From genes to function: the *C. elegans* genetic toolbox

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This review aims to provide an overview of the technologies which make the nematode *Caenorhabditis elegans* an attractive genetic model system. We describe transgenesis techniques and forward and reverse genetic approaches to isolate mutants and clone genes. In addition, we discuss the new possibilities offered by genome engineering strategies and next-generation genome analysis tools. © 2011 Wiley Periodicals, Inc.

INTRODUCTION

The modern era of *Caenorhabditis elegans* research began <50 years ago when Sydney Brenner settled on the small nematode to tackle an ambitious goal laid out in his seminal paper published in 1974: 'Some eight years ago, [...] we decided that what was needed was an experimental organism which was suitable for genetical study and in which one could determine the complete structure of the nervous system. *Drosophila*, with about 10^5 neurons, is much too large, and, looking for a simpler organism, our choice eventually settled on the small nematode *Caenorhabditis elegans*'.[1] From the start, Brenner outlined a methodology that still prevails today: 'One experimental approach to these problems is to investigate the effects of mutations on nervous systems. In principle, it should be possible to dissect the genetic specification of a nervous system in much the same way as was done for biosynthetic pathways in bacteria [...]'.[1] In the following years, Brenner and his colleagues completed a series of major projects that laid the foundations for *C. elegans* research: (1) they established the genetics of *C. elegans* and performed the first large-scale mutant screens[1]; (2) they described the complete and largely invariant lineage of all 959 somatic *C. elegans* cells (plus 131 cells that

die by programmed cell death) using standard light microscopy[2,3]; and (3) they achieved the complete reconstruction of the adult nervous system by using electron micrographs of serial sections.[4]

Over the years, a number of discoveries added considerable attractiveness to *C. elegans* as a biological model organism. One important advance was the generation of transgenic animals, achieved through an efficient transgenesis technique developed in 1991 by Mello et al.[5] and still in use to this day. In 1994, GFP reporter technology introduced by Martin Chalfie allowed the visualization of gene expression patterns, as well as protein localization in live animals.[6] In 1998, the discovery of genetic interference by double-stranded RNA (dsRNA)[7] enabled alternative approaches for large-scale genetic screens. In the same year, the completion of the *C. elegans* genome sequence[8] provided the first comprehensive view over the complete genomic landscape of a metazoan organism. This review aims to describe the genetic toolbox available to *C. elegans* researchers by focusing on classical and novel techniques used for forward and reverse genetic analysis and recent developments in genome engineering by homologous recombination.

FORWARD GENETICS

Forward genetic screens in *C. elegans* have provided key mechanistic insights into deeply conserved developmental, homeostatic, and pathogenic processes (http://www.wormbook.org). We will describe here the different methods which can be used to generate and clone mutants from forward genetic screens. Previous monographs have discussed some of the more traditional methods in more detail (http://www.wormbook.org and Refs 9, 10).

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Random Mutagenesis with Chemical and Physical Mutagens

Random mutations can be generated by exposing worms to mutagenic agents, such as ethyl methane-sulfonate (EMS), *N*-ethyl-*N*-nitrosourea (ENU), or trimethylpsoralen followed by ultraviolet light activation (TMP/UV).[9] EMS is the most popular mutagen in *C. elegans*. It alkylates primarily guanine residues leading to mispairing during replication: alkylated G pairs to T instead of C. The resulting mutations are mainly G/C to A/T transitions. EMS also introduces deletions, yet at much lower frequency than point mutations. ENU is also a potent mutagen which generates transversions in addition to transitions. Mutagenesis by EMS and ENU was compared in a genetic suppressor screen of the rubber band phenotype induced by the *unc-93(e1500)* mutation.[11] Both mutagens produced revertants at similar frequencies but the types of alleles and the number of alleles per known suppressor gene were different. In particular, 90% of EMS point mutants were G/C to A/T transitions, which are more likely to produce stop codons rather than missense mutations. In contrast, the rate of A/T to G/C transitions was greatly increased with ENU. This analysis may be partially biased by the A/T richness of *unc-93* but has been largely confirmed by examining whole-genome sequencing (WGS) data.[12,13] Interestingly, UV/TMP treatment appears to induce all types of transitions and transversions, with almost no bias.[12] EMS is the most powerful mutagen causing 1.5–2 times more mutations than ENU and more than 3 times more variations than UV/TMP. Which mutagen to choose will therefore be determined by the type of desired mutations. EMS remains the best choice to obtain null alleles; however, ENU generates the biggest diversity of missense alleles which can sometimes be more informative for functional studies. γ, UV, and X-ray irradiation cause major chromosomal rearrangements and have been mostly used to generate duplications, inversions, deficiencies, and translocations.[9]

Classical Mutant Mapping

Classically, mutants have been mapped to a genetic interval by a combination of the following techniques (extensively reviewed in 'Genetic mapping and manipulation', www.wormbook.org): (1) two- or three-point mapping with genetic markers[14]; (2) mapping with deficiencies and duplications[15]; and (3) single nucleotide polymorphism (SNP) mapping.[16]

Two- or three-point mapping with genetic markers relies on the measure of the genetic distance (i.e., recombination frequency) between phenotypic mutants (with known physical positions) and the gene to be mapped. This approach can be used to rapidly assign a given mutation to a chromosomal region; however, it lacks resolution because of the relatively small number of such 'landmark' mutants. In addition, genetic interactions between the landmark mutant and the target gene can sometimes complicate mapping. Similarly, deficiencies and duplications are not always precisely defined at their extremities. Classical genetic mapping was therefore rapidly supplanted by the introduction of SNP mapping techniques.

SNP mapping in *C. elegans* is based on the sequence divergence between the reference strain N2 *Bristol* and the CB4856 *Hawaiian* strain.[17] SNPs occur in, on average, every 1000 bp. Most SNPs are indeed single nucleotide changes, but small insertions or deletions are also common. SNPs which lead to restriction-fragment length polymorphisms can be easily detected by restriction digests while 'silent' SNPs need to be sequenced. SNP mapping can rapidly assign mutants to a chromosome[18] and narrow down the position of a mutation of interest to relatively few candidate open reading frames (ORFs). Note, however, that an analysis of the CB4856 *Hawaiian* strain by array comparative genome hybridization has revealed 141 deletions removing 1.54 Mb of DNA and 483 predicted genes from CB4856's genome.[19] In addition, complex genetic interactions[20] and phenotypic variation of mutants in the *Bristol* or *Hawaiian* background can sometimes confuse mapping results. These and other limitations have encouraged the development of novel gene cloning techniques described in the next section.

New Approaches for Mapping Mutants

Traditional mapping strategies can be time-consuming undertakings depending on the phenotypic complexity (e.g., tedious to score, variable phenotype, low penetrance) and genetic nature (e.g., suppressor or enhancer mutant) of the studied mutant. Two 'next-generation' technologies circumvent these problems.

Array Comparative Genome Hybridization

Array comparative genome hybridization (aCGH) was first developed in *C. elegans* to accelerate the identification of 'indel' (insertion/deletion) mutants for the *C. elegans* Knockout Consortium.[19] It uses high-density oligonucleotide microarrays ($\sim$380,000 probes/array) to detect sequence polymorphisms (SNP, indels) for the entire genome or specific regions of interest. The great sensitivity of aCGH allows the detection of heterozygous deletions as small as 141 bp. The resolution is directly related to the extent of the overlap between

50 mer oligonucleotides on the array. In addition, aCGH can map a mutation to a small chromosomal interval (~200–400 kb) by analyzing the SNP distribution in homozygous mutants resulting from a single genetic cross of *Hawaiian* and N2 strains.[21] In theory, this approach should also be able to resolve polygenic traits.

Beyond SNP and deletion mapping, aCGH can directly detect homozygous single nucleotide mutations.[22] To maximize the chances of detection, a maximum probe distance of <5 nucleotides is recommended. Given the probe capacity of the array, a mutation should therefore be mapped to a 1 Mb interval prior to aCGH, in order to achieve the highest probe density on both strands. Alternatively, multiple sub-arrays could be used to further increase coverage. For reference, aCGH has been successfully used to detect two single base lesions in the promoter of the *cog-1* gene.[23]

Whole-Genome Sequencing

The most recent paradigm shift for *C. elegans* researchers is the development of WGS methods. The first *C. elegans* mutant cloned by WGS (*lsy-12(ot177)*) was reported in 2008.[24] *lsy-12(ot177)* was initially mapped by a single SNP mapping cross to a 4-Mb region of chromosome V containing ~1100 genes. Paired-end Illumina sequencing generated 3.1 Gb of mapped reads resulting in ~28-fold genome coverage. Analysis and filtering of these sequences produced a list of 80 candidate variants which were tested by Sanger sequencing. This list was further reduced to four candidates by focusing on exonic mutations and by comparing variants between the *lsy-12(ot177)* mutant strain and the N2 strain used for outcrossing. Only one of the four polymorphisms was a nonsense mutation and mapped to the *lsy-12* gene. This demonstrated for the first time the feasibility of gene cloning by rough mapping and WGS.

The strengths of WGS are evident. (1) It is fast. Sequencing currently takes ~5 days and data analysis a few hours using optimized bioinformatic tools such as MAQgene[25] or galign.[26] (2) It is highly cost-effective. Given the comparatively small size of the *C. elegans* genome (~100 Mb) and recent technology advances, a genome can be sequenced for less than US$1000. This expense compares favorably with the time, personnel, and reagent cost involved for classical mapping and is also expected to drop even much further. (3) It greatly simplifies cloning of mutants with subtle, low penetrance, or difficult to score phenotypes. Multigenic, quantitative, or behavioral genetic traits which are sensitive to genetic backgrounds and therefore hard to map by conventional

approaches will be much more accessible with WGS. (4) In addition to point mutations, WGS is also able to detect deletions depending on sequencing depth.[13] (5) Sequencing errors are negligible when sufficient coverage is reached, reducing the risks of false negatives. (6) As it is relatively easy to obtain sufficient amounts of DNA, the technology can also be used for analyzing lethal mutants. For example, a homozygous lethal mutant in the *lsy-22* gene could be cloned by sequencing DNA extracted from a few hundred hand-picked, sterile worms.[27]

Chemical mutagenesis generates hundreds of sequence variants per genome.[12,13] Initially, genetic mapping information was therefore considered crucial, unless independent alleles of the same gene were available for WGS. A novel one-step strategy that combines SNP mapping and WGS has recently been described (Figure 1).[28] Instead of focusing only on a few manually analyzed SNPs, this approach can theoretically take advantage of every SNP in the genome. The mutant strain isolated in an N2 *Bristol* background is first crossed with the polymorphic CB4856 *Hawaiian* strain and 20–50 mutant F2 recombinants are picked to single plates. These *Bristol/Hawaiian* recombinant lines are then pooled and analyzed by WGS. Chromosomal regions which are linked to the mutation are characterized by a strong increase in the *Bristol* to *Hawaiian* SNP ratio. In this proof-of-principle study, the SNP mapping information defined a ~2 Mb region, which only contained three protein-coding sequence variants.[28]

Another strategy takes advantage of the fact that phenotype-relevant EMS mutations should cluster around the mutation of interest after outcrossing (Figure 1).[29] Rather than using the polymorphic *Hawaiian* strain, mutants retrieved from a genetic screen are backcrossed four to six times with the original strain used for mutagenesis. Their complete genome sequences are aligned to the N2 reference genome and variants which are common between at least two mutant strains (i.e., background mutations) are filtered out. Hotspots containing typical EMS mutations can then be detected by plotting sequence variants along chromosomes, highlighting the likely position of the mutant gene (Figure 1). Finally, analysis of individual variants provides a list of candidate genes which can be validated by other approaches such as phenotypic rescue, RNA interference (RNAi) or analysis of additional alleles by Sanger sequencing.

Transposon-Based Insertional Mutagenesis

Transposons are mobile DNA elements present in the genomes of all living organisms. Although mobile
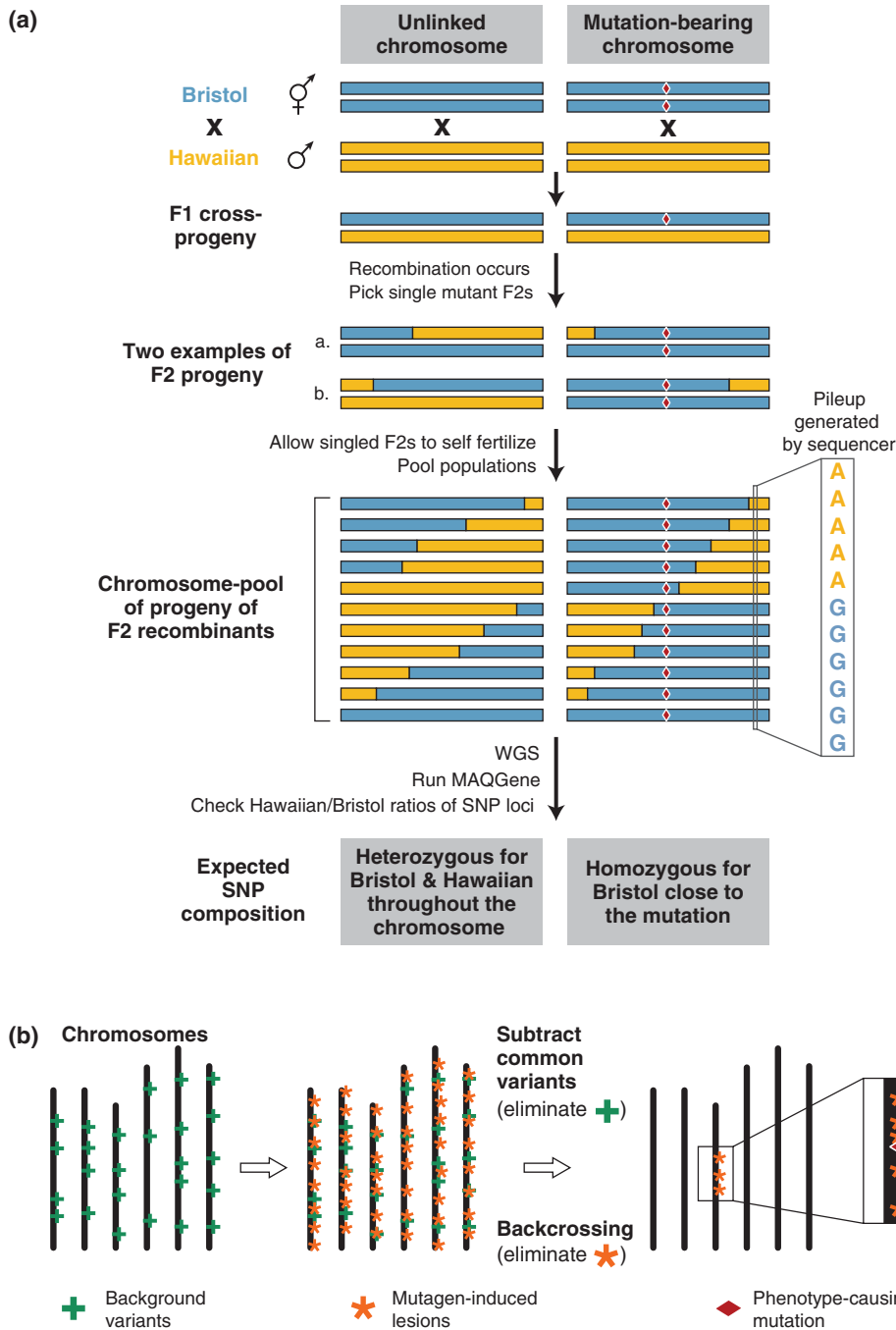
FIGURE 1 | Mutant mapping by whole-genome sequencing (WGS). (a) WGS-single nucleotide polymorphism (WGS-SNP) mapping. A homozygous mutant strain (mutation denoted by a red diamond) in a Bristol background is crossed with a SNP-containing Hawaiian strain. Mutant worms segregating from this cross are reselected in the F2 generation. These mutant lines carry chimeric chromosomes (containing Bristol and Hawaiian SNPs) generated by meiotic recombination. When DNA from multiple F2 recombinant lines is pooled and sequenced, then the relative number of Bristol versus Hawaiian SNP at a given location in the genome will be a reflection of the relative distribution of recombinants in the pool. Therefore, only Bristol SNPs will be found close to the mutation of interest. (Reprinted with permission from Ref 28. Copyright 2010 Public Library of Science) (b) Mapping based on mutagen-induced DNA variation density across the genome. Mutagenized strains contain not only mutagen-induced damage (orange asterisks) but also carry background variants (green crosses) present in the mother strain before mutagenesis. By backcrossing a candidate mutant line to the strain used for the original mutagenesis, it is possible to eliminate most mutagen-induced lesions except for those which are closely linked to the phenotype-causing mutation (red diamond). In addition, background variation present in the unmutagenized strain can be identified by comparing independent backcrossed mutant lines. After filtering, the genomic region linked to the phenotype-causing mutation will be highlighted by a high-density cluster of mutagen-induced lesions. (Reprinted with permission from Ref 29. Copyright 2010 Genetics Society of America)

elements are less efficient than chemical mutagens (see below), the resulting mutants can be more easily cloned because the transposon sequence serves as a molecular tag. This advantage will be less striking in the future, given the rapid development of WGS. However, transposons have unique and powerful downstream applications, i.e., generating custom deletions and engineering the genome.

Endogenous Transposons

Tc1 was the first transposable element identified in *C. elegans* and a founding member of the *Tc1/mariner* superfamily.[30] These class II elements move throughout the host genome via a 'cut-and-paste' mechanism in which a specific transposon-encoded transposase binds the two terminal inverted repeats of the transposon and catalyzes its excision and reinsertion into a TA dinucleotide. In wild-type strains, *Tc* transposons are only mobile in somatic cells. However, 'mutator' backgrounds can lift germline silencing[31] and allow insertional mutagenesis using *Tc* elements. In 1985, long before any of the genome project tools were available, Iva Greenwald cloned the first *C. elegans* gene, *lin-12*/Notch, using a transposon-tagging and chromosome walking approach.[32] Subsequently, many genetic screens were performed with *Tc1* and the localization of mutagenic *Tc1* insertions was further simplified by the development of a 'transposon-insertion display' technique.[33–36]

Although *Tc1* has been a useful tool for forward genetic screens, some limitations have hampered its routine use. (1) Transposition in mutator backgrounds is an uncontrolled process. Most mutator backgrounds derepress all classes of *Tc1/mariner* transposons and lead to an accumulation of transposon copies in the genome, making it harder to identify the mutagenic insertion. (2) *Tc* mutants are inherently unstable in mutator backgrounds. Spontaneous mobilization of a mutagenic insertion can sometimes lead to imprecise excision of the transposon leaving a DNA footprint which can remain mutagenic but is no longer linked to a transposon. (3) *Tc1* transposons can be spliced out of pre-mRNAs.[37] This results in a mature mRNA that either contains small insertions of *Tc1* sequences and/or lacks gene sequences at the spliced junctions.

Given these limitations, a number of labs have attempted to mobilize heterologous transposons in the *C. elegans* germline (*Himar*, *Sleeping Beauty*, *Minos*). However, all of these transposons could only be mobilized in somatic cells so far. However, one publication reports heritable insertions of the medaka fish transposon *Tol1*.[38] Although this system needs to be further characterized, it could serve as the basis for the gene delivery system because, contrary to *Tc1/mariner* transposons, *Tol1* can carry exogenous sequences and still be reasonably active.[39] To date, the only efficient transposon mutagenesis strategy using a heterologous transposon is based on the mobilization of the *Drosophila mauritiana* element *Mos1*.[40,41]

Mos1 Transposon Mutagenesis

Mos1 is a member of the *Tc1/mariner* transposon family. It only requires its transposase but no host cell factor for transposition. Therefore, it has been possible to mobilize *Mos1 in vitro* and in a number of evolutionarily distant species.[42,43] The *Mos1* sequence is absent from the *C. elegans* genome making it a unique molecular feature which can be easily identified by standard molecular biology techniques (Figure 2).[44] *Mos1*-containing strains can be outcrossed against N2 or any other N2-derived background, removing all unlinked copies. *Mos1* transposons are active only when the *Mos1* transposase is provided. Therefore, *Mos1* insertions are stable in the genome once the source of transposase has been lost. Finally, a study of over 900 insertions demonstrated no strong insertional bias of *Mos1* except for a site on chromosome I.[45]

Mos1 mutagenesis is based on a binary system (Figure 2).[40,44] Two extrachromosomal transgenes are used: one that contains multiple copies of the *Mos1* transposon (transposon array), and another that provides the transposase under the control of a heat-shock inducible promoter (transposase array). When the transposase activity is induced in doubly transgenic worms, *Mos1* can jump from the 'transposon array' into the genome. The extrachromosomal arrays are lost in subsequent generations, eliminating the risk for *Mos1* re-excision. *Mos1* is approximately 10 times less efficient than EMS as a mutagen under optimal conditions.[46] This low efficiency has to be contrasted with the ease, speed, and precision with which *Mos1* can be located.[44] *Mos1* transposons in a given strain can be quickly mapped by inverse PCR and sequencing of the *Mos1*-flanking genomic regions, revealing their location with single base pair precision (Figure 2). On average, 2.5 insertions (range from 1 to 10) can be found per animal, making it easy to genetically associate a given insertion with a mutant phenotype. *Mos1* has been used successfully in different forward genetic screens.[47–50] In addition, *Mos1* mutagenesis can also be used in *C. briggsae*, a related nematode species useful for comparative functional studies.[51] Most importantly, each *Mos1* insertion can be used as a starting point to engineer the genome, as described in detail later in this review.
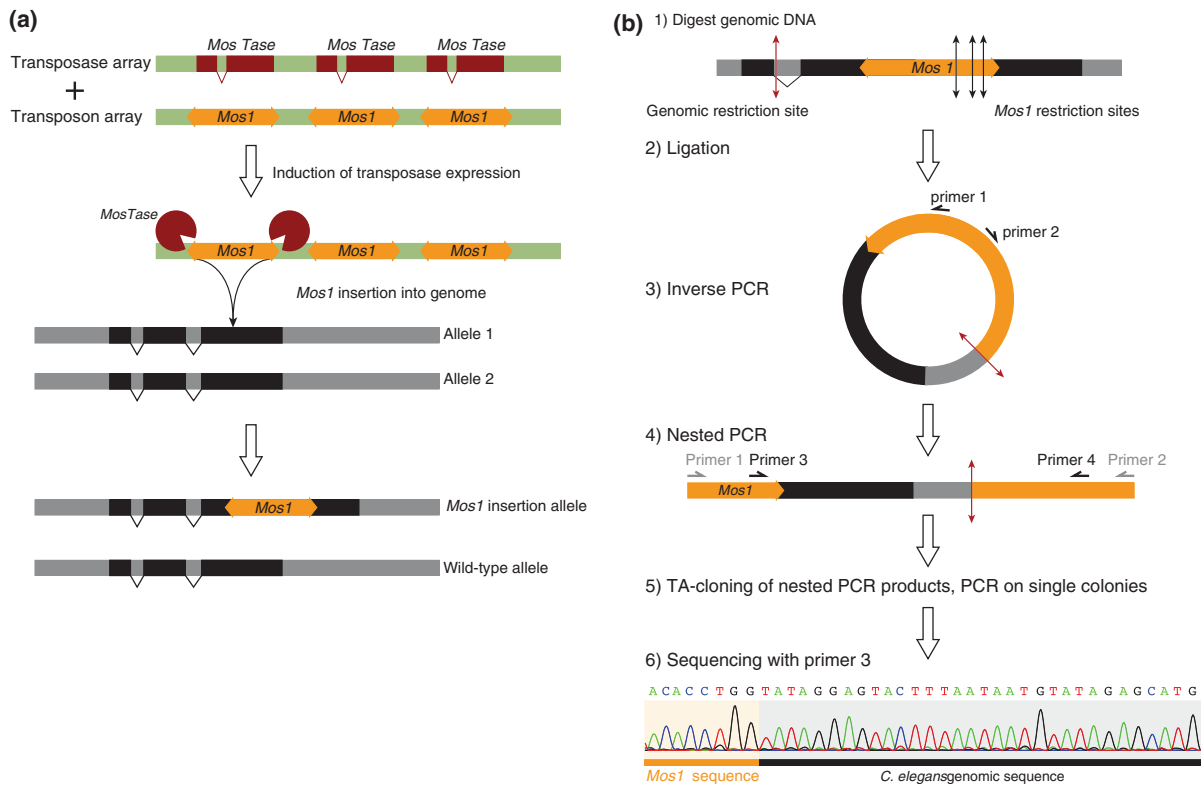
FIGURE 2 | *Mos1*-based insertional mutagenesis. (a) *Mos1* can jump from an extrachromosomal transgene carrying multiple copies of the transposon into the genome, following the expression of the *Mos1*-specific transposase via a heat-shock inducible transgene. *Mos1* insertions are generally random in the genome but always take place at a TA dinucleotide. (b) *Mos1* insertions can be rapidly mapped with single nucleotide resolution by sequencing the flanking genomic sequences using an inverse PCR strategy. (Reprinted with permission from Ref 44. Copyright 2007 Nature Publishing Group)

Automating Genetic Screens

There are many clever ways to perform genetic screens in *C. elegans*[52] but many of them still rely on a researcher sitting for innumerable hours behind a microscope sifting through mountains of worms. In contrast, 'high-throughput' screens make it possible to (1) approach genetic saturation, (2) use less efficient mutagens (e.g., transposons), and (3) increase the chances of finding rare and unusual alleles (e.g., temperature-sensitive, hyper-, anti-, or neomorphic alleles). Recently, certain screens have been automated using 'worm sorters' and microfluidic devices.

The COPAS (Complex Object Parametric Analyzer and Sorter) Biosort machine (Union Biometrica) can sort hundreds of worms per minute, based on fluorescence, optical density, or length. It is used to distribute worms into multiwell plates, for example, for high-throughput screening of chemical libraries[53,54] or to generate *C. elegans* libraries.[55] But its most powerful feature is the ability to measure fluorescence profiles along the length of the animal at two different wavelength. This has allowed, for example, to analyze spatiotemporal promoter activity,[56] identify mutants required for the execution of neuronal cell fates,[57] or screen for genes involved in the innate immune response of *C. elegans* to pathogens.[58]

Although the COPAS sorter is able to distinguish fluorescence changes along the body axis, it is not adapted for high-resolution analysis. Microfluidic devices have now been developed that allow automated screening of live *C. elegans*.[59,60] Worms can be automatically loaded into 'worm chips', mechanically immobilized, imaged at high resolution, and sorted based on complex phenotypic criteria with high accuracy. However, these elaborate systems require more processing time, with throughput still being significantly smaller than the COPAS worm sorter. Alternatively, approaches combining high-resolution confocal microscopy and image analysis software could be adapted for some screening approaches. By taking advantage of the reproducibility of the *C. elegans* cell lineage and anatomy, it was possible, for example, to automatically assess the expression profiles of ~100 genes in ~400 cells of L1 stage larvae[61] and to analyze gene expression during embryonic development with single-cell resolution.[62]

REVERSE GENETICS

With the completion of the worm's genome sequence over a decade ago,[8] reverse genetic approaches have become increasingly popular. Instead of searching for genes implicated in a given process by phenotype ('forward genetics'), the goal of reverse genetics is to characterize the function of a selected gene by inactivating the expression of the gene either by RNAi or by isolating a loss-of-function allele.

Manipulating Gene Activity by RNAi

RNAi was first discovered in *C. elegans*,[7,63] rapidly generalized to all other model systems from plants to vertebrates and recognized as a revolutionary approach to modify gene expression with potential medical applications. RNAi immediately became an attractive gene knock-down technology in *C. elegans*. With RNAi and the complete genome sequence, it was theoretically possible for the first time to easily and rapidly test the function of a large number of genes in a systematic fashion. This led to remarkable large-scale screens for cytokinesis,[64] essential genes and cell polarity,[65] lifespan,[66] fat regulation,[67] and gene function at the whole-genome level.[68,69] Hundreds of genes gained new functions through RNAi-based studies. However, it also became clear that not all genes were affected by RNAi and affected genes often did not show phenotypes as strong as corresponding genetic null mutants. Intriguingly, RNAi seemed to be much more efficient in certain tissues, with neurons being often refractory to RNAi. This surprising result led researchers to investigate the basis for this tissue specificity and identify sensitized mutant backgrounds which display increased RNAi sensitivity.[70–75]

RNAi can be applied at any stage during development, which makes it easier to study essential genes or to study a gene's function at different stages of development. dsRNA can be efficiently delivered in worms by four protocols.[76,77] Worms can either be (1) injected with *in vitro*-transcribed dsRNA[7]; (2) soaked in buffer containing concentrated *in vitro*-transcribed dsRNA[78]; (3) fed bacteria expressing dsRNA from an engineered plasmid[79–81]; (4) transformed with a vector expressing dsRNA in a given tissue.[82–84] Injection of dsRNA into the gonad of young adult hermaphrodites, yields the greatest number of affected progeny. Surprising at first, injection into the intestine or body cavity also produces phenotypes in the progeny due to systemic RNAi.[85] Younger larvae may also be injected when the effect of RNAi needs to be tested on the injected animal and not its progeny. Following injection, the effect of RNAi lasts several days, affecting the adult progeny of an injected worm. Recently, an approach has been developed which allows temperature-dependent induction of RNAi using the *mec-8*-dependent regulation of *mec-2* intron 9 splicing.[86] In addition, expression of the transmembrane protein SID-1[85] in neurons has been found to increase neuronal response to dsRNA delivered by feeding.[87] Finally, clones for ∼94% of all *C. elegans* genes are available from RNAi-feeding libraries generated by the Ahringer and Vidal labs and can be procured from commercial suppliers (Source BioScience LifeSciences and Open Biosystems, respectively).

However, a few limitations of RNAi have to be kept in mind. (1) A major disadvantage of RNAi compared to genetic mutation is the variable efficiency and potency of RNAi depending on the experimental conditions. (2) It is not possible to assess unambiguously the degree to which a gene's function is knocked down by RNAi. This is a significant caveat when interpreting RNAi results. (3) As discussed previously, some tissue types are insensitive to RNAi in the wild-type context but sometimes also in sensitized backgrounds. (4) The efficiency of RNAi decreases rapidly when multiple genes are targeted at once, limiting genetic interactions tests based solely on RNAi. (5) A given RNAi clone can sometimes target more than one gene, based on sequence similarity.[88] If no mutant allele is available to confirm an RNAi result, it is important to conduct RNAi with distinct non-overlapping dsRNA sequences derived from the target gene to avoid off-target effects.[89]

Inhibition of MicroRNA Function by Antisense Oligoribonucleotides

MicroRNAs (miRNA) constitute a large number of specialized, non-protein coding regulatory genes.[90] Their function cannot be easily manipulated by conventional RNAi. When no genetic mutant is available for an miRNA of interest, its function can be inhibited by injecting antisense 2′-*O*-methyl oligoribonucleotides. Injection of these antisense compounds into larvae phenocopies *let-7* loss-of-function defects.[91] However, injection into adult hermaphrodite gonads does not result in efficient miRNA inhibition suggesting that antisense oligoribonucleotides are not transmitted to the progeny efficiently. This approach was recently optimized by cross-linking antisense 2′-*O*-methyl oligoribonucleotides to dextran, a soluble polysaccharide.[92] These novel antisense conjugates can be conveniently injected into hermaphrodite gonads and their effects assessed in the progeny up to late larval stages. Conjugated oligoribonucleotides
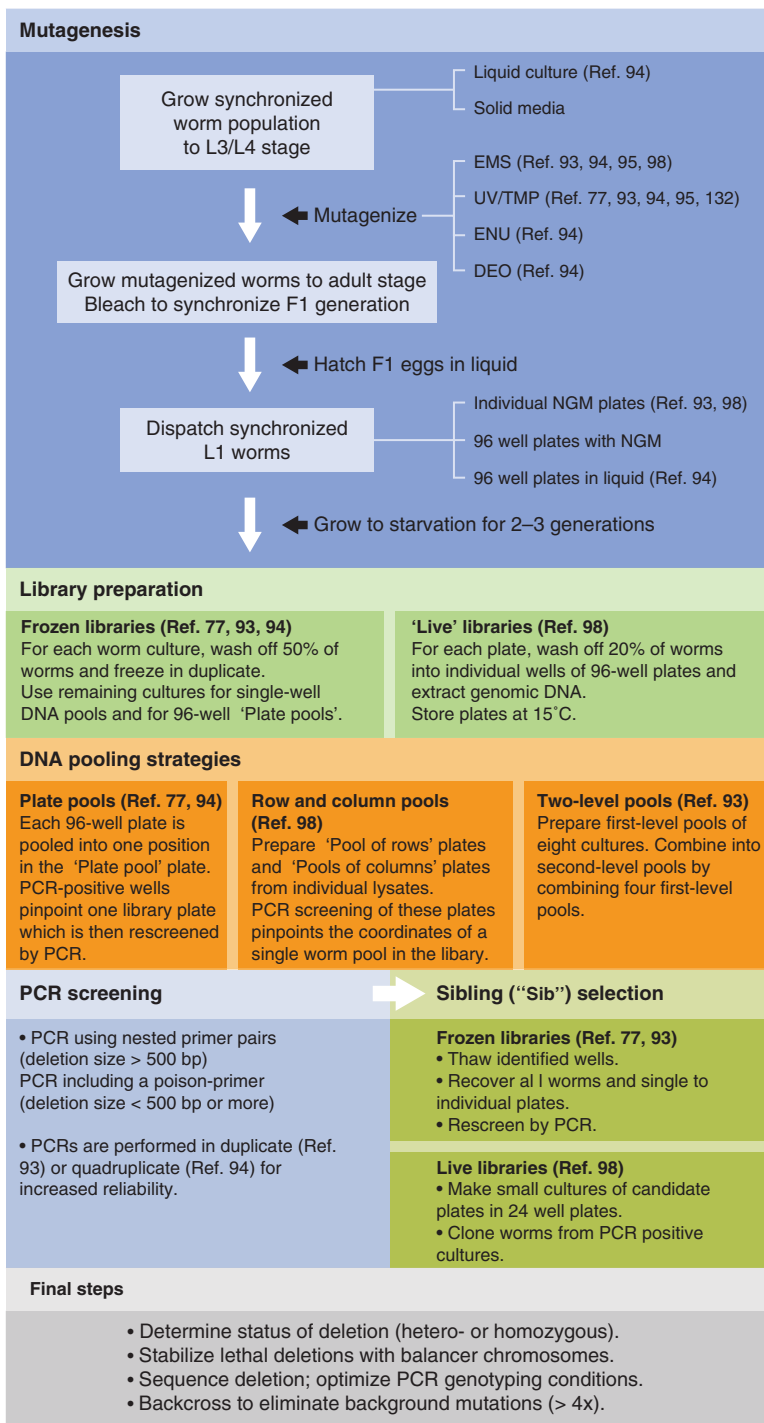
Mutagenesis

Grow synchronized worm population to L3/L4 stage
- Liquid culture (Ref. 94)
- Solid media

Mutagenize
- EMS (Ref. 93, 94, 95, 98)
- UV/TMP (Ref. 77, 93, 94, 95, 132)
- ENU (Ref. 94)
- DEO (Ref. 94)

Grow mutagenized worms to adult stage
Bleach to synchronize F1 generation

Hatch F1 eggs in liquid

Dispatch synchronized L1 worms
- Individual NGM plates (Ref. 93, 98)
- 96 well plates with NGM
- 96 well plates in liquid (Ref. 94)

Grow to starvation for 2–3 generations

Library preparation

Frozen libraries (Ref. 77, 93, 94)
For each worm culture, wash off 50% of worms and freeze in duplicate.
Use remaining cultures for single-well DNA pools and for 96-well 'Plate pools'.

'Live' libraries (Ref. 98)
For each plate, wash off 20% of worms into individual wells of 96-well plates and extract genomic DNA.
Store plates at 15°C.

DNA pooling strategies

Plate pools (Ref. 77, 94)
Each 96-well plate is pooled into one position in the 'Plate pool' plate. PCR-positive wells pinpoint one library plate which is then rescreened by PCR.

Row and column pools (Ref. 98)
Prepare 'Pool of rows' plates and 'Pools of columns' plates from individual lysates. PCR screening of these plates pinpoints the coordinates of a single worm pool in the library.

Two-level pools (Ref. 93)
Prepare first-level pools of eight cultures. Combine into second-level pools by combining four first-level pools.

PCR screening

• PCR using nested primer pairs (deletion size > 500 bp)
PCR including a poison-primer (deletion size < 500 bp or more)

• PCRs are performed in duplicate (Ref. 93) or quadruplicate (Ref. 94) for increased reliability.

Sibling ("Sib") selection

Frozen libraries (Ref. 77, 93)
• Thaw identified wells.
• Recover all worms and single to individual plates.
• Rescreen by PCR.

Live libraries (Ref. 98)
• Make small cultures of candidate plates in 24 well plates.
• Clone worms from PCR positive cultures.

Final steps

• Determine status of deletion (hetero- or homozygous).
• Stabilize lethal deletions with balancer chromosomes.
• Sequence deletion; optimize PCR genotyping conditions.
• Backcross to eliminate background mutations (> 4x).

FIGURE 3 | Generating deletion mutants by library screening. Populations of randomly mutagenized worms can be established using different mutagens and different culture strategies. Mutant strains are either preserved in a frozen library or conserved on NGM plates in a 'live' library. To optimize library screening, DNA samples extracted from all worm cultures can be pooled according to different pooling schemes. Deletions with sizes ranging from 500 to 1500 bp are detected by nested PCR screening. Shorter deletions can be detected more easily by including a poison-primer. Once a candidate pool has been identified, the corresponding well is thawed from the frozen library and all recovered worms are cloned on individual plates for secondary rescreening. Similarly, worms from a 'live' library are recultured in small pools and single worms are picked from PCR-positive pools. Once a single worm harboring a deletion has been isolated, a few steps should be undertaken before the strain can be used for functional studies.

have been shown to efficiently inhibit miRNA function in hypodermis, vulva, and neurons. Their effect is dose-dependent and highly specific. In addition, multiple miRNA can be targeted at once by mixing antisense reagents conjugated with different antisense oligoribonucleotides. This could facilitate the study of miRNA families and address potential functional redundancy.

Generating Deletion Mutants
Obtaining Mutants by Deletion Library Screening

Deletion libraries contain large numbers of worms organized in small pools that have been mutagenized with a deletion-inducing agent, such as UV/TMP or EMS (Figure 3).[93–95] When a deletion is of sufficient size, it can be detected by PCR with primers flanking

the region of interest. Once a pool is found that reproducibly tests positive for a deletion, the worms in the pool are separated and rescreened until a single animal carrying the deletion is isolated. Two groups in the *C. elegans* community generate such mutants upon request: the Mitani Lab[96] and the *C. elegans* gene knock-out consortium[97] in Vancouver. So far, alleles for over 6000 genes have been generated by these two groups (Don Moerman, personal communication). Mutant strains are available from these consortia at a small fee and/or can be ordered from the CGC strain repository. In addition, individual labs can use published protocols to setup libraries to screen for gene deletions using EMS[98] or TMP/UV mutagenesis.[77,99] Building and screening a deletion library is a fairly large-scale project which is best suited when multiple genes are targeted.

Deletion mutants are very useful but a few issues need to be kept in mind: (1) worms retrieved from a library have been heavily mutagenized and usually carry many mutations in their genetic background, sometimes closely linked with the deletion of interest. (2) The size of the deletion is random and usually in the range of few hundred bases, which can be a problem for large genes and result in alleles which are not molecular nulls. (3) The end-points of the deletion cannot be chosen, but only framed by the position of the screening primers. (4) Some deletions causing sterility or lethality can be difficult to recover and need to be stabilized using genetic balancers.[99] (5) Some deletions can be complex rearrangements that are difficult to characterize.

Obtaining Deletion Mutants by Transposon Mobilization
Spontaneous Tc1 Excision
Although *Tc1* elements are fairly large DNA sequences which should disrupt gene function entirely, this is not always the case (see above). It is therefore important to derive a deletion mutant by remobilization of the transposon. In mutator backgrounds, deletions are generated at a low frequency after imprecise excision of the transposon. Most likely, these events result from interrupted gene conversions with the sister strand.[100] This can be used as an alternative to deletion library screening when a *Tc1* transposon insertion is already available at an appropriate position in the gene. Such insertions can come from *Tc1*-based screens, specific PCR-based strategies targeted to a gene of interest,[101] or from *Tc1* mutant collections (CGMC, Lyon, France).[36] This technique offers little to no control over the final structure of the deletion. In addition, PCR screening can be complicated by the frequent mobilization of somatic *Tc1* insertions which can be

confused with heritable germline events and require additional sib-selection steps. Finally, uncontrolled jumping of *Tc1/mariner* transposons in the mutator background is accompanied by increased morbidity of the progeny and the possibility of novel transposon insertions closely linked to the deletion site. To circumvent some of these limitations, a protocol has been developed to precisely engineer genomic deletions or *gfp* insertion alleles by transgene-instructed gene conversion.[102] This strategy is based on an earlier protocol[103] with a few optimizations: (1) two mutator backgrounds with increased *Tc1* excision rates (*mut-2* and *mut-7* vs *mut-6*) were used; (2) the total length of sequence homology was increased from 3 to 9 kb. These modifications increased the frequency of transgene-instructed gene conversion events significantly (4×10^{-4}); however, no further study has been published since this initial report.

Mos1 Transposon Remobilization
Generating deletion alleles through *Mos1* transposon mobilization (MosDEL) is, in our opinion, the superior technique for this purpose (Figure 5). In order to put this technique in context with other *Mos1*-related techniques (*Mos*TIC and MosSCI), we will discuss this approach in the Section *Genome Engineering*.

Obtaining Spontaneous Deletions in the dog-1 *Mutant Background*
G4 DNA (guanine-quadruplex DNA) is a sequence motif that can fold into quadruplex structures *in vitro*. *In vivo*, such stable secondary structures can be problematic for DNA and RNA polymerases. Consistently, G4 DNA motifs have been identified as highly mutagenic sites in *dog-1* mutant animals.[104,105] *dog-1* is the *C. elegans* ortholog of the mammalian DNA helicase FANCJ, which is mutated in Fanconi anemia patients. There are 2907 sites matching the G4 DNA signature in the *C. elegans* genome. In a proof-of-principle study, candidate sites were chosen to isolate deletion mutants in 10 genes. In brief, 96 pools of *dog-1(pk2247)* mutant worms were grown to starvation. After DNA isolation, each pool was screened by PCR for deletions at 5′ of various G4 DNA sites. This approach led to the isolation of 11 deletion alleles for the 10 targeted genes. This strategy could be generalized to the 1642 genes that are located 5′ to the 2907 G4 DNA sites. Approximately, three quarters of these genes had no available alleles at the time of the study.[105]

Generating Targeted Deletions by Spontaneous Gene Conversion
Spontaneous recombination events between an engineered DNA template and a targeted genomic locus
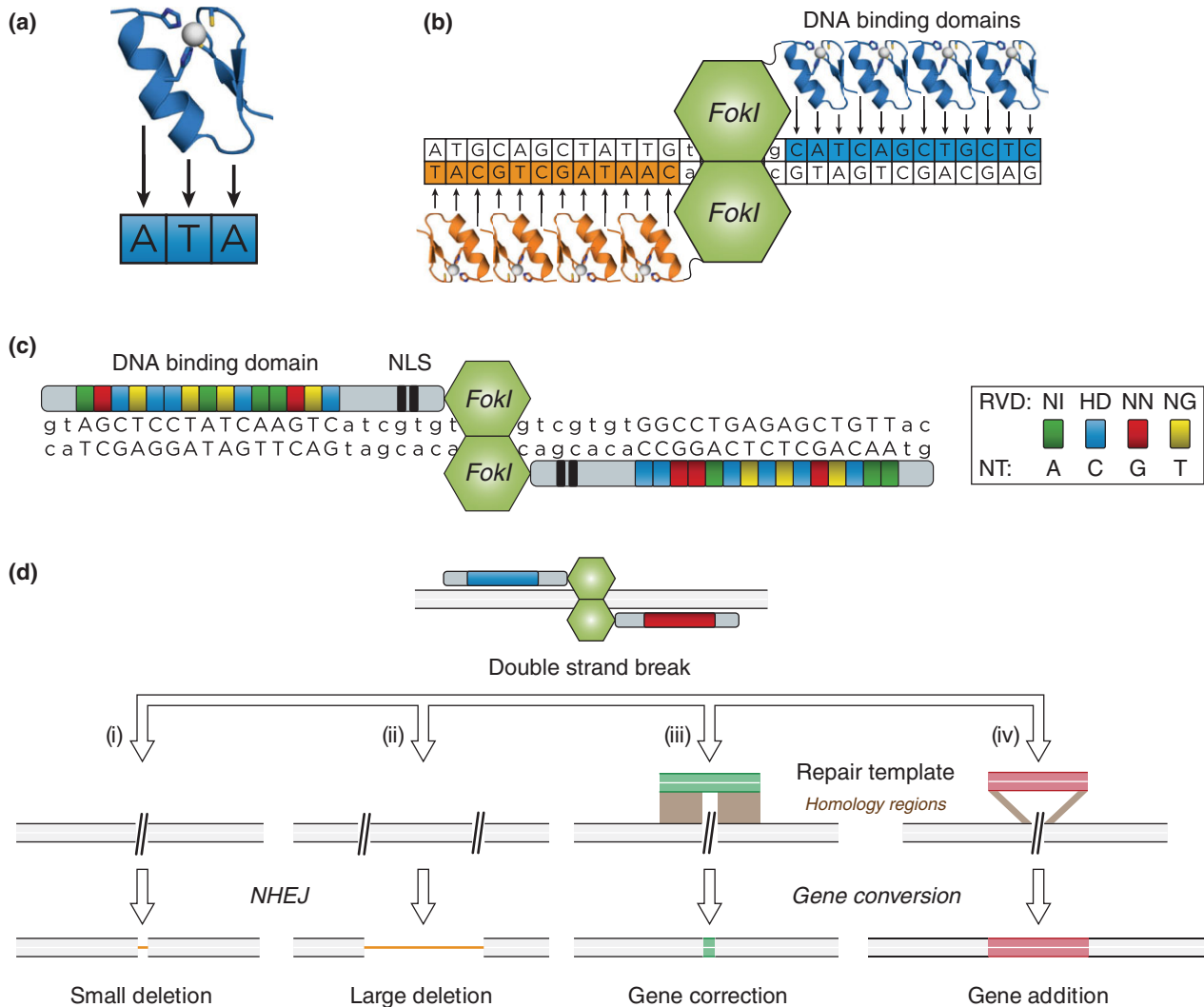
FIGURE 4 | Genome editing with engineered nucleases. (a) Structure of a zinc finger (ZF) DNA-binding module. Each individual ZF domain is optimized to recognize a 3-bp sequence. Two cysteines and two histidines coordinate a zinc atom in each module. (b) ZF nucleases (ZFN) are composed of at least four ZF domain repeats, linked to the *FokI* endonuclease domain. Specificity is increased by using obligatory dimers of two ZFN recognizing two sites which flank the targeted double-strand break (DSB) site. (c) Structure of TALE nucleases (TALEN). A central array of tandem repeats forms the specific DNA-binding domain. Two nucleotides (repeat-variable di-residues, RVD) in each repeat determine the sequence preference of each module. Examples of four RVDs that recognize all four nucleotides (NT) are indicated. Two TALEN monomers bind to their target sites and allow the dimerization of the *FokI* endonuclease domain. TALENs also contain a nuclear localization signal (NLS). (d) ZFNs and TALENs allow different types of genome editing strategies. A DSB can be resolved by different pathways. (i) A single DSB can be repaired by non-homologous end-joining (NHEJ) and result in a small deletion of a few base pairs. Alternatively, (ii) two simultaneous DSB can generate a larger deletion allele. When a repair template with homology regions is provided, the DSB is resolved by gene conversion to (iii) insert a small sequence (as small as a single nucleotide, i.e., 'gene correction') or (iv) a larger transgene ('gene addition').

are extremely rare in *C. elegans*, most likely, because injected DNA preferably forms extrachromosomal arrays and is therefore less available from homologous recombination. Early on, spontaneous gene conversion was obtained by injecting DNA into meiotic oocytes of individual worms which is technically challenging.[106,107] Another strategy has used microparticle bombardment to deliver a recombination template to a very large number of worms with little hands-on work.[108,109] The recombination template is composed of two homology arms which define the deletion end-points and flank a positive selection marker (*unc-119*(+)). In practice, this approach relies on generating hundreds of integrated lines in a *unc-119(ed3)* mutant background and selecting the rare homologous recombination events. When this strategy was used to disrupt the *unc-54* and *unc-22* genes, 1 and 3 homologous recombination events

from 400 and 274 integrated lines were retrieved, respectively.[108] This strategy was recently refined by 'tagging' the bombardment plasmid with a *gfp* reporter gene (located outside the homology arms), which makes it easy to distinguish recombination events from non-homologous integrations events.[110] In addition, FLP recombination sites were added upstream and downstream of *unc-119*(+) to create 'clean' mutants by removing the cassette using tissue-specific FRT expression. Unfortunately, it was impossible to delete the *unc-119* cassette in the germline and no further report using the technique has been published since.

Genome-Scale Identification of Point Mutations

Although chemical mutagens such as EMS can generate deletions (generally small and at a low frequency), they overwhelmingly induce point mutations in the genome. Two approaches have been devised to identify point mutants in individual genes throughout the entire genome.

TILLING: Targeting-Induced Local Lesions in Genomes

TILLING aims to identify single nucleotide changes in target genes in a library of randomly mutagenized worms. PCR-amplified DNA from control and mutagenized worms is hybridized and digested with a single-strand-specific nuclease. Digested fragments are detected via the fluorescently labeled primers used for amplification. The position of the mutation can be estimated by determining the size of the resulting DNA fragment on a denaturing polyacrylamide LI-COR gel. Using a library of 1500 mutagenized worms, TILLING was successfully used to identify 71 mutations in 10 target genes; 3% are putative null alleles and 59% result in missense mutations. The remaining mutants are silent.[111] TILLING appears not to have been used in *C. elegans* since this initial study.

Target-Selected Mutagenesis by High-Throughput Resequencing

Nonsense mutations can be identified from a library of mutagenized worms by cost-optimized high-throughput resequencing of target genes.[112] First, a clonal library of 6144 mutagenized F1 animals was generated and cryopreserved. Next, gene regions of interest were amplified by nested PCR and sequenced. Calculations based on a pilot screen suggest that knockout mutations for over 90% of *C. elegans* genes are present in this library. Furthermore, if every non-synonymous amino-acid change is taken into account,

then every eighth amino of the proteome should be mutated. In order to reduce the screening complexity, the authors propose to only target genomic positions called NIMs (nonsense introducing mutations). As 93% of EMS mutations are G to A transitions and based on the size of the ORFeome (~24 Mb), only 500,000 NIMs should be targeted in the genome. Theoretically, this could now be achieved by aCGH or even WGS.

Genome Editing Using Sequence Specific Nucleases: ZFNs and TALENs

Genome editing using *in vitro*-engineered Zn finger nucleases (ZFNs)[113] and TALE-based hybrid nucleases (TALENs)[114,115] has been recently demonstrated in *C. elegans*.[116] These proteins combine a tailor-made DNA-binding module with the endonuclease domain of the *FokI* enzyme (Figure 4). Recognition of specific DNA sequences by a nuclease dimer is followed by DNA cleavage between the two DNA-binding sites. Imprecise repair of this double-strand break (DSB), usually through error-prone non-homologous end-joining (NHEJ), results in small deletions or insertions of a few base pairs at the target locus, thereby likely disrupting the function of the targeted sequences, such as genes or *cis*-regulatory elements.[116]

Genome editing in *C. elegans* is a four step process. First, two complementary nucleases that recognize a specific site in the genome must be designed, constructed and validated. Next, 5′ and 3′ UTR sequences favorable for germline translation are added to maximize the expression of the enzymes in the *C. elegans* germline. This artificial ORF is then transcribed *in vitro* to obtain highly concentrated, 5′ capped, messenger RNA. Finally, RNA mixtures of the two complementary nucleases are directly injected into the gonad using the same procedure as for DNA germline transformation. Mutant alleles can then be selected in the F1 or F2 progeny of an injected animal. In the proof-of-principle study, mutant alleles were found in 1% of the total progeny of an injected animal (5%, if an optimal 4-h time window was considered).[116] Whole-genome analysis of one mutant strain revealed no off-target, ZFN-related lesions, confirming the very high specificity of this strategy. ZFN-based editing was also successfully applied to generate mutations in the related nematode species *C. briggsae*.[116]

Even though operated by similar overall principles, the TALE-based nucleases are more straightforward to design, hence more cost-effective and will probably be the future method of choice. Different genome editing strategies which are used in

other model systems[113] could be adapted for *C. elegans* to allow for very precise genome engineering (Figure 4(d)).

Engineering the Genome: from Single Base Changes to Complex Gene Modification

The goal of genome engineering is to mutate a gene of interest or to modify a genomic locus by introducing *in vitro*-engineered sequences that, for example, contain affinity or reporter tags or alter the activity or localization of a gene. As mentioned previously, spontaneous gene conversion events are extremely rare in *C. elegans*. One efficient way to increase the rate of homologous recombination is to induce DSBs by reexcising a transposon. Two types of transposons have been successfully used in *C. elegans*: endogenous *Tc1* and *Drosophila Mos1*.

Chromosomal DSBs are catastrophic events which can be resolved using multiple cellular mechanisms.[117] In brief, DSBs are repaired either by using homology of DNA sequences flanking the breakpoint (gene conversion) or by religation of free DNA ends (NHEJ). If the break produces cohesive ends, NHEJ will restore the wild-type sequences. If the lesion is blunt, partial digestion and religation by NHEJ will introduce small footprints. When a homologous recombination pathway is used, 5′–3′ exonucleases digest DNA to generate single-stranded DNA regions which can carry out homology scanning. If this single-stranded region anneals inappropriately within the same chromosome, DNA synthesis from this position and subsequent religation will generate a deletion around the DSB site. However, this single-stranded region can also anneal with a homologous donor on a different DNA fragment such as sister chromatids, homologous chromosomes, or engineered homologous templates. In the later case, artificial sequences can then be incorporated into the chromosome at the DSB site via gene conversion, resulting in a modified genomic locus and engineered alleles.

Transgene-Instructed Genomic Engineering upon Tc1 Excision

Genome engineering in a template-dependent manner was first reported by Plasterk and Groenen.[103] A strain containing a mutagenic *Tc1* insertion in the *unc-22* locus was microinjected with a wild-type repair template containing silent sequence polymorphisms close to the *Tc1* insertion site. These markers were flanked by 1.5 kb of homologous sequences to drive recombination. Phenotypic revertants of the Unc-22 phenotype were selected from the progeny of these transgenic animals and analyzed. The observed reversion frequency was low (2×10^{-5}) but three quarters of the revertants had incorporated sequence polymorphism up to ∼200 bp from the DSB. This approach and an optimized protocol[102] have, however, remained proof-of-principle experiments.

MosTIC: Mos1 Excision Induced Transgene-Instructed Gene Conversion

*Mos*TIC is the first robust strategy that allows genome engineering in *C. elegans* in a targeted and controlled fashion (Figure 5).[117] *Mos1* excision is used to create a genomic DSB at the engineered locus. This DSB is then repaired by gene conversion using an *in vitro*-engineered template, which is provided on an extrachromosomal transgene. A detailed protocol has been published recently.[118] *Mos*TIC is preceded by a *Mos1* mutagenesis screen (see above) which provides the necessary transposon insertions. Alternatively, a large collection of *Mos1* alleles has been generated and mapped by the NemaGENETAG consortium (searchable on WormBase).[55,119] For efficient *Mos*TIC, *Mos1* insertions should be chosen as close as possible to the target site. Indeed, the frequency of *Mos*TIC events decreases in a bell-shaped curve as a function of the distance from the *Mos1* insertion site. Point mutations have been introduced up to 3 kb away from the insertion site but with a 20-fold lower efficiency than at the insertion site itself.[117] A point mutation contained within a 1-kb-long region centered on the DSB will be transferred into the genome in 50% of *Mos*TIC events.

The *Mos*TIC repair template is composed of two homology arms of 1.5 kb that flank the target region and the *Mos1* insertion site. The rate of *Mos*TIC does not seem to increase when longer homology sequences are used.[117] This template is injected into the *Mos1* carrying strain with one of the two vectors used to trigger *Mos1* transposase expression in the germline: (1) a heat-shock inducible construct using the *Phsp-16.48* promoter (*Phsp-16.48::MosTase*); (2) a construct using the constitutive germline promoter *Pglh-2* (*Pglh-2::MosTase*). In the first case, transgenic lines are established and amplified. Once sufficient transgenic animals are available, adult worms are heat shocked and their progeny is screened for gene conversion events. *Mos*TIC frequency generally ranges from 10^{-3} to 10^{-5} events per offspring of the heat-shocked animals. In the second case, gene conversion events can be detected in the F1 progeny of injected animals but more animals need to be injected because at best 1/10 will produce a *Mos*TIC progeny.[118]

*Mos*TIC events can be detected either by phenotypic reversion[117] or by PCR screening.[49] Note that not all phenotypic reversion events will be *bona fide Mos*TIC. Indeed, DSBs repaired by NHEJ can

sometimes regenerate functional alleles. In the case of PCR screening strategies, false positives can arise from PCR jumping.[117] Therefore, PCR screening conditions should be optimized carefully beforehand.[118] *Mos*TIC has been used successfully to insert point mutations,[117] fluorescent tags,[49,117] epitopes for commercial antibodies,[49] and affinity tags (unpublished). Such *Mos*TIC alleles seem to replicate gene expression and protein localization with high accuracy and fidelity.[49]

MosDEL: Mos1-Mediated Deletion

The goal of MosDEL is to generate *in vitro*-designed deletions around a *Mos1* insertion site in the genome (Figure 5).[120] On the basis of available strains in WormBase (>14,000), 99.4% of the 20,160 *C. elegans* genes are within 25 kb of at least one *Mos1* insertion making them theoretically all targetable. Of these, 8803 have no other genes between them and *Mos1*, so only the targeted gene would be removed. In addition, all *Mos1* alleles obtained by insertional mutagenesis are usable for MosDEL.

The first step of MosDEL is to combine a *Mos1* strain with the *unc-119(ed3)* mutant. These worms are then injected with a DNA mix containing (1) mCherry-expressing vectors (used to identify false positives because of extrachromosomal array formation), (2) the source of *Mos1* transposase (P*glh-2*::*MosTase*), and (3) the deletion template. The deletion template contains the *C. briggsae unc-119*(+) gene flanked by two homology arms which define the planed deletion end-points. When the *Mos1*-induced DSB is repaired using this transgene, the target region is deleted and *Cbr-unc-119*(+) is copied into the locus. This rescues the *unc-119(ed3)* mutation and results in a perfectly balanced deletion allele (Figure 5).

Approximately 100 P$_0$ worms should be injected for MosDEL. In a proof-of-principle experiment aimed at deleting a 25-kb region close to the *dpy-13* locus, injection of 81 animals resulted in seven stable lines carrying a *Cbr-unc-119*(+) fragment (9%).[120] Four of these seven lines were complete deletions of the 25-kb region (5%). However, no complete deletion of 35 or 50 kb could be recovered. MosDEL currently appears as the most efficient tool to engineer precise gene deletions in the *C. elegans* genome.

Recent experiments show that antibiotics neomycin[121] and puromycin[122] can be used as efficient selection markers in worms. Current efforts to facilitate MosDEL focus on using antibiotic markers (Christian Frøkjær-Jensen and Erik M. Jorgensen, personal communication), which will eliminate the need to carry an *unc-119* mutation in the background.

EXPRESSING GENES IN *C. ELEGANS*

There are numerous reasons to introduce genes in *C. elegans*, including reporter gene analysis, transformation rescue of mutant phenotypes, manipulation of cell function, etc.

Classical *C. elegans* Transgenesis

Transformation of *C. elegans* was first reported in the early 1980 in a study of the sex determination gene *tra-3*.[123] Kimble et al. microinjected an amber codon-suppressing tRNA (*sup-7(st5)*) into the hermaphrodite gonad of *tra-3* mutants carrying an amber allele which resulted in suppression of the Tra-3 phenotype in the progeny. Integrative[107] and non-integrative[124] DNA-mediated transformation was derived from this approach soon after.

DNA transformation by microinjection is a very fast, simple, inexpensive, and efficient way to generate transgenic *C. elegans* strains.[5,10,124] Injection requires little to no preparation and only few worms need to be injected to obtain transgenic lines. The *C. elegans* hermaphrodite germline is a syncytium containing hundreds of mitotically active oocytes at different stages of maturation and almost all nuclei share a common cytoplasm. Different DNA species can be co-injected (plasmids, PCR products, cosmids, fosmids, BACs, YACs) with selectable injection markers (fluorescent reporter genes, dominant or recessive phenotypic markers). This DNA mix will be processed by DNA end ligation and homologous recombination into large, heritable DNA concatemers called extrachromosomal arrays.[5,124] These episomes are recognized by the replication machinery and usually segregate like chromosomes during cytokinesis and meiosis. Detailed transgenesis protocols can be found in Refs 10 and 125 (see Jin's work in Ref 10).

The ease with which these transgenes can be generated has to be balanced against the limitations of this approach. (1) Classical transgenesis offers little to no control over expression levels. Extrachromosomal transgenes generally contain hundreds of copies of the transforming DNA and therefore almost always result in overexpression. To obtain a more physiological result, the concentration of a given DNA species can be reduced by diluting it with more carrier DNA. (2) The highly repetitive nature of extrachromosomal transgenes leads to silencing in the germline.[126] This effect can be limited by generating 'complex arrays' by co-injecting digested genomic DNA, preferably from a bacterial source. (3) Transgenes can rarely recreate the full genomic context and regulatory sequences necessary for accurate gene expression. Strikingly, the linear or circular nature of the injected DNA

can influence the resulting expression patterns, with linear, vector-free DNA producing more accurate results.[127] (4) Extrachromosomal arrays can evolve over time. In particular, their expressivity and stability can change rapidly between generations.[124] It is therefore recommended to freeze transgenic lines as soon as possible and return to the original isolate if necessary. (5) Extrachromosomal arrays are semi-stable and not all progeny of a transgenic animal will inherit the transgene. Therefore, they require manual maintenance unless a selectable co-injection marker is used. For example, transgenic animals can be generated in a *pha-1(e2123ts)* mutant background. These *pha-1* mutants are viable at 15°C but require the wild-type *pha-1* gene to survive at 25°C.[128] Very recently, two groups have developed antibiotic selection systems that can be used in any genetic background. Neomycin (or G-418)[121] and puromycin[122] are translation inhibitors which prevent the larval development of *C. elegans*. Ubiquitous expression of the corresponding resistance genes allows hands-off selection of transgenic animals and robust enrichment of large transgenic populations.

Stable Integration of *C. elegans* Transgenes

Integrative transgenesis was first achieved by injecting DNA into the nuclei of maturing oocytes.[106,107] Some integration events have also been reported when single-stranded oligonucleotides of 50 bases are co-injected with double-stranded DNA.[5] However, these approaches are inefficient or technically challenging. Therefore, transgene integration is generally achieved by irradiating extrachromosomal lines with γ or UV radiation (see Jin's work in Ref 10 and 125). Transgene sequences are randomly integrated into the genome, most likely, following erroneous repair of a chromosomal break. The resulting stable lines need to be extensively outcrossed to remove background mutations. Moreover, it is recommended to obtain independent integrated lines because some variability in expression can be observed, probably due to positional effects and the amount of integrated DNA.

Stable Transformation by Microparticle Bombardment

Microparticle bombardment has been used successfully in *C. elegans* to generate single to low-copy integrated transformant.[129,130] Gold beads coated with DNA are projected at high velocity at the sample allowing penetration into cells. The small amount of DNA on each bead likely decreases the chances of extrachromosomal array formation, therefore favoring integration events. Bombardment lines show more reproducible gene expression than extrachromosomal lines, but some variability has been reported.[131] This approach also allows expression of transgenes in the germline because the low-copy number circumvents germline-silencing observed for multicopy extrachromosomal arrays.[126] The low-integration frequency ($\sim 5 \times 10^{-5}$) requires a positive selection strategy (usually *unc-119(ed3)* rescue) and the use of large numbers of synchronized worms.[129] Detailed instructions for transformation by microparticle bombardment can be found in Ref 132.

MosSCI: Generating Single-Copy Transgenes by Homologous Recombination

The goal of MosSCI (*Mos1*-mediated single-copy insertion) is to generate stable single-copy transgenic strains by transgene-instructed gene conversion (Figure 5).[131] The MosSCI approach is based on *Mos*TIC[117] with some specific modifications: (1) MosSCI is performed at one of two genomic sites (on chromosome II and IV) which are located in the 3′ regions of two opposing genes. Therefore, insertions there should minimally disrupt gene function. (2) MosSCI uses positive and negative selection strategies to identify *bona fide* gene conversion events. (3) A toolbox of vectors is available to rapidly generate MosSCI constructs (http://sites.google.com/site/jorgensenmossci/), in particular, for germline expression.[133]

The first step for MosSCI is to build the recombination template. This vector contains two 1.5-kb-long homology arms corresponding to one of the two genomic insertion sites (Figure 5). These sequences flank the *C. briggsae unc-119*(+) gene used for positive selection and a multiple cloning site or two gateway *att* recombination sequences which are used to clone the transgene. Transgenes of 3–8 kb have been inserted into the genome using these vectors[131] and unpublished results indicate that insert sizes can be as large as 17 kb (Frøkjær-Jensen and Jorgensen, personal communication). Transgene insertions generated by MosSCI should be designated by 'Si' (following the unique laboratory identifier).

Two protocols can be used for MosSCI. (1) The direct insertion protocol: the MosSCI vector is co-injected with mCherry-expressing vectors and the *Pglh-2*::*MosTase* plasmid and recombination events are selected directly in the F2–F3 progeny of injected animals. This requires to inject at least 25–50 P_0 animals, of which 2–5 will generate a MosSCI allele. On average, 60% of these lines will contain complete, single-copy inserts. (2) The selection-based insertion protocol: the MosSCI vector is co-injected with

the *Phsp-16.48::MosTase* construct, multiple vectors expressing *mCherry* in different tissues and a muscle-expressed gain-of-function mutant of the *twk-18* potassium channel. MosSCI is triggered by heat shocking these transgenic animals and *mCherry* and *twk-18* are used as counterselection markers to eliminate animals which rescue the Unc-119 phenotype but are not true MosSCI lines. The direct insertion protocol is generally much faster (~10 days vs 1 month) and requires less hands-on time. However, the selection-based insertion protocol can be scaled up significantly and displays higher success rates.

MosSCI recombination events are detected by the phenotypic rescue of *unc-119(ed3)*. However, *unc-119(ed3)* mutant are fairly unhealthy and difficult to inject. Recently, an alternative strategy usable in any genetic background and based on neomycin resistance has been used successfully.[121]

Up-to-date information, detailed instructions, descriptions of reagents, and strains can be found in the Website (http://sites.google.com/site/jorgensenmossci/). Upcoming developments of this technique are: (1) vectors and strains for five new genomic sites; (2) an improved insertion method with a success rate above 50%; (3) a new, heat-shock inducible negative selection marker that simplifies the selection of insertion events; (4) in-depth characterization of each site regarding its permissiveness for germline expression (Frøkjær-Jensen and Jorgensen, personal communication).

Spatial and Temporal Manipulation of Gene Expression

Cell-specific gene expression can be achieved easily in *C. elegans* using specific, heterologous promoter fragments, and gene expression can be controlled temporally using broadly, if not ubiquitously, expressed heat-shock-induced promoters. One approach to achieve both temporal *and* spatial control has been to combine cell-specific rescue of a mutant defective in the heat-shock response (*hsf-1(sy441)*) with a transgene expressing a gene of interest under the control of a heat-shock-responsive promoter.[134] Gene induction is therefore restricted to the *hsf-1*-expressing cells. This two component system was used to express *gfp* in the pharynx and neurons. It functions at all developmental stages and requires only a 30-min heat-shock treatment. GFP expression was seen 1 h after heat shock and remained visible for 24 h. This system can also be used to study gene function as exemplified by the rescue of the dye filling defect of a mutant defective in neuron cilium formation and function.[134]

A second approach for controlled gene expression is based on the *mec-8*-dependent alternative splicing of *mec-2*.[86] MEC-8 is an RNA-binding protein that regulates the formation of the long *mec-2a* mRNA by promoting exon 9–10 over 9–9′ splicing. Hence, inclusion of the ninth *mec-2* intron into another gene confers *mec-8*-dependent regulation. Using the temperature-sensitive *mec-8(ut218ts)* allele, it is possible to control splicing and gene expression by shifting worms from the restrictive (25°C) to the permissive (15°C) temperature. This approach was used to activate *yfp* expression in neurons, hypodermis, and intestine. However, although *mec-8* is ubiquitously expressed early in development, it is restricted to some tissue types at later stages. Therefore, *gfp* expression was seen in head and tail neurons but not in the ventral nerve cord, somewhat limiting this approach. A temperature-sensitive *mec-8(ut218ts)* allele could be ectopically expressed in the targeted tissue to circumvent this limitation. By extension, this approach was used to selectively express genes in cells for which no specific promoters are known by using promoters with partially overlapping expression domains to express *mec-8(ut218ts)* and the targeted gene fused to the ninth *mec-2* intron.[86]

Another approach is based on the use of FLP recombinases which allows for permanent induction of gene expression in any genetic background.[110,135,136] The FLP enzyme catalyzes the intramolecular excision of DNA located between two FRT sites when they are oriented in the same direction ('FLP-out'). FLP-out activity in *C. elegans* was demonstrated by removing a cassette containing a fluorescent protein and a transcriptional termination sequence flanked by FRT sites.[135,136] Induction of FLP expression using a heat-shock promoter resulted in total or partial loss of the FRT-flanked sequences and the 'activation' of the downstream ORF. This system was used as a lineage tracing tool, to induce cell-specific RNAi, express a dominant negative transcription factor[136] and to silence neurotransmission in a temporally and spatially controlled fashion using tetanus toxin.[135] Both publications provide vector toolkits that can be used to adapt these technologies to specific biological questions. Recently, somatic FLP-mediated recombination has been demonstrated in the context of a genomic knock-in allele generated by biolistic transformation.[110]

Building Reporter Gene Constructs by Fosmid Recombineering

Determining a gene's expression pattern is a necessary step in the characterization of any *C. elegans* gene. This can be achieved by using transcriptional or translational *gfp* or LacZ reporters.[6] Reporter
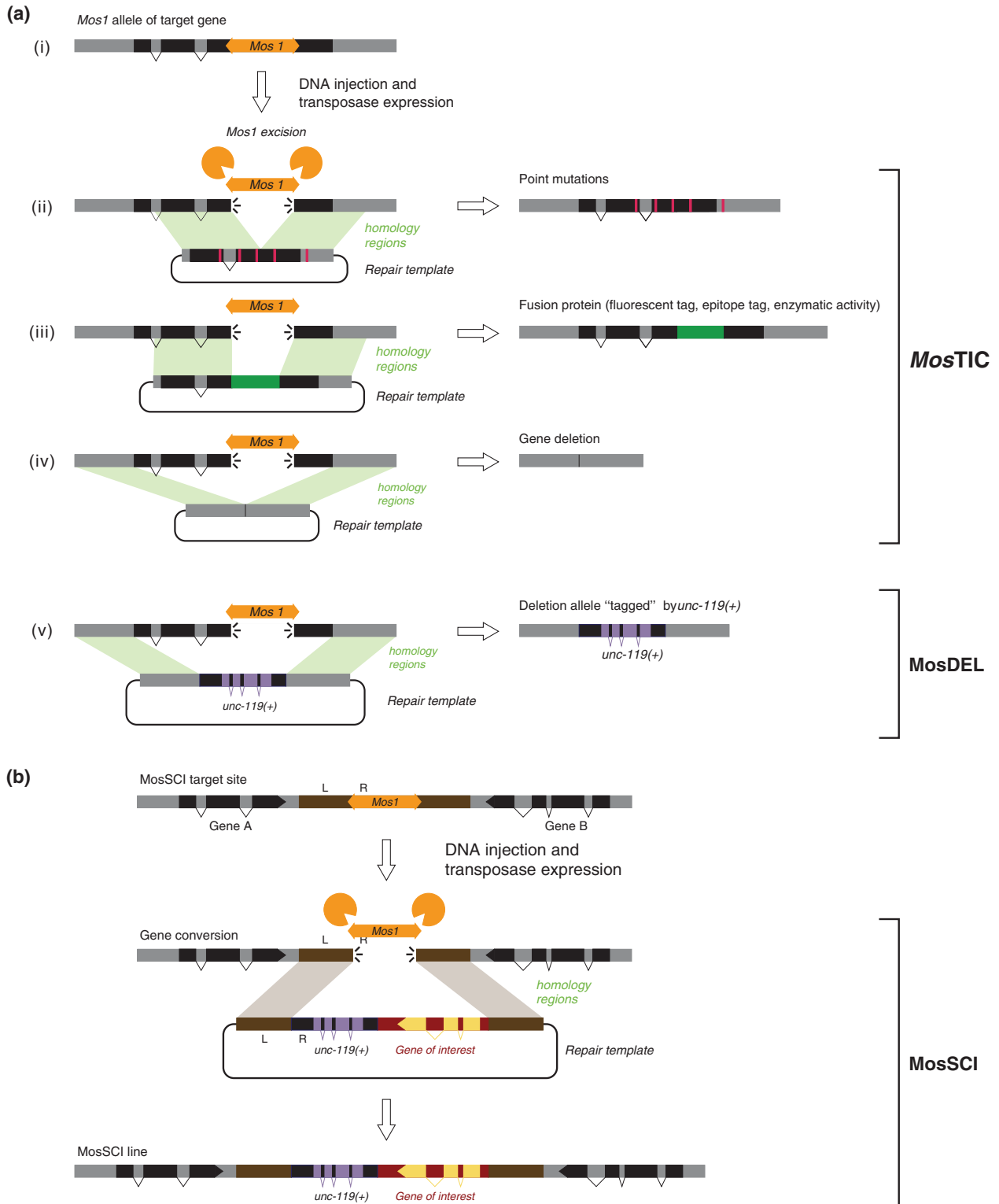
FIGURE 5 | *Mos*TIC, MosDEL and MosSCI strategies. (a) The double-strand break (DSB) induced by the excision of *Mos1* (i) can be repaired by *Mos*TIC using different repair templates to engineer point mutations (ii), fusion proteins (iii), or gene deletions (iv). In the case of MosDEL (v), a *unc-119*(+) mini-gene is used as a positive selection marker. (b) The goal of MosSCI is to engineer single-copy transgenes at a defined location in the genome. A DSB induced by *Mos1* excision is repaired by homologous recombination with an *in vitro*-engineered template which contains the desired transgene in combination with the *unc-119*(+) mini-gene.
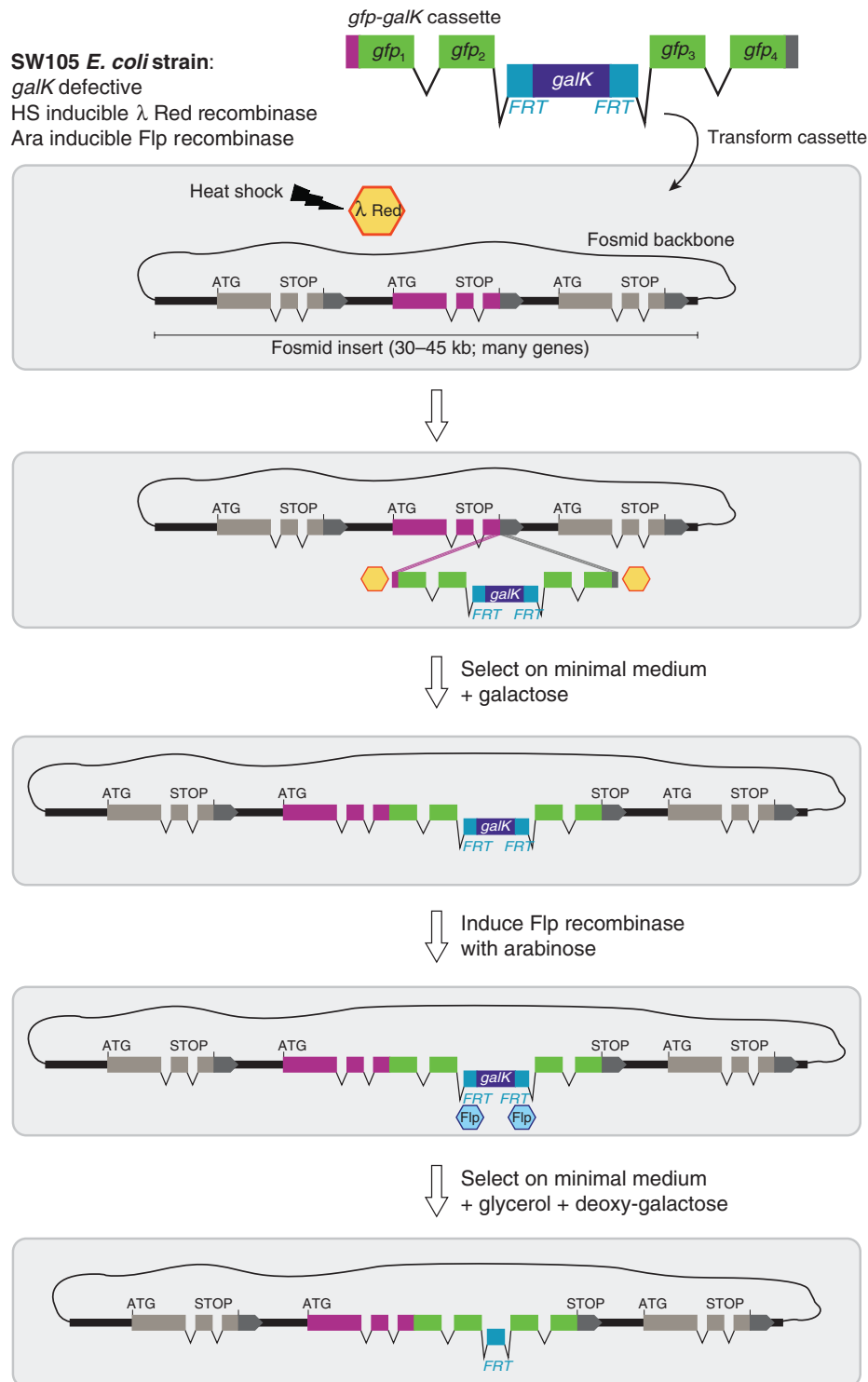
FIGURE 6 | *Caenorhabditis elegans* fosmid recombineering. Recombineering can be used to efficiently engineer a gene of interest in the context of a fosmid genomic clone. For example, a cassette containing a GFP tag and the FRT-*galK*-FRT selection module (FgF) can be inserted by λ Red recombinase-mediated homologous recombination into any position of a gene. Recombination is carried out in the SW105 *E. coli* strain. Recombinant clones are selected on minimal medium with galactose. Excision of the FgF selection module is carried out by Flp recombinase (induced by addition of arabinose) and can be selected for in medium containing deoxy-galactose. Note that the FRT 'scar' remaining after *galK* excision resides in an intron and therefore has no impact on protein-coding sequences. Additional applications of fosmid recombineering include: sequence deletion, sequence replacement, and fosmid extension. A comprehensive set of recombineering cassettes including different fluorescent reporters, affinity tags, nuclear localization signals, mutant FRT sites, and gene replacement cassettes is available. (Reprinted with permission from Ref 140. Copyright 2009 Public Library of Science)

gene strategies have been discussed in great detail in Ref 137. High-throughput approaches were also made possible by the use of Gateway compatible vectors, in particular with the completion of the ORFeome[138] and Promoterome projects.[139] However, most of these reporters only contain a few kilobases of gene regulatory sequence, because of the technical limitations encountered when trying to manipulate much larger DNA sequences. Recently, three novel methods have been developed which use recombineering of fosmid clones or BACs to engineer more comprehensive reporter constructs (Figure 6).[140–142]

A fosmid library containing over 15,000 clones, each carrying ∼40-kb-long genomic fragments, is now available for *C. elegans* (Don Moerman, personal communication). Clones can be searched on WormBase or using a dedicated tool (http://elegans.bcgsc.ca/perl/fosmid/CloneSearch/), and obtained through Source BioScience LifeSciences. Given the good genome coverage (∼×6) of this library, it is usually possible to obtain a fosmid in which the gene of interest is at the center, flanked by large portions of genomic DNA, therefore including most of the likely regulatory sequences. The most recently developed recombineering protocol[140] is fast (∼1 week), robust (owing to positive and negative selection steps), and requires little more than a freely available bacterial strain, minimal media, and a diverse set of cassettes including fluorescent proteins, affinity tags, or bicistronic fluorescent reporters.[140] Injection of fosmid as complex arrays [typically digested recombineered fosmid at 10–15 ng/μL, digested co-injection marker (2–7 ng/μL) and digested bacterial genomic

DNA (120–150 ng/μL)] generates arrays that usually show excellent germline expression.[140]

CONCLUSION

C. elegans has not only been at the forefront as a model system to understand biological processes, but also at the forefront for establishing widely applicable technologies. GFP reporter technology and RNAi are already classic examples. A number of techniques presented here will surely be further developed and significantly fine-tuned. For example, the recent introduction of antibiotic selection markers[121,122] offers much promise. Anticipated significant drops in the cost of WGS will further popularize forward genetic screens but also provide reverse-generated (i.e., non-phenotype driven) allelic versions of many/most/all genes in the genome. The development of engineered *Mos1* transposable elements, able to carry large cargo, will greatly simplify the generation of single-copy transgenes and allow gene trapping strategies. Finally, efficient genome editing and engineering will open the door to a range of new experimental approaches. So far, only *Mos1*-based genome engineering was sufficiently efficient to be used routinely. The promise of ZFNs and TALENs lies in their ability to cause DSBs with high efficiency and accuracy. Once this is combined with transgene-instructed repair, these nucleases and possibly other nucleases such as meganucleases[143] will become the premier tools to mutate, modify, and restore genes and gene function in *C. elegans* and related nematodes.

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FURTHER READING

Sequence information, mutants, expression patterns. (www.wormbase.org)

Comprehensive anatomy resource. (www.wormatlas.org)

www.wormbook.org

Books/Monographs

Wood WB, ed. C. elegans *I : The Nematode* Caenorhabditis elegans. Cold Spring Harbor Monograph Series 17.1988, 667 pp. ISBN 978-087969433-3.

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Where to get strains: Caenorhabditis Genetics Center (CGC) (http://www.cbs.umn.edu/CGC/), Mitani lab (http://www.grs.nig.ac.jp/c.elegans/), Oklahoma Knockout Consortium (http://celeganskoconsortium.omrf.org/), Moerman Lab (http://www.zoology.ubc.ca/~dgmweb/), BC Genome Science Center (http://elegans.bcgsc.bc.ca) NemaGENETAG (http://elegans.imbb.forth.gr/nemagenetag/).

Promoterome and ORFeome clones (http://worfdb.dfci.harvard.edu).

BC *C. elegans* Gene Expression Consortium: Gene expression project, Fosmid library (http://elegans.bcgsc.bc.ca)