Genetic and Genomic Tools for the Marine Annelid *Platynereis dumerilii*

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ABSTRACT The bristle worm *Platynereis dumerilii* displays many interesting biological characteristics. These include its reproductive timing, which is synchronized to the moon phase, its regenerative capacity that is hormonally controlled, and a slow rate of evolution, which permits analyses of ancestral genes and cell types. As a marine annelid, *Platynereis* is also representative of the marine ecosystem, as well as one of the three large animal subphyla, the Lophotrochozoa. Here, we provide an overview of the molecular resources, functional techniques, and behavioral assays that have recently been established for the bristle worm. This combination of tools now places *Platynereis* in an excellent position to advance research at the frontiers of neurobiology, chronobiology, evo-devo, and marine biology.

"MAN is but a worm" is the punch line of a caricature published in the Punch Almanack in 1881, lampooning the, at that time, so obviously ridiculous idea that men and earthworms could share evolutionary roots. One hundred thirty years later it is clear that research on annelid worms, the group to which earthworms belong, has led to scientific advances in several areas of biology. Hypotheses like Anton Dohrn's "annelid theory"—suggesting that vertebrates indeed originate from annelid-like ancestors (Dohrn 1875; Nübler-Jung and Arendt 1994)—led to many ideas and conceptual progress in evolutionary and developmental biology. The advent of molecular biology now allows revisiting and testing of such ideas with the help of molecular markers. Here, the nereidid *Platynereis dumerilii* (Nereididae, Annelida) (Figure 1 and Figure 2) has emerged as a well-suited reference species. Its protein sequences, as well as the number and position of introns in its genome, show lower divergence from those of vertebrates than that of other protostomes (Raible *et al.* 2005). Consistently, *Platynereis* contains the orthologs of many protein-coding and

micro-RNA genes present in vertebrates, which have been lost from the genomes of faster-evolving species, such as *Caenorhabditis elegans*, *Drosophila*, or *Ciona* (Raible *et al.* 2005; Hui *et al.* 2009; Christodoulou *et al.* 2010). This strongly suggests that *Platynereis* evolves molecularly with a slow rate. On the cellular level, it possesses several cell types that share common ancestry with cell types present in other vertebrates and invertebrates. This conclusion is based on the transcription factors and cell-type-differentiation markers, cellular morphologies, and position in the axonal scaffold and has been investigated in detail for the photo-sensory and neurosecretory systems (Arendt *et al.* 2002; Arendt *et al.* 2004; Fritzsch *et al.* 2005; Tessmar-Raible *et al.* 2007; Jekely *et al.* 2008) and the mushroom bodies (Tomer *et al.* 2010). The patterning of its central nervous system also shares many similarities with the molecular events taking place during vertebrate nervous system development (Denes *et al.* 2007; Kulakova *et al.* 2007; Kerner *et al.* 2009; Steinmetz *et al.* 2011; Demilly *et al.* 2013). As discussed in more detail previously (Tessmar-Raible and Arendt 2003), an indication for evolutionary ancestrality is the lack of group-specific (derived) characters or traits. Following this reasoning, the analyses of *Platynereis* suggest that genes and cell types of this bristle worm are evolutionarily ancestral, *i.e.*, representative of what was present in the bilaterian ancestors. A similar picture emerges when considering the bristle worm's overall morphology. Features such as its rope-ladder-like nervous
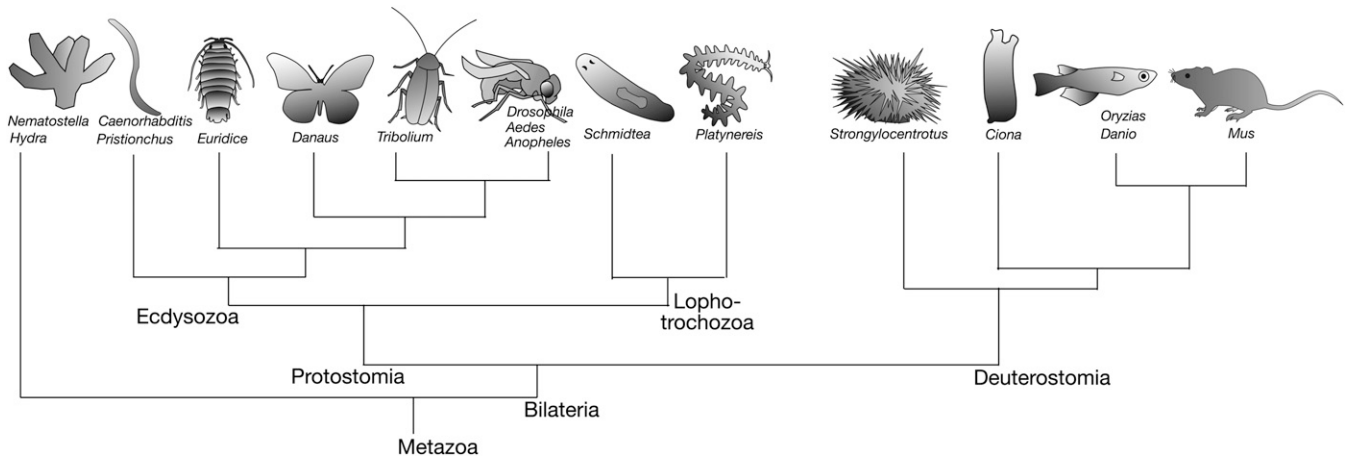
Figure 1 The phylogenetic position of *P. dumerilii*. A schematized phylogenetic tree of Bilateria with its three main branches: Ecdysozoa, Lophotrochozoa, and Deuterostomia (Aguinaldo *et al.* 1997; de Rosa *et al.* 1999). *Platynereis* is an annelid worm positioned within the Lophotrochozoan group. The species represented in the tree have at least one functional tool established (transgenesis, RNAi, morpholino-based gene knockdown, genome mutagenesis using Zn-fingers, TALENs or Cas9/CRISR).

system show a prototypical design (Bullock and Horridge 1965; Tessmar-Raible and Arendt 2003). Thus, multiple strings of evidence converge to suggest that *Platynereis* represents a slowly evolving species with an ancestral-type body plan.

In addition to its suitability as an evolutionary and developmental model system, *Platynereis* exhibits fascinating biological features. A remarkable property of the species is that its reproductive cycles synchronize with the natural moon phases. Recognized already in the beginning of the 20th century (Hempelmann 1911), this phenomenon of lunar-controlled reproductive periodicity led to the establishment of *Platynereis* as a laboratory model in the 1950s. Whereas the synchronization of gonadal maturation and spawning is typical for many marine animals (reviewed in Naylor 2010; Tessmar-Raible *et al.* 2011), experiments on these *P. dumerilii* laboratory cultures revealed for the first time that nocturnal light is sufficient for the synchronization of a monthly spawning cycle (Hauenschild 1954, 1955, 1956, 1960). This early work also suggested that the monthly reproductive rhythm is under the control of an endogenous timer or circalunar clock. While this suggestion was initially disputed (Hauenschild 1960; Palmer 1974), recent work has shown that *P. dumerilii* indeed possesses such an endogenous monthly timer, the maintenance of which is not dependent upon the oscillations of the worm's circadian clock (Zantke *et al.* 2013).

A third line of research for which nereidid annelids have played a critical role is the field of neuroendocrinology. *Nereis virens* was among the first invertebrates for which the concept of neurosecretory cells was proposed (Scharrer 1936). Later transplantation studies, inspired by pioneering work in the earthworm (Harms 1948), indicated that one or more neurohormonal components produced by the brains of *Platynereis* and other nereidids (commonly referred to as "brain hormone activity") promote regeneration in juveniles

and, conversely, block transition into the maturing form (Hauenschild 1956; Hofmann 1976). Despite significant biochemical efforts (Cardon *et al.* 1981), the molecular identities of the respective neurohormonal activities still remain enigmatic.

For all of the outlined research directions, a deeper mechanistic understanding requires precise molecular interrogation of the system. Here we review recent progress in the development of genetic and genomic tools for *Platynereis* that allow researchers to address important questions spanning evolution, development, gene regulation, chronobiology, and behavior on a functional level. We conclude by briefly summarizing both classical and more recently developed assays that can be used for phenotype analyses of larval and adult *Platynereis*.

Basic Genetic Features of *P. dumerilii*

Strains and culture

P. dumerilii (Figure 1 and Figure 2) belongs to the phylum Annelida (segmented worms), one of the major phyla residing within the lophotrochozoan superphylum. The Lophotrochozoa form one of the three major branches of Bilateria (the others being Ecdysozoa and Deuterostomia) (Aguinaldo *et al.* 1997; de Rosa *et al.* 1999). Worms can be continuously bred in the lab throughout the year using a well-developed culture protocol (Hauenschild and Fischer 1969; a detailed description in English is available at http://www.uni-giessen.de/~gf1307/breeding.htm). Breeding of a single male and female provides hundreds of transparent embryos that undergo synchronous development (Fischer and Dorresteijn 2004). *Platynereis* embryos undergo spiralian cleavage typical for Lophotrochozoans and develop through sequential planktonic, trochophore, and metatrochophore larval stages [swimming 20–80 hr postfertilization
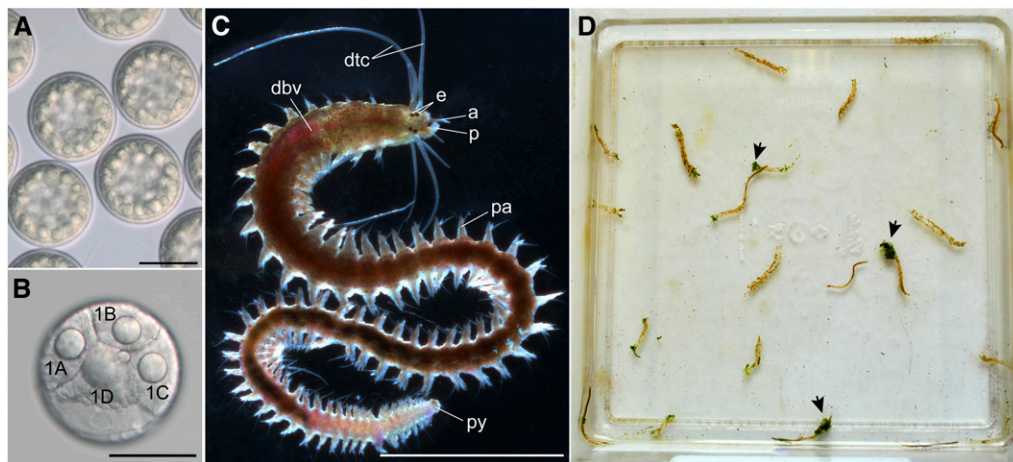
Figure 2 The annelid *P. dumerilii*. (A and B) Early developmental stages *of P. dumerilii*. (A) Unfertilized eggs. (B) Cleaving embryo at stereoblastula stage (6 hpf) at 18° ±1°, 4-cell stage (anterior view, blastomeres 1A, 1B, 1C, 1D). The refractive spherical structures in A and B are lipid droplets. (C) Premature adult worm removed from the tube, dorsal view: a, antennae; dbv, dorsal blood vessel; dtc, dorsal tentacular cirri; e, eyes; p, palps; pa, parapodia; py, pygidium. (D) Adult worms >2 months of age in worm box (25 × 25 cm) living in self-made tubes feeding on spinach (arrows). Scale bars: A and B, 100 μm; C, 0.5 cm.

(hpf)] before settling to the bottom and beginning to crawl during the transition from nectochaetal larval to juvenile stages (80 hpf onward) (Hauenschild and Fischer 1969; Fischer and Dorresteijn 2004; Fischer *et al.* 2010). *P. dumerilii* are gonochoristic; *i.e.*, male and female sexes are separate, with sexual dimorphism becoming morphologically apparent during the last few weeks prior to spawning. The generation time of *Platynereis* in the laboratory is 3–4 months, a fate map for the three-segmented young worm exists (Ackermann *et al.* 2005), and a detailed staging system has been developed to characterize and define *Platynereis* development (Fischer *et al.* 2010).

One important aspect to consider for functional techniques, as well as for behavioral analyses, is the level of naturally occurring polymorphisms. Genomes of several other marine organisms revealed relatively high rates of sequence polymorphism. For single nucleotide polymorphisms (SNPs), for instance, reported values can reach 2–4% (Sodergren *et al.* 2006; Small *et al.* 2007; Putnam *et al.* 2008). Similarly, non-inbred *P. dumerilii* cultures or newly caught worms exhibit significant allelic variation. For 3′-untranslated regions of coding genes, SNP frequencies are ~2%, whereas for coding sequence, the respective value is <1% (Christodoulou *et al.* 2010; C. Lohs and F. Raible, unpublished results). Such variance can pose problems for functional tools relying on sequence-specific recognition, such as phosphorodiamidate morpholino oligonucleotide (morpholino)-mediated knockdown, customized nucleases (*e.g.*, transcriptional activator-like effector nucleases: TALENs), or homologous recombination.

Systematic inbreeding and maintenance of inbred strains are therefore key prerequisites for reverse genetic applications in *Platynereis*. The animals originally chosen for such systematic inbreeding (started at the European Molecular Biology Laboratory in Heidelberg) were retrieved from a culture maintained in Berlin (lab of Hans-Dieter Pfannenstiel). This population is reported to descend from worms originally isolated from the Bay of Naples in the 1950s (Hauenschild and Fischer 1969) and subsequently maintained as closed cultures in several labs in Germany and France. Inbreeding has been further continued in Vienna, resulting in distinct inbred lines of >10 inbred generations that are currently kept as closed cultures and typically contain one or two alleles per gene. Interestingly, these distinct inbred strains show phenotypic variations. When investigating the fraction of reproducing animals over the course of a lunar month, worms of the lines PIN, ORA, and VIO exhibit rather unimodal spawning peaks, whereas BLU and FL2 strains tend to exhibit more bimodal peaks (Figure 3). A clearly non-unimodal spawning curve also exists for descendants of animals collected from Ischia/Naples in 2009 (called Naples) (Figure 3) and is reflected by the spawning data presented by Hempelmann(1911). Offspring from the Naples strain displays higher levels of allelic variation than systematically inbred strains, with up to eight alleles isolated for the same locus. These alleles encompass the alleles isolated from the inbred strains, consistent with the notion that the cultured and subsequently inbred strains originally derive from the same location (C. Lohs and F. Raible, unpublished results).

Genomics

The genome of *P. dumerilii* has a size of ~1 Gbp and is organized into 2*n* = 28 chromosomes (Jha *et al.* 1995). Joint community sequencing efforts have generated a high-coverage, still-unpublished reference genome for this organism, which was derived from sperm DNA of FL2 line male worms (Simakov *et al.* 2013; http://4dx.embl.de/ platy/). Additional large, high-quality data sets of expressed sequenced tags (EST), and sequences of individual genomic BAC clones (Raible *et al.* 2005), have been generated by multiple labs. The complete mitochondrial genome of *P. dumerilii* (Boore 2001) is a valuable source of data for phylogenetic relationships analyses (*e.g.*, Larget *et al.* 2002; Hassanin 2006).
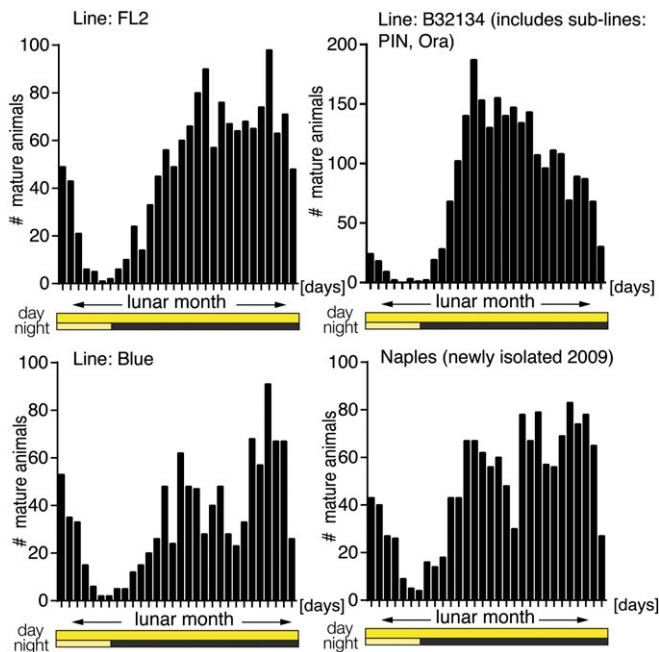
Figure 3 Circalunar reproductive periodicity of different *P. dumerilii* strains. Maturation curves under the displayed light regime. The *y*-axis represents the number of mature spawning animals recorded each day of the lunar month (duration ~29.5 days). The *x*-axis shows the days of the lunar month and the illumination conditions for day (top bar) and night (bottom bar). Yellow, daylight; black, nights without moon (new moon, NM); light yellow; nights with dim light simulating full moon (FM). FL2 and Blue strains share a common origin, but were subsequently independently inbred. Vio, PIN, and Ora share a common origin (B3213); Ora and PIN diverged one generation later (B32134). Maturation data are pooled from data collected over the course of several months. For further details on the illumination regime and scoring see Zantke *et al.* (2013).

Molecular Analyses Techniques

In addition to sequence resources, techniques for molecular analyses have now been established for *P. dumerilii*. Robust protocols exist for immunohistochemistry (Dorresteijn *et al.* 1993), as well as whole-mount *in situ* hybridization (WMISH) for embryonic and early larval stages (Arendt *et al.* 2001; Tessmar-Raible *et al.* 2005), posterior regenerates (Prud'homme *et al.* 2003), and adult worms (Backfisch *et al.* 2013). For detection of tissue hybridization signal by confocal microscopy, a special reflection technology has been developed (Jekely and Arendt 2007). Fluorescent *in situ* hybridization (FISH) has been established for both tissue and chromosome stainings (Tessmar-Raible *et al.* 2005; Hui *et al.* 2007). For the comparison of gene expression patterns, expression profiling by image registration has paved the way for systematic comparisons of expression patterns, at least for larval stages (Tomer *et al.* 2010). Finally, qPCR (Dray *et al.* 2010; Zantke *et al.* 2013) facilitates accurate investigation of temporal gene expression changes. (See Table 1.)

Treatment with Drugs and Signaling Molecules

Due to its aquatic lifestyle, *Platynereis* is easily accessible for experimental treatments using water-soluble drugs and signaling proteins. This has already been explored to test the relevance of distinct signaling pathways and cell structural components for *Platynereis* development, chronobiology, or behavior. Successful examples include the small molecules cyclopamine and SANT-1, known antagonists of the hedgehog signaling pathway in vertebrates, which provided evidence that hedgehog signaling is involved in segment formation outside of the arthropod phylum (Dray *et al.* 2010). The microtubule-depolymerizing drug nocodazole and the actin-depolymerizing drug cytochalasin B were employed in a study investigating the mechanisms underlying convergent extension movements in the morphogenesis of the trunk nervous system; moreover, two inhibitors of Jun N-terminal kinase, SP600125 and AS601245, revealed a relevance of this pathway in convergent extension, presumably under control of noncanonical Wnt signaling (Steinmetz *et al.* 2007). Similarly, the Wnt/β-catenin inhibitors endo-IWR-1 and PNU-74654 have been used to reveal the relevance of Wnt/β-catenin signaling for the differentiation of neural progenitor cells (Demilly *et al.* 2013). In the same study, Rho-associated kinase (Rok) signaling was found to play a role in morphogenetic movement of the neuroectoderm, based on experiments with Y-27632, a Rok inhibitor. A role for β-catenin in the establishment of animal *vs.* vegetal cell fates during early cleavages was shown by the use of the GSK-3β inhibitors 1-azakenpaullone or alsterpaullone. Treatment with these inhibitors led to stabilization of β-catenin and subsequent alterations of cell fates (Schneider and Bowerman 2007). Treatment of *Platynereis* embryos with estradiol affects the number of the worms' primordial germ cells (Lidke *et al.* 2014). Finally, *Platynereis* has been shown to be sensitive to the mammalian casein kinase 1δ/ε inhibitor PF670462 that impairs the function of the circadian clock, but does not have marked effects on circalunar spawning periodicity (Zantke *et al.* 2013). All the above-mentioned examples suggest that *P. dumerilii* is an experimental system that is very amenable to chemical screens.

In addition to incubations with small molecule inhibitors, signaling molecules and peptides have been added to *Platynereis* cultures. Incubation of young embryos with exogenous zebrafish BMP4 suggests a dose-dependent regulation of mediolateral patterning genes by BMP-type ligands (Denes *et al.* 2007). Various neuropeptides were found to affect ciliary locomotor control of the swimming larvae, establishing *Platynereis* as a model for addressing the principles underlying vertical migration in the water column (Conzelmann *et al.* 2011). Similarly, a recent study indicated a role of myoinhibitory peptide (MIP)/allatostatin-B in the process of larval settling (Conzelmann *et al.* 2013).

Micro-injection and Transgenesis

Micro-injection

The first injection protocol for *Platynereis* zygotes and early embryos was established to facilitate a detailed analysis of cell lineages and cleavage patterns (Ackermann 2003) and has since been further modified (Backfisch *et al.* 2013; Conzelmann *et al.* 2013; Bannister *et al.* 2014). An experienced injector can now inject 200 embryos/hr with a survival

Table 1 Functional toolkit of P. dumerilii

Technique	Reference
Whole-mount *in situ* hybridization (WMISH) in larvae, regenerates and adults; fluorescent WMISH	Arendt *et al.* (2001); Prud'homme *et al.* (2003); Tessmar-Raible *et al.* (2005); Backfisch *et al.* (2013)
Chromosomal FISH mapping	Hui *et al.* (2007)
Reflection microscopy	Jekely and Arendt (2007)
Cellular profiling by image registration	Tomer *et al.* (2010)
Laser-assisted cell ablation; conditional mtz/Ntr-mediated cell ablation	Jekely *et al.* (2008); Veedin Rajan *et al.* (2013)
Gene knockdown with morpholino-oligomers	Conzelmann *et al.* (2013)
Transgenesis (transient/stable)	Backfisch *et al.* (2013, 2014)
Targeted mutagenesis using TALE nucleases	Bannister *et al.* (2014)

rate of 70–80% at 24 hpf. Of these survivors 30–60% of worms reach adulthood, almost all of which will go on to mature and spawn (Bannister *et al.* 2014).

Transient and stable transgenesis

Transposon-based integration systems are powerful tools for genetic analyses and are widely used for transgenesis in vertebrate and invertebrate model species. Protocols for transposon-mediated transient and stable germline transgenesis have recently been established in *P. dumerilii* (Backfisch *et al.* 2013, 2014).

At least two transposon systems have excision and transposition activities in *Platynereis*, the Tc1/mariner-type element Mos1 and the hAT/Tol2 element (Backfisch *et al.* 2013, 2014). Tol2 co-injected transgenes are integrated early on and exhibit robust expression in the G0 generation. eGFP constructs driven by a ubiquitous enhancer reveal that through Tol2-mediated integration, the complete animal can be transgenically modified (Backfisch *et al.* 2014). In contrast, Mos1-mediated transgenesis produces more mosaic expression. Tol2-mediated transgenesis is therefore the current method of choice for evaluating the complete repertoire of expression patterns regulated by a specific enhancer. However, whereas transgenes integrated via the Tol2 transposase system can still be detected in the genome of the G1 generation using molecular techniques, none of these animals exhibit any detectable transgene expression, suggesting that Tol2-mediated integrations are most likely silenced during germline transmission (Backfisch *et al.* 2014).

In contrast to Tol2-mediated transgenesis, Mos1-mediated transgene insertions are stably expressed in the following generations. The use of Mos1 has therefore facilitated the creation of the first stable transgenic lines in *Platynereis* (Figure 4, A and B; Backfisch *et al.* 2013, 2014; Veedin Rajan *et al.* 2013).

Resources for Gene Regulatory Analyses

The size of the *P. dumerilii* genome of ~1 Gbp approximates the average observed for eukaryotic metazoans (for comparisons see http://www.genomesize.com). Compared with many conventional invertebrate molecular model species, however, this genome size is rather large and therefore presents a challenge for the identification of gene regulatory elements that may be located at considerable distances from the corresponding promoter. Genome size estimates for two related polychaete species, *P. massiliensis* (sampled in Naples) and *N. virens* (sampled in Newcastle), revealed genomes of 0.39 and 0.45 Gbp, respectively, and thus less than half the size of the *P. dumerilii* genome (T. Gregory, personal communication). This has raised the question whether the genomes of these species might be useful for phylogenetic footprinting. Phylogenetic footprinting is a technique used for the identification of DNA sequences involved in gene regulation based on their conservation between evolutionarily distant species (Gumucio *et al.* 1996). For footprinting to be efficient, overall sequence conservation between two species needs to be high enough to maintain regulatory sequences, but low enough to allow evolutionary sequence modification of less-constrained DNA regions. Comparison of selected gene loci between *P. massiliensis* and *P. dumerilii* suggests that the genome sequences of these two species are too closely related for efficient phylogenetic footprinting. In contrast, *N. virens* and *P. dumerilii* appear to have a suitable distance for the identification of conserved elements via phylogenetic footprinting (Willmann 2007; V.B. Veedin-Rajan, F. Raible, and K.Tessmar-Raible, unpublished results). A BAC library for *N. virens* is available. In light of the small genome size of both *P. massiliensis* and *N. virens*, an interesting idea is to use the respective organisms for the generation of smaller enhancer constructs to be used in *P. dumerilii*.

The use of gene regulatory elements of other nereidid species will certainly facilitate *in vivo* labeling of specific cell types. For detailed gene regulatory analyses, sequences of other nereidids will in many cases at least serve as a proxy for *P. dumerilii* gene regulatory elements. The parallel analysis of gene regulatory sequences across different nereidid species (including *P. dumerilii*) promises to be a powerful approach to understanding gene regulation in this molecularly slowly evolving organism.

Functional Cell Ablation

Cell-specific ablation is a powerful tool to study the functional requirement of individual cells or groups of cells.
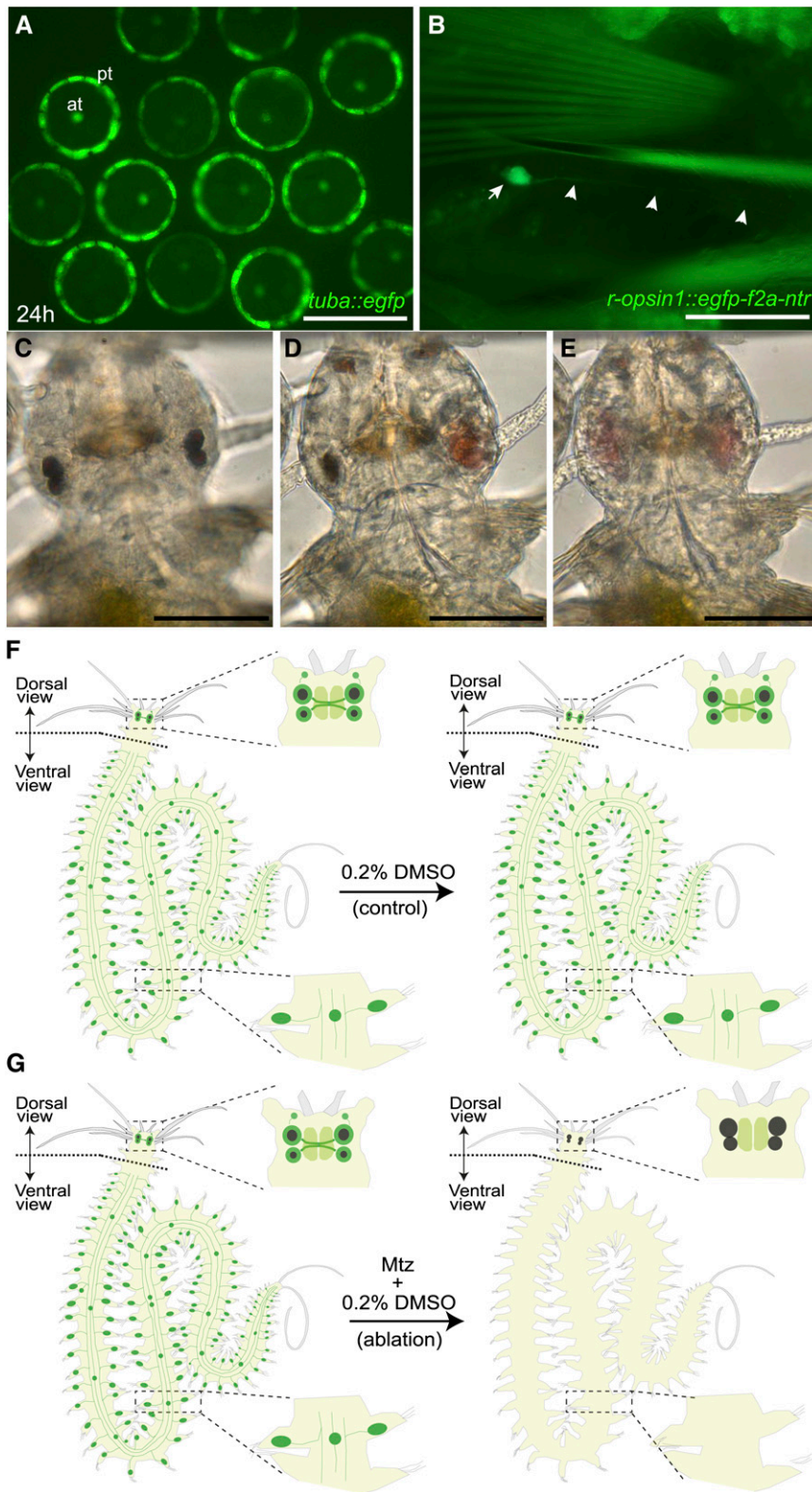
Figure 4 Transgenesis and specific cell ablation in *P. dumerilii*. (A and B) Examples of cells marked by stable transgenesis with EGFP. (A) Trochophore larvae (24 hpf, apical view): larval protroch (pt) and apical tuft (at) cells are labeled by a construct containing the regulatory region of the *Platynereis* α-tubulin locus (*tuba*::*egfp*; Backfisch *et al.* 2013, 2014). (B) Peripheral neuron (arrow, soma; arrowheads, neurite) in appendage of an immature adult worm highlighted by an *r-opsin1*::*egfp-f2a-ntr* construct (Backfisch *et al.* 2013, 2014; Veedin Rajan *et al.* 2013). Orientation: ventral view, anterior to the top. (C–E) Laser ablation of *Platynereis* adult eyes performed on a 2- to 3-week-old juvenile worm. (F and G) Scheme of chemical ablation using metronidazole (mtz) to induce apoptosis in cells expressing eGFP and nitroreductase (Ntr) from an integrated *r-opsin1*::*egfp-f2a-ntr* transgene (Veedin Rajan *et al.* 2013). Cells expressing *r-opsin1*::*egfp-f2a-ntr* are depicted in green. (F) DMSO control. (G) Incubation with mtz. Scale bars: A and B, 100 μm; C–E, 50 μm.

In *Platynereis*, two main methods of cell ablation have been applied: laser-assisted cell ablation and chemically mediated induction of apoptosis. Both methods allow the investigation of cellular functions at any given time throughout the life cycle of the worm.

Laser cell ablation

Laser-assisted cell ablation has been a very useful tool in invertebrate model species such as *C. elegans* (Bargmann and Avery 1995) and *Drosophila* (Farrell and Keshishian 1999). In larval stages of *Platynereis*, laser ablation of the

eyespots has helped to dissect the coupling of sensory input to locomotor ciliary cells (Jekely *et al.* 2008). We have successfully established UV-laser ablation for adult *Platynereis* specimens and ablated the worms' adult eyes, comprising both photoreceptors and nonphotoreceptive pigment cells (Figure 4, C–E; Keplinger 2010).

Metronidazole/Nitroreductase-Mediated chemical ablation

Whereas laser-mediated cell ablation is a straightforward tool to study the function of individual cells, the ablation of larger numbers of cells, for instance, cells expressing a given molecular marker or ablation of cells in many individuals, can be tedious and impractical with this method. In such cases, chemically mediated cell ablation is a suitable alternative. A powerful method of chemical cell ablation is the induction of apoptosis using metronidazole and nitroreductase (mtz/Ntr). Mtz/Ntr-mediated cell ablation is a combination of chemical and genetic tools, originally established for the analysis of tissues and cell types in zebrafish (Curado *et al.* 2008). The method is based on transgenic expression of the bacterial enzyme nitroreductase (Ntr) in restricted cell populations. The *ntr* coding sequence is typically linked to a fluorescent reporter gene, which allows for monitoring the specific cells *in vivo*. The Ntr enzyme converts the nitroimidazole compound metronidazole (mtz) into a highly toxic DNA interstrand cross-linking agent, which subsequently induces cell death (Curado *et al.* 2008).

We established mtz/Ntr-mediated cell ablation in *Platynereis* by generating *ntr*-expressing transgenic worms (Veedin Rajan *et al.* 2013). Co-expression of *ntr* and *egfp* on a single, polycistronic mRNA under the control of the *r-opsin1* enhancer allowed us to both visualize *r-opsin1*+ photoreceptor cells and induce death of the same cells upon addition of metronidazole (schematized in Figure 4, F and G). Adjacent cell types, such as the pigment cells or other neuron types, were not affected, nor was the neurite scaffold, providing strong evidence for the specificity of the technique. It is noteworthy that the exact concentration of mtz, as well as the incubation time, varied according to the size of the animals (as measured by segment number), likely reflecting differences in how well mtz can penetrate the cuticle. Similarly, we observed that cells expressing lower amounts of eGFP (and thus also Ntr) needed longer incubation times for full ablation. Taking both aspects into consideration, we optimized effective concentrations for treating worms at different stages, which can serve as a guideline for future *ntr*-expressing constructs in *Platynereis* and other organisms (Veedin Rajan *et al.* 2013).

Interfering with Gene Function

Targeted genome mutagenesis using TALENs

Recently, new technologies that use customized nucleases to facilitate targeted modification of genomic DNA have energized the field of genome engineering (Gaj *et al.* 2013). These technologies involve different methods for generating customized DNA-binding proteins that are coupled to an endonuclease domain (Kim *et al.* 1996). The utility of customized nucleases for genome editing lies in their ability to generate double-strand breaks (DSBs) in genomic DNA. DSBs are the most severe form of DNA damage and stimulate DNA repair, which can occur via homology-directed or nonhomologous end-joining (NHEJ) mechanisms (Goodarzi and Jeggo 2013). By generating DSBs at specific sites in the genome, the frequency of erroneous DNA repair by NHEJ is increased, resulting in insertions or deletions (indels) that generate mutations at the target site. Exogenously provided DNA can be used as a repair template (by, *e.g.*, possessing sequence homologous to the sequence neighboring the cut), promoting the integration of exogenous sequences at the target site via homology-directed repair (for review, see Gaj *et al.* 2013, 2014). In this way, streamlined nuclease approaches can also be used as an alternative approach for generating transgenic animals.

The three major technologies currently used for custom nuclease-based genome engineering are zinc finger nucleases (ZFNs) (Kim *et al.* 1996), TALENs (Cermak *et al.* 2011), and Cas9/clustered regularly interspaced short palindromic repeats (CRISPR) systems (Jinek *et al.* 2012; Cong *et al.* 2013; Mali *et al.* 2013a). ZFNs and TALENs are both customizable DNA-binding proteins, which are fused to FokI nuclease domains and function in pairs by binding to tandem-oriented binding sites separated by short spacer sequences (Kim *et al.* 1996; see also Gaj *et al.* 2013 for review). TALENs feature modular DNA-binding domains composed of 34 amino acid repeats. Each repeat interacts with a single nucleotide, the specificity of which is determined by two amino acids, the so-called repeat variable di-residues (RVDs). Using the established RVD code (Boch *et al.* 2009; Moscou and Bogdanove 2009), TALE proteins can be assembled to recognize any given sequence of interest, the only restriction for target site selection being the presence of a thymidine (T) immediately 5′ of each recognition sequence (Cermak *et al.* 2011; Reyon *et al.* 2012). Alternatively, the Cas9/CRISPR system uses small chimeric guide RNAs (sgRNAs) that are complementary to the genomic target site at the 5′ end and contain 3′ secondary structures that are bound by the Cas9 endonuclease. The sgRNAs thus anchor Cas9 to a specified genomic sequence, which then cleaves the DNA at a defined distance from the recognition site (Jinek *et al.* 2012).

The use of ZFNs, TALENs, and Cas9/CRISPR technologies for targeted genome engineering has already been demonstrated in all major molecular model systems, including yeast (Cermak *et al.* 2011), *Drosophila melanogaster* (Liu *et al.* 2012), *Xenopus* (Lei *et al.* 2012; Nakayama *et al.* 2013), zebrafish (Huang *et al.* 2011; Moore *et al.* 2012; Hwang *et al.* 2013), mice (Davies *et al.* 2013; Qiu *et al.* 2013; Wang *et al.* 2013), *C. elegans* (Wood *et al.* 2011; Friedland *et al.* 2013; Lo *et al.* 2013), sea urchin (Ochiai *et al.* 2010; Hosoi *et al.* 2013), and human cells (Hockemeyer *et al.* 2011; Miller *et al.* 2011; Cong *et al.* 2013; Mali *et al.* 2013b).

EENdb also collates published data for ZFN, TALEN, and Cas9/CRISPR-mediated genome engineering for diverse

organisms: http://eendb.zfgenetics.org/ (Xiao *et al.* 2013). Most importantly, these techniques are not only applicable to the conventional molecular model systems, but also have the immense potential to facilitate reverse genetics studies in less conventional and emerging models. For example, ZFNs were used to demonstrate the role of *cry2* as a transcriptional repressor of the circadian clock in the monarch butterfly (Merlin *et al.* 2013), while both ZFNs and TALENs have been used for targeted mutagenesis in the cricket *Gryllus bimaculatus* (Watanabe *et al.* 2012).

We have recently established highly efficient genome mutagenesis using TALENs in *P. dumerilii* (schematized in Figure 5; Bannister *et al.* 2014). As for any method that relies on efficient sequence-specific binding, the relatively high polymorphism rate in the *Platynereis* genome presents a challenge. We circumvent high polymorphism rates at the TALEN-binding sites by working with inbred strains, as well as carrying out additional genotyping analyses, to screen for polymorphisms at the genomic regions we aim to target (Bannister *et al.* 2014). Using this approach, we have now generated mutations for five *Platynereis* genes and have so far confirmed germline transmission of TALEN-induced mutations for two of these (Figure 5; Bannister *et al.* 2014). (Other mutation-positive G0 animals are not yet old enough to reproduce; thus, the number is expected to increase within the next months.) Mutant carrier G0 animals are outcrossed to wild-type worms of inbred lines to remove possible nonspecific mutations in the genome, which might occur as a result of nonspecific TALEN cleavage. However, it should be noted that recent studies revealed that the specificity of the TALEN system is high, especially when using heterodimeric FokI endonuclease domains (Dahlem *et al.* 2012). As judged by the evidence from other organisms, this appears to be one advantage of the TALENs over the more straightforward Cas9/CRISPR system, which can tolerate greater degrees of sequence mismatch and was recently reported to induce high-frequency off-target mutations (Fu *et al.* 2013). Off-target effects can be reduced in the Cas9/CRISPR system by using two CRISPR guide RNAs that bind opposite DNA strands, combined with a mutated Cas9 enzyme that functions as a nickase (Ran *et al.* 2013). The use of truncated guide RNAs also reduces off-target mutation frequencies (Fu *et al.* 2014).

Due to the *ab initio* high specificity of TALENs, their versatility to target any given sequence, with the fact that the Cas9 proteins used for genome editing are encoded by relatively long transcripts, ~4.1 kb (Jinek *et al.* 2012; Esvelt *et al.* 2013) (which might matter for genome editing efficiency in *Platynereis*), we have primarily focused our efforts on developing TALENs for use in *P. dumerilii*.

Morpholinos and RNAi

Alternative methods to gaining insight into a gene's function target post-transcriptional stages. One fast and flexible technique takes advantage of morpholinos that can be used to block either splicing or translation of mRNA (reviewed in Eisen and Smith 2008). In other model species such as

amphibians and fish, gene knockdown by morpholinos has allowed major progress in understanding gene function, particularly during early development. As morpholinos will eventually be diluted out (reviewed in Eisen and Smith 2008) and effects are not inheritable, the analysis of potential knockdown phenotypes is usually restricted to a relatively short time window in the injected (G0) generation. Experimental repetitions and design of scrambled negative controls are used to ensure that a given phenotype is not caused by the injection itself, or by developmental artifacts within a given batch. Morpholino-based gene knockdown has already proven useful to investigate the function of one of the two sex peptide receptor orthologs of *Platynereis* during larval stages (Conzelmann *et al.* 2013). Hence, morpholinos are a viable and useful tool for analyzing gene functions during embryonic and larval development in *Platynereis*.

Injection of dsRNA leads to reduced mRNA levels in injected *Platynereis* larvae, as detected by WMISH, consistent with functioning RNA interference (RNAi) process (reviewed in Tessmar-Raible and Arendt 2003). However, neither effective parameters for dsRNA/siRNA/shRNA design nor *in vivo* RNAi expression systems have been developed in *Platynereis*.

## Tools for the Study of Behavior and Chronobiology

As outlined above, there is now an array of technologies to monitor and manipulate genes and cell types in *Platynereis*. In addition to the study of molecular readouts, several tools allow an assessment how the respective manipulations affect animal behavior. Especially the last years have also seen progress in the analysis of *Platynereis* behavior.

Larval swimming and settling assays have been established and fruitfully been combined with laser-assisted nanosurgery and gene knockdown assays to gain insight into the regulation of larval behavior (Jekely *et al.* 2008; Conzelmann *et al.* 2013). For postlarval stages, we have recently developed a behavioral assay with which to monitor the locomotor activity of adult worms during different time points and light conditions (Zantke *et al.* 2013). As the behavior of worms is recorded via a digital video camera, different aspects of their behavior can be (re) analyzed in various ways: for example, assessing rhythmic components using periodogram analyses. Using this assay for future studies, in combination with methods to chemically or genetically influence gene expression or to mediate cell ablation, will help to characterize cell types and genes specifically involved in the generation of daily and lunar rhythms. So far, the behavioral scoring is performed manually. While this provides a high degree of accuracy, it is also highly time consuming. Thus, an immediate aim for expanding the use of behavioral assays in the future will be to develop software to automate the scoring procedure and speed up the data analysis process.

Future Directions

The proof-of-principle that TALE nucleases mediate targeted genome mutagenesis now opens the door for further use in
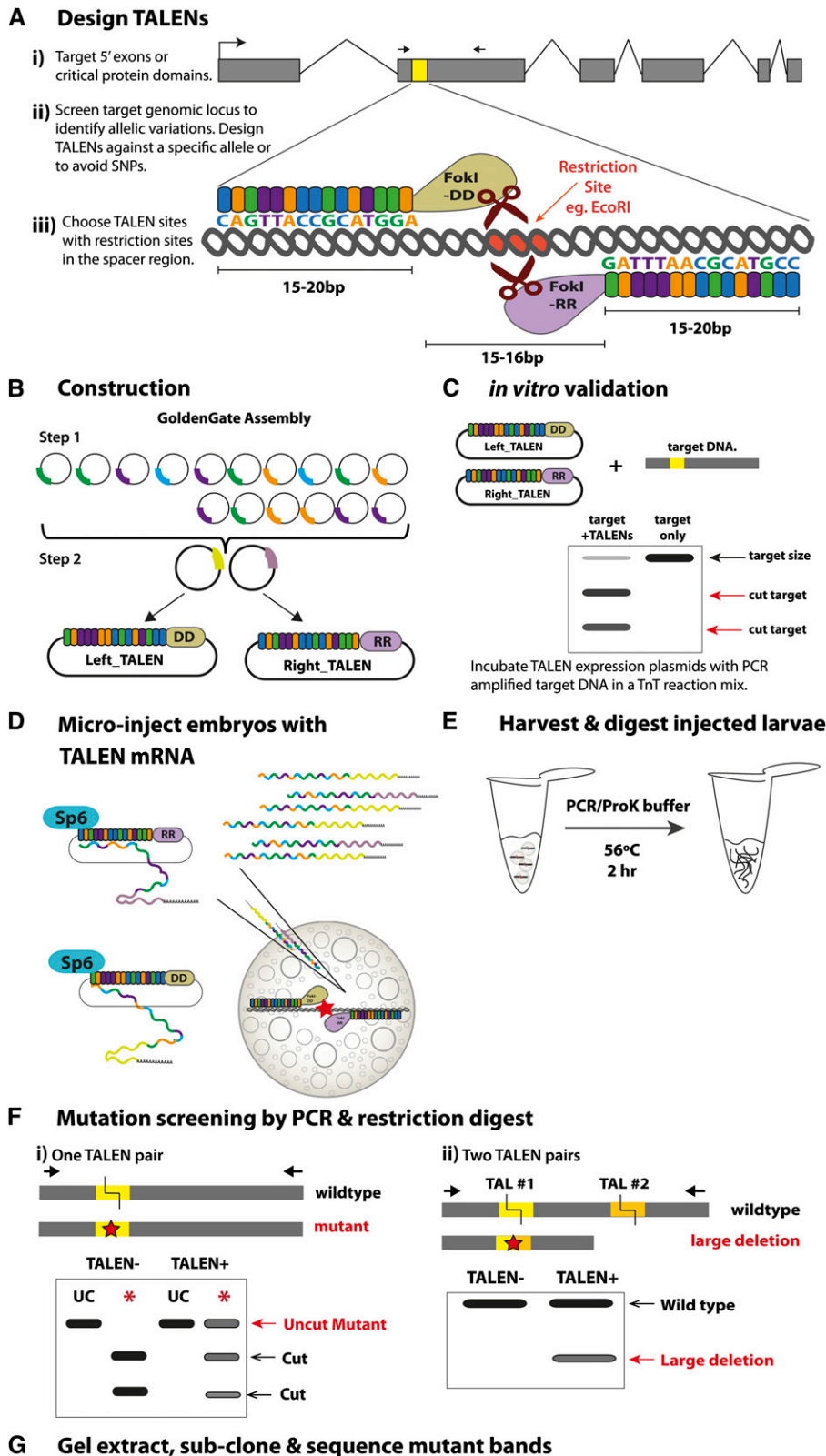
Figure 5 Workflow for targeted mutagenesis using TALENs in *P. dumerilii*. (A) TALENs can be designed to target specific DNA sequences given their modular composition: each repeat in the DNA binding domain contains a repeat variable di-residue (RVD) that recognizes a single nucleotide (blue, HD = C; yellow, NI = A; purple, NG = T; green, NN = G). (i) TALEN pairs are designed to recognize a specified locus using a web-based prediction tool (https://tale-nt.cac.cornell.edu/node/add/talen). (ii) Genotyping PCR of target loci (arrows, primers) to test for SNPs at target site (yellow). (iii) Target sites comprise 15- to 20-bp binding sites for each TALEN separated by a 15- to 16-bp spacer, with unique restriction endonuclease sites in the spacer, to facilitate screening. (B) TALEN expression plasmids are constructed via two-step GoldenGate (Cermak *et al.* 2011). (C) *In vitro* cleavage assay to validate that the constructed TALENs are catalytically active against the intended target. TALEN activity is confirmed by the presence of smaller (gray, "cut target") bands visible by gel electrophoresis (red arrows). (D) Equal amounts of left and right TALEN mRNA are delivered into *Platynereis* zygotes via micro-injection. (E) Single or small pools of injected larvae are digested with proteinase K to produce a DNA lysate used as template for screening PCR. Genomic DNA from adult worm tail-clip tissue samples are prepared by conventional genomic DNA extraction kits. (F) Mutation screening is performed by PCR amplification of the target locus (yellow) and (i) digestion of the PCR product with the restriction enzyme cutting within the wild-type spacer sequence (jagged line): if mutations are present in the spacer that disrupts the restriction site, some or all of the PCR product will be resistant to digestion, resulting in "uncut" bands. (ii) Larger deletions are detected as smaller PCR bands (red arrow). (G) Subcloning of uncut bands or smaller deletion bands and subsequent sequencing is used to confirm the presence of mutations centered at the target site.

Platynereis. The most immediate application, aside from generating loss-of-function mutations, will be to use donor DNA for engineering precise modifications, including transgene insertion. A further application of this technology will be the use of custom TAL effectors to manipulate gene expression *in vivo*. TALE proteins can be engineered to bind next to the promoter of a gene of interest and fused to transcriptional activators such as VP16 to generate custom

transcription factors (Miller *et al.* 2011). The modulation of gene expression using TALE proteins can also be inducible: light inducible transcriptional effectors (LITE) are TALE proteins fused to a modified Cry2 protein from *Arabidopsis thaliana* and are designed to bind to enhancer sequences in human cells. Upon illumination with blue light, they induce the expression of a downstream gene (Konermann *et al.* 2013).

In addition to a further exploration of the TALE-based system, a complementary establishment of the Cas9/CRISPR system will certainly be highly useful. First, Cas9/CRISPR might be able to be used for genome engineering in cases for which the TALEN system fails and vice versa. Second, due to more straightforward design of the guide RNA as the DNA recognition unit, the Cas9/CRISPR system is more easily adaptable for genome-wide screen approaches (Heintze *et al.* 2013; Shalem *et al.* 2014; Wang *et al.* 2014).

The possibility of performing unbiased reverse or forward genetic screens still remains a technology gap in *P. dumerilii*. Establishing the tools required for reverse or forward genetics is certainly a challenge. Is the expectable scientific gain worth the considerable effort required for establishment? When aiming to study the molecular mechanisms underlying any aspects of *Platynereis*' unquestionably interesting biology, genome-wide reverse or forward genetic screens provide the advantage of a nonbiased approach, which could uncover entirely novel and unexpected players.

Conclusion

P. dumerilii has been maintained as an experimental animal for several decades. But as many organisms that were not easily accessible to molecular and genetic tools, it has so far existed outside the limelight and attention of many fields of biology. However, its slowly evolving genome and transcriptome, an ancestral-type cell-type repertoire, a neurohormone repertoire close to vertebrates, a hormonally regulated regeneration ability, and its circadian and circalunar rhythms make *Platynereis* an interesting and useful model organism. This interest has driven the establishment of new molecular sequence resources and tools. The availability of morpholino-mediated knockdown, transgenesis, inducible cell ablation, and targeted genome mutagenesis has greatly expanded the genetic tractability of this species, enabling the community to address new and more detailed functional questions. These, with new approaches and technologies for studying behavior, will continue to move *Platynereis* research into new directions and further our understanding of the molecular mechanisms and cell types that underpin its fascinating biology.

Acknowledgments

Note added in proof: See Bannister *et al.* 2014 (pp. 77–89) in this issue for a related work.

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