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Contribution to the establishment of an RNAi ressource for Caenorhabditis elegans and its use toward understanding the TGF-B pathway

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Contribution to the establishment of an RNAi ressource for *Caenorhabditis elegans* and its use toward understanding the TGF- β pathway

Mémoire présenté pour l'obtention du grade de

licencié en Sciences biologiques

Violaine GOIDTS

Juin 2003

Facultés universitaires Notre Dame de la Paix
FACULTE DES SCIENCES
Secrétariat du Département de Biologie
Rue de Bruxelles, 61 – 5000 Namur
Téléphone: +32(0)81.72.44.18 – Téléfax: + 32(0)81.72.44.20
E-mail: joelle.jonet@fundp.ac.be – http://www.fundp.ac.be/fundp.html

Contribution to the establishment of an RNAi resource for *Caenorhabditis elegans* and its use toward understanding the TGF-β pathway

GOIDTS Violaine

Résumé

La voie de signalisation de TGF-β (Transformation Growth Factor) est impliquée dans de nombreux processus biologiques tels que la régulation de la prolifération cellulaire et la différenciation.
L'activité des récepteurs TGF-β entraîne la phosphorylation d'une protéine du groupe Smad qui hétérodimérise avec une autre protéine (Smad 4). Ce complexe est importé dans le noyau pour activer la transcription. Ces protéines ont été identifiées dans différents organismes tels que *Drosophila* et le ver *Caenorhabditis elegans*. Ce dernier a été un modèle type pour l'étude de telles voies de transduction en raison de l'homologie qu'elles présentent avec celles de l'humain.
Chez le ver, suite à la présence de phéromone, d'une température élevée (27°C) et d'une diminution de la quantité de nourriture, le cycle de développement s'arrêtera au stade L2. Cette larve dite « dauer » pourra vivre plusieurs mois avec une activité métabolique réduite. Ce phénomène est régulé par la voie de TGF-β dont les protéines se dénomment Daf (pour « dauer larva formation ») chez le ver.
Ces protéines responsables du développement de la larve en « dauer » contrôlent aussi l'expression de nombreux gènes chémorécepteurs exprimés dans différents neurones. En intégrant les informations que percevront et transmettront ces récepteurs, l'animal adapte sa physiologie au milieu.

Notre hypothèse de travail est que non seulement les protéines reconnues pour jouer dans la voie TGF-β mais aussi certains de leurs interacteurs découverts par double-hybride au laboratoire pourraient affecter l'expression des gènes des chémorécepteurs.
Pour tester cette hypothèse, nous avons utilisé un ver transgénique, porteur d'un rapporteur en fusion transcriptionnelle avec le promoteur du gène str-2 d'un chémorécepteur.
Par la technique du RNAi, nous avons testé l'effet du « knock down » de ces gènes candidats régulateurs sur l'expression du rapporteur.
Nos résultats ont permis de mettre en évidence deux interacteurs de la voie de DAF-7, DX13 et DX41, qui affectent l'expression du rapporteur, mais dans une région pharyngienne et non dans les neurones. Cette observation demande confirmation.

Mémoire de licence en Sciences biologiques

Juin 2003

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Et finalement, je vous remercie, vous, lecteurs et membres du jury, qui allez prêter attention à ce travail.

You have made your way from worm to man,
and much within you is still worm.

Friedrich Nietzsche, Zarathustra's Prologue, 3

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List of abbreviations.

AD	Activation Domain
Amp	Ampicillin
BP reaction	Bacteria-Phage reaction
cDNA	Complementary DNA
C. elegans	*Caenorhabditis elegans*
DB	DNA Binding Domain
dNTP	DeoxyriboNucleotide TriPhosphate
dsRNA	Double-Strand RNA
E. coli	*Escherichia coli*
EST	Expressed Sequence Tag
GFP	Green Fluorescent Protein
GST	Gluthation-S-Transferase
HF	HiFi
HIF	Integration Host Factor
Int	Integrase
IPTG	Isopropyl-β-D thiogalactopyranoside
kb	Kilobase pairs
LB	Luria Bertani
LR reaction	Left-Right reaction
NGM	Nematode Growth Medium
ORF	Open Reading Frame
OST	Open Reading Frame Sequence Tag
PCR	Polymerase Chain Reaction
PTGS	Post Transcriptional Gene Silencing
RNAi	Interfering RNA
RNase	Ribonuclease
SOB	Salt Optimized Broth
SOC	Salt Optimized Carbon
TBE	Tris Borate EDTA
TE	Tris EDTA
Tet	Tetracycline
TGF-β	Transcription Growth Factor-β
Xis	Excisionase

Introduction

Contribution to the establishment of an RNAi resource for *Caenorhabditis elegans* and its use toward understanding the TGF-β pathway.

Introduction.

1. Letter to Max Perütz

Dear Max,

These notes record and extend our discussions on the possible expansion of research activities in the Molecular Biology Laboratory.
First, some general remarks. It is now widely realized that nearly all the "classical" problems of molecular biology have either been solved or will be solved in the next decade. The entry of large numbers of American and other biochemists into the field will ensure that all the chemical details of replication and transcription will be elucidated. Because of this, I have long felt that the future of molecular biology lies in the extension of research to other fields of biology, notably development and the nervous system.[...]
The great difficulty about these fields is that the nature of the problem has not yet been clearly defined, and hence the right experimental approach is not known. There is a lot of talk about control mechanisms, and very little more than that.
It seems to me that, both in development and in the nervous system, one of the serious problems is our inability to define unitary steps of any given process. Molecular biology succeeded in its analysis of genetic mechanisms partly because geneticists had generated the idea of one gene-one enzyme, and the apparently complicated expressions of genes in terms of eye color, wing length and so on could be reduced to simple units which were capable of being analyzed. Molecular biology succeeded also because there were simple model systems such as phages which exhibited all the essential features of higher organisms so far as replication and expression of the genetic material were concerned, and which simplified the experimental work considerably. And, of course, there were the central ideas about DNA and protein structure.

In the study of development and the nervous system, there is nothing approaching these ideas at the present time. It is possible that the repressor/operator theory of Jacob and Monod will be the central clue, but there is not very much to suggest that this is so, at least in its simple form. There may well be insufficient information of the right kind to generate a central idea, and what we may require at the present is experimentation into these problems.

Our success with bacteria has suggested to me that we could use the same approach to study the specification and control of more complex processes in cells of higher organisms. As a first stage, I would like to initiate studies into the control of cell division in higher cells, in particular to try to find out what determines meiosis and mitosis. In this work there is a great need to "microbiologize" the material so that one can handle the cells as one handles bacteria and viruses.

[...] I would like to tame a small metazoan organism to study development directly. My ideas on this are still fluid and I cannot specify this in greater detail at the present time [...]

Sydney Brenner.
5 June, 1963

2. Functional genomics, a future of biology[1].

In the early days of molecular biology, genes were first defined through the description of their mutant phenotype. That's the FORWARD GENETICS. It has the advantage that the phenotype of the mutant gives a clue to the function of the gene. But with the advent of large-scale genome sequencing, the situation is different. Indeed, thousands of genes have been predicted, but we know nothing about their function. So, since the complete genome sequences for Human and other organisms are available, it is expected to change the way we formulate and address biological questions. That's where the REVERSE GENETICS, which is the genetic analysis that proceeds from genotype to phenotype by gene-manipulation techniques, can now be considered as the most effective way to assess the function of a gene. Indeed, with nearly all genes in hand, the conventional approach of studying one gene at a time can now be complemented by more global or integrative approaches that consider all genes at once (see figure 1). So understanding life at the molecular level will require the description not only of each protein individually but also of the full complement of proteins involved in particular biological processes (1, 12).

[1] The main content of these introductory pages is inspired and sometimes literally transcribed from published or unpublished papers from the Vidal lab.

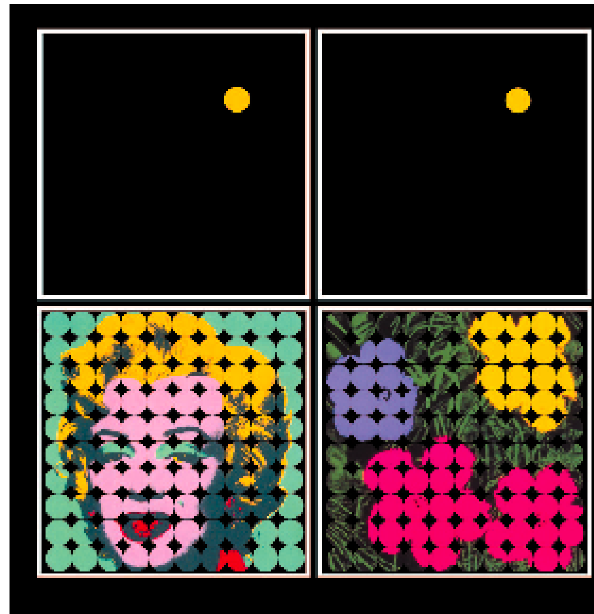


Figure 1: An illustration of the power of functional genomics.
The top two "experiments" reveal the same results, but from the global views below, it is clear that the pictures are different. By analogy, classical molecular biology gives information on only a single gene while functional genomics gives information potentially on all of the genes in the organisms (2).

The complexity inherent to considering tens of thousands of proteins to formulate integrative biological questions is such that biologists will need more and more different maps that indicate information on protein function. In the post genome era, molecular biologists will have the opportunity to navigate through larger parts of uncharacterised territories of the proteome. Thus, in the continuation of the genome mapping, new functional mapping projects appears for the transcriptome, the proteome, ... So, to facilitate the use of those tools, it is important that the maps describing different aspects of protein function should be compiled into a "biological atlas" (3).
By integrating the information contained in the atlas, meaningful biological hypothesis could be formulated. However, it should be kept in mind that these hypotheses will still need to be tested back in the context of relevant biological settings.
One way for the "future of biology" is the integration of data from various high-throughput experiments to obtain global views of biological processes.
So gene functions are being determined by annotation of the genome, by expression analysis using DNA microarrays, by finding protein-binding interactions using for instance a global yeast two-hybrid approach, by the structural and biochemical genomics, by genetic screens using RNAi,...(see figure 2). Together, these approaches will deliver information on the basic properties of gene function: sequence, mutant phenotype, expression profile and protein-binding interaction, protein localisation,... (4)

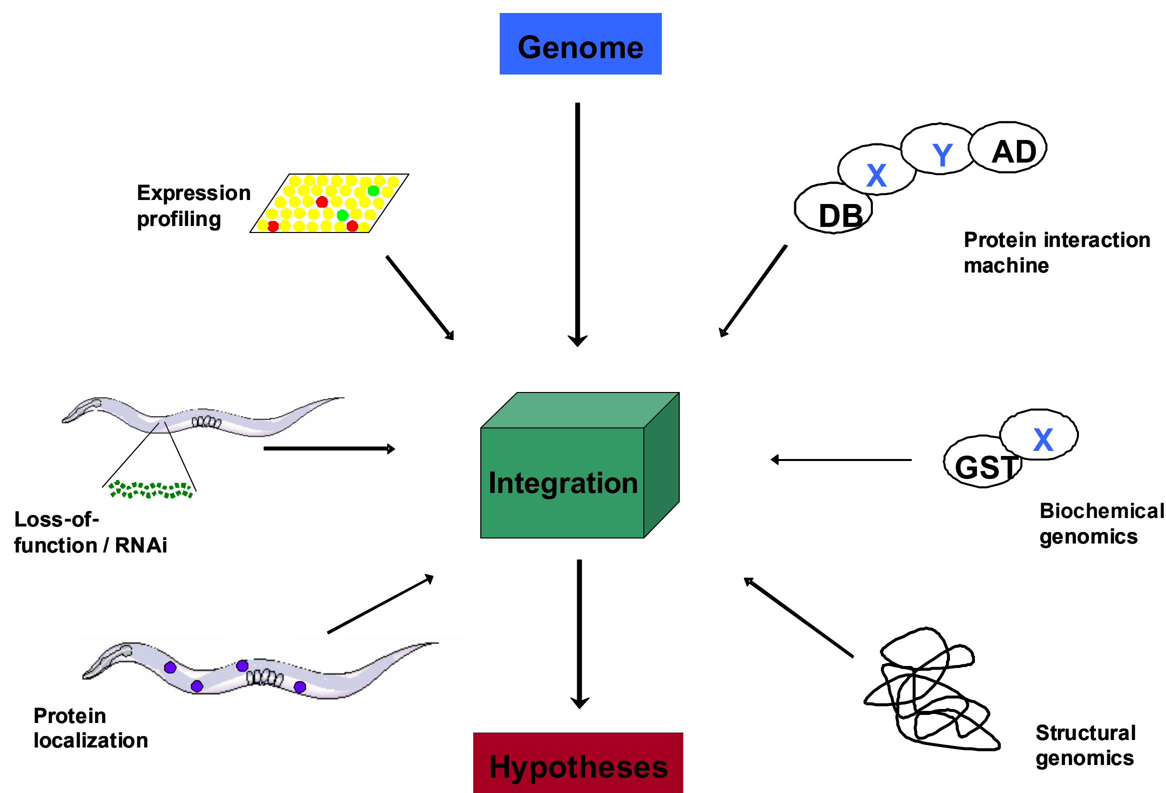


Figure 2: Schematic illustration of a functional genomic approach.
By integrating several large-scale functional genomic projects it should be possible to formulate increasingly meaningful hypotheses.

3. *Caenorhabditis elegans* as a model for functional genomics.

3.1. Some basic knowledge about *C. elegans (7)*.

The nematode worm *Caenorhabditis elegans*, which was the first metazoan to have its genome (100 Mb) sequenced is now taking a leading role in functional genomics.
Indeed, defining the mechanism of the action of Human genes can be extremely difficult, because of the complexity of Human biology and the lack of reliable tools to selectively alter genes *in vivo*, not to mention obvious ethical constraints. The evolutionary conservation from this small roundworm to Human, at the molecular level, is remarkable (about 35 % of *C. elegans* genes have Human homologs (5)), fully justifying the use of this model system.
Importantly, *C. elegans* although it is a primitive organism nonetheless shares many of the essential biological characteristics that are central problems of human biology (6).
Embryogenesis, morphogenesis, development, nerve function, behaviour and aging, and how they are determined by genes: the list includes most of the fundamental mysteries of modern biology. *C. elegans* exhibits these phenomena, yet is only 1 mm long and may be handled as a microorganism (it is usually grown on Petri plates seeded with bacteria). All 959 somatic cells of its transparent body are visible with a microscope, and its average life span is a mere 2-3

weeks. Thus *C. elegans* provides the researcher with the ideal compromise between complexity and tractability.
Thus since entire protein complexes and biochemical pathways appear to be conserved between humans and relatively simple model as *C. elegans*, many laboratories have turned to such organisms, with the goal of establishing functional models that can ultimately teach us about human biology.
Most importantly at a practical point of view, the mutant strains of *C. elegans* can be stored as stocks at -80°C in glycerol, thereby saving the laborious task to maintain living stocks as it is the case for *Drosophila*.

The worm is conceived as a single cell which undergoes a complex process of development, starting with embryonic cleavage, proceeding through morphogenesis and growth to the adult (see figures 3 and 4). It has a nervous system with a “brain” (the circumpharyngeal nerve ring). It exhibits behaviour and is even capable of rudimentary learning. It produces sperm and eggs, mates and reproduces. After reproduction, it gradually ages, loses vigour and finally dies.

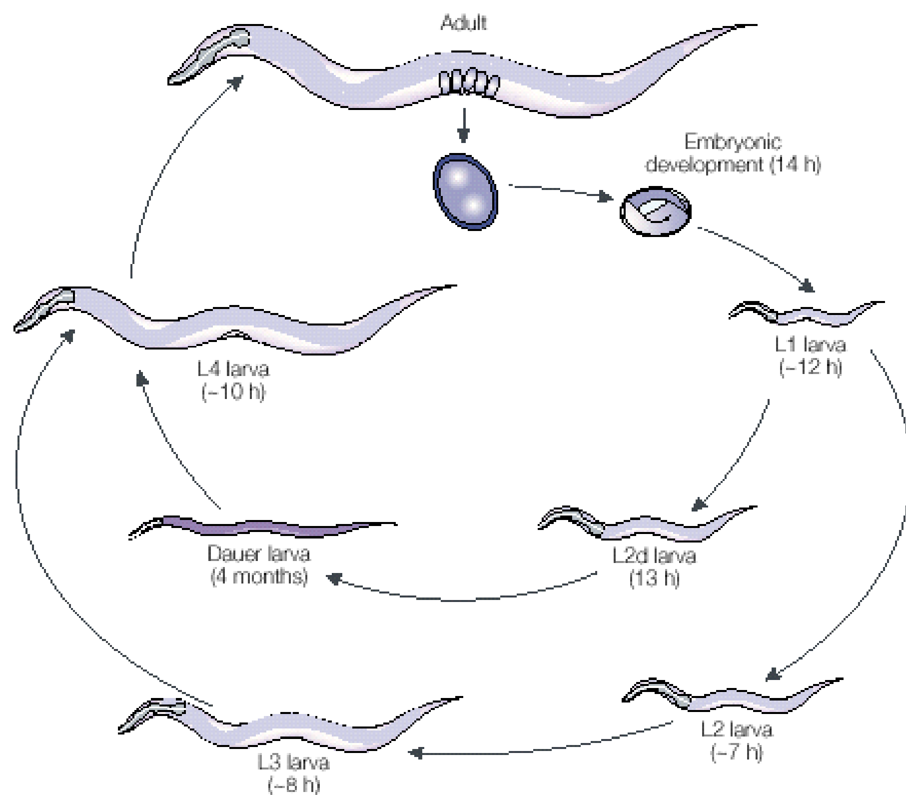


Figure 3: The life cycle of *C. elegans (27)*.

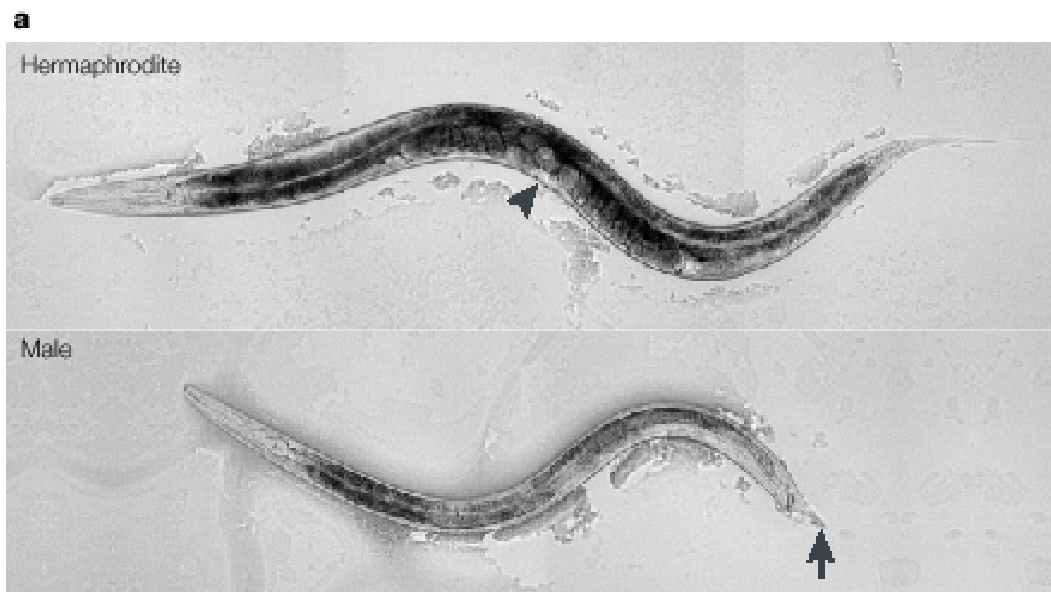


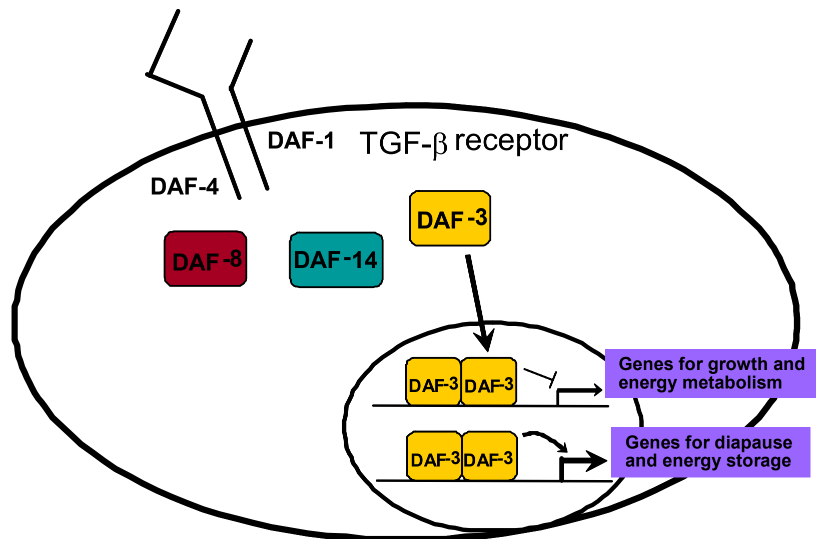
Figure 4: The adult stage of *C. elegans*.
The arrows show the particularity of the hermaphrodite and the male worm. Hermaphrodites can be distinguished by the presence of a vulva (arrowhead); males can be distinguished by the fan-like tail (arrow) (27).

3.2. A homolog to the human TGF-β pathway controls developmental decision in *C. elegans*.

The embryo develops through a series of invariant cell divisions that occur during the first 5 hours of embryonic development at 25 ºC. After about 14 hours of development in the egg case, the larva hatches from the eggshell. The animal then passes through four larval stages (from L1 to L4) (See figure 3). With, high concentration of pheromone (a constituvely secreted substance which is an indicator of population density), high temperature and with limited food, the L1 larvae can enter an alternative developmental stage called dauer stage (a facultative diapause).
The word “dauer” comes from the German: “Dauer”, meaning “endurance” or “duration”. Whereas adult worms have an average life span of 2-3 weeks, dauer larvae survive for months. If given food after such a period, they develop into adults that have a normal life span. For this reason, the dauer larva is considering non-aging. Dauers look different from the other larval stages: they are thinner, relative to their lengths and their bodies are darker (due to nutrient storage granules in the intestinal and hypodermal cells). They also have relatively impermeable cuticles, and are non-feeding (7).
Mutant genes controlling the ability to form dauer larvae interact in a way that allows them to be ordered into pathways. The mutant phenotypes suggest that the pathway corresponds to neural processing of environmental stimuli. A pheromone that influences both entry into and exit from the dauer larva stages have been characterized, and specific chemosensory functions have been correlated with both entry and exit pathways. During dauer larva development, information about the environment, received by sensory organs, is transduced by the nervous system and somehow translated into a developmental transformation.
In studying these processes a number of questions central to biology can be addressed as how is information carried by the nervous system conveyed to the whole organism to bring about a developmental change?

Genetically, there are three pathway that regulate the decision to go or not into dauer stage. One of them is the DAF-7/transforming growth factor (TGF)-β pathway (see figure 5).

(a)



(b)

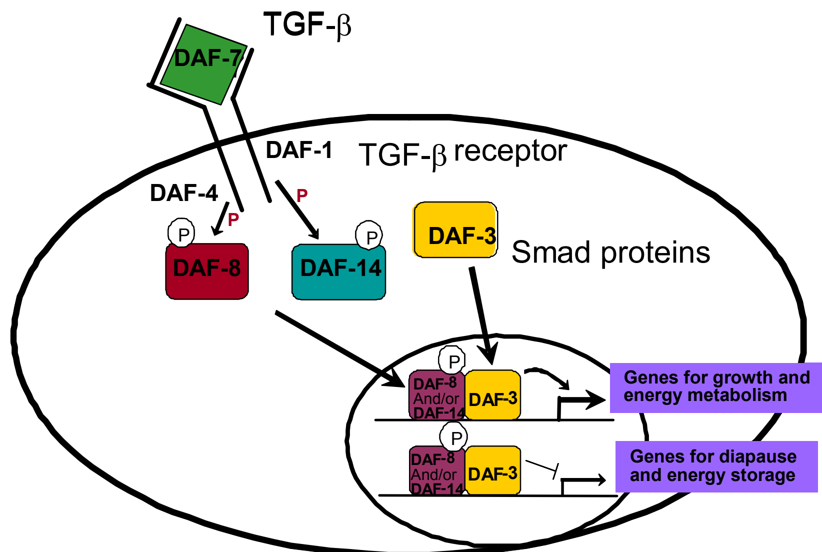


Figure 5: A model of the DAF pathway.
(**a**) Dauer growth induction in absence of DAF-7.
(**b**) Reproductive growth induction in the presence of DAF-7 (8).
For further informations, refer to the text.

Two genes, *daf-1* and *daf-4*, encode transmembrane receptor protein kinases. DAF-4 is a type II receptor in the TGF-β receptor family and DAF-1 is a candidate type I receptor. A third gene, *daf-7*, encodes a secreted protein that is a novel member of the TGF-β superfamily. This protein is a candidate ligand for the DAF-1/DAF-4 receptors. The *daf-4* mutants form dauer

larvae constituvely, and they also have a small adult body size. *daf-1* and *daf-7* mutants share the same dauer phenotype, but have normal body size. Hence, *daf-1*, *daf-4*, *daf-7* are involved in dauer signaling, and *daf-4* is also involved in determination of body size. A set of sma (small) genes specifies other components of the body size pathway.
Transcription of the *daf-7* gene is inhibited by the dauer-inducing pheromone. Gene products mediating the receptor signal include three Smad proteins, encoded by *daf-3*, *daf-8*, *daf-14*. This family of DNA binding proteins acts downstream of TGF-β family receptors.
In absence of the *daf-7* gene, *daf-3* forms a homodimer and, through a signalling pathway, induces the expression of the genes that are required for the dauer state and the energy storage.

This type of pathway is important to study in such model as *C. elegans* because TGF-β in the mammals is a powerful growth inhibitor; it is involved in tumor suppression. The TGF-β type II receptor is usually inactivated in hereditary nonpolyposis colorectal cancer, and mutations in Smad 4 (a homolog to the DAF proteins) occur in 50 % of human pancreatic cancers, presumably through a lack of response to growth-influencing cellular communication signals.

4. General principles and specific tools for the genetic dissection of developmental processes in *C. elegans*.

The general approaches of a biological process are roughly classified, as pointed earlier in the introductory chapter, in two categories. The first one is the so called classical “forward genetics”. Starting from the phenotype, the gene responsible for it is named, mapped and finally cloned. “Reverse genetics” on the contrary goes from the sequences to the function by creating the corresponding mutant and analysing its phenotype. As will be seen below, *C. elegans* offers a powerful combination of both approaches.

4.1. The classical genetic screening approach.

C. elegans came to prominence as an important model system in biology because of its suitability for forward genetics, that is to say, the characterisation of a gene starting from the mutant phenotype. This forward genetic approach was of prominent importance since mutagenesis could be induced and mutagenesis screens yielded collections of mutants with specific phenotypes resulting from the disruption of a specific biological process. Thereby, the mutations responsible for the phenotype identified genes with key roles in the processes. It should be remembered that an unusual feature of this nematode is that it is a hermaphrodite (see figure 4). This means that an interesting mutant can be transferred to a fresh plate and, in three days, the self progeny can be inspected to see if the phenotype breeds true.
However, males are also produced and are essential for moving mutations between strains.

What should be of interest to mention here is that two breakthroughs occurred recently in the field of *C. elegans* research with the effect of completely changing the way genetics can be done. First, *C. elegans* was the firs metazoan whose genome was completely sequenced and, second and quite unexpectedly, the RNAi phenomenon was discovered empirically in the worm. The knowledge of the genome sequence and the availability of the RNAi technique open the way to a very easy "reverse genetics" approach. In addition, if a complete RNAi resource could be established, even whole genome screening approaches become also possible.

4.2. Large-scale functional genomic approaches.

4.2.1. The ORFeome resource.

4.2.1.1. The concept and the technical challenges.

In 1998, the genome of the nematode *C. elegans* was completely sequenced, and about 19,000 proteins encoding ORFs (Open Reading Frame) (see figure 6) were predicted. But at that time, the function of approximately 1,200 has been characterized.

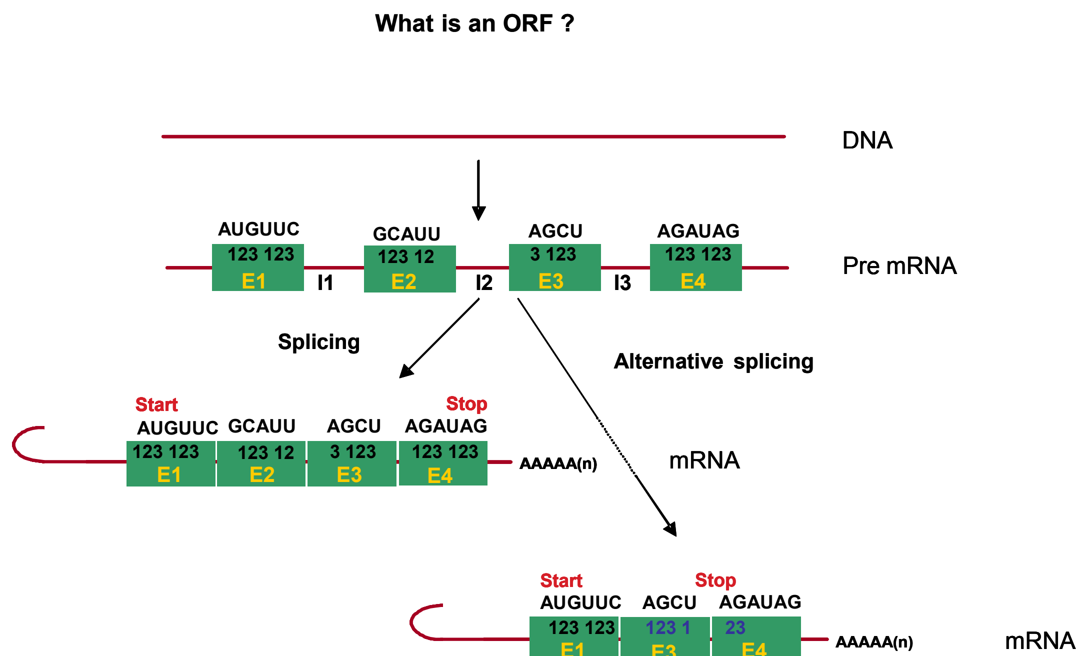


Figure 6: Schematic representation of the production of ORFs by splicing and alternative splicing.
An ORF, at the DNA level, is strictly defined as "a set of triplets between two STOP codons". Usually one considers an ORF as a protein encoding sequence between an AUG and a terminal codon (9).

So, the functional genomics emerging, it is crucial to develop standardized assays and high-throughput procedures that allow the functional characterization of large numbers of genes and /or proteins simultaneously (10,11). As a prerequisite for the characterisation of the

proteins of an organism and their functional interplay in the course of its development and physiology, an important challenge arises. Indeed, one has to be able to express nearly all predicted proteins of a proteome, under different conditions and in various hosts, to allow the development of diverse large-scale functional genomic approaches (cloning in two-hybrid vectors, tagging for localisation immuno-precipitation etc ...). The unambiguous identification of the true "ORFs" from the genome appears as a very first step towards this goal.
So far, the challenges of experimentally finding genes expressed in multicellular organisms have been addressed mainly by large-scale sequencing of random cDNAs, or expressed sequence tags (ESTs) (see figure 7), in the context of various "transcriptome" projects.
However, those full-length cDNA projects are limited (10). Indeed, first, the random cDNA picking limits the detection of genes expressed at relatively low levels. For example, the proportion of *C. elegans* predicted genes that have been experimentally verified by ESTs is about 60 % (although they all have been showed to exist (9)).
Second, randomly picked cDNA clones can rarely be used directly for protein expression, because their 5' end is not cloned in the appropriate reading frame, their 3' end is not compatible with the expression of C-terminal fusion proteins or both. In addition, for diverse large-scale functional genomic approaches which use the expression of fusion's proteins between the protein of interest and a tag, it is very important to control the sequence of the cloned DNA and particularly to determine the open reading phase and the presence or not of the Stop codon.

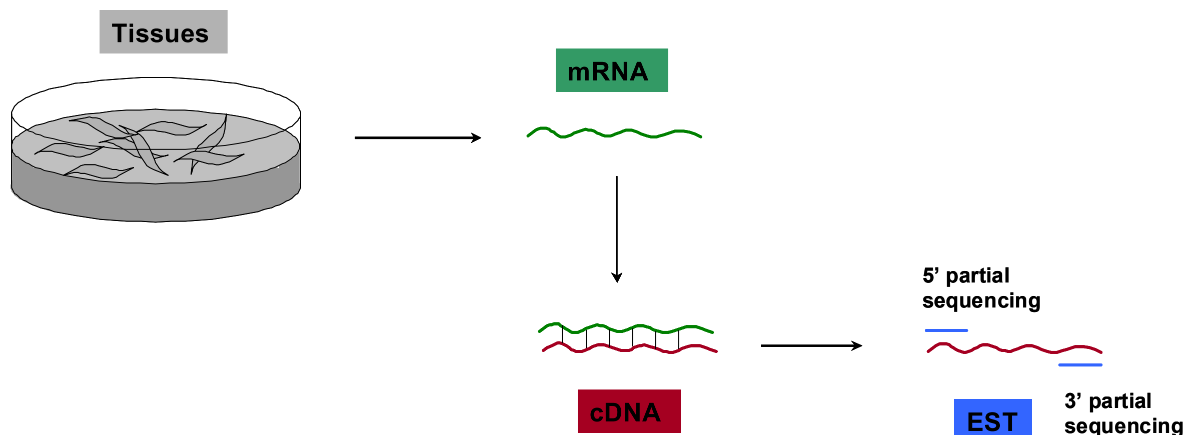


Figure 7: Scheme representing the major steps of the EST project.
The EST project consists on generating a sequence tag from a cDNA in order to verify the existence of the transcriptome.

And finally, most cDNAs identified in transcriptome projects are not available in vectors that allow the protein-encoding sequences to be transferred to a variety of expression vectors by automated, high throughput methods. Projects using predicted ORF sequences as a starting point for the exogenous production and characterization of the corresponding encoded products have been limited so far mainly because it remains technically difficult to clone, precisely, a large number of ORF into different vectors. To resolve these three problems and to verify experimentally the information predicted by the *C. elegans* Sequencing Consortium and simultaneously clone the ORF in different expression vectors, the ORFeome team of the Vidal's lab designed an alternative strategy using the Gateway™ technology. The Gateway™ recombinational cloning provides a solution for large-scale cloning of ORFs into many different expression vectors.

4.2.1.2. The way to clone an ORFeome: the Gateway™ procedure.

The Gateway™ recombinational cloning (12) mimics the recombination reactions which integrate or excise the phage λ into the *E. coli* genome (see figure 9).
The integration involves the recombination of the *att*P site of the phage DNA with the *att*B site located in the bacterial genome. This generates an integrated prophage genome flanked by *att*L and *att*R.
The integration reaction requires two enzymes: the phage protein integrase (Int) and the bacterial protein integration host factor (IHF) (referred as "BP clonase").
One of the main advantages of the Gateway™ cloning is that the recombination reaction is reversible. The phage DNA can be excised from the bacterial genome by recombination between the *att*L and *att*R sites. This excision reaction requires Int, HIF, and excisionase (Xis), an additional phage enzyme (referred as "LR clonase").
To make the Gateway™ useful, the recombination reactions have been modified in different ways (see figure 8).

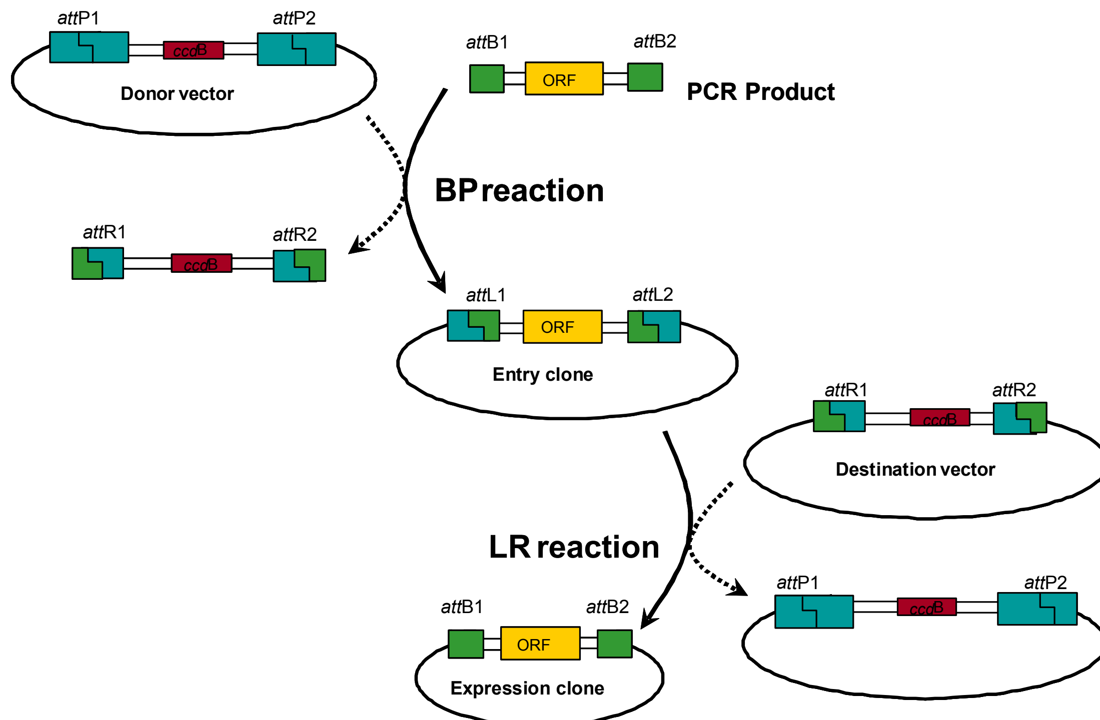


Figure 8: The scheme for ORF cloning by Gateway™ is as follow: a PCR product containing the ORF of interest is recombined into a "Donor vector", using the BP reaction. The resulting "Entry clone" is then used to recombine the ORF by the LR reaction into one or more "Destination vectors", generating "Expression clones" (13).

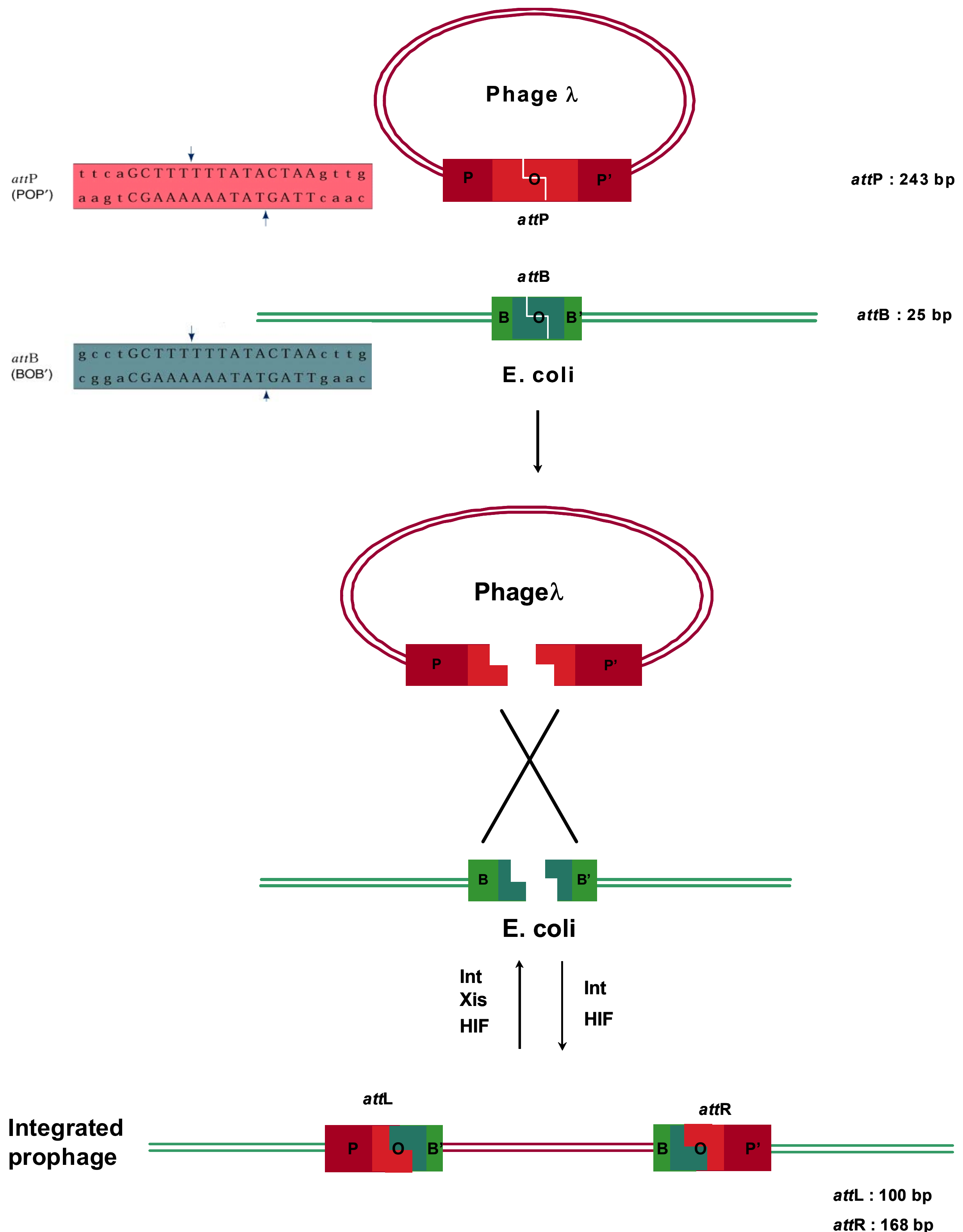


Figure 9: The phage lambda recombination in *E. coli*

First, both BP and LR clonases have been purified, allowing the Gateway™ reactions to take place *in vitro*. Second, the *att* sites have been mutated to generate pairs of derivatives: *att*B was modified into *att*B1 and *att*B2, *att*P into *att*P1 and *att*P2, *att*L into *att*L1 and *att*L2, and finally *att*R into *att*R1 and *att*R2. These sites were designed to allow the recombination

reaction to take place only between *att*B1 and *att*P1, *att*B2 and *att*P2, *att*L1 and *att*R1, *att*L2 and *att*R2. So the duplication of *att* sites allows two independent recombination reactions to take place in the same molecules, one at the 5' end of the ORF and the other at the 3' end. Third, the sequence of the *att*B1 and *att*B2 sites was selected such that each frame is open and thus both N- and C- terminal fusion proteins can be generated.

Usually, the coding sequence (ORF) is amplified from a cDNA library by PCR with a DNA polymerase having a high fidelity. Those ORF amplified by PCR are cloned in a donor vector. We can then talk about ORFeome to designate the library of cloned ORF thus generated. Because the Gateway™ reaction is reversible, the cloned fragments can be next sub-cloned very simply in destination vectors chosen according to the functional study which is wished to be done (see figure 10).

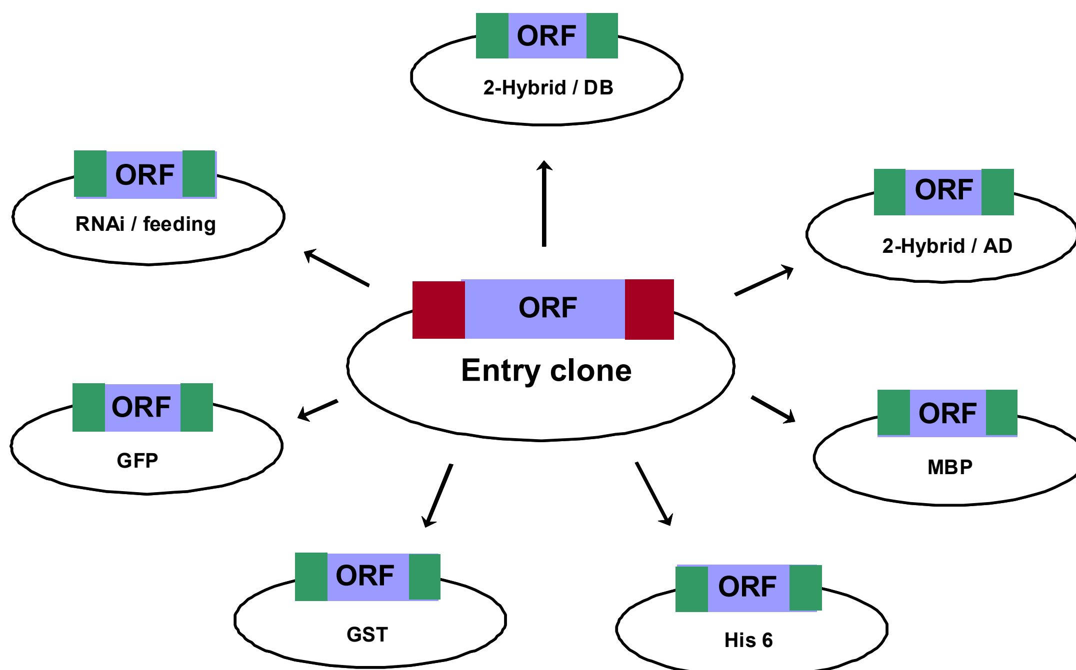


Figure 10: Scheme representing the main advantage of the GATEWAT™ technology: the subcloning.
Once an ORF is cloned into an entry vector, it can be transferred by recombination cloning into different destination vectors including the AD, the DB, the pL4440 for the RNAi, GFP, GST, ... and other vectors.

Many proteins require interactions with other proteins to function properly. So the identification of interactions partners of a protein of known or unknown function might help to understand its molecular role. Combination of such interaction data and results obtained from other functional approaches (protein localization, developmental expression p*att*erns and deletion analysis) should allow valuable functional predictions. The objective of the Vidal's lab is to construct a protein interaction map for the *C. elegans* proteome. They choose *C. elegans* as a model organism because it was the first multicellular organism for which the complete genome was sequenced.

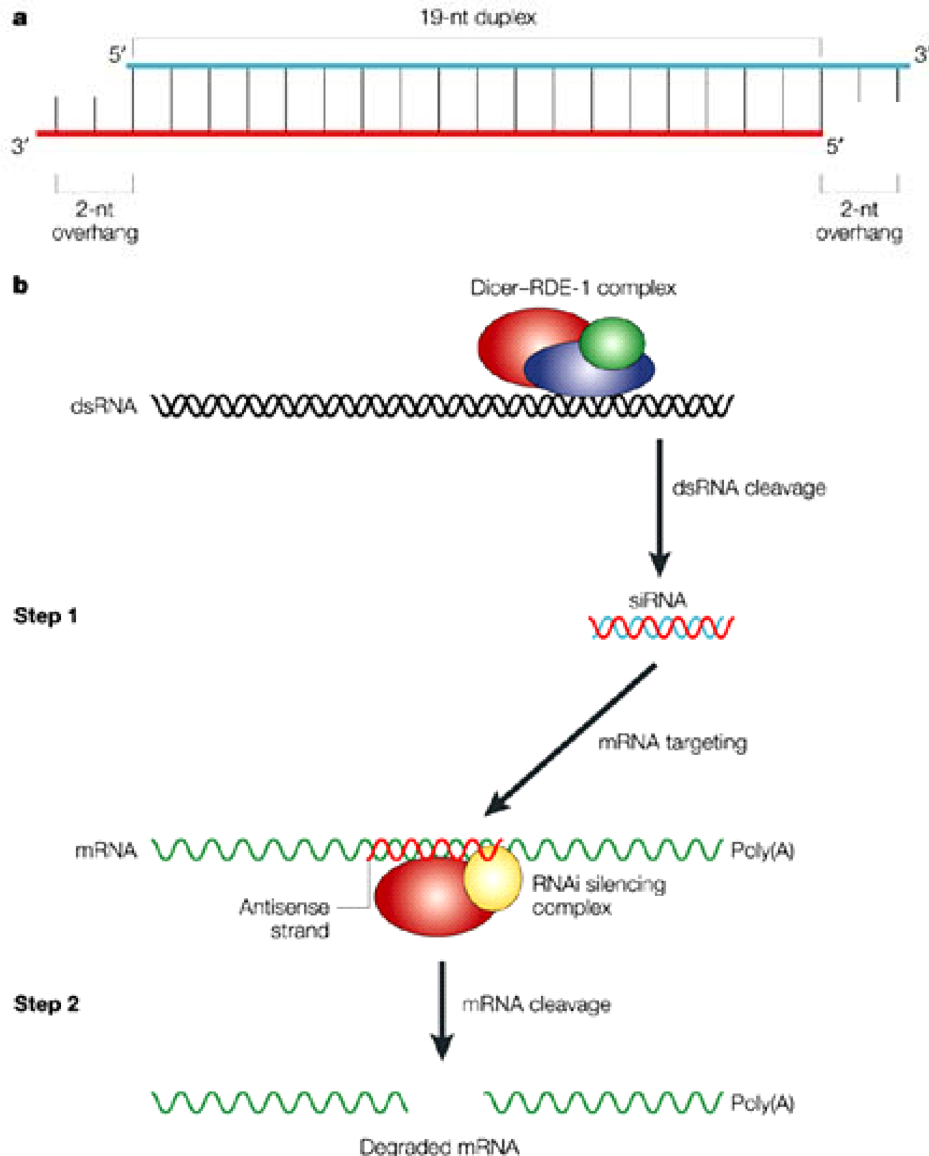


Figure 11: Scheme representing the processes of mRNA degradation by short interfering RNAs (siRNAs).

a) siRNAs typically consists of two 21 nucleotide (nt) single-stranded RNAs that form a 19-bp duplex with 2-nt 3' overhangs.

b) Long double strand RNAs (dsRNA; typically > 200 nt) can be used to silence the expression of target genes in a variety of organisms and cell types.

Upon introduction, the long dsRNAs enter a cellular pathway that is commonly referred to as the RNA interference (RNAi) pathway. First, the dsRNA get processed into 20-25 nucleotide (nt) small interfering RNAs (siRNAs) by an RNase III-like enzyme called Dicer (it's the initiation step). Then, the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs). The siRNA strands are then unwound to form activated RISCs. The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA (this is the effecter step) (14)

4.2.2. The RNAi-ome resource.

In 1998, and almost at the achievement of the *C. elegans* genome project, Andrew Fire and Craig Mello made a surprising discovery (15) which revolutionized the *C. elegans* "reverse genetics": by the introduction of a double strand RNA (dsRNA) including coding sequences of almost any known gene we are able to disrupt specifically the function of that gene. In many species, expression of a particular dsRNA sequence has been shown to cause the specific degradation of the corresponding mRNA, resulting in nearly complete loss-of-function phenotypes (16) (see figure 11).
This discovery of RNAi silencing is related to previous work on post-transcriptional gene silencing (PTGS) in plants and the so called "quelling" in fungi.

RNAi works exceptionally well in *C. elegans*, and the dsRNA can be delivered by injection, by soaking worms in a solution of dsRNA, or by feeding (17) the worms with bacteria expressing dsRNA (see figure 12).

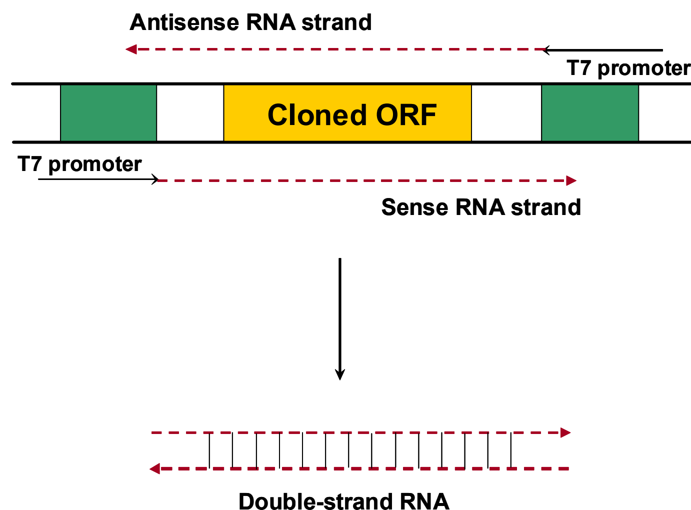


Figure 12: Scheme representing the production of dsRNA by the T7 promoters.
RNA is easily established by action of a RNA polymerase that recognizes phage promoters in expression vectors. The phage T7 promoters enable the expression of two complementary strands. So the T7 promoter on the left can drive the expression of an antisense RNA strand whereas the other T7 promoter makes a sense RNA strand. Those two strands anneal to make double-stranded RNA.

Actually, the dsRNA appears to disperse freely within the worm. Hence it is not necessary to inject dsRNA directly into the gonad to get progeny that exhibit RNAi effect. This indicates that the introduced dsRNA species must be able to move across cell boundaries freely. Even more astonishing, nematodes can be soaked in dsRNA or can be fed plasmids that make dsRNA and consequently exhibit RNAi effects.
The entrance of RNAi around the time of fertilization leads to the synchronised depletion of both maternal and zygotic mRNAs. This has advantages for cell biological studies, because all gene functions are interrogated at the same developmental point, whereas early developmental defects can be missed with conventional mutations because of persistent maternal mRNAs (18).

Delivering dsRNA by feeding is particularly useful, because it is less labour intensive than micro-injection and could thus be used in high throughput experiments.
Unfortunately, RNAi doesn't always work. As described by the Ahringer and Hyman groups (18- 20), only 50 % of genes with known phenotypes that should have been detected were detected in the RNAi screens.
Moreover, there has been a general concern that some tissues are more resistant to RNAi than others: neurons and sperm are resistant to RNAi.
Although, as already mentioned in the introduction chapter, RNAi is able to induce a "Knock down" of a target gene at once and is thus considered as a "reverse genetics" tool, the complete RNAi library resource offers a mean to perform genetic screens as usually accomplished in classical "forward genetics".
Many studies have illustrated the power of RNAi (18-21).
Therefore, in order to make high-throughput studies, the Vidal's lab decided to create an RNAi library which will allow large-scale RNAi screenings.
It should be underlined that such genetic screens using RNAi have an advantage over conventional mutagenesis approaches, as the genetic map position and molecular nature of the genes are already known. So a mutant phenotype can immediately be related to the sequence of the gene that induced it. Unlike a classical genetic screen, where positional cloning of the mutated gene can take months, the molecular nature of genes identified in an RNAi-based reverse genetics screen is known at the onset. Of additional invaluable interest is the possibility to perform double perturbation experiments in *C. elegans*, e.a to apply a RNAi procedure for one target gene in a strain already mutated (LOF) for another gene. This kind of analysis will yield the epistatic relations that could exist between the tested genes, as usually done (but in this case it's a very painful task) in classical genetics.
Of course the data obtained by these combined genetic approaches will be an incredibly useful resource for those working directly on *C. elegans*. And since basic gene functions are conserved, the data will also be informative for those working on related genes in other species.

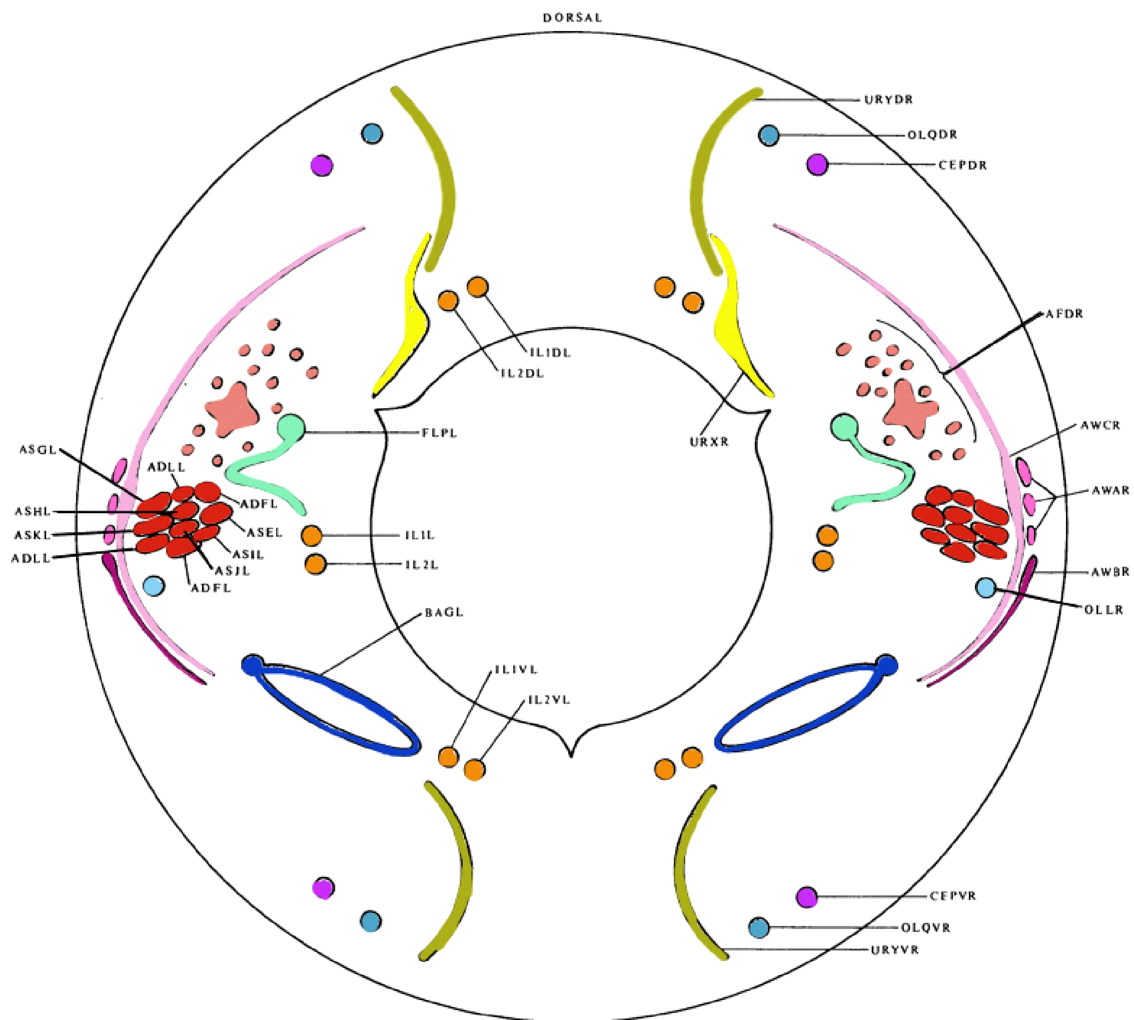


Figure 13: Sensory receptors in the head (in an idealized section, near the tip of the head) (22).

5. The aim of the project.

The nematode *C. elegans* provides an excellent model system in which we can study the mechanisms by which olfactory behaviours are modulated. Nematodes exhibit sensitive olfactory responses to multiple chemicals using a small and well-defined number of chemosensory neurons. The DAF-7 TGF-β pathway which controls the dauer developmental "decision" is also involved in the expression of chemoreceptors known to be expressed in the ASI chemosensory neurons, as well as chemoreceptor expressed in the ASH sensory neurons.
The project is actually an example of gene screening. We will use strains that contained a promoter of a candidate chemosensory receptor gene that drives the expression of a GFP reporter in different sensory neurons in the head (see figure 13) and in the tail. GFP expression is thus the reporter of the activity of the candidate receptor gene and can be used as a read-out of the transcriptional regulatory control exerted upon this gene. By RNAi we thus will search for the genes involved in the regulation of this chemosensory receptor in the neurons. This regulation will be evidenced by a change in the level expression of the GFP (see figure 14). The candidate regulatory genes will be chosen in the known DAF-7 TGF-β pathway, but we are also interested to test possible function of some two-hybrid interactors of this pathway previously discovered in the lab(see figure 23).

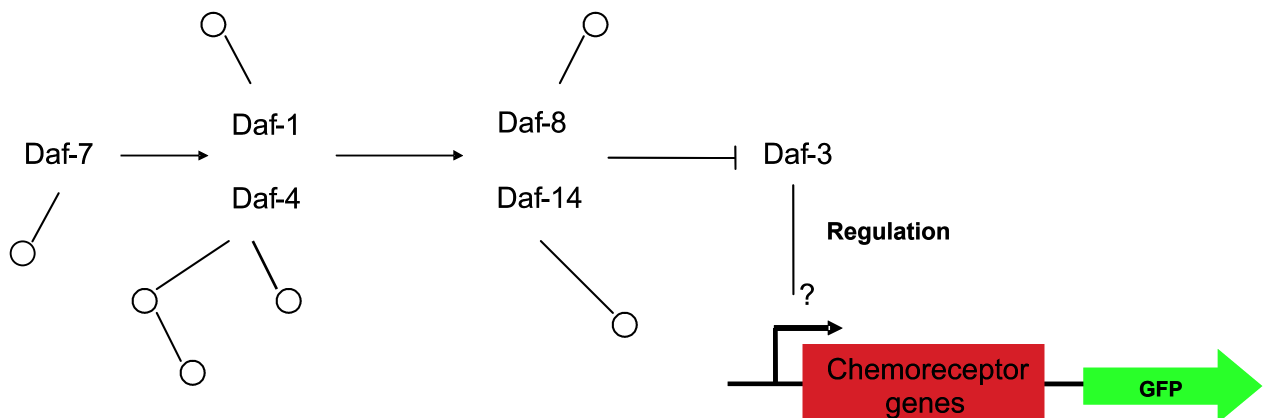


Figure 14: Model for regulation of chemoreceptor expression.
We would like to show a diminution or an augmentation (depending on the positive or negative regulation of the chemoreceptor gene by the DAF-7 pathway) by knocking down the genes involved in the DAF pathway (or genes (open circles) of proteins interacting in a two-hybrid test with the *daf* genes) using RNAi.

Results and discussion

Results and discussion.

The results I present here will be divided in two parts. The first one reports the establishment of an RNAi resource corresponding to the complete ORFeome of *C elegans*. Since the ORFeome is the prerequisite for obtaining a complete RNAi library, we briefly recall what the ORFeome is and how it was used in the making of the RNAi-ome. Since at the time I joined the Vidal lab, this work was nearly achieved; my contribution was limited in helping the final set up of the resource.

The second part of this report is devoted to the results we obtained in using this resource as a tool to revisit the TGF-β pathway.

1. The establishment of the RNAi resource.

1.1. The ORFeome project, the first step to the constitution of the RNAi library.

The genome sequences of *C. elegans* have been predicted by the GeneFinder to contain about 20,800 ORFs. 12,376 ORFs have been verified by ESTs (the so called "touched" ORFs). But 8,424 ORFs were at that time still unconfirmed.
Those ORFs might have been undetectable with the EST method because they belong to a class of weakly expressed genes.
Considering this problem, the Vidal's lab reasoned that the ORFeome strategy should be more sensitive than the EST approach because it is based on a PCR amplification of the ORFs, starting from a cDNA library established as already explained

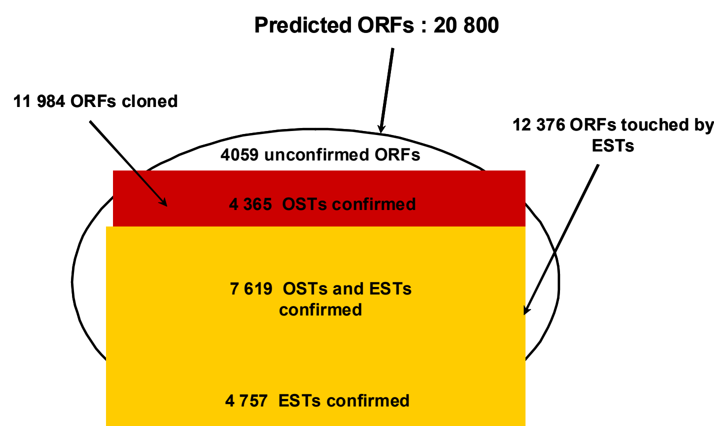


Figure 15: Scheme showing the overall distribution of the total predicted number of ORFs into four distinct categories, namely the confirmed ESTs, the OSTs and EST confirmed, the confirmed OST and the unconfirmed ORFs (11).

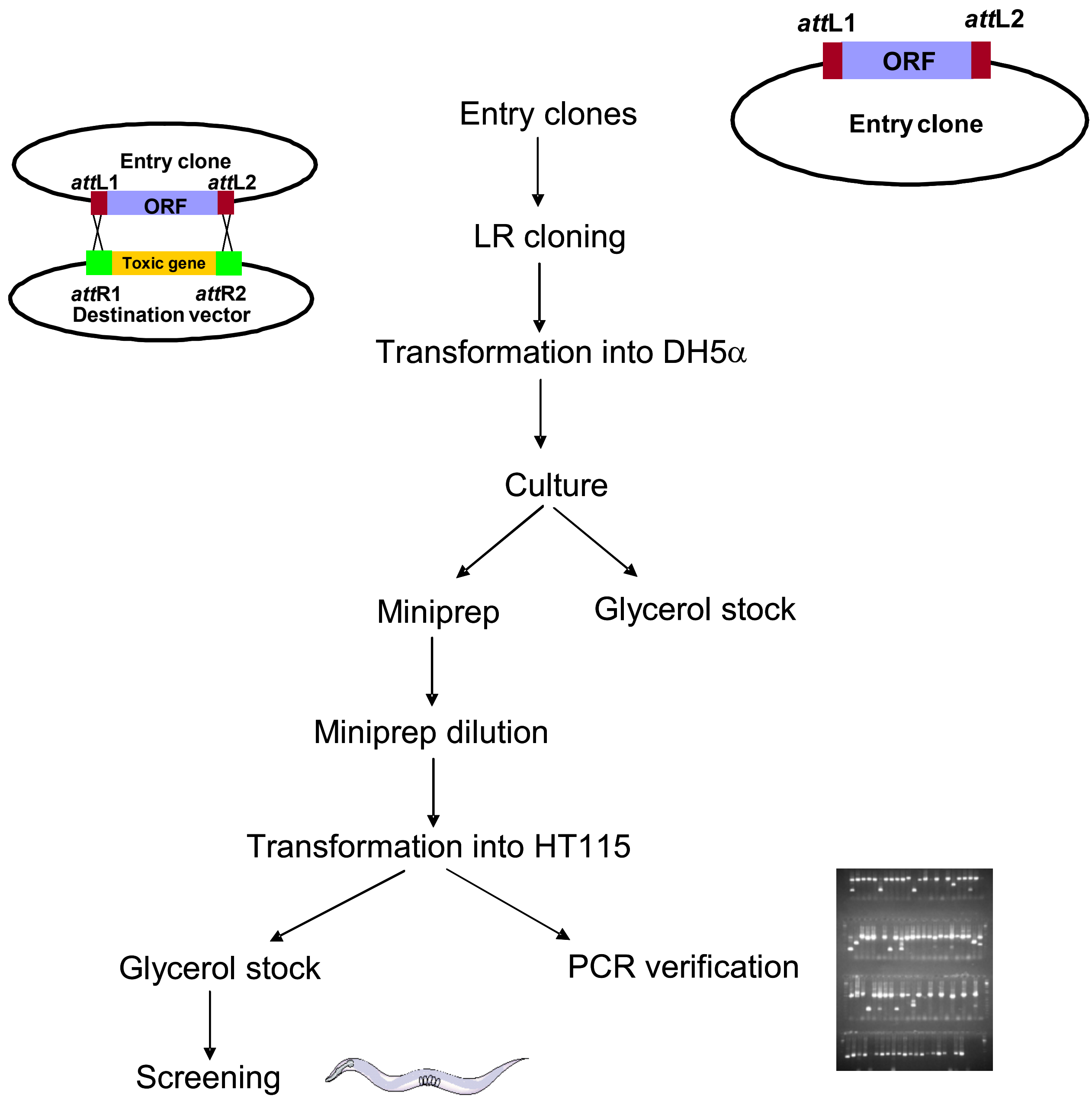


Figure 16: General flow chart of the constitution of the RNAi library.

Indeed, the first result of the *C. elegans* ORFeome was the experimental proof of the existence of 4,365 ORFs that had not been identified yet by any EST. This increased the number of *C. elegans* genes experimentally confirmed up to, 16,741 (4,365 previously untouched ORFs cloned by the ORFeome team in the Vidal's lab plus 12,376 ORFs already touched by ESTs) (see figure 14)(11).
Furthermore, it has been noticed that the OSTs are in the right reading frame and so the entire coding sequence of the organism is cloned and available for expression tagging.
Nevertheless, although the OST is therefore a tool of importance for functional genomics, this resource can be established, as already mentioned, only if a high quality sequence and very good ORF predictions for the genome of interest are available.

1.2. Experimental procedures to establish the RNAi resource (See figure 16).

1.2.1. The Gateway™ technology.

As mentioned in the introductory chapter, the ORFeome project for *C elegans* was based on a main technique which is the Gateway™ technology. This technique is a universal cloning and expression platform that provides a rapid and high efficiency route to protein expression, functional analysis, and transfer of DNA segment across multiple vectors. Gateway™ Technology uses lambda phage-based site-specific recombination instead of restriction enzyme to insert a gene of interest into an expression vector. The DNA recombination sequences (*att*L, *att*R, *att*B and *att*P) and the LR and BP clonase enzyme mixtures are the basics of the Gateway™ Technology. Transferring a gene into a destination vector is accomplished in just two steps: the first step is to clone the gene of interest into an entry vector (see upstream in the text). The second one is to transfer *in vitro* the gene of interest from the entry clone to the appropriate destination vector using the Gateway™ LR clonase enzyme mix (Int, HIF, and excisionase (Xis)). Site-specific recombination between the *att* sites (*att*B x *att*P ↔ *att*R x *att*L) generates an expression vector clone and a by-product. The expression clone bears the gene of interest in the recombined destination vector backbone. Following transformation and selection in *E. coli*, the expression clone is ready to use for expression in the appropriate host.
The Gateway™ Technology saves time, increases productivity, makes possible the cloning without the need for restriction endonuclease or ligase, and, most importantly, it allows the transfer of DNA sequences into multiple destination vectors in parallel while maintaining reading frame and directionality.
So the first part of the project was to design the corresponding primers for each ORF. An important feature of those primers is that they are tailed on each side with the 25 nucleotides stretch (*att*B1 and *att*B2), to allow the recombination with the vector P201DNR in which the ORFs are to be cloned (see figure 17).

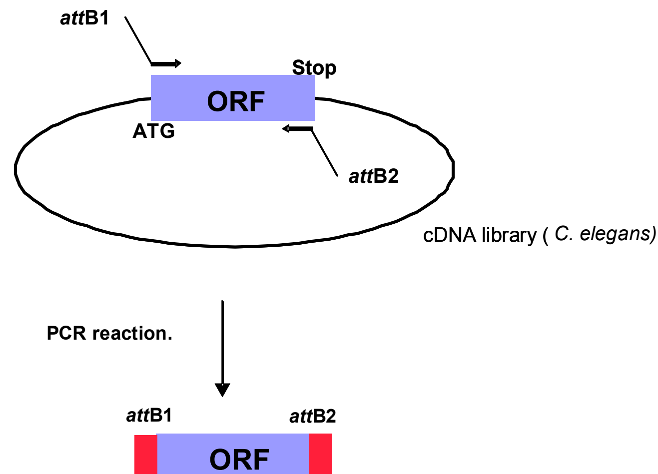


Figure 17: Initial PCR amplification of a set of ORF Gateway™ compatible from a cDNA library.

Once the amplified product obtained, the homologous recombination is performed between the specific tails *att*B1, *att*B2 of the ORF and the Pl and P2 sites of the donor vector, to integrate the ORF in the vector (replacing the *ccdB* toxic gene in P201DNR). It is important to notice that the efficiency of the recombination is size dependent. So the bigger the ORF is, the less efficient the cloning. This reaction is catalyzed by the BP clonase (Int, IHF) (see figure 18).

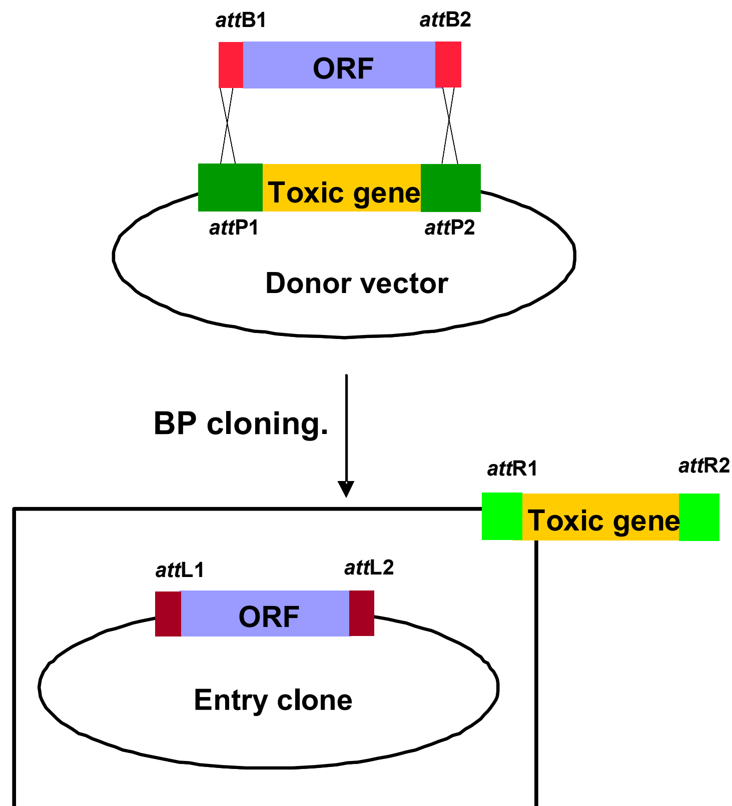


Figure 18: BP cloning reaction of the amplified ORFs.

The presence of the *ccdB* gene allows negative selection of the donor and destination vectors in *E coli*. The CcdB protein interferes with *E. coli* gyrase (23), thereby inhibiting growth of most *E. coli* strains. Thus, because of the lethal effects of the CcdB protein, all the Gateway™ vectors containing the *ccdB* gene must be propagated in an *E. coli* strain which contains a gyrase mutation that renders it resistant to the ccdB effects.
When recombination occurs between a destination vector and an entry clone or between a donor vector and an *att*B-PCR product (our case), the *ccdB* gene is replaced by the gene of interest. This allows high-efficiency recovery of the desired clones. Indeed, cells that take up unreacted vectors carrying the *ccdB* gene or by-product molecules retaining the *ccdB* gene will not grow which means that they have been negatively selected.
For a high throughput, and to obtain the maximum number of cloned ORFs, especially for the longer than 1 kb (the efficiency of the cloning is size dependent), high efficiency chemically competent DH5a cells (10^8 colonies / µg pUC19), which are sensitive to the effect of CcdB, are used.

1.2.2. PCR verification of the products obtained.

After the transformation, a plasmid DNA miniprep was performed with the Biorobot 96000, using the Qiagen 96-well kit.
A PCR verification with the HiFi Taq polymerase (from Invitrogen) was done on 3 µl on the DNA preparation using external primers localized right before L1 and right after L2 on the vector:

- P201DNR For : 5’ TGGATAACCGTATTACCGCC 3’
- P201DNR Rev : 5’ GTTTTCCCAGTCACGACGTT 3’

The analysis of the PCR product enables to control the size of the ORF cloned (the size observed should correspond to: expected size of the predicted ORF + 2 x 100bp for *att*L1 and *att*L2) and the quality of the cloning (the presence or not of by-products). The PCR products will be also used for sequencing and so the ORF will be confirmed by the OST method.

1.2.3. The LR reaction in order to establish the RNAi library.

Once the ORFeome library is established, it is now possible to use it and for example to make a library of RNAi. To that purpose, we used the major feature of the Gateway™ technology: we transfer the entry clone into a destination vector (see figure 19) that can express the RNAi.

So the first part of the constitution of the RNAi library is to perform the LR reaction which is the homologous recombination between the tails *att*L1, *att*L2 of the P201DNR and the *att*R1 and *att*R2 sites of the PL4440DEST, replacing the *ccdB* toxic gene into the destination vector. We used the PL4440DEST (24) because it contains the bacteriophage T7 RNA polymerase gene, under the transcriptional control of the *lac* promoter induced by addition of IPTG, and therefore allows the expression of dsRNA corresponding to the gene inserted into the expression clone.
The products are transformed in the DH5α bacteria for the reasons of efficiency already mentioned.

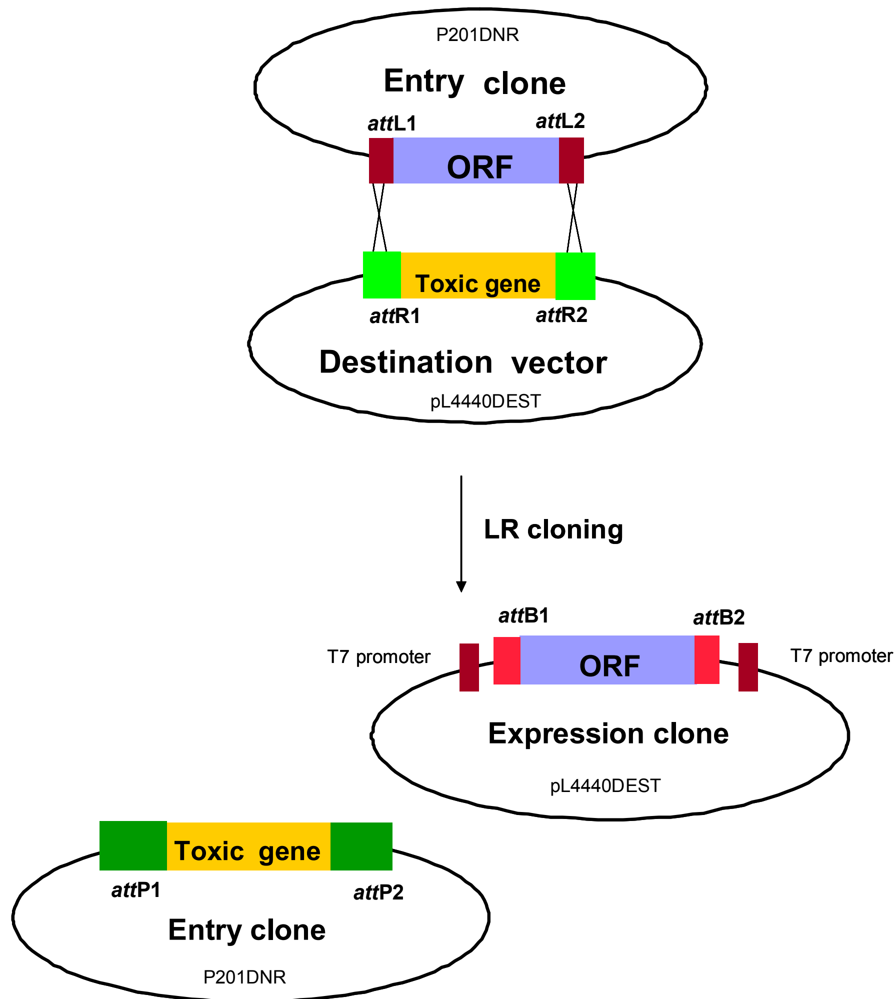


Figure 19: LR cloning reaction of the amplified ORFs.
The ORF was cloned in an expression clone containing two T7 promoters in order to express the dsRNA.

1.3. Results obtained for a sample of RNAi clones.

1.3.1. Results of the transformations in DH5α.

The table 1 is an example of the presentation of the transformation's results of the destination vectors into DH5α.
The homogeneity of the liquid culture allowed the validation of the results as well as the control of the efficiency and the number of colony on solid plates.

Plate	Type of control on solid plate	Concentration of pUC (ng/ml)	Efficiency (average) (number of colonies/mg)	Number of colonies	Liquid culture
TRF-057	G12	0,04	$2{,}5.10^7$	864	Looks good
TRF-058	F12			2400	Looks good
TRF-061	G12	0,04	$2{,}5.10^7$	1040	Looks good
TRF-063	F12			4000-5000	Looks good
TRF-066	G12	0,04	$2{,}5.10^7$	696	Looks good
TRF-043	G12	5	7.10^6	~1400	Looks good
TRF-059	F12				Looks good
TRF-060	G12	5	7.10^6	~1400	Looks good
TRF-068	F12				Looks good
TRF-069	G12	5	7.10^6	~1400	Looks good
TRF-070	F12				Looks good
TRF-071	G12	5	$3{,}5.10^7$	~7000	Looks good
TRF-072	F12				Looks good
TRF-074	G12	5	$3{,}5.10^7$	~7000	Looks good
TRF-077	F12				Looks good
TRF-078	G12	5	$3{,}5.10^7$	~7000	Looks good
TRF-079	F12				Looks good
TRF-081	G12	5	$3{,}5.10^7$		Looks good

Table 1: Presentation of the validation of the transformation's results of the expression clone into DH5α.
The plates received an identity: the name of the products they contain and a number to order them. The transformations are grouped by experiments. The plates TRF-057, TRF-058, TRF-061, TRF-063, and TRF-066 were done the same day with the same conditions.
As an example of the results we got, the transformation of the plate TRF-058 (TRansFormation-ID number) gave us an efficiency of $2{,}5.10^7$, using pUC 19 at the concentration of 0,04 ng/ml, and 864 colonies appeared on the solid plate.

1.3.2. Results of the transformation in HT115.

The DH5α bacteria are the typical bacteria used for cloning and are highly efficient but we need a strain that enables the production of the dsRNA. We used the bacteria *E. coli* HT115 which is RNase III deficient, it has an IPTG-inducible T7 polymerase activity. In addition, there is the Tn10 transposon interrupting the rncl4 gene and this transposon carries a tetracycline resistant gene. The bacteria should thus be subjected to tetracycline selection to maintain the RNase deficiency.
To transform the miniprep products into HT115, we proceeded the same way than for the transformation in DH5α. The results of a sample of transformations are shown in the table 2.

Plate	Type of control on solid plate	Concentration of pUC (ng/ml)	Efficiency (average) (number of colonies/mg)	Number of colonies	Liquid culture
TRF-023	G12	5	1.10^2 (too low)	~1	Not homogenous
TRF-044	F12			~10	Not homogenous
TRF-051	G12	5	1.10^2 (too low)	~1	Not homogenous
TRF-062	F12			~10	Not homogenous
TRF-039	G12			372	Looks good
TRF-039	G12	5	Too low	1	Looks good
TRF-044	F12			12	Looks good
TRF-044	G12	5	Too low	0	Looks good
TRF-039	F12	5	$\sim 1.10^3$	1	Looks good
TRF-041	G12			23	Looks good
TRF-044	F12	5	$\sim 1.10^3$	6	Looks good
TRF-046	G12			230	Looks good
TRF-047	F12	1	$\sim 1.10^3$	0	Looks good
TRF-051	G12			1900	Looks good
TRF-054	F12	5	$\sim 1.10^3$	11	Looks good
TRF-062	G12			400	Looks good
TRF-048	F12	5	$\sim 1.10^3$	4	Looks good
TRF-052	G12			40	Looks good
TRF-058	G12	5	$\sim 1.10^3$	12	Looks good
TRF-061	G12			145	Looks good
TRF-041	F12	5	5.10^3	82	Looks good
TRF-043	G12			161	Looks good
TRF-057	G12	5	5.10^3	16	Looks good
TRF-059	F12			153	Looks good
TRF-060	G12	5	5.10^3	43	Looks good
TRF-063	F12			0	63 F12 to redo
TRF-066	G12	5	5.10^3	49	Looks good
TRF-068	F12			632	Looks good
TRF-070	G12	5	6.10^4	860	Looks good
TRF-072	F12			3760	Looks good
TRF-072	G12				No contamination
TRF-074	F12	5	6.10^4	428	Looks good
TRF-077	G12			~4000	Looks good
TRF-077	G12				No contamination
TRF-079	G12	5	6.10^4	468	Looks good
TRF-081	G12			~4500	Looks good
TRF-081	G12				No contamination

Table 2: Presentation of the validation of the transformation's results of the Miniprep products into HT115.
The transformations are grouped by experiments and were done the same day with the same conditions.
We didn't keep the results of the TRF-051. During the preparation of the glycerol stocks, the GenMate robot made a mistake and we didn't want to take the risk of any contamination.
The transformation of the plate 039, 044, 051, 062 was too less efficient. Even if the liquid cultures look good, we will have to redo it.
We did a test Tet^R for the plates 048, 052, 058, 061. We plated on solid LB + Tet, 4 transformations of the MPR products in HT 115 and also one Entry clone as a negative control. The results were as following: the bacteria containing the MPR products grew but nothing appeared on the place of the Entry clone. We thus validated the results.
The plates 070, 072, 074, 077, 079, 081 gave a high efficiency of transformation. To justify these results, there is the hypothesis that it is due to the freshness of the cells. Indeed, those competent cells were freshly done compare to the others that were kept at -80°C for days.

From the culture, we extracted 3 µl to perform a PCR verification on the transformants to make sure that the product has the predicted size. The remaining part of the liquid culture is stored as a glycerol stock at -80°C.

1.3.3. PCR control of the amplicon's size obtained.

To control the size of the ORF cloned, we compared the size of the amplicons obtained with the size of the predicted ORFs. The size observed should correspond to the expected size of the predicted ORF + 2 x 100 bp for *att*B1 and *att*B2.
The table 3 and the figure 20 show an example of the analysis of the results of the PCR. When the results we got didn't match with the size expected we performed the PCR until we amplified the right products.
The table 4 presents a summary of the results of each PCR done.

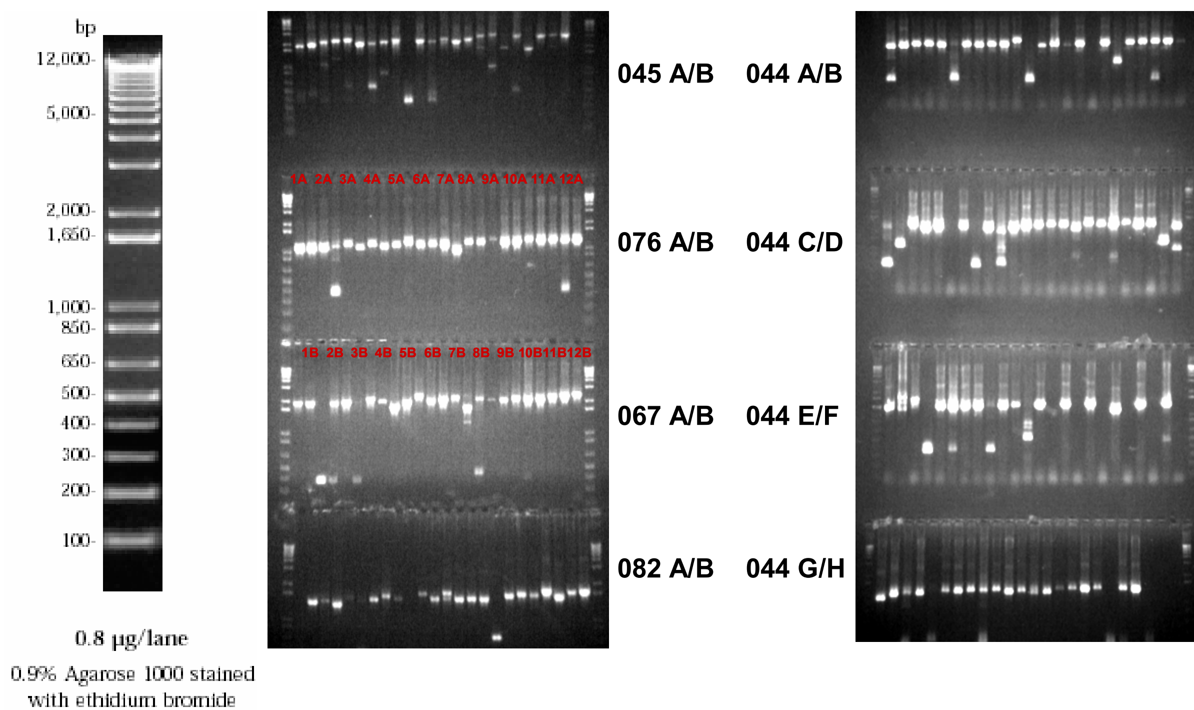


Figure 20: An example of results obtained for a PCR verification on HT115 -045, -067, -076, -082, -044.

Plate	#	Name of the ORF	Size of the ORF obtained (bp) (approximation)	Size of the predicted ORF (bp)
PCR ver-oo-HT115-067 (A [1-12] / B [1-12])	A1	ZC443.6	1800	1605
	A2	Y111B2G.J	To redo	1695
	A3	Y32B12C.3	1900	1671
	A4	Y113G7B.14	1800	1623
	A5	Y105E8E.t	1900	1689
	A6	Y32F6A.2	> 2000	1914
	A7	ZC513.3	> 2000	1860
	A8	Y59A8B.g	2000	1821
	A9	D1065.1	2000	1758
	A10	C12D8.1b	2000	1836
	A11	M04G12.3	> 2000	2061
	A12	T16G1.1	> 2000	2088
	B1	C44H9.1	1800	1605
	B2	T01G5.2	1800	1608
	B3	F45F2.5	To redo	1671
	B4	T18H9.5	1900	1680
	B5	F25G6.6	1900	1656
	B6	Y105E8E.c	1900	1731
	B7	R03H4.1	> 2000	1929
	B8	Y51A2D.4	2000	1821
	B9	Y113G7A.8	2000	1758
	B10	Y111B2G.e	> 2000	1887
	B11	ZC376.2	> 2000	2064
	B12	Y38H6C.20	> 2000	2094
PCR ver-oo-HT115-076 (A [1-12] / B [1-12])	A1	F53H4.3	1000	780
	A2	T18D3.6	1000	816
	A3	R07E3.4	1100	897
	A4	F31F6.2	1100	897
	A5	F52D10.4	1100	855
	A6	C05E7.2	1100	861
	A7	F54B11.7	1100	864
	A8	T03G11.6	1100	891
	A9	F46C8.7	1200	869
	A10	C33D3.3	1200	939
	A11	C24H10.2	1200	984
	A12	F43C9.1	1200	963
	B1	B0310.3	1000	795
	B2	C17G1.1	1100	843
	B3	T22B2.5	1100	849
	B4	F23A7.3	1100	825
	B5	C54H2.1	1100	855
	B6	C32A9.1	1100	882
	B7	K02D3.2	1100	891
	B8	F55D10.4	1200	924
	B9	C18B12.3	1200	933
	B10	F21G4.5	1200	981
	B11	T24C2.4	1200	987
	B12	R04D3.7	1200	924

Table 3: Example of the analysis we did before we validated the results of a PCR verification. Comparison of the size of the amplicon we obtained (cfr. Figure 20) with the appropriate size of the predicted ORF. There is a difference between the two sizes of approximately 200 bp which correspond to B1 and B2.

Plate	Comments	Number of attempts	Validation
PCR ver-oo-HT115-021		1	OK
PCR ver-oo-HT115-023	The first time we did the PCR on this sample, none of the samples of the lane A amplified.	3	OK
PCR ver-oo-HT115-030	The first time we did the PCR, nothing amplified.	2	OK
PCR ver-oo-HT115-031		1	OK
PCR ver-oo-HT115-032	The first time we did the PCR, the results weren't perfect.	2	OK
PCR ver-oo-HT115-034	The first time we did the PCR on this sample, some of the samples of the lane A and B didn't amplified.	2	OK
PCR ver-oo-HT115-035		1	OK
PCR ver-oo-HT115-036		1	OK
PCR ver-oo-HT115-038		1	OK
PCR ver-oo-HT115-039		1	OK
PCR ver-oo-HT115-041	One lane on two showed up so we redid it.	2	OK
PCR ver-oo-HT115-043		1	OK
PCR ver-oo-HT115-044	(cfr. Figure)	1	OK
PCR ver-oo-HT115-045		1	OK
PCR ver-oo-HT115-046		1	OK
PCR ver-oo-HT115-047		1	OK
PCR ver-oo-HT115-048		1	OK
PCR ver-oo-HT115-050	The results weren't really clear the first time.	2	OK
PCR ver-oo-HT115-051		1	OK
PCR ver-oo-HT115-052		1	OK
PCR ver-oo-HT115-054		1	OK
PCR ver-oo-HT115-057	3 wells amplified (A1, A2, A12)		OK
PCR ver-oo-HT115-058	One well amplified	2	OK
PCR ver-oo-HT115-059		1	OK
PCR ver-oo-HT115-062	It looked strange because the size was too low compare to the one predicted.	2	OK
PCR ver-oo-HT115-064		1	OK
PCR ver-oo-HT115-066		1	OK
PCR ver-oo-HT115-067	(cfr. Figure)	1	OK
PCR ver-oo-HT115-068		1	OK
PCR ver-oo-HT115-070	5 wells amplified (B2, B3, B9, B10, B12)	1	To redo
PCR ver-oo-HT115-072		1	OK
PCR ver-oo-HT115-074	A lot of wells didn't amplify.	1	To redo
PCR ver-oo-HT115-076	(cfr. Figure)	1	OK
PCR ver-oo-HT115-077		1	OK
PCR ver-oo-HT115-079	A lot of wells didn't amplified	1	To redo
PCR ver-oo-HT115-081	An half of the B wells didn't amplified.	1	To redo
PCR ver-oo-HT115-082		1	OK

Table 4: Presentation of the results of a sample of PCR verification performed on HT115.

1.4. Validation of the RNAi resource.

The RNAi library is in its way to be completed really soon. On the 10,600 "in frame" ORFs, a few hundred are still to be cloned and 1,400 "out of frame" ORFs still remained to be coined. It will constitute the final library containing 12,000 ORFs cloned in RNAi feeding bacteria.
It is interesting to compare the results obtained with the Vidal's lab library, with the results coming from other RNAi libraries and/or delivery methods (table 5). Indeed, those validate the RNAi library generated in the Vidal's lab.

Lab	Number of clones	Methods of delivery	DNA template	% of hits (phenotype different from wild type)	Number of hits
Vidal/van den Heuvel	12000	Feeding	ORF	14,1 (with 4228 screened)	1692 (expected)
Ahringer	16800	Feeding	Genomic DNA	10,3	1722
Hyman	2174	Injection	Genomic DNA	12,9	281
Sugimoto	2479	Soaking	cDNA	27,2	675
Piano/Kemphues	751	Injection	cDNA	42,8	322

Table 5: Comparison of the different approaches of RNAi screens and of different RNAi libraries.
Those results validate the quality of the library and show how the different RNAi approaches (different libraries, delivery strategies, human eyes ....) are complementary.

1.5. Some perspectives offered by the RNAi resource.

The van den Heuvel's lab (MGH Cancer Center in Charlestown, Massachusetts) aims at finding proteins interacting with the Retinoblastoma tumor suppressor protein (pRb). Therefore a synthetic lethal test is performed in *C. elegans*. The idea is to use a viable null mutant of the gene lin-35 (which is a true ortholog of pRb) and to look for genetic interactors. The mutant is feeded with an *E. coli* RNAi library. If lethality or synergy results it will mean that Lin 3 and the colethal mutated gene interact together. In the same way a *C. elegans* ortholog of an oncogene whose mutation is observed at high frequency in a particular human cancer may be found synthetic lethal with a gene detected in an RNAi screen. Assuming true “interology” between the human and worm genes involved in the lethality test, one possible application of such results could be for instance the search for drugs targeting the ortholog of that interacting gene in order to kill specifically *in vivo* the human tumor cells.

2. Use of the RNAi resource to analyse the TGF-β pathway in *C. elegans*.

2.1. Some basics about the TGF-β signaling pathway (25-26).

One of the primary signaling pathways is the Transforming Growth Factor-β pathway (TGF-β).
Following extracellular ligand action, the signaling is mediated by two receptor serine-threonine kinases that heterodimerize and transduce the signal to downstream components. Two classes of receptor kinases have been identified: the type I and II that are both essential to this role. A distinguishing feature of type I receptors is the existence of a glycine-serine rich stretch of residues, the GS domain, preceding the kinase region.
A model of receptor activation and signal transduction has been developed for TGF-β receptors in mammals. According to this model, the ligand binds first the type II receptor, creating a receptor-ligand combination that has high affinity for the type I receptor. A tetrameric complex is formed in which the type II receptor phosphorylates the type I receptor. In this manner, the active receptor complex is able to transduce the signal from the ligand to downstream intracellular effectors (the Smad proteins). The discovery and characterization of the Smad protein family has shown that they are integral signal transducing elements in diverse TGF-β-like pathway. The Smad members have been identified thus far in several organisms, including *C. elegans*, *Drosophila*, and vertebrates.

2.1.1. The Smad pathway in mammals.

Concerning the mammals, the TGF-β signaling system is important in early embryonic development, where it is part of the pathways that lead to development of specific tissues (typically bone formation and the development of mesoderm). Also, because TGF-β pathway acts as a powerful growth inhibitor, this pathway is involved in tumor suppression.
The TGF-β signaling system is a pathway in which the phosphorylation at the membrane triggers migration of transcription factor to the nucleus (see figure 21). The members of the TGF-β superfamily of paracrine factors activate members of the Smad family of transcription factors. The ligand binds to receptors that consist of two types of subunits. Once the active complex has formed, the type I receptor phosphorylates a member of the cytosolic Smads family (Smad 2,3 or Smad 1,5). This causes it to form a dimer with the common partner Smad 4. The heterodimer is imported into the nucleus, where it binds to DNA and activates transcription.

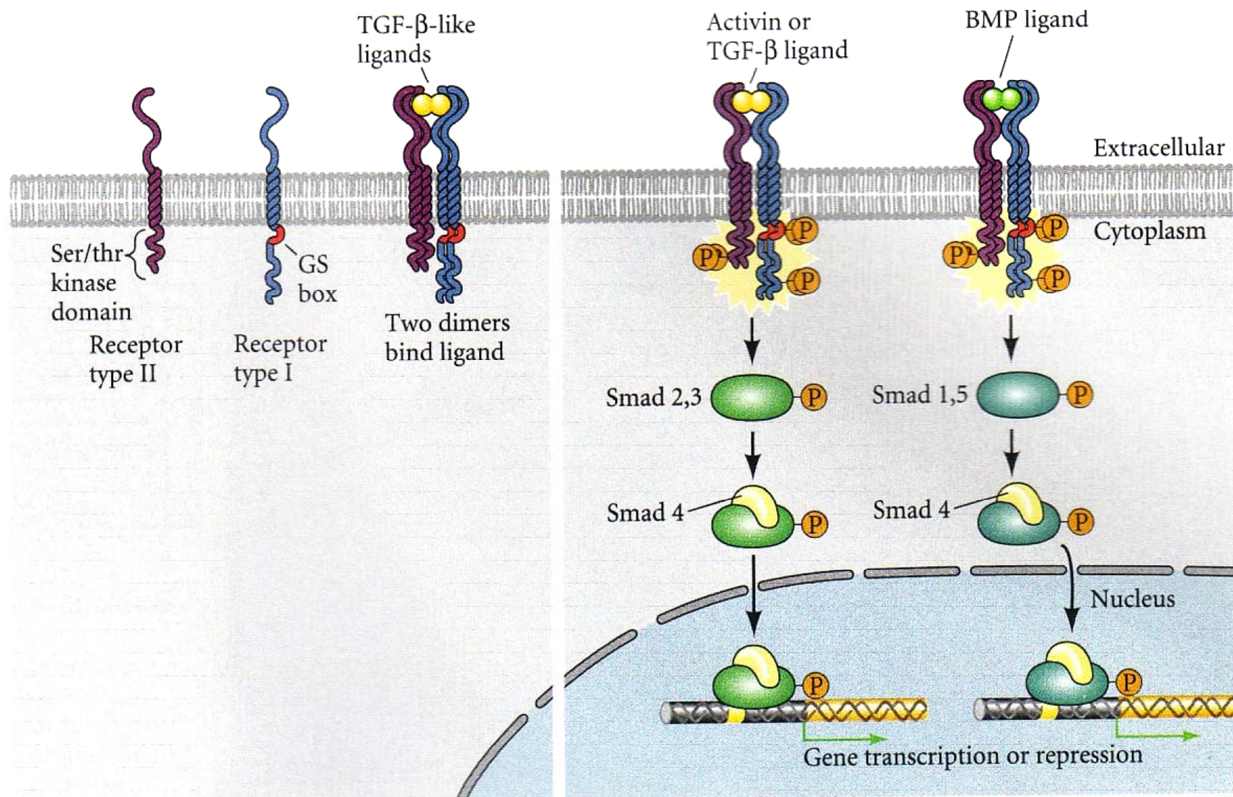


Figure 21: The Smad pathway activated by TGF-β superfamily ligands (27).
An activation complex is formed by the binding of the ligand by the type I and type II receptors. This allows the type II receptor to pbosphorylate the type I receptor on particular serine or threonine residues (of the "GS box"). The phosphorylated type I receptor protein can now phosphorylate the Smad proteins (Smad 2,3 or Smad 1,5). These Smads can complex with Smad 4 to form active transcription factors.

2.1.2. A homolog to the TGF-β pathway in *C. elegans*.

In *C. elegans*, there are two well-characterized TGF-β-like pathways, the Sma/Mab and dauer pathways.

2.1.2.1.The Sma/Mab (small and male abnormal) pathway.

The Sma pathway is involved in the body size regulation and in the male tail development. Three genes have been identified to be required for *daf-4* mediated developmental processes: *sma-2*, *sma-3* and *sma-4* (see figure 22). These three genes define a protein family, the dwarfins, that includes the *Mad* gene product, which participates in the *ddp* (decapentaplegic) TGF-β like pathway in *Drosophila*. In fact, dwarfins have been isolated in vertebrates, which indicates that these components are common to invertebrates and vertebrates.

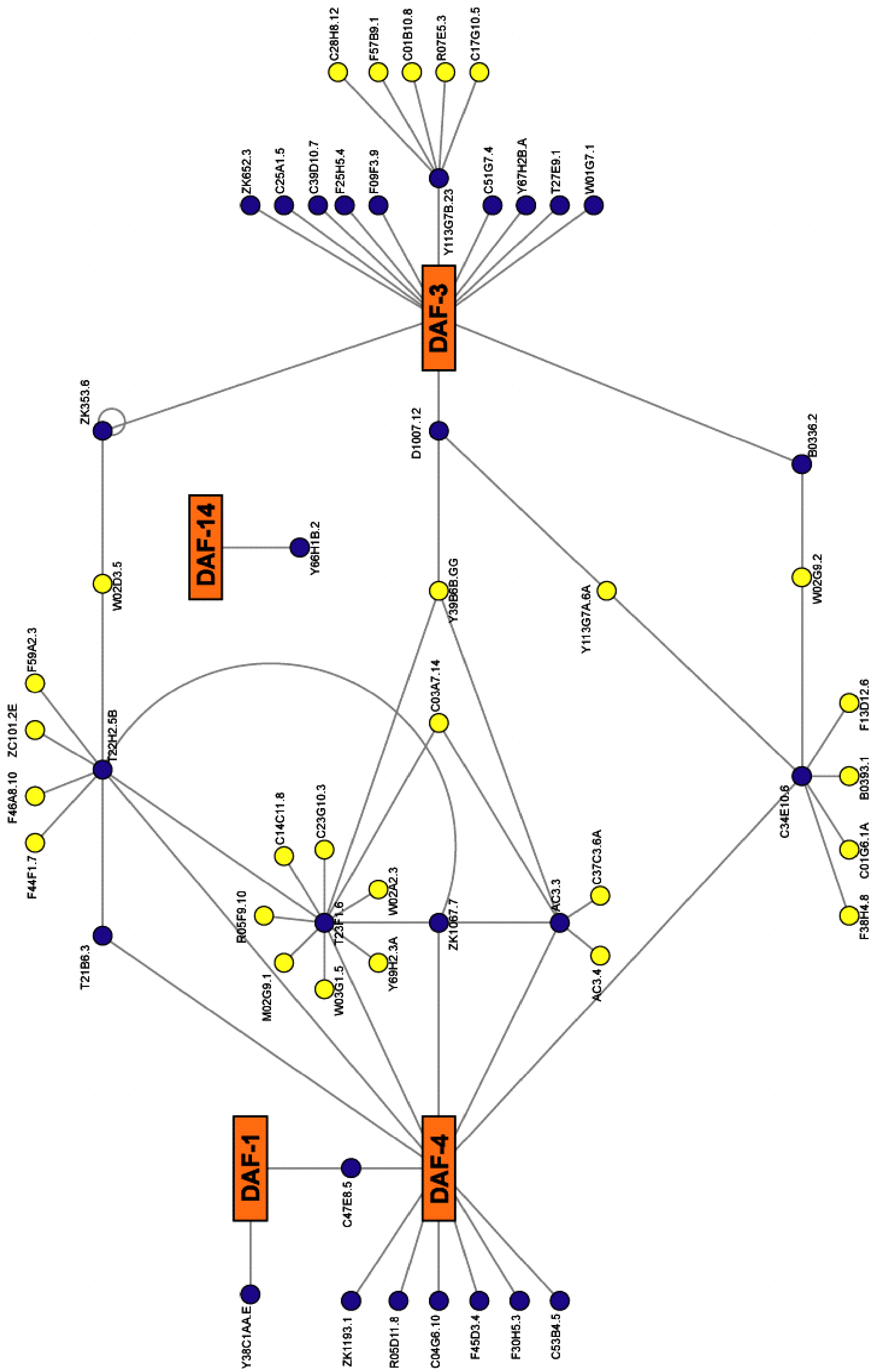


Figure 23: Map realised thanks the yeast two-hybrid system, showing the protein interaction between the potential candidates component of the daf-7 pathway (Tewari et al., submitted).

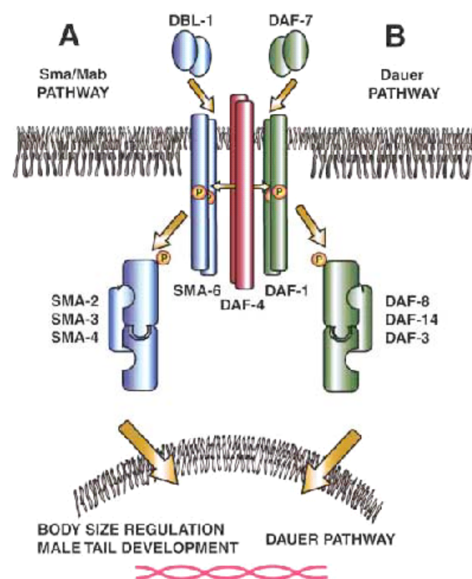


Figure 22: A model for TGF-β superfamily signaling in *C. elegans (25)*.
The type II receptor DAF-4 is shared between both developmental pathways. SMA-6, a type I receptor, transduces the extracellular signal to the downstream effectors SMA-2, SMA-3, and SMA-4 which regulate the expression of downstream targets.

2.1.2.2.The DAF pathway (28).

The dauer, as explained in the introductory chapter, is a developmentally arrested stage larva of the nematode *C. elegans*.

Genetic analysis of dauer formation in *C. elegans* has focused on two classes of mutations. The first class showed the Daf-c (dauer formation constitutive) phenotype. The second
class exhibits the Daf-d (dauer formation defective) phenotype. Together, these genes encode a TGF-β-like signaling cascade. The actual specific components are DAF-7 (TGF-β/BMP related ligand), DAF-1 (type I receptor serine/threonine kinase), DAF-4 (type II receptor serine/threonine kinase), DAF-8, DAF-14, and DAF-3 (Smad protiens).
By analogy with the vertebrates and Drosophila TGF-β like signaling, ligand binding is thought to induce multimerization of receptors and the activation of the type I kinase trough phosphorylation. Activated type I kinase in turn probably phosphorylates downstream Smad proteins, which transduces the signal to the nucleus and regulate transcription.
It has been showed that there are interactors of this pathway (see figure 23). Thanks the two-hybrid system a map of them could be drawn. In addition, some epistatic relations between each others are defined.

2.2. Are there additional genes that function in daf-7 pathway to regulate chemoreceptor gene expression?

Our hypothesis is the following: knocking down a gene whose expression is involved in the regulation of another will alter the expression from the latter. If the daf-7 pathway regulates the expression of the chemoreceptors of the chemosensory neurons, it is speculated that interactors of the members of this pathway could possibly also affect the expression of this pathway, thereby allowing us to further extend it.

2.2.1. The experimental procedures to perform the RNAi screening.

2.2.1.1. Choice of the strains expressing GFP fusion proteins at a significant level.

In order to analyse candidates for a role in daf-7 pathway, we used 5 strains of *C. elegans* which contained a specific construction: all of them carried a chemosensory receptor gene with a promoter which drives the expression of GFP reporter in the neurons (see table 6). The screening of microscopic phenotypes has been greatly aided by the introduction into worms of green fluorescent protein (GFP) (27). It can be used to mark a protein, cell or subcellular compartment, and changes in GFP expression can be screened. In this case, this GFP reporter will thus enable us to monitor the regulation exerted via the daf-7 pathway.

Chemoreceptor gene in fusion with the GFP.	Neuron in which the GFP is expressed.
srd-1::GFP	ASI neurons
str-2::GFP	ASI neurons (weakly) and in AWC neurons (strongly)
str-3::GFP	ASI neurons
sra-6::GFP	ASH and in the ASI neurons
str-1::GFP	AWB neurons

Table 6: characteristics of the strains used (28-30).
Each particular strain contains the fusion between the promoter of a chemosensory gene and the GFP allowing expression in specific neurons. Identity of the chemosensory genes is mentioned etc...The name and the mutant (??) allele of each strain are detailed in the experiments procedure.

In order to test the genes on strains exempted of contamination, we picked a few worms per plate and replaced them on another plate containing feeding bacteria.
Another solution was to chunk them which means to cut a small square (1 cm^2) of agar from the plate contaminate and place it gently on another plate containing the feeding bacteria.
This transfer from one plate to another enables also to have different generations on the plates and to make sure that the worms have food enough to not go into the dauer stage. This diversity of worms at different stage is important since we do the experiments only on L4 worms; it would be at that time that the RNAi has the most effect and it is the last stage before the adults and so the progeny will have the best chance to show the RNAi effects (17).

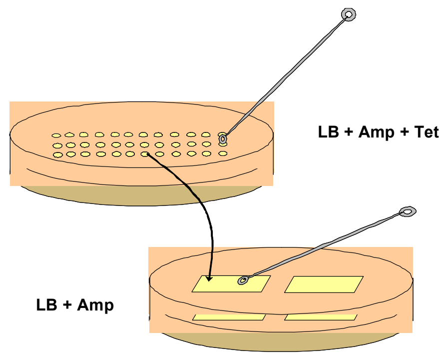


Figure 24: Scheme representing the technique used to transfer the feeding bacteria from the LB-Amp-Tet plate to the LB-Amp plate.
Every week, a new LB-Amp-Tet plate containing the feeding bacteria was made to ensure the freshness of the bacteria.

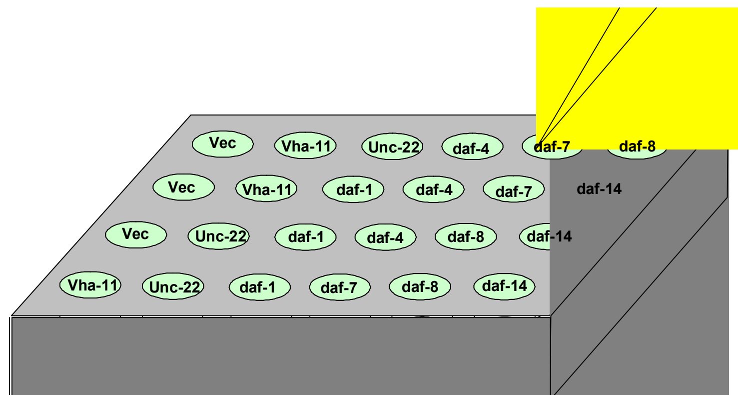


Figure 25: Scheme representing an example of the distribution of the feeded bacteria in a 24-well plate.
From each bacteria three replicates were done.

During all the experiments, the worms were stocked in their plates at 20°C (the optimal temperature).

We decided to do 3 controls and to do 3 replicates per genes.
We observed the strains under the microscope and we choose to keep only two of them because either ones didn't express the GFP at all; either the signal wasn't clear or low.

2.2.1.2.The different media used to grow the feeded bacteria.

In order to cultivate fresh bacteria containing the RNAi of the genes of interest and to prepare them to feed the worms, we prepared plates containing LB and antibiotics. For the plates that will contain fresh bacteria, we used the antibiotics tetracycline (Tet) (12,5 µg/ml) and ampicillin (Amp) (50 µg/ml). Because the HT115 bacteria strains transformed with the plasmid L4440 is a RNase III-deficient *E. coli* strain and, as explained earlier, has the rnc14 gene disrupted by transposition via Tn10 carrying a tetracycline-resistance gene, the maintenance of the RNase III deficient character depend on continuous growth under tetracycline selection conditions.
The bacteria ready to feed the worms, were plated on LB-Amp plates because as shown in previous experiments, the inclusion of tetracycline in feeding plates results in a weaker RNAi effect perhaps because the bacteria grew very poorly (17).

2.2.1.3.The bacteria used to feed the worms.

2cm² of the feeding bacteria, from the LB-Tet-Amp plate, were patched on the LB-Amp plates and we let them grow up overnight (see figure 24).
After 18 hours we scraped off the patch of feeding bacteria and diluted them in an eppendorf containing 250 µl of LB liquid. In a 24-wells NGM plate (see figure 25) that we kept in 4°C, we distributed 50 µl of the bacteria in each well.

2.2.1.4.The feeding of the worms.

Once bacteria were shared out in each well of the 24-well plate, we let those plates dry for an hour, at least, under the hoot. When the plates were dry, we picked a worm at the L4 stage and placed it in each well. We let the worms for 1 up to 2 days at 15°C. After those 2 days, we took the parents (P0) out in order to obtain a single progeny generation of worms being at almost the same stage.
The plates containing the F1 generation will be stored 2 more days at 15°C.
So after those 4 days at 15°C, we looked at the worms and we searched for a difference in the GFP expression under the microscope.

2.2.2. Analysis of the results of the RNAi screening.

2.2.2.1.The genes used as controls to verify the results obtained from the screening.

For the screening, we used three controls: a negative control which was the vector pL4440 alone in the bacteria HT115 (DE3) (Vec).

A positive control: the gene vha11. The dsRNA of this gene is responsible to produce a sterility's phenotype.
Another positive control: the *C. elegans* gene *unc-22* which encodes an abundant muscle filament protein. The injection of *unc-22* dsRNA is known to produce a characteristic and uniform twitching phenotype in which the animals can sustain only transient muscle contraction (24).
An additional positive control has been chosen: an RNAi vector containing a sequence of the gene of the GFP. As we know that the RNAi by feeding doesn't work well in the neurons (29), this new control will enable us to see how the chemoreceptor genes react to RNAi in our experiment.
We compared the results of the screening obtained with the level expression of the GFP on the negative control (the vector alone) (see figure 26 and 27).

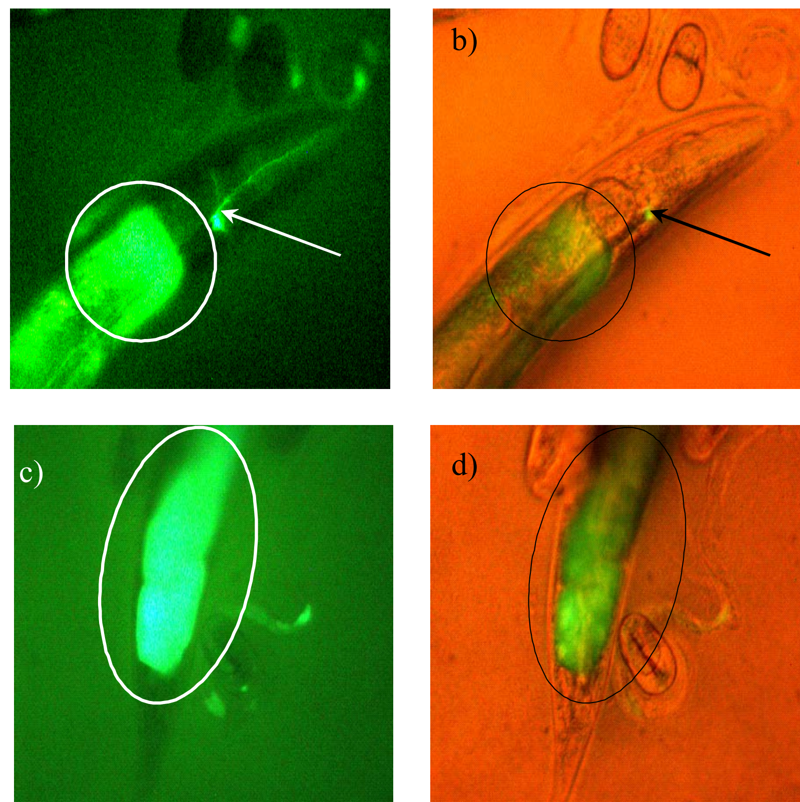


Figure 26: Pictures of the negative control on the strain CX3427 with the GFP in fusion with str-2.
The picture (a) shows a green patch around the beginning of the intestine area (the white circle) The AWC neuron is also visible (the arrows). This picture had been taken with the blue filter and without any light. The picture (b) is actually the same but it was taken with the light and the blue filter.
The picture (c) and (d) shows another green patch of GFP on the tail (see the white and the black circles).

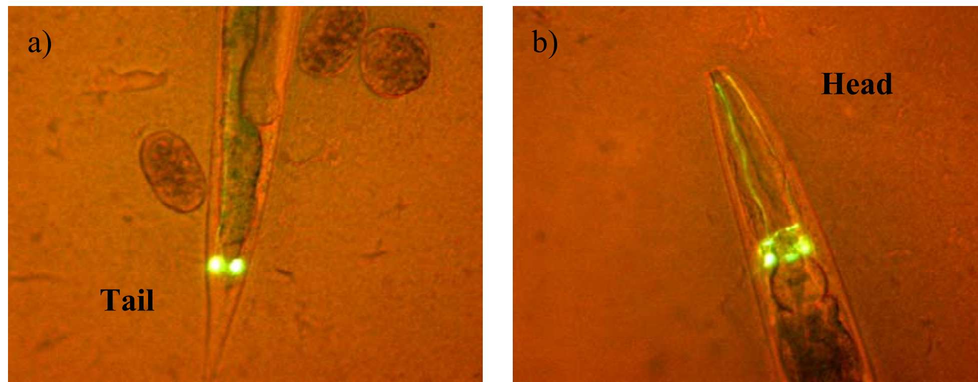


Figure 27: Pictures of the negative control on the strain PY1058.
There are no patches of GFP, only the ASI and ASH neurons are visible in the head (b) and in the tail (a).

The positive control with the *vha11* gave 100% of success. Indeed, in each well containing that feeding bacteria, no progeny appeared. It was the same for the other strain, the PY1058. That ensures us that the RNAi feeding procedure of the worms was successful.
The other positive control (*unc-22*) was also to verify the efficiency of the RNAi feeding of the worms.
And the twitching phenotype produced as observed in 60% of the worms. What is interesting to notice is that the GFP's spots were still visible.
The last positive control (the GFP RNAi) worked only in the strain CX3427: only the spots around the tail and the intestine area are switched off. The AWC neuron is still green by the expression of the GFP (see figure 28).
In the PY1058 strain, the GFP control didn't have any effect on the level expression of the GFP (data not shown).

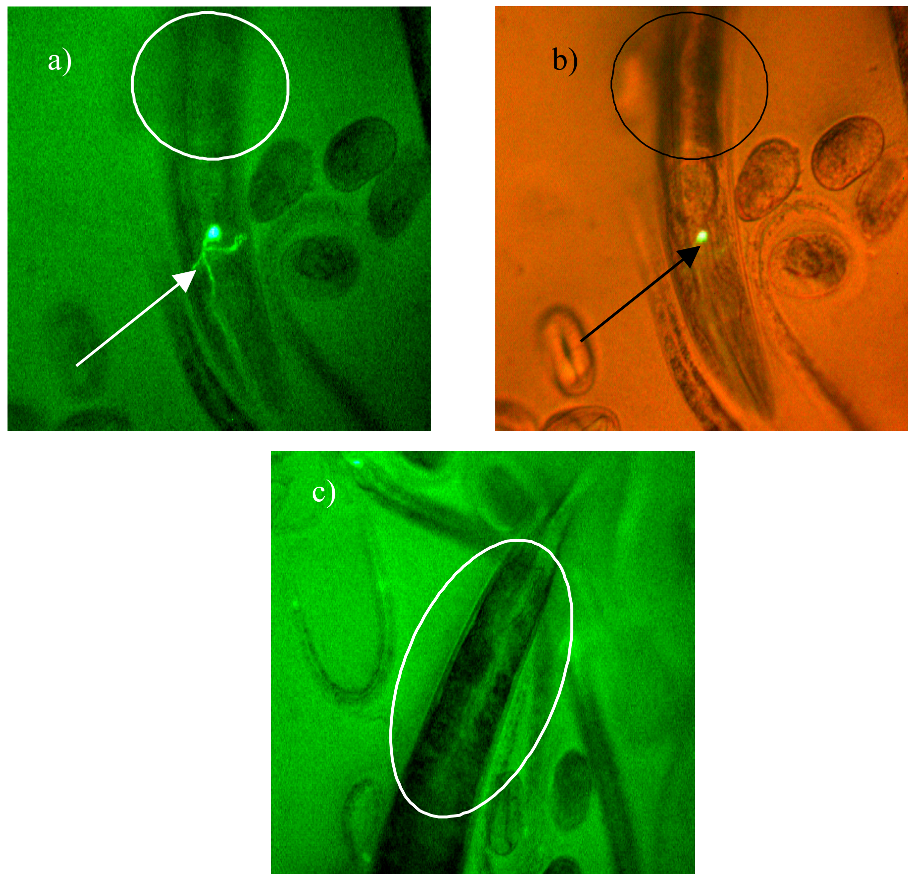


Figure 28: Pictures of the positive control (the GFP control) on the strain CX3427.
The picture (a) shows us that the green patch around the beginning of the intestine area disappeared (the white circle) The AWC neuron is still visible (the arrows). This picture had been taken with the blue filter and without any light. The picture (b) is actually the same but it was taken with the light and the blue filter.
The picture (c) shows also that the green patch disappeared from the tail (see the white circle).

2.2.2.2. The genes implicated in the DAF-7 pathway, tested in the RNAi screening.

We tested the genes directly implicated in the dauer pathway, i.e. *daf-1, daf-4, daf-7, daf-8, daf-14*. Unfortunately, none of the worms showed a difference in the level expression of the GFP (data not shown).
After testing all those genes at least one time, we decided to try the experiment on the second generation of worms. So from each well of the RNAi plates containing worms already feeded with the bacteria, we picked a worm and transferred it in another well containing the corresponding bacteria. We actually hoped that we would get more results if the RNAi acted on the second generation. But unfortunately it didn't make any difference (data not shown).

The 48 potential interactors of the DAF pathway found in the Vidal's lab have shown a particular importance in the dauer formation. By RNAi screening, some interactors yielded higher score of worms going into dauer (Tewari et al., submitted).
For each gene, we did three replicates. The gene DX41 as well as the DX13 gene showed a difference in the GFP level expression.

The gene DX41 gave us two phenotypes. The patch around the intestine area disappeared (picture not shown) in 35% of the adults worms and a diminution of the intensity level of the GFP was observed in 30% still of adults worms. The last 35% of that stage didn't show any difference in the level expression of the GFP (see figure 29).
The green spot still appeared around the tail (picture not shown).
Out of those results, we can conclude that DX41 has an effect on that *str-2* chemoreceptor gene but only in the adult worms.

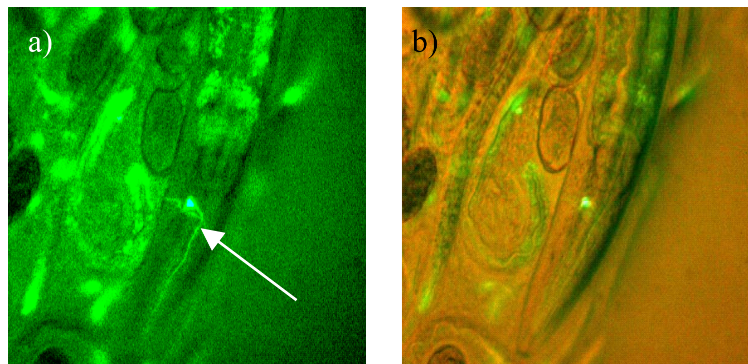


Figure 29: Pictures of the effect of the DX41 gene, an interactor of the daf-7 pathway, on the strain CX3427.
The pictures (a) and (b) shows a diminution of the intensity of the GFP. As in the other pictures, the AWC neuron is still visible (the arrow). The green patch around the tail still appears but is not shown on that picture.

The second gene that gave us interesting results is the DX13.
We observed also two phenotypes. All the worms from the progeny were blocked between the stage L1 and L2 and didn't show any green patch around the intestine area (see figure 30).
Thus from those results, we could already conclude that this interactor plays a role in the development of the worms.
We could wonder if the interactor is implicated in a pathway acting on the chemoreceptor gene or if it is direct acting on the GFP (by knocking down a gene important for its stability, …). Because the GFP control showed that the two patches disappeared, if the DX13 gene was also acting on the GFP, we wouldn't see the green patch around the tail anymore, as noticed, this tail patch is still present.

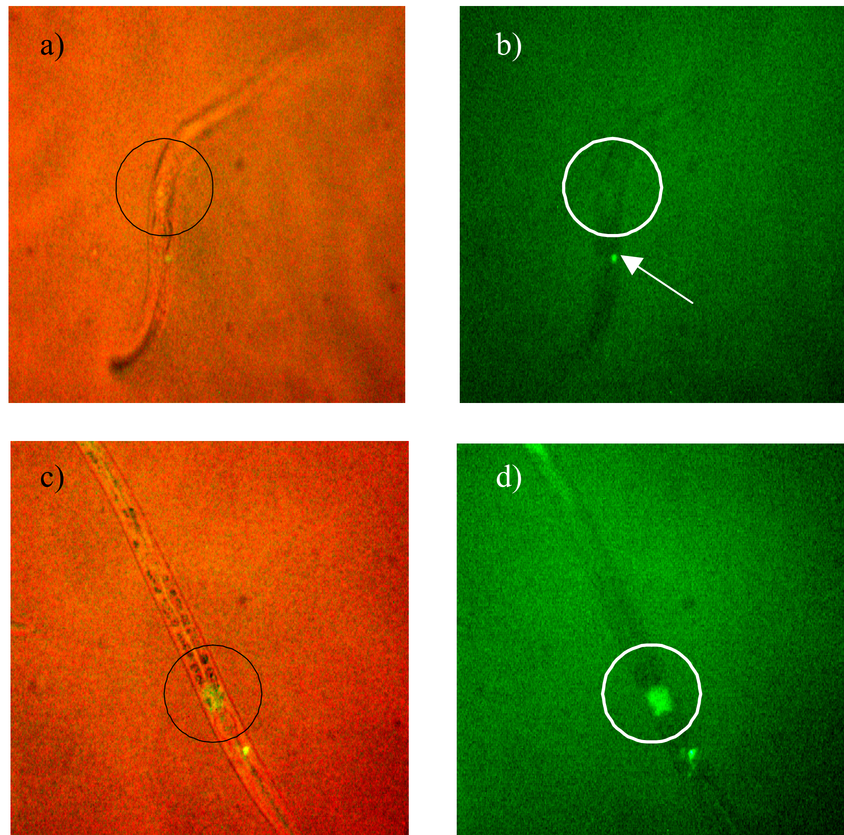


Figure 30: Pictures of the effect of the DX13 gene, an interactor of the DAF-7 pathway, on the strain CX3427.
The picture (a) shows that the green patch around the beginning of the intestine area disappeared (the black circle) The AWC neuron is still visible (the white arrow) on the picture (b). The green patch around the tail still appears but is not visible on that picture.
The pictures (c) and (d) is a worm at the same stage than those on the pictures (a) and (b) but feeded with the vector alone (the negative control). On that picture the green patch appears clearly around the intestine area.

Gene	ORF name	Strain			
		str-2::GFP		sra-6::GFP	
DX1	T21B6.3	20/20	100%	20/20	100%
DX2	F30H5.3	20/20	100%	20/20	100%
DX3	F45D3.4	20/20	100%	20/20	100%
DX4	Y38C1AA.E	20/20	100%	20/20	100%
DX5	ZK353.6	20/20	100%	20/20	100%
DX6	D1007.12	20/20	100%	20/20	100%
DX7	T23F1.6	20/20	100%	20/20	100%
DX8	ZK1067.7	20/20	100%	20/20	100%
DX9	Y67H2B.A	20/20	100%	20/20	100%
DX10	T27E9.1	20/20	100%	20/20	100%
DX11	ZK652.3	20/20	100%	20/20	100%
DX12	BO336.2	20/20	100%	20/20	100%
DX13	C25A1.5	04/20	20%	20/20	100%
DX14	RO5D11.8	20/20	100%	20/20	100%

DX15	ZK1193.1	20/20	100%	20/20	100%
DX16	F28D1.7	20/20	100%	20/20	100%
DX17	C34E10.6	20/20	100%	20/20	100%
DX18	T22H2.5b (WS)	20/20	100%	20/20	100%
DX19	C53B4.5	20/20	100%	20/20	100%
DX20	F09F3.9	20/20	100%	20/20	100%
DX21	T10B10.3	20/20	100%	20/20	100%
DX22	F25H5.4	20/20	100%	20/20	100%
DX23	Y113G7B.23	20/20	100%	20/20	100%
DX24	AC3.3	20/20	100%	20/20	100%
DX25	C51G7.4	20/20	100%	20/20	100%
DX26	C39D10.7	20/20	100%	20/20	100%
DX27	W01G7.1	20/20	100%	20/20	100%
DX28	Y110A7A.10	20/20	100%	20/20	100%
DX29	C47E8.5	20/20	100%	20/20	100%
DX30	W02D3.5	20/20	100%	20/20	100%
DX31	C17G10.5	20/20	100%	20/20	100%
DX32	R05F9.10	20/20	100%	20/20	100%
DX33	F44F1.7	20/20	100%	20/20	100%
DX34	W02A2.3	20/20	100%	20/20	100%
DX35	C01B10.8	20/20	100%	20/20	100%
DX36	Y69H2.4	20/20	100%	20/20	100%
DX37	R07E5.3	20/20	100%	20/20	100%
DX38	G_YK7273	20/20	100%	20/20	100%
DX39	G_YK1666	20/20	100%	20/20	100%
DX40	Y66H1B.2	20/20	100%	20/20	100%
DX41	W03G1.5	07/20	35%	20/20	100%
DX42	C04G6.10	20/20	100%	20/20	100%
DX43	W02G9.2	20/20	100%	20/20	100%
DX44	C03A7.14	20/20	100%	20/20	100%
DX45	C14C11.8	20/20	100%	20/20	100%
DX46	AC3.4	20/20	100%	20/20	100%
DX48	Y39B6A.1	20/20	100%	20/20	100%
DX49	Y71H2B.10	20/20	100%	20/20	100%

Table 7: resume of the results obtained for the 48 interactors of the DAF-7 pathway.

2.2.3. The conclusion.

As showed in the table 7, we got interesting data for the genes DX13 and DX41. For the gene DX41, 7 worms out of 20 still showed the GFP fluorescence around the intestine area. In the 13 others, we saw either a diminution of the intensity of the GFP, either a complete disappearance of it.

For the gene DX13, on 4 worms out of 20, it was still possible to observe the GFP fluorescence around the intestine area.

Globally, it is important to notice that these results should be retested. Indeed, we had the time to repeat once only the experiment with DX13 and DX41 but the other genes have not been retested.
We should also implement the experiment with other conditions. Indeed, the temperature for instance can be an important factor which influences the results, since it regulates the decision to go or not into dauer. Because the DX13, for instance, obviously blocks the worm at the stage L1 at 15°C, what about its activity at 22°C or better, at 27°C which is the temperature that is inducing the dauer stage?

Our results indicate that the difference in the GFP expression is specifically due to dsRNA of a particular gene and not to a decrease in the stability of the GFP. If the stability of the GFP was involved in the change in the expression level we would have observed a diminution or a complete disappearance in both patches (around the tail and the intestine area).
It should be mentioned that recent results of the Vidal's group (Tewari et al., submitted) show that the RNAi phenotype of DX13 and DX41 on dauer arrest pathway is the strongest among the various interactors of this pathway they have tested.
Indeed, the RNAi phenotype of those genes is 100 % dauer, which is an effect similar to what is observed for *daf-7* LOF (loss of function) mutation with result in a dauer constitutive (dc) phenotype. Interestingly, when they performed an assay with double perturbation by RNAi feeding DX13 in a *daf-12* (m20) background they observed a strong suppression of the dauer phenotype which means that DAF-12 is epistasic to DX13. That is to say that, based on genetic data, DX13 is placed upstream of DAF-12.

We were unable to evidence any alteration in the GFP fused expression of str-2 in any neuron (ASH, ASI, AWC) as anticipated; this can be due to the well established relative insensivity of neurons toward RNAi in general. Surprisingly, the effect we observed was at the level of unidentified cellular structures of the pharynx.

What seems unclear from our own data on the control of str-2, is the exact relationship with the classical TGF-β pathway in *C. elegans*. This pathway starts from DAF-7 to DAF-3 and DAF-12 which are factors acting at the transcription level of the target genes involved in dauer formation and in the control of the expression of chemoreceptors. From previous published data (Nolan et al., 2002), it seems that multiple modes of regulation (DAF-7 dependent or independent) alter the expression pattern of chemoreceptors as a function of developmental stage, environmental conditions, as well as in a cell dependent manner.

Since DX13 and DX41 are two-hybrid interactors of this pathway (respectively with *daf-12* or as a secondary interactor of *daf-4*) and (at least for DX13) exhibit genetic interaction with the dauer determining gene, they seem to be part of this pathway. Nevertheless, it cannot be excluded that these genes have a possible role at the interplay of the interconnected signalling cascades which are known to participate in the dauer effect (namely the TGF-β signalling pathway or DAF-7 pathway, the cyclic nucleotide signalling or DAF-

11-DAF-21 pathway) or are somehow connected to it (insulin-like receptor pathway or DAF-2 pathway) in an ill defined manner.

Undoubtedly, the high complexity of TGF-β pathway itself and, on the other hand, the various related phenotype (egg laying, sterility, dwarfism, chemosensitivity, ...) often overlapping with dauer effect indicate that this high level of pleiotropy and complexity needs further data to be fully appreciated.

We feel that the approach tempted here, namely, the use of transgenic worms expressing a gene of interest GFP-tagged challenged with specific RNAi's, is capable to give new insights in the wiring of these intricate pathways.

Materials and methods

Materials and methods.

1. Establishment of the RNAi resource.

1.1. Characteristics of the vectors.

The Gateway™ technology that was used to clone ORF in different vectors is based on the bacteriophage lambda site-specific recombination system which facilitates the integration of lambda into the E. coli chromosome.

1.1.1. pDONR™201

pDONR™201 (see figure 31) is the Donor Vector that they used to clone the PCR products flanked by *att*B sites to generate entry clones. When PCR fragments modified with *att*B sites are recombined with the pDONR vector in a BP reaction, they yield an entry clone.

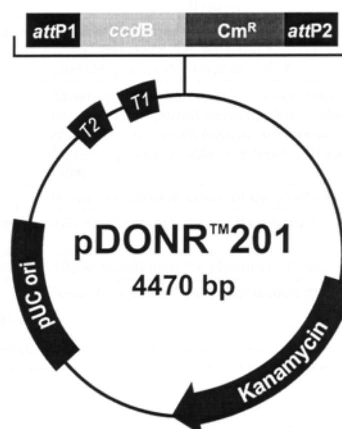


Figure 32: the map of the pDONR™201 showing its different elements.
The rrnB T1 and T2 transcription terminators protect the cloned gene from expression by vector-encoded promoters, thereby reducing possible toxicity.
The *ccdB* toxic gene permits a negative selection of the plasmid
The kanamycin resistance gene allows selection of the plasmid in E. coli.
The pUC origin enables the plasmid to replicate and to maintain in E. coli.

1.1.2. pL4440

In order to constitute the RNAi library, the ORF were cloned in an expression vector that expresses the dsRNA. The destination vector that we used to recombine with the entry clone in an LR reaction is the pL4440.
The pL4440 was furnished by the Fire's lab. In order to be Gateway™ compatible, the Vidal's lab modified it: they added the *att*R1 and the *att*R2 sites to allow the recombination with the *att*L1 and *att*L2 sites of the Entry clone.
That vector contains two T7 promoters in inverted orientation flanking the multiple cloning site and is Ampicillin resistant.

1.2. Characteristics of the bacteria.

To enable recombinational cloning and efficient selection of entry or expression clone, most Gateway™ vectors contain two *att* sites flanking a cassette containing the *ccdB* gene for negative selection. The CcdB protein interferes with *E. coli* DNA gyrase, thereby inhibiting growth of most *E. coli* strains. Thus, in order to realize a selection of the bacteria, we used the DH5α *E. coli* strain wild type to transform the ORF cloned into the destination vector pL4440.

In order to produce the dsRNA to constitute the RNAi library, we transformed the ORF into another strain of *E. coli* which enables the production of this particular RNA. Therefore we used HT115 (DE3) which is an RNase III-deficient *E. coli* strain with IPTG-inducible T7 polymerase activity.

Strain	Genotype	Comments
DH5α	F-, θ80*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K-, m_K+), *sup*E44, *rel*A1, *deo*R, Δ(*lac*ZYA-*arg*F)U169	Common host for cDNA cloning.
HT115 (DE3)	*F*-, *mcr*A, *mcr*B, *IN* (*rrn*D-rrnE)1, λ-, *rnc*14::*Tn10*(DE3 *lysogen*: *lac*UV5 *promoter*-T7*polymerase*) (IPTG-inducible T7 polymerase (RNase III minus).	Growth on LB plates, tetracycline resistant.

Table 8: description of the genotype of the strains we used to transform the LR products and to constitute the RNAi library.

1.3. The LR cloning.

The LR reactions facilitate the recombination of an *att*L substrate (entry clone) with an *att*R substrate (destination vector) to create an *att*B-containing expression clone. This reaction is catalysed by LR clonase enzyme mix: the bacteriophage λ Integrase (Int), the E. coli Integration Host Factor (HIF) and the Excisionase (Xis) proteins.
This LR clonase is a fragile enzyme and therefore should be stored at -80°C (or on dry ice) before using, and should be thawed on ice just before using it.

The mix for one reaction is as following:

5 x LR buffer	2 µl
Destination vector (2 µg/µl) digested	150 ng
LR clonase	2 µl
TE	Up to get a final volume of 10 µl

Aliquot on ice 8 µl of this mix in each well of a PCR plate using a dispenser
Add the 2 µl of entry clone (from a DNA miniprep dilution) with a micro multichannel, mix up and down 5 times. And seal with aluminium tape.
The plates must be incubated at 25°C overnight (at least 2 hours).
Seal with the Marsh plate sealer, incubate 10 min. at 37°C.

1.4. The transformations.

Remove the frozen competent cells from -80°C and thaw them on ice. The delicates is really important because the competent cells are really fragile.
On ice aliquot 20 µl of competent cells to each well of 96-well plate (MARSH AB0800), with the following identity: TRF-### (TRansFormation-ID number).
2 µl of LR products were added.
As controls we used the wells F12 and G12 that we plated on LB solid plate to verify easily the efficiency of the transformation. The well F12 contained the products of the transformation and enabled us to count the colonies (it is impossible to get a good idea of the transformation's results in a liquid culture). The well G12 was a control of the efficiency in which we put 2µl of pUC 19 (at different concentration). If the number of plate allowed us to do, we left the well H12 without any cells as a negative control to verify the level of contamination.
The cells were left on ice for 30 minutes and were heat shocked for 45 seconds using the MJ Research Thermal Cycler preheated to 42°C. We incubated them on ice for 5 minutes and than 105 µl of SOC were added.
We sealed the plate with a green sheet (Costar 6524) and let them incubate 1 hour at 37°C.
The controls were platted on an individual plate containing solid LB and the appropriate antibiotic (100 µg/ml Amp because the pL4440DEST vector is resistant to the ampicilin, therefore a selection of the plasmid in *E. coli* is possible).
For the liquid culture, the GenMate robot transferred the 125 µl of *E. coli* transformants in SOC into a deep 96-well plate containing 400 µl of LB and Amp (100µg/ml) per well.
The plates were sealed with an AirPore microporous tape sheet (Qiagen, 19571) which promotes gas exchange during culturing. We let the cells incubate overnight at 37°C. For maximum growth, we used the Vibra –Translator (Union Scientific Corporation).

<u>Solutions:</u>

- The SOC is composed of -SOB : 2% bacto tryptone
  0,5% yeast extract
  10mM NaCl
  2,5 mM KCL
  10 mM $MgCl_2$
  10 mM $MgSO_4$
  pH 6.7 - 7.0
  -and Glucose 20mM.

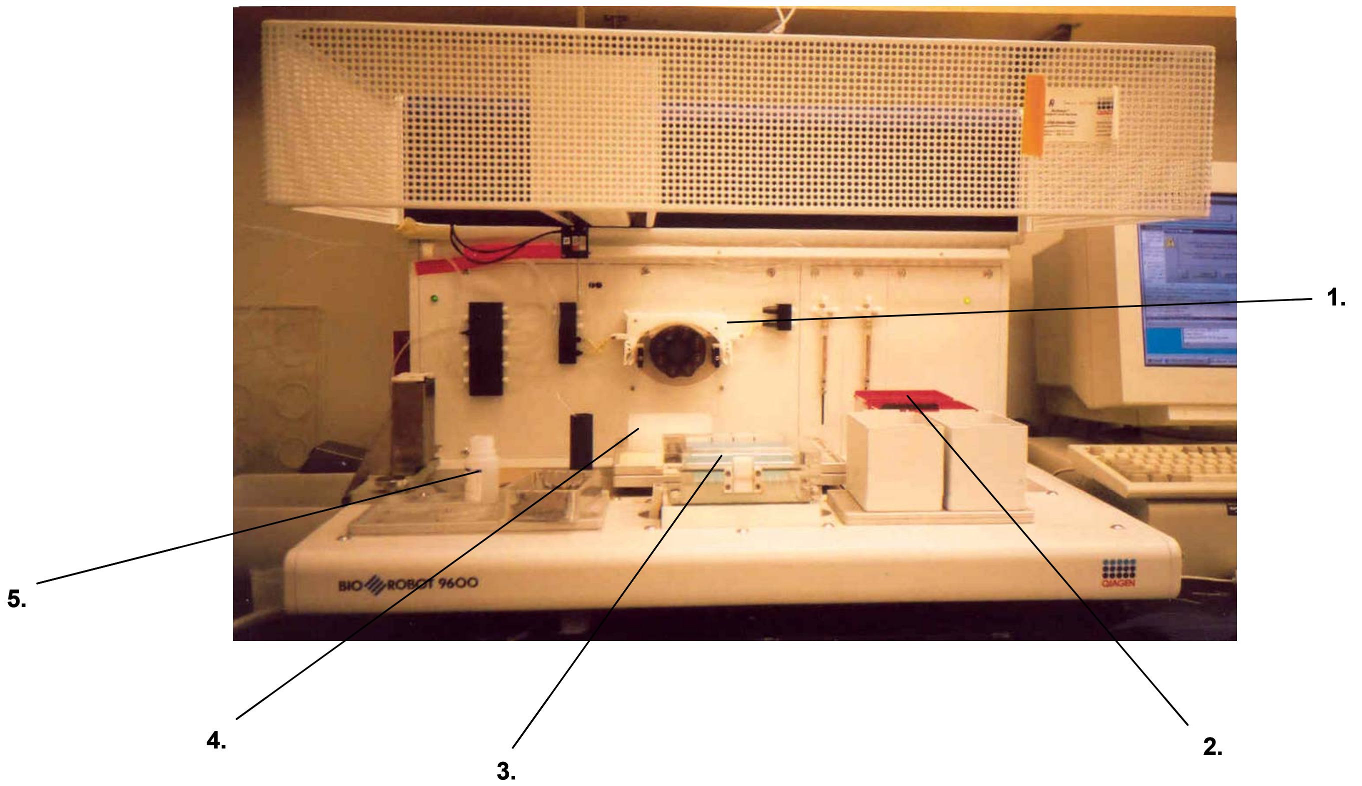


Figure 33: The Bio-Robot 96000 used to perform the plasmid DNA Miniprep.

1. The peristaltic pump.
2. The tip racks.
3. The blue DNA binding column (QIAprep 96 plate) in the manifold base with the white filter (TurboFilter 96 plate).
4. The flat bottom block with bacteria pellet in back left position.
5. EB in slot F3.

- The LB media:

Capsules of LB	4
H2O	1 l.

Composotion of one capsule of LB (Q-Bio gene):

Tryptone	10 gr.
Yeast extract	5 gr.
NaCl	10 gr.

- The LB solid plate:

Agar	1,5 %
H2O	1 l.
Capsqules of LB	4

1.5. The miniprep.

This protocol (see figure 34) is designed for high-throughput plasmid DNA minipreps using TurboFilter 96 and QIAprep 96 plates on QIAvac 96. The kit accommodates up to 96 parallel preparations of up to 20 µg oh high-copy plasmid DNA from 1-5 ml overnight cultures of *E. coli* grown in LB medium. This DNA purification with the QIAprep 96 Turbo Miniprep Kit was automated with the QIAGEN BioRobot 9600 System (see figure 33). Up to 96 minipreps were processed in 70 minutes.
Fill in the bottle for P1, P2, P3 and PE, and plug them to the corresponding tubes.
P1 should be kept at 4°C when not in use.
Make sure that the cassette of peristaltic pump is well fitted.
Place blue DNA binding column (QIAprep 96 plate) in the manifold base with the white filter (TurboFilter 96 plate).
Place the flat bottom block with bacteria pellet in back left position.
Fill the tip racks with 1000 µl tips in slot 1 and in slot 2.
Insert EB in slot F3.
Turn on the robot.

1. The robot resuspend pelleted bacterial cells in 250 µl Buffer P1. No cell clumps should be visible after resuspension of the pellet.
2. 250 µl of Buffer P2 were added and incubate it at room temperature for 5 min.
3. The robot add 350 µl Buffer N3 to each sample. The solutions should become cloudy.
4. The robot pipets the lysates from step 3 (850 µl per well) into the wells of the TurboFilter. The all the samples should be vacuumed and passed through TurboFilter. Optimal flow rate is 1-2 drops/sec.
5. Discard TurboFilter, place QIAprep into vacuum manifold top plate. Make sure that the QIAprep is well fitted so that the vacuum can work. The flow-through is collected in the waste tray.
6. The vacuum is switched off and the QIAprep is washed by adding 1 ml Buffer PE to each well and applying vacuum.
7. Repeat step 6.

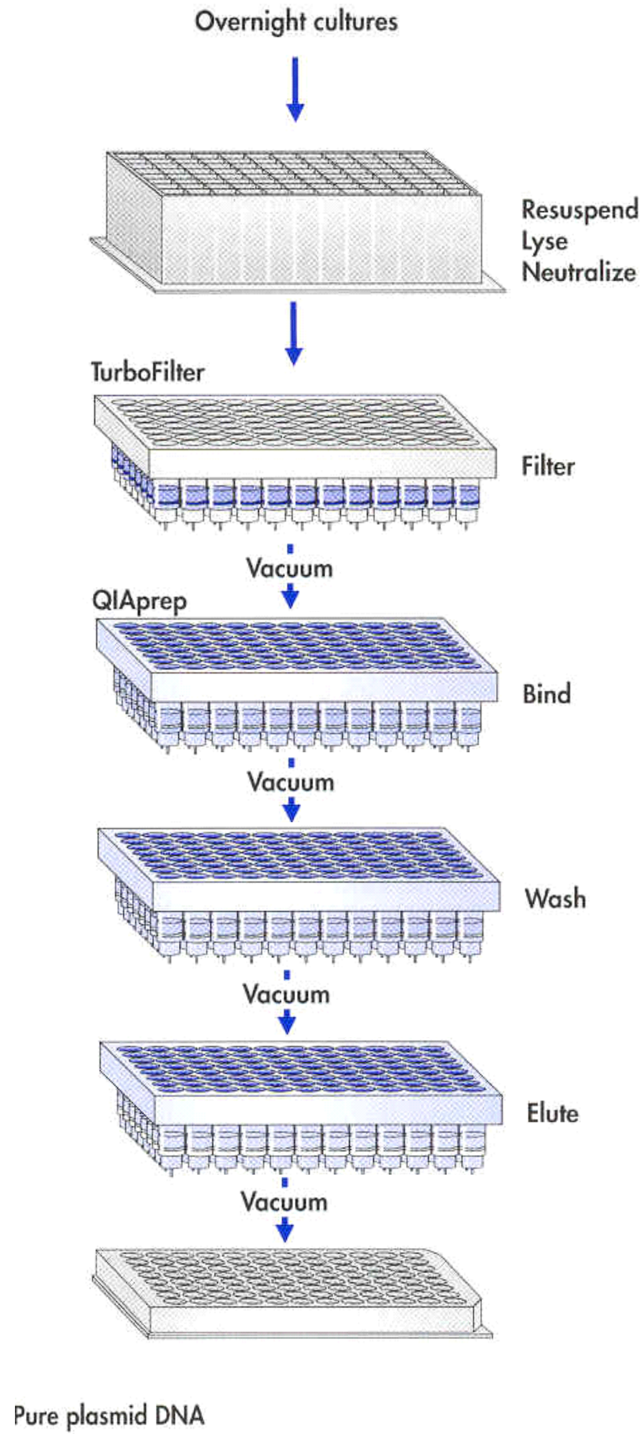


Figure 34: QIAprep 96 Turbo Procedure.

8. After PE has been drawn through all wells, maximum vacuum is applied for an additional 5 min. to dry the membrane. Thus step removes residual Buffer PE from the membrane.
9. The vacuum is switched off. Tap vigorously QIAprep on a stack of absorbent paper until no drops come out. Remove ethanol in the vacuum manifold base, place the collection tube rack into manifold base, well aligned under QIAprep.
10. To elute DNA, the robot adds 100 µl Buffer EB (10 mM Tris-Cl, pH 8.5) and vacuums for 5 min.

1.6. Constitution of the Glycerol stock

For each plate three glycerol stocks were prepared, using the round bottom culture cell plates (Corning, 3799).
Using the Labsystems Multidrop DW, aliquot 96 x 50 µl of 40% sterile glycerol. The GenMate Robot added 50 µl of the cell overnight culture. Before to seal the plates with an aluminium tape (Costar, 6570) 80°C resistant, we took 3 µl aliquot of the culture and put it in a Marsh plate. It will be used for the PCR verification.
The three sealed plates have to be stored at -80°C to ensure a good conservation of the samples.

1.7. The PCR verification.

A PCR verification was performed on the samples (3 µl of the bacteria culture) with the HiFi Taq polymerase (Invitrogen), using the external primers localized right before *att*B1 and right after *att*B2 on the vector.

- pL4440 For NEW: 5' TGGATAACCGTATTACCGCC 3'
- pL4440 Rev NEW: 5' GTTTTCCCAGTCACGACGTT 3'

The analysis of the PCR products enables to control the size of the ORF cloned (the size observed should correspond to the expected size of the predicted ORF + 2 x 100 bp for *att*B1 and *att*B2). The PCR products will also be used for the sequencing.

<u>PCR mix for 1 reaction:</u>

10X buffer	5 µl
100 mM dNTP each	0,4 (0,1 µl each)
50 mM MgSO4	2 µl
Primer For (240 µM)	0,08 µl
Primer Rev (240 µM)	0,08 µl
Platinum HF (Invitrogen, 5UI/µl)	0,2 µl
H2O	40,24 µl

Total	48 µl
Out of mix: Bacteria	2-3 µl

48 µl of PCR mix were aliquoted in each well of a 96-well plate.

To facilitate the PCR product size analysis and to more conveniently adjust the PCR reaction elongation times, the samples were organized in order of increasing size of the predicted ORFs

The PCR program used is as following:

1- 94ºC	2’
2- 94ºC	45 sec
3- 56ºC	1’
4- 68ºC	1’/kb
5- go to step 2	35 times
6- 68ºC	5’
7- 4ºC	For ever

1.8. Agarose gel electrophoresis.

This technique allows the separation of DNA fragments depending to the size of the bands. Thanks its capacity to become fluorescent when integrated in between the double strands of DNA, the ethidium bromide is used to visualised the fragments under UV (λ = 300 nm).

Solutions:

- TBE :

Tris base (890mM)	54 gr.
Boric acid (890mM)	27,2 gr
EDTA (0,5 M)	20 ml

pH 8.0

- Agarose (gel 1 %):

Agarose	2,5 gr.
TBE	250 ml.
EtBr (10 mg/ml)	15 µl

- Molecular weight marker :

1 Kb Plus DNA ladder from GibcoBRL™, which enables an estimation of the fragment’s size between 100bp and 12 Kb.

- Loading buffer :

Tris-HCl 100 mM
EDTA 25 mM
Glycerol 60 %
Orange pigment.

Loading of the gel:

5 µl of the orange loading buffer were aliquoted to each well of the PCR plate containing 10 µl of the PCR products.
Those 15 µl were loaded on a 96-well gel.
On each side of each lane of the gel, 10 µl of the DNA ladder were loaded.
Using a multichannel pipette, the lane A of the plate was loaded in wells 1-3-5-7… and the lane B in between in wells 2-4-6-8…

2. RNAi screening on *C. elegans*.

2.1. The strains

In order to do the RNAi screening, we received five strains of C. elegans from the Katie Nolan's lab which express the GFP in the neurons.

Strain name	Mutant allele	Origin
CX3427	kyls86 (srd-1::GFP)	Katie Nolan / Sengupta Lab / Bargmann
CX3427	kyls139 (str2::GFP)	Katie Nolan / Sengupta Lab / Bargmann
CX3596	str-3::GFP, X	Katie Nolan / Sengupta Lab / Bargmann
PY1058	oyls14, V (sra-6::GFP)	Katie Nolan / Sengupta Lab / Bargmann
PY1988	kin-29 (oy39) str-1::GFP, X	Katie Nolan / Sengupta Lab / Bargmann

Table 9: Description of the different strains used for the RNAi screening.

2.2. The clones.

The plasmids containing the fragments designated for RNAi were obtained from the RNAi library previously constituted. The HT115 *E. coli* strain was previously shown by Timmons and A. Fire to be beneficial for RNAi by feeding.
The single colonies of HT115 bacteria containing cloned L4440 plasmids were picked and grown overnight at 37°C on LB plate with 50 µg/ml Amp and 12,5 µg/ml Tet.
When we decided to begin the screening, we patched the bacteria on a LB + Amp (50 µg/ml) plate and let it grow overnight at 37°C.

Gene	ORF name	Gene	ORF name
daf-1	F29C4.1	DX23	Y113G7B.23
daf-4	C05B2.1A	DX24	AC3.3
daf-7	B0412.2	DX25	C51G7.4
daf-8		DX26	C39D10.7
daf-14	F01G10.8	DX27	W01G7.1
DX1	T21B6.3	DX28	Y110A7A.10
DX2	F30H5.3	DX29	C47E8.5
DX3	F45D3.4	DX30	W02D3.5
DX4	Y38C1AA.E	DX31	C17G10.5
DX5	ZK353.6	DX32	R05F9.10
DX6	D1007.12	DX33	F44F1.7
DX7	T23F1.6	DX34	W02A2.3
DX8	ZK1067.7	DX35	C01B10.8
DX9	Y67H2B.A	DX36	Y69H2.4
DX10	T27E9.1	DX37	R07E5.3
DX11	ZK652.3	DX38	G_YK7273
DX12	BO336.2	DX39	G_YK1666

DX13	C25A1.5	DX40	Y66H1B.2
DX14	RO5D11.8	DX41	W03G1.5
DX15	ZK1193.1	DX42	C04G6.10
DX16	F28D1.7	DX43	W02G9.2
DX17	C34E10.6	DX44	C03A7.14
DX18	T22H2.5b (WS)	DX45	C14C11.8
DX19	C53B4.5	DX46	AC3.4
DX20	F09F3.9	DX48	Y39B6A.1
DX21	T10B10.3	DX49	Y71H2B.10
DX22	F25H5.4		

Table 10: List of the clones used for the RNAi screening.

2.3. The worms cultures techniques.

C. elegans worms are raised in the laboratory in covered Petri plates containing agar medium overlaid with a "lawn" of *E. coli*, which the worms eat. Overgrowth of the bacteria is controlled by limiting the amount of uracil in the medium and seeding the plates with a mutant strain of *E. coli* bacteria (OP-50) deficient in uracil production. The bacteria grow until the uracil is used up and then stop so that a fine lawn is produced.
Since wild bacteria and molds can also grow on the culture plates, sterile techniques must be used.
Keep the plates covered as much as possible.

2.4. RNAi by feeding.

2.4.1. Preparation of the bacteria used to feed the worms.

DAY 1: Plate single colonies of feeding bacteria on a LB-Tet-Amp solid plate. Incubate at 37°C overnight, not exposed to the light (Tet is sensible to the light). Those plates can be stored at 4°C for a few days. “Fresh” cells tend to work best. Bacteria that have been stored on plates at 4°C for a long period of time (more than one week) often loose effectiveness.

DAY 2: Patch the bacteria of interest (2 cm^2) on a LB-Amp solid plate. Incubate overnight at 37°C.

2.4.2. Feeding of the worms.

DAY 3: Dilute a patche of $2cm^2$ of bacteria in 250 µl of LB and distribute 50 µl onto the NGM plate. The ratio of bacteria to worms is important: if the plates starve out, RNAi will not be effective. In addition, the bacterial lawn should not be allowed to continue to grow. Cells that do grow on plates after induction are generally cells that have lost the ability to produce T7 polymerase, or cells that are contaminants.
The open seeded plates were allowed to dry at room temperature for 1-2 hours under the hoot.
Add L4-stage hermaphrodite worms to 24 well plate and incubate at 15°C for 48 hours. Worms can be added by hand-picking or by adding chunks onto plates, containing NGM agar

+ 5 mM IPTG, that have been allowed to dry after seeding. In this case were added by hand-picking, using a platinum wire attached to a glass pipette.

With the aid of a binocular dissecting microscope and a "worm pick", the following procedure is used to transfer worms:

- Sterilize the worm pick by briefly flaming; then cool completely by gently pressing against the agar surface.
- Coat the underside of the cool sterile pick with "sticky" bacteria.
- Using the dissecting microscope, gently place the pick upon the desired worm. Incredibly the worms stick to the bacteria.
- Using the dissecting microscope, place the pick, with the worm underneath, gently on the agar surface of the destination plate. In a few moments the worm should crawl out and the pick can then be removed.

Important to notice: Sterile technique must be exercised at all times to prevent foreign contamination. Keep the lids on the plates except when absolutely necessary to remove them. Be sure not to damage the agar surface because the worms will burrow into the holes making them impossible to observe and transfer later.

DAY 5: The parents of each well were excluded from the plates and the plates were placed back at 15°C for another 36 to 48 hours.

DAY 6-7: Worms can be observed under the fluorescent microscope: GFP fluoresces bright green when illuminated with the correct wavelength of light, and thus can be visualized in live animals.
First, the mercury lamp on the microscope is warmed up for 15 min before use. A sliding barrier prevents the light from reaching the specimen until the bench scientist is ready to observe the fluorescence. The worms are located on the slide using bright field microscopy. After the worm is in focus using the 40× objective, the bright field light source is covered. Next, the barrier that blocks the light from the mercury bulb from reaching the sample is slid away. The blue cube is slid into the path of the light so that the sample is excited by blue light. The pattern of fluorescence in the worm is observed.
To allow a shot of the worms, freeze them with a few microliters of sodium azide (50 µl are enough).

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