Conditional gene expression in invertebrate animal models

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Abstract

A mechanistic understanding of biology requires appreciating spatiotemporal aspects of gene expression and its functional implications. Conditional expression allows for (ir)reversible switching of genes on or off, with the potential of spatial and/or temporal control. This provides a valuable complement to the more often used constitutive gene (in)activation through mutagenesis, providing tools to answer a wider array of research questions across biological disciplines. Spatial and/or temporal control are granted primarily by (combinations of) specific promoters, temperature regimens, compound addition, or illumination. The use of such genetic tool kits is particularly widespread in invertebrate animal models because they can be applied to study biological processes in short time frames and on large scales, using organisms amenable to easy genetic manipulation. Recent years witnessed an exciting expansion and optimization of such tools, of which we provide a comprehensive overview and discussion regarding their use in invertebrates. The mechanism, applicability, benefits, and drawbacks of each of the systems, as well as further developments to be expected in the foreseeable future, are highlighted.

Keywords

Conditional gene expression; Spatiotemporal control; Invertebrate; Model organism; *Drosophila melanogaster*; *Caenorhabditis elegans*

1. Introduction

The field of genetics owes a great debt to invertebrate animal models, which have repeatedly proven to be very powerful models to study biological processes. Their generally short life cycles and large numbers of offspring, combined with relatively easy genetic manipulation, allow for the generation and screening of mutants on a large scale and within modest time frames. Because many genes are well conserved between invertebrates and humans, their study has also vastly expanded our knowledge of human biology. Because of their extensive use in the field of functional genetics, it is not surprising that the tools available to study genetic regulation of biological processes in such model organisms continue to diversify.

For several years now, it has become relatively easy to constitutively (in)activate genes using (targeted) mutagenesis, and publicly available collections of mutated model organisms keep expanding as valuable resources for functional research. However, not all research questions can be approached by this rationale as mutating some genes can lead to unwanted side effects. Typical examples include mutations leading to infertility or lethality, which tend to complicate functional genetic experiments. In several cases, it can therefore be useful to gain temporal control over gene expression, for example, when aiming to uncouple the role(s) of a gene in an adult organism from its role(s) during development. While several tools for conditional gene expression are conceptually shared with those used in vertebrate model organisms (*e.g.*, reviewed in the studies by Lewandoski, 2001; Maltzman and Turka, 2007; and Kallunki et al., 2019), the appeal of fast and easy genetics of invertebrate animal models has led to tool kit specification and expansion for these organisms.

With this review, we aim to present a comprehensive overview of the current methods for temporal control of transcription that are available for invertebrate animal models, including their advantages and disadvantages, with a major focus on *Drosophila melanogaster* and *Caenorhabditis elegans*, as most tools were developed in these model organisms. We here consider both unidirectional and bidirectional control, meaning either irreversibly or reversibly switching the expression of a target gene on or off, via heat, compounds, or light, as well as propose potential future developments relevant to the field.

2. Heat shock promoters enable temperature-based control over gene expression

The oldest and perhaps most straightforward system for inducible gene expression is based on the use of endogenous heat shock responsive promoters to drive transgene expression. At elevated temperatures, constitutively expressed but inactive heat shock factors (HSFs) trimerise, allowing them

to bind specific heat shock promoters (*hsp*) that activate the transcription of heat shock genes (Fig. 1). The canonical heat shock response is present across organisms, first characterized by Ritossa (1962) in *D. melanogaster*. In 1983, an *hsp-70p::hsp-70::lacZ* fusion was shown to be expressed in extracts from heat-shocked but not from non-heat-shocked *D. melanogaster* through a β -galactosidase activity assay (Lis et al., 1983), and in 1992, the same was seen in *C. elegans* using an *hsp-16p::hsp-16::lacZ* hybrid (Stringham et al., 1992), validating that these systems can be used for rapid expression of a protein of interest. Since then, the value of the endogenous heat shock response for conditional transgene expression has been demonstrated in many other invertebrate representatives, including experiments in sea urchin embryos (McMahon et al., 1984), the sea squirt *Ciona intestinalis* (Kawaguchi et al., 2015), and cell lines of the beetle *Tribolium castaneum* (Silver et al., 2015). The system is also promising for use in many nonmodel invertebrates, primarily insects (Chen et al., 2020).

When relying on an endogenous heat shock promoter, transgene expression is typically activated in diverse tissues simultaneously. In *C. elegans* (Stringham and Candido, 1993) and *D. melanogaster* (Halfon et al., 1997), it is possible to add a spatial element of control by using a laser to restrict the area of heat shock induction, even to individual cells. An additional possibility in the nematode involves working in a *hsf-1* knockout background and reintroducing *hsf-1* cell-specifically (Bacaj and Shaham, 2007). Many other systems of conditional expression still rely on heat shock promoters for temporal control, as will be discussed in the following sections.

There are several significant disadvantages to using heat shock promoters for conditional gene expression. First of all, a major drawback is the necessity of exposing experimental animals to high temperatures. This can lead to unwanted effects when examining phenomena influenced by temperature, such as life span, reproduction, and behavior (Lithgow et al., 1994; Hercus et al., 2003; Hill et al., 2014; Nikitina et al., 2014). Second, the response is transient, meaning transgene expression will disappear again after some time (*e.g.*, Didomenico et al., 1982; Fortin et al., 2014), which is problematic when sustained expression within a specific time frame is desired. Third, leaky expression has been observed even at lower temperatures, when no expression is wanted (Steller and Pirrotta, 1985). Finally, unless using lasers to induce the heat shock or having to work with specific genetic backgrounds, which may bring along difficulties of their own, there is no spatial control. Therefore, current tools for conditional expression that rely on heat shock promoters tend to use these in combination with other systems, or have been completely replaced by other systems, as described in the following sections.

3. Site-directed recombination unidirectionally switches expression on or off using heat, compounds, or light

Two inducible expression systems that are widely adopted are based on site-specific recombinases. These recombinase enzymes can bind and bring together two DNA recognition sites and then cleave, exchange, and rejoin the DNA strands (Fig. 2). This leads to the removal or reversal of the DNA sequence in between these two sites. The Cre-*LoxP* system was adapted from P1 bacteriophages by Sauer (1987) and Sauer and Henderson (1988) for use in *Saccharomyces cerevisiae* and mammalian cells, respectively, whereas the Flp-*FRT* system was derived from yeast by Golic and Lindquist (1989), who applied it in *D. melanogaster*. In both cases, a site-specific recombinase (Cre, encoded by a locus originally named *Causes recombination* [Sternberg, 1981], or Flp, short for flippase [Broach and Hicks, 1980]) is able to excise or invert a sequence flanked by a pair of 34-bp recognition sites (*loxP*, locus of crossover in P1 [Sternberg, 1981], or *FRT*, flippase recognition target [McLeod et al., 1986]).

Recombinases can also be used to insert DNA sequences into genomic DNA containing the recognition sequence, but this will not be discussed here because it is of little relevance to conditional gene expression. When the recognition sites occur in the same orientation, the sequence contained between them is excised, whereas opposite orientations result in an inversion. The former is often used for gene inactivation (Golic and Lindquist, 1989; Hoier et al., 2000; Vázquez-manrique et al., 2010) (Fig. 2A), while the latter allows for inactivation by inverting a sequence to its correct orientation (Vázquez-manrique et al., 2010; Flavell et al., 2013) (Fig. 2B). Other methods of gene activation are the *lox-stop-lox* or Flp-out systems, which are based on excision of genetically introduced stop cassettes, containing a stop codon flanked by *loxP* or *FRT* sites upstream of a sequence of interest. Upon activation of the recombinase, the stop cassette is excised, and the sequence can be transcribed (Struhl and Basler, 1993; Davis et al., 2008; Voutev and Hubbard, 2008) (Fig. 2C). All this can be carried out spatially (tissue-specific promoter) (Golic and Lindquist, 1989; Hoier et al., 2000), temporally (heat shock promoter) (Newsome et al., 2000; Voutev and Hubbard, 2008), or by both means (Struhl and Basler, 1993; Davis et al., 2008; Voutev and Hubbard, 2008). Many variants have been designed for various end goals, as described in more detail in the following paragraphs.

Cre-*LoxP* was first introduced in *D. melanogaster* in 1996 to introduce transgenes at specific allelic sites (Siegal and Hartl, 1996) and in *C. elegans* in 2000 to inactivate a gene postembryonically (Hoier et al., 2000). Flp-*FRT* was used in *C. elegans* for the first time in 2008 for spatial and temporal gene activation (Davis et al., 2008; Voutev and Hubbard, 2008). These recombinase systems have been applied in a

number of insect species, such as the pest *Anastrepha suspensa* (Schetelig and Handler, 2013), the disease vector *Aedes aegypti* (Morris et al., 1991), and the economically important lepidopteran *Bombyx mori* (Long et al., 2012), with the latter also having seen the use of stop cassettes (Duan et al., 2013). Besides Cre-LoxP and Flp-FRT, Hubbard (2014) gives a comprehensive overview of the use of Cre-LoxP and Flp-FRT in *C. elegans* up until 2014, while Germani et al. (2018) provide a similar summary for Flp-FRT applications in *D. melanogaster* up until 2018. We here provide an updated summary of these tools' main applications in these organisms, specifically in the context of conditional expression.

For *D. melanogaster*, Flp-FRT seems more successful than Cre-LoxP as toxic effects have been reported when expressing the Cre recombinase in this fruit fly (Heidmann and Lehner, 2001), including in studies directly comparing both systems (Nern et al., 2011; Frickenhaus et al., 2015). FRT sites have been integrated into all major chromosome arms, allowing for *trans*-chromosomal recombination resulting in mosaic animals. This can be used to investigate recessive mutations that are lethal when homozygous in fly embryos or larvae (Xu and Rubin, 1993) or to determine whether intervening with gene expression in one cell affects the biology of the neighboring cells (Heitzler and Simpson, 1991) (Fig. 2D). Further developments of Flp-FRT for conditional gene expression in *D. melanogaster* include Mutant Analysis by Rescue Gene Excision, in which a cassette containing a rescuing gene for a homozygous mutation can be excised, leading to conditional loss of expression (Zhou et al., 2016); FlpStop, wherein a cassette consisting of disruptive elements can be inverted irreversibly to halt gene expression (Fisher et al., 2017); and Flip-flop, which functions similar to FlpStop but expresses EGFP when gene expression is on and mCherry when it is off (Manivannan et al., 2019). The latter two depend on a flip excision (FLEx) switch for their irreversibility, using two incompatible pairs of FRT sites (Fig. 2E). Another interesting adaptation is the compound-regulated Flp-DD, which contains a destabilizing domain (DD) that results in Flp degradation, until addition of the stabilizing agent trimethoprim (Fig. 2F). Thus, recombinase activity is uncoupled from heat shock, instead of being regulated by feeding with a small molecule (Sethi and Wang, 2017). Although Cre recombinase is less popular in *D. melanogaster* owing to its toxicity, this has been circumvented by creating a nontoxic fusion of Cre to the ligand-binding domain (BD) of the human estrogen receptor. This Cre-estrogen BD (Cre-EBD) fusion allows for recombination upon addition of estrogen (Fig. 2G); however, the reaction may not occur in all cells where Cre-EBD is expressed (Heidmann and Lehner, 2001), and Flp-FRT has remained the recombinase of choice in *D. melanogaster*, as evidenced by the number of Cre/LoxP and Flp/FRT strains available at the Bloomington *Drosophila* Stock Center (± 20 vs ± 450). Nevertheless, some researchers have managed to apply Cre successfully and inventively in the fruit fly: for example, a photoactivatable split-Cre system that allows dimerization

of two Cre halves upon blue light illumination, consequently enabling recombination and gene (in)activation (Boulina et al., 2013) (Fig. 2H). An important distinction with the other recombination-based techniques is that this makes it possible to activate Cre enzyme activity, rather than the transcription of the Cre gene, which should benefit temporal dynamics. Finally, it should be noted that Flp/*FRT* and Cre/*LoxP* are not the only recombinase systems for conditional expression in the fruit fly. For example, PhiC31 is a phage-derived recombinase that acts upon *attB* and *attP* sites, to invert, excise, or insert a sequence unidirectionally owing to its asymmetric landing sites, and can be applied in *D. melanogaster* (Groth, 2004; Knapp et al., 2015; Luo et al., 2019). Moreover, its use has been expanded to other insects, which are listed in Table 1 (Nimmo et al., 2006; Labbé et al., 2010; Meredith et al., 2011; Yonemura et al., 2013; Haghighat-Khah et al., 2015). Other recombinases from different yeast species that also show strong activity and no toxicity in *D. melanogaster* are KD, R, B2, and B3 recombinases. These can be used in combination with each other, or with Flp, as they do not recognize each other's target sequences (excluding the combination R and B2) (Nern et al., 2011).

In *C. elegans*, both Cre-*LoxP* and Flp-*FRT* have been used regularly. In cases where desired cell-specific promoters are unavailable, using different tissue-specific promoters for the recombinase and target genes permits to further restrict target gene expression to the intersecting cells of both promoters (Macosko et al., 2009). In this context, Kage-Nakadai et al. (2014) created a source of tissue-specific and heat shock-driven Cre recombinase strains with multicopy expression, which can be crossed into strains carrying *loxP*-flanked sequences of interest. While many experiments used to rely on such multicopy transgenic strains (either carrying the recombinase extrachromosomally or relying on DNA constructs that were integrated randomly into the genomic DNA), targeted genome editing technologies greatly facilitated single-copy expression of recombinase and/or target transgenes from the genome (Lo et al., 2013; Chen et al., 2018). Consequently, Muñoz-jiménez et al. (2017) generated a set of single-copy transgenic *C. elegans* strains expressing Flp recombinase reliably upon heat induction or in specific tissue types. The newest adaptation of the Cre system in *C. elegans* is Laser Targeted Activation of Cre, based on a photoactivatable version of Cre, which uses infrared light instead of heat to achieve activation. It does so by cell-specifically incorporating a noncanonical photocaged amino acid into the active site of Cre, allowing for both spatial and temporal control of gene expression (Davis et al., 2020) (Fig. 2I). As mentioned as an advantage of photoactivatable Cre in *D. melanogaster*, this also does not depend on temporal Cre transcription, but rather on temporal control over the functional reconstitution of available Cre protein.

Despite their many advantages, site-directed recombinases are genetic tools that entail their own risks and limitations. One well-known risk would be the possibility of unpredictable side effects of the recombinase enzyme, as is clearly evident for Cre in *D. melanogaster* (Heidmann and Lehner, 2001; Nern et al., 2011; Frickenhaus et al., 2015), and it is also a major issue in mammals, where it is very popular nonetheless (Schmidt-Supprian and Rajewsky, 2007). Moreover, most systems still rely on temporal control via heat shock promoters, whose limitations have been listed previously. Spatial control on the other hand relies on the availability of appropriate tissue-specific promoters, and it is important to verify their assumed specificity under the appropriate experimental conditions, which may differ from those of reported expression patterns. In addition, it is essential to always confirm that the intended inversion or excision event has actually taken place, and the inclusion of a reporter is thus recommended. On top of this, working with inversions is a little more challenging because DNA fragments flanked by inverted recognition sites may continue to randomly reverse back and forth while the recombinase is active. A technique that prevents this is the FLEEx switch (Schnütgen et al., 2003), applying incompatible pairs of *loxP* or *FRT* sites, resulting in stable, single inversion. In *D. melanogaster*, this has currently only been used in the FlpStop and Flip-Flop systems, and in *C. elegans*, the method is yet to be successfully introduced. Unlike inversions, excisions tend to be practically irreversible (thanks to the loss of the circular reaction product), meaning that excisable stop cassettes do not suffer from this issue. Although there seems to be a general consensus that read-through of stop cassettes leading to low-level target expression in the absence of the matching recombinase is not uncommon, there are few published data supporting this (in mice, very low and rare read-through has been reported [Kuhlman and Huang, 2008], whereas other articles refer to unpublished observations [Kaczmarczyk, 2001]). Research into the frequency and level of this leaky expression (in invertebrates) would be of great interest. Inversely, incomplete removal of the stop cassette leading to variable levels of expression has also been demonstrated in mice (Bapst et al., 2020), and the incidence of this phenomenon in invertebrates would also be useful to quantify. Although the irreversibility of the ultimate result of recombinase systems can be advantageous in some cases, it can also be a major disadvantage in and of itself as it is a one-way street: once Cre or Flp has been induced and executed the recombination reaction, target expression is permanently turned on or off. Systems allowing for bidirectional control offer a greater range of experimental uses—the systems described in the following sections (in principle) permit such applications.

4. Tetracycline-controlled transcriptional activation depends on small molecules for temporal control

Many transcription factors rely on small molecules as inducers or activators, which provides a window of opportunity to develop conditional gene expression systems with improved temporal control: as opposed to use of heat shock and/or spatio-specific promoters, use of compounds also permits deliberately stopping an effect after it has been induced. One such group of genetic tools is based on a transcriptional activator that is regulated by tetracycline (Tet) and its derivatives (such as doxycycline [Dox]). The Tet-Off system was adapted from *Escherichia coli* by Gossen and Bujard (1992) for application in mammalian cells and shortly after expanded to the Tet-On system by Gossen et al. (1995). Both use a chimeric protein consisting of the Tet repressor (TetR) and the transactivator virus protein 16 (VP16), which together function as a transcriptional activator (Fig. 3). In the Tet-Off system, this chimeric protein is called the tetracycline transactivator (tTA). It can be expressed in a tissue of choice, where it can then bind *tet* operator (*tetO*) sequences in the DNA to activate transcription of a downstream cytomegalovirus (CMV) minimal promoter. A combination of one or several operator sequences and the minimal promoter is called a tetracycline response element (*TRE*), and this can be placed upstream of a sequence of interest by standard genetic manipulation. When Tet or Dox is added, they will bind tTA, causing it to dissociate from the *TRE*, thus halting expression (Fig. 3A). The Tet-On system instead uses a slightly different chimeric protein, the reverse tetracycline transactivator (rtTA), which can only bind *TRE* and activate transcription when in the presence of Tet or Dox (Fig. 3B). By restricting the expression of tTA or rtTA to a tissue or cell type of interest and adding Tet or Dox at a specific time point, these systems allow for both spatial and temporal control of gene (in)activation. The technique has limited use in *C. elegans* owing to the canonical Tet system being mostly inactive in the nematode (Mao et al., 2019), but has been applied more often in *D. melanogaster*, since its first use in these fruit flies in 1998 (Bello et al., 1998; Bieschke et al., 1998). Beyond these two leading invertebrate models, Tet systems have also been applied in diverse insect species, such as the malaria mosquito *Anopheles stephensi* (Lycett et al., 2004) and the silkworm *B. mori* (Karasaki et al., 2009; Tatsuke and Lee, 2013), and tTA-EGFP vectors have been designed for future applications in a wide range of insect species (Hara et al., 2008, 2009).

The Tet-On system has further been improved for use in *D. melanogaster*, with the rtTA-M2-alt version combining a second-generation rtTA that is more sensitive to Dox, is more stable in eukaryotes, and has no background expression (rtTA-M2) (Urlinger et al., 2000) with a fly-optimized rtTA (rtTA-alt). In the fruit fly, rtTA-M2-alt shows lower basal expression levels as well as faster and higher expression upon induction (Stebbins et al., 2001), although there is conflicting evidence concerning the reported higher expression levels (Ford et al., 2007). An additional development is the combination of the Tet system

with Gal4-*UAS* (Stebbins et al., 2001; Stebbins and Yin, 2001), as will be further considered when discussing combinatorial techniques later in this manuscript. Only recently has the system been adapted for application in *C. elegans*, when used in combination with the Q system (Mao et al., 2019), as will also be discussed in more detail in the section on combinatorial approaches.

A disadvantage of all Tet systems is the necessity for the continuous presence of Tet or analogues such as Dox, which could entail side effects and must thus be controlled for in any experimental setup. In addition, expression has been reported in the absence of the drug (Bieschke et al., 1998), although these levels are very low for rtTA-M2-alt (Stebbins et al., 2001). Finally, while in principle, these systems are bidirectional as addition of Tet/Dox can be stopped and thus transcriptional (in)activation would in theory be reversed, recovery periods are uncharacterized for current applications described in invertebrates.

5. Gal4-*UAS* and LexA-*lexO* systems allow for heat- or compound-regulated bidirectional control

Two conceptually alike binary expression tools are Gal4-*UAS* and LexA-*lexO*, which function through use of a transgenic transcription factor and its target sequence (Fig. 4). The Gal4-*UAS* system is especially popular in the fly community and was first tailored from yeast for use in mammalian cells (Kakidani and Ptashne, 1988; Webster et al., 1988). Gal4 is a transcription activator protein that can bind an enhancer, the upstream activation sequence (*UAS*), which is followed by a basal promoter (*hsp70* and *pes-10* minimal promoters for *D. melanogaster* and *C. elegans*, respectively), and drives transcriptional activation upon binding (Brand and Perrimon, 1993; Wang et al., 2017a). In the literature, *UAS* usually refers to the combination of the enhancer and basal promoter, so we will also adhere to this notation throughout the review. By expressing Gal4 under control of a tissue-specific or heat shock promoter and placing *UAS* upstream of a target sequence of interest, the latter can be expressed in a spatially or temporally restricted manner (Fig. 4A). The end result should be much like what it would be when the target sequence is under direct control of these promoters, avoiding the Gal4-*UAS* intermediate step—in an ideal scenario, it is identical, and in practice, it entails a few controls. As for other binary expression systems, the many advantages of building a flexible resource for use by the international research community have been a major driving force in the continued development of the Gal4-*UAS* system (del Valle Rodríguez et al., 2012) (more details in the following paragraphs). In 1993, it was applied in *D. melanogaster* (Brand and Perrimon, 1993), and only much later, in 2016, *C. elegans* followed (Wang et al., 2017a). The system has further been adapted for use in the red flour beetle *T. castaneum*, a genetic model more closely related to many insect pest species than *D. melanogaster* (Schinko et al.,

2010). The technology has also been exploited in various insect representatives, such as *A. aegypti* (Kokoza and Raikhel, 2011), *Anopheles gambiae* (Lynd and Lycett, 2012), *A. stephensi* (Brochta et al., 2012), and *B. mori* (Imamura et al., 2003). Gal4 has also seen use in sea urchins (see Table 1 for different species), although only for its DNA binding and transcription activation capacities, and not yet in the context of conditional gene expression (Mao et al., 1996; Bulgakov et al., 2002).

As mentioned previously, the LexA-*lexO* system functions similar to Gal4-*UAS*, with a chimeric LexA transcription factor binding and activating its operator *lexO* (Fig. 4A). In 2000, LexA-*lexO* was adapted for use in *D. melanogaster* using the LexA BD fused to the Gal4 activation domain (AD) (Szuts and Bienz, 2000), and later versions instead fused it to the VP16 AD (Lai and Lee, 2006). Its use is more limited owing to the popularity of Gal4-*UAS* and its plethora of already available driver and reporter lines, but the systems can be applied in parallel to achieve induction of two distinct transgenes in one mosaic animal (Lai and Lee, 2006). Recently, it has been demonstrated that the LexA-*lexO* system is also active in *C. elegans* (Nonet, 2020).

In *D. melanogaster*, Gal4 is usually expressed under the regulation of tissue-specific promoters, allowing for spatial control of gene activation. These driver lines can then be crossed with effector lines containing *UAS* upstream of a sequence of interest, allowing transcriptional activation in the progeny. Proteins that are toxic, are lethal, or hinder fertility can be investigated this way as the parental driver and effector lines do not express these (Duffy, 2002). Thousands of tissue- or cell-specific Gal4 driver lines have been made available for use by the community (Hayashi et al., 2002; Gohl et al., 2011; Jenett et al., 2012; Tirian and Dickson, 2017; Dionne et al., 2018). For temporal control, a heat shock promoter can be used to express Gal4, but this would imply loss of spatial restriction as brought about by tissue-specific promoters. A technique that circumvents this is Temporal And Regional Gene Expression Targeting (TARGET), which uses the temperature-sensitive Gal4 repressor Gal80^{ts} under control of a tissue-specific promoter (McGuire et al., 2003) (Fig. 4B). Heat-dependent versions of Gal4^{ts} can also be used (Mondal et al., 2007). Another widely adopted method is the GeneSwitch system. This uses a modified Gal4 protein that is only active in the presence of the synthetic steroid mifepristone (administered through feeding or larval bathing), which allows for spatiotemporal control when combined with a tissue-specific promoter (Osterwalder et al., 2001; Scialo et al., 2016) (Fig. 4C). A newer compound-regulated version of Gal4-*UAS* depends on Gal80-DD, which is similar to Flp-DD discussed previously: Gal80-DD is stable when flies are fed with trimethoprim, but is degraded upon withdrawal from trimethoprim, leading to activation of transcription (Sethi and Wang, 2017) (Fig. 4D). Other options

for spatiotemporal control include localized heat shock through use of a laser (Halfon et al., 1997) or photoactivation of caged Gal4 (Cambridge, 1997) (Fig. 4E). The Gal4 repressor Gal80 can also be used for refinement of spatial control. Through use of specific promoter combinations for Gal4 and Gal80, expression of the target sequence can be restricted to a smaller subset of cells (Suster et al., 2004). Another technique for intersectional control of gene expression involves splitting Gal4 into its DNA-BD and transcription AD, each fused to a leucine zipper fragment to allow reconstitution of the two parts (Fig. 4F). This split-Gal4 makes it possible for each half to be expressed under a different promoter, enabling a more precise spatial control over conditional gene expression (Luan et al., 2006; Pfeiffer et al., 2010; Dionne et al., 2018). Split-Gal4 has been further developed to include the expression of a Killer Zipper (KZip⁺) repressor under a third promoter of choice, disrupting the formation of split-Gal4 dimers and subsequent Gal4 activity in a subset of cells (i.e., expressing both Gal4 halves, but not the KZip⁺ repressor), allowing for even more accurate spatial regulation (Dolan et al., 2017) (Fig. 4F). Alongside these various possibilities for Gal4 driver lines, different *UAS* effector lines have been developed with a myriad of uses. Beside standard gain-of-function or rescue constructs, Gal4-*UAS* has been used for gene knockdown through RNA interference (Kennerdell and Carthew, 2000), for which resources are available containing *UAS*-driven inverted repeats targeting thousands of genes (Dietzl et al., 2007; Ni et al., 2011). Other applications include cell ablation (*UAS::hid* and *UAS::rpr*) (Zhou et al., 1997), attenuation of electrical transmission (*UAS::EKO*) (White et al., 2001), and inhibition of synaptic vesicle release (*UAS::tetanus toxin light chain*) (Sweeney et al., 1995). LexA-*lexO* has also seen several interesting adaptations, such as a Gal-80 suppressible form (when fused to the Gal4 AD) (Lai and Lee, 2006), a split-LexA system (Ting et al., 2011), and, perhaps most interestingly of all, a photoactivatable split-LexA system switched on by blue light (Chan et al., 2015) (Fig. 4G).

C. elegans is a relative newcomer to the world of Gal4-*UAS* as early attempts to use the system in this nematode were unsuccessful, in part owing to its lower (~20°C) culturing temperatures (Wang et al., 2017a). Several alterations in both Gal4 and the *UAS* resulted in cGAL, a Gal4-*UAS* system applicable in *C. elegans* that works robustly at temperatures of 15–25°C. The system has been verified for use in tissue-specific rescue and gain-of-function experiments (Wang et al., 2017a). Driver lines are available for multiple tissues and individual neurons, and effector lines exist for fluorescent labeling, cell ablation, calcium indication, neuronal activation and inhibition, and inhibition of synaptic vesicle release (Wang et al., 2017a). Similar to *D. melanogaster*, a split-cGAL has also been developed, using intein domains (which associate, split out, and ligate adjoining peptides) instead of leucine zippers (Wang et al., 2018).

As mentioned previously, the LexA-*lexO* system can also be applied in *C. elegans* (Nonet, 2020), but no further adaptations are available yet in the nematode.

The canonical Gal4-*UAS* system does not allow for temporal control, making it less useful for flexible conditional expression. In contrast, temperature-dependent systems or the use of heat shock promoters comes with their own issues, as already mentioned. The GeneSwitch system has the limitation of having to add and remove a compound, which has slow activation compared with TARGET: the first is detectable after 3–5 h vs 30 min for the latter, and the GeneSwitch maximal signal was observed after 21–48 h vs 6 h for TARGET. GeneSwitch has even slower deactivation (up to 6 days vs 1.5 days for TARGET) (Osterwalder et al., 2001; Roman et al., 2001; McGuire et al., 2003) and requires the consideration of leaky expression of a sequence of interest in the absence of mifepristone (McGuire et al., 2003). This is especially true when GeneSwitch is used to induce RNA interference, in which case total protein depletion was observed even when no mifepristone was present (Scialo et al., 2016). A direct experimental comparison between the kinetics, leakiness, and efficiency of the TARGET and GeneSwitch systems would be of interest to allow the community to balance practical considerations for specific experimental purposes. As a final note, when creating new drivers or effectors, it must always be confirmed that these do not affect the process of interest.

6. Q system enables reversible regulation under control of a small molecule

A binary expression system comparable with Gal4-*UAS* is the Q system, which was developed for mammalian cells and *D. melanogaster* by Potter et al. (2010). Its components—QUAS, QF, and QS—originate from the *qa* gene cluster of the fungus *Neurospora crassa* (Potter et al., 2010). The cluster encodes a backup system when glucose levels are low as it allows *N. crassa* cells to catabolize quinic acid (QA) as an alternative carbon source (Giles et al., 1991). QF is the transcriptional activator of this gene cluster and is able to recognize and bind a 16-bp repeated enhancer sequence, *QUAS* (Patel et al., 1981; Baum et al., 1987), which in the Q system is intended to be transgenically placed upstream of a basal promoter (*hsp70* and *pes-10* minimal promoters for *D. melanogaster* and *C. elegans*, respectively) and a sequence of interest (Potter et al., 2010; Wei et al., 2012). In conditions wherein QA is absent (or only present at low concentration), the transcriptional suppressor QS can block QF-mediated transcription by binding QF, preventing its interaction with *QUAS* (Huiet and Giles, 1986). However, when present at higher concentrations, QA is able to (reversibly) sequester QS, thereby abolishing its interaction with QF and thus (temporally) restoring transcription from QF-regulated sequences (Giles et al., 1991) (Fig. 5A). The Q system is conceptually identical to the GeneSwitch system. Despite differences in their setup,

sequences, proteins, and compounds, both allow for temperature-independent temporal control of gene expression through the addition of a small molecule (QA for the Q system [Potter et al., 2010] or mifepristone for GeneSwitch [Osterwalder et al., 2001]). Both systems therefore provide the same advantage over the canonical Gal4-*UAS* system, but they have not yet been directly compared. Such a comparison could be valuable to the field because it may help researchers decide to choose which system is best suited for their specific research organism or question. To obtain temperature-independent temporal control of gene expression with the Q system, QA can simply be added to the food source or growth medium of the model organism. Moreover, QA is nontoxic, even in high doses and/or during longtime exposure (Potter et al., 2010). Wei et al. (2012) successfully adapted it for use in *C. elegans*, and later adoptions of the Q system in other organisms include the disease-carrying mosquito species *A. gambiae* (Riabinina et al., 2016), *Anopheles coluzzii* (Afify et al., 2019), and *A. aegypti* (Matthews et al., 2019), although without inclusion of its temporal control components, that is, QS and QA were not used.

Although temperature-independent temporal control of gene expression was an option, when developed, the Q system in *D. melanogaster* was mainly used for constitutive tissue- or cell-specific transgene expression, mosaic analysis, and cell lineage tracking, as an equivalent to or in combination with the Gal4-*UAS* system (Potter and Luo, 2011; Mann et al., 2013; Pérez-Garijo et al., 2013; Silies et al., 2013; Riabinina and Potter, 2016). For this purpose, flies in which QF expression is spatially limited to a subset of cells are crossed with flies carrying a transgene under control of *QUAS* and, if needed, further crossed with flies expressing QS (Potter et al., 2010; Potter and Luo, 2011). In addition, to obtain more targeted spatial control over gene expression, QF can be divided into two fragments, which is referred to as the split-Q system and works similar to split-Gal4 (Luan et al., 2006; Wei et al., 2012; Riabinina et al., 2019) (Fig. 5B). Furthermore, the spatial control of QF-induced gene activation can also be used in the context of conditional gene expression (*i.e.*, in combination with QS and QA) to obtain spatiotemporal control (Potter et al., 2010; Potter and Luo, 2011). However, when expressed too broadly *in vivo* (*e.g.*, pan-neuronally or ubiquitously), QF appears to be lethal (Potter et al., 2010). To overcome this problem, improved versions of QF have been designed, QF2 and QF2^{w(eaker)}, which no longer contain the apparent major source of *in vivo* toxicity: the QF middle domain. Both improved versions remain fully active, yet QF2^w is a weaker transcriptional activator than QF2, and it is more susceptible to repression by QS. Ubiquitous expression of the stronger activator QF2 appears to still have deleterious health effects, suggesting that next to global expression, the degree of QF activity also contributes to its *in vivo* toxicity. Together, these results imply QF2^w is of more general use as it is more

suitable for broad expression, whereas QF2 may be preferred only when weaker tissue-specific promoters are used (Riabinina et al., 2015). All versions of the transcriptional activator (QF, QF2, and QF2^w) can be used in the context of the previously mentioned split-Q system and are suitable for application in conditional gene expression experiments as they are all prone to QS-induced repression, which can be reversed by QA (Riabinina et al., 2015, 2019). The extent of the effect of QA-induced gene activation is dose dependent, but the temporal on-dynamics are relatively constant: in adult flies, expression can be observed after 6 h, reaching relatively high levels after 24 h, and after about 3 days, saturation occurs (Potter et al., 2010; Riabinina and Potter, 2016).

Application of the Q system in *C. elegans* did not require major adaptations, except for the incorporation of the $\Delta pes-10$ minimal promoter downstream of the *QUAS* repeats, to allow for initiation of QF-induced transcription (Wei et al., 2012). Indeed, recent publications report the successful application of the improved versions of QF, QF2, and QF2^w, in *C. elegans* (Aoki et al., 2018; Nonet, 2020). Analogous to *D. melanogaster*, by using tissue- or cell-specific promoters, the Q system also allows for spatial control of gene expression in *C. elegans*. In fact, the split-Q system was initially developed in the latter organism (Wei et al., 2012). Regarding temporal dynamics of transcriptional derepression by QA, experiments in *C. elegans*—perhaps unsurprisingly so—outperform those in *D. melanogaster*, that is, expression of QF-regulated genes can be observed after 6 h of exposure, with saturation occurring after 24 h (Wei et al., 2012). Yet, for some biological processes, this time frame is not sufficient (*e.g.*, developmental events or rapid cellular processes). Therefore, in *C. elegans*, the Q system tool kit was extended with the possibility to use a version of QF to which the human glucocorticoid receptor (GR) ligand-BD is fused. The activity of this so-called QF-GR is regulated by the presence of dexamethasone, a glucocorticoid. This ligand-gated QF-mediated transcription is more rapid than the conventional derepression of gene expression by QS-QA: using dexamethasone, gene expression can already be observed *two hours* after starting the treatment (Fig. 5C). The available version of this system still suffers from considerable uninduced activation of QF-GR (Monsalve et al., 2019), which is why it currently relies on additional repression by QS (and derepression by QA), irrespective of the presence of dexamethasone. Once this un-induced activation would be resolved, an exciting prospect of this system is a more refined spatial restriction of expression by driving QS (slow dynamics) and QF-GR (fast dynamics) by distinct tissue- or cell-specific promoters, that is, dexamethasone should then only activate QF-GR in cells where QS is absent.

The Q system is independent of temperature for temporal control, avoiding several common drawbacks as explained before. Instead, after administration, QA is taken up by the cells where it induces QF-based transcription through interaction with QS (Potter et al., 2010). However, not all cells may be able to take up QA equally efficiently, which is especially the case in organisms with a blood-brain barrier as this impedes diffusion of QA (Riabinina and Potter, 2016). For example, in *D. melanogaster*, the peripheral neurons, which are in direct contact with hemolymph, exhibit a stronger QA-induced effect than central brain neurons (Riabinina et al., 2015; Riabinina and Potter, 2016). In addition, it is still undefined what time it might take for the QA-induced transcription to be effectively repressed again by QS after QA is removed. This is especially of interest for experiments in which a transgene needs to be switched on only for a short period of time. It has also been reported that QS does not fully suppress QF when present in a 1:1 ratio, which could easily lead to leaky expression from QF-regulated genes (Potter et al., 2010). On the other hand, too great an excess of QS may be suboptimal as well as it has already been demonstrated that derepression of transcription by QA is not highly efficient, that is, QA is not able to fully inactivate QS (Potter et al., 2010; Wei et al., 2012). Therefore, finding the right balance between QF and QS is a crucial part when implementing and using the Q system.

7. Combinatorial approaches broaden the applicability of conditional gene expression

The genetic tool kits of invertebrate model organisms include various systems whose distinct components can be used interchangeably, thereby generating combinatorial systems that further improve the individual systems (Fig. 6).

7.1 Combining Tet and Gal4-UAS systems for increased spatial (and temporal) control in *D. melanogaster*

A major drawback of the Gal4-UAS system is the lack of temperature-independent temporal control of gene expression. While this issue is largely overcome in the GeneSwitch (Osterwalder et al., 2001; Scialo et al., 2016) system, in some *D. melanogaster* tissues Gal4 is relatively unresponsive to mifepristone-mediated activation, as there appears to be leaky activity of the transcriptional activator (Poirier et al., 2008). Combinations of elements from two conditional systems can alternatively be used to enable temperature-independent temporal control, for example, thanks to a Tet-Off driven Gal80 transgene. In the absence of an inducer (Tet or Dox), tTA is able to bind the *tet* operator, from which it promotes transcription of the Gal4 repressor, Gal80. As such, transcriptional inhibition of the Gal4-regulated transgene can be lifted by addition of Tet or Dox, which prevent tTA from interacting with *tetO* (Barwell et al., 2017) (Fig. 6A). This Tet-Off Gal80 system is not the only example of Tet and Gal4-UAS systems

combinations. In 2001, Stebbins et al. already reported on flies carrying spatially restricted Gal4 that drives the expression of tTA (Stebbins and Yin, 2001) or rtTA (Stebbins et al., 2001), which are able to regulate transcription of a sequence of interest downstream of *tetO*. The rationale to establish this combination was to tackle the issue of the relative lack of (r)tTA driver lines compared to the abundant presence of Gal4 driver lines at that time, and thus significantly increase the options for spatial control over gene expression when temporal control is already secured via the Tet system. Making use of the available Gal4 resource avoided the need for the labor-intensive engineering of countless new (r)tTA drivers (Stebbins et al., 2001; Stebbins and Yin, 2001).

7.2. Combining Tet and Q systems for more robust control in *C. elegans* with reduced leaky expression

A considerable issue with the Q system is the inefficiency of transcriptional derepression, that is, suppression by QS is not fully abolished after treatment with QA (Potter et al., 2010; Wei et al., 2012). To tackle this problem, Mao et al. (2019) adapted the Tet system for application in *C. elegans* by combining it with components of the Q system, resulting in a hybrid system that appears to be more robust and flexible than both the individual predecessors. The rationale for this combination with the Tet system is the relatively highly efficient interaction between (r)tTA and Dox or Tet compared with the QS-QA interaction. However, as mentioned earlier, the canonical Tet-controlled system appears to be largely inactive in the nematode, presumably because the VP16 transactivation domain of (r)tTA and the CMV minimal promoter that are combined with the *tet* operator are derived from human viruses. In other words, both components have probably evolved to specifically interact with the human transcription machinery, making them less functional in nematodes (Mao et al., 2019). Therefore, in the Tet/Q Hybrid system, the VP16 transactivation domain and the CMV minimal promoter are replaced by components that are known to be active in *C. elegans* (Wei et al., 2012), allowing the system to be applicable in this worm: the hybrid reverse transactivator, rtTA(Q), consists of the Dox-inducible reverse TetR fused to the AD of QF, and the modified *TRE* includes *tetO* upstream of the *Δpes-10* minimal promoter (Mao et al., 2019). To avoid leaky expression from the modified *TRE* in the absence of Dox, a tetracycline-regulated transcriptional silencer (tTS) is introduced, which consists of TetR fused to PIE-1, an inhibitor of RNA polymerase II (Ghosh and Seydoux, 2008; Mao et al., 2019). As such, the general activity of the Tet/Q Hybrid system is controlled by Tet or Dox. Their presence induces rtTA(Q) and suppresses tTS, allowing mRNA production from the modified *TRE*, whereas in their absence, transcription is not activated and even effectively silenced by tTS (Mao et al., 2019) (Fig. 6B). Furthermore, because rtTA(Q) comprises the QF AD, an additional level of control can be achieved by

coexpressing a (spatially restricted) QS repressor construct, of which the activity can be temporally regulated by QA (Wei et al., 2012; Mao et al., 2019).

7.3 *D. melanogaster* chimeric Q systems exchange temperature for QA as means of temporal control

Analogous to rtTA(Q) of the Tet/Q Hybrid system, other chimeric transactivators have been generated through fusion of a DNA-BD to the activation domain of QF, including Gal4QF (Riabinina et al., 2015) and LexAQF (Riabinina et al., 2015). Both hybrid components are of particular interest as they allow for a GAL80_(ts)-independent suppression of UAS-controlled transcription, that is, through QS (Fig. 6C). As such, temporal control over gene expression can be obtained by adding QA instead of by increasing temperature, which is a significant improvement for the Gal4-UAS and LexA-lexO systems in *D. melanogaster* (Riabinina et al., 2015).

7.4 Temporal control over recombinase activation

In a previous section, it was mentioned that Cre-LoxP and Flp-FRT do not allow for temporal control, unless used in combination with a heat shock promoter. However, with the myriad of inducible expression techniques available nowadays, this statement is not entirely correct: expression of the recombinase enzyme could be temporally controlled by all previously described systems as well. Yet, to date, there have only been reports of Flp or Cre recombinase genes being expressed under control of Gal4-UAS in *D. melanogaster* (Frickenhaus et al., 2015) and the Tet/Q Hybrid system in *C. elegans* (Mao et al., 2019). Although the result of the enzymatic action remains irreversible, in these animals, Flp-mediated DNA recombination is induced upon temperature-dependent inactivation of Gal80 (Frickenhaus et al., 2015) (Fig. 6D) or Cre is activated by addition of Dox (Mao et al., 2019).

8. Post-transcriptional approaches provide an additional level of control

So far, we have discussed techniques with a direct effect at the genetic (Cre-LoxP and Flp-FRT) or transcriptional (heat shock-related induction, Tet, Gal4-UAS and Q system) level of gene expression. Yet, conditional RNA or protein availability can also be established through post-transcriptional control. We here wish to discuss—albeit briefly—the various post-transcriptional techniques that are available for use in invertebrate models. Some such techniques are already commonly used (e.g., reviewed in the studies by Nance and Frøkjær-Jensen, 2019 and Trauth et al., 2019); although others are relatively new to the field, they show great promise for more widespread use.

One aspect of the post-transcriptional level of gene expression is the conditional control of RNA availability, which can be obtained using riboswitches or RNA interference. On the other hand, there is the protein aspect, which includes two main approaches: targeted degradation and trapping of proteins, both of which comprise a various number of techniques, as will be briefly introduced in the following sections.

8.1 Tight bidirectional control over mRNA availability by using a self-cleaving ribozyme switch in *C. elegans*

Riboswitches are regulatory elements in mRNA that are able to control the availability of their respective strand. Because they exist as secondary structures, their regulatory function can be affected by structural changes induced by external factors, including certain ligands that are able to bind a riboswitch-associated aptamer domain (Batey, 2006). By consequence, bidirectional conditional control over a sequence of interest can be obtained by transgenically incorporating a riboswitch sequence. Although an exciting prospect for conditional control over mRNA availability, only a single riboswitch-based tool has been reported in invertebrate model organisms so far: a Tet-dependent ribozyme switch adapted from human cell culture for use in *C. elegans* (Wurmthaler et al., 2019). This specific riboswitch exists as an aptamer-containing self-cleaving ribozyme in the 3'-untranslated region of a sequence of interest, and its mechanism of action allows for tight bidirectional control over gene expression. In the absence of the ligand, Tet, the enzymatic activity of the ribozyme is induced, causing it to make a single cut in one of its own stem-loop structures, eventually resulting in mRNA decay of the sequence of interest. However, in the presence of Tet, ribozyme activity is repressed and the mRNA remains intact, allowing it to be translated into protein (Wurmthaler et al., 2019). The main advantage of using a riboswitch over conditional gene expression is that typically fewer genetic manipulations are required than the approaches mentioned before. Yet, a considerable drawback is that riboswitches are inherently limited to the temporal level of control, that is, transgenic incorporation of a tissue-specific promoter would be required to spatially restrict the expression of the target gene.

8.2 Targeted mRNA degradation through RNA interference

A frequently used technique that could be considered a form of post-transcriptional conditional expression is RNA interference, in which double-stranded RNA sequences are used to target and degrade specific mRNAs of interest. This approach is widespread and applied in practically all (invertebrate) model organisms. There are several ways to conditionally induce RNA interference,

including via the conditional expression systems described earlier in this review. For an explanation of the mechanism of action and application potential of RNA interference itself, we refer the reader to existing thorough reviews by Xu et al. (2019) and Ahringer (2006).

8.3 Protein degradation bypasses transcription and translation to rapidly inactivate target proteins

While the systems discussed up to now focus on regulating the production of a protein of interest, there is also the possibility to control their presence by focusing on the degradation of existing proteins. The most popular way to achieve this is through tagging with a degron domain. This degron is recognized and bound by an F-box protein, recruiting it to the E3 ubiquitin-protein ligase complex that ubiquitinates the protein, thus targeting it to the proteasome for breakdown. An early example in both *D. melanogaster* and *C. elegans* is deGradFP: a protein of interest is transgenically tagged with a GFP degron, which is targeted by a GFP nanobody fused to a component of the E3 complex, allowing ubiquitination and subsequent degradation (Caussinus et al., 2012; Wang et al., 2017b). In another *C. elegans* system, proteins tagged with zinc-finger domain 1 are bound by zinc finger-interacting factor 1, which forms part of the E3 ubiquitin-protein ligase complex and thus leads to protein degradation (Armenti et al., 2014). Protein degradation can also be controlled by compounds, heat, or light. A widely used compound-based system applicable in both *D. melanogaster* and *C. elegans* is the plant-derived auxin-inducible degradation system. In this system, the auxin-activated F-box protein TIR1 is responsible for detecting degron sequences, leading to ubiquitination and protein depletion in the presence of the plant hormone auxin (Zhang et al., 2015; Trost et al., 2016). A deGradFP variant in *C. elegans* allows for drug-induced protein depletion. In this method, the GFP nanobody and E3 complex are separated and fused to chemically induced dimerization domains, which allow for ligation upon induction by rapamycin, thus resulting in depletion of the target protein (Deng et al., 2020). Alternative to degrons, DDs can be used, which are only stable when in the presence of trimethoprim and lead to the degradation of any attached protein upon removal of the ligand. This has been applied in both *D. melanogaster* and *C. elegans* (Cho et al., 2013; Sethi and Wang, 2017). As for heat-regulated techniques, a low-temperature-controlled degron has been designed for *D. melanogaster*, which induces degradation of the protein it is fused to when switching from 15°C to 29°C (Faden et al., 2016). A *C. elegans* temperature-driven technique depends on the tagging of proteins with a LPETG peptide sequence, the ubiquitous expression of a heptamutant *Staphylococcus aureus* Sortase A (SrtA_{7m}), and the heat shock promoter-regulated expression of a tri-glycine tagged F-box protein (G₃-F-box). Upon heat shock, G₃-F-box is expressed. SrtA_{7m} will attach this G₃-F-box to the tagged protein of interest,

marking it for degradation by the proteasome (Wu et al., 2017). Finally, light can be used as a stimulus. In *C. elegans*, there is a degron-independent light-regulated system that relies on fusing a miniature singlet oxygen generator (miniSOG) to a protein of interest to enable chromophore-assisted light inactivation. When illuminated with blue light, miniSOG generates reactive oxygen species, which in this setup destroy the protein that miniSOG is attached to (Lin et al., 2013). A more recent *C. elegans* light-based system uses a photosensitive degron, which activates protein degradation upon low-intensity blue light illumination, and is more specific than the miniSOG system, which can unwantedly affect the nearby proteins (Hermann et al., 2015). The major advantage of protein degradation over all the other techniques described is its speed. While other systems rely on transcription, translation, and protein turnover, protein degradation directly targets and inactivates the final product. However, these systems are often more complex, requiring specific transgenic strains for each experiment, sometimes containing multiple transgenes. Thus, there are no vast libraries of genetic resources available, as is the case for recombinase or Gal4-UAS-based systems. Moreover, these systems generally rely on fusing the protein of interest to a degron or other tag, which can in itself interfere with the stability or function of the protein (Kerk et al., 2017; Patel and Hobert, 2017).

8.4 Protein trapping as alternative to degradation

Instead of completely degrading a protein of interest, the protein can also be conditionally targeted to an abnormal subcellular location, thus interfering with its usual activity. This can be useful to investigate whether the localization of the protein is important to its function. The morphotrap system in *D. melanogaster* and *C. elegans* relies on a membrane-tethered GFP nanobody that traps GFP-tagged proteins of interest, keeping them at the membrane (Harmansa et al., 2015; Pani and Goldstein, 2018). In *C. elegans*, this has been further developed into a photoinducible system. Here, the protein of interest is tagged with a light-activated membrane anchor, localizing it to the membrane upon blue light illumination (Fielmich et al., 2018). The (dis)advantages of this system are similar to those described previously for protein degradation, with the added consideration that the protein may have residual or altered function at its ectopic location.

9. Future developments promise a further expansion of the invertebrate genetic tool kit

A large variety of methods for transcriptional control of gene expression is currently available for use in invertebrate model organisms (summarized in Table 1). However, a drawback of the most novel method among them is the relative lack of available driver lines and/or reporter strains (Stebbins et al., 2001;

Gohl et al., 2011; Wei et al., 2012; Lin and Potter, 2016; Riabinina and Potter, 2016). Nevertheless, as these systems have already proven their efficacy in these organisms, this issue will likely resolve itself in the near future, as has formerly been the case, for example, with the already well-established Gal4-UAS system (Hayashi et al., 2002; Gohl et al., 2011; Jenett et al., 2012; Tirian and Dickson, 2017; Dionne et al., 2018). Moreover, as current methods permit exchange of transcription factors of existing driver lines with those of other systems, this could proceed even faster (Gohl et al., 2011; Lin and Potter, 2016). We can thus expect to see a continued growth of the invertebrate strain collection to support experiments relying on transcriptional conditional genetics. Furthermore, new post-transcriptional techniques acting at the RNA and protein level are providing an additional means to control protein availability, with their own set of novel applications and challenges.

Many of the systems described here have not yet reached their full potential in (all or even major only) invertebrate model organisms. Site-directed recombinases are particularly popular in mice and mammalian cells, where they have seen a plethora of further adaptations not yet fully exploited in invertebrates. A frequently used example is the previously described FLEEx switch (Schnütgen et al., 2003), which has seen limited use in *D. melanogaster* and has to our knowledge not yet been applied in *C. elegans*. Other examples include the use of inverted-repeat *loxP* site variants that can only recombine once and are then locked in their new orientation (Araki et al., 1997), combining Cre-*LoxP* and Flp-*FRT* for more precise control of gene expression (Kim et al., 2009) and using a drug-dependent Cre fusion protein for temporal control (Feil et al., 1997). Gal4-UAS has already seen extensive use in *D. melanogaster* but has only recently been adapted for *C. elegans*. With cGAL now available, we can expect to see many further variations of the system in *C. elegans* that have already been developed in *D. melanogaster*, such as the GeneSwitch system and the combination with Gal80. Although it was first introduced in *D. melanogaster* (Potter et al., 2010), the Q system tool kit is a little more extensive in *C. elegans*, in which QF-GR allows for additional ligand-gated control of transcription (Monsalve et al., 2019). In the near future, this LBD-fused QF will presumably be adapted for use in other organisms as well. Similarly so, combinatorial approaches are generally relatively recent techniques, which to date have mainly been used in only one organism (Stebbins et al., 2001; Stebbins and Yin, 2001; Riabinina et al., 2015; Mao et al., 2019). We therefore predict that their application will shortly be expanded to a broader range of invertebrate model organisms. Finally, the post-transcriptional approaches have also got a lot of potential left for further development, which we expect will be taken advantage of in the near future. For example, to date, only a single riboswitch-based tool has been adapted for use in

invertebrates (only in *C. elegans*), whereas multiple such systems have been developed for use in vertebrates (Felletti and Hartig, 2017).

Most conditional expression systems rely on heat or small compounds for temporal control, which limit their use in experimental setups. With the advent of optogenetics, new possibilities arise, especially in transparent invertebrates such as *C. elegans* or the translucent larval stages of *D. melanogaster*. Already developed are the photoactivatable split-Cre (Boulina et al., 2013), Gal4 (Cambridge, 1997), and LexA (Chan et al., 2015) systems in *D. melanogaster* and the photoactivatable Cre (Davis et al., 2020), miniSOG (Lin et al., 2013), and degron (Hermann et al., 2015) systems in *C. elegans*. In vertebrate cell cultures, a plethora of such systems have been developed (Hörner et al., 2017), based on light-inducible recombinases (Weinberg et al., 2019), Tet transactivators (Müller et al., 2014; Yamada et al., 2018), Gal4 (Shimizu-Sato et al., 2002; Wang et al., 2012), diverse other transcriptional activators (Polstein and Gersbach, 2012; Konermann et al., 2013), and clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) (Nihongaki et al., 2015; Polstein and Gersbach, 2015). Optogenetic systems have even been combined to create a multichromatic expression tool that can activate three separate genes in one cell culture depending on the wavelength of light used (Müller et al., 2013). Some of the aforementioned systems have also been applied *in vivo* in mammalian models, either through optical implants (Konermann et al., 2013; Yamada et al., 2018) or through direct external illumination (Wang et al., 2012); however, this is more technically challenging than is the case for translucent organisms. Adapting these systems for invertebrates would deliver important advantages compared with most current tools for conditional genetics: less unwanted side effects, rapid on-off switching, and spatial precision using focused light sources. The transparency of *C. elegans* and translucency of larval *D. melanogaster* is an additional great benefit, and we look forward to seeing the future applications of optogenetic gene expression in these invertebrate models.

A gene editing tool that has revolutionized the world of molecular biology over the past decade is CRISPR-Cas9, in which a complex consisting of a Cas9 nuclease and guide RNA is directed to a specific genomic site where the nuclease cuts the DNA, allowing for highly targeted mutagenesis. Recent years have seen the adaptation of CRISPR for conditional gene expression, especially in mammalian cell lines and mice. A first way to achieve this is by simply regulating the expression of Cas9 or guide RNA using one of the already discussed systems, such as the Tet system (Aubrey et al., 2015; Dow et al., 2015), recombinases (Chylinski et al., 2019), or ribozymes (Tang et al., 2017). A second approach involves modifying the Cas9 protein or guide RNA itself, making it inducible by a small molecule (Davis et al.,

2015; Zetsche et al., 2015; Wang et al., 2020) or by light (Nihongaki et al., 2015; Polstein and Gersbach, 2015; Jain et al., 2016). These concepts are also spreading to invertebrate communities, primarily to research relying on *D. melanogaster*. As in mammals, existing systems can be applied to obtain conditional control over Cas9 or guide RNA; so far, this has been carried out using Gal4-UAS (Port and Bullock, 2016) and TARGET (Port et al., 2020b). In addition, Gal4-UAS has been applied to drive CRISPR-Cas9-based transcriptional activation (CRISPRa). This novel approach depends on a UAS-controlled nuclease-dead Cas9 (dCas9) fused to a transcriptional activator, which can bind a target DNA sequence complementary to a guide RNA, where it activates transcription of a sequence of interest (Lin et al., 2015). A more recent combination of Gal4-UAS and CRISPRa in *D. melanogaster* is the synergistic activation mediator technique (flySAM), which consists of a UAS-driven dCas9 activator, a modified guide RNA containing two hairpin aptamers, and a tripartite fusion of an aptamer-binding subunit, a trans-activating subunit, and an AD. The addition of this latter fusion protein greatly improves transcriptional activation (Jia et al., 2018). In addition, engineering Cas9 in such a way that it can act conditionally, independently of any other systems, is making its way into invertebrate research. For example, Cas9 can be fused to the progesterone ligand-BD, resulting in a mifepristone-inducible Cas9 protein, similar to the GeneSwitch system (Huynh et al., 2018). In yet another conditional CRISPR system, a temperature-dependent Cas12a is used, which is inactive at 18°C and active at 29°C (Port et al., 2020a). In *C. elegans*, only heat shock promoters have been used to conditionally express Cas9 (Shen et al., 2014), although simply feeding these worms with guide RNA at a specific moment allows for temporal control as well (Liu et al., 2014). These conditional CRISPR-Cas9 systems allow for a wider variety of interventions than classical conditional systems—instead of simply turning an entire gene on or off, single nucleotides, specific regulatory elements, or protein domains can be targeted in a temporally restricted manner. For example, by mutating only the catalytic site, protein function can be abrogated without having to get rid of the protein altogether. Owing to this broader application potential, we can expect to see the systems already available in vertebrates being introduced in invertebrates, as well as those already established in *D. melanogaster* spreading to other invertebrate organisms.

In current research relying on invertebrates, most of the techniques are primarily applied in *D. melanogaster* and *C. elegans*, which makes sense as these represent the largest research communities. However, in recent years, the availability of cheap genome sequencing has led to an explosion in the number of available genomes. Together with the expansion of genetic methodologies, functional genetics is now possible in organisms other than the ‘classical’ model organisms, which forms

a great advantage to scientific research. An essential future development would therefore be to adapt the techniques for conditional gene expression—classic and novel—that are already well established in a few mammalian or invertebrate representatives, to be applicable in a much wider range of organisms. The most basic forms of all the methods mentioned here have already been applied in some other invertebrate species, and it is likely for the adapted versions to transition into applications in such other species as well. Considerations that must be taken into account when applying these systems in a ‘new’ model include the absence of endogenous recognition sequences when using transgenic enzymes and transcription factors, the potential toxicity of enzymes involved in these systems in their new cellular and organismal contexts, and possible side effects of heat, compounds, or light used for (in)activation.

The combination of fast genetics in invertebrates and the extensive tool kit for conditional gene expression reviewed here has already shown to be of great value to the research community. Many of these systems are rapidly gaining popularity, indicating their importance to the field, that is, there is a myriad of research questions for which these systems can be used. However, we will only be able to address several other research questions when further improving the temporal resolution and spatial precision of control, that is, despite the extensiveness of the tool kit, many of the available systems have too slow dynamics or act too widely to be able to tackle extremely fast and/or local processes. We therefore predict that the future of the field lies not in the use of compounds, but rather in the use of light (optogenetics), which is fast and can be administered with high precision. By controlling gene expression as tightly as possible, we will be able to answer even more research questions, thus further unraveling the fundamentals of life.

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System	Stimulus	Control	Directionality	Organism
Heat shock promoter				
- Endogenous	Heat	Temporal	Bidirectional	<i>Dmel</i> (Lis et al., 1983), <i>Cele</i> (Stringham et al., 1992), <i>Spur</i> (McMahon et al., 1984), <i>Cint</i> (Kawaguchi et al., 2015), <i>Tcas</i> (Silver et al., 2015), <i>Sfru</i> (Chen et al., 2020)
- Laser	Heat (laser)	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Halfon et al., 1997), <i>Cele</i> (Stringham and Candido, 1993)
- <i>hsf-1</i> background	Heat	Temporal + Spatial	ON (bidirectional)	<i>Cele</i> (Bacaj and Shaham, 2007)
Recombinases				
- Cre- <i>LoxP</i>	Heat	Temporal + Spatial	ON or OFF	<i>Dmel</i> (Siegal and Hartl, 1996), <i>Cele</i> (Hoier et al., 2000), <i>Asus</i> (Schetelig and Handler, 2013), <i>Bmor</i> (Duan et al., 2013)
- Flp- <i>FRT</i>	Heat	Temporal + Spatial	ON or OFF	<i>Dmel</i> (Golic and Lindquist, 1989), <i>Cele</i> (Davis et al., 2008; Voutev and Hubbard, 2008), <i>Aaeg</i> (Morris et al., 1991), <i>Bmor</i> (Long et al., 2012)
- <i>lox-stop-lox</i> / Flp-out	Heat	Temporal + Spatial	ON	<i>Dmel</i> ²⁸ , <i>Cele</i> (Davis et al., 2008; Voutev and Hubbard, 2008), <i>Bmor</i> (Duan et al., 2013)
- FLEx	Heat	Temporal + Spatial	ON or OFF	<i>Dmel</i> (Fisher et al., 2017)
- Flp-DD	Compound (trimethoprim)	Temporal + Spatial	ON or OFF	<i>Dmel</i> (Sethi and Wang, 2017)
- Cre-EBD	Compound (oestrogen)	Temporal + Spatial	ON or OFF	<i>Dmel</i> (Heidmann and Lehner, 2001)
- Photoactivatable Split-Cre	Light (blue)	Temporal + Spatial	ON or OFF	<i>Dmel</i> (Boulina et al., 2013)
- PhiC31	Heat	Temporal + Spatial	ON or OFF	<i>Dmel</i> (Groth, 2004), <i>Aaeg</i> (Nimmo et al., 2006), <i>Bmor</i> (Yonemura et al., 2013), <i>Agam</i> (Meredith et al., 2011), <i>Aalb</i> (Labbé et al., 2010), <i>Pxyl</i> (Haghighat-Khah et al., 2015)
- KD, R, B2 and B3	Heat	Temporal + Spatial	ON or OFF	<i>Dmel</i> (Nern et al., 2011)
- Laser-TAC	Light (UV)	Temporal + Spatial	ON or OFF	<i>Cele</i> (Davis et al., 2020)
Tet system				
- Tet-Off	Compound (Tet/Dox)	Temporal + Spatial	OFF (bidirectional)	<i>Dmel</i> (Bello et al., 1998), <i>Aste</i> (Lycett et al., 2004)
- Tet-On	Compound (Tet/Dox)	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Bieschke et al., 1998), <i>Aste</i> (Lycett et al., 2004), <i>Bmor</i> (Karasaki et al., 2009)
- rtTA-M2-alt	Compound (Tet/Dox)	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Stebbins et al., 2001)

Gal4-UAS system				
- Gal4-UAS	Heat	Temporal or Spatial	ON (bidirectional)	<i>Dmel</i> (Brand and Perrimon, 1993), <i>Cele</i> (H. Wang et al., 2017), <i>Tcas</i> (Schinko et al., 2010), <i>Aaeg</i> (Kokoza and Raikhel, 2011), <i>Agam</i> (Lynd and Lycett, 2012), <i>Aste</i> (Brochta et al., 2012), <i>Bmor</i> (Imamura et al., 2003), <i>Spur</i> (Mao et al., 1996), <i>Lpic</i> (Mao et al., 1996), <i>Sint</i> (Bulgakov et al., 2002), <i>Smir</i> (Bulgakov et al., 2002)
- TARGET	Heat	Temporal + Spatial	Bidirectional	<i>Dmel</i> (McGuire et al., 2003)
- Gal4 _{ts}	Heat	Temporal + Spatial	Bidirectional	<i>Dmel</i> (Mondal et al., 2007)
- GeneSwitch	Compound (mifepristone)	Temporal + Spatial	Bidirectional	<i>Dmel</i> (Osterwalder et al., 2001)
- Gal80-DD	Compound (trimethoprim)	Temporal + Spatial	Bidirectional	<i>Dmel</i> (Sethi and Wang, 2017)
- Laser	Heat (laser)	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Halfon et al., 1997)
- Photocaged Gal4	Light (UV)	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Cambridge, 1997)
- Split-Gal4	Heat	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Luan et al., 2006), <i>Cele</i> (Wang et al., 2018)
- Split-Gal4-KZip ⁺	Heat	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Dolan et al., 2017)
- LexA- <i>lexO</i>	Heat	Temporal or Spatial	ON (bidirectional)	<i>Dmel</i> (Lai and Lee, 2006), <i>Cele</i> (Nonet, 2020)
- Gal80-repressible LexA	Heat	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Lai and Lee, 2006)
- Split-LexA	Heat	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Ting et al., 2011)
- Photoactivatable LexA	Light (blue)	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Chan et al., 2015)
Q system				
- Q system	Compound (QA)	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Potter et al., 2010), <i>Cele</i> (Wei et al., 2012)
- Split-Q	Compound (QA)	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Riabinina et al., 2019), <i>Cele</i> (Wei et al., 2012)
- QF2	Compound (QA)	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Riabinina et al., 2015), <i>Cele</i> (Nonet, 2020), <i>Agam</i> (Riabinina et al., 2016), <i>Acol</i> (Afify et al., 2019), <i>Aaeg</i> (Matthews et al., 2019)
- QF2 ^w	Compound (QA)	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Riabinina et al., 2015), <i>Cele</i> (Aoki et al., 2018)
- QF-GR	Compound (dexamethasone/QA)	Temporal + Spatial	ON (bidirectional)	<i>Cele</i> (Monsalve et al., 2019)

Combinatorial				
- Tet-Off-Gal80	Compound (Tet/Dox)	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Barwell et al., 2017)
- Gal4-Tet-Off	Compound (Tet/Dox)	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Stebbins and Yin, 2001)
- Gal4-Tet-On	Compound (Tet/Dox)	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Stebbins et al., 2001)
- Tet-Q	Compound (Tet/Dox)	Temporal + Spatial	ON (bidirectional)	<i>Cele</i> (Mao et al., 2019)
- Q-Gal4 + Q-LexA	Compound (QA)	Temporal + Spatial	ON (bidirectional)	<i>Dmel</i> (Riabinina et al., 2015)
- Gal4-FLP + Gal4-Cre	Heat	Temporal + Spatial	(ON or) OFF	<i>Dmel</i> (Frickenhaus et al., 2015)
- Tet-On-Cre	Compound (Tet/Dox)	Temporal + Spatial	ON or OFF	<i>Cele</i> (Mao et al., 2019)
Post-transcriptional				
- Riboswitch	Compound (Tet)	Temporal	ON (bidirectional)	<i>Cele</i> (Wurmthaler et al., 2019)
- RNA interference	Double-stranded RNA	Temporal + Spatial	OFF	<i>Dmel</i> (Xu et al., 2019), <i>Cele</i> (Ahringer, 2006) ^a
- deGradFP	GFP nanobody expression ^b	Temporal + Spatial	OFF (bidirectional)	<i>Dmel</i> (Caussinus et al., 2012), <i>Cele</i> (S. Wang et al., 2017)
- ZF1	ZIF-1 expression ^b	Temporal + Spatial	OFF (bidirectional)	<i>Cele</i> (Armenti et al., 2014)
- AID	Compound (auxin)	Temporal	Bidirectional	<i>Dmel</i> (Trost et al., 2016), <i>Cele</i> (Zhang et al., 2015)
- DiPD	Compound (rapamycin)	Temporal	Bidirectional	<i>Cele</i> (Deng et al., 2020)
- DD	Compound (trimethoprim)	Temporal	Bidirectional	<i>Dmel</i> (Sethi and Wang, 2017), <i>Cele</i> (Cho et al., 2013)
- It-degron	Heat	Temporal	Bidirectional	<i>Dmel</i> (Faden et al., 2016)
- SrtA _{7m}	Heat	Temporal	Bidirectional	<i>Cele</i> (Wu et al., 2017)
- CALI	Light (blue)	Temporal + Spatial	Bidirectional	<i>Cele</i> (Lin et al., 2013)
- psd	Light (blue)	Temporal + Spatial	OFF (bidirectional)	<i>Cele</i> (Hermann et al., 2015)
- Morphotrap	GFP nanobody expression ^b	Temporal + Spatial	OFF (bidirectional)	<i>Dmel</i> (Harmansa et al., 2015), <i>Cele</i> (Pani and Goldstein, 2018)
- Photo-trapping	Light (blue)	Temporal + Spatial	Bidirectional	<i>Cele</i> (Fielmich et al., 2018)

Table 1. Overview of systems for conditional gene expression. *Dmel*, *Drosophila melanogaster* (common fruit fly); *Cele*, *Caenorhabditis elegans* (nematode); *Spur*, *Strongylocentrotus purpuratus* (purple sea urchin); *Cint*, *Ciona intestinalis* (vase tunicate); *Tcas*, *Tribolium castaneum* (red flour beetle); *Sfru*, *Spodoptera frugiperda* (fall armyworm); *Asus*, *Anastrepha suspensa* (Caribbean fruit fly); *Bmor*, *Bombyx mori* (silkworm); *Aaeg*, *Aedes aegypti* (yellow fever mosquito); *Agam*, *Anopheles gambiae* (African malaria mosquito); *Aalb*, *Aedes albopictus* (Asian tiger mosquito); *Pxyl*, *Plutella xylostella* (diamondback moth); *Aste*, *Anopheles stephensi* (Indian malaria mosquito); *Lpic*, *Lytechinus pictus* (painted urchin); *Sint*, *Strongylocentrotus intermedius* (sea urchin); *Smir*, *Scaphechinus mirabilis* (sea urchin); *Acol*, *Anopheles coluzzii* (African malaria mosquito); CALI, chromophore-assisted light inactivation; It-degron, low-temperature-controlled degron; DD, destabilizing domain; DiPD, drug-induced protein depletion; ZF1, zinc-finger domain 1; AID, auxin-inducible degradation; psd, photosensitive degron; Tet, tetracycline; Dox, doxycycline; TARGET, Temporal And Regional Gene Expression Targeting; Cre-EBD, Cre-estrogen binding domain; Laser-TAC, Laser Targeted Activation of Cre; FLEx, flip excision. Systems discussed in this review are listed alongside their (in)activation stimulus, level, and directionality of control and in which organisms they are applicable (with a reference to their first use in each organism). When parentheses are used in the directionality column, the direction mentioned is theoretically possible but has not yet been tested *in vivo*. ^a Applied in several organisms; refer to cited reviews for more details. ^b The stimulus depends on the system driving expression.

Fig. 1. Conditional expression using heat shock promoters. The heat shock factor gene (*hsf*) is constitutively expressed under its endogenous promoter (*pro*) and is thus present in its inactive, monomeric form throughout the organism. A sequence of interest (*SOI*) under the control of a heat shock responsive promoter (*hsp*) is transgenically introduced. Upon heat shock, heat shock factor (HSF) trimerizes, allowing it to bind *hsp* and drive expression of the *SOI*. Alternatively, *hsf* can be rescued under the control of a tissue-specific promoter in an *hsf* knockout background, adding spatial control.

Fig. 2. Recombinase-based conditional expression relying on Cre-*LoxP* or Flp-*FRT*. Cre or Flp recombinase are transgenically expressed under the control of a heat shock or tissue-specific promoter (*pro*). *LoxP* or *FRT* sites are present flanking a sequence of interest (*SOI*). Depending on their orientation, the recombinase will **(A)** excise or **(B)** invert the *SOI*, which can lead to gene (in)activation. **(C)** Alternatively, in the *lox-stop-lox* or Flp-out systems, a stop cassette preventing transcription is present upstream of the *SOI*, flanked by *loxP* or *FRT* sites. Upon recombinase expression, the stop cassette is excised, allowing transcription of the *SOI*. **(D)** When *loxP* or *FRT* sites are integrated on different chromosome arms, *trans*-chromosomal recombination can be used to create mosaic animals. **(E)** A flip excision (FLEX) switch avoids a *SOI* inverting back to its original orientation, by relying on two pairs of different recognition sites. After inversion, one of these sites is excised, preventing further inversions. **(F)** A destabilized Flp (Flp-DD) is only stable—thus, able to drive recombination—upon addition of trimethoprim. **(G)** A compound-regulated Cre-estrogen binding domain (Cre-EBD) can only bind and drive recombination upon addition of estrogen. **(H)** Split-Cre dimerizes to drive recombination upon illumination with blue light. **(I)** Photocaged Cre (TAC-Cre) is uncaged and able to drive recombination upon illumination with UV light.

1310

1311 Fig. 3. Tetracycline-dependent conditional expression. **(A)** In the Tet-Off system, tetracycline
 1312 transactivator (*tTA*) is transgenically expressed under the control of a tissue-specific promoter (*pro*). *tTA*
 1313 dimers can bind and activate a tetracycline response element (*TRE*) upstream of a sequence of interest
 1314 (*SOI*), allowing transcription. Addition of tetracycline or doxycycline inactivates *tTA*, preventing
 1315 transcription. **(B)** In the Tet-On system, reverse tetracycline transactivator (*rtTA*) is transgenically
 1316 expressed under a tissue-specific promoter. *rtTA* dimers can only bind and activate *TRE* and the *SOI* in
 1317 the presence of tetracycline or doxycycline.

Fig. 4. The binary Gal4-*UAS* system as used for conditional gene expression. **(A)** Transcriptional activator Gal4 or LexA is transgenically expressed under the control of a heat shock or tissue-specific promoter (*pro*). It can bind an upstream activation sequence (*UAS*) or LexA operator (*lexO*), driving expression of a downstream sequence of interest (*SOI*). A temperature-sensitive version of Gal4 can also be used, which is inactivated at higher temperatures. **(B)** Gal80, a repressor of Gal4, can be expressed under a second tissue-specific promoter. In Temporal And Regional Gene Expression Targeting (TARGET), temperature-sensitive Gal80ts is used, and heat shock can inactivate the repressor, allowing binding of Gal4 to the *UAS* and activation of transcription. **(C)** In the GeneSwitch system, a modified form of Gal4 is used, which is sensitive to the steroid mifepristone. GeneSwitch can only bind *UAS* and activate transcription upon addition of mifepristone. **(D)** A destabilized Gal80 (Gal80-DD) is stable while in the presence of trimethoprim and thus blocks activation by Gal4, but destabilizes upon addition of trimethoprim, thus allowing expression. **(E)** A photocaged Gal4 can be used that is uncaged (and thus able to bind *UAS* and activate expression) upon illumination with UV light. **(F)** In Split-Gal4, the activation domain (AD) and binding domain (BD) are each expressed under a different promoter, fused to an adaptor. Only where expression overlaps will the two domains combine via the adaptor and activate expression. In addition, when expressing the Killer Zipper (KZip+) from a third promoter, the formation of split-Gal4 dimers will be disrupted in a subset of cells, preventing Gal4 activity there. **(G)** In the photoactivatable split-LexA system, the VP16 AD and LexA BD are each fused to an adaptor, which dimerizes upon illumination with blue light, enabling transcription of a *SOI* upstream of a *lexO* site.

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1339 Fig. 5. Reversible control over gene expression by means of the Q system. **(A)** Transcriptional activator
 1340 QF and its repressor QS are transgenically expressed under the control of a tissue-specific promoter
 1341 (*pro*). QS binds and suppresses the activity of QF. Upon addition of quinic acid (QA), QS is sequestered
 1342 and QF can bind to the *QUAS* enhancer sequence that is followed by a basal promoter, driving
 1343 expression of a downstream sequence of interest (*SOI*). **(B)** In Split-Q, the activation domain (AD) and
 1344 binding domain (BD) of QF are each expressed under a different promoter, fused to an adaptor. Only
 1345 where expression overlaps will the two domains combine via the adaptor and activate expression (in the
 1346 presence of QA). **(C)** QF can be fused to a glucocorticoid receptor ligand-BD (QF-GR) so that it is only
 1347 active in the presence of dexamethasone. This can be combined with QS to avoid uninduced activation
 1348 of QF-GR or for more spatial refinement.

Fig. 6. Combinatorial approaches integrate components of different systems for conditional gene expression. **(A)** In the Tet/Gal4 system, both the tetracycline transactivator (tTA) and Gal4 are transgenically expressed under the control of a tissue-specific promoter (*pro*). tTA dimers can bind and activate a tetracycline response element (*TRE*) upstream of Gal80, which is then expressed and will bind and repress Gal4. Upon addition of tetracycline or doxycycline, tTA is repressed and Gal80 transcription is halted. This allows Gal4 dimers to bind the upstream activation sequence (*UAS*), driving expression of a downstream sequence of interest (*SOI*). **(B)** In the Tet/Q system, a fusion of the reverse tetracycline transactivator (rtTA) and the QF activation domain (rtTA(Q)) is transgenically expressed under the control of a tissue-specific promoter (*pro*), as is a tetracycline-regulated transcriptional silencer (tTS). rtTA(Q) is inactive, while TS binds *TRE* and inhibits expression of a downstream *SOI*. Addition of tetracycline 68 or doxycycline inactivates the tTS and activates rtTA(Q), which will bind *TRE* and activate expression of the downstream *SOI*. **(C)** In the Gal4/Q system, a fusion of the Gal4 binding domain and the QF activation domain (Gal4QF) is transgenically expressed under the control of a tissue-specific promoter (*pro*), as is its repressor QS. Gal4QF activity is repressed by QS until addition of QA, upon which Gal4QF dimers can bind *UAS*, driving expression of a downstream *SOI*. **(D)** In the Gal4/Cre and Gal4/Flp systems, Gal4 is transgenically expressed under the control of a tissue-specific promoter (*pro*), as is its repressor Gal80ts. Gal4 activity is repressed by Gal80ts until heat shock, upon which Gal4 dimers can bind *UAS*, driving expression of downstream Cre or Flp recombinase. This in turn will allow for recombination and (in)activation of a *loxP*- or *FRT*-flanked *SOI*.