2019 Oct 7

Decoding the clockwork underlying sex-specific cell fate switches in C. elegans.

Albert Martí I Sabarí, Dr. Carla Lloret Fernández, Dr. Richard Poole. Department of Cell and Developmental Biology, University College London.

Contents

1. SUMMARY ... 2
2. INTRODUCTION ... 3
2.1. Cell fate plasticity during development ... 3
2.2. *C. elegans* as a model for transdifferentiation ... 3
2.3. Timing of cell fate switches ... 4
2.4. Proneural genes control of neurogenesis ... 5
3. MAIN AIM ... 5
3.1. Specific aims ... 5
4. MATERIALS AND METHODS ... 5
4.1. *C. elegans* strains ... 5
4.2. Maintenance of strains ... 7
4.3. Crosses ... 7
4.4. Genotyping ... 7
4.4.1. Primers ... 7
4.4.2. Genomic DNA extraction (whole plate worm lysis) ... 8
4.4.3. Polymerase Chain Reaction ... 8
4.4.4. Electrophoresis ... 9
4.4.5. DNA Purification ... 10
4.4.6. Sequencing ... 10
4.5. Scoring using the fluorescence microscope ... 10
4.5.1. Image acquisition and processing ... 10
5. RESULTS ... 11
5.1. Generation of wild type and mutant strains with the same genetic background ... 11
5.1.1. Heterochronic genes ... 11
5.1.2. *Lin-48* control of neurogenesis ... 13
5.2. Scoring the panneuronal reporter *rab-3::YFP* in *lin-48(sa469)* mutant background ... 15
6. DISCUSSION AND FUTURE PERSPECTIVES ... 16
6.1. Heterochronic genes and timing ... 16
6.2. Proneural genes and neurogenesis ... 16
7. CONCLUSION ... 17
8. ACKNOWLEDGEMENTS ... 18
9. BIBLIOGRAPHY ... 18

1. SUMMARY

C. elegans is a nematode worm widely used in cell biology research. Recently, the Poole Lab has identified two glia-to-neuron cell fate switches[1] that only occur in males, one in the head and one in the tail. The first one self-renews a pair of socket glial cells[2] (called AMso) and produces a new neuron (MCM) for each. In the tail, the socket glial cells (called PHso1) become neurons (PHD) without any cell division. Both described events are examples of transdifferentiation[3], which is regulated by complex cell signalling events.

In this project, we attempt to shed light on the cellular mechanisms that control proneural gene[4] expression, as well as the timing (developmental stage) in which they are expressed. *Lin-48* is a transcription factor[5] expressed in the AMso and PHso1 socket cells since they are born, and it is hypothesized it may play an early role in the development of the MCM and PHD neurons. *Hlh-14* is a proneural gene known to be necessary for the described fate switches, but it is unknown if it may be regulated by *lin-48*. Both events take place during the sexual maturation of the males, and a good study candidate to understand the onset of these changes are the heterochronic pathway genes. This pathway is conserved throughout evolution and it regulates the timing of the gene expression changes that occur during the different developmental stages.

The results indicate that *lin-48* does not participate in the formation of the PHD, but it is essential for the MCM to have a neuronal fate. However, even after producing a worm strain with a non-functioning *lin-48*, the MCM had neuron-like characteristics in some worms. This could mean that it is in an intermediate step with glia-like and neuron-like traits simultaneously. The fact that the MCM and PHD have reacted differently to the deletion of *lin-48* means both events may be regulated differently. Interestingly, in some worms two additional socket-like cells were observed in the head of both males and hermaphrodites[6]. This could mean *lin-48* also affects other types of glial cell lineages.

The *hlh-14* mutant strain was successfully produced and genotyped[7]. However, it has not been scored for results.

The heterochronic gene crosses have all failed to produce new strains due to the sever effect it has on the characteristics of the worm, causing infertility and temperature sensitivity among others.

---

[1] A change from one cell type to another.
[2] A type of cell that is usually associated to neurons to protect and sustain them.
[3] A process in which a mature cell becomes a mature cell of a different type. In this case, a glia becomes a neuron.
[4] Gene that promotes neurogenesis.
[5] A molecule that controls the activity of a gene by promoting or inhibiting its expression.
[6] *C. elegans* has two sexes: males (X0) and hermaphrodites (XX).
[7] Genotyping is investigating the genetic constitution of the worms. As a result, the relevant genes are known to be homozygous (2 copies), meaning the strain is stable and will not lose these traits.

2. INTRODUCTION

2.1. Cell fate plasticity during development

Traditionally, development has been viewed as a unidirectional process where embryonic stem cells progressively lose plasticity as they commit to increasingly restrictive cell fates. This is believed to occur through the action of transcription factors and epigenetic mechanisms that regulate gene expression[1]. However, over the last decades numerous experiments have proven that the identity of a differentiated cell can be changed under the right conditions both *in vitro* ([2],[3],[4]) and *in vivo*[5] in a process known as transdifferentiation ([6],[7]). In fact, it has been observed in different types of tissue across several species as part of their natural developmental programmes ([8],[9],[10]). Despite its prevalence, the fundamental principles underlying natural transdifferentiation *in vivo* remain poorly understood.

2.2. *C. elegans* as a model for transdifferentiation

The nematode *Caenorhabditis elegans* is a well-established model organism due to its numerous advantages[11,12]. In the laboratory, *C. elegans* can be cultured on a diet of *Escherichia coli* on a nutrient agar surface. Its small size (1 mm in length), rapid generation time (three and a half days at 20 °C) and large brood size (~300 offspring) facilitates the culture of large populations. *C. elegans* is primarily self-fertilising (hermaphrodite XX), which assures an isogenic background, although males (XO) also appear with a frequency lower than 0.2 %. In addition, *C. elegans* is highly amenable to genetic studies. Mutants are readily generated and are made available upon request to the research community by multiple consortia. Importantly, the whole genome sequence is available ([13],[14]). Furthermore, very relevant to this work, it allows single cells to be easily traced during its development thanks to its transparent body and invariant cell lineage. In this regard, fluorescent reporters can be readily used to study cell morphology and gene expression *in vivo*.

Recently, Dr. Richard Poole's laboratory at UCL has identified and characterised two sexually dimorphic (i.e. occur exclusively in males) glia-to-neuron transdifferentiation events in *C. elegans*. They showed that the pair of amphid socket glial cells (AMso) in the head asymmetrically divide to produce the mystery cells of the male (MCM) interneurons[15]. Moreover, the pair of phasmid socket glial cells (PHso1) in the tail become Phasmid D (PHD) neurons without division, through a direct transdifferentiation[16]. The following figure pictures the aforementioned cells.

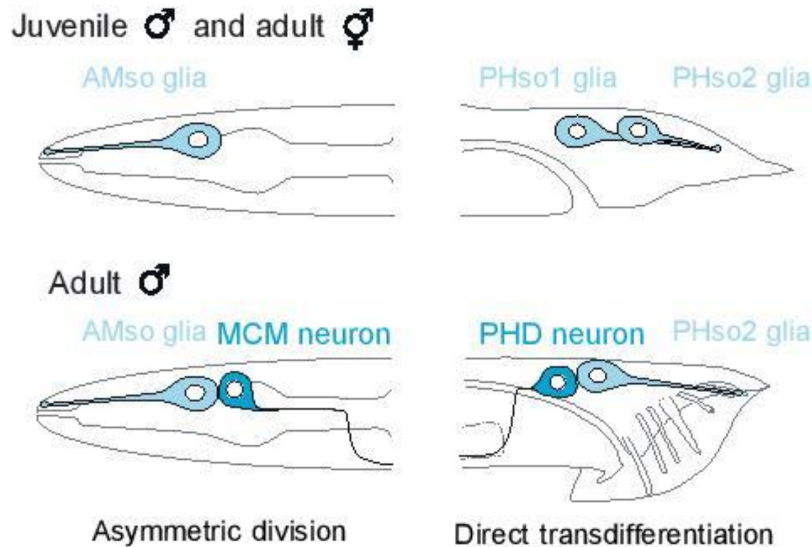

Figure 1. Schematic representing the head and tail anatomy of juvenile and adult male and hermaphrodite worms. The PHso2 is another phasmid socket glial cell present on the posterior side of the PHso1 or PHD.

These two sexually dimorphic transdifferentiation events take place during sexual maturation, along numerous developmental events that result in a male nervous system composed of 387 neurons and a hermaphroditic composed of 302 neurons[17]. The Poole Lab has determined that these glia-to-neuron transdifferentiation events are under the cell-autonomous control of the evolutionary conserved sex-determination pathway ([15],[16]). However, the mechanisms regulating where (specific cells) and when (developmental stage) these cell fate switches occur remain obscure.

2.3. Timing of cell fate switches

A suitable candidate to study the timing of transdifferentiation are the heterochronic pathway genes. The pathway was discovered as a mechanism to control cell division patterns in the ectoderm and larval transitions of *C. elegans*[18], and it is conserved throughout the animal kingdom. Specifically, the pathway consists of a microRNA-target cascade that regulates the timing of the transcriptional changes that occur between developmental stages. Therefore, heterochronic mutations do not affect the cellular or spatial identity of cells but the timing of their cell fate switch, resulting in either precocious or retarded phenotypes. It has been recently shown that heterochronic genes (*lin-28, let-7, lin-41* and *lin-29*) (figure 2) are required for some neuronal sexual dimorphisms in *C. elegans*, together with the sex specific pathway and neuron fate-specific transcription factors[19]. Their possible role in the timing of AMso/MCM and PHso/PHD transitions remains to be determined.

Figure 2. Diagram representing the main mechanisms of the heterochronic pathway. The lines connecting the different transcription factors signify inhibition.

2.4. Proneural genes control of neurogenesis

Proneural genes act as developmental switches that control neural fate during early development, instructing blast cells to commit to neuronal differentiation[20]. They belong to the basic helix-loop-helix (bHLH) transcription factor family and are preserved throughout evolution, including *C. elegans*. Preliminary data from the Poole Lab shows that *hlh-14* is functionally required for the described glia-to-neuron fate switches. However, very little is known about upstream regulators of proneural genes, particularly in the context of AMso/MCM and PHso1/PHD during late development.

The ovo-related LIN-48 transcription factor is expressed in the AMso and PHso1 glial cells since they are born, as well as in duct cells and a few additional uncharacterised cells. This is one of the few transcription factors known to be expressed in AMso and PHso1 cells prior to the cell fate switch. Additionally, it is expressed in the new-born MCM and PHD neurons (larval stage 4, L4), and its expression is downregulated in adult animals. *Lin-48* has been described to play a role in epithelial differentiation and shaping, and in hindgut and male tail morphogenesis ([21],[22],[23]) but has never been implicated in neurogenesis. However, its expression pattern is suggestive of *lin-48* playing an early role in the commitment of AMso and PHso1 into a neuronal fate. Furthermore, it raises the possibility that it could be activating the expression of *hlh-14* in these cells.

3. MAIN AIM

The aim of this project is to identify the molecular mechanisms that regulate the timing of the AMso/MCM and PHso1/PHD cell fate switches.

3.1. Specific aims

1.- Explore the role of the heterochronic pathway in the cell fate switch of AMso/MCM and PHso1/PHD during male sexual maturation.
2.- Explore the role of *lin-48* as early regulator of the cell fate switch of AMso/MCM and PHso1/PHD, acting through the *hlh-14* proneural gene.

4. MATERIALS AND METHODS

4.1. *C. elegans* strains

Mutant strains were provided by the CGC (Caenorhabditis Genetics Center), which is the main *C. elegans* strain repository. The rest of strains were either generated previously in the Poole Lab or built in this work.

Strain name	Genotype	Description	Origin
N2	*C. elegans* wild type		CGC
MT7626	*let-7(n2853) ts* X	Heterochronic pathway gene. Substitution G>A	CGC
MT1524	*lin-28(n719)* I	Heterochronic pathway gene. Substitution G>A	CGC
VT132	*sqt-1(sc13) lin-29(n333)/mnC1 [dpy-10(e128) unc-52(e444)]* II	Heterochronic pathway gene. Substitution G>A	CGC

MT7897	*lin-41(n2914)/unc-29(e1072) lin-11(n1281)* I	Heterochronic pathway gene. Substitution G>A	CGC
CB4088	*him-5(e1490)* V	High incidence of males. Substitution G>A	CGC
CB1489	*him-8(e1489)* IV	High incidence of males. G>A	CGC
CHL100	*drpIs3 [lin-48p::tdtomato]* I; *otIs291 [rab-3(prom1)::2xNLS::YFP + rol-6(su1006)], him-5(e1490)* V	Wild type strain carrying a transcriptional reporter that labels specific glial cells (*drpIs3*) and a transcriptional reporter that labels all neuronal cells (*otIs291*). *him-5* mutation increases the number of males in the population.	Poole Lab
CHL101	*drpIs3 [lin-48p::tdtomato]* I; *gmIs20 [hlh-14prom::hlh-14::gfp rol-6(+)]* II; *him-8(e1489)* IV	Wild type strain carrying a transcriptional reporter that labels specific glial cells (*drpIs3*) and a translational reporter of the HLH-14 transcription factor (*gmIs20*). *him-5* mutation increases the number of males in the population.	Poole Lab
CHL103	*drpIs3 [lin-48p::tdTomato]* I; *inIs179 [ida-1p::gfp]* II; *lin-48 (sa469)* III; *him-5 (e1490)* V	LIN-48 transcription factor mutant, carrying a transcriptional reporter that labels specific glial cells (*drpIs3*) and a transcriptional reporter that labels all neuronal cells (*otIs291*). *him-5* mutation increases the number of males in the population. Substitution A>G	Poole Lab
CHL104	*drpIs3 [lin-48p::tdtomato]* I; *lin-48 (sa469)* III; *otIs291 [rab-3(prom1) ::2xNLS::YFP + rol-6(su1006)], him-5(e1490)* V	LIN-48 transcription factor mutant, carrying a transcriptional reporter that labels specific glial cells (*drpIs3*) and a transcriptional reporter that labels all neuronal cells (*otIs291*). *him-5* mutation increases the number of males in the population. Substitution A>G	Generated in this work
CHL105	*drpIs3 [lin-48p::tdtomato]* I; *otIs291 [rab-3(prom1) ::2xNLS::YFP + rol-6(su1006)], him-5(e1490)* V	Wild type strain with the same genetic background as CHL104	Generated in this work
CHL106	*drpIs3 [lin-48p::tdtomato]* I; *gmIs20 [hlh-14prom:: hlh-14::gfp rol-6(+)]* II;	LIN-48 transcription factor mutant, carrying a transcriptional reporter that labels specific glial cells	Generated in this work

	lin-48(sa469) III; *him-8(e1489)* IV	(*drpIs3*) and a translational reporter of the HLH-14 transcription factor (*gmIs20*). *him-8* mutation increases the number of males in the population. Substitution A>G	
CHL107	*drpIs3 [lin-48p::tdtomato]* I; *gmIs20 [hlh-14prom:: hlh-14::gfp rol-6(+)]* II; *him-8(e1489)* IV	Wild type strain with the same genetic background as CHL106	Generated in this work

Table 1. List of *C. elegans* strains used.

4.2. Maintenance of strains

Worms were cultured in Nematode Growth Medium (NGM) (1.7% (w/v) Agar, 50 mM NaCl, 0.25% (w/v) Peptone, 1 mM $CaCl_2$, 5 µg/ml Cholesterol, 25ng/ml nystatin, 25 mM KPO_4, 1 mM $MgSO_4$), seeded with 250 µl of OP50 *E. coli* bacterial strain. These were periodically chunked to prevent the worms from starving, which could have interfered in the mating success and gene expression. Despite the plates also containing the antifungal agent nystatin, they occasionally got contaminated. Plates were bleached (0.2M NaOH, 0.16M NaClO), which killed any present microbes while leaving the eggs inside gravid hermaphrodites intact, allowing new worms to be born in a clean environment.

4.3. Crosses

In order to obtain the desired fluorescent reporter in the desired mutant background, worm crosses were set in the following way. A small lawn of bacteria was added at the centre of an agar plate, together with worms from two different strains (one containing the reporter, and the other containing the mutation). To ensure there was cross-progeny more males that hermaphrodites were added to the cross, in a 4:12 ratio. For every new mutant strain generated an additional strain without the mutation was kept, in order to score animals that otherwise share the same genetic background. This is important in order to avoid possible effects on reporter expression from other elements of the genome.

The crosses are further explained in the results section (figures 6&7).

4.4. Genotyping

4.4.1. Primers

The following list contains the primers used for the PCR and Sequencing protocols.

Primer name	Sequence	Tm (ºC)	Product size (bp)	Description
oCLF8	CAACAATGGAGCATACGGAGACG	59	563	

oCLF9	CGTCTCCGTATGCTCCATTGTTG			Genotype *let-7(n2853)* mutation
oCLF10	TTCAGTCACCAACACCTCGATAC	58	524	Genotype *lin-28(n719)*
oCLF11	GCATTCTCAGAATTTCGGATTCCC			
oCLF12	GGACATCCAGGAGAGCAAGG	n.d.	593	Genotype *sqt-1(sc13),* linked to *lin-29(n333)* (indirectly genotype *lin-29*)
oCLF13	GCTGATGTCGGGTTAATTGGATG			
oSGP11	AGTTCCAAGATTCATTAACCTGTCAC	58	688	Genotype *lin-48(sa469)* mutation
oSGP12	CTTACTACGACGCTCTTTGTAGGC			
oSGP13	AGGTGCACGTTTCAAGAGTC	57	239	Genotype *him-5(e1490)* mutation
oSGP14	TGCGTCTATCAGCTTCGTCA			
oSGP15	AATATTCAATGTCATTTCCCGG	53	214	Genotype *him-8(e1489)* mutation
oSGP16	GTGATAACGCATTTGACGG			

Table 2. List of primers used for PCR and genotyping. (n.d.= non-determined.)

4.4.2. Genomic DNA extraction (whole plate worm lysis)

The worms were collected from their culture plates using M9 buffer (22mM KH_2PO_4, 42mM Na_2HPO_4, 86mM NaCl, 1mM $MgSO_4$) and transferred to a 0.5ml tube. The pellet of worms were lysed using a lysis solution (50mM KCl, 10mM Tris-HCl – pH 8.3, 2.5mM $MgCl_2$, 0.45% (v/v) Triton X-100, 0.45% (v/v) Tween 20) with 1% proteinase K. This enzyme was allowed to act during 2 hours at 65 ºC and then inactivated 30 minutes at 95 ºC.

4.4.3. Polymerase Chain Reaction

The polymerase chain reaction (PCR) was used to genotype the newly generated mutant and wild type strains. As described in Table 1, all mutants used in the work are point mutations, so primers were designed to amplify this region of the genome. The genomic DNA obtained from the lysates was used as a template, and specific pair of primers (listed in table 2) were used to amplify the different genes.

PCR Standard Mix	
2 µL	DNA template (from lysate)
1 µL	Primer direct (200 nM; Sigma-Aldrich)
1 µL	Primer reverse (200 nM; Sigma-Aldrich)
10 µL	Buffer 5x PCRBIO HiFi polymerase (3 mM MgCl2, 1 mM dNTPs; PCR Biosystems)
0.5 µL	PCRBIO HiFi polymerase (0.02 u; PCR Biosystems)
35.5µL	Nuclease free water
50 µL	Total volume

Table 3. Example of standard PCR mix used in this work. (): Final concentration in the mix.

Step	T (ºC)	Time (min)	Cycles
Initial denaturation	95	1	1
Denaturation	95	0.5	
Annealing	x	1	35
Extension	72	1 min / kb	
Final elongation	72	10	1
Incubation	10	∞	

Table 4. Example of standard PCR programme used in this work. Annealing temperature (x) is oligo pair specific.

4.4.4. Electrophoresis

Electrophoretic gels were prepared using 1% agarose in 1X TAE (40 mM Tris-acetate; 1 mM EDTA, pH 8.3) and ethidium bromide (EtBr; 0.5 mg/ml). Ethidium bromide is a fluorescent dye that intercalates double-stranded DNA and RNA and is used to label DNA samples. The PCR samples were mixed with loading dye (New England Biolabs), which contains glycerol that confer density and a purple dye to identify the DNA migration, before being loaded in the gel. A DNA ladder was used to visualise the size of the DNA band (1 kb, New England Biolabs).

The following pictures are representative examples of electrophoretic gels for the some of the genes used in this work.

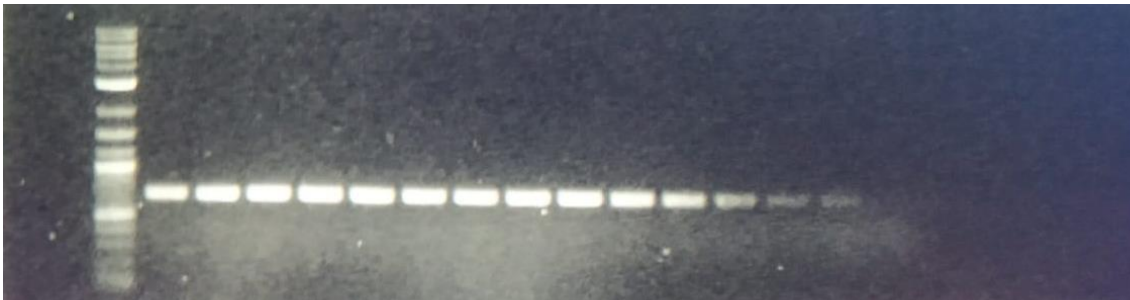

Figure 3. Electrophoresis gel for the *lin-48(sa469)* gene, using oSGP11 and oSGP12 primers, which render a 688 bp band. Lanes 1-12 correspond to different lysates from a newly generated cross. Lane 13 corresponds to control N2 (wild type) DNA. Lane 14 corresponds to control mutants (*lin-48(sa469)*) DNA. Lane 15 is the negative control (water).

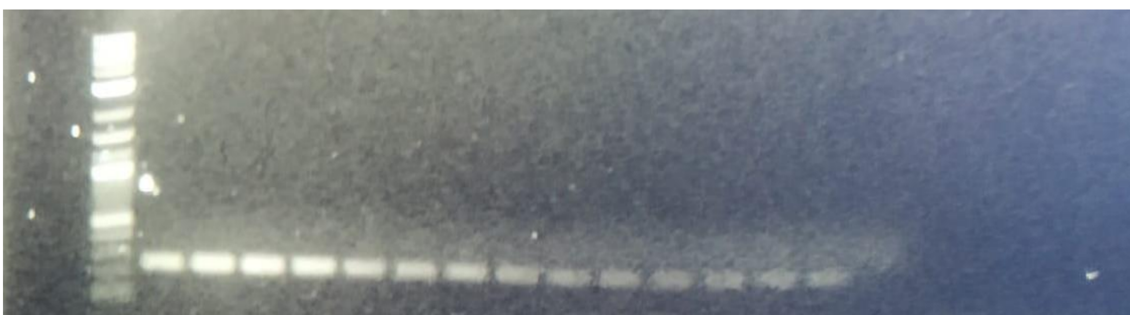

Figure 4. Electrophoresis gel for the *him-5(e1490)* gene, using oSGP13 and oSGP14 primers, which render a 239 bp band. The lane identities are equivalent to figure 3.

Figure 5. Electrophoresis gel for the *him-8(e1489)* gene, using oSGP15 and oSGP16 primers, which render a 214 bp band. The lane identities are equivalent to figures 3&4.

4.4.5. DNA Purification

The DNA was purified using a Monarch DNA Cleanup Kit. Briefly, a PCR sample is mixed with a buffer that allows DNA to bind to a column. Then DNA is washed with an ethanol-based buffer and eluted from the column using water. DNA was quantified using a Nanodrop.

4.4.6. Sequencing

DNA samples were sequenced by Source Bioscience company, using Sanger Sequencing. Previously, samples and primers were prepared at the desired concentrations. Sequencing results are represented as chromatograms that allow us to distinguish between a strain that is homozygote for the mutation, heterozygote for the mutation, or homozygote for the wild type (WT) allele.

4.5. Scoring using the fluorescence microscope

Worms were anesthetised using 50mM sodium azide to impede their movement and mounted on 5% agarose pads on glass slides. The scoring was done by sides, meaning two sides per worm. In the case where the fluorescence corresponding to the side further away from the eyepiece was not clearly visible, that side was not considered. Each strain was usually scored on different days to assess the reproducibility of the results. Mutant strains were scored in parallel to wild type reporter strains.

Scoring was performed using males ranging late L4 stage (when tail retraction was already observed) and young adults. Worms were maintained at 20ºC. Lack of GFP signalling was considered an 'OFF' phenotype. Percentages of fluorescence (YFP or tdtomato) expression in the cells were calculated as the total number of fluorescence positive cells over the total number of cells scored. Two-wailed Fisher Exact Test was applied for statistical analysis, considering a significant difference values where $p<0.05$. Calculations were performed using Graphpad QuickCalcs online software (www.graphpad.com/quickcalcs/).

4.5.1. Image acquisition and processing

Images were acquired on a Zeiss AxioImager using a Zeiss Colibri LED fluorescent light source and custom TimeToLive multichannel recording software (Caenotec).

Representative images are shown following maximum intensity projections of 2-10 1µm z-stack slices and were performed and edited in ImageJ.

5. RESULTS

5.1. Generation of wild type and mutant strains with the same genetic background

5.1.1. Heterochronic genes

In order to analyse the effects of the heterochronic pathway genes in the glia-to-neuron switch in the AMso/MCM and PHso1/PHD cells, we attempted to cross different genes of the pathway with fluorescent reporters that label the cells. We used the *drpIs3[lin-48 prom::tdtomato]* transgene that labels in red the AMso, MCM, PHso1 and PHD, and additional glial cells in the head. Therefore, we will name this reporter glia subtype specific. Additionally, we used the *otIs291[rab-3 prom::YFP]* transgene that labels in green all of the neurons in the worm, so we refer to it as panneuronal. Additionally, the panneuronal reporter contains a nuclear localisation signal (NLS) that directs YFP expression exclusively in the nucleus of the cells. In contrast, the glia subtype reporter is expressed in the cytoplasm and all cell structures including axons and dendrites. They are both transcriptional reporters, which means that the gene promoter has been fused to a fluorescent reporter.

C. elegans has 5 autosomes and an allosome that has 2 sexes (XO males and XX hermaphrodites), predominantly hermaphroditic. The *him-5* and *him-8* mutations promote chromosome X non-disjunction, resulting in a higher incidence of males in the population. These mutations are vital for this project as we are analysing males.

The following (figure 6) represents the cross design for the *lin-29* gene with the glia (*lin-48::tdt)* and panneuronal reporters (*rab-3::YFP)*. Similar crosses were performed with the *let-7, lin-41*and *lin-28* genes. In the particular case of the *lin-29* (n333) mutation, animals are sterile and do not produce any progeny. In order to be able to stably maintain the mutation in a strain, it must be balanced with the mnC1 element. Therefore, the strain we are looking for must contain a copy of the n333 allele and the mnC1 balancer.

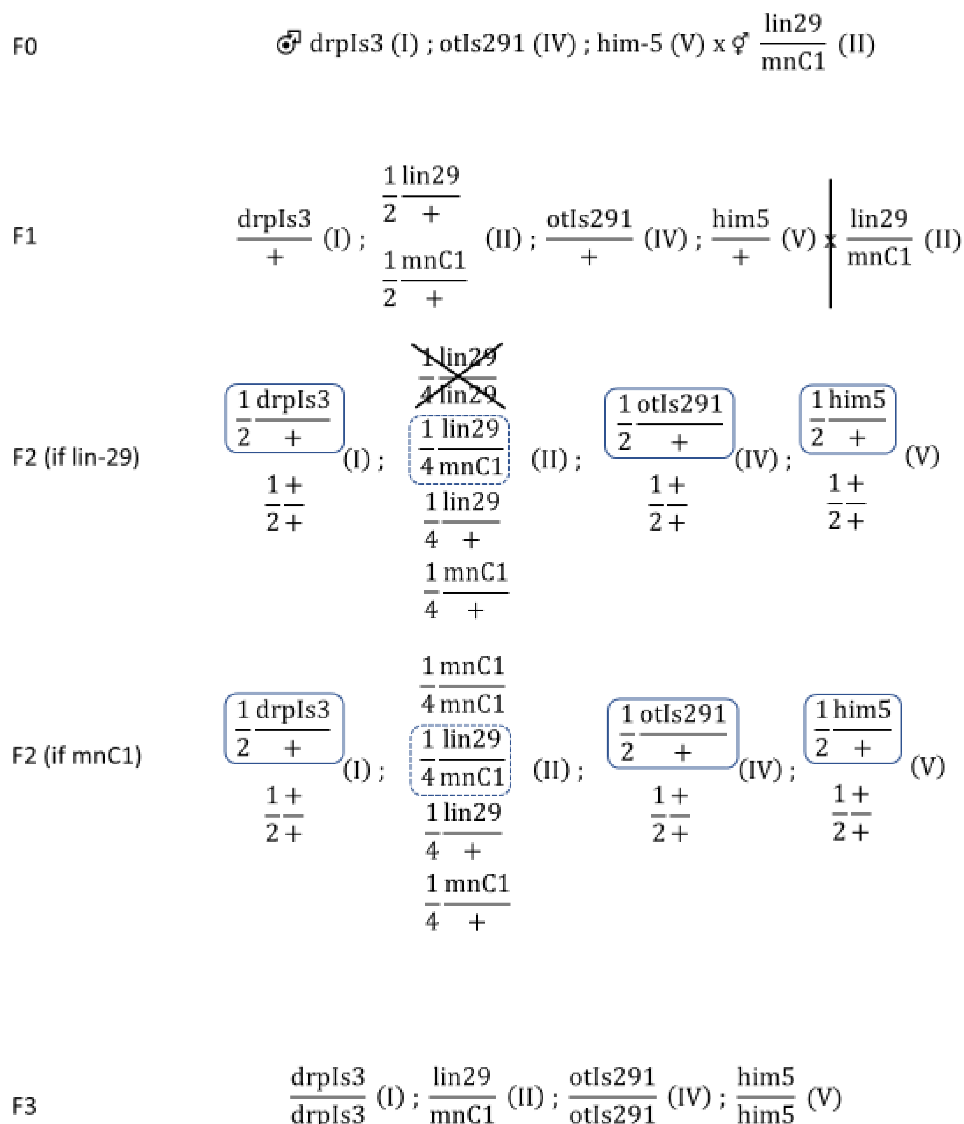

Figure 6. Cross design to obtain a strain with the *rab-3::YFP* and *lin-48::tdt* reporter transgenes in the *lin-29 (n333)* mutant background. The black line at the F1 stage discriminates the offspring from the strain it is backcrossed with. The black cross represents the lethality of homozygous *lin-*29 (which is exceptional to this heterochronic gene). The blue squares mark the selected alleles. Blue boxes indicate the desired genotype that is possible to identify phenotypically using a fluorescence dissecting scope. Dotted blue boxes indicate the desired genotype that cannot be identified by eye and needs to be genotyped by sequencing. Roman numbers in brackets indicate the chromosome where the reporter or mutation is located in the worm genome. The figure contains two F2 generations, depending on the genotype for each F1 generation plate.

In the P0 generation, males carrying the reporter of interest were crossed with hermaphrodites carrying the relevant mutation. Based on Mendelian genetics, everything is in heterozygosis in the F1 generation. In Figure 6, the F1 males are backcrossed with the original mutant strain. In the F2 generation, 30-40 singles were made selecting *drpls3* (red), *otls291* (green, roll) and *him-5* (heterozygous if multiple males but under 40% and

homozygous is over 40% male population). There was no F3 progeny due to the severity of the mutations and therefore the strain was not successfully built.

Heterochronic genes control the timing of vulval development in the *C. elegans* hermaphrodite. Thus, mutants have a reduced fitness and important defects in vulva morphogenesis, often showing multi-vulva or protruding vulva phenotypes, and also fertility defects. For these reasons, we were unsuccessful in obtaining the desired crosses. However, the experiment will be reattempted in the Poole Lab in the future.

5.1.2. *Lin-48* control of neurogenesis

Crosses were performed to investigate the effect of the loss-of-function *lin-48(sa469)* mutant in MCM and PHD neurogenesis. In order to achieve this, the mutants were crossed with the same *drpIs3* glial subtype reporter and *otIs291* panneuronal reporter. In parallel, the *lin-48* mutants were also crossed with the proneuronal gene reporter *gmIs20[hlh-14 prom::GFP, rol-6 (+)]*. Contrary to the previously described reporters, this is a translational reporter, meaning the gene has been fused in frame to a fluorescent protein. An additional feature of this reporter is that it carries a co-marker (*rol-6*) that affects worm locomotion, making them move in circles. This allows to easily select worms carrying the transgene of interest, even if heterozygous, without the need of fluorescent dissection scopes.

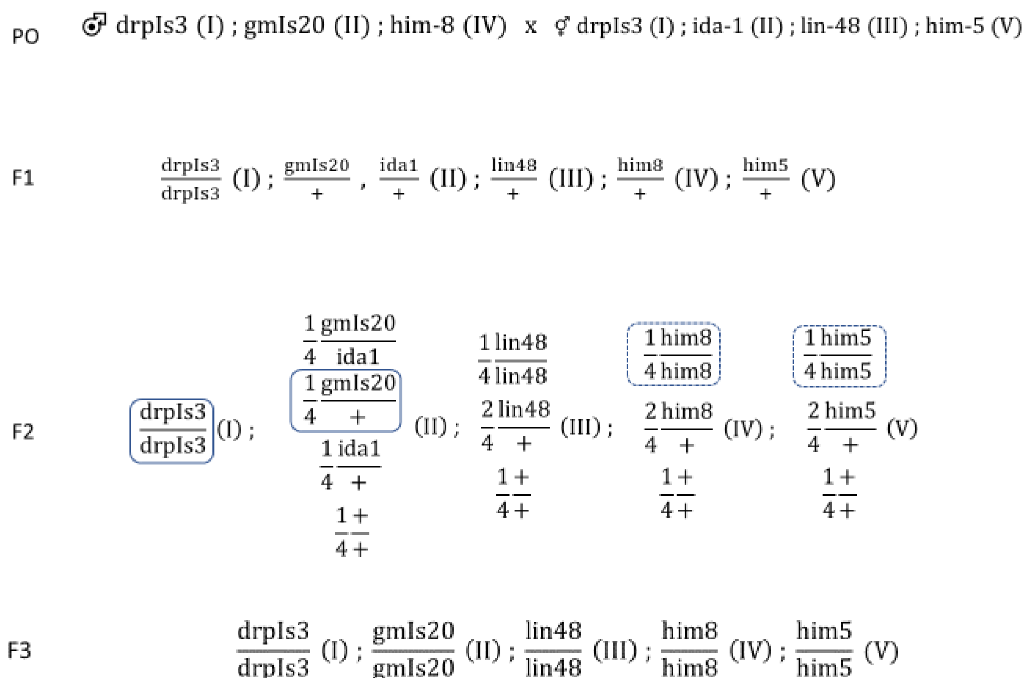

Figure 7. Cross design to obtain a strain with the *hlh-14::GFP* reporter transgene in the *lin-48 (sa469)* mutant background. Blue boxes indicate the desired phenotype that is possible to identify phenotypically using the fluorescence dissecting scope. Dotted blue boxes indicate the desired phenotype cannot be identified by eye and needs to be genotyped by sequencing. Roman numbers in brackets indicate the chromosome where the reporter or mutation is located in the worm genome.

In the P0 generation, males carrying the reporter of interest were crossed with hermaphrodites carrying the mutation. Based on Mendelian genetics, the entire F1

generation was heterozygous (except for *drpIs3*, which was already present in both strains and therefore homozygous). About 30-40 singles were made from F2 worms, selecting for gmIs20 (green) and with either of the *him* genes, selecting plates with around 40% male population. The F3 progeny from the F2 plates was lysed and genotyped for *lin-48, him-5* and *him-8*. The mutant plates were then singled (10-20 plates) selecting for homozygous reporters to produce the strain.

Lin-48 mutants (CHL106) and WT (CHL107) strains were successfully produced in a common genetic background containing a *hlh-14* translational reporter, *lin-48* transcriptional reporter and *him-8* mutation (table 1) (figure 8). The scoring has not been done yet, but the strains are now available for the Poole Lab to work with.

In the same manner, *lin-48* mutants (CHL104) and WT (CHL105) strains were successfully produced in a common genetic background containing a *rab-3* panneuronal marker, *lin-48* transcriptional reporter and *him-5* mutation (table 1). The *lin-48 (sa469)* mutation was confirmed by sequencing. Figure 8 shows the sequencing results corresponding to three plates from the *lin-48 (sa469)*, *drpIs3, rab-3, him-5* newly generated strain, sent to sequence for the *sa469* allele. This allele is a single nucleotide substitution where the WT A changes to the mutant G (represented as A>G).

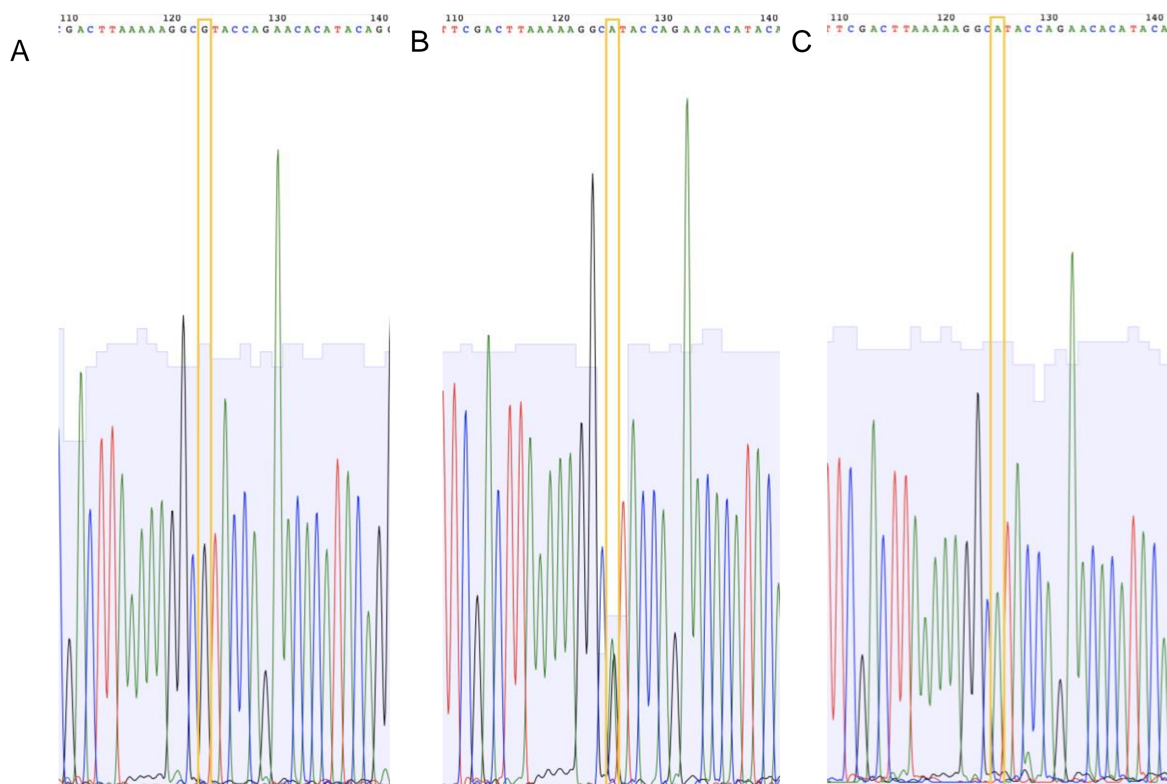

Figure 8. Chromatograms for *lin-48(sa469)* genotyping. Peaks corresponding to the nucleotide affected in the *sa469* allele are highlighted with the yellow box. A: Mutant strain (G), B: heterozygote strain (G and A overlapping peaks), C: wild type (A).

5.2. Scoring the panneuronal reporter *rab-3::YFP* in *lin-48(sa469)* mutant background

Firstly, the WT expression pattern of the *drpIs3[lin-48::tdtomato]* and *otIs291[rab-3::YFP]* reporters was characterised. This is represented in the following figure.

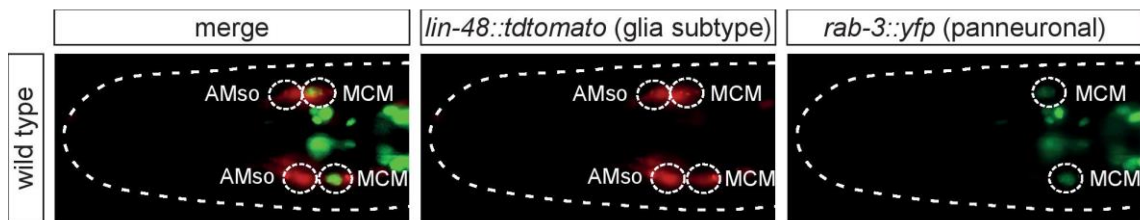

Figure 9. Images of a WT L4-adult male. The AMso and MCM have been labelled using white circles. The first image is a combination of the other two, showing both the glial subtype and panneuronal fluorescent markers.

Lin-48::tdt (red) was observed in the AMso in the head and in the PHso1 in the tail of hermaphrodites and in young male larvae. In larval stage 4 (L4) and adult animals, it was also observed in the MCM in the head, as well as the already transdifferentiated PHD in the tail. Both the MCM and the PHD showed *rab-3* (green) panneuronal expression in all the worms scored (figure 9). Expression was only quantified in males.

Second, we analysed the expression of the reporters in the *lin-48 (sa469)* mutant background. Focusing first in the tail, the mutant strain showed no significant defect, with the only exception of one worm where one PHso1 cell had a developing axon but did not express the panneuronal marker *rab-3*. Regarding the head, following the *drpIs3* reporter, we observed that the AMso glial cell divided to give rise to the MCM in most cases. In 13% of the scored sides there was no AMso/MCM division, although this percentage is not significantly different ($p>0.05$).

Moreover, 86% of the *lin-48::tdt* positive MCMs did not express the panneuronal marker *rab-3* ($p=0.0001$) (figure 11). Furthermore, two additional cells were observed in the head of some males and hermaphrodites which resembled socket cells but extended longer towards the posterior region of the worm. In some cases, this caused a displacement of at least one AMso.

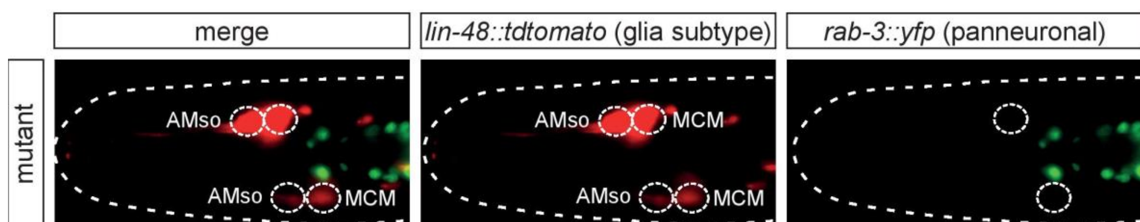

Figure 10. Images of a mutant L4-adult male. The AMso and MCM have been labelled using white circles. The first image is a combination of the other two, showing both the glial subtype and panneuronal fluorescent reporters. In this picture, the MCM are not marked by *rab-3* due to the *lin-48* loss-of-function mutation.

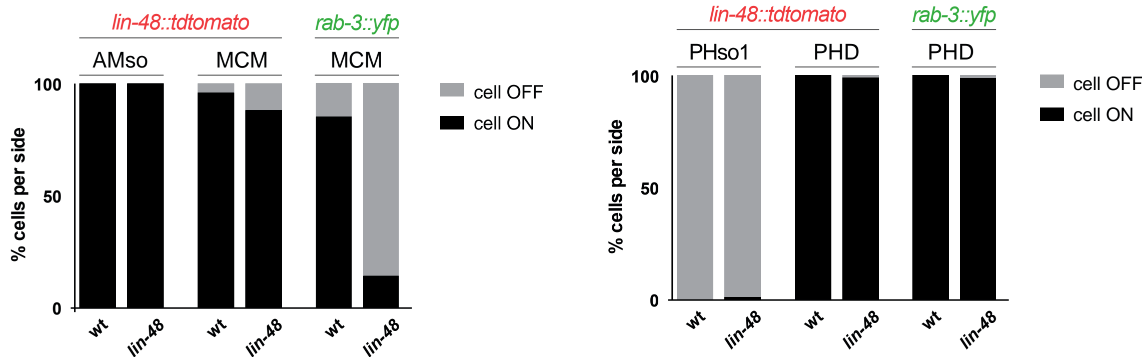

Figure 11. Bar charts representing the percentage of ON or OFF cells expressing the reporters respectively for each strain and cell. Cell counts: WT head (n=80), WT tail (n=66), *lin-48* mutant head (n=80), *lin-48* mutant tail (n=78).

6. DISCUSSION AND FUTURE PERSPECTIVES

6.1. Heterochronic genes and timing

The heterochronic mutant crosses did not result in any newly constructed strains, but generated tools (primers) and genotyping strategies for the Poole Lab in its future research.

Heterochronic genes remain a candidate as regulators of the glia-to-neuron transdifferentiation events described. If precocious neurogenesis was observed, that could result in an early loss of *lin-48::tdt* and/or a precocious expression of *rab-3*. On the other hand, if the event was delayed, perhaps late or no expression of *rab-3* at all would suggest it is regulated by heterochronic genes. It is possible that these genes regulate the expression of proneural genes like *hlh-14* to allow the glia-to-neuron fate switches. If an overexpression of *hlh-14* could revert the effect of the heterochronic pathway, it would prove this link. Otherwise, it is possible they act in parallel pathways.

6.2. Proneural genes and neurogenesis

The results show *lin-48* mutants have a significant phenotype in the MCM where it does not express the *rab-3* panneuronal marker. The functional consequences of losing this fate switch have not been described yet and could potentially influence the behaviour (especially during mating) of males. Based on preliminary data from the Poole Lab, the loss of *hlh-14* through RNAi knockdown blocks neurogenesis (the MCM is born but does not express neuronal features such as the ion channel *ida-1*). Both phenotypes are remarkably similar, which would support the hypothesis that *lin-48* may be regulating neurogenesis through proneural gene action. The newly created strain CHL106 will allow the hypothesis to be tested.

On the other hand, *rab-3* is expressed in a small portion of the worms, suggesting the MCM have an uncertain fate where they may not share the same function as the WT, or even not fully acquire a neuronal fate. Exploring other neuron-specific features of the MCM such as the *ida-1* ion channels or *pdf-1* neuropeptides could define the characteristics of the *lin-48* mutant MCM. Furthermore, setting crosses with other glial reporters would be of interest to find out if the MCM sometimes keeps its glial features or, on the contrary, if it is a cell with no fixed identity (does not express *rab-3* panneuronal marker nor other glia-specific reporters).

In some cases, the AMso did not seem to divide, which may link *lin-48* to an earlier role in neuronal specification, already in the AMso. Alternatively, sa469 is a point mutation which causes a loss of function but not a null mutation. Using a deletion mutant may increase the frequency of non-dividing AMso or non-neuronal MCM.

As for the additional socket-looking cells, it is likely that *lin-48* is affecting other glial lineages. Since these are observed in both males and hermaphrodites, it assures it is not an additional MCM.

With regards to the tail, it is unexpected that the mutation had no effect on the PHD. This puts forward the idea that there may be cell-specific mechanisms regulating transdifferentiation, even if both cells express the transcription factor *lin-48*.

Finally, the fact that the *lin-48* mutation does not affect the expression of drpIs3 (*lin-48::tdt)* indicates that it does not autoregulate itself. Autoregulation is a feature associated to many transcription factors which expression must be maintained through development.

7. CONCLUSION

Due to compromised fertility and morphological defects affecting mating, the heterochronic gene crosses have been unsuccessful, therefore not yielding any new strains. The *lin-48* mutant strain has been successfully generated with the *rab-3* panneuronal reporter and the proneural *hlh-14* reporter. The lin-48 mutation does not affect PHD neurogenesis, but it does affect the MCM as they sometimes express *lin-48::tdt* but not *rab-3::YFP*. This indicates that different molecular mechanisms regulate the glia-to-neuron switch in MCM and PHD neurons. Moreover, a small percentage of *lin-48* mutants does not experience division of the AMso, suggesting an additional earlier role of the transcription factor in neuronal specification. Finally, some *lin-48* mutants have shown two additional socket-like cells in the head, meaning it likely affects other glial lineages. Regarding the *hlh-14* proneural reporter, the strain will be scored shortly in the Poole Lab. The results of this project shed light on the molecular mechanisms controlling proneural gene expression.

8. ACKNOWLEDGEMENTS

I want to thank the Laidlaw Foundation for completely funding my research and for the opportunity to post my research output in their journal.

I would like to acknowledge the Poole Lab for hosting this research project, especially Dr. Poole and Carla Lloret Fernández for supervising it and for the support.
Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

9. BIBLIOGRAPHY

1. Waddington, C. H. The strategy of the genes: a discussion of some aspects of theoretical biology. 1957.
2. Weintraub, H. *et al.* Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. *Proc. Natl. Acad. Sci. U. S. A.* **86**, 5434–5438 (1989).
3. Takahashi, K. & Yamanaka, S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell* **126**, 663–676 (2006).
4. Tursun, B., Patel, T., Kratsios, P. & Hobert, O. Direct conversion of C. elegans germ cells into specific neuron types. *Science* **331**, 304–308 (2011).
5. GURDON, J. B., ELSDALE, T. R. & FISCHBERG, M. Sexually mature individuals of Xenopus laevis from the transplantation of single somatic nuclei. *Nature* **182**, 64–65 (1958).
6. Okada, T. S. & Okada, T. *Transdifferentiation: flexibility in cell differentiation.* (Oxford University Press on Demand, 1991).
7. Eguchi, G. & Kodama, R. Transdifferentiation. *Curr. Opin. Cell Biol.* **5**, 1023–1028 (1993).
8. Selman, K. & Kafatos, F. C. Transdifferentiation in the labial gland of silk moths: is DNA required for cellular metamorphosis? *Cell Differ.* **3**, 81–94 (1974).
9. Red-Horse, K., Ueno, H., Weissman, I. L. & Krasnow, M. A. Coronary arteries form by developmental reprogramming of venous cells. *Nature* **464**, 549–553 (2010).
10. Jarriault, S., Schwab, Y. & Greenwald, I. A Caenorhabditis elegans model for epithelial-neuronal transdifferentiation. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 3790–3795 (2008).
11. Brenner, S. The genetics of Caenorhabditis elegans. *Genetics* **77**, 71–94 (1974).
12. Sulston, J. E. & Brenner, S. The DNA of Caenorhabditis elegans. *Genetics* **77**, 95–104 (1974).
13. Genome Sequence of the Nematode C. elegans: A Platform for Investigating Biology. *Science (80-.).* **282**, 2012 LP – 2018 (1998).
14. Waterston, R. & Sulston, J. The genome of Caenorhabditis elegans. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 10836–10840 (1995).
15. Sammut, M. *et al.* Glia-derived neurons are required for sex-specific learning in C. elegans. *Nature* **526**, 385 (2015).
16. Molina-Garcia, L. *et al.* A direct glia-to-neuron natural transdifferentiation ensures nimble sensory-motor coordination of male mating behaviour. *bioRxiv* 285320 (2018). doi:10.1101/285320
17. Altun, Z.F., Herndon, L.A., Wolkow, C.A., Crocker, C., Lints, R. and Hall, D. H. WormAtlas. (2019).

18. Ambros, V. & Horvitz, H. R. Heterochronic mutants of the nematode Caenorhabditis elegans. *Science (80-.).* **226**, 409 LP – 416 (1984).
19. Pereira, L. *et al.* Timing mechanism of sexually dimorphic nervous system differentiation. *Elife* **8**, e42078 (2019).
20. Bertrand, N., Castro, D. S. & Guillemot, F. Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517–530 (2002).
21. Menoret, D. *et al.* Genome-wide analyses of Shavenbaby target genes reveals distinct features of enhancer organization. *Genome Biol.* **14**, R86–R86 (2013).
22. Roca, H. *et al.* Transcription factors OVOL1 and OVOL2 induce the mesenchymal to epithelial transition in human cancer. *PLoS One* **8**, e76773–e76773 (2013).
23. Aue, A. *et al.* A Grainyhead-Like 2/Ovo-Like 2 Pathway Regulates Renal Epithelial Barrier Function and Lumen Expansion. *J. Am. Soc. Nephrol.* **26**, 2704–2715 (2015).