Molecular mechanism involved in the regulation of Mouth-Form Dimorphism in the Nematode *Pristionchus pacificus*

Dissertation

der Mathemathisch-Naturwissenschaftlichen Fakultät

der EBERHARD KARLS UNIVERSITÄT TÜBINGEN

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Suryesh Kumar Namdeo

aus Garhakota, India

Tübingen

2018

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen
Fakultät der EBERHARD KARLS UNIVERSITÄT TÜBINGEN.

Tag der mündlichen Qualifikation: 23/10/2018

Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	Prof. Dr. Ralf J. Sommer
2. Berichterstatter:	Prof. Dr. Alfred Nordheim

Contents

1. Summary

The ability of an organism to produce different phenotypes in response to environmental signals has been described as phenotypic plasticity. Plastic traits are ubiquitous in nature, and can have adaptive advantages as the increased variability in phenotypes provide more raw material for natural selection to act upon. In addition, plasticity can get genetically accommodated, and has been proposed to result in the origin of novel traits, and speciation. Polyphenisms are extreme forms of discrete plasticity that can be controlled by developmental switches. The dimorphism in mouth morphology of the nematode *Pristionchus pacificus* has been studied to decipher the mechanistic control of plasticity. This nematode can either form a narrow stenostomatous (St) mouth or a wide eurystomatous (Eu) mouth. Many environmental, genetic and epigenetic factors have been characterized to influence the decision to form one of two alternative phenotypes. The developmental switch mechanism controlling mouth-form plasticity involves a sulfatase EUD-1, and a nuclear hormone receptor NHR-40. Moreover, the polyphenism in the mouth morphology of *P. pacificus* is maternally influenced, and exhibits both condition dependent and stochastic regulation.

The research described in this dissertation furthers the current mechanistic understanding of the mouth-form polyphenism in two major areas. First, characterization of a genetic locus that regulates the maternal influence, and exhibits a complex transcriptional activity, is performed. The alternatively spliced antisense long non-coding RNAs transcribed from this locus are also proposed to be involved in the stochastic regulation of plasticity. Second, the role of two independent sulfation processes in the regulation of mouth-form dimorphism is described. I identified a sulfotransferase that acts downstream, and independent of the previously characterized sulfatase EUD-1 to influence the mouth-form decision. This establishes the differential sulfation of biomolecules as a mechanism that can control expression of the phenotypically plastic traits.

2. Zusammenfassung

Die Fähigkeit eines Organismus verschiedene Phänotypen als Reaktion auf Signale aus der Umwelt auszubilden wird als phänotypische Plastizität bezeichnet. Plastische Merkmale sind in der Natur allgegenwärtig und können Vorteile bei der Anpassung haben, da die erhöhte phänotypische Variabilität Rohmaterial für die natürliche Selektion bietet. Darüber hinaus kann Plastizität genetisch akkomodiert werden und damit zur Entstehung neuer Merkmale und Arten beitragen. Polyphänismen sind eine extreme Form der diskreten Plastizität, die durch Schaltergene während der Entwicklung kontrolliert werden. Der Mundhöhlen-Dimorphismus im Nematoden *Pristionchus pacificus* wird untersucht um die mechanistische Kontrolle der Plastizität zu entschlüsseln. Dieser Fadenwurm kann entweder einen engen stenostomaten (St) oder einen breiten eurystomaten (Eu) Mund bilden. Zahlreiche ökologische, genetische und epigenetische Faktoren beeinflussen die Entscheidung, welcher der beiden alternativen Phänotypen gebildet wird. Zwei wesentliche Schaltergene kodieren für die Sulfatase EUD-1 und den nukleären Hormonrezeptor NHR-40. Darüber hinaus ist der Polyphänismus in der Mund Morphologie maternal beeinflusst und weist sowohl eine konditionale als auch eine stochastische Regulation auf.

Die in dieser Dissertation beschriebene Forschung trägt zum mechanistischen Verständnis des Polyphänismus der Mundform in zwei Bereichen bei. Zunächst wird eine Charakterisierung eines genetischen Lokus durchgeführt der den mütterlichen Einfluss reguliert und eine komplexe Transkriptionsaktivität aufweist. Es wird auch vorgeschlagen, dass die alternativ gespleißten, langen, nicht-codierenden antisense-RNAs, die von diesem Lokus transkribiert werden, an der stochastischen Regulation der Plastizität beteiligt sind. Zweitens, wird die Rolle von zwei unabhängigen Sulfatierungs-Vorgängen bei der Regulation des Mundhöhlen-Dimorphismus beschrieben. So identifizierte ich eine Sulfotransferase, die unabhängig von der zuvor charakterisierten Sulfatase EUD-1 die Mundform-Entscheidung beeinflusst. Diese Befunde etablieren die differentielle Sulfatierung von Biomolekülen als einen Mechanismus, der die Expression der phänotypischen Plastizität zentral kontrollieren kann.

3. Acknowledgements

4. List of Publications

Namdeo, S., Moreno, E., Rödelsperger, C., Baskaran, P., Witte, H., and Sommer, R.J. (2018). Two independent sulfation processes regulate mouth-form plasticity in the nematode *Pristionchus pacificus*. Development *145*, dev166272.

Serobyan, V., Xiao, H., **Namdeo, S.**, Rödelsperger, C., Sieriebriennikov, B., Witte, H., Röseler, W., and Sommer, R.J. (2016). Chromatin remodelling and antisense-mediated up-regulation of the developmental switch gene *eud-1* control predatory feeding plasticity. Nat. Commun. 7, 12337.

Werner, M.S., Sieriebriennikov, B., Loschko, T., **Namdeo, S.**, Lenuzzi, M., Dardiry, M., Renahan, T., Sharma, D.R., and Sommer, R.J. (2017). Environmental influence on *Pristionchus pacificus* mouth form through different culture methods. Sci. Rep. *7*.

Sommer, R.J., Dardiry, M., Lenuzzi, M., **Namdeo, S.**, Renahan, T., Sieriebriennikov, B., and Werner, M.S. (2017). The genetics of phenotypic plasticity in nematode feeding structures. Open Biol. 7, 160332.

5. Introduction

5.1 Evolutionary developmental biology

'How does the great diversity of life forms come about?'

This is one of the fundamental questions humans have been wondering about probably since the very beginning of civilization. Over the period of thousands of years many theories, both scientific and unscientific have been developed to explain the vast diversity of life forms on earth. In the early 19th century, the first fully formed scientific theory of evolution was proposed by Jean-Baptiste Lamarck in the early 19th century (de Lamarck 1809). His theory on 'Transmutation of Species' was influential in the 19th and early 20th centuries, but was slowly abandoned with the rediscovery of the Mendelian inheritance, and increase in evidences supporting the theory of evolution by natural selection, proposed by Charles Darwin and Arthur Russel Wallace in 1858. It states that new species arise due to natural selection acting upon the inheritable variation in various traits of an organism (Darwin 1859). In the 1920s and 30s, Darwinian natural selection was combined with recently discovered and tested principles of population genetics, and the concept of genetic mutation to form a more unified theory called 'Modern Synthesis' or 'Neo Darwinian Synthesis' (Haldane and Fisher 1931; Dobzhansky 1937). However, the Modern synthesis failed to take into account developmental biology, which caused it to have difficulties in describing the origin of organismal form in mechanistic terms, an important shortcoming that has received a lot of attention in the last three decades (Müller 2007).

Evolutionary developmental biology or evo-devo emerged in the 1980s as an attempt to reconcile the advances in the fields of developmental biology and molecular genetics with evolutionary theory. It provides a mechanistic model for evolution by showing how alteration in developmental process can cause phenotypic change on which natural selection can act upon (Alberch and Gale 1985; Gilbert et al. 1996; Arthur 2002; Rudel and Sommer 2003). In contrast to the population centric approach of the modern synthesis, evo-devo

is focused on the development of the individual organism. Evo-devo also brings the environment to the forefront as the environment can influence developmental trajectories, which can lead to phenotypic variation (Van Valen 1973). It establishes that the environment, in addition to the genotype, is involved in the production of phenotypic variation, in contrast to the earlier theories that described it to only act as a 'selection sieve' (Davidson 2006; Sommer 2009; Gilbert and Epel 2009).

The major contributions of evo-devo to evolutionary theory are characterized by the terms - evolvability, emergence and organisation (Müller 2007). **1)** Evolvability is defined as the capacity of a system for adaptive evolution. The intrinsic potential of an organism to produce heritable, selectable phenotypic variance makes it evolvable. Heritable phenotypic variance can be generated through adaptive genetic variance that exists because of mutation, recombination and drift (Kirschner and Gerhart 1998; Pigliucci 2008; Brookfield and F.Y Brookfield 2009). Evo-devo argues that the expression of variation capacity of the genotype is concomitant with development and hence evolvability can be expounded in terms of developmental variation and plasticity (Wagner 2005). **2)** Emergence: Evo-devo not only confines itself by addressing the generation of phenotypic variance, but also explicate on the emergence of novel traits by appraising development as a potent locus of innovation (Müller and Newman 2005; Hallgrímsson et al. 2012). It argues that novelty results from the specific dynamics of developmental systems, which undergo modification and are subject to selection (Newman and Müller 2000). **3)** Organization: Different features of evo-devo such as modularity, homology, homoplasy and body plans contribute to the organizational structure that is omnipresent in organismal biology. This organizational structure also provides the raw material for phenotypic evolution (Raff 1996). Hence, the phenotypic organization is not only just an outcome of evolution, but also an attribute that can have a profound effect on further evolution (Love and Raff 2006).

5.2 Phenotypic plasticity

Phenotypic plasticity is the phenomenon where different phenotypes can be produced from the same genotype in response to distinct environmental inputs. Even though it has a relatively straightforward and simple definition and has been around in some form for a long time (Baldwin 1896; Johannsen 1911), it received very little attention during the first half of the twentieth century. Bradshaw, based on his review on progress made in plant biology postulated the adaptive role of phenotypic plasticity and suggested that it must be genetically controlled (Bradshaw 1965). While most of the well-studied examples are of adaptive plasticity, plasticity can also be non-adaptive, resulting from passive and often short-term adjustment in behaviour, physiology, and/or morphology in response to environmental conditions (Ghalambor et al. 2007).

Conceptually, phenotypic plasticity is often understood in relation to Reaction norm, which is simply a function that relates the environment to which a genotype is exposed and the phenotypes that can be produced by the genotype (Woltereck 1913; Pigliucci 2001). Phenotypic plasticity is represented as the slope of this phenotype-environment mapping function for a given genotype (Fig 1A). Different genotypes can be represented as different functions on the phenotype-environment map, which can have different slopes i.e. phenotypic plasticities (Fig 1B). This variability in phenotypic plasticity of different genotypes can also act as raw material for selection to act upon, potentiating evolvability of phenotypic plasticity (Pigliucci and Schlichting 1998; West-Eberhard 2003). The evolutionary transition between plastic and robust development has been documented in different phylogenetic levels. For example, erosion of plasticity in head size for snakes (Aubret and Shine 2009) and the evolution of varying degrees of genetic caste determination for ants (Schwander et al. 2010). Phenotypic plasticity can also have huge implications on phenotypic diversification, the origin of novel traits and speciation (West-Eberhard 2003; Pfennig et al. 2010). Moreover, it can promote accumulation of cryptic genetic variation by shielding the genetic variation from natural selection. This heritable cryptic

genetic variation when released can provide raw material for adaptive evolution (Schlichting 2008).
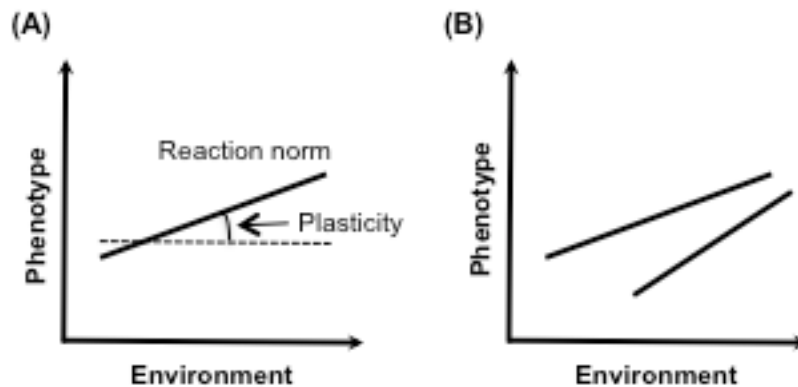
Fig 1: Reaction norm and phenotypic plasticity. (A) Reaction norm as a phenotype-environment function. Phenotypic plasticity is represented as the slope of this function. (B) Different genotypes can result in different reaction norms. (Adapted from Pigliucci 2001)

Phenotypic plasticity can produce traits that can be subcategorized as continuous and discontinuous. Most of the studied examples of plasticity are of continuous traits such as changing pigmentation of *Drosophila* abdomen in response to the varied temperature (David et al. 1990). In contrast, the discontinuous plasticity, termed polyphenism, produces discrete alternative traits. In last few years many genetic mechanisms have been described that control polyphenism (Projecto-Garcia et al. 2017). One well-studied example for this is of caste polyphenism found in honey bees, as a fate of being a queen or worker bee is decided based on whether larvae receive royal jelly or not (Weaver 1957). Another prominent example is of polyphenism found in winged/wingless forms and sexual/parthenogenetic modes of reproduction in pea aphids (Mittler and Sutherland 1969). Temperature-dependent sex determination, observed in reptiles, turtles and fish is investigated in the context of evolution of the sex chromosomes, and speciation in vertebrates (Matsumoto and Crews 2012; Merchant-Larios and Díaz-Hernández 2013). A predator-induced polyphenism is observed in the water flea *Daphnia pulex* as it can form neckteeth in response to kairomones released by larvae of its predator *Chaoborus* (Imai et al. 2009).

One important concept in the context of phenotypic plasticity is canalization, the capacity of an organism to produce the same phenotype

despite allelic or environmental variability (Waddington 1942). Originally, it was used to describe the phenomenon of genetic assimilation observed in Waddington's famous heat shock experiments on *Drosophila* pupae (Waddington 1953). Genetic assimilation is genetic fixation of an environmentally induced phenotype, so that it is expressed even in the absence of the environmental cue, resulting in the loss of plasticity. In contrast, a relatively recent but broader term, genetic accommodation indicates the evolutionary processes by which the target phenotype varies its sensitivity to the environmental or genetic variation (West-Eberhard 2003). In other words, genetic accommodation delineates transgenerational mechanisms of quantitative genetic change that can both fine tune phenotypic plasticity or canalize development (Beldade et al. 2011). In this context, it has been argued that an evolutionary pulse of plasticity and evolutionary responsiveness is followed by genetic canalization or accomodation, and both phases together are seen as facilitators of phenotypic diversification (West-Eberhard 2003). Finally, 'developmental switching', a concept often studied in the context of polyphenism, is a genetic mechanism, which is responsible for the decision to produce one out of two or more alternative phenotypes (Mather 1953; Golden and Riddle 1984).

Two different categories of non mutually exclusive molecular mechanisms, epigenetic and hormonal regulation, are often studied in the context of connecting environmental sensing to the regulation of phenotypic plasticity. For example, DNA methylation has been associated with caste determination of different species of Hymenoptera with highly conserved and and complex methylation system present in certain groups (Wang et al. 2006; Weiner and Toth 2012). A recent study highlights the role of a histone demethylase in temperature dependent sex determination, a phenotypically plastic trait in a turtle species (Ge et al. 2018). Another study on the locust *Locusta migratoria*, known for its alternative solitarius and gregarious phenotypes, reveal that miRNAs regulate phase transition by controlling the production of the hormone dopamine (Yang et al. 2014). Epigenetic information can also be transmitted transgenerationally, which can allow the environment to have multi-generational phenotypic influence through epigenetic processes (Jablonka and Raz 2009).

Hormones are in a unique position to regulate plastic traits as they can act both as sensors of the environment and regulators of the postembryonic development, having the potential to connect external environmental information with developmental switches (Nijhout 1998). Insect juvenile hormone and ecdysteorides have been implicated in regulating various plastic traits, such as castes in social hymenoptera and seasonal polyphenism in butterfly wing patterns (Wheeler 1986; Brakefield et al. 1998). Insulin/insulin like growth factor signaling has been observed in many cases of polyphenism including wing polyphenism in the brown planthopper *Nilaparvata lunges*, and the red-shouldered soapberry bug *Jadera haematoloma* (Xu et al. 2015; Fawcett et al. 2018). In general, hormonal signalling is involved in multiple physiological and developmental processes and often different sensitivity thresholds and sensitivity periods govern their specific phenotypic effects (Moczek and Nijhout 2002; Bento 2010). The environmental signals can bring about changes in the dynamics of hormonal production and targeting, which can influence gene expression. This could happen either via nuclear hormones receptors that have transcription regulator activity or via hormone mediated changes in the chromatin (Baniahmad and Tsai 1993; Lu et al. 1998).

Despite these advances, until recently our understanding of the mechanistic basis of phenotypic plasticity has suffered due to lack of a suitable model that has genetic and biochemical tools developed to understand plasticity in the ecological and evolutionary context. A free living nematode, *Pristionchus pacificus*, is now being used to study the genetic and environment interaction that regulate plastic traits, and the evolutionary potential of phenotypic plasticity as facilitator of diversity.

5.3 The nematode *Pristionchus pacificus* as a model system

Caenorhabditis elegans has proved to be a very successful nematode model system in the last half a century. This is due to many fundamental advantages of *C. elegans*, including the short generation time and life span, small size, relatively large brood size, the possibility of genetic crosses, small and specific number of cells, transparent body, and hermaphroditic mode of reproduction (Brenner 1974). It is important to note here that many of these features are conserved across nematodes. *C. elegans* has been at the center of many seminal discoveries including the neural connectome map (White et al. 1986), genetic control of apoptosis (Ellis 1986), genetics of ageing (Friedman and Johnson 1988), miRNAs (Lee et al. 1993), transgenic expression of fluorescent protein (Chalfie et al. 1994), RNAi (Kamath et al. 2003), and the first genome of a multicellular organism (C. elegans Sequencing Consortium 1998). Despite all these advantages, there are some major constraints with *C. elegans* such as limited understanding of its natural ecology and population dynamics as well as it not being a good representation of its phyla in terms of distribution and abundance (Schulenburg and Félix 2017). These factors in combination prevent *C. elegans* from becoming an ideal system for integrative and comparative evolutionary studies. However, many other nematode systems, especially *Pristionchus pacificus,* does not suffer from these limitations and have been used in the context of the integrative studies of evolutionary, ecological and developmental processes (Sommer 2015).

Pristionchus pacificus was first introduced in the 1990s as a satellite model of *C. elegans* for the comparative study of vulva development (Sommer and Sternberg 1996)*.* This comparison demonstrated that even though the vulva of *C. elegans* and *P. pacificus* are morphologically and functionally similar, their development is governed by different signalling pathways, result of a process named 'Developmental systems drift' (Wang and Sommer 2011). Most animals are self fertilizing hermaphrodites (XX), but there are occasional males (XO) arising because of meiotic non-disjunction. The occurrence of spontaneous males, which can then be maintained, allow for genetic crosses that enables complex genetic analysis. The life cycle is similar to *C. elegans*

with four juvenile stages, and a facultative dauer stage. It has a four day generation time and a large brood size (150-200 eggs), making it easy to culture and maintain in the lab (Sommer et al. 1996). The ability of cryopreservation of *P. pacificus* enables the long term storage of different strains. *P. pacificus* is a free living nematode with a cosmopolitan distribution (Herrmann et al. 2010). Many wild isolates of *P. pacificus* and closely related species were collected from all over the world, providing ample opportunity for both micro and macro evolutionary analysis and comparative genomics (Rödelsperger et al. 2014; Kanzaki and Giblin-Davis 2015; Ragsdale et al. 2015). In the wild, the species has been isolated from soil as well as from the back of oriental beetle *Exomala orientalis* and other scarab beetles of several genera including *Oryctes, Adoretus, Maladera and Hoplia* (Herrmann et al. 2007; Herrmann et al. 2010)*.* It exhibits a necromanic association with beetles, that is, it lives in an arrested dauer stage until the beetle dies the worm exits the dauer stage and starts to feed on the microbes growing on the carcasses (Herrmann et al. 2007).

Many genetic, biochemical, and genomic tools have been developed in *P. pacificus,* which have immensely contributed to our understanding of the system*.* This include forward genetics (Sommer et al. 1996), a genetic linkage map (Srinivasan et al. 2002), whole genome sequencing (Dieterich et al. 2008), transgenesis (Schlager et al. 2009), reverse genetics (Tian et al. 2008; Witte et al. 2015), in situ hybridization (Tian et al. 2008), endo and exo metabolome analysis (Yim et al. 2015), and transcriptomics and proteomics techniques (Borchert et al. 2010; Sinha et al. 2012). Whole genome sequencing followed by the gene annotation revealed many distinct features including the presence of a huge fraction of recently evolved genes (Prabh and Rödelsperger 2016). These tools have enabled the molecular and genetic characterization of several traits of ecological and evolutionary significance in *P. pacificus* (Sommer and McGaughran 2013). This includes molecular characterization of phenotypes such as vulva development (Zauner and Sommer 2007; Wang and Sommer 2011), olfaction (Hong and Sommer 2006), dauer development (Mayer and Sommer 2011), mouth-form polyphenism (Bento et al. 2010; Ragsdale et al. 2013), predatory feeding behaviour (Wilecki et al. 2015), and oxidative stress response (Moreno et al.

2017). The focus of this dissertation is on the genetic regulation of polyphenism observed the mouth morphology of *P. pacificus.*

5.4 Mouth-form dimorphism in *Pristionchus pacificus*

The center of this thesis is the molecular mechanisms that regulate the phenotypic plasticity observed in the mouth structure of *P. pacificus*. Phylogenetic studies have revealed that dimorphism in the mouth structure is a morphological innovation restricted to the Diplogastridae family, the family *P. pacificus* is a member of (Hirschmann 1951; Susoy et al. 2015). The two different mouth morphs in *P. pacificus*, characterized by their distinct morphological features, are known as Eurystomatous (Eu, 'wide mouthed'), and Stenostomatous (St, 'narrow mouthed') (Fig 2). They differ in the mouth size, number and topology of the denticles, and morphology of the buccal cavity. Eu animals exhibit a shallow but broad buccal cavity, a claw-shaped dorsal tooth, and a subventral tooth. On the other hand, in the St animals the buccal cavity is narrow and deep, the dorsal tooth is flint-shaped, and they lack the subventral tooth (Hirschmann 1951; von Lieven and Sudhaus 2000). The developmental decision to produce one of two alternative morphs is executed in the J4 larval stage and is irreversible then (Bumbarger et al. 2013). Eu mouth is adapted for predatory feeding, whereas animals with St mouth form are strict microbe feeders and, develop faster (Serobyan et al. 2013; Serobyan et al. 2014). A systematic examination of multiple *P. pacificus* strains established mouth form dependent predatory behaviour, demonstrating that only Eu and not St animals are able to kill other nematodes (Wilecki et al. 2015). Thus, this dimorphism might provide a competitive advantage to *P. pacificus* over other nematodes, which shares the same natural habitat and resources.
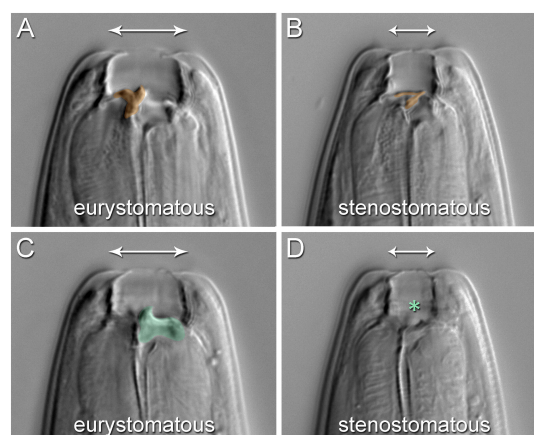
Fig 2: Mouth-form dimorphism in *P. pacificus*. (Adapted from Ragsdale et. al. 2013) (A) and (C) are a single Eu hermaphrodite in two focal planes, whereas (B) and (D) are a single St hermaphrodite in the corresponding planes. (A) and (B) are sagittal, (C) and (D) right sublateral planes. The Eu form bears a claw-like dorsal tooth (A, false-colored orange) and an opposing, claw-like right subventral tooth (C, green), whereas the St form has a thin, flint-shaped dorsal tooth (B, orange) and no subventral tooth (D, asterisk).

The regulation of *P. pacificus* mouth form expression is dependent on both, stochastic and conditional factors (Susoy and Sommer 2016). *P. pacificus* shows high levels of intra-genotypic variability even in a common environment. For example, under laboratory conditions about 80-90% of hermaphrodites of *P. pacificus* Californian strain RS2333 representing the wild type strain used for laboratory studies, exhibit Eu mouth morphology, whereas 10-20% of hermaphrodites are St. This phenotypic heterogeneity in a stable environment resulting from the inherent stochasticity in the system can provide an adaptive advantage as a bet hedging strategy to cope up with unpredictable changes in its natural habitat (Susoy and Sommer 2016). Some bet hedging strategies are common in microbes, and plants that experience unpredictable environment (Veening et al. 2008; Gremer and Lawrence Venable 2014). The mouth-form dimorphism is also sex dependent because males are more likely to be St (Serobyan et al. 2013). Also, the ratio of Eu to St animals can vary substantially among different wild isolates, indicating towards the variability in plasticity that might exist for this trait in nature (Ragsdale et al. 2013).

This polyphenism can be influenced by several environmental cues including starvation, population density, bacterial diet and culture condition (Bento et al. 2010; Werner et al. 2017; Akduman et al. 2018). Certain pheromones, which may be acting as sensors of population density, can influence the mouth-form decision in a dose-dependent manner, resulting in more Eu animals (Bento et al. 2010; Bose et al. 2012). Out of all the compounds that were synthesized and tested, dasc#1 showed the strongest Eu form inducing capacity, with other compounds including pasc#9, ascr#1 and npar#1 showing much weaker Eu form inducing effect (Bose et al. 2012). A conserved endocrine signaling system involving a nuclear hormone receptor DAF-12 and its steroid hormone ligand Δ7- dafachronic acid (DA) influence mouth form ratio (Bento et al. 2010). Interestingly, this 'dafachronic

acid - DAF-12' system is also involved in the plastic decision of whether to enter dauer stage or not, in both *P. pacificus* and *C. elegans*, which demonstrates a partial overlap of molecular pathways governing two distinct plastic phenotypes (Motola et al. 2006; Ogawa et al. 2009). This overlap is only partial because a dauer promoting transcription factor DAF-16/FOXO, which is downstream of DAF-12, has no effect on mouth-form phenotype (Ogawa et al. 2011).

In order to identify factors that can switch the mouth form either completely to Eu or St, a forward genetic approach using EMS mutagenesis was applied, leading to the discovery of many genetic regulators of mouth-form plasticity. EUD-1, a sulfatase was identified to act as a dosage dependent, lineage specific, and completely penetrant developmental switch of mouth form plasticity (Ragsdale et al. 2013). A null mutation in *eud-1* results in all St animals (*Eud:* Eu form defective), whereas animals over-expressing *eud-1* are always Eu. *eud-1* is expressed in amphid neurons indicating its probable involvement in the environmental perception. In a suppressor screen of *eud-1*, another mouth-form regulator NHR-40, a nuclear hormone receptor, was isolated and characterized (Kieninger et al. 2016). In contrast to *eud-1*, mutations in *nhr-40* results in all Eu animals, while overexpression leads to all-St animals. Both, *eud-1* and *nhr-40* have been described as part of the developmental switch mechanism. Recently it was described that *eud-1* and its genetic neighbour *nag-1*, encoding for a N-acetyl acetylglucosaminidases, show diplogastridae lineage specific duplication and their duplicated genes are located next to each other on the opposite strand. They collectively form, what has been described as a multi-gene locus as the other genes *nag-1, nag-2*, and *sul-2.2.1* also show mouth form related phenotypes (Sieriebriennikov et al. 2018). In addition, a conserved protein chaperone HSP-90 was identified to buffer naturally occuring polyphenism by controlling canalization of the developmental decision (Sieriebriennikov et al. 2017).

In spite of these advances, there are some major questions regarding the control and execution of developmental switches that remain to be addressed: 1) How do different developmental switch genes interact at the molecular and genetic level, 2) how is environmental information transmitted

to the switch mechanism (upstream factors), and 3) how does the developmental switch result in different mouth morphologies (downstream factors). Moreover, the molecular mechanism that regulate the stochastic expression of mouth-form dimorphism also remains to be elucidated. Further understanding of the mechanism regulating mouth-form dimorphism require a systematic genetic approach followed by biochemical analysis. The following passages provide a brief introduction to various genetic and biochemical concepts that turned out to be important for mouth-form regulation in the course of my research.

5.5 Sulfation of biomolecules

Sulfation and desulfation of biomolecules, first described in 1876, are among the most fundamental biochemical processes (Baumann 1876). Sulfation is an enzyme, sulfotransferase, driven conjugation of a sulfo group ($-SO_3^-$) to biomolecules that could be steroids, proteins, liposaccharides or metabolites, usually resulting in increased water solubility (Lipmann 1958; Strott 2002). In sulfation, 3'-phosphoadenosine-5'-phosphosulfate (PAPS) acts as the donor of the sulfo group. In contrast, the reverse process, desulfation is cleavage and removal of the sulfo group from the biomolecule by an enzyme named sulfatase, resulting in decreased hydrophilicity (Diez-Roux and Ballabio 2005). The reversible sulfation of biomolecules is entailed in the biological processes such as hormonal regulation, cell signaling, detoxification, molecular recognition and viral entry into cells (Buono and Cosma 2010; Mueller et al. 2015; Barbeyron et al. 2016; Soares da Costa et al. 2017).

The sulfotransferases, based on their substrate specificity, are broadly classified into aryl-sulfotransferase and hydroxysteroid sulfotransferase families (Dooley 2000). The aryl-sulfotransferases catalyze the sulfation of phenol and amine groups, whereas hydroxysteroid- sulfotransferases sulfate estrogen and other steroid compounds (Li 2001; Falany et al. 2009). Based on their subcellular localization, sulfotransferases can be categorized as cytosolic or membrane bound. The membrane bound sulfotransferases are mostly localized on the golgi membrane and are responsible for sulfation of proteins, glycolipids and proteoglycans (Kasinathan et al. 1991; Skelton et al. 1991). In contrast, the cytosolic sulfotransferases sulfate low molecular weight compounds such as phenols and steroids (Suiko et al. 2017).

Most of the research on the reversible sulfation processes has been focused on their association with human health, which has contributed immensely to our understanding of the sulfation biochemistry, and the progression of various diseases (Coughtrie et al. 1994; Klüppel 2010). However, this leaves a big gap in our understanding of the role of that reversible sulfation plays in the organismal development. In order to fill this gap, various organismal models including the fruit fly, zebrafish and

nematodes are being used in last one decade (Mizuguchi et al. 2009; Kamimura et al. 2011; Xi et al. 2016). Nematodes, in particular can be very useful in this regard due to their fast and well described development along with the availability of the various biochemical and genetic tools.

5.6 Alternative splicing

Alternative splicing is defined as a regulated post transcriptional process that involves selective removal of exons and/or introns from maturing RNAs, thereby generating distinct transcripts from the same gene (Berget et al. 1977; Early et al. 1980). The process is heavily regulated, involving both cis regulatory elements, and trans acting splicing factors (Kelemen et al. 2013) and the disruption of the process has been linked to various diseases (Scotti and Swanson 2016). Although alternative splicing is present in all multicellular organisms, it seems to be more abundant in more complex organisms with humans having over 95% of multi exon genes that are alternatively spliced (Kim et al. 2007; Pan et al. 2008; Wang et al. 2008). Alternative splicing has been shown to be involved in a wide range of functions from cellular to organismal level (Kalsotra and Cooper 2011; Kelemen et al. 2013). One advantage of alternative splicing over changes in the coding regions of the genes is that while the later affects all transcripts containing the region, the former allows specific transcripts to be expressed in a cell type or developmental stage-specific manner. Consistently, genes with developmental roles are more likely to be alternatively spliced highlighting the contribution of alternative splicing to development (Bush et al. 2017).

One of the molecular consequences of alternative splicing is the modifications of the properties of proteins by, for example altering the domain composition and thereby affecting protein stability, localization, binding properties and enzymatic activities (Resch et al. 2004; Stamm et al. 2005). Alternative splicing can also generate novel non coding RNAs, which can further contribute to the emergence of novel phenotypes and complexities (McFarlane and Wilhelm 2009; Boivin et al. 2018). For example, large human proteome experiments on different cell and tissue types indicated that only a minor fraction of detected polypeptides come from alternatively spliced transcripts (Tress et al. 2017), which reinforces the notion that the majority of alternatively spliced isoforms do not translate into functional proteins but may still be acting as non coding RNAs (Leoni et al. 2011).

Alternative splicing has been suggested to aid in the emergence of novel phenotypes as small mutations on splice sites can lead to alternative

isoforms containing novel combinations of exons (Nilsen and Graveley 2010; Krawczak et al. 1992). Alternative splicing is often compared with gene duplication, another major source of phenotypic innovation, and is suggested as an inversely correlated evolutionary mechanism as alternative splicing has been shown to be reduced in gene duplication, where the duplicated copies are retained. (Kopelman et al. 2005; Talavera et al. 2007). Alternative splicing can also espouse subfunctionalization of duplicated genes, hence preventing them from becoming pseudogenes (Lynch and Force 2000). The contrarity that exists between cell type diversity and total gene numbers is often termed as G- value paradox (Hahn and Wray 2002). The increase in cell type number is often linked to the evolution of organismal complexity (Schad et al. 2011). Along with other factors, like non coding RNAs and epigenetic modifications, alternative splicing is considered a prime candidate in explaining the G-value paradox (Chen et al. 2014; Xing and Lee 2007).

5.7 Antisense long non-coding RNAs

It is now established that non-coding RNAs are abundantly transcribed, and they are involved in a multitude of biological functions (Djebali et al. 2012; Chen and Aravin 2015; Schmitz et al. 2016). Non-coding RNAs that are more than 200 nucleotides in size are defined as long non coding RNAs (lncRNAs). These RNAs share many similarity with mRNAs as they are transcribed by RNA pol II, as well as they can be polyadenylated, caped and spliced (Guttman and Rinn 2012). However, unlike most protein coding transcripts, the show low evolutionary conservation, and are mostly expressed at much lower levels (Katayama et al. 2005; Carninci et al. 2005). A large fraction of lncRNAs localize in the nucleus, consistent with their role as epigenetic modulators (Magistri et al. 2012). In a study in humans and mice, lncRNAs were found to predominantly originate from the vicinity of the protein-coding genes, suggesting some lncRNAs may depend on the same promoter/enhancer regions as their nearby protein-coding genes (Khachane and Harrison 2010).

Antisense transcription of lncRNAs, a common phenomenon in eukaryotes, is defined as transcription from the opposite strand of a protein coding gene (Yelin et al. 2003; Numata and Kiyosawa 2012). In comparison to their sense counterparts, the antisense transcripts exhibit much lower expression levels, and are primarily cell type specific (Magistri et al. 2012). Antisense lncRNAs have been described as both positive and negative regulator of gene expression (Su et al. 2012; Huang et al. 2016). They are also involved in mammalian X chromosome inactivation, imprinting and epigenetic regulation (Brown et al. 1992; Pandey et al. 2008; Neumann et al. 2018).

Antisense RNAs mediated gene regulation can occur in two ways, either in *cis* or in *trans.* While in *cis,* the antisense transcripts interact with transcription from the sense strand on same locus; in *trans,* they can influence transcriptional regulation at distant loci (Osato et al. 2007; Werner 2013). They can mediate gene regulation at all three levels: pre-transcriptional, co-transcriptional, and post-transcriptional (Faust et al. 2012; Clark and Blackshaw 2014). The molecular mechanisms involving antisense lncRNAs

based regulation can be subcategorized into lncRNA-DNA interaction, lncRNA-RNA interaction and lncRNA-protein interaction (Villegas and Zaphiropoulos 2015). In lncRNA-DNA interaction, an RNA-DNA duplex or triplex can associate with regulatory proteins to affect neighbouring gene (Bierhoff et al. 2010). Sense RNA and antisense lncRNA can hybridize to form RNA duplexes, resulting in different post-transcriptional outcomes for the sense mRNAs (Poliseno et al. 2010; Modarresi et al. 2011). Antisense lncRNAs can also influence the function and localization of various gene regulatory proteins by acting as molecular decoys (Jeon and Lee 2011; Villegas et al. 2014).

Despite all these advances in our understanding of antisense lncRNAs, there are many limitations in their functional characterizations due to their genetic and biochemical nature (Bassett et al. 2014). First, the majority of them are expressed at low levels in cell specific manner, which makes it difficult to ascertain changes in their expression levels after genetic knockdown or editing (Eißmann et al. 2012). Second, molecular function of many lncRNAs can be dependent on the act of transcription, rather than transcripts themselves (Kornienko et al. 2013). Third, only in a few cases mutations in antisense lncRNAs produce any observable phenotype, which could be due to small mutations might fail to significantly affect secondary and tertiary RNA structure, and localization (Goyal et al. 2017). Also, little evolutionary conservation of most antisense lncRNAs prevents micro and macro evolutionary analysis (Bush et al. 2018). In order to better understand molecular mechanism and function of antisense lncRNAs, further analysis in an organismal system is warranted.

5.8 **Maternal effect on plastic traits**

Maternal effects have been shown to influence several aspects of early and late development. The mother is the source of extensive sensing of the external environment and transmitting the signal to the progeny during embryogenesis. Early embryos are transcriptionally inactive since more resources are devoted to rapid and synchronous cell cycles resulting in expeditious amplification of DNA and the number of nuclei, while the cytoplasmic content remains constant (Glover 1991). These transcriptionally inactive early embryos are regulated by cytoplasmic mRNAs from the mother. The early maternal transcriptional control of embryonic development is then gradually replaced by the zygotic transcriptional control in a process known as maternal to zygotic transition (Schier 2007). In addition, maternal effects could also be transmitted to the progeny by other means such as epigenetic and cytoplasmic inheritance. Epigenetic inheritance, parental effect over the epigenome profile of the progeny, is in part, mediated by maternally controlled DNA methylation (Weaver et al. 2009), histone modifications (Sankar et al. 2017, Cao et al. 2017) and small RNAs (Dallaire and Simard 2016). On the other hand, cytoplasmic inheritance is mediated by maternal organelles, metabolites, RNAs and proteins that are present in the zygote (Motomura et al. 2010). Furthermore, the maternal hormonal status has a huge impact on embryonic development and many life history traits of the offspring (Meylan et al. 2012).

Maternal effects have been demonstrated to be associated with developmental plasticity, and have been argued to influence adaptive evolution (Mousseau and Fox 1998; Uller 2008). In many cases, the environment in which offspring develop may be a direct consequence of maternal phenotypes (Plaistow et al. 2007). Furthermore, the maternal phenotype is considered one the most important environmental factors during early development in many animals, which might result in the coadaptation of maternal phenotypes and offspring development (Wolf and Brodie 1998). In humans, maternal effects via nutritional, hormonal, or epigenetics profile of the mother, can also alter the susceptibility to various non communicable diseases (Hanson et al. 2011).

In nematodes, maternal effects have been observed during dauer formation decision and the sexual polymorphism of *Rhabditis sp.* SB347. This organism exhibits a sexual polymorphism by producing males, females and hermaphrodites (Felix 2004). In this nematode, instead of being dependent on the environment, the larval decision to become dauer correlates with the age and sex of the mother. Older parents are more likely to produce progeny that go through the dauer stage and become hermaphrodites. Also, hermaphrodite mothers produce more female offsprings compared to female mothers (Chaudhuri et al. 2011; Chaudhuri et al. 2015). In *P. pacificus* maternal influence has been observed in the mouth-form phenotype of the male progeny after crosses. If the maternal phenotype is Eu then male progeny are 35% Eu, whereas if the maternal phenotype is St then there are almost no Eu males (Serobyan et al. 2013). However, the molecular players involved in mediating this maternal influence still remain to be elucidated.

5.9 Aim of the Research

The main objective of my research focuses on the identification and characterization of the genetic players that govern the polyphenism observed in mouth morphology of the nematode *P. pacificus.* The mouth-form dimorphism in *P. pacificus* was used as model to understand genetic regulation that underlies phenotypic plasticity. In particular, I focused on the identification and characterization of factors that 1) Act upstream of developmental switch mechanism and might be responsible for sensing the environment. 2) Act downstream of the developmental switch and are involved in relaying the information about the mouth-form decision, and ultimately result in Eu or St mouth formation. The genetic players involved in the maintenance and regulation of the maternal influence on *P. pacificus* mouth form dimorphism has been the center of my research. In addition, I focused on the regulators of sulfation biochemistry and their involvement in the regulation of the mouth-form polyphenism. My research led to the discovery and characterization of two genetic regulators of the polyphenism in *P. pacificus* mouth form: a sulfotransferase coding gene, *sult-1,* and a locus with complex transcriptional activity involving a sense and two antisense transcripts, *mfra-1*.

6. Results and Discussion

6.1 Two Independent sulfation processes regulate mouth-form dimorphism in nematode *Pristionchus pacificus*

Suryesh Namdeo, Eduardo Moreno, Christian Rödelsperger, Praveen

Baskaran, Hanh Witte & Ralf J. Sommer

6.1.1 Synopsis

Phenotypic plasticity, the property of a genotype to form distinct phenotypes in response to the environment, represents a core concept in developmental biology. However, genetic inroads into developmental plasticity are scarce because systematic environmental perturbations are difficult to achieve under laboratory conditions. The nematode model organism *Pristionchus pacificus* shows phenotypic plasticity of its feedings structures, which is controlled by conditional and stochastic factors, the latter of which allow the isolation of monomorphic mutants by genetic manipulation. *Pristionchus* has either a eurystomatous (Eu) mouth form with two teeth enabling predation, or a stenostomatous (St) form with a single tooth that can only feed on microbes. While wild-type animals form a mixture of both mouth forms when fed *Escherichia coli*, mutants in the sulfatase-encoding gene *eud-1* are all-St. In contrast to *eud-1* mutants, *eud-1* overexpression results in all-Eu populations, indicating that *eud-1* is a developmental switch, a finding that confirmed long-standing predictions that plasticity must be controlled by switch mechanisms.

Here, we describe the results of a pharmacological screen identifying that Bisphenol A, Tyramine and Dopamine influence mouth-form ratios. As these phenolic compounds are known substrates of sulfotransferases, we systematically knocked out five cytosolic sulfotransferases in *P. Pacificus* and identified *sult-1* to control mouth-form plasticity. *sult-1* mutants have all or

preferential Eu mouth forms in both sexes and under distinct environmental conditions. When testing the assumption that the *sult-1*/sulfotransferase and *eud-1*/sulfatase form a sulfation module that has identical substrates, we found strong evidence for two independent sulfation processes during mouth-form regulation. Epistasis and expression pattern analysis and further pharmacological assays with Bisphenol A strongly suggest that *eud-1* and *sult-1* act at different levels in the genetic hierarchy of mouth-form plasticity. Thus, mouth-form plasticity in *P. pacificus* provides a model to study the role of sulfation in fundamental organismal and cellular processes.

6.1.2 Own Contribution:

I designed, performed and analysed all the experiments except for phylogenetic analysis, and *sult-1* reporter lines. The phylogenetic analysis was performed by Dr. Christian Rödelsperger and Dr. Praveen Baskaran. The reporter lines for *sult-1* were generated by Dr. Eduardo Moreno. I prepared and edited the manuscript with Prof. Dr. Ralf Sommer. My overall contribution to this study correspond to about 90%.

6.2 Chromatin remodelling and antisense-mediated up-regulation of the developmental switch gene *eud-1* control predatory feeding plasticity

Vahan Serobyan, Hua Xiao, **Suryesh Namdeo**, Christian Rödelsperger, Bogdan Sieriebriennikov, Hanh Witte, Waltraud Röseler & Ralf J. Sommer

6.2.1 Synopsis

Developmental plasticity, the capacity to produce different phenotypes from the same genotype has been suggested to represent a facilitator of novel phenotypes and adaptive evolution. Polyphenism or discontinuous plasticity has been proposed to act though developmental switches. However, the molecular and genetic mechanisms of how developmental switches work have not been well elucidated. *Pristionchus pacificus,* a diplogastridae nematode model, has been intensively studied for the polyphenism observed in its mouth morphology. In *P. pacificus,* the same genotype can produce either a wide and complex Eurystomatous (Eu) mouth form, or a narrow and simpler Stenostomatous (St) mouth morphology. The animals with Eu mouth morphology display the ability for predatory feeding, which is not observed in St animals. EUD-1, a sulfatase, was identified to act as a developmental switch that regulates this polyphenism.

Here, we describe two chromatin modifiers, a histone acetyltransferase LSY-12, and a methyl binding protein MBD-2 that regulates the mouth-form decision. Mutations in both genes result in Eu-form defective (*Eud*) phenotype. The mutations also cause down regulation of *eud-1;* and defects in the histones marks H3K4me2, H3K4me3, H3K9ac, and H3K27ac. We also identified and characterised an antisense transcript at the *eud-1* locus. This *as-eud-1* is downregulated in *lsy-12* mutant indicating *lsy-12* might be acting through *as-eud-1.* Based on our analysis of *as-eud-1* over-expression and report constructs, we conclude that *as-eud-1* acts as a positive regulator of *eud-1.* Thus, this study describes the epigenetic regulation of a developmental switch gene.

6.2.2 Own Contribution

I optimized and performed RNA FISH experiments, which revealed that *eud-1* and *as-eud-1* transcripts are co-expressed. My experimental results are illustrated in figure 4 of the paper. My overall contribution is about 15% in this study.

6.3 A locus with complex transcriptional activity regulates polyphenism in mouth structure of *P. pacificus*

Suryesh Namdeo, Vahan Serobyan, Christian Rödelsperger, Hanh Witte, Waltraud Röseler & Ralf J. Sommer

6.3.1 Introduction

The mouth-form dimorphism in *P. pacificus* is sex linked as most hermaphrodites have Eu mouth morphology, whereas most males have St morphology (Ragsdale et al. 2013; Serobyan et al. 2013). For males, it is also influenced by the maternal phenotype, as the male offspring of St mothers are predominantly St. In contrast, the male offspring of Eu mothers maintain much higher Eu frequency (Fig 3A; Serobyan et al. 2013). Mendelian inheritance fails to explain this distribution in the context of phenotypic plasticity. The short generation time and the available genetic tools of *P. pacificus* allowed us to perform forward genetic screens to study this complex genetic trait. In a forward genetic screen, two maternal influence defective (*Mid*) mutants, *mid-1* and *mid-2* were isolated (Serobyan 2015). Both, *mid-1* and *mid-2* mutants show reduced maternal effect as the male progeny of St mothers show much higher Eu frequency compared to their wild type counterparts (Fig 3B; Serobyan 2015). The genetic mapping followed by RACE experiments established that *mid-1* is on a predicted gene Contig2-snapTAU.253 (gene annotation: Sinha et al. 2012) on chromosome III of *P. pacificus.* Further analysis confirmed that the associated gene encodes a serine/arginine rich protein kinase that acts upstream of the developmental switch gene *eud-1* (Serobyan 2015). The *C. elegans* homolog, SPK-1, is an essential component of the pre-mRNA splicing machinery and involved in germ line development and early embryogenesis (Kuroyanagi et al. 2000). Genetic mapping of the second mutant allele *mid-2* localized it to the X chromosomal gene Contig4-snapTAU.369 (gene annotation: Sinha et al. 2012). The mutation lesion of *mid-2* is a single nucleotide change from A to G, in an intron of this gene (Serobyan 2015). The associated gene was named *mfra-1* for *m*outh *f*orm *r*elated *a*bnormal-*1*.
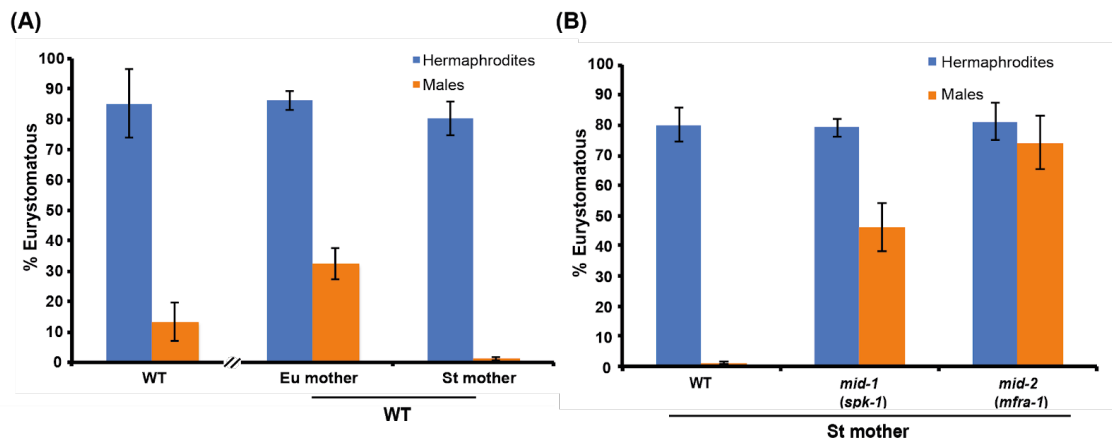
Fig 3: Maternal influence on the mouth form of *P. pacificus*. (A) Mouth-form ratio of *P. pacificus* male progeny is influenced by the maternal phenotype (Adapted from Serobyan et al. 2013). (B) Maternal influence defective mutants *mid-1* and *mid-2* were isolated using EMS mutagenesis (Adapted from Serobyan 2015).

6.3.2 *mfra-1* exhibit complex transcriptional activity that involves a sense and two antisense RNAs

In order to characterize the transcriptional activity at the *mfra-1* locus, RACE experiments were conducted for both DNA strands. We found that both strands show transcriptional activity with sense transcribing a single RNA named *mfra-1-SF1*, while antisense transcription resulting in two alternatively spliced RNAs named *mfra-1-AS1* and *mfra-1-AS2* (Fig 4A). The mutation is in an intron of the sense transcript and in exons of both antisense transcripts. The sense transcript encodes for a protein that has an uncharacterized ortholog in *C. elegans.* Both antisense transcripts *mfra-1-AS1* and *mfra-1-AS2* were characterized as long non-coding RNAs based on two lines of evidences. First, both *mfra-1-AS1* and *mfra-1-AS2* do not code for any polypeptide that has been detected in previously published proteomics studies of *P. pacificus* (Borchert et al. 2010; Borchert et al. 2012). Second, RNA coding potential estimation tools CPC2 and CPAT assign both *mfra-1-AS1* and *mfra-1-AS2* as long non-coding RNAs (lncRNAs) based on their Fickett scores, ORF lengths and integrity, and other chemical properties (Wang et al. 2013; Kang et al. 2017). Specifically, CPC2 assigned *mfra-1-AS1*, a 979 bp long RNA, coding probability of 0.109; and *mfra-1-AS2*, an 885 bp long RNA, coding probability of 0.160. In comparison, *mfra-1-SF1*, the sense protein-coding transcript was assigned coding probability 1, which is consistent with

the detection of protein coded by it in *P. pacificus* proteomics data (UniprotKB id: H3FEE9) and the presence of its *C. elegans* ortholog (C09B8.3, Wormbase).

(A)

(B)
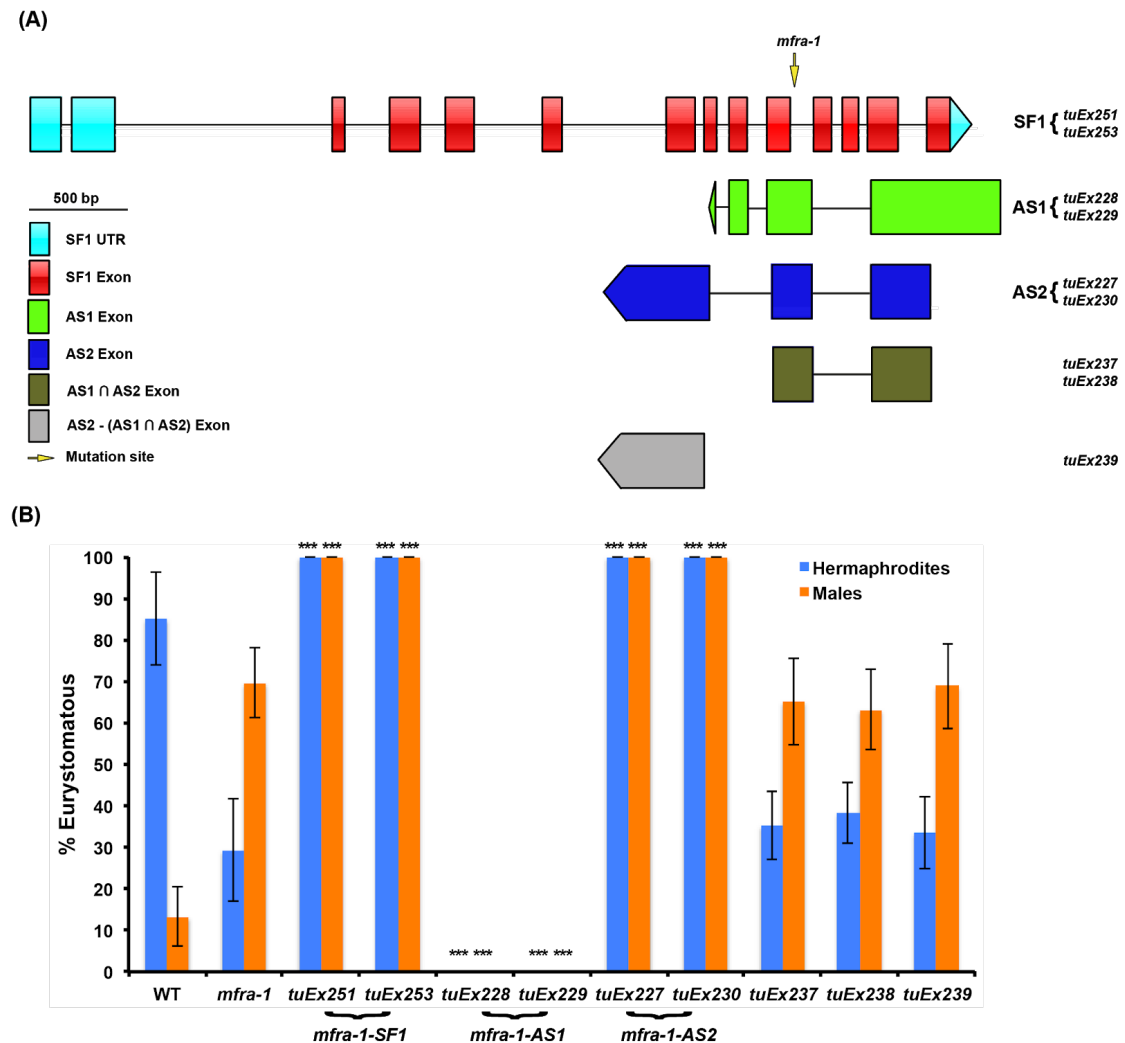
Fig 4: Different RNAs from *mfra-1* locus influence mouth form of *P. pacificus*: (A) Three transcripts from *mfra-1* locus are illustrated along with the mutation site and two additional shared/unshared antisense exonic regions that were used to create transgenic alleles. (B) Eu form frequency in different transgenic animals for both sexes. They are compared with corresponding *mfra-1* values as they are all in *mfra-1* background. *** $p<0.001$, two tailed student t test.

6.3.3 Sense and antisense transcripts of *mfra-1* have an influence on the mouth-form dimorphism

In order to decipher the function of different RNAs transcribed from *mfra-1* locus, we generated transgenic animals with *mfra-1-SF1*, *mfra-1-AS1*, or *mfra-1-AS2* transgenes in the *mfra-1* mutant background (Fig 4A). These

transgenes contained *mfra-1-SF1*, *mfra-1-AS1*, or *mfra-1-AS2* cDNA, and their respective promoter sequences (see methods). The transgenic animals having *mfra-1-SF1* (*tuEx251* and *tuEx253*) and *mfra-1-AS2* (*tuEx227* and *tuEx 230*) cDNAs show 100% Eu frequency (Fig 4B). In contrast, the animals expressing *mfra-1-AS1* transgene (*tuEx228* and *tuEx229*) show only the alternative all-St phenotype (Fig 4B). Since *mfra-1-AS1* and *mfra-1-AS2* are lncRNAs they might have some regions or motifs that can be sufficient for their specific function. To ascertain if this is the case here, we generated transgenic lines that have only some parts of the *mfra-1-AS1* and *mfra-1-AS2* cDNAs fused to the shared promoter of *mfra-1-AS1* and *mfra-1-AS2* (Fig 4A). The transgenic animals having only the shared exons of *mfra-1-AS1* and *mfra-1-AS2* (*tuEx237* and *tuEx238*) fail to show any difference in Eu frequency compared to *mfra-1* (Fig 4B)*.* Furthermore, the transgenic animals with only the *mfra-1-AS2* specific exon (*tuEx239*) show mouth form frequencies similar to *mfra-1* (Fig 4B). Taken together, these findings suggest that whole RNAs of *mfra-1-AS1* and *mfra-1-AS2* are required for their mouth form related function.

It is surprising that *mfra-1-SF1* elicits such a strong effect on the mouth-form phenotype, because the *mfra-1* mutation is in an intron of *mfra-1-SF1* and hence, it does not directly affect the RNA transcript or the resulting protein. In addition, no evidence was found for altered splicing of sense transcripts due to the mutation (data not shown). One simple explanation could be that *mfra-1-AS1* and/or *mfra-1-AS2* act through *mfra-1-SF1* to elicit their phenotypic effect.

6.3.4 *mfra-1-SF1* encodes a transmembrane protein and its expression is influenced by *mfra-1-AS2*

In order to understand the nature of the previously uncharacterized protein coded by *mfra-1-SF1*, we used various online available tools including Interpro, UniprotKB, HHpred and NCBI protein blast with different settings (Altschul et al. 1997; Finn et al. 2017; The UniProt Consortium 2017; Zimmermann et al. 2017). Based on the predictions, the 429 amino acids containing protein has a signal peptide at the N terminus, followed by a

cytoplasmic TPM domain, a transmembrane domain, and a C terminal, unorganized extracellular domain (Fig 5A). The closest characterized homolog is MOLO-1 present in *C. elegans.* MOLO-1 is described as an auxiliary subunit of levamisole-sensitive acetylcholine-gated ion channel complex in neurons (Boulin et al. 2012). The presence of this transmembrane protein on neurons might be an indicative of its role in sensing the environment.

(A)

(B)

(C)
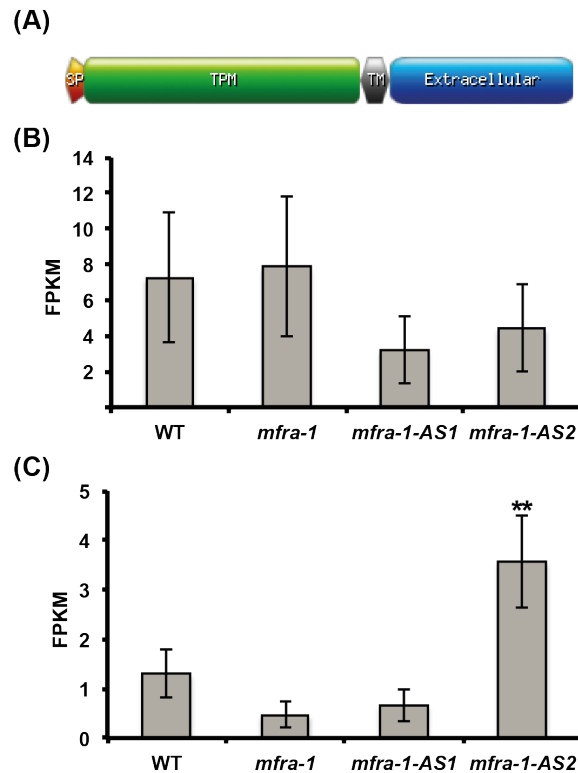
Fig 5: *mfra-1-AS2* induces the expression of sense transcript in stage specific manner. (A) Protein domain organization of SF1 protein. The orange, green, gray, and blue domains indicate signal peptide, TPM domain, trans membrane domain, and extracellular domains respectively. Expression levels of *mfra-1-SF1* in (B) egg-J1, and (C) J2 developmental stages are shown for WT, *mfra-1*, *mfra-1-AS1* (*tuEx228*), and *mfra-1-AS2* (*tuEx230*). Error bars are based on the estimated confidence interval of two independent biological replicates. **p<0.01 based on FDR corrected p values generated by cuffdiff (see methods).

Next, the levels of *mfra-1-SF1* in *mfra-1-AS1* and *mfra-1-AS2* transgenic lines were examined in early developmental stages (egg-J1, and J2) in order to assess if the antisense transgenes can influence the sense expression. The expression levels were tested in the early developmental stages, as that is the time window when the maternal influence likely affects mouth-form decision. We found that in the egg-J1 stage the expression of *mfra-1-SF1* is not altered in *mfra-1-AS1*, and *mfra-1-AS2* transgenic lines (Fig

41

5B). However, in J2 stage worms, *mfra-1-SF1* is upregulated in the *mfra-1-AS2* transgenic line (*tuEx230*), whereas it is not affected in the *mfra-1-AS1* transgenic line (*tuEx228*) (Fig 5C). This is consistent with the 100% Eu phenotype observed in *mfra-1-AS2* and *mfra-1-SF1* transgenes. There are three lines of evidence that when taken together suggest *mfra-1-AS2* acts through *mfra-1-SF1* to exert its Eu form inducing effect. First, the *mfra-1* mutation does not affect *mfra-1-SF1* directly, but only the antisense RNAs. Second, both *mfra-1-AS2* and *mfra-1-SF1* transgenes induce 100% Eu phenotypes. Third, *mfra-1-SF1* is upregulated by *mfra-1-AS2* transgene. In contrast, the mode of action of *mfra-1-AS1* might be independent of *mfra-1-SF1* transcript or its translated protein.

6.3.5 *mfra-1-AS2* and *mfra-1-SF1* co-localize in the pharynx

In order to examine the expression pattern of all three RNAs, we performed single molecule RNA FISH analysis. We found that *mfra-1-AS2* and *mfra-1-SF1* are expressed in either in the same cells, or in close proximity, whereas *mfra-1-AS1* is also expressed in the neighboring pharyngeal cells and does not co-localize with *mfra-1-SF1* (Fig 6). It is interesting that all three RNA transcripts are expressed in the pharynx, the body part involved in sensing the environment and executing the mouth-form decision. The co-localization of *mfra-1-AS2* and *mfra-1-SF1* further might indicate towards their possible molecular interaction.
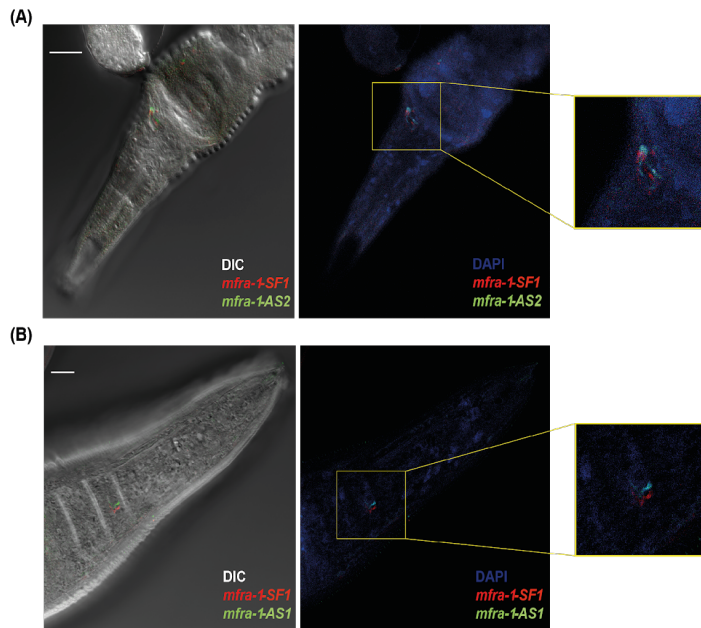
Fig 6: *mfra-1-AS2* and *mfra-1-SF1* **partially co-localize in the pharynx.** RNA FISH experiments illustrate that (A) *mfra-1-SF1* and *mfra-1-AS2* are proximally localized in the worm body, (B) *mfra-1-SF1* and *mfra-1-AS1* show at least partial co-localization in the pharyngeal cells. The scale bar indicates 10 µm.

6.3.6 Antisense lncRNAs on *mfra-1* locus influence transcription of regulators of mouth-form plasticity

Next, we examined if the antisense RNAs can influence the expression of other molecular regulators of the mouth-form polyphenism. Interestingly, the developmental switch gene *eud-1* is downregulated in *mfra-1-AS1* transgenic line (*tuEx228*) and shows more that 100-fold upregulation in *mfra-1-AS2* transgenic line (*tuEx230*) in both the egg-J1, and the J2 stage (Fig 7A,B). This finding is consistent with the previous results showing overexpression of *eud-1* causing Eu form induction (Ragsdale et al. 2013) as the *mfra-1-AS2* transgenic line has 100% Eu frequency. Moreover, downregulation of *eud-1* in *mfra-1-AS1* transgenic animals could explain the all-St phenotype observed in these animals. It also suggests that *eud-1* probably acts downstream of both *mfra-1-AS1* and *mfra-1-AS2*. In contrast, the expression of the nuclear hormone receptor *nhr-40* is not affected (Fig

7C). *nhr-40* is also a developmental switch gene that is expressed in many different tissue and cell types (Kieninger et al. 2016). It is probably involved in many different functions making it less surprising that its expression is not affected in the transgenic lines.

Further, we examined the expression of the other genes of the *eud-1* multi-gene locus on the X chromosome, which controls the mouth-form polyphenism (Sieriebriennikov et al. 2018). We found that all three of these genes (*nag-1*, *nag-2*, and *sul-2.2.1*) fail to show differential expression in the egg-J1 stage. The expression of *sul-2.2.1* in the J2 stage is higher in *mfra-1-AS2* compared to *mfra*-1 (Fig 7D), which is in line with it having a mild Eu form inducing effect (Sieriebriennikov et al. 2018). However, we found that in the J2 stage both N-acetylglucosaminidases *nag*-1 and *nag*-2 are differentially regulated in animals expressing the *mfra-1-AS2* transgene (Fig 7E,F). Interestingly, these genes are differentially regulated in opposite directions in *mfra-1-AS2* transgenic animals, which is surprising considering the fact that they both have similar St form inducing phenotypic effect (Sieriebriennikov et al. 2018). In order to resolve this contradiction, we looked closer at the coverage profiles of different genes on this locus. We found that in *mfra-1-AS2* transgenic line (*tuEx230*) only the exons (and introns) of *nag-1* that are closest to the highly expressed neighboring *eud-1* show upregulation, which could be a result of a highly transcriptionally active, open chromatin around *eud-1* instead of specific transcriptional regulation of *nag-1* (Fig 7F). This is supported by the fact that even the intergenic region between *eud*-1 and *sul-2.2.1* exhibit high transcriptional activity (Fig 7F). Interestingly, except for *eud-1*, the transcriptional status of no other gene is affected by *mfra-1-AS1* transgene. There are two possibilities that could result in this outcome. First, *mfra-1-AS1* only acts through *eud-1* and does not interact with other known genes in the pathway. Second, *mfra-1-AS1* regulates these genes in later developmental stages.
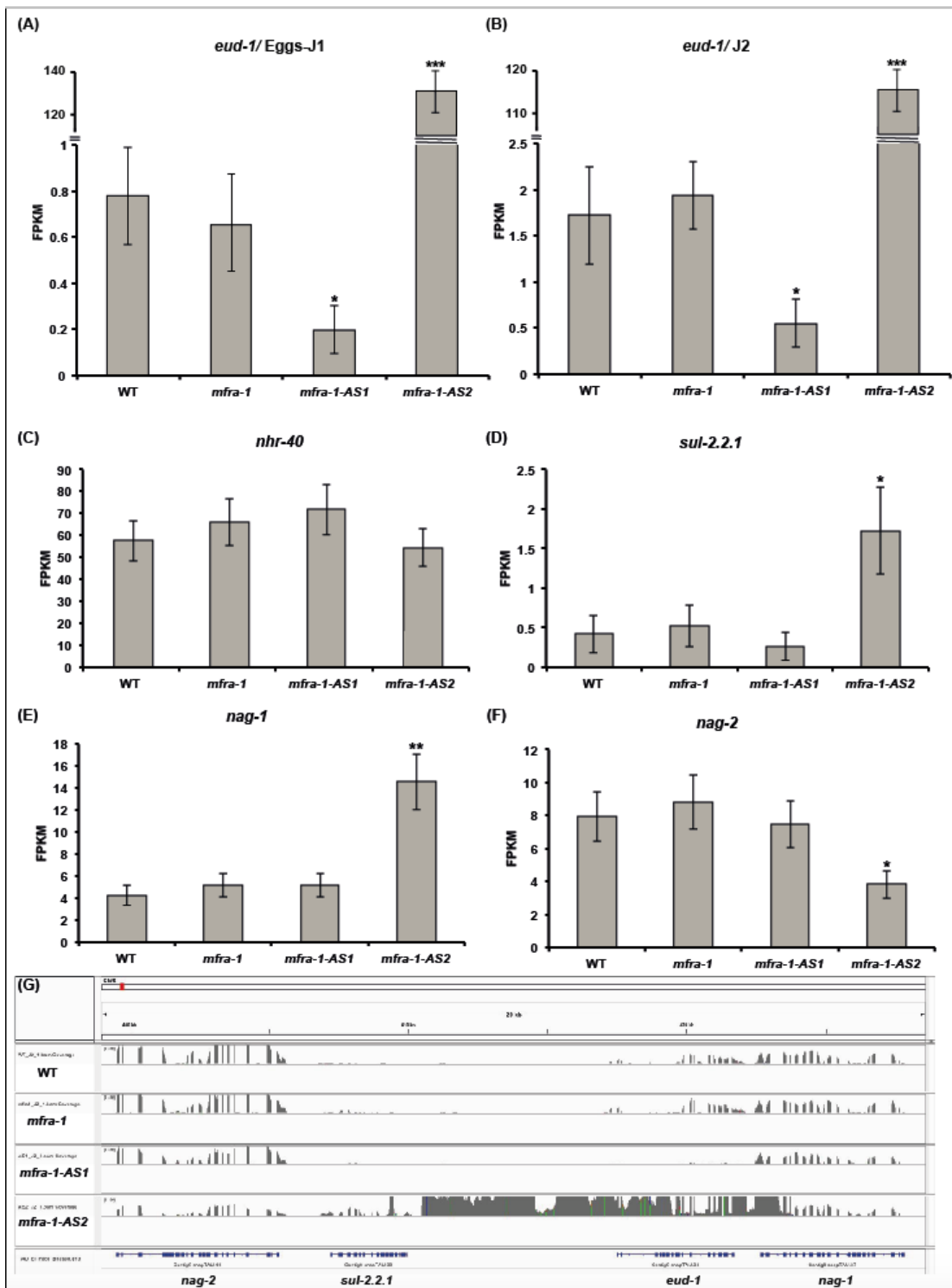
Fig 7: Antisense lncRNAs on *mfra-1* locus influence transcription of regulators of mouth form plasticity. Expression level of *eud-1* in WT, *mfra-1*, *mfra-1-AS1* (*tuEX228*), and *mfra-1-AS2* (*tuEX230*) animals during (A) egg-J1, and (B) J2 developmental stages are shown. Expression levels of (C) *nhr-40*, (D) *sul-2.2.1*, (E) *nag-1*, and (F) *nag-2* in WT, *mfra-1*, *mfra-1-AS1* (*tuEx228*), and *mfra-1-AS2* (*tuEx230*) animals during egg-J1 developmental stages are shown. (F) Coverage profile representing normalized raw reads of the multi-gene locus that regulates mouth form plasticity. The image shown is a snapshot of the locus on Integrative Genomics Viewer (IGV). Data range is set constant at 0-80 for all samples. For A-

F the error bars are based on the estimated confidence interval of two independent biological replicates. *p<0.05, **p<0.01, ***p<0.005 based on FDR corrected p values generated by cuffdiff (see methods).

6.3.7 CRISPR/Cas9 induced mutant library

In order to further understand the role of different transcripts in the regulation of the mouth-form dimorphism, we generated a CRISPR/cas9 induced mutant library by mutating different transcripts specifically and in combination (Fig 8A). The mutant alleles generated by CRISPR/Cas9 and their mouth form related phenotypic effects are listed in Table 1 as well as illustrated in Fig 8B. We obtained three alleles that specifically target different exons of *mfra-1-SF1* (wild type protein: 429 amino acids). The first *mfra-1-SF1* specific allele, *tu627*, is a 31 bp deletion that targets the N terminal intracellular TPM domain of the protein and leads to a premature stop codon resulting in truncated protein (172 amino acids). This mutation is homozygous lethal with animals being sick and hard to maintain even as heterozygotes. Owing to these limitations the mutant animals could not be screened for their mouth-form phenotype. The second allele, *tu1106*, is a 5 bp deletion in exon 9 of *mfra-1-SF1* and also results in truncated protein (284 amino acids). No significant difference was observed for the mouth-form phenotype compared to wild type for this allele (Fig 8 A,B). The third *mfra-1-SF1* specific allele, *tu1255*, has a 11 bp deletion in the exon 10 of *mfra-1-SF1*, which corresponds to the unorganized extracellular domain. This mutation also creates a premature stop codon resulting in a truncated protein (319 amino acids). However, unlike other *mfra-1-SF1* specific allele, *tu1106*, *tu1255* exhibits a significant decrease in the Eu frequency (Fig 8 A,B). This prevalence of the St animals in *mfra-1-SF1* specific mutant allele is consistent with the earlier results from *mfra-1-SF1* overexpressing transgenic animals showing 100% Eu mouth form (Fig 3B).

sgRNA	Sequence	Remarks	Allele	Mutation
CRIS-1	tttcaatgagaaaacgtaca	Shared region between *mfra-1-SF1*, and *mfra-1-AS2*	-	
CRIS-2	aacaccggaaggaaaagaca	Specific to *mfra-1-AS2*	-	
CRIS-3	tccgacaagaacgacgaaat	Specific to *mfra-1-SF1*	*tu627*	31 bp deletion
CRIS-4	gagatgagtgaatcaagcac	Shared region between *mfra-1-SF1*, and *mfra-1-AS1*	*tu1052*	4 bp deletion
CRIS-5	ttgggagatcggcatggcat	Shared region between *mfra-1-SF1*, and *mfra-1-AS1*	*tu1053*	13 bp deletion
CRIS-7	tttattcaagcttcacggtt	Shared exon between *mfra-1-AS1*, and *mfra-1-AS2*	-	
CRIS-9	ccacaactgatgctacttcg	Specific to *mfra-1-AS1*	-	
CRIS-10	gtggaatgctcggagaatcg	Specific to *mfra-1-SF1*	*tu1106*	5 bp deletion
CRIS-11	aattcttccgtcatttttcg	Specific to *mfra-1-AS2*	-	
CRIS-12	aataaagtcatcgatgagat	Shared region between *mfra-1-SF1*, and *mfra-1-AS2*	-	
CRIS-13	ttccagggcctcacaacgac	Specific to *mfra-1-SF1*	*tu1255*	11 bp deletion
CRIS-15	aataccttgaaattcaacat	Shared exon between *mfra-1-AS1*, and *mfra-1-AS2*	*tu1254* / *tu1256*	74bp insertion / 56 bp deletion

Table 1: single guide RNAs (sgRNAs) used for CRISPR/Cas9 and resultant alleles. sgRNAs used for the CRISPR/Cas9 library are shown. All alleles are in wild-type (RS2333) background. The sequence of sgRNAs, and the length of the genetic lesion for mutant alleles are indicated.
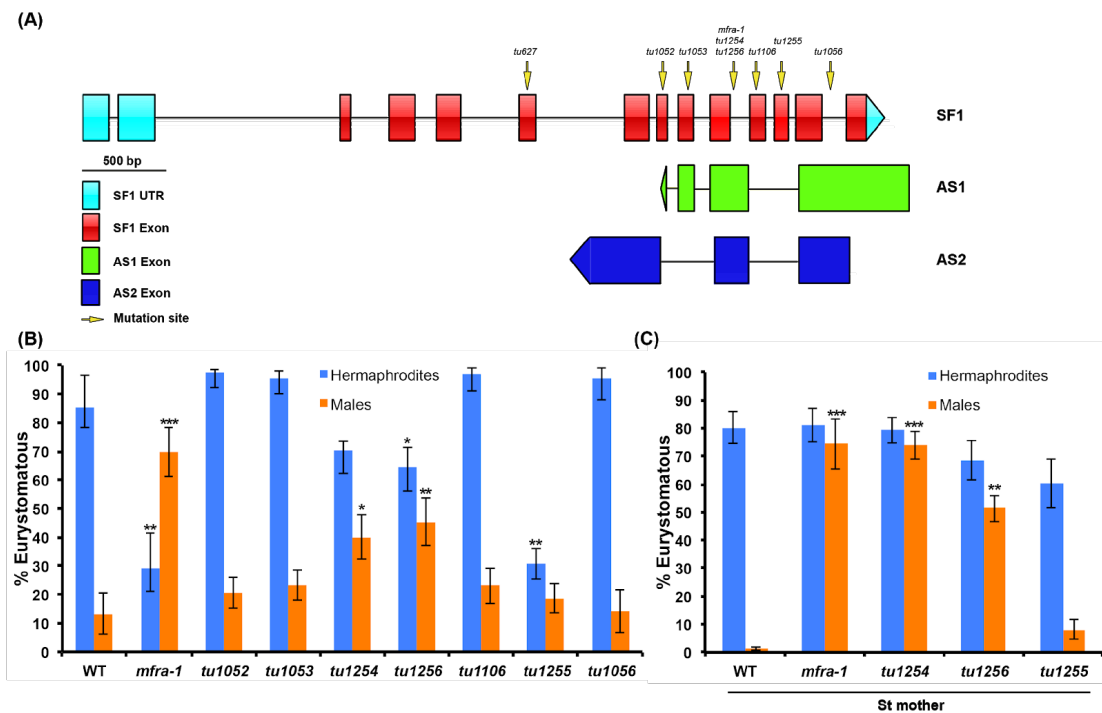
Fig 8: CRISPR/Cas9 directed mutagenesis. (A) Three transcripts from *mfra-1* locus are illustrated along with the mutation sites that are indicated by arrows. (B) Eurystomatous form frequency in different mutant alleles for both sexes. They are compared with corresponding WT values. (C) Examination of *Mid* phenotype in different mutant alleles. *p<0.05, **p<0.01, *** p<0.005, two tailed student t test.

There are several possibilities that could explain the phenotypic difference between two *mfra-1-SF1* specific alleles. First, the truncated protein in *tu1106* might still maintain its function or parts of it, unlike the truncated protein in *tu1255*. Second, instead of the translated protein, the RNA of *mfra-1-SF1* itself is important for the mouth form related function. The small lesions

47

present in both alleles might not influence the *mfra-1-SF1* RNA secondary and tertiary structure, and its function. Third, nonsense-mediated decay (NMD) can act differently on different transcripts resulting from these mutations. In theory, the difference in NMD of different mutated transcripts could also cause the observed difference in phenotype. Finally, there could be more functional splice variants that are low expressed, and hence remain undetected with RACE or RNA sequencing experiments. Unlike *tu1255*, the mutation in *tu1106* might not be affect the other functional splice variants.

Next, we attempted to knock out the antisense transcripts *mfra-1-AS1* and *mfra-1-AS2* specifically. However, despite trying with several single guide RNAs (listed in Table 1), we failed to generate any *mfra-1-AS1*, or *mfra-1-AS2* specific mutations with CRISPR/Cas9. This could be due to their relative importance in early embryonic development so that even heterozygous mutant animals are lethal. However, we could obtain mutant alleles that have mutations in the shared region of *mfra-1-AS1* and *mfra-1-AS2* (*tu1058*, *tu1254*, *tu1256*), and the shared region of *mfra-1-AS1* and *mfra-1-SF1* (*tu1052*, *tu1053*) (Fig 8A). Interestingly, out of these, only the alleles that have large mutation around the original *mfra-1* mutant site (*tu1254*: 74 bp insertion, and *tu1256:* 56 bp deletion) exhibit a significant decrease in the Eu frequency in hermaphrodite (Fig 8B). These two alleles also show an increase in Eu frequency in males, which is similar to *mfra-1.* This result indicates that the shared exon of *mfra-1-AS1* and *mfra-1-AS2* that covers the *mfra-1* mutation site is probably the most important for *mfra-1-AS1* and *mfra-1-AS2* function. This is also supported by the fact already a single nucleotide change in that region is sufficient to result in *Mid* phenotype as observed in *mfra-1* mutant animals.

In order to ascertain if the mutations that significantly alter the mouth-form frequency also affect the maternal influence, we examined the male progeny of St mother from *tu1254*, *tu1255*, and *tu1256* mutant alleles (Fig 8C). We found that both mutations around *mfra-1* (*tu1254* and *tu1256*) show the *Mid* phenotype. In comparison, the *mfra-1-SF1* specific mutation in *tu1255* fails to produce *Mid* phenotype. This could be due to only antisense lncRNAs *mfra-1-AS1* and/or *mfra-1-AS2* being important for carrying the maternal information to the progeny unlike *mfra-1-SF1*.

6.3.8 *mfra-1-AS2* downregulates dosage compensation gene *dpy-21*

Many genes encoding mouth-form regulators such as *eud-1, nag-1, nag-2, nhr-40,* and *mfra-1* itself are located on X chromosome. In addition, *eud-1* escapes dosage compensation, as we know from males having lower expression of *eud-1* compared to hermaphrodites (Ragsdale et al. 2013). In order to assess if dosage compensation is linked to mouth-form dimorphism, we examined the levels of a key regulator of nematode dosage compensation complex, *dpy-21*. In *C. elegans* DPY-21 regulates dosage compensation by enriching X chromosome of somatic cells with H4K20me1, which affects higher order chromatin structure and results in the downregulation of X linked genes (Brejc et al. 2017). Furthermore, DPY-21 is linked to the phenotypically plastic decision of dauer formation in *C. elegans* as it promotes dauer arrest by repressing a X-linked gene *ins-9* that is only expressed in sensory neurons (Delaney et al. 2017). We find that in *mfra-1-AS2* transgenic line *dpy-21* levels are lower compared to wild type (Fig 9). This is consistent with higher levels of the Eu form promoting genes *eud-1* and *mfra-1-SF1* in the *mfra-1-AS2* transgenic line (Fig 5B, 7)*.* In contrast, the levels of St form promoting genes *nhr-40* and *nag-2* are not affected in *mfra-1-AS2* (Fig 7). However, other St form promoting gene *nag-1* is upregulated in *mfra-1-AS2* but that could be caused by excessive transcriptional activation of only the first few exons that are closest to *eud-1* (Fig 6F)*.* Taken together, this might be an indication of X-linked Eu form promoting genes escaping dosage compensation in *mfra-1-AS2* transgenic line via downregulation of *dpy-21.* However, further experimentation and analysis is required to confirm and establish this.
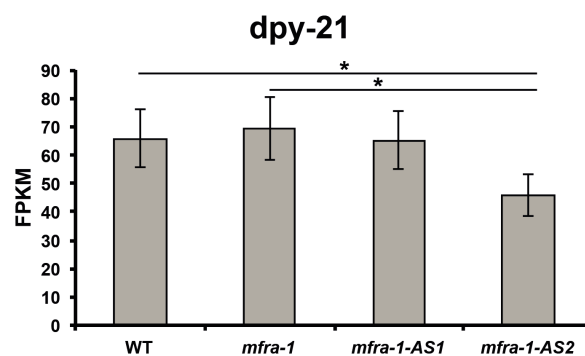
Fig 9: *mfra-1-AS2* **downregulates dosage compensation regulator gene** *dpy-21.* RNA sequencing results showing expression levels of *dpy-21* in WT, *mfra-1*, *mfra-1-AS1*

(*tuEx228*), and *mfra-1-AS2* (*tuEx230*) animals. Error bars are based on the estimated confidence interval of two independent biological replicates. *p<0.05 based on FDR corrected p values generated by cuffdiff (see methods).

6.3.9 Discussion

Here, we studied and established the role of a X-linked locus *mfra-1* in the mouth-form dimorphism in *P. pacificus.* The two antisense lncRNAs *mfra-1-AS1* and *mfra-1-AS2* exert strong and opposite phenotypic effects as their transgenes induce all-St and all-Eu phenotypes respectively. The transgene from the sense RNA *mfra-1-SF1* also has an Eu form inducing effect and it might be downstream of, and upregulated by *mfra-1-AS2.* The mode of action of *mfra-1-AS1* is not clear but there are several possibilities. First, *mfra-1-AS1* might be acting independently of the molecular function of the other RNAs in the locus. Second, *mfra-1-AS1* acts through either *mfra-1-AS2* or *mfra-1-SF1* to exert its phenotypic effect. Third, *mfra-1-AS1* is downstream of *mfra-1-AS2*, and/or *mfra-1-SF1* in the mouth-form determination pathway. Lastly, the act of transcription of *mfra-1-AS1* itself can influence the transcriptional activity of other RNAs via affecting the availability and activity of transcriptional activation and elongation factors in a process known as transcriptional interference (Xue et al. 2014; Nevers et al. 2017). The protein SF1 with a neuronal localization, and a predicted potential function of modulation of acetylcholine gated ion channel might be involved in sensing the environment. However, the antisense lncRNAs might also act independently of their SF1 related effect.

The transcription of alternatively spliced antisense lncRNAs with different phenotypic effects might also be the key for understanding the stochasticity that is observed with respect of the mouth-form phenotypes in *P. pacificus.* It can be hypothesized that depending on the relative dosage of *mfra-1-AS1* and *mfra-1-AS2* worms can choose to develop either Eu or St mouth morphology. However, the lack of ability to examine the antisense transcript levels due to the presence of overlapping exons of different RNAs as well as some repetitive sequences has hindered our ability to test this assumption directly. In the future, advanced techniques in RNA expression analysis such as digital droplet PCR can be applied to resolve this issue.

One major issue with our CRISPR/Cas9 induced mutant screen has been the absence of any *mfra-AS1* and *mfra-1-AS2* specific mutant allele with a significant phenotypic effect. This is not surprising if we consider that small lesions in most lncRNAs fail to produce any observable phenotypic difference because of small mutations do not always result in significant difference in higher order RNA structure and RNA localization (Goyal et al. 2017). Another problem with lncRNAs is the lack of information about their functional motifs, which makes it difficult to design guide RNAs that can be used to target these functional motifs and produce phenotype. In our case large mutations (*tu1254*: 74 bp insertion, and *tu1256*: 56 bp deletion) in the shared exons of *mfra-1-AS1* and *mfra-1-AS2* resulted in a *Mid* like phenotype (Fig 8C), which is an indication towards this shared exon containing the functional motif for both *mfra-1-AS1* and *mfra-1-AS2* lncRNAs. Furthermore, the possibility of small peptides being translated from antisense lncRNAs and the production of functional small RNAs cannot be fully denied. Again, the complex transcriptional organization of *mfra-1* makes it difficult to disentangle the role of each transcript separately and in combination.

Transmission of the maternal information can happen through accumulation of RNAs in the eggs that can not only control early embryonic development, but also have a huge impact on later development (Zhang and Smith 2015). It is possible that the maternal accumulation of either *mfra-1-AS1* or *mfra-1-AS2* in eggs can pre-empt the progeny towards one of two alternative developmental paths that ultimately result in different mouth morphs. Here, dosage dependent accumulation of two antisense lncRNAs can provide the basis for stochasticity that is observed with respect to mouth-form polyphenism in a constant environment. The downregulation of the dosage compensation regulator DPY-21 in *mfra-1-AS2* could also impact early development, more specifically affecting the developmental decision regarding mouth formation. Further experiments in early developmental stages are required to decipher the mechanistic details of antisense lncRNAs action.

In short, the maternal influence on mouth-form phenotype is in part transmitted by *mfra-1.* The complex transcriptional activity of *mfra-1* produces multiple transcripts including alternatively spliced antisense lncRNAs that

through intragenic and intergenic interactions regulate the mouth-form polyphenism in *P. pacificus.*

6.3.10 Materials and methods

Culture conditions: For culturing worms on agar plates, all *P. pacificus* strains, including the wild type strain RS2333, all mutants as well as transgenic lines generated in this study, were grown on 6 cm plates containing Nematode Growth Medium (NGM) Agar. Worms were fed on a 300 μl bacterial lawn containing *Escherichia coli* OP50 strain grown in LB Broth. All cultures were maintained at 20°C.

Phenotypic scoring: Mouth-form phenotypes were scored in agar cultures using a method described earlier (Bento et al. 2010). In short, adults were washed, gently pelleted and transferred to 4% agar pads (containing 10 mM sodium azide) with 5-8 μl of M9 and observed under differential interference contrast (DIC) microscope (Zeiss Axioskop) at 40-100 x magnifications. Discrete characters were used to discriminate between Eu and St individuals, respectively: the presence *vs.* absence of a subventral tooth, and a claw-like *vs.* flint-like or triangular dorsal tooth, which were together sufficient to distinguish the two forms.

Rapid amplification of cDNA elements (RACE) analysis: RACE analysis was performed to identify and characterize all the transcripts from the *mfra-1* locus. The total RNA isolation from mixed stage worms was done using PureLink RNA micro kit (Invitrogen, Cat no. 12183-016) following the manufacturer's instructions. The RACE experiments including RACE ready cDNA synthesis and the RACE PCR were performed using SMARTer RACE kit (Clontech Laboratories, Cat. no 634858), following the manufacturer's instruction. The amplified product was cloned into a pUC19 vector using Infusion cloning kit (Clontech Laboratories, Cat no. 639648). The PCR primers used for RACE amplification of sense and antisense RNAs at *mfra-1* locus are listed in table 2.

RACE primers		
	Name	**Sequence**
5' RACE sense	R1GSP1	cgacttctttccgtcgatttcaggtgag
	R1NGSP1	ctcgattctccgagcattccacgaa
3' RACE sense	R1GSP2	ctgactctgctccatccgaatgctactc
	R1NGSP2	tcctgctagcaaagcgaattcacacg
5' RACE antisense	midasF2	gctcattaccgatgttgaatttcaaggt
	midasF1	ggcccaaaccgtgaagcttgaa
3' RACE antisense	midasR1	ggtcccacctagcgttgtcttacgac
	midasR2	tagcgttgtcttacgaccgcttgacacc

Table 2: PCR primers used for RACE analysis of *mfra-1*.

CRISPR/Cas9 induced mutagenesis: We generated various mutant alleles specifically targeting different regions of the *mfra-1* locus using a previously described method (Witte et al. 2015). In short, different single guide RNAs (sgRNAs) were ordered from Integrated DNA Technologies (IDT). For inducing the mutations, sgRNAs were co-injected with Cas9 nuclease (NEB, Cat no. #M0386M). Heterozygous mutant carriers were identified and singled out by analyzing high-resolution melting curves using a quantitative PCR system (Roche Lightcycler 480 II) with separate primers for each gene. The sgRNAs sequences are listed in table 1, and primers used are listed in table 3.

Allele	sgRNA	Forward primer	Reverse Primer
tu627	CRIS-3	gactggccgaatctccagtcttctatac	ccgctcaagtactttcatccctca
tu1052	CRIS-4	ggggaaccaattaaacctaccaatttgc	gcttcacggtttgggcctgttgaatg
tu1053	CRIS-5	ggggaaccaattaaacctaccaatttgc	gcttcacggtttgggcctgttgaatg
tu1106	CRIS-10	cgacttctttccgtcgatttcaggtgag	gaaatgagacttaagcttacct
tu1254 *tu1256*	CRIS-15	cagaacaattcaattcgatgattcgc	tgaaccaatatataggcctgctc

Table 3: Primers used for quantitative PCR to amplify mutated site in CRISPR generated alleles.

Genetic transformation: Transgenic animals were generated as previously described (Schlager et al. 2009). To obtain transgenic lines for *mfra-1-AS1*, *mfra-1-AS2*, and *mfra-1-SF1*, germ lines of *mfra-1* adult hermaphrodites were injected with a mix of cDNA construct of respective transcripts (10 ng/µl), the marker *egl-20*::*TurboRFP* (10 ng/µl), and genomic carrier DNA (60 ng/µl) from the *mfra-1* animals. The cDNA constructs had a 2 Kb immediate promoter region followed by cDNA of the transcripts, and a respective small (<500 bp) 3' UTR region. Fragments were then fused and amplified by overlapping

extension PCR. All amplified fragments were verified by sequencing. We used the restriction enzymes PstI (Thermo scientific, Cat no. #ER0611), KasI (Thermo scientific, Cat no. #ER2191), and XmaI (NEB, Cat no. #R0180S) for digestion of different constructs and genomic host DNA depending on the sequences. Except for transgene containing *mfra-1-AS2* specific exon (*tuEx23*), all experiments two independent transgenic lines were generated. The primers used for amplification and fusion of respective promoter, cDNA, and 3# UTR for different constructs are listed below in table 4.

Transgene	Sequence	Remark
	agaaaagaggagaggaaaatgaaggccggcattcg	Promter forward
	aggagggataacatcctgaaacggatataatatatttc	Promote reverse+cDNA
tuEx251	ttatatccgtttcaggatgttatccctcctgaatctc	cDNA forward
tuEx253	caatcgttgataaatcagacaaagctggacgatttcattag	cDNA reverse
	tccagctttgtctgatattatcaacgattgtagatttcgc	cDNA+3' UTR forward
	taattggcgcctcatttcgcaccttttctcgcctc	3' UTR reverse
	tcgtgcccgggtggctctcgatcatttcctc	Promter forward
	cgctaggtgggacccactggaaaggttgatagaattatta	Promote reverse+cDNA
tuEx228	atcaacctttccagtgggtcccacctagcgttgtcttacgac	cDNA forward
tuEx229	actagagacgactgaggagatgagtga	cDNA reverse
	ctcctcagtcgtctctagtacagcttcg	cDNA+3' UTR forward
	aaacccgggcgcgctgtgagacgcaaaag	3' UTR reverse
	tcgtgcccgggtggctctcgatcatttcctc	Promter forward
	cgctaggtgggacccactggaaaggttgatagaattatta	Promote reverse+cDNA
tuEx227	atcaacctttccagtgggtcccacctagcgttgtcttacgac	cDNA forward
tuEx230	ggctatttcttagagaaacagagggtttagtaaatataat	cDNA reverse
	ctaaaccctctgtttctctaagaaatagccatcagctata	cDNA+3' UTR forward
	aaacccgggcgcgctgtgagacgcaaaag	3' UTR reverse
	tcgtgcccgggtggctctcgatcatttcctc	Promter forward
	cgctaggtgggacccactggaaaggttgatagaattatta	Promote reverse+cDNA
tuEx237	atcaacctttccagtgggtcccacctagcgttgtcttacgac	cDNA forward
tuEx238	ggctatttcttagagaacgctcatcgtgcttctcattgta	cDNA reverse
	agcacgatgagcgttctctaagaaatagccatcagctata	cDNA+3' UTR forward
	aaacccgggcgcgctgtgagacgcaaaag	3' UTR reverse
	tcgtgcccgggtggctctcgatcatttcctc	Promter forward
	ccactaccaccgaagcactggaaaggttgatagaattatt	Promote reverse+cDNA
tuEx239	atcaacctttccagtgcttcggtggtagtgggctggaaga	cDNA forward
	ggctatttcttagagaaacagagggtttagtaaatataat	cDNA reverse
	ctaaaccctctgtttctctaagaaatagccatcagctata	cDNA+3' UTR forward
	aaacccgggcgcgctgtgagacgcaaaag	3' UTR reverse

Table 4: Primers used for PCR for amplification and fusion of different transgenic constructs.

Gene, and Protein domain structure visualization: The visualization of the gene structure was performed using IBS (illustrator for biological sequences) online tool (Liu et al. 2015). The visualization of the protein domains was performed using the online tool PROSITE (Sigrist et al. 2013).

Single molecule RNA FISH: Single molecule RNA FISH was performed using a previously protocol described for nematodes (Ji and van Oudenaarden 2012). Biosearch Technologies Stellaris FISH online platform was used to design and order RNA FISH probes for sense and antisense RNAs of *mfra-1. mfra-1-SF1*, *mfra-1-AS1*, and *mfra-1-AS2* probes were designed for specificity and they were coupled with Quasar 670, FAM and FAM fluorescent dyes respectively.

Imaging: Image acquisition was performed at Leica SP8 confocal system using settings to maximize the detection of fluorescent RNA FISH probes labelled with Quasar 670 and FAM. At least 15 animals were imaged for each sample type. Image analysis was performed using Fiji (is just image J) software (Schindelin et al. 2012).

RNA sequencing experiments: The RNA sequencing experiments was performed using a previously described method (Serobyan et al. 2016). In short, WT (RS2333), *mfra-1*, *spk-1*, *mfra-1-AS1*, and *mfra-1-AS2* worms were synchronously grown. For egg-J1 samples, this was followed by treatment with mix of 1:2, Bleach and 5M NaOH. This treatment kills all the animals except for egg-J1, which are protected due to their eggs shells. For J2 stage samples, a 20µm filter was used that allows only the passage of J2 worms for their collection. Following the separate collection of egg-J1 and J2 worms, the total RNA was extracted from them using the method described above. NEBNext Ultra II directional RNA Library prep Kit (Cat. # E7760S) was used for library preparation. Two biological replicates were performed for each sample. RNA-Seq libraries were sequenced as 2 × 100-bp paired-end reads on an Illumina HiSeq 3000.

Raw Illumina reads were aligned against the P. pacificus reference genome (version El Paco) (Rödelsperger et al. 2017) with the help of the

TopHat alignment program (version 2.0.14, default options) (Kim et al. 2013). For both stages (egg-J1 and J2), gene expression levels were quantified and calls of significant differential expression between all pairwise comparisons (FDR corrected p-value < 0.05) were generated by cuffdiff (version 2.2.1, default options) (Trapnell et al. 2013). Analysis was repeated for two different versions of P. pacificus annotations (version TAU2011 (Sinha et al. 2012), El Paco annotations v1 (Rödelsperger et al. 2017). For visualization purposes, alignment files in bam format were converted into sam format by the samtools view program (version 0.1.19, default options) (Li et al. 2009) and alignments were separated according to strand-specificity based on samflags (forward strand (147,99), reverse strand (83,163)) and converted into bam format again with the help of samtools view. Alignments files in bam format were visualized using the Integrative Genomics Viewer (Robinson et al. 2011).

Statistical analyses: All phenotypic data show percentage Eu frequency calculated from total individuals screened in three biological replicates. The total sample size is illustrated on graphs. Significant differences were tested by two-tailed student t-test. Statistical analysis performed for RNA sequencing data is described in the previous passage.

6.3.11 Own Contribution

I designed and conducted most of the experiments described in this study. Dr. Vahan Serobyan performed the mutant screen and mapped the *mid* mutations to their respective genes. Dr. Christian Rödelsperger performed the initial analysis of the RNA sequencing data. Hanh Witte performed the microinjections for genetic transformation and CRISPR/Cas9 mutagenesis. Waltraud Röseler prepared the library for RNA sequencing. My overall contribution is about 85% in this study.

7. References:

Akduman, N., Rödelsperger, C., and Sommer, R.J. (2018). Culture-based analysis of Pristionchus-associated microbiota from beetles and figs for studying nematode-bacterial interactions. PLoS One *13*, e0198018.

Alberch, P., and Gale, E.A. (1985). A developmental analysis of an evolutionary trend: digital reduction in amphibians. Evolution *39*, 8–23.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. *25*, 3389–3402.

Arthur, W. (2002). Editorial: Questions of Complexity. Evolution and Development *4*, 165–166.

Aubret, F., and Shine, R. (2009). Genetic assimilation and the postcolonization erosion of phenotypic plasticity in island tiger snakes. Curr. Biol. *19*, 1932–1936.

Baldwin, J.M. (1896). A New Factor in Evolution. Am. Nat. *30*, 441–451.

Baniahmad, A., and Tsai, M.J. (1993). Mechanisms of transcriptional activation by steroid hormone receptors. J. Cell. Biochem. *51*, 151–156.

Barbeyron, T., Brillet-Guéguen, L., Carré, W., Carrière, C., Caron, C., Czjzek, M., Hoebeke, M., and Michel, G. (2016). Matching the Diversity of Sulfated Biomolecules: Creation of a Classification Database for Sulfatases Reflecting Their Substrate Specificity. PLoS One *11*, e0164846.

Bassett, A.R., Akhtar, A., Barlow, D.P., Bird, A.P., Brockdorff, N., Duboule, D., Ephrussi, A., Ferguson-Smith, A.C., Gingeras, T.R., Haerty, W., et al. (2014). Considerations when investigating lncRNA function in vivo. Elife *3*, e03058.

Baumann, E. (1876). Ueber Sulfosäuren im Harn. Ber. Dtsch. Chem. Ges. *9*, 54–58.

Beldade, P., Mateus, A.R.A., and Keller, R.A. (2011). Evolution and molecular mechanisms of adaptive developmental plasticity. Mol. Ecol. *20*, 1347–1363.

Bengtson, S. (1997). Fossilized Metazoan Embryos from the Earliest Cambrian. Science *277*, 1645–1648.

Bento, G.N. (2010). Evolution and Development of Pristionchus Pacificus Mouth Form Dimorphism.

Bento, G., Ogawa, A., and Sommer, R.J. (2010). Co-option of the hormone-signalling module dafachronic acid-DAF-12 in nematode evolution. Nature *466*, 494–497.

Berget, S.M., Moore, C., and Sharp, P.A. (1977). Spliced segments at the 5′ terminus of adenovirus 2 late mRNA. Proceedings of the National Academy of Sciences *74*, 3171–3175.

Bierhoff, H., Schmitz, K., Maass, F., Ye, J., and Grummt, I. (2010). Noncoding transcripts in sense and antisense orientation regulate the epigenetic state of ribosomal RNA genes. Cold Spring Harb. Symp. Quant. Biol. *75*, 357–364.

Boivin, V., Deschamps-Francoeur, G., and Scott, M.S. (2018). Protein coding genes as hosts for noncoding RNA expression. Semin. Cell Dev. Biol. *75*, 3–12.

Borchert, N., Dieterich, C., Krug, K., Schütz, W., Jung, S., Nordheim, A., Sommer, R.J., and Macek, B. (2010). Proteogenomics of Pristionchus pacificus reveals distinct proteome structure of nematode models. Genome Res. *20*, 837–846.

Borchert, N., Krug, K., Gnad, F., Sinha, A., Sommer, R.J., and Macek, B. (2012). Phosphoproteome of Pristionchus pacificus provides insights into architecture of signaling networks in nematode models. Mol. Cell. Proteomics *11*, 1631–1639.

Bose, N., Ogawa, A., von Reuss, S.H., Yim, J.J., Ragsdale, E.J., Sommer, R.J., and Schroeder, F.C. (2012). Complex small-molecule architectures regulate phenotypic plasticity in a nematode. Angew. Chem. Int. Ed Engl. *51*, 12438–12443.

Boulin, T., Rapti, G., Briseño-Roa, L., Stigloher, C., Richmond, J.E., Paoletti, P., and Bessereau, J.-L. (2012). Positive modulation of a Cys-loop acetylcholine receptor by an auxiliary transmembrane subunit. Nat. Neurosci. *15*, 1374–1381.

Bradshaw, A.D. (1965). Evolutionary Significance of Phenotypic Plasticity in Plants. In Advances in Genetics, pp. 115–155.

Brakefield, Brakefield, Kesbeke, and Koch (1998). The Regulation of Phenotypic Plasticity of Eyespots in the Butterfly Bicyclus anynana. Am. Nat. *152*, 853.

Brejc, K., Bian, Q., Uzawa, S., Wheeler, B.S., Anderson, E.C., King, D.S., Kranzusch, P.J., Preston, C.G., and Meyer, B.J. (2017). Dynamic Control of X Chromosome Conformation and Repression by a Histone H4K20 Demethylase. Cell *171*, 85–102.e23.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71–94.

Brookfield, J.F.Y., and F.Y Brookfield, J. (2009). Evolution and evolvability: celebrating Darwin 200. Biol. Lett. *5*, 44–46.

Brown, C.J., Hendrich, B.D., Rupert, J.L., Lafrenière, R.G., Xing, Y., Lawrence, J., and Willard, H.F. (1992). The human XIST gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. Cell *71*, 527–542.

Bumbarger, D.J., Riebesell, M., Rödelsperger, C., and Sommer, R.J. (2013). System-wide rewiring underlies behavioral differences in predatory and bacterial-feeding nematodes. Cell *152*, 109–119.

Buono, M., and Cosma, M.P. (2010). Sulfatase activities towards the regulation of cell metabolism and signaling in mammals. Cell. Mol. Life Sci. *67*, 769–780.

Bush, S.J., Chen, L., Tovar-Corona, J.M., and Urrutia, A.O. (2017). Alternative splicing and the evolution of phenotypic novelty. Philos. Trans. R. Soc. Lond. B Biol. Sci. *372*.

Bush, S.J., Muriuki, C., McCulloch, M.E.B., Farquhar, I.L., Clark, E.L., and Hume, D.A. (2018). Cross-species inference of long non-coding RNAs greatly expands the ruminant transcriptome. Genet. Sel. Evol. *50*, 20.

Carninci, P., Kasukawa, T., Katayama S., G.J., Frith M.C., M.N., Oyama, R., Ravasi, T., Lenhard, B., and Wells, C. (2005). The Transcriptional Landscape of the Mammalian Genome. Science *309*, 1559–1563.

C. elegans Sequencing Consortium (1998). Genome sequence of the nematode C. elegans: a platform for investigating biology. Science *282*, 2012–2018.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W., and Prasher, D. (1994). Green fluorescent protein as a marker for gene expression. Science *263*, 802–805.

Chaudhuri, J., Kache, V., and Pires-daSilva, A. (2011). Regulation of Sexual Plasticity in a Nematode that Produces Males, Females, and Hermaphrodites. Curr. Biol. *21*, 1548–1551.

Chaudhuri, J., Bose, N., Tandonnet, S., Adams, S., Zuco, G., Kache, V., Parihar, M., von Reuss, S.H., Schroeder, F.C., and Pires-daSilva, A. (2015). Mating dynamics in a nematode with three sexes and its evolutionary implications. Sci. Rep. *5*, 17676.

Chen, Y.-C.A., and Aravin, A.A. (2015). Non-coding RNAs in Transcriptional Regulation. Current Molecular Biology Reports *1*, 10–18.

Chen, L., Bush, S.J., Tovar-Corona, J.M., Castillo-Morales, A., and Urrutia, A.O. (2014). Correcting for differential transcript coverage reveals a strong relationship between alternative splicing and organism complexity. Mol. Biol. Evol. *31*, 1402–1413.

Clark, B.S., and Blackshaw, S. (2014). Long non-coding RNA-dependent transcriptional regulation in neuronal development and disease. Front. Genet. *5*, 164.

Coughtrie, M.W., Bamforth, K.J., Sharp, S., Jones, A.L., Borthwick, E.B., Barker, E.V., Roberts, R.C., Hume, R., and Burchell, A. (1994). Sulfation of endogenous compounds and xenobiotics--interactions and function in health and disease. Chem. Biol. Interact. *92*, 247–256.

Dallaire, A., and Simard, M.J. (2016). The implication of microRNAs and endo-siRNAs in animal germline and early development. Dev. Biol. *416*, 18–25.

Darwin, C. (1859). On the origin of species by means of natural selection, or, The preservation of favoured races in the struggle for life /.

David, J.R., Capy, P., and Gauthier, J.-P. (1990). Abdominal pigmentation and growth temperature in Drosophila melanogaster: Similarities and differences in the norms of reaction of successive segments. J. Evol. Biol. *3*, 429–445.

Davidson, E.H. (2006). Gene Regulatory Networks and the Evolution of Animal Body Plans. Science *311*, 796–800.

Delaney, C.E., Chen, A.T., Graniel, J.V., Dumas, K.J., and Hu, P.J. (2017). A histone H4 lysine 20 methyltransferase couples environmental cues to sensory neuron control of developmental plasticity. Development *144*, 1273–1282.

Dieterich, C., Clifton, S.W., Schuster, L.N., Chinwalla, A., Delehaunty, K., Dinkelacker, I., Fulton, L., Fulton, R., Godfrey, J., Minx, P., et al. (2008). The Pristionchus pacificus genome provides a unique perspective on nematode lifestyle and parasitism. Nat. Genet. *40*, 1193–1198.

Diez-Roux, G., and Ballabio, A. (2005). Sulfatases and human disease. Annu. Rev. Genomics Hum. Genet. *6*, 355–379.

Djebali, S., Davis, C.A., Merkel, A., Dobin, A., Lassmann, T., Mortazavi, A., Tanzer, A., Lagarde, J., Lin, W., Schlesinger, F., et al. (2012). Landscape of transcription in human cells. Nature *489*, 101–108.

Dobzhansky, T. (1937). Genetics and the Origin of Species. Ann. Entomol. Soc. Am. *30*, 641–642.

Early P, Rogers J, Davis M, Calame K, Bond M, Wall R, Hood L (1980). Two mRNAs can be produced from a single immunoglobulin mu gene by alternative RNA processing pathways. Cell 313–319.

Eißmann, M., Gutschner, T., Hämmerle, M., Günther, S., Caudron-Herger, M., Groß, M., Schirmacher, P., Rippe, K., Braun, T., Zörnig, M., et al. (2012). Loss of the abundant nuclear non-coding RNA MALAT1 is compatible with life and development. RNA Biol. *9*, 1076–1087.

Ellis, H. (1986). Genetic control of programmed cell death in the nematode C. elegans. Cell *44*, 817–829.

Falany, C.N., He, D., Li, L., Falany, J.L., Wilborn, T.W., Kocarek, T.A., and Runge-Morris, M. (2009). Regulation of hepatic sulfotransferase (SULT) 1E1 expression and effects on estrogenic activity in cystic fibrosis (CF). J. Steroid Biochem. Mol. Biol. *114*, 113–119.

Faust, T., Frankel, A., and D'Orso, I. (2012). Transcription control by long non-coding RNAs. Transcription *3*, 78–86.

Fawcett, M.M., Parks, M.C., Tibbetts, A.E., Swart, J.S., Richards, E.M., Vanegas, J.C., Cenzer, M., Crowley, L., Simmons, W.R., Hou, W.S., et al. (2018). Manipulation of insulin signaling phenocopies evolution of a host-associated polyphenism. Nat. Commun. *9*, 1699.

Felix, M.-A. (2004). Alternative morphs and plasticity of vulval development in a rhabditid nematode species. Dev. Genes Evol. *214*, 55–63.

Finn, R.D., Attwood, T.K., Babbitt, P.C., Bateman, A., Bork, P., Bridge, A.J., Chang, H.-Y., Dosztányi, Z., El-Gebali, S., Fraser, M., et al. (2017). InterPro in 2017-beyond protein family and domain annotations. Nucleic Acids Res. *45*, D190–D199.

Frederik Nijhout, H. (1998). Insect Hormones (Princeton University Press).

Friedman, D.B., and Johnson, T.E. (1988). A mutation in the age-1 gene in Caenorhabditis elegans lengthens life and reduces hermaphrodite fertility. Genetics *118*, 75–86.

Ge, C., Ye, J., Weber, C., Sun, W., Zhang, H., Zhou, Y., Cai, C., Qian, G., and Capel, B. (2018). The histone demethylase KDM6B regulates temperature-dependent sex determination in a turtle species. Science *360*, 645–648.

Ghalambor, C.K., McKAY, J.K., Carroll, S.P., and Reznick, D.N. (2007). Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. Funct. Ecol. *21*, 394–407.

Gilbert, S.F., and Epel, D. (2009). Ecological developmental biology: integrating epigenetics, medicine, and evolution (Sinauer Associates).

Gilbert, S.F., Opitz, J.M., and Raff, R.A. (1996). Resynthesizing Evolutionary and Developmental Biology. Dev. Biol. *173*, 357–372.

Glover, D. (1991). Mitosis in the Drosophila embryo — in and out of control. Trends Genet. *7*, 125–132.

Golden, J.W., and Riddle, D.L. (1984). A pheromone-induced developmental switch in Caenorhabditis elegans: Temperature-sensitive mutants reveal a wild-type temperature-dependent process. Proceedings of the National Academy of Sciences *81*, 819–823.

Goyal, A., Myacheva, K., Groß, M., Klingenberg, M., Duran Arqué, B., and Diederichs, S. (2017). Challenges of CRISPR/Cas9 applications for long non-coding RNA genes. Nucleic Acids Res. *45*, e12.

Grantham, M.E., and Brisson, J.A. (2018). Extensive differential splicing underlies phenotypically plastic aphid morphs. Mol. Biol. Evol.

Gremer, J.R., and Lawrence Venable, D. (2014). Bet hedging in desert winter annual plants: optimal germination strategies in a variable environment. Ecol. Lett. *17*, 380–387.

Guttman, M., and Rinn, J.L. (2012). Modular regulatory principles of large non-coding RNAs. Nature *482*, 339–346.

Hahn, M.W., and Wray, G.A. (2002). The g-value paradox. Evol. Dev. *4*, 73–75.

Haldane, J.B.S., and Fisher, R.A. (1931). The Genetical Theory of Natural Selection. The Mathematical Gazette *15*, 474.

Hallgrímsson, B., Jamniczky, H.A., Young, N.M., Rolian, C., Schmidt-Ott, U., and Marcucio, R.S. (2012). The generation of variation and the developmental basis for evolutionary novelty. J. Exp. Zool. B Mol. Dev. Evol. *318*, 501–517.

Hanson, M., Godfrey, K.M., Lillycrop, K.A., Burdge, G.C., and Gluckman, P.D. (2011). Developmental plasticity and developmental origins of non-communicable disease: theoretical considerations and epigenetic mechanisms. Prog. Biophys. Mol. Biol. *106*, 272–280.

Harr, B., and Turner, L.M. (2010). Genome-wide analysis of alternative splicing evolution among Mus subspecies. Mol. Ecol. *19 Suppl 1*, 228–239.

Herrmann, M., Mayer, W.E., Hong, R.L., Kienle, S., Minasaki, R., and Sommer, R.J. (2007). The nematode Pristionchus pacificus (Nematoda: Diplogastridae) is associated with the oriental beetle Exomala orientalis (Coleoptera: Scarabaeidae) in Japan. Zoolog. Sci. *24*, 883–889.

Herrmann, M., Kienle, S., Rochat, J., Mayer, W.E., and Sommer, R.J. (2010). Haplotype diversity of the nematode Pristionchus pacificus on Réunion in the Indian Ocean suggests multiple independent invasions. Biol. J. Linn. Soc. Lond. *100*, 170–179.

Hirschmann, H. (1951). Über das Vorkommen zweier Mundhöhlentypen bei Diplogaster lheritieri Maupas und Diplogaster biformis n. sp. und die Entstehung dieser hermaphroditischen Art aus Diplogaster lheritieri. Zool. Jahrb. *80*, 132–170.

Hong, R.L., and Sommer, R.J. (2006). Chemoattraction in Pristionchus nematodes and implications for insect recognition. Curr. Biol. *16*, 2359–2365.

Huang, B., Song, J.H., Cheng, Y., Abraham, J.M., Ibrahim, S., Sun, Z., Ke, X., and Meltzer, S.J. (2016). Long non-coding antisense RNA KRT7-AS is activated in gastric cancers and supports cancer cell progression by increasing KRT7 expression. Oncogene *35*, 4927–4936.

Imai, M., Naraki, Y., Tochinai, S., and Miura, T. (2009). Elaborate regulations of the predator-induced polyphenism in the water flea Daphnia pulex: kairomone-sensitive periods and life-history tradeoffs. J. Exp. Zool. A Ecol. Genet. Physiol. *311*, 788–795.

Jablonka, E., and Raz, G. (2009). Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. Q. Rev. Biol. *84*, 131–176.

Jeon, Y., and Lee, J.T. (2011). YY1 tethers Xist RNA to the inactive X nucleation center. Cell *146*, 119–133.

Ji, N., and van Oudenaarden, A. (2012). Single molecule fluorescent in situ hybridization (smFISH) of C. elegans worms and embryos. WormBook 1–16.

Johannsen, W. (1911). The Genotype Conception of Heredity. Am. Nat. *45*, 129–159.

Kalsotra, A., and Cooper, T.A. (2011). Functional consequences of developmentally regulated alternative splicing. Nat. Rev. Genet. *12*, 715–729.

Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., et al. (2003). Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature *421*, 231–237.

Kamimura, K., Maeda, N., and Nakato, H. (2011). In vivo manipulation of heparan sulfate structure and its effect on Drosophila development. Glycobiology *21*, 607–618.

Kang, Y.-J., Yang, D.-C., Kong, L., Hou, M., Meng, Y.-Q., Wei, L., and Gao, G. (2017). CPC2: a fast and accurate coding potential calculator based on sequence intrinsic features. Nucleic Acids Res. *45*, W12–W16.

Kanzaki, N., and Giblin-Davis, R.M. Diplogastrid systematics and phylogeny. In *Pristionchus Pacificus*, pp. 43–76.

Kasinathan, C., Liau, Y.H., Murty, V.L., Slomiany, B.L., and Slomiany, A. (1991). Identification of gastric mucosal mucus glycoprotein sulfotransferase. Biochem. Int. *24*, 43–49.

Katayama, S., Tomaru, Y., Kasukawa, T., Waki, K., Nakanishi, M., Nakamura, M., Nishida, H., Yap, C.C., Suzuki, M., and Kawai, J. (2005). Antisense Transcription in the Mammalian Transcriptome. Science *309*, 1564–1566.

Kelemen, O., Convertini, P., Zhang, Z., Wen, Y., Shen, M., Falaleeva, M., and Stamm, S. (2013). Function of alternative splicing. Gene *514*, 1–30.

Khachane, A.N., and Harrison, P.M. (2010). Mining mammalian transcript data for functional long non-coding RNAs. PLoS One *5*, e10316.

Kieninger, M.R., Ivers, N.A., Rödelsperger, C., Markov, G.V., Sommer, R.J., and Ragsdale, E.J. (2016). The Nuclear Hormone Receptor NHR-40 Acts Downstream of the Sulfatase EUD-1 as Part of a Developmental Plasticity Switch in Pristionchus. Curr. Biol. *26*, 2174–2179.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S.L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. *14*, R36.

Kim, N., Alekseyenko, A.V., Roy, M., and Lee, C. (2007). The ASAP II database: analysis and comparative genomics of alternative splicing in 15 animal species. Nucleic Acids Res. *35*, D93–D98.

Kirschner, M., and Gerhart, J. (1998). Evolvability. Proc. Natl. Acad. Sci. U. S. A. *95*, 8420–8427.

Klüppel, M. (2010). The roles of chondroitin-4-sulfotransferase-1 in development and disease. Prog. Mol. Biol. Transl. Sci. *93*, 113–132.

Kopelman, N.M., Lancet, D., and Yanai, I. (2005). Alternative splicing and gene duplication are inversely correlated evolutionary mechanisms. Nat. Genet. *37*, 588–589.

Kornienko, A.E., Guenzl, P.M., Barlow, D.P., and Pauler, F.M. (2013). Gene regulation by the act of long non-coding RNA transcription. BMC Biol. *11*, 59.

Krawczak, M., Reiss, J., and Cooper, D.N. (1992). The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. Hum. Genet. *90*, 41–54.

Kuroyanagi, H., Kimura, T., Wada, K., Hisamoto, N., Matsumoto, K., and Hagiwara, M. (2000). SPK-1, a C. elegans SR protein kinase homologue, is essential for embryogenesis and required for germline development. Mech. Dev. *99*, 51–64.

de Lamarck, J.B.P.A. de M. (1809). Philosophie zoologique: ou Exposition des considérations relative à l'histoire naturelle des animaux.

Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell *75*, 843–854.

Leoni, G., Le Pera, L., Ferrè, F., Raimondo, D., and Tramontano, A. (2011). Coding potential of the products of alternative splicing in human. Genome Biol. *12*, R9.

Li, X. (2001). Characterization of human liver thermostable phenol sulfotransferase (SULT1A1) allozymes with 3,3',5-triiodothyronine as the substrate. J. Endocrinol. *171*, 525–532.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., and 1000 Genome Project Data Processing Subgroup (2009). The Sequence Alignment/Map format and SAMtools. Bioinformatics *25*, 2078–2079.

von Lieven, A.F., and Sudhaus, W. (2000). Comparative and functional morphology of the buccal cavity of Diplogastrina (Nematoda) and a first outline of the phylogeny of this taxon*. J. Zoolog. Syst. Evol. Res. *38*, 37–63.

Lipmann, F. (1958). Biological sulfate activation and transfer. Science *128*, 575–580.

Liu, W., Xie, Y., Ma, J., Luo, X., Nie, P., Zuo, Z., Lahrmann, U., Zhao, Q., Zheng, Y., Zhao, Y., et al. (2015). IBS: an illustrator for the presentation and visualization of biological sequences. Bioinformatics *31*, 3359–3361.

Love, A.C., and Raff, R.A. (2006). Larval ectoderm, organizational homology, and the origins of evolutionary novelty. J. Exp. Zool. B Mol. Dev. Evol. *306B*, 18–34.

Lu, B.Y., Ma, J., and Eissenberg, J.C. (1998). Developmental regulation of heterochromatin-mediated gene silencing in Drosophila. Development *125*, 2223–2234.

Lynch, M., and Force, A. (2000). The probability of duplicate gene preservation by subfunctionalization. Genetics *154*, 459–473.

Magistri, M., Faghihi, M.A., St Laurent, G., 3rd, and Wahlestedt, C. (2012). Regulation of chromatin structure by long noncoding RNAs: focus on natural antisense transcripts. Trends Genet. *28*, 389–396.

Mather, K. (1953). Genetical control of stability in development. Heredity *7*, 297–336.

Matsumoto, Y., and Crews, D. (2012). Molecular mechanisms of temperature-dependent sex determination in the context of ecological developmental biology. Mol. Cell. Endocrinol. *354*, 103–110.

Mayer, M.G., and Sommer, R.J. (2011). Natural variation in Pristionchus pacificus dauer formation reveals cross-preference rather than self-preference of nematode dauer pheromones. Proc. Biol. Sci. *278*, 2784–2790.

McFarlane, L., and Wilhelm, D. (2009). Non-Coding RNAs in Mammalian Sexual Development. Sex Dev. *3*, 302–316.

McGinnis, W., Garber, R.L., Wirz, J., Kuroiwa, A., and Gehring, W.J. (1984). A homologous protein-coding sequence in drosophila homeotic genes and its conservation in other metazoans. Cell *37*, 403–408.

Merchant-Larios, H., and Díaz-Hernández, V. (2013). Environmental Sex Determination Mechanisms in Reptiles. Sex Dev. *7*, 95–103.

Meylan, S., Miles, D.B., and Clobert, J. (2012). Hormonally mediated maternal effects, individual strategy and global change. Philos. Trans. R. Soc. Lond. B Biol. Sci. *367*, 1647–1664.

Mittler, T.E., and Sutherland, O.R.W. (1969). Dietary influences on aphid polymorphism. Entomol. Exp. Appl. *12*, 703–713.

Mizuguchi, S., Dejima, K., and Nomura, K. (2009). Sulfation and related genes in Caenorhabditis elegans. Trends Glycosci. Glycotechnol. *21*, 179–191.

Moczek, A.P., and Nijhout, H.F. (2002). Developmental mechanisms of threshold evolution in a polyphenic beetle. Evol. Dev. *4*, 252–264.

Modarresi, F., Faghihi, M.A., Patel, N.S., Sahagan, B.G., Wahlestedt, C., and Lopez-Toledano, M.A. (2011). Knockdown of BACE1-AS Nonprotein-Coding Transcript Modulates Beta-Amyloid-Related Hippocampal Neurogenesis. Int. J. Alzheimers. Dis. *2011*, 929042.

Moreno, E., Sieriebriennikov, B., Witte, H., Rödelsperger, C., Lightfoot, J.W., and Sommer, R.J. (2017). Regulation of hyperoxia-induced social behaviour in Pristionchus pacificus nematodes requires a novel cilia-mediated environmental input. Sci. Rep. *7*.

Motola, D.L., Cummins, C.L., Rottiers, V., Sharma, K.K., Li, T., Li, Y., Suino-Powell, K., Xu, H.E., Auchus, R.J., Antebi, A., et al. (2006). Identification of ligands for DAF-12 that govern dauer formation and reproduction in C. elegans. Cell *124*, 1209–1223.

Motomura, T., Nagasato, C., and Kimura, K. (2010). Cytoplasmic inheritance of organelles in brown algae. J. Plant Res. *123*, 185–192.

Mousseau, T.A., and Fox, C.W. (1998). Maternal Effects As Adaptations (Oxford University Press on Demand).

Mueller, J.W., Gilligan, L.C., Idkowiak, J., Arlt, W., and Foster, P.A. (2015). The Regulation of Steroid Action by Sulfation and Desulfation. Endocr. Rev. *36*, 526–563.

Müller, G.B. (2007). Evo-devo: extending the evolutionary synthesis. Nat. Rev. Genet. *8*, 943–949.

Müller, G.B., and Newman, S.A. (2005). Editorial: evolutionary innovation and morphological novelty. J. Exp. Zool. B Mol. Dev. Evol. *304*, 485–486.

Neumann, P., Jaé, N., Knau, A., Glaser, S.F., Fouani, Y., Rossbach, O., Krüger, M., John, D., Bindereif, A., Grote, P., et al. (2018). The lncRNA GATA6-AS epigenetically regulates endothelial gene expression via interaction with LOXL2. Nat. Commun. *9*, 237.

Nevers, A., Doyen, A., Malabat, C., Neron, B., Kergrohen, T., Jacquier, A., and Badis, G. (2017). Antisense transcriptional interference mediates condition-specific gene repression in budding yeast.

Newman, S.A., and Müller, G.B. (2000). Epigenetic mechanisms of character origination. J. Exp. Zool. *288*, 304–317.

Nilsen, T.W., and Graveley, B.R. (2010). Expansion of the eukaryotic proteome by alternative splicing. Nature *463*, 457–463.

Numata, K., and Kiyosawa, H. (2012). Genome-wide impact of endogenous antisense transcripts in eukaryotes. Front. Biosci. *17*, 300–315.

Ogawa, A., Streit, A., Antebi, A., and Sommer, R.J. (2009). A conserved endocrine mechanism controls the formation of dauer and infective larvae in nematodes. Curr. Biol. *19*, 67–71.

Ogawa, A., Bento, G., Bartelmes, G., Dieterich, C., and Sommer, R.J. (2011). Pristionchus pacificus daf-16 is essential for dauer formation but dispensable for mouth form dimorphism. Development *138*, 1281–1284.

Olds, B.P., Coates, B.S., Steele, L.D., Sun, W., Agunbiade, T.A., Yoon, K.S., Strycharz, J.P., Lee, S.H., Paige, K.N., Clark, J.M., et al. (2012). Comparison of the transcriptional profiles of head and body lice. Insect Mol. Biol. *21*, 257–268.

Osato, N., Suzuki, Y., Ikeo, K., and Gojobori, T. (2007). Transcriptional interferences in cis natural antisense transcripts of humans and mice. Genetics *176*, 1299–1306.

Pan, Q., Shai, O., Lee, L.J., Frey, B.J., and Blencowe, B.J. (2008). Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. Nat. Genet. *40*, 1413–1415.

Pandey, R.R., Mondal, T., Mohammad, F., Enroth, S., Redrup, L., Komorowski, J., Nagano, T., Mancini-Dinardo, D., and Kanduri, C. (2008). Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. Mol. Cell *32*, 232–246.

Pfennig, D.W., Wund, M.A., Snell-Rood, E.C., Cruickshank, T., Schlichting, C.D., and Moczek, A.P. (2010). Phenotypic plasticity's impacts on diversification and speciation. Trends Ecol. Evol. *25*, 459–467.

Pigliucci, M. (2001). Phenotypic Plasticity: Beyond Nature and Nurture (JHU Press).

Pigliucci, M. (2008). Is evolvability evolvable? Nat. Rev. Genet. *9*, 75–82.

Pigliucci, M., and Schlichting, C.D. (1998). Phenotypic Evolution: A Reaction Norm Perspective. J. Evol. Biol. *11*, 285.

Plaistow, Plaistow, St. Clair, J.J.H., Grant, J., and Benton (2007). How to Put All Your Eggs in One Basket: Empirical Patterns of Offspring Provisioning throughout a Mother's Lifetime. Am. Nat. *170*, 520.

Poliseno, L., Salmena, L., Zhang, J., Carver, B., Haveman, W.J., and Pandolfi, P.P. (2010). A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Nature *465*, 1033–1038.

Prabh, N., and Rödelsperger, C. (2016). Are orphan genes protein-coding, prediction artifacts, or non-coding RNAs? BMC Bioinformatics *17*.

Projecto-Garcia, J., Biddle, J.F., and Ragsdale, E.J. (2017). Decoding the architecture and origins of mechanisms for developmental polyphenism. Curr. Opin. Genet. Dev. *47*, 1–8.

Raff, R.A. (1996). The Shape of Life – Genes, Development and Evolution of Animal Forms, by Rudolf A. Raff. University of Chicago Press 1996. 544 pages. ISBN 0 226 70265 0 (cloth), 0-226-70266-9 (paper). Price £43.95 (55) cloth, £23.95 (29.95) paper. Genet. Res. *68*, 263.

Ragsdale, E.J., Müller, M.R., Rödelsperger, C., and Sommer, R.J. (2013). A Developmental Switch Coupled to the Evolution of Plasticity Acts through a Sulfatase. Cell *155*, 922–933.

Ragsdale, E.J., Kanzaki, N., and Herrmann, M. Taxonomy and natural history: the genus Pristionchus. In *Pristionchus Pacificus*, pp. 77–120.

Resch, A., Xing, Y., Modrek, B., Gorlick, M., Riley, R., and Lee, C. (2004). Assessing the impact of alternative splicing on domain interactions in the human proteome. J. Proteome Res. *3*, 76–83.

Robinson, J.T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011). Integrative genomics viewer. Nat. Biotechnol. *29*, 24–26.

Rödelsperger, C., Neher, R.A., Weller, A.M., Eberhardt, G., Witte, H., Mayer, W.E., Dieterich, C., and Sommer, R.J. (2014). Characterization of genetic diversity in the nematode Pristionchus pacificus from population-scale resequencing data. Genetics *196*, 1153–1165.

Rödelsperger, C., Meyer, J.M., Prabh, N., Lanz, C., Bemm, F., and Sommer, R.J. (2017). Single-Molecule Sequencing Reveals the Chromosome-Scale Genomic Architecture of the Nematode Model Organism Pristionchus pacificus. Cell Rep. *21*, 834–844.

Rudel, D., and Sommer, R.J. (2003). The evolution of developmental mechanisms. Dev. Biol. *264*, 15–37.

Sankar, A., Kooistra, S.M., Gonzalez, J.M., Ohlsson, C., Poutanen, M., and Helin, K. (2017). Maternal expression of the histone demethylase Kdm4a is crucial for pre-implantation development. Development *144*, 3264–3277.

Schad, E., Tompa, P., and Hegyi, H. (2011). The relationship between proteome size, structural disorder and organism complexity. Genome Biol. *12*, R120.

Schier, A.F. (2007). The maternal-zygotic transition: death and birth of RNAs. Science *316*, 406–407.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9*, 676–682.

Schlager, B., Wang, X., Braach, G., and Sommer, R.J. (2009). Molecular cloning of a dominant roller mutant and establishment of DNA-mediated transformation in the nematodePristionchus pacificus. Genesis *47*, 300–304.

Schlichting, C.D. (2008). Hidden reaction norms, cryptic genetic variation, and evolvability. Ann. N. Y. Acad. Sci. *1133*, 187–203.

Schmitz, S.U., Grote, P., and Herrmann, B.G. (2016). Mechanisms of long noncoding RNA function in development and disease. Cell. Mol. Life Sci. *73*, 2491–2509.

Schulenburg, H., and Félix, M.-A. (2017). The Natural Biotic Environment ofCaenorhabditis elegans. Genetics *206*, 55–86.

Schwander, T., Lo, N., Beekman, M., Oldroyd, B.P., and Keller, L. (2010). Nature versus nurture in social insect caste differentiation. Trends Ecol. Evol. *25*, 275–282.

Scotti, M.M., and Swanson, M.S. (2016). RNA mis-splicing in disease. Nat. Rev. Genet. *17*, 19–32.

Serobyan, V. (2015). Modes of Inheritance and Adaptive Values of Mouth-form Dimorphism in the Nematode Pristionchus Pacificus. Eberhard Karls Universität Tübingen Press.

Serobyan, V., Ragsdale, E.J., Müller, M.R., and Sommer, R.J. (2013). Feeding plasticity in the nematode Pristionchus pacificus is influenced by sex and social context and is linked to developmental speed. Evol. Dev. *15*, 161–170.

Serobyan, V., Ragsdale, E.J., and Sommer, R.J. (2014). Adaptive value of a predatory mouth-form in a dimorphic nematode. Proceedings of the Royal Society B: Biological Sciences *281*, 20141334–20141334.

Sieriebriennikov, B., Markov, G.V., Witte, H., and Sommer, R.J. (2017). The Role of DAF-21/Hsp90 in Mouth-Form Plasticity in Pristionchus pacificus. Mol. Biol. Evol. *34*, 1644–1653.

Sieriebriennikov, B., Prabh, N., Dardiry, M., Witte, H., Röseler, W., Kieninger, M.R., Rödelsperger, C., and Sommer, R.J. (2018). A Developmental Switch Generating Phenotypic Plasticity Is Part of a Conserved Multi-gene Locus. Cell Rep. *23*, 2835–2843.e4.

Sigrist, C.J.A., de Castro, E., Cerutti, L., Cuche, B.A., Hulo, N., Bridge, A., Bougueleret, L., and Xenarios, I. (2013). New and continuing developments at PROSITE. Nucleic Acids Res. *41*, D344–D347.

Sinha, A., Sommer, R.J., and Dieterich, C. (2012). Divergent gene expression in the conserved dauer stage of the nematodes Pristionchus pacificus and Caenorhabditis elegans. BMC Genomics *13*, 254.

Skelton, T.P., Hooper, L.V., Srivastava, V., Hindsgaul, O., and Baenziger, J.U. (1991). Characterization of a sulfotransferase responsible for the 4-O-sulfation of terminal beta-N-

acetyl-D-galactosamine on asparagine-linked oligosaccharides of glycoprotein hormones. J. Biol. Chem. *266*, 17142–17150.

Soares da Costa, D., Reis, R.L., and Pashkuleva, I. (2017). Sulfation of Glycosaminoglycans and Its Implications in Human Health and Disorders. Annu. Rev. Biomed. Eng. *19*, 1–26.

Sommer, R.J. (2009). The future of evo–devo: model systems and evolutionary theory. Nat. Rev. Genet. *10*, 416–422.

Sommer, R.J. (2015). Pristionchus pacificus: A Nematode Model for Comparative and Evolutionary Biology (BRILL).

Sommer, R.J., and McGaughran, A. (2013). The nematode Pristionchus pacificus as a model system for integrative studies in evolutionary biology. Mol. Ecol. *22*, 2380–2393.

Sommer, R.J., and Sternberg, P.W. (1996). Evolution of nematode vulval fate patterning. Dev. Biol. *173*, 396–407.

Sommer, R., Carta, L.K., Kim, S., and Sternberg, P.W. Morphological, genetic and molecular description of Pristionchus pacificus sp. n.(Nematoda: Neodiplogasteridae). Fundam. Appl. Nematol. *19*, 511–522.

Srinivasan, J., Sinz, W., Lanz, C., Brand, A., Nandakumar, R., Raddatz, G., Witte, H., Keller, H., Kipping, I., Pires-daSilva, A., et al. (2002). A bacterial artificial chromosome-based genetic linkage map of the nematode Pristionchus pacificus. Genetics *162*, 129–134.

Stamm, S., Ben-Ari, S., Rafalska, I., Tang, Y., Zhang, Z., Toiber, D., Thanaraj, T.A., and Soreq, H. (2005). Function of alternative splicing. Gene *344*, 1–20.

Strott, C.A. (2002). Sulfonation and molecular action. Endocr. Rev. *23*, 703–732.

Su, W.-Y., Li, J.-T., Cui, Y., Hong, J., Du, W., Wang, Y.-C., Lin, Y.-W., Xiong, H., Wang, J.-L., Kong, X., et al. (2012). Bidirectional regulation between WDR83 and its natural antisense transcript DHPS in gastric cancer. Cell Res. *22*, 1374–1389.

Suiko, M., Kurogi, K., Hashiguchi, T., Sakakibara, Y., and Liu, M.-C. (2017). Updated perspectives on the cytosolic sulfotransferases (SULTs) and SULT-mediated sulfation. Biosci. Biotechnol. Biochem. *81*, 63–72.

Susoy, V., and Sommer, R.J. (2016). Stochastic and Conditional Regulation of Nematode Mouth-Form Dimorphisms. Frontiers in Ecology and Evolution *4*.

Susoy, V., Ragsdale, E.J., Kanzaki, N., and Sommer, R.J. (2015). Rapid diversification associated with a macroevolutionary pulse of developmental plasticity. Elife *4*.

Talavera, D., Vogel, C., Orozco, M., Teichmann, S.A., and de la Cruz, X. (2007). The (in)dependence of alternative splicing and gene duplication. PLoS Comput. Biol. *3*, e33.

The UniProt Consortium (2017). UniProt: the universal protein knowledgebase. Nucleic Acids Res. *45*, D158–D169.

Tian, H., Schlager, B., Xiao, H., and Sommer, R.J. (2008). Wnt signaling induces vulva development in the nematode Pristionchus pacificus. Curr. Biol. *18*, 142–146.

Tovar-Corona, J.M., Castillo-Morales, A., Chen, L., Olds, B.P., Clark, J.M., Reynolds, S.E., Pittendrigh, B.R., Feil, E.J., and Urrutia, A.O. (2015). Alternative Splice in Alternative Lice. Mol. Biol. Evol. *32*, 2749–2759.

Trapnell, C., Hendrickson, D.G., Sauvageau, M., Goff, L., Rinn, J.L., and Pachter, L. (2013). Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat. Biotechnol. *31*, 46–53.

Tress, M.L., Abascal, F., and Valencia, A. (2017). Alternative Splicing May Not Be the Key to Proteome Complexity. Trends Biochem. Sci. *42*, 98–110.

Uller, T. (2008). Developmental plasticity and the evolution of parental effects. Trends Ecol. Evol. *23*, 432–438.

Van Valen, L. (1973). body size and numbers of plants and animals. Evolution *27*, 27–35.

Veening, J.-W., Smits, W.K., and Kuipers, O.P. (2008). Bistability, epigenetics, and bet-hedging in bacteria. Annu. Rev. Microbiol. *62*, 193–210.

Villegas, V.E., and Zaphiropoulos, P.G. (2015). Neighboring gene regulation by antisense long non-coding RNAs. Int. J. Mol. Sci. *16*, 3251–3266.

Villegas, V.E., Rahman, M.F.-U., Fernandez-Barrena, M.G., Diao, Y., Liapi, E., Sonkoly, E., Ståhle, M., Pivarcsi, A., Annaratone, L., Sapino, A., et al. (2014). Identification of novel non-coding RNA-based negative feedback regulating the expression of the oncogenic transcription factor GLI1. Mol. Oncol. *8*, 912–926.

Waddington, C.H. (1942). canalization of development and the inheritance of acquired characters. Nature *150*, 563–565.

Waddington, C.H. (1953). Genetic Assimilation of an Acquired Character. Evolution *7*, 118.

Wagner, A. (2005). Robustness and Evolvability in Living Systems (Princeton: Princeton University Press).

Wang, X., and Sommer, R.J. (2011). Antagonism of LIN-17/Frizzled and LIN-18/Ryk in nematode vulva induction reveals evolutionary alterations in core developmental pathways. PLoS Biol. *9*, e1001110.

Wang, E.T., Sandberg, R., Luo, S., Khrebtukova, I., Zhang, L., Mayr, C., Kingsmore, S.F., Schroth, G.P., and Burge, C.B. (2008). Alternative isoform regulation in human tissue transcriptomes. Nature *456*, 470–476.

Wang, L., Park, H.J., Dasari, S., Wang, S., Kocher, J.-P., and Li, W. (2013). CPAT: Coding-Potential Assessment Tool using an alignment-free logistic regression model. Nucleic Acids Res. *41*, e74.

Wang, Y., Jorda, M., Jones, P.L., Maleszka, R., Ling, X., Robertson, H.M., Mizzen, C.A., Peinado, M.A., and Robinson, G.E. (2006). Functional CpG methylation system in a social insect. Science *314*, 645–647.

Weaver, N. (1957). Effects of Larval Age on Dimorphic Differentiation of the Female Honey Bee1. Ann. Entomol. Soc. Am. *50*, 283–294.

Weaver, J.R., Susiarjo, M., and Bartolomei, M.S. (2009). Imprinting and epigenetic changes in the early embryo. Mamm. Genome *20*, 532–543.

Weiner, S.A., and Toth, A.L. (2012). Epigenetics in social insects: a new direction for understanding the evolution of castes. Genet. Res. Int. *2012*, 609810.

Werner, A. (2013). Biological functions of natural antisense transcripts. BMC Biol. *11*, 31.

Werner, M.S., Sieriebriennikov, B., Loschko, T., Namdeo, S., Lenuzzi, M., Dardiry, M., Renahan, T., Sharma, D.R., and Sommer, R.J. (2017). Environmental influence on Pristionchus pacificus mouth form through different culture methods. Sci. Rep. *7*.

West-Eberhard, M.J. (2003). Developmental Plasticity and Evolution (Oxford University Press).

Wheeler, D.E. (1986). Developmental and Physiological Determinants of Caste in Social Hymenoptera: Evolutionary Implications. Am. Nat. *128*, 13–34.

White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1986). The structure of the nervous system of the nematode Caenorhabditis elegans. Philos. Trans. R. Soc. Lond. B Biol. Sci. *314*, 1–340.

Whitehead, A., Galvez, F., Zhang, S., Williams, L.M., and Oleksiak, M.F. (2011). Functional genomics of physiological plasticity and local adaptation in killifish. J. Hered. *102*, 499–511.

Wilecki, M., Lightfoot, J.W., Susoy, V., and Sommer, R.J. (2015). Predatory feeding behaviour in Pristionchus nematodes is dependent on phenotypic plasticity and induced by serotonin. J. Exp. Biol. *218*, 1306–1313.

Witte, H., Moreno, E., Rödelsperger, C., Kim, J., Kim, J.-S., Streit, A., and Sommer, R.J. (2015). Gene inactivation using the CRISPR/Cas9 system in the nematode Pristionchus pacificus. Dev. Genes Evol. *225*, 55–62.

Wolf, J.B., and Brodie, E.D., 3rd (1998). the coadaptation of parental and offspring characters. Evolution *52*, 299–308.

Woltereck, R. (1913). Weitere experimentelle untersuchungen über Artänderung, speziell über das Wesen quantitativer Artunterschiede bei Daphniden. Z. Indukt. Abstamm. Vererbungsl. *9*, 146–146.

Xi, Y., Seyoum, H., and Liu, M.-C. (2016). Role of SULT-mediated sulfation in the biotransformation of 2-butoxyethanol and sorbitan monolaurate: A study using zebrafish SULTs. Aquat. Toxicol. *177*, 19–21.

Xing, Y., and Lee, C. (2007). Relating alternative splicing to proteome complexity and genome evolution. Adv. Exp. Med. Biol. *623*, 36–49.

Xu, H.-J., Xue, J., Lu, B., Zhang, X.-C., Zhuo, J.-C., He, S.-F., Ma, X.-F., Jiang, Y.-Q., Fan, H.-W., Xu, J.-Y., et al. (2015). Two insulin receptors determine alternative wing morphs in planthoppers. Nature *519*, 464–467.

Xue, Z., Ye, Q., Anson, S.R., Yang, J., Xiao, G., Kowbel, D., Glass, N.L., Crosthwaite, S.K., and Liu, Y. (2014). Transcriptional interference by antisense RNA is required for circadian clock function. Nature *514*, 650–653.

Yang, M., Wei, Y., Jiang, F., Wang, Y., Guo, X., He, J., and Kang, L. (2014). MicroRNA-133 inhibits behavioral aggregation by controlling dopamine synthesis in locusts. PLoS Genet. *10*, e1004206.

Yelin, R., Dahary, D., Sorek, R., Levanon, E.Y., Goldstein, O., Shoshan, A., Diber, A., Biton, S., Tamir, Y., Khosravi, R., et al. (2003). Widespread occurrence of antisense transcription in the human genome. Nat. Biotechnol. *21*, 379–386.

Yim, J.J., Bose, N., Meyer, J.M., Sommer, R.J., and Schroeder, F.C. (2015). Nematode signaling molecules derived from multimodular assembly of primary metabolic building blocks. Org. Lett. *17*, 1648–1651.

Zauner, H., and Sommer, R.J. (2007). Evolution of robustness in the signaling network of Pristionchus vulva development. Proc. Natl. Acad. Sci. U. S. A. *104*, 10086–10091.

Zhang, K., and Smith, G.W. (2015). Maternal control of early embryogenesis in mammals. Reprod. Fertil. Dev. *27*, 880.

Zimmermann, L., Stephens, A., Nam, S.-Z., Rau, D., Kübler, J., Lozajic, M., Gabler, F., Söding, J., Lupas, A.N., and Alva, V. (2017). A Completely Reimplemented MPI Bioinformatics Toolkit with a New HHpred Server at its Core. J. Mol. Biol. 430, 2237-2243.

8. *Curriculum Vitae*

Personal details:

Name: Suryesh Kumar Namdeo

Date of Birth: 11/08/1991

Nationality: Indian

Email Address: suryeshn@gmail.com

Education:

2014-	Max Planck Institute for Developmental Biology, Tübingen, Germany
2009-2014	Indian Institute for Science Education and Research, Pune, India
2007-2009	Saraswati Gyan Mandir, Garhakota, Sagar, Madhya Pradesh, India

Publications:

Namdeo, S., Moreno, E., Rödelsperger, C., Baskaran, P., Witte, H., and Sommer, R.J. (2018). Two independent sulfation processes regulate mouth-form plasticity in the nematode *Pristionchus pacificus*. Development *145*, dev166272.

Serobyan, V., Xiao, H., **Namdeo, S.**, Rödelsperger, C., Sieriebriennikov, B., Witte, H., Röseler, W., and Sommer, R.J. (2016). Chromatin remodelling and antisense-mediated up-regulation of the developmental switch gene *eud-1* control predatory feeding plasticity. Nat. Commun. 7, 12337.

Werner, M.S., Sieriebriennikov, B., Loschko, T., **Namdeo, S.**, Lenuzzi, M., Dardiry, M., Renahan, T., Sharma, D.R., and Sommer, R.J. (2017). Environmental influence on Pristionchus pacificus mouth form through different culture methods. Sci. Rep. 7.

Sommer, R.J., Dardiry, M., Lenuzzi, M., **Namdeo, S.**, Renahan, T., Sieriebriennikov, B., and Werner, M.S. (2017). The genetics of phenotypic plasticity in nematode feeding structures. Open Biol. 7, 160332.

Conferences and Symposia:

Poster: Namdeo S. and Sommer R.J. ''Interplay between two non-coding antisense RNAs from same loci governs phenotypic plasticity'' EMBO workshop 'Non coding RNAs in embryonic development and cell differentiation' Rehovot, Israel (8-11 April 2018).

Talk: Science Slam (Winner) organized by MNF Graduiertenakademie, Eberhard Karls Universität Tübingen. "The Tale of Two Morphs: A Worm Story" (21 February 2018).

Participation: 'Vision in Science – Discover New Perspectives' organized by Max Planck PhDNet in Berlin (29-30 September 2017).

Talk: Science Slam at 'Vision in Science – Break the Enigma' conference organized by Max Planck PhDNet in Berlin (16-18 September 2016).

Poster: Namdeo S. and Sommer R.J. ''Role of potential non-coding RNAs in regulation of mouth-form phenotypic plasticity in *Pristionchus pacificus*'' EMBO symposium ''The Non-coding Genome'' Heidelberg, Germany (18-21 October 2015).

Poster: Namdeo S. and Sommer R.J. ''Role of potential non-coding RNAs in regulation of mouth-form phenotypic plasticity in *Pristionchus pacificus*'' EMBO conference ''Chromatin and epigenetics'' Heidelberg, Germany (6-10 May 2015).

9. Appendix

The Company of
Biologists

RESEARCH ARTICLE

Two independent sulfation processes regulate mouth-form plasticity in the nematode *Pristionchus pacificus*

Suryesh Namdeo, Eduardo Moreno, Christian Rödelsperger, Praveen Baskaran, Hanh Witte and Ralf J. Sommer*

ABSTRACT

Sulfation of biomolecules, like phosphorylation, is one of the most fundamental and ubiquitous biochemical modifications with important functions during detoxification. This process is reversible, involving two enzyme classes: a sulfotransferase, which adds a sulfo group to a substrate; and a sulfatase that removes the sulfo group. However, unlike phosphorylation, the role of sulfation in organismal development is poorly understood. In this study, we find that two independent sulfation events regulate the development of mouth morphology in the nematode *Pristionchus pacificus*. This nematode has the ability to form two alternative mouth morphologies depending on environmental cues, an example of phenotypic plasticity. We found that, in addition to a previously described sulfatase, a sulfotransferase is involved in regulating the mouth-form dimorphism in *P. pacificus*. However, it is unlikely that both of these sulfation-associated enzymes act upon the same substrates, as they are expressed in different cell types. Furthermore, animals mutant in genes encoding both enzymes show condition-dependent epistatic interactions. Thus, our study highlights the role of sulfation-associated enzymes in phenotypic plasticity of mouth structures in *Pristionchus*.

KEY WORDS: *Pristionchus pacificus*, Developmental plasticity, Sulfotransferases, Developmental switch gene, *Eud-1*/sulfatase

INTRODUCTION

Phenotypic plasticity is the ability of an organism to develop different phenotypes from the same genotype in response to environmental cues, and has been suggested to facilitate the evolution of phenotypic novelty and diversity (Pigliucci, 2001; West-Eberhard, 2003; Moczek et al., 2011; Susoy and Sommer, 2016). Often also referred to as 'developmental plasticity', the phenomenon is widespread in nature, and is best known in plants and insects. For example, beetle horn development, butterfly wing polyphenisms, phase transition in locusts and the formation of castes in social insects all represent plastic traits, indicating the importance of plasticity for the physiology, ecology and evolution of these organisms (Moczek, 1998; Beldade and Brakefield, 2002; Ernst et al., 2015; Corona et al., 2016; Fischman et al., 2017). Not surprisingly, plasticity has become a prominent idea extensively discussed in the literature (Bateson et al., 2017).

Max Planck Institute for Developmental Biology, Department for Integrative Evolutionary Biology, Max-Planck-Ring 9, 72076 Tuebingen, Germany.

*Author for correspondence (ralf.sommer@tuebingen.mpg.de)

C.R., 0000-0002-7905-9675; R.J.S, 0000-0003-1503-7749

Experimental studies of phenotypic plasticity have been restricted, owing to the scarcity of model organisms that provide genetic and molecular tools for mechanistic insight. However, several recent studies in insects provide molecular mechanisms of plasticity (Wang and Kang, 2014). In addition, investigations in the nematode model organism *Pristionchus pacificus* focus on phenotypic plasticity in the context of the formation of alternative feeding structures (Sommer and McGaughran, 2013; Sommer et al., 2017). *P. pacificus* has two alternative and discontinuous mouth-form morphologies, the eurystomatous (Eu) and stenostomatous (St) forms (Bento et al., 2010). The Eu mouth form is wider and has a large claw-like dorsal tooth and an opposing right subventral tooth, whereas the St mouth form is narrower, having only a flint-shaped dorsal tooth (Fig. 1A,B). Mouth morphology once formed is irreversible. The dimorphism is associated with predatory feeding behavior, as Eu worms have the extraordinary ability to prey on other nematodes including *Caenorhabditis elegans* (Fig. 1C), whereas St worms are strictly bacteriovorous (Serobyan et al., 2014; Wilecki et al., 2015; Lightfoot et al., 2016).

P. pacificus is amenable to the genetic analysis of mouth-form plasticity for several reasons (Sommer et al., 2017). First, *P. pacificus* is a self-fertilizing hermaphrodite, which can be propagated as isogenic strains, thereby simplifying the analysis of plasticity. Similar to the distantly related *C. elegans*, *P. pacificus* has a fast generation time of 4 days under laboratory conditions (see Materials and Methods) and can be grown on *Escherichia coli* OP50, all of which resulted in the development of forward genetic protocols for mutagenesis (Sommer and Carta, 1996). Second, a chromosome-scale genome assembly, as well as methods for transgenesis and CRISPR/Cas9 engineering are available for *P. pacificus* (Dieterich et al., 2008; Rödelsperger et al., 2017; Schlager et al., 2009; Witte et al., 2014). Third, the wild-type strain RS2333 of *P. pacificus* exhibits mouth-form plasticity that – in addition to conditional factors, such as starvation and pheromones (Serobyan et al., 2013; Bose et al., 2012) – also depends on stochasticity (Susoy and Sommer, 2016). Regardless of the mouth form of the hermaphroditic mother, offspring will have 70-90% Eu and 10-30% St mouth forms under standard laboratory conditions with only OP50 as food (Serobyan et al., 2014). Thus, even in the absence of environmental perturbations, both plastic traits are developed in parallel, making *P. pacificus* an ideal system for genetic screens and the identification of monomorphic mutants.

Over the years, several genetic, epigenetic and environmental factors were identified to play a role in mouth-form determination of *P. pacificus* (Fig. 1D). Environmental cues such as starvation and crowding can shift the mouth-form ratio towards more Eu animals (Serobyan et al., 2013). In earlier studies, it was shown that treatment with the steroid hormone dafachronic acid results in fewer animals with Eu form (Bento et al., 2010), whereas treatment with the pheromone dasc#1 induces Eu forms (Bose et al., 2012). More
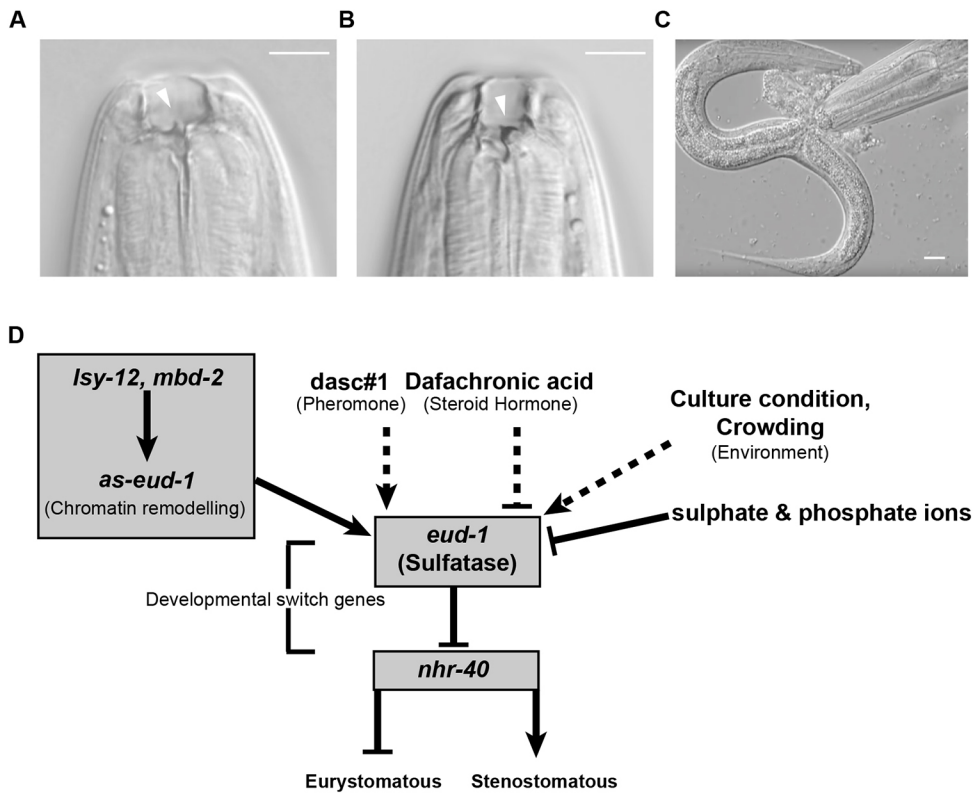
DEVELOPMENT

1

Fig. 1. *P. pacificus* exhibits mouth-form dimorphism. (A-C) Representative images of eurystomatous mouth form of *P. pacificus* (A), stenostomatous mouth morph of *P. pacificus* (B), *P. pacificus* killing and feeding on *C. elegans* (C). The arrowhead in A indicates the subventral denticle of an Eu animal, whereas the arrowhead in B indicates the dorsal tooth in an St animal. The smaller worm in C is an L2-stage *C. elegans*. Internal body material is coming out of the *C. elegans* owing to the *P. pacificus* (larger worm) biting it. (D) Representation of relationships among various known factors that affect mouth form. Scale bars: 10 µm.

recently, liquid culture of worms in S-medium was identified as a growth condition that also lowers the abundance of Eu animals (Werner et al., 2017).

Using the forward genetic tools available in *P. pacificus* with its hermaphroditic mode of reproduction resulted in the discovery and characterization of several genes involved in mouth-form regulatory pathways. *eud-1*, a gene coding for an aryl-sulfatase, was described as a developmental switch because *eud-1* mutants are all-St animals, whereas worms overexpressing *eud-1* all have the Eu mouth form (Ragsdale et al., 2013). The discovery of a developmental switch gene in the regulation of plasticity confirmed a long-standing theory that was originally developed in the context of caste differentiation in wasps and other hymenopterans (West-Eberhard, 2003). *eud-1* is expressed in neurons and is thought to be involved in either environmental sensing or decision making in the mouth-form determination process. NHR-40, a nuclear hormone receptor was identified in a suppressor screen of *eud-1* (Kieninger et al., 2016). *nhr-40* null mutants have only Eu mouth forms, whereas animals overexpressing *nhr-40* have only St forms, indicating that the developmental switch of *P. pacificus* mouth-form plasticity is not a single gene but a genetic network. *nhr-40* is also expressed in neurons and, similar to *eud-1*, acts in cells far away from those that form the teeth in the mouth (Kieninger et al., 2016).

Finally, multiple epigenetic factors – including the histone acetyltransferase *lsy-12*, the methyl binding protein *mbd*-2 and an antisense RNA at the *eud-1* locus, as-*eud-1* – were also shown to affect mouth-form plasticity (Serobyan et al., 2016). Interestingly, all these factors positively regulate *eud-1* levels suggesting that the switch gene *eud-1* is a primary target of mouth-form regulation. In agreement with this observation, EUD-1 protein is sensitive to small molecules. For example, treatment of animals with small molecules like sulfate and phosphate ions has been shown to induce the St form, and is thought to act by inhibition of EUD-1 (Ragsdale et al.,

2013). However, only a very small number of bioactive compounds were tested for their potential role in mouth-form regulation.

The key role of the sulfatase EUD-1 in controlling mouth-form plasticity hints at the importance of sulfation processes. Sulfation, also described as sulfonation or sulfoconjugation, is one of the most fundamental biochemical modifications in various biomolecules including proteins, steroids, glycolipids and glycoproteins (Strott, 2002). It is present in organisms ranging from bacteria to humans, and has been shown to be essential for a multitude of biological processes, such as hormone metabolism, xenobiotic metabolism, and intra- and extracellular localization of sulfated molecules (Strott, 2002; Kauffman, 2004). The sulfation pathway is considered to be reversible, and it consists of two major enzyme families, sulfotransferases and sulfatases (Coughtrie et al., 1998). Sulfotransferases are responsible for the transfer of a sulfonate or sulfo group ($-SO_3^-$) to a substrate. They are further divided into cytosolic and membrane-bound categories. The cytosolic sulfotransferases are responsible for sulfation of small molecules including xenobiotics, whereas their membrane-bound counterparts are associated with Golgi membranes and catalyze sulfation as a post-translational modification of proteins (Negishi et al., 2001). In general, sulfation of xenobiotics by cytosolic sulfotransferases is essential for detoxification and elimination of these compounds. In contrast, sulfatases catalyze the hydrolysis of sulfate esters formed by the action of a sulfotransferase (Hanson et al., 2004). This enzymatic module of sulfation (sulfotransferase-sulfatase) is well studied in various cancers, especially breast and ovarian cancers, for which tumor growth is often associated with steroid metabolism (Rižner, 2016). However, unlike the phosphorylation module (kinase-phosphatase), sulfation has not been studied extensively in the context of model systems and, as a result, relatively little is known about the involvement of sulfation in organismal processes in the context of development or ecology. One of the possible

functions could be the perception of environmental cues based on the known role of sulfation in steroid and hormonal metabolism, and the fact that many environmental biochemical signals are lipophilic in nature. Another potential function could be a role in intracellular signaling, because sulfation can change the nature and activity of various bioactive compounds in a cell.

Here, we performed a pharmacological screen by treating *P. pacificus* with several bioactive compounds and examined their effect on mouth-form ratio. We found that bisphenol A, tyramine and dopamine induce St mouth forms. As all three of these phenolic compounds are potential substrates of cytosolic sulfotransferases, we examined a potential role of sulfotransferases in regulating mouth-form plasticity. We generated knockout mutants in all five cytosolic sulfotransferases in *P. pacificus* (arbitrarily named *sult-1* to *sult-5*), and found that *sult-1* mutants resulted in all or preferentially Eu animals. Surprisingly, *sult-1* expression studies and epistasis analysis between *sult-1* and *eud-1* suggest that both genes act in different cells. Our analysis provides the first evidence for the function of a sulfotransferase in the regulation of phenotypic plasticity and indicates that at least two independent sulfation processes are involved in mouth-form determination in *P. pacificus*.

RESULTS
Bisphenol A, tyramine and dopamine induce St mouth forms
To extend previous studies on the effect of bioactive compounds on the regulation of mouth-form plasticity (Ragsdale et al., 2013), we decided to study the effect of a series of small molecules. We treated the highly Eu wild-type strain RS2333 from California with 23 different bioactive compounds (Fig. 2A). Because we would not be able to identify Eu form-inducing chemicals in this screen, we performed similar assays on another *P. pacificus* strain, RSC019, from La Réunion Island, which has equal frequencies of Eu and St animals (Fig. 2B). For all assays, three J4 hermaphrodites were placed on agar plates containing *E. coli* OP50 and one of the compounds. The progeny of these three hermaphrodites were scored for mouth-form phenotypes once they reached adulthood. Animals of both RS2333 and RSC019 strains showed strongest reduction in Eu form frequency in bisphenol A, tyramine and dopamine, out of all the compounds tested, at the applied concentration (10 µM) (Fig. 2A,B, Fig. S1). In addition, synephrine, octopamine, insulin and S-adenosyl methionine showed a relatively weak reduction of Eu form frequency in RS2333, but not in RSC019. Some molecules (cyclic AMP, chondroitin sulfate and acetyl-CoA) have a Eu form-inducing effect in RSC019 only (Fig. 2B). For this study, we focused on molecules that induce the strongest reduction of Eu frequency in both the RS2333 and RSC019 strains, i.e. bisphenol A, tyramine and dopamine. Interestingly, all three phenolic compounds are potential substrates of cytosolic sulfotransferases (Brix et al., 1999; Hattori et al., 2006; Yasuda et al., 2007). For example, in *C. elegans*, the sulfotransferase *ssu-1* has been described to most efficiently sulfate bisphenol A out of all the endogenous and xenobiotic compounds tested (Hattori et al., 2006). This intrigued us because the sulfatase *eud-1* was already described as a developmental switch gene for mouth-form regulation (Ragsdale et al., 2013), and thus a sulfation module consisting of a sulfatase and a sulfotransferase might modify common substrates during mouth-form regulation. Also, *C. elegans* *ssu-1* (*Cel-ssu-1*) is expressed in neurons including amphid neurons, which are related to those cells expressing *P. pacificus* *eud-1* (Ragsdale et al., 2013; Carroll et al., 2006). Therefore, we targeted cytosolic sulfotransferases in *P. pacificus* as potential regulators of mouth-form plasticity.

A mutant library of *P. pacificus* cytosolic sulfotransferases
Next, we examined the presence and phylogenetic relationship of sulfotransferase genes in the nematodes *P. pacificus*, *C. elegans* and *Caenorhabditis briggsae* using the fruit fly *Drosophila melanogaster* as an outgroup for phylogenetic reconstruction (Fig. 3). *P. pacificus* has 17 sulfotransferases, a massive expansion in comparison to *C. elegans* and *C. briggsae*, which have seven genes each. In particular, *P. pacificus* has a large number of paralogs to the single *C. elegans* cytosolic sulfotransferase *ssu-1*. Five *P. pacificus* genes are most closely related to *Cel-ssu-1* and four additional genes cluster with a *Drosophila*-specific expansion. Given these phylogenetic clusters, we decided to systematically study the five closest paralogs of *Cel-ssu-1* in *P. pacificus*. For this, we arbitrarily named these five genes *sult-1* to *sult-5*. We employed the CRISPR/Cas9 technique to obtain knockout mutants for all five genes (Fig. 4). In total, we isolated ten mutants as described in Fig. 4, Fig. S2 and Table 1. Only nonsense alleles or those resulting in frame-shift mutations were selected for phenotypic characterization.

sult-1 has a strong mouth-form phenotype
To study the potential role of *sult-1* to *sult-5* in mouth-form regulation, we have grown all mutant lines under two different culture conditions that generate preferentially Eu or St mouth forms in wild-type animals, respectively (Werner et al., 2017). Additionally, we tested hermaphrodites and males separately, as wild-type RS2333 males are highly St, unlike hermaphrodites (Serobyan et al., 2013). When grown on standard nematode growth medium (NGM) agar plates, hermaphrodites of all mutant lines showed highly Eu mouth-form ratios that were not different from RS2333 animals (Fig. 5A). In contrast, we found that in males, alleles of *sult-1* are either completely Eu (*tu1061*) or highly Eu (*tu1232*), whereas wild-type males are highly St (Fig. 5B). Similarly, *sult-2(tu1063)* mutant males also had a significant increase in Eu frequency compared with wild type (Fig. 5B). Therefore, we employed liquid culture conditions for scoring mouth-form phenotypes because such conditions were previously shown to reduce Eu frequency in hermaphrodites (Werner et al., 2017). Indeed, *sult-1(tu1061)* and *sult-1(tu1232)* mutant hermaphrodites exhibited a strong Eu phenotype in liquid culture, with 100% of the observed animals being Eu (Fig. 5C). In contrast, mutant alleles of the other Sult genes resulted in high St phenotypes as in wild-type animals. Similarly, mouth-form ratios of *sult-1(tu1061)* and *sult-1(tu1232)* mutant males grown under liquid culture conditions were highly biased towards the Eu form, whereas wild-type males and mutants of the other four genes were Eu defective (Fig. 5D). Together, these experiments suggest a role of *sult-1* in mouth-form regulation in *P. pacificus*. It is important to note that both *sult-1* alleles have frame-shift mutations that result in premature stop codons (Fig. S3). However, the *sult-1* guide RNA was designed to target the central part of the coding region to avoid translation from several known alternative open reading frames when targeting more 5′ regions of the gene (Fig. S2). Therefore, these frame-shift mutations most likely result in reduction-of-function, rather than loss-of-function, alleles.

Given these results, we overexpressed *sult-1* in a wild-type background using two genomic constructs with a 2.2 kb and an 8 kb promoter region, respectively. Indeed, we found that hermaphrodites overexpressing *sult-1* have significantly lower Eu frequency compared with wild-type animals in NGM agar plates (Fig. 5E). This phenotype is contrary to the *sult-1* knockout phenotype, further indicating that *sult-1* is an important regulator of mouth-form plasticity in *P. pacificus* and promotes the St mouth form.
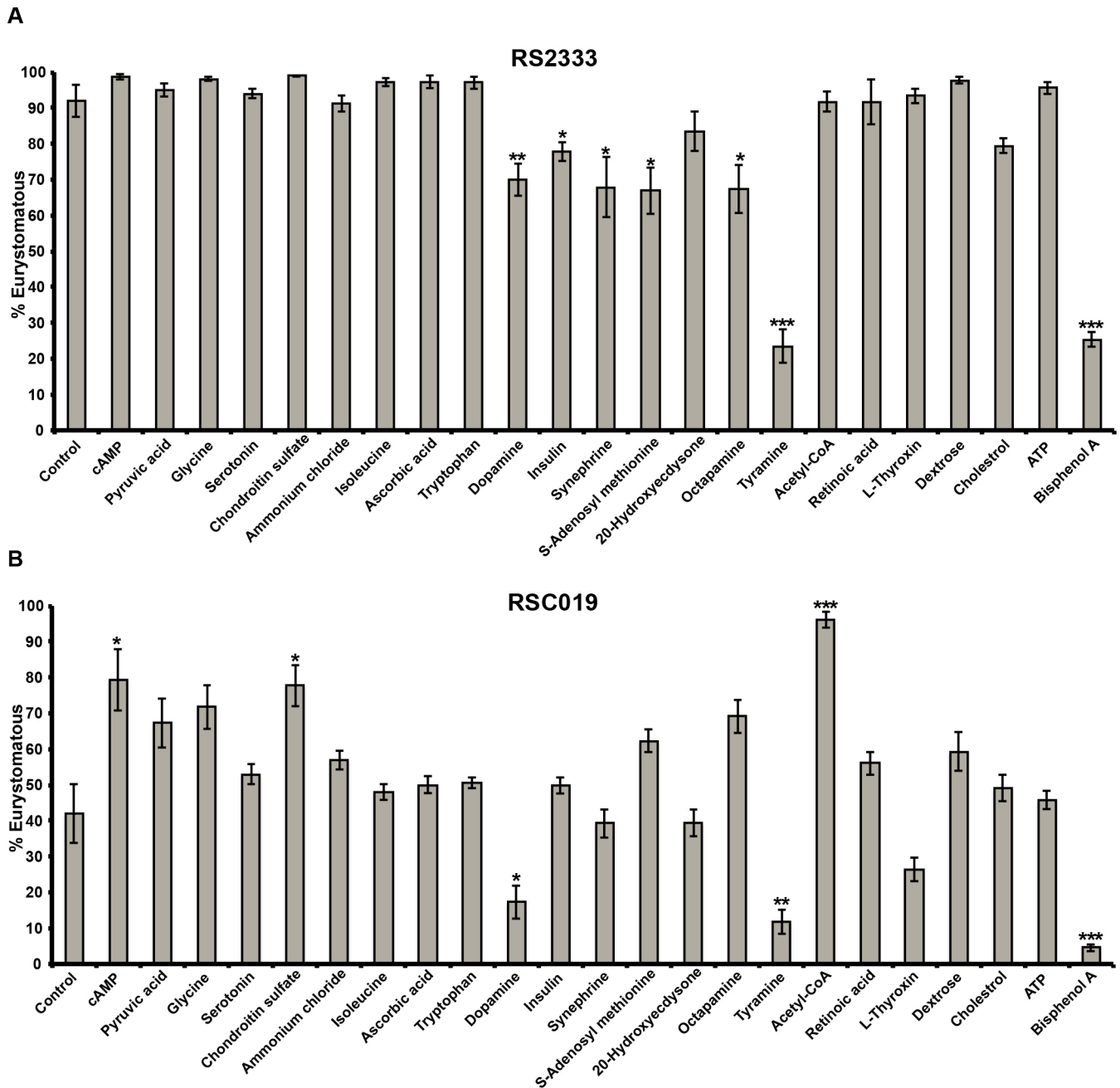
DEVELOPMENT

Fig. 2. Pharmacological screening reveals that bisphenol A, tyramine and dopamine have strongest St form-inducing effects.
(A,B) Pharmacological screening with different bioactive compounds (10 µM) on RS2333 (wild-type) (A) and RSC019 (B) animals. For each compound and each strain (RS2333 and RSC019), more than 200 animals were scored for their mouth morphology phenotype. At least three biological replicates were performed, with each having more than 50 animals. Error bars represent standard deviation from all the biological replicates. *$P<0.01$, **$P<0.005$, ***$P<0.001$, two-tailed Student's t-test, with respect to control values. Only the compounds showing strongest St form-inducing effects were further analyzed.

Epistasis analysis of *sult-1* and *eud-1* shows strong conditional effects

The identification of the sulfotransferase *sult-1* as a regulator of mouth-form plasticity, in addition to the sulfatase *eud-1* and their opposing phenotypes, raises the question of their potential genetic and biochemical interaction. In theory, SULT-1 and EUD-1 could form a sulfation module that acts on identical target molecules, i.e. hormone ligands that can be activated or de-activated by changing their sulfate moieties. Alternatively, SULT-1 and EUD-1 might have different target molecules acting in parallel pathways, or in the same pathway, resulting in epistatic relationships. To unravel the

functional relationship between *sult-1* and *eud-1*, we first performed epistasis tests by creating a *sult-1(tu1061);eud-1(tu455)* double mutant. The *sult-1(tu1061)* mutation weakly suppressed the Eu-defective phenotype of *eud-1(tu455)* in the double mutant when grown on NGM agar plates (Fig. 5F). Strikingly, however, epistasis between *sult-1* and *eud-1* is conditional as different Eu form-inducing factors can strongly influence the mouth-form ratio. First, animals were treated with dasc#1, a pheromone earlier found to increase the ratio of Eu animals (Bose et al., 2012). Wild-type and *sult-1(tu1061)* single mutant animals are 100% Eu after dasc#1 treatment, whereas *eud-1(tu455)* mutants remain 0% Eu (Fig. 5F).
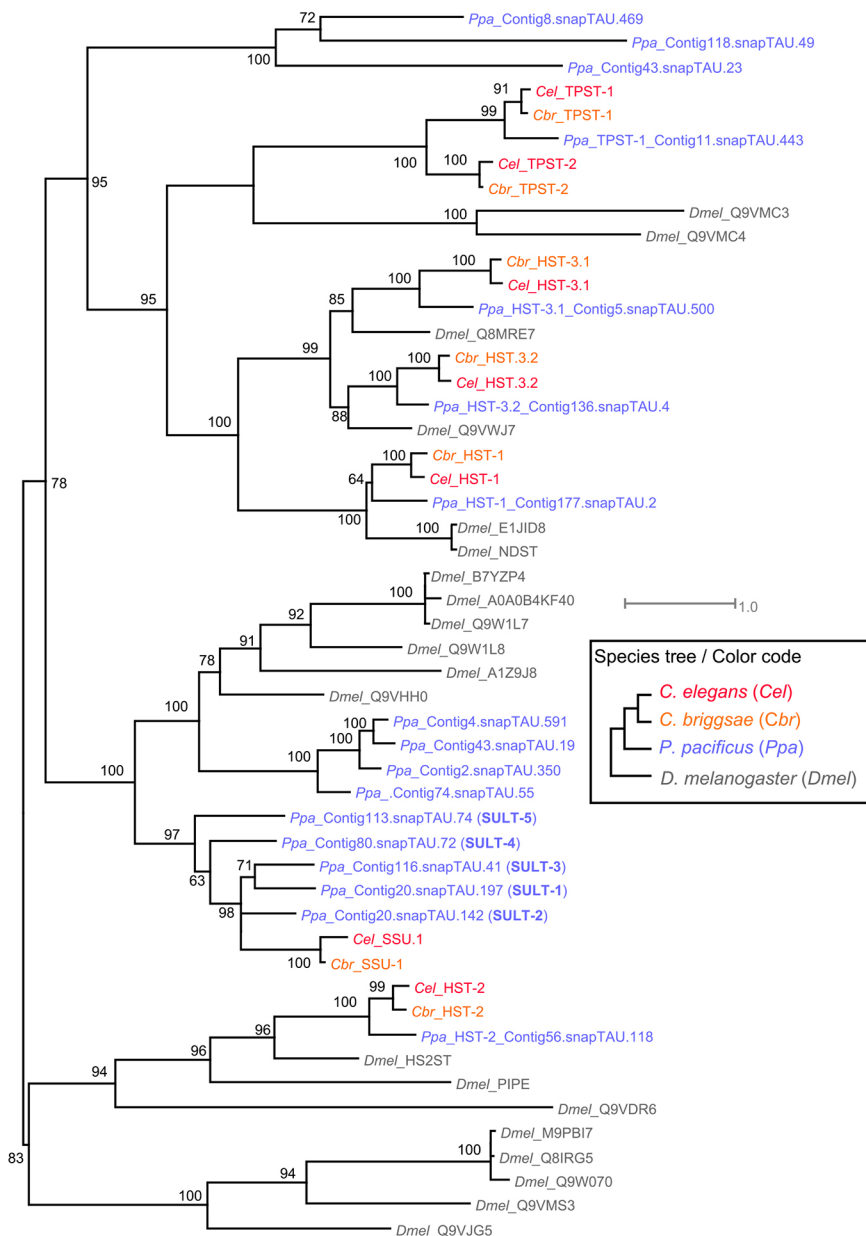
72
*Ppa*_Contig8.snapTAU.469
*Ppa*_Contig118.snapTAU.49
100
*Ppa*_Contig43.snapTAU.23
91 *Cel*_TPST-1
99 *Cbr*_TPST-1
*Ppa*_TPST-1_Contig11.snapTAU.443
100 100 *Cel*_TPST-2
*Cbr*_TPST-2
95
100 *Dmel*_Q9VMC3
*Dmel*_Q9VMC4
100 *Cbr*_HST-3.1
*Cel*_HST-3.1
100
*Ppa*_HST-3.1_Contig5.snapTAU.500
85
*Dmel*_Q8MRE7
95
100 *Cbr*_HST.3.2
100 *Cel*_HST.3.2
*Ppa*_HST-3.2_Contig136.snapTAU.4
99
88 *Dmel*_Q9VWJ7
100 *Cbr*_HST-1
64 *Cel*_HST-1
100
*Ppa*_HST-1_Contig177.snapTAU.2
100 100 *Dmel*_E1JID8
*Dmel*_NDST
100
*Dmel*_B7YZP4
100 *Dmel*_A0A0B4KF40
*Dmel*_Q9W1L7
92
*Dmel*_Q9W1L8
91
*Dmel*_A1Z9J8
78
*Dmel*_Q9VHH0
100
100 *Ppa*_Contig4.snapTAU.591
100 *Ppa*_Contig43.snapTAU.19
*Ppa*_Contig2.snapTAU.350
100
*Ppa*_.Contig74.snapTAU.55
100
*Ppa*_Contig113.snapTAU.74 (**SULT-5**)
97
*Ppa*_Contig80.snapTAU.72 (**SULT-4**)
71 *Ppa*_Contig116.snapTAU.41 (**SULT-3**)
63 *Ppa*_Contig20.snapTAU.197 (**SULT-1**)
98 *Ppa*_Contig20.snapTAU.142 (**SULT-2**)
*Cel*_SSU.1
100 *Cbr*_SSU-1
99 *Cel*_HST-2
100 *Cbr*_HST-2
96 *Ppa*_HST-2_Contig56.snapTAU.118
96
*Dmel*_HS2ST
94
*Dmel*_PIPE
*Dmel*_Q9VDR6
78
83
*Dmel*_M9PBI7
100 *Dmel*_Q8IRG5
94 *Dmel*_Q9W070
100
*Dmel*_Q9VMS3
*Dmel*_Q9VJG5

1.0

Species tree / Color code
C. elegans (*Cel*)
C. briggsae (*Cbr*)
P. pacificus (*Ppa*)
D. melanogaster (*Dmel*)

Fig. 3. *P. pacificus* has an expansion of cytosolic sulfotransferases. The tree shows the phylogenetic relationships between sulfotransferase genes in *C. elegans*, *C. briggsae*, *P. pacificus* and *D. melanogaster*. Although the number of sulfotransferases in *Caenorhabditis* nematodes remained rather constant, the *P. pacificus* lineage showed multiple independent expansions.

In contrast, *sult-1(tu1061);eud-1(tu455)* double mutants have significantly higher percentages of Eu animals when compared with untreated animals (Fig. 5F).

Ongoing experiments in our laboratory had shown that when nematodes are grown on bacteria isolated from the wild instead of on *E. coli* OP50, the ratio of the two mouth forms in the population does change. For example, the bacterial isolate *Pseudomonas* sp. LRB26 increases the ratio of Eu animals, such that in the case of wild-type animals, no St forms have ever been seen on *Pseudomonas* sp. LRB26 (Fig. 5F). Therefore, we tested the *sult-1(tu1061);eud-1(tu455)* double mutant on these bacteria and found a significant increase in the percentage of Eu worms, whereas *eud-1(tu455)* single mutants are unaffected at 0% Eu (Fig. 4F). Finally, similar patterns were observed when *sult-1(tu1061); eud-1(tu455)* worms were grown for 10 days under starvation conditions on agar plates, another condition known to increase the ratio of Eu worms (Serobyan et al., 2013). In all the mentioned Eu form-inducing conditions, *eud-1(tu455)* single mutant worms

remained St, whereas *sult-1(tu1061);eud-1(tu455)* double mutants formed condition-specific ratios of Eu animals. Although the epistasis tests are likely influenced by the fact that the available *sult-1* mutant represents a reduction-of-function allele, these results suggest first, that *sult-1* is partially epistatic over *eud-1*, and second, that *sult-1(tu1061);eud-1(tu455)* double mutants are sensitized to environmental factors. This finding would be compatible with several hypotheses of the molecular interactions of SULT-1 and EUD-1, including one in which both proteins act in different cellular contexts. Therefore, we subsequently tested where *sult-1* is expressed in *P. pacificus*.

sult-1 and *eud-1* exhibit distinct expression profiles
To examine the spatiotemporal expression pattern of *sult-1*, we created two reporter lines, *tuEx282* and *tuEx283*, carrying an Ex[*sult-1*::Venus] construct in a wild-type background, each containing a 9 kb upstream fragment of *sult-1* fused to Venus fluorescent protein. We then compared the expression of *sult-1* with
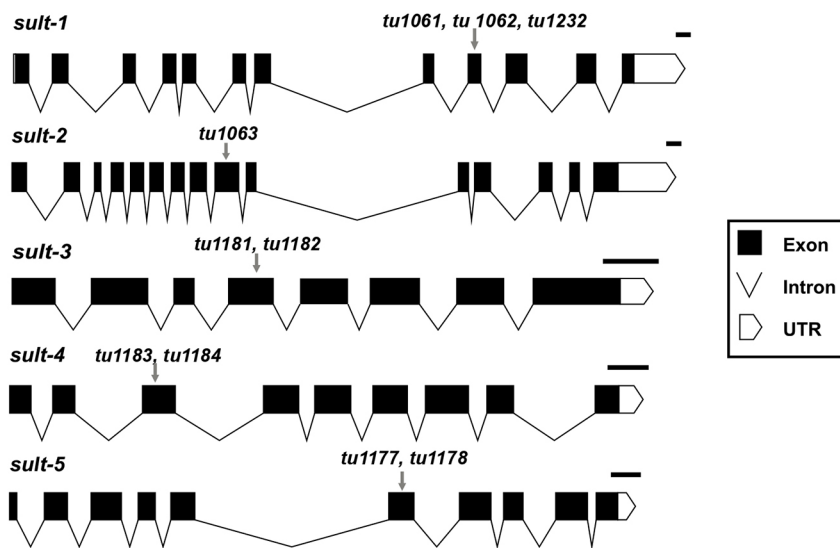
Fig. 4. CRISPR/Cas9-mediated knockout of cytosolic sulfotransferases: gene structures of sulfotransferases that were selected for knockout using CRISPR/Cas9 technique. The arrow indicates the mutated site. Scale bars: 100 bp. Black boxes represent exons, lines are introns and empty boxes are UTRs.

that of *eud-1*, which is expressed in several head neurons (Werner et al., 2017; Serobyan et al., 2016). Both reporter lines of *sult-1* resulted in similar expression patterns. Surprisingly, we found that *sult-1* and *eud-1* are expressed in different cells throughout the development of the worm. We confirmed the earlier reported expression of *eud-1* in sensory neurons, more specifically in amphid neurons (Fig. 6A). Surprisingly, however, we found that *sult-1* is expressed in pharyngeal muscle cells. Specifically, *sult-1* is expressed in cells homologous to *C. elegans* pm1, pm2 and pm3 (Fig. 6B). *sult-1* expression in pharyngeal muscle cells is seen throughout development, from the early juvenile stage (J2), and remains visible in adults. Thus, *sult-1* and *eud-1* are not co-expressed, which makes it unlikely that they compete for common target compounds. Instead, these findings indicate that *sult-1* expression in pharyngeal muscle cells might be involved in the execution of mouth formation and of the mouth structure itself. This would suggest that the mouth form-related function of *sult-1* is genetically downstream of or in parallel to *eud-1*.

sult-1 is downregulated in nhr-40 mutant animals

The experiments described above revealed that (1) bisphenol A decreases the frequency of Eu hermaphrodites on agar plates, (2) *sult-1* mutants are highly Eu in both sexes and under different culture conditions, and (3) *eud-1* and *sult-1* act on different cells. These findings, together with our previous characterization of *nhr-40* as a suppressor of *eud-1*, and studies in humans and rodents

indicating that sulfotransferases are regulated by nuclear hormone receptors (Kodama and Negishi, 2015), are consistent with a model in which *sult-1* is a transcriptional target of NHR-40. Consistent with this model, *sult-1(tu1061)* and *nhr-40(tu505)* mutants have similar Eu phenotypes in *P. pacificus*.

To determine whether *nhr-40* can affect *sult-1* at the transcriptional level, we examined *sult-1* transcription in the *nhr-40(tu505)* mutant (Fig. 7A). Indeed, we observed that *sult-1* is significantly downregulated in *nhr-40(tu505)* mutant animals relative to wild type. Thus, *sult-1* might act downstream of *nhr-40*, further supporting an independent function of SULT-1 and EUD-1. In a most parsimonious model, *eud-1*, *nhr-40* and *sult-1* are part of a linear genetic pathway, in which *eud-1* inhibits *nhr-40*, which acts as a transcriptional activator of *sult-1*. This model for *Ppa*-NHR-40 function would show resemblance to the related human receptor HNF4α, which activates the transcription of the cytosolic sulfotransferase SULT1E1 (Kodama et al., 2011). However, the role of bisphenol A would remain unclear in this model, because bisphenol A is known from other systems to be inactivated by sulfotransferases, and acts as xenobiotic ligand of mammalian nuclear hormone receptors, such as the estrogen receptor during breast cancer formation (Kodama and Negishi, 2015; Sui et al., 2012; Xu et al., 2017).

sult-1 and nhr-40 mutations abolish the effect of bisphenol A on mouth-form plasticity

Given the known biochemical interactions of bisphenol A as targets of sulfotransferases and xenobiotic ligands of nuclear-hormone-receptors in mammals, we wanted to identify the interaction of bisphenol A with the mouth-form regulatory machinery in *P. pacificus*. To this end, we performed pharmacological assays with wild-type and mutant animals. If a sulfotransferase is involved in the inactivation and detoxification of bisphenol A, mutations in the corresponding gene should increase the effect of bisphenol A because it can no longer be inactivated. On the contrary, if bisphenol A acts upstream of a given factor, mutants for the corresponding genes would be unaffected by bisphenol A. Using bisphenol A in assays on agar plates as described above, we found that mutants in *sult-3*, *sult-4* and *sult-5* showed frequencies of St animals similar to wild type, whereas *sult-2(tu1063)* mutant animals showed an even greater increase of St frequencies relative to wild type (Fig. 7B). In contrast, *sult-1(tu1061)* mutant animals remained completely Eu after bisphenol A treatment, indicating that bisphenol A acts through

Table 1. Mutant alleles of cytosolic sulfotransferases in P. pacificus

Gene	Allele	Mutation	Type of mutation
sult-1	tu1061	10 bp deletion	frame-shift
sult-1	tu1232	4 bp deletion	frame-shift
sult-1	tu1062	3 bp insertion	in frame
sult-2	tu1063	8 bp deletion	nonsense
sult-3	tu1181	31 bp deletion	nonsense
sult-3	tu1182	11 bp deletion	nonsense
sult-4	tu1183	43 bp insertion+6 bp deletion	nonsense
sult-4	tu1184	5 bp deletion+1 bp insertion	nonsense
sult-5	tu1177	8 bp deletion	nonsense
sult-5	tu1178	7 bp deletion	nonsense

All the mutant alleles generated using the CRISPR/Cas9 system are shown. All alleles are in wild-type (RS2333) background. The length of the genetic lesion and type of mutation for each allele are indicated.
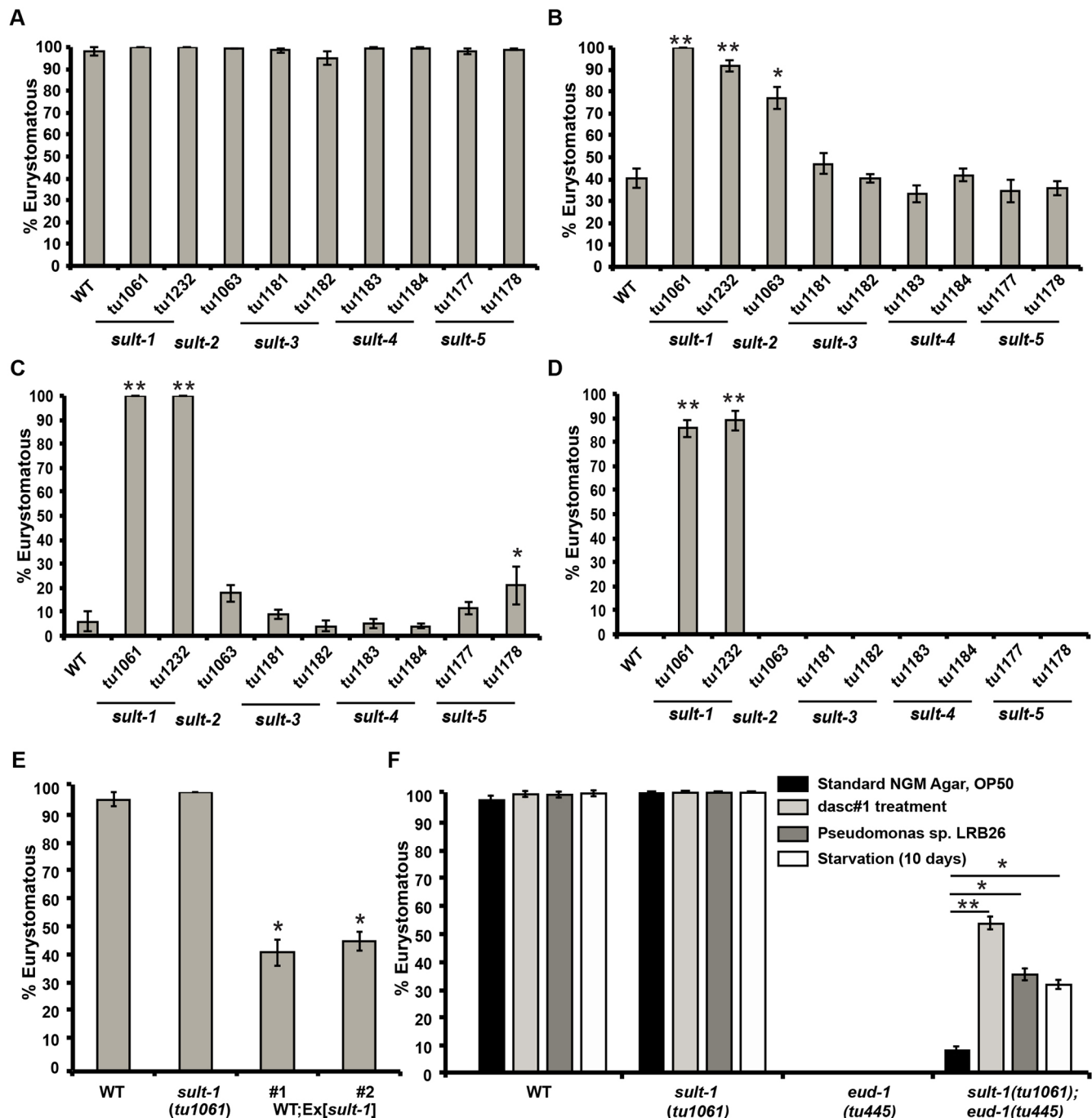
DEVELOPMENT

Fig. 5. *sult-1* is important for mouth-form determination. (A) Mouth-form ratios presented as % Eu for hermaphrodites of wild type (RS2333) and sulfotransferase mutants in agar (solid medium) culture. The total number of animals examined in three biological replicates (each with at least 100 worms) is greater than 300. (B) Mouth-form ratios for males of RS2333 and sulfotransferase mutants in agar (solid medium) culture. The total number of animals examined in three biological replicates (each with at least 50 worms) is greater than 150. (C) Mouth-form ratios for hermaphrodites of wild type and sulfotransferase mutants in liquid culture condition. The total number of animals examined in three biological replicates (each with at least 60 worms) is greater than 200. (D) Mouth-form ratios for males in liquid culture condition. The total number of animals examined in three biological replicates (each with at least 30 worms) is greater than 100. (E) Mouth-form ratios of wild type, *sult-1* (*tu1061*) and *sult-1* overexpression lines Ex[*sult-1*] #1 (*tuEx266*) and #2 (*tuEx281*). The total number of animals examined in three biological replicates (each with at least 50 worms) is greater than 200. (F) Mouth-form ratios of wild type, *sult-1*, *eud-1* and *sult-1;eud-1* in Eu form-inducing conditions: dasc#1 treatment, starvation and worm culture on *Pseudomonas* sp. LRB26. The total number of animals examined in three biological replicates (each with at least 50 worms) is greater than 200 for each condition. Error bars represent standard deviation. *$P<0.05$, **$P<0.01$, two-tailed Student's *t*-test with respect to wild-type values, except in F, where it is with respect to % Eu form of the double mutant in standard NGM agar condition.

SULT-1 (Fig. 7B). This finding suggests that bisphenol A is not a substrate of SULT-1 for mouth-form regulation, which is contrary to our starting assumption and the findings for *Cel-ssu-1* (Hattori et al.,

2006). Interestingly, bisphenol A has also no effect on mouth morphology in *nhr-40* mutant animals, which remained all Eu (Fig. 6B). Together, these results suggest that bisphenol A is not a
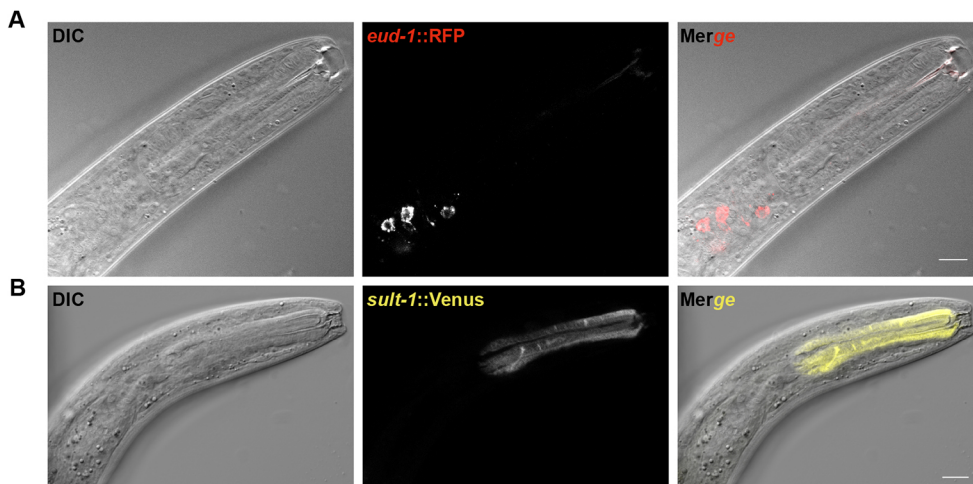
Fig. 6. *sult-1* and *eud-1* exhibit distinct expression profiles. (A,B) Representative images of expression pattern of *eud-1* in *eud1*::RFP reporter line (*tuEx177*) (A), expression pattern of *sult-1* in *sult-1*::Venus reporter line (*tuEx282*) (B). More than 15 animals of different stages were observed and imaged for A and B. Scale bars: 10 μm.

target of SULT-1, and that it acts upstream of, or at the level of, NHR-40 itself. Thus, bisphenol A might act as a xenobiotic ligand of NHR-40 during *P. pacificus* mouth-form regulation.

DISCUSSION

We have identified the influence of bisphenol A and other phenolic compounds on mouth-form plasticity in *P. pacificus* and subsequently delineated the role of the sulfotransferase *sult-1* in mouth-form specification. Although the investigation of a potential role of sulfotransferases in mouth-form regulation was initiated under the assumption that a SULT enzyme is required for the inactivation of bisphenol A, the subsequent experiments involving the characterization of the *sult-1* mutant and the treatment of various mutants with bisphenol A resulted in several unexpected findings. Together, our work highlights the importance of sulfation processes for the regulation of developmental processes independent of disease contexts and results in three major conclusions.

First, pharmacological screens remain a powerful tool to investigate biological processes such as phenotypic plasticity. With regard to mouth-form plasticity in *P. pacificus*, the effects of bisphenol A, tyramine and dopamine extend the number and type of bioactive compounds involved in mouth-form regulation. Previous pharmacological and genetic analyses had indicated that bioactive compounds are involved in regulating mouth-form plasticity at various levels of the molecular network. While the hormone dafachronic acid and the small molecule dasc#1 were shown to act genetically upstream of *eud-1*, sulfate and phosphate molecules most likely directly inhibit the sulfatase EUD-1, similar to effects described for vertebrate sulfatases (Glössl et al., 1979; Ragsdale et al., 2013). The effects of bisphenol A and tyramine resulted in the identification of the sulfotransferase *sult-1* and its role in mouth-form regulation. It should be noted, however, that the effects of dopamine might rely on a different mechanism. Although dopamine is known as a substrate of sulfotransferases (Yasuda et al., 2007), it is also a known neurotransmitter, and as such it might be involved in neuronal environmental perception. Interestingly, recent studies have shown that in *P. pacificus* animals, serotonin, but not dopamine, is involved in predatory feeding, the physiological and behavioral consequence of mouth-form plasticity (Wilecki et al., 2015; Okumura et al., 2017). Thus, neurotransmitters generated by related enzymatic pathways, such as serotonin and dopamine, have distinct functions in the context of mouth-form plasticity and predation.

Second, our analysis of sulfotransferases indicated that *P. pacificus* has undergone a massive expansion of cytosolic sulfotransferases relative to *C. elegans*. By the use of the CRISPR/Cas9 technology we have identified *sult-1* to be involved in mouth-form regulation, demonstrating how lineage-specific duplications can generate genes that can be incorporated into networks regulating novel phenotypes. *sult-1* mutants are highly Eu under most tested conditions. Only liquid culture conditions in males did not result in all-Eu *sult-1* mutant animals, but still in very high Eu frequencies (85% in *tu1061* and 89% in *tu1232*). This male trait could either be caused by partial redundancy with other sulfotransferases under certain growth conditions, or the incomplete penetrance of the reduction-of-function alleles available for *sult-1*. Thus, the *sult-1* gene encodes a sulfotransferase regulating mouth-form plasticity. This finding highlights the role of sulfation-desulfation for *P. pacificus* plasticity.

It should be noted that *sult-2* mutants also showed altered mouth-form ratios relative to wild type; however, with much smaller effects than *sult-1*. On the one hand, *sult-2* mutant males are highly Eu on agar plate. On the other hand, the effect of bisphenol A on mouth-form regulation is enhanced in *sult-2* mutants, resulting in less than 10% of animals being Eu (Figs 5 and 7). These observations would be consistent with a role of SULT-2 in the sulfation and inactivation of bisphenol A. However, given the fact that *sult-2* mutants did not show any effect in other test conditions, i.e. growth of hermaphrodites and particularly males in liquid culture, the specific role of this sulfotransferase might simply be minor.

In contrast to our original assumption, we did not obtain any evidence for EUD-1 and SULT-1 forming a sulfation module that acts in the same tissues or cells. First, *sult-1* is expressed in pharyngeal muscles cells, whereas *eud-1* is expressed in sensory neurons. Second, epistasis analysis indicates that while *sult-1;eud-1* double mutants are largely St under agar growth conditions, other conditions cause double mutants to adopt more Eu mouth forms. Such a pattern was never seen in *eud-1* single mutants. Together, these observations result in the third major conclusion of our study, suggesting that two independent sulfation processes regulate mouth-form plasticity. In general, *P. pacificus* mouth-form regulation represents a rare example of sulfation processes in normal developmental processes in invertebrates. Although sulfation processes are commonly seen in disease contexts, such as breast cancer (Martinez et al., 2013; Jamil et al., 2017), little is known about the role of sulfation in organismal development. Many studies in invertebrate model systems such as *D. melanogaster* and
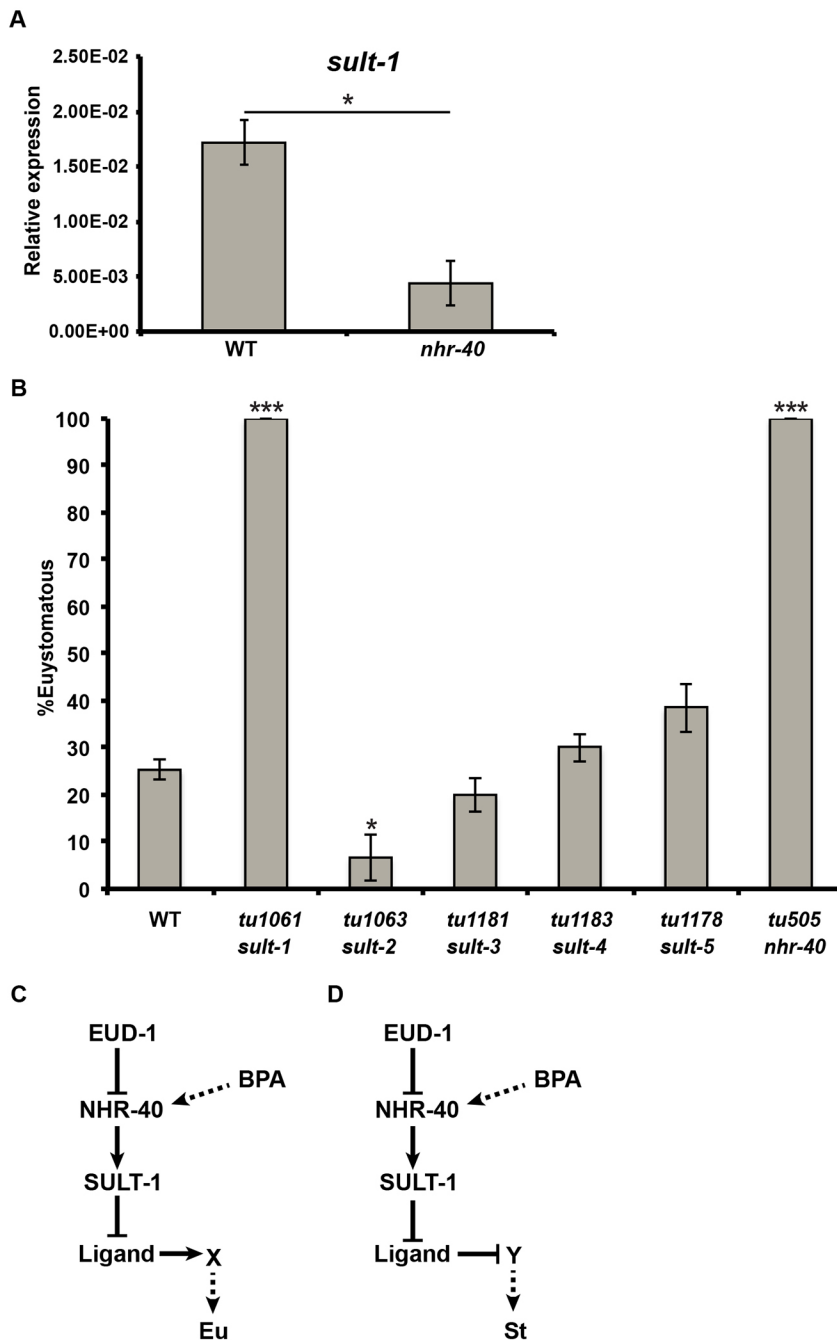
Fig. 7. Bisphenol A acts through NHR-40 to influence mouth morphology. (A) Expression levels of *sult-1* in *nhr-40* mutant line. (B) Bisphenol A treatment was performed on wild type (RS2333), sulfotransferase mutants and *nhr-40* animals. Mouth-form ratios presented as % Eu. The total number of animals phenotyped is greater than 200. Three biological replicates were performed, each having at least 50 animals. (C,D) Representations of the two most parsimonious models, showing the genetic factors involved in mouth morph determination in *P. pacificus*. *P<0.05, ***P<0.001, two-tailed Student's *t*-test with respect to wild-type values.

C. elegans provided examples for the role of phosphorylation in development with strong phenotypes of mutants in specific kinases and phosphatases (Zielinska et al., 2009; Chen and Jiang, 2013). In contrast, enzymes regulating sulfation have rarely been found in model system approaches (Lin et al., 1999). This is surprising because sulfation is a common modification in biological systems and, in principle, the inactivation of bioactive compounds through sulfation would be an obvious target for regulatory processes in development and physiology.

Many mechanisms have been identified to control developmentally plastic traits in different organisms. For example, in *C. elegans*, sensing of the environment using the calcium-dependent kinase CMK-1 can regulate DAF-7/TGF-β and insulin-like protein DAF-28/ILP, which control dauer entry (Ren et al., 1996; Neal et al., 2015). In the brown planthopper *Nilaparvata lunges*, the insulin receptors are linked to a developmental plastic decision between short-winged and long-winged morphs (Xu et al., 2015). This study, in combination with the earlier studies on sulfatase, EUD-1, establishes sulfation of biomolecules as a mechanism that can regulate developmentally plastic traits.

Future studies will address the targets of SULT-1 during the regulation of mouth-form plasticity in *P. pacificus*. Currently, the available genetic data and the *sult-1* expression in pharyngeal muscles cells place *sult-1* downstream of, or in parallel to, other mouth-form regulators. Therefore, we speculate that SULT-1 targets a bioactive compound that interacts with a downstream or parallel regulator of mouth-form plasticity acting in the pharyngeal muscle cells. Given the known roles of sulfotransferases in the inactivation of xenobiotic or endobiotic bioactive compounds, two different models are similarly possible given the current data. SULT-1 either

DEVELOPMENT

9

inhibits or inactivates a ligand of a mouth-form regulator X that promotes the Eu mouth form (Fig. 7C). In the absence of *sult-1*, this ligand is overactivated resulting in all-Eu mouth forms. Alternatively, *sult-1* might control a mouth-form regulator Y that promotes St development by inhibiting or deactivating the ligand of factor Y (Fig. 7D). Although the distinction between these two models is currently impossible, the identification of the molecular nature of the factor(s) acting downstream of *sult-1* might be elucidated, given the powerful genetic tools available in *P. pacificus*. Thus, this nematode model organisms and its unusual example of phenotypic plasticity represent a promising system to investigate sulfation processes in invertebrate model organisms.

In summary, our findings add an additional layer of regulation of mouth-form plasticity that points towards the role of a second sulfation process. Most importantly, *sult-1* is the first gene to be identified in mouth-form regulation that acts in the pharyngeal muscle cells. This is a striking finding because the pharyngeal muscles cells pm1, pm2 and pm3 are known to be involved in the secretion of the extracellular matrix that eventually forms the teeth-like structures in *P. pacificus* and other diplogastrid nematodes (Baldwin et al., 1997; Jay Burr and Baldwin, 2016). Thus, mouth-form regulation involves multiple regulatory levels and cell types, from neurons involved in environmental perception to pharyngeal muscle cells involved in mouth-form specification.

MATERIALS AND METHODS
Culture conditions
For culturing worms on agar plates, all *P. pacificus* strains, including the wild-type strain RS2333, all mutants generated in this study and transgenic worms were grown on 6 cm plates containing NGM agar. Worms were fed on a 300 μl bacterial lawn containing *E. coli* OP50 strain grown in LB broth. All cultures were maintained at 20°C.

For culturing worms in liquid we used S medium, a standard protocol to obtain high frequency of St animals as reported earlier (Werner et al., 2017). In short, for each sample, three agar plates in which worms had eaten all the OP50 on plates were washed with M9 buffer into 15 ml conical tubes. We added bleach (5 M NaOH in a 2:1 ratio) to a final volume of 30%. This mix was left on a rotor for 9 min with gentle rotation at room temperature. Carcasses were filtered through a 120 μm nylon net (Millipore) fixed between two rubber gaskets in a plastic funnel, washed by applying 3 ml M9 drop-wise on the filter, then pelleted at 500 g for 1 min at room temperature. Remaining eggs-J1 larvae were washed again with 3 ml M9 and centrifuged at 500 g for 1 min at room temperature. The pelleted eggs-J1 larvae were then suspended in 50 ml volume autoclaved Erlenmeyer flasks containing 10 ml S medium. To this bacterial pellet (centrifuged at 3000 g for 30 min at 4°C), an empirically determined amount of 100 ml, grown overnight on OP50 in LB medium (at an optimal density at 600 nm of 0.5), was added. Nystatin (20 μg/ml, final concentration) was added to prevent fungal contamination. Liquid cultures were incubated at 20°C and shaken at 180 rpm (INFORS HT Multitron standard) for 4 days.

Because mouth-form ratios are influenced by several environmental conditions (Bose et al., 2012; Werner et al., 2017), all experiments included their own wild-type control for Eu frequency.

Phenotypic scoring
Mouth-form phenotypes were scored in agar cultures using a method described earlier (Bento et al., 2010). For liquid culture, worms were phenotyped for mouth form by filtering using a 20 μm filter. Adults were then gently pelleted and transferred to 4% agar pads (containing 10 mM sodium azide) with 5-8 μl M9 and observed under a differential interference contrast (DIC) microscope (Zeiss Axioskop) at 40-100× magnifications. Discrete characters were used to discriminate between Eu and St individuals: the presence versus absence of a subventral tooth, and a claw-like versus flint-like or triangular dorsal tooth, respectively, which were together sufficient to distinguish the two forms.

Pharmacological screening
To test the effect of different bioactive compounds on mouth morphology, pharmacological screening was performed on RS2333 and RSC019 worms and mutants of sulfotransferases and *nhr-40*. All compounds tested and described in Fig. 2 were separately dissolved in either ethanol or water and thereafter mixed with melted NGM agar to bring chemicals to a final concentration of 10 μM. Control treatments consisted of agar mixed with the corresponding volumes of ethanol or water, and they did not show any significant difference compared with non-treated worms. Six-centimeter plates containing 10 ml agar were seeded with 300 μl OP50 and 10 μM of the test chemical, and were then incubated overnight at room temperature to allow bacterial growth. Three J3-J4 hermaphrodites were picked to each plate from the same well-fed source plate. Plates were kept at 20°C for 1 week. Adult animals in the next generation were screened for the mouth-form phenotype. Experiments were conducted in at least three replicates for each treatment type.

Phylogenetic analysis
Sulfotransferase domains (type I PF00685 and type II PF03567) of *C. elegans*, *C. briggsae* and *P. pacificus* were identified using the hmmsearch program of the HMMER package (version 3.1b2, e-value <0.001). *D. melanogaster* sequences were taken from the sequence alignments of the corresponding Pfam profiles. Manual curation, multiple sequence alignment and tree reconstruction were performed as previously described (Baskaran et al., 2015). The final tree (Fig. 3) represents a maximum-likelihood tree under the LG substitution model, with a correction for invariant sites and four rate classes that follow a gamma distribution. The robustness of internal nodes was measured as the number of 100 bootstrap replicates that would support a given topology.

Generation of CRISPR-induced mutants for sulfotransferases
We generated mutant alleles for the five closest paralogs of the *C. elegans* cytosolic sulfotransferase *ssu-1* using the CRISPR/Cas9 technique following the protocol described previously (Witte et al., 2014). The gene structures are based on earlier published RNA sequencing results and gene annotation (Ragsdale et al., 2013; Rödelsperger et al., 2017). Single guide RNAs (sgRNAs) for five sulfotransferases were ordered from Integrated DNA Technologies (see Table S1 for sequences). For inducing mutations, sgRNAs were co-injected with Cas9 nuclease (M0386M, NEB). Heterozygous mutant carriers were identified and singled out by analyzing high-resolution melting curves using a quantitative PCR system (Lightcycler 480 II, Roche) with separate primers for each gene (Table S2).

Conditional epistasis of *sult-1* and *eud-1*
Worms were first treated with the pheromone dasc#1 using a method described previously (Bose et al., 2012). In short, dasc#1 was first dissolved in ethanol and then added to 10 ml NGM to obtain 1 μM final concentration. The melted NGM was added to 6 cm plates, which were then seeded with 300 μl OP50 culture in LB medium. Control treatments consisted of agar mixed with the corresponding volumes of ethanol. These plates were incubated overnight at 20°C and the next day, three J4 hermaphrodites were picked to each plate from the same well-fed source plate. Adult offspring of these worms were phenotyped in 1 week for mouth morphology. Experiments were conducted in at least three replicates.

Pseudomonas sp. LRB26 was isolated from the scarab beetle *Oryctes borbonicus* found in La Réunion Island. To examine the effect of growth on *Pseudomonas* sp. LRB26, we seeded a 6 cm NGM plate with 300 μl *Pseudomonas* sp. LRB26 in LB medium. These plates were incubated overnight at 20°C and the next day, three J4 hermaphrodites were transferred to each plate from the same well-fed source plate. Offspring of these worms were phenotyped in 1 week for mouth-form morphology. Experiments were conducted in at least three replicates.

To examine the effects of starvation, we transferred three J4 hermaphrodites from a well-fed source plate to 6 cm NGM plates seeded with 300 μl OP50 in LB. Plates were kept at 20°C for 10 days. Adults were phenotyped for mouth morphology from these 10-days-starved plates. Experiments were conducted in three replicates.

DEVELOPMENT

Genetic transformation

Transgenic animals were generated as previously described (Schlager et al., 2009). To obtain overexpression lines for *sult-1*, germ lines of adult hermaphrodites were injected with a mix of genomic construct of *Ppa-sult-1* (10 ng/µl), the marker *Ppa-egl-20*::*TurboRFP* (10 ng/µl), and genomic carrier DNA (60 ng/µl) from the RS2333 strain. The *Ppa-sult-1* genomic construct had a 2.2 kb promoter for the generation of the first line Ex[*sult-1*]#1 (*tuEx266*) and an ∼8 kb promoter for the second Ex[*sult-1*]#2 (*tuEx281*). Transgenic animals were scored over multiple generations involving at least 200 transgenic animals per line.

To generate reporter lines for *sult-1*, cDNA of fluorescence protein Venus was transcriptionally fused to a ∼9 kb long *sult-1* promoter and 3′ UTR sequence of the gene *rpl-23* to create a 12 kb long *sult-1*::Venus construct. Fragments were then fused and amplified by overlapping extension PCR. All amplified fragments were verified by sequencing. The *sult-1*::Venus construct (10 ng/µl) was injected along with the co-injection marker *egl-20*::Venus (10 ng/µl), and genomic carrier DNA (60 ng/µl) from the wild-type strain. Two independent transgenic lines Ex[*sult-1*::Venus]#1 (*tuEx282*) and Ex[*sult-1*::Venus]#2 (*tuEx283*) were generated. For *eud-1*, a reporter line (*tuEx177*) with TurboRFP generated in an earlier study (Ragsdale et al., 2013) was used.

In all cases, we used the restriction enzymes *Pst*I (Thermo Fisher Scientific) for digestion of the respective construct and genomic host DNA. For both experiments, two independent transgenic lines were generated.

Quantitative reverse transcription PCR experiments

Mixed-stage worms were washed from at least five crowded plates and filtered using 20 µm nylon filter (Millipore) to collect J2-stage worms. Worms were pelleted (20,817 ***g*** for 1 min at room temperature) and re-suspended in Trizol. Total RNA was isolated using a PureLink (Invitrogen) RNA micro kit following the manufacturer's protocol. Reverse transcription was performed with 1 µg total RNA using superscript II reverse transcriptase (18064, Invitrogen) following the manufacturer's instructions. The quantitative reverse transcription PCR experiments were performed using SyberGreen I mastermix (Roche Diagnostics) following a previously described method (Schuster and Sommer, 2012), on a Roche Lightcycler 480 system. *cdc-42* and *β-tubulin* were used as reference genes to calculate ΔCt values. The sequences of the primers used are listed in Table S3. Expression levels were analyzed with advanced relative quantification on the Roche Lightcycler 480 system according to the manufacturer's instructions. At least three biological replicates were performed for each experiment.

Imaging

Image acquisition was performed on a Leica SP8 confocal system using settings to maximize the detection of fluorescent protein tags TurboRFP and Venus. At least 15 animals were imaged for each sample type. Image analysis was performed using Fiji (ImageJ) software (Schindelin et al., 2012).

Statistical analyses

All phenotypic data show percentage Eu frequency calculated from total individuals screened in three biological replicates. Total sample size is illustrated on graphs. Significant differences were tested by two-tailed Student's *t*-test.

Author contributions

Conceptualization: S.N., R.J.S.; Methodology: S.N., E.M., C.R., P.B., H.W.; Investigation: S.N., E.M.; Resources: C.R., P.B.; Data curation: C.R.; Writing - original draft: E.M., C.R., R.J.S.; Writing - review & editing: S.N., R.J.S.; Supervision: R.J.S.; Funding acquisition: R.J.S.

References

Baldwin, J. G., Giblin-Davis, R. M., Eddleman, C. D., Williams, D. S., Vida, J. T. and Thomas, W. K. (1997). The buccal capsule of Aduncospiculum halicti (Nemata: Diplogasterina): an ultrastructural and molecular phylogenetic study. *Can. J. Zool.* **75**, 407-423.

Baskaran, P., Rödelsperger, C., Prabh, N., Serobyan, V., Markov, G. V., Hirsekorn, A. and Dieterich, C. (2015). Ancient gene duplications have shaped developmental stage-specific expression in Pristionchus pacificus. *BMC Evol. Biol.* **15**, 185.

Bateson, P., Cartwright, N., Dupré, J., Laland, K. and Noble, D. (2017). New trends in evolutionary biology: biological, philosophical and social science perspectives. *Interface Focus* **7**, 20170051.

Beldade, P. and Brakefield, P. M. (2002). The genetics and evo-devo of butterfly wing patterns. *Nature* **3**, 442-452.

Bento, G., Ogawa, A. and Sommer, R. J. (2010). Co-option of the hormone-signalling module dafachronic acid–DAF-12 in nematode evolution. *Nature* **466**, 494-497.

Bose, N., Ogawa, A., Von Reuss, S. H., Yim, J. J., Ragsdale, E. J., Sommer, R. J. and Schroeder, F. C. (2012). Complex small-molecule architectures regulate phenotypic plasticity in a nematode. *Angew. Chemie Int. Ed.* **51**, 12438-12443.

Brix, L. A., Barnett, A. C., Duggleby, R. G., Leggett, B. and McManus, M. E. (1999). Analysis of the substrate specificity of human sulfotransferases SULT1A1 and SULT1A3: site-directed mutagenesis and kinetic studies. *Biochemistry* **38**, 10474-10479.

Carroll, B. T., Dubyak, G. R., Sedensky, M. M. and Morgan, P. G. (2006). Sulfated signal from ASJ sensory neurons modulates stomatin-dependent coordination in Caenorhabditis elegans. *J. Biol. Chem.* **281**, 35989-35996.

Chen, Y. and Jiang, J. (2013). Decoding the phosphorylation code in Hedgehog signal transduction. *Cell Res.* **23**, 186-200.

Corona, M., Libbrecht, R. and Wheeler, D. E. (2016). Molecular mechanisms of phenotypic plasticity in social insects. *Curr. Opin. Insect Sci.* **13**, 55-60.

Coughtrie, M. W. H., Sharp, S., Maxwell, K. and Innes, N. P. (1998). Biology and function of the reversible sulfation pathway catalysed by human sulfotransferases and sulfatases. *Chem. Biol. Interact.* **1**, 3-27.

Dieterich, C., Clifton, S. W., Schuster, L. N., Chinwalla, A., Delehaunty, K., Dinkelacker, I., Fulton, L., Fulton, R., Godfrey, J., Minx, P. et al. (2008). The Pristionchus pacificus genome provides a unique perspective on nematode lifestyle and parasitism. *Nat. Genet.* **40**, 1193-1198.

Ernst, U. R., Van Hiel, M. B., Depuydt, G., Boerjan, B., De Loof, A. and Schoofs, L. (2015). Epigenetics and locust life phase transitions. *J. Exp. Biol.* **218**, 88-99.

Fischman, B. J., Pitts-Singer, T. L. and Robinson, G. E. (2017). Nutritional regulation of phenotypic plasticity in a solitary bee (Hymenoptera: Megachilidae). *Environ. Entomol.* **46**, 1070-1079.

Glössl, J., Truppe, W. and Kresse, H. (1979). Purification and properties of N-acetylgalactosamine 6-sulphate sulphatase from human placenta. *Biochem. J.* **181**, 37-46.

Hanson, S. R., Best, M. D. and Wong, C. H. (2004). Sulfatases: structure, mechanism, biological activity, inhibition, and synthetic utility. *Angew. Chem. Int. Ed. Engl.* **43**, 5736-5763.

Hattori, K., Inoue, M., Inoue, T., Arai, H. and Tamura, H.-O. (2006). A novel sulfotransferase abundantly expressed in the dauer larvae of Caenorhabditis elegans. *J. Biochem.* **139**, 355-362.

Jamil, Q. U. A., Jaerapong, N., Zehl, M., Jarukamjorn, K. and Jäger, W. (2017). Metabolism of curcumin in human breast cancer cells: impact of sulfation on cytotoxicity. *Planta Med.* **83**, 1028-1034.

Jay Burr, A. H. and Baldwin, J. G. (2016). The nematode stoma: homology of cell architecture with improved understanding by confocal microscopy of labeled cell boundaries. *J. Morphol.* **277**, 1168-1186.

Kauffman, F. C. (2004). Sulfonation in pharmacology and toxicology. *Drug Metab. Rev.* **36**, 823-843.

Kieninger, M. R., Ivers, N. A., Rödelsperger, C., Markov, G. V., Sommer, R. J. and Ragsdale, E. J. (2016). The nuclear hormone receptor NHR-40 acts downstream of the sulfatase EUD-1 as part of a developmental plasticity switch in pristionchus. *Curr. Biol.* **26**, 2174-2179.

Kodama, S. and Negishi, M. (2015). Sulfotransferase genes: regulation by nuclear receptors in response to xeno/endo-biotics. *Drug Metab. Rev.* **27**, 320-331.

Kodama, S., Hosseinpour, F., Goldstein, J. A. and Negishi, M. (2011). Liganded pregnane X receptor represses the human sulfotransferase SULT1E1 promoter through disrupting its chromatin structure. *Nucleic Acids Res.* **39**, 8392-8403.

Lightfoot, J. W., Wilecki, M., Okumura, M. and Sommer, R. J. (2016). Assaying predatory feeding behaviors in pristionchus and other nematodes. *J. Vis. Exp.* e54404.

Lin, X., Buff, E. M., Perrimon, N. and Michelson, A. M. (1999). Heparan sulfate proteoglycans are essential for FGF receptor signaling during Drosophila embryonic development. *Development* **126**, 3715-3723.

DEVELOPMENT

Martinez, P., Vergoten, G., Colomb, F., Bobowski, M., Steenackers, A., Carpentier, M., Allain, F., Delannoy, P. and Julien, S. (2013). Over-sulfated glycosaminoglycans are alternative selectin ligands: insights into molecular interactions and possible role in breast cancer metastasis. *Clin. Exp. Metastasis* **30**, 919-931.

Moczek, A. P. (1998). Horn polyphenism in the beetle Onthophagus taurus: larval diet quality and plasticity in parental investment determine adult body size and male horn morphology. *Behav. Ecol.* **9**, 636-641.

Moczek, A. P., Sultan, S., Foster, S., Ledon-Rettig, C., Dworkin, I., Nijhout, H. F., Abouheif, E. and Pfennig, D. W. (2011). The role of developmental plasticity in evolutionary innovation. *Proc. R. Soc. B Biol. Sci.* **278**, 2705-2713.

Neal, S. J., Takeishi, A., O'Donnell, M. P., Park, J. S., Hong, M., Butcher, R. A., Kim, K. and Sengupta, P. (2015). Feeding state-dependent regulation of developmental plasticity via CaMKI and neuroendocrine signaling. *Elife* **4**, e10110.

Negishi, M., Pedersen, L. G., Petrotchenko, E., Shevtsov, S., Gorokhov, A., Kakuta, Y. and Pedersen, L. C. (2001). Structure and function of sulfotransferases. *Arch. Biochem. Biophys.* **390**, 149-157.

Okumura, M., Wilecki, M. and Sommer, R. J. (2017). Serotonin drives predatory feeding behavior via synchronous feeding rhythms in the nematode pristionchus pacificus. *G3(Bethesda)* **7**, 3745-3755.

Pigliucci, M. (2001). *Phenotypic Plasticity: Beyond Nature and Nurture: Syntheses in Ecology and Evolution*. Baltimore, MD; The Johns Hopkins University Press.

Ragsdale, E. J., Müller, M. R., Rödelsperger, C. and Sommer, R. J. (2013). A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell* **155**, 922-933.

Ren, P., Lim, C.-S., Johnsen, R., Albert, P. S., Pilgrim, D. and Riddle, D. L. (1996). Control of C. elegans larval development by neuronal expression of a TGF-beta homolog. *Science* **274**, 1389-1391.

Rižner, T. L. (2016). The important roles of steroid sulfatase and sulfotransferases in gynecological diseases. *Front. Pharmacol.* **7**, 30.

Rödelsperger, C., Meyer, J. M., Prabh, N., Lanz, C., Bemm, F. and Sommer, R. J. (2017). Single-molecule sequencing reveals the chromosome-scale genomic architecture of the nematode model organism pristionchus pacificus. *Cell Rep.* **21**, 834-844.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B. et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676-682.

Schlager, B., Wang, X., Braach, G. and Sommer, R. J. (2009). Molecular cloning of a dominant roller mutant and establishment of DNA-mediated transformation in the nematode Pristionchus pacificus. *Genesis* **47**, 300-304.

Schuster, L. N. and Sommer, R. J. (2012). Expressional and functional variation of horizontally acquired cellulases in the nematode Pristionchus pacificus. *Gene* **506**, 274-282.

Serobyan, V., Ragsdale, E. J., Müller, M. R. and Sommer, R. J. (2013). Feeding plasticity in the nematode pristionchus pacificus is influenced by sex and social context and is linked to developmental speed. *Evol. Dev.* **15**, 161-170.

Serobyan, V., Ragsdale, E. J. and Sommer, R. J. (2014). Adaptive value of a predatory mouth-form in a dimorphic nematode. *Proc. R. Soc. B Biol. Sci.* **281**, 20141334-20141334.

Serobyan, V., Xiao, H., Namdeo, S., Rödelsperger, C., Sieriebriennikov, B., Witte, H., Röseler, W. and Sommer, R. J. (2016). Chromatin remodelling and antisense-mediated up-regulation of the developmental switch gene eud-1 control predatory feeding plasticity. *Nat. Commun.* **7**, 12337.

Sommer, R. and Carta, L. (1996). Morphological, genetic and molecular description of Pristionchus pacificus sp. n. (Nematoda:Neodiplogastridae). *Fundam. Appl. Nematol.* **19**, 511-521.

Sommer, R. J. and McGaughran, A. (2013). The nematode Pristionchus pacificus as a model system for integrative studies in evolutionary biology. *Mol. Ecol.* **22**, 2380-2393.

Sommer, R. J., Dardiry, M., Lenuzzi, M., Namdeo, S., Renahan, T., Sieriebriennikov, B. and Werner, M. S. (2017). The genetics of phenotypic plasticity in nematode feeding structures. *Open Biol.* **7**, 160332.

Strott, C. A. (2002). Sulfonation and molecular action. *Endocr. Rev.* **23**, 703-732.

Sui, Y., Ai, N., Park, S.-H., Rios-Pilier, J., Perkins, J. T., Welsh, W. J. and Zhou, C. (2012). Bisphenol A and its analogues activate human pregnane X receptor. *Environ. Health Perspect.* **120**, 399-405.

Susoy, V. and Sommer, R. J. (2016). Stochastic and conditional regulation of nematode mouth-form dimorphisms. *Front. Ecol. Evol.* **4**, 1-7.

Wang, X. and Kang, L. (2014). Molecular mechanisms of phase change in locusts. *Annu. Rev. Entomol.* **59**, 225-244.

Werner, M. S., Sieriebriennikov, B., Loschko, T., Namdeo, S., Lenuzzi, M., Dardiry, M., Renahan, T., Sharma, D. R. and Sommer, R. J. (2017). Environmental influence on Pristionchus pacificus mouth form through different culture methods. *Sci. Rep.* **7**, 7207.

West-Eberhard, M. J. (2003). Developmental plasticity and evolution. *Nature* **424**, 794.

Wilecki, M., Lightfoot, J. W., Susoy, V. and Sommer, R. J. (2015). Predatory feeding behaviour in Pristionchus nematodes is dependent on phenotypic plasticity and induced by serotonin. *J. Exp. Biol.* **218**, 1306-1313.

Witte, H., Moreno, E., Rödelsperger, C., Kim, J., Kim, J. S., Streit, A. and Sommer, R. J. (2014). Gene inactivation using the CRISPR/Cas9 systemin the nematode Pristionchus pacificus. *Dev. Genes Evol.* **225**, 55-62.

Xu, H.-J., Xue, J., Lu, B., Zhang, X.-C., Zhuo, J.-C., He, S.-F., Ma, X.-F., Jiang, Y.-Q., Fan, H.-W., Xu, J.-Y. et al. (2015). Two insulin receptors determine alternative wing morphs in planthoppers. *Nature* **519**, 464-467.

Xu, F., Wang, X., Wu, N., He, S., Yi, W., Xiang, S., Zhang, P., Xie, X. and Ying, C. (2017). Bisphenol A induces proliferative effects on both breast cancer cells and vascular endothelial cells through a shared GPER-dependent pathway in hypoxia. *Environ. Pollut.* **231**, 1609-1620.

Yasuda, S., Liu, M.-Y., Suiko, M., Sakakibara, Y. and Liu, M.-C. (2007). Hydroxylated serotonin and dopamine as substrates and inhibitors for human cytosolic SULT1A3. *J. Neurochem*. **103**, 2679-2689.

Zielinska, D. F., Gnad, F., Jedrusik-Bode, M., Wiśniewski, J. R. and Mann, M. (2009). Caenorhabditis elegans has a phosphoproteome atypical for metazoans that is enriched in developmental and sex determination proteins. *J. Proteome Res.* **8**, 4039-4049.

DEVELOPMENT

Chromatin remodelling and antisense-mediated up-regulation of the developmental switch gene *eud-1* control predatory feeding plasticity

Vahan Serobyan[1], Hua Xiao[1,†], Suryesh Namdeo[1], Christian Rödelsperger[1], Bogdan Sieriebriennikov[1], Hanh Witte[1], Waltraud Röseler[1] & Ralf J. Sommer[1]

Phenotypic plasticity has been suggested to act through developmental switches, but little is known about associated molecular mechanisms. In the nematode *Pristionchus pacificus*, the sulfatase *eud-1* was identified as part of a developmental switch controlling mouth-form plasticity governing a predatory versus bacteriovorous mouth-form decision. Here we show that mutations in the conserved histone-acetyltransferase *Ppa-lsy-12* and the methyl-binding-protein *Ppa-mbd-2* mimic the *eud-1* phenotype, resulting in the absence of one mouth-form. Mutations in both genes cause histone modification defects and reduced *eud-1* expression. Surprisingly, *Ppa-lsy-12* mutants also result in the down-regulation of an antisense-*eud-1* RNA. *eud-1* and antisense-*eud-1* are co-expressed and further experiments suggest that antisense-*eud-1* acts through *eud-1* itself. Indeed, overexpression of the antisense-*eud-1* RNA increases the *eud-1*-sensitive mouth-form and extends *eud-1* expression. In contrast, this effect is absent in *eud-1* mutants indicating that antisense-*eud-1* positively regulates *eud-1*. Thus, chromatin remodelling and antisense-mediated up-regulation of *eud-1* control feeding plasticity in *Pristionchus*.

[1] Department for Evolutionary Biology, Max-Planck-Institute for Developmental Biology, Spemannstrasse 35, 72076 Tübingen, Germany. † Present address: University of Missouri-Columbia, Division of Biological Sciences 103 Tucker Hall, Columbia, Missouri 65211 USA. Correspondence and requests for materials should be addressed to R.J.S. (email: ralf.sommer@tuebingen.mpg.de).

Developmental (phenotypic) plasticity has been suggested to facilitate morphological novelty and diversity[1–5], but little is known about the molecular aspects of developmental switch mechanisms that underlie plasticity. The nematode *Pristionchus pacificus* is a potential model system to study the molecular and mechanistic details of developmental plasticity because it can be easily cultured in the laboratory by feeding on bacteria, but in the wild it lives in a necromenic interaction with beetles[6,7]. Specifically, the necromenic life style of *P. pacificus* and related nematodes is facilitated by dynamic feeding mode switching between bacterial grazing and the predation of other nematodes (Fig. 1a,b; ref. 7). This feeding diversity relies on the presence of moveable teeth and *Pristionchus* nematodes exhibit two distinct morphs— stenostomatous (St, narrow-mouthed) or eurystomatous (Eu, wide-mouthed) —that differ in the number and shape of associated teeth and the size and form of the buccal cavity[8] (Fig. 1c,d). When fed on *Escherichia coli* OP50 bacteria under lab conditions, *P. pacificus* California reference strain RS2333 hermaphrodites have a stable 70:30% Eu:St ratio, but this can be influenced by starvation, crowding and pheromone signalling[8–10]. Because *P. pacificus* hermaphrodites reproduce primarily by selfing, strains are genetically homogeneous, and the presence of two distinct morphs thus represents an example of developmental plasticity, which was also demonstrated experimentally[8].

The existence of developmental switch mechanisms is essential for the irreversible control of plasticity and has long been anticipated by evolutionary theory[1], but associated mechanisms are largely unknown. We have recently identified the sulfatase *eud-1* as part of a genetic network that constitutes the developmental switch for the *P. pacificus* mouth-form decision[6]. In *eud-1* mutants, the Eu form is absent (*eud*, *eu*rystomatous-form-*d*efective), whereas overexpression from transgenes fixes the Eu form, thus confirming that EUD-1 acts as a developmental switch[6]. *eud-1* is X-linked and dosage-dependent, and it regulates differences in mouth-form frequency between hermaphrodites and males, among *P. pacificus* strains, and between *Pristionchus* species[6]. Interestingly, *P. pacificus eud-1* derives from a recent duplication that resulted in two neighbouring gene copies arranged in a head-to-head orientation (Fig. 1e). *eud-1* is expressed in a small number of *P. pacificus* head neurons, where its expression is sufficient to induce the execution of the Eu mouth-form[6]. However, while *eud-1* expression is highly regulated, the underlying mechanisms that control this developmental switch gene remain unknown.

Here we show that mutations in the conserved histone acetyltransferase *Ppa-lsy-12* and the methyl-binding-protein *Ppa-mbd-2* result in the absence of the Eu mouth-form similar to mutants in *Ppa-eud-1*. Mutations in both genes cause histone modification defects that result among others, in reduced *eud-1* expression. In addition, in *Ppa-lsy-12* mutants an antisense-*eud-1* RNA is also down-regulated. Overexpression of the antisense-*eud-1* RNA from transgenes increases the *eud-1*-sensitive mouth-form and results in increased *eud-1* expression. In contrast, this effect is absent in *eud-1* mutants indicating that antisense-*eud-1* positively regulates *eud-1*. These epigenetic mechanisms provide a theoretical framework for linking genetic regulation to environmental input.

Results

Two pleiotropic mutants with mouth-form defects.
To study the regulation of *eud-1*, we searched for pleiotropic mutants with a Eud phenotype in hermaphrodites, similar to *eud-1*. By screening more than 30 already-established mutant strains with egg laying- or vulva defects, we identified two mutants, *tu319*

and *tu365*, with a nearly complete loss of the Eu form (Fig. 2a). While *tu319* was previously molecularly uncharacterized, *tu365* represents a deletion allele in the methyl-binding protein family member *Ppa-mbd-2* (ref. 11). *Ppa-mbd-2(tu365)* is recessive, homozygous viable, and displays both a fully penetrant egg-laying defect and a complete absence of the Eu mouth-form (Fig. 2a). *Ppa-mbd-2(tu365)* contains a 1.7 kb deletion that removes four of six exons, suggesting that the absence of the Eu form results from a strong reduction-of-function or even null mutation in this gene. Thus, the phenotype of *mbd-2* in *P. pacificus* reveals the existence of pleiotropic regulators of mouth-form plasticity.

A conserved histone-acetyltransferase regulates plasticity.
In *tu319* mutants, only 2% of hermaphrodites have a Eu mouth-form (Fig. 2a). *tu319* was isolated in a screen for vulva-defective mutants and represents one of three alleles of the previously genetically characterized *vul-2* (*vul*valess) locus[12]. Interestingly, only *tu319* but not the two other alleles, *tu18* and *tu30*, show mouth-form defects indicating that the effect of *vul-2* on mouth-form regulation is allele-specific. We mapped *tu319* to the tip of chromosome IV in proximity to the marker S210 (Supplementary Fig. 1a). Sequencing of fosmid clones of this gene poor region resulted in the identification of a histone-acetyltransferase orthologous to the *Caenorhabditis elegans* gene *lsy-12* (Supplementary Fig. 1b,c; ref. 13. Sequencing of *lsy-12* identified mutations in all three alleles; *tu319* and *tu30* have splice-site mutations, whereas *tu18* contains a 598 bp deletion in the putative 3′-end of the gene (Supplementary Fig. 1b). *Ppa-lsy-12* is a complex gene with more than 30 predicted exons, and rapid amplification of cloned/cDNA ends (RACE) and RNA seq experiments provide strong evidence for extensive alternative splicing (Supplementary Fig. 1b). *Ppa-lsy-12* has a typical MYST domain in the 5′ part of the gene encoded by exons 5–13 (Supplementary Fig. 1b), which is present in the majority of transcripts. Interestingly, *tu319* affects the splice site of exon 7, whereas the two other alleles affect the 3′ part of the gene, which is not associated with known protein domains and is not present in the majority of transcripts.

To attempt phenotypic rescue, we generated a construct of *Ppa-lsy-12* containing exons 1–20 (see Methods) and obtained three independently transformed lines carrying the *Ppa-lsy-12* construct alongside an *egl-20::rfp* (red fluorescent protein) reporter. All three transgenic lines rescued both the vulvaless defect and the mouth-form defect of *tu319* (Supplementary Fig. 1c,d). Specifically, in transgenic animals the mouth-form was 71% Eu (versus 2% Eu in *tu319* worms) and, in one line studied in greater detail, 90% of the vulva precursor cells were induced to form vulval tissue (versus 33% in *tu319* worms). These results indicate that *vul-2* is indeed identical to *Ppa-lsy-12* and we renamed the gene accordingly. Taken together, two evolutionarily conserved genes, *Ppa-lsy-12* and *Ppa-mbd-2*, are pleiotropic regulators of mouth-form plasticity and mutations in these genes result in a strong reduction or absence of the Eu mouth-form.

mbd-2 and *lsy-12* mutants cause histone modification defects.
The molecular nature of *Ppa-lsy-12* suggests that chromatin remodelling may control the developmental switch mechanism that underlies the *P. pacificus* mouth dimorphism. Chromatin remodelling proteins regulate numerous developmental processes[14], but nothing is known of a potential role for chromatin remodelling in the regulation of developmental plasticity. Therefore, we first asked if histone modifications are indeed altered in *lsy-12* and *mbd-2* mutants. We isolated proteins from mixed stage cultures of wild-type, *mbd-2*, and *lsy-12* mutant
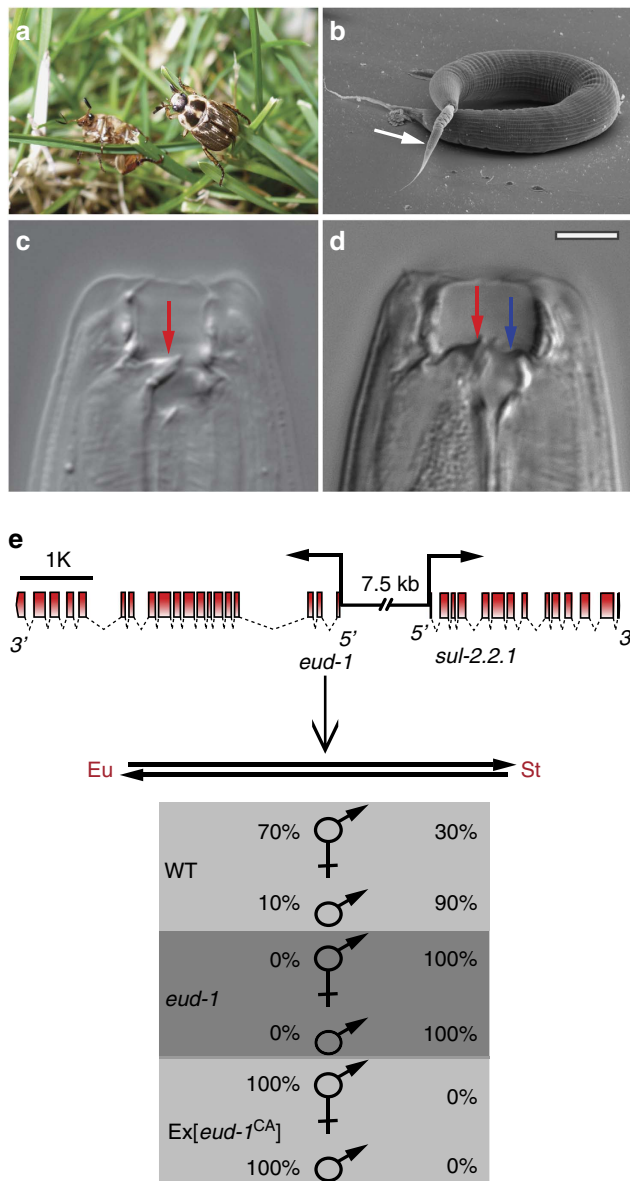
Figure 1 | Developmental plasticity in *P. pacificus* and its regulation by the developmental switch gene *eud-1*. (**a**) The oriental beetle *Exomala orientalis* is one of the beetle hosts with which *P. pacificus* lives in a necromenic association. (**b**) Scanning electron micrograph showing *P. pacificus* predatory feeding on a small larva of *C. elegans* (white arrow). (**c,d**) Mouth dimorphism of *P. pacificus* enabling a switch between bacterial grazing and predatory feeding. Stenostomatous (St) animals (**c**) have a narrow buccal cavity and a flint-like dorsal tooth (red arrow), but miss the subventral tooth. In contrast, eurystomatous (Eu) animals (**d**) have a wide buccal cavity, a claw-like dorsal tooth (red arrow) and an additional subventral tooth (blue arrow). Scale bars, 10 μm. (**e**) Molecular organization of the *eud-1* locus and effect of *eud-1* function on mouth-form ratios. *eud-1* derives from a recent gene duplication, with the neighbouring sulfatase *sul-2.2.1* arranged in a head-to-head orientation. The two genes are separated by a 7.5 kb intergenic region that when used as promoter drives the expression of *eud-1* in various head neurons. In wild-type animals, hermaphrodites and males form ∼70% and 10% Eu animals, respectively. In *eud-1* mutants, both sexes are completely St, whereas *eud-1* overexpression causes both genders to form only Eu animals indicating that EUD-1 functions as developmental switch.
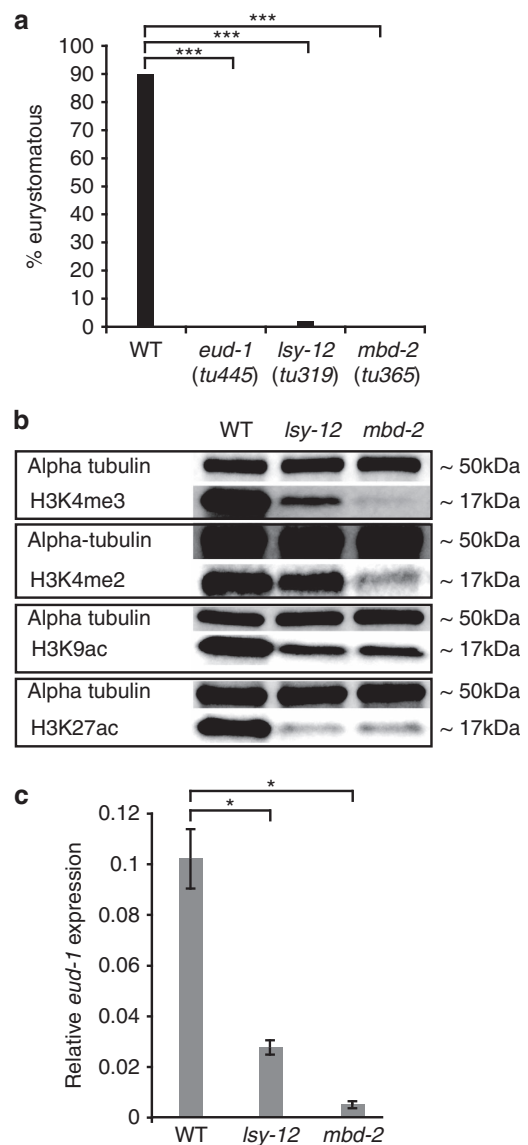

Figure 2 | Mouth-form defects of two pleiotropic mutants and their effect on histone modification and *eud-1* expression. (**a**) *Ppa-lsy-12(tu319)* and *Ppa-mbd-2(tu365)* result in the (nearly) complete absence of Eu hermaphrodites, similar to *eud-1* mutants. Data are presented as the total Eu frequency, $n > 100$ for all strains. (**b**) *Ppa-lsy-12* and *Ppa-mbd-2* mutants result in severe histone modification defects. This experiment has been replicated three times. (**c**) qRT–PCR experiments reveal down-regulation of *eud-1* expression in *Ppa-lsy-12* and *Ppa-mbd-2* mutants relative to wild type in J2 larvae. This experiment has been replicated three times. Error bars are defined as s.e.m. *$P < 0.05$ and ***$P < 10^{-5}$, Kruskal–Wallis test.

animals and found changes of four histone marks using antibody staining (Fig. 2b). H3K4me3 is strongly reduced in both *mbd-2* and *lsy-12* mutant animals, whereas H3K4me2 is reduced only in *mbd-2* mutants (Fig. 2b). In contrast, H3K4me1 and several other histone marks are not affected (Supplementary Fig. 2a). In addition to H3K4 methylation, the acetylation of H3K27 is strongly, and that of H3K9 moderately, reduced in both mutants (Fig. 2b). These findings demonstrate a genome-wide role for MBD-2 and LSY-12 in histone modifications in *P. pacificus*. Furthermore, because H3K4 methylation and acetylation of various H3 lysines are often found as gene activation marks[14], these results suggest that the effects of *mbd-2* and *lsy-12*

on mouth-form developmental plasticity is a consequence of chromatin remodelling-mediated transcriptional regulation.

eud-1 expression is down-regulated in lsy-12 mutants. Next, we tested the developmental switch gene *eud-1* as a potential candidate target of chromatin remodelling by LSY-12 and MBD-2. First, we studied *eud-1* expression by performing quantitative reverse transcription (qRT)–PCR experiments in wild-type and mutant hermaphrodites in different developmental stages. Indeed, *eud-1* is significantly down-regulated in *mbd-2* and *lsy-12* mutants, in J2 worms, the larval stage at which the mouth-form is determined (Fig. 2c). In addition, we also observed *eud-1* down-regulation in adult stages, suggesting that *eud-1* expression is similarly controlled throughout development (Supplementary Fig. 2b). These results suggest that the mouth-form defects of *mbd-2* and *lsy-12* mutants result from down-regulation of *eud-1*. Interestingly, these effects of *mbd-2* and *lsy-12* mutants on *eud-1* expression levels and the mouth-form frequency qualitatively match the patterns seen in *P. pacificus* males and highly St wild isolates[6]. Altogether, our findings indicate that reduction-of-function or loss-of-function mutations in *mbd-2* and *lsy-12* cause genome-wide changes in histone modifications, which alter, among other target genes, the expression of *eud-1* throughout development.

An antisense RNA associated with the eud-1 locus. To further explore the function of chromatin remodelling on the regulatory network controlling the developmental switch, we used ultra-directional RNAseq to compare the transcriptomes of wild-type and *Ppa-lsy-12* mutant animals (Fig. 3a). In total, we found 309 genes to be differentially expressed (Supplementary Data 1). This includes, consistent with our qRT–PCR results *eud-1* expression, which was heavily down-regulated in *Ppa-lsy-12* worms ($P < 10^{-8}$, Fisher exact test). Surprisingly, however, we also found a strong effect on previously uncharacterized antisense reads at the *eud-1* locus (Fig. 3a). Indeed, additional RT–PCR experiments identified an antisense *eud-1* transcript, termed as-*eud-1*. The as-*eud-1* RNA consists of three exons with a total size of 863 nucleotides, some of which cover *eud-1* exons, such as exons 7–10 and exon 19 (Fig. 3a). When we searched for short open reading frames we did not observe any evidence for coding potentials and putative micropeptides longer than 10 amino acids. Thus, as-*eud-1* has no obvious open reading frame suggesting that as-*eud-1* encodes a long non-coding (lnc) RNA (Supplementary Fig. 3).

eud-1 and as-eud-1 are co-expressed. Next, we tried to determine the expression pattern of as-*eud-1*. First, we used various constructs containing a putative 3.5 kb as-*eud-1* promoter fragment fused to turbo-RFP but they did not reveal specific expression. Therefore, we established RNA fluorescent in-situ hybridization (FISH) protocols. Indeed, we were able to detect *eud-1* RNA in the same head neurons as previously reported for a 7 kb *eud-1* promoter fragment driving RFP expression (Fig. 4a, Supplementary Fig. 4a; ref. 6). FISH of the as-*eud-1* RNA and *eud-1* RNA revealed that both transcripts are indeed expressed at the same site (Fig. 4b, Supplementary Fig. 4b; Supplementary Movie). Thus, our experiments suggest that *eud-1* and as-*eud-1* are co-expressed.

Overexpression of as-eud-1 increases the Eu mouth-form. To study the functional significance of this lnc RNA for the mouth-form decision, we performed overexpression experiments of as-*eud-1*. Specifically, we generated transgenic animals in which the as-*eud-1* complementary DNA (cDNA) is placed under the *eud-1* promoter, because the putative as-*eud-1* promoter

region itself does not drive specific expression of the antisense transcript (see above). Given that *eud-1* and as-*eud-1* are co-expressed the use of the *eud-1* promoter presumably results in overexpression of as-*eud-1*. We generated transgenic animals in a wild-type background in order to be able to score the potential effects of as-*eud-1*. We obtained three independent transgenic lines, all of which showed a strong masculinization phenotype resulting in more than 95% of animals being males. These transgenic lines showed no embryonic lethality and transgenic males were successfully mated indicating that the high incidence of males result from as-*eud-1*-induced X chromosome-specific non-disjunction, a phenomenon known from various *C. elegans* mutants such as *him-8* (ref. 15). We, therefore, used the male mouth-form frequency to study the influence of as-*eud-1*. In contrast to hermaphrodites, spontaneous wild-type males are only 10–20% Eu because *eud-1* is X-linked and dosage-sensitive (Fig. 1e; refs 6,10). The male mouth-form phenotype should be shifted towards more St animals in case of a negative effect and towards higher Eu frequencies in case of a positive function of the as-*eud-1*RNA.

We made the remarkable finding that as-*eud-1* has a positive function on the Eu versus St mouth-form decision and *eud-1* expression (Fig. 3b,c), whereas most cases of antisense-mediated regulation results in transcriptional down-regulation[16]. Four observations result in this conclusion. First, the frequency of the *eud-1*-sensitive mouth-form was dramatically increased in transgenic lines carrying as-*eud-1* in a wild-type background (from 20 to 64% Eu males) (Fig. 3b). Second, qRT–PCR experiments revealed a strong up-regulation of *eud-1* in as-*eud-1* transgenic males (Fig. 3c). Third, *eud-1* RNA can be detected in the J1 stage in *eud-1*::as-*eud-1* transgenic animals, an early expression that is never seen in wild-type animals (Fig. 4c). Finally, as-*eud-1* transgenes in a *eud-1* mutant background also caused a high incidence of males, but without affecting male mouth-form. Specifically, *eud-1(tu445);Ex(as-eud-1)* males were completely St (Fig. 3b), indicating that as-*eud-1* acts through *eud-1*. Taken together, these experiments suggest that chromatin remodelling acts through antisense-mediated up-regulation of *eud-1*.

Finally, we used the recently developed CRISPR/Cas9 technology in *P. pacificus* (ref. 17) to generate mutations that would specifically affect as-*eud-1*, but not *eud-1*. Therefore, we targeted the small exon 2 of as-*eud-1*, but were unable to generate a deletion/insertion in this 26 bp exon (Fig. 3a). In contrast, we succeeded in generating two mutations in the putative promoter of as-*eud-1* (Fig. 3a). Specifically, *tu520* eliminates a 4 bp fragment, whereas *tu522* contains a 44 bp insertion. Both mutant lines show a wild-type mouth-form ratio. However, the *tu522* mutant shows significantly reduced *eud-1* expression as observed by qRT–PCR experiments (Fig. 3d). In contrast, qRT–PCR experiments with as-*eud-1* failed to reveal transcripts above background level, a phenomenon known from other lnc RNAs[18]. Altogether, these experiments provide further evidence that as-*eud-1* up-regulates *eud-1* expression and additionally, they suggest that as-*eud-1* expression is itself driven by distal regulatory elements that are unaffected in the *tu520* and *tu522* mutants.

Discussion
Developmental switching represents an appealing concept to link genetic and environmental influences on phenotypically plastic traits. Our studies of the sulfatase *eud-1* —its function as a developmental switch, its role in micro- and macroevolutionary divergence and, here its regulation—provide such mechanistic insights. Previous characterization of *eud-1* resulted in several surprising findings, that is its recent origin by gene duplication and its epistasis to other factors controlling feeding plasticity[6].
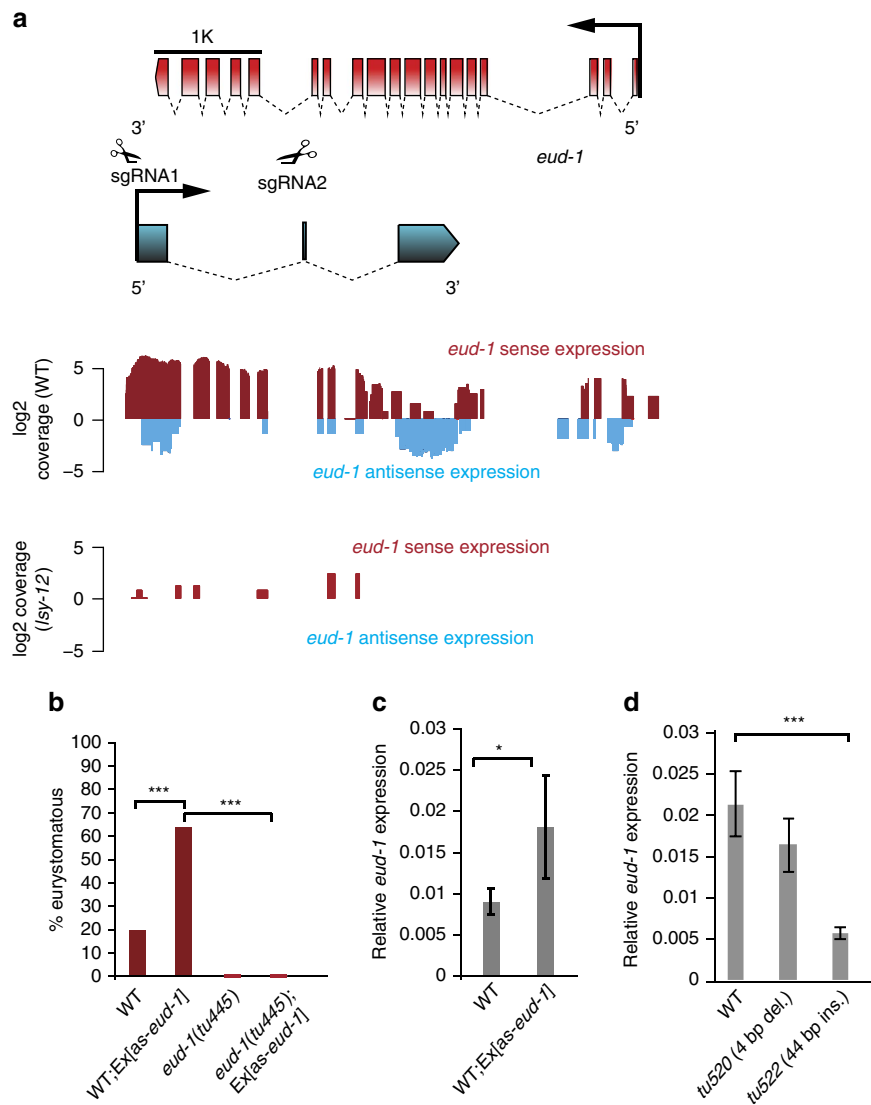
Figure 3 | Molecular organization and function of as-eud-1. (**a**) Organization of the *eud-1* and antisense *eud-1* (as-*eud-1*) locus and RNAseq experiments of wild-type and *Ppa-lsy-12* mutant animals. The long noncoding RNA as-*eud-1* consists of three exons that span large parts of the *eud-1* coding region. The structure of as-*eud-1* was identified in RT–PCR experiments and revealed the existence of a short exon, which went undetected in RNAseq. Other antisense reads obtained at lower frequency in the RNAseq experiment, were not confirmed to be part of as-*eud-1* in RT–PCR experiments with mixed stage wild-type animals. Subsequent panels show sense and antisense expression as measured for wild-type (wt) and *Ppa-lsy-12* mutant animals. Note that nearly no reads of *eud-1* and as-*eud-1* were observed in *Ppa-lsy-12* mutants. sgRNA1 and sgRNA2 in the 5′ untranslated region (UTR) and exon 2 of as-*eud-1*, respectively, were used to induce mutations by CRISPR. (**b**) Transformation of wild-type hermaphrodites with as-*eud-1* cDNA induced a high incidence of males and a Eud phenotype in male progeny. In contrast, transformation of *eud-1(tu445)* mutant animals with as-*eud-1* did not result in a Eud phenotype, although the high incidence of males was similar to the transformation of wild-type animals. Two independent transgenic lines were generated each, *n* > 100 for all strains. (**c**) qRT–PCR experiments reveal an up-regulation of *eud-1* in as-*eud-1* transgenic males relative to wild-type males. This experiment has been replicated three times. Error bars are defined as s.e.m. (**d**) qRT–PCR experiments reveal that *eud-1* is significantly down-regulated in the as-*eud-1* promoter mutant *tu522* that contains a 44 bp insertion. This experiment has been replicated three times. Error bars are defined as s.e.m. *$P < 0.05$ and ***$P < 10^{-5}$, Kruskal–Wallis test.

We have now shown that two evolutionarily conserved genes, *mbd-2* and *lsy-12*, are involved in genome-wide histone modifications that also influence transcription of *eud-1*, providing first insight into the molecular mechanisms underlying the regulation of developmental switches. In particular, the involvement of antisense-mediated up-regulation of *eud-1* indicates an unexpected complexity and results in three major conclusions. First, our findings demonstrate that the role of *eud-1* involves complex regulation of its own transcription. We previously observed that the coding region of *eud-1* is subject to strong purifying selection, and our new findings support

and extend these conclusions regarding the importance of regulatory versus structural changes. Second, we demonstrate the involvement of chromatin remodelling in the developmental switch mechanism regulating mouth-form plasticity in *P. pacificus*. We speculate that chromatin remodelling represents a powerful epigenetic mechanism that might link environmental signals to transcriptional regulation of plasticity. Third, we provide evidence for an antisense RNA in up-regulation. Transcriptional surveys of many eukaryotes have uncovered hundreds of noncoding transcripts[19] and though many of these function as transcriptional regulators, most do so
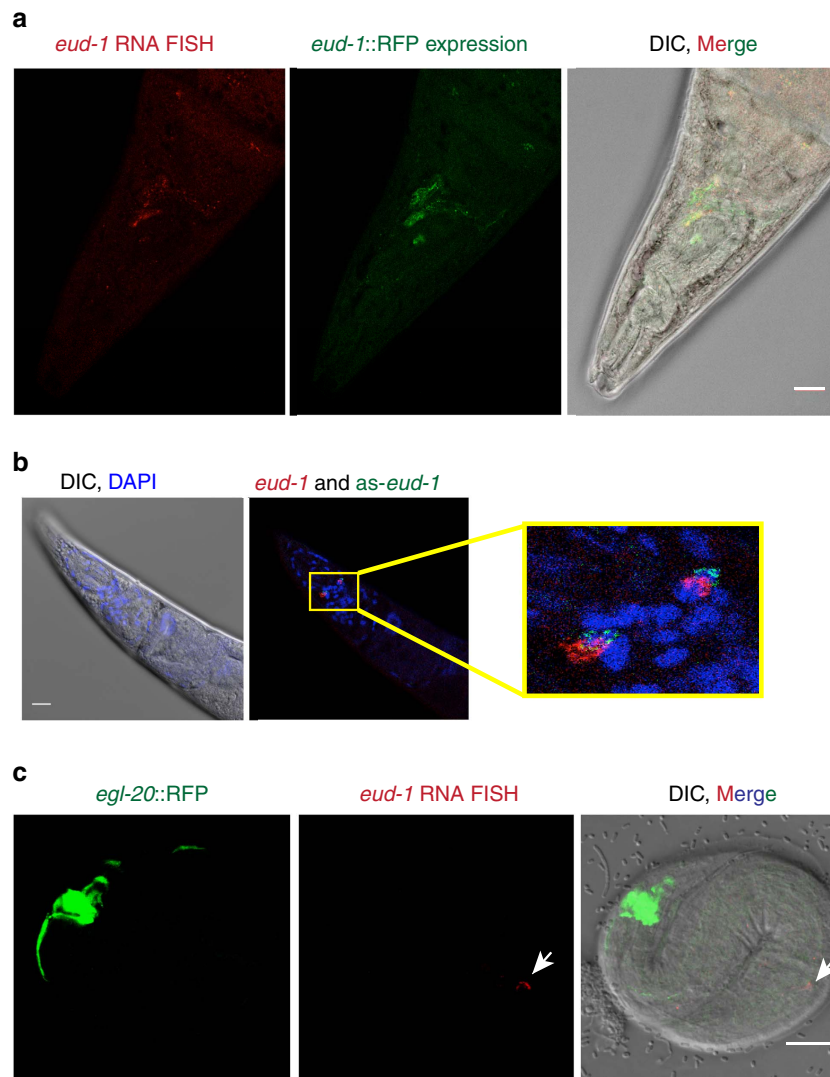
Figure 4 | Fluorescent *in situ* hybridization (FISH) of *eud-1* and as-*eud-1* reveals co-expression of both transcripts. FISH probes were designed as described in the Methods section. Photographs in **a** and **b** show adult animals, photographs in **c** show a J1 stage larvae, which in *P. pacificus* is still in the egg shell. (**a**) *eud-1* FISH (red, left image) and an *eud-1*::RFP reporter construct (green, central image) show the same expression pattern in several head neurons. The image at the right represents a merger of both and differential interference contrast (DIC) microscopy. Note that not all *eud-1*-expressing cells are visible in this plane of focus. (**b**) Head area of an adult worm with DIC and 4,6-diamidino-2-phenylindole staining (left image) and co-expression of *eud-1* (red) and as-*eud-1* (green) as revealed by FISH probes. Both transcripts are expressed at multiple foci, two of which are shown in this plane of focus (inset). Overlapping fluorescence (yellow) was seen in all animals observed, but not in all cells. The expression pattern was highly consistent among multiple adults ($n > 20$). See Supplementary Movie for additional details of the partially overlapping expression of both transcripts. (**c**) Transgenic animals carrying an *eud-1*::as-*eud-1* construct show *eud-1* expression in head neurons already in the J1 stage, which is never seen in wild-type animals. *egl-20*::RFP (green, left image) is used as transformation marker. The same *eud-1* FISH probe (red, central image) was used as above. The image at the right represents a merger and DIC microscopy. Supplementary Fig. 4 provides additional photographs for **a** and **b**. Scale bars, 10 μm.

as inhibitors. Conversely, antisense-mediated transcriptional activation or maintenance has only rarely been described[18,20]. Thus, the example of as-*eud-1* regulation of *eud-1* reveals complex regulatory mechanisms that can serve as model to link genetic and environmental control of developmental plasticity in future studies.

Methods

Culture conditions. All wild-type worms were *P. pacificus* reference strain RS2333. All *Pristionchus* strains were kept on 6-cm plates with nematode growth medium agar and were fed with a lawn of *E. coli* OP50 grown in 400 μl L-broth. Cultures were maintained at 20 °C. Because the mouth-form ratio is sensitive to unknown environmental factors[6], all experiments include their own controls for the wild-type Eu frequency. Also, to minimize the potential for laboratory

evolution of the trait, a new culture of the California (RS2333) strain was revived annually from a frozen voucher.

Phenotype scoring. The mouth-form phenotype was scored using the following characters to discriminate between Eu and St individuals, respectively, (i) the presence versus absence of a subventral tooth, (ii) a claw-like versus flint-like or triangular dorsal tooth, and (iii) a wide versus narrow stoma (mouth). Characters (i) and (ii) were discrete, non-overlapping, and sufficient to distinguish the two forms. Apparent intermediates between the two forms were rare ($< 0.1\%$) and were not included in counts. Phenotypes could be scored using Zeiss Discovery V.12 stereomicroscopes and were supplemented where necessary with differential interference contrast (DIC) microscopy on a Zeiss Axioskop.

Mapping of *vul-2(tu319)* and mutant identification. For genetic mapping, mutants in the California background were crossed to the Washington strain

(PS1843). F2 progeny were cloned and screened for two generations to confirm the mutant phenotype and the homozygosity of mutations. Genomic DNA of outcrossed mutant lines was extracted for genetic mapping. Simple-sequence conformation polymorphism markers were tested against 30–40 outcrossed mutant lines and detected as previously described[21,22]. *vul-2* was mapped to the tip of chromosome IV close to the marker S210. Further mapping localized *vul-2* to the bacterial-artificial-chromosome clone BACPP16-M16 and the fosmid subclones 525-J06, 543-P16 and 558-O23. Light shotgun sequencing of these fosmid clones resulted in the identification of *Ppa-lsy-12* as candidate gene for *vul-2*. To prepare samples for whole-genomic sequencing, DNA was extracted from all three alleles *tu18*, *tu30* and *tu319* and mutations were identified in all three alleles.

Alternative splicing of *Ppa-lsy-12*. Following preparation of mixed-stage RNA libraries for *P. pacificus* RS2333, coding DNA (cDNA) was amplified by reverse transcription PCR and sequenced. 5′ and 3′ RACE experiments were performed by SMARTer RACE cDNA Amplification Kit following the manufacturer's protocol (Life Technologies). The full list of gene-specific primers that were designed according to the available genomic sequence for *Ppa-lsy-12* is provided in Supplementary Table 1.

RNA-sequencing experiments. The presence and levels of gene expression were measured by whole-transcriptome sequencing (RNA-Seq) of *Ppa-lsy-12(tu319)* mutants and *P. pacificus* wild type. Culture populations were allowed to grow until their food was exhausted, immediately after which the cultures were processed for sequencing. Five mixed-stage plates were washed with 40 ml M9, centrifuged immediately at $1300\,g$ for 4 min, rinsed with 40 ml 0.9% NaCl treated with 40 μl ampicillin and 40 μl chloramphenicol and shaken gently for 2 h, and finally concentrated into a pellet by centrifugation and immediately frozen in liquid nitrogen. NEBNext Ultradirectional RNA Library Kit (Cat # E7420L) was used to prepare libraries. RNA-Seq libraries were sequenced as 2×100-bp paired-end reads on an Illumina HiSeq 2000, yielding 30.6 million reads for the wild type and 31.6 million reads for the *lsy-12* sample. Raw reads were aligned to the reference genomes of *P. pacificus* (Hybrid1) (www.pristionchus.org), using the software Tophat v.2.0.3 (ref. 23). Expression levels were estimated and tested for differential expression using the programs Cufflinks and Cuffdiff v.2.0.1 (ref. 23) resulting in 95 significantly differentially expressed genes including *eud-1* (FDR corrected *P* value < 0.01). The equivalent test for down-regulation of as-*eud-1* was not significant despite no evidence of as-*eud-1* expression in *Ppa-lsy-12* animals, which is probably due to the reduced statistical power for differential expression detection resulting from the low expression of as-*eud-1* even in wild-type animals.

qRT-PCR. Total RNA from synchronized cultures was isolated using TRIzol (ambion by life technologies). For reverse transcription Superscript II reverse transcriptase (Invitrogen, Cat. No: 18064) was used following the manufacturer's instructions. We used 1 μg total RNA. The qRT–PCR experiments were performed on a LightCycler 480 system; using SybrGreen (Roche Diagnostics) with a reaction set up described elsewhere[24]. To detect *eud-1* expression we used VSe13F GATGATCGAGTCACACAGATCCG forward and VSe13R ATGTGTAGTAGGAGA GTTGAGCAGCG reverse primers. *Ppa-cdc-42* and *Ppa-Y45F10D.4* were used as reference genes as previously described[25]. PCR efficiencies were determined using external standards on plasmid mini-preparation of cloned PCR-products. Expression levels were analysed by basic relative quantification. We performed 3–6 biological replicates for different experiments.

as-*eud-1* transcript analysis. RNAseq reads of wild-type worms cover the majority of *eud-1* exons to a similar extent. In addition, we observed antisense readsat the *eud-1* locus that were previously uncharacterized. These antisense reads are expressed at very low levels and cannot be detected in qRT–PCR experiments, which otherwise are used as a standard procedure in *P. pacificus* (see above). We used many different PCR primer combinations (Supplementary Table 2) in a variety of nested PCR experiments to study which of the antisense reads if any are present in a potential as-*eud-1* cDNA. These experiments revealed the existence of one transcript of 863 nucleotides that consists of three exons (Fig. 3, Supplementary Fig. 5). The two larger exons cover exactly those reads that were most abundantly found in the RNAseq experiment of wild-type worms. However, exon 2 consists of only 26 nucleotides and went unnoticed at the RNAseq level. Similar to noncoding (nc) RNA (ncRNA) in other systems[18], as-*eud-1* is expressed at very low levels.

Genetic transformation. For phenotypic rescue of *vul-2*, the germ line of *vul-2(tu319)* mutant animals was injected with a 17 kb genomic construct containing exons 1-20 of *Ppa-lsy-12* and 4.5 kb of flanking regulatory region (2 ng ul⁻¹), the marker *Ppa-egl-20::TurboRFP* (10 ng ul⁻¹) and genomic carrier DNA (60 ng ul⁻¹ from the recipient strain[26]). To study the as-*eud-1* lnc RNA, we generated a 7.5 kb construct consisting of ~6.5 kb promoter element and the 860 bp cDNA fragment of as-*eud-1*. This construct was injected (2 ng ul⁻¹) with the *Ppa-egl-20::TurboRFP* (10 ng ul⁻¹) marker and genomic carrier DNA

(60 ng ul⁻¹) of *P. pacificus* RS2333 and *Ppa-eud-1(tu445)*, respectively. For all constructs, at least two independent transgenic lines were generated and transgenic animals were scored over multiple generations involving at least 100 transgenic animals per line. Transgenic lines containing the as-*eud-1* lnc RNA yielded more than 90% male progeny and all lines were kept at least until the tenth generation. No embryonic lethality was observed in association with these transgenes. Transgenic males were crossed with *Ppa-pdl-1* and wild-type hermaphrodites and cross-progeny was readily observed.

CRISPR/Cas9 induced mutations. To generate CRISPR/Cas9 induced mutations, sgRNAs were co-injected with Cas9 protein[17]. We used the following sites for single-guided (sg) RNAs (sgRNA):
    sgRNA1: 5′CAGTTGAAGAACAAAACACACGG3′.
    sgRNA2: 5′GTCGTAATCAAGCTAACAGCTGG3′.

Statistical analyses. All phenotypic data show Eu frequency calculated from total individuals screened. Total sample size is illustrated on graphs. Significant differences were tested by Fisher's exact test. For the expression data we performed Kruskal–Wallis test. All statistical analyses were implemented in the program Statistica v. 9 (Statsoft).

Western blotting and antibodies. Proteins were extracted from mixed stage cultures. Concentration was determined by Neuhoff's Dot-Blot assay[24]. Proteins were equally loaded and separated in polyacrylamid gels. Proteins were transferred to polyvinylidene difluoride transfer membrane and incubated overnight with primary antibodies (Supplementary Table 3), and were then incubated for an hour in secondary antibodies (Anti-rabbit IgG, horseradish peroxidase-linked antibody, Cell Signaling Technology, Cat. #7074S and Anti-mouse IgG, horseradish peroxidase-linked antibody, Cell Signaling Technology, Cat. #7076S). For dilution of primary antibodies see Supplementary Table 3. The secondary antibody was diluted 1:1,000. The detection was done by Bio-Rad Clarity western enhanced chemiluminescent (ECL) substrate using Peqlab FUSION Xpress multi-imaging system. We provide an uncropped scan of the most important blot as Supplementary Fig. 5.

Single molecule RNA FISH. Single molecule RNA FISH was performed using a protocol described earlier for *C. elegans*[25]. Biosearch Technologies Stellaris FISH online platform was used to design and order *eud-s* and as-*eud-1* probes. They were coupled with Quasar 670 and TAMRA fluorescent dyes, respectively. Image acquisition was performed on Leica SP8 confocal system using settings to maximize detection of fluorescent dyes. Image J software was used for Image analysis.

Data availability. All relevant data, including mutant and transgenic lines, constructs and plasmids are available upon request from the corresponding author[26].

References

1. West-Eberhard, M.-J. *Developmental Plasticity and Evolution* (Oxford University Press, 2003).
2. Moczek, A. P. *et al.* The role of developmental plasticity in evolutionary innovation. *Proc. R. Soc. B* **278,** 2705–2713 (2011).
3. Nijhout, H. F. *Insect Hormones* (Princeton University Press, 2003).
4. Schlichting, C. D. Origins of differentiation via phenotypic plasticity. *Evol. Dev.* **5,** 98–105 (2003).
5. Pigliucci, M. *Phenotypic Plasticity: Beyond Nature and Nurture* (John Hopkins University, 2001).
6. Ragsdale, E., Müller, M., Roedelsperger, C. & Sommer, R. J. A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell* **155,** 922–933 (2013).
7. Herrmann, M. *et al.* The nematode Pristionchus pacificus (Nematoda: Diplogastridae) is associated with the Oriental beetle *Exomala orientalis* (Coleoptera: Scarabaeidae) in Japan. *Zool. Sci.* **24,** 883–889 (2007).
8. Bento, G., Ogawa, A. & Sommer, R. J. Co-option of the endocrine signaling module Dafachronic Acid-DAF-12 in nematode evolution. *Nature* **466,** 494–497 (2010).
9. Bose, N. *et al.* Complex small molecular architectures regulate phenotypic plasticity in a nematode. *Angew. Chemie.* **51,** 12438–12443 (2012).
10. Serobyan, V., Ragsdale, E., Müller, M. & Sommer, R. J. Feeding plasticity in the nematode Pristionchus pacificus is influenced by gender and social context and is linked to developmental speed. *Evol. Dev.* **15,** 173–182 (2013).
11. Gutierrez, A. & Sommer, R. J. Functional diversification of the *mbd-2* gene between Pristionchus pacificus and Caenorhabditis elegans. *BMC. Genet.* **8,** 57 (2007).
12. Sigrist, C. & Sommer, R. J. Vulva formation in Pristionchus pacificus relies on continuous gonadal induction. *Dev. Genes Evol* **209,** 451–459 (1999).

13. Sarin, S. *et al.* Analysis of multiple ethyl methanesulfonate-mutagenized *Caenorhabditis elegans* strains by whole-genome sequencing. *Genetics* **185,** 417–430 (2010).

14. Musselman, C. A., Lalonde, M.-E., Cote, J. & Kutateladze, T. G. Perceiving the epigenetic landscape through histone readers. *Nat. Struct. Mol. Biol.* **19,** 1218–1227 (2012).

15. Hodgkin, J. A., Horvitz, H. R. & Brenner, S. Nondisjunction mutants on the nematode *Caenorhabditis elegans. Genetics* **91,** 67–94 (1979).

16. Rinn, J. L. & Chang, H. Y. Genome regulation by long noncoding RNAs. *Annu. Rev. Biochem.* **81,** 145–166 (2012).

17. Witte, H. *et al.* Gene inactivation using the CRISPR/Cas9 system in the nematode *Pristionchus pacificus. Dev. Genes Evol.* **225,** 55–62 (2015).

18. Dimitrova, N. *et al. LincRNA-p21* activates *p21* in *cis* to promote polycompb target gene expression and to enforce the G1/S checkpoint. *Mol. Cell* **54,** 1–14 (2014).

19. Guttman, M. *et al.* Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. *Nature* **458,** 223–227 (2009).

20. Sakurai, I. *et al.* Positive regulation of *psbA* gene expression by cis-encoded antisense RNAs in *Synechocystis* sp. PCC6803. *Plant. Physiol.* **160,** 1000–1010 (2012).

21. Srinivasan, J. *et al.* A bacterial artificial chromosome-based genetic linkage map of the nematode *Pristionchus pacificus. Genetics* **162,** 129–134 (2002).

22. Srinivasan, J. *et al.* An integrated physical and genetic map of the nematode *Pristionchus pacificus. Mol. Genet. Genomics* **269,** 715–722 (2003).

23. Trapnell, C. *et al.* Differential gene and transcript expression analysis of RNA-Seq experiments with TopHat and Cufflinks. *Nat. Protoc.* **7,** 562–578 (2012).

24. Neuhoff, V., Philipp, K., Zimmer, H.-G. & Mesecke, S. A simple, versatile, sensitive and volume-independent method for quantitative protein determination which is independent of other external influences. *Hoppe-Seyler's Z. Physiol. Chem.* **360,** 1657–1670 (1979).

25. Ji, N. & van Oudenaarden, A. *Single Molecule Fluorescent In Situ Hybridization (smFISH) of C. elegans Worms and Embryos.* The *C. elegans* Research Community. Wormbook, ed. (2012).

26. Schlager, B., Wang, X., Braach, G. & Sommer, R. J. Molecular cloning of a dominant Roller mutant and establishment of DNA-mediated transformation in the nematode model *Pristionchus pacificus. Genesis* **47,** 300–304 (2009).

Additional information

Accession Codes: The RNA-seq data are available at the NCBI sequence read archive under accession codes SRX1609204, SRX1858662, SRX1858663.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no conflict of interest.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Serobyan, V. *et al.* Chromatin remodelling and antisense-mediated up-regulation of the developmental switch gene *eud-1* control predatory feeding plasticity. *Nat. Commun.* 7:12337 doi: 10.1038/ncomms12337 (2016).