	39.8	53.0	21.7	22.9	3.6	4.8	83
let-418 (s1617)	58.0^{+}	70.0	64.0°	36.0	22.0^{+}	18.0^{+}	50
let-418 (RNAi)	60.0^{+}	71.1	53.3°	40.0	24.4°	15.6^{+}	45
hda-1 (RNAi)	57.8^{+}	66.7	53.3°	35.6	15.6^{+}	13.3	45
lin-15A (n767)	51.1	51.1	26.7	35.6	6.7	4.4	45
lin-15A (n767) ; let-418(RNAi)	46.7	60.0	57.8°	35.6	22.2^{+}	17.8^{+}	45
lin-1 (RNAi)	51.1	75.6^{+}	57.8°	37.8	17.8^{+}	13.3	45

Expression frequency of the *lin-39::gfp* reporter gene in the P3.p to P8.p cells of late L2 larvae grown at 25°C.
$^{+}$p value < 0.05
$^{\circ}$p value < 0.001
p values were derived from comparing data from mutants or RNAi depleted animals to those from wild-type in a Fisher's exact test.

To further assess the differences in *lin-39* expression, we performed a real time RT-PCR experiment with total RNA isolated from wild-type, *let-418* and *lin-1* late L2 larvae before vulval induction. As an internal standard the housekeeping gene *gpd-1* was used. In agreement with the previous results, we found a significant increase of *lin-39* mRNA in *let-418(s1617)* and *lin-1(sy254)* mutants as compared to wild-type animals (Fig. 1). Since the difference (4.2±0.9SE fold for *let-418* and 5.5±2.0SE fold for *lin-1*, Fig. 1) was higher than expected from the modest increase observed in the VPCs, we compared the overall expression pattern of our *lin-39::gfp* reporter in wild-type, *let-418(RNAi)* and *lin-1(RNAi)* mutant animals of different developmental stages. The reporter gene was not only expressed in the VPCs, but also in other cells of the central body region, including cells of the ventral nerve cord, the myoblasts and their descendents, a few neurons in the region of the pharynx and some intestinal cells. Consistent with the RT-PCR results, we noticed an increase of *lin-39::gfp* expression in many of these cells in *let-418* and *lin-1* depleted animals as compared to wild type worms (result not shown). No obvious ectopic expression, however, was observed. We have also compared wild-type and *let-418* mutant animals stained with anti-LIN-39 antibodies. The staining pattern corresponded to that of the *lin-39::gfp* reporter. In agreement with the results from the *lin-39::gfp* expression study and the real time RT-PCR experiment, we noted an increased staining in many of the *lin-39* expressing cells in *let-418* and *lin-1* depleted animals (data not shown). An increase of LIN-39 expression in *lin-1* mutant animals stained with anti-LIN-39 antibodies was previously reported (Maloof and Kenyon, 1998). In summary, our results suggested that LET-418 and LIN-1 control the level of *lin-39* expression in the VPCs, as well as in other *lin-39* expressing cells during development.

Figure 1

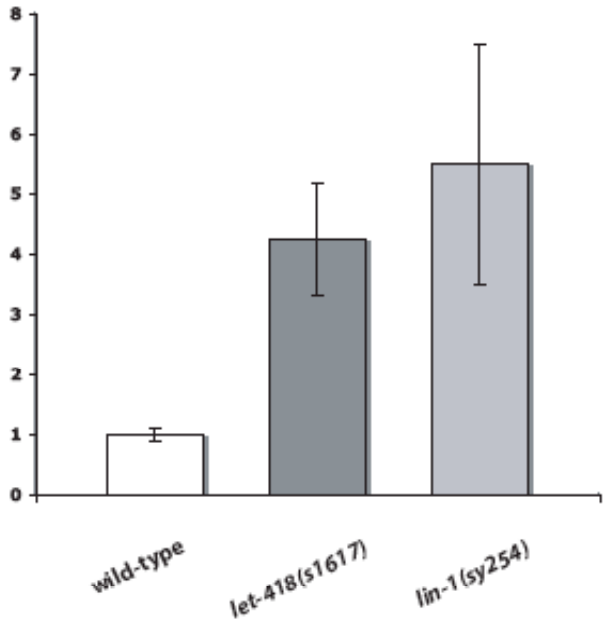


Figure 1. ***lin-39*** **expression is increased in** ***let-418(s1617)*** **and** ***lin-1(sy254)*** **mutants**

Quantitative RT-PCR experiments revealed a significant increase of the *lin-39* mRNA levels in *let-418(s1617)* (4.2±0.9SE) and *lin-1(sy254)* (5.5±2SE) mutants as compared to wild-type animals. The mean values for each genotype were obtained from two independent experiments and normalized against those of the housekeeping gene *gpd-1* used as internal standard (see materials and methods). Bars represent fold enrichment relative to the wild-type value that has been set to 1 for clarity.

lin-39 is a direct target of LET-418

Vertebrate and fly Mi-2 proteins have been proposed to exhibit a transcriptional repressor activity through chromatin remodelling (Marhold et al., 2004; Xue et al., 1998; Zhang et al., 1999). In order to test whether LET-418 directly regulates *lin-39* expression by binding to its promoter, we performed chromatin immunoprecipitation (ChIP) experiments. First we searched for putative regulatory regions in the promoter

of *lin-39* by comparing the genomic sequences of the *C. elegans lin-39* gene and its ortholog in the related species *C. briggsae* (Genome Sequencing Centre, Washington University, St. Louis, MO, USA). Whereas intergenic sequences have diverged considerably between the two nematode species, conservation of both sequence and function of regulatory elements has been shown for a number of genes (Gilleard et al., 1997; Kennedy et al., 1993; Krause et al., 1994; Xue et al., 1992). Alignment of the *C. elegans* and *C. briggsae* genomic sequences using the LALIGN (Huang X., 1991) and the VISTA (Mayor et al., 2000) online software revealed several significantly conserved regions with sequence identities of over 50% (not shown) that were located within the first 6 kb of upstream promoter sequences of *lin-39*. Regions III (approximately 6000 to 5700 bp upstream of the START codon) and IV (approximately 3600 to 3200 bp upstream) were chosen to test for LET-418 binding (Fig. 2A). Region IV contained the most conserved sequences in the promoter of lin-39 and region III was identified by Wagmeister et al. (2006) as a RTK/Ras responsive element necessary for LIN-39::GFP expression in P6.p . As negative controls we used two DNA fragments located about 12 and 8kb upstream of the *lin-39* coding region that did not contain conserved sequences (region I and II, Fig. 2A) and a fragment of the unrelated gene *ntl-1* (Collart and Struhl, 1994; Tucker et al., 2002) (Fig. 2C).

The ChIP experiments were performed by immunoprecipitating total sonicated chromatin with anti-LET-418 antibodies. We found an association of LET-418 with the two conserved regions III and IV in wild-type animals (see Fig. 2B). This binding was specific, since PCR amplifications of the *lin-39* promoter regions I and II and the control fragment of the gene *ntl-1* revealed no DNA bands (Fig. 2B). To further confirm the specificity of the association of LET-418 with fragments III and IV, we performed ChIP experiments with *let-418(ts)* animals. As expected, PCR bands were

present only at the permissive temperature of 15°C, but not at the restrictive temperature of 25°C (Fig. 2B). Altogether, our data show that LET-418 associates specifically with regions III and IV of the *lin-39* promoter, suggesting a direct transcriptional control.

Figure 2

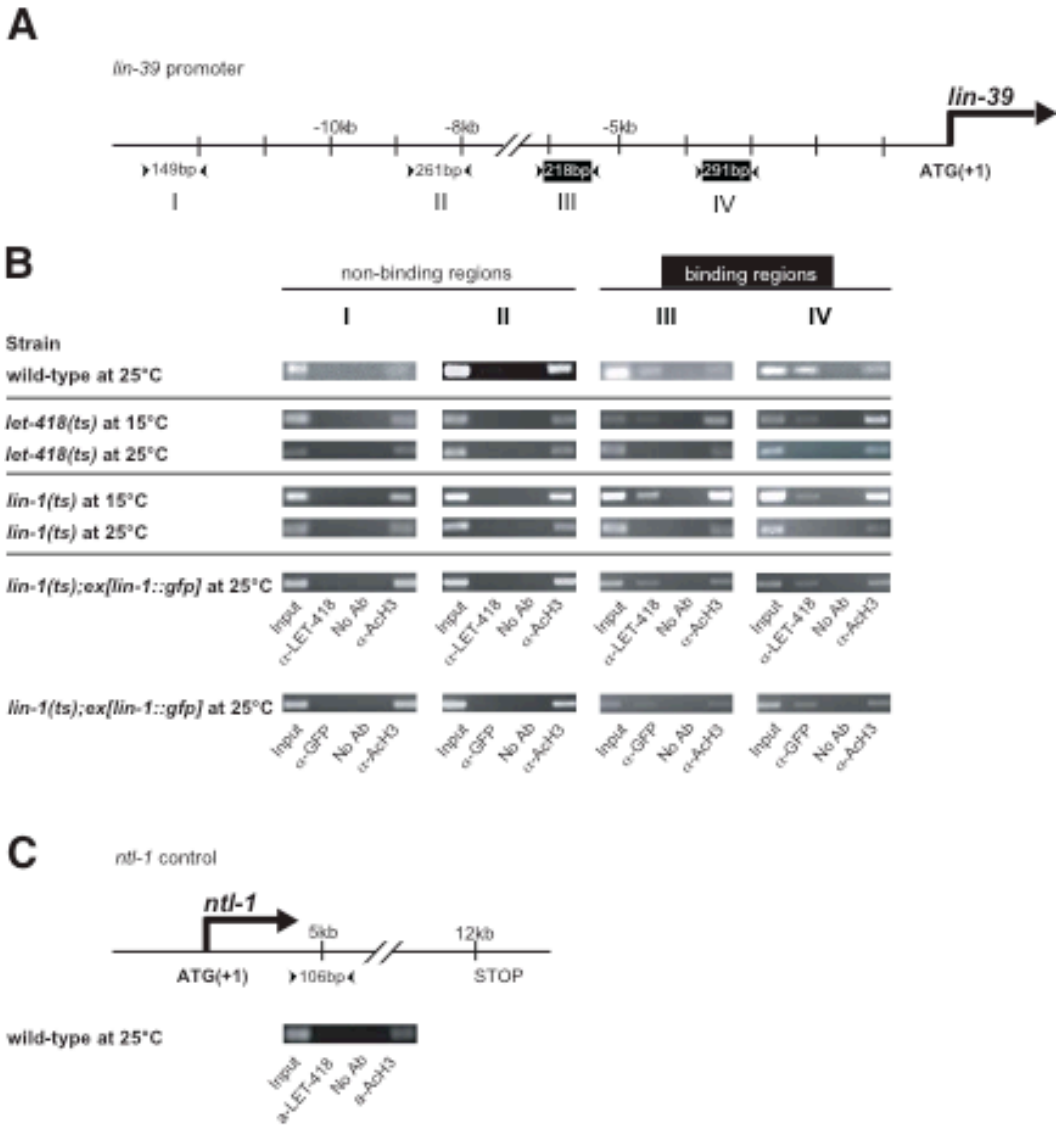


Figure 2. The *C. elegans* protein LET-418 associates specifically with at least two conserved regions of the *lin-39* promoter.

(A) The promoter sequences of the Hox gene *lin-39* from *C. elegans* and *C. briggsae* share several conserved regions with over 50% similarity that are likely to represent important regulatory elements. Two of them (regions III and IV) were chosen to test for LET-418 binding. Regions I and II, located

about 12 and 8 kb upstream of the *lin-39* START codon, contain no obvious conserved sequences and were therefore used as negative controls for the ChIP experiments. Localisation and length of the *lin-39* promoter fragments used for the ChIP experiments are indicated.

(B) The *C. elegans* protein LET-418 associates specifically with the *lin-39* promoter regions III and IV, and this association depends on LIN-1. DNA from chromatin immunoprecipitated with rabbit anti-LET-418 was amplified by PCR and analyzed on agarose gels. Chromatin precipitated with anti-acetyl–histone H3 antibodies, that recognized acetylated K9 and K14, was used as positive control for the ChIP experiments. LET-418 binds *lin-39* promoter regions III and IV, but not the non-conserved control regions I and II. In *let-418(n3536ts)* animals, association of LET-418 with regions III and IV was observed only at the permissive temperature of 15^{o}C, but not at the restrictive temperature of 25^{o}C. Binding of LET-418 depended on the activity of LIN-1, since in *lin-1(e1275ts)* animals LET-418 associated with the fragments III and IV only at the permissive temperature of 15^{o}C but not at the restrictive temperature of 25^{o}C. The rescuing construct *lin-1::gfp*, however, was able to restore LET-418 binding in these animals at restrictive temperature. Consistently, the LIN-1::GFP fusion protein bound to promoter fragments III and IV, but not to the control fragments I and II.

(C) LET-418 does not bind randomly to genomic DNA as shown by the coding region of the gene *ntl-1*.

LET-418 is targeted to the *lin-39* promoter by the transcription factor LIN-1 /ETS

How is LET-418 targeted to the promoter of *lin-39*? It has been proposed that the vertebrate and insect Mi-2 complexes are brought to their sites of repression by the action of specific transcription factors (Bowen et al., 2004; Ng and Bird, 2000; Struhl, 1998). Since the transcription factor LIN-1/ETS and LET-418 are both required for the negative control of *lin-39* expression, and since depletion of either of them results in de-repression of *lin-39* transcription (Fig. 1 and Table 3), we hypothesized that a LET-418 containing repressor complex could be recruited to the promoter of *lin-39* by the action of LIN-1. To test this hypothesis, we first checked whether LET-418

interacts with LIN-1. Since we were unable to obtain reliable anti-LIN-1 antibodies, we generated a translational *lin-1::gfp* fusion construct that contains a 15 kb long genomic *lin-1* fragment (see materials and methods). On Western blots loaded with total protein extracts from *lin-1(n1275ts);ex[lin-1::gfp]* animals, anti-GFP-antibodies detected a faint band corresponding to the predicted length of the LIN-1::GFP fusion protein (data not shown). Furthermore, the *lin-1::gfp* transgene was able to rescue the Muv phenotype of *lin-1(e1275ts)* animals, suggesting that the LIN-1::GFP fusion protein is functional. To eliminate endogenous LIN-1, we shifted the *lin-1(n1275ts); ex[lin-1::gfp]* animals for two generations at the restrictive temperature of 25˚C before performing the co-immunoprecipitation experiments. Using anti-GFP antibodies we were able to co-immunoprecipitate LET-418, demonstrating that the two proteins interact *in vivo* (Fig. 3A).

In the VPCs, LIN-1 binds to the forkhead-related winged helix transcription factor LIN-31, and the LIN-1/LIN-31 complex inhibits vulval induction (Tan et al., 1998). We reasoned that, if LET-418 formed an inhibitory complex with LIN-1, it should also associate with LIN-31. Using specific anti-LIN-31 antibodies (Miller et al., 2000; Tan et al., 1998), we were able to co-immunoprecipitate LET-418 (Fig. 3B), suggesting that LIN-1/LIN-31/LET-418 co-exist in the same complex. If LIN-1 recruits LET-418 to the promoter of *lin-39*, we expected that LET-418 binding should be abolished in *lin-1* mutants. We tested this assumption by performing ChIP experiments using *lin-1(ts)* animals at both, permissive and restrictive temperature. Whereas LET-418 bound the *lin-39* promoter regions III and IV in *lin-1(ts)* extracts at 15°C, this association was abolished in *lin-1(ts)* animals at 25°C (Fig. 2B). The rescuing translational fusion construct *lin-1::gfp* was able to restore LET-418 binding in the *lin-1(ts)* background at 25ºC, and as expected, LIN-1::GFP bound to the

promoter regions III and IV (Fig. 2B). Altogether, the results suggested that a complex containing LET-418 is targeted by the transcription factor LIN-1 to the promoter of *lin-39* to mediate transcriptional repression.

Figure 3

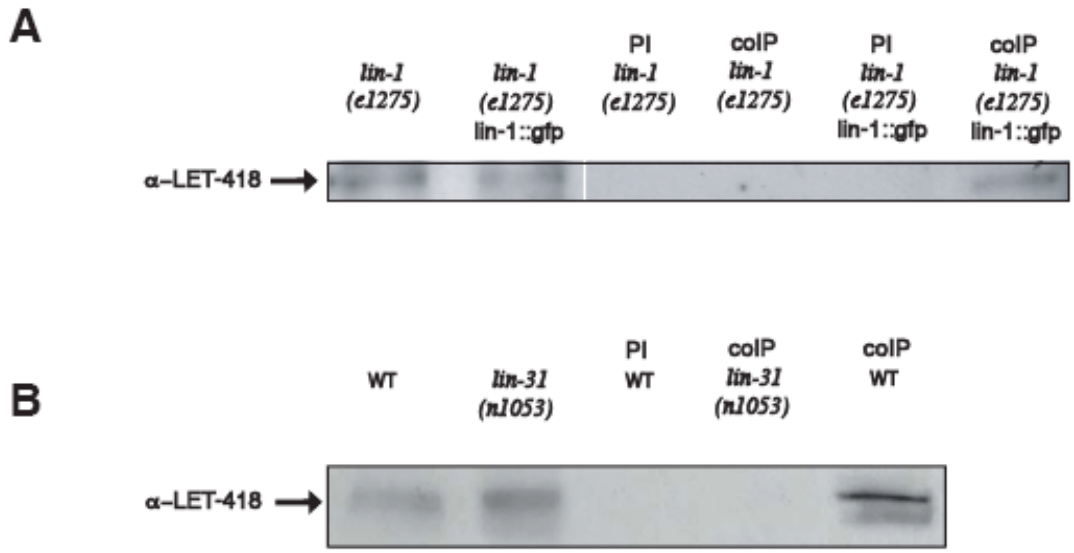


Figure 3. LET-418 forms a complex with LIN-1 and LIN-31

(A) LET-418 co-immunprecipitates with LIN-1::GFP. Western blot probed with rabbit anti-LET-418 antibodies. Lane 1: total extract from *lin-1(e1275ts)* animals (2% input); lane 2: total extract from *lin-1(e1275ts);lin-1::gfp* animals (2% input); lane 3: total *lin-1(e1275ts)* worm extracts immunoprecipitated with non-immune total mouse IgG; lane 4: total *lin-1(e1275ts)* mutant extracts immunoprecipitated with mouse anti-GFP antibodies; lane 5: total *lin-1(e1275ts);lin-1::gfp* worm extracts immunoprecipitated with non-immune total mouse IgG. lane 6: total *lin-1(e1275ts);lin-1::gfp* worm extracts immunoprecipitated with mouse anti-GFP antibodies.

(B) LET-418 co-immunoprecipitates with LIN-31. Western blot probed with rat anti-LET-418 antibodies. Lane 1: total extract from WT animals (5% input); lane 2: total extract from *lin-31(n1053)* animals (5% input); lane 3: total WT worm extracts immunoprecipitated with non-immune total rabbit IgG; lane 4: total *lin-31(n1053)* mutant worm extracts immunoprecipitated with rabbit anti-LIN-31 antibodies; lane 5: total WT worm extracts immunoprecipitated with rabbit anti-LIN-31 antibodies.

Discussion

In this study we examine the role of the class B synMuv gene *let-418*, a *C. elegans* ortholog of the mammalian chromatin remodelling protein Mi-2, during vulval formation. It has been suggested that *let-418* antagonizes the inductive RTK/Ras signalling pathway in the VPCs (von Zelewsky et al., 2000). Here we show that *let-418* negatively regulates the activity of the Hox gene *lin-39*, a key regulator for vulval development. Using genetic and biochemical approaches, we provide evidence that LET-418 controls the level of *lin-39* transcription in the VPCs and perhaps in other *lin-39* expressing cells during development by directly interacting with its promoter. Moreover, we show that the interaction of LET-418 with the *lin-39* promoter depends on the transcription factor LIN-1/ETS, a direct downstream target of the inductive RTK/Ras signalling pathway. Our data suggest that LIN-1 links RTK/Ras signalling with chromatin remodelling via a LET-418 containing complex.

LET-418 LIN-1negatively control the expression of *lin-39*

The Hox gene *lin-39* is regulated at least in part by the RTK/Ras signalling pathway and functions downstream of *lin-1* (Chen and Han, 2001; Maloof and Kenyon, 1998). We found that RNAi depletion of *let-418* was able to partially rescue the VPC fusion defects and the vulval induction phenotype of the weak *lin*-39 allele *n709ts*, indicating that *let-418* suppresses *lin-39* activity in the VPCs during vulval development. By using different approaches we demonstrated that *let-418* negatively controls *lin-39* expression. We have analyzed the expression pattern of an integrated rescuing *lin-39::gfp* construct that was variably expressed in P3.p to P6.p and at lower levels in P7.p and P8.p during the late L2 stage of wild-type worms. Depletion of *let-418* resulted in a significant increase of the number of *lin-39::gfp* expressing VPCs (Table

3). An increase of the expression levels was also observed in other *lin-39::gfp* expressing cells of *let-418* depleted animals, but interestingly no obvious ectopic expression was found. Enhanced levels of *lin-39* expression in *let-418* depleted animals were confirmed by using anti-LIN-39 antibody staining (results not shown) and by performing whole animal real time RT-PCR (Fig. 1). Similar results were obtained with *lin-1* depleted animals (results not shown; Maloof and Kenyon, 1998). In summary, we found that *let-418* and *lin-1* negatively control the *lin-39* expression levels not only in the VPCs, but probably also in other cells normally expressing *lin-39* during development.

LET-418 suppresses *lin-39* transcription by LIN-1/ETS mediated promoter binding

By performing ChIP experiments we found that LET-418 associates with the *lin-39* promoter fragments III and IV (see Fig. 2B). This binding was specific, and no LET-418 association was found with two control fragments located approximately 8 and 12 kb upstream of the LIN-39 coding sequence (fragments I and II, see Fig. 2B) or with a control fragment from the coding region of the unrelated gene *ntl-1*. LET-418 binding depends on the presence of the transcription factor LIN-1/ETS. No LET-418 association with the *lin-39* promoter was observed in *lin-1(ts)* mutants at restrictive temperature of 25ºC, but the rescuing fusion construct *lin-1::gfp* was able to restore LET-418 binding in these animals. Furthermore, ChIP experiments confirmed *in vivo* binding of LIN-1::GFP to the *lin-39* promoter (see Fig. 2B). This is in agreement with the finding of Wagmeister et al. showing that LIN-1 and LIN-31 bind *in vitro* to several sites within the LET-418 associating promoter fragment III of *lin-39* (Wagmeister et al ,2006b). LIN-1/ETS is a negative regulator of vulval cell fate and

DNA binding is essential for its function (Miley et al., 2004). Both proteins, LET-418 and LIN-1, are required for the negative control of the *lin-39* activity, and depletion of either of them results in de-repression of *lin-39* transcription (Fig. 1 and Table 3). LET-418 associates *in vivo* with LIN-1 and with the forkhead related winged-helix transcription factor LIN-31 (see Fig. 3 and B), that was previously shown to form an inhibitory heterodimer with LIN-1 to repress vulval induction in the absence of RTK/Ras signalling (Tan et al., 1998). In summary, these data provide compelling evidence for a model in which an inhibitory dimer of LIN-1 and LIN-31 binds to specific regions in the promoter of *lin-39* and recruits LET-418 as co-repressor .

The vertebrate protein Mi-2 exerts its inhibitory function on gene expression as a member of the NuRD complex (Xue et al., 1998; Zhang et al., 1999). Since the genome of *C. elegans* encodes several homologs of vertebrate NuRD components, the existence of a worm NuRD-like complex has been suggested but was never shown. We found that one of these homologs, HDA-1, co-immunoprecipitated with LET-418 (results not shown) and also repressed *lin-39* expression in the VPCs (see Table 3, Chen and Han, 2001). Therefore, it is likely that the two proteins control *lin-39* expression as members of the same repressor complex. Moreover, the class B synMuv gene *lin-53*/RbAp48, that encodes another *C. elegans* ortholog of a mammalian NuRD component, also negatively regulates *lin-39* expression (Chen and Han, 2001). Taken together, these findings provide indirect evidence for a *C. elegans* NuRD-like complex that binds to the promoter of *lin-39* and negatively controls its transcription in the non-induced P(3-8).p cells through histone modification and chromatin remodelling. Besides LET-418, HDA-1 and LIN-53/RbAp48, this NuRD-like complex is likely to involve other proteins encoded by the synMuvB pathway, however its exact composition remains to be determined.

Transcriptional repression by ETS family proteins through chromatin structure and DNA accessibility has previously been shown to occur in a considerable number of different processes and organisms (Hsu and Schulz, 2000; Li et al., 2000; Mavrothalassitis and Ghysdael, 2000). The human LIN-1 ortholog ELK-1, e.g., recruits the HDAC containing chromatin-remodelling complex mSin3 to silence specific target genes (Kukushkin et al., 2002; Yang et al., 2001). ELK-1 is, as LIN-1, a direct target of the RTK/Ras pathway, and phosphorylation of ELK-1 disrupts its repressive activity (Yang et al., 2003a; Yang et al., 2003b; Yang and Sharrocks, 2004). Our data presented here suggest that in *C. elegans* LIN-1 acts via a similar mechanism to remodel chromatin in a targeted manner.

LIN-1 provides a direct link between RTK/Ras signalling and chromatin structure

Activation of the Ras pathway in P6.p triggers the phosphorylation and subsequent disruption of the LIN-1/LIN-31 dimer. This may result in the release of the LET-418 containing NuRD-like repressor complex, thereby allowing de-condensation of the chromatin and up-regulation of *lin-39* transcription (see model in Fig. 4). At present the mechanisms involved in this process remain unknown. Besides its role as inhibitor, LIN-1 was recently found to have also an activating role for vulval induction (Tiensuu et al., 2005). However, it seems not to have a critical role in the transcriptional activation of *lin-39*, since depletion of *lin-1* still results in high levels of *lin-39* transcription (see Fig. 1 and Table 3). Activation of *lin-39* may, however, involve the action of LIN-31 that acts, upon RTK/Ras mediated phosphorylation by MPK-1, as transcriptional activator, thereby promoting the vulval cell fate (Tan et al.,

1998, Wagmeister et a., 2006). Recently, it has been shown that the transcription factor LIN-25 is required for *lin-39* transcriptional upregulation in P6.p following Ras pathway interaction. Since LIN-25 interacts with SUR-2, it is likely that a mediator complex facilitates *lin-39* upregulation. Finally, Wagmeister et al. (2006) found that LIN-39 binds in vitro to the promoter region III that is required for lin-39::gfp expression in P6.p. In agreement with their finding, our preliminary ChIP experiments revealed LIN-39 binding to several regions in the *lin-39* promoter (results not shown). Altogether, these data suggest that LIN-39 may autoregulate its own expression.

LET-418 associates to two different promoter regions of *lin-39* (III and IV, see Fig. 2B), and region III has at least three different sites that can bind LIN-1 *in vitro* (Wagmeister et al., 2006). This suggests that a LET-418 containing NuRD-like complex mediates silencing by binding to multiple sites within the *lin-39* promoter. Repression of *lin-39* transcription by the LIN-1/LET-418 complex, however, is not complete, and non-induced VPCs still have basic levels of *lin-39* expression that are required for normal vulval development. Moreover, mutations in *let-418* resulted in the up-regulation of *lin-39* in the VPCs and in other cells that normally express *lin-39* during development, but no obvious ectopic expression was seen. Based on these data we propose that *let-418* acts as part of a control system, that prevents the VPCs (and perhaps other cells) from being ectopically induced in the absence of an inducing RTK/Ras signal. In the VPCs, e.g., a LET-418 containing NuRD-like complex establishes a high activation threshold for *lin-39* and perhaps for other key genes required for vulval development. This threshold distinguishes the responses of cells that receive from those of cells that do not receive inductive signal. In synMuv mutants, this threshold reduces in P(3–8).p to a level that can be exceeded even by

unstimulated, that is, LIN-3-independent, Ras pathway activity (Ceol and Horvitz, 2004).

***lin-39* is necessary but not sufficient for induction**

We observed that depletion of *lin-1* increased *lin-39* expression to the same level as depletion of *let-418* (see Table 3). However, whereas *lin-1(lf)* animals are Muv, loss of *let-418* can only produce a Muv phenotype in the background of a synMuvA mutation. This suggests that the increase of *lin-39* expression alone is not sufficient for vulval induction. Consistent with our findings, Maloof and Kenyon (1998) have previously reported that high uniform levels of *lin-39* alone cannot trigger vulval development in absence of RTK/Ras signalling. LIN-39 may need co-factors or modifications for its function, so that increased expression alone is not sufficient for vulval induction. Therefore, in addition to the induction of high levels of *lin-39* expression, the RTK/Ras pathway and its downstream effector LIN-1 must have other important functions for vulval development (Howard and Sundaram, 2002; Jacobs et al., 1998). It is also interesting to note that mutations in the synMuvA gene *lin-15A* do not affect the expression level of *lin-39* (Table 3 and Chen and Han, 2001), suggesting that the synMuvA pathway does not act through *lin-39*. Furthermore, at least three class B synMuv genes (*lin-35*/Rb, *dpl-1*/DP *and efl-1*/E2F) have no inhibitory effect on *lin-39* expression (Chen and Han, 2001), indicating that they must have another function during vulval differentiation. Moreover, a recent discovery demonstrated that the activity of the class B synMuv gene *lin-35*/Rb is required in the hypodermal syncytium (Myers and Greenwald, 2005) to inhibit vulval fates. Altogether, these results suggest that the synMuv genes are likely to control redundant and/or independent activation pathways for vulval development that are complex and

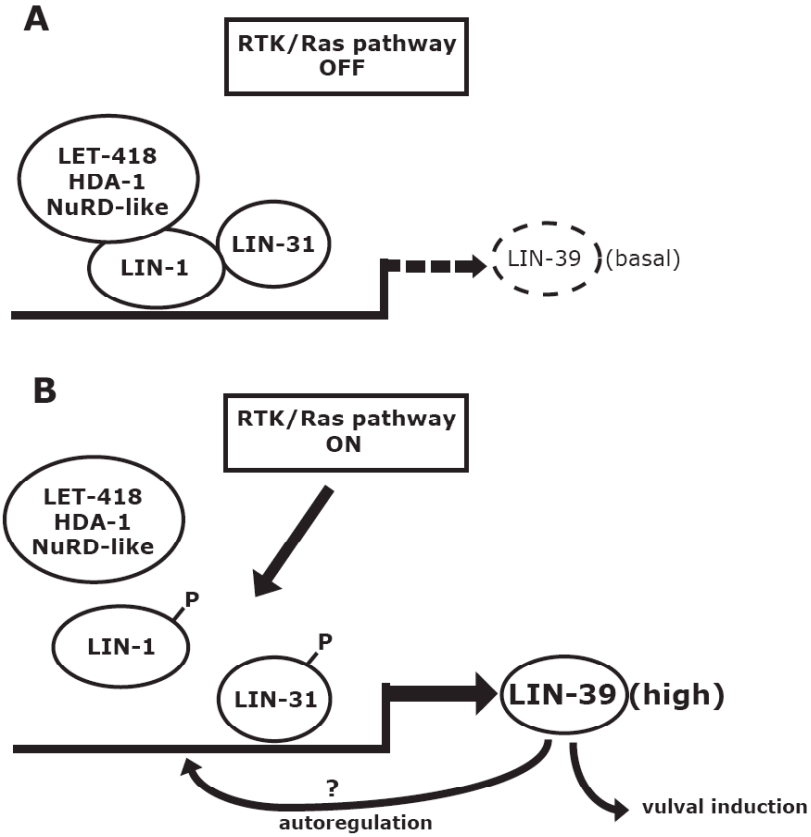
have different cellular foci. The determination of their exact functions will be crucial for the clarification of their roles in VPC specification.

Figure 4. A model for the transcriptional regulation of *lin-39* by the LET-418/NuRD-like complex.

(A) In absence of RTK/Ras signalling in the VPCs, a LIN-1/LIN-31 inhibitory complex binds to the promoter of *lin-39* and recruits a LET-418 and HDA-1 containing NuRD-like complex. This complex acts as co-repressor of LIN-1 to repress *lin-39* transcription by affecting histones and condensing the chromatin. The repression, however, can not be complete, since basal levels of LIN-39 are required for normal vulval development.

(B) Upon RTK/Ras signalling in the cells P(5-7).p, LIN-1 is phosphorylated and releases the repressive NuRD-like complex. This allows de-condensation of the chromatin and up-regulation of *lin-39* transcription required for vulval cell fate induction. The increase of *lin-39* may involve autoregulation, or the action of the transcription activator LIN-31, or both.

Figure 4



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Acknowledgements

We thank Craig Ceoal, Robert Horvitz and Frank Stegmaier for the ts allele n3536 of *let-418*, Alex Hajnal for the *C. elegans* strain AH30, Cynthia Kenyon and Stuart Kim for anti-LIN-39 and anti-LIN-31 antibodies. We are grateful to Florence Moulin, Tibor Vellai, Marco Belfiore, Karin Brunschwig, Chantal Wicky, Myriam Passannante, and all members of the Müller Lab for reagents, fruitful discussions or critical reading of the manuscript. We thank Yolande Molleyres, Laurence Buillard and Verena Zimmerman for excellent technical help. Some of the strains used in this work were supplied by the *C. elegans* Genetics Center funded by the NIH. This work was supported by the Swiss National Science Foundation grant numbers 3100-068240 and 3100-056853.

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Development of a protocol for single worm quantitative RT-PCR

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Summary

One of many advantages to use *C.elegans* as an experimental model is the availability of thousands of genetic mutants. They are a quite precious source for the characterization of the function of specific genes. Of particular interest is the identification of target genes of regulatory proteins. However, many of these genes are lethal or show maternal lethality, thus limiting the isolation of large amount of mRNA for high throughput assays, e.g. microarray analysis. Here we describe the use of different RNA isolation methods in combination with both semi-quantitative RT-PCR and real time quantitative RT-PCR to analyze the putative target genes of proteins of interest.

Introduction

1. Comparative quantitation by using Semi-quantitative RT-PCR

The transcription levels of target genes of proteins of interest can change due to different genetic backgrounds, at different environments or different drug treatment. The relative abundance of a transcript in different samples can be estimated by semi-quantitative or relative RT (reverse transcription)-PCR. Typically, the signal from the RT-PCR product is normalized to the signal from an internal control in all samples and co-amplified at the same time as the target. Generally, the use of a house keeping gene as internal control (IC) improves the reliability of the quantitative result of the PCR reaction of putative target of interesting proteins after reverse transcription. Transcripts of housekeeping genes are frequently chosen as an internal control because they are abundantly expressed at relatively constant rates in different

samples. Because of their high abundance, relatively low internal control primer concentrations may be helpful to minimize the preferential amplification of the internal controls. In this multiplex PCR, several PCR products from different primer pairs are amplified simultaneously in a single tube. The length of PCR product is generally < 500bp with a size difference of approximately 150 bp-200 bp between them to be sure that the products can be easily distinguished on an agarose gel. If possible, the designed primers or probes should bridge two exons so that they cannot amplify any contamining genomic DNA (http://www.QIAGEN.com). Furthermore, amplification efficiency should be biased minimally by size differences. The PCR amplification should end in the exponential phase for both the internal control reference and the PCR product of interest (PI). Thus, the quantity of PCR products should still increase in an exponential fashion along the last amplification cycles. Since this assay is linear only over a very short range, rare targets will possibly be below the limit of detection, while abundant targets will be past the exponential phase. In order to extend the linear range, duplicate reactions may be performed for a greater or smaller number of cycles or on serial dilutions of the sample. Once optimal conditions are settled, each reaction can be tested by checking that the PI/IC ratio is constant for PCR performed by using serial dilution of the sample. In summary, it's critical to determine the RT-PCR conditions that are suitable for both amplicons by varying the amount of template, the number of cycles, the annealing temperature, and the extension time. Finally, the normalized data from different samples can be compared and are informative for the characterization of particular target gene of interesting protein.

2.Comparative quantitation by using Real time RT-PCR

Quantitative real-time RT-PCR is based on detection of a fluorescent signal produced proportionally during the amplification of a PCR product. This allows to visualize directly the exponential part of the PCR reaction.

Principle of quantitative real time PCR

Quantitative real-time PCR is based on the detection of a fluorescent signal produced during the amplification of a PCR product. This allows to visualize directly the exponential part of the PCR reaction using TaqMan or SYBRGreen (http://www.appliedbiosystems.com/molecularbiology/about/pcr/sds/)

2.1 Detection system for the TaqMan and SYBRGreen RT-PCR (see Figure 1)

In brief, the detection system for the TaqMan and SYBRGreen RT-PCR consists of a thermal cycler connected to a laser and charge coupled device (CCD) optics system.

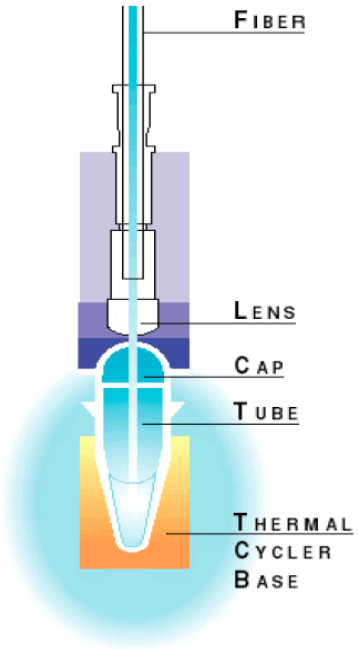


Figure 1 Scheme of the detection system

An optical fiber inserted through a lens is positioned over each well, and laser light is directed through the fiber to excite the fluorochrome in the PCR solution. Emissions are sent through the fiber to the CCD camera, where they are analyzed by the software's algorithms. Collected data are subsequently sent to the computer.

The software calculates the threshold cycle (Ct) for each reaction with which there is a linear relationship to the amount of starting DNA. Ct is the threshold cycle i.e. the cycle number at which the reporter dye emission intensities rises above background noise (Figures 2-3) The Ct is determined at the most exponential phase of the reaction and is more reliable than end-point measurements of accumulated PCR products used by traditional PCR methods. The Ct is inversely proportional to the copy number of the target template; the higher the template concentration, the lower the threshold

cycle measured. There are many advantages to quantifying gene sequences using this technology, foremost being precision and sensitivity. This precision exists because the quantification of the gene sequence is determined by the Ct which is calculated during the exponential phase of the reaction (http://www.QIAGEN.com).

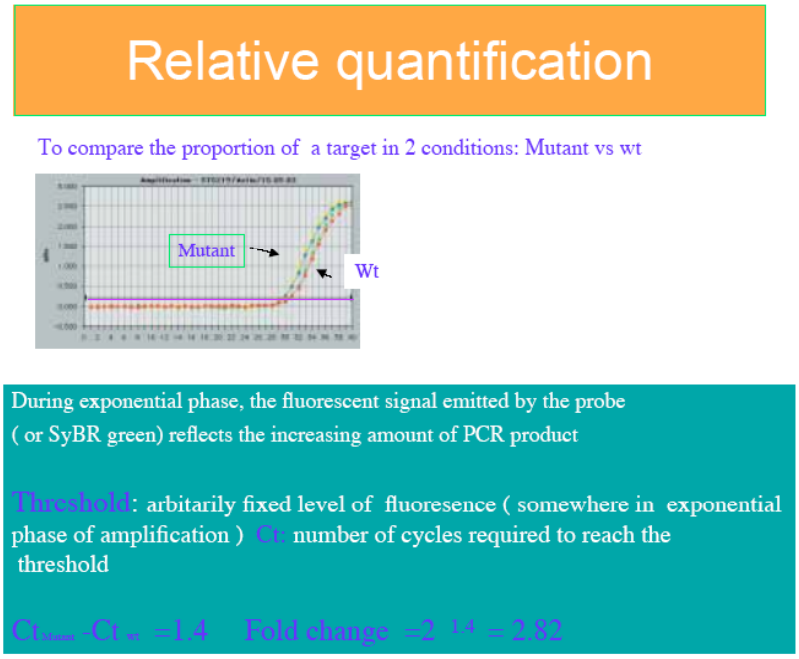


Figure 2 Principle of relative quantification

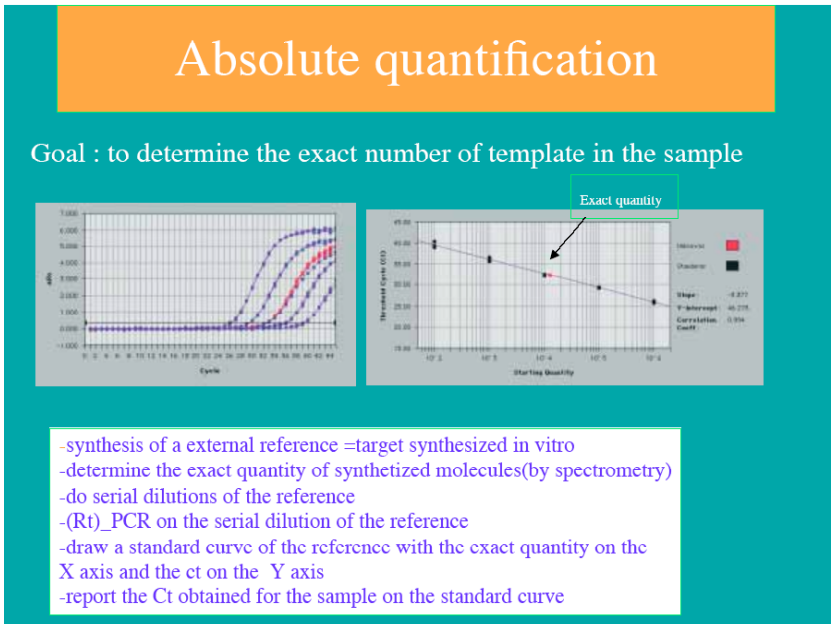


Figure 3 Principle of absolute quantification

These windows show the amount of fluorescence obtained in each amplification cycle for each reaction. The threshold cycle (Ct) is shown by the darker horizontal line.

2.2 Real-time Reporters

SYBR® Green, TaqMan®, and Molecular Beacons:
All real-time PCR systems rely upon the detection and quantification of a fluorescent reporter, the signal of which increases in direct proportion to the amount of PCR product in a reaction.

SYBR® Green

In the simplest and most economical format, that reporter is the double-strand DNA-specific dye SYBR® Green (Molecular Probes). SYBR Green binds double-stranded DNA, and upon excitation emits light (Figure 4). Thus, as a PCR product accumulates, fluorescence increases. The advantages of SYBR Green are that it's inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration. For single PCR product reactions with well designed primers, SYBR Green can work extremely well, with spurious non-specific background only showing up in very late cycles.

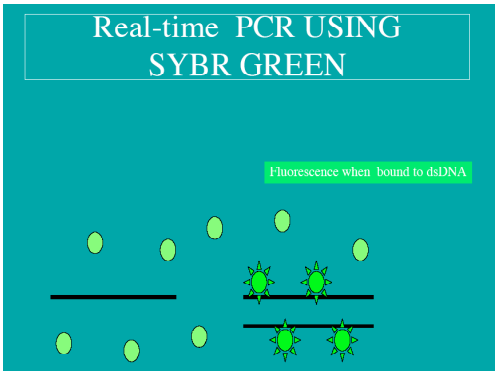


Figure 4 SYBR® Green binds double–stranded DNA

At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers, and the dye. The unbound dye molecules weakly fluoresce, producing a

minimal background fluorescence signal which is subtracted during computer analysis. After annealing of the primers, a few dye molecules can bind to the double stranded DNA. DNA binding results in a dramatic increase of the SYBR Green I molecules to emit light upon excitation. During elongation, more and more dye molecules bind to the newly synthesized DNA. If the reaction is monitored continuously, an increase in fluorescence is viewed in real-time. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls.

TaqMan® and molecular beacons

Both TaqMan and molecular beacons are hybridization probes relying on fluorescence resonance energy transfer (FRET) for quantification. The probe is designed to anneal to the target sequence between the traditional forward and reverse primers (Figure 6). TaqMan Probes are oligonucleotides that contain a reporter fluorochrome (usually 6- carboxyfluorescein [6-FAM]) and a quencher fluorochrome (6-carboxy-tetramethyl-rhodamine [TAMRA]) added at any T position or at the 3' end. When irradiated, the excited fluorochrome transfers energy to the nearby quenching dye molecule rather than fluorescing, resulting in a nonfluorescent substrate. TaqMan probes are designed to hybridize to an internal region of a PCR product and to have a higher Tm than the primers, but during the extension phase, the probe must be 100% hybridized for success of the assay. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5' exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent and quenching dyes and FRET no longer occurs. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle. Molecular beacons also contain fluorescent and quenching dyes, but FRET only occurs when the quenching dye is directly adjacent to the fluorescent dye. Molecular beacons are designed to adopt a hairpin structure while free in solution, bringing the fluorescent dye and quencher in close proximity. When a molecular beacon hybridizes to a target, the fluorescent dye and quencher are separated, FRET does not occur, and the fluorescent dye emits light upon irradiation. Unlike TaqMan probes, molecular beacons are designed to remain intact during the amplification reaction, and must rebind to target in every cycle for signal

measurement. TaqMan probes and molecular beacons allow multiple DNA species to be measured in the same sample (multiplex PCR), since fluorescent dyes with different emission spectra may be attached to the different probes. Multiplex PCR allows internal controls to be co-amplified. These hybridization probes afford a level of discrimination impossible to obtain with SYBR Green, since they will only hybridize to true targets in a PCR and not to primer-dimers or other spurious products.

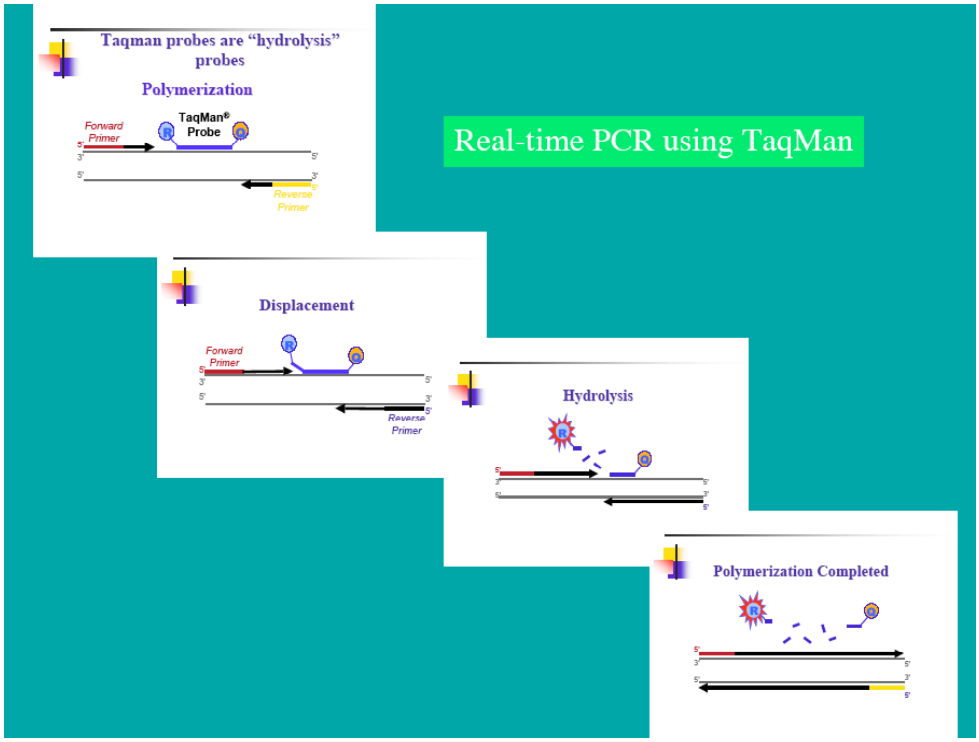


Figure 5 Fluorogenic 5' nuclease chemistry

1. Forward and reverse primers are extended with Taq polymerase as in a traditional PCR reaction. A probe with two fluorescent dyes attached, a reported (R) and a quencher (Q), anneals to the gene sequence between the two primers.
2. When both dyes are attached to the probe, reporter dye emission is quenched. As the polymerase extends the primer, the probe is displaced.
3. An inherent nuclease activity in the polymerase cleaves the reporter dye from the probe.
4. Once separated from the quencher, the reporter dye emits its characteristic fluorescence.

Melting curve analysis can show specificity of reactions

The peak for higher temperature shows the specificity. The non-specific primer dimer can be shown by the peak corresponding to the lower temperature (Figure 6).The different lines represent different samples with the same primers.

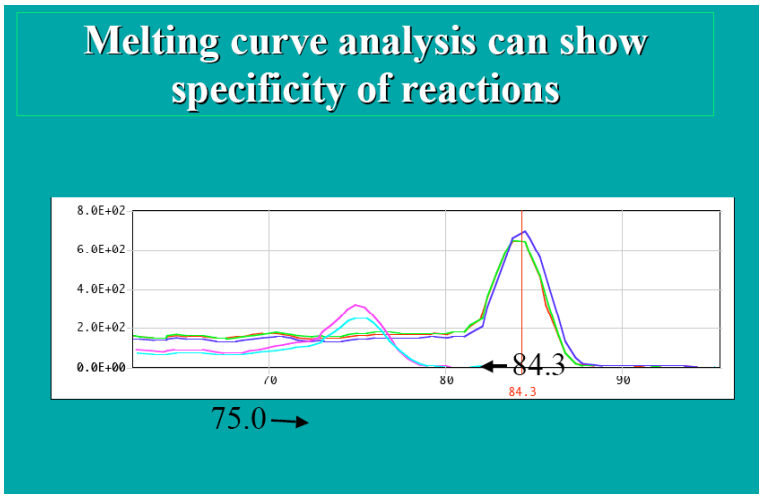


Figure 6 Melting curve analysis

2.3 Instruments

Real-time PCR requires an instrumentation platform that consists of a thermal cycler, computer, optics for fluorescence excitation and emission collection, and data acquisition and analysis software. These machines, available from several manufacturers, differ in sample capacity (some are 72-well standard format, others process fewer samples or require specialized glass capillary tubes), method of excitation (some use lasers, others broad spectrum light sources with tunable filters), and overall sensitivity. There are also platform-specific differences in how the software processes data. SYBR is a registered trademark of Molecular Probes. TaqMan is a registered trademark of Roche Molecular Systems. ABI PRISM is a registered trademark of PE Applied Biosystems. For cost -effective multiplex real – time PCR, Plexor technology (Promega) could be applied. Plexor™ chemistry in multiplex reactions, one of the primers for each target must have a different fluorescent label. The types and number of fluorescent labels that can be used depend upon the detection capabilities of the real-time instrument used. Besides, the Universal ProbeLibrary from Roche (https://www.roche-applied-science.com) for *C.*

elegans could create a functional, highly specific, optimized assay that can be performed overnight using pre-validated universal probes.

Material and method

Development of single worm real time quantitative RT-PCR (step by step protocol)

The availability of thousands of genetic mutants is a quite precious source for the characterization of the function of specific genes. However, many of these genes are lethal or show maternal lethality in *C.elegans*, thus limiting the isolation of large amount of mRNA for high throughout assays, e.g. microarray analysis. Here we describe the use of different RNA isolation methods in combination with real time quantitative RT-PCR to analyze the putative target genes of LET-418 protein on a few worms or single worm.

Preparation of staged worms for real time quantitative RT-PCR

To characterize the function of a protein of interest, the transcriptional regulation of its downstream target genes generally needs to be studied. To assay the target genes of LET-418, we synchronized the worms (both test and reference samples) for several generations using the following treatment: Pick young adults and let them lay eggs. The adults are removed. Let the eggs hatch and develop to young adults. Repeat this synchronization cycle several times. In general, we keep these worms synchronized continuously, since lack of synchronization leads to more isogenic or epigenetic variations among the worms. The changes in gene expression in *C.elegans* can result from differences in developmental time, age, environmental factors, or other experimental manipulations. Particular attention must therefore be given to the experimental design to ensure that the only difference between the samples is the one that the experimenter intends.

We synchronized worms at least twice for assaying the target genes of LET-418 by using the following precedure:

1. Pick 30-40 L3 staged wildtype (WT) and *let-418(ts)* worms;
2. After about one day at 15°C, pick 20-30 identically staged L4 or young adult

worms;

3. After 5-6 hrs, most adults begin to lay eggs; Pick 20-30 egg-laying adults and transfer them on new plates for 30 min until they have produced about 150 eggs;
4. The eggs are grown to the young adult stage;
5. Repeat step1-3 until the worms reach the late L1 stage. The WT and *let-418(ts)* worms were then transferred to 25°C. After about one day, we picked 110 early L4 lavae and transferred them to lysis buffer.

Trizol based isolation of total RNA for single worm quantitative RT-PCR

By using the Trizol reagents (Chomczynski P, 1993), we can easily isolate total RNA from single or a few worms. It is powerful but care in pipeting should be taken due to the many steps. Other procedures could be basically applied, such as column chromatography based total RNA isolation or single worm in one tube RT-PCR. For low abundance transcripts, we found that the Trizol–based total RNA isolation works better than others in our hands (unpublished).

1. Put 3-30 worms into 40 μl M9 (1.7 ml eppendorf tube).
2. Add 360 μl of fresh Trizol reagent . Vortex gently, 3 x freeze – thaw.
3. Invert tube to solubilize and lyse worms. Incubate at RT for 5 min.
4. Spin at14 K at 4 ºC for 10 min in a microfuge to remove insoluble material.
5. Remove the supernatant liquid to a fresh RNase free eppendorf tube, add 80 μl CHCl3 (chloroform) to each tube.
6. Invert /vortex for 15 sec. Incubate at RT for 2-3 min.
7. Spin 15 min at 14k at 4 ºC to separate phases.
8. Remove upper aqueous phase and transfer into a fresh tube. Add 200 μl isopropanol and mix.
9. Incubate 10 min at RT to precipitate RNA. Recover RNA by spinning at 14 K for 10 min at 4 ºC.
10. Carefully remove aqueous solution away from the pellet.
11. Wash pellet with 40 μl 75% EtOH in DEPC (diethyl pyrocarbonate) treated H_20. Vortex briefly.
12. Spin at 10K for 5 min at 4 ºC.

13. Remove supernatant and air dry pellets for 5-10 min.
14. Dissolve pellets in 25-50 μl DEPC H_20. To help dissolve, heat at 60°C for 10 min.
15. Take 1 μl to measure OD value to evaluate the concentration and purity.

The removal of genomic DNA

The isolated total RNA is briefly incubated in gDNA wipeout buffer (QIAGEN) at 42°C for 5 minutes to effectively eliminate contaminating genomic DNA.

In general, the classical DNase treatment can work for the removal of genomic DNA. Specifically we add a DNase treatment in chromatography column based total RNA isolation in our hands (not shown). However, the treatment using gDNA wipeout buffer is simple and fast.

The reverse transcription of total RNA

Add genomic DNA-removed template RNA to each tube containing reverse–transcription master mix (QIAGEN). Mix thoroughly by vortexing for no more than 5s. Centrifuge briefly to collect residual liquid from the sides of the tubes. Incubate for 30 min at 42°C. Then incubate for 3 min at 95°C to inactivate Quantiscript Reverse Transciptase. Add an aliquot of each finished reverse-transcription reaction to real-time PCR mix. Store reverse-transcription reactions on ice and proceed directly with real-time PCR, or for long–term storage, store reverse-transcription reactions at -20°C.

Running the real time quantitative PCR

We used the Rotor-gene 3000 real time PCR machine (Corbett Inc.) to perform the real time quantitative PCR according to the manufactor's recommendation.

Results and discussion

Application of single worm agarose gel semi-quantitative RT-PCR

The Down syndrome (DS), caused by trisomy 21, is the most common genetic cause of mental retardation, with an incidence of approximately 1 in 700 live births. There are numerous other phenotypes associated with DS, such as mental retardation and facial characteristics, heart disease, early onset Alzheimer disease, and so on. In

collaboration with Dr. Guipponi M group, *C.elegans* was used for to study the functional/phenotypical effects of inactivation and overexpression of *C21orf80*. Here, we report on initial characterization of the *C.elegans* ortholog of *C21orf80*, a potential new protein O-fucosyltransferase that maps to Hsa21.To gain insight into the biological role of *C21orf80* and its potential role in DS, *C. elegans* ortholog, *pad-2*, overexpression experiments have been done.

Analysis of *pad-2* overexpression was performed by semi-quantitative RT-PCR.
Total RNA was isolated from 30 wild-type, *hs::pad-2* transgenic, or K10G9 transgenic L4-stage larvae. *pad-2* and *ama-1* mRNAs were amplified by the Qiagen one-step RT-PCR system using the following primers 5', AAATTCGAGAAACGGAGCTG-' 3 and 5'-TACTCCTTCTCGCCTTCCAG-'3 for *pad-2*; 5'-CAGTGGCTCATGTCGAGT-'3 and 5'CGACCTTCTTTCCATCAT-'3 for *ama-1*. The PCR products were separated on an agarose gel and transgene expression levels were estimated by measuring the respective intensity of the ethidium bromide bands using the Multi-Analyst software (Bio-Rad). We performed this experiment twice and basically it gave the same results (Figure7, and Menzel O et al., 2004)

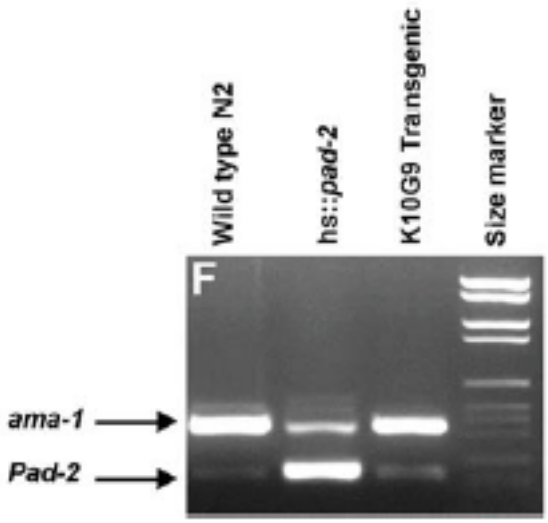


Figure 7 Agarose gel electrophoresis of *pad-2* mRNA. *ama-1* mRNA was used as internal control for semi-quantitative RT-PCR

Lane 1: Wild type animals;

Lane 2: Animal transgenic for *hs::pad-2* after heat shock (33°C, 30 minutes) ;

Lane 3: Animals transgenic for the cosmid K10G9;

Lane 4: Molecular weight marker (pBR328/*Hind*III +pBR328/*Bgl* I)

Levels of transgene expression were estimated by semiquantitative PCR that showed increased dose of *pad- 2* mRNA in both transgenic strains (*hs::pad-2* and cosmid K10G9) compared to wild-type animals. This was confirmed by the corresponding phenotype characterized by embryonic lethality associated with various morphological defects (Menzel O et al., 2004).

Application of semi-quantitative and real-time RT-PCRs of the *lag-2* mRNA levels

A recent work on the *hda-1*/*gon-10* mutations result in up-regulation of *lag-2* mRNA levels (Dufourcq et al., 2002). Since LET-418 and HDA-1 are members of the same nucleosome-remodelling complex, we expected that LET-418 negatively controls *lag-2* expression. Furthermore, we observed ectopic expression of *lag-2::gfp* in *let-418 (RNAi)* animals. Semi-quantitative and real-time PCR experiments were performed to test this hypothesis and we found that *lag-2 mRNA* levels indeed increase in *let-418(lf)* and *let-418(lf);chd-3(lf)* animals (Figure 8) but not *chd-3(lf)* animals (not shown).

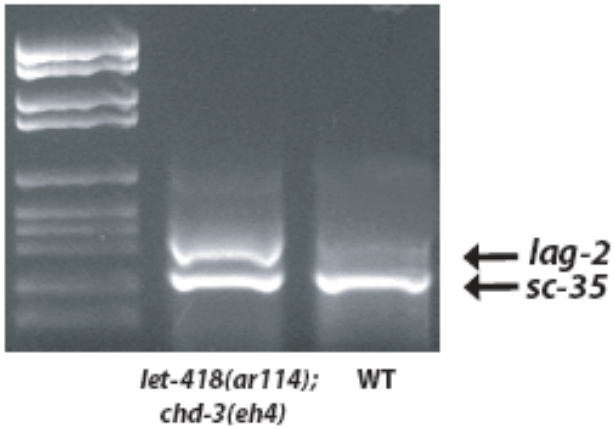
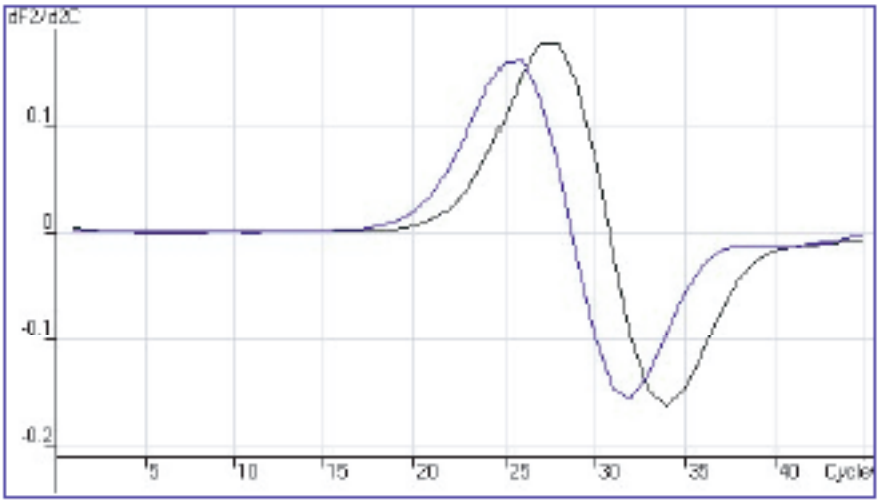


Figure 8a Semi-quantitative RT-PCRs of the *lag-2* mRNA levels

Gel analysis of the *lag-2* RT-PCR amplification products from *let-418(ar114);chd-3(eh4)* and wild-type L4 stage animals. A significantly higher amount of *lag-2* transcript is found in *let-418(ar114);chd-3(eh4)* than in wild-type animals. For comparison and normalization, the amplification of the gene *sc-35* (splicing factor) was used.



Colour	Genotype	Takeoff	Amplification
	wild-type	23. 4	1 .7 3
	let-418(ar114)	21. 5	1 .6 9

Figure 8b Real-time RT-PCRs of the *lag-2 mRNA* levels
Quantification of the *lag-2* mRNA levels by real time PCR. *lag-2* amplification was significantly higher in *let-418(ar114)* than in wild-type animals: the fold of increasement for *let-418(ar114)* against WT: $2^{\Delta Ct} = 2^{23.4--21.5} = 3.73$; repeated twice.

The increase of *lag-2 mRNA* levels in *let-418(lf)* and *let-418(lf);chd-3(lf)* animals could be proven by either agarose-gel semi-quantitive RT-PCR or real time RT-PCR method.

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Identification of differentially expressed target genes of LET-418/Mi-2 using the technique of SSH (Suppression subtractive hybridization)

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Summary

It is expected that Mi-2/NuRD (Nucleosome-Remodeling Deacetylase complex) regulates critical genes involved in distinct pathways. Some of these genes may be expressed at a low level. We have isolated differentially expressed downstream genes of *C. elegans* LET-418/Mi-2 in *let-418(ts)* mutants using SSH (suppression subtractive hybridization) genome-wide search, a method that monitor differential gene expressions. It can provide invaluable informations about important cellular and developmental processes. The method includes a normalization step to equalize the abundance of cDNAs within the target population, making it possible to detect low abundant transcripts. We prepared two SSH subtracted libraries using cDNA isolated from *let-418(ts)* mutants and wild type animals. Some of the genes that we identified to be differentially expressed can be divided into groups of functionally related genes. Among them are members of metabolic pathways, the IGF-1 pathway, RNAi and IP3 signaling. They also include chromatin modifier genes, ribosomal protein genes, germline-specific genes and genes encoding proteins involved in ubiquitination and protein degradation, *etc.* Similar expression patterns of such functionally related genes emphasize their importance in LET-418/Mi-2-NuRD-regulated processes. The functions of these candidate genes were preliminarily studied by using RNA interference with a specifically engineered RNAi T-vector. The *let-418* dependent regulation of some representative target genes (*sqt-2*, *sqt-3*, *clp-1*, *rps-2*, *etc*) were further confirmed by real time RT-PCR. Comparison of our results with those of a recent genome-wide RNAi screen for suppressors of synMuv highlight the possibility that the overlapping genes are good candidates for being transcriptionally regulated by LET-418/Mi-2.

Introduction

In eukaryotes, cellular and biological processes such as cellular growth, cell fate determination and organogenesis are mediated by differential gene expression. To understand the regulation of these processes, differentially expressed genes of interest must be identified, cloned, and characterized in detail.

Techniques involving functional genomics such as DNA microarrays (Lipshutz RJ et al., 1995; Schena M. et al., 1995; Chee M et al., 1996), SAGE (Serial Analysis of Gene Expression; Velculescu VE, et al., 1995), RDA (representational difference analysis) cDNA substraction (Lisitsyn N and Wigler M., 1993; Lisitsyn NA, 1995) and SSH (suppression subtractive hybridization (Diatchenko L et al., 1996; 1999) are powerful tools to profile differential gene expression and provide invaluable information for the cellular and developmental processes they are involved. The high throughput DNA microarray technology is limited for transcripts of low abundance. However, SSH has a normalization step to equalize the abundance of cDNAs within the target population, thus enabling the detection of low abundance transcripts. A "case study" has shown that about two thirds of the transcripts identified using SSH methodology were not identified using GeneChip microarrays alone (Cao W, et al., 2004). This points out that SSH is a powerful method for the identification and analysis of differentially expressed cDNAs and can be used as an alternative to transcript profiling with Affymetrix GeneChip microarrays, especially in identifying novel genes or transcripts of low abundance.

DNA microarrays

DNA microarray, first introduced in 1995, has been extensively employed for large-scale analysis of gene expression in the field of functional genomics (Schena et al., 1995). This high-capacity system can measure in a single experiment the relative quantities of specific mRNAs of ten thousands of genes in two or more tissue samples. Two types of *C.elegans* microarray exist. The first contains PCR amplified fragments that are spotted as shown in Figure 1. The second type is the commercial Affymetrix platform (Lockhart et al., 1996) that contains 20-25 bp long

oligonucleotides synthesised directly on the surface by photolithography and solid-phase chemistry. Multiple probe pairs (one perfect matching oligonucleotide is paired with a mismatch oligonucleotide) for different regions of each gene are designed, allowing for mean values of signal intensities to be calculated. DNA microarray technology, however, is limited by its insensitivity to transcripts of low abundance.

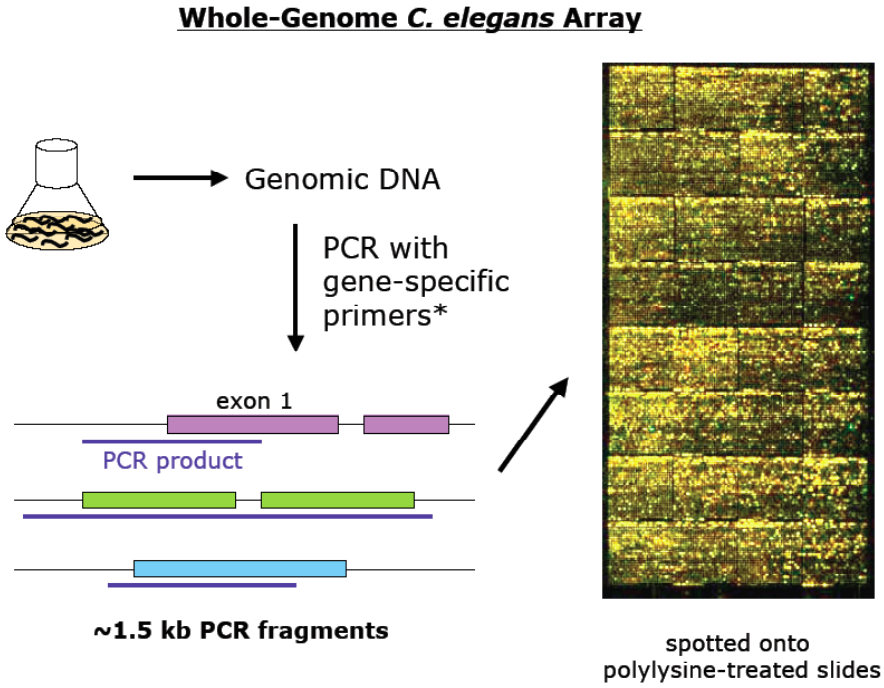


Figure 1a. Outline of PCR amplified cDNA microarray

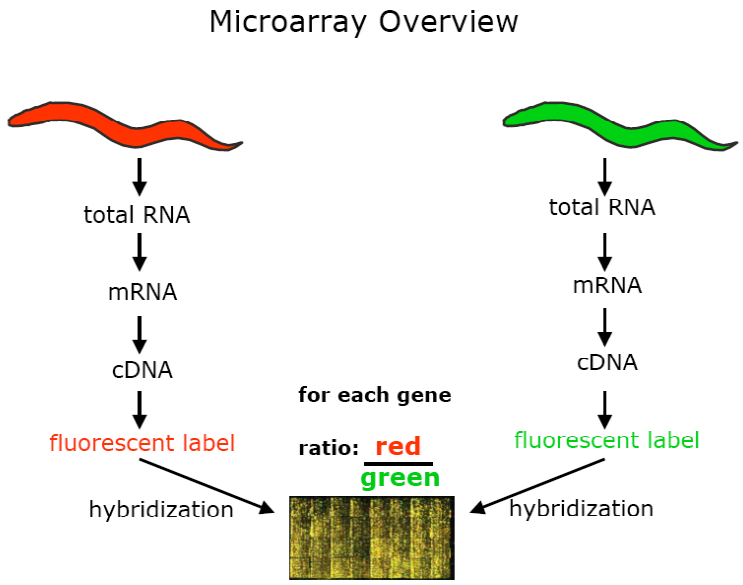


Figure 1b. Overview of cDNA microarray assay

Suppression Subtractive Hybridization (SSH)

Initially, Suppression Subtractive Hybridization (SSH) was developed for cDNA comparisons (Lukyanov SA et al., 1994, 1996; Gurskaya NG et al., 1996). The SSH method is based on PCR suppression by inverted terminal repeats (PCR suppression) (Lukyanov SA et al., 1994; Siebert PD et al., 1995), which is shown in Figure 2. Upon initiation of PCR after the denaturation phase, the single-stranded (ss) DNA fragments flanked by inverted terminal repeats (ITR) forms either self-annealing "pan-like" structures (preventing the primer binding to its complementary binding sites and suppressing the PCR) or DNA/primer hybrid structures. In the latter case, if the primer corresponding to the outer part of ITR is used, the DNA synthesis by Taq-polymerase restores the original structure, ensuring the persistence of suppression during further PCR cycles.

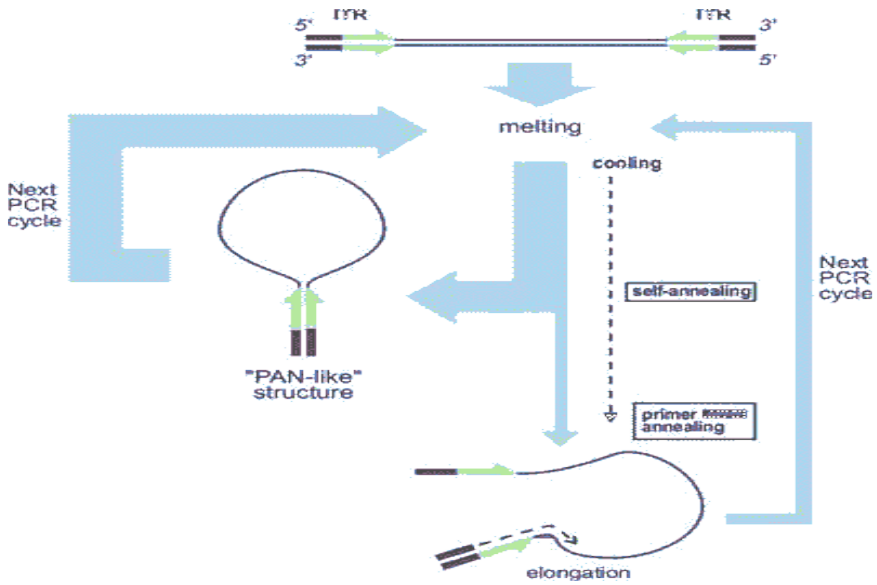


Figure 2 PCR suppression by inverted terminal repeats

In complex mixtures, the PCR suppression allows precise amplification only of molecules that are flanked by different adapters at opposing termini (asymmetrically flanked molecules). This principle is used in Suppression Subtractive Hybridization (SSH): the molecules of interest are driven to the asymmetrically flanked state, leaving the major fraction symmetrically flanked, i.e. without sites for primer annealing, as shown in Figure 3. The substrate for SSH consists of melted double-stranded (ds) cDNA (the tester) containing specifically expressed sequences to

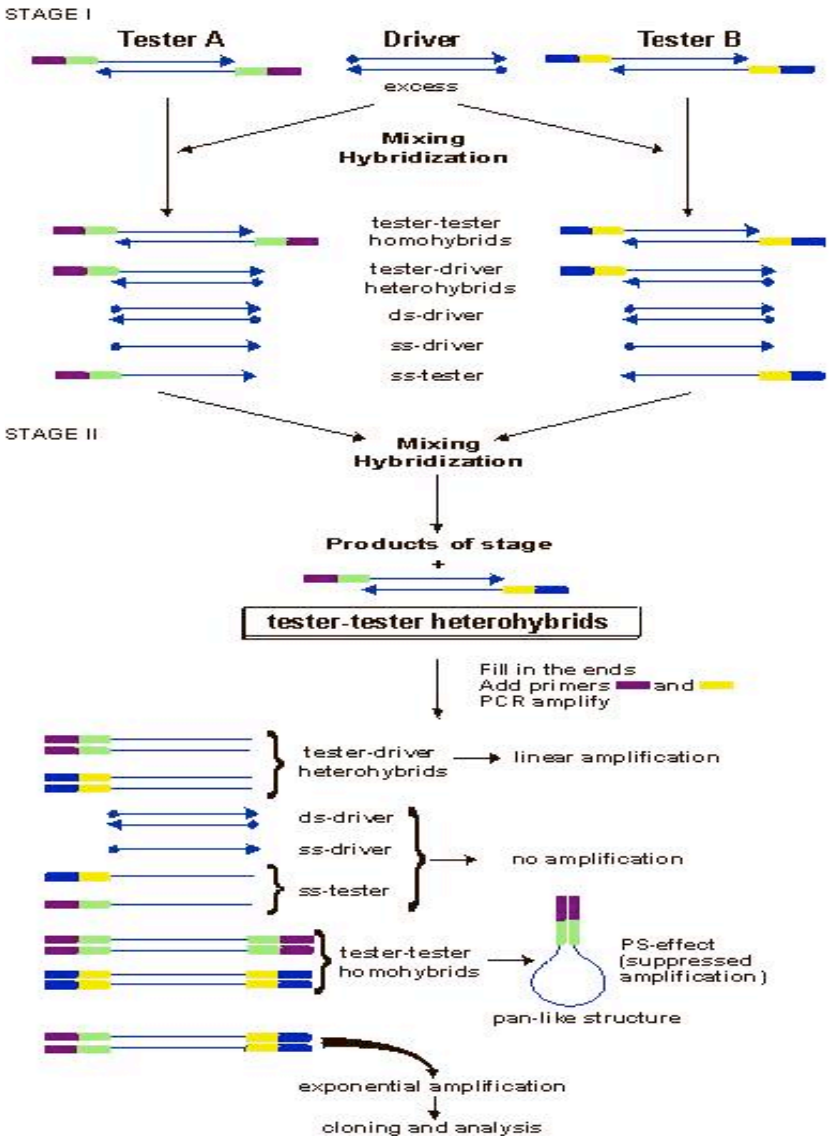


Figure 3 Schematic diagram of SSH

Yellow and green boxes represent the inner parts of adapters. Blue and violet boxes represent the outer parts of adapters.

be extracted (the target) and melted ds-cDNA lacking the target sequences (the driver) that is used for comparison. First, the cDNA is digested with a blunt cutting restriction enzyme. Then, the resulting tester cDNA fragments are subdivided into two batch samples (A and B) that are equal in all respects and ligated to the corresponding different "pseudo-double-stranded" adapters A and B at their 5' ends in separate tubes. The two different sets of linker containing long, inverted terminal repeats (Lukyanov et al., 1995) result in two tester populations.

During the first stage of subtraction, excess driver is added to each sample of tester

and the samples are heat-denatured and allowed to re-anneal in a first hybridisation. In this step, tester molecules corresponding to the driver (termed from this point "redundant molecules") form driver-tester heterohybrids removing them from the ss-fraction. Due to the second-order kinetics of the hybridization reaction, it is highly effective for the abundant redundant molecules, leading to the approximate equalization of concentrations of various types of redundant molecules. Meanwhile, target molecules are not affected by "driver pressing" and the remaining ss-tester becomes target enriched. Since target molecules form homohybrids with each other (re-associate), and since re-association progresses much more rapidly for abundant targets than for rare sequences, the concentration of target molecules in the ss-tester is equalized.

During the second stage of subtraction, samples A and B (having different ligated adaptors) are mixed and allowed to re-associate as well as new driver to allow for the possibility that ds DNA may be formed, originating from both tester populations. This leads to the creation of novel molecules from the subtracted ss-DNA that are by definition asymmetrically flanked by adapter A at one end and adaptor B at the other. These molecules may now be isolated by suppression PCR using primers corresponding to the outer part of the two linkers that recognize the outer parts of adapters A and B. Amplification of symmetrically flanked testers from the first stage will be suppressed by forming stable hairpin-like structures after each denaturation-annealing PCR step, and hybrids formed with the driver will also not amplify due to the lack of one or both primer annealing sites, leading to preferential amplification of the asymmetrically flanked sequences of interest, i.e. in this manner, only rare target (or differentially expressed) fragments are enriched.

Some studies of downstream targets of different eukaryotic Mi-2/NuRD

The downstream targets of Mi-2/NuRD are poorly understood, although a few studies have advanced our understanding. The Mi-2 protein is the central component of the recently isolated NuRD (nucleosome remodelling and histone deacetylase) complex. Two *C. elegans* Mi-2 homologues, LET-418 and CHD-3 possess both shared and unique functions during vulval cell fate determination. These include antagonism of the *Ras* signalling pathway required for vulval cell fate induction and the proper execution of the 2 degrees of cell fate of vulval precursor cells, a process under the control of LIN-12/ Notch signaling (von Zelewsky T et al., 2000).The *C. elegans*

NuRD complex negatively regulates *lin-39* Hox activity, likely by down-regulating its expression (Chen Z and Han M, 2001; Guerry et al., in preparation). The *lag-2* gene, which plays a role in gonadogenesis and vulval development encodes a Notch ligand that is derepressed in *gon-10 /hda-1* animals. This suggests that *lag-2* may be a target of HDA-1 (Dufourcq P et al., 2002). Recently, we found that transcripton of *lag-2* was regulated by LET-418 (Zhang et al., in preparation). Human MTA3 was identified as an estrogen-dependent component of the Mi-2/NuRD transcriptional corepressor in breast epithelial cells. The absence of estrogen receptor or of MTA3 leads to aberrant expression of the transcriptional repressor Snail, a master regulator of epithelial to mesenchymal transitions (Fujita et al., 2003). Latest, a global gene expression profiling study using *C. elegans* embryos identified tissue-specific and extracellular matrix (ECM)-related genes as major HDA-1 targets (Whetstine JR et al., 2005). In *C. elegans*, the absence of CHD-3/Mi-2 in genetic null mutants has no obvious phenotype, but LET-418/Mi-2 causes developmental arrest at the L4 larval stage in the presencce of maternal LET-418 proteins or L1 larval arrest without maternal LET-418 proteins (von Zelewsky T et al., 2000). *let-418 (n3536ts)* animals are thermosensitive. At permissive temperature of 15°C they behave like wild type animals and at the restrictive temperature of 25°C, they display a mutant phenotype (i.e. everted vulva, sterile with maternal LET-418 and L1 arrested without maternal protein). In order to search for downstream targets, we performed suppression subtractive hybridization to obtain two subtracted cDNA libraries of putative targets of LET-418/Mi-2 by using *let-418 (n3536ts)*.

SMART cDNA synthesis technology

let-418 mutants are maternally lethal, thus limiting the isolation of large amount of RNAs. A good quality of RNA, as well as a high percentage of full length cDNA after reverse transcription (RT) are critical to assay for differentially expressed genes. Most of the commonly used cDNA synthesis methods result in an underrepresentation of the 5' ends of genes cDNA populations, especially for long mRNAs or for mRNAs having a persistent secondary structure. In contrast, the SMART method is able to preferentially enrich for full-length cDNAs. SMART cDNA synthesis starts with nanogram amounts of total RNA. A modified oligo (dT) primer (3' SMART CDS Primer II A, AAGCAGTGGTATCAACGCAGAGTACT(30)MN (M = A, C, G, or T; N-1 = A, G, or C) primes the first-strand synthesis reaction (Figure 4). When RT

reaches the 5' end of the mRNA, the enzyme's terminal transferase activity adds a few additional nucleotides, primarily deoxycytidine, to the 3' end of the cDNA. The SMART TM oligonucleotide, which has an oligo (G) sequence at its 3' end, base pairs with the deoxycytidine stretch, thus creating an extended template. RT then switches template and continues replicating to the end of the oligonucleotide (Chenchik et al., 1998). The resulting full-length, single-stranded (ss) cDNA contains the complete 5' end of the mRNA, as well as sequences that are complementary to the SMART oligonucleotide. In cases where RT pauses before the end of the template, the addition of deoxycytidine nucleotides is much less efficient than with full-length cDNA-RNA hybrids, thus the overhang needed for base pairing with the SMART oligonucleotide is absent. The SMART anchor sequence and the poly A sequence serve as universal priming sites for end-to-end cDNA amplification. In contrast, cDNA without these sequences such as prematurely terminated cDNAs, contaminating genomic DNA, or cDNA transcribed from poly A RNA, will not be exponentially amplified. Therefore we combined this technique with SSH to isolate the differentially expressed downstream targets of LET-418/ Mi-2 in *C.elegans*.

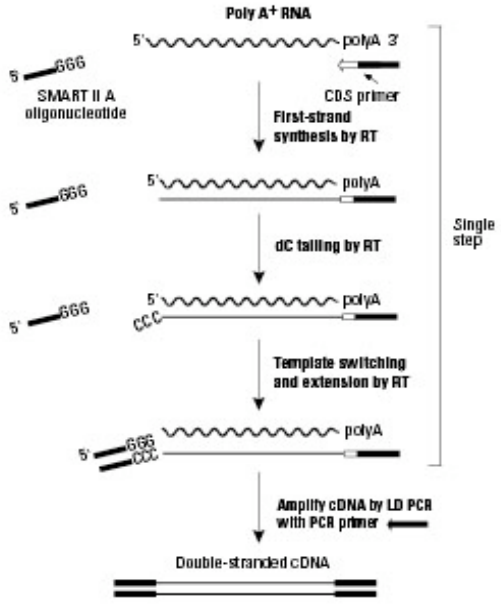


Figure 4 Flowchart of SMART (Switching Mechanism At 5' end of RNA Transcript)

Material and methods (see Step by step protocol)

Results

The preparation of worms for driver and tester cDNA populations

Von Stein et al. (1997, 2001) observed a positive rate of 94% in their SSH study, and suggested that confirmation of differential expression by Northern blot analysis for each clone obtained was unnecessary. However, the success rate of the SSH technique relies on many critical steps that need to be well monitored. Organism-wide changes in gene expression in *C.elegans* can result from differences in developmental time, age, environmental factors, or other experimental manipulations. Particular attention must therefore be given to ensure that the only difference between the samples is the one that the experimenter intends, since very subtle differences in culture conditions, age and other factors can lead to an unacceptably high number of biologically false positives.

Ideally, the preparation of worms for driver and tester cDNA populations should not introduce any artificial difference and minimize secondary developmental difference between two samples (e.g. wild type & mutant). This could be achieved by carefully checking the development time windows referring to life cycles at different temperatures and lineage (Figure 5 and Figure 6).

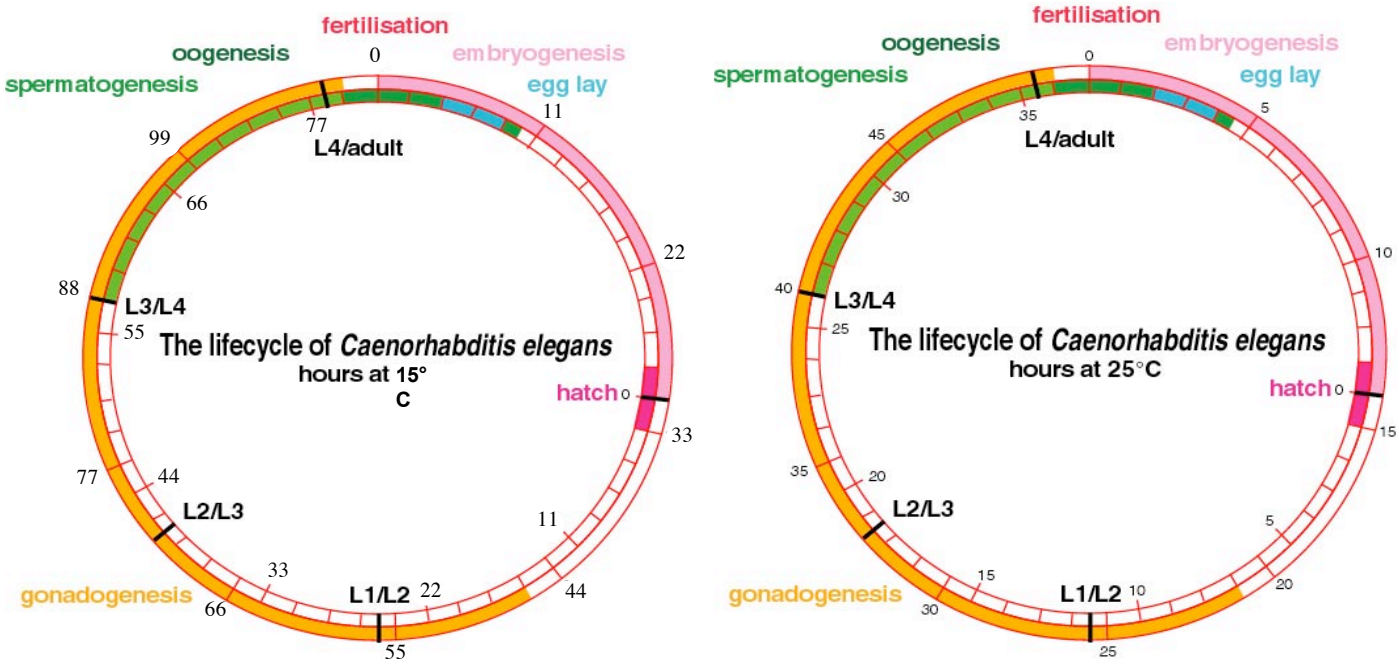


Figure 5 *C.elegans* life cycle at 15 °C and 25°C

Numbers at the outside of the circles: time of development after fertilisation in hours;

Numbers at the inside of the circles: time of development after hatching in hours

For the SSH subtracted cDNA library, we synchronized worms twice by using the following precedure:

1. Pick 30-40 L3 staged wildtype (WT) and *let-418(ts)* worms;
2. After about one day at 15°C, pick 20-30 identically staged L4 or young adult worms;
3. After 5-6 hrs, most adults begin to lay eggs; Pick 20-30 egg-laying adults and transfer them on new plates for 30 min until they have produced about 150 eggs;
4. The eggs are grown to the young adult stage;
5. Repeat this above mentioned procedure (step1-3) until reach late L1 stage (see Figure 6, green line), the WT and *let-418(ts)* worms were then transferred to 25°C;

After about one day, we picked 110 early L4 lavae (see Figure 6, blue line) and transferred them to lysis buffer.

Prior to harvest the worms in lysis buffer, we have carefully studied the phenotypical difference between WT and *let-418(ts)* worms. After having shifted one aliquot of worms from 15°C to 25°C in parallel, we observed that *let-418*(*ts)* worms incubated at 25°C for 24 hours behaved more or less wild type like; however after incubated for further 3 hours, they became smaller and thinner than wild type animals. This indicted that incubation at 25°C longer than 24 hours may result in secondary developmental difference.

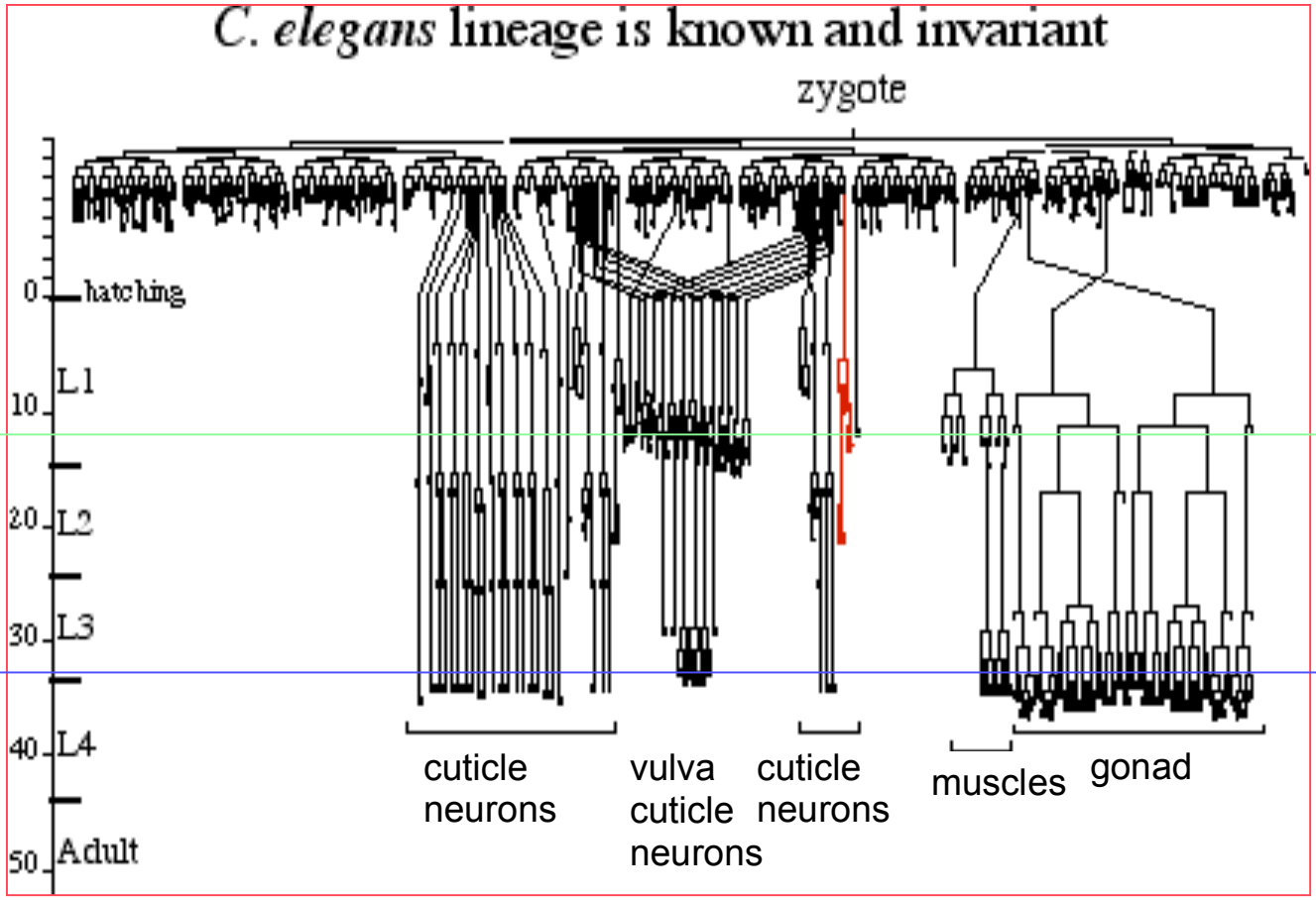


Figure 6 ***C.elegans*** **cell lineage**

Adapted from The *C.elegans* book (edited by Wood WB; Sulston JE and Horvitz HR,1977; Sulston JE *et al* ,1983). The green line indicates the developmental time point when L1 larvae were transferred to 25°C (see text); the blue line indicates the time point to harvest the worms at 25°C for early L4 larvae.

Optimization of SMART cDNA amplification

The SMART ds cDNA PCR amplification method had to be optimized before it could be used for SSH (Figure 7-9). Total mRNA was isolated from about 110 staged *let-418(ts)* and N2 early L4 larvae (see above) and converted by reverse transcription by using the SMART PCR amplification technique into double stranded cDNA. In the subsequent subtraction experiment, we referred to the *let-418(ts)* cDNA that contains presumably up-regulated transcripts as "tester", and to the wild type cDNA as "driver".

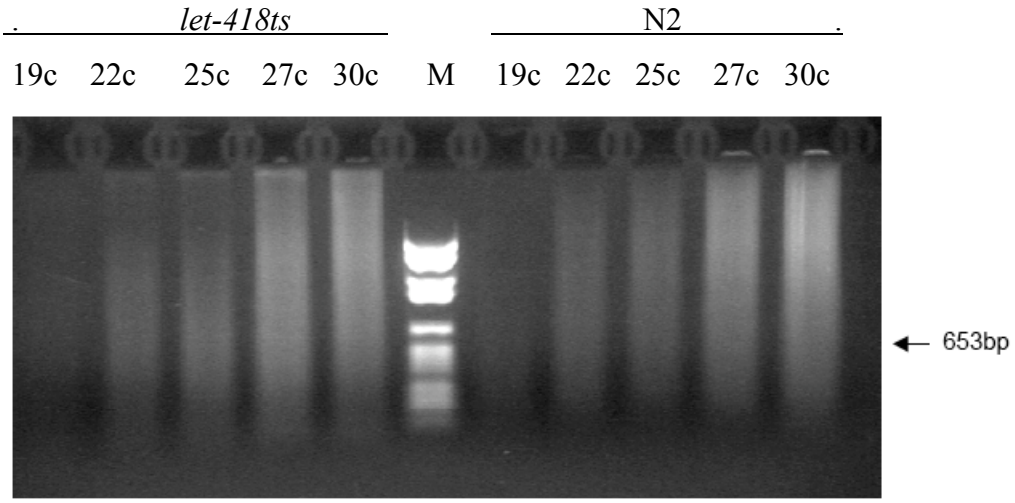


Figure 7 Agarose gel electrophoresis for optimization of the PCR parameters

The number of PCR cycles ranged from 12 to 30. 2.5% of cDNAs after reverse transcription step (see text for details) were used for each PCR reaction (totally 50 µl), 5 µl of PCR products of each time point were loaded on the agarose gel and visualized by EtBr staining. The amplified cDNAs correspond to a smear of 0.1 to 3 kb length on the gel. Between 27 and 30 cycles, the non-specific amplification in the high molecular weight range of the gel became obvious.

Left half: Optimization of the PCR parameters for the *let-418(ts)* sample using 19,22,25,27 and 30 cycles.
Middle: Molecular weight marker (pBR328/*Hind*III +pBR328/*Bgl* I).
Right half: Optimization of PCR parameters for the N2 sample using 19,22,25,27 and 30 cycles.

In parallel, one fraction of the cDNA products were amplified and monitored by real time quantitative PCR using *gpd-1* (the *C.elegans* homogue of the mammalian house–keeping gene GAPDH). For optimal results, the parameters of the SMART PCR reaction were optimized so that the ds cDNA remained in the exponential phase of amplification. When the yield of PCR products stops to increase with more cycles, the reaction has reached its plateau. Overcycled cDNA can result in a less representative probe. Undercycling, on the other hand, results in a lower yield of cDNA. The optimal number of cycles was determined as to be one cycle below the number that is needed to reach the plateau. Generally, the analysis by agarose gel electrophoresis for this optimization is sufficient. However, we used real time quantitative PCR to be more precise for this optimization. Based on these experiments, 19 cycles were finally

chosen for the SMART cDNA amplification step.

No.	Colour	Name	Takeoff	Amplification
1		gpd1on12c2-80ul-50ul10wm	22.6	1.79
2		gpd1on15c2-80ul-50ul10wm	20.2	1.59
3		gpd1on18c2-80ul-50ul10wm	18.6	1.56
4		gpd1on19c2-80ul-50ul10wm	17.7	1.61
5		gpd1on21c2-80ul-50ul10wm	17.7	1.79
6		gpd1on22c2-80ul-50ul10wm	17.7	1.76
7		gpd1on24c2-80ul-50ul10wm	17.6	1.72
8		gpd1on27c2-80ul-50ul10wm	17.2	1.71
9		gpd1on30c2-80ul-50ul10wm	17.4	1.67
10		gpd1onNtc	37.7	1.83
11		gpd1on12c2-80ul-50ul10wm	22.2	1.76
12		gpd1on15c2-80ul-50ul10wm	19.9	1.57
13		gpd1on18c2-80ul-50ul10wm	18.6	1.50
14		gpd1on19c2-80ul-50ul10wm	17.7	1.52
15		gpd1on21c2-80ul-50ul10wm	17.1	1.61
16		gpd1on22c2-80ul-50ul10wm	17.9	1.66
17		gpd1on24c2-80ul-50ul10wm	18.1	1.72
18		gpd1on27c2-80ul-50ul10wm	17.8	1.75
19		gpd1on30c2-80ul-50ul10wm	17.6	1.67

Figure 8 Optimization of the SMART cDNA PCR amplification by real time quantitative PCR using N2 template DNA

In each qPCR reaction in Figure 8, 2 microliter PCR products from 12, 15, 18, 19, 21,22,24,27,30 cycle for the above mentioned PCR optimization was used as template and ran in duplicate using Rotor-gene 2000 and following the manual description. This Figure was extracted from the machine after it finished the program of these experiments. The melting curves were monitored using Rotor-gene software to ensure that only a single product was amplified (see also Figure 9) using the specific *gpd-1* primers. The panel shows the Ct values and amplification efficiency. Ntc: no template control

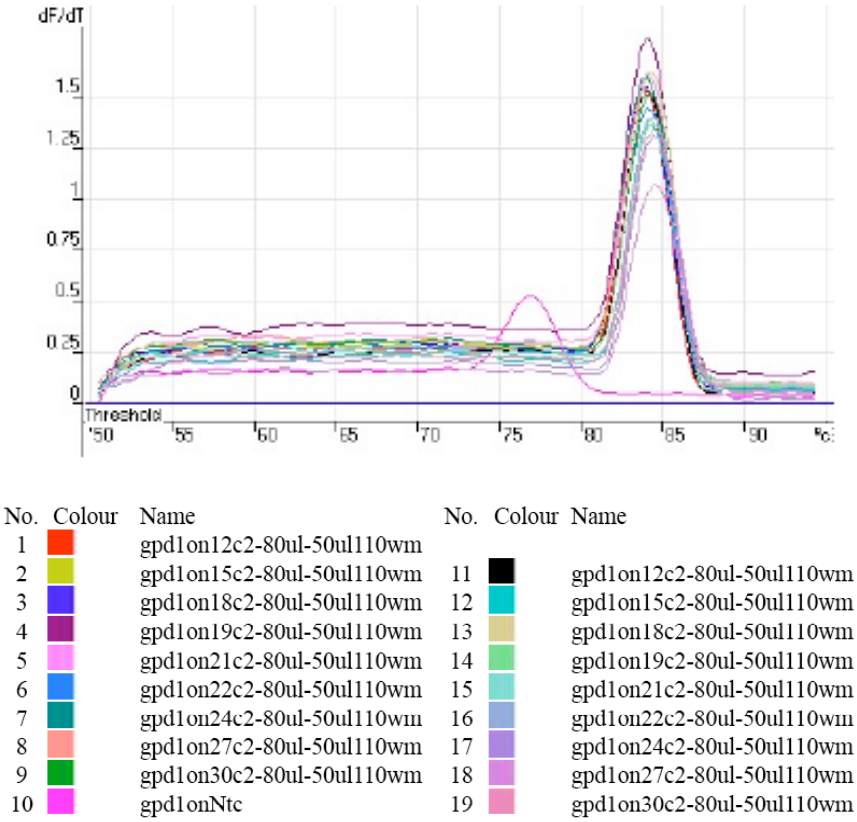


Figure 9 Melting curve shows the specificity of *gpd-1* primers

The melting curves were monitored using Rotor-gene software to show that only a single product was amplified (unique peak at 84°C; the NTC control shows another peak for primer dimer). All PCR reactions are the same as shown in Figure 8.

Construction of SSH cDNA libraries

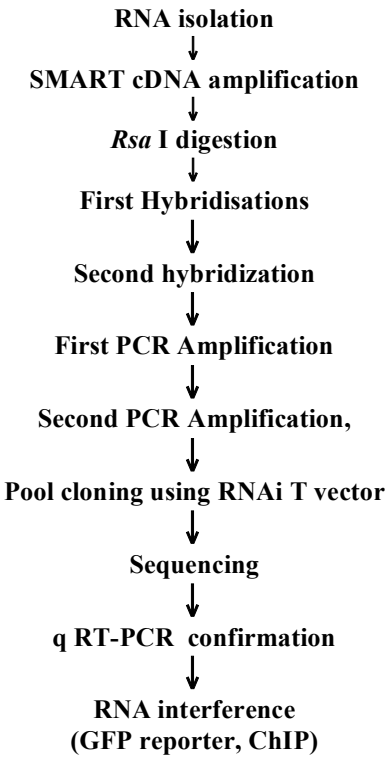


Figure 10 Outline of genome-wide screening of targets using SMART and SSH PCR
This scheme focus on a combination of SMART (RNA isolation and SMART full –length cDNA synthesis), SSH PCR (*Rsa* I digestion, Hybridisations and Amplifications), RNAi T vector cloning, RNA interference, sequencing and qRT-PCR confirmation.

Rsa I digestion

After purification on a QIAGEN PCR purification kit, the SMART cDNA samples were digested with *Rsa* I to obtain blunt-end cDNA molecules. Two different subtraction experiments were then performed (for details, see step by step SSH protocol as adapted from Diatchenko et al., 1999). For a direct subtraction library, *let-418 (ts)* cDNA was used as tester and wild type cDNA as driver, whereas for a reversed subtraction library, *let-418(ts)* cDNA was used as driver and wt cDNAs as tester.

Adaptor ligation and ligation efficiency analysis

For each library, two tester populations were generated by ligation of adaptors. For a

direct subtraction library, *let-418(ts)* cDNA was used and ligated to adaptor1 or adaptor2 (tester1-1 and tester1-2). For a reversed subtraction library, wt cDNA was used as tester and ligated with adaptor1 or adaptor2 (tester2-1 and tester2-2)(Table 1-2). The driver cDNAs remained with naked ends.

Table 1The adaptors and primers for ligation efficiency analysis

Name	Sequences	Description
Adaptor 1	5'CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT3' 3'GGCCCGTCCA5'	5' end non-phosphorylated
Adaptor 2	5'CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT3' 3'GCCGGCTCCA5'	
PCR primer1	5'CTAATACGACTCACTATAGGGC3'	adaptor - outside specific
gpd-1 5' Primer	5'AAAGGACACGGTTCAAGTGG 3'	no *Rsa* I site in this amplicon. *gpd-1* is the homologue of the mammalian *GAPDH*. Other genes can be used for this ligation efficiency analysis
gpd-1 3' Primer	5'CTAATACGACTCACTATAGGGC3'	

Table 2 Definition of tester populations

	Name	Description
Direct subtraction library	tester1-1	*let-418 (ts)* cDNA was used as tester, ligation involving the adaptor 1
	tester1-2	*let-418 (ts)* cDNA was used as tester, ligation involving the adaptor 2
Reverse subtraction library	tester2-1	N2 cDNA was used as tester, ligation involving the adaptor 1
	tester2-1	N2 cDNA was used as tester, ligation involving the adaptor 2

The ligation efficiency was checked by PCR. For the direct subtraction library, the ligation efficiency analysis is shown (Figure 11). Two *let-418(ts)* tester populations were created by adding adaptor1 or 2 , the driver cDNAs, isolated from N2 worms, remained with naked ends. The PCR products were produced by either using

Figure 11 Results of the ligation efficiency analysis on a 2% agarose/ EtBr gel

Lane 1: PCR amplification with Tester 1-1 cDNA as template and *gpd-1* 3' Primer and PCR Primer 1.

Lane 2: PCR amplification with Tester 1-1 cDNA as template, and *gpd-1* 3' and 5' Primers.

Lane 3: PCR amplification with Tester 1-2 cDNA as template, and *gpd-1* 3' Primer and PCR Primer 1.

Lane 4: PCR amplification withTester 1-2 cDNA as template, and *gpd-1* 3' and 5' Primers.

Lane M: molecular weight size marker (pBR328/*Hind* III +pBR328/*Bgl* I).

the *gpd-1* gene specific primer (*gpd-1* 3' Primer) in combination with the PCR Primer 1 or two gene-specific primers (*gpd-1* 3' and 5' Primers)(Figure 11). The size of the resulting PCR products using the *gpd-1* 3' Primer and PCR Primer 1(note: only ligated cDNA with adaptor can be amplified!) is bigger than the fragment produced with the two gene-specific primers *gpd-1* 3' and 5' Primers (e.g. Lane 1 in comparison with Lane 2). Moreover, both classes of PCR products (those with ligation and those without ligation) had similar intensities, suggesting that the ligation reaction was efficient. In parallel, an identical experiment was performed for the reverse subtraction library that showed a similar ligation efficiency (not shown).

Two hybridizations and two PCR amplifications

Next, for each SSH library, two hybridization reactions were performed. In the first one, a 30 fold excess in DNA concentration of driver was added to each sample of tester and the kinetics lead to equalization and enrichment of differentially expressed sequences (see step by step SSH protocol). A second hybridization was made by mixing the two first hybridization samples together without denaturing and adding fresh denatured driver, finally followed by annealing (see step by step SSH protocol). Using suppression PCR (Figure 2), only differentially expressed sequences were amplified exponentially. During the second nested PCR amplification, the background is reduced and specific differentially expressed sequences further enriched (Figure 3, Figure10 and Figure 13; and for details, see step by step SSH protocol).

The mock experiment

In parallel, we performed a mock experiment ("tester" was *ØX*174 /*Hae* III plus worm cDNA: "driver" was worm cDNAs only. The aim of this experiment is to ensure that the subtraction procedure works well (see step by step SSH protocol). The *ØX*174 /*Hae* III DNA fragments are present in "tester" and absent in "driver". After subtraction, we expected to find only the *ØX*174 /*Hae* III DNA fragments on agarose gel electrophoresis rather than the complex banding pattern observed with both *ØX*174 /*Hae* III plus worm cDNAs.

This result is shown in Figure 12, we observed several individual bands in the subtracted cDNA samples (Figure 12, Lane 1) corresponding to the positive control (i.e *ØX*174 /*Hae* III), whereas a quite different smear was present in the non-subtracted samples (*ØX* 174 /*Hae* III plus worm cDNAs). This clearly confirmed that the subtraction procedure worked well.

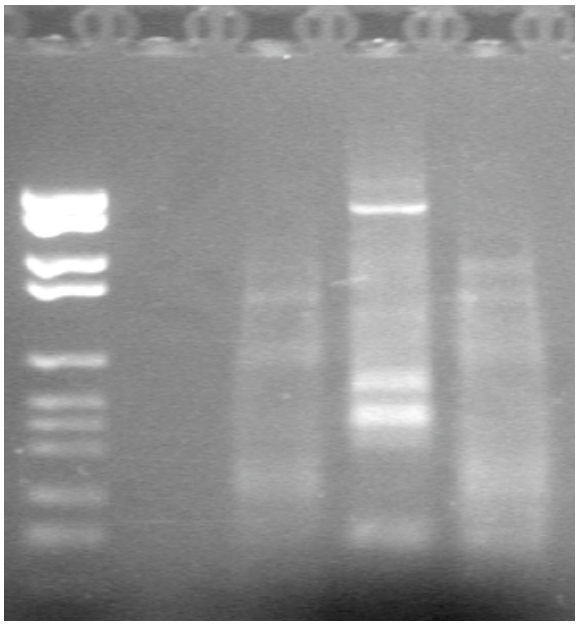


Figure 12 The control mock subtraction experiment

Lane M: Molecular weight marker (pBR328/*Hind* III +pBR328/*Bgl* I);

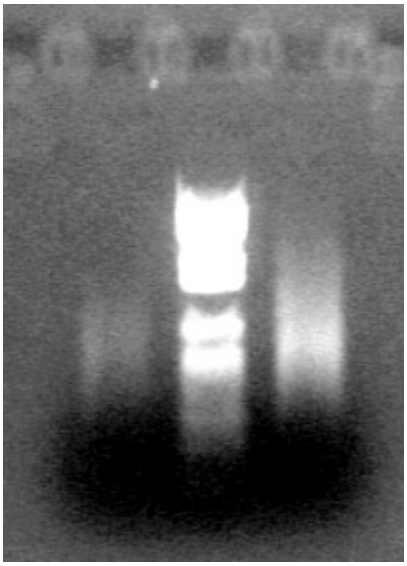
Lane 1: subtracted DNA in this mock experiment; among which subtracted *ØX*174 /*Hae* III DNAs are amplified.

Lane 2: non-subtracted DNA in this mock experiment; among which both *ØX*174 /*Hae* III DNAs and worm cDNAs are amplified.

Lane 3: PCR amplified *ØX*174 /*Hae* III DNAs as positive control.

The final experimental subtractive cDNAs

Parallel to the mock experiment (Figure 12), we constructed two final experimental subtractive cDNA populations (Figure 13, see details in the step by step protocol).



1 M 2

Figure 13 The final subtraction cDNAs

Lane 1: SSH subtracted DNAs, the *let-418(ts)* cDNAs were used as the tester and N2 cDNA were used as the driver; putatively up-regulated in *let-418* animals;

Lane M: Molecular weight marker (pBR328/*Hind* III +pBR328/*Bgl* I);

Lane 2: SSH subtracted DNAs, the N2 cDNAs were used as the tester and *let-418(ts)* cDNA were used as the driver; putatively down-regulated in *let-418* animals.

Cloning of the final experimental subtraction cDNAs

Finally, the amplified cDNA enriched in sequences from differentially expressed *let-418* target genes was directly ligated in the engineered RNAi T vector pYZ101 (Figure 14), and HB10b competent *E. coli* cells were transformed to yield two different libraries.

Figure 14 Construction of blue –white selection pYZT RNAi T vector

The vector pYZT was constructed by ligating the *Xcm* I site containing adaptor sequences 5' gatccaccaagcttcccttggtgtacccaggtagct 3' and 5' cacctgggtaaccaagggaagcttggtg 3' into *BamH*I / *Sac*I digested LITMUS 28i, i.e. two partially complementary DNA oligonucleotides bearing the 5' *Bam*H I and *Sac* I overhang sequence, were therefore synthesized, annealed, and ligated with plasmid LITMUS 28i that was previously digested with *Bam*H I and *Sac* I. Upon digestion with *Xcm* I, the resulting vector, pYZT, yielded the desired unpaired 3' deoxythymidylate residues required for cloning of PCR fragments.

The colony PCR screening

Colony PCR screening was then performed (see detailed step by step protocol, Figure15-16). After cloning the SSH cDNAs into pYZ101 RNAi T-vector, we picked individual white colonies using tooth-picks, and put them into 5 microliter MiliQ water. The tooth-picks were stirred. Then 2.5 μl of each sample were transferred to 14

ml Falcon tubes containing LB and Ampicillin for standard HB10b culture and shaked at 37°C over night for further experiments. The remaining 2.5 µl of each sample was added into 20 µl PCR reaction mixture. After PCR amplification (5 min 95°C for the first denaturation step; 20 sec, 94°C for denaturation; 30 sec at 57°C for annealing, 1min 30sec at 72°C for extension, totally 32 cycles; 30min at 72°C for last extension. As primers, we used LacZ.L (5'CGTTGTAAAACGACGGCCA3') and LacZ.R (5'AGCGGATAACAATTTCACACAGG3'). Finally, the PCR products were run on a 1.2 % Agarose gel, visualized by EtBr and photographed using a Bio-rad Gel–doc instrument. Clones with an insert >250bp were considered to be positive and sequenced for further characterization.

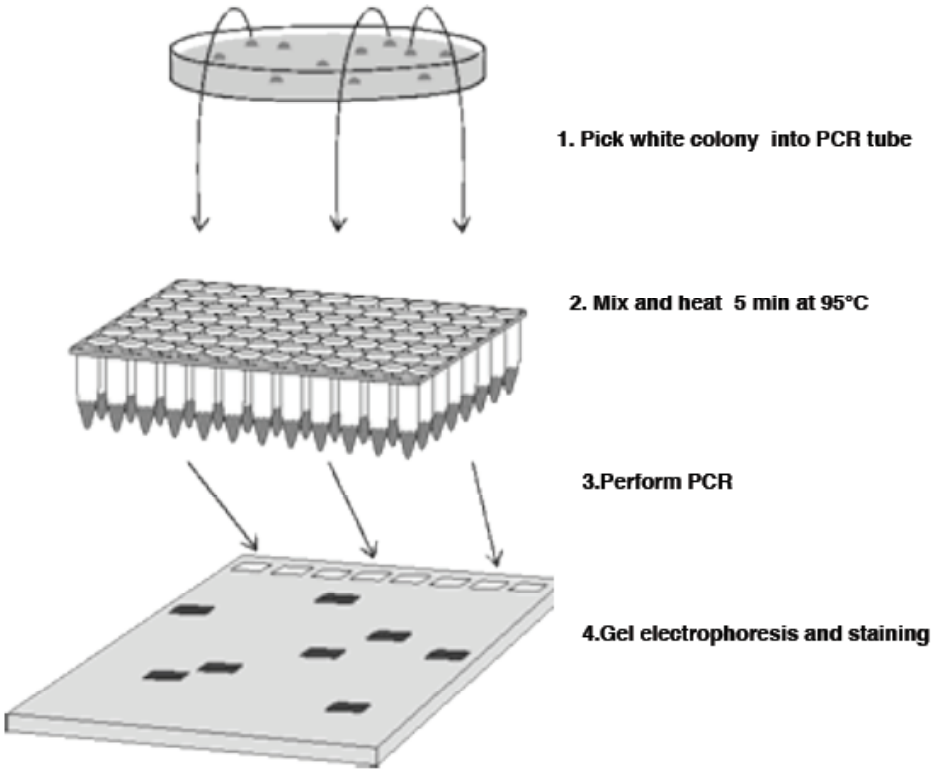


Figure 15. The Colony PCR Screening without the need for DNA purifications.

The direct subtractive cDNA library is expected to contain sequences complementary to genes that are up-regulated in *let-418* animals (Figure16a) whereas the reverse subtractive cDNA library is expected to contain sequences complementary to down-regulated genes in *let-418* animals (Figure16b).

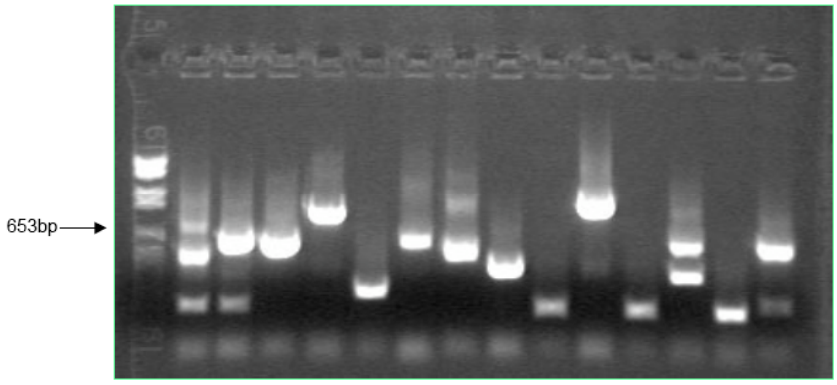


Figure 16a Colony PCR screening for positive clones with inserts of direct subtractive cDNAs

This shows an agarose gel electrophoresis of the PCR products from different white colonies using T - vector specific primers.

Lane 1: Molecular weight marker (pBR328/*Hind* III +pBR328/*Bgl* I);

Lane 2-9,11,13,15: colonies with a size of PCR products bigger than that of vector backbone. (K06A5.4, F22A3.6, R09B3.3, F41E7.5, F53B3.6, *sqt-2*, B0280.3, F41F3.3, Y41D4B.16, Y65B4BL.5, *rps-2*, *rps-8*, *glp-3/eft-3*)

Lane 10,12,14: colonies with a size of PCR products similar to vector backbone PCR products (no true inserts)

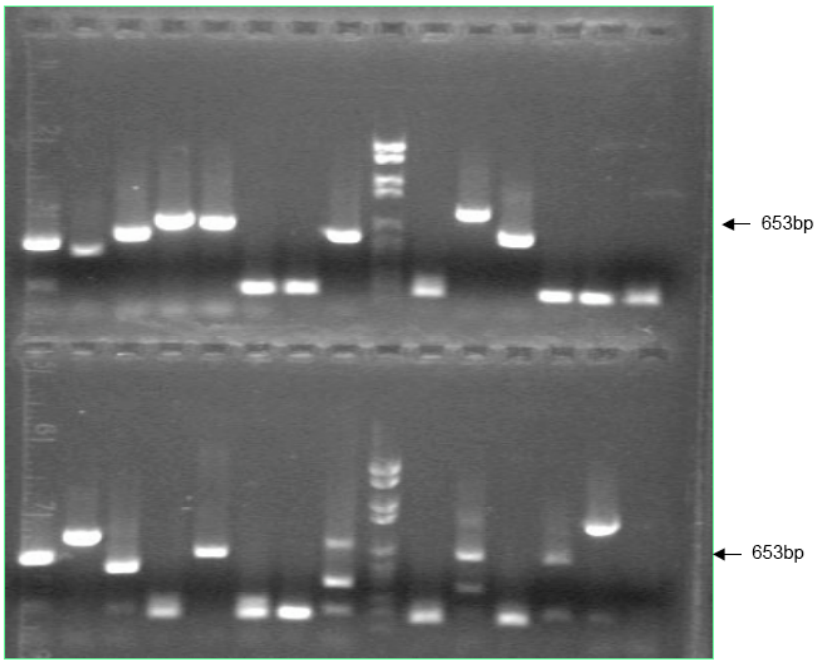


Figure 16b Colony PCR screening for positive clones with inserts of reverse subtractive cDNAs derived from genes that may be up -regulated in *let-418* animals

Agarose gel electrophoresis of the PCR products from white colonies using T-vector specific primers. Colonies with a size of PCR products bigger than that of vector backbone.

Upper Lane 1-5:	F36F2.3 (Rbbp6), F59A2.4 (*clp-1*), C04F12.1, *sqt-3, bli-1*,
Upper Lane 8, 11-12:	C08B11.6, Y37E3.8, F41F3.3,
Bottome Lane 1-3,5,8,11,13,15:	*gpd-2*, F32H2.5, *rpl-13*, C01B10.3, C32E8.11, *ari-1*, Y54G2A.18, *cyn-6*, B0261.6, *nlp-33*)
Lane 9:	Molecular weight marker (pBR328/*Hind* III +pBR328/*Bgl* I).
Upper Lane 6-7,10,13-15:	colonies with a size of PCR products similar to vector backbone
Bottom Lane 4,6-7,10,12:	PCR products (no true inserts)

Sequencing of the positive clones

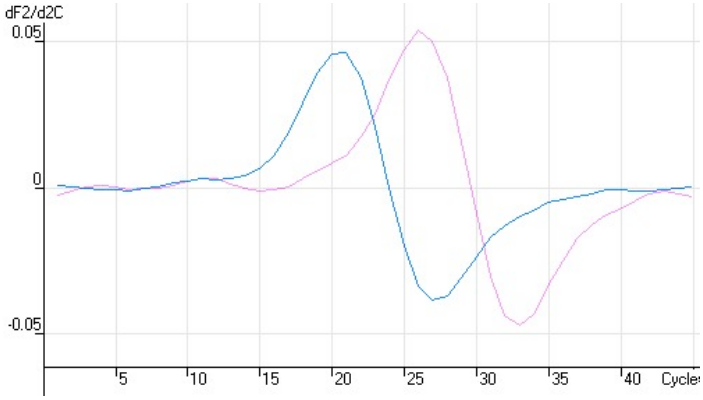
Sequencing was done by using pYZ101 vector-specific primers, such as M13 sequencing primer, LacZ.L (5'CGTTGTAAAACGACGGCCA3'), or LacZ.R (5'AGCGGATAACAATTTCACACAGG3'). The list of genes, whose sequences were analyzed by BLAST search homology against WormBase is shown in Table 4. The comprehensive list of all sequenced clones is shown in the Appendix.

Real time quantitative PCR to evaluate the quality and efficiency of subtracted cDNA libraries

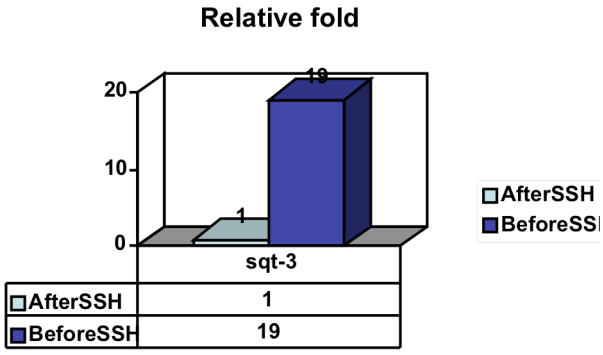
In general, either standard PCR or hybridization analysis can be used to estimate the efficiency of subtraction. In both cases, this is done by comparing the abundance of known cDNAs before and after subtraction. Ideally this is done by comparing a non-differentially expressed gene (e.g., a housekeeping gene), with a gene known to be differentially expressed between the two RNA sources. PCR amplification provides a quicker test than hybridization analysis.

However, not all housekeeping gene transcripts are subtracted evenly. Although they should be subtracted very efficiently, there are sometimes exceptions. For this reason, using real time qPCR, we tested the subtraction efficiency of *sqt-3* (a putative *let-418* down-regulated gene) and expected a reduced relative abundance of the *sqt-3* cDNA following the SSH selection procedure in direct subtractive library and an enhanced abundance in the reverse subtractive library. In the mock subtraction experiment, the agarose gel banding pattern of the *ΦX1*74/*Hae* III digest (Figure 12, Lane 1) has already indicated that our subtractions were successful.

Because we performed subtraction in both directions and therefore had unsubtracted controls for both the subtraction and the reverse subtraction, we had the chance to do two qPCR analyses to test the subtraction efficiency for both subtractive libraries. Both analyses consistently demonstrated a difference in *sqt-3* abundance in the *let-418(ts)* and N2 cDNA samples. Since quantifications of *sqt-3* cDNA before and after subtraction showed an about 19 or 36 fold difference, these experiments (Figure 18-19) not only confirmed that *sqt-3* is down–regulated in *let-418* animals, but also indicated that the quality and efficiency of subtracted cDNA libraries are good.



(a)



(b)

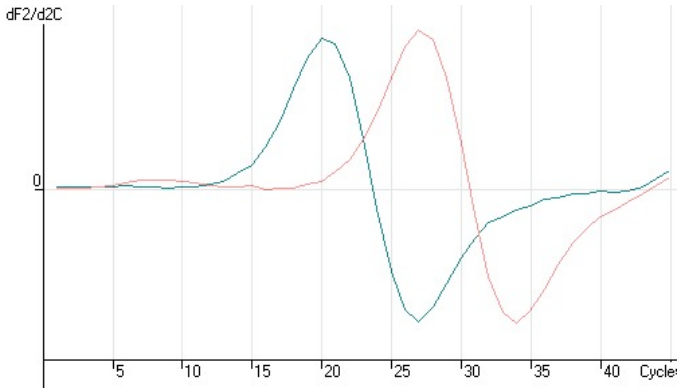
No.	Colour	Name	Takeoff	Amplification
5		*sqt-3* after SSH	21.9	1.73
6		*sqt-3* before SSH	16.5	1.72

(c)

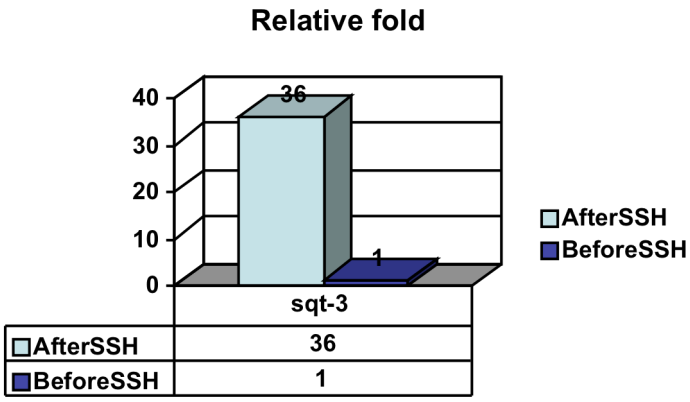
Figure 17 Evaluation of the quality and efficiency of direct subtracted cDNA library (*let-418(ts)* cDNA as tester and N2 cDNA as driver) and confirmation of down–regulation of *sqt-3* in *let-418 (ts)* by using real time qPCR

The melting curves were monitored using Rotor-gene software to ensure that only a single product was amplified. The upper left panel (a) shows the results of a real-time comparative quantitation report, the upper right panel (b) shows the *sqt-3* cDNA level normalized to that of *sqt-3* after SSH. The bottom panel (c) shows the Ct values and amplification efficiency. The final value of the *sqt-3* after SSH control was arbitrarily set to 1. PCR was performed on subtracted (*sqt-3* after SSH) or unsubtracted (*sqt-3* before SSH) secondary PCR product with the *sqt-3* 5' and 3' primers.

sqt-3 after SSH: DNAs from the direct subtracted cDNA library (*let-418 (ts*) cDNA as "tester " and N2 cDNA as "driver" for SSH)
sqt-3 before SSH: DNAs from the unsubtracted cDNA library (*let-418 (ts)*cDNA as "tester "and N2 cDNA as "driver" for SSH)



(a)



(b)

No.	Colour	Name	Takeoff	Amplification
7		*sqt-3* after SSH	16.1	1.68
8		*sqt-3* before SSH	22.8	1.68

(c)

Figure 18 Evaluation of the quality and efficiency of reverse subtracted cDNA library library (*let-418(ts)* cDNA as driver and N2 cDNA as tester)and confirmation of down–regulation of *sqt-3* in *let-418* ts by using real time qPCR

The melting curves were monitored using Rotor-gene software to ensure that only a single product was amplified. The upper left panel (a) shows the results of a real-time comparative quantitation report, the upper right panel (b) shows the *sqt-3* cDNA level normalized relatively to *sqt-3* Before SSH. The bottom panel (c) shows the Ct values and amplification efficiency. The final value of the *sqt-3* Before SSH was arbitrarily set to 1. PCR was performed on subtracted (*sqt-3* After SSH) or unsubtracted (*sqt-3* Before SSH) secondary PCR product with the *sqt-3* 5' and 3' primers.

sqt-3 After SSH: DNAs from the reverse subtracted cDNA library (N2 cDNA as "tester "and *let-418 ts* cDNA as "driver" for SSH);
sqt-3 Before SSH: DNAs from the unsubtracted cDNA library (N2 cDNA as "tester "and *let-418 ts* cDNA as "driver" for SSH).

Confirmation of some putative target genes using real time qRT-PCR

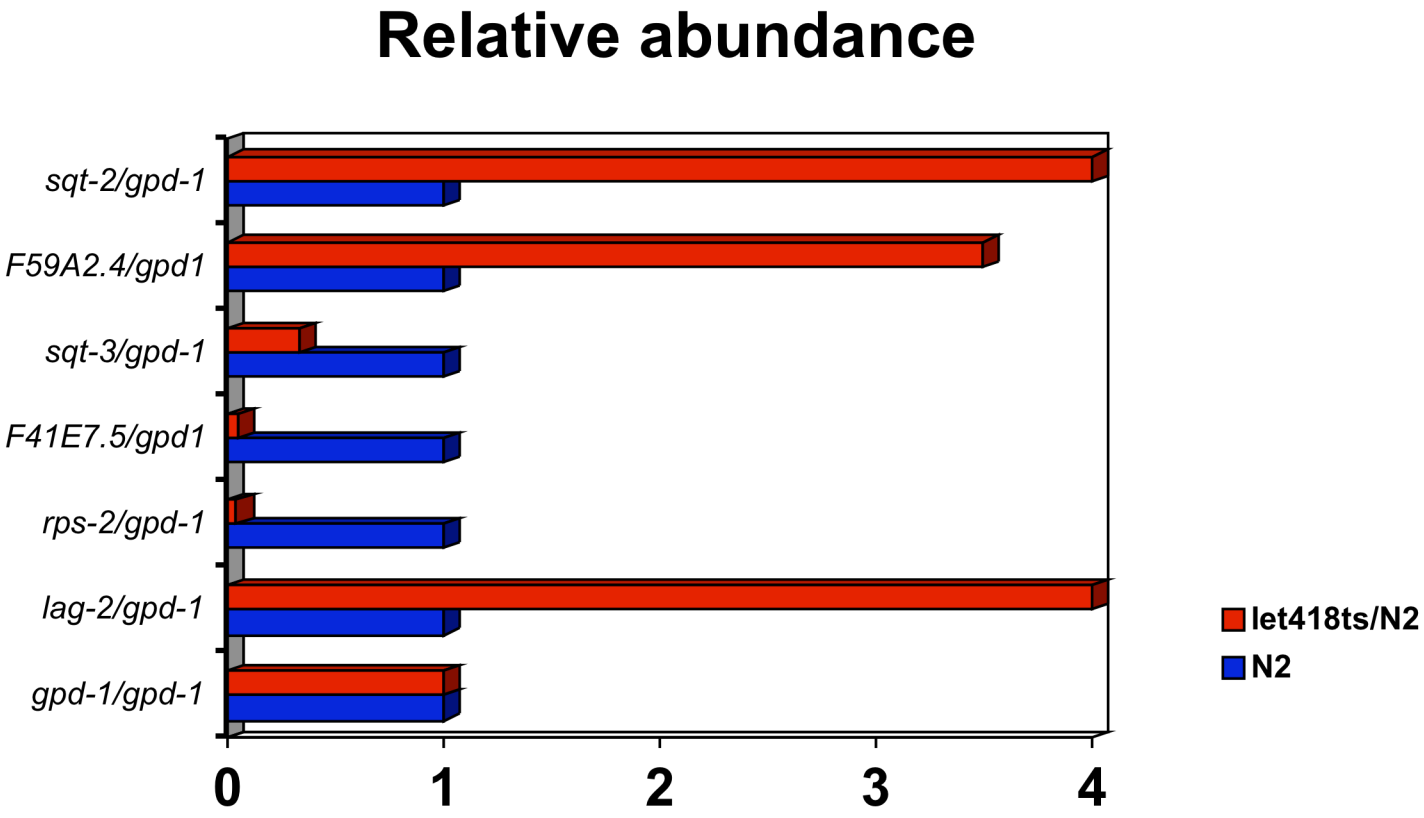
We performed real time qRT-PCR to confirm the dis-regulations of some putative targets in *let-418* mutant animals (Figure19, and Table 4). As a condidate, we have tested the *lag-2* cDNA has previously been found to be differentially expressed as a

direct target gene of LET-418/Mi-2 (Zhang *et al*., in preparation) was used as positive control. The preparation of 110 worms was exactly as described previously in SSH, Trizol Reagent was used for total RNA preparation. RNA was DNase-treated with DNA-free reagent in column. 2.5 % of cDNAs after reverse transcription of the isolated RNAs from both *let-418 (ts)* and control N2 were used for each SYBR Green dye real time quantitative PCR in 20 μl reaction with gene-specific primers. Reactions were run in duplicates or triplicates on the Rotor-gene 2000 Real-Time PCR machine. Relative fold changes in the expression patterns were calculated by using the $2^{-\Delta\Delta Ct}$ method. Primer sets were designed by using the PRIMER 3 software. The primer sequences are shown in Table 3.

Table 3 The sequences of primers for mRNAs

Name	Forward/reverse	sequences
rps-2	F	5' gctttcgttgctatcggaga3'
	R	5' tccgatcttgttaccccagt3'
sqt-3	F	5' caggaaagccaggaaagc3'
	R	5' ctcctggtggtcctggtg3'
sqt-2	F	5' ggaccacacctattccatcg3'
	R	5' catttccgtcatctccaggt3'
gpd-1	F	5'aaaggacacggttcaagtgg3'
	R	5' acaacgaaatcggctttgac3'
F59A2.4 *(clp-1)*	F	5' tggttatcatggagcccagt3'
	R	5' ccgtgacaagacaaaatcca3'
lag-2	F	5' ggcttcatcggtgaattctg3'
	R	5' gctgtctcatcggcttgaac3'
F41E7.5	F	5'gaccaccattatccgtggag3'
	R	5'atcctccatttcctccgaat3'

The QRT-PCR experiments confirmed that *lag-2*, *sqt-2* and *F59A2.4 (clp-1)* mRNA levels are up-regulated, whereas *rps-2, F41E7.5* and *sqt-3* mRNA levels are down–regulated in *let-418(ts)* mutants relative to N2 animals (Figure 19).



	gpd-1/gpd-1	lag-2/gpd-1	rps-2/gpd-1	F41E7.5/gpd1	sqt-3/gpd-1	F59A2.4/gpd1	sqt-2/gpd-1
let418ts/N2	1	4	0.04	0.05	0.333	3.5	4
N2	1	1	1	1	1	1	1

Figure 19 Regulations of *lag-2, sqt-2, clp-1, rps-2* and *F41E7.5* in *let-418 (ts)* animals determined by using real time qRT-PCR

The reactions were run in duplicates or triplicates on a Rotor-gene 2000 real time qPCR machine. To ensure that only a single product was amplified, the melting curves were monitored using Rotor-gene software. Based on the results of a real-time comparative quantitation report from Rotor-gene software, the *lag-2, sqt-2, sqt-3, F59A2.4 (clp-1), rps-2* and *F41E7.5* mRNA level were normalized relatively to the mRNA levels of *gpd-1*. The final value of the N2 was arbitrarily set to 1. The table at the bottom indicates the corresponding numbers.

Preliminary characterization of functions of these putative target genes using

RNAi by feeding

The putative functions of the candidate genes were categorized (Figure 20) and systematically studied by RNAi using the engineered RNAi T-vector pYZT. *C. elegans* worms were fed with HT115 (DE3) bacteria containing the pYZT plasmid with various cloned SSH cDNAs. The RNAi feeding procedure was done as described (Tabara et al., 1999). Adult worms were placed on bacteria expressing dsRNA, and the phenotype of their embryos, larvae (L1to L4) and adults was investigated: most of them had either no obvious phenotype or showed embryonic lethality. The results are presented in Table 4 ("phenotype")

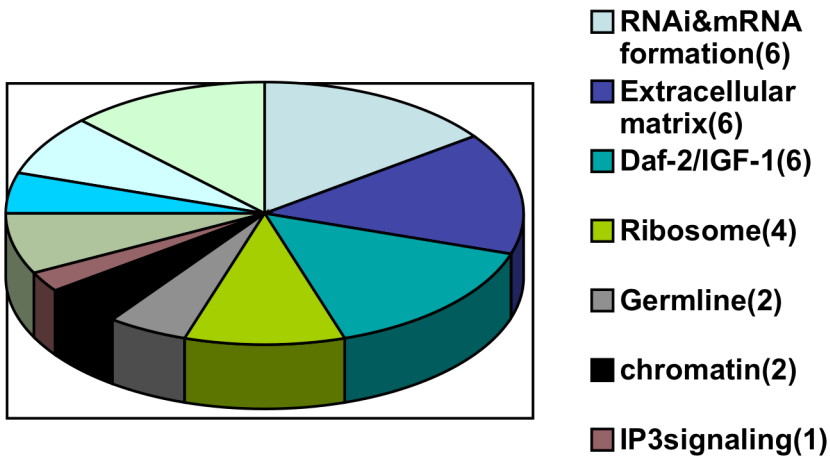


Figure 20 Summary of categories of putative target genes

Discussion

For the construction of our SSH cDNA libraries, we used mRNA isolated from WT and *let-418 (ts)* animals. During the optimization of the SMART cDNA amplification, we inserted a real time qPCR step to further ensure the quality of amplified cDNAs (Figures 8 -9). Finally, an aliquot of each library was cloned in the pYZT vector (Figure16a, b) and the positive clones were sequenced: 18 out of totally 24 clones

from the up-regulated subtracted cDNA library (*let-418(ts)* cDNA as tester, N2 cDNA as driver) had inserts (positive colonies; Figure16a). 20 out of totally 27 clones from down-regulated subtracted cDNA library (*let-418(ts)* cDNA as driver, N2 cDNA as tester) had inserts (positive colonies, Figure16b); the rest of the clones had either polyT stretches and could not to be sequenced or contained just orphans, i.e. sequences not contained in Wormbase. Finally, the inserts of some clones clearly targeted more than one gene (see Appendix) probably either due to overlapping PCR reactions during the PCR amplification step or to cloning artifacts. Only one of the putative targets was cloned independently twice, suggesting that the SSH libraries are not saturated. The analysis of the subtraction efficiency was performed using the gene *sqt-3* (Figure17-18). The results show that the quality and efficiency of subtracted cDNA libraries are good because a quantification of *sqt-3* cDNA before and after the subtraction shows a difference of 19 to 36 fold difference. Alltogether the data show that the quality of this work meets the requirements for efficient suppression subtractive hybridization.

The confirmation of putative targets was performed by real time RT-PCR (Figure19, and Table 4); the enrichments were measured by normalisation with the constitutively expressed gene *gpd-1* as internal control and relative fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method. Firstly, we confirmed that *lag-2* cDNAs, which by a previous candidate approach was found to be a differentially expressed direct target gene of LET-418/Mi-2 (Zhang et al., in preparation). We also confirmed that *sqt-2* and *clp-1* mRNA levels are up-regulated in *let-418(ts)* animals relative to N2 (wild type) worms.

Table 4 LET-418/Mi-2 putative targets and Associated phenotypes

Name	Description	Phenotype
RNA interference pathway, 3' end mRNA formation, cleavage & polyadenylation factor		
F36F2.3(Rbbp6) +	(Retinoblastoma binding protein 6) , E3 ubiquitin ligase ,RING finger essential conserved subunit of CPF (cleavage and polyadenylation factor), plays a role in 3' end formation of mRNA via the specific cleavage and polyadenylation of pre-mRNA, contains a putative RNA-binding zinc knuckle motif	L3 lethal (mutant)
F59A2.4 *(clp-1)* + ;confirmed by qRT-PCR	mRNA cleavage and polyadenylation factor I /IIA complex, subunit clp1; plays a role in 3' end formation of mRNA , required for soma transgenic silencing; presumably also for required for RNA interference	weak long (RNAi)
R09B3.3 -	mRNA cleavage and polyadenylation factor I complex, subunit RNA15 (RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain)	WT (RNAi)
C04F12.1 +	Translation initiation factor 2C (eIF-2C) and related proteins; required for RNA interference & stem cell renewal	WT (RNAi)
F41E7.5 -,confirmed by qRT-PCR	Nucleolar protein, component of the small subunit processome complex, required for processing of pre-18S rRNA; has similarity to mammalian fibrillarin	WT(RNAi)
F53B3.6 -	Splice Isoform 4 of U1 small nuclear ribonucleoprotein 70 kDa	WT (RNAi)
Extracellular matrix /Collagen		
F23H12.4(*sqt-3*) -confirmed by qRT-PCR	cuticular collagen ,Proline-rich, actin-associated protein involved in cytoskeletal organization and cytokinesis; related to mammalian Wiskott-Aldrich syndrome protein (WASP)-interacting protein (WIP) Yeast two- hybrid interactor of *sma-4*	left-handed roller(mutant) roller (RNAi)
C01B12.1(*sqt-2*) +,confirmed by qRT-PCR	Collagens (type IV and type XIII), affecting alae formation and body morphology such that mutants exhibit in slightly dumpy phenotype and the bodies of mutants are helically twisted. yeast RNA-binding protein that carries poly(A)+ mRNA from the nucleus into cytoplasm; phosphorylation by Sky1p in the cytoplasm may promote release of mRNAs	right-handed roller(mutant) roller (RNAi)
C09G5.6 *(bli-1)* +	Collagens (type IV and type XIII), required for proper strut formation within the unique medial layer of adult cuticle; bli-1 interacts genetically with other cuticular collagens such as bli-2 and rol-1, and may be processed for secretion by BLI-4, a Kex2 /subtilisin serine endoproteinase; consistent with its role in adult cuticle formation ; in yeast , Actin assembly factor, activates the Arp2/3 protein complex that nucleates branched actin filaments; localizes with the Arp2/3 complex to actin patches; homolog of the human Wiskott-Aldrich syndrome protein (WASP)	blistered,esp.head(mutant) bli (RNAi)
F41F3.3 +/-	cuticlin, keratin 10, Epstein-Barr nuclear antigen 1 (EBNA-1).	WT (RNAi)
Y41D4B.16 -	Membrane mucin MUC17, in yeast, ER membrane protein involved in the translocation of soluble secretory proteins & insertion of membrane proteins into the ER membrane; may also have a role in the stress response but has only partial functional overlap with WSC1-3	WT (RNAi)
K06A4.3 +	Actin regulatory proteins (gelsolin/villin family), Advillin (p92) Actin-binding protein DOC6)	WT (RNAi)

Daf-2/daf-16 IGF signaling pathway and metabolism

Y65B4BL.5 -	Long-chain acyl-CoA synthetases (AMP-forming)	WT (RNAi)
Y76A2B.3 +	Long-chain acyl-CoA synthetases (AMP-forming) puatative direct target of daf-16	WT (RNAi)
K10B3.8*(gpd-2)* +	glyceraldehyde-3-phosphate dehydrogenases (GAPDHs); by homology, GPD-2 is predicted to reversibly catalyze the oxidation and phosphorylation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate during glycolysis; in C. elegans, GPD-2 and GPD-3 constitute the major GAPDH isoenzymes and are expressed at highest levels during postembryonic development, confirmed target of daf-16 ; Yeast two- hybrid interactor of Daf-d *unc-119*	WT (RNAi)
F32H2.5 +	Animal-type fatty acid synthase and related proteins, Lipid transport and metabolism	WT (RNAi)
K10B2.2 +	Serine carboxypeptidases (lysosomal cathepsin A)	WT (RNAi) .
B0280.3 -	Ribose 5-phosphate isomerase ; Ribose-5-phosphate ketol -isomerase, catalyzes the interconversion of ribose 5-phosphate and ribulose 5-phosphate in the pentose phosphate pathway; participates in pyridoxine biosynthesis	Unc/ WT (RNAi)

Ribosomal proteins

C49H3.11*(rps-2)* -confirmed by qRT-PCR	40S ribosomal protein S2/30S ribosomal protein S5 essential for control of translational accuracy; has similarity to E. coli S5 . and rat S2 ribosomal proteins , putative target of *daf-2*	Emb (RNAi)
F42C5.8*(rps-8)* -	small ribosomal subunit S8 protein; by homology, RPS-8 is predicted to function in protein biosynthesis; in C. elegans, RPS-8 activity is required for germline development and the . overall health of the animal, putative target of *daf-2*	Emb/Lva (RNAi)
rpl-13 +	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl13Ap; not essential for viability; has .similarity to rat L13 ribosomal protein , putative target of *daf-2*	Emb(RNAi)
Y37E3.8 +	60s ribosomal protein L15/L27 ,Ribosomal protein L29 of the large (60S) ribosomal subunit, has similarity to E. coli L15 and rat L27a ribosomal proteins; may have peptidyl transferase activity; . can mutate to cycloheximide resistance	Emb(RNAi)

Germline specific gene

F36F2.3 (above)+ F31E3.5*(glp-3/eft-3)* -	translation elongation factor 1-alpha homolog EF-1 alpha/Tu that is required for embryonic viability, fertility, germline .maintenance , putative target of *daf-2*	Bmd/Emb (RNAi) sterile, no germ line (mutant)

Chromatin modifier complex

C08B11.6 +	Actin-related protein - Arp6p Nuclear actin-related protein involved in chromatin remodeling, component of chromatin-remodeling enzyme .complexes, probably in SWR1 HAT complex	Bmd/Emb (RNAi)
C01A2.4 +	Predicted assembly/vacuolar sorting protein, snf7 domain, .Charged multivesicular body protein 2b (Chromatin-modifying proteins2b)	WT (RNAi)

IP3 signalling

C01B10.3 +	Inositol polyphosphate 5-phosphatase, type I,Type I inositol-1,4,5 .-trisphosphate 5-phosphatase (EC 3.1.3.56)s(5PTase)	WT (RNAi)
Ubiquitination		
C32E8.11 +	N-end rule pathway, recognition component UBR1, Ubiquitin-protein ligase (E3) that interacts with Rad6p/Ubc2p to ubiquitinate substrates of the N-end rule pathway; binds to the Rpn2p, Rpt1p, .and Rpt6p proteins of the 19S particle of the 26S proteasome	WT (RNAi)
F36F2.3 (above)		
ari-1 +	E3 ubiquitin ligase ,in mouse, Ariadne-1 protein homolog (ARI-1) (Ubiquitin-conjugating enzyme E2-sbinding protein 1) (UbcH7 .-binding protein) (UbcM4-interacting proteins77)	WT (RNAi)
Apoptosis		
Y54G2A.18 +	B-cell receptor-associated protein and related proteins , Endoplasmic .reticulum transmembrane protein, homolog of human BAP31 protein	WT (RNAi)
F22A3.6 -	.Lysozyme (EC 3.2.1.17) (1,4-beta-N-acetylmuramidase) (Fragments).	Emb/WT (RNAi)
Secretory pathway		
K06A5.4 -	Essential protein involved in intracellular protein transport, coiled-coil protein necessary for transport from ER to Golgi; required for assembly .of the ER-to-Golgi SNARE complex	Emb/Ste (RNAi)
cyn-6 +	Peptidyl-prolyl cis-trans isomerase ,cyclophilin most similar to human secreted cyclophilin type B isoforms that is functional when expressed in E. coli; it is expressed in the intestine. Peptidyl-prolyl cis-trans isomerase (cyclophilin), catalyzes the cis-trans isomerization of peptide bonds N . -terminal to proline residues; has a potential role in secretory pathway	WT (RNAi)
B0261.6 +	Partially redundant sensor-transducer of the stress-activated PKC1-MPK1 signaling pathway involved in maintenance of cell wall integrity and recovery from heat shock; secretory pathway Wsc2p is required for the arrest of secretion response; interestingly localizes the intron but opposite direction of transcription of *ceTor/let-363*	WT (RNAi)
Others		
F40F8.5 +	RNA polymerase I enhancer binding protein; DNA binding protein which binds to genes transcribed by both RNA polymerase I and RNA polymerase II; required for termination of RNA polymerase I transcription	WT (RNAi)
wrt-10 +	.WaRThog (hedgehog-like family)	WT (RNAi).
hmp-1 +	Alpha-catenin	Emb/WT (RNAi)
nlp-33 +	Splice Isoform Long of TATA-binding protein associated factor 2N, .RNA-binding region RNP-1 (RNA recognition motif)	WT (RNAi).
mlc-1 +	Myosin regulatory light chain, EF-Hand protein superfamily [muscle regulatory myosin light chain that is nearly identical to MLC-2 and functions redundantly with MLC-2 in the body-wall and in pharyngeal muscle to affect L1 larval viability, elongation, and pharyngeal and body -wall muscle development, and also affects locomotion and growth; expressed in the body-wall muscles, pharyngeal muscles, and vulval muscles. calmodulin; Ca++ binding protein that regulates Ca++ independent processes (mitosis, bud growth, actin organization, endocytosis, etc.) and Ca++ dependent processes (stress-activated pathways), targets include Nuf1p, . Myo2p and calcineurin	Unc, Lva (RNAi)

Furthermore *rps-2, F41E7.5* and *sqt-3* mRNA levels are down-regulated in *let-418(ts*) animals relative to N2 (wild type) worms. Among the other positive clones (see Table 4), C08B11.6 has already been identified as a synMuv suppressor gene in a RNAi screen (Cui et al., 2006). C08B11.6 encodes a protein homologous to a component of the yeast SWR1 complex (Cui et al., 2006). *C08B11.6* **(**RNAi), like *mes-4*, rescued the L1/L2 larval lethality, the weak germline desilencing and partial rescue of the ectopic expression of *lag-2* in the intestine of *mep-1 (q660)* mutant animals (Cui et al., 2006). The protein MEP-1 was previously shown to form a complex with LET-418 (Unhavaithaya et al. 2002). The 3 putative targets of LET-418 (F36F2.3 (Rbbp6), F59A2.4 (*clp-1*) and R09B3.3) are homologues of essential components of transcription machinery in mammals and yeast (Gross S and Moore C, 2001). Furthermore, *clp-1* was previously found in a genome-wide suppressor screen for synMuv genes in *C.elegans* (Zhong W and Sternberg P, 2006; Cui et al., personal communication). Interestingly, *sqt-1, sqt-2* and *sqt-3* function genetically in a common pathway. *sqt-1* can suppress the Lon defects in *dbl-1* or *lon-3* mutants. *dbl-1* and *lon-3* are two important genes of the *C.elegans* TGFβ pathways (Nystrom J et al., 2002). Therefore we speculate that LET-418/Mi-2 may involve the TGFβ pathways by controlling the transcription of its targets, at least *clp-1*, *sqt-2* and *sqt-3*.

According to their functions, we can divide the putative LET-418 target genes in several groups (Table 4):

I) Genes involved in RNA interference, in 3' end mRNA formation and cleavage and polyadenylation of mRNAs, such as F36F2.3 (Rbbp6), F59A2.4 (*clp-1*), R09B3.3, C04F12.1, F41E7.5 and F53B3.6.

II) Genes encoding proteins of the ECM or collagens, such as F23H12.4 (*sqt-3*), C01B12.1 (*sqt-2),* C09G5.6 (*bli-1*), F41F3.3, Y41D4B.16 and K06A4.3.

III) Genes involved in the DAF-2/IGF-1 signaling pathway and in metabolism, such as Y65B4BL.5, Y76A2B.3, K10B3.8 (*gpd-2*), F32H2.5, K10B2.2 and B0280.3.

IV) Ribosomal proteins, such as C49H3.11 (*rps-2*), F42C5.8 (*rps-8*), *rpl-13* and Y37E3.8.

V) Germline -specific genes, such as F36F2.3; F31E3.5 (*glp-3/eft-3*).

VI) Chromatin modifiers, such as C08B11.6, C01A2.4.

VII) Genes involved in IP3 signalling, such as C01B10.3.

VIII) Genes for ubiquitination and protein degradation, such as C32E8.11, F36F2.3 and *ari-1*.

IX) Genes involved in apoptosis, such as Y54G2A.18 and F22A3.6.

X) Genes involved in the secretory pathway, such as K06A5.3, *cyn-6* and B0261.6.

XI) Other genes, such as F40F8.5, *wrt-10, hmp-, nlp-33*, *mlc-1*, etc.

So far no data are available from DNA microarray studies on LET-418/NuRD targets. We do not know if the results of our approach differ markedly from those of DNA microarray studies. A recent DNA microarray study on targets of HDA-1 during embryogenesis also found many collagen genes and genes related to the extracellular matrix (Whethwine et al., 2005). However, the majority of our putative LET-418 target genes are distinct from the HDA-1 target genes. This may be explained by the fact that HDA-1 is a member of different complexes; whereas LET-418 is only involved in the NuRD complex. A major advantage of SSH compared to microarray analysis is that a cDNA normalization step can equalize the abundances of downstream targets of interest. Therefore, SSH may detect also some low abundantly expressed genes that could remain undetected in microarray analysis. Many putative candidates are related to metabolisms (Table 4), some of which are even proven to act downstream of *daf-2* or *daf-16* (Murphy et al., 2003, Halaschek-Wiener et al., 2005), although further confirmation are required, our results are potentially useful for understanding human aging as well as other processes.

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Competing financial interests: declared none.

Genetics

Influence of TOR kinase on lifespan in *C. elegans*

The group of enzymes known as TOR (for 'target of rapamycin') kinases regulates cell growth and proliferation in response to nutrients and hormone-dependent mitogenic signals[1,2]. Here we show that TOR deficiency in the nematode *Caenorhabditis elegans* more than doubles its natural lifespan. This new function for TOR signalling in ageing control may represent a link between nutrition, metabolism and longevity.

In *C. elegans*, the absence of LET-363/TOR activity causes developmental arrest at the L3 larval stage[3]. We examined nematodes bred as *let-363/CeTor* genetic null mutants and nematodes that had been depleted of TOR by using RNA interference to block *let-363* expression (termed *let-363*-(RNAi) worms), and found that these animals had a strikingly extended mean lifespan (Fig. 1a, squares and triangles, respectively). At 25.5 °C, the mean lifetime was 25 days in *let-363* mutants compared with 10 days in wild-type animals. This is all the more intriguing in light of the fact that TOR-deficient worms existed as arrested L3 larvae. In comparison, L3 larval arrest induced by starvation persisted for only 14 days on average in wild-type animals (Fig. 1a, diamonds).

Strong inhibition of mitochondrial respiration also arrests development at the L3 stage, whereas weaker inhibition permits growth to adulthood and extends adult lifespan, but only if it occurs during larval development[4]. In contrast, treatment with *let-363* double-stranded RNA starting from the first day of adulthood lengthens lifespan to a comparable extent when RNAi treatment is initiated at hatching (Fig. 1a, open triangles). This indicates that TOR has a role in ageing control during adulthood and that the long-lived phenotype of *let-363*-(RNAi) adults cannot be explained by reduced mitochondrial activity.

Longevity in *C. elegans* is controlled hormonally by a conserved signalling pathway that involves insulin and insulin-like growth factor (IGF)[5,6]. Mutants with reduced DAF-2/IGF signalling activity live twice as long as the wild type[5,6]. The DAF-2/IGF cascade also acts during adulthood to influence ageing[7]. The remarkable similarity in the developmental stage at which ageing rate is affected, and our finding that the extended lifespan of *daf-2(e1370)* mutants is not increased further by treatment with *let-363* RNAi (results not shown) — as it is with RNAi blocking expression of respiratory-chain components[4] — raise the possibility that TOR and the DAF-2/IGF pathway are related in controlling lifespan.

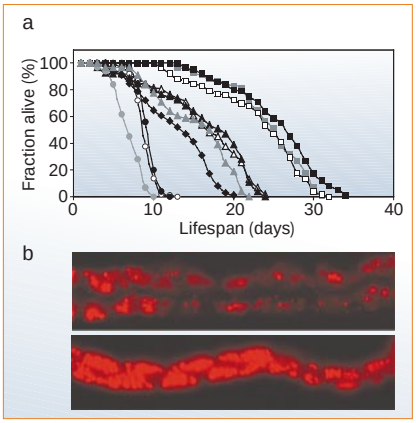

Figure 1 TOR deficiency in the nematode *Caenorhabditis elegans*. **a,** Lifespan of TOR-deficient worms compared with the wild type at 25.5 °C: wild type (filled circles); *daf-16(mg50)* (open circles); *dpy-5(e61) unc-13(e450)* double mutant (shaded circles); TOR-deficient triple mutants *let-363(h114) dpy-5(e61) unc-13(e450)* (open squares), *let-363(h111) dpy-5(e61) unc-13(e450)* (filled squares) and *let-363(h131) dpy-5(e61) unc-13(e450)* (shaded squares); *let-363*-RNAi-treated worms from hatching (open triangles) or from the first day of adulthood (shaded triangles); *let-363*-RNAi-treated *daf-16(mg50)* worms (filled triangles); starving-arrested wild-type L3 larvae (filled diamonds). Disruption of TOR by RNAi (triangles) seems to be incomplete, as lifespan is not extended as much as in *let-363* mutants (squares). **b,** Nile Red staining of lipid droplets in a wild-type L3 larva (top) and an L3 larva arrested by *let-363*-(RNAi) treatment (bottom). Images were obtained with the same exposure time.

This idea is compatible with results indicating that the insulin/IGF cascade regulates protein synthesis and cell growth in mammals and *Drosophila* through the activity of nutrient-sensing TOR (reviewed in refs 1, 2, 8). We have also noted that *let-363*-(RNAi) animals share certain features of the pleiotropic Daf-2(−) phenotype, such as lipid accumulation mainly in intestinal cells[9] (Fig. 1b), as well as reduced fertility[10] (mean brood sizes: *let-363*-(RNAi) adults, 68 ± 6.4; wild type, 191 ± 14.5) and reduced viability[10] (embryonic/early larval arrest: *let-363*-(RNAi), 40.3%; wild type, 5.4%).

Strong mutations in DAF-2/IGF signalling cause a long-lived phenotype, together with a state of developmental diapause known as dauer that is triggered by starvation and crowding in the wild type[11]. According to our results (not shown), *let-363(h111)* animals bearing the thermosensitive *daf-2(e1370ts)* mutation were able to form dauers at the restrictive temperature. Furthermore, *let-363*-(RNAi) enhanced dauer formation in *daf-2(e1370)* animals. At 20 °C, only 4.6% (29 out of 630) of *daf-2(e1370)* mutants entered into the dauer stage, compared with 17.9% (146 out of 817) of *daf-2(e1370); let-363*-(RNAi) animals (results not shown). This indicates a genetic interaction between *let-363/CeTor* and *daf-2*. These results show that in *C. elegans* the TOR and DAF-2/IGF signalling pathways could be related in controlling ageing, metabolism and reproductive growth.

Lifespan extension in *daf-2(e1370)* mutants requires the activity of the forkhead transcription factor DAF-16 (refs 5, 6). Mutations in *daf-16*, however, do not suppress the long-lived phenotype of *let-363*-(RNAi) worms (Fig. 1a, filled triangles), indicating that TOR may be acting downstream or independently of DAF-16, and that it is interacting with the insulin endocrine system. Although the detailed signalling connections require clarification, our findings point to TOR as a possible mediator of lifespan regulation by insulin signalling and nutrient sensing.

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Competing financial interests: declared none.

Metabolism

Ecology shapes bird bioenergetics

The basal rate of metabolism of birds and mammals is the lowest rate that is compatible with endothermic temperature regulation, balancing the heat generated with the heat lost by the product of thermal conductance and the temperature differential with the environment[1]. Here I measure the bioenergetics of 13 species and 9 genera of birds of paradise (Paradisaeidae) and

Appendix

Step by step protocol for SMART and SSH PCR

Developed from (Diatchenko L, Methods Enzymol. 1999; and Clontech Inc.)

SMART PCR

• x μl **SMART II A Oligonucleotide** (12 μM)
5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'
Rsa I
• x μl **3' SMART CDS Primer II A** (12 μM)
5'-AAGCAGTGGTATCAACGCAGAGTACT(30)MN-3'
(N = A, C, G, or T; M = A, G, or C) *Rsa* I
• x μl **PowerScriptTM Reverse Transcriptase**
• x μl **5X First-Strand Buffer**
250 mM Tris-HCl (pH 8.3)
375 mM KCl
30 mM MgCl2
• x μl **5' PCR Primer II A** (12 μM)
5'-AAGCAGTGGTATCAACGCAGAGT-3'
•x μl **dNTP Mix** (10 mM of each dNTP)
• x μl **Dithiothreitol** (DTT; 100 mM)
• x ml **Deionized H2O**
β-mercaptoethanol (Sigma Cat.# M6250)
• RNase Inhibitor (20 U/μl)
• DNA size markers (1-kb DNA ladder)
• 50X TBE or
TAE electrophoresis buffer
242.0 g Tris base
57.1 ml glacial acetic acid
37.2 g Na2EDTA•2H2O
Add H2O to 1 L.

A. Isolation of total RNAs

B. First-Strand cDNA Synthesis

1. For each sample RNA, combine the following reagents in a sterile 0.5-ml reaction tube:
1–50 μl RNA sample*
(2–1,000 ng of total RNA)
7 μl 3' SMART CDS Primer II A (12 μM)
7 μl SMART II A Oligonucleotide (12 μM)
x μl Deionized H2O
64 μl Total volume
* start with ≥10 ng of total RNA.
2. Mix contents and spin the tube briefly in a microcentrifuge.
3. Incubate the tube at 65°C in a hot-lid thermal cycler for 2 min, then reduce the temperature to 42°C.

4. Add the following to each reaction tube:
20 µl 5X First-Strand Buffer
2 µl DTT (100 mM)
10 µl 50X dNTP (10 mM)
5 µl RNase Inhibitor (20 U/ µl)
5 µl PowerScript Reverse Transcriptase
42 µl Total added per reaction
5. Gently pipet up and down to mix, then spin the tubes briefly in a microcentrifuge.
6. Incubate the tubes at 42°C for 90 min* in a hot-lid thermal cycler.
7. Add 2 µl of 0.5 M EDTA to stop the reaction. If necessary, cDNA can be stored at -20°C until ready to proceed with QIAgen column chromatography

C.cDNA Amplification by LD PCR

1. Preheat the PCR thermal cycler to 95°C.
2. For each reaction, aliquot the appropriate volume (5 µl -10 µl) of each first-strand cDNA into a labeled 0.5-ml reaction tube. If necessary, add deionized H2O to adjust the volume to 80 µl.
3. Prepare a Master Mix for all reaction tubes, plus one additional tube.
Combine the following components in the order shown:
per rxn (3 x)
4 µl Deionized H2O
10 µl 10X Advantage 2 PCR Buffer
2 µl 50X dNTP (10 mM)
2 µl 5' PCR Primer II A (12 µM)
2 µl 50X Advantage 2 Polymerase Mix
20 µl Total volume
4. Mix well by vortexing and spin the tube briefly in a microcentrifuge.
5. Aliquot 21 µl of the PCR Master Mix into each tube from Step 2.
6. Cap the tube, and place it in the preheated thermal cycler. If necessary, overlay the reaction mixture with two drops of mineral oil.
7. Commence thermal cycling using the following:
• 95°C 1 min • 95°C 1 min
• x cycles:
95°C 15 sec 65°C 30 sec 68°C 6 min
Subject all tubes to 17 cycles. Then, divide the PCR reaction mix between the "Experimental" and "Optimization" tubes, using the Optimization tube for each reaction to determine the optimal number of PCR cycles, as described in Step 8 (below). Store the Experimental tubes at 4°C.
8. Subject each tube to 17 cycles, then pause the program. Transfer 30 µl from each tube to a second 0.5-ml reaction tube labeled "Optimization".
Store the "Experimental" tubes at 4°C. Using the Tester PCR tube,
determine the optimal number of PCR cycles
a. Transfer 5 µl from the 17-cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
b. Return the Optimization tubes to the thermal cycler. Run one additional cycle (for a total of 18) with the remaining 25 µl of PCR mixture.
c. Transfer 5 µl from the 18-cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).

d. Run three additional cycles (for a total of 21) with the remaining 20 μl of PCR mixture.
e. Transfer 5 μl from the 21-cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
f. Run three additional cycles (for a total of 24) with the remaining 15 μl of PCR mixture.
g. Transfer 5 μl from the 24-cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
h. Run three additional cycles (for a total of 27) with the remaining 10 μl of PCR mixture.
i. Transfer 5 μl from the 27-cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
j. Run three additional cycles (for a total of 30) with the remaining 5 μl of PCR mixture.
9. Electrophorese each 5-μl aliquot of the PCR reaction alongside 0.1 μg of 1-kb DNA size markers on a 1.2% agarose/EtBr gel in 1X TBE /TAE buffer. Determine the optimal number of cycles required for each experimental and control sample (see last SSH chapter, Figure 8). Optional: the real time PCR using *gpd-1* primer pairs could further confirm the optimization of number of cycles (see last SSH chapter , Figure 8).
10. Retrieve the 15-cycle Experimental PCR tubes from 4°C, return them to the thermal cycler, and subject them to additional cycles, if necessary, until reach the optimal number.
11. When the cycling is completed, analyze a 5-μl sample of each PCR product alongside 0.1 μg of 1-kb DNA size markers on a 1.2% agarose/ EtBr gel in 1X TBE or TAE buffer.

D. SMART cDNAs purification using QIAGEN PCR purification kit

To ensure to have sufficient cDNA for application, to estimate the yield of SMART cDNA by UV spectrophotometry (final concentration >300 ng/ μl)

SSH PCR

Material and reagents

Endonuclease digestion
• x μl 10X *Rsa* I Restriction Buffer
100 mM Bis Tris Propane-HCl (pH 7.0)
100 mM MgCl2
1 mM DTT
• x μl *Rsa* I (10 units/μl)

Adaptor ligation
• x μl T4 DNA Ligase (400 units/μl; contains 3 mM ATP)
• x μl 5X DNA Ligation Buffer
250 mM Tris-HCl (pH 7.8)
50 mM MgCl2
10 mM DTT
0.25 mg/ml BSA
• x μl Adaptor 1 (10 μM)
• x μl Adaptor 2 (10μM)

Hybridization
• 200 μl 4X Hybridization Buffer
• 1.4 ml Dilution buffer (pH 8.3)
20 mM HEPES (pH 6.6)
20 mM NaCl
0.2 mM EDTA (pH 8.0)

PCR amplification
• x μl PCR Primer 1 (10 μM)
• x μl Nested PCR primer 1 (10 μM)
• x μl Nested PCR primer 2 (10 μM)
• x μl *gpd-1* 5' Primer (10 μM)
• x μl *gpd*-1 3' Primer (10 μM)

General reagents
• x μl dNTP Mix (10 mM each dATP, dCTP, dGTP, dTTP)
• x μl 20X EDTA/Glycogen Mix (0.2 M EDTA; 1 mg/ml glycogen)
• x μl NH4OAc (4 M)
• x ml Sterile H2O

• ***Hae* III digest of bacteriophage *ΦX174*** (#6310-1,-2)
DNA size markers for agarose gels
• **0.5-ml PCR reaction tubes**.
• **80% Ethanol & 96% Ethanol**

• **50X PCR enzyme mix**
Advantage® cDNA Polymerase Mix (#8417-1; also

Also provided in the Advantage cDNA PCR Kit [#K1905-1,-y]).

• 10X PCR buffer
Use the 10X reaction buffer supplied with DNA polymerase or mix (included with Advantage cDNA Polymerase Mix and Advantage cDNA PCR Kit).
• dNTP Mix for PCR (10 mM each dATP, dCTP, dGTP, dTTP)
• 50X TBE electrophoresis buffer
or TAE electrophoresis buffer
242 g Tris base
57.1 ml Glacial acetic acid
37.2 g Na2EDTA•2H2O
Add H2O to 1 L. For 1X TAE buffer, dilute 50X stock solution 1:49 with H2O.

E. *Rsa* I Digestion

1. Add the following reagents into the tube:
ds cDNA 43.5 μl
10X *Rsa* I Restriction Buffer 5.0 μl
Rsa I (10 units/μl) 1.5 μl
2. Mix by vortexing and centrifuging briefly.
3. Incubate at 37°C for 1.5 hr.
4. Set aside 5 μl of the digest mixture to analyze the efficiency of *Rsa* I digestion. Check *Rsa* I-digested cDNA from Step E.4 using agarose/EtBr gel electrophoresis
5.QIAgen PCR purification kit, *Rsa* I digested cDNAs dissolve in 5.5 -10 μl ddH2O. These samples of *Rsa* I digested cDNA will serve as experimental driver cDNA.

F. Adaptor Ligation

To perform subtractions in both directions, to prepare tester cDNA corresponding to each of poly A+ RNA samples. To perform a positive control mock subtraction. In step 2 (below), prepare tester cDNA for this control subtraction by mixing the worm cDNA with ΦX174/*Hae* III DNA and diver cDNA using the worm cDNA only. Three adaptor ligations must be performed for each experimental tester cDNA, as well as the control mock tester cDNA. Each cDNA is aliquotted into two separate tubes: one aliquot is ligated with Adaptor 1 (Tester 1-1, 2-1, and 3-1), and the second is ligated with Adaptor 2 (Tester 1-2, 2-2, and 3-2). After the ligation reactions are set up, portions of each tester tube are combined so that the cDNA is ligated with both adaptors (Unsubtracted tester control 1-c, 2-c, and 3-c).

Adaptors will not be ligated to the driver cDNA.

1. Dilute 1 μl of each *Rsa* I-digested experimental cDNA (Step E.5) with 5 μl of sterile H2O.
2.Prepare a mock positive control tester cDNA
a. Briefly centrifuge the tube containing Control DNA (*Hae III*-digest of φX174 / *Hae* III DNA [3 ng/ μl]).
b. Dilute 2 μ l of the Control DNA with 38 μl l of sterile H2O (to 150 ng/ml).
c. Mix 1 μ l of control worm cDNA (or gDNA) (Step E.5) with 5 μl l of the diluted φX174/ *Hae* III Control DNA (150 ng/ml).

Prepare your adaptor-ligated tester cDNA:

3. Prepare a ligation Master Mix by combining the following reagents in a 0.5-ml microcentrifuge tube. To ensure that you have sufficient Master Mix, prepare enough for all ligations plus one additional reaction.
per rxn
Sterile H2O 3μl
5X Ligation Buffer 2 μl (NEB)
T4 DNA Ligase (400 units/μl) 1 μl (NEB)
Note: The ATP required for ligation is in the T4 DNA Ligase (3 mM initial, 300 μM final).
4. For each experimental tester cDNA, combine the reagents in Table I in the order shown in 0.5-ml microcentrifuge tubes. Pipet mixture up and down to mix thoroughly.

Table I Setting up the ligation reactions

Component	Tube #: 1 Tester 1-1* (μl)	2 Tester 1-2* (μl)
Diluted tester cDNA	2	2
Adaptor 1 (10 μM)	2	–
Adaptor 2R (10 μM)	–	2
Master Mix	6	6
Final volume	10	10

* Use the same setup for Tester 2-1 and 2-2, 3-1 and 3-2.

5. In a fresh microcentrifuge tube, mix 2 μl of Tester 1-1 and 2 μl of Tester 1-2. After ligation is complete, this will be Unsubtracted tester mock
control 1-c. Do the same for each additional experimental tester cDNA. After ligation, approximately 1/3 of the cDNA molecules in each Unsubtracted tester control tube will bear two different adaptors.
6. Centrifuge tubes briefly, and incubate at 16°C overnight.
7. Stop ligation reaction by adding 1 μl of EDTA/Glycogen Mix.
8. Heat samples at 72°C for 5 min to inactivate the ligase.
9. Briefly centrifuge the tubes. Preparation of experimental Adaptor-Ligated Tester cDNAs and Unsubtracted tester controls is now complete.
10. Remove 1 μl from each Unsubtracted tester control (1-c, 2-c, 3-c) and
dilute into 1 ml of H2O. These samples will be used for PCR (Section.I).
11. Store samples at –20°C.
Perform the ligation efficiency analysis (see last SSH chapter, Figure 9)

G. First Hybridization

Note: Before begin the hybridization, warm the 4X Hybridization buffer to room temperature for at least 15–20 min.

1. For each of the experimental subtractions, combine the reagents in Table II (below) in 0.5-ml tubes in the order shown.
2. Overlay samples with one drop of mineral oil and centrifuge briefly.
3. Incubate samples in a thermal cycler at 98°C for 1.5 min.
4. Incubate samples at 68°C for 8 hr, then proceed **immediately** to

Section H.

Table II Setting up of the first hybridization

Component	Hybridization sample 1(μl)	Hybridiazion sample 2(μl)
***Rsa I*-digested driver cDNA(E.5)**	**1.5**	**1.5**
Adaptor 1-ligated tester 1-1*	**1.5**	**-**
Adaptor 2R-ligated tester 1-2*	**-**	**1.5**
4X Hybridization Buffer	**1.0**	**1.0**
Final volume	**4.0**	**4.0**

***use the same setup for tester 2-1 and 2-2,3-1 and 3-2**

H. Second Hybridization

1. Add the following reagents into a sterile tube:
Driver cDNA (Step E.5) 1 ul
4X Hybridization Buffer 1 ul
Sterile H2O 2ul
2. Place 1 ul of this mixture in a 0.5-ml microcentrifuge tube and overlay it with 1 drop of mineral oil.
3. Incubate in a thermal cycler at 98°C for 1.5 min.
4. Remove the tube of freshly denatured driver from the thermal cycler.
a. Set a micropipettor at 15 ul.
b. Touch the pipette tip to sample interface of the tube containing hybridization sample 2.
c. Carefully draw the entire sample partway into the pipette tip.
d. Remove the pipette tip from the tube, and draw a small amount of air into the tip, creating a slight air space below the droplet of sample.
e. Repeat steps b–d with the tube containing the freshly denatured driver. The pipette tip should now contain both samples separated by a small pocket of air.
f. Transfer the entire mixture to the tube containing hybridization sample 1.
g. Mix by pipetting up and down.
5. Briefly centrifuge the tube.
6. Incubate reaction at 68°C overnight.
7. Add 200 μl of dilution buffer to the tube and mix by pipetting.
8. Heat in a thermal cycler at 68°C for 7 min.
9. Store at –20°C.

I. PCR Amplification

A minimum of 6 PCR reactions are recommended: (1) forward-subtracted experimental cDNA, (2) the unsubtracted tester control (1-c), (3) reverse-subtracted experimental cDNA, (4) the unsubtracted tester control for the reverse subtraction (2-c), (5) mock subtracted experimental cDNA, (6) the unsubtracted mock tester control (3-c), using digested ΦX174 DNA/*Hae*III as a positive control when run–gel. To perform a standard PCR control to ensure that your enzyme is performing efficiently (see last SSH chapter, Figure 10 a,b).

1. Prepare the PCR templates:

a. Aliquot 1 µl of each diluted cDNA (i.e., each subtracted sample from Step H.9 and the corresponding diluted Unsubtracted tester control from Step F.10) into an appropriately labeled tube.
b. Aliquot 1 µl of the PCR control subtracted cDNA into an appropriately labeled tube.
2. Prepare a Master Mix for all of the primary PCR tubes plus one additional tube. For each reaction planned, combine the reagents in

Table III Preparation of Primary PCR master mix in the order shown:

Reagent	Amount per reaction (µl)	For a 7-rxn Experiment (µl)*
Sterile H_2O	19.5	156.0
10X PCR reaction buffer	2.5	20.0
dNTP Mix (10 mM)	0.5	4.0
PCR Primer 1 (10 µM)	1.0	8.0
50X Advantage cDNA Polymerase Mix	0.5	4.0
Total volume	24.0	192.0

* For each additional experimental cDNA, prepare Master Mix for one additional reaction.

3. Mix well by vortexing, and briefly centrifuge the tube.
4. Aliquot 24 µl of Master Mix into each of the reaction tubes prepared in step 1.
5. Overlay with 50 µl of mineral oil.
6. Incubate the reaction mix in a thermal cycler at 75°C for 5 min to extend the adaptors. (Do not remove the samples from the thermal cycler.)
7. Commence thermal cycling using the following:
• 95°C 1 min • 95°C 1 min
• 27 cycles:
95°C 20 sec 66°C 30 sec 72°C 2 min
8. Analyze 8 µl from each tube on a 2.0% agarose/EtBr gel run in 1X TBE/TAE buffer. Alternatively, set these 8-µl aliquots aside and run them on the same gel used to analyze the secondary PCR products (step 16).
9. Dilute 3 µl of each primary PCR mixture in 27 µl of H2O.
10. Aliquot 1 µl of each diluted primary PCR product mixture from Step 9 into an appropriately labeled tube.
11. Prepare Master Mix for the secondary PCRs plus one additional reaction by combining the reagents in Table IV in the order shown:

Table IV Preparation of secondary PCR master mix

Reagent	Amount per reaction (µl)	For a 7-Rxn Experiment (µl)*
Sterile H_2O	18.5	148.0
10X PCR reaction buffer	2.5	20.0
Nested PCR primer 1 (10 µM)	1.0	8.0
Nested PCR primer 2R (10 µM)	1.0	8.0
dNTP Mix (10 mM)	0.5	4.0
50X Advantage cDNA Polymerase Mix	0.5	4.0
Total volume	24.0	192.0

* For each additional experimental cDNA, prepare Master Mix for one additional reaction.

12. Mix well by vortexing, and briefly centrifuge the tube.
13. Aliquot 24 µl of Master Mix into each reaction tube from step 10.

14. Overlay with 1 drop of mineral oil.
7. Commence thermal cycling using the following:
• 95°C 1 min • 95°C 1 min
• 12 cycles:
95°C 20 sec 68°C 30 sec 72°C 2 min
16. Analyze 8 µl from each reaction on a 2.0% agarose/EtBr gel run in 1X TBE/TAE buffer.
17. Store reaction products at –20°C.

Step by step protocol for blue-white selection RNAi T-vector cloning

Transformation protocol 1

1. Gene X one-tube PCR/ RT-PCR

0.5-1 hours at last elongation step 72°C, more A overhangs

2. Ligation of gene X RT-PCR products with pYZT vectors.

In 1.5 ml eppendorf tube, add:

3 μl	*gene X* RT-PCR /PCR purified products
0.5 μl	pYZ101 T -vector (Mini-prep)
4.5 μl	2 X T4 DNA RAPID ligation buffer
1 μl	T4 DNA ligase (Promega)

Gently mix, then incubate at RT for 1-2 hours

Note: To reduce background, 1 unit *Xcm* I could be included in ligation mix if the insert without *Xcm* I restriction site)

3. Transformation

1) Preparation of HT115 eletrophoration competent cells:
   a) Shake HT115 cells at 37°C overnight
   b) Transfer 0.5 ml and dilute to 8 ml
   c) Shake at 37°C for 3-4 hrs
   d) Centrifuge at 5000rpm for 5 min at 4 °C
   e) Wash once with 1 ml ice-cold water and repeat another twice
   f) Keep 40-50μl water rest
2) 2μl the above rapid ligation mixture is used for electroporation of HT115 competent cells
3) Shake at 37°C over night

4. Colony PCR 2.5 Hrs

1. Preparation of PCR mixture:

10 μl	10 x PCR buffer
7 μl	dNTPs (each 2.5 mM)
4 μl	MgCl2 (25 -40mM)
0.3 μl	LacZ L primer (100 μM)
0.3 μl	LacZ R primer (100 μM)
1-2 μl	Taq polymerase (5 unit / μl)
X μl	Mili-Q water
100 μl	

2. Pick individual white colonies using tooth-picks, and put them into 5 μl miliQ water. Stir the tooth-picks.
3. Transfer 2.5 μl of each sample 14 ml Falcon tubes containing LB and Ampicillin for standard HB10b culture and shake at 37°C over night.
4. Add 20 μl PCR reaction mixture in the remaining 2.5 μl of each sample. After PCR amplification (5 min 95°C for the first denaturation step; 20 sec, 94°C for denaturation; 30 sec at 57°C for annealing, 1min 30sec at 72°C for extension, totally 32 cycles; 30min at 72°C for last extension. As pimers, used LacZ.L (5'CGTTGTAAAACGACGGCCA3') and LacZ.R (5'AGCGGATAACAATTTCACACAGG3').
5. Run the PCR products on a 1.2 % Agarose gel, visualized by EtBr and photographed using a Bio-rad Gel–doc instrument.
6. Keep clones with an insert >250bp (i.e.considered to be positive).

Transformations protocol 2

1. **Gene X one-tube RT-PCR**

2. **Ligation of gene X RT-PCR products with pYZT vector**

In 1.5 ml eppendorf tube, add:

7	μl	*gene X* RT-PCR purified products
1	μl	pYZ101 T -vector (Mini-prep)
1	μl	10 X T4 DNA ligation buffer
1	μl	T4 DNA ligase (Promega)

Gently mix, then incubate at 15°C for 16 hours

or, in 1.5 ml eppendorf tube, add:

3	μl	*gene X* RT-PCR /PCR purified products
0.5	μl	pYZ101 T -vector (Mini-prep)
4.5	μl	2 X T4 DNA RAPID ligation buffer
1	μl	T4 DNA ligase (Promega)

Gently mix, then incubate at RT for 1-2 hours

3. **Optional -Transformation**

1μl of the above mixture is used for electroporation of *E.coli* HB10b cells. Shake at 37°C over night.

4. **Colony PCR**

1. Preparation of PCR mixture:

10 µl	10 x PCR buffer
7 µl	dNTPs (each 2.5 mM)
4 µl	MgCl2 (25 -40mM)
0.3 µl	LacZ L primer (100 µM)
0.3 µl	LacZ R primer (100 µM)
1-2 µl	Taq polymerase (5 unit /µl)
X µl	Mili-Q water
100 µl	

2. Pick individual white colonies using tooth-picks, and put them into 5 µl miliQ water. Stir the tooth-picks.
3. Transfer 2.5 µl of each sample 14 ml Falcon tubes containing LB and Ampicillin for standard HB10b culture and shake at 37°C over night.
4. Add 20 µl PCR reaction mixture in the remaining 2.5 µl of each sample. After PCR amplification (5 min 95°C for the first denaturation step; 20 sec, 94°C for denaturation; 30 sec at 57°C for annealing, 1min 30sec at 72°C for extension, totally 32 cycles; 30min at 72°C for last extension. As pimers, used LacZ.L (5'CGTTGTAAAACGACGGCCA3') and LacZ.R (5'AGCGGATAACAATTTCACACAGG3').
5. Run the PCR products on a 1.2 % Agarose gel, visualized by EtBr and photographed using a Bio-rad Gel–doc instrument.
6. Keep clones with an insert >250bp (i.e.considered to be positive).
7. Mini preparation of plasmid DNA using kit.
8. Re-transformation
   0.2µl of the above mini-prep plasmid DNA is used for heat shock CaCl2 chemical or electroporation transformation of *E.coli* HT115 competent cells.
9. Shake at 37°C over night.

Alternative step by step protocol: chromotography column based total RNA isolation for single worm quantitative RT-PCR

It's convenient, in comparison with Trizol based method, although it's somehow limited in detection of low abundance transcript even the abuandant internal control works well in our hands (not shown).

1. Washed by M9 and 70% ethanol, pick single worm into 350μl RLT (QIAGEN), frozen at -70°C, then thawn for 3 times.
2. Centrifuge the lysate for 3 min at 14000rpm at 4°C. Carefully transfer the supernatant to a new eppendorf tube.
3. Add 350μl of 70% ethanol to the lysate, and mix immediately by pipetting.
4. Apply up to 700μl of sample to an RNeasy mini column placed in a 2 ml collection tube. Close the tube tightly, then centrifuge for 15s at 14000rpm. Discard the flowthrough. Re-use the collection tube in step 5.
5. Add 700μl RW1 (QIAGEN), to the RNeasy column (QIAGEN). Close the tube gently, and centrifuge for 15s at 14000rpm. Discard the flowthrough and collection tube.
6. Transfer the RNeasy column into a new 2 ml collection tube. Pipet 500μl RPE (QIAGEN) onto the RNeasy column. Close the tube and centrifuge for 15s at 14000rpm. Discard the flowthrough and re-use the collection tube.
7. Add another 500 μl RPE to the RNeasy column. Close the tube gently, and centrifuge for 3min at 14000 rpm .
8. To elute, transfer the RNeasy column to a new 1.7 ml collection tube. Pipet 30-50μl RNease-free water directly onto the RNeasy membrane. Close the tube gently, and centrifuge for 1 min at 14000rpm to elute.

Alternative step by step protocol for Chromatin IP

Modified from (Chu, DS et al. 2002,Genes and Development).

Wash

- Collect the worms in M9
- Wash 3x in M9 to discard the maximum of bacteria
- Let the worm incubate 30 min in M9 (bacteria in intestines)
- Centrifuge and discard the maximum of s/n

Fixation

- Fixation with ***2% formaldehyde in M9*** (v/v)
- Add ~20 x vol of formaldehyde (1ml of worm = 20ml of formaldehyde). Important !!!
- Let incubate at RT during 30 min on a wheel. Be careful, the temperature must be 20-26°C. If higher, incubate a shorter time. This step is very important and sensitive! It depends also of the stage of worm (larvae or adult, bigger cuticle)

Wash

- Centrifuge and remove the formaldehyde
- Wash 2x (3min) with ***stop solution*** (= 0.1M Tris-HCl pH = 7.5)
- Remove the solution, then wash 3x with M9 (3min)
- Remove max of M9

Lysis

- Add 1 vol of freshly made ***lysis solution*** (50mM HEPES-KOH, pH 7.6, 1mM EDTA, 140mM KCl, 0.5% NP-40, 10% glycerol, 5mM DTT, protease inhibitor cocktail). 1 tablet for 6-8ml of solution (1/2 life = 30min). Keep the solution on ice
- We can stop at this step (better results with): Add the lysis solution, then snap-freeze into EtOH/ CO_2 ice before storing @ -70°C
- Warning: If continue directly: Incubate the worms in lysis solution on ice for

at least 10min (the worms had to start to lyse

Sonication

- Thaw out the worm gently on ice. During this time, prepare ¼ of complete mini tablet (make powder with a tip, or if there is less worms, break in "big" part, it can help for sonication, like beads). Add 1ml of fixed worm into sterile ED containing the tablet. Keep on ice

- Prepare ice + EtOH for cooling. Sonication: Put the sonicator nearly the bottom of the tube (touch the bottom and go up for 1-2 mm). Use program 2: 20s sonication, 1min pause (8x). Put the temperature thermometer into ice. Sonication depends principally of:

    - Temperature
    - Position of the tip
    - Foaming, bubbles (if present, bigger bands)

- Once done, centrifuge directly @ 4°C at 14'000 rpm during 20 min.

- Remove s/n (=SONICATED LYSATE). Be sure to take no waste. The s/n should be clear. Once done, snap-freeze and keep @ -70°C

- Re-centrifuge the waste for 5 min. Take s/n in a new ED (= SONICATION CONTROL). Add 5M NaCl (20µl for 500µl solution) and let incubate @ 65°C for at least 6h → o/n (=reversion of cross-linking) (can do that in PCR machine if the volume is small, better, no evaporation)

Sonication control

- Mix 20µl sonication control + 1µl SDS 10% +0.5µl Prot K. Incubate 1 hour @ 37°C followed by 2 hours @ 65°C (digestion of prot)

- Extract with phenol/chloroform

- Then 2 possibilities:

    - Rapid: Use PCR purification kit. Let migrate the DNA on a 1% agarose gel. Important ! Load always the same among of DNA (8µl first) and run the gel for same period

    - More sensitive:
        - precipitation in EtOH with 10µg/ml glycogen (final conc)
        - resuspend DNA in 20µl TE
        - treat with 40µg/ml RNAse for 1 hour @ 37°C
        - run on agarose gel with appropriated ladder

- If the shearing is ok, continue. If the bands are too big, can sonicate once more (2x 20s, test after that). If there is only smear, restart with new batch of worms

Pre-cleared

- Defreeze and thaw the sonicated lysate (pool the 2 lots). Add the protease inhibitor tablet (dissolve in ChIP buffer)
- Centrifuge the sonicated lysates once at top speed @ 4°C for 20 min. Take the s/n
- Divide them in 6 unique tubes (special with "nose bottom") equally by volume
- Dilute them to 1ml by adding ***ChIP buffer***
- Add 30µl of Prot G beads (wash before 2x with ChIP buffer) per tube
- Add tablet (in solution), rotate the tubes @ 4°C for 1 hour (it's the minimum, can be longer)
- Centrifuge down the beads. Take the s/n carefully
- Repeat the incubation with beads
- Centrifuge once more at top speed @ 4°C for 20min
- Take s/n carefully
- Pool all s/n (= PRE-CLEARED LYSATE)

Ab incubation / Ab test (if not already done)

- The used Abs have to be tested on Western first !!!. They must be specific and have one band (best). If many bands, they had to be well separated unless we can say nothing
- Take 1ml of pre-cleared lysate per tube for sample Ab / no Ab. Snap freeze the rest (about 1ml) @ -70°C
- Add 4µg Ab per tube (AcH3: 2µg). Use tips with white filter
- Rotate the tubes @ 4°C O/N

Beads binding

- Wash bead 2x with ChIP buffer. Add 40µl beads per tube
- Add ¼ of complete mini tablet per tube
- Rotate @ 4°C for 2 hours (min 1 hour, best 2)

Wash

- Centrifuge the beads (2000 rpm 30'', rotate 180°, 30'')
- Take s/n to new labeled tubes (keep for snap-freeze later)
- Wash the beads:
    - 2x 1ml of ***ChIP buffer I (100mM KCl)*** for 3-5min on wheel @ 4°C
    - 2x 1ml of ***ChIP buffer II (1M KCl)***
    - 2x 1ml of ***TE***. Last wash: Discard the maximum of s/n (important !!!)
- Be careful with the beads: Use special long tip to avoid discard beads

Elution

- Add once 200µl ***elution buffer*** (Tris-HCl 10mM pH8, 1% (w/v) SDS)
- Vortex for 30'' and rotate 30 min @ RT on wheel.
- Centrifuge, take s/n (= ELUATES) in a new ED
- Redo the elution once (20% of recuperation). Add to the ED (final vol 400µl of eluates)
- Keep the eluates @ -20°C. Snap-freeze the beads (not necessary)

Reverse of cross-linking

- Defreeze the snap-frozen pre-cleared lysate, take 19µl of lysate (snap-freeze & keep the rest)
- Add 400µl ***elution buffer*** (as INPUT control). Glean all samples (400µl of eluates) plus INPUT control
- Add 5M NaCl (20µl for 500µl of lysate → 16µl) Incubate @ 65°C for 6 hours (min 4 hours) Possibility to freeze @ -20°C after this step
- Add 8µl EDTA 0.5M, 16µl Tris-HCl pH7 and 1.6µl Prot K (1µl for 250µl lysate). Incubate 1 hour @ 45°C

Purification

- 2 possibilities for purify: as before (precipitation (for smaller fragment) or PCR kit)
    - If PCR purification column: Filter in 3 times the solution + PB buffer (because of the big volume)
- Finally, elute DNA into 30µl Elution buffer. Use 2µl per 25µl PCR reaction

PCR amplification

- PCR procedure:

95°C	2’	
95°C	30’’	} 21 cycles
57°C	30’’	
72°C	1’	
72°C	3’	

Or PCR procedure for wonder Taq

95°C	3’		DNA	2µl
			Buffer 10x	2.5µl
93°C	35’’		dNTPs	(20mM) 0.25µl
57°C	1’		Primer 1	(2µM) 2.5µl
72°C	1’		Primer 2	(2µM) 2.5µl
			Taq	0.25µl
72°C	3’		Water	15µl

- If no signal, 4 cycles added, etc
- Primer (1µM): 20-24bp, 50% of CG, producing a 200-500 bp fragment

Detection

- Detect the PCR products (10-15µl) separated on 2% agarose gels or 5-6% non denaturing PAA gels

Materials:

Solutions:

1) Lysis buffer:

50 mM HEPES-KOH, pH 7.6
1 mM EDTA
140 mM KCl
0.5% NP-40
10% glycerol
5 mM DTT (add when use. DTT degrades rapidly!!!)
Protease inhibitor cocktail (Roche: 1 for 6-8ml)

2) ChIP washing buffer I:

Lysis buffer (see the above) at 100 mM KCl

3) ChIP washing buffer II:

Lysis buffer at 1M KCl

4) ChIP washing buffer III:

TE 1x

5) Elution buffer:

10mM Tris-HCl (pH8), 1% (w/v) SDS

6) NaCl 5M

7) 0.5M EDTA

Materials:

1) rec-Protein G-Sepharose 4B conjugate beads (Zymed): 50% suspension (2ml of beads + 2ml PBS)

2) Complete, Mini, Protease Inhibitor Cocktail Tablets (Roche)

Appendix

1) List of sequences (5'-3'):

Puatative targets of LET-418/Mi-2

F45D3.3, F45D3.4* and *F59A2.4
AAGGTCTAAGGCTTGACTAGAGTNTGTACAGAGCTTGCGTGCAGTGGCACGTGTACGCGGGCATGAGACAAAGCT
GGTTATCATGGAGCCCAGTGCCGACATTAAACATCATTTGTTCGCGTTCTCGCGGAGCACGAAAGCCGACGAAAA
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TCTGCCCACAGCGTACAACTCCATCTGGTTGCTCAAATTCATCCTTTACTTTCTTTTGTCCTGGAAGTTTCTCGGATT
TCTTTGGAGTCATTGGAACAACTCCGTCGACATTTCCATTTCCATCGGCAAAATGCCCATCTCCCATGGCACCGAG
GAACAGGTCAAGATCGAAGGAGTCATCTGGGTTTTTCAG

Y64H9A.2
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ACCCTTTCAGNCCAGC

W04G3.1 W04G3.3
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F20A1.6a F20A1.6b
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eft-3, eft-4
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ACTTGACATTCAGTG

eft-3, eft-4
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Y64H9A.2
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gpd-2.gpd-3,gpd-4
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C01A2.4 snf7
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Y76A2B.3
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F21C10.4
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TACGTCA

C01B10.3 IP3
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F45D3.3 F59A2.4
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rpl-13
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wrt-10
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C32E8.11

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F32H2.5

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F56F10.1

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K10B2.2 and F22G12.1

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Y37E3.8 rpl-15/27

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C

Y37E3.8 rpl-15/27

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rps-13 or Y102A5C or F52C6.13

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F45D3.3 F59A2.4

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W07G9

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mlc-1 and K06A4.3

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act-4 or act-5

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hmp-1

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C47D12.2

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Y73B6BL.24 Y73B6BL.38 puf-3

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W04G3.1
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AC

cyn-6 cyn-5 cyn-1
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nlp-33
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F53B3.6
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B0261.6
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rrn-3.1
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A

sqt-3,col-43,col-124,col-112,
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C04F12.1
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F41E7.5,grl-25,
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Y41D4B.15,npp-8
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rps-2 (C49H3.3)
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sqt-2
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ari-1:
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F41F3.3
CCTCACCACNGAACNCGAGCAACGCATCCAANNAAACCATATGACATGATTACGCAACGCTAGGTTTATACGACT
CACTATAGGGCAGCATCTTCGATATTTGGCATCGCGCGCACCGGTACGTCTCGAGGAATTCTGCAGTGATATCTGG
ATCCACCAAGCTTAGCGTGGTCGCTGGACAGAGGTACGCGGGCGGATACGCCGTGAGCCGACCCCAGACCTGAAG
GAGCTTACGCCGGAAGAAAGTAGATGATGACGAATCTGATTGTATAAGTGTTGTACAACTTGCAACTATAATTTA
GCTGTGGTATATTTNGAGAAAAACCCTTATCAGGAGATGAATTACGTTTGNAT

xbx-4 , C23H5.2, C33E10.8
CCTAGGTTGAAAGATCAATATAGGGAGATCTTCGATTTAAAATTTGGCGCACCGTACGTTNCGAGGAATTCTGCAG
GATATTGGATCCACCAAGCTTAGACGTGGTNGCGGGCGAGGTACTTATTTANTTTTATTNTTAATGTTTATAGTTN
ATNTTATTTATTTTATATTGTTTTTTTTATTTTATGANATTTTTTNATTTAGATAATGATA

C08B11.6 Arp6p
GCTATGACATGATTACGCACGCTAGTTNTAACGACTCACTATAGGGCACGATCTTCGAGAATGCATCGCGCGCACC
GTACGTCTCGAGGAATTCTGCAGGATATCTTGGATCCACCAAGCTTGGCGGCCGCCCGGGCAGGTACGCGGGGAA
GGACCGGTCGCCTTACGTGGGAATCCTAATTTTTAAGAAGCATATGTAACATTCGTAAAAACGCATAGATTTTCAA
TTCAATTCCAATAACCGGGTTGTTTCTTGTGTGCCTGATACTTTCATGAATATTGTGATA

Y54G2A.5 , Y54G2A.18
AACGCTATGACATGATTACGCCACCGCTACGTTTATACGACTCACTATAGGGCAGATCTTCGAATGCATCGCGCGC
ACCGTACGTCTCGAGGAATTCCTGCAGGATATCTGGATCCACCAAGCTTAGCGTGGTCGCGGCCGAGGTACGCGG
GGAGAGCTCAAGTCCGCCAACACTGACCGTGACACTCTCAAGAAGCAATCCGAAGGGCTCCAGCGCGAGTTCGAC
CGCGTCTCCGATCTTCTCAGAGATTCTGCCGAATCCAAGGGTGACAAGAAGAGGACTAATCGATTCTCATTTCGCG
TTTTGTTTTTTCTATTATCAAAATCACAATTTTCTCAAGCCGCCTATATATTTGTTCTGTATAGAATCGCTCAGTTTT
CGAGTGAATAAAATGTA

F36F2.3
GGCAGAATTTGAAAACATGGACGAAGCAGAACGTCTCAAGTCATATCAGAGACCAGTCGACAAGAGCATACGAT
CAATCAAACTTTCGTCGGCCGGTCAGCCAGGCATTATGACAGGACCACCTCCACCAACATATACATGTAATCGTTG
TAGTCAACCAGGACATTGGTAC

F36F2.3

ACGTATGACATGATTACGCCAACCCTAGTTGTAAGACTCACTATAGGGCACATCTTCGATTAGCATCGCGCGCACC
GTACGTCTCGAGGAATTCTGCAGGATATCTGGATCCACCAAGCTTTCGAGCGGCGCCCGGGCAGGTCCGGGCAGG
TACGCGGGCAGAATTTGAAAACATGGACGAAGCAGAACGTCTCAAGTCATATCAGAGACCAGTCGACAAGAGCA
TACGATCAATCAAACTTTCGTCGGCCGGTCAGCCAGGCATTATGACAGGACCACCTCCACCAACATATACATGTAA
TCGTTGTAGTCAACCAGGACATTGGTACCTCGGCCGGACCACGCTAGCTCTGGTACCCTCTAGTCAANGCCTATAG
TGAGTCGTATTACGGAC

ntl-1
GGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGATTTTTTCTGCTAAA
AAGTTTCGACATCGATAACGCGGATTTTCAGAATTCAAACTCTGGCTGGCATGCTGCTCCGGCACCACAAAGACCA
TCCGGACCACCAACGCCACAACAGCAAATGGATTTTCGCGGTCAGATTCAAGAGTTTGCACCGCAGGGACCTCAT
CAATTGCAACGAAGCGGAAGTGTCACCGGCAGATCTATGGGAATTGTTAATCACTAGTGAATTCGCGGCCGCCTG
CAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCT
TGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGG
AAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCT
TTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATT
GGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACT
CAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAA
AAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAA
AATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATNAAGATACCAGGCGTTTCCCCCTGGAAGCTCC
CTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCNTTTCTCCNTTCGGNAAGCGTGGCGCT
TTTCTCATAGCTCA

Sequences of sequenced RNAi constructs or deletion mutant

p66
GGGCGGAACAGCTCACTATAGGCTTGACTAGAGGGTACAGAGCTCACCTAGGTATCTAGAACCGGTGACGTCACC
ATGGGAAGCTTCGTGGATCCAGATTAATCTTCTGGAAAGCTCTCTTCAACAAAGCTGTCTGATCTGTTCGATTTTTC

CTTTTTTGTGCTGATCGTACACAATTTTCACAATAAAGATGAAGATCATCTTGTGTACTTCCAATAGCTTTCCAAGT
GTGAGCACATTCGGTTTTACATTCTTCACATTCGTATGATGGTCCGGCATATGGTGTCTCATTAGATCTATCTTTGA
GAACATGCTGAACCACTAGATCCAATCCATGCAATGCCAGGAATGCAGAAGCATTCGGATTCGGTAGGAAAGTGA
TATCATGAGGTTGACTTTTTTGTGTTGGAATTGTTGTCAATTGCTTATCAGCTTGAGCTCTGAAAGCCAGCCGAGCT
GCTTGTATCCTTTGTGCAGGTGTCTGTTGATTTAGAAGTTTTGATTGTGTGATTTGAGCCTGTTGTGGTTGCTGTGCT
TGTTGAGAAATCCTGCAGGAATTCCTCGAGACGTACGGTGCGCGCCGAATGCATTTCGAAGAATCCTTGCCCCC

rrf-3
GACTCACTATAGTGANTAGAGGGTACCCGACTGGAGATGGCATAGCGTATTTTACTACTTCGAGGTATTCATCTTG
GCAGCTCTCCACCACCTTCACCAAACTTTTGCTGACTTTTTCTGAAGATAACTTTAGGCATATTTATAATATCATCG
ATTGTTGGATCAATAACTAGAATTCCTTTGAAACCTTTAAATCGCACTTGGAAACAAGCTGGTACTTCCTTCAATT
GGAGAATCTTAGATATATGTGTTGCCAGTTTGATAGAAATTCGACCACAACCATCAGAAAAACAATATTTGTTCTC
TACACCACCTTCAATGTCAGGTTCACGGGGTACCAGAGCTCACCTGGGTAACCAAGGGAAGCTTGGTGGATCCAG
ATATCCTGCAGGAATTCCTCGAGACGTACGGTGCGCGCGATGCATTCGAAGATCTGCCCTATAGTGAGTCGTATTA
CGTAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATAC
GAGCCGGAAGCATTAAAG

hda-1
GGGTACCAGACCTCACCTAGGTATCTAGAACCGGTGACGTCACCATGGCAATTTTCATTGCTGCATTCGACGACTC
GATGTGAAGTCGGTAGTTTGGTCCAAAGTATTCGAAGTAGTCATTGTATGGCAACTCATTTGGAACTTCCTTATCG
ACGGCAATCGAGGTTTCGTACGTCCAACAACGTGCCACATTTCTTGGAGTGTATCCACCTCCACCGACCATCATAA
GTGGAACGTTGTAGCTTCGGAAGAAACGAGCACATTCTCCGTGGCCTTTCAAGGTCAGATTGAATGGTCCAAGTCT
ATCTCCGTTGAGAGAATCAGCTCCACATTGAAGAACAACAGCACAGGGATCAAATCTCTCCATAACCTTTGTCATG
ATTGGTTTAAAAATACTCTGGTAAGAGACGTCGGTGATTCCATCGCGAAGTGGAACATTGACTGAATAGAGCTTTC
TTTTTCCAGCTCCTATATCTTTCAGGTCTCCGGTTCCTGGGAAGAAATCTCCATATTTATGGAATGACACTGTCATT
ACTCGATCAGTCGTATAGAACGCCTCCTCTACTCCATCTCCGTGATGAACATCAATATCGACGTAAAGTACTCGCT
TGTGGTACTTGAGAAGCTCGAGAATACCGAGAACGATGTCATTGGTGTAACAGAATCCGGACGCCTCGCTTTTCTT
GGCGTGATGGAGGCCTCCCATCCAATTGATAGCAATGTCCACCTTCTGCTTGTTCAATTTAGTGGCAGCAGCCAGA
GAACCTCCCGAACTGAGTTGGCAGAACTCATAAAGACCATCAAAGAGAGGACAATCTTCTCCAACATTGAACTTA
AGCATTTGTTTGTTGAAGGGATTTCAGATTATCTGGATTCCGCACNTCTTCAAAAACGGTCATGTACTNCGTCGCT
GTGAAAACNAGTCATGTTCTTNGANTGAATNCNAGGGGAAATGGGCGGGAAAAACCTNTAAATT

rrf-1del
TTATTGATGGTAGAAGCGAATCGGATCAATTTTGAAGGTTGTGTTTTTTGCATATTTTTTGATAATTTTTTTAAAAA
TTTTTCAGTGCCTAGATAAAATTTGCCAAAAACGTGAGGTTATGAAACTGGTTAATGGTTTAACAGAAAAGGAGA
GCATAGAAGGATATCAACGAGTTCGAAAAGTTATCTTCACACCTACGAGAGTCATCTACATTGCACCAGAGACGA
TCATGGGAAATCGAGTTCTACGGAAATTCGATAAAGATGGCACCCGGGTGCTTAGAGTAACATTTCGAGATGATA
ATAATAAGAAAATGAGGATTTTCGAGGCTGTCGATATTCCAGAACTTCATCACATGTGTGATGTAGTAGTATTCCC
ACAACATGGACCGAGGCCTCATCCTGATGAAATGGCTGGATCTGATTTGGATGGAGATGAATATTCTATCATTTGG
GATCAGGAATTACTTCTTGAAAGAAATGAAGAACCTTTCGATTTTGCTGTTGAAAAGATTAAAGTACCATATGATA
GAGAGAAGCTTGTTAGTTTTTTTTTTGTNCATTTTTTTTTAAACCTTNGGNGNAANACCAATTTNAGGCCNNCTNGN
NCCGNGGGTTTNATGNCANANNTCTAAANTTAGCCCCCGTNGGNCAAANCCCGAAACAGTTNGNTNAANAAAGG
GGNTCCCCCTAANNCNCTGGNGGCCCCCAANAAANNNTTTNCCCCCAAAA

lin-1
GACTACTTAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTG
CAGCCCGGGGGATCCACGCGTCACGTGGCTAGCCATGGAACCGGTGGATCCACTAGTTCTAGAGCGGCCGCGGGA
ATTCGATTCATCATCCGTCGACTCAATCATAACCCTGTGGCAATTCCTTCTAGAACTACTGCAACAAGACCAAAAT
GGTGATATAATCGAATGGACACGCGGAACGGACGGCGAATTCCGACTGATTGATGCAGAAGCCGTGGCGAGAAA
GTGGGGACAACGGAAGGCGAAACCGCATATGAATTATGATAAACTGTCGAGAGCGTTACGATATTATTATGAGAA
GAATATTATTAAGAAGGTGATCGGCAAAAAGTTCGTATATCGCTTTGTAACTACTGACGCCCACGCTCCGCCGACC
GCCGACTTTTCCTCAAATATGAACATGAAGATGTGTTATGTCAAAGACGAGAAGGACATTCGACACGAGATTCCG
TCGTTTATGACGTCATTACAAGCACCGCCGCCGCCGCCTCCACCACCTCAAAATCCACGTGGCAACACGGATTTCT
CGGCGCTGAGCCTTCTTGGGACGGATTCACCGACGACGCACAGTGTCAGCACACCAAGTCCAACAGATAGTGTGT
GCTCCCCGTCAAGCAGTGTGGCCTCTTCGGCGACTCCATCCACCTCATCCCCTGTAGATGAGTCCCGACATTGCCG
AAAACGATCCCTATCGCCCTCCACGACGTCATCGACGACTGCACCGCCGCCGCCGCCGCAGCCGCAACGAAAAAA
GGAATGANGCCGACCCCCTGACCTGACAGCAACNTCGANTTTNTCCTACAACCGTCANTCTCGTCTCCNCTTTTGA
AGCTTCAGCAACNCNTTAAAACTNCCCGCTTTTCCAGCCCCAATCAGTCACTGTCCCCTNCCCNCGCTGGGTTTGC
CNGGCTTTNTGGNCN

lin-15A
AGGCCTTGACTAGAGGGTACCAGAGCTGAGACTGGTCGGTCTCCATCTCGGAGACGGAAAATCGAGGATATTCTT
GACAACAAGTTTGCTGCTGCAGCTGCAAAAGCTTATCGAGATCACAGTGAAGATGCACCTAGTGAACCGTATATT
CCCAATCAAAGTGAGATGCAGAACACTGTAGAACGCAGAAAGCGAAAGCTTCATTCGCCTGAACAGGATGATGCT
GGATCATCGAGTATCTCATGGAACGCAAAAAAAACGAAAACTCCAATTGACTATGTTCACCTAGCAACGCGTGTT
TTGGAAGGTCATTCAATTGCCGACGAGGCGCTTTTGCACAAATCCAAAGTTTCTTATGCTCGTAATGCATTTGGTG
AGAAGCCAAGCTCCCCCACTCCGCCATCTGCGCCACTAAAGTTCTGTGTTGTCAATGGAAAGAAATACTTGCGTTT
TGAAAACGGAACCGGACCTCCGAAAGTTGTAGTTCAAGGGAATGTCGTTCTTCGCACTAATACTCTTAAAGACGC
ATTGACTACAGCGCCACGTGCACAAAACCAGCCTTCTACGTCCACAGACTCATCAAGCTCATCCGAGATGGAGGG
AATACGTCAATCATTTGGGGCGCCTCAAAAGGAAGAAGAAGAAGAAGANTTAGTACCTACGCTTCTTCAAAACAA
ACCTACCCACGTGGAATCTTCGAGCCCCGTTGAAAAAAANCCNCCAACCNNANACGAACGTATNGGAANCNGGC
CGTTCCCTNTTNGGAAAAATGTTTACCGACTTGCCATTTGGTNCNANTGGANTCACCCNCCAAAGNAAANCNGGT
AAAAAANAGAGGGGGTNNTTTTNAAATTNAGCCCAACCTTTTGNNNNNTNCCCNGNGNCCCAATNGGCTTAAAAG
GTNGGGNNCNAAACCCAAATCCNNGNCC

List of primers (Sequence: 5'-3')

Genomic primer sets

Name	Forward/Reverse	Sequences
F59A2.4end	F	5’ TGG TTA TCA TGG AGC CCA GT 3’
	R	5’ CCG TGA CAA GAC AAA ATC CA 3’
F59A2.4pr151	F	5’ AAC ACT GCT TCC CTT GTT CG 3’
	R	5’ CTT CGA TCT CAC GGT TCA GG 3’
lin-39prJW5.1-218bp	F	5’ AATCAGTCACGGCTGGAAGT 3’
	R	5’ CTCCAAACGCTCCAAACAGT 3’
egl-17a	F	5’ CAAAACCAATAGCGCTCAAA 3’
	R	5’ AAATTGGCGTACGCTTTCTG3’
egl-17b	F	5’ CGGTGTTCGTTGGAAGAAAT 3’
	R	5’ ACTCCAGAAGGGGATTTTTG 3’
lag-2L	F	5’ TTC CAA CCG TCC TCC AAT AA3’
	R	5’ CAC TTT AGC GCC GGA AGA TA3’
GFP	F	5’ TGC TGA AGT CAA GTT TGA AGG 3’
	R	5’ TTC TTT TGT TTG TCT GCC ATG A 3’
lag2pr173-7.5k	F	5’ TGT TTC GGA AGA ATG AAG TGA G 3’
	R	5’ TCG ATT GTA AAT TGG CAA ACA C3’
lag-2med151 bp	F	5’ AAG TGC TCA TGC GAG AAC G 3’
	R	5’ GGA ACT GAT TGA AGC CGA AA 3’
elt-5 intron site2-204bp	F	5’ GGGCGAACAAAGGAAAGATA 3’
	R	5’ ACAAGGGTTTCCCCAGAAGT 3’
elt-5intron Site3280bp	F	5’ AAGCTCCCAAAGTTGCTCAG 3’
	R	5’ ATGTGGTGGAGGTCTGGAAG 3’
ntl1-107bp	F	5’ CCA CTT ACT CAA CGG CAG GT 3’
	R	5’ CCA CTT ACT CAA CGG CAG GT 3’
LacZ	F	5’ CGT TGT AAA ACG ACG GCC A 3’
	R	5’ AGC GGA TAA CAA TTT CAC ACA GG 3’
tba-1.ChIP 150bp	F	5’ TTT TCA GGT TCG TTT TCA ACA 3’
	R	5’ CAT CGG CTT GTT GGT CTG AT 3’
act-4pr150bp	F	5’ GCG TGC AGC AGT ATA AAT AGG C 3’
	R	5’ ATC AAC TCA CCT TGC ACA TTC C 3’

mRNA primer sets

Name	Forward/Reverse	Sequences
rps-2	F	5’ gctttcgttgctatcggaga 3’
	R	5’ tccgatcttgttaccccagt 3’
lin-53	F	5’ attcatcggctcattcttgg 3’
	R	5’ ttatcgtcaccaaccgatcc 3’
p66	F	5’ aataaggaaccggatgaggaa 3’
	R	5’ ctttgagaacatgctgaacca 3’
lin-15A	F	5’ gagactgtcggtctcggagacggagacggaaaatc 3’
	R	5’ gagaccgacagtctgttggcggaatatgttttgg3’
hda-1	F	5’ attatggtcaagggcacgtc 3’
	R	5’ ttttcatttgctgcattcgac 3’
lin-15B	F	5’ tgttggcctactggggtta 3’
	R	5’ tttgcgctcgtacaaatcct 3’
ama-1	F	5’ cagtggctcatgtcgagt 3’
	R	5’ cgaccttctttccatcat 3’
pad-2	F	5’ aaattcgagaaacggagctg 3’
	R	5’ tactccttctcgccttccag3’
lag-2end	F	5’ ggcttcatcggtgaattctg 3’
	R	5’ gctgtctcatcggcttgaac 3’
lin-39 mRNA	F	5’ cctggaaggagacgatgatg3’
	R	5’ cgcgtgaacctcctgtagtt 3’
gpd-1	F	5’ aaaggacacggttcaagtgg 3’
	R	5’ acaacgaaatcggctttgac 3’
lin-35	F	5’ ttggaatcgaacaatcaaagtct3’
	R	5’ ttgaaccatttgacgacttagc3’
lin-1	F	5’ tgaatcacattgacctttgaag3’
	R	5’ aacgctctcgacagtttatca 3’
sqt-2	F	5’ ggaccacacctattccatcg 3’
	R	5’ catttccgtcatctccaggt3’
sqt-3	F	5’ caggaaagccaggaaagc3’
	R	5’ ctcctggtggtcctggtg 3’
egl-27	F	5’ atcaacgcattacgccatttc 3’
	R	5’ tgcgtgatgccagtctttac3’
R05D3.11	F	5’ aatgtcgcttcgacaaatgat 3’
	R	5’ tcaagtccaaatcagcgaagt 3’
egr-1	F	5’ tggaagaggacgaagaatgc3’
	R	5’ aatccttgcccaccttatcc 3’
rrf-3	F	5’ cggggtaccccgtgaacctgacattgaaggtg 3’
	R	5’ cggggtaccccgactggagatggcatagcgta 3’
	R1	5’ cggggtaccccgttcggaaaatctacagctactgc3’

Oligonucleotides

SMART IIA	5’AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG3’
3'SMART CDS Primer II A	5’AAGCAGTGGTATCAACGCAGAGTACT(30)MN3’ (N = A, C, G, or T; M = A, G, or C)
5'PCR primer II A	5’AAG CAG TGG TAT CAA CGC AGA GT3’
pYZT XcmI adaptors	5’GATCCACCAAGCTTCCCTTGGTGTACCCAGGTAGCT3’ 5’CACCTGGGTAACCAAGGGAAGCTTGGTG3’

CURRICULUM VITAE
Yue ZHANG

Personal data

Birthday 26 May 1970
Birthplace Hunan, P. R. China
Status Married
Nationality Chinese

Education

2001-2005 PhD candidate in Cellular and Developmental biology (Supervisor: Prof. Dr. Fritz Muller), at the Institute of Zoology, Faculty of Sciences, University of Fribourg, Switzerland

2000-2001 Visit scholar (Supervisor: Prof. Dr. Fritz Muller), Institute of Zoology, University of Fribourg, Switzerland

1998-1999 Postgraduate program in Fish Genetics and Aquaculture (Supervisors: Prof. Jianren LUO and Prof. Sifa LI, Chinese Academy of Fishery sciences and Shanghai Fisheries University

1994-1996 Bachelor of Sciences in International Trade, (Supervisor: Prof.Huanfa HUANG) at the College of Commerce sciences, Nanjing University, P. R. China

1988-1992 Bachelor of Sciences in Genetics (Supervisor: Prof. Dr. Ruiyang CHEN), at the College of Life sciences, Nankai University, P. R. China

Poster Presentation at conferences

European *C.elegans* Meeting (Interlaken, Switzerland): p043, poster, 2004

14th International *C.elegans* Meeting (UCLA): p666. poster award 2003

European *C.elegans* Meeting (Paestum, Italy): p193, 2002

13th International *C.elegans* Meeting (Los Angels) abstract 522, 2001

Publications

Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L, Müller F (2003)
Genetics: influence of TOR kinase on lifespan in *C. elegans*.
Nature. 426(6967):620.

Guerry F*, Zhang Y*, Marti CO*, Moroni P, Müller F
The Mi-2 nucleosome-remodeling homolog LET-418 is targeted to the promoter of *lin-39*/Hox to regulate vulval cell fusion and fate determination in *C. elegans* (in revision, *co–first authors)

Vellai T, Takacs-Vellai K, Zhang Y, Guerry F, Chen E, Stern MJ, Müller F (2006)
Transcriptional control of Notch signaling by a HOX and a PBX/EXD protein during vulval development in *C.elegans* Developmental biology (in press)

Zhang Y, Guerry F, Marti CO, Müller F
The roles of *C. elegans* Mi-2 homologue LET-418 in LIN-12/Notch signaling during the development (in preparation)