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A versatile strategy for gene trapping and trap conversion in emerging model organisms

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SUMMARY

Genetic model organisms such as *Drosophila*, *C. elegans* and the mouse provide formidable tools for studying mechanisms of development, physiology and behaviour. Established models alone, however, allow us to survey only a tiny fraction of the morphological and functional diversity present in the animal kingdom. Here, we present iTRAC, a versatile gene-trapping approach that combines the implementation of unbiased genetic screens with the generation of sophisticated genetic tools both in established and emerging model organisms. The approach utilises an exon-trapping transposon vector that carries an integrase docking site, allowing the targeted integration of new constructs into trapped loci. We provide proof of principle for iTRAC in the emerging model crustacean *Parhyale hawaiensis*: we generate traps that allow specific developmental and physiological processes to be visualised in unparalleled detail, we show that trapped genes can be easily cloned from an unsequenced genome, and we demonstrate targeting of new constructs into a trapped locus. Using this approach, gene traps can serve as platforms for generating diverse reporters, drivers for tissue-specific expression, gene knockdown and other genetic tools not yet imagined.

KEY WORDS: *Parhyale*, Gene trapping, ϕC31 integrase, Regeneration, Transgenesis, iTRAC

INTRODUCTION

Although established genetic models offer unmatched resources for genetic analysis, there is strong motivation to develop genetic tools in new species. This motivation stems from the diversity that is evident in development, morphology and physiology, which means that many questions cannot be addressed in the few well-established models. The development of transgenesis in emerging animal models, such as *Nematostella vectensis* (Renfer et al., 2010), *Parhyale hawaiensis* (Pavlopoulos and Averof, 2005), *Tribolium castaneum* (Berghammer et al., 1999) and *Ciona intestinalis* (Sasakura et al., 2007), represents the first step for establishing sophisticated genetic techniques in these species. One such technique, gene trapping, captures gene expression at the site of transgene insertion. Transposon-mediated gene trapping allows the implementation of unbiased genetic screens to identify new genes and provides valuable markers for in vivo imaging and phenotypic characterisation (Bellen, 1999; Bellen et al., 1989).

In *Drosophila*, gene traps are also used to generate GAL4 drivers, powerful tools that exploit endogenous genes to direct gene expression with spatial and temporal specificity (Brand and Perrimon, 1993). In principle, gene trapping can be used to introduce a wide range of genetic tools, such as alternative expression drivers, recombinases, specialised markers and knockdown constructs, into a trapped locus. In practice, this is difficult to achieve because each application relies on a different transgene construct, and traps derive from unique insertions that cannot be reproduced with each construct. Replacing one type of construct with another, at a given locus, is possible but technically challenging and restricted to highly developed genetic models (Sepp and Auld, 1999).

We present a new approach termed integrase-mediated trap conversion (iTRAC) that allows primary gene traps to be adapted for diverse applications through transgene conversion. The approach uses a primary exon-trapping vector, based on the *Minos* transposon, that incorporates an *attP* docking site for the ϕC31 integrase. Once a trap has been generated and selected, a virtually unlimited range of secondary constructs carrying the cognate *attB* site can be integrated specifically into the docking site at the trapped locus (Fig. 1). As a proof of principle, we demonstrate iTRAC in *Parhyale hawaiensis*, a crustacean that has emerged as an attractive model for developmental studies (Browne et al., 2005; Extavour, 2005; Gerberding et al., 2002; Liubicich et al., 2009; Ozhan-Kizil et al., 2008; Pavlopoulos and Averof, 2005; Pavlopoulos et al., 2009; Price et al., 2010; Rehm et al., 2009; Vargas-Vila et al., 2010).

MATERIALS AND METHODS

Gene-trapping constructs

The 1.3 kb *PhHsp70a* fragment (accession FR749989) was isolated by inverse PCR from *Parhyale* genomic DNA using a previously described approach (Pavlopoulos and Averof, 2005; Pavlopoulos et al., 2009) and cloned upstream of the *DsRed/SV40polyA* reporter cassette to obtain plasmid pSL(PhHsp70a-DsRed).

A 230 bp *Spe*I fragment containing the ϕC31 *attP* site from pTA-attP (Groth et al., 2000) was cloned into the *Spe*I site of pSL(PhHsp70a-DsRed) to generate pSL(attP;PhHsp70a-DsRed). The *attP-PhHsp70a-DsRed* construct was cloned as an *Asc*I fragment into the *Minos* vectors pMi{3xP3-DsRed} and pMi{3xP3-EGFP} (Pavlopoulos and Averof, 2005; Pavlopoulos et al., 2004), generating pMi{3xP3-DsRed;attP;PhHsp70a-DsRed} and pMi{3xP3-EGFP;attP;PhHsp70a-DsRed}.

The transcription initiation and splice sites of *PhMS*, *PhHS* and *PhHsp70a* were mapped by 5′ RACE from transgenic animals carrying stable insertions of *PhMS-DsRed* (Pavlopoulos and Averof, 2005), *PhHS-DsRed* (Pavlopoulos et al., 2009) and *PhHsp70a-DsRed* (*Distal*^{DsRed} trap),

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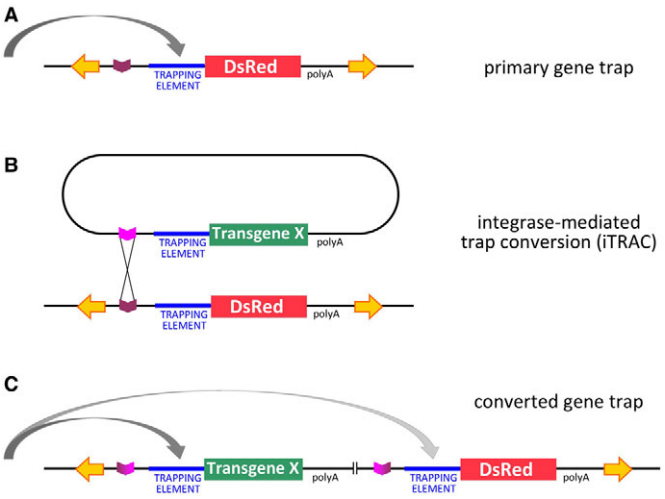

Fig. 1. Gene-trap and trap conversion strategy. (**A**) Primary gene-trapping construct based on a transposon vector (yellow inverted arrows), carrying a ϕC31 *attP* recognition site (purple) and a trapping element (core promoter or splice acceptor) upstream of a reporter gene (*DsRed*). The vector may also include additional markers (not shown). Depending on its insertion site in the genome, the reporter might come to be expressed under the influence of nearby sequences (grey arrow). (**B**) Once a gene trap has been isolated, integrase-mediated trap conversion (iTRAC) utilises *attP* as a docking site for integrating new constructs into the trapped locus. Constructs carrying the cognate *attB* site (magenta) are introduced into the locus by integrase-mediated site-specific recombination. A wide range of secondary constructs for different types of applications can be envisaged (see text); in this example, the DsRed trap is converted into one that expresses a hypothetical transgene X. (**C**) Integration mediated by single *attP* and *attB* sites results in displacement of the original trapping construct by the new construct. Complete replacement is also feasible using flanking pairs of *attP* and *attB* sites.

respectively, using SMART-RACE (Clontech) and a reverse primer targeting the coding sequence of DsRed (5′-CTTGGTCACCTTC-AGCTTGGCGGT-3′).

iTRAC constructs

The EGFP coding sequence was excised as a *Nco*I-*Not*I fragment from pMi{3xP3-EGFP} and cloned into *Nco*I and *Not*I cut pSL(PhHS-DsRed) (Pavlopoulos et al., 2009) to generate pSL(PhHS-EGFP).

Plasmid pBS(MiL;attB;PhHS-EGFP) was generated as follows: we removed the right inverted repeat of *Minos* from pMi{3xP3-DsRed} by *Avr*II and *Nhe*I digestion and religation; we excised *DsRed* with *Sgr*BI and *Not*I and replaced it with a *Sgr*BI-*Not*I fragment of pSL(PhHS-EGFP) carrying *PhHS-EGFP*; we then excised *3xP3* using *Sal*I and replaced it with a *Sal*I fragment containing *attB* from plasmid pTA-attB (Groth et al., 2000).

Plasmid pBS(MiL;attB;PhHsp70a-EGFP) was generated by excising *PhHS* from pBS(MiL;attB;PhHS-EGFP) using *Sma*I and *Nco*I and replacing it by *PhHsp70a* obtained by *Sma*I and partial *Nco*I digestion of pMi{3xP3-DsRed;attP;PhHsp70a-DsRed).

Parhyale gene traps

Plasmid pMi{3xP3-DsRed;attP;PhHsp70a-DsRed} was injected with *Minos* transposase mRNA and stable transgenic lines were isolated as described previously (Pavlopoulos and Averof, 2005). Traps were imaged on a Leica MZ16F epifluorescence stereoscope.

Trapped genes were identified by 5′ RACE on each line using the SMART-RACE Kit (Clontech) with reverse primers DsRed-SMART (5′-CTTGGTCACCTTCAGCTTGGCGGT-3′) or hsp70aDsRedR (5′-GAGGCCATGGTTGTGGATT-3′). Additional cDNA sequences from the *Distal* locus were obtained by 3′ RACE on wild-type animals using forward primers targeting the sequences already determined (accession FR821313). In situ hybridisation using the *Distal* cDNA probe was carried out on wild-type embryos as described previously (Rehm et al., 2009). The $Distal^{DsRed}$ line carries additional transgene insertions that do not give visible traps.

Splicing to *PhHsp70a-DsRed* and normal splicing at the *Distal* locus were measured by quantitative RT-PCR using a common forward primer targeting the *Distal* 5′ UTR (5′-TGACAGTCGCTGCGAAATAG-3′) and two reverse primers targeting *DsRed* (5′-GGGTGCTTCACGTACACCTT-3′) and a *Distal* 3′ exon (5′-GTCTGCTCGTCTTCCTTTGC-3′). RNA was isolated from populations of 20-30 heterozygous and homozygous $Distal^{DsRed}$ embryos, reverse transcribed using oligo(dT) primers and amplified in triplicate on the MJ Research Opticon real-time PCR machine; PCR efficiency with each set of primers was 1.98 and 1.72, respectively. Trapped and normally spliced products were detected at a ratio of 0.09 (s.e.=0.18) in heterozygous embryos and 0.15 (s.e.=0.32) in homozygous embryos.

ϕC31 interplasmid assay, integration and iTRAC

In vitro synthesised ϕC31 integrase mRNA was prepared as described previously (Groth et al., 2004). For the interplasmid assay, 1- or 2-cell stage *Parhyale* embryos were injected with plasmids carrying the *attP* and *attB* sites and ϕC31 integrase mRNA at 500 ng/μl, 500 ng/μl and 100 ng/μl, respectively. Surviving embryos were collected 24 hours after injection and nucleic acids were isolated by mechanical disruption in Holmes-Bonner buffer (100 mM Tris-HCl pH 7.5, 10 mM EDTA, 300 mM NaCl, 2% SDS, 7 M urea), triple phenol/chloroform extraction and ethanol precipitation. Recombination events were detected by PCR using two forward primers (5′-AGGAAGGGAAGAAAGCGAAA-3′ and 5′-CCAATTTCTATCTTAGCCCAACC-3′) and a common reverse primer (5′-GGGTGCTTCACGTACACCTT-3′), as illustrated in Fig. 4A.

Genomic integration via ϕC31 integrase was tested by injecting plasmid pBS(MiL;attB;PhHS-EGFP) and integrase mRNA (300 ng/μl and 100 ng/μl, respectively) into 1- or 2-cell stage embryos of a line carrying multiple *attP* insertions. Out of 512 injected embryos, 207 survived to late embryogenesis and 104 expressed EGFP after heat shock. Seventeen individuals with uniform expression were raised to adulthood; all produced progeny carrying the *PhHS-EGFP* marker. Wild-type embryos lacking *attP* insertions were injected as controls; out of 619 injected embryos, 255 survived as late embryos, but none expressed EGFP after heat shock.

iTRAC was tested by injecting plasmid pBS(MiL;attB;PhHsp70a-EGFP) and ϕC31 integrase mRNA (300 ng/μl and 100 ng/μl, respectively) into 1- or 2-cell stage embryos of the $Distal^{DsRed}$ line. Out of 389 injected embryos, 90 survived to late embryogenesis and 47 expressed EGFP in at least some limbs and in the characteristic pattern of *Distal*; 12 of these embryos showed bilateral replacement of DsRed fluorescence by EGFP. Four individuals were raised to adulthood, of which three gave rise to $Distal^{EGFP}$ progeny.

RESULTS AND DISCUSSION

The gene-trapping vector

Our *Minos* gene-trapping vector carries the *3xP3-DsRed* or *3xP3-EGFP* transformation marker (Berghammer et al., 1999; Pavlopoulos and Averof, 2005), the ϕC31 *attP* site, and a ‘trapping element’ (described below) upstream of the *DsRed* reporter and the early mRNA polyadenylation sequence of SV40. The two types of trapping strategies commonly employed, i.e. enhancer trapping and exon trapping, make use of a core promoter or a splice acceptor to capture the activity of cis-regulatory elements or splice donors of trapped genes, respectively. To create a trapping system that is widely applicable, we sought to identify promoters or splice acceptors capable of gene trapping in a range of species. First, we tested two core promoter elements, the *Drosophila hsp70* basal promoter and an artificial ‘super core promoter’ (which combines

several core promoter motifs) (Juven-Gershon et al., 2006), for enhancer trapping activity in *Drosophila*, *Tribolium* and *Parhyale*, but neither was found to work across the species tested (Schinko et al., 2010) (data not shown). Next, we searched for core promoters and splice acceptors among sequences that lie upstream of *Parhyale hsp70* genes. Among the sequences tested, two elements were capable of efficient gene trapping in *Parhyale*: the heat-inducible element *PhHS* (Pavlopoulos et al., 2009) and a fragment named *PhHsp70a*. Both fragments could drive expression patterns specific to individual transgene insertions without any need for a heat shock (Fig. 2B-L). Using 5′ RACE on cDNA prepared from transgenic lines, we determined that *PhHS* contains a core promoter upstream of the transcription start site and a large intron within the 5′ UTR, whereas *PhHsp70a* is a truncated 5′ UTR sequence with a splice acceptor site that becomes spliced to the exons of trapped genes (see Fig. S1 in the supplementary material). We decided to focus on exon trapping mediated by *PhHsp70a*.

In our first experiment using the *Minos{3xP3-DsRed;PhHsp70a-DsRed}* vector, we recovered at least six independent traps from ~250 injected *Parhyale* embryos. Using the same vector, we also obtained four independent exon traps in *Drosophila* from ~350 injected embryos, suggesting that this construct can mediate exon trapping efficiently in diverse arthropods.

Imaging of developmental and physiological processes

A variety of exon traps have been generated to date using the *PhHsp70a-DsRed* trapping construct in *Parhyale* (Fig. 2). These include traps with expression in the central nervous system, mesoderm, appendages, mouthparts, gills and other patterns. Most lines have been propagated through many generations over 4-5 years, demonstrating that the transgenes are stable, with continuing activity and no detrimental effects on reproduction and survival.

In emerging model organisms, gene traps are likely to be first used as markers for visualising specific tissues or cell types, providing a means to follow dynamic cell behaviours, to study physiological processes in vivo and to assess phenotypes following experimental manipulations. Some of our traps mark well-recognised organs, such as the nervous system, gills or paragnaths (Fig. 2D,F,H,K), whereas others mark complex populations of cells and previously undescribed cell types. For instance, one trap marks a previously uncharacterised cell type on the dorsal epidermis of late embryos, juveniles and adults (Fig. 2C) that is associated with specific sensory or structural elements in the epidermis of *Parhyale* (Fig. 3A).

Another trap allows us to image cardiac function. *Parhyale* has a typical arthropod heart, consisting of a muscular tube with three pairs of lateral inflow valves and an anterior outflow valve (Fig. 3B). Using this trap, we were able to observe heart function and to visualise the opening and closing of valves in unprecedented detail (Fig. 3C and see Movie 1 in the supplementary material).

A third trap, which we named *Distal*^{DsRed}, marks the distal part of all *Parhyale* limbs in embryos, larvae and adults (Fig. 2E,L). We have used this line to monitor limb regeneration following amputation in *Parhyale* (Fig. 3D,D′).

Cloning trapped genes and mutagenic effects

In emerging model organisms, transposon-based exon trapping is one of the most straightforward ways to isolate genes through unbiased genome-wide screens. The trapped gene of interest can be cloned easily by primer extension on cDNA from the trapped line, even when the genome is unsequenced. To demonstrate this, we cloned a cDNA from the gene trapped in the *Distal*^{DsRed} line of *Parhyale*. The cDNA contains a long open reading frame with no similarity to known proteins. A corresponding probe revealed the same expression pattern as *Distal*^{DsRed} in embryos (see Fig. S2 in the supplementary material). Similarly, we have cloned cDNAs of several other genes trapped by *PhHsp70a-DsRed* in *Parhyale* and *Drosophila*. Sequencing of these cDNAs led to our discovery of trans-splicing in *Parhyale* (Douris et al., 2010).

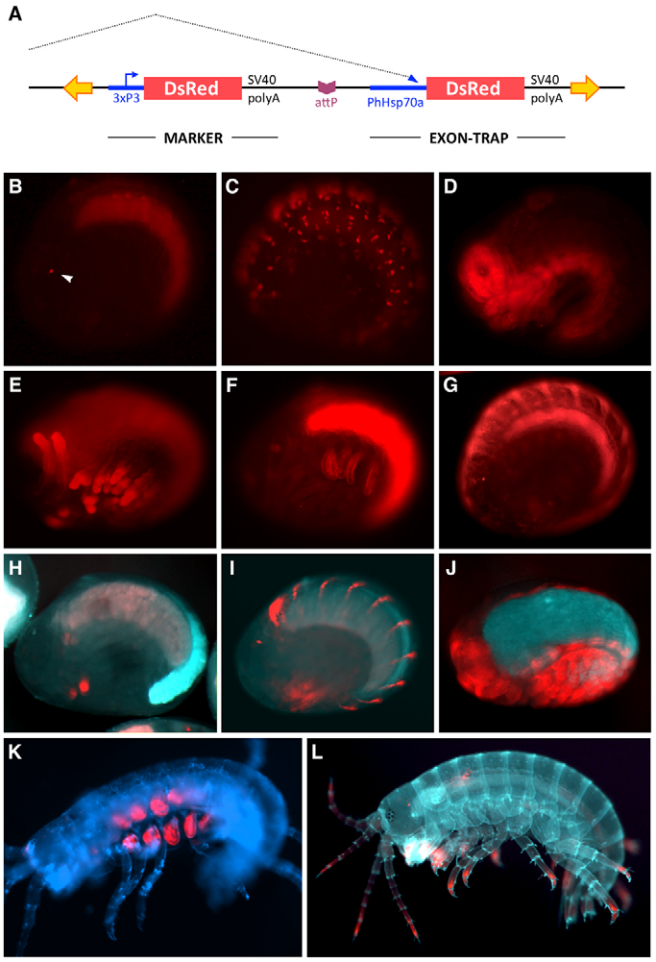

Fig. 2. Gene trapping in *Parhyale hawaiensis*. (**A**) Our primary trapping vector consists of the transformation marker *3xP3-DsRed*, the ϕC31 *attP* site and the exon-trapping element *PhHsp70a-DsRed*, flanked by *Minos* inverted repeats (yellow inverted arrows). A splice acceptor in *PhHsp70a* mediates exon trapping (dotted arrow). (**B**) Embryo expressing the *3xP3-DsRed* marker in the absence of gene trapping (arrowhead); the autofluorescence of yolk is also seen (red crescent). (**C-J**) Embryos with gene traps expressing DsRed in a variety of patterns, including specific epidermal cell types (C), brain and ventral nerve cord (D), limbs (E), gills (F), paragnaths (H), segmental stripes (I), as well as more widespread patterns (G,J). (**K**,**L**) Hatchlings with gene traps expressing DsRed in the gills (K) and limbs (L). All images show lateral views (except H,K,J, which are tilted) with anterior to the left. In H-L, DsRed fluorescence images are merged with corresponding UV autofluorescence or brightfield images (in cyan or blue) to highlight morphological features. The traps were obtained using the *PhHsp70a-DsRed* trapping construct, except those shown in H and K, which were obtained using *PhHS-DsRed*.

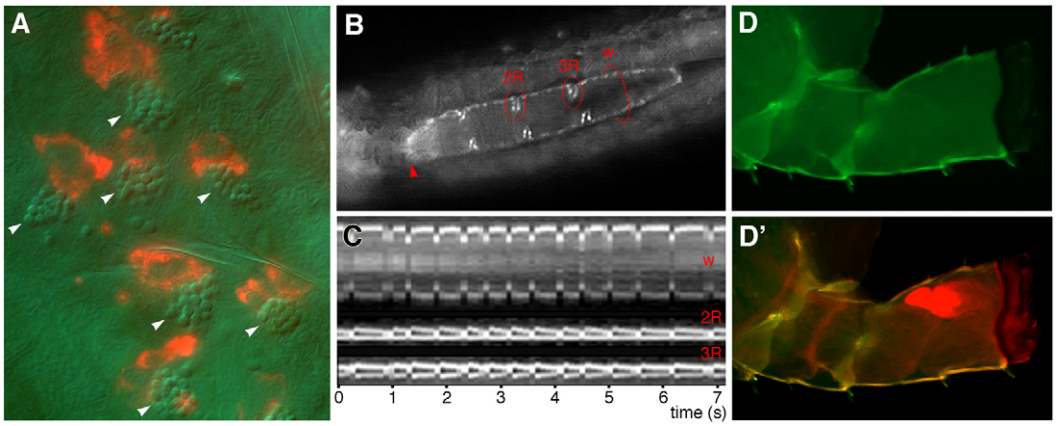

Fig. 3. Using gene traps to visualise physiology and development in *Parhyale*. (**A**) Gene trap marking a previously undescribed cell type on the dorsal epidermis of a late embryo. DsRed-marked cells are of irregular shape and are associated with refractile structures located below the cuticle (arrowheads, visualised with Nomarski optics). (**B**,**C**) A gene trap allows us to study the function of the heart and valves by live imaging (see Movie 1 in the supplementary material). (B) Frame from live recording of a *Parhyale* hatchling, showing the outline of the heart tube and the three pairs of inflow valves; the outflow valve, at the anterior end of the heart (arrowhead), is out of focus. The length of the heart is 450 μm. Red circles mark the regions where kymograms were recorded. (C) Kymograms depicting successive contractions of the heart wall (w) and inflow valves 2 and 3 of the heart. (**D**,**D′**) The *Distal*^{DsRed} trap helps to visualise regenerating limbs, which are not normally visible prior to moulting. The regenerated limb (red in D′) can be clearly seen folded within the amputation stump (green).

In exon-trapped genes, splicing of the endogenous transcript onto the gene-trap cassette generates a chimaeric mRNA that expresses, at most, the N-terminal portion of the endogenous protein. If splicing to *PhHsp70a-DsRed* were 100% efficient, traps would be mutagenic and we would expect to detect a loss-of-function phenotype in animals homozygous for the trap. However, the majority of exon traps obtained using *PhHsp70a-DsRed* do not cause a detectable phenotype in homozygous animals. We used quantitative RT-PCR to examine splicing to *PhHsp70a-DsRed* relative to normal splicing at the *Distal*^{DsRed} locus. We found that only some transcripts are spliced to the *PhHsp70a-DsRed* trapping cassette, whereas a large proportion are still spliced onto the endogenous downstream exon. Thus, *PhHsp70a-DsRed* allows for sensitive trap detection with little disruption of endogenous gene function. This is helpful for maintaining stocks in the absence of balancer chromosomes. iTRAC, described below, provides the means to convert such traps into mutagenic insertions.

Integrase-mediated trap conversion (iTRAC)

The *attP* site in the trapping vector is a platform for integrating new constructs into trapped loci so as to generate new markers and tools for genetic analysis. ϕC31 integrase has never been used before in *Parhyale*. First, we devised a rapid PCR-based assay for ϕC31 integrase activity in vivo, which demonstrated efficient integrase-dependent recombination of *attP* and *attB* sites across two plasmids injected into early *Parhyale* embryos (Fig. 4A).

Next, we tested the ability of *attB*-bearing plasmids to integrate at *attP* sites inserted in the *Parhyale* genome in the presence of ϕC31 integrase mRNA. A plasmid carrying *attB* and a heat-inducible EGFP marker (*PhHS-EGFP*) integrated with high efficiency into a transgenic line carrying multiple copies of the *attP* sequence (104 of 207 injected embryos expressed EGFP after heat shock). No integration events were recovered by injection into wild-type embryos (0 out of 255 embryos). Integration of the *PhHS-EGFP* transgene also occurred in the germline, as judged from its transmission to the next generation.

Finally, we were able to demonstrate the conversion of a DsRed exon trap into one that expresses EGFP in the same pattern. A plasmid carrying *attB-PhHsp70a-EGFP* was injected with ϕC31 integrase mRNA into early embryos of the *Distal*^{DsRed} transgenic line. A high proportion of these embryos (47 out of 90 survivors) showed EGFP fluorescence replacing DsRed fluorescence in at least a subset of limbs (Fig. 4B). In ~20% of injected survivors, iTRAC also occurred in the germline and a stable DsRed to EGFP conversion was evident in subsequent generations (Fig. 4C). In *Distal*^{EGFP} lines, DsRed expression could not be detected. These results indicate that iTRAC can be implemented with high efficiency in *Parhyale*.

Conclusions

Model organisms are defined by the experimental approaches they offer to address biological questions of broad interest. Candidate gene approaches have, until now, been the main avenue for applying knowledge gained in established models to other species of interest, but these approaches are biased and incomplete. The establishment of transgenesis in new species that span the phylogenetic tree of animals, from cnidarians to protostomes and chordates, sets the stage for developing forward and reverse genetics tools and approaches in a wide range of organisms. iTRAC provides a shortcut for implementing these approaches.

The versatility of iTRAC opens opportunities for a wide range of downstream applications: (1) the generation of markers for different types of microscopy, ranging from fluorescence-based live imaging to electron microscopy; (2) the implementation of binary systems for gene expression, such as the GAL4, LexA, tTA and Q systems; (3) the genetic marking and manipulation of clonal populations of cells using FLP or Cre recombinase; (4) the conversion of gene traps into gene knockouts, for instance by insertion of strong transcriptional terminators; (5) genetic cell ablation using cell-autonomously acting toxins; and (6) chromosome engineering. Conceivably, the same collections of traps could be used in the future to implement tools that are not yet

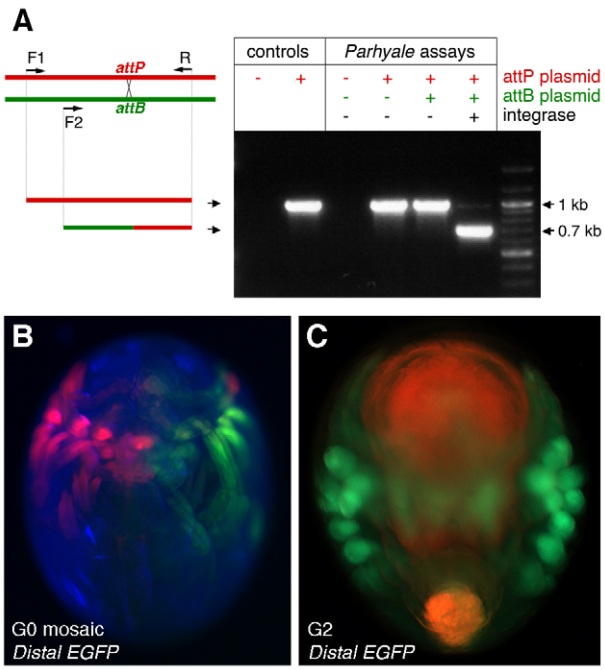

Fig. 4. Integrase-mediated trap conversion (iTRAC) in *Parhyale*. (**A**) The interplasmid assay for ϕC31 integrase-mediated recombination involves injecting plasmids carrying an *attP* or an *attB* site (red and green, respectively) with ϕC31 integrase mRNA into *Parhyale* embryos, and assaying recombination by PCR using primers F1, F2 and R. Each gel lane represents a single experiment, in which different combinations of plasmids and integrase mRNA were injected. The 1 kb band results from amplification of the *attP* plasmid fragment (red), whereas the 0.7 kb band results from amplification of a hybrid fragment created by recombination between *attP* and *attB* sites (green-red). The 0.7 kb fragment is strictly dependent on the presence of ϕC31 integrase. The identity of this fragment was also verified by sequencing. (**B**) Ventral view of a *Distal*^{DsRed} embryo (G0) injected with ϕC31 integrase mRNA and a plasmid carrying *attB* and *PhHsp70a-EGFP*. Integration at the 2-cell stage resulted in a mosaic in which the *Distal*^{DsRed} trap was converted to *Distal*^{EGFP} on one side of the embryo; the other side retained *Distal*^{DsRed} expression. (**C**) Ventral view of *Distal*^{EGFP} embryo, two generations after conversion (G2). DsRed fluorescence is not detectable in limbs; red autofluorescence of the yolk is shown for contrast.

realised. Thus, iTRAC may serve as a genetic Swiss army knife, allowing the exploitation of gene traps in a virtually endless number of ways.

The approach and the vectors presented here are likely to be applicable in a broad range of animal models, as all the constituents are known to work in widely divergent species: the *Minos* transposon is an excellent vector for gene trapping not only in arthropods, but also in mammals and in *Ciona* (Pavlopoulos et al., 2007; Sasakura et al., 2007); the ϕC31 integrase system has found application in *Drosophila*, zebrafish, *Xenopus*, mouse and human (Allen and Weeks, 2005; Groth et al., 2004; Groth et al., 2000; Lister, 2010), and we have shown here that it works very efficiently in *Parhyale*; the *PhHsp70a* element can mediate exon trapping in *Parhyale* and *Drosophila* and, given the wide conservation of splice acceptor sites, is likely to be useful more broadly.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
Z.K. established the ϕC31 integrase system and implemented iTRAC in *Parhyale*; A.P. cloned the *hsp70* sequences and established gene trapping in *Parhyale*; A.K. built and tested alternative trapping vectors; N.K. studied limb regeneration using gene traps; A.K. and N.K. determined promoter and splice sites in the *hsp70* sequences; Z.K., V.D. and A.K. cloned *Distal* and assessed splicing at that locus; M.A. conceived iTRAC, imaged gene traps, supervised the project and wrote the paper; all authors discussed the results and commented on the manuscript.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.066324/-/DC1

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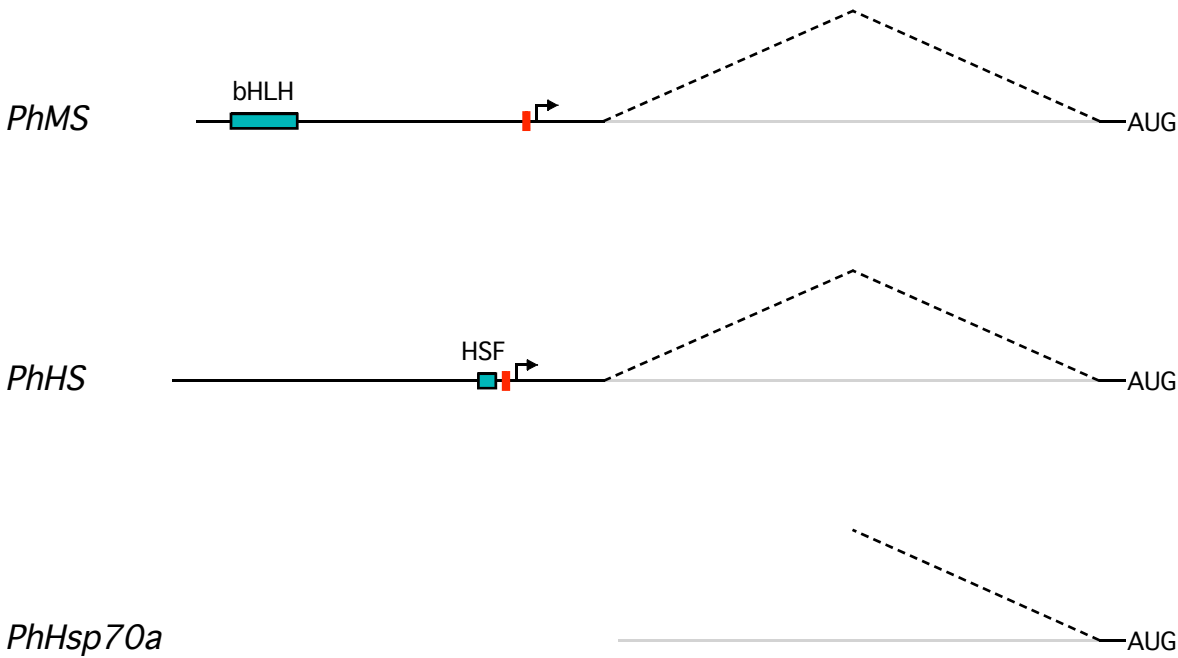
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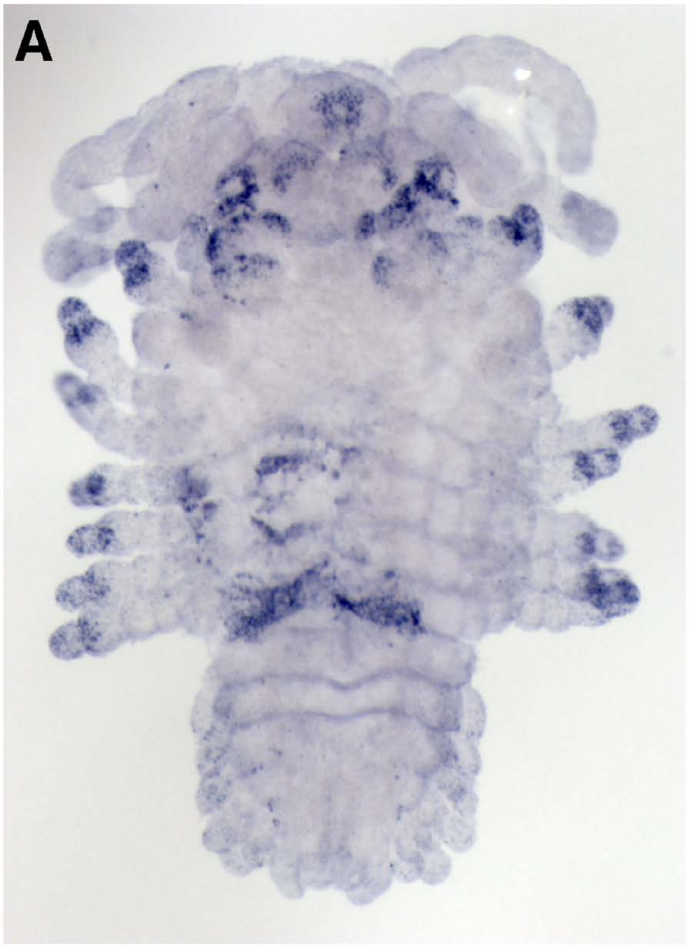
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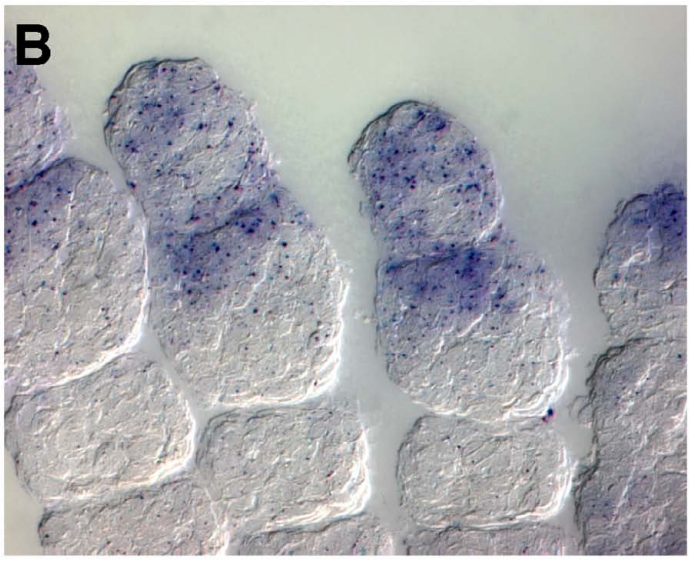
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