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RESEARCH ARTICLE

TECHNIQUES AND RESOURCES

A targeted gene expression system using the tryptophan repressor in zebrafish shows no silencing in subsequent generations

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ABSTRACT

The ability to visualize and manipulate cell fate and gene expression in specific cell populations has made gene expression systems valuable tools in developmental biology studies. Here, we describe a new system that uses the *E. coli* tryptophan repressor and its upstream activation sequence (TrpR/*tUAS*) to drive gene expression in stable zebrafish transgenic lines and in mammalian cells. We show that TrpR/*tUAS* transgenes are not silenced in subsequent generations of zebrafish, which is a major improvement over some of the existing systems, such as Gal4/*gUAS* and the Q-system. TrpR transcriptional activity can be tuned by mutations in its DNA-binding domain, or silenced by Gal80 when fused to the Gal4 activation domain. In cases in which more than one cell population needs to be manipulated, TrpR/*tUAS* can be used in combination with other, existing systems.

KEY WORDS: Gal4/UAS, Gene expression system, Silencing, Tryptophan repressor, Zebrafish

INTRODUCTION

Bipartite gene expression systems allow selective gene expression in a tissue-specific manner *in vivo* (reviewed by del Valle Rodríguez et al., 2012; Elliott, 2008; Halpern et al., 2008). They consist of two parts: a driver line and an effector/reporter line. In the driver line, a tissue-specific promoter drives a transcriptional activator, while in the effector/reporter line a target gene is placed under control of the binding site [upstream activation sequence (*UAS*)] of the transcriptional activator. The cross between the driver and effector/reporter lines allows expression of target genes exclusively in tissues in which the specific promoter is functional. The advantage of these bipartite systems is that the same effector can be expressed in different tissues simply by crossing to different driver lines. Similarly, the same driver can easily be used to promote the expression of various effectors. In addition, effector lines for potentially deleterious gene products can be maintained without expression until crossed to driver lines.

Gal4/*UAS* was the first gene expression system to be developed in *Drosophila* (Brand and Perrimon, 1993). It uses the yeast Gal4 transcription factor, which coordinates the expression of genes needed for utilization of galactose through a common *UAS* (*gUAS*; Fig. 1A,B). The Gal4 transcriptional activator was integrated randomly in the *Drosophila* genome, landing at times adjacent to enhancers expressed in specific tissues and cell populations, thereby creating *Drosophila* lines in which Gal4 was expressed in a tissue-specific manner, which was termed 'enhancer trapping'. Additionally, reporter/effector lines were generated, in which either *lacZ* (a reporter gene) or *even-skipped* (an effector gene) was placed downstream of *gUAS*. By crossing the enhancer lines with the reporter/effector lines it became possible not only to visualize the 'trapped' cell populations but also to misexpress *even-skipped* in specific cell populations in an effort to determine its role in their development. Since its establishment, the Gal4/*gUAS* system has facilitated a wide variety of techniques, including gene overexpression and misexpression, targeted gene knockouts, targeted cell ablation, disruption of neuronal synaptic transmission, and *in vivo* cell tracing followed by time-lapse microscopy during development (del Valle Rodríguez et al., 2012; Elliott, 2008; Halpern et al., 2008). Owing to its usefulness, this system has been adopted in several other model organisms, such as *Arabidopsis* (Engineer et al., 2005), *Xenopus* (Hartley et al., 2002), *Medaka* (Grabher and Wittbrodt, 2004), zebrafish (Asakawa et al., 2008; Scheer and Campos-Ortega, 1999; Scott et al., 2007), mouse (Hu et al., 2004; Ornitz et al., 1991; Rowitch et al., 1999) and human cell culture (Webster et al., 1988).

A serious disadvantage of the Gal4/*gUAS* system is that the *UAS* is silenced in subsequent generations in vertebrates due to methylation at CpG nucleotides (Akitake et al., 2011; Goll et al., 2009) (Fig. 1B). This leads to the silencing of the *UAS*-regulated effector/reporter gene as early as the first (F1) generation and necessitates continual reestablishment of these lines. To circumvent this problem, we have developed a new bipartite gene expression system that employs the tryptophan repressor (TrpR) and its *UAS* (*tUAS*), which are responsible for tryptophan biogenesis in *E. coli* (Gunsalus and Yanofsky, 1980). The minimal *tUAS* lacks CpGs (Fig. 1D) (Li et al., 1995), suggesting that it would not be silenced by methylation. We created *tUAS* effector/reporter zebrafish lines and found no indication of silencing as far as the fourth (F4) generation. Taking advantage of the wealth of data on the structure and function of TrpR, we identified TrpR mutants with reduced transcriptional activity in zebrafish, for use in cases where lower levels of effector protein expression are desired. Finally, we found that the TrpR/*tUAS* system works well in mammalian cell culture, demonstrating that this approach will be broadly applicable. The TrpR/*tUAS* system is an excellent alternative to the Gal4/*gUAS* system, and it can also be combined with Gal4/*gUAS* to permit combinatorial regulation of effector expression *in vivo*.

RESULTS

Design of the TrpR system and establishment of driver and reporter lines

To make the driver construct, we fused the entire TrpR coding region to the Gal4 activator domain (G4AD) (Fig. 1C), which can

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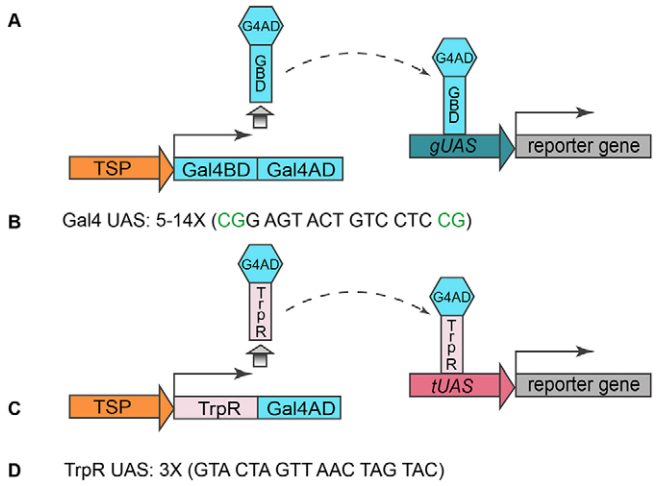

Fig. 1. Gal4/*gUAS* and TrpR/*tUAS* gene expression systems.
(A) Expression from a tissue-specific promoter (TSP) of the fusion protein consisting of Gal4 binding (GBD) and activation (G4AD) domains generates a transcriptional activator that can bind to the upstream activation sequence (*UAS*) of Gal4 and initiate transcription of a reporter gene. (B) Gal4 *UAS* contains CpG sites (green) that can be methylated, leading to the silencing of reporter lines. (C) The TrpR/*tUAS* system combines full-length TrpR with the Gal4 activation domain to drive the expression of reporter genes under 3× TrpR-UAS (*tUAS*). (D) *tUAS* has no CpG dinucleotides and is therefore predicted not to be silenced by methylation.

be inhibited by Gal80 (Fujimoto et al., 2011; Traven et al., 2006) and thus affords an additional level of transcriptional regulation (see below). We added a nuclear localization signal (nls) and placed the nlsTrpR-Gal4AD fusion construct under the *CMV* promoter (p*CMV*:nlsTrpR-G4AD). For the reporter construct, we used a multimerized (3×) *TrpR UAS* (*tUAS*) (Fig. 1D) to drive DsRed fluorescent protein (p*tUAS*:DsRed). When we transfected the constructs into HEK 293 cells or injected them into zebrafish embryos, we found that TrpR/*tUAS* constructs were able to induce transcription of the DsRed reporter gene both in the cell line and in embryos (data not shown). This indicated that it would be feasible to create transgenic animals and test this expression system in stable lines.

To build the constructs used for generating zebrafish transgenic lines, we used the Tol2 Gateway cloning system (Kwan et al., 2007; Villefranc et al., 2007), which will make it easy to swap promoters and effector/reporter genes in the future (Table 1). For the driver construct, we chose *myosin 6b* (*myo6b*) (Obholzer et al., 2008) and *ribeye A* (*ribA*; *ctbp2a* – Zebrafish Information Network) (Odermatt et al., 2012) promoters to drive nlsTrpR-G4AD (p*myo6b*:nlsTrpR-G4AD and p*ribA*:nlsTrpR-G4AD, respectively). In zebrafish, the *myo6b* promoter is expressed in auditory, vestibular and lateral line hair cells, whereas *ribA* is also expressed in these plus photoreceptor, bipolar and pineal cells. Since the transgenic driver lines are not visible until crossed to transgenic reporter lines, we added to the destination constructs the *alpha A crystallin* promoter (Hesselson et al., 2009) driving the expression of the red fluorescent protein Cherry (*cryaa*:Cherry, abbreviated CC), which promotes the expression of Cherry in the lens, allowing us to easily identify transgenic fish. For the reporter constructs, we used *tUAS* (Fig. 1D) to drive tagRFP (p*tUAS*:tRFP) (Merzlyak et al., 2007) or the nuclear-localized photoconvertible protein nlsEos (p*tUAS*:nlsEos) (Curran et al., 2010; Wiedenmann et al., 2004), which is particularly useful for lineage tracing (McGraw et al., 2012). We added *cryaa*:Venus (abbreviated CV) to identify transgenic carriers using yellow fluorescent protein expression in the lens. We injected each DNA construct together with Tol2 transposase RNA into one-cell stage zebrafish embryos (Asakawa et al., 2008), grew the embryos to adulthood and screened for stable insertions.

We isolated one insertion for *Tg(CV,tUAS:tRFP)*^{w80} and several insertions for each of *Tg(CV,tUAS:nlsEos)*^{w81}, *Tg(CC,myo6b:nlsTrpR-G4AD)*^{w83} and *Tg(CC,ribA:nlsTrpR-G4AD)*^{w85} (Fig. 2). Both tRFP and nlsEos appeared to be ubiquitously expressed when 5 pg *nlsTrpR-G4AD* mRNA was injected into one-cell stage transgenic embryos (Fig. 2A,B). However, when the *Tg(CV,tUAS:tRFP)*^{w80} reporter line was crossed to the *Tg(CC,myo6b:nlsTrpR-G4AD)*^{w83} driver line, only a subset of hair cells was labeled in double-transgenic embryos (Fig. 2E, Fig. 4A). Since this pattern was not observed when the *Tg(CV,tUAS:nlsEos)*^{w81} reporter line was crossed to *Tg(CC,myo6b:nlsTrpR-G4AD)*^{w83} (Fig. 2D), we concluded that *Tg(CV,tUAS:tRFP)*^{w80} is variegated due to an insertion-specific position effect. We also noticed that *Tg(CC,ribA:nlsTrpR-G4AD)*^{w85} could induce expression of nlsEos when crossed with *Tg(CV,tUAS:nlsEos)*^{w81} (Fig. 2F), but was unable to induce tRFP expression when crossed with *Tg(CV,tUAS:tRFP)*^{w80} (data not shown). Since the *ribA* promoter is weak, we suspect that the insertion site of p*tUAS*:tRFP in our reporter line requires relatively high levels of TrpR-G4AD to activate transcription. We are in the process of screening for new *Tg(CV,tUAS:tRFP)*^{w80} founders that lack insertion-specific effects.

Table 1. Constructs used to generate transgenic lines

Destination constructs	Driver constructs	Reporter constructs
pDestTol2CV*	p5E-*myo6b*	p5E-*tUAS*
pDestTol2CC‡	p5E-*ribA*	pME-tRFP
	p5E-*neuroD*	pME-nlsEos
	pME-nlsTrpR-G4AD	p3E-pA
	pME-nlsTrpR_T81M-G4AD	pTol2-CV,*tUAS*:tRFP
	pME-nlsTrpR_T81A-G4AD	pTol2-CV,*tUAS*:nlsEos
	p3E-pA	
	pTol2-CC,*myo6b*:nlsTrpR-G4AD	
	pTol2-CC,*ribA*:nlsTrpR-G4AD	
	pTol2-CC,*neuroD*:nlsTrpR-G4AD	
	pTol2-CC,*neuroD*:nlsTrpR_T81M-G4AD	
	pTol2-CC,*neuroD*:nlsTrpR_T81A-G4AD	

Gateway technology was applied to create all constructs used to generate transgenic lines in this study (see Materials and Methods for further details).
*CV, *cryaa*:Venus.
‡CC, *cryaa*:Cherry.

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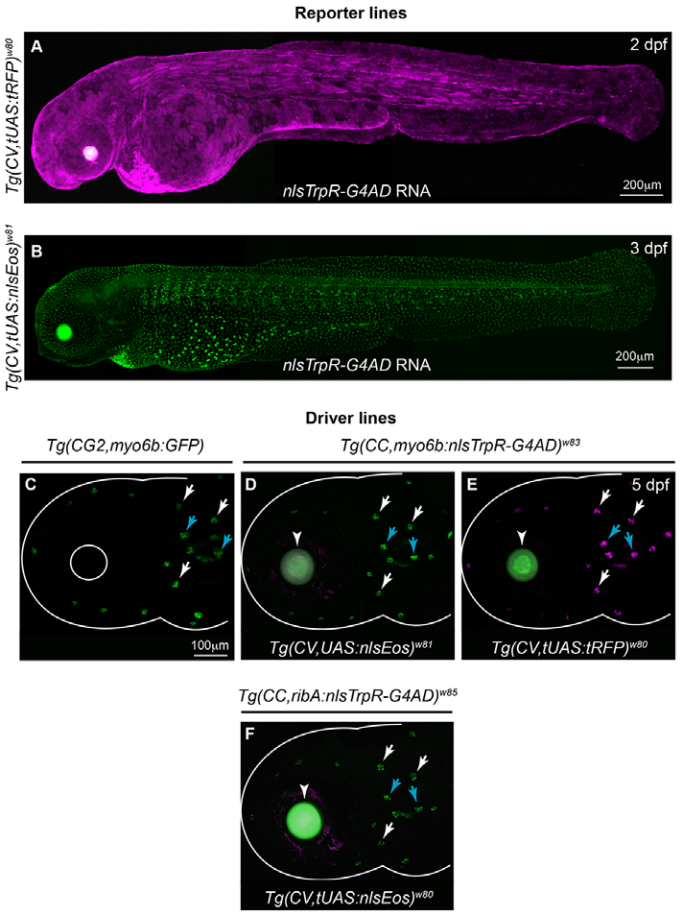

Fig. 2. Transgenic zebrafish lines. (A,B) Two and 3 day post-fertilization (dpf) embryos from reporter lines visualized by injection of *nlsTrpR-G4AD* mRNA into one-cell stage embryos. (C) Expression pattern of the *myo6b* promoter visualized by GFP in a transgenic line. (D-F) Driver lines crossed to reporter lines. Heads of 5 dpf double-transgenic zebrafish larvae showing expression of reporter genes in hair cells. The *myo6b* promoter drives expression in vestibular (blue arrows) and auditory hair cells and lateral line hair cells (white arrows) (D,E), whereas the *ribA* promoter drives expression in vestibular (blue arrows) and auditory hair cells, lateral line hair cells (white arrows), bipolar and photoreceptor cells (obscured by retinal pigment cells) and pineal cells (not shown) (F). The lens expression is also visible in D-F (white arrowheads).

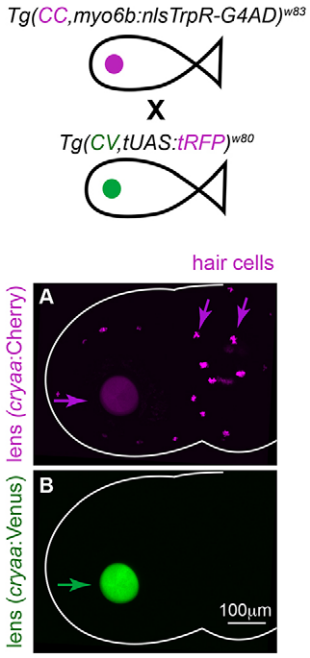

Fig. 3. *tUAS* is not silenced, as determined by assessing individuals from the F3 generation of the *Tg(CV,tUAS:tRFP)*^{w80} reporter line. Eighteen adults from the F3 generation of *Tg(CV,tUAS:tRFP)*^{w80} were crossed to the F2 generation of *Tg(CC,myo6b:nlsTrpR-G4AD)*^{w83}. Progeny double positive for red lenses (indicating the driver line; A, arrow) and green lenses (indicating the reporter line; B, arrow) were sorted out and scored for overall hair cell expression. A single embryo is presented (A, red channel; B, green channel) showing lens expression (A,B) and hair cell expression (A). Of the combined progeny double positive for red and green lens expression, 24.8% (171/690) were also positive for hair cell expression. None was positive for both lens markers but negative for hair cell expression.

The reporter line does not become silenced with subsequent generations

Since the minimal *tUAS* has no CpG dinucleotides (Fig. 1D), we predicted that it would not be silenced by methylation. To determine whether silencing occurs in our transgenic reporter lines, we raised four generations of *Tg(CV,tUAS:tRFP)*^{w80} and three generations of *Tg(CV,tUAS:nlsEos)*^{w81} from our F0 founders. We then tested for silencing in two ways. First, we outcrossed different individuals from the F3 generation of *Tg(CV,tUAS:tRFP)*^{w80} to individuals from the F2 generation of the driver line *Tg(CC,myo6b:nlsTrpR-G4AD)*^{w83}, and scored the progeny for the presence of transgenic markers: the lens markers (indicating the presence of the transgenes) and hair cell expression (indicating tissue-specific expression of the reporter gene). We reasoned that if the *tUAS* of the reporter line was silenced, we would see no hair cell expression in some of the progeny that are positive for both the red and green lens markers (embryos transgenic for both the driver and reporter line). Conversely, if we always saw hair cell expression in embryos that were double positive for red and green lens markers that would indicate that *tUAS* is not silenced. With this in mind, we outcrossed 18 F3 *Tg(CV,tUAS:tRFP)*^{w80} adults to F2 *Tg(CC,myo6b:nlsTrpR-G4AD)*^{w83} adults and found that 24.8% of the combined progeny (171/690) had red and green lens expression and were also positive for hair cell expression (Fig. 3). We found no cases of progeny positive for both lens markers but negative for hair cell expression, demonstrating that no silencing of the reporter line had occurred in the F3 generation of the reporter line.

Second, we assessed silencing by looking at individual lateral line neuromasts, which consist of hair cell clusters, within an embryo and by scoring hair cell expression in outcrosses of adults from different generations of the reporter line. We crossed a single adult carrier from each F1-F4 generation of *Tg(CV,tUAS:tRFP)*^{w80} or F1-F3 generation of *Tg(CV,tUAS:nlsEos)*^{w81} to an F2 or F3 adult carrier from the *Tg(CC,myo6b:nlsTrpR-G4AD)*^{w83} line (Fig. 4). We sorted for double-transgenic embryos, as indicated by the presence of red and green lenses, and scored for tRFP (Fig. 4A-D) or nlsEos (Fig. 4F-H) expression in neuromast hair cells. We additionally stained for Parvalbumin, which labels all mature hair cells within the neuromasts. We found that tRFP was consistently expressed in a subset of mature neuromast hair cells due to the insertion-specific effect of the pTol2-CV,*tUAS*:tRFP construct. However, the ratio of hair cells expressing tRFP to the total number of neuromast hair cells (tRFP/Parvalbumin) across the four *Tg(CV,tUAS:tRFP)*^{w80} generations was not statistically different (Fig. 4E), confirming that *tUAS* is not silenced. Similarly, when we assessed nlsEos expression across three *Tg(CV,tUAS:nlsEos)*^{w81} generations, we found that all mature hair cells (Parvalbumin positive) also expressed nlsEos, again proving that

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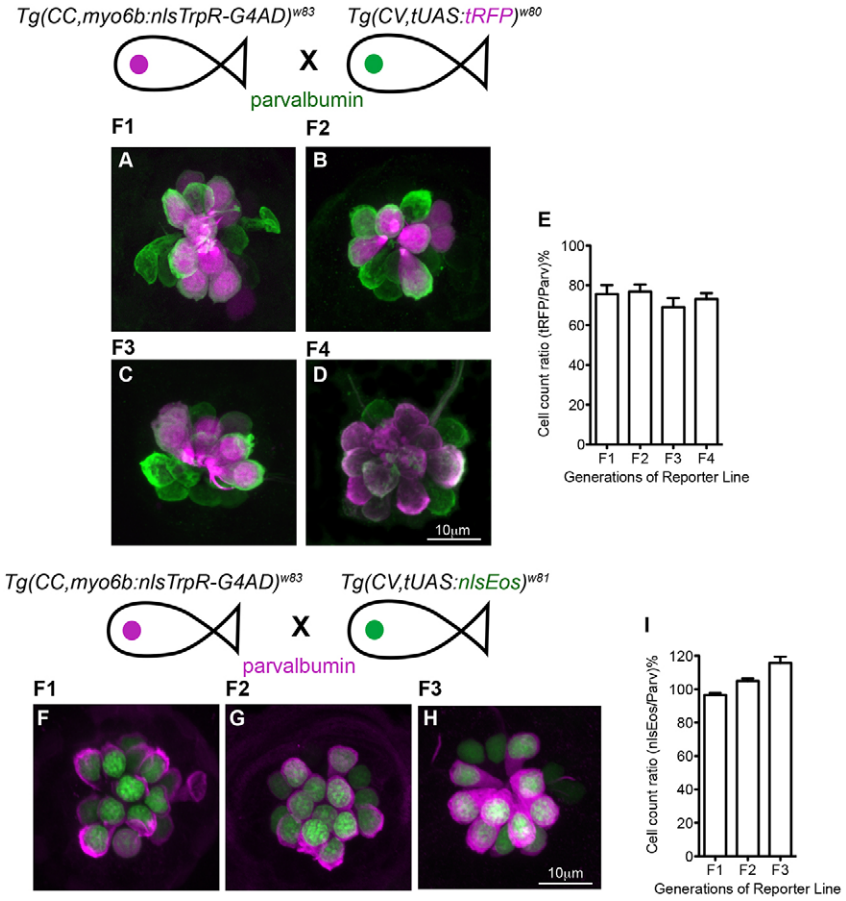

Fig. 4. *tUAS* is not silenced across generations of two reporter lines. One adult from each F1-F4 generation of the *Tg(CV,tUAS:tRFP)*^{w80} reporter line or F1-F3 generations of the *Tg(CV,tUAS:nlsEos)*^{w81} reporter line was crossed to an F2 or F3 adult from *Tg(CC,myo6b:nlsTrpR-G4AD)*^{w83}. Progeny double positive for the lens markers were sorted out and stained for Parvalbumin, which labels mature hair cells. Head neuromasts, which consist of hair cell clusters, were scored for tRFP (magenta) and Parvalbumin (green) expression (A-D) or nlsEos (green) and Parvalbumin (magenta) expression (F-H). In each case, 5 neuromasts from each of 5 embryos at 5 dpf were analyzed producing a total of 25 total neuromasts. (A-D) A representative neuromast across four generations *Tg(CV,tUAS:tRFP)*^{w80} showing tRFP and Parvalbumin (Parv) expression. In each case, the representative neuromast was located over the otic vesicle. (E) The ratio of tRFP/Parv positive hair cells was not statistically different across the generations of the reporter line, showing that tUAS is not silenced. One-way ANOVA, F=0.7655, R^2=0.02336, P=0.5161. Tukey's pairwise column comparison post-test shows no significant differences between any of the columns. (F-H) A representative neuromast showing nlsEos and Parv expression across three generations of *Tg(CV,tUAS:nlsEos)*^{w81}. (I) The ratio of nlsEos/Parv positive hair cells was always equal or above 100%, showing that across generations all mature cells (Parv positive) also expressed nlsEos. Because Parvalbumin is a late marker for mature hair cells, some nlsEos-positive cells were Parv negative, which most likely indicates the presence of immature hair cells in these samples. Error bars show s.e.m.

tUAS is not silenced (Fig. 4H). We noted that some nlsEos-positive cells were negative for Parvalbumin, as shown by an nlsEos/Parv ratio greater than 100% in the F2 and F3 crosses, probably indicating the presence of some immature hair cells in the neuromasts. We assessed the variability of expression within a clutch of embryos in each generation and obtained similar results (supplementary material Fig. S1). From these experiments we conclude that *tUAS* reporter lines are not silenced across generations.

Gal80 can be used with the TrpR/*tUAS* system to regulate expression

An advantage to using the Gal4 activator domain in nlsTrpR-G4AD is that we can regulate its ability to activate transcription by employing the Gal4 inhibitor Gal80 (Carrozza et al., 2002; Traven et al., 2006; Wu et al., 1996). This would be a useful feature in cases in which the manipulation of subgroups of cells is needed. For example, using a promoter that drives Gal80 expression in a partially overlapping manner with that used to drive nlsTrpR-G4AD, it would be possible to limit the tissues in which nlsTrpR-G4AD activates expression (Fujimoto et al., 2011). To determine whether this is feasible in our system, we crossed the *Tg(CC,myo6b:nlsTrpR-G4AD)*^{w83} line to the *Tg(CV,tUAS:nlsEos)*^{w81} line. We injected some of the one-cell stage embryos with pTol2-CG2,*myo6b*:Gal80IREStRFP DNA, a construct that expresses Gal80 under the *myo6b* promoter and marks the cells that received the plasmid using an internal ribosomal entry site (IRES) sequence to drive tRFP expression (Fig. 5A-B″). As predicted, cells expressing Gal80, as indicated by the presence of cytoplasmic tRFP, show inhibition of Eos expression in the nucleus, whereas cells negative for cytoplasmic tRFP show Eos expression in the nucleus (Fig. 5B-B″). Note that, because the larvae were not reared in the dark, nlsEos is detected both in the green and red channel due to some level of protein photoconversion. This experiment demonstrates that Gal80 can be used together with the TrpR/tUAS system to further control gene expression.

Modulation of TrpR transcriptional activity

There are many cases in which it is advantageous to drive an effector protein at submaximal levels, e.g. if a biological sensor affects the cell when expressed at higher levels. Fortunately, there is a large body of work analyzing mutations in TrpR. In one study, a key residue in the DNA-binding domain [threonine 81 (T81)] was sequentially changed to 19 alternative amino acids and the activity of TrpR then measured in *E. coli* (Pfau et al., 1994). This produced a series of TrpR mutants with varying activity from mildly affected to virtually inactive. We tested a variety of these mutants in our system. In general, we found that the effects of each of the mutations were considerably less severe in zebrafish than reported for *E. coli*, although the same trend was observed (data not shown). From this analysis we were able to identify two mutants that had reduced activity compared with wild type but still retained significant activity using a luciferase assay in early zebrafish embryos. We found that T81M was ~5× less active than wild type, and that T81A was ~11× less active (Fig. 6A,B). These results demonstrate that the ability of TrpR to regulate transcription can be 'tuned' using mutations that affect DNA binding.

TrpR works in mammalian systems

The problem of Gal4 *UAS* methylation exists not only in zebrafish but also in all vertebrates and plants. To explore whether TrpR

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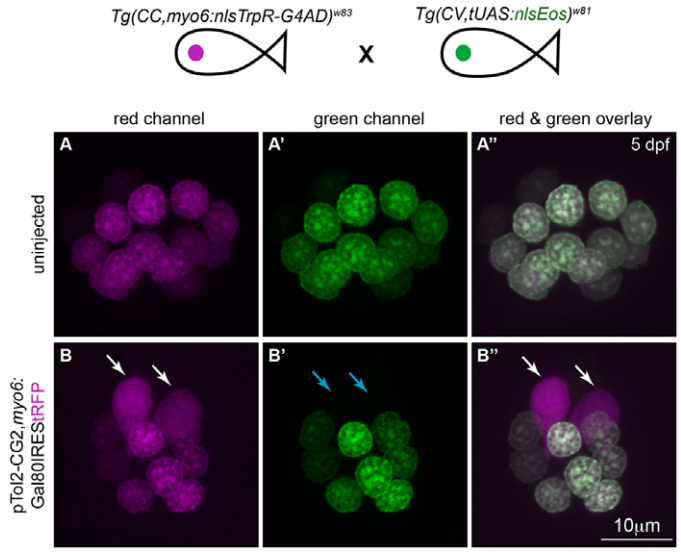

Fig. 5. Gal80 can inhibit nlsTrpR-G4AD. Adults from F2 generations of *Tg(CC,myo6b:nlsTrpR-G4AD)*^{w83} and *Tg(CV,tUAS:nlsEos)*^{w81} were crossed, and a subset of embryos was injected with pTol2-CG2,*myo6*:Gal80IREStRFP. (A-A″) Hair cell nuclei, as visualized by nlsEos, of one neuromast in an uninjected larva. Note that, because the larvae were not reared in the dark, a portion of nlsEos, which is usually detected in the green channel, was photoconverted and nlsEos was detected in both the green channel (A′) and in the red channel (A). (B-B″) A neuromast in a larva injected with pTol2-CG2,*myo6*:Gal80IREStRFP. Cells with cytoplasmic tRFP (white arrows, B,B″), which express Gal80, have no nlsEos expression (blue arrows, B′), showing that Gal80 can inhibit Gal4 in the TrpR/*tUAS* system.

could be used in systems other than zebrafish, we examined the ability of TrpR to drive luciferase expression in human HEK 293 cells. As shown in Fig. 7, TrpR is a very potent activator of transcription in mammalian cells. Similar to results obtained in zebrafish embryos, the T81M mutant was ~5× less effective and the T81A mutant was ~14× less effective than wild type. Thus, the TrpR system will be effective in other vertebrate systems in addition to zebrafish.

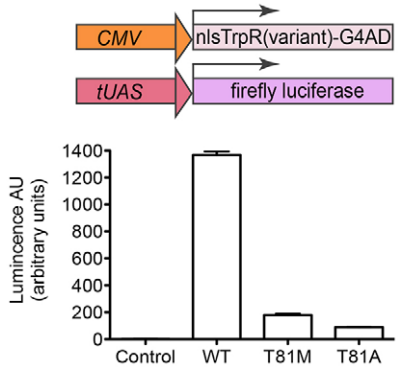

Fig. 7. TrpR works in a mammalian system. Luciferase reporter assay was used to assess transcriptional activity of the TrpR/*tUAS* system. (A) HEK 293 cells were transfected with the reporter construct (p*tUAS*:firefly luciferase) plus one of driver construct variants: p*CMV*:nlsTrpR-G4AD (WT), p*CMV*:nlsTrpR_T81M-G4AD (T81M) or p*CMV*:nlsTrpR_T81A-G4AD (T81A). Empty driver construct was used as control. Luciferase activity was assayed 24 hours post-transfection. All driver constructs were able to activate transcription of luciferase, with the T81M variant being ~5× less efficient and the T81A variant ~14× less efficient than WT. Readings were made in triplicate. Error bars show s.d.

DISCUSSION

We describe a new bipartite gene expression system that relies on the use of the tryptophan repressor (TrpR). Unlike the commonly used Gal4/*gUAS* system, our TrpR system is not subject to gene silencing as its *UAS* does not contain CG sequences. We have demonstrated that expression is stable for three to four generations in two zebrafish reporter lines, even when expression is examined at the cellular level. Furthermore, we have shown that TrpR works in mammalian cell culture; consequently, we expect that it will be useful in all vertebrate systems, and there is no reason to believe that it will not work in invertebrates as well. The activity of TrpR can be 'tuned' using mutations in the DNA-binding region, which is particularly valuable for proteins such as biological sensors that have to be expressed at a level at which they are useful but which does not disrupt development. Finally, owing to use of the Gal4AD,

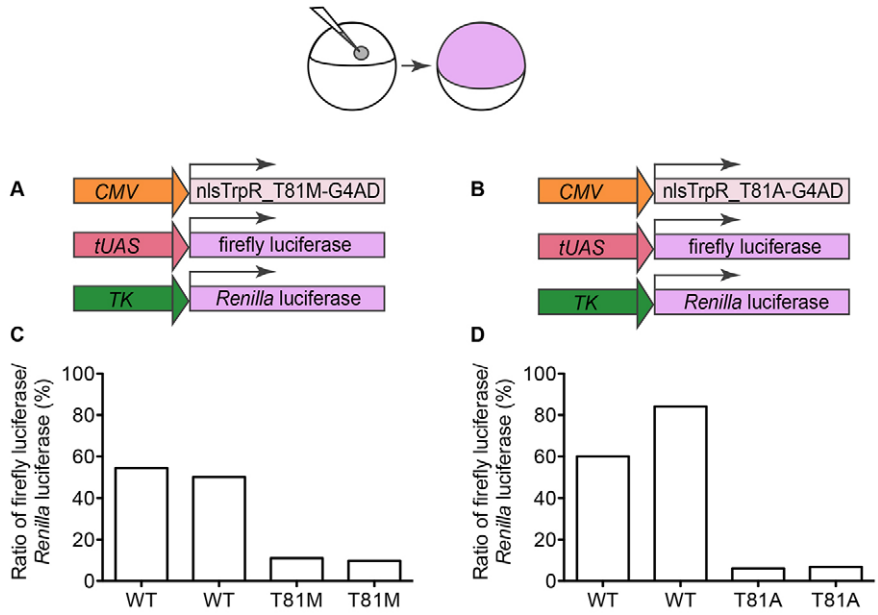

Fig. 6. Mutations in the TrpR DNA-binding domain reduce transcriptional activation of nlsTrpR-G4AD. A dual-luciferase reporter assay was used to assess transcriptional activity of two TrpR DNA-binding domain mutations: threonine 81 to methionine (T81M) (A) and threonine 81 to alanine (T81A) (B). Constructs were injected into one-cell zebrafish embryos and then luciferase activity was assayed at 70% epiboly. *Renilla* luciferase driven by the *thiamine kinase* (*TK*) promoter was used as a control. (C,D) The average fluorescence ratio in two sets of 30 embryos for each condition was plotted, showing that T81M is on average 5× less potent than wild-type TrpR (WT), and that T81A is 11× less potent. Readings were made in duplicate for each set.

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Gal80 can be used to shut off gene expression temporally in specific spatial domains.

The existence of an alternative bipartite system is also useful for combinatorial experiments. For example, using one promoter to drive Gal4 and another to drive TrpR it is possible to express combinations of effectors in overlapping domains. We note that TrpR is not the only other bipartite system available. The Q-system, which utilizes the regulatory genes normally needed for quinic acid catabolism in *Neurospora crassa*, has also been studied (Potter et al., 2010), although its *UAS* also contains CpG sites for methylation (GGGTAAT**CG**CTTATCC). LexA has been used in zebrafish to drive expression (Emelyanov and Parinov, 2008) and, in principle, the *lexA UAS* should not be silenced, although this system has not been studied over multiple generations as we have done here.

Although the TrpR/*tUAS* system worked well with a moderate level promoter, such as *myo6b*, we observed problems with the very strong promoter *neuroD* (McGraw et al., 2012). The embryos showed correct reporter expression at earlier developmental stages; however, we observed neuronal cell death and a general pericardial and periocular edema by 5 days post-fertilization. Although this does not preclude studies of the first few days of development using transient injections of driver constructs, it is problematic for the establishment of stable driver lines using strong promoters and analysis of larvae and adults. The observed toxicity could be due to a general titration of transcription factors from strong transcriptional activators known as 'squelching' (Gill and Ptashne, 1988; Habets et al., 2003) or to specific toxicity resulting from the TrpR protein spuriously binding a sequence in the zebrafish genome and activating a gene that is toxic to the embryo. When we generated fusion constructs of different transcriptional activators [Gal4AD and partially crippled VP16 (Asakawa et al., 2008; Distel et al., 2009)], with either TrpR or Gal4 and injected the *in vitro* transcribed mRNAs into embryos, we saw that *TrpR* mRNA was consistently more toxic than *Gal4* mRNA regardless of which transcriptional activator was fused to it (data not shown). This suggests that the TrpR transcriptional activator is toxic to cells when expressed from strong promoters and implies that, currently, TrpR/*tUAS* is compatible only with moderate or weak promoters. Since there is wide variability in the expression strength of constructs produced using the Tol2 system according to the site of integration, one solution to the problem of strong promoters is to screen F0 carriers for those that do not show toxicity due to lower levels of expression from the promoter. Alternatively, a suboptimal translation initiation site (Kozak sequence) could be placed in front of TrpR with strong promoters, thereby reducing TrpR levels. Although this does place some limitations on the system, many promoters are moderate to weak and will be well suited for this approach, and solutions are available with promoters that are naturally strong. This might also be a peculiarity of zebrafish, as we did not observe toxicity when expressing TrpR in HEK 293 cells.

In conclusion, the lack of silencing and the ability to tune transcriptional activation with different TrpR mutants makes TrpR/*tUAS* a valuable alternative to the existing Gal4/*gUAS* system. We have already produced two reporter lines (tRFP and nlsEOS), which will be accessible through the Zebrafish International Resource Center (ZIRC), and additional effector and driver lines can readily be made using our Gateway system clones.

MATERIALS AND METHODS

Constructs

p*tUAS*:DsRed

Using pDsRed-Express-N1 (Clontech) as a template, the whole *CMV* promoter was replaced by a *Hin*dIII-*Bgl*II-*Eco*RI linker using (5′-3′) TAAGCTTAGATCTGAATTCA and CCGGTGAATTCAGATCTAAGCT followed by insertion of three copies of the *tUAS* linker using GATCTGTACTAGTTAACTAGTACTCAGTCAGTCAGT and GATCACTGACTGACTGAGTACTAGTTAACTAGTACA into the *Bgl*II site. A minimal *CMV* promoter containing only the TATA box was PCR amplified from pDsRed-Express-N1 using primers GGGGAATTCTAGGCGTGTACGGTGGGA and GGGACCGGTGCGATCTGACGGTTCACTAAA and inserted into the *Eco*RI-*Age*I sites of multimerized (3×) TrpR *UAS* (*tUAS*)-containing construct.

p*tUAS*:firefly luciferase

The *UAS* region was amplified from p*tUAS*:DsRed using primers GGCGGCGGTACCGGATAACCGTATTACCGCCATGC and GGCGGCGGATCCGGTGGCGACCGGTGC, which add a *Kpn*I site to the 5′ end and a *Bam*HI site to the 3′ end, then cloned into the *Kpn*I and *Bgl*II sites of pGL3-Basic.

p*CMV*:nlsTrpR-G4AD

TrpR was amplified from *E. coli* using primers (the initiation codon is underlined) CGAATTCAGGATGGCACCCAAGAAGAAGAGGAAGGCCCAACAATCACCCTATTCAGC and CGTCGACCCATCGCTTTTCAGCAACACCTCTTC, which add *Eco*RI and *Sal*I sites along with a nls at the N-terminus. It was cloned into the CS2 vector, which contains a *CMV* promoter, after mutating an internal *Sal*I site without changing the protein sequence. The Gal4AD along with an SV40 polyadenylation site (Distel et al., 2009) were inserted between the *Sal*I and *Not*I sites to produce the fusion protein nlsTrpR-G4AD.

p*CMV*:nlsTrpR_T81M-G4AD and p*CMV*:nlsTrpR_T81A-G4AD

These plasmids were generated by mutagenizing p*CMV*:nlsTrpR-G4AD with CGGCGCAGGCATCGCGAtGATTACGCGTG and GGCGCAGGCATCGCGgCGATTACGCGTG (the lowercase base indicates the introduced mutation).

pDestTol2CV

cryaa:venus (abbreviated CV) was PCR amplified from p*ins*:cre_*cryaa*:venus plasmid (gift of D. Stainier, University of California, San Francisco, CA, USA) using primers GGCGGCAGATCTATTAATAGTGTGCATTCAGTGCAG and GGCGGCAGATCTCACCGCGGTGGCG, which add *Bgl*II sites, then cloned into the *Bgl*II sites of pDestTol2pA2 (Kwan et al., 2007).

pDestTol2CC

cryaa:cherry (abbreviated CC) was PCR amplified from p*hsp70l*:loxP-mCherry-STOP-loxP-H2B-GFP_*cryaa*:Cherry plasmid (gift of D. Stainier), then cloned as with pDestTol2CV using the same primers used for *cryaa:venus*.

pDestTol2CG2

A gift from K. Kwan and C. B. Chien (University of Utah, Salt Lake City, UT, USA) (Kwan et al., 2007).

p5E-*myo6b* and p5E-*neuroD*

Gifts form T. Nicolson (Oregon Health and Science University, Portland, OR, USA) (McGraw et al., 2012; Obholzer et al., 2008).

p5E-*ribA*

The *ribA* promoter (1.8 kb) was amplified from a p*ribeyeA:*ribeyeCherry plasmid (Odermatt et al., 2012) (gift from L. Lagnado, University of Cambridge, Cambridge, UK) using primers GGGGACAACTTTGTATAGAAAAGTTGCCAGGCTTTGAAGTCGTCACTC and GGGGACTGCTTTTTTGTACAAACTTGCTATACCTTACTCACAGGGAAG and Gateway cloned into pDONRP4-P1R.

p5E-t*UAS*

Multimerized (3×) TrpR *UAS* (*tUAS*) was PCR amplified from p*tUAS*:DsRed using primers GGGGACAACTTTGTATAGAAAAGTTGGGATGCATTAGTTATTAAGCTTAGATC and GACGTTCTCGGAGGAGGCCTGCAGGGCGACCGGTGCGATCTGA, which add *Hin*dIII and *Pst*I sites, and cloned into p5E MCS (Kwan et al., 2007) using *Hin*dIII and *Pst*I.

pME-nlsTrpR-G4AD

nlsTrpR-G4AD was PCR amplified from p*CMV*:nlsTrpR-G4AD using primers GGGGACAAGTTTGTACAAAAAAGCAGGCTGGACCATG-GCACCCAAGAAG and GGGGACCACTTTGTACAAGAAAGCTG-GGTGTGGTTTGTCCAAACTCATCAATG and Gateway cloned into pDONR221.

pME-gal80

A gift from J. Bonkowsky (University of Utah, Salt Lake City, UT, USA) (Fujimoto et al., 2011).

pME-nlsTrpR_T81M-G4AD and pME-nlsTrpR_T81A-G4AD

These were generated by site-directed mutagenesis of pME-nlsTrpR-G4AD as described above.

pME-nlsEos

This construct was described previously (Prendergast et al., 2012).

pME-tRFP, p3E-pA and p3E-IREStRFP

Gifts from K. Kwan and C. B. Chien (Kwan et al., 2007).

pTol2 constructs

pTol2-CV,*tUAS*:tRFP, pTol2-CV,*tUAS*:nlsEos, pTol2-CC,*myo6b*:nlsTrpR-G4AD, pTol2-CC,ribA:nlsTrpR-G4AD, pTol2-CC,*neuroD*:nlsTrpRG4AD, pTol2-CC,*neuroD*:nlsTrpR_T81M-G4AD, pTol2-CC,*neuroD*:nlsTrpR_T81A-G4AD and pTol2-CG2,*myo6b*:gal80IREStRFP were generated using the constructs above and Gateway technology (Kwan et al., 2007; Villefranc et al., 2007).

Transgenic lines

One-cell zebrafish embryos were microinjected with 25 pg DNA constructs and 25 pg Tol2 transposase RNA to generate *Tg(CV,tUAS:tRFP)*^{w80}, *Tg(CV,tUAS:nlsEos)*^{w81}, *Tg(CC,myo6b:nlsTrpR-G4AD)*^{w83} and *Tg(CC,ribA:nlsTrpR-G4AD)*^{w85} germline transgenics as previously described (Fisher et al., 2006). *Tg(CG2,myo6b:gfp)* was kindly provided by A. Coffin (Washington State University-Vancouver, Vancouver, WA, USA) and will be described elsewhere.

Luciferase assay

Dual-Luciferase Reporter Assay (Promega) was used to assess the efficiency of TrpR modulators. One-cell stage zebrafish embryos were injected with 10 pg driver construct (p*CMV*:nlsTrpR-G4AD, p*CMV*:nlsTrpR_T81M-G4AD or p*CMV*:nlsTrpR_T81A-G4AD), 20 pg p*tUAS*:firefly luciferase and 5 pg p*TK*:*Renilla* luciferase. They were grown to 70% epiboly, ground and luminescence was measured using a Victor plate reader (PerkinElmer) sequentially after application of firefly substrate and *Renilla* substrate.

Human cell line assay

The HEK 293 cell line was transfected in 24-well dishes using Lipofectamine 2000 (Life Technologies) and standard procedures with 1 ng driver construct (p*CMV*:nlsTrpR-G4AD, p*CMV*:nlsTrpR_T81M-G4AD or p*CMV*:nlsTrpR_T81A-G4AD) and 50 ng p*tUAS*:firefly luciferase reporter construct per well plated with ~100,000 cells per well the day before transfection. Luminescence was measured as described above.

Immunohistochemistry and confocal microscopy

Larvae were fixed with 4% paraformaldehyde for 2 hours at room temperature or overnight at 4°C, washed three times for 20 minutes each with PBST (0.1% Tween 20 in PBS) and incubated for 1 hour in distilled water. They were placed in block solution (1% BSA, 1% DMSO and 0.02% sodium azide in PBST, 10% normal goat serum) for 1 hour and then incubated with anti-Parvalbumin antibody (Millipore MAB1572; 1:400) overnight at 4°C. After four 20 minutes washes with PBST, they were incubated with secondary antibody (mouse anti-IgG1 Alexa488; Invitrogen) for 3 hours at room temperature, washed four times for 10 minutes each in PBST, and cleared in 50% glycerol/PBS. Embryos were imaged using an Olympus FV1000 confocal microscope.

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Competing interests

The authors declare no competing financial interests.

Author contributions

A.S. developed the approach, performed experiments, analyzed data and prepared the manuscript. A.D.G. developed the approach, performed experiments, analyzed data and edited the manuscript. D.W.R. developed the approach, analyzed data and prepared the manuscript. D.K. developed the approach, performed experiments, analyzed data and prepared the manuscript.

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Supplementary material

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Supplemental Figure 1

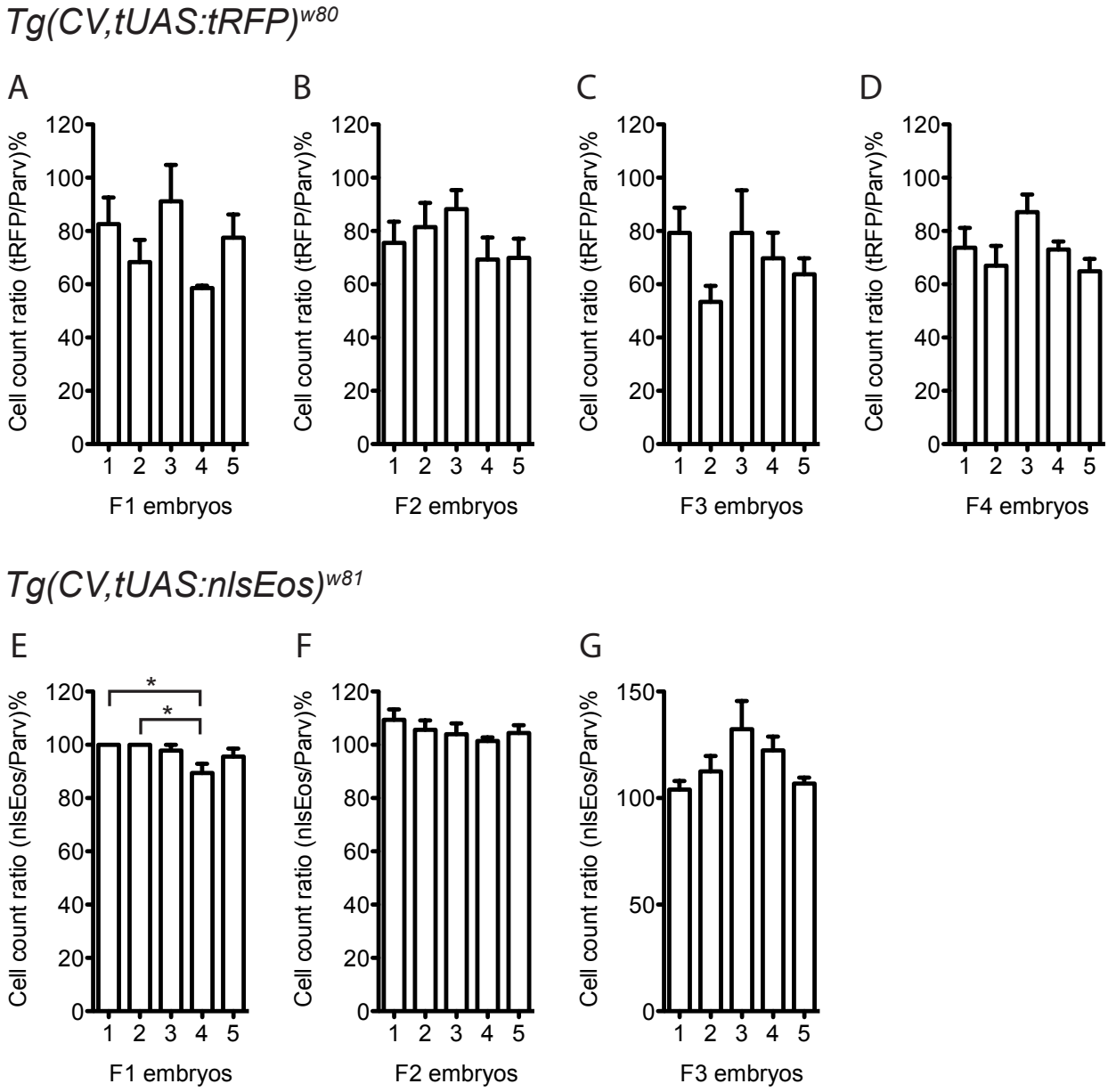

Fig. S1. *tUAS* expression is consistent between larvae of the same clutch. To determine if tUAS is silenced within a clutch we compared tRFP and nlsEos expression in five different larvae for each cross between F2 or F3 of *Tg(CC,myo6b:nlsTrpR-G4AD)*^{w83} driver line with F1-F4 of *Tg(CV,tUAS:tRFP)*^{w80} reporter line (A-D) or with F1-F3 of *Tg(CV,tUAS:nlsEos)*^{w81} reporter line (E-G). Progeny double positive for the lens markers were sorted out and stained for Parvalbumin, which labels mature hair cells. Head neuromasts, consisting of hair cell clusters, were scored for tRFP and Parvalbumin expression or nlsEos and Parvalbumin expression. In each case, hair cell expression in 5 neuromasts from each embryo at 5 dpf were analyzed and graphed. There is no statistical difference between expression in embryos of the same clutch in F1-F4 generations of *Tg(CV,tUAS:tRFP)*^{w80} and F2-F3 generations of *Tg(CV,tUAS:nlsEos)*^{w81}. We see a difference in F1 embryos of *Tg(CV,tUAS:nlsEos)*^{w81} (E), although this difference might be due to multiple insertions present in the F1 generation. In all cases, statistical analysis was 1 way ANOVA with Tukey's pair-wise column comparison post-test. Error bars show SEM. In (E), F=3.741, R^2=0.428, P=0.0198.