Transgenesis in *Hydra* to characterize gene function and visualize cell behavior

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The freshwater polyp *Hydra* is a cnidarian used as a model organism in a number of fields, including the study of the origin and evolution of developmental mechanisms, aging, symbiosis and host-microbe interactions. Here, we describe a procedure for the establishment of stable transgenic *Hydra* lines by embryo microinjection. The three-stage protocol comprises (i) the design and preparation of a transgenic construct, (ii) the microinjection of the vector into early embryos of *Hydra vulgaris*, and (iii) the selection and enrichment of mosaic animals in order to develop uniformly transgenic clonal lines. The preparation of a transgenic construct requires ~2 weeks, and transgenic lines can be obtained within 3 months. The method allows constitutive or inducible gain- and loss-of-function approaches, as well as in vivo tracing of individual cells. *Hydra* polyps carrying transgenic cells reveal functional properties of the ancestral circuitry controlling animal development.

**Introduction**

The freshwater polyp *Hydra* is a representative of the phylum Cnidaria, which diverged from its sister group Bilateria ∼600–700 million years ago. Observations in *Hydra*, therefore, allow insights into ancient gene regulatory mechanisms that were present in the common ancestor of cnidarians and bilaterians. *Hydra* offers a morphologic simplicity and experimental accessibility unmatched by other cnidarian models. In particular, the cell-type composition and differentiation processes are understood in *Hydra* better than in any other non-bilaterian animal. This knowledge allows investigation of the molecular mechanisms behind morphogenesis and pattern formation, stem-cell control and aging, and gene–environment interactions. The rich and well described behavioral repertoire generated and controlled by a small and morphologically simple nervous system makes *Hydra* a unique model for understanding important neurobiological questions. Similarly, *Hydra*’s stable associated microbiome is amenable to extensive manipulations and therefore should enable deep insight into the fundamental principles of host–microbe interactions, as well as the evolution of immunity. All these studies rely on in vivo labeling and tracing of cells, and on the functional analysis of genes by means of transgenesis. *Hydra* genomic and transcriptomic resources are available. In addition, an embryo microinjection technique has been used to develop stable transgenic lines, increasing the possibilities of *Hydra* research. The diversity of available construct designs ensures a broad application range of the transgenic *Hydra* technology and allows constitutive gene gain- and loss-of-function analyses, as well as conditional gene manipulation, dissection of cis-regulatory sequences, differential labeling and in vivo visualization of the entire repertoire of cell types in a polyp. Complemented by recently developed techniques for manipulating *Hydra*’s symbiotic microbiota, the protocol described here provides a valuable tool for dissecting the ancient holobiont *Hydra* in all its complexity. Although transgenesis should be possible in other cnidarian model systems as well, *Hydra* remains a strategic experimental system that is suitable for the study of basic biological principles with high translational relevance.

**Overview of the transgenesis technology in *Hydra***

The *Hydra* embryo microinjection technique, originally published by Wittlieb et al. and amended with some modifications by Juliano et al. , embodies the following steps (Fig. 1a). A transgenesis vector containing a fluorescent reporter gene is generated by means of standard genetic engineering techniques, propagated in suitable bacterial strains, purified as a supercoiled plasmid DNA, and injected into blastomeres of two- to four-cell embryos of *H. vulgaris* strain AEP.
using a microinjection manipulator (Fig. 1b–d, Supplementary Video 1). The injected embryos are screened for fluorescence in the next 3–5 weeks until they hatch (Fig. 1e,f). After hatching, mosaic transgenic polyps are clonally propagated and selected to achieve uniformly transgenic lines (Figs. 1g–i and 2). Since 2006, thousands of embryos have been microinjected, and nearly 200 transgenic lines have been established in the Hydra Transgenic Facility at the University of Kiel (http://www.transgenic-hydra.org). Over the past decade, the method has proven to be efficient, with >25% of injected embryos giving rise to stable transgenic lines\(^7\)\(^{–}\)\(^{25}\), and to be highly versatile, which is due to a flexible design of the transgenic vector. Here, we provide guidelines for the design of transgenic constructs tailored to address various research questions. Our experience of the past decade has uncovered three steps in the protocol that require particular attention: (i) isolation of a highly purified plasmid and the preparation of an injection solution; (ii) development of a large-scale Hydra culture for sustained collection of embryos for microinjection; and (iii) selection of mosaic transgenics and appropriate control lines.

**Construct design and applications of transgenesis in Hydra**

The original construct for Hydra transgenesis was generated by cloning the GFP coding sequence (CDS) into a commercial pUC19 vector. The reporter gene was flanked by 1,386 bp of the \(H. \text{ vulgaris}\) \(\beta\)-actin 5′-regulatory sequence, referred to as the actin promoter, that comprises a transcription start site, a native initiator codon and the first 10 amino acids of \(H. \text{ vulgaris}\) \(\beta\)-actin. The GFP reporter
gene was also flanked by the 3′-genomic region of the β-actin gene, which includes a termination/polyadenylation signal and is referred to as the actin terminator sequence. Since then, GFP has been replaced by a more efficient eGFP gene, resulting in the hoT G construct published by Wittlieb et al. (Fig. 3a). Later, multiple restriction sites were introduced into this ‘backbone’ vector by K. Khalturin (University of Kiel; Fig. 3b), giving rise to the LigAF family of constructs used by the Bosch lab, whereas recombination of the expression cassette from the hoT G vector into the pBluescript plasmid by R. Steele (University of California, Irvine) gave rise to the pHyVec vector family (GenBank accession no. AY561434, https://www.addgene.org/Rob_Steele/), including a particularly interesting pHyVec7 with an operon configuration. A catalog of published constructs and transgenic lines developed at the Transgenic Hydra Facility in Kiel is available at http://www.transgenic-hydra.org. All in all, the rich toolkit of diverse constructs allows functional analysis of virtually any gene in Hydra, as well as labeling and functional characterization of populations of Hydra cells.

Gain-of-function analysis
To generate gain-of-function phenotypes, the overexpression of a gene of interest (GOI) is achieved by cloning its coding sequence in-frame up- or downstream of the eGFP CDS, resulting in the expression of a fusion protein N- or C-terminally coupled to the eGFP (Fig. 3c,d). Importantly, if certain residues on the N terminus of the protein of interest are functionally essential (such as a signal peptide in secreted proteins), we recommend leaving this portion intact and attaching the eGFP to the protein’s C terminus. Inversely, if a C-terminal domain (such as the CaaX-motif of the Lamin protein) must remain intact, N-terminal fusion is advisable.

The so-called actin promoter, originally present in the hoT G vector, is a strong, ubiquitously active regulatory region that drives expression in both ectodermal and endodermal epithelial cells along the entire polyp (Fig. 2). Within the interstitial cell lineage, actin promoter activity is weak in stem cells and germ cells but strongly increases in differentiated nerve cells and nematocytes. The use of the actin promoter allows a substantial gene upregulation in all three Hydra stem cell lineages, and ectopic expression of a GOI, as clearly demonstrated, for instance, by Boehm et al. In this study, the transcription factor FoxO, whose expression is normally restricted to stem cells, was
ectopically expressed in mature nematocytes, resulting in the activation of stem-cell-specific genes in these terminally differentiated cells.

Coupling the eGFP sequence to the coding sequence of a GOI allows fluorescent labeling of the overexpressed protein and thus a convenient screening of mosaic animals and the expansion of the transgenic cell pool to be monitored. However, as the presence of eGFP may compromise the...
function of the overexpressed chimeric protein, one should also consider replacing eGFP with a smaller non-fluorescent tag, such as a myc or flag sequence. In our experience, the use of a short myc epitope for tagging small secreted proteins is particularly effective (A.K., X. Xiang and T.C.G.B., data not shown). Alternatively, to generate a line that overexpresses a protein that is not fluorescently tagged but enables the transgenic tissue to be tracked, one can use an operon vector backbone, pHyVec11, developed in the lab of R. Steele31 (available at https://www.addgene.org/Rob_Steele/). In this case, a single promoter drives expression of a polycistronic transcript, which is then separated by trans-spliced leader addition, resulting in independent translation of two transcripts.

**Loss-of-function analysis**

For loss-of-function approaches, an RNA-interference-mediated knockdown can be achieved using a so-called ‘hairpin’ cassette—composed of two segments of a CDS cloned in both sense and anti-sense orientations, with a short non-homologous linker between them—that is inserted into the backbone vector downstream from the eGFP CDS5,7,20–22 (Fig. 3e). Similar to the gain-of-function approach, the ubiquitous actin promoter is typically used to drive the knockdowns, allowing effective down-regulation of GOI expression to 5–15% of normal levels5,7,11,22. Transgenic lines with strikingly strong eGFP signal resulting from the injection of a hairpin construct often show little or no down-regulation of the GOI, most likely due to only partial integration of the expression cassette into the genome. By contrast, lines with an efficient gene knockdown are typically characterized by a rather weak eGFP fluorescence. Most likely, formation of a dsRNA structure by the hairpin and its further degradation by the shRNA machinery destabilizes the eGFP transcript, reduces its half-life, and compromises the production of the fluorescent marker. Therefore, we encourage selecting the least fluorescent lines and discarding the strongly fluorescent lines, in spite of their convenience for screening.

A similar loss-of-function approach has been used by Juliano et al.35 to downregulate the piwi gene expression in *Hydra*, yet the construct design was different. First, the actin intron sequence was used as a spacer between two homologous parts of the hairpin. Second, the authors used an operon construct to drive expression of both a short hairpin and a DsRED coding sequence, as two transcripts split apart after transcription. This construct design allowed a comparable downregulation of gene expression to be achieved (to 15–25% of the normal expression level). Importantly, the stability of the DsRED mRNA was probably not compromised by the hairpin processing, thus yielding a stable, strong DsRED signal35.

We recommend cloning only a 350-bp-long fragment of a GOI into a hairpin, as the use of short sequences seems to increase the specificity of the knockdown and eliminates possible ‘off-target’ effects. Moreover, to increase the specificity of the hairpin’s effect, we avoid using conserved sequences in the construct. This approach was effectively used in the study by Boehm et al.5 with a hairpin that suppressed the expression of the foxO gene by 80% but exerted only minor effects on another forkhead transcription factor gene, e.g., foxA2. We therefore encourage controlling for the off-target effects of a hairpin by estimating the expression level of the GOI’s closest homologs using qRT–PCR. However, as an in silico analysis cannot guarantee the absence of off-target effects, we recommend developing multiple transgenic lines with different shRNA hairpins that target different parts of the same GOI. Common changes in phenotype observed in these lines point to specific effects of the GOI knockdown, whereas the alterations inherent only in the lines bearing one of the hairpins can be attributed to nonspecific off-target effects. Recently, we have used this approach to rule out nonspecific off-target effects of the HyLMN knockdown.

An shRNA hairpin can be exploited not only to downregulate a single gene in *Hydra*, but also to silence the expression of multiple members of a gene family. For instance, Franzenburg et al.21 simultaneously downregulated six members of the arminin gene family of antimicrobial peptides with a single hairpin. Remarkably, the efficiency of the knockdown was proportional to the degree of similarity between the sequences of the arminin family members and the hairpin.

An alternative way to perform a loss-of-function gene analysis in *Hydra* is by overexpressing a mutated version of a protein of interest in which a functionally essential domain is removed1–7 (Fig. 3f). In contrast to the shRNA approach, in this case, the normal endogenous protein remains expressed in the transgenic cells, yet the activity of the actin promoter allows for an at least twofold excess of a non-functional protein over its normal counterpart7.

**Conditional gain- and loss-of-function analysis**

Until very recently, functional gene analysis in *Hydra* was restricted by the use of constitutively active promoters. As a major drawback, the analysis of genes vitally important for *Hydra* was virtually
impossible, as any manipulation of these genes resulted in lethal phenotypes. To overcome this drawback of constitutively expressed constructs, we developed an inducible tetracycline-dependent gene expression system. The backbone vector is derived from the hoT G plasmid by adding several CDSs and replacing the promoter sequences (Fig. 3g). Specifically, the eGFP is cloned under the control of a synthetic tetracycline-sensitive promoter (tetO-P, for details, see ref. 7). The actin 5′-flanking region drives the expression of a tetracycline-responsive transcriptional transactivator, rTetR, which is fused to the fluorescent reporter DsRED. Only in the presence of doxycycline does the DsRED–rTetR fusion protein bind the tetO-P promoter and activate the GOI expression (Fig. 3g). So far, the inducible system has been tested in two applications: to temporarily drive eGFP expression and to achieve a conditional downregulation of the hyLMN gene. In the latter case, only a transient activation of the shRNA cassette allowed the embryonic lethality to be bypassed, enabling mass culture of the corresponding transgenic polyps and analysis of the phenotype in the adult clonally growing polyps. Similarly to the constitutively active constructs, the inducible transgenes are randomly integrated into the genome and may occasionally come under the influence of a nearby promoter or enhancer. The inducible cassettes, however, appear to be particularly sensitive to the local chromatin environment into which the construct integrates, most likely because of the synthetic nature of the short regulatory region (tetO-P). Therefore, it is essential to generate multiple transgenic lines for each inducible construct and to carefully test them for leaky expression in the non-induced state. We were able to generate lines with no evidence for ‘leakiness’ in the non-induced state, as eGFP transcripts and fluorescent signals are not detectable before doxycycline induction. The inducible system is highly sensitive—responding to doxycycline concentrations as low as 1 μM—and also reversible.

Analysis of cis-regulatory elements
Besides the analysis of protein-coding sequences, the transgenesis technology allows functional dissection of the cis-regulatory elements in Hydra by using reporter constructs (Fig. 3h). The actin promoter in the backbone vector is replaced by a 500- to 2,000-bp-long sequence flanking a GOI from the 5′ end and directly adjacent to the transcription start site, referred to as the ‘promoter’ for simplicity. The eGFP CDS remains intact (Fig. 3h). Surprisingly, in most cases, such a short 5′-regulatory sequence was sufficient to fully recapitulate the expression pattern of a gene. This indicates that, in contrast to other model organisms such as mice, in which trans-regulatory sequences located many megabase pairs apart from the CDS are essential for establishing specific gene expression patterns, in Hydra the gene expression seems to be controlled primarily by rather proximal cis-regulatory elements. A particularly detailed dissection of the regulatory elements controlling wnt3 expression in Hydra was performed by Nakamura and co-authors. By cloning a 2,219-bp-long wnt3 5′-regulatory region and performing subsequent mutation analysis, they revealed that two modules, the activator and the repressor, are necessary for localized wnt3 expression in the hyposome of a polyp. Similarly, the reporter construct with the β-catenin promoter allowed in vivo monitoring of the dynamic activity of the bcat gene in various developmental and pharmacological contexts. Importantly, the eGFP version used so far in the reporter constructs appears to be very stable and, once synthesized, disappears only slowly from the cells. Therefore, to increase the temporal resolution of the analysis, it may be advisable to replace the eGFP with a destabilized, degradation-prone version. Another pitfall of the analysis of the cis-regulatory elements is the influence of the chromatin context on the promoter activity. As the transgene integrates into the genome randomly, it may come under the influence of a nearby promoter or enhancer. This local chromatin environment may have differential and unpredictable effects on the activity of the expression of a reporter construct. We therefore highly recommend generating multiple transgenic lines for each reporter construct to uncover consistent patterns of cis-regulatory element activity.

In vivo tracing of individual cells and lineages
The specific activity of certain cis-regulatory elements allows distinct cell populations to be labeled in Hydra. For instance, Boehm et al. replaced the actin promoter in the backbone vector with the cnnos1 promoter, which is normally active only in interstitial stem cells and germ cell precursors. In addition, a second expression cassette made up of a DsRED CDS driven by an actin promoter was added to the construct. This resulted in a differential labeling of interstitial stem cells by eGFP and ectodermal epithelial cells by DsRED (Fig. 3i). Currently, we implement a similar approach (A.K. and T.C.G.B., data not shown) and combine the cnnos1-driven eGFP expression with an actin-driven DsRED expression to label the interstitial stem cells green, proliferating precursor cells weak yellow
and differentiated neurons strong red. The differential fluorescent labeling of cells is a powerful tool for monitoring the behavior of different cell populations in vivo, such as the migration of interstitial stem cells and their committed progeny, the establishment of the germline or the transdifferentiation of gland cells, and the localization of the stem cells within their specific niche (Fig. 3i). Moreover, the fluorescent labeling provides a unique opportunity to selectively isolate cell populations using FACS sorting and to perform deep transcriptomic analysis in ‘bulk’ or with single-cell resolution (A.K. and T.C.G.B., data not shown).

Establishment and maintenance of Hydra cultures
A stable, large-scale culture of H. vulgaris strain AEP is absolutely essential for sustained collection of the embryos necessary for generating transgenic Hydra lines. The basic method for Hydra culture has been described elsewhere. Here, and in the Procedure, we summarize the most essential steps in facilitating the establishment and maintenance of Hydra cultures by newcomers to the field.

Multiple factors, including temperature, photoperiod, medium composition and pH, feeding intensity and regime, and culture density, affect the physiology of Hydra polyps and particularly their gamete production. Therefore, before starting a Hydra culture, minimal initial investments into basic infrastructure and equipment are necessary. A simple incubator, such as a wine cooler set to 18 °C, provides sufficient control over the temperature. Alternatively, for large-scale culture of numerous lines, a temperature-controlled chamber is advisable. As Hydra is extremely sensitive to heavy metals and detergents, only artificial Hydra medium based on deionized water should be used for culturing the polyps. Moreover, all detergents must be avoided while washing the glassware or any other equipment used for Hydra culture. Plasticware, such as cultivation dishes, must be tested for toxicity. Finally, a robust procedure for the regular feeding and cleaning of the cultures must be established.

Cultures should be monitored daily for their health status. Shortening of tentacles, permanent body-column contraction, appearance of colorless debris on the dish bottom and aggregation of polyps into dense clumps joined by fungal hyphae are indicators that polyps are in poor health. Diseased polyps should be removed immediately, followed by thorough cleaning of the corresponding Hydra culture.

Parallel cultivation of the brine shrimp Artemia salina as a food source is required to maintain Hydra cultures long-term. Importantly, the hatched Artemia nauplii must be ≤24 h old at the time they are fed to Hydra. It is also essential to clear nauplii of the seawater in which they develop, as any residual salt transferred to a Hydra culture is detrimental to the polyps.

Overall, establishment and long-term maintenance of a Hydra culture is simple and inexpensive, and does not require any particular training of personnel.

Hydra mass culture for efficient embryo collection and preparation of zygotes
To obtain sufficient numbers of embryos for microinjection experiments, we use a mixed culture of males and females of the H. vulgaris strain AEP. The number of fertilized eggs available at a given time point is a major limiting factor for scaling up the microinjection technique. In contrast to other cnidarian model organisms, such as Nematostella vectensis in which tens of thousands of fertilized eggs can be obtained in 1 d, female Hydra polyps produce only 1–3 oocytes per polyp in a week. Therefore, to obtain an optimal number of zygotes, we usually maintain three to six mixed cultures of male and female H. vulgaris strain AEP polyps at a high density (each with >4,000 polyps per standard dish (20 cm × 20 cm × 6 cm); Fig. 4a, see Procedure for details) and feed four times per week before induction. To achieve synchronized egg production, the feeding regime is changed from four to three feeds per week. In addition, a drop in the culture density by a factor of two to three may increase the efficiency of induction. 10–12 d after induction, the polyps start producing sperm and eggs (Fig. 4b–d), with the highest yield of 10–12 zygotes per dish per day being achieved ~6 weeks after induction. With time, the egg production in a given mass culture will decline, and virtually no zygotes can be observed in a dish 4 months post induction.

A particular challenge for sustained collection of embryos is the gradual sex reversal in the mass culture, known as masculinization. After 1 year of culture and intensive gamete release, egg production drops markedly, as the majority of polyps in a culture have switched to males (Fig. 4d). Masculinization is based on competition between the male and female germ precursor cells within an individual polyp and is irreversible. To minimize the effect of masculinization and the subsequent decline in egg production, we recommend changing the culture every 10–12 months. For that, a new
Fig. 4 | High-density culture and regular intensive feeding before sex induction allow maximization of the yield of fertilized eggs from a mass culture of Hydra. a, Hydra polyps are cultured at high density (>4,000 polyps per standard dish (20 cm × 20 cm × 6 cm)). b, 12 d after a change in the feeding regime, female polyps bearing eggs (red arrowheads) appear in the culture. c, Females with fertilized eggs (white arrowheads) are isolated for microinjection. d, Egg production rises 12 d after induction (Ind.) of the culture, reaches its maximum 4 weeks later, and gradually declines. The same culture can be induced again, yet because of masculinization, the maximum egg production drops in each successive induction cycle. After three to four induction cycles (red line), a culture must be replaced with a new one (blue line), thus allowing sustained collection of embryos. e, The first embryos hatch 12 d after injection (green curve). A total of 227 embryos were injected with LigAF vector (1.00 μg/μl). The hatching rate peaked on day 29 post injection, with 17 embryos hatched in a day. f, The first embryos hatch 12 d after injection (green curve). A total of 227 embryos were injected with LigAF vector (1.00 μg/μl). The hatching rate peaked on day 29 post injection, with 17 embryos hatched in a day. All viable embryos (52% of total injected zygotes) hatched within seven weeks post injection (blue line, cumulative hatching). g, >60% of injected embryos show eGFP fluorescence, and >30% of embryos give rise to stable transgenic eGFP lines. The efficiency remains stable over years. Results of five injection rounds over 6 years (LigAF vector (1.00 μg/μl); injected embryos: n = 140; center line represents median value; box limits show the first and third quartiles; whiskers correspond to the highest and lowest values).

Hydra mass culture must be established from a single female founder polyp and optimally propagated to a density of 4,000 polyps per dish before induction of gametogenesis (Fig. 4d).

Embryos from the first cleavage stage up to the four-cell stage are collected from the mass culture daily and injected with a plasmid DNA. Although the microinjection of embryos at a later (up to eight-cell) stage is technically feasible, we strongly recommend injecting zygotes or embryos during or immediately after the first cleavage for the following reasons: (i) as the size of a zygote is larger compared to that of later blastomeres, a higher volume of plasmid solution can be injected, thus delivering a higher dose of a transgene per genome; (ii) the use of later stages requires the injection of each blastomere within an embryo and therefore causes intensive damage, which decreases the embryo's survival; (iii) the injection of an embryo soon after fertilization increases the chance of an early chromosomal integration of the construct and obtainment of a transgenic line in which two or even all three stem cell lineages are transgenic.

Preparation of a transgene construct

All transgene constructs are generated and propagated in standard Escherichia coli strains. As originally described by Wittlieb et al.,25 the supercoiled plasmid DNA is extracted from bacteria using the QIAfilter Plasmid Midi Kit and is resuspended in water. We note that the purity of the plasmid prepared strictly following the manufacturer’s instructions may not be sufficient to ensure a high transgenesis efficiency in Hynda. In particular, the presence of any contaminant from the bacteria (cell wall components) or from the kit (MOPS, KAc, and NaCl salts) in the injected solution compromises the survival of the embryos. Therefore, we recommend inclusion of an additional ethanol precipitation step after the plasmid extraction (see Procedure) that also concentrates the plasmid to 2–4 μg/μl. Any impurities, such as dust particles or parts of an undissolved plasmid, will clog the
microinjection pipette, resulting in the need to disassemble the injection equipment and replace the capillary, thus, resulting in the loss of selected fertilized eggs. In addition to taking particular care while drying the pellet and resuspending it in water, we advise a centrifugation step (see Procedure) to remove any particles from the solution, which will be loaded into the microinjection capillary.

On the basis of our experience, there is a clear dependence of transgenesis efficiency on the plasmid concentration in the injected solution: the number of eGFP+ hatchlings is almost three times higher for embryos injected with 1 μg/μl as compared with lower concentrations (0.13 and 0.25 μg/μl, Fig. 4e). As an increase of the plasmid concentration to 2 μg/μl does not improve the transgenesis efficiency, we recommend injecting the constructs at a 1 μg/μl concentration.

Screening transgenic animals and propagation of clonal cultures
After injection, maintain the embryos separately in 12-well plates and monitor daily. The embryos that have survived injection will start hatching 12 d after the injection. The hatching rate culminates at ~3–4 weeks post injection and then declines. If embryos do not hatch within 7 weeks after injection (Fig. 4f), we do not consider them to be viable.

Our observations over the past decade revealed that, under optimal conditions, a rather high transgenesis efficiency can be achieved. For instance, >60% of embryos injected (n = 140) with the LigAF vector were eGFP+, and the majority of them hatched as eGFP+ polyps (Fig. 4g). These values remained remarkably stable over years of observation.

Similar to other animals obtained by embryo microinjection, such as mice or Nematostella, the Hydra polyps hatching from injected embryos are initially mosaic (Figs. 1f,g and 5a,b). Yet in Hydra, unlike in mouse and the sea anemone, it is not necessary to carry out laborious breeding to produce uniformly transgenic animals. Mitotic activity of the transgenic cells in the stem-cell compartment of a polyp results in expansion of the transgenic cell pool and their propagation into buds25. By selecting for buds enriched in transgenic cells (Fig. 5a–c), one eventually ends up with a polyp in which any of the three cell lineages or all of them are uniformly transgenic (Fig. 5a,d). Most frequently, a transgene integrates into one of the epithelial cell lineages, whereas integration into the interstitial lineage occurs in only 3% of cases25. Even less often, the construct integrates into two lineages. So far, we have never observed integration of a transgene into all three stem cell lineages simultaneously. To obtain a Hydra line carrying a particular transgene in all three stem cell lineages, breeding of polyps from a line harboring the transgene in the interstitial lineage and the germ cells (see next section) is advisable.

Equally essential is the establishment of proper controls for each transgenic line. At the ‘dawn’ of Hydra transgenesis, transgenic polyps were compared with a wild-type Hydra AEP line or with polyps expressing eGFP in a corresponding cell line3,21,33. However, independent observations made over recent years uncovered substantial differences between clonal lines originating from the same parental culture. For instance, even among several non-transgenic lines, a marked variation in the average polyp size is observed (J. Taubenheim, A.K. and T.C.G.B., data not shown). Therefore, we recommend development of a control line for each transgenic line and comparing them pairwise
produced by the fully transgenic F1 polyps again showed no transgene in the epithelial lineages is its transmission via germline. Surprisingly, the gametes (Fig. 6b) and demonstrated homogeneous expression of eGFP in all interstitial stem cells (Fig. 6f, g). Embryos generated by sexual reproduction of these lines were, from their hatching, fully transgenic (Fig. 5). Such a control line, called ‘empty’, is generated from the same injected embryo by selecting the progeny with the least number of transgenic cells until a polyp is obtained with no transgenic cells (Fig. 5a, d). Importantly, the control line is genetically identical to the transgenic line, except for the integration of the construct. Therefore, a pairwise comparison allows separation of the specific effects of the transgene from sporadic developmental defects (caused, for instance, by recombination errors in gametogenesis) that would be expressed in both the transgenic and empty lines. In our experience, empty transgenic lines (Fig. 5) are very useful, and we recommend them as controls.

Genomic integration and germline transmission of the transgene
The transgenic Hydra lines generated by embryo microinjection remain stable for years with no change in phenotype. For instance, the lines published by Wittlieb et al. are still available. This stability clearly indicates the genomic integration of the microinjected transgene. At a first glance, this a bit surprising, as the injected constructs are not linearized and do not harbor any sites favoring genomic integration. Southern blot analyses performed by Wittlieb et al. clearly demonstrated integration of the hoT G construct into the Hydra genome. Others also repeatedly referred to unpublished observations indicating that genome sequencing confirmed integration of a single transgene copy into the genome (see, for instance, refs. 29, 53).

The most convincing evidence for a stable genomic integration comes from germline transmission of the transgene. Several earlier studies reported that a given transgene can be detected in the germ cells and further transmitted to the F1 generation upon sexual reproduction of the corresponding parental transgenic line. For instance, Khalturin et al. demonstrated expression of GFP in differentiating sperm cells. Glauber et al. reported that a self-crossing of polyps from a Hydra line with a transgene construct integrated into the interstitial cell lineage resulted in establishment of homozygous diploid lines in which all three stem cell lineages are transgenic. However, three major points must be considered when using germline-mediated transgenesis for functional genetics in Hydra. First, the prime prerequisite for any germline transmission is that the transgene integrates into the interstitial stem cell lineage, which occurs relatively rarely. In our experience, injection of at least 30 embryos is necessary to obtain one line with a transgenic construct integrated into the interstitial cell lineage. Injecting hundreds of embryos may help to bypass this limitation, yet often this is technically not feasible.

Second, to obtain transmission of the transgene into virtually every F1 generation embryo via the germline, the entire interstitial stem cell population must be uniformly transgenic. Although multipotent interstitial stem cells give rise to germ cells in cloning experiments and peduncle regeneration experiments, this happens only rarely in normal polyps. Nishimiya-Fujisawa and Kobayashi provide an elegant solution to enrich the population of transgenic interstitial cells in a polyp and elicit formation of new germ cells from the transgenic multipotent cells.

Finally, some constructs appear to be silenced specifically in germline precursor cells and mature gametes, even if they are strongly active in the multipotent stem cells. For instance, microinjection of a construct with eGFP expression driven by a stem-cell-specific nanos1 promoter (nos1-P, Fig. 6) resulted in transgenic lines with numerous eGFP+ interstitial cells, yet the gametes were always devoid of the GFP signal (Fig. 6c, c’, d, d’). The expression of the second fluorescent reporter, DsRED, was controlled by the actin promoter and therefore the red fluorescent signal was present only in the differentiating neurons (Fig. 6c, c’, e, e’). However, most of the polyps (10/12) that developed from embryos generated by sexual reproduction of these lines were, from their hatching, fully transgenic (Fig. 6h) and demonstrated homogeneous expression of eGFP in all interstitial stem cells (Fig. 6f, g). Notably, the second fluorescent marker was present in the cells of all three lineages, including the epithelial ectodermal and endodermal cells (Fig. 6b, h). The only scenario that explains appearance of the transgene in the epithelial lineages is its transmission via germline. Surprisingly, the gametes produced by the fully transgenic F1 polyps again showed no fluorescent signal (Fig. 6b, f, f’, g, g’, h, h’), supporting the hypothesis that the construct is specifically silenced in the germline. The mechanism of silencing and ways to overcome this limitation remain to be uncovered.

Advantages and limitations of the technique
The embryo microinjection technique became widely used for two reasons. First, clonal propagation and selection of polyps enriched for transgenic cells allows the establishment of uniformly transgenic lines within several months, without extensive back-crossing. Second, owing to the chromosomal integration of a construct, transgenic Hydra lines are stable and can be cultured and maintained as
clonal lines for years. The main challenge of the technique is to have sufficient numbers of embryos available at a given time. This also makes the use of high-throughput approaches such as morpholino or siRNA screenings, which are feasible in other model cnidarians, difficult in Hydra. One of the major limitations of the current system—and a serious obstacle in performing basic genetic engineering techniques using, e.g., the Cre/loxP or Gal4 technologies—is the difficulty in establishing Hydra lines in which the construct is integrated into the germline. Moreover, our knowledge of the genomic integration of the constructs remains very limited. No systematic observation of partial construct integration, number of transgene copies incorporated into the genome or the chromatin context of the integration is available to date. Finally, the currently available technology allows genetic manipulations only at the transcriptional level. Knockout or genome-editing approaches are not feasible so far in Hydra. It is therefore particularly important that fast and efficient technologies for genome editing, such as CRISPR–Cas9, be developed.

### Alternative methods

Multiple efforts to develop an efficient Hydra transgenesis technology have been made over the past 30 years. They differ mainly in the method of gene construct delivery. For instance, particle bombardment technique, whole-polyp electroporation, and DNA injection into the gastric cavity were previously used to overexpress genes in Hydra. Moreover, ingestion of bacterially expressed dsRNA and an enhanced uptake of siRNA in low-pH solutions were proposed to allow gene silencing. Although many of these efforts provided interesting biological insights, they allowed only transient transgenesis and did not facilitate the generation of transgenic polyps with stable integrated transgene expression vectors. As microinjecting the same constructs into clonal lines for years. The main challenge of the technique is to have sufficient numbers of embryos available at a given time. This also makes the use of high-throughput approaches such as morpholino or siRNA screenings, which are feasible in other model cnidarians, difficult in Hydra. One of the major limitations of the current system—and a serious obstacle in performing basic genetic engineering techniques using, e.g., the Cre/loxP or Gal4 technologies—is the difficulty in establishing Hydra lines in which the construct is integrated into the germline. Moreover, our knowledge of the genomic integration of the constructs remains very limited. No systematic observation of partial construct integration, number of transgene copies incorporated into the genome or the chromatin context of the integration is available to date. Finally, the currently available technology allows genetic manipulations only at the transcriptional level. Knockout or genome-editing approaches are not feasible so far in Hydra. It is therefore particularly important that fast and efficient technologies for genome editing, such as CRISPR–Cas9, be developed.

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### Future developments

#### Identification of cis-regulatory sequences

Currently, the number of functionally tested Hydra promoters is disappointingly low, mainly because a full genome sequence is available only for the H. vulgaris species (the most recent genome
assembly is available at https://research.nhgri.nih.gov/hydra/). As H. vulgaris differs in several aspects from the H. vulgaris strain AEP used for transgenesis, genome sequencing of H. vulgaris AEP in combination with the study of the cis-regulatory elements in Hydra promises to bring this model to a new level by allowing the investigation of cross talk between different signaling pathways and the dependency of transcription on external stimuli such as temperature, light, food and presence of microbes. These studies may shed light on how environmental signals are integrated to affect developmental programs. Moreover, the identification of novel cis-regulatory sequences will foster the development of new vectors with specific non-ubiquitous promoters, which are much needed to enable manipulation of a GOI in a particular cell population or body compartment. The ongoing transcriptomic studies with single-cell resolution are a step toward the identification of cell-type-specific (e.g., neuronal) genes and their promoters.

Dissecting the functions of cell populations
The further development of transgene constructs could make it possible to move beyond the functional analysis of single genes to address the functions of cell populations. A promising observation toward this goal was recently published by Dupre et al. The authors overexpressed a Ca\(^{2+}\)-sensitive reporter GCaMP in the interstitial cell lineage of Hydra and monitored in vivo the activity of the entire nervous system of a polyp. Further developments of this principle, such as the use of highly dynamic Chameleon proteins or voltage sensors, may fuel neurophysiology studies on Hydra. Even more powerful would be the development of techniques for manipulating target cell populations, such as optogenetics and cell-suicide systems.

Expanding the range of species
Although transgenesis is currently performed only in the H. vulgaris strain AEP, other lines and species of Hydra are used to address various biological problems. For instance, naturally occurring tumors were described in H. oligactis and Pelmatohydra robusta, and further molecular studies of these models would greatly benefit from adapting the transgenesis technique to them. Polyps of the green Hydra viridissima species provide insights into the nature of symbiotic interaction with algae. Transgenesis would allow movement from merely describing the interactions to proof of their significance and causality. It would be very useful if, in the more distant future, the principles developed and tested in Hydra could be transferred to phylogenetically more distant model organisms such as the sponge Suberites domuncula.

Materials

**Biological materials**
- H. vulgaris strain AEP (the strain can be obtained from multiple groups worldwide and from the authors of this paper upon request) !CAUTION Appropriate national laws and institutional regulatory board guidelines must be followed when working with transgenic Hydra in the laboratory. The generation and culture of transgenic animals must be approved by the relevant authorities. Our work was approved by the Ministry of Environment, Nature and Forestry of the State Schleswig-Holstein, Germany.

**Reagents**
- Transgenesis construct. A plasmid containing a promoter, a reporter gene and a terminator sequence, as well as multiple endonuclease restriction sites for directed cloning. Backbone plasmids LigAF and indGFP (Fig. 2) and their sequences are available from the authors upon request. Multiple HyVec-derived vectors can be obtained from Addgene (http://www.addgene.org)
- QIAfilter Plasmid Midi Kit (Qiagen, cat. no. 12243)
- Isopropanol (Carl Roth, cat. no. 6752.4) !CAUTION Isopropanol is flammable and may cause serious eye damage or eye irritation.
- Ethanol (Carl Roth, cat. no. 9065.4)
- Potassium acetate (3 M, pH 5.5; Fisher Scientific, cat. no. AM9610)
- CaCl\(_2\) · 2H\(_2\)O (Carl Roth, cat. no. 5239.1)
- NaCl (Carl Roth, cat. no. 3957.2)
- MgSO\(_4\) · 7H\(_2\)O (Carl Roth, cat. no. P027.2)
- NaHCO\(_3\) (Carl Roth, cat. no. A135.1)
- K\(_2\)CO\(_3\) (Carl Roth, cat. no. P743.1)
- Sea Salt Aquarium Systems Reef Crystals (Zoo Zajac, cat. no. AS209908)
Lyophylized *A. salina* cysts (Mrutzek Meeres-Aquaristik, cat. no. 114416)

Milli-Q water (produced using the water purification station Merck, model Milli-Q Reference)

Sodium hydroxide (NaOH; Carl Roth, cat. no. 6771.3)

**Equipment**

- Centrifuge (Eppendorf, model no. 5417R)
- Centrifuge rotor (Eppendorf, model no. F45-30-11)
- Dissecting microscope (Wild, model no. M3)
- Fluorescence dissecting stereomicroscope with GFP (excitation (Ex)460–490/emission (Em)510–), GFPA (Ex460–495/Em510–550) and RFP (Ex530–550/Em575–) filter sets (Olympus, model no. SZX16)
- Inverted microscope (Zeiss, model no. Axiovert 100)
- Air-pressure microinjector pump (Eppendorf, model no. FemtoJet 4)
- Micromanipulator system (Eppendorf, model no. InjectMan 4)
- Holding device (Eppendorf, CellTram Air model)
- Pipette puller (Sutter Instrument, model no. P-97)
- Filament (Sutter Instrument, cat. no. FB255B)
- Borosilicate glass capillaries (custom: 1.2-mm outer diameter, 0.1-mm wall thickness, 100-mm length; BioMedical Instruments)
- Grid head for capillaries (1.2–1.3 mm; Eppendorf, cat. no. 5196083008)
- Borosilicate glass capillaries (custom: 200-µm outer diameter, 150-µm inner diameter, length of the angular segment = 1,000 µm, bent angle = 35°; BioMedical Instruments)
- Microloader pipette (0.5–10.0 µl; Eppendorf, cat. no. 3123000020)
- Microloader tips (Eppendorf, cat. no. 5242956003)
- Incubator (set to 18 °C; Liebherr, model no. WKT-5552)
- Petri dishes (60 × 15 mm; Sarstedt, cat. no. 82.1194.500)
- Flat-bottom 12-well tissue culture plates (Sarstedt, cat. no. 83.3921)
- Microscope slide (26 × 76 mm; Karl Hecht, cat. no. 42406110)
- Secure-Seal imaging spacers (20-mm diameter, 0.12-mm depth; Grace Bio-Labs, cat. no. 654006)
- Glass Pasteur pipettes (1.1-mm diameter, 150-mm length; Eydam, cat. no. 9411015)
- Scalpel blades (Eydam, cat. no. 9409921)
- *Hydra* culture dishes (20 × 20 × 6 cm; Westmark, cat. no. 3134-01)
- Nitrile gloves (powder free, accelerator free; Starlab International, cat. no. SG-N)
- Plastic or metal sieves (500 µm; Rösle Marktoberdorf, cat. no. 95162; or 560 µm; Dohse Aquaristik, cat. no. 21630)
- Plastic sieves (120 µm; Dohse Aquaristik, cat. no. 21620)
- Aquarium pump (Eheim, cat. no. 5321010)
- UV spectrophotometer (Eppendorf, model BioPhotometer 6131)

**Reagent setup**

*Hydra* medium

*Hydra* medium is prepared from the following two stock solutions:

- **Solution 1** (CaCl$_2$ 1,000×): dissolve 42.180 g of CaCl$_2$ · 2H$_2$O in 1 liter of deionized water and mix thoroughly. Store at 4 °C indefinitely.
- **Solution 2** (100×): dissolve 8.116 g of MgSO$_4$ · 7H$_2$O, 4.238 g of NaHCO$_3$ and 1.096 g of K$_2$CO$_3$ in 1 liter of deionized water; mix thoroughly. Store at 4 °C indefinitely.

To make ready-to-use *Hydra* medium, mix 10 ml of solution 1 and 100 ml of solution 2 and fill up to 10 liters with dH$_2$O. Ready *Hydra* medium can be stored at 18 °C for up to 1 month (to avoid growth of algae).

*Artificial seawater*

Dissolve 36 g of Sea Salt Aquarium Systems Reef Crystals in 1 liter of deionized water (35–36 p.p.t.) and stir overnight at room temperature (20–22 °C). Ready seawater can be stored at 18 °C for up to 1 month (to avoid growth of algae).
**Preparation of Artemia for Hydra feeding**  
**Timing 24–75 h**

1. **Add 2 g of *A. salina* cysts to 400 ml of artificial seawater (see ‘Reagent setup’) in a 500-ml glass bottle.**
   
   **CRITICAL STEP** Lyophilized stocks of *Artemia* cysts should be stored at 4 °C (for up to 6 months); otherwise, they lose viability. We also noticed substantial variability between batches from the same provider. We recommend purchasing several small packages of cysts from different lots and testing them for hatching efficiency before ordering large amounts of stock.

2. Subject the solution to continuous aeration, using an aquarium pump with an attached Pasteur pipette completely submerged in the liquid. Incubate the culture at 18 °C. At this temperature, most of the cysts hatch within 60–75 h. Alternatively, the culture can be incubated in a water bath at 28–30 °C. This results in accelerated hatching of nauplii (within 24–30 h).

3. After 60–75 h, cease aeration; this allows the hatched nauplii to become concentrated at the bottom of the bottle; un­hatched cysts will float on the surface. Aspirate 40–100 ml of concentrated nauplii, avoiding un­hatched cysts, and place into a sieve with a 120-μm mesh. Wash the nauplii thoroughly with running tap water to eliminate all the salt. Rinse with Hydra medium and decant into a glass beaker before feeding the *Artemia* to *Hydra*. The sieve should be rinsed thoroughly in tap water and then Hydra medium after each use.

4. Feed the nauplii to the *Hydra* immediately after collection with a clean Pasteur pipette.

   **CRITICAL STEP** The *Artemia* nauplii must be <24 h old when fed to the *Hydra*. A milky appearance of the water and low motility of larvae indicate an intensive growth of fungi and bacteria. Using nauplii in these conditions for feeding will probably compromise the *Hydra* health. Discard old (>30 h after hatching) and contaminated cultures immediately.

**Establishment and maintenance of *Hydra* cultures**  
**Timing 1 month**

5. Obtain a few founder polyps of the *H. vulgaris* AEP strain from a reliable source. The strain identity can be verified by means of a microscopic analysis of nematocytes or analysis of molecular markers\(^65,69,70\). The strain can be obtained from the authors upon request.

6. Keep *Hydra* polyps in square plastic dishes (20 × 20 × 6 cm) filled approximately two-thirds full with Hydra medium. Dishes should be loosely covered to allow gas exchange.

7. Maintain the cultures at 18 °C with a photoperiod of 12 h light:12 h darkness.

8. Feed the culture three to four times per week with *Artemia* nauplii immediately after they hatch (from Step 4). On average, 7–10 brine shrimp larvae per polyp are sufficient.

   **CRITICAL STEP** Avoid overfeeding the polyps, as excessive nauplii will not be caught by polyps, will quickly die in the low-salt *Hydra* medium, and will eventually start to decompose, thus compromising the health of the *Hydras*.

9. Approximately 8 h after feeding, the debris from dead shrimp and remains expelled undigested by *Hydra* must be removed. First, detach the polyps from the dish by gently scraping the walls and bottom with a finger in a latex glove.

   **CRITICAL STEP** The gloves must be powder free and tested for toxicity on *Hydra* in a small-scale experiment. Soaking a piece of a glove material in a culture dish with 10–50 polyps and incubating for at least 5 d will reveal whether any component of the glove has toxic effects on *Hydra*. Shortening of tentacles (Supplementary Fig. 1) and appearance of debris on the dish bottom are the first signs of adverse effects on *Hydra* health. In our experience, powder-free nitrile gloves cause no harm to the polyps.

**TROUBLESHOOTING**

10. Pour the medium out of the dish through a 500-μm mesh metal or plastic sieve. Wash the culture dish under running tap water while intensively rubbing the surface with a finger. Do not use any detergents. Rinse the dish with *Hydra* medium.

11. Rinse the polyps in a sieve with running *Hydra* medium, invert the sieve and decant the polyps into the clean culture dish. Flush the sieve with *Hydra* medium to collect all the polyps retained on the walls. Fill the culture dish to two-thirds with *Hydra* medium and remove any debris or damaged polyps with a clean Pasteur pipette.

   **CRITICAL STEP** Delayed or incomplete washing of the culture will result in growth of fungi and bacteria. First evident as a slimy biofilm on the dish surface, this contamination will eventually compromise the health of the polyps and the collected embryos.
**Preparation of a transgene construct**

12 Assemble a plasmid containing an expression cassette, using standard molecular cloning techniques and a published vector (such as LigAF\textsuperscript{25} or indGFP\textsuperscript{9}) as a backbone. A cassette should include the 5′-flanking region containing a transcription start site and other cis-regulatory elements (for simplicity, referred to as a ‘promoter’), a fluorescent marker gene, and a 3′-flanking region necessary for transcript polyadenylation and stability. Use restriction enzymes to exchange the elements in the backbone vector.

**CRITICAL STEP** If cloning with restriction enzymes becomes infeasible (e.g., due to the presence of internal restriction sites), it is advisable to use a commercial gene-synthesis service. It saves time, ensures proper sequence of the construct and ensures excellent performance in terms of transcript stability and optimal protein expression.

13 Propagate the plasmid in the standard DH5\textsubscript{a} E. coli strain and purify it, using a QIAfilter Plasmid Midi Kit and following the manufacturer’s instructions. Elute the plasmid with 5 ml of Buffer QF (from the kit), precipitate by adding isopropanol and centrifuge immediately at 15,000 \text{g} for 30 min at 4 °C. Wash the pellet twice with 2 ml of 70% (vol/vol) ethanol, air-dry the DNA for 5 min and re-dissolve in 100 μl of Milli-Q water. Incubate the solution overnight at 4 °C to ensure that the plasmid dissolves completely. Determine the yield and DNA concentration by UV spectrophotometry.

CRITICAL STEP The resulting plasmid solution must contain no salts, in order to be highly purified (absorbance ratios A\textsubscript{260}/280 > 1.8, A\textsubscript{260}/230 > 1.8) and highly concentrated (>1 μg/μl DNA). An additional precipitation step is recommended to increase the DNA concentration and improve its purity. Add 10 μl of 2.5 M KAc and 250 μl of 96% (vol/vol) ethanol to 100 μl of plasmid solution, mix intensively, incubate for 2 h at –20 °C, spin down at 15,000 g for 20 min at 4 °C, and wash with 1 ml of 70% (vol/vol) ethanol. Finally, air-dry the pellet for 5 min and dissolve in Milli-Q water.

**Pause Point** Purified plasmid at a concentration of 1–3 μg/μl in Milli-Q water can be stored indefinitely at –20 °C.

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**Preparation of equipment and injection solution**

14 Prepare the injection plate by fixing a 20-mm Secure-Seal imaging spacer to a standard glass slide. Place a 2-ml drop of *Hydra* medium into the circle limited by the spacer.

15 Use the P97 pipette puller to pull an injection needle from a 1.2-mm borosilicate capillary with 0.1-mm wall thickness. We recommend the following settings: heat, 810; pull, 90; velocity, 100; and time, 190. However, the optimal values depend on the shape and thickness of the filament. We use the FB255B filament. Refer to the Pipette Cookbook\textsuperscript{22} for detailed recommendations on puller settings. Mount the injecting capillary on the micromanipulator at 35° to the horizontal plane of the injecting plate.

**Caution** The injection capillaries are sharp and must be handled with care. Dispose of the capillaries according to appropriate safety procedures.
**CRITICAL STEP** The length and the shape of the needle are essential to a successful injection success. If the needle is too thin, it tends to bend instead of piercing the embryo. An injection needle with too big a diameter results in damage to the zygote and leakage of injection solution from both the pipette and the injected embryo.

16 Mount the holding capillary on the CellTram Air holding device attached to a micromanipulator. Set the micromanipulator at an angle of 35° to the injection plate. As the holding capillary is pre-bent to 35°, its tip is thus positioned parallel to the bottom of the injection plate. This minimizes damage to embryos upon injection. Clean the capillary with 1 M NaOH and flush with Milli-Q water, using the CellTram Air device. Air-dry the capillary.

17 Ensure that both capillaries are submerged in the *Hydra* medium and are at the same height above the glass surface. Arrange the tips of the capillaries in the center of the visible field.

**CRITICAL STEP** The microscope with micromanipulators must be mounted on a solid table to reduce vibrations. For the same reason, we recommend allocating an isolated room for the microinjection facility.

**PAUSE POINT** The microinjection equipment can remain set up for several hours while the injection solution and embryos are prepared.

18 Defrost an aliquot of the plasmid solution from Step 13. Adjust the concentration to 1 μg/μl by adding Milli-Q water and centrifuge the solution at 15,000 g for 90 min at 4 °C. Collect the supernatant and load 3 μl into the microloader with a 10-μl pipette. Place the tip of the microloader into the injection capillary in such a way that it reaches the end of the sharp needle and releases the sample. Capillary force will pull the solution into the tip of the injection needle.

19 Place the needle into the grip head and ensure that its tip is aligned with the holding capillary opening in the middle of the field of view. Touch the holding capillary with the injection needle to break its tip.

20 Connect the tube attached to the injection needle to the FemtoJet microinjector and flush it twice, using the 'clean' button. Under the microscopic control, ensure that there is a constant minimal discharge of the sample from the needle. Adjust the compensation pressure to 10–30 hPa, if necessary. Set the injection pressure to 180–200 hPa.

**CRITICAL STEP** If the injection needle opening is too wide, major pressure adjustments are necessary.

**Hydra mass culture for embryo collection** **Timing** 2 weeks, after induction; 3 months to raise a dense culture

21 Use several female *H. vulgaris* AEP polyps to start a mass culture. Propagate the culture in the standard 20 × 20 × 6-cm dishes at 18 °C as described in Steps 6–11. Feed the polyps four times per week with freshly hatched *A. salina* nauplii, prepared as described in Steps 1–4.

**CRITICAL STEP** It is essential to keep all conditions (feeding frequency and intensity, temperature, photoperiod) very stable before induction. Any minor change elicits spontaneous gametogenesis in some polyps and makes the further synchronous induction less effective.

22 Determine the density of the culture by counting polyps within a randomly selected 5 × 5-cm square (equals 1/16 of the dish surface) and multiplying it by 16. Once a culture reaches a density of >4,000 polyps per dish, switch the feeding regime to three times per week. In addition, transferring half of the polyps to a new dish and thus decreasing the culture density increases the induction efficiency. 10–14 d post induction, the females should start producing eggs.

**CRITICAL STEP** The same mass culture can be induced many times. However, owing to polyp masculinization, egg production declines, and after three cycles of induction, egg production becomes too low for daily injections. Therefore, we recommend exchanging the mass culture with a new one, raised from few females, at least every 12 months.

**TROUBLESHOOTING**

**Preparation of embryos for injection** **Timing** 1–4 h

23 Screen the induced culture dish for the presence of female polyps developing eggs and use a Pasteur pipette to transfer them to a 60-mm Petri dish containing *Hydra* medium. Add an equal number of male polyps to the dish to ensure fertilization.

24 Over the next 1–4 h, screen the females under a dissecting microscope and select only those with embryos that are at or have not yet reached the four-cell stage. Appearance of a first cleavage furrow on the otherwise round and smooth egg surface indicates a successful fertilization.
Immediately before an injection, detach a fertilized egg from the female polyp by cutting the egg holder with a blade and transfer it to the injection plate with Hydra medium. Return the female polyp to the mass culture.

**CRITICAL STEP** As zygotes and early embryos do not have any hard shell, manipulate them very carefully to avoid damaging them. Blunting sharp Pasteur pipette tips over a gas flame is recommended.

### Injection of embryos  ◆ **Timing 2–3 h**
26 Place a single embryo in the middle of the field of view and immobilize it at the holding capillary tip by gently applying aspiration with the CellTram Air device.
27 Pierce the zygote or a blastomere of an embryo with the injection needle and insert the needle until it reaches the center of a cell. Use the ‘manually injecting’ mode of the FemtoJet and press the manipulator’s button. Release the button when a slight bloating of the cell becomes visible.
28 Remove the injection needle from the cell and repeat the injection of all blastomeres in an embryo. Transfer an injected embryo to a well of a 12-well plate filled with 3 ml of Hydra medium.
29 Return to Step 26 and repeat the injection cycle. Inject as many zygotes and early embryos as possible, repeating Steps 26–28.

**CRITICAL STEP** Before injecting the next blastomere or a new embryo, ensure that the injection needle is not clogged and that the plasmid solution drains slowly from the tip.

### Screening of transgenic animals and propagation of clonal cultures  ◆ **Timing 2–3 months, with ~10–20 min hands-on time per d**
31 Collect injected embryos in the wells of a 12-well plate (up to four zygotes per well) and keep them at 18 °C. During the first week of development, replace the Hydra medium in the plates daily. Monitor the expression of the reporter gene under a fluorescence dissecting stereomicroscope, starting from the second day post injection.

**CRITICAL STEP** On the day after injection, detach the embryos from the bottom with a gentle stream of water from a Pasteur pipette and remove the debris from the degrading egg holders.

**CRITICAL STEP** A hard cuticle shell should appear around each embryo about 48 h after injection, indicating successful development. If a shell is not formed within 72 h, an embryo will normally degenerate within the next 2 d. It must be removed from the well to avoid bacterial growth. All further developmental processes (cleavage, gastrulation) remain hidden under the shell, until a juvenile polyp (so-called hatching) emerges from the shell.

32 Keep the developing embryos in darkness for 2 weeks post injection, and then transfer them to normal light conditions. This results in synchronous hatching, with up to 90% of all primary polyps appearing within 2–4 d after the switch. Transfer each newly emerged polyp to a single well with Hydra medium.

**TROUBLESHOOTING**
33 Start feeding the polyps 24–48 h after hatching, once their tentacles become extended and ‘armed’ with nematocytes. Feed the animals four times per week with freshly hatched *A. salina* nauplii and replace the Hydra medium 12 h after each feeding. Once the bottom of a well becomes opaque, indicating a bacterial biofilm growth, transfer the polyp to a new well. Under optimal conditions, a polyp starts budding 5 d after hatching. Feed the polyp intensively and keep it in the well until a clone reaches seven to ten polyps.

34 Screen each clone under the fluorescence stereomicroscope. Specific eGFP fluorescence should be localized in cells and be visible upon microscopy through a narrow-pass filter set (GFPA: Ex460–495/Em510–550). Nonspecific fluorescence appears as a diffuse yellowish glow and is visible only when using a broad-band GFP filter set (Ex460–490/Em510–), but can be eliminated by using the narrow-pass GFPA filter. Identify and separate the polyps with expanding patches of transgenic cells. Propagate them asexually by budding and screen them every week until a uniformly transgenic line is achieved. Simultaneously, identify and separate the polyps with the least density of transgenic cells. Propagate them and select until complete depletion of the reporter signal to use as controls. Discard all polyps with intermediate phenotype.
35 Verify the transgenesis by real-time qRT–PCR, immunocytochemical staining, in situ hybridization or western blot, following the guidelines presented in refs. \(^1,5,7,21\).  

**Troubleshooting**

Troubleshooting advice can be found in Table 1.

### Table 1 | Troubleshooting table

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>The polyps look sick: tentacles are shortened or have rounded knobs at their ends (Supplementary Fig. 1), polyps stay in a permanently contracted state, fungal hyphae surround the polyps' peduncles or a colorless debris appears on the dish bottom</td>
<td>The culture was not properly washed after feeding or was exposed to some toxic chemical from the media or equipment</td>
<td>Discard all the stock solutions and media, and prepare fresh ones. Isolate the affected Hydro culture and treat it separately from other dishes. Intensively wash the dish and the culture, removing any debris or dying polyps. In our experience, feeding a sick culture only makes the state worse, and we strongly advise washing the culture twice a day for several days before commencing normal feeding</td>
</tr>
<tr>
<td>22</td>
<td>Few to no female polyps are observed in an induced mass Hydra culture, while numerous male polyps are present</td>
<td>The culture went through several induction cycles and is masculinized</td>
<td>Start a new mass culture from a single female polyp; induce it three times in the next year and then discard</td>
</tr>
<tr>
<td>29</td>
<td>Injection needle is plugged; injection is not possible</td>
<td>The plasmid preparation is not sufficiently clean and homogeneous</td>
<td>Avoid dust particles upon drying of the pellet. Centrifuge the stock plasmid solution (Step 18) at high speed and take the supernatant</td>
</tr>
<tr>
<td>32</td>
<td>Mortality of injected embryos exceeds 50%</td>
<td>The injected volume is too high</td>
<td>Inject less plasmid solution. Stop delivering the solution into a zygote or a blastomere immediately once it appears slightly distended</td>
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<tr>
<td></td>
<td></td>
<td>The injection solution contains impurities (e.g., salt)</td>
<td>Use different cleanup procedures; precipitate the DNA again, and wash the pellet intensively with 70% (vol/vol) ethanol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intensive bacterial growth around the injected embryos with cloudy debris being visible in the wells</td>
<td>Remove the remains of an egg holder from the embryos. Replace the medium and, if necessary, the plates</td>
</tr>
<tr>
<td>35</td>
<td>High mortality of transgenic polyps or disappearance of transgenic cells</td>
<td>The transgene affects the activity of an essential gene, causing adverse effects in the transgenic cells or whole polyps</td>
<td>Consider an alternative construct design, e.g., overexpress a mutant version of a protein instead of knockdown. Consider using an inducible system</td>
</tr>
<tr>
<td></td>
<td>Weak fluorescence of transgenic embryos and polyps</td>
<td>The GFP transcript or protein is degraded</td>
<td>Analyze the predicted transcript structure for the presence of internal splicing sites and for transcript stability, using available software. Consider N-terminal tagging instead of C-terminal fusion of GFP. Avoid putative proteolysis sites (e.g., dibasic sites Lys-Arg or Arg-Arg) in the sequence of an overexpressed fusion protein. Consider using an affinity tag (myc tag)</td>
</tr>
</tbody>
</table>

**Timing**

Steps 1–4, preparation of *Artemia* for *Hydra* feeding: 24–75 h  
Steps 5–11, establishment and maintenance of *Hydra* cultures: 1 month  
Steps 12 and 13, preparation of a transgene construct: 2 weeks  
Steps 14–20, preparation of equipment and injection of solution: 95 min  
Steps 21 and 22, *Hydra* mass culture for embryo collection: 2 weeks (after induction); 3 months to raise a dense culture  
Steps 23–25, preparation of embryos for injection: 1–4 h  
Steps 26–30, injection of embryos: 2–3 h  
Steps 31–34, screening of transgenic animals and propagation of clonal cultures: 2–3 months, with ~10–20 min of hands-on time per d
Anticipated results

Microinjection of the plasmid into *Hydra zygotes* results in integration of the transgene construct into the genome. Although the integration mechanisms and preferential integration sites remain unknown, this integration only rarely causes adverse effects on the phenotype. Typically, >60% of injected embryos show expression of a transgene construct from a few days after injection. In many cases, however, the transgenesis is transient, and the polyps lose expression of the reporter construct soon after hatching. Nevertheless, a substantial fraction of embryos (on average, 30% of all injected embryos) become stably transgenic. This transgenesis rate is remarkably high and contrasts with the efficiency of transgenesis in other model organisms. The success rate of transgenesis can vary substantially, for reasons that are not fully understood. Besides the aforementioned purity and concentration of the plasmid, the size of a construct, and the nature of the GOI and the regulatory sequences used may affect the transgenesis efficiency. As the construct integrates into the genome, the clonally propagated lines obtained by embryo microinjection remain transgenic for years without changing their phenotype. This allows for great control and consistency in experiments performed on these lines over the years.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data generated or analyzed during the current study are included in this paper. The raw data used in the example results are available from the corresponding authors upon reasonable request.

References


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Author contributions
A.K. and T.C.G.B. conceived the paper. J.W. and A.K. generated the data and took photos. A.K., J.W. and T.C.G.B. wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41596-019-0173-3.
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Correspondence and requests for materials should be addressed to A.K. or T.C.G.B.
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Corresponding author(s):

Alexander Klimovich, Thomas C.G. Bosch

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Our web collection on statistics for biologists may be useful.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

<table>
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<tr>
<th>Sample size</th>
<th>Sample size was not predetermined. Here we report observations collected over 12 years.</th>
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<td>Data exclusions</td>
<td>No data were excluded from the analysis.</td>
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<tr>
<td>Replication</td>
<td>The findings presented in the paper are based on observations made by monitoring over 17,000 injected embryos within 12 years. Figures 3d,f show representative cases of egg production (d) and hatching (f) dynamics. The Figure 3g reports the transgenesis efficiency of 6 independent injection series of 140 embryos made within a range of 6 years.</td>
</tr>
<tr>
<td>Randomization</td>
<td>Randomization was not relevant for the study. No control groups have been used in this study.</td>
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<tr>
<td>Blinding</td>
<td>Blinding was not relevant for the study. No control groups have been used in this study.</td>
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Reporting for specific materials, systems and methods

Materials & experimental systems

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<td>✗</td>
<td>Animals and other organisms</td>
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<td>Human research participants</td>
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Methods

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<td>Flow cytometry</td>
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<tr>
<td>✗</td>
<td>MRI-based neuroimaging</td>
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</table>

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

The plasmids reported in this manuscript and their sequences are available upon request. Transgenic lines are also available.

Animals and other organisms

Policy information about studies involving animals. ARRIVE guidelines recommended for reporting animal research

Laboratory animals

Hydra vulgaris strain AEP, males and females

Wild animals

The study did not involve wild animals.

Field-collected samples

The study did not involve samples collected from the field.