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Review

Using transgenic reporter assays to functionally characterize enhancers in animals☆

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ABSTRACT

Enhancers or *cis*-regulatory modules play an instructive role in regulating gene expression during animal development and in response to the environment. Despite their importance, we only have an incomplete map of enhancers in the genome and our understanding of the mechanisms governing their function is still limited. Recent advances in genomics provided powerful tools to generate genome-wide maps of potential enhancers. However, most of these methods are based on indirect measures of enhancer activity and have to be followed by functional testing. Animal transgenesis has been a valuable method to functionally test and characterize enhancers *in vivo*. In this review I discuss how different transgenic strategies are utilized to characterize enhancers in model organisms focusing on studies in *Drosophila* and mouse. I will further discuss recent large-scale transgenic efforts to systematically identify and catalog enhancers as well as highlight the challenges and future directions in the field.

Contents

1. Introduction

The first transcriptional enhancer was characterized more than 30 years ago, when a remarkable viral DNA sequence was shown to activate transcription of the rabbit hemoglobin beta1 gene over a long distance independent of its orientation and position relative to the basal promoter [1]. In that pioneering paper the authors hypothesized: "it appears possible that classes of different enhancers are involved in the developmental, as well as tissue specific, expression of genes" [1]. Indeed enhancers were later found in fruit flies [2–4], mammals [5], as well as in other metazoans (e.g., [6–9]) and were shown to be the major regulators of developmental gene expression (reviewed in

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[10–12]). A typical enhancer is from 200 bp to 1 kb in length and is usually located in inter- and intra-genic non-coding regions of the genome [10]. Some enhancers can operate over very long distances up to 1 Mb [13]. Enhancers contain multiple binding sites for different transcription factors (TFs) and function as information hubs integrating input from all bound TFs, which results in the activation of target gene transcription in a spatially and temporally specific manner. Sequence changes in enhancers are hypothesized to be the most prevalent drivers of the phenotypic divergence within and between species [14,15]. Moreover, enhancer malfunction has been associated with a variety of human disorders such as cancer and heart disease (reviewed in [12,16,17]). Despite the importance of enhancers for evolution and disease, our understanding of the mechanisms governing their function is still limited and the characterization of their genomic location, activity, target genes and function has been a major goal in biology.

While the first properties of enhancers were characterized in cell culture-based assays, the introduction of transgenesis based on transposable elements in fruit flies [18] greatly improved our knowledge of how enhancers orchestrate gene expression during animal development. In the pioneering work focusing on the *even-skipped* (*eve*) locus, a combination of enhancer–reporter fusion constructs and *P*-element-mediated transformation were used to show that the sequence of the *eve* stripe 2 enhancer is sufficient to drive expression of a reporter gene within the limits of the endogenous *eve* stripe 2 [4,19]. These experiments also demonstrated that the *eve* stripe 2 enhancer contained binding sites for more broadly expressed activators and repressors whose combined inputs resulted in the striped expression of *eve*. The same mechanism was shown to be true for other enhancers, and it is now accepted as a fundamental principle of how all enhancers establish gene expression domains during animal development (reviewed in [10, 12]).

The enhancer–reporter setup in a transgenic experiment uses a defining property of an active enhancer as a DNA sequence able to activate transcription of a reporter from a minimal promoter [1,11]. It therefore provides a direct read-out of enhancer activity in an *in vivo* based system. Reporter transcription can be detected directly using RNA in situ hybridization [20] or live imaging of nascent RNA [21], or detected indirectly using, for example, lacZ staining [22] or fluorescent proteins such as GFP [23]. Indeed, transgenic reporters are widely used to study regulation of gene expression in model organisms such as the nematode, *Caenorhabditis elegans*, the fruit fly, *Drosophila melanogaster,* the Zebrafish, *Danio rerio,* the mouse, *Mus musculus* and others. This review aims to provide an overview of the vast array of transgenic methods available to functionally characterize enhancers in animals and to discuss their relevance with respect to the recent advances in regulatory genomics, focusing on *in vivo* studies in *Drosophila* and mouse but also covering other major animal model organisms.

2. Importance of functional transgenic assays to characterize enhancers

Recent development of high-throughput methods for mapping protein–DNA and DNA–protein–DNA interactions has provided a relatively straightforward way to obtain instant genome-wide pictures of potential regulatory regions in any tissue or cell type of an animal (reviewed in [11,24]). However, this has raised a challenge of how to interpret these data and, more specifically, whether most of the predicted regulatory regions have any biological significance [25–27], which highlights the importance of functional transgenic assays to validate and characterize enhancers *in vivo*.

One of the most common methods for high-throughput identification of enhancers is chromatin immunoprecipitation (ChIP) using antibodies directed against co-activators (e.g., CBP/P300), TFs or enhancer-associated histone marks (e.g., H3K4me1 and H3K27ac). This technology is followed by DNA microarray hybridization (ChIP-on-chip) or deep sequencing (ChIP-seq) from whole organisms or tissues to identify a genome-wide set of potential binding sites [28–34]. Another class of methods is based on the fact that regulatory proteins create low nucleosome density regions upon binding to the DNA. Using different strategies such as DNase-seq [35], MNase-seq [36], ATAC-seq [37], or FAIRE-seq [38] these regions can be separated from the rest of the chromatin, deep-sequenced and mapped back to the genome, providing a genome-wide map of open chromatin regions. Recently, the ENCODE and modENCODE consortia used a combination of DNase-seq and ChIP-based methods to create unprecedented catalogs of potential regulatory elements in *Drosophila* [39], *C. elegans* [40], mouse [41,42], and human [43–45]. A different set of methods ('3C-based': 3C, 4C, 5C, Hi-C and ChIA-PET; reviewed in [46]) utilizes nuclear proximity ligation to assess physical contacts between different genomic regions and can be used to map enhancer–promoter interactions. Among them, 4C has the highest resolution [46] and has been used to predict locations of enhancers for developmental genes in *Drosophila* [47] and mouse tissues [48].

Despite the vast amount of invaluable information provided by this new generation of genomic assays, most of them are based on indirect measures of enhancer activity and must be followed by functional validation. For example, a typical TF ChIP-seq experiment can yield from several thousand [49] to tens of thousands of high-confidence protein-bound regions [50]. Indeed, many TF binding sites identified by ChIP were shown to be negative when tested for *in vivo* enhancer activity [51, 52], which can either reflect an artifact of the method [53,134] or the promiscuous nature of TF binding. Interestingly, regions that are strongly bound by TFs and cofactors, or bound by multiple TFs are more likely to be active in a transgenic reporter assay [32,52].

A more widely used method for genome-wide annotations of enhancers is based on mapping of enhancer-associated H3K4me1 and H3K27ac histone marks [30,34,42,43,54]. Nevertheless, a recent systematic survey of ENCODE chromatin-based enhancer predictions showed that only ~26% of predicted regions had regulatory activity [55], suggesting that care must be taken when using enhancer-associated histone marks for interpreting enhancer activity [11,55–57].

Results of 3C-based methods should also be interpreted with caution, as there are discrepancies between fluorescence in situ hybridization (FISH) and 3C-based methods, which indicates that the identified contacts might not always reflect spatial proximity [58]. Furthermore, the majority of identified enhancer–promoter contacts appear to be stable during development and often do not reflect enhancer activity at the time-point or in the cell-type of interest [47,48,59].

Transgenic assays, despite being relatively low throughput, represent an entirely orthogonal approach to those described above and, therefore, provide means to address biological significance of predicted regulatory regions.

3. Current transgenic strategies to characterize enhancers *in vivo*

Transgenic reporters can be utilized to characterize gene regulatory landscapes in three conceptually different ways. One way is to randomly probe different parts of the genome to detect accumulative activity of enhancers (enhancer-trap). Another way is to directly test enhancers for *in vivo* activity by placing them in a reporter vector (enhancer–reporter). The third way is to test enhancers in the native context of a much longer DNA sequence using, for example, BAC transgenesis. Below I discuss different methods for enhancer characterization that utilize one of these strategies in more detail.

3.1. Enhancer-trap-based methods

In an enhancer-trap a vector containing a minimal promoter and a reporter gene is randomly integrated into the genome. The resulting reporter expression is a cumulative activity of all regulatory sequences around the insertion site, typically within the regulatory domain of the target gene (Fig. 1a, b). Such regulatory domains often coincide

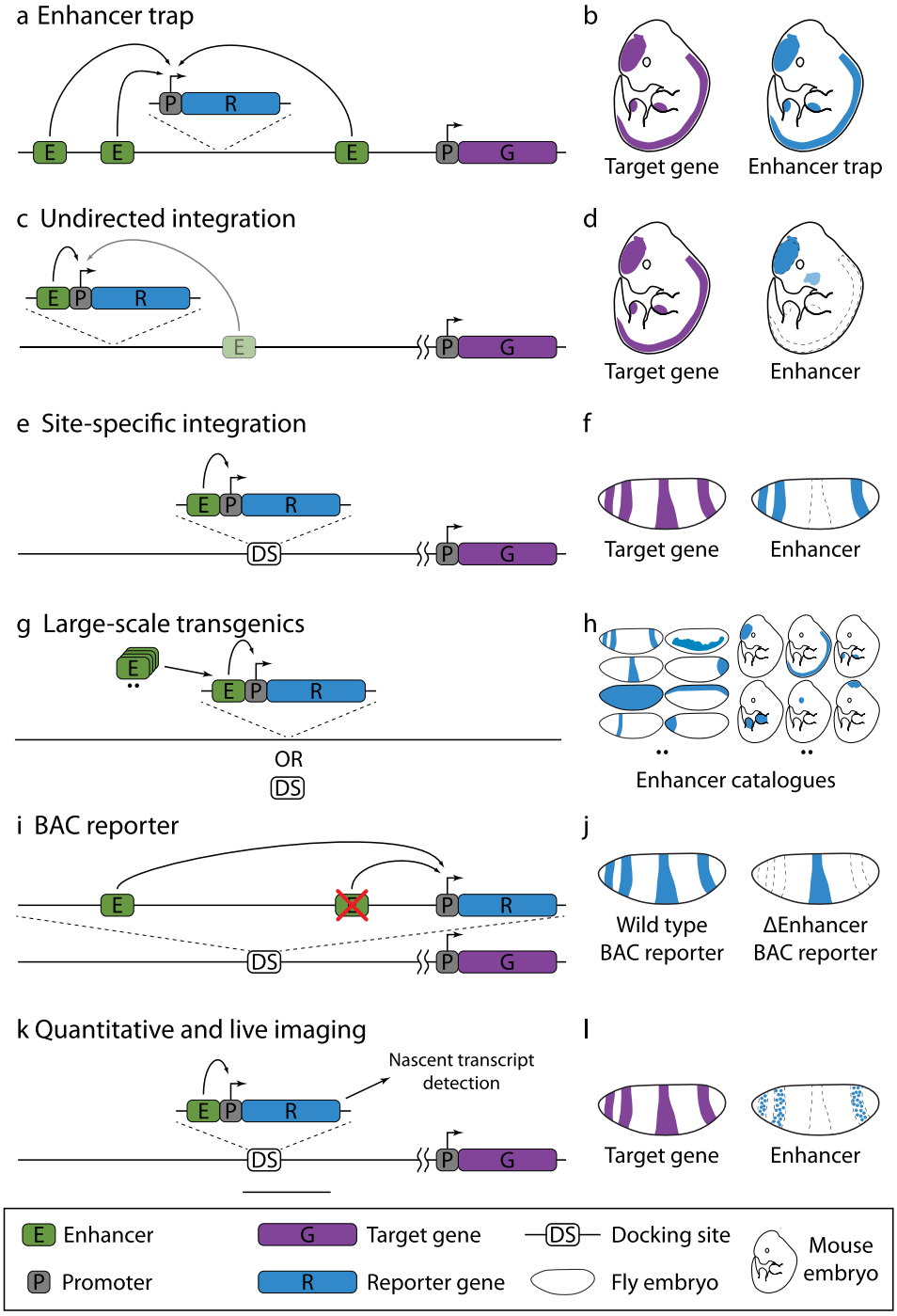

Fig. 1. Schematic overview of different transgenic strategies for enhancer characterization. a–b, *Enhancer-trap* uses a transposon-based sensor construct containing minimal promoter followed by a reporter gene (blue box) integrated into the genome. The reporter gene transcription is activated by all enhancers (green boxes) surrounding the integration site. The resulting reporter expression pattern closely recapitulates the expression pattern of a target gene (the gene which is normally regulated by these enhancers; purple). The left mouse embryo displays a schematic expression pattern of a hypothetical gene active in the forebrain, limbs and neural tube. The activity of the enhancer-trap reporter in the embryo is shown on the right. c–d, *Undirected integration* of enhancer–reporter DNA into the genome results in the reporter transcription pattern that typically corresponds to endogenous enhancer activity and recapitulates parts of the target gene's expression pattern. Chromatin environment or other enhancers surrounding insertion site (faded green boxes) can affect reporter activity resulting in additional ectopic domains of expression (faded blue domain of expression in a mouse embryo on the right). e, *Site-specific integration*. The enhancer–reporter DNA is integrated into a "docking site" (DS) located in the genome – the process, which is mediated by a site-specific phage integrase φC31 [91]. f, Shown on the left is a schematic expression pattern of a gene active in four characteristic stripes in the early *Drosophila* embryo. One of the enhancers is sufficient to recapitulate three of the four distinct stripes of the gene expression in the early embryo shown on the right. g–h, *Large scale transgenesis*. To create enhancer catalogs, a large set of enhancers is introduced into a reporter vector one-by-one, and each construct is subsequently integrated into the genome using conventional transgenesis (site-specifically on randomly). The resulting library of transgenic animals is screened for enhancer activity in entire embryo or tissue using different reporter detection systems. i–j, The *BAC reporter* construct typically contains the whole regulatory region of the gene of interest. If the gene within the BAC is replaced or tagged by a reporter, the resulting reporter expression pattern will typically closely recapitulate endogenous gene expression (left embryo; shown is the expression pattern for the hypothetical gene from (f)). A variant BAC reporter with deleted enhancer results in the full loss of the three stripes indicating necessity of enhancer for driving gene expression in these stripes (right embryo). k–l, *Quantitative and live imaging* in combination with site-specificly integrated enhancer and BAC reporters can be used to detect nascent reporter transcripts within developing embryo with a very high spatio-temporal resolution. The right *Drosophila* embryo shows nascent reporter transcription activated by the enhancer (single blue dots inside actively transcribed nuclei). Schematic gene and enhancer expression patterns are drawn after refs [11,52,78].

with topologically associated domains (TADs) inferred from chromosomal interactions [60–63]. Enhancer-trap vectors based on transposable *P*-elements have been first introduced in *Drosophila* to characterize developmental gene expression [20,64,65] and to drive ectopic reporter gene expression in a spatially and temporally regulated fashion [66]. In addition to a minimal promoter and a reporter gene (typically *LacZ* or *Gal4*), *P*-element-based vectors contain a marker gene and transposase recognition sequences [20]. The resulting artificial transposon is microinjected together with *P*-element transposase into the germline of the fly embryo, and the progeny are screened for transformants with insertion events based on a marker. With the availability of transgenes based on novel transposable elements (e.g., *Sleeping Beauty* (*SB*), *piggyBAC* or *Minos* transposons), similar enhancer-trap strategies have been successfully applied in vertebrates [67–69]. Most transposons use "cut-and-paste" mechanism to move from one location of the genome to another. This ability can be utilized to create additional insertions of a transposon-based vector in other genomic locations without the need for complicated manipulation of the early embryos or zygotes [69,70].

Interestingly, depending on where the enhancer-trap transgene integrates within the regulatory domain, the cumulative activity of neighboring enhancers can vary and is not always simply additive [60,71]. Enhancer-trap-based methods therefore provide means to study the regulatory architecture of a given locus [60]. It was particularly useful for studying the organization of complex long-range regulatory domains of mouse *fibroblast growth factor 8* (*Fgf8*) and *sonic hedgehog* (*Shh*) developmental genes [71,72]. "Enhancer trap"-based methods do not provide information about the location and activity of individual enhancers. Therefore, they have to be combined with more direct methods for enhancer characterization.

3.2. Undirected integration of the enhancer–reporter vector

In this setup enhancer is placed directly upstream of a reporter gene in a vector containing transposon recognition sequences. The resulted transgene is randomly integrated into the genome via embryo microinjection. The assessment of multiple independent integration events allows selecting a representative reporter activity pattern that in most cases recapitulates part of the expression pattern of the target gene and corresponds to endogenous enhancer activity (Fig. 1c, d) [2,3]. If several non-related enhancers are combined in one reporter construct, the resulted activity pattern will be a sum of individual activities, which indicates modularity and functional autonomy of enhancers [73]. As transposable elements exist in many animals (both vertebrates and invertebrates) they represent versatile tools to directly characterize enhancer activity and have been applied in *Drosophila* [2], zebrafish [68], *Xenopus* [74], chicken [75], mouse [76] and other animals. In zebrafish and mouse enhancer–reporter vectors can be randomly integrated into the genome without the help of a transposase by direct microinjection of the DNA into a zygote. In mouse, due to the high efficiency of integration (10–20% depending on the size of the construct and transgenic facility) this method is more widely used compared to transposon-mediated transgenesis [22,77,78]. Many human developmental enhancers were characterized this way in mouse [12,77].

3.3. Transient transgenesis of the enhancer–reporter vector

Transient transgenesis is commonly used in the nematode *C. elegans*, the sea squirt *Ciona intestinalis* and in chick embryos. A transgenic vector containing the desired enhancer–reporter, typically in form of plasmids, is injected into gonads (in *C. elegans* [79]) or simply electroporated into the developing embryos (in *Ciona* [80] and chicken [81]). The enhancer–reporter vector does not incorporate into the genome and remains extrachromosomal during embryogenesis (in *Ciona* and chicken), and can even be maintained in subsequent generations (in *C. elegans*). Because animals can be analyzed for reporter expression directly, transient transgenesis is relatively high-throughput and has been useful for identifying numerous developmental enhancers in these species (e.g., [8,9,82]).

3.4. Site-specific integration of the enhancer–reporter vector

Transient transgenesis and random integration of enhancer–reporter vector have limitations. Direct microinjection of linearized DNA results in integration into more than one genomic locus and often in formation of head-to-tail repeats (concatemers) in both mice [22,83] and zebrafish [84]. This makes it difficult to detect quantitative differences between the different tested enhancers. Similar arrays are also formed extrachromosomally during *C. elegans* transient transformation [79]. Even if enhancer–reporter vector integration occurs only in one copy (for example *P*-element-mediated transformation in *Drosophila*), it often results in additional domains of expression due to position effects (Fig. 1c, d) [85]. This complicates interpretation of the results from experiments and requires analysis of several independent integration events. In *Drosophila*, flanking enhancer–reporter vector with insulator sequences was shown to reduce position effects and to increase specific reporter expression dramatically [86,87]. However, to be able to quantitatively compare different enhancer activities, it is desirable to insert reporter constructs into the same chromosomal location (Fig. 1e, f).

One method uses a combination of Cre/lox and FLP/FRT recombination systems and two reporters in the same *Drosophila P*-element-based vector [88]. One reporter is removed upon Cre expression, while the other is removed upon FLP expression, which results in two different transgenic lines with different reporters integrated in the same genomic location. This system was successfully used to show an enhancer–promoter specificity mediated by different types of core promoters [89]. One limitation of this method is that only two reporters can be tested at a time, and it is not possible to control the placement of the *P*-element-mediated insertion. This can result in activation or repression of a reporter by regulatory elements surrounding the insertion site.

The most efficient and widely used system for site-specific integration of enhancer–reporter constructs is based on phage integrase φC31 [90]. This integrase mediates irreversible recombination between two sequences, *attP* and *attB*. If one of the sequences (typically *attP*) is placed on a chromosome (using conventional *P*-element-mediated transgenesis) and another (*attB*) – on a plasmid, the addition of φC31 will result in site-specific integration of the entire plasmid (Fig. 1e, f). This strategy has been successfully applied to create transgenic animals with site-specifically integrated enhancer–reporter constructs in both *Drosophila* [91,92] and mouse [93,94]. In *Drosophila,* over 80 *attP* landing sites were created in different genomic locations, covering all chromosomes via conventional *P*-element- and *piggyBAC*-mediated transformation [87,91,95,116]. Hence, it is possible for researchers to choose desirable integration locations that are permissive for reporter expression and lack any ectopic expression [87,92].

Homologous recombination can be also used to insert enhancer–reporter DNA into a single genomic location. In mouse, embryonic stem (ES) cell targeting of an enhancer-reporter to the hypoxanthine phosphoribosyltransferase (*Hprt*) locus allows direct assessment and comparison of enhancer activities in various mouse embryonic and adult tissues [135–137]. However, the efficiency of this method in comparison to conventional transgenic approaches is very low. The frequency of homology driven integration can be increased through introduction of a double stranded break at the insertion site. In *C. elegans,* for example, this has been achieved through transposase-mediated transposon excision that induced breaks at the insertion site [96]. Recently developed CRISPR/Cas9 system allows to introduce double stranded breaks at any desired location of the genome, and it appears to be highly efficient in a wide range of organisms [97,98]. Combined with homologous recombination, this technology has the potential to become the preferred method for site-specific integration of

reporters not only in well-studied model organisms [99,100] but also in other animals.

3.5. Increasing the scale of transgenesis: systematic large-scale approaches

Up until recently, different research groups characterized enhancers one by one and reported them in individual publications, which complicated a systematic computational analysis. Curated databases of published enhancers such as REDfly (*Drosophila* enhancers) [101], CAD (*Drosophila* embryonic enhancers) [30], or ANISEED (*Ciona* enhancers) [102] collected all published transgenic experimental data in comprehensive searchable databases that allow to easily retrieve DNA sequences, target genes and expression patterns of experimentally validated enhancers. With the increase in efficiency and throughput of transgenesis and efforts to create large libraries of transgenic animals it has also become possible to generate large systematic datasets of *in vivo* validated enhancers that use consistent promoters, reporter genes, time-points of development and reporter activity detecting setups (Fig. 1g, h). For *Drosophila* two such resources exist: Fly Enhancers (http://enhancers.starklab.org/; 7793 enhancer candidates that are ~2 kb on average) [103] and FlyLight (http://flweb.janelia.org/; 7128 enhancer candidates that are ~3 kb on average) [104–107]. Together these resources represent the largest collection of enhancers functionally tested in any animal organism to date. Fly Enhancers focuses on enhancer characterization in all cell types and tissues throughout *Drosophila* embryogenesis and is a representative set with respect to different classes of genes. FlyLight enhancers are characterized with higher resolution, but only in adult, larval and embryonic *Drosophila* central nervous system [104,105,107] and larval imaginal discs [106]. Both resources use the same enhancer–reporter setup based on a minimal *Drosophila* synthetic core promoter (DSCP) and yeast *Gal4* as a reporter gene [92]. All reporters were integrated into the same location of the *Drosophila* genome, which allows direct assessment and comparison of enhancer activities across all fragments. Enhancer activity was detected directly via in situ hybridization against reporter *Gal4* transcript (Fly Enhancers), or indirectly, after combining GAL4 protein with the *UAS-GFP* reporter (FlyLight). The UAS-GAL4 system detects amplified signal, but results in a lag in the onset of GFP protein expression compared to the onset of *GAL4* transcription [108].

In vertebrates the major resource for transgenic enhancer data is the mouse VISTA Enhancer Browser (http://enhancer.lbl.gov/; 2192 enhancer candidates that are ~2 kb on average) [78]. Candidates are tested for enhancer activity in transgenic E11.5 mouse embryos (sometimes in earlier or later stages as well) using DNA microinjection and classical whole mount β-galactosidase (lacZ) staining [22]. In addition to the whole embryo pictures, many forebrain enhancers are characterized at higher resolution through histological brain sectioning [109]. The database also contains many human enhancers tested in an *in vivo* based mouse assay, which can serve as a resource to facilitate human genetics and disease-related studies.

Large sets of experimentally characterized animal enhancers serve as an important basis for many types of downstream computational, evolutionary and developmental studies. Both positively and negatively tested fragments can be used as training sets for computational enhancer prediction and discovery [31,103,110–112]. These datasets also add some important numbers and generalize principles from previous single enhancer studies. For example, it was estimated that the *Drosophila* genome contains approximately 50,000 to 100,000 enhancers located without any particular preference upstream, within and downstream of their target gene [92,103]. However, 12 to 21% of enhancers appear to skip the most proximal gene and regulate a more distal neighbor [103].

3.6. Large construct-based transgenic reporters to study enhancer function

When tested in isolation outside of their genomic context, enhancers typically recapitulate their endogenous activity pattern (i.e., tissue-specific activity that enhancer exert on their native target gene in the context of the genome) [103,110]. However, at least a fraction of enhancers is modulated by the endogenous sequence or chromatin context resulting in an altered pattern in transgenic reporter assays [56,71,103]. It is also well documented that some enhancers have a preference for certain classes of core promoters and do not work with others (e.g., [89,113]). Transgenes based on large yeast, bacterial or P1-derived artificial chromosomes (YACs, BACs or PACs respectively) [114] help to overcome these limitations, as they direct gene and reporter expression from native promoters at levels very close to endogenous. BACs are more commonly used, and genome-wide BAC libraries are available for most model organisms and many newly sequenced species (e.g., [115,116] for *Drosophila*). The average size of a BAC from a library is ~200 kb, which is typically less than the length of an average *Drosophila* or even vertebrate gene locus and will likely contain all regulatory regions. If a BAC gene is replaced or tagged with a reporter (using, for example, BAC recombineering), the resulting expression of the reporter will closely recapitulate endogenous gene expression (Fig. 1i, j) [52,117]. For small gene loci (<20 kb) a regular plasmid can be also used to create such transgenes (e.g., [118]). The ability to introduce mutations and deletions of enhancers into large transgenes before transgenesis makes it a very powerful platform to study enhancers in a context close to endogenous (Fig. 1i, j). This experimental design has been often used in studying enhancer requirements for gene transcription in *Drosophila* [52,119,120], zebrafish [121], and mouse [122].

3.7. Methods based on detection of nascent reporter transcription

Development of new imaging and transcript detection techniques increased the resolution with which reporter gene transcripts can be visualized within the developing embryo (Fig. 1k, l) [123,138,139]. In *Drosophila*, a combination of site-specific integrated enhancer–reporter constructs, BAC transgenesis, intronic RNA in situ probes detecting nascent reporter transcription and quantitative confocal imaging were used to monitor enhancer activity in early embryos at a cellular resolution [120]. These results provided fundamental insights into how redundant 'shadow' enhancers coordinate gene transcription [120]. Recently, a live imaging method was developed to examine enhancer activity in the living embryo at unprecedented temporal resolution. Nascent transcripts from enhancer reporter insertion were visualized via MS2 RNA stem loops and MS2 coat protein fused to GFP [21]. Enhancer activity was surprisingly transient, but the cumulative dynamic activity of a reporter closely resembled the known pattern obtained by conventional RNA in situ hybridization [21]. Live imaging methods will become a useful tool to visualize enhancer activity with high spatio-temporal resolution not only in the *Drosophila* embryo but also in other model systems.

4. Future directions and conclusion

Transgenesis has been an invaluable tool in studying the basic principles of gene regulation by transcriptional enhancers. Recent advances in genomics, and especially deep sequencing, not only provided biologists with tools to study regulation of gene expression across entire genomes, but also highlighted the importance of downstream functional validation in investigating genome function. In this review, I provided an overview of different transgenic strategies to functionally characterize enhancers in different animal model organisms.

Over the last 30 years of enhancer research thousands of developmental enhancers have been validated *in vivo* on a one-by-one basis and through systematic approaches [30,78,101–107]. However we are still very far from characterizing all enhancers. At the moment the most comprehensive collection of elements tested for developmental enhancer activity covers only 13.5% of *Drosophila* non-coding non-repetitive genome [103]. Mammalian genomes are an order of magnitude larger than *Drosophila,* and indirect methods indicate that they possibly contain many more (millions) enhancers [41–44]. Thus, it is

important to continue to experimentally characterize this predominant class of functional non-coding elements [78].

One way to increase the throughput of enhancer testing is to parallelize it using deep sequencing. This strategy has been successfully applied to cell cultures and tissues both in *Drosophila* and mammals [56,124–129], but has been challenging at the level of entire organisms. Typically thousands to millions of candidate enhancer reporters are introduced into cell cultures or tissues via electroporation or transfection and enhancers are identified via sequencing after cell sorting or sequencing of a transcribed barcode. In *Drosophila* cell culture, it was possible to screen the entire genome for cell type specific enhancer activity [56]. Enhancer reporter libraries can be also introduced to developing embryos via site-specific integration and screened for activity using FACS of dissociated cells. This approach has been applied to *Drosophila* where one marker was used to label cells of a specific cell type, and another marker was a reporter [130], which allows screening of hundreds of enhancers that are active in a tissue or a cell type of interest. Similar high-throughput strategies will be useful for identifying new enhancers in species with efficient transient transgenesis of embryos *en masse* (for example in *Ciona* [80]).

While transgenesis remains the primary method for functional enhancer characterization in animal organisms, it is also important for us to move beyond enhancer catalogs and explore the functional necessity of enhancers for gene expression and organismal function through enhancer deletion or enhancer modification experiments at their endogenous location [131–133]. This type of experiments, although invaluable for understanding enhancer function, has been challenging and time-consuming due to low efficiency of targeting. The recent development of highly efficient CRISPR/Cas9 genome editing technology [97,98] should further facilitate these studies and will be a powerful complementation to current genomics and transgenesis methods for enhancer characterization.

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Appendix A. Supplementary data

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