Chapter 10

Transgenesis in Non-model Organisms: The Case of *Parhyale*

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Abstract

One of the most striking manifestations of Hox gene activity is the morphological and functional diversity of arthropod body plans, segments, and associated appendages. Among arthropod models, the amphipod crustacean *Parhyale hawaiensis* satisfies a number of appealing biological and technical requirements to study the Hox control of tissue and organ morphogenesis. *Parhyale* embryos undergo direct development from fertilized eggs into miniature adults within 10 days and are amenable to all sorts of embryological and functional genetic manipulations. Furthermore, each embryo develops a series of specialized appendages along the anterior–posterior body axis, offering exceptional material to probe the genetic basis of appendage patterning, growth, and differentiation. Here, we describe the methodologies and techniques required for transgenesis-based gain-of-function studies of Hox genes in *Parhyale* embryos. First, we introduce a protocol for efficient microinjection of early-stage *Parhyale* embryos. Second, we describe the application of fast and reliable assays to test the activity of the *Minos* DNA transposon in embryos. Third, we present the use of *Minos*-based transgenesis vectors to generate stable and transient transgenic *Parhyale*. Finally, we describe the development and application of a conditional heat-inducible misexpression system to study the role of the Hox gene *Ultrabithorax* in *Parhyale* appendage specialization. Beyond providing a useful resource for Parhyalists, this chapter also aims to provide a road map for researchers working on other emerging model organisms. Acknowledging the time and effort that need to be invested in developing transgenic approaches in new species, it is all worth it considering the wide scope of experimentation that opens up once transgenesis is established.

Key words Arthropods, Crustaceans, *Parhyale hawaiensis*, *Minos* transgenesis, Microinjections, Heat-shock promoter, Conditional gene misexpression, Hox genes, *Ultrabithorax*, Appendage development

1 Introduction

The enormous diversity of body plans, segments, and associated appendages is considered one of the cornerstones underlying the evolutionary success of the arthropod phylum, comprising chelicerates, myriapods, crustaceans, and insects [1]. Among these extant arthropod groups, crustaceans (barnacles, copepods, ostracods, shrimps, lobsters, crabs, and their kin) exhibit the most impressive

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diversity in appendage morphology and function that is evident not only across crustacean species but also within the same species. Until recently, it has not been possible to explore the molecular and cellular basis of segmental specialization and appendage diversification in any crustacean species due to the lack of suitable experimental approaches. During the last decade, the amphipod *Parhyale hawaiensis* has emerged as the most powerful available crustacean model for developmental genetic and molecular cell biology studies [2, 3].

Considering the sister group relationship of crustaceans and insects, it is not surprising that *Parhyale* research has benefited enormously from the wealth of methodologies and knowledge available in insect models like *Drosophila melanogaster*, *Tribolium castaneum*, and others. In this respect, *Parhyale* studies are contributing to our understanding of how developmental mechanisms have changed or remained conserved over macroevolutionary time scales [4–8]. More importantly, *Parhyale* has proven not only suitable for comparative developmental studies but also a powerful model system in its own right. Current research demonstrates the strengths of *Parhyale* in addressing key processes in animal development, like germ layer specification, cell fate specification, head and central nervous system development, organ morphogenesis, and regeneration [9–14]. It should also be noted that amphipods exhibit diverse lifestyles and associated morphological and physiological adaptations. In addition to *Parhyale*, ongoing developmental studies in other amphipod species (e.g., *Orchestia cavimana* [15, 16], *Caprella scaura* [17], *Gammarus minus* [18]) hold great promise in establishing the Amphipoda as a group where one could study evolution on smaller time scales.

Parhyale is a marine amphipod crustacean with a worldwide tropical distribution living in shallow aquatic habitats [2, 3]. It was put forward as an attractive model organism by William Browne and Nipam Patel in the late 1990s. Since then, several labs in the USA and Europe have joined a growing *Parhyale* community, attracted by the easiness to grow and maintain this species in dense cultures, the relatively short generation time (2 months), and the accessibility of embryos at all stages of development all year round. *Parhyale* embryogenesis and early cell lineages have been described in detail [2, 19, 20], and an increasing number of experimental resources are being developed at a fast pace. *Parhyale* embryos can be subjected to various embryological manipulations, developmental genetic techniques, and molecular and cell biology approaches, including cell microinjection, isolation and ablation [10, 21, 22], cell lineage tracing [19, 20, 23], in situ hybridization and immunohistochemistry [24–26], RNA interference and morpholino-based gene knockdown [12, 27], transposon and integrase-mediated genetic transformation [11, 28], conditional gene misexpression [14], and live imaging using transmitted light

or fluorescence microscopy ([10, 19, 29]; see also http://www.cell.com/pictureshow/lightsheet2). Transcriptomic and genomic resources have also been made available by high-throughput sequencing of BAC clones and cDNA libraries [30–32], as well as by ongoing efforts to sequence and assemble the relatively large *Parhyale* genome that is estimated at 3 Gb.

Parhyale is a sexually dimorphic species; sexually mature males are easily distinguishable from females by their enlarged grasping appendages on the third thoracic segment [2]. Males grasp and hold females for one or more days until mating occurs. The released females molt and oviposit 5–50 eggs (depending on the age) in a ventral brood pouch. Adults breed year-round every 2–3 weeks and can be set routinely in single crosses to generate inbred lines. The embryos in each brood develop almost synchronously and can be dissected from females at any stage and cultured in Petri dishes in artificial seawater. After 10–11 days of embryogenesis at 25–26 °C, the hatched juveniles resemble miniature adults since *Parhyale* is a direct developer. Hatchlings increase in size through successive molts and need about 7–8 weeks at 25–26 °C to reach sexual maturity.

The optical properties of *Parhyale* embryos allow detailed microscopic inspections of constituent cells with exceptional spatial and temporal resolution ([10, 19, 29]; see also http://www.cell.com/pictureshow/lightsheet2). Early cleavages of fertilized eggs are holoblastic [2, 20]. The first cleavage is slightly unequal and generates two blastomeres, each contributing to the ectodermal and mesodermal lineages of either the left or the right side of the animal. The second cleavage is also slightly unequal, while the third cleavage is highly unequal producing a stereotyped arrangement of four macromeres and four micromeres that are uniquely identifiable based on their relative position, size, and contacts. Already at the 8-cell stage, the fate of each blastomere is restricted to a single germ layer (although blastomeres exhibit some regulative capacity too [22]); three macromeres give rise to the ectoderm, the fourth macromere gives rise to the visceral and anterior mesoderm, two micromeres form the somatic mesoderm, one micromere forms the endoderm, and one micromere forms the germline [2, 20]. Later cell divisions segregate the superficial layers of the embryo from the yolk and cells aggregate to form the embryo rudiment.

Ectodermal cells in the growing embryo become organized into the head lobes anteriorly and into regular rows posteriorly, forming a grid-like pattern typical for amphipods and other malacostracan crustaceans [2, 20, 33]. Similar to *Drosophila* and other arthropods, *Parhyale* embryos exhibit initially a parasegmental organization; each row of cells in the grid corresponds to one parasegment [2, 34]. The parasegmental rows undergo stereotyped divisions that, together with the progressive addition of more parasegmental rows

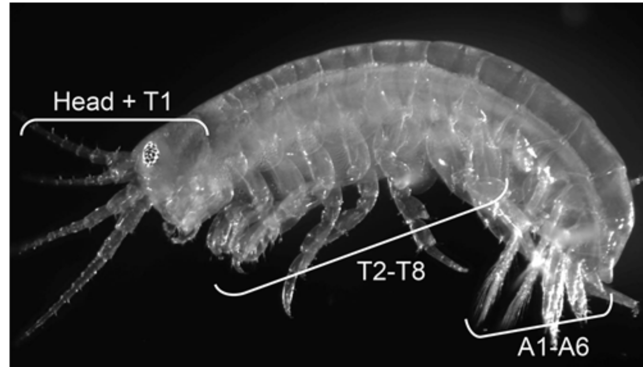

Fig. 1 The body plan of the crustacean amphipod *Parhyale hawaiensis*. The head is fused to the first thoracic segment (T1), the thorax is composed of seven segments (T2–T8), and the abdomen consists of six segments (A1–A6)

posteriorly, lead to the axial elongation of the embryo. Subsequent cell divisions disrupt the regularity of the grid and contribute to formation of segmental units and appendage bud outgrowths. *Parhyale* axial patterning and growth occur in an anterior-to-posterior progression; more anterior structures form and elaborate earlier than more posterior ones. As appendages extend and differentiate along their proximodistal axis, the yolk gets sequestered into the developing midgut, and the head region becomes clearly distinguishable from the trunk of the embryo. Towards the end of embryogenesis, the pigmented compound eyes form, the dorsal heart starts beating, and muscles start twitching shortly before hatching [2].

The segmented body of *Parhyale* consists of the head, the thorax, and the abdomen [2, 14] (Fig. 1). The head is composed of six segments bearing five pairs of sensory and feeding appendages (antennae 1, antennae 2, mandibles, maxillae 1, maxillae 2) and is fused to the first thoracic segment (T1) bearing a pair of segmented feeding appendages, known as maxillipeds. The next seven thoracic segments (T2–T8) develop larger segmented appendages; T2 and T3 bear subchelate grasping appendages, called gnathopods, and T4–T8 bear elongated walking appendages called pereopods. The abdomen is subdivided into two regions with three segments each: the pleon with three pairs of biramous paddling appendages (A1–A3 pleopods) and the urosome with three pairs of reduced thickened appendages (A4–A6 uropods). This striking specialization of *Parhyale* appendages along the anterior–posterior body axis offers exceptional material to study the molecular and cellular basis of organ patterning, growth, and morphogenesis.

This chapter introduces step by step all the methodologies and techniques that we established for transgenesis-based functional studies of Hox genes in *Parhyale* [14, 28]. First, we describe a robust and easily adaptable protocol for microinjection of early-stage *Parhyale* embryos.

Second, we describe the application of quick and sensitive extrachromosomal assays, known as excision and transposition assays, to examine whether the selected DNA transposon (in this case the *Minos* transposon) can be mobilized efficiently in a transposase-dependent manner in the cellular environment of interest (in this case in early-stage *Parhyale* embryos) [28, 35, 36]. The excision assay tests the ability of a *Minos* transposon to excise from a donor plasmid, when provided with a suitable source of transposase (in this case with the *Minos* transposase provided as mRNA or as plasmid). The transposition assay tests the ability of the *Minos* element to transpose by the cut-and-paste mechanism from its original site in the donor plasmid into a new site in a target plasmid in a transposase-dependent manner.

Third, we present the use of *Minos*-based transposon vectors for the generation of stable transgenic and transient transgenic *Parhyale* [28, 36]. Stable transgenesis (a.k.a. germline transformation) involves insertion of exogenous DNA constructs (transgenes) carried by the transposon into the germline of the injected animal (G0), so that transgenes can be transmitted down the generations. The offspring (G1) that will emerge from fertilization of a transformed gamete will be stably transformed (transgenic); all its somatic cells will carry the same insertion(s) of the transgene. The transformation efficiency is typically relatively low; only a small percentage of the injected G0s will acquire a small percentage of transformed gametes, and will give rise to a small percentage of transformed progeny. For this reason, *Minos* vectors are engineered to carry a transformation marker gene, in addition to the transgene of interest (Fig. 3b). Expression of the transformation marker allows the straightforward identification of transgenic individuals among a large number of untransformed animals. Nowadays, the most commonly used transformation markers in arthropod transgenesis, including *Parhyale*, combine fluorescent proteins with an artificial *cis*-regulatory element responsive to the Pax6 transcription factor, known as *3xP3* [28, 37–39].

In *Parhyale* transgenesis, expression of the transformation marker genes and of the transgenes is not only detected in transgenic animals (G1s, G2s, etc.) but also in a substantial fraction of the injected G0 animals [14, 28]. The ability to obtain genomic integration events of *Minos* in early-stage *Parhyale* embryos enables to produce G0 animals with very low levels of mosaicism (transient transgenesis). In such a transient transgenic animal, a large proportion (or even all) of the descendant cells from the injected blastomere carry the *Minos* insertion(s). It should be stressed here that transposition is a stochastic process; the stage and cell at which the injected transposon integrates into the genome, as well as the number and genomic loci of integration, vary dramatically among injected embryos. Therefore, transient transgenic *Parhyale* embryos can exhibit diverse patterns and levels of marker gene/transgene expression [14]. Despite this caveat, injections at the 1-cell stage

can produce bilateral marker gene/transgene expression, while single blastomere injections at the 2-cell stage often result in unilateral expression of marker genes/transgenes expressed in the ectoderm and somatic mesoderm [28]. The prescreening of G0s for marker gene/transgene expression is an extremely useful feature in *Parhyale* transgenesis. It provides an early and accurate indication about the potential success or failure of the experiment, and also provides information about transgene expression and function, months before stable transgenics are available for analysis.

Fourth, we describe a gain-of-function approach based on conditional heat-inducible misexpression of transgenes, and the application of this system to study the role of the Hox gene *Ultrabithorax* in *Parhyale* appendage specialization [14]. This method allows to assess the function of the gene of interest by misexpressing it in cells and at developmental stages that normally do not experience its product. A number of other transgenesis-based functional approaches not discussed in this chapter have also been realized in *Parhyale*, including trapping of genes in unbiased genetic screens and trap conversion by targeted integration of new constructs into trapped loci [11].

2 Materials

2.1 Microinjection of Early-Stage Parhyale Embryos

1. Sea salt (Tropic Marin).
2. Flake food for tropical fish (TetraMin).
3. Crushed coral.
4. Air or water pumps.
5. Artificial seawater (ASW): Dissolve sea salt in purified water (about 34 g/l) to a specific gravity of about 1.022.
6. Filtered artificial seawater (FASW): ASW filtered through a 0.22 μm filter.
7. Filtered artificial seawater with antibiotics and antimycotics (FASWA): FASW with penicillin-streptomycin (diluted 1/100) and fungizone-amphotericin B (diluted 1/200).
8. Penicillin-Streptomycin (Gibco).
9. Fungizone-Amphotericin B (Gibco).
10. Blunt dissecting forceps.
11. Fine paintbrushes.
12. Micropipettes and tips.
13. Pasteur pipettes.
14. Collection baskets: Can be made from cut 50 ml Falcon tubes with a fine Nytex mesh sealed at one opening or bought ready-made (Corning).
15. Plain 150, 90, and 60 mm Petri dishes.

16. Tissue culture grade coated 60 and 35 mm Petri dishes (Nunc).
17. Clove oil (Sigma).
18. Bovine serum albumin.
19. Glass slides.
20. Agarose.
21. Ready-made microinjection needles (Eppendorf Femtotips).
22. Borosilicate glass capillaries 1 mm O.D. × 0.58 mm I.D. with inner filament (Clark Electromedical Instruments).
23. Microloading pipette tips (Eppendorf).
24. Incubator set at 25–26 °C.
25. CO_2 station.
26. Flaming-Brown micropipette puller (Sutter Instrument Company).
27. Beveler (Narishige).
28. Injector (Narishige or Eppendorf).
29. Micromanipulator (Leica or Narishige).
30. Dissecting stereoscope with external light source.
31. Upright microscope.

2.2 Testing the Activity of DNA Transposons with Excision and Transposition Assays

1. Materials described in Subheading 2.1.
2. Nuclease-free ddH_2O.
3. Microcentrifuge tubes.
4. Filter micropipette tips.
5. Plasmid midi or maxi prep kit (Qiagen or Nucleobond).
6. NotI restriction endonuclease (NEB).
7. T7 mMESSAGE mMACHINE kit (Ambion).
8. 3 M sodium acetate solution pH 5.2.
9. Isopropanol.
10. Absolute ethanol.
11. 70 % ethanol RNase-free (mix absolute ethanol with DEPC-treated ddH_2O).
12. Phenol red solution (Sigma).
13. Holmes–Bonner solution: 100 mM Tris–HCl pH 7.5, 10 mM EDTA, 350 mM NaCl, 2 % SDS, 7 M urea (store at −20 °C, mix well before use).
14. Disposable RNase-free tubes and pestles (Kontes).
15. Phenol:chloroform:isoamyl alcohol 25:24:1.
16. Chloroform:isoamyl alcohol 24:1.
17. High-specificity/sensitivity *Taq* DNA polymerase and PCR buffer (Taq 2000 from Agilent or Platinum from Invitrogen).

18. Purified custom oligos.
19. 10 mM dNTP mix.
20. DNA molecular weight ladder.
21. pGEM-T Easy vector system (Promega).
22. High-efficiency *E. coli* electrocompetent cells (ElectroMAX DH5α-E cells from Invitrogen).
23. SOC medium.
24. LB medium.
25. LB + Cm plates: LB plates with 30 μg/ml chloramphenicol.
26. LB + Cm + Suc plates: LB plates with 30 μg/ml chloramphenicol and 10 % sucrose.
27. LB + Cm + Tet plates: LB plates with 30 μg/ml chloramphenicol and 12 μg/μl tetracycline.
28. Gel electrophoresis setup.
29. Benchtop cooling microcentrifuge.
30. Nanodrop spectrophotometer.
31. Standard microbiological equipment: Culture flasks and tubes, disposable sterile pipettes, 37 °C shaker and incubator, centrifuge with rotor and tubes.
32. PCR thermal cycler.

2.3 Transposon-Based Stable and Transient Transgenesis in *Parhyale*

1. Materials described in Subheadings 2.1 and 2.2.
2. Fluorescence stereoscope equipped with appropriate filter sets for detection of fluorescent proteins.
3. Equipment and reagents for Southern blot analysis, detailed in [28, 40, 41].
4. Equipment and reagents for inverse PCR, detailed in [40, 42, 43].

2.4 Conditional Heat-Inducible Misexpression of Hox Genes in Transient and Stable Transgenic *Parhyale*

1. Materials described in Subheadings 2.1–2.3.
2. Cloning reagents: PCR reagents, RACE kit, restriction enzymes, ligase, phosphatase.
3. Equipment and reagents for Northern blot analysis, detailed in [28, 40, 41].
4. RNA extraction reagent (TRIzol from Invitrogen) or kit (Qiagen).
5. Reverse transcription kit (SuperScript III from Invitrogen).
6. DNase I (amplification grade from Invitrogen).
7. Real-time PCR kit (SYBR Green I from Roche).
8. Equipment and reagents for in situ hybridization and antibody staining, detailed in [14, 24–26].

9. Glutaraldehyde.
10. Osmium tetroxide.
11. Formaldehyde.
12. 10× PBS pH 7.4: 18.6 mM NaH_2PO_4, 84.1 mM Na_2HPO_4, 1.75 M NaCl (store at room temperature).
13. Triton X-100.
14. Hoyer's medium/lactic acid (1:1) solution.
15. Sylgard 184 silicone elastomer kit (Dow Corning).
16. Fine dissecting forceps.
17. Incubator set at 37 °C.
18. Real-time PCR instrument.
19. Heating plate set at 60 °C.
20. Compound microscope.
21. Critical point dryer.
22. Sputter coater.
23. Scanning electron microscope.

3 Methods

3.1 Microinjection of Early-Stage *Parhyale* Embryos

3.1.1 Collection of *Parhyale* Embryos

Parhyale are maintained easily in dense cultures using standard aquarium equipment in plastic containers with a bottom layer of crushed coral covered in artificial seawater (2–3 l ASW in 10–20 l container). Large cultures with thousands of animals are maintained on standard fish flake food or other diets at 22–26 °C, are aerated with a submerged water or air pump, and are kept waste free with phosphate- and nitrate-absorbing bags and weekly to monthly water changes (for more details refer to [3] or visit http://www.extavourlab.com/protocols/Parhyale%20hawaiensis%20culture.pdf). A few of these cultures provide daily accessibility to hundreds of embryos at all stages of development all year round.

1. Adult *Parhyale* form mating pairs. The day before injecting, collect 50 or more pairs from the main cultures with a basket or by sucking them up using a Pasteur pipette with a large opening. Transfer pairs into 150 mm Petri dishes with ASW and few corals.
2. Many of the collected *Parhyale* pairs will have separated the day of injections. Gravid females with eggs in their ventral brood pouch are easily identifiable by eye. Transfer gravid females into a basket immersed in FASW using a Pasteur pipette or by picking up with blunt forceps the piece of coral they are sitting on.
3. Anaesthetize gravid females by bubbling CO_2 gas for 30–60 s into the filtered artificial sea water (FASW) or by transferring

the basket into FASW with 1:2,000 diluted clove oil. Wait for 1–2 min until completely still and transfer anaesthetized females with a paintbrush (or with a Pasteur pipette or by grabbing the females with blunt forceps from their antennae) into 60 mm tissue culture Petri dish in filtered artificial seawater with antibiotics and antimycotics (FASWA).

4. Hold each female gently on its back under a dissecting scope with one pair of blunt forceps. Place another pair of blunt forceps (or a blunted rounded end of a Pasteur pipette) between the embryos and the pouch, and nick the pouch along the ventral midline by lifting the forceps. Then place the forceps below the eggs and push them gently out of the nicked pouch. Continue with the rest of anaesthetized females, collecting all embryos into the 60 mm tissue culture Petri dish in FASWA. Transfer dissected females into another Petri dish with ASW, wait until they are fully awake and mobile, and return them into the main *Parhyale* cultures.
5. Sort the collected embryos under the dissecting scope according to their developmental stage [2].
6. Repeat **steps 2–5** every 4 h. This way you can collect a large number of 1-cell-stage embryos and stage them according to experimental requirements (*see* **Note 1**).

3.1.2 Preparation of Needles for Microinjection

The quality of microneedles is one the most important parameters for successful microinjections. Commercially available microneedles are well suited for microinjection of *Parhyale* embryos, but are relatively expensive. Alternatively, microneedles can be prepared from glass capillaries on a Flaming-Brown needle puller, and then beveled on a rotating microbeveler (*see* **Note 2**).

1. We prepare microneedles from borosilicate glass capillaries on a Sutter P-87 puller with a box filament using the following settings: heat 850, pull 10, velocity 150, time 250, pressure 680. These parameters need to be adjusted for each puller, filament, and capillary type used (*see* **Note 2**).
2. For each pull cycle, mount a glass capillary onto the puller. Start the program to turn on heating of the filament. The glass heats up and a weak pull draws the glass out until it reaches the programmed velocity. The heat turns off and after a time delay the hard pull is executed. A puff of air is delivered at a certain pressure and period of time to quickly cool the glass. Remove needles from the puller and store in a 150 mm Petri dish on a stripe of plasticine.
3. Pulled needles are then beveled to reproducibly sculpt pointy tips for smooth eggshell penetration. Alternatively, the tip can be broken off randomly by touching it against a glass surface or by cutting it with scalpel or forceps.

4. To bevel needles, place the microbeveler under a dissecting scope and focus on the rotating platform. Clean the dust of the rotating platform by wiping with ethanol and spraying pressurized air. Load a microneedle at an angle of 30°. Use the beveler's controller to move the needle down until the tip just touches the platform. Move a bit further down so that the needle is just slightly bent. Leave beveling for at least 30 min before using the needle for microinjection.

3.1.3 Preparation of Agarose Steps

Parhyale eggs are extremely sensitive to desiccation and should be kept wet during microinjection. Placing the embryos on agarose steps provides a convenient solution for immobilizing the embryos while keeping them wet (Fig. 2a).

1. Prepare the template for the agarose steps by sticking two glass slides together with tape, so that their long edges protrude 1 mm. Place slides into a 90 mm Petri dish.

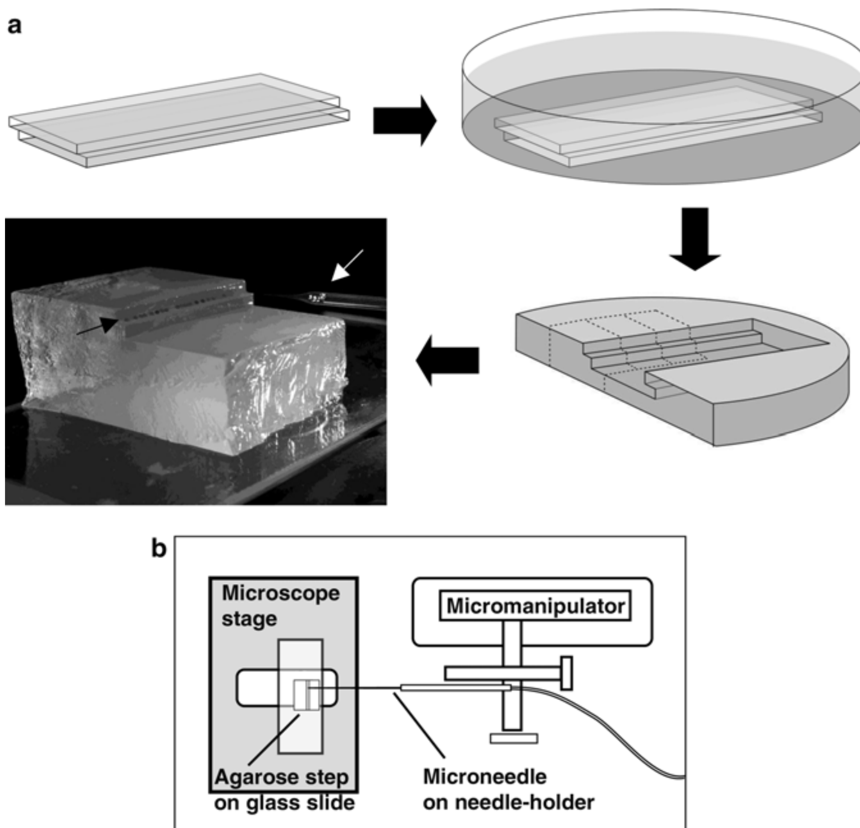

Fig. 2 Setup for *Parhyale* embryo microinjection. (**a**) Preparation of custom-made agarose steps as described in Subheading 3.1.3. *Parhyale* embryos (*black arrow*) are kept wet on the agarose step that faces the microneedle coming from the side (*white arrow*). (**b**) The *Parhyale* microinjectory is composed of a microscope focused on the agarose step with the embryos, a micromanipulator controlling the movement of the microneedle through a needle holder, and an injector (not shown) that delivers small volumes of the injection mix through the microneedle

2. Dissolve 2 % agarose in FASW by bringing to boil under continuous stirring. Let it cool down to 40–50 °C and pour 30 ml into Petri dish with slides.
3. Wait until agarose has solidified and carefully detach the slides from the agarose. Remove unwanted agar with a scalpel keeping the part that has formed a narrow step.
4. Agarose steps can be stored at 4 °C covered in FASWA.

3.1.4 Microinjections

1. The *Parhyale* microinjectory is composed of an injector, a micromanipulator, and an upright microscope equipped with a 5× or a 10× dry objective (an inverted microscope or dissecting scope could do as well) (Fig. 2b). The injector allows small liquid volumes to be delivered precisely through the microneedle by applying a regulated pressure for a certain period of time. The pressure is supplied from a compressed gas cylinder containing air or nitrogen. The microneedle is mounted on a needle holder, the movement of which is controlled by a micromanipulator with three knobs or a joystick to move the needle in the *x*-, *y*-, and *z*-axes. The agarose step is placed on a glass slide so that its long edge faces the needle coming from the side of the microscope (Fig. 2a). Triggering of injection is accomplished either with a push button or more conveniently with a foot switch. Short surges of maximum pressure can be delivered to clear a clogged microneedle and restore flow rate. A regulated holding pressure (balance pressure) is applied to the microneedle in between injections to prevent dilution of the injected material by the inflow of seawater due to capillary forces.
2. Backfill the needle with 2–3 μl of the injection mix using a microloading pipette tip. Mount the filled needle onto the needle holder and the micromanipulator at a small downward angle or horizontally (*see* **Note 3**).
3. Bring the agarose step into focus using the microscope focusing knobs. Withdraw the step from the field of view (away from the needle) using the stage controllers. Bring the needle tip into focus in the center of the field of view using the micromanipulator controllers. Bring the agarose step back into the field of view; it should be level with the tip of the needle. The movement of the needle towards the egg (*x*-axis) and all minor up and down corrections (*z*-axis) should be done using the micromanipulator. Repeat this step whenever the eggs or the needle go out of focus over the course of injections.
4. Pipet one drop of FASWA onto the agarose step. The tip of the needle should be covered with FASWA. Looking through the eyepieces, apply maximum pressure to the needle. Repeat a few times until the injection mix flows out of the needle. Keeping the needle tip underwater, adjust the holding pressure (balance) to prevent inflow of seawater into the needle. A slight outflow of the injection mix is acceptable.

5. Remove the needle tip out of FASWA (with *x*-axis micromanipulator controller) and adjust the injection pressure or time of each pulse so that it delivers the right amount to be injected (about 100 pl in the case of early-stage *Parhyale* embryos; *see* **Note 4**). Remove the needle tip from FASWA only for a short while to check the flow rate. Always return and keep it underwater to avoid clogging.
6. Cut the end of a pipette tip and fit it onto a micropipette. Coat the plastic tip by sucking up a solution of bovine serum albumin (BSA) to prevent eggs from sticking to the plastic. Use the micropipette with the coated tip to transfer the embryos to be injected from the Petri dish onto the agarose step.
7. Suck up 2–10 embryos (depending on the experience) in 3–4 μl of FASWA and pipet them onto the agarose step. If required, arrange the embryos on the step one next to the other with a fine paintbrush.
8. Use the stage controllers to move the agarose step sideways to center each egg for injection. Center the first egg and move the needle tip onto its middle with the *x*-axis micromanipulator controller. The eggshell should retract slightly and then expand again engulfing the needle tip. Apply the injection pressure while staying close to the egg cortex, and then withdraw needle from the embryo with the *x*-axis micromanipulator controller. The water surface tension will hold the embryo on the agarose step.
9. Center the next embryo with the stage controllers and proceed as described in the previous step. To correct the contact point of the needle tip on the egg, move the needle up (for a higher contact point) or down (for a lower contact point) using the *z*-axis controller of the micromanipulator. Do not forget to check the flow rate frequently as described in **step 5**. When finished, use a fine paintbrush to transfer embryos to a 35 mm tissue culture Petri dish in FASWA.
10. Repeat **steps 7–9** for all embryos. Transfer about 30 injected embryos in each 35 mm coated Petri dish and incubate them at 25–26 °C. Surviving injected embryos should be transferred every second day to a new 35 mm tissue culture dish with FASWA.
11. Under optimal microinjection conditions, at least 30 % of injected *Parhyale* embryos should hatch 10–13 days later.

3.2 Testing the Activity of DNA Transposons with Excision and Transposition Assays

Both activity assays involve co-injecting a donor plasmid (carrying the *Minos* transposon) and a target plasmid—with or without a source of transposase—into early-stage *Parhyale* embryos, incubating these for 24 h and extracting total nucleic acids. The excision assay is based on PCR reactions to determine whether *Minos* has excised from the donor plasmid using primers flanking the

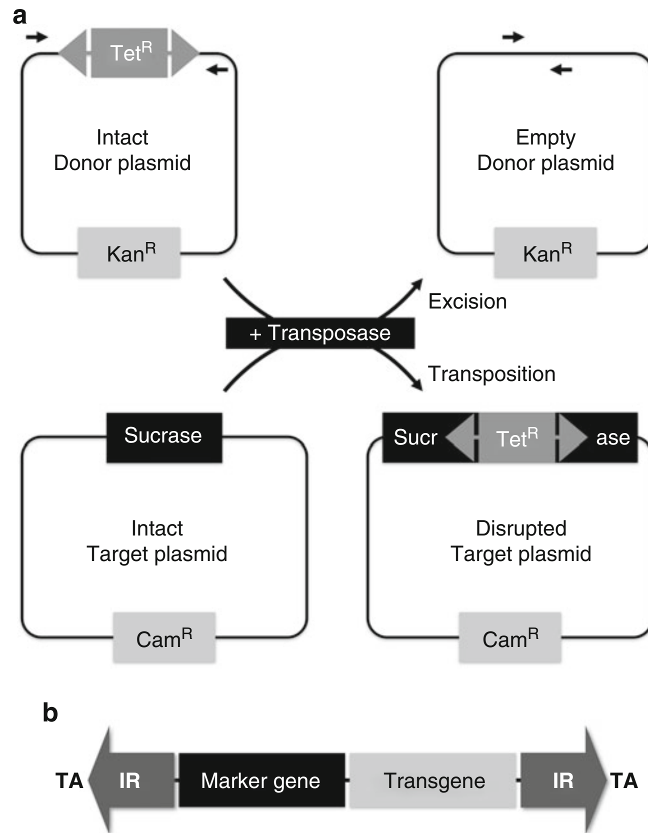

Fig. 3 Overview of activity assays and transgenesis vectors based on the *Minos* DNA transposon. (**a**) The excision and transposition assays test the ability of the *Minos* transposon to excise from a donor plasmid, when provided with a source of *Minos* transposase, and transpose by the cut-and-paste mechanism into a target plasmid. Please refer to Subheading 3.2 for more details. (**b**) Schematic representation of engineered transgenesis vectors: the *Minos* inverted repeats (IR) flank the transgene of interest and a transformation marker gene that allows identification of transgenic individuals. *Minos* transposition occurs exclusively into a TA target dinucleotide that is duplicated upon insertion on either side of the inverted repeats

transposon on the donor plasmid (Fig. 3a). The transposition assay involves transforming bacteria with the recovered plasmids and screening these for the presence of target plasmids containing the transposed *Minos* element based on bacterial marker selection (Fig. 3a).

3.2.1 Preparation of Plasmid DNA

The quality of injected plasmid DNA is important for efficient *Minos* mobility in transgenic experiments and in excision and transposition assays. The majority of purified plasmid DNA molecules

should be in the supercoiled configuration. Plasmids prepared on a medium or a large scale from commercially available ion-exchange columns are used routinely in our laboratories.

1. Extract plasmid DNA from 50 to 500 ml of bacterial culture according to the manufacturer's instructions and dissolve purified plasmid DNA in nuclease-free water at a concentration of at least 1 μg/μl.
2. Centrifuge dissolved plasmid DNA at >12,000 × *g* for 30 min at 4 °C to precipitate any insoluble particles that might clog the microneedle.
3. Pipet plasmid DNA into a new tube and store at −20 °C. Plasmids used in multiple rounds of injections over several days should be stored in aliquots to avoid multiple freeze-thaw cycles.

3.2.2 Preparation of Capped mRNA

In vitro synthesized capped mRNA encoding the *Minos* transposase (helper mRNA) is our preferred transient source of transposase in *Parhyale* transgenic experiments and in excision/transposition assays (*see* **Note 5**). The plasmid template pBlueSKMimRNA contains a T7 promoter driving the expression of the *Minos* transposase coding sequence with 5′ and 3′ UTR flanking sequences from the *Drosophila hsp70* and *inflated* (αPS2 integrin) genes, respectively [43, 44].

1. Set up a digest in a tube at 100 μl with the NotI restriction enzyme to linearize 5–10 μg of the pBlueSKMimRNA plasmid template. After 2 h of incubation, analyze 500 ng by 1 % agarose gel electrophoresis (next to uncut vector) to confirm complete linearization.
2. Extract linearized pBlueSKMimRNA once with equal volume (100 μl) phenol–chloroform–isoamyl alcohol and once with chloroform–isoamyl alcohol. Add 1/10th volume (10 μl) sodium acetate solution and two volumes (200 μl) ice-cold absolute ethanol. Incubate at −80 °C for at least 30 min and precipitate linearized plasmid by centrifuging at >12,000 × *g* for 20 min at 4 °C.
3. Discard supernatant and wash pellet with RNase-free 70 % ethanol. Air-dry pellet for 2–3 min until it becomes completely transparent and dissolve it in 10 μl nuclease-free water. Quantify concentration on a nanodrop spectrophotometer and use as template for capped mRNA synthesis using the T7 mMESSAGE mMACHINE kit (Ambion).
4. In an RNase-free tube pipet 10 μl 2× NTP/CAP mix, 2 μl 10× T7 reaction buffer, 1 μg linearized plasmid, and 2 μl T7 enzyme mix, and add nuclease-free water to a final reaction volume of 20 μl. Incubate for 2 h at 37 °C. Remove template plasmid by adding

1 μl RNase-free DNase I to the reaction and incubating for 15 more minutes at 37 °C.

5. Stop reaction by adding 115 μl nuclease-free water and 15 μl ammonium acetate stop solution. Extract once with equal volume (150 μl) phenol–chloroform–isoamyl alcohol and once with chloroform–isoamyl alcohol. Add equal volume (150 μl) isopropanol, mix well, make aliquots depending on the use (about 20–30 μl), and store at −20 °C. In our experience, each in vitro transcription reaction yields about 30 μg of capped *Minos* transposase mRNA.
6. Just before use in microinjections, precipitate mRNA by spinning at >12,000 × *g* for 20 min at 4 °C. Discard supernatant and wash pellet with RNase-free 70 % ethanol. Air-dry pellet until it becomes completely transparent and dissolve it in about 5 μl nuclease-free water (actual volume depends on the yield and final concentration needed in downstream application). Quantify on a nanodrop spectrophotometer.

3.2.3 Preparation of the Microinjection Mix

Prepare 10–20 μl of the injection mix just before microinjection. Special care should be taken in the preparation of the mix to avoid clogging of the fine needle tip.

1. Donor plasmids carrying the *Minos* transposons are injected at a concentration never exceeding 1 μg/μl, usually between 300 and 500 ng/μl.
2. The mRNA or the plasmid DNA encoding the *Minos* transposase (referred to as helper mRNA or helper plasmid, respectively) are usually injected at a ratio 1:2 to 1:5 relative to the donor plasmid, i.e., at a final concentration of 100–300 ng/μl.
3. In the case of *Parhyale*, the different injection components are mixed together in water. All injection mixes also include the inert dye phenol red (1/10 dilution of stock), which allows better visualization of the injected material.
4. Centrifuge injection mix at >12,000 × *g* for 20 min at 4 °C to precipitate any insoluble material that might clog the needle, and keep tube on ice throughout microinjections. Use 2–3 μl of the injection mix to backfill the needle using a microloading pipette tip fitted onto a micropipette.

3.2.4 Microinjections and Nucleic Acid Extraction

1. Collect 1- to 16-cell-stage *Parhyale* embryos and proceed with microinjections as described in Subheading 3.1.
2. Inject pools of at least 50 embryos with each one of the following mixes (*see* **Note 6**):

# Mix	Donor plasmid pMiLRTetR(L)	Transposase source	Target plasmid pBC/SacRB
1	–	–	–
2	150 ng/μl	–	300 ng/μl
3	150 ng/μl	mRNA (75 ng/μl)	300 ng/μl
4	150 ng/μl	mRNA (150 ng/μl)	300 ng/μl
5	150 ng/μl	mRNA (300 ng/μl)	300 ng/μl
6	150 ng/μl	Plasmid (300 ng/μl)	300 ng/μl

3. Incubate pools of injected embryos for 1 day at 25–26 °C.
4. Transfer each pool of embryos into a microcentrifuge tube with a micropipette. Remove excess seawater and proceed with the next step or flash freeze in liquid nitrogen and store at −80 °C.
5. In each tube, add 100 μl of Holmes–Bonner solution and homogenize with a pestle for 1 min.
6. Add 100 μl more Holmes–Bonner solution and extract with 200 μl of phenol–chloroform–isoamyl alcohol, mixing gently on a rotating platform for 10 min.
7. Centrifuge at >12,000 × *g* for 5 min at room temperature. Transfer upper aqueous phase to a new tube.
8. Repeat extractions (**steps 6**, **7**), twice with phenol–chloroform–isoamyl alcohol and twice with chloroform–isoamyl alcohol.
9. Add 1/10th volume (20 μl) sodium acetate solution and two volumes (400 μl) ice-cold absolute ethanol. Incubate at −80 °C for at least 30 min and precipitate nucleic acids by spinning at >12,000 × *g* for 20 min at 4 °C.
10. Discard supernatant and wash pellet twice with 70 % ethanol.
11. Air-dry pellet for 2–3 min until it becomes completely transparent and dissolve it in 10 μl of nuclease-free water.
12. Quantify on a nanodrop spectrophotometer and analyze 1 μl from each sample by 1 % agarose gel electrophoresis. The genomic DNA and ribosomal RNA bands should be visible on the gel.

3.2.5 Minos Excision Assay

1. For the excision assay, use an equal amount of extracted nucleic acids (about 20 ng) from each sample as template in PCR reaction with a high-specificity/sensitivity Taq DNA polymerase according to the manufacturer's instructions with primers MiR-hydei (5′-TGCATTCTCTATGCT-3′) and MiL-Lorist (5′-CCAGCTGGCTTATCGAAA-3′) [35]. For example, set

50 µl reactions in 1× Taq buffer, 1.25 Units Taq, 200 µM each dNTP, 0.5 µM each primer, and 1.5–2 mM $MgCl_2$ using the following cycling program.

Initial denaturation:	98 °C for 2 min	
Main cycling program:	Denaturation	98 °C for 30 s
(35 cycles)	Annealing	55 °C for 30 s
	Extension	72 °C for 60 s
Final extension:		72 °C for 5 min

2. Analyze 10 µl of each PCR reaction by 1 % agarose gel electrophoresis next to a DNA molecular weight ladder.
3. Amplification of the non-excised *Minos* transposon from intact pMiLRTetR(L) donors produces a 2.2 kb band, whereas amplification from "empty" donors after *Minos* excision produces a 211 bp band (*see* **Note 7**). The relative abundance of the 2.2 kb and 211 bp bands between samples provides a qualitative assessment of *Minos* excision activity between conditions assayed [28, 35] (*see* **Note 8**).
4. To verify the specificity of *Minos* excision, gel-purify the 211 bp band, clone it in a T-Vector, and sequence individual clones. Transposase-mediated excision leaves behind characteristic footprints in empty donor plasmids consisting of the four terminal nucleotides of either end of the *Minos* transposon flanked by the duplicated TA target site (TAcgagTA or TActcgTA; [35, 45]).

3.2.6 *Minos* Transposition Assay

1. For the transposition assay, use an equal amount of extracted nucleic acids from each sample (about 50 ng) to transform high-efficiency *E. coli*-competent bacteria by electroporation according to the manufacturer's instructions.
2. Transfer each electroporated bacterial suspension in 1 ml SOC medium in a 15 ml snap-cap tube and shake for 1 h at 225 rpm at 37 °C.
3. Spread 5 % of cells (50 µl) on LB + Cm plates and the rest 95 % of cells (950 µl) on LB + Cm + Suc plates (*see* **Note 9**). Incubate plates at 37 °C until colonies reach a diameter of 1–2 mm (about 16 h).
4. Make replica patches of chloramphenicol and sucrose-resistant colonies grown on LB + Cm + Suc plates onto LB + Cm + Tet replica plates and grow overnight at 37 °C (*see* **Note 9**).
5. Count the number of colonies recovered on LB + Cm plates, and the number of chloramphenicol, tetracycline, and sucrose triple-resistant clones grown on the LB + Cm + Tet replica plates.

6. For each injected mix, calculate transposition efficiency as the percentage of target plasmids disrupted by *Minos* insertion. In this calculation, divide the number of chloramphenicol, tetracycline, and sucrose triple-resistant clones (disrupted targets) with the number of colonies grown on LB + Cm multiplied by 19 (total number of target plasmids assayed) (*see* **Note 10**).
7. Inoculate liquid LB + Cm + Tet cultures and extract plasmid DNA on a small scale from a subset of chloramphenicol, tetracycline, and sucrose triple-resistant clones that are considered independent interplasmid transposition events. Validate clones by digesting with the NotI restriction enzyme 0.5–1 μg of each extracted plasmid DNA (*see* **Note 11**). Check by 1 % agarose gel electrophoresis for the presence of two diagnostic bands with a total size of about 8 kb (6 kb pBC/SacRB + 2 kb *Minos* transposon).
8. Verify the specificity of *Minos* transposition by determining the insertion site of the *Minos* transposon in each disrupted target plasmid. Sequence the nucleotides flanking the *Minos* left and right inverted repeats with primers 309_reverse (5′-GATTCCGTTACATTAGTTGC-3′) and 1500_forward (5′-TAAGTATGATAGTAAATCAC-3′), respectively [35]. *Minos* transposition occurs exclusively into a TA target dinucleotide that is duplicated upon insertion and should be found on either side of the sequenced inverted repeats (Fig. 3b) [35, 45].

3.3 Transposon-Based Stable and Transient Transgenesis in *Parhyale*

The methodologies described in this Subheading apply to any *Minos*-based construct that needs to be inserted into the *Parhyale* genome. However, the original demonstration of germline transformation in a new species of interest is typically done with a simple vector made of the transformation marker flanked by the transposon's inverted repeats. For this reason, we describe here mainly the use of the donor plasmid pMi{3xP3-DsRed} [28]. This plasmid contains the *Minos*{*3xP3-DsRed*} transposon, in which the *Minos* inverted repeats flank the *3xP3-DsRed* transformation marker (*see* **Note 12**). Many more *Minos*-based vectors are available containing other fluorescent proteins under *3xP3* control, like pMi{3xP3-mTFP1}, pMi{3xP3-EYFP}, pMi{3xP3-EGFP}, and others [36]. As described in Subheading 3.4.2, all these *Minos* vectors have unique restrictions sites for cloning the transgenes to be delivered into the *Parhyale* genome.

1. Prepare the donor plasmid pMi{3xP3-DsRed} and *Minos* transposase helper mRNA according to Subheadings 3.2.1 and 3.2.2, respectively.
2. Prepare 10–20 μl of the injection mix containing 500 ng/μl (or 300 ng/μl) of the donor plasmid, 300 ng/μl (or 100 ng/μl) of the helper mRNA, and 0.1 volumes phenol red as described in Subheading 3.2.3.

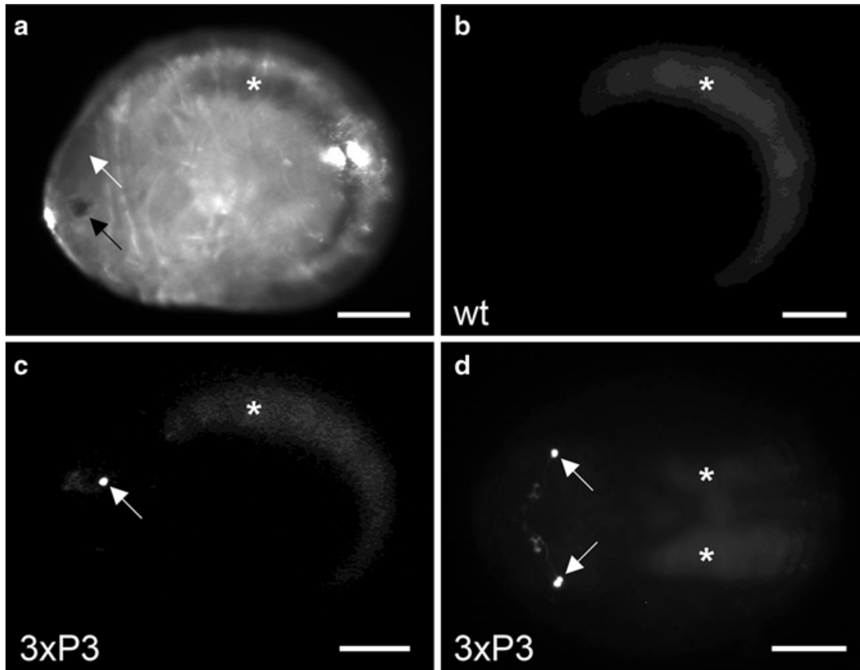

Fig. 4 *3xP3*-driven transformation marker gene expression in transgenic *Parhyale* embryos. (**a**) Bright-field image of a late-stage *Parhyale* embryo. (**b**) Fluorescent image of a wild-type late-stage *Parhyale* embryo. (**c**, **d**) Fluorescent images of late-stage transgenic *Parhyale* embryos. Yolk autofluorescence produces a dorsal crescent of fluorescence in the gut (*asterisks*) in transgenic and non-transgenic embryos. Transgenic embryos exhibit the *3xP3*-driven spot of strong fluorescence in each side of the head (*white arrows*) located posterior to the pigmented compound eye (*black arrow*). Fluorescently labeled neuronal projections are visible in panel (**d**) extending from each spot (cell body) towards the brain. Panels (**a**–**c**) are lateral views, panel (**d**) is dorsal view, anterior is to the *left* in all panels. Scale bars are 100 μm

3. Collect at least 200 1- and 2-cell-stage *Parhyale* embryos and proceed with microinjections as described in Subheading 3.1. In the case of 2-cell stage embryos, inject the smaller blastomere that will give rise to the germline [20].
4. Transfer injected embryos in FASWA in 35 mm tissue culture Petri dishes, aiming for about 30 embryos per dish. Label the lids with the mix injected, the stage of injection and number of embryos in the dish, and the date and time of injection.
5. Incubate dishes with embryos at 25–26 °C. Check the dishes daily and remove dead embryos to avoid microbial contamination. Surviving injected embryos should be transferred every second day (using a micropipette with a BSA-coated plastic tip) into a new 35 mm tissue culture dish in FASWA.
6. Screen late-stage *Parhyale* embryos 9–10 days after injection (their formed compound eyes should be pigmented; Fig. 4a) for *3xP3*-driven DsRed expression under a fluorescence stereoscope equipped with the DsRed filter set. Screen both sides of each embryo. In each side, *3xP3* drives a spot of fluorescence

in the posterior head region behind the compound eye (Fig. 4b, c) [28]. In strongly expressing *Parhyale* embryos, it is also possible to detect fluorescence in a neuronal projection extending from this spot (the cell body) towards the brain (Fig. 4d) (*see* **Note 13**).

7. Store embryos exhibiting the characteristic *3xP3* pattern in separate 35 mm dishes in FASWA. Detection of *3xP3* expression in a G0 is a good predictor for its germline transformation and its potential to produce transgenic G1s! In our experience, about 30–40 % of embryos injected at the 1-cell and 2-cell stage with pMi{3xP3-DsRed} were transient transgenics with bilateral or unilateral *3xP3* fluorescent patterns [28] (*see* **Note 14**).
8. From day 10 onwards, screen dishes twice daily for embryos hatched. Under optimal microinjection conditions, at least 30 % of *Parhyale* embryos should hatch 10–13 days after injection.
9. Using a micropipette with a BSA-coated plastic tip, transfer hatchlings into Petri dishes in FASW with a couple of pieces of coral and only few pieces of ground fish flakes. Keep hatchlings with bilateral or unilateral *3xP3* expression individually in 60 mm tissue culture Petri dishes. Hatchlings without *3xP3* expression can also be grown separately in 60 mm Petri dishes or in groups of five animals in 90 mm Petri dishes. Label lids accordingly.
10. Change surviving G0s regularly every 4 days into new Petri dishes with fresh FASW and ground fish flakes using a micropipette or by picking up with blunt forceps the piece of coral they are sitting on. To reduce plastic consumption, recycle used Petri dishes by cleaning them with tap and purified water (no soap water).
11. Repeat **step 10** for about 2 months until G0s grow to about 1 cm and reach sexual maturity. At this stage, G0 males are distinguishable from G0 females based on the size of grasping appendages on the third thoracic segment, which are greatly enlarged in males [2]. Females can also be distinguished by their paired ovaries (oblong and opaque) visible through the cuticle in the dorsal thorax.
12. Set single backcrosses of sexually mature G0s to 2–3 similar-sized wild-type *Parhyale* adults of the opposite sex in 90 mm Petri dishes in FASW with a couple of pieces of coral and ground fish flakes. To reduce the amount of labor, non-*3xP3*-expressing G0s (that have a lower probability of transformed germlines) can be first screened in intercrosses of G0 individuals. Change FASW and food regularly as described in **step 10**. Assign a unique ID to each G0 and label lid with the sex and ID of the G0(s) crossed (e.g., Female#1, Male#2, etc.).
13. Check crosses daily for gravid females. Pick up gravid females and grow them separately for 9 days in 60 mm Petri dishes in FASW with few pieces of coral and ground fish flakes. Label lid with the sex and ID of the associated G0.

14. Dissect 9–10-day-old embryos (G1s) from each gravid female in a 35 mm tissue culture Petri dish with FASWA as described in **steps 3** and **4** in Subheading 3.1. The same female can be crossed again one day later as described in **step 12**.
15. Screen G1s for *3xP3*-driven DsRed expression under a fluorescence stereoscope. Stable transgenic animals will display a bilateral *3xP3* fluorescent pattern (Fig. 4d). Discard non-*3xP3*-expressing G1s. Screen at least 50 G1s from each G0 and discard G0s that do not produce transgenic progeny.
16. Grow *3xP3*-expressing G1 siblings (derived from the same G0 parent) to adulthood separately or in groups as described in **steps 9–11**.
17. The ratio of *3xP3*-expressing to non-expressing G1s provides a first hint about the abundance of *Minos* insertions transmitted by their G0 parent; the higher the number of *Minos* insertions in the germline of a G0, the higher the proportion of its transgenic G1 progeny. The actual number of insertions transmitted can be identified by Southern blot analysis on genomic DNA isolated from pools of transgenic G1 siblings [28, 43]. Analyze *Minos*{*3xP3-DsRed*} insertions by digesting genomic DNA with SacI and using as probe the *DsRed* coding sequence. For detailed protocols on genomic DNA preparation, digestion, Southern blotting, and hybridization, please refer to these other sources [28, 40, 41] (*see* **Note 15**).
18. To analyze the segregation and stability of *Minos* insertions, compare the Southern blot pattern between individual transgenic G1 parents and G2 offspring [28]. To do this, backcross adult G1s individually to 2–3 similar-sized wild-type *Parhyale* as described in **step 12**. Assign a unique ID to each G1 and label lid with the sex and ID of the G1 crossed (e.g., transgenic G1 siblings from G0 Female#1 can be labeled Female#1.1, Male#1.2, etc.). Screen for transgenic G2 progeny as described in **steps 13–15** and grow them to adulthood separately or in groups as described in **steps 9–11**. Carry out Southern blot analysis on genomic DNA isolated from a single adult G1 parent and from single adult G2 offspring as described in **step 17**. Each G2 offspring should exhibit a subset or all of the bands (but not different bands) present in the G1 parent.
19. Assess the specificity of *Minos* transposition from the donor plasmid into the *Parhyale* genome by the cut-and-paste mechanism.
    - First, confirm integration of intact transposons into the genome: Check that all bands detected in the Southern blots described in **steps 17** and **18** exceed a minimum size expected for intact transposons [28, 43].
    - Second, confirm specific integration of the *Minos* transposon without flanking sequences from the donor plasmid: Check

that the Southern blot pattern of transgenic animals differs from the Southern blot pattern of the donor plasmid [28, 43]. If required, repeat hybridization of the Southern blot membranes using the plasmid backbone as probe.

- Third, verify the target site specificity of the *Minos* transposase for the TA dinucleotide: Recover the DNA sequences upstream and downstream of *Minos* insertions by inverse PCR and sequencing as detailed elsewhere [40, 42, 43]; the inverted terminal repeats should be flanked by the characteristic TA dinucleotide, followed by sequences unrelated to those of the plasmid backbone.

20. Establish *Parhyale* transgenic lines by repeated rounds of inbreeding to drive transposon insertion(s) to homozygosity (*see* **Note 16**). Select G1 siblings with the strongest *3xP3* expression that are presumably inheriting multiple common *Minos* insertions, and grow them to adulthood as described in **steps 15** and **16**. Set intercrosses between two G1 siblings of the opposite sex (e.g., Female#1.1 × Male#1.2) as described in **steps 12–14**. Select G2 siblings with the strongest *3xP3* expression that are presumably homozygous for one or more of the *Minos* insertions. Grow selected G2s to adulthood and use two animals of the opposite sex as founders to establish the transgenic line.
21. Establish three or more independent transgenic lines in parallel, derived from different G0s. In the long term, keep the lines that exhibit homogeneous transgene expression among sampled individuals. Keep small-scale cultures in small plastic containers on a bottom layer of crushed coral covered in ASW at 22–26 °C (100–200 ml ASW in 0.5–1 l container). No aeration is required if the seawater and food are changed regularly, at least once a week.

3.4 Conditional Heat-Inducible Misexpression of Hox Genes in Transient and Stable Transgenic Parhyale

The establishment of genetic transformation in *Parhyale* has opened several possibilities for functional genetic approaches in this emerging model organism. We describe here a gain-of-function approach based on conditional heat-inducible misexpression to study the role of the Hox gene *Ultrabithorax* (or any other developmental regulatory gene of interest) in *Parhyale* appendage specification.

3.4.1 PCR-Based Isolation of Parhyale Heat-Inducible cis-Regulatory Sequences

Note that these protocols are only briefly outlined here, because extensive descriptions can be found in other sources [14, 40].

1. Amplify by degenerate PCR from *Parhyale* genomic DNA part of the coding sequence of a *heat-shock protein 70* gene (*Phhsp70*) with primers Hsp70F (5′-ACIACITAYTCITGYGTIGG-3′) and Hsp70R (5′-AAIGGCCARTGYTTCAT-3′).

2. Verify by Northern blot analysis using as probe the amplified coding sequence the conditions for *Phhsp70* heat inducibility in wild-type *Parhyale* incubated for 1 h at different temperatures. This analysis shows that *Phhsp70* transcripts become strongly induced at 37 °C and are not detectable at lower temperatures tested [14].
3. Recover the heat-inducible *cis*-regulatory sequence upstream of *Phhsp70* start codon (5′UTR, promoter sequence and heat-responsive enhancer) piecemeal by repeated rounds of inverse PCR (*see* **Note 17**).
4. Amplify by conventional PCR from *Parhyale* genomic DNA a contiguous fragment containing the heat-inducible *cis-regulatory* sequence (called *PhHS*) with primers Phhsp70F (5′-TTACTGTAACCGCAGGGGCAAAAGA-3′) and Phhsp70R (5′-ACAGCATCCTTCACGTCTCCTCCAA-3′).

3.4.2 Analysis of cis-Regulatory Sequences with Reporter Constructs in Transgenic Parhyale

1. Clone *PhHS* upstream of the *DsRed* fluorescent reporter and the *SV40* polyadenylation sequence in the versatile subcloning vector pSLfa1180fa to generate plasmid pSL-PhHS-DsRed [14, 46]. To place any other gene of interest under *PhHS* control for heat-inducible misexpression in *Parhyale*, remove the *DsRed* coding sequence by NcoI/NotI digest of pSL-PhHS-DsRed, and replace it with the coding sequence of interest digested with NcoI (or BspHI or PciI) encompassing the start codon in its 5′ end and with NotI (or PspOMI) after the stop codon in its 3′ end (*see* **Note 18**).
2. Digest pSL-PhHS-DsRed with AscI, gel-purify the *PhHS-DsRed-SV40polyA* reporter cassette, and clone it in an AscI-digested *Minos* vector (e.g., pMi{3xP3-EGFP}) to generate donor plasmid pMi{3xP3-EGFP;PhHS-DsRed}. The resulting transposon construct contains the *3xP3-EGFP* transformation marker and the *PhHS-DsRed-SV40polyA* reporter flanked by the *Minos* inverted repeats.
3. Prepare 10–20 μl of an injection mix containing 300 ng/μl of the donor plasmid pMi{3xP3-EGFP;PhHS-DsRed}, 100 ng/μl of the *Minos* transposase helper mRNA, and 0.1 volumes phenol red as described in Subheadings 3.2.1–3.2.3.
4. Microinject at least 200 1- and 2-cell-stage *Parhyale* embryos as described in Subheading 3.1, and grow injected embryos in FASWA to late stages as described in **steps 4** and **5** in Subheading 3.3.
5. Screen 9–10-day-old G0 embryos for transformation marker gene expression (*3xP3*-driven EGFP fluorescence) and single out embryos exhibiting the characteristic *3xP3* pattern as described in **steps 6** and **7** in Subheading 3.3.

6. Prescreen both *3xP3*-expressing and non-expressing G0s for transgene activity, i.e., for heat-inducible *PhHS*-driven DsRed fluorescence. To heat-shock *Parhyale*, pipet embryos with a BSA-coated plastic tip into 35 mm tissue culture Petri dishes with prewamed FASWA at 37 °C. Incubate for 1 h at 37 °C and transfer dishes with heat-shocked embryos back to 25–26 °C. Screen embryos for DsRed fluorescence 12 h after heat-shock.
7. Establish independent transgenic lines with *Minos*{*3xP3-EGFP*;*PhHS-DsRed*} insertions as described in **steps 8–17** and **20–21** in Subheading 3.3. During inbreeding, select G1 and G2 siblings with the strongest *3xP3-EGFP* and *PhHS-DsRed* (after heat-shock) expression.

3.4.3 Characterization of the Parhyale Heat-Inducible System

The properties of the heat-inducible *PhHS* system are assessed in transgenic *Minos*{*3xP3-EGFP*;*PhHS-DsRed*} *Parhyale* lines. The reader is also referred below to other sources for more detailed protocols.

1. Determine the spatiotemporal aspects of heat-inducible embryonic expression; transgene expression with *PhHS* in *Parhyale* embryos can be induced robustly from early germband stage onwards uniformly in all cells and tissues [14].
2. Examine the on kinetics of *PhHS* at the transcriptional level with quantitative RT-PCR. Extract total RNA from pools of about ten late-stage transgenic embryos heat-shocked for varying periods of time at 37 °C as described in [14]. Perform relative quantification of *DsRed* transcript levels (relative to the highest expressing sample) on reverse-transcribed cDNA with primers that hybridize on *SV40polyA* (SV40F 5′-CCACATTTG TAGAGGTTTTACTTGC-3′andSV40R5′-TGAGTTTGGAC AAACCACAACTA-3′). In each sample, normalize *DsRed* transcript abundance against housekeeping *Parhyale* ribosomal genes *PhRpL21* and *PhRpL32* (with primer pairs PhRpL21F 5′-CCGAGGCTTCAAGAAGAATG-3′ and PhRpL21R 5′-AA AATCCGGCCTCGTACTCT-3′; PhRpL32F 5′-CCAGCATT GGTTATGGTTCA-3′ and PhRpL32R 5′-TTGAGCTTAGC CTTGCCATT-3′).
3. Examine the off-kinetics of *PhHS* at the transcriptional level with quantitative RT-PCR on samples collected at various time points after a 1-h heat-shock at 37 °C. Analysis of the on/off kinetics of *PhHS* shows that misexpressed transcripts peak 1–2 h after the start of heat-shock at 37 °C, have a half-life of about 6 h, and fade away within 10–12 h after the end of the heat-shock [14].
4. Analyze misexpressed transcript and protein accumulation/ localization by whole-mount in situ hybridization and anti-

body staining, respectively, in heat-shocked transgenic *Parhyale* embryos [14, 24–26]. This time-course study shows that nascent transcripts appearing as nuclear dots are detectable from 0 to 2 h after a 1-h heat-shock at 37 °C. Transcription ceases about 2 h post-heat-shock, and cytoplasmic transcripts and protein accumulate 3–4 h post-heat-shock (*see* **Note 19**).

3.4.4 Cloning, Expression, and Functional Analysis of Parhyale Hox Genes

The steps described here focus on the Hox gene *Ultrabithorax* (*PhUbx*) and its role in *Parhyale* appendage specialization.

1. Extract total RNA from mixed-stage *Parhyale* embryos and reverse transcribe into cDNA as described elsewhere [14].
2. Use this cDNA to amplify by PCR part of the *PhUbx* homeobox with degenerate primers encoding the conserved ELEKEF and WFQNRR amino acid sequences of the homeodomain. Clone the PCR product in a T-Vector and sequence individual clones.
3. Recover the full coding sequence of *PhUbx* by 5′ and 3′ RACE. Clone RACE products in a T-Vector and sequence individual clones. This analysis identified two *PhUbx* splice variants, I and II, which differed in their first few N-terminal amino acids [12]. Each of these isoforms was studied separately following the steps described below [14]. For simplicity, we will refer collectively to both isoforms here as *PhUbx* (*see* **Note 20**).
4. Analyze *PhUbx* expression pattern during normal embryogenesis by in situ hybridization using *PhUbx*-specific riboprobes, and by immunostaining using raised PhUbx-specific antibodies or the cross-species-reactive monoclonal FP6.87 [12, 24–26]. PhUbx mRNA and protein are expressed at high levels in the walking appendages of thoracic segments 4–8 (T4–T8) and at lower levels in the T2 and T3 gnathopods, but are not expressed in the T1 maxillipeds and more anterior head appendages (Fig. 6a) (*see* **Note 21**).
5. Examine appendage morphology in the thorax and posterior head of wild-type *Parhyale* hatchlings by scanning electron microscopy (SEM) and cuticle preparations (Fig. 5a) [14]. Thoracic segments T4 to T8 develop elongated segmented walking appendages (pereopods), each with a cuticular plate (coxal plate) and a gill (except T8) at its base. The more anterior T2 and T3 gnathopods (that facilitate mating and grasping) differ from pereopods by the distinct size and shape of their segments, the presence of characteristic sensory bristles, and in the case of T2 also by the absence of a gill and the different shape of the coxal plate. The developing maxillipeds on T1 are extensively modified to function in feeding. Maxillipeds develop a main segmented limb branch with the same number of segments as the more posterior thoracic appendages, but are

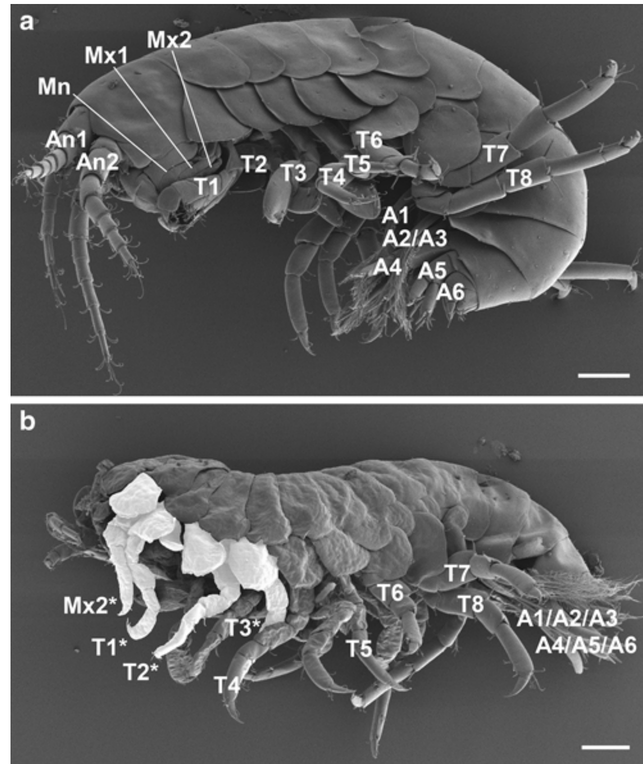

Fig. 5 Scanning electron microscopy of wild-type and homeotically transformed *Parhyale* hatchlings. (**a**) Appendage morphology of a wild-type *Parhyale*. (**b**) Homeotic transformation of feeding and grasping appendages towards walking legs (shown in white and marked with an *asterisk*). Abbreviations of appendages indicated on the *left side* of each specimen: An1 (antenna 1), An2 (antenna 2), Mn (mandible), Mx1 (maxilla 1), Mx2 (maxilla 2), T1–T8 (thoracic appendages 1–8), A1–A6 (abdominal appendages 1–6). Both panels show lateral views with anterior to the *left*. Scale bars are 100 μm

highly reduced in size, medially fused, and lack coxal plates and gills; maxillipeds develop prominent proximal outgrowths to manipulate food, similar to the more anterior gnathal appendages (maxillae 2 and 1).

- For SEM analysis, fix hatchlings in 1 % glutaraldehyde in FASW for 1 h and then in 1 % osmium tetroxide in FASW for 1 h, wash several times in FASW, and dehydrate through an ethanol series. If required, store specimens in 90 % ethanol; otherwise proceed immediately with three washes in absolute ethanol, critical point drying, coating with gold or platinum, and observation under a scanning electron microscope.
- For cuticle preparations, fix hatchlings in 3.7 % formaldehyde in FASW for 1 h and wash several times in 1× PBS with

0.1 % Triton X-100 (PTx). Dissect individual appendages with fine forceps in PTx on a Sylgard plate, dehydrate through an ethanol series, mount in Hoyer's medium/lactic acid (1:1) solution, clear overnight on a 60 °C plate, and observe under a compound microscope.

6. Clone the *PhUbx* coding sequence under the control of the heat-inducible *PhHS* in a *Minos* transposon, as detailed in Subheading 3.4.2, **steps 1** and **2**. For example, the *PhUbx-II* coding sequence was amplified from a full-length cDNA clone with primers PhUbxII_BspHI_F (5′-TTAGTCATGAACT CCTACTTTGAAC-3′) and PhUbx_NotI_R (5′-TATTGCG GCCGCTTAGTTTTGTCCGGGGTT-3′), digested with BspHI/NotI, and cloned downstream of *PhHS* in NcoI/NotI-digested pSL-PhHS-DsRed. The resulting plasmid pSL-PhHS-PhUbxII was digested with AscI, and the gel-purified *PhHS-PhUbxII-SV40polyA* cassette was cloned into AscI-digested pMi{3xP3-EGFP} to generate donor plasmid pMi{3xP3-EGFP;PhHS-PhUbxII} [14].
7. Microinject 1,500–2,000 *Parhyale* embryos at the 1- and 2-cell stage with an injection mix containing 300 ng/μl of the donor plasmid pMi{3xP3-EGFP;PhHS-PhUbx}, 100 ng/μl of the *Minos* transposase helper mRNA, and 0.1 volumes phenol red as described in Subheading 3.4.2, **steps 3** and **4**.
8. Subject injected embryos to daily heat-shocks (or every 12 h) for 1 h at 37 °C, starting from stages 12–13 onwards (72 h of embryogenesis at 25–26 °C) and continuing until stage 28 (day 9 of embryogenesis at 25–26 °C) [2].
9. Grow embryos to hatching, anaesthetize them as described in **step 3** in Subheading 3.1.1, and examine their morphology under a stereomicroscope. Keep note of the number of embryos injected, the number of embryos hatched, and the number of hatchlings with wild-type and abnormal phenotypes [14].
10. Examine appendage morphology in affected hatchlings by scanning electron microscopy and cuticle preparations as described in **step 5**. Classify the types and frequencies of abnormal phenotypes observed [14]. In the case of *PhUbx* misexpression, the following appendage transformations were observed, sorted out in descending order of frequency: maxilla 2-to-maxilliped transformation, antennae-to-thoracic appendage transformation, maxilla 2/maxilliped-to-gnathopod transformation, and maxilla 2/maxilliped/gnathopod-to-walking appendage transformation (Fig. 5b) (*see* **Note 22**).
11. To associate the induced homeotic transformations with the pattern and intensity of *PhUbx* misexpression, repeat microinjection and heat-shock of about 500 embryos as described in **steps 8** and **9**, up to stages 23–24 (days 6–7 of embryo-

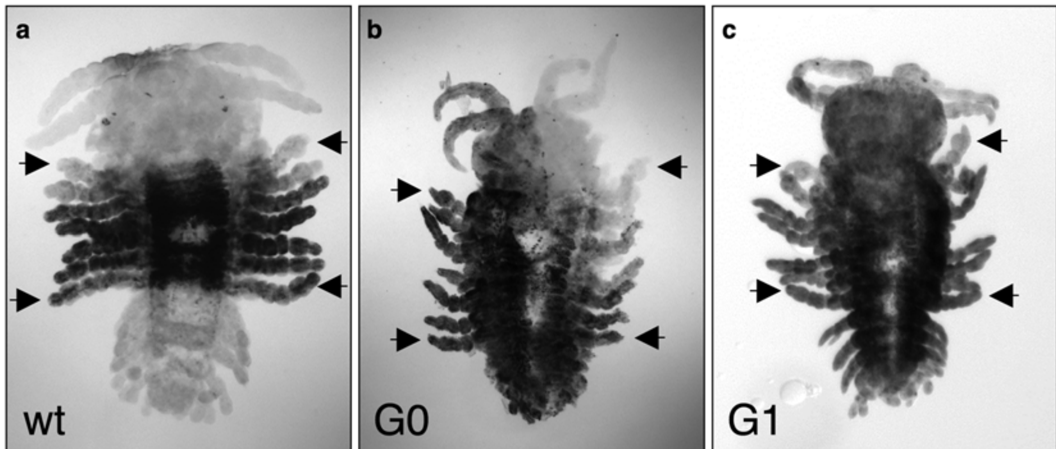

Fig. 6 PhUbx expression in wild-type and transgenic *Parhyale*. (**a**) Wild-type expression pattern of *PhUbx* detected by in situ hybridization. (**b**) Transient transgenic G0 embryo exhibiting unilateral ectopic PhUbx expression in half the head region detected by FP6.87 antibody staining. (**c**) Stable transgenic G1 embryo exhibiting uniform ectopic PhUbx expression detected by FP6.87 antibody staining. In each embryo, *arrows* indicate the normal anterior (in T2) and posterior (in T8) border of PhUbx expression in developing appendages. Note that the FP6.87 monoclonal antibody detects also the abdominal-A Hox protein in the more posterior abdominal segments. In all panels, anterior is to the *top*

genesis at 25–26 °C) when appendage morphogenesis is almost complete [2]. Fix surviving embryos 3–4 h after the last heat-shock and analyze PhUbx expression pattern and levels by immunostaining using a raised PhUbx-specific antibody or the cross-species-reactive monoclonal FP6.87 (Fig. 6b) [14, 24–26]. To also observe embryo morphology, counterstain the DNA of immunostained embryos (e.g., by DAPI staining) and mount embryos in 70 % glycerol for microscopy (*see* **Note 23**).

12. To achieve homogeneous rather than mosaic *PhUbx* misexpression, establish stable transgenic lines with the *Minos*{*3xP3-EGFP;PhHS-PhUbx*} transposon as described in Subheading 3.3. The effects of ectopic *PhUbx* can be then analyzed in these transgenic lines with the approaches described in **steps 8–11** (Fig. 6c). However a number of important considerations should be taken into account when misexpressing pleiotropic genes like *PhUbx* that function at many different stages and processes during development [14] (*see* **Note 24**).

4 Notes

1. Freshly oviposited 1-cell-stage embryos are extremely fragile and should be dissected from the brood pouch 1–2 h later once they have hardened. After oviposition, *Parhyale* embryos spend 0–4 h at the 1-cell stage, 4–6 h at the 2-cell stage,

6–7.5 h at the 4-cell stage, and 7.5–9 h at the 8-cell stage at 26 °C [2]. These times can be increased or decreased by incubating the embryos at lower or higher temperatures, respectively, within the 18–30 °C range.

2. In principle, needles should be rigid enough to penetrate the *Parhyale* eggshell without bending, should have a fine pointy tip to avoid yolk leakage through the induced hole, and should allow sufficient injection flow without frequent clogging. The table below shows how the different adjustable parameters affect tip morphology on a Sutter P-87 puller with a box filament (Sutter Instrument Company). Please refer to these other sources for more information on microneedle preparation ([47]; Sutter Pipette Cookbook: http://www.sutter.com/PDFs/pipette_cookbook.pdf).

Parameter	Range	Increase	Decrease
Heat	0–999	Smaller tips Longer taper	Larger tip Shorter taper
Pull	0–255	Smaller tips Longer taper	Larger tip Shorter taper
Velocity	0–255	Smaller tips	Larger tip
Time	0–255	Shorter taper	Longer taper
Pressure	0–730	Shorter taper	Longer taper

3. Never try to mount or change the needle when applying pressure, because this might shoot the needle. When removing a needle always disconnect the needle holder from the injector and keep it upright, directed away from you and your colleagues.
4. One can estimate the amount injected by injecting the aqueous mix into oil and measuring the diameter of the drop under the microscope.
5. The helper mRNA provides a ready-to-use transposase source that has been shown to increase *Minos* transposition rates in various arthropod species compared to helper plasmids [43, 44]. It also alleviates the need to characterize functional promoters to drive the expression of transposase from helper plasmids [28].
6. For the excision and transposition assays, we describe here the use of the original donor plasmid pMiLRTetR(L) that carries a 2 kb *Minos* transposon with the tetracycline resistance gene [28, 35]. Any plasmid carrying a *Minos* transposon can be tested for excision in the excision assay with appropriate flanking primers to amplify by PCR a small diagnostic band.

However, amplification efficiency of the non-excised *Minos* transposon in PCR reactions will depend on transposon size, i.e., smaller transposons will be amplified more efficiently than bigger ones. The use of pMiLRTetR(L) as donor plasmid is required for the transposition assay that is based on bacterial marker selection. The helper plasmid can carry the *Minos* transposase coding sequence under the control of any *cis*-regulatory sequence of interest, like a constitutive or a heat-inducible promoter isolated from *Parhyale* or other species.

7. Additional bands of intermediate size may appear in samples containing the 2.2 kb band [28]. Most likely, these bands represent extra conformations of the 2.2 kb band containing the long *Minos* inverted repeats.
8. The excision and transposition assays are very convenient to assess and compare various aspects of *Minos* transposition, like *Minos* activity in new species of interest, the mobility of transposons with different sizes, and the activity of alternative transposase sources (e.g., mRNA helpers with different 5′ and 3′ UTRs or helper plasmids with different promoter elements). Importantly, injection of the donor plasmid without transposase provides a first clue about the possibility of *Minos* cross-mobilization by endogenous *Minos*-related transposases encoded by the targeted species. This possibility needs to be investigated and excluded in every new species of interest, because it has important implications for the stability of *Minos* insertions and propagation of established transgenic lines.
9. The *Minos* transposition assay involves screening for interplasmid transposition events of the *MiLRTetR* transposon from the pMiLRTetR(L) donor plasmid into the sucrase gene of the pBC/SacRB target plasmid [28, 35]. The screen is done in bacteria by positive selection for the chloramphenicol and tetracycline resistance genes carried by the pBC/SacRB target plasmid and the *MiLRTetR* transposon, respectively, and by rescuing *E. coli* lethality induced by the sucrase gene (*sacB* from *Bacillus subtilis*) in the presence of sucrose. The function of the sucrase gene can be disrupted due to insertional mutagenesis by the *MiLRTetR* transposon. The chloramphenicol, tetracycline, and sucrose triple-resistant clones are selected stepwise by first screening for chloramphenicol and sucrose double-resistant clones, and then screening these for chloramphenicol and tetracycline resistance (removing sucrose-resistant clones that are not caused by *Minos* insertion). Selection in the first step for chloramphenicol and tetracycline double-resistant clones is not recommended, because it is very stringent and may lead to loss of true positives.

10. Multiplying with this factor of 19 normalizes for the fact that 5 % of transformed bacteria are grown on LB-Cm plates and 95 % of transformed bacteria (19 times more cells) are subjected to the triple selection. For example, if 5 % of cells spread on LB + Cm plates produced 500 colonies, while 95 % of cells spread on LB + Cm + Suc plates and then replica patched onto LB + Cm + Tet produced 95 triple-resistant clones, the calculated transposition efficiency is 1 %.
11. The pBC/SacRB target plasmid and the *MiLRTetR* transposon each carries a NotI site. The NotI digestion pattern will vary between digested triple-resistant clones depending on the landing TA site, but the total size should be about 8 kb (except in the case of multiple *Minos* insertions in the same target plasmid) [28, 35].
12. The choice of the fluorescent protein to be coupled to *3xP3* depends on the signal-to-noise ratio detected in the embryos and tissue of interest. Originally, *DsRed* was chosen over *EGFP* in *Parhyale*, because embryos exhibited a stronger *3xP3*-driven signal and lower embryo autofluorescence during DsRed detection compared to EGFP detection. Once the *3xP3* pattern became known, we have employed alternative, spectrally distinct fluorescent markers under *3xP3* control. This way, we have expanded the number of different transgenes that can be combined in the same transgenic *Parhyale* animal.
13. The *Parhyale 3xP3* pattern differs from that of transgenic insects where fluorescence is driven in the photoreceptors of compound eyes and in other tissues [28, 39, 48]. This discrepancy may result from the basal promoter sequences that the *3xP3* element is coupled to, which are derived from a *Drosophila hsp70* gene. When *3xP3* is cloned in the vicinity of *Parhyale hsp70* promoter sequences, expression is not only detected in the posterior head region but also in the photoreceptors and optic lobes of *Parhyale* [14]. In either case, *3xP3* represents a very convenient transformation marker gene: first, it is a reliable marker to identify transgenic *Parhyale*, and second, it exhibits a highly localized expression pattern that does not interfere with the detection of transgene expression.
14. Transient transgenic embryos injected at the 1-cell stage can exhibit either bilateral or unilateral expression of the marker gene/transgene depending on the timing of *Minos* insertion into the genome. Transient transgenic embryos injected at the 2-cell stage can exhibit only unilateral expression of the marker gene/transgene, because each blastomere at this stage is fated to give rise either to the left or the right half of the ectoderm and somatic mesoderm [20, 28]. The percentage of transient transgenic embryos recovered varies between experiments and tends to decrease with increasing transposon size.

15. In our transgenic experiments with pMi{3xP3-DsRed}, some *3xP3*-expressing G0 parents gave rise exclusively to *3xP3*-expressing G1 progeny [28]. Southern blot analysis on pools of transgenic G1 siblings revealed that their G0 parents carried dozens of *Minos* insertions in their germlines. Each G1 had inherited a subset of these insertions. The number of insertions transmitted by G0s and the percentage of their transgenic G1 offspring drop with increasing transposon size and with decreasing concentration of the injected donor plasmid and helper mRNA.
16. Establishing stable *Parhyale* transgenic lines by inbreeding is a time-consuming process that takes at least 6 months from the time of injections. An alternative faster approach is to set up cultures with transgenic G1 siblings. However, in this case the *Minos* insertion(s) will be fluctuating in the population, and cultures need to be selected and enriched for *3xP3*-expressing (and/or transgene-expressing) individuals every 6 months.
17. As a faster alternative to the inverse PCR methodology, it may be possible to isolate by standard PCR the intergenic heat-inducible *cis*-regulatory sequences from *hsp70* genes that are physically linked in the genome [40]. In either case, the presence of clusters of putative binding sites (GAANNTTC) for the Heat shock factors (HSFs) in the isolated *cis*-regulatory sequence is a good evidence for its heat responsiveness [14].
18. The subcloning vector pSLfa1180fa contains a super-polylinker (with multiple cloning sites) flanked by two oligos recognized by the rare 8-cutter restriction endonucleases AscI and FseI [46]. Transgene constructs are routinely assembled in pSL-fa1180fa, digested by AscI, gel-purified, and cloned in AscI-digested *Minos* vectors.
19. The expression dynamics of heat-inducible DsRed (or any other transgene fused to a fluorescent protein) can also be imaged live in intact transgenic embryos under a fluorescence microscope, a laser scanning confocal microscope, or a fluorescence light-sheet microscope ([14] see also http://www.cell.com/picture-show/lightsheet2).
20. The two *PhUbx* splice variants exhibited identical expression patterns and their misexpression resulted in similar homeotic transformation. However, expression of *PhUbx-II* was stronger than *PhUbx-I*, and the penetrance and severity of induced transformations were much higher with *PhUbx-II* [14]. Interestingly, the evolutionarily conserved NSYF motif required for transcriptional activation [49] is present in *PhUbx-II* and absent in *PhUbx-I* [12].
21. There is a considerable lag between the appearance of PhUbx transcripts and protein [12]; although PhUbx transcripts start

being detected from germband stage onwards (stage 12), the protein comes up about a day later at the early limb bud stage (stage 17).

22. The observed frequencies of homeotic transformations reflect *PhUbx* expression levels required for specification of each appendage type [14]. In particular, ectopic gnathopods that require low *PhUbx* expression are more frequently induced than ectopic walking appendages that require high *PhUbx* expression. The most frequent phenotype of maxilla 2-to-maxilliped transformation is an indirect effect of *PhUbx* misexpression through downregulation of the more anteriorly expressed Hox gene *Sex combs reduced* (*PhScr*). *PhScr* is normally expressed at high levels in maxillae 2 and at lower levels in maxillipeds, and is a sensitive target of *PhUbx*. Even low levels of ectopic *PhUbx* can reduce *PhScr* levels in developing maxillae 2 transforming them into maxillipeds. Antennae develop normally in the absence of any Hox input and acquire thoracic leg identity (gnathopod or pereopod) when they misexpress *PhUbx*.

23. Because of the stochastic and mosaic nature of transient transgenesis, each transient transgenic embryo experiences a unique spatial and temporal pattern of *PhUbx* misexpression. Yet, within each embryo analyzed, there is good association between the pattern and levels of ectopic PhUbx detected and the type of homeotic transformations induced [14]. Furthermore, classification of immunostained embryos based on signal intensity results in class frequencies that are consistent with the frequencies of morphological transformations observed with SEM and cuticle prep analyses (described in **Note 22**). For example, the majority of immunostained embryos express low levels of ectopic PhUbx resulting in the most abundant maxilla 2-to-maxilliped transformation, while few embryos express high levels of ectopic Ubx resulting in rare cases of ectopic walking appendages [14].

24. Homogeneous and prolonged misexpression of *PhUbx* at wild-type levels in stable transgenic embryos subjected to multiple heat-shocks results in embryonic lethality before the effects on appendage morphogenesis can be scored [14]. Misexpression of *PhUbx* at lower levels is tolerated by stable transgenic embryos, but induces only certain phenotypes like maxilla 2-to-maxilliped transformation or antennae-to-thoracic appendage transformations [14]. For this reason, analysis of stable transgenic lines is not sufficient to recover the full spectrum of homeotic transformations induced by *PhUbx*. The full range of homeotic transformations can be recovered with transient transgenics that are genetically mosaic and can express wild-type levels of *PhUbx* locally in the affected appendages and survive to hatching.

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