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Chicken β -globin insulators fail to shield the *nkx2.5* promoter from integration site effects in zebrafish

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Abstract

Genetic lineage tracing and conditional mutagenesis are developmental genetics techniques reliant on precise tissue-specific expression of transgenes. In the mouse, high specificity is usually achieved by inserting the transgene into the locus of interest through homologous recombination in embryonic stem cells. In the zebrafish, DNA containing the transgenic construct is randomly integrated into the genome, usually through transposon-mediated transgenesis. Expression of such transgenes is affected by regulatory features surrounding the integration site from general accessibility of chromatin to tissue-specific enhancers. We tested if the 1.2 kb cHS4 insulators derived from the chicken β -globin locus can shield a transgene from chromosomal position effects in the zebrafish genome. As our test promoters, we used two different-length versions of the zebrafish *nkx2.5*. We found that flanking a transgenic construct by cHS4 insulation sequences leads to overall increase in the expression of *nkx2.5:mRFP*. However, we also observed a very high degree of variability of mRFP expression, indicating that cHS4 insulators fail to protect *nkx2.5:mRFP* from falling under the control of enhancers in the vicinity of integration site.

Keywords

Zebrafish; Transposon; Position effect; Insulator; Transgenesis; *nkx2.5*

Introduction

Relative ease of maintenance and breeding combined with the fast external development of optically transparent embryos are among the biological advantages, which enabled rapid ascent of zebrafish as the developmental biology model system of choice. This ascent was greatly facilitated by the development of efficient transgenesis techniques, from the first

expression of the green fluorescent protein (GFP) (Chalfie et al. 1994; Amsterdam et al. 1995) to the application of transposable elements (Raz et al. 1998; Kawakami and Schima 1999; Kawakami et al. 2000; Davidson et al. 2003; Balciunas et al. 2004), to recent advances in bacterial artificial chromosome (BAC) transgenesis (Suster et al. 2009, 2011; Bussmann and Schulte-Merker 2011), and insertional mutagenesis (Köster and Fraser 2001; Golling et al. 2002; Kawakami et al. 2004; Parinov et al. 2004; Sivasubbu et al. 2006; Koga et al. 2008; Clark et al. 2011; Maddison et al. 2011). Transgenic lines are typically generated using cassettes consisting of three primary components: a promoter, a transgene of interest (such as a fluorescent protein or a site specific recombinase) and a transcriptional termination/polyadenylation sequence. Early transgenesis experiments in mouse embryos clearly demonstrated that the genomic context of an integration site influences such transgenes both in terms of level of expression, as well as tissues in which the transgene is expressed (see examples in Palmiter and Brinster 1986). The ability of a reporter transgene to recapitulate the expression pattern of a nearby gene was adapted and extensively used in enhancer detection (enhancer trapping) in *Drosophila melanogaster* (Bellen et al. 1989; Bier et al. 1989; reviewed in Bellen 1999). Naturally, the same phenomenon was observed when scientists started generating transgenic zebrafish (Bayer and Campos-Ortega 1992). Transgenic lines arising from such serendipitous events sometimes turn out to be quite valuable. One of the very first GFP-transgenic zebrafish lines used to facilitate an organ-specific chemical mutagenesis screen—*gutGFP^{SS84}*—is known to be an enhancer trap (Field et al. 2003). For practical applications, high predictability and specificity of expression in transgenic lines is preferred over the low probability of finding new and exciting transgene expression patterns. Since it is not yet feasible to routinely “knock-in” a transgene into the locus of interest in zebrafish, the solution of highest fidelity may be to recombineer the transgene into a BAC, and then use the BAC to establish a transgenic line. However, even with recent advances in BAC recombineering and transgenesis (Suster et al. 2009, 2011; Bussmann and Schulte-Merker 2011), the process is still too labor intensive for many laboratories. Furthermore, BACs may be subject to position effects, too, as enhancers are known to act across long distances (Ellingsen et al. 2005; Jeong et al. 2006; Ragvin et al. 2010).

We decided to test if flanking a transgene with insulator elements derived from the chicken β -globin locus hypersensitive site-4 (cHS4 hereafter) would improve the fidelity of expression in zebrafish. This insulator has been shown to protect against position effect in *Drosophila* (Chung et al. 1993). Furthermore, cHS4 was demonstrated to limit the ability of enhancers to activate a minimal promoter in zebrafish (Bessa et al. 2009). Even though the main (but not all) insulating activity of the cHS4 element has been demonstrated to reside within a fragment of 250 bp (Chung et al. 1997), we opted to use a more complete 1.2 kb sequence. As our “model” promoter, we chose the zebrafish *nkx2.5* promoter for two reasons: first, we were interested in using it as early cardiac-specific driver for the expression of Cre recombinase and other transgenes. Second, we wanted to use a promoter with a complex regulatory input, and studies of the mouse *Nkx2.5* promoter provided evidence that multiple enhancers are responsible for expression in different parts of the developing heart and other organs. At least some of the mouse regulatory elements were present within a 4 kb region upstream of ATG (Searcy et al. 1998; Lien et al. 1999; Reecy et

al. 1999). An even more complete expression pattern of *Nkx2.5* was revealed by analysis of transgenic BAC-GFP mice carrying a 120 kb region of the *Nkx2.5* gene (Chi et al. 2003). The data uncover the existence of additional distant transcription regulatory regions of the *Nkx2.5* gene and again confirm the complexity of genetic regulatory elements. Several transgenic zebrafish lines using *nkx2.5* to drive expression of different reporters were generated by others, while our study was ongoing: *Tg(nkx2.5:EGFP)* (Witzel et al. 2012), *Tg(nkx2.5:GAL4-VP16)* (Choe et al. 2013), *TgBAC(-36nkx2.5:ZsYellow)* (Zhou et al. 2011), *TgBAC(-36nkx2.5:ERT2CreERT2)*, *TgBAC(-36nkx2.5:Kaede)* (Guner-Ataman et al. 2013).

We constructed two transgenes containing the single intron and genomic fragments encompassing 3.3 and 6.1 kb upstream of the *nkx2.5* ATG, intron 1, part of exon 2, and mRFP reporter. We found that flanking *nkx2.5:mRFP* transgenes with insulator elements results in increased transgene expression, but does not protect the transgene from nearby enhancers. In contrast, cHS4-flanked transgenes appear to be especially susceptible to regulation by enhancers in the vicinity of integration site.

Materials and methods

Transgene construction and transgenesis

Exact details on transgene construction are available upon request. All transgenic constructs are based on miniTol2 (Balciunas et al. 2006) and contain GFP under the lens-specific gamma-crystalline promoter (*crygc*) (Davidson et al. 2003) as a transgenesis selection marker. The 4.8 kb *nkx2.5* promoter (Fig. 1a) is flanked by PCR primers NotI-Nkx2.5-3F3 (5'-GCGGCCGCGTAGACTGAGTATTCGTTGCGTTAT-3') and Nkx2.5-R11 (5'-ACCCGGGCTTCCTGACAACAGCCGA-3'). The 7.6 kb *nkx2.5* promoter (Fig. 1a) is flanked by PCR primers NotI-NKX2.5-6F1 (5'-gcccgcgcgagaaatcagtcacgtg ggc-3') and Nkx2.5-R11 (5'-ACCCGGGCTTCCTGACA ACAGCCGA-3'). Two chicken β -globin insulator fragments were amplified by PCR using primers cHS4/Swa-F1 (5'-gATTTAATagctcacggggacagccccc-3') and cHS4/Not-R1 (5'-tGCGGCCGCACTAGTaatattctcactgactccgtc-3') (Swa-Not fragment) or cHS4/Nhe-F1 (5'-aGCTagctcacggggacagccccc-3') and cHS4:Swa-R1 (5'-gATTTAaatattctcactgactccgtc-3') (Nhe-Swa fragment). Both PCR fragments were sequentially cloned into miniTol2 (Balciunas et al. 2006) to generate *Tg(CHS4,-3.3nkx2.5:RFP,crygc:GFP,CHS4)* (*-3.3nkx2.5:RFP/INS* hereafter) and *Tg(CHS4,-6.1nkx2.5:RFP,crygc:GFP,CHS4)* (*-6.1nkx2.5:RFP/INS* hereafter) constructs. The *Tg(-6.1nkx2.5:RFP-pp(A),crygc:GFP)* (*-6.1nkx2.5:RFP-pp(A)* hereafter) plasmid was made by removing both insulators from *-6.1nkx2.5:RFP/INS*. The 5' insulation sequence was removed with *SacI* and *NotI* restriction enzymes. The lost Tol2 fragment was reconstituted from pDB773 plasmid. Next, the 3' insulator was cut out with *ApaI* and *BstBI* enzymes. The *Tg(-6.1nkx2.5:RFP-zp(A),crygc:GFP)* (*-6.1nkx2.5:RFP-zp(A)* hereafter) construct was generated by replacing ocean pout antifreeze protein transcription termination/polyadenylation sequence (polyA) in *-6.1nkx2.5:RFP-pp(A)* with zebrafish β -actin polyadenylation sequence from pDB713 using *AvrII* and *AatII* restriction enzymes.

We used standard miniTol2-mediated transgenesis protocols (Balciunas et al. 2006). All plasmids were prepared using the Qiagen miniprep kit including the optional PB wash step. Immediately before injection, plasmids were diluted to 10 ng/μL in nuclease-free water (Ambion) and 8 μL of the dilution was mixed with a 2 μL aliquot of in vitro transcribed Tol2 mRNA. Yolks of 1-cell zebrafish embryos were injected with 3 nL of the DNA/RNA mixture. Embryos were screened for GFP positive lens and raised until adulthood. Adult fish were incrossed and their progeny screened to identify three F₀ founders with germline transmission of the transgene. Three F₁ families from three different founders were produced for each transgene. Three F₁ fish from each family were outcrossed, and their progeny was analyzed for RFP expression patterns at 3 *d*pf.

Image acquisition and manipulation

All images were acquired on a Zeiss AxioImager microscope with a Spot RT3 camera. All mRFP fluorescence images were taken using identical illumination and settings of Spot Advanced software, and all GFP fluorescence images were taken using identical illumination and settings on Spot Advanced software. Furthermore, all RFP images were identically enhanced in Adobe Photoshop (brightness increased 85 %, contrast increased 30 %) so that the images on the computer screen would subjectively correspond what is observed on the microscope. Identical conditions of photography and identical image manipulation enable direct comparison between different figures.

Results

To test if chicken β-globin hypersensitive site 4 (cHS4), a well-characterized insulator element (Chung et al. 1993), is able to protect a transgene from position site effects, we built four different constructs (Fig. 1). The first construct, *-3.3nkx2.5:RFP/INS* for brevity, contains a 4.8 kb promoter, exon 1 and intron 1 of *nkx2.5* driving mRFP followed by ocean pout antifreeze protein (p(A)) containing a potential boundary element (Gibbs and Schmale 2000) (Fig. 1b). It also contains a lens-specific *crygc:GFP* cassette from pT2/Cry:GFP (Davidson et al. 2003) as a transgenesis marker. Both cassettes are flanked by 1.2 kb cHS4 elements in direct orientation. The second construct, *-6.1nkx2.5:RFP/INS*, is similar to *-3.3nkx2.5:RFP/INS*, but has a longer 7.6 kb *nkx2.5* promoter/intron fragment (Fig. 1b). The third construct, *-6.1nkx2.5:RFP-pp(A)*, is similar to *-6.1nkx2.5:RFP/INS*, but lacks cHS4 insulator elements. Finally, *-6.1nkx2.5:RFP-zp(A)* is similar to *-6.1nkx2.5:RFP-pp(A)*, but the ocean pout antifreeze protein polyA is replaced with zebrafish β-actin polyA from GBT-R15/pDB713 (Petzold et al. 2009), thus removing the potential boundary element. All constructs were built in miniTol2 transposon (Balciunas et al. 2006).

All four transgenes were injected into the yolks of 1-cell zebrafish embryos using standard Tol2-mediated transgenesis techniques (Balciunas et al. 2006; Balciuniene and Balciunas, in press). Injected embryos displaying RFP and GFP expression were raised to adulthood and screened for germline transmission of transgenes by incross. For each of the four constructs, fluorescence-positive embryos were obtained from three independent incrosses; thus, representing at least three independent integrations into the genome for each construct (Table 1).

We observed significant differences in mRFP expression patterns among transgenic F1 embryos obtained with both constructs flanked by insulator sequences (Fig. 2). Most of the transgenic embryos generated with the shorter $-3.3nkx2.5$ promoter/intron element displayed mRFP expression in the heart, indicating that the 4.8 kb promoter/intron fragment is likely to have some heart-specific regulatory elements (Fig. 2a–g). However, the differences in mRFP expression patterns were extremely pronounced, ranging from very broad (Fig. 2c, e, i), to highly specific (Fig. 2d, f, h, j). Thus, the 4.8 kb $nkx2.5$ promoter/intron fragment appears to be highly susceptible to the influence from genomic enhancers. This observation was strengthened further when we outcrossed one of the F1s obtained with $-3.3nkx2.5:RFP/INS$. This F1 harbors multiple unlinked transgene integrations, as 40/43 (93 %) of embryos obtained from the outcross were positive for RFP and lens GFP. We recovered 5 distinct mRFP expression patterns among the F2 embryos (Fig. 2i–m).

All transgenics with the longer 7.6 kb promoter/intron element displayed mRFP expression in the heart (Fig. 2p–w), supporting the notion that the $-6.1nkx2.5$ promoter/intron part contains one or more heart-specific enhancers. When compared with the shorter $nkx2.5$ promoter, we observed a lower degree of variation in mRFP expression, suggesting that the larger promoter is less susceptible to position effects. Nonetheless, we did recover lines with mRFP expression in the nervous system, indicative of position effects (Fig. 2t, u).

Insulators have been demonstrated to shield transgenes from surrounding repressive chromatin (Chung et al. 1993; Emery et al. 2000; Rivella et al. 2000; Rincón-Arano et al. 2007; Yahata et al. 2007; Ochiai et al. 2011; Sharma et al. 2012; Uchida et al. 2013). Therefore, we decided to test if insulator-flanked transgenes display stable expression of mRFP and GFP in zebrafish. We identified an F1 fish with the $-3.3nkx2.5:RFP/INS$, which produced 50 % of embryos positive for GFP and RFP, indicating a single-copy integration. We then compared the expression of mRFP and GFP among F2 embryos (Fig. 3). To our surprise, we observed that while most embryos displayed robust, somewhat variable mRFP and GFP expression (Fig. 3a–c), mRFP expression was highly reduced in some embryos. Reduced mRFP expression correlated with reduced GFP expression (Fig. 3d–f). Thus, cHS4 insulators do not offer complete protection from repressive chromatin.

Next, we tested if a transgene lacking cHS4 insulators would be as susceptible to position effects. We also wanted to test if replacing ocean pout antifreeze protein polyA, thought to contain a putative boundary element, with zebrafish β -actin polyA, would further increase susceptibility to position effects. We decided to use the longer $-6.1nkx2.5$ promoter because it displayed higher specificity in previous experiments (compare variability in Fig. 2a–o to Fig. 2p–w). We found mRFP expression from transgenes lacking cHS4 insulators to be significantly lower and more restricted to the heart (Fig. 4), consistent with observation by others (Witzel et al. 2012). However, some embryos displayed a low level of mRFP expression in the hindbrain, forebrain, branchial arches and ear (Fig. 4c), again indicating susceptibility to position effects. Finally, we noted that the overall level of mRFP and GFP expression was higher when ocean pout polyA was used compared with the zebrafish beta actin polyA (Fig. 4a–f), consistent with previous observations made during construction of GBT-RP2 (Clark et al. 2011; Balciunas and Ekker, unpublished observation).

Discussion

We tested the ability of chicken HS4 insulators to shield a promoter from position effects in the developing zebrafish embryo. As our test promoter, we used two different-length variants of *nkx2.5*, *-6.1nkx2.5* and *-3.3nkx2.5*, containing exon 1, intron 1 and the beginning of exon 2 of the gene. To test for heart-specific expression, we fused these potential promoter regions to mRFP. We found that both *-6.1nkx2.5* and *-3.3nkx2.5* regions contain one or more enhancers required to drive transgene expression in the embryonic heart. All transgenic embryos generated with *-6.1nkx2.5:RFP* had mRFP expression in the heart, while two of the transgenic lines generated with *-3.3nkx2.5:RFP* lacked RFP expression in the heart, suggesting that there may be a heart-specific enhancer missing in the smaller construct. A larger number of transgenic animals would need to be generated to validate this observation. We also noted that even with the larger promoter fragment, mRFP fluorescence in the hearts of transgenic F2 embryos was relatively weak. This weakness of expression may be attributed to the way our transgenes were designed, or it may reflect inherent weakness of the tested promoter fragments. With regard to transgene design, we opted to fuse mRFP to the 112-amino acid N-terminal peptide of Nkx2.5 to include the intron of the gene. This may impede folding of mRFP or may affect the stability of the fusion protein. We also compared two nearly identical transgenes, only different in that one contained zebrafish β -actin polyA from the GBT-R15 gene trap and the second contained ocean pout antifreeze protein polyA from the GBT-RP2 gene trap (Petzold et al. 2009; Clark et al. 2011). Consistent with previous observations (Balciunas and Ekker, unpublished), mRFP and GFP expression was markedly stronger when ocean pout antifreeze protein polyA was used. Increased expression of mRFP may be readily explained by improved polyadenylation (and thus stability) of transcript. However, increased expression of GFP indicates that the presence of ocean pout polyA with a putative boundary element improved the transcriptional availability of gamma-crystallin promoter (*crygc*).

We chose the *nkx2.5* promoter to test enhancer-blocking ability of cHS4 insulators based on two premises. First, based on experiments in the mouse, we expected that *nkx2.5* would respond to a complex regulatory input (Lien et al. 1999; Reecy et al. 1999; Chi et al. 2003). Second, we expected that promoters with complex regulatory input might be more susceptible to falling under the control of nearby enhancers upon integration into the genome. In line with our expectation, we found that both *-6.1nkx2.5* and *-3.3nkx2.5* are highly susceptible to upregulation in the ectopic tissues, presumably due to enhancers nearby the integration site. The behavior of *-3.3nkx2.5* and *-6.1nkx2.5* is markedly different from the behavior of the *Xenopus laevis crygc* promoter included to mark our transgenes. We did not observe ectopic expression GFP under the control of *crygc*, indicating that this promoter is not susceptible to falling under the control of nearby enhancers. However, the strength of *crygc:GFP* expression was clearly different between different transgenic lines, suggesting that strength of expression of *crygc:GFP* does depend on general features of surrounding chromatin.

We were surprised to find that flanking a transgene with full-length (1.2 kb) cHS4 insulators did not abrogate position effects. The *-6.1nkx2.5:RFP* construct was at least as, if not more susceptible to falling under the control of nearby enhancers when flanked by cHS4

insulators. Although we did not test the $-3.3nkx2.5:mRFP$ construct without insulators, an insulator-flanked version was exceptionally susceptible to nearby enhancers. It is likely that a different experimental setup resulted in apparent differences between our results and those of Bessa et al. (2009). Bessa and colleagues used an experimental setup testing the ability of a defined small enhancer element to activate a minimal promoter and carried out the majority of their experiments in injected F0 zebrafish embryos. In contrast, we used a relatively large weak promoter and probed its susceptibility to genomic enhancers in transgenic F1/F2 embryos. Since we only tested a single promoter, it remains to be determined whether cHS4 insulators would be able to protect other large, complex promoters from position effects.

It is also important to note that flanking the $-6.Inkx2.5:RFP$ with cHS4 insulators resulted in markedly increased expression of mRFP. This observation is quite consistent with the most frequent use of cHS4 insulators in mouse genetics: to increase transgene expression and reduce silencing of transgenes (Emery et al. 2000; Rivella et al. 2000; Yannaki et al. 2002; Yao et al. 2003; Rincón-Arango et al. 2007; Yahata et al. 2007; Ochiai et al. 2011; Sharma et al. 2012; Emery et al. 2013; Uchida et al. 2013). Insulators are most often tested for this activity in cell culture-based assays and our observation of increased transgene expression is completely consistent with the outcomes of these experiments. However, it is nearly impossible to test tissue specificity of transgene expression in cell culture experiments. Our *in vivo* assays clearly demonstrate that additional elements of the cHS4 locus are required to achieve complete blockage of enhancer activity and argue for more cautious interpretation of experiments performed *in vitro*.

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Fig. 1.

Diagrams of zebrafish *nkx2.5* genomic region (**a**) and different transgenes used in this study (**b**). Solid maroon boxes denote exons of *nkx2.5*, with an ATG translation start codon and TGA termination codon indicated. Black pentagons are miniTol2 inverted terminal repeats (Balciunas et al. 2006). Light pink and maroon are *nkx2.5* genomic regions. A red arrow is monomeric RFP (*mRFP*), a gray box (*pp(A)*) is ocean pout antifreeze protein transcriptional termination and polyadenylation sequence (Gibbs and Schmale 2000; Clark et al. 2011), a yellow box (*zp(A)*) is zebrafish β -actin transcriptional termination and polyadenylation sequence (Petzold et al. 2009; Clark et al. 2011). All transgenes are marked by a *crygc:GFP* cassette (Davidson et al. 2003) consisting of *Xenopus laevis* gamma-crystallin promoter (orange box), GFP (green arrow) and SV40 polyadenylation site (white box)

Fig. 2.

Different mRFP expression patterns in transgenic embryos carrying insulator-flanked $-3.3nkx2.5:RFP/INS$ and $-6.1nkx2.5:RFP/INS$ transgenes. All embryos are shown from the left side, with the anterior positioned to the left and the posterior to the right. **a–g** F1 embryos with $-3.3nkx2.5:RFP/INS$ (**a** progeny of parent A; **b–d** parent B; **e–g** parent C). **h–o** F2 progeny with $-3.3nkx2.5:RFP/INS$ (**h** F2 progeny from parent A; **i–m** parent B; **n, o** parent C). **p–s** F1 progeny with $-6.1nkx2.5:RFP/INS$ (**p** parent A; **r** parent B; **s** parent C). **t–w** F2 progeny with $-6.1nkx2.5:RFP/INS$ (**t** parent A; **u, v** parent B; **w** parent C). A yellow arrow points to heart-specific mRFP. *ba* branchial arches, *e* eye, *fb* forebrain, *h* heart, *hb* hindbrain, *mb* midbrain, *sc* spinal cord

Fig. 3.

Variegation of expression in F2 embryos obtained from an F1 with a single-copy *-3.3nkx2.5:RFP/INS* integration. All embryos are shown from the left side, with the anterior positioned to the left and the posterior to the right. *The insert in the upper right corner of each picture demonstrates lens GFP fluorescence.* Note the variability of mRFP expression from high (**a–c**) to very weak (**d–f**) and a correlation between strength of mRFP and GFP expression. A yellow arrow points to heart-specific mRFP. *ba* branchial arches, *e* eye, *fb* forebrain, *h* heart, *hb* hindbrain, *mb* midbrain, *sc* spinal cord

Fig. 4.

Transgenes with $-6.1nkx2.5:RFP$ without insulators exhibit lower levels and more heart-restricted mRFP expression. All embryos are shown from the left side, with the anterior positioned to the left and the posterior to the right. *The insert in the upper right corner of each picture demonstrates lens GFP fluorescence.* The presence of ocean pout antifreeze protein polyadenylation sequence (*pp(A)*) leads to higher expression of both transgenes as compared to zebrafish β -actin polyadenylation sequence (*zp(A)*). **a–c** Progeny of F1s with $-6.1nkx2.5:RFP-pp(A)$ (**a** parent A; **b** parent B; **c** parent C). **d–f** Progeny of F1s with $-6.1nkx2.5:RFP-zp(A)$ (**d** parent A; **e** parent B; **f** parent C). A yellow arrow points to heart-specific mRFP. *h* heart, *hb* hindbrain, *mb* midbrain

Table 1

Transgenesis and copy number data for all *nkx2.5:mRFP* constructs used

Transgene	F1 family	# of F2 embryos		# of mRFP patterns	
		Total #	%	Lens GFP+	%
<i>Tg(CHS4, -3.nkx2.5:RFP, crygc:GFP, CHS4)</i>	A	32	21	65.62	11
	B	103	97	95.09	5
	C	43	40	93.02	3
	D	68	34	50	34
<i>Tg(CHS4, -6.1nkx2.5:RFP, crygc:GFP, CHS4)</i>	A	50	35	70	15
	B	146	107	73.29	39
	C	23	8	34.78	15
<i>Tg(-6.1nkx2.5:RFP-pp(A), crygc:GFP)</i>	A	41	38	92.68	3
	B	28	26	92.86	2
	C	29	26	89.66	3
<i>Tg(-6.1nkx2.5:RFP-zp(A), crygc:GFP)</i>	A	87	45	51.72	42
	B	136	24	17.65	112
	C	34	17	50	17

For each construct, F1 generation from at least three independent F0 crosses was obtained (Families A–D, second column). From each F1 family, one F1 was chosen for outcross. Obtained F2 embryos were analyzed for lens GFP fluorescence at 3 *dpf* (columns 3–6) and the number of different mRFP expression patterns observed at 1–3 *dpf* was noted (column 7)