The *Caenorhabditis elegans* Transgenic Toolbox

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ABSTRACT The power of any genetic model organism is derived, in part, from the ease with which gene expression can be manipulated. The short generation time and invariant developmental lineage have made *Caenorhabditis elegans* very useful for understanding, *e.g.*, developmental programs, basic cell biology, neurobiology, and aging. Over the last decade, the *C. elegans* transgenic toolbox has expanded considerably, with the addition of a variety of methods to control expression and modify genes with unprecedented resolution. Here, we provide a comprehensive overview of transgenic methods in *C. elegans*, with an emphasis on recent advances in transposon-mediated transgenesis, CRISPR/Cas9 gene editing, conditional gene and protein inactivation, and bipartite systems for temporal and spatial control of expression.

KEYWORDS *C. elegans*; CRISPR/Cas9; genetic engineering; recombinases; transgenesis; transposon; WormBook

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CAENORHABDITIS elegans has made significant contributions to our understanding of the genetic basis of multicellular biology. Early discoveries were based predominantly on forward genetic screens with mutations induced by chemical mutagens combined with careful microscopy and behavioral assays. In contrast, biochemistry has played a modest role in *C. elegans* experimentation. Clever forward genetic mutagenesis screens (Jorgensen and Mango 2002) will undoubtedly continue to uncover fascinating biology; however, reverse genetic experimentation is increasingly being facilitated by sophisticated and accessible transgenic techniques with a range of methods to manipulate individual or large classes of genes. Occasionally, novel methods fundamentally change how we perform experiments. One such example was the realization that ingested dsRNA can silence complementary genes (Timmons *et al.* 2001), and the ensuing construction of a genome-wide library of bacteria expressing dsRNA for systematic loss-of-function screens (Ahringer 2006). Currently, we are in the midst of a similar fundamental change precipitated by the versatile use of CRISPR/Cas9 to modify the genome in ways previously unimaginable.

Here, we present a comprehensive overview of both gradually refined and transformational transgenic methods currently in use in *C. elegans*, with an emphasis on recently developed methods for manipulating genes and their expression. Our aim is to lower the entry barrier for students or researchers new to *C. elegans*, while also describing recent advances of interest to the seasoned worm geneticist. First, we describe some of the intricacies of transgenes optimized for *C. elegans* expression, and the use of multicopy transgene arrays. These sections include recently developed methods to optimize transgenes for expression in the germline, and protocols to generate targeted integrations of multicopy arrays. Next, we describe the use of Mos1 transposons for random and targeted single-copy transgene insertions. We proceed to give an overview of current CRISPR/Cas9 techniques for editing endogenous genes, but refer readers interested in more detail to a recent comprehensive WormBook chapter (Dickinson and Goldstein 2016) and several recent reviews (Chen *et al.* 2016; Farboud 2017). We then present methods for conditional gene deletion using FLP and CRE recombinases, as well as several newly developed conditional protein degradation methods that utilize degron tags. Finally, we describe how bipartite systems such as FLP and CRE, the Q-system, and cGAL can be used for temporal and cell-specific gene expression. Each section includes a discussion of when a given technique may be advantageous, notes on experimental considerations, and, with the risk of looking foolish a few years from now, occasional guesses about likely near-future technical developments.

C. elegans Genes and Transgene Structures

Some conventions for designing transgenes for use in *C. elegans* differ from other genetically tractable organisms and mammalian cell culture. These design rules are based on >30 years of cumulative experience that has often been shared through informal, nonpeer-reviewed publications such as the comprehensive documentation distributed with the Fire lab vector kits (Andrew Fire and colleagues, unpublished reagents, Addgene Kit # 1000000001), meeting abstracts, or Worm Breeders Gazette articles (wbg.wormbook.org). We point readers to two *C. elegans*-specific venues for communicating technical advances. First, "micropublications" describe, among other things, technical advances or resources in microPublication Biology by Caltech library (www.micropublication.org). These manuscripts are peer-reviewed but are at present not indexed by PubMed or Google Scholar. Second, the discussion forum associated with Wormbase (forums.wormbase.org) is a rich resource for browsing prior discussions and asking questions of a technical nature.

Experimental considerations

Choosing a starting point for generating transgenes: Before designing a new transgene, it is useful to decide on a

backbone scaffold for the final construct. A variety of cloning vectors that facilitate transgene construction for *C. elegans* are available at a nominal cost through public repositories such as Addgene, which maintains a list of worm-specific resources (https://www.addgene.org/worm-expression/). The Fire laboratory vector kits (Andrew Fire and colleagues, unpublished reagents) contain common fluorophores and vectors for tissue-specific expression. Mos1 vectors allow targeted single-copy integration by MosSCI (Frøkjær-Jensen *et al.* 2008, 2012) or random integration using the miniMos transposon (Frøkjær-Jensen *et al.* 2014) (see *Transposon-mediated genetic engineering*). Reagents for CRISPR/Cas9 genetic engineering include cloning vectors to express sgRNAs and fluorophores with selection markers for tagging genes (Dickinson *et al.* 2013, 2015; Arribere *et al.* 2014; Ward 2015a; Schwartz and Jorgensen 2016) (see *CRISPR/Cas9-mediated transgenesis*).

Fluorescent proteins: Continuous improvement by directed evolution and rational engineering has resulted in a large palette of fluorescent proteins with a multitude of properties (*e.g.*, color, brightness, stability, and photo-conversion) (Rodriguez *et al.* 2017). A useful interactive overview is curated at FPbase.org (Lambert 2019). Most commonly used fluorophores in *C. elegans* have been optimized for worm expression (see below), as the properties of fluorescent proteins in worms can differ substantially from those *in vitro* or when expressed in other organisms. For example, in *C. elegans*, the S65C variant of GFP is preferred to eGFP (Fire *et al.* 1998), and mNeon is not as bright *in vivo* as predicted (Heppert *et al.* 2016). Validated fluorophores include GFP variants [Fire laboratory kits 1995, 1997, 1999, (unpublished reagents)], mCherry (Green *et al.* 2008), mCardinal (Chu *et al.* 2014), silencing-resistant GFP and mCherry (Frøkjær-Jensen *et al.* 2016; Zhang *et al.* 2018a), wrmScarlet (El Mouridi *et al.* 2017), Dendra2 (Gallo *et al.* 2010), fluorophore cassettes with selection markers (Dickinson *et al.* 2015, 2018; Schwartz and Jorgensen 2016), fluorophores coupled to auxin degradation (Zhang *et al.* 2015), and "split" superfolderGFP for amplified or intersectional expression (Noma *et al.* 2017; Hefel and Smolikove 2019; He *et al.* 2019). In addition to spectral properties, an important consideration when choosing a fluorescent protein is the time required for its maturation *in vivo*. The maturation time of different fluorescent proteins can vary substantially, and slowly maturing proteins may be unsuitable for applications such as transcriptional reporters to determine when a gene is first expressed. It is worth noting that fluorescent protein maturation times are often determined in bacteria or cells grown at 37°, and proteins can behave quite differently when expressed in worms. GFP matures fairly rapidly in *C. elegans*, and is a good choice if fast maturation is an important experimental consideration.

Large-scale transgenesis for systems biology: Larger plasmid collections for systems biology approaches ("omic" collections) that contain genome-scale cDNAs (Reboul *et al.* 2003), promoters (Dupuy *et al.* 2004), 3′ UTRs (Mangone *et al.* 2010), and curated transcription factors (Vermeirssen *et al.* 2007) are all commercially available (Figure 1B).

Designing transgenes for effective expression

Transgene structure is important for expression and should be designed with care. It is central to the design process that transgenes should incorporate an understanding of how transcription and translation of endogenous genes are regulated. To complement a previous review on transgene design (Boulin *et al.* 2006), here, we describe transgene features shown to increase expression in worms (Figure 1A).

Promoters: A number of well-characterized promoters can be used for specific expression in most tissues (*e.g.*, muscles, intestine, neurons, or germ line), and, in some cases, even individual neurons (for neuronal expression, see Hobert (2005)). In addition to commonly used canonical promoters, many expression patterns have been characterized by *in situ* hybridization (Shin-i and Kohara 1999) or promoter::GFP fusions (Hunt-Newbury *et al.* 2007).

The *C. elegans* genome is relatively compact (*C. elegans* Sequencing Consortium 1998), with most enhancers located within a few kilobases (kb) of a gene's start codon (McGhee and Krause 1997). This proximity of transcriptional elements makes it relatively simple to generate a short promoter that approximates the expression of the gene. There are several important caveats to this statement. First, although enhancers are frequently located in proximity to the start codon, a recent study on genome-wide DNA accessibility identified many putative distal enhancers with experimental verification of several enhancers located up to 10 kb away from the transcriptional start site (Daugherty *et al.* 2017). Second, early introns can contain enhancers (Okkema *et al.* 1993), which can sometimes be identified by visual inspection via the UCSC genome browser PhastCon comparative genomics track (Siepel *et al.* 2005). Third, ~70% of *C. elegans* genes are *trans*-spliced—a process by which a part of the 5′ mRNA is replaced in a splicing reaction with a 22 nt SL1 or SL2 spliced leader RNA (Blumenthal 2005). *Trans*-splice sites identified from global RNA sequencing data do not always map uniquely to the 5′ end of a gene—nearly 20% of genes contain multiple *trans*-splice sites, revealing the presence of alternative 5′ exons (Allen *et al.* 2011). The exact transcriptional start site (TSS) of many genes is therefore not well defined, although nascent RNA sequencing prior to *trans*-splicing has allowed identification of the TSSs for many genes (Chen *et al.* 2013b; Kruesi *et al.* 2013). Fourth, many genes (~15% of all genes) reside in operons where genes are cotranscribed from a single upstream promoter (Blumenthal 2005). Generally, the first gene in an operon is SL1-spliced while subsequent genes are SL2-spliced (Spieth *et al.* 1993) with the subsequent genes expressed at significantly lower levels (Cutter *et al.* 2009). Furthermore, promoter elements are frequently (~25%) located in intergenic sequences and generate "hybrid operons" where gene expression of later genes in the operon are driven by the combined influence of

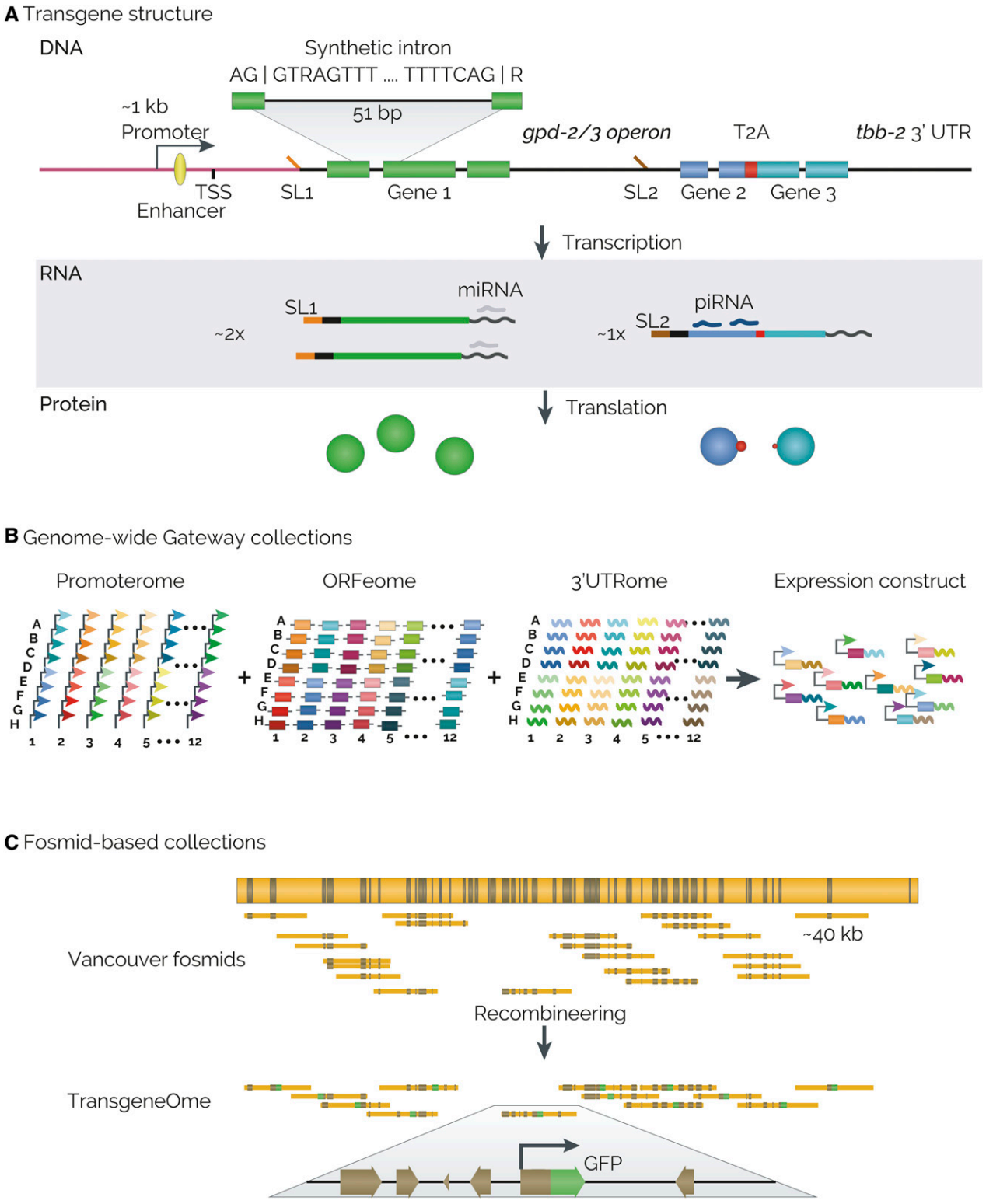

Figure 1 Multicopy transgenesis. (A) Schematic transgene with many common regulatory elements indicated. Promoters commonly contain all regulatory elements within 1 kb. The transcriptional start site (TSS) is frequently removed in the mature mRNA in a *trans*-splicing reaction. Short synthetic introns are routinely included to stimulate expression, and longer introns with PATCs can prevent silencing in the germ line (not shown). Bicistronic expression can be achieved by linking genes with an intergenic operon sequence (often from *gpd-2/3*) or a 2A viral peptide (here, T2A). Genes separated by an operon sequence are split into two RNAs during transcription, with the first gene expressed on average at twice the level of the second gene. Genes separated by 2A sequences are split into two proteins during translation and are expressed in a stoichiometric ratio with short peptides appended to both proteins. Small RNAs (*e.g.*, piRNAs and microRNAs) can repress transcription and translation by imperfect base-pairing with the primary sequence. Codon optimization is frequently used to improve transgene expression and remove small RNA homology. (B) Genome-wide plasmid

two promoters (Huang *et al.* 2007; Allen *et al.* 2011). For these reasons, it is challenging to generate promoter fusions that fully capture gene expression.

Introns: Intron splicing is integral to transcription and nuclear mRNA export across eukaryotes (Le Hir *et al.* 2003). So, although expression of transgenes with no introns (cDNAs) is possible, inclusion of introns substantially enhances expression in *C. elegans* (Okkema *et al.* 1993). *C. elegans* introns have a few unusual characteristics to be aware of: introns are relatively short (often <60 nt) and have an extended, highly conserved 3′ splice sequence (Blumenthal and Steward 1997). For practical reasons, fluorophores, genetically encoded sensors, and genome editing enzymes often include three short synthetic introns in their coding regions based on the initial design of LacZ and GFP constructs from the Fire lab vector kits (which additionally contained introns in the 5′ and 3′ UTRs).

Primary sequence: Gene synthesis is increasingly affordable and simple, allowing optimization of coding sequences for improved expression. The effects of codon optimization on gene expression are debated across different organisms (Quax *et al.* 2015); nonetheless, most synthetic transgenes used in *C. elegans* are optimized in a series of steps that minimize the use of rare codons, remove cryptic splice sites, incorporate short synthetic introns, and optimize ribosome binding (Green *et al.* 2008). A "one-stop" codon-adaptation algorithm for *C. elegans* changes codons to tune transgene expression levels (Redemann *et al.* 2011). Although it is unclear whether this algorithm accurately modulates expression across all tissues and stages, the algorithm is easy to use via a convenient web interface and is frequently used as a helpful first step to improve expression (Redemann *et al.* 2011).

3′ UTRs: The 3′ UTR frequently carries regulatory information in addition to terminating transcription and stimulating polyA synthesis. *C. elegans* 3′ UTRs have a median length of 140 nt but genes frequently use several 3′ UTR isoforms that may reflect tissue-specific regulation (Mangone *et al.* 2010). Importantly, 3′ UTRs are a platform for post-transcriptional regulation. MicroRNAs repress transcription and translation by binding to partially complementary sequences to regulate developmental timing (Lee *et al.* 1993; Wightman *et al.* 1993) and nervous system asymmetry (Johnston and Hobert 2003), for example. A second mode of regulation is via RNA-binding proteins that can bind to sequence motifs in the 3′ UTR to repress translation (Lee and Schedl 2006). Posttranscriptional regulation by 3′ UTRs appears to be the main mode of spatially regulating expression in the germ line (Merritt *et al.* 2008), so 3′ UTR choice is a critical consideration when designing any germline transgene.

For ectopic expression of transgenes, the main concern is to utilize a 3′ UTR that is not under strong regulation or poorly expressed. The *unc-54* 3′ UTR has frequently been used, partially because the short intron in the 3′ UTR improves expression (Fire lab vector kit 1995). More recently, the *tbb-2* 3′ UTR has been used for genome editing (Mos1 and Cas9) due to its presumed ubiquitous expression and general permissiveness for germline expression (Merritt *et al.* 2008). Modification of "neutral" 3′ UTRs has furthermore been used to generate 3′ UTR sensors that detect piRNAs (Bagijn *et al.* 2012; Lee *et al.* 2012) and RNA binding proteins (Theil *et al.* 2018).

Co-expression of genes: In some cases, it is useful to coexpress two transgenes from a bicistronic cassette without having a physical link between the two proteins. For example, the cellular expression of a transgene [*e.g.*, a transcriptional activator (Wei *et al.* 2012)] can be monitored by a coexpressed soluble fluorophore.

Two methods for generating bicistronic cassettes are frequently used in *C. elegans*. One method separates transgenes by an intergenic operon sequence, often *gpd-2/3*, that contains a 3′ UTR and an SL2 splice donor; the two transcripts are expressed from the same promoter but cleaved into two transcripts by *trans*-splicing (Blumenthal 2005). *Trans*-splicing has the advantage that no extraneous peptides are appended to coexpressed proteins, and operons are a "natural" transcriptional mechanism in worms. A potential drawback of using intercistronic operon sequences is that coexpressed genes are not necessarily expressed at the same level. For endogenous genes, downstream genes in an operon are expressed, on average, at half the level of the first gene (Cutter *et al.* 2009). A second method links two (or more) transgenes with viral 2A self-cleaving peptide sequences (*e.g.*, T2A). 2A peptide sequences are ~20 amino acids long, and result in the release of the upstream gene product during translation, including most of the 2A peptide (Luke *et al.* 2008). Several 2A peptides resulted in efficient cleavage in *C. elegans* and were used to coexpress up to five different genes (Ahier and Jarriault 2014). Proteins joined by 2A sequences are generally expressed stoichiometrically, but, in mammalian cell culture, some translation events resulted in translationally fused proteins or early termination before translation of the downstream protein (Liu *et al.* 2017b); these effects were infrequently observed in worms (Ahier and Jarriault 2014).

Reducing transgene silencing in the germ line

In contrast to somatic cells, genome-defense mechanisms act at multiple regulatory steps to strongly limit expression of

collections distributed in multi-well plates contain Gateway compatible promoters (Promoterome; Dupuy *et al.* 2004), genes (ORFeome; Reboul *et al.* 2003), 3′ untranslated regions (3′UTRome; Mangone *et al.* 2010), and transcription factors (not shown; Vermeirssen *et al.* 2007) which can be recombined to generate expression vectors. (C) The Vancouver fosmid collection (Perkins 2011) contains ~40 kb genomic clones covering most of the *C. elegans* genome. The fosmids can be modified by recombineering. The TransgenOme project generated a large collection with ~15,000 GFP-tagged genes (Sarov *et al.* 2012).

transgenes in the germline (Kelly *et al.* 1997). Inhibitory mechanisms include repressive chromatin, transcriptional and post-transcriptional control, and gene structure [*e.g.*, number of introns (Akay *et al.* 2017)]. Because of the redundant and overlapping nature of these pathways, specific considerations are necessary to ensure germline expression. Silencing mechanisms are partially copy-number dependent (Kelly *et al.* 1997), and act via RNAi pathways (Kim *et al.* 2005) and germline-specific piwi-interacting small RNAs (piRNAs) (Ashe *et al.* 2012; Lee *et al.* 2012). Expression from extrachromosomal arrays can occur at low levels in the germ line, and this method was historically used to rescue mutations in germline genes (*e.g.*, Varkey *et al.* 1995), but expression is typically lost within several generations (Kelly *et al.* 1997). Improved germline expression from extrachromosomal arrays is possible by coinjection of the transgene with complex genomic carrier DNA (Kelly *et al.* 1997), but these arrays are difficult to generate and maintain, although maintenance at 25° can reduce silencing (Strome *et al.* 2001). Low- or single-copy genomic insertions created by biolistic transformation (Praitis *et al.* 2001; Merritt 2010), MosSCI (Frøkjær-Jensen *et al.* 2008), or CRISPR/Cas9 (Dickinson *et al.* 2013) are more frequently expressed in the germ line but these insertions are also not fully resistant to silencing (Shirayama *et al.* 2012; Fielmich *et al.* 2018).

Recently, two complementary approaches were shown to minimize germline silencing, as described in the following sections.

Modifying non-coding DNA: This approach is based on the observation that endogenous genes expressed in the germ line from repressive chromatin environments are highly enriched for a pervasive, noncoding DNA structure called Periodic A_n/T_n Clusters (PATCs) (Fire *et al.* 2006). Incorporating PATC-rich DNA into introns of transgenes largely eliminated epigenetic germline silencing of single-copy fluorophore insertions (Frøkjær-Jensen *et al.* 2016; Zhang *et al.* 2018a) and CRISPR-tagged endogenous genes (Fielmich *et al.* 2018). The mechanistic basis for this effect is unknown, but, presumably, transgenes resemble endogenous genes, and bypass silencing pathways tuned to detect foreign DNA.

Optimizing the coding sequence: Two codon optimization algorithms were specifically developed to improve germline expression. One algorithm removes homology to piRNAs (Zhang *et al.* 2018a), a class of small RNAs that negatively regulates expression in the germ line (Ashe *et al.* 2012; Bagijn *et al.* 2012). The algorithm combines a recent understanding of piRNA targeting rules (Zhang *et al.* 2018a) with a catalog of all known expressed piRNAs (Batista *et al.* 2008; Gu *et al.* 2012). Using this algorithm, *gfp* and *mCherry* transgenes became significantly more resistant to silencing in extrachromosomal arrays, and an integrated codon-optimized Cas9 improved genome editing efficiency (Zhang *et al.* 2018a). Transgenes can be optimized with the piRNA-based algorithm using a simple web interface (Wu *et al.* 2018). A second approach ("Dickinson algorithm") improves germline expression by recoding transgenes using 12 nt "words" enriched in germline-expressed genes (Fielmich *et al.* 2018). This algorithm also allowed stable germline expression of most tested transgenes, and a simple web interface to recode sequences is publicly accessible (Fielmich *et al.* 2018).

Incorporating PATCs, optimizing codons, and removing piRNA binding sites synergistically improve germline expression (Frøkjær-Jensen *et al.* 2016; Fielmich *et al.* 2018; Zhang *et al.* 2018a). If possible, combining the three optimization steps is likely to improve germline transgene expression the most. Recoding transgene sequences is the most straightforward approach as inclusion of PATCs in transgenes is relatively complicated and increases their size substantially. It should also be noted that the impact of these manipulations on expression outside of the germ line has not been extensively tested and could have adverse effects, although preliminary experiments suggest this is not the case (Frøkjær-Jensen *et al.* 2016).

Recombineering fosmids to retain regulatory information

Some concerns caused by using arrays, such as the omission of nearby regulatory information or cross-talk between injected plasmids, can be mitigated by using larger (~40 kb) fosmid transgenes, predominantly based on the Vancouver *C. elegans* fosmid library (Perkins 2011) (Figure 1C). Large DNA fragments can be manipulated by "recombineering," a method based on homologous recombination in bacteria (Zhang *et al.* 1998). Several recombineering protocols for *C. elegans* transgenesis have been developed (Dolphin and Hope 2006; Sarov *et al.* 2006; Tursun *et al.* 2009). Each of these methods relies on inducing homologous recombination between a fosmid and a "cassette" with flanking DNA homology arms by expressing a recombinase (lambda Red). Dolphin and Hope (2006) demonstrated the benefits of fosmids by tagging three genes within fosmids with fluorescent proteins and validating expression patterns. Sarov *et al.* (2006) developed a highly scalable recombineering protocol that was initially used for the even larger *C. briggsae* bacterial artificial chromosomes (BACs). This method was subsequently applied to *C. elegans* fosmids, resulting in a comprehensive genome-scale resource ("TransgeneOme") containing ~15,000 C-terminally GFP-tagged genes (Sarov *et al.* 2012) (Figure 1C). Another protocol for manipulating individual fosmids was developed by Tursun *et al.* (2009) along with a set of useful cassettes containing various fluorophores for C-, N-, and bi-cistronic gene tagging (available at Addgene). Although CRISPR/Cas9 techniques have made fosmid engineering less crucial, fosmids continue to be a useful resource for rescuing mutant phenotypes and for transgenesis with unusually large genes, *e.g.*, to identify expression patterns of microRNAs (Drexel *et al.* 2016). Additionally, no other such large-scale resource of high-quality GFP-tagged transgenes for *C. elegans* currently exists (Sarov *et al.* 2012).

Multicopy Transgenesis

For over 35 years, germline injection of DNA to generate multicopy extrachromosomal arrays has been the workhorse

of *C. elegans* transgenesis (Kimble *et al.* 1982; Stinchcomb *et al.* 1985; Fire 1986; Mello *et al.* 1991; Mello and Fire 1995). In contrast to most other genetically tractable systems, injection of either plasmids or linear DNA fragments into the *C. elegans* germ line results in the formation of hereditary extrachromosomal DNA structures estimated to contain >100 plasmids (Mello *et al.* 1991). Observing array formation and inheritance by microscopy, Yuen *et al.* (2011) detected 1–2 arrays composed of ~1 Mb of DNA per mitotic cell, and showed that arrays were inherited at higher frequency in embryonic cells than during mitotic proliferation and meiotic segregation. Detailed experiments to understand episomal DNA as artificial chromosomes and *de novo* centromere formation determined that arrays are formed after fertilization by a combination of homologous recombination and nonhomologous end-joining (NHEJ) (Yuen *et al.* 2011; Zhu *et al.* 2018). *In vivo* recombination of injected overlapping DNA fragments has been exploited to generate repair templates for CRISPR/Cas9 gene editing (Paix *et al.* 2016).

Despite some limitations, such as variable expression and silencing in the germ line, extrachromosomal and integrated arrays have been invaluable tools for scientific discovery using *C. elegans*. More recent and precise transgenic methods based on transposons and nucleases will be covered in the following sections, but arrays continue to be very useful tools for certain applications. These include easy testing of transgenes for use in manipulating endogenous genes or for overexpressing transgenes. Several published reviews and instructional videos cover methods to generate transgenic worms by injection (Mello and Fire 1995; Evans 2006; Berkowitz *et al.* 2008), and biolistic transformation (Hochbaum *et al.* 2010; Praitis and Maduro 2011; Schweinsberg and Grant 2013). Here, we discuss some considerations for generating extrachromosomal arrays and describe novel methods for site-specific array integration.

When to use multicopy transgenes

Multicopy transgene arrays are simple to generate, maintain, and detect through phenotypic markers; DNA injected at sufficiently high concentration together with a screenable cotransformation marker will readily form arrays. The following sections list some typical examples of when arrays are useful.

Arrays contain several hundred copies of the injected plasmids (Stinchcomb *et al.* 1985) and are frequently expressed at high levels compared to low-copy biolistic (Praitis *et al.* 2001), or single-copy (Frøkjær-Jensen *et al.* 2008; Dickinson *et al.* 2013) transgene integrants. Standard, tissue-specific promoters have typically been characterized with extrachromosomal arrays, and their expression from low-copy integrants may not be sufficiently high to enable reliable detection, *e.g.*, on a fluorescence dissection microscope. In cases where high expression is necessary, *e.g.*, when fluorescence is used as a marker for structural chromosome changes (balancers), when cellular structures such as nuclei and the plasma membrane are used as cytological markers, or when genetically encoded sensors are used to measure cellular activity, there is currently no good alternative to arrays.

Arrays solve what is a significant problem for gene editing in many other biological systems: how to transiently deliver DNA for gene expression (*e.g.*, Mos1 transposase or Cas9 + sgRNAs) and templates for genome editing (*e.g.*, a source of Mos1 transposons or DNA repair templates). The high copy number of arrays and their heritable, yet transient, nature should, in principle, allow highly multiplexed gene editing and transcriptional control.

Extrachromosomal transgenes are particularly useful for an initial determination of the cellular site of action by using tissue-specific promoters to rescue mutant phenotypes, or by mosaic analysis where unequal transgene segregation and rescue are compared (Yochem and Herman 2003). Because of overexpression and heterogeneous expression, as well as the difficulties in including all relevant regulatory elements important for endogenous expression in a transgene (see *C. elegans Genes and Transgene Structures*), extrachromosomal transgenes should generally not be trusted "*as the sole means to determine the physiological expression pattern of an endogenous gene*" as strongly cautioned by Fire and colleagues (Fire laboratory vector kit documentation 1995).

Experimental considerations

An initial consideration is whether to inject linear DNA fragments or plasmids, as both readily generate hereditary extrachromosomal arrays by homologous recombination and nonhomologous end-joining (Mello *et al.* 1991; Yuen *et al.* 2011). Some of the more common considerations an experimenter will need to decide on before injection include:

Carrier DNA and transformation efficiency: DNA injection most frequently leads to animals that are transgenic in the first generation (F1), but that fail to generate stable lines with heritable arrays. Increased DNA concentration stimulates stable array formation with most injections using a minimum concentration of 100 ng/μl, resulting in inheritance in ~10% of F1 transgenic animals (Mello *et al.* 1991). Frequently, the transgene comprises only a small fraction of the injection mix (~5–10 ng/μl are common concentrations); the remaining required DNA concentration can be a combination of selection markers and simple stuffer DNA, *e.g.*, a DNA ladder for gel electrophoresis (with the dye omitted). Complex *C. elegans* genomic DNA can also be used as carrier DNA, which increases germline expression (Kelly *et al.* 1997) (see *C. elegans Genes and Transgene Structures*). However, in practice, genomic DNA is rarely used since stable arrays are not readily formed, and the advantages of complex DNA arrays are limited.

Selection markers: A variety of selection markers to identify and select for arrays have been developed and described in detail elsewhere (Praitis and Maduro 2011). Some markers confer a visible and dominant phenotype [*e.g.*, *rol-6* (Kramer *et al.* 1990)) whereas others rescue a mutant phenotype (*e.g.*, *lin-15*(ts) (Clark *et al.* 1994) or *unc-119* (Maduro and Pilgrim 1995)]. Bright co-injected fluorescence markers expressed in the pharynx, body wall muscle, or nervous system can also be

used to identify and select, either for or against, transgenic animals under fluorescence illumination (*e.g.*, Frøkjær-Jensen *et al.* 2008). Fluorescence markers are the preferred method for detecting arrays when their presence needs to be scored in embryos, as most markers producing visible phenotypes (*e.g.*, *rol-6* or *unc-119* rescue) are only scorable in larvae and adults. Positive selection markers using resistance to the antibiotics puromycin (Semple *et al.* 2010), neomycin (Giordano-Santini *et al.* 2010), and hygromycin (Radman *et al.* 2013) enable transgenesis in wildtype animals. Visible markers require continuous manual selection, whereas mutant rescue and antibiotics facilitate propagation; however, the selection may influence the phenotype of transgenic animals.

Linear DNA fragments: It is relatively easy, and sometimes preferable, to generate PCR products for injection. This is true for rescue experiments where a genomic locus can be rapidly amplified or for a popular "PCR stitching" method where promoters and fluorophores are fused by PCR (Hobert 2002). One drawback of linear DNA is that the injected DNA is nonclonal, which may mask mutations or misassembly from fragments stitched together by PCR.

Integrating arrays

Integrated, multicopy arrays are, in some cases, preferable to extrachromosomal arrays because integrants are not readily lost during mitosis or meiosis, and show less variable expression (Evans 2006). Spontaneous DNA integration is stimulated by injection into oocytes, as opposed to the much larger syncytial gonad arms (Fire 1986), or by co-injecting oligos (Mello *et al.* 1991), but, for practical reasons, these approaches have not been widely adopted. Instead, many laboratories have utilized a two-step method based on first generating animals with stable extrachromosomal arrays, and subsequent integration into random chromosomal locations using gamma (Mello and Fire 1995) or UV irradiation (Evans 2006). Integration by irradiation is relatively efficient when using selection or easily scored cotransformation markers, and animals with stable and high levels of somatic expression can be recovered. The disadvantages of this method include potential mutations and genome rearrangements resulting from irradiation and the necessity to map integration sites.

Several recent papers describe improved methods to integrate arrays. The Mitani laboratory, known for generating knock-out strains (*C. elegans* Deletion Mutant Consortium 2012) and genetic balancers (Iwata *et al.* 2016; Dejima *et al.* 2018) for the community, has also recently developed two methods for integrating plasmids. One method utilizes trimethylpsoralen mutagenesis and UV irradiation coupled with positive (*vps-45* rescue) and negative (benomyl sensitivity) selections to generate single or low-copy integrations at random locations (Kage-Nakadai *et al.* 2012). A second method utilizes CRISPR/Cas9 to cut the array and the genome at defined sequences, resulting in site-specific integration of arrays (Yoshina *et al.* 2016). This second approach was generalized by cutting arrays within a common antibiotic selection marker (ampicillin) that has no homology to the insertion site, showing that arrays already in use can be integrated into defined locations. Finally, Noma and Jin (2018) recently developed a one-step method to generate integrated arrays directly from injection. This method relies on inducing DNA double-strand breaks soon after injection (6 hr) by light activation of a germline-expressed mini singlet oxygen generator (miniSOG), thereby bypassing the need to first establish array lines and subsequently generate insertions.

All three methods (UV/trimethylpsoralen, CRISPR/Cas9, and miniSOG) can integrate arrays; however, CRISPR/Cas9 has the substantial advantage relative to UV or miniSOG that secondary mutations are limited and the integration site can be selected. These methods fill an important gap between extrachromosomal arrays and single-copy insertions when high somatic expression is required and should, for example, allow the creation of well-characterized driver and effector lines for FLP, CRE, or cGAL mediated control of expression (see *Conditional Gene and Protein Inactivation* and *Bipartite Systems for Temporal and Spatial Control of Expression*).

Transposon-Mediated Genetic Engineering

Transposons are mobile genetic elements that can be experimentally controlled to induce double-strand DNA breaks (Boulin and Bessereau 2007). Active, endogenous transposons contain DNA sequences (inverted terminal repeats) flanking an enzyme (the "transposase") that catalyzes transposition; endogenous transposons are frequently under stringent repressive control (Bessereau 2006). In contrast, experimentally controlled transposons do not encode a functional transposase and instead often carry a transgene flanked by the inverted terminal repeats. Transposition is controlled by using a non-native transposon and supplying the transposase in *trans* (commonly from a co-injected plasmid). In several genetically tractable systems, fruit flies, in particular, transposons carrying a genetic marker have been extensively used for random mutagenesis and gene identification [reviewed in Kanca *et al.* (2017)]. With knowledge of the transposon sequence, it is relatively simple to identify sequences adjacent to the inserted transgene using PCR and Sanger sequencing, particularly if only one copy of the transposon is present in the genome. Here, for an important historical context, we briefly describe how endogenous (Tc1) transposons were used to understand gene repair pathways, and, thereby, form the background for our current ability to manipulate endogenous genes. Subsequently, we describe the use of a transposon from *Drosophila mauritiana* (Mos1) for inserting transgenes into well-defined or random genomic locations.

Common uses for transposon-mediated transgenesis

Some common situations when transposons, especially Mos1, can be advantageous for transgenesis are described in the following sections.

Expressing transgenes in the germ line: Transgenes located on most repetitive arrays are silenced in the germ line (Kelly *et al.* 1997) (see *C. elegans Genes and Transgene Structures*). Insertion of single-copy transgenes into well-defined genomic locations allows stable germline expression of most genes and can be used to identify regulators of germline expression.

Comparing transgenes: In cases where transgenes are used to determine structure–function relationships, *e.g.*, by mutating individual amino acids or modifying whole domains, it is beneficial to maintain a constant (single) transgene copy number and genomic environment so that different gene modifications can be compared.

Studying effects of genome organization: The genome is divided into large- and small-scale chromatin environments that can influence gene expression (*e.g.*, Rockman and Kruglyak 2009; Gu and Fire 2010; Liu *et al.* 2011). For instance, chromosome centers are enriched for permissive chromatin marks, arms are enriched for repressive marks, and the X-chromosome is silenced in the germ line and dosage-compensated in somatic cells (Kelly *et al.* 2002; Meyer 2005). Position-effect variegation screens have been used successfully in flies to identify chromatin modifiers (Girton and Johansen 2008), and similar screens have recently become possible in worms (Frøkjær-Jensen *et al.* 2014, 2016).

Developing methods for endogenous gene editing

In yeast, plasmid DNA can lead to integration into homologous genomic regions (Hinnen *et al.* 1978)— a process that is stimulated by linear DNA with free ends (Orr-Weaver *et al.* 1981). In flies, injection of linear DNA is not enough to efficiently stimulate transformation at endogenous locations (Gloor *et al.* 1991). Instead, repair from injected plasmids (Gloor *et al.* 1991) or oligonucleotides (Banga and Boyd 1992) is stimulated by generation of free DNA ends via excision of a genomic *P*-element. Alternatively, an elegant but technically difficult approach allowed homologous editing in flies by *in vivo* excision and linearization of a circular, genomic repair template (Rong and Golic 2000). The unifying theme is that DNA with free ends stimulates the DNA repair pathway; however, until recently (see *CRISPR/Cas9-mediated transgenesis* – CRISPR/Cas9) it has been exceedingly difficult to generate targeted DNA breaks at arbitrary genomic locations.

In worms, Tc1 was the first transposon to be used widely for transposon-derived mutations (Korswagen *et al.* 1996; Plasterk and van Luenen 1997). Tc1 is an endogenous DNA transposon, which transposes by a "cut and paste" mechanism, leading to transposon excision and a double-strand DNA break at the original locus before insertion at a new locus. The Bristol *C. elegans* strain carries ~30 Tc1 elements, but Tc1 normally transposes infrequently due to germline defense mechanisms that silence transposons (*e.g.*, Vastenhouw *et al.* 2003). Strains with impaired silencing machinery ("mutators") or natural isolates such as Bergerac, have high transposition frequencies, and can be used for transposon-mediated mutagenesis screens (Moerman and Waterston 1984). Tc1 insertions in well-defined loci with clear phenotypes were used to develop the first methods for efficient endogenous gene editing (Figure 2A). The first examples generated double-strand DNA breaks with Tc1 excision and inserted short polymorphic DNA templated from extrachromosomal arrays (Plasterk and Groenen 1992), or created random nontemplated indels (insertion, deletion, or a combination) by nonhomologous end-joining (Zwaal *et al.* 1993). Gene editing was not confined to short, polymorphic DNA, as Barrett *et al.* (2004) used Tc1 excision to demonstrate that a larger DNA fragment (*e.g.*, a fluorophore) could be copied into the genome by endogenously tagging *frm-3* with *gfp*. These two processes (templated and untemplated gene edits) are still the basis for most targeted gene editing protocols in *C. elegans*; the following 35 years have focused on how to more efficiently generate double-strand DNA breaks and identify gene edits.

Mos1-mediated modification of endogenous loci

Developing methods to control the non-native Mos1 transposon greatly advanced our ability to engineer the *C. elegans* genome. Mos1 elements could be inserted into the genome from extrachromosomal arrays and re-excised to create targeted double-strand DNA breaks (Bessereau *et al.* 2001). However, the promise of using Mos1 as a general tool for mutagenesis and for uncovering regulatory elements (*e.g.*, "enhancer traps") was largely unfulfilled for practical reasons (Bessereau *et al.* 2001; Williams *et al.* 2005; Boulin and Bessereau 2007). Mos1 transposition frequency was relatively low, and only small transgenes (<400 bp) were compatible with transposition, which ruled out transposition with "cassettes" to select for insertions or to identify expression patterns. Instead, Mos1 has predominantly been used to modify the genome using several techniques derived from Mos1 excision-induced transgene-instructed gene conversion (mosTIC)—a method initially used to generate small deletions and insertions (Robert *et al.* 2008) (Figure 2A). Gene editing required a nearby Mos1 transposon (within a few kilobases) (Robert *et al.* 2008). This significant limitation was addressed by the NemageneTag project, a collaboration of six European laboratories to develop Mos1-based techniques and an extensive collection (~13,000) of transposon insertions (Duverger *et al.* 2007; Bazopoulou and Tavernarakis 2009; Vallin *et al.* 2012). A shortcoming of the collection was that pools of Mos1-containing animals had been frozen with no selection markers, often making it difficult to recover individual Mos1 insertions. With the advent of CRISPR/Cas9, the NemageneTag collection lost its relevance and strain distribution was discontinued in 2017. Regardless, the NemageneTag resource was a significant step forward that enabled endogenous gene editing, generation of large deletions (Frøkjær-Jensen *et al.* 2010), and sites for transgene insertion (Frøkjær-Jensen *et al.* 2008).

Inserting single-copy transgenes using Mos1 (mosSCI and miniMos)

Variable expression, overexpression, and germline silencing preclude the use of multicopy arrays for some types of

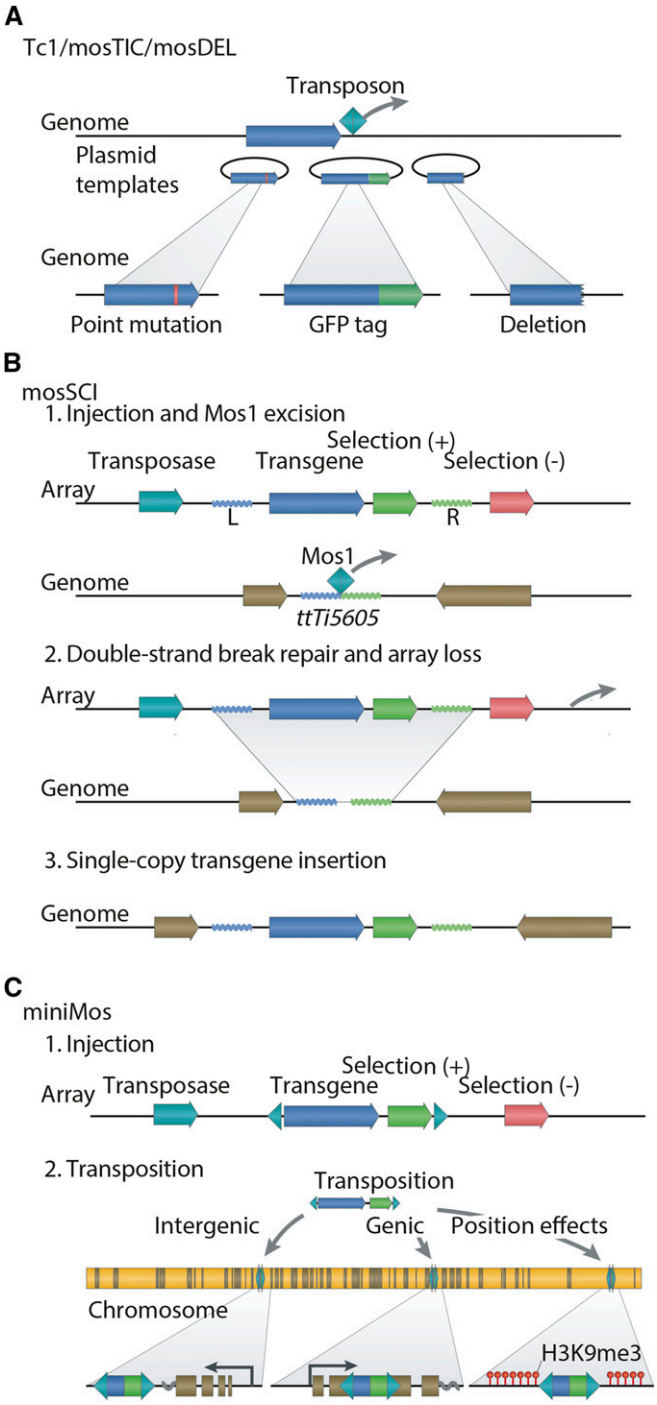

Figure 2 Transposon-mediated transgenesis. (A) Transposon (Tc1 or Mos1) excision enables gene editing near the double-strand breakpoint generated by transposition. Edits include single-basepair mutations, tagging endogenous genes with GFP, or making targeted deletions. (B) Mos1 excision from a "safe landing site" (here the frequently used *ttTi5605* site) allows insertion of single-copy transgenes at the DNA breakpoint guided by left (L) and right (R) homologous regions. Positive (typically *unc-119*(+) or antibiotic resistance] and negative selection (typically, red fluorophores and an inducible toxin) facilitate isolation of genomic insertions and loss of extrachromosomal arrays generated during injection. (C) miniMos transposition allows random genome insertion of single-copy DNA embedded within a transposon. Transposons insert into genic and intergenic regions, with some regions under strong repressive control by histone modifications, such as H3K9me3.

experiments, *e.g.*, determining how small RNAs regulate expression (Lehrbach *et al.* 2009), using transgenes to determine structure–function relationships (Hollopeter *et al.* 2014), or studying dosage-dependent processes (Barkoulas *et al.* 2013). To facilitate such experiments, Frøkjær-Jensen *et al.* (2008) developed a method to insert single-copy transgenes into a "safe harbor" genomic landing site after Mos1 excision (Mos1-mediated single copy insertion, or MosSCI) (Figure 2B). MosSCI relies on a set of standardized vectors targeting a few insertion sites, as well as positive (*e.g.*, *unc-119*) and negative [*e.g.*, mCherry, *peel-1* toxin, or histamine-induced paralysis (Pokala *et al.* 2014)] selection markers to identify insertions. Using single-copy insertions, it was possible to express proteins at near-native levels and achieve stable germline expression (Frøkjær-Jensen *et al.* 2008), although subsequent experiments showed that some single-copy insertions are stochastically silenced in the germ line (Lee *et al.* 2012). Transgene insertions can be generated at relatively high efficiency in a single-step injection (Frøkjær-Jensen *et al.* 2012). Development of antibiotic selection methods and MosSCI-compatible vectors expanded the utility of MosSCI by allowing insertion into any genetic background (Giordano-Santini *et al.* 2010; Semple *et al.* 2010). Initially, every transgene insertion required cloning into a new targeting vector—a cumbersome process for generating lines with several transgenes. To solve this, a subsequent iteration of the method generated a set of "universal" insertion sites, where many (13) genomic insertion sites across chromosomes and different chromatin environments could be targeted by a single vector (Frøkjær-Jensen *et al.* 2014, 2016). However, transgene insertion into these universal insertion sites is generally more difficult, possibly because of duplicated Mos1 sequences at the landing sites (C. Frøkjær-Jensen, unpublished results).

In some cases, random insertion of single-copy DNA is desirable. For example, random insertion of *P*-elements has been used extensively in fruit flies to disrupt genes (Spradling *et al.* 1999), identify enhancers (Bellen *et al.* 1989), and study chromatin structure (Wallrath and Elgin 1995). A 2-kb transgene abolished transposition of the native Mos1 transposon (Bessereau *et al.* 2001), but *in vitro* assays suggested that sequence modifications to the transposon might allow Mos1 to carry cargo (Casteret *et al.* 2009). Equivalent modifications significantly improved *in vivo* transposition of Mos1 with cargo in *C. elegans* (Frøkjær-Jensen *et al.* 2014). Insertions can be generated at high frequency, often with several independent insertions from a single injected animal, and the modified Mos1 ("miniMos") can carry large cargo, including fosmids (~40 kb) (Frøkjær-Jensen *et al.* 2014) (Figure 2C). Because miniMos transposition is random, the insertions may disrupt endogenous genes and need to be mapped by molecular techniques ("inverse PCR"). However, as a result of the high transposition frequency and easy identification of the insertion site, it was possible to generate a relatively large number of strains (~300) with bright fluorescent markers at specific genomic locations, which can facilitate strain construction in genetic crosses (see curated list at

wormbuilder.org). In one example, random miniMos insertions were used to characterize somatic and germline position-effect variegation (Frøkjær-Jensen *et al.* 2016) that follow patterns corresponding to genome compartmentalization based on recombination frequency (Rockman and Kruglyak 2009) and chromatin modifications (Liu *et al.* 2011).

Which transposon approach to use and future perspectives

Although Tc1 transposition and Mos1-mediated editing of endogenous genes form the basis for recent transgenic methods, these methods are no longer in use as they have been superseded by the rapid development of CRISPR/Cas9-mediated gene editing (see *CRISPR/Cas9-Mediated Transgenesis*). MosSCI remains useful for generating transgenic lines expressing "sensor" DNAs, such as fluorophores under the regulation of elements in the 3′ UTR. Universal insertion sites targeted by a single transgene, and habit, are probably the only reason to continue using MosSCI; CRISPR/Cas9 can generate insertions at safe landing sites at a similar frequency (Dickinson *et al.* 2013) but does not require Mos1 elements and the potential effects arising from second-site Mos1 insertions. Random single-copy insertions with Mos1 (miniMos) is perhaps the easiest of the techniques and has the advantage that many insertions can be generated rapidly. The most obvious use for the miniMos transposon is "gene-traps" or "enhancer-trap" experiments, which cannot be accomplished by chemical mutagenesis or with current CRISPR/Cas9-based approaches. Until now, no such experiments have been described, probably because it is difficult to envision generating a large-scale miniMos collection by injection alone and mapping by Sanger sequencing. Efforts to allow efficient transposition from arrays, coupled with next-generation sequencing, may enable such collections or large-scale screens in the future.

CRISPR/Cas9-Mediated Transgenesis

Common uses for CRISPR/Cas9

The advantages of editing genes at their endogenous loci have long been appreciated. Important regulatory information contained in enhancers, gene structure, or genomic location is frequently lost from transgenes manipulated *in vitro* and reintroduced into the organism. As described in the section on transposons, manipulating endogenous loci requires a nearby double-strand break. The repurposed CRISPR/Cas9 bacterial immunity system is the current method of choice for genetic engineering due to the ease of generating targeted double-stranded breaks. The first *in vitro* demonstration of DNA cleavage guided by a chimeric, single guide RNA targeted a plasmid (which, curiously, encoded the standard Fire laboratory *C. elegans* GFP). Rapidly thereafter, Cong *et al.* (2013) and Mali *et al.* (2013a) demonstrated that CRISPR/Cas9 could be used to edit the genome of human and murine cell lines. In the blink of an eye, CRISPR/Cas9 has enabled gene editing in most commonly used cell lines, commercial crops, and genetic model organisms (reviewed in Mali *et al.* 2013b; Zhang *et al.* 2014; Doudna and Charpentier 2014). The first demonstration of Cas9 activity in *C. elegans* generated mutations by NHEJ after expressing Cas9 and sgRNAs from plasmid templates (Friedland *et al.* 2013). Within a year, many laboratories explored editing efficiency by varying the source of Cas9 (DNA, mRNA, and protein), sgRNA (transcribed *in vivo* from the U6 promoter or synthesized *in vitro*), and repair templates (plasmids and oligos), as well as methods to detect edits (phenotypes, fluorescent markers, antibiotics, and drugs) (Chen *et al.* 2013b,c, 2014; Dickinson *et al.* 2013; Katic and Großhans 2013; Lo *et al.* 2013; Tzur *et al.* 2013; Waaijers *et al.* 2013; Arribere *et al.* 2014; Kim *et al.* 2014; Liu *et al.* 2014; Paix *et al.* 2014; Shen *et al.* 2014; Zhao *et al.* 2014). A recent, comprehensive study by Farboud *et al.* (2019) systematically optimized Cas9/CRISPR editing and characterized repair processes and timing.

Targeting any arbitrary base is now limited mainly by requirements for unique sequences and protospacer adjacent motifs (PAMs)—a limitation that has been reduced by re-engineering Cas9 (Kleinstiver *et al.* 2015; Bell *et al.* 2016; Hu *et al.* 2018; Nishimasu *et al.* 2018) and identification of alternative endonucleases, such as Cpf1 (Zetsche *et al.* 2015; Ebbing *et al.* 2017), which use other PAMs. Furthermore, targeting a "nuclease-dead" Cas9 (dCas9) that does not create DNA breaks to specific genomic locations enables many perturbations; *e.g.*, base editors can revert mutations, transcriptional activators (CRISPRa) and inhibitors (CRISPRi) can alter gene expression, chromatin readers and writers can change the epigenome, and fluorophores can be targeted to genomic regions for dynamic imaging of chromosomal domains (reviewed by Dominguez *et al.* 2016).

We refer readers to several recent reviews covering the use of CRISPR/Cas9 for genome editing in *C. elegans* germ cells (Xu 2015; Chen *et al.* 2016; Dickinson and Goldstein 2016; Farboud 2017), in somatic cells (Li and Ou 2016), and in other nematode species (Ward 2015b; Sugi 2016; Zamanian and Andersen 2016; Lok *et al.* 2017). In particular, a comprehensive WormBook chapter by Dickinson and Goldstein (2016) discusses practical considerations of various CRISPR/Cas9 methodologies, which are rapidly evolving. We note that other nucleases, such as zinc finger nucleases (Kim *et al.* 1996) and transcription activator-like effector nucleases (TALENs) (Boch *et al.* 2009; Moscou and Bogdanove 2009), had been used to develop nuclease-mediated genome editing protocols in *C. elegans* and related nematodes (Wood *et al.* 2011; Lo *et al.* 2013), but that CRISPR/Cas9 has rendered these nucleases mostly obsolete, except for specialized cases such as manipulating mitochondrial DNA. For completeness, in this section, we briefly outline two commonly used CRISPR/Cas9 methodologies and discuss potential future applications of CRISPR-based genome editing in *C. elegans*.

When to use

The following sections list some common uses for CRISPR/Cas9.

Generating specific designer alleles: Traditional alleles generated by mutagenesis, including loss-of-function and dominant gain-of-function mutations, often carry linked passenger mutations that are not easily removed even with extensive outcrossing. Increasingly, new and "classic" alleles are generated *de novo* with Cas9, presumably with fewer unintended background mutations (*e.g.*, Chiu *et al.* 2013; Paix *et al.* 2014). Standard out-crossing and validation is good practice as no comprehensive evaluation of off-target effects has been undertaken for *C. elegans*.

Tagging proteins within native genome context: Endogenous genes tagged with fluorophores, affinity tags, or degron tags retain all regulatory elements and can be used for determining cellular expression patterns, for protein purification, and for elucidating functional roles. However, it should be stressed that introducing a tag may impair gene regulation or function in many ways. In addition to direct effects of the tag on protein function or localization (*e.g.*, Noma *et al.* 2017), sequences encoding the tag could affect splicing, transcriptional initiation, or silencing by small RNAs. In some cases, interference will be immediately apparent if the gene's loss-of-function phenotype is known. However, more subtle effects may not be obvious. Currently, there is no universally accepted methodology to validate the functionality of endogenously tagged genes.

Inserting transgenes with a minimal mutational load: Inserting transgenes at safe landing sites is efficiently mediated by nucleases, including single-copy insertions or extrachromosomal array integration. Using a nuclease avoids mutations induced by transposable elements or chemical mutagens. This approach is particularly useful in nematode species that have less well-developed transgenic tools, as protein and synthetic single guide RNAs (sgRNAs) injection requires minimal or no species-specific adaptations.

Engineering structural genomic variants: The efficiency of generating DNA breaks with CRISPR/Cas9 has enabled researchers to engineer not only changes to specific genes but also large-scale genome rearrangements such as chromosome inversions and translocations. Such rearrangements are useful tools for a variety of experiments; in particular, they frequently suppress meiotic recombination over large regions of the genome and are thus useful as "balancers" for mutations that strongly reduce fitness or fertility. Several groups have used this approach to engineer novel balancers with desirable properties, including precisely defined breakpoints, an absence of background mutations, and readily scored markers.

Experimental considerations

CRISPR-based genetic engineering requires a unique ~20 nt binding sequence and a proximal adjacent motif (PAM). To make specific edits, an exogenous template with homology to the DNA breakpoint is also necessary. Plasmid DNA, PCR product, or ssDNA oligos can be used as repair templates.

Proximity of cleavage site to desired genome modification: Oligo-mediated repair is limited to the length of the oligo, and even for long oligos (~200 nt), repair mostly occurs within 10 bp of the cleavage site (Farboud *et al.* 2019). Plasmid-based templates with long (~1 kb) homology regions enable repair much further from the cleavage site, but point mutations are harder to isolate, and unintended structural variants in the inserted DNA are relatively common (Dickinson *et al.* 2013). Cas9 variants or orthogonal CRISPR nucleases, *e.g.*, Cpf1 that recognize different PAMs, allow more flexibility in sgRNA design near the intended mutation.

Tagging endogenous genes can interfere with function: Tagging a gene at its endogenous locus may perturb the gene, as described above. Identifying localization signals, *e.g.*, nuclear localization signals or lipid membrane insertion signals, adding flexible linkers between tags and the coding sequence, and determining protein conservation by comparison with orthologs can minimize the effects of tagging. C-terminal tags commonly label all isoforms of a gene since the stop codon is generally shared. In contrast, N-terminal tags can be used for both transcriptional and translational fusions, but because of the frequent occurrence of alternative 5′ ends (see *C. elegans Genes and Transgene Structures*), often do not tag all isoforms. In contrast to common practice, tagging genes internally at splice junctions appears to cause the least overall perturbation, at least in flies, as introns frequently separate functional domains (Kanca *et al.* 2017).

Expression in some tissues may not tolerate foreign tags: Endogenous tagging does not guarantee full or persistent germline expression. For example, a set of relatively small tags (ePDZ and LOV), as well as fluorescent proteins, tagged to mitotic spindle proteins consistently resulted in strong germline silencing (Fielmich *et al.* 2018); transgene design can reduce silencing (see *C. elegans Genes and Transgene Structures*).

CRISPR/Cas9 methodologies

As this review is focused primarily on transgenes, here, we will focus mainly on considerations for inserting larger DNA fragments (>1 kb), *i.e.*, transgenes that cannot currently be encoded by oligos. Two or three components are necessary for CRISPR/Cas9 experiments: the Cas9 nuclease, an sgRNA to guide the nuclease, and an optional DNA repair template. Although many permutations of CRISPR/Cas9 editing have been developed, two main distinctions separate protocols: Ribonucleoprotein (RNP) based protocols use mainly commercially available reagents (Cas9 protein, synthetic RNA oligos, and single-strand DNA oligos for repair or amplification of fluorophores), and identify relatively frequent gene edits by fluorescence or PCR-based screening. Plasmid-based protocols express Cas9 and sgRNAs from injected plasmids and build repair templates that include selection markers to identify relatively rare insertions. Each protocol has distinct advantages and disadvantages that we discuss in the next

two subsections. In the following section, we discuss insertion of shorter tags for conditional gene expression.

Ribonucleoprotein-based CRISPR/Cas9 genome editing

Ribonucleoprotein (RNP)-based editing relies almost entirely on commercially available reagents (*i.e.*, Cas9 protein, sgRNAs, and oligos), which minimizes the hands-on time before injection (Figure 3A) [although Cas9 protein can be purified at significant cost savings (Fu *et al.* 2014)]. RNP-based methods generally generate edits at high frequency and allow scarless gene editing because selection markers are not co-inserted. Drawbacks of RNP-based methods include the cost of commercial reagents for medium- to large-scale projects; the frequent necessity of identifying edits by PCR and Sanger sequencing; and the difficulty in isolating lethal or sterile mutants because positive selection markers are not inserted. Two methodological developments have facilitated RNP-based editing: first, the use of "co-CRISPR" approaches to enrich for edited animals based on the modification of a secondary locus; and second, the discovery that linear DNA with short homology regions is an efficient repair template.

Co-CRISPR introduces gene edits at a secondary locus, which result in a visible phenotype; these edited animals are enriched for edits at the primary locus (which may not have a phenotype). This observation makes intuitive sense: if Cas9 and oligos from an injection mix successfully entered the nucleus of a germ cell, then it is likely that all other injected components were also present in that nucleus. Therefore, co-CRISPR significantly reduces the number of animals that need to be screened to identify successful edits. Several co-CRISPR markers can be used: a recessive allele of *unc-22* (Kim *et al.* 2014), dominant alleles of *dpy-10*, *sqt-1*, and *unc-58* (Arribere *et al.* 2014), reversion of a nonfluorescent *gfp* or a loss-of-function *unc-119* allele (Zhang and Glotzer 2014), reversion of a temperature-sensitive *pha-1* allele (Ward 2015a), or reversion of a temperature-sensitive *zen-4* allele and a dominant benomyl resistance allele of *ben-1* (Farboud *et al.* 2019). Edits at the primary locus are subsequently identified by PCR, fluorescence of inserted fluorophores, or phenotype.

Linear DNA fragments with short homology regions are attractive repair templates for transgene insertion because they are easily generated by PCR. Paix *et al.* (2014) were the first to demonstrate the insertion of small tags and *gfp* based on short, linear repair templates and Cas9/sgRNAs encoded by plasmids. Edits are generally limited to within ~10 bp of the nuclease cut site (Arribere *et al.* 2014; Paix *et al.* 2015; Ward 2015a), although large deletions can be generated between two cut sites (Paix *et al.* 2015). Small tags were introduced by long oligos, whereas *gfp* tags were provided by PCR products containing a minimum of 30 bp homology arms (Paix *et al.* 2014). Subsequent improvements to the protocol increased efficiency by utilizing Cas9 protein and synthetic RNAs (tracrRNA and crRNA) (Paix *et al.* 2015), as well as a fully cloning-free method that relies on *in vivo* recombineering with oligos bridging the insertion site and tag (Paix *et al.* 2016). Although very promising, inconsistent efficiencies of repair from linear templates with short homology have been reported, possibly because high concentrations of RNP result in toxicity (Dokshin *et al.* 2018) and injection needles can clog (Prior *et al.* 2017). A recent publication developed an easy method to titrate RNP concentration to avoid toxicity and also demonstrated improved insertion frequencies with long, partially single-stranded repair templates created by annealing asymmetric PCR products (Dokshin *et al.* 2018). Also, constitutive germline expression of an integrated Cas9 significantly increases gene editing frequency (Zhang *et al.* 2018a) and bypasses potential problems caused by toxicity of injected Cas9 protein. It has been proposed that short homology arms favor insertion of small (up to ~1 kb) inserts via a short-range repair pathway (Dickinson and Goldstein 2016). However, at least in some cases, short targeting homology (45 bp) is sufficient to insert larger fragments (>3 kb) at high frequency (Schwartz and Jorgensen 2016).

In summary, gradual but constant improvements in RNP-mediated genome editing, as well as in DNA and RNA synthesis, allow most laboratories to routinely edit the genome and insert tags with minimal plasmid cloning. RNP-based methods can be used with virtually no upfront experimental effort before injection and require injection of few animals. However, the choice of sgRNA is more constrained, as not all synthesized sgRNAs cut the genome efficiently, and, in such cases, it often requires more effort to identify edits. RNP-based methods have the added advantage that commercial reagents are continuously developed, such as sgRNAs (or crRNAs and tracrRNAs) that are chemically stable, or purified proteins that encode other CRISPR nucleases, for example, the single-guide CRISPR nuclease Cpf1 (Ebbing *et al.* 2017).

Plasmid-based CRISPR/Cas9 genome editing

An advantage of plasmid-based methods is that selection markers are co-inserted, which generally allows less labor-intensive identification of insertions and the insertion of larger transgene cassettes (Figure 5B). Furthermore, by acting as genetic balancers, selection markers allow for the isolation and maintenance of severe mutations that cause lethal or sterile phenotypes. Drawbacks to plasmid-based engineering are that edits are rarely "scarless," selection markers often need to be removed in a second step, and targeting cassettes generally require some cloning to generate.

Dickinson *et al.* (2013) developed the first successful plasmid-based method to insert large DNA fragments using CRISPR/Cas9 and a positive selection marker (*unc-119*). To reduce the possible influence of the selection marker, *unc-119* was flanked by *LoxP* recombination sites for subsequent excision upon injection of a plasmid expressing Cre recombinase in the germ line (Dickinson *et al.* 2013). The same authors developed an optimized method based on a large self-excising cassette (SEC), which contains a fluorophore, a positive selection marker (HygroR), a negative selection

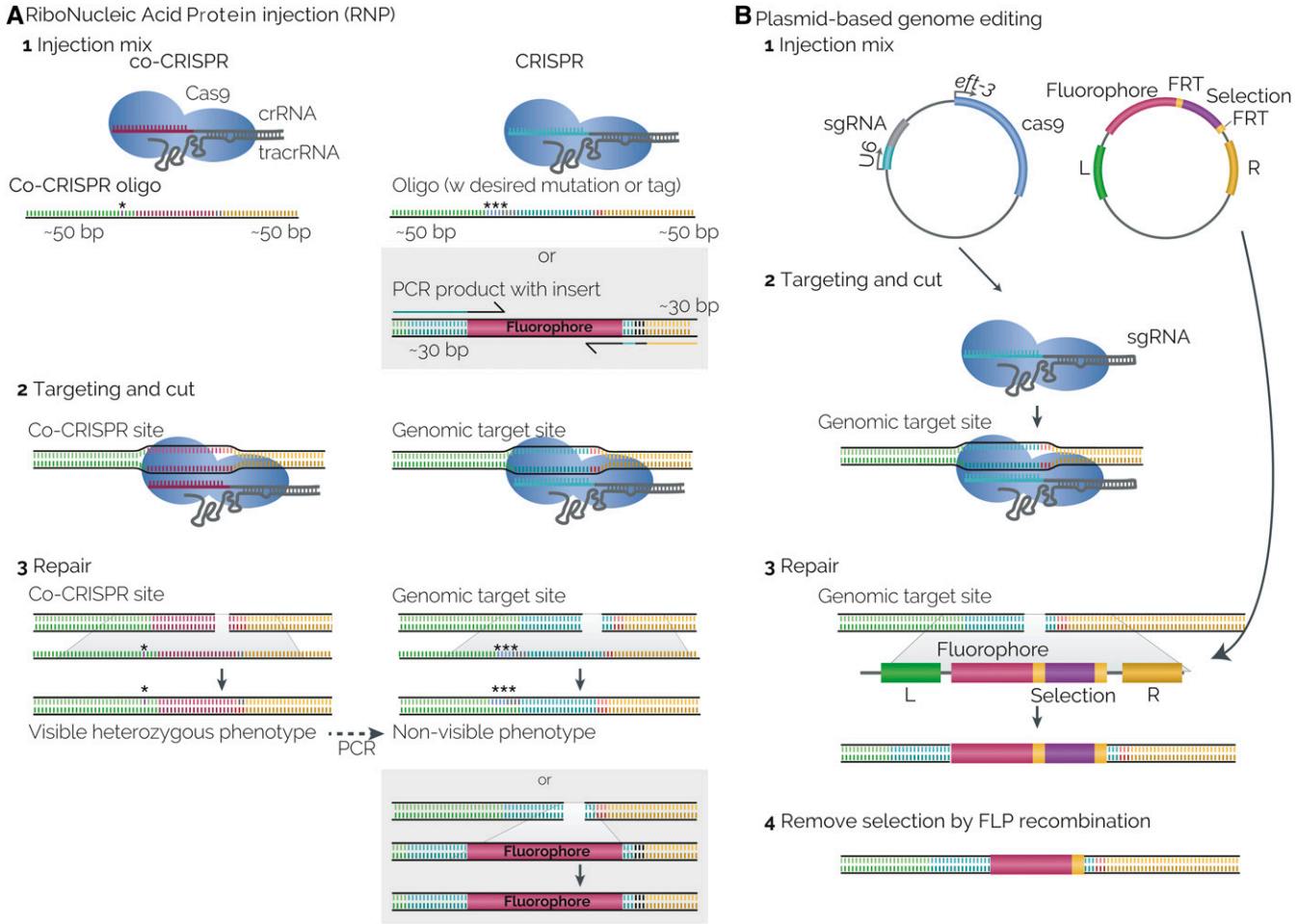

Figure 3 Cas9-mediated transgenesis. (A) RiboNucleic acid Protein (RNP) based transgenesis. The injection mix consists of Cas9 protein, synthetic crRNA, and tracrRNA, and oligos encoding the desired gene edit. Alternatively, a larger fragment is inserted, such as a fluorophore, by PCR-amplifying the fragment with site-specific oligos (gray box). The genome is targeted, and cut at specific sites guided by the crRNA sequence. Commonly, a co-CRISPR approach is used, in which editing at a secondary locus results in a visible phenotype; these worms are enriched for edits at the primary site, which are identified by PCR, phenotype, or fluorescence. (B) Plasmid-based transgenesis. The injection mix consists of plasmids expressing Cas9 under a strong Pol II promoter, the single guide RNA (sgRNA) under a U6 pol III promoter, and a repair template plasmid containing a fluorophore (or other tag) next to a positive selection marker, both of which are flanked by homologous recombination regions ("L" and "R"). The genome is targeted and cut at a specific site guided by the sgRNA and repair is templated from the plasmid. The negative selection marker can be removed in a subsequent step by recombination with FLP recombinase at FRT sites or CRE recombinase at *Lox*P sites, if they are included in the vector (not shown).

gene (*sqt-1*, causing a Rol phenotype), and a heat-shock promoter driving Cre recombinase (Dickinson *et al.* 2015). Heat-shock expression of Cre excises the cassette except for the fluorophore, which saves reinjecting the strain but also results in occasional spontaneous excision of the cassette. This optimized method relies on shorter homologous regions (~500 bp), with much lower gene editing frequencies observed when using 35–40 bp homology arms (Dickinson *et al.* 2015). An alternative method, SapTrap, named for its use of the *Sap*I restriction enzyme to "trap" plasmid components in a particular order, encodes homology arms with short oligos (Schwartz and Jorgensen 2016). SapTrap uses Golden Gate assembly (Engler *et al.* 2008), which allows complex assemblies of targeting vectors from a toolkit of plasmids containing various tags and fluorophores (Schwartz and Jorgensen 2016). Short (~45 bp) homology arms are almost as efficient as longer homology arms (~150 or 400 bp) using the SapTrap method (Schwartz and Jorgensen 2016), which is seemingly at odds with observations by Dickinson *et al.* (2015).

An advantage of plasmid-based methods is that they do not appear to be sensitive to sgRNA cutting efficiency, possibly because repair, rather than cutting, is the rate-limiting step (Dickinson and Goldstein 2016). Plasmid-based protocols that incorporate selection markers appear to be more robust for fluorophore-sized insertions, at the expense of a more difficult build process and not entirely scarless DNA insertion. The SEC and SapTrap methods are under continuous development with a set of SEC vectors incorporating Golden Gate cloning (Dickinson *et al.* 2018), software to design SapTrap oligos (Schwartz and Jorgensen 2018a), and SapTrap vectors for generating point mutations (Schwartz and Jorgensen 2018b).

Which CRISPR/Cas9 method to use?

Which CRISPR/Cas9 method to choose for genetic engineering? At present, the RNP-based methods are used predominantly for making point mutations and for inserting small tags, whereas the plasmid-based methods have been favored for fluorophore tagging.

To generate point mutations and insert small tags, RNP-mediated gene editing is the obvious choice. Similarly, to simultaneously generate transcriptional and translational fluorophore fusions, the plasmid-based methods are the obvious choice. For insertion of a fluorophore, the answer is more complicated and will often be determined by individual laboratory preferences; *e.g.*, one author's laboratory favors RNP-based insertion (J.N.) whereas the other's prefers plasmid-based (C.F.J.). The ease of generating reagents compared to the cost and relative difficulty in isolating insertions for the two methods must be balanced by every laboratory. Rapid development of optimized protocols suggests that this balance will be continuously shifting.

Future directions for CRISPR-based genome manipulation

It is difficult to overestimate the influence that CRISPR/Cas9-based editing has already had on experiments in *C. elegans*. Deleting genes, generating mutant alleles, and inserting tags can be done in a matter of a few weeks, with success virtually guaranteed. Of course, CRISPR/Cas9 has revolutionized biology broadly, and has, perhaps, made traditional genetic model organisms including *C. elegans* less relevant because genetic experiments can now be done with ease in "higher" model organisms or "nonmodel" organisms. For example, mammalian cell culture experiments coupled with sgRNAs encoded by massively scaled oligo synthesis allow rapid interrogation of entire pathways (Shalem *et al.* 2015). *C. elegans* researchers have been quick to use CRISPR/Cas9 for techniques that had already been developed, such as gene editing and plasmid insertion, now just with considerably less difficulty. However, whereas novel uses of CRISPR/Cas9 to perform previously impossible experiments have been developed in other systems, such advances have, so far, been used only sporadically in *C. elegans*. Interesting new methods developed using nuclease-dead dCas9 in other systems that have potential applications in *C. elegans* are detailed in the following sections:

Functional genomic screens: Transcriptional inhibitors and activators coupled to dCas9 allow interrogation of loss-of-function or gain-of-function phenotypes of individual genes or pairs of genes in cell culture. Large-scale screens are generally conducted using oligo-derived sgRNAs synthesized on chips with functional effects assessed by comparing the representation of sgRNAs at the beginning and end of experiments using next-generation sequencing. Large-scale screens could possibly be applied to *C. elegans* to study multicellular phenotypes, such as development, behavior, or aging. This would be particularly useful for uncovering genetic and epistatic interactions, which are typically poorly studied by currently used functional genomic methods in *C. elegans* such as RNA interference. Expression of dCas9 coupled to the KRAB repressor or VP160 activator in *C. elegans* modestly repressed or activated endogenous genes, respectively, and would need to be improved substantially (Long *et al.* 2015).

Engineering cellular signaling pathways: Transcriptional activators coupled to dCas9 allow gene overexpression or misexpression. Genetic dissection of cellular pathways has proceeded mainly by knocking out pathway components, but biological engineering ("synthetic biology") argues that underlying systems can also be understood by building new functions. In the process, cells with useful properties can be generated (Gordley *et al.* 2016). T-cells that recognize and kill cancer cells as a result of re-engineering chimeric antigen receptors (CARs) are a prime example. If technically possible, large-scale re-engineering of signaling pathways or neuronal circuits in *C. elegans* may uncover design principles that allow multicellular organisms to develop and function and may allow us to imbue an organism with novel properties.

Epigenome editing: Chromatin writers coupled to dCas9 allow perturbation of chromatin marks in a genome to interrogate epigenetic inheritance. *C. elegans* is frequently used to study transgenerational epigenetic inheritance, in part because the short generation time allows for experiments over many generations (reviewed by Houri-Zeevi and Rechavi 2017). However, by necessity, most experiments have focused on quantifying the global landscape of chromatin marks and knocking out chromatin "writers" or small RNA pathways to assign functional relevance to individual chromatin modifications. Targeted chromatin editing should allow rigorous tests of models describing how epigenetic information is passed on to future generations.

Conditional Gene and Protein Inactivation

The function of a protein can be revealed by inactivating its encoding gene or mRNA, or by inhibiting the protein directly. In this section, we discuss conditional methods to delete genes using recombinases and to degrade proteins using degrons. Inhibiting mRNA using RNAi has been reviewed in detail previously, and will not be discussed here (Ahringer 2006).

Conditional gene deletion using Cre and FLP recombinases

Genes can be conditionally deleted by using site-specific recombinases to excise the coding sequence. Cre and FLP recombinases have both been optimized for use in *C. elegans* (Hubbard 2014). Each enzyme catalyzes recombination between a pair of 34 bp target sequences (*lox*P sites for Cre, *FRT* sites for FLP), which can be used to invert or excise the intervening sequence depending on the relative orientation of recombination sites. Recombination of target sites in

inverted orientation results in inversion of the intervening sequence and preserves both targets. To excise a gene, *lox*P or *FRT* sites are inserted in direct orientation flanking the coding sequence, or within introns flanking a critical exon(s). Recombinase expression in somatic cells can be used to produce mosaic animals with gene deletions in specific tissues (using a tissue-specific promoter) or at a specific developmental stage (using a heat-shock promoter), whereas recombinase expression in the germ line results in heritable deletions. An advantage of deleting genes is that the null phenotype is revealed once existing mRNA and protein gene product decays. This is also a liability of the approach, as mRNA and protein from some genes can persist for lengthy periods, masking phenotypes. In such cases, targeted protein degradation (see *Conditional protein degradation using degrons*) is the preferable genetic tool.

It is advisable to characterize any Cre or FLP-expressing "driver" line by first crossing it with a "reporter" line, which expresses a fluorescent protein upon excision of a stop cassette. This cross will indicate whether the driver is effective in promoting excision and will reveal when, and in which tissues, excision takes place. The latter information is essential for interpreting experiments, as some "tissue-specific" promoters may be active transiently in other cell types during development (Ruijtenberg and van den Heuvel 2015). The excision efficiency of the intended target gene itself should also be tested directly. The gold standard would be to use an engineered allele that contains a fluorescent protein or tag within or downstream of the region to be excised (assuming the excision produces a frameshift). Such a strategy would allow one to simultaneously determine knockout efficiency from both alleles and judge how long any protein or mRNA present at the time of the excision perdures. In addition to excising endogenous loci, recombinases can be used to excise genes from a rescuing transgene. However, recombinases are unlikely to excise all copies of the gene within a high-copy array (Davis *et al.* 2008; Voutev and Hubbard 2008), so single-copy transgene insertions should be used for this approach.

Collections of driver and reporter plasmids and transgenic lines have been developed for both FLP and Cre (Davis *et al.* 2008; Voutev and Hubbard 2008; Kage-Nakadai *et al.* 2014; Ruijtenberg and van den Heuvel 2015; Muñoz-Jiménez *et al.* 2017). A compilation of integrated Cre and FLP driver lines, is presented in Table 1.

Conditional protein degradation using degrons

In certain cases, it may be necessary to rapidly remove an essential gene's function to uncover its role in a given process. Traditional tools such as nonconditional mutants or RNAi may prove insufficiently slow for this task given that perdurance of the wild-type protein can mask phenotypes until it decays. In such cases, the preferable method is to target the protein itself for rapid degradation using one of several recently developed methods. Although the degradation methods differ in important ways, each involves genetically tagging a protein with a degron, which allows the fusion protein to be conditionally recruited to an E3 ubiquitin ligase complex for ubiquitylation and subsequent proteasome-mediated degradation. In this section, we discuss when protein degradation is the best genetic tool and compare three methods that have been used to conditionally degrade proteins.

When to use conditional protein degradation

Here are some common situations when conditional protein degradation may be the best loss-of-function genetic approach:

Circumventing maternal contribution. Many genes are expressed in the maternal germ line (Reinke *et al.* 2004), and their mRNA or protein products can persist well into embryogenesis or even larval stages. Maternal contribution presents a problem when using mutants to study essential genes during embryogenesis since homozygous mutant embryos must be obtained from heterozygous mothers, which load wild-type gene product into the egg.

Satisfying earlier developmental requirements. Unless one is examining the first developmental stage when an essential gene functions, it may not be possible to analyze or interpret mutant phenotypes at later developmental stages. This could be because mutant (or RNAi) embryos arrest at an earlier stage, or have developmental defects that indirectly affect the process being studied.

Minimizing indirect effects caused by long-term gene inhibition. Disrupting some genes can lead to a cascade of indirect effects on cellular events that they do not directly control (*e.g.*, interfering with vesicular trafficking pathways). Rapid inhibition of the gene product is the best way to minimize these indirect effects.

Experimental considerations

To degrade proteins conditionally, a specific degron sequence is appended to a protein either by modifying the endogenous locus or a rescuing transgene. Subsequent expression of a protein that binds the degron and brings the tagged protein to an E3 ligase complex leads to its degradation. Regardless of the specific method employed, there are several important things to consider when planning experiments:

The degron tag itself may interfere with the function of the protein: As with all tagged proteins, functionality of the fusion protein should be assessed prior to using it in genetic experiments. Even if the protein appears functional, genetic comparisons should be made between worms expressing the tagged protein before and after degradation, not between wild-type worms and worms with degraded protein.

The degron must be present in the same cellular compartment as the E3 ligase complex: Each of the methods described below can target cytoplasmic proteins or

Table 1 Integrated Cre and Flp driver lines

Promoter	Reported Expression	Copy number	Reference
Cre recombinase			
ges-1	intestine	multiple	Kage-Nakadai *et al.* (2014)
myo-2	pharynx	multiple	Kage-Nakadai *et al.* (2014)
scm-1	body wall muscles	multiple	Kage-Nakadai *et al.* (2014)
dpy-7	epidermis	multiple	Kage-Nakadai *et al.* (2014)
unc-122	coelomocytes	multiple	Kage-Nakadai *et al.* (2014)
hsp-16.2	heat shock	multiple	Kage-Nakadai *et al.* (2014)
rgef-1	pan-neuronal	multiple	Kage-Nakadai *et al.* (2014)
unc-4	DA and VA	multiple	Kage-Nakadai *et al.* (2014)
unc-25	DD	multiple	Kage-Nakadai *et al.* (2014)
eat-4	glutamatergic neurons	multiple	Kage-Nakadai *et al.* (2014)
che-2	sensory neurons	multiple	Kage-Nakadai *et al.* (2014)
ttx-3	AIY	multiple	Kage-Nakadai *et al.* (2014)
mec-4	touch receptor neurons	multiple	Kage-Nakadai *et al.* (2014)
fig-1	glia	multiple	Kage-Nakadai *et al.* (2014)
myo-3	body wall muscles	single	Ruijtenberg and van den Heuvel (2015)
elt-2	intestine	single	Ruijtenberg and van den Heuvel (2015)
hlh-8	M mesoblast	single	Ruijtenberg and van den Heuvel (2015)
FLP recombinase			
hsp-16.41	heat shock	multiple	Voutev and Hubbard (2008)
hsp-16.2	heat shock	multiple	Voutev and Hubbard (2008)
dat-1	dopaminergic neurons	single	Muñoz-Jiménez *et al.* (2017)
dpy-7	epidermis	single	Muñoz-Jiménez *et al.* (2017)
elt-2	intestine	single	Muñoz-Jiménez *et al.* (2017)
hlh-8	M mesoblast	single	Muñoz-Jiménez *et al.* (2017)
hsp-16.41	heat shock	single	Muñoz-Jiménez *et al.* (2017)
lag-2	multiple including distal tip cells	single	Muñoz-Jiménez *et al.* (2017)
mec-7	mechanosensory neurons	single	Muñoz-Jiménez *et al.* (2017)
myo-2	pharyngeal muscles	single	Muñoz-Jiménez *et al.* (2017)
myo-3	body wall muscles	single	Muñoz-Jiménez *et al.* (2017)
nhr-82	seam cell lineage	single	Muñoz-Jiménez *et al.* (2017)
rgef-1	pan-neuronal	single	Muñoz-Jiménez *et al.* (2017)
tph-1	serotonin-producing neurons	single	Muñoz-Jiménez *et al.* (2017)
unc-47	GABAergic motor neurons	single	Muñoz-Jiménez *et al.* (2017)

transmembrane proteins that contain a cytoplasmic domain. Nucleoplasmic proteins have also been targeted. It is unclear whether secreted proteins lacking a cytoplasmic domain, or proteins that are imported into organelles such as mitochondria, would have access to the E3 ligase complex responsible for degrading the protein.

Degradation should be monitored: Depletion of every degron-tagged protein should be monitored by using a specific antibody or by adding a small epitope or fluorescent protein. Monitoring is also necessary to learn how quickly the tagged protein degrades. In addition to differences in degradation rates and extent that are intrinsic to each method, characteristics of the tagged protein, such as abundance and accessibility within the cell, also influence degradation. Similar to RNAi, depleting a degron-tagged protein may or may not produce a null phenotype.

Degron methods

ZF1-mediated degradation: ZF1-mediated degradation takes advantage of an endogenous *C. elegans* degron, ZF1 (Zinc Finger 1). The 36 amino acid ZF1 domain was identified for its role in targeting the germline protein PIE-1 for degradation in somatic cells of the early embryo (Reese *et al.* 2000). PIE-1 degradation is mediated by ZIF-1, a SOCS-box containing substrate recognition subunit of an ECS (Elongin-C–Cullin–SOCS-box) E3 ubiquitin ligase complex (DeRenzo *et al.* 2003); ZIF-1 binds to the ZF1 domain and targets PIE-1 for ubiquitylation and degradation. Fusing the ZF1 domain to other maternally expressed proteins causes the fusion protein to degrade in early embryonic somatic cells, revealing loss-of-function phenotypes (Nance *et al.* 2003). ZF1-tagged proteins do not normally degrade at later stages of development (Nance *et al.* 2003), likely because ZIF-1 is absent or inactive beyond early embryogenesis. Accordingly, expressing *zif-1* transgenically is sufficient to induce the degradation of ZF1-tagged proteins at later stages (Armenti *et al.* 2014) (Figure 4A). This strategy, in which *zif-1* is expressed from a tissue-specific or heat-shock promoter, provides a method for conditionally degrading proteins in apparently any somatic cell (Armenti *et al.* 2014). ZF1-tagging has been used to deplete numerous essential proteins in the early embryo (taking advantage of endogenous *zif-1*

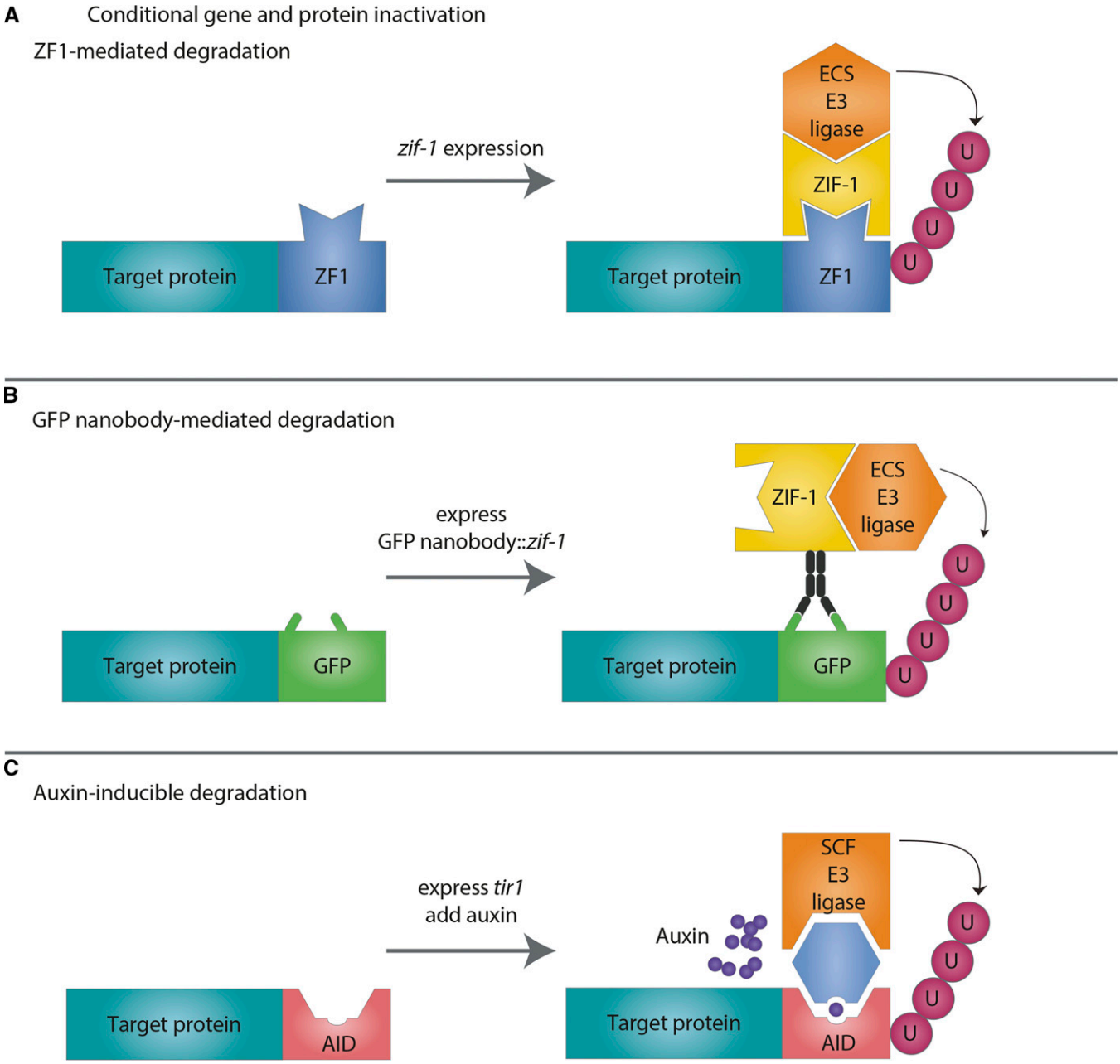

Figure 4 Conditional protein degradation. (A) ZF1-mediated degradation. Target proteins tagged with the ZF1 degron are inducibly degraded upon expression of ZIF-1. (B) GFP nanobody-mediated degradation. Target proteins tagged with GFP are inducibly degraded upon expression of a GFP nanobody fused to ZIF-1. (C) Auxin-inducible degradation. Target proteins tagged with the AID domain are inducibly degraded upon expression of TIR1, which binds the AID domain only if auxin is present.

expression) (Nance *et al.* 2003; Anderson *et al.* 2008; Achilleos *et al.* 2010; Chihara and Nance 2012; Feldman and Priess 2012; Chan and Nance 2013; Fazeli *et al.* 2016, 2018; Walck-Shannon *et al.* 2016; Zilberman *et al.* 2017; Beer *et al.* 2018) or at later stages (using conditional *zif-1* expression) (Armenti *et al.* 2014; Lim *et al.* 2016; Sallee *et al.* 2018; Soulavie *et al.* 2018), and can produce a rapid loss-of-function phenotype. Degradation of target proteins to undetectable levels can occur in well under an hour (Armenti *et al.* 2014).

A limitation of the ZF1-tagging approach is that it should not be used in the germ line in order to avoid degrading endogenous PIE-1 and several other proteins that have similar Zinc Finger domains. An additional limitation is that endogenous ZIF-1 in early embryos will clear maternally expressed ZF1-tagged proteins, complicating the analysis of proteins required for cell viability or division. This problem was recently circumvented by performing experiments in *zif-1* mutants, which are viable and healthy (Sallee *et al.* 2018).

GFP nanobody-mediated degradation: This method is a variation of the deGradFP approach, in which GFP itself is used as a degron (Caussinus *et al.* 2011). deGradFP utilizes a camelid single-domain antibody fragment that specifically binds GFP ("GFP nanobody") (Saerens *et al.* 2005), and which is fused to an F-box domain from the *Drosophila* Slmb E3 ligase substrate recognition component. Upon conditional expression, the nanobody binds to GFP within a GFP fusion protein and targets the GFP fusion protein for ubiquitylation by an SCF (Skp1–Cullin–F-Box) E3 ligase complex (Caussinus *et al.* 2011). To get the system to work in worms, the Slmb F-box was replaced with *C. elegans* ZIF-1 (see above) (Wang *et al.* 2015, 2017b) (Figure 4B). This recently developed method has been used to degrade several endogenously tagged GFP fusion proteins, revealing loss-of-function phenotypes (Chuang *et al.* 2014; Wang *et al.* 2017b; Kim *et al.* 2018; Kurup *et al.* 2018). Degradation of proteins to a level below detection can be accomplished and occurs quickly, although at least one abundantly expressed protein could not be depleted entirely using this method (Wang *et al.* 2017b). A strength of this approach is that it takes advantage of the expanding number of GFP knock-in alleles being created by CRISPR/Cas9. Like ZF1-tagging, this method should not be used in the germ line, where endogenous ZF1-domain proteins could be targeted by the GFP nanobody::ZIF-1 fusion protein.

Auxin-inducible degradation (AID): This method offers the advantage of combined spatial and temporal control of protein degradation. Adapted from *Arabidopsis* and optimized for *C. elegans* (Zhang *et al.* 2015), AID utilizes the substrate recognition F-box protein TIR1, a component of an SCF E3 ubiquitin ligase complex. The twist is that TIR1 binds its 44 amino acid AID degron substrate only in the presence of auxin (Dharmasiri *et al.* 2005; Kepinski and Leyser 2005). Proteins fused to the AID degron can be degraded in specific cells by expressing TIR1 from a tissue-specific promoter, but only when auxin is present (Figure 4C). Fortunately, auxin enters worms readily, and can even cross the eggshell (Zhang *et al.* 2015). Because auxin can be washed away, the system is also potentially reversible, although restoration to full protein levels may require a lengthy recovery. Like the methods described above, AID can degrade proteins rapidly and has been used to study the loss-of-function phenotype of several different types of proteins (Zhang *et al.* 2015, 2018b; Kerk *et al.* 2017; Liu *et al.* 2017a; Patel and Hobert 2017; Pelisch *et al.* 2017; Yu *et al.* 2017; Ferrandiz *et al.* 2018; Kasimatis *et al.* 2018; Serrano-Saiz *et al.* 2018; Shen *et al.* 2018). One potential issue is that addition of the AID degron tag has been reported in a few cases to interfere with function or stability of the tagged protein, even in the absence of TIR1 and auxin (Kerk *et al.* 2017; Patel and Hobert 2017; Schmidt *et al.* 2017). However, this concern applies to all of the degradation methods described here, which each rely on a tag that must be appended to the target protein. AID is currently the only method that can be used to conditionally degrade proteins in the germ line.

Which approach to use?

Conditional protein degradation methods are just starting to be used in *C. elegans*, so strengths and limitations of each method will become more apparent over time. In particular, different E3 ligase complexes are used for ZIF-1-mediated (ECS complex) and AID-mediated (SCF complex) degradation, and it is possible that these complexes have varying activities in different cellular compartments, cell types, or developmental stages. For degradation in somatic cells, one strategy could be to append multiple degrons (GFP, ZF1, AID) to a protein of interest and try each of the three methods. For experiments in which combined temporal and spatial control are needed, or if one wishes to target a protein in the germ line, the AID method is the only suitable choice so far. However, it should be feasible to use heat-shocked Cre or FLP recombinases to add a temporal aspect to either ZF1-mediated or GFP nanobody-mediated degradation (see *Bipartite Systems for Temporal and Spatial Control of Expression*), and an auxin-inducible GFP nanobody has been recently developed for use in human cells and zebrafish (Daniel *et al.* 2018).

Other methods for conditionally inhibiting proteins

In addition to degron-tagging, several other methods have been described that allow proteins to be conditionally degraded or damaged. These include sortase-mediated ligation of an F-box directly to a target protein, causing it to be recruited to an E3 ubiquitin ligase complex and degraded (Wu *et al.* 2017); destabilizing domains, which cause constitutive protein degradation that can be blocked by the addition of a worm-permeant drug (Cho *et al.* 2013a); chromophore-assisted light inactivation (CALI), in which a protein is tagged with a genetically encoded chromophore that emits ROS upon illumination, oxidizing nearby residues on the protein (Lin *et al.* 2013); and light-induced protein degradation, in which a C-terminal degron is exposed upon blue-light illumination, causing much of the tagged protein to degrade (Hermann *et al.* 2015). Diffusible proteins can also be inactivated by trapping them at an ectopic site, for example by using a GFP nanobody (Pani and Goldstein 2018) or by light-inducible protein mislocalization (Fielmich *et al.* 2018). Finally, tools have been developed to conditionally disrupt specific endogenous proteins. While these must be custom-developed for each protein of interest, some could prove particularly useful. For example, conditional expression of a peptide that disrupts actin polymerization (DeAct) has been used to interfere with the morphogenesis of a specific neuron (Harterink *et al.* 2017).

Bipartite Systems for Temporal and Spatial Control of Expression

Bipartite systems have two main advantages. First, gene expression can be controlled in time and space. Spatial control is useful for determining a gene's site of action and temporal

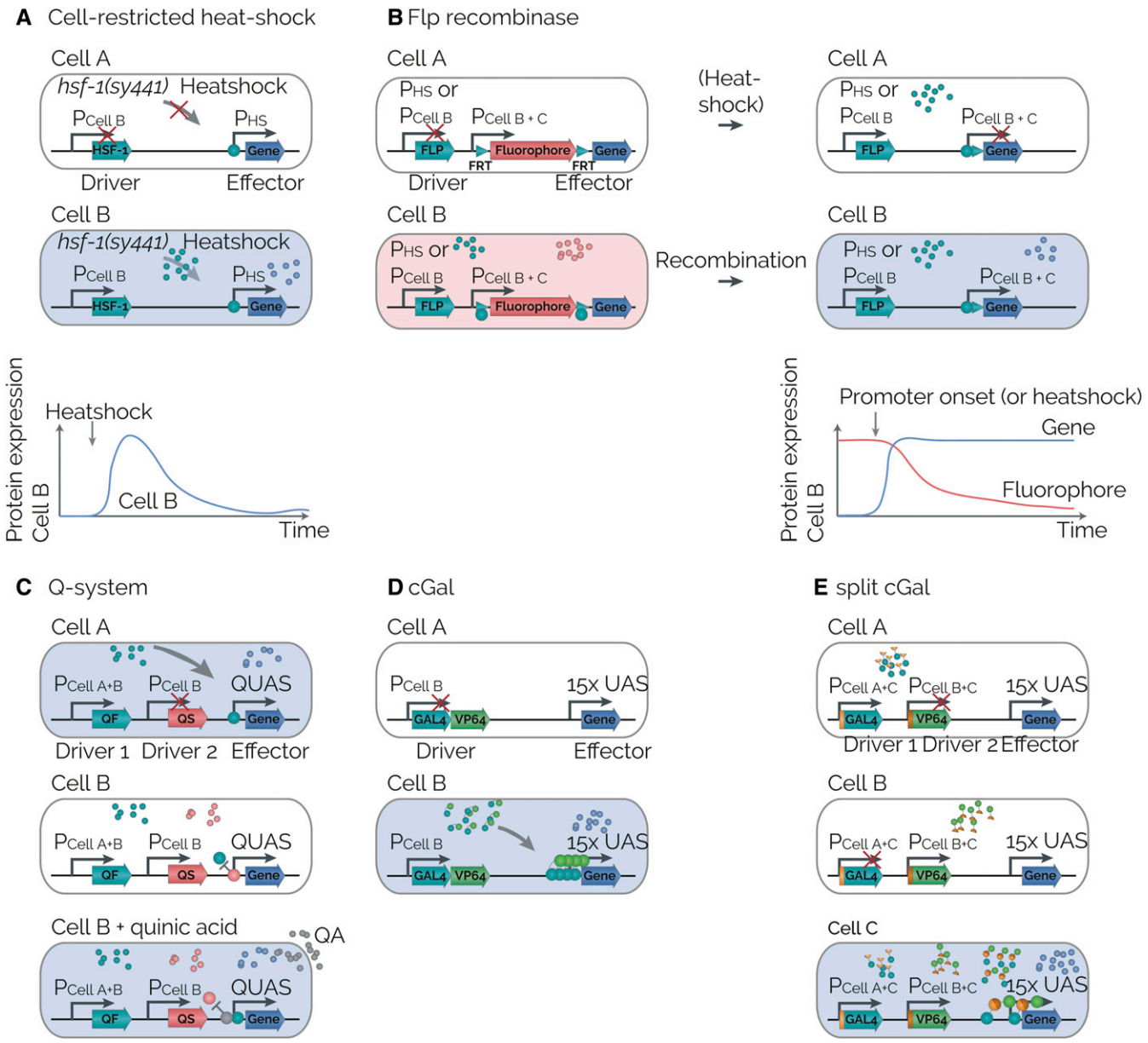

Figure 5 Bipartite expression systems. (A) Cell-restricted heat-shock. Cell-specific heat shock responses are achieved using a heat shock defective mutant, *hsf-1(sy441)*, and reintroducing HSF-1 protein in specific cells (Bacaj and Shaham 2007). Effector transgenes under a heat-shock (HS) promoter are only expressed in cells where the HSF-1 protein has been reintroduced, typically using a cell-specific promoter (here, a promoter for cell B). Transgene expression is transient using this method. (B) FLP (or CRE) recombinases. Inducible (*e.g.*, by heat shock, HS) or cell-specific (*e.g.*, using a promoter specific for cell B) expression of FLP can be combined with effector transgenes that are prevented from expression from a second promoter by a "stop" cassette (Davis *et al.* 2008; Voutev and Hubbard 2008). Following recombinase expression, the stop cassette is excised, allowing expression in a subset of cells where both promoters are active. Transgene expression is continuously turned on after stop cassette excision using this method. (C) Q-system. The Q-system relies on transcriptional activation, with the QF activator (green circles) binding to a QUAS sequence upstream of the transgene (Wei *et al.* 2012). Additional spatial and inducible control is conferred by the inhibitor QS (red circles), which prevents QF from activating the transgene and quinic acid (gray circles) supplied exogenously, which neutralizes the QS inhibitor. (D) cGAL system. The cGAL system relies on transcriptional activation, with GAL4 (cyan circles) fused to a transcriptional activator VP64 (green circles), which bind to a 15× UAS sequence upstream of the effector transgene (Wang *et al.* 2017a). The spatial expression is conferred via promoter-specific expression of cGAL and can be transient or constitutive. (E) Split cGAL system. The split cGAL system expresses the GAL4 DNA binding protein (cyan circle) and the VP64 activator protein (green circle) from individual promoters (Wang *et al.* 2018). A functional cGAL is reconstituted by intein splicing in cells where both are expressed, leading to transcriptional activating of the effector transgene.

control for determining a gene's role in development or homeostasis. A second important advantage of bipartite systems is the ability to reuse components (driver and effector lines), which reduces the workload in individual labs and furthers reproducibility between labs. Bipartite methods overcome limitations of individual promoters and the broad activity of inducible promoters. Although the methods differ in important aspects, they all rely on at least two components (hence,

bipartite) consisting of an activator and an effector. Generally, bipartite systems confer spatial and temporal expression by utilizing two components that regulate gene expression under different promoters. Often, expression requires both components but in some cases, gene expression is repressed by one of the components, with additional regulation conferred by drugs (Figure 5). In this section, we discuss when bipartite systems may be useful and describe three qualitatively different bipartite techniques.

When to use bipartite systems

The following sections list some common examples using bipartite systems.

Restricting expression to single cells. Frequently, there are no known promoters that drive expression in single cells. Single-cell expression is often necessary for measuring or perturbing activity of individual neurons using genetically encoded sensors or light-activated ion channels, respectively.

Generating cellular loss-of-function without disrupting development. Cells in multicellular organisms develop using cues and inputs from other cells. Therefore, removing a cell early in development is likely to perturb the development of other cells, making it difficult to determine the isolated role of the removed cell. For example, neuronal laser ablation in early larval stages may disrupt how neuronal networks are established and lead to compensatory synaptic connections.

Experimental considerations

Bipartite systems function by separating activator and effector functions. Typically, the activator is necessary for transcription of the effector protein, for example by expressing a transcriptional activator that binds to DNA sequences upstream of the effector. There are several important considerations to keep in mind when designing experiments utilizing bipartite systems.

Characterization of levels, cellular pattern, and onset of expression: The two-step cascade used in bipartite systems makes it difficult to *a priori* predict expression levels of the effector protein. Therefore, it is necessary to test activator (driver) lines with standardized GFP effector lines to quantify expression levels and cellular expression pattern. This is a particular concern with integrated arrays, which frequently show cryptic expression in nonspecific tissues. For inducible activator lines, it is important to quantify how rapidly the effector protein is induced, and how long the effector perdures; induction can be monitored by proxy using a fluorophore linked to the effector protein with an operon or T2A peptide.

Single-cell expression is limited by promoters: Often, the main motivation for using bipartite systems is that sufficiently restricted expression is not possible with individual promoters. It is unlikely that bipartite expression can target some cells individually (*e.g.*, individual body wall muscle cells), whereas neurons with restricted expression of neurotransmitters and receptors are good candidates for single-cell expression. FLP, Cre, and cGAL have all been used for single neuron expression. Regardless of cell-type, single-cell expression is constrained by the availability of specific, intersecting promoters. In addition to WormBase, there are several useful online tools for identifying candidate promoters, *e.g.*, in the nervous system [http://www.wormweb.org/ (Bhatla 2016)] and early embryo [http://tintori.bio.unc.edu/ (Tintori *et al.* 2016)].

The availability of ready-made reagents: A large advantage of bipartite systems is the potential availability of standardized driver and effector lines, which typically carry integrated arrays. In addition to minimizing the work in individual laboratories of generating and characterizing lines, scientific reproducibility across different laboratories increases with standardized reagents. Before deciding which bipartite system to use, it may be useful to search through currently available reagents (FLP and Cre reagents listed in Table 1, cGAL listed in Table 2).

Split fluorescent reporters: A qualitatively different type of bipartite system relies on split fluorescent proteins. In this approach, two complementary GFP fragments are expressed individually; each fragment is nonfluorescent, but assembly of the two fragments reconstitutes a fluorescent GFP reporter (Ghosh *et al.* 2000). This approach has been used to determine coexpression patterns (Zhang *et al.* 2004) and spatial colocalization of two tagged proteins (Feinberg *et al.* 2008). Increased fluorescence signal can be achieved by using a superfolder GFP variant and multimerized domains (Feng *et al.* 2017). These fluorophores have recently been used to develop strains that express the "bulkier" GFP fragment under a strong cell-specific promoter, and tagging endogenous genes with one or more short GFP fragments to reduce interference from the tag (Noma *et al.* 2017; Hefel and Smolikove 2019), or to amplify the fluorescence signal and to facilitate cell identification (He *et al.* 2019).

Cell-restricted heat-shock response

Bacaj and Shaham (2007) developed a system for temporal and spatial transgene expression based on the heat-shock response. Heat-shock responsive promoters, *e.g.*, *hsp-16.2* or *hsp-16.41*, require trimerization of the HSF-1 transcription factor to initiate transcription in response to elevated temperature (Hajdu-Cronin *et al.* 2004). The *hsf-1(sy441)* loss of function allele prevents heat-shock induced transcription, but the response can be restored by ectopic expression of HSF-1. By tissue-specific expression of HSF-1, an effector gene under control of a heat-shock promoter can be expressed in response to elevated temperature in a cell-specific manner (Figure 5A). This approach was successfully used to express GFP in individual neurons and pharyngeal

Table 2 cGAL driver and effector lines

Promoter	Reported Expression	Transgene	Reference
cGAL4::VP64 driver lines			
myo-2	Pharynx	integrated	Wang *et al.* (2017) and Walton *et al.* (2017)
nlp-40	Intestine	integrated	Wang *et al.* (2017) and Walton *et al.* (2017)
myo-3	Body muscle	integrated	Wang *et al.* (2017) and Walton *et al.* (2017)
rab-3	Neuronal	integrated	Wang *et al.* (2017) and Walton *et al.* (2017)
unc-47	GABA neurons	integrated	Wang *et al.* (2017) and Walton *et al.* (2017)
hsp16.41	Heat-shock inducible	integrated	Wang *et al.* (2017) and Walton *et al.* (2017)
cGAL(DBD) split driver lines			
hsp16.41	Heat-shock inducible	integrated	Wang *et al.* (2018)
myo-2	Pharynx	integrated	Wang *et al.* (2018)
rab-3	Neuronal	extrachrom	Wang *et al.* (2018)
unc-17	Cholinergic	extrachrom	Wang *et al.* (2018)
cGAL(VP64) split driver lines			
Pmyo-2	Pharynx	integrated	Wang *et al.* (2018)
Peft-3	Ubiquitous	extrachrom	Wang *et al.* (2018)
Pceh-19b	MC, ADF, PHA neurons	extrachrom	Wang *et al.* (2018)
cGAL(VP64) driver lines			
Punc-17 + Pceh-19b	MC neurons	integrated	Wang *et al.* (2018)
15x UAS effector lines			
Effector	**Utility**	**Transgene**	**Reference**
GFP	Cell labeling	integrated	Wang *et al.* (2017) and Walton *et al.* (2017)
GFP::H2B	Cell labeling	integrated	Wang *et al.* (2017) and Walton *et al.* (2017)
mCherry::H2B	Cell labeling	integrated	Wang *et al.* (2017) and Walton *et al.* (2017)
mKate2	Cell labeling	integrated	Wang *et al.* (2017) and Walton *et al.* (2017)
hChR2(Y134R)::YFP	Neuronal activation	integrated	Wang *et al.* (2017) and Walton *et al.* (2017)
HisCl1::SL2::GFP	Neuronal inhibition	integrated	Wang *et al.* (2017) and Walton *et al.* (2017)
caspase ICE	Cell ablation	integrated	Wang *et al.* (2017) and Walton *et al.* (2017)
tetanus toxin	Synaptic inhibition	integrated	Wang *et al.* (2017) and Walton *et al.* (2017)
GCaMP6s::SL2::mKate2	Calcium sensing	integrated	Wang *et al.* (2017) and Walton *et al.* (2017)
PKA(DN)::SL2::GFP	Dominant negative PKA	extrachrom	Wang *et al.* (2018)

muscles and to determine how ciliated neurons are maintained in adult animals (Bacaj and Shaham 2007). The main limitations of this system are that expression is transient, that high temperatures (25°) kill *hsf-1* mutants, and that preventing cryptic misexpression of HSF-1 is difficult (Bacaj and Shaham 2007). Although the system is conceptually elegant, it has not yet found widespread use, and few HSF-1 driver lines are readily available.

Spatial and temporal expression using recombinases

Here, we highlight how FLP (Davis *et al.* 2008; Voutev and Hubbard 2008) and Cre (Macosko *et al.* 2009) recombinases can be used to generate conditional gene expression (reviewed by Hubbard 2014). In this application, transgene expression is blocked by separating transgene and promoter (P1) with a "stop cassette" containing a fluorophore flanked by *FRT* or *Lox*P recombination sequences (Figure 5A). When FLP or Cre is expressed from a second tissue-specific promoter (P2), the transgene will be expressed only in cells where P1 is active and where P2 is, or has previously been, active (*e.g.*, during development, leading to early cassette excision). If FLP or Cre is expressed from a broad inducible promoter, such as the heat-shock promoter, then the transgene will be expressed continuously after the heat-shock in all cells where P1 is active. Thus, an important difference compared to "normal" or tissue-specific heat-shock (Bacaj and Shaham 2007) is that recombinase expression can turn on continuous expression (Figure 5B). As a proof-of-principle for FLP recombinase, Davis *et al.* (2008) demonstrated heat-shock inducible expression of GFP in muscle (pharyngeal and body-wall) and tetanus toxin expression at the intersection of two promoters to inactivate neurotransmission in GABAergic neurons. Voutev and Hubbard (2008) used FLP and intersectional expression from two promoters as a lineaging tool, and induced expression of a dominant-negative allele of *hlh-12* in distal tip cells. Both implementations of "FLP-on" used multicopy transgene arrays and observed incomplete excision of the stop cassette (Davis *et al.* 2008; Voutev and Hubbard 2008). However, incomplete expression is unlikely to be a significant concern when the goal is to overexpress transgenes. FLP and Cre have primarily been used in the nervous system to narrow expression to single neurons by intersecting two promoters (Ezcurra *et al.* 2011; Schmitt *et al.* 2012; White and Jorgensen 2012; Chen *et al.* 2013a; Li *et al.* 2014). Recombination-based methods are facilitated by Gateway compatible stop cassettes (Davis

et al. 2008; Voutev and Hubbard 2008), integrated FLP/Cre driver lines (Table 1), and SapTrap-compatible cassettes for conditional expression of endogenous genes (Schwartz and Jorgensen 2016).

Transcriptional control with Q-system and cGAL

More recent methods for achieving spatial and temporal control are based on transcriptional activation, an approach used widely in flies (Brand and Perrimon 1993). In these systems, inducible or constitutive expression of a transcriptional activator binds to a sequence element upstream of an effector to drive expression (Figure 5, C–E).

The Q system is derived from the fungus *Neurospora crassa* (Potter *et al.* 2010), and was adapted for transcriptional activation in *C. elegans* (Wei *et al.* 2012). The Q-system has several components that allow detailed spatial and temporal control over expression (Figure 5C). Transgene expression is driven by the QF activator binding to a QUAS sequence upstream of the transgene. Transgene expression can be blocked by expression of an inhibitor (QS), but this inhibition can, in turn, be suppressed with a drug (quinic acid) (Wei *et al.* 2012). Wei *et al.* (2012) demonstrated controlled expression in a subset of motor neurons by expressing QF ("activator"), QUAS:GFP ("effector"), QS ("inhibitor"), and by adding quinic acid. From arrays, the Q-system behaved as expected but lacked tight regulation, *e.g.*, not all neurons were fully labeled in the QF "on" state and expression persisted in the QS "off" state. These experiments expose the Achilles heel of Qs-mediated expression; tight control over expression was achieved only using single-copy transgene insertions, but at the cost of overall expression levels (Wei *et al.* 2012).

The cGAL system is an optimized GAL4-UAS system developed specifically for *C. elegans* (Wang *et al.* 2017a). To implement the GAL4 system, Wang *et al.* (2017a) systematically improved three components of the system: the activation domain (VP64), the optimal number of GAL4 binding sites (15× UAS), and a cryophilic GAL4 DNA binding domain from the yeast *Saccharomyces kudriavzevii*. These components were sufficient to drive tissue-specific expression in the intestine, body wall muscles, and several neuron classes. In contrast to the Q-system, the cGAL system does not include negative regulation via a second inhibitor protein. This limitation was mitigated with the development of a split cGAL system, in which the cGAL DNA binding domain and the VP64 activation domain are expressed from two different promoters; the two functional domains are fused *in vivo* by intein splicing, resulting in transcriptional activity only in cells where both promoters are active (Wang *et al.* 2018). This is important because splitting cGAL in two components allows expression at the intersection of two promoters in adult animals, such as a single pair of MC neurons (Wang *et al.* 2018). Since no stop cassette is irreversibly removed in the cGAL approach, concerns about uncharacterized promoter expression in early development (resulting in expression in all derived lineages) are minimized.

Other methods for conditional gene expression

Although not widely used, several other specialized techniques for conditional gene expression have been developed for *C. elegans*. For example, Calixto *et al.* (2010) developed a method for temperature-sensitive transgene expression based on MEC-8 dependent splicing of a transplanted *mec-2* intron and used the technique to generate a strain with temperature-dependent RNA interference. In an approach that requires specialized instrumentation, Churgin *et al.* (2013) induced single-cell heat-shock responses with a laser. If the sole purpose of using a bipartite system is to label individual cells that lack a specific promoter, then reconstituted GFP fragments expressed from intersecting promoters can also be utilized with success (Zhang *et al.* 2004; Kamiyama *et al.* 2016). Also, for any effector protein that can be split into two inactive modules, the gp41-1 intein (Wang *et al.* 2018) could potentially be used to reconstitute activity in individual cells. Inducible control of the Q-system was recently achieved by fusing the QF activating domain to a steroid receptor ligand-binding domain (Monsalve *et al.* 2019). Here, the target gene is expressed upon addition of the ligand (dex) by translocating the chimeric activation domain to the nucleus. Although promising, this approach showed significant background expression and variable induction. Finally, (Mao *et al.* 2019) recently developed a hybrid method that combines the Q-system with tetracycline-controlled transcription. This very promising approach demonstrated high levels of inducible expression and a large degree of experimental control but has yet to be widely tested and used.

Conclusions

Gene synthesis, transgene assembly, and technologies to manipulate endogenous genomic loci are advancing rapidly, and this progress is only likely to accelerate. More than ever, novel biological insights will be limited only by imagination, with fewer and fewer technical barriers to experimentation. We hope this overview of current transgenic methods may inspire and facilitate ingenious experiments in new and experienced worm laboratories alike.

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