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Novel vectors for functional interrogation of *Xenopus* ORFeome coding sequences

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Abstract

The current *Xenopus* ORFeome contains ~10,250 validated, full-length cDNA sequences without stop codons from *Xenopus laevis* and ~3,970 from *Xenopus tropicalis* cloned into Gateway-compatible entry vectors. To increase the utility of the ORFeome, we have constructed the Gateway-compatible destination vectors pDXTP and pDXTR, which in combination can control the spatial and temporal expression of any open reading frame (ORF). pDXTP receives a promoter/enhancer of interest, which controls the spatial expression of a doxycycline-inducible transcription factor rtTA. pDXTR receives an ORF of interest, which is controlled by a tetracycline response element enabling temporal control of ORF expression via rtTA activation by simple addition of doxycycline to the rearing water at any desired time point. These vectors can be integrated into the genome via well-established microinjection-based SceI, tol2, or phi-C31 transgenesis procedures and contain fluorescence reporters to confirm transgene integration. Cell-autonomous verification of ORF expression occurs via red nuclear fluorescence due to an mCherry-histone H2B fusion protein that is cleaved from the ORF during translation. Function of all essential features of pDXTP and pDXTR has been experimentally validated. pDXTP and pDXTR provide flexible molecular cloning and transgenesis options to accomplish tissue-specific inducible control of ORF expression in transgenic *Xenopus*.

KEYWORDS
doxycycline, ORFeome, rtTA, tetracycline response element, *Xenopus* transgenesis

1 | INTRODUCTION

The *Xenopus* ORFeome is composed of full-length, validated, open reading frame (ORF) clones representing an estimated 10,250 genes for *Xenopus laevis* and 3,970 genes for *Xenopus tropicalis*, and is fully searchable on Xenbase, the *Xenopus* model organism database (Karimi et al., 2018). The production of the *Xenopus* ORFeome was a major advance in the ability of *Xenopus* as a model system to reveal developmental roles of proteins by promising to greatly facilitate protein function studies (Grant et al., 2015; Horb et al., 2019). The rate-limiting step in protein function studies can often be subcloning ORFs into different types of expression plasmids (e.g., CMV or SP6 promoters) with the appropriate epitope tags (e.g., GFP, flag, etc.) for the many different types of functional experiments that researchers perform. To facilitate cloning, the *Xenopus* ORFeome was constructed in Gateway-based plasmids, which streamlines the cloning process by utilizing efficient in-vitro reactions with precise unidirectional cloning enabling rapid in-frame transfer of the ORFs into a wide variety of Gateway-compatible expression vectors for functional experiments (Katzen, 2007). There are many destination vectors, some specifically designed for use in *Xenopus*, including the pTransgenesis system (Love et al., 2011), and several produced in association with the *Xenopus*

ORFeome (Grant et al., 2015). However, no Gateway-compatible vectors capable of receiving ORFs from the ORFeome for the purpose of genome engineering were previously available.

To address this need and facilitate functional genomics in *Xenopus*, we generated two destination vectors for transgenesis, pDXTP and pDXTR, to enable tissue-specific and inducible control of ORF expression (Figure 1). The destination vectors reported here use the Tet-On expression system shown to work in *Xenopus* (Das & Brown, 2004; Kerney, Brittain, Hall, & Buchholz, 2012; Rankin, Zorn, & Buchholz, 2011), which is comprised of: (a) a promoter component controlling tissue specificity and (b) an ORF component regulating inducible expression of a transgene of interest. A significant advantage of binary expression systems comes from the combinatorial possibilities of having separate promoter and ORF transgenic lines, which multiplies the combinations of tissue specificity and transgenes of interest readily available to researchers. Here, we describe the construction and use of these Gateway-compatible *Xenopus* ORFeome destination vectors.

2 | RESULTS AND DISCUSSION

2.1 | Design of pDXTP and pDXTR

The motivation to make pDXTP and pDXTR (pDestination *Xenopus* Transgenesis Promoter/Red) stemmed from the desire to enable Gateway-compatible transfer of ORFs from the *Xenopus* ORFeome into transgenesis plasmids capable of tissue-specific inducible control of ORF expression using the rtTA-TRE transgene expression system (Figure 1). The vector pDXTP (GenBank accession MN044709) was designed to receive promoters via Gateway cloning from the pTransgenesis set of plasmids to control expression of rtTA (Figure 1b; Love et al., 2011). The vector pDXTR (GenBank accession MN044710) was designed to receive an ORF via Gateway cloning from the *Xenopus* ORFeome under control of the TRE promoter, which is inducibly activated by rtTA and doxycycline (Figure 1c). pDXTR also has a multiple cloning site to insert an optional promoter controlling rtTA. ORFs can be induced from pDXTR alone (with a promoter and an ORF) or ideally from the combination of both pDXTP (with a promoter) and pDXTR

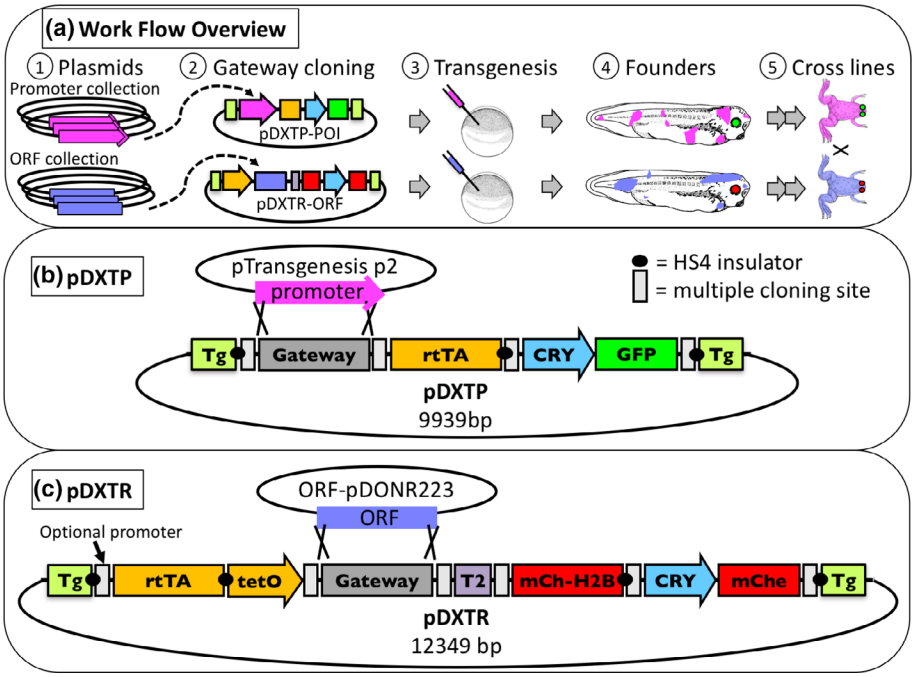


FIGURE 1 pDXTP and pDXTR workflow and plasmid diagrams. (a) Work flow overview. (1 and 2) a promoter of interest (POI) from a gateway-compatible promoter plasmid (e.g., from pTransgenesis) and an ORF from the *Xenopus* ORFeome are cloned into pDXTP and pDXTR, respectively. (3 and 4) these transgenesis plasmids are injected into eggs to make mosaic founders that may be analyzed in the F0 generation. (5) transgenic lines may be characterized and then crossed with each other to obtain double-transgenic offspring capable of tissue-specific and inducible control of ORF expression. (b) pDXTP plasmid diagram. pDXTP receives promoters via restriction enzyme or gateway cloning, and these promoters control expression of the doxycycline-responsive transcription factor rtTA. (c) pDXTR plasmid diagrams. pDXTR receives ORFs from the *Xenopus* ORFeome housed in pDONR223 vectors via gateway cloning. The ORF is under inducible control by virtue of the tetracycline-responsive element (TRE) upstream of the ORF. Gateway cloning puts the ORF in frame with peptidase recognition sequence T2A and mCherry and *Xenopus* histone H2B. Both pDXTP and pDXTR plasmids contain transgenesis (Tg) cassettes capable of phiC31, tol2, and SceI methods of transgenesis. The three black dots in pDXTP and the four black dots in pDXTR represent chicken HS4 insulator sequences to protect against undesired enhancer activity from the genomic insertion site and from the promoter controlling rtTA. The gray boxes represent multiple cloning sites to insert promoters, exchange N- or C-terminal tags, and transgenesis reporter cassettes. pDXTP has the transgenesis reporter cassette driving green fluorescent protein (GFP) in the lens of the eye from the gamma crystallin promoter (CRY), and pDXTR has the transgenesis reporter cassette driving mCherry fluorescent protein in the lens of the eye. The rtTA in pDXTR is not expressed unless a promoter is cloned into the MCS upstream of it

TABLE 1 Annotated pDXTP feature list and position on plasmid

1. 5′ transgenesis cassette	22–1,045
a. Transgenic features (to insert plasmid into genome)	
i. φC31 integrase *attB* site (34 bp minimal sequence)	22–55
ii. I-SceI 5′ site (TAGGG/ATAA\CAGGGTAAT)	56–73
iii. Tol2 5′ sequence (200 bp minimal sequence)	74–273
iv. FRT site (flanked by BsmBI sites)	931–978
v. loxP site (flanked by BstAPI sites)	1,001–1,034
b. Insulator sequences (to reduce insertion site variegation)	
i. cHS4 core	274–505
ii. cHS4 3′ 400 bp	506–919
Note: cHS4 core + cHS4 3′ 400 bp provides insulating capability closer to that of a full length cHS4 insulator compared to two consecutive cHS4 core sequences	
c. Restriction sites (to remove FRT and/or loxP sites)	
i. BsmBI (CGTCTCn\nnnn/)	920–930, 979–989
This pair of BsmBI sites surrounds the FRT site in the 5′ transgenesis cassette; another pair resulting in a different overhang sequence surrounds the FRT3 site in the 3′ transgenesis cassette. A single BsmBI digest followed by ligation will remove both the FRT and the FRT3 sites and will maintain the correct orientation of the inserts	
ii. BstAPI (GCAnnn\nTGC)	990–1,000, 1,035–1,045
This pair of BstAPI sites surrounds the loxP site in the 5′ transgenesis cassette; another pair resulting in a different overhang sequence surrounds the lox2272 site in the 3′ transgenesis cassette. A single BstAPI digest followed by ligation will remove both the loxP and the lox2272 sites and will maintain the correct orientation of the inserts	
2. MCS I (to clone in promoter controlling rtTA expression)	1,046–1,088
AscI, AgeI, SbfI, SalI, Bsu36I	
3. Gateway cloning cassette (site of ORF insertion)	1,089–2,793
a. *attR1*	1,089–1,213
b. CmR: Chloramphenicol resistance for counter selection	1,322–1981
c. ccdB: Toxin targeting *E. coli* DNA gyrase	2,323–2,628
d. *attR2*	2,669–2,793
e. BbvCI, BmgBI RE sites (remove gateway cloning cassette)	2,795–2,807
4. rtTA expression cassette	2,817–4,679
a. rtTA-TRE elements	
i. Tet-ON 3G (3rd generation of rtTA inducible activator)	2,817–3,563
ii. SV40 polyA	3,578–4,033
b. Insulator sequences (protect ORF from transgenic promoter)	
i. HS4 core (see Note 1.b.)	4,034–4,265
ii. HS4 3′ 400 bp	4,266–4,679
5. MCS IIa (exchange of transgenic reporter cassette with MCS IIb)	4,696–4,717
PacI, EcoRI, SwaI (DraI)	
Note: Works in conjunction with MCS IVb (below)	
6. Transgenesis reporter cassette (to detect transgenic animals)	4,718–5,995
a. γ-Crystallin minimal promoter: Drives expression in the eye	4,718–5,031
b. GFP3	5,032–5,748
c. SV40 polyA signal	5,798–5,995
7. MCS IIb (see MCS IIa annotation)	6,070–6,097
AsiSI, AvrII, PmeI, BglII	

(Continues)

TABLE 1 (Continued)

8. 3′ transgenesis cassette	6,025–7,037
a. I-SceI 3′ site (ATTACCCTG\TTAT/CCCTA)	6,025–6,042
b. Restriction sites (see 1.c. annotation)	
i. BstAPI	6,098–6,108, 6,143–6,153
ii. BsmBI	6,154–6,164, 6,213–6,223
c. Insulator sequences (to reduce insertion site variegation)	
i. cHS4 core (see Note 1.b.)	6,224–6,455
ii. cHS4 3′ 400 bp	6,456–6,869
d. Transgenic features	
i. lox2272 site	6,109–6,142
ii. FRT3 site	6,165–6,212
iii. tol2 3′ sequence (150 bp minimal sequence)	6,870–7,019
iv. I-SceI 3′ site (ATTACCCTG\TTAT/CCCTA)	7,020–7,037
9. pGEM-T vector backbone	7,038–21
a. AatII and MluI (cloning sites in pGEM-T MCS)	16–21, 7,038–7,043
b. SP6 RNA polymerase promoter	7,063–7,082
c. pUC/M13 reverse sequencing primer binding site	7,100–7,116
d. β-Lactamase coding region (ampicillin resistance)	8,261–9,121
e. pUC/M13 forward sequencing primer binding site	9,880–9,896
f. T7 RNA polymerase promoter (−17 to +3)	9,923–3

(with an ORF and no promoter). Mosaic analysis can be done in the F0 generation, and offspring of founder transgenic individuals harboring pDXTP or pDXTR can be characterized to produce separate promoter and ORF transgenic lines, which can then be crossed to obtain offspring with the capacity for tissue-specific inducible control of ORF expression (Figure 1a).

pDXTR was constructed on the pGEM-T backbone and the main design considerations included methods of transgenesis, transgene insertion position effect, Gateway cloning, N-terminal and C-terminal tags, transgenesis reporter, and inducible system (see Tables 1 and 2 for annotated feature lists for pDXTP and pDXTR). For transgenesis, a pair of transgenesis cassettes proximal to the pGEM-T backbone houses sequences enabling phiC31, tol2, and Sce-I meganuclease transgenesis methods (Figure 2a; Allen & Weeks, 2005; Hamlet et al., 2006; Ogino, McConnell, & Grainger, 2006; Ogino & Ochi, 2009). Within the transgenesis cassettes, loxP/lox2272 and FRT/FRT3 sites, which can be removed with restriction enzymes, allow transgenic animals made with pDXTP or pDXTR to be used for target site insertion using CRE or FLP recombinase (Turan et al., 2011). The well-known insulator sequences, chicken hypersensitivity site 4 (HS4), flank the entire ORF expression and transgenesis marker cassettes to reduce potential influence from the genomic insertion site (Aker et al., 2007). Additional HS4 insulators are downstream of the tissue-specific promoter to prevent it from influencing expression of the ORF and transgenesis marker (Kerney et al., 2012). Likely due to these repetitive insulator sequences, pDXTR and its derivatives have the tendency to recombine (see Section 3 for discussion and solution of this issue).

The *Xenopus* ORFeome is housed in Gateway entry vectors that can be easily cloned into Gateway-compatible destination vectors (Grant et al., 2015). We produced the pDXTR destination vector for the purpose of expressing ORFs in transgenic animals. The attR1 and attR2 integrase recognition sequences in pDXTR correspond to the attL1 and attL2 sequences on the *Xenopus* ORFeome vectors which enable LR clonase to shuttle ORFs into pDXTR (Figure 2b,c). ORFs in the *Xenopus* ORFeome are full length but missing the STOP codon. Upon recombination of the ORF into pDXTR, the ORF will then be in frame with a C-terminal tag (Figure 2c). In effect, transcription and translation of the ORF will result in a single translated polypeptide starting with the start codon of the ORF sequence followed by T2A (Sipieter et al., 2015) followed by mCherry then *Xenopus* histone H2B. During translation, endogenous endopeptidases will cleave the T2A sequence resulting in two proteins: (a) the ORF with a C-terminal 17 amino acid sequence tag from T2A (for which commercial antibodies are available) and (b) a protein starting with the 18th and last amino acid of T2A fused to mCherry (monomeric version) fused to *Xenopus* histone H2B. Thus, induced expression of the ORF will be in molar expression equivalent with a tagged histone visible as red fluorescence in each nucleus in which the ORF is expressed. If desired, this fusion protein can be replaced with a different C-terminal tag or simply a STOP codon via conventional restriction enzyme cloning. Also, an N-terminal tag can be added using restriction enzyme sites at the N-terminus. Detection of pDXTR in the genome irrespective of ORF expression is made possible by the transgenesis reporter mCherry controlled by the constitutive lens-specific promoter gamma crystallin. This transgenesis reporter can be replaced using restriction

TABLE 2 Annotated pDXTR feature list and position on plasmid

1. 5′ transgenesis cassette	22–1,045
a. Transgenic features (to insert plasmid into genome)	
i. φC31 integrase *attB* site (34 bp minimal sequence)	22–55
ii. I-SceI 5′ site (TAGGG/ATAA\CAGGGTAAT)	56–73
iii. Tol2 5′ sequence (200 bp minimal sequence)	74–273
iv. FRT site (flanked by BsmBI sites)	931–978
v. loxP site (flanked by BstAPI sites)	1,001–1,034
b. Insulator sequences (to reduce insertion site variegation)	
i. cHS4 core	274–505
ii. cHS4 3′ 400 bp	506–919
Note: cHS4 core + cHS4 3′ 400 bp provides insulating capability closer to that of a full length cHS4 insulator compared to two consecutive cHS4 core sequences	
c. Restriction sites (to remove FRT and/or loxP sites)	
i. BsmBI (CGTCTCn\nnnn/)	920–930, 979–989
This pair of BsmBI sites surrounds the FRT site in the 5′ transgenesis cassette; another pair resulting in a different overhang sequence surrounds the FRT3 site in the 3′ transgenesis cassette. A single BsmBI digest followed by ligation will remove both the FRT and the FRT3 sites and will maintain the correct orientation of the inserts	
ii. BstAPI (GCAnnn\nTGC)	990–1,000, 1,035–1,045
This pair of BstAPI sites surrounds the loxP site in the 5′ transgenesis cassette; another pair resulting in a different overhang sequence surrounds the lox2272 site in the 3′ transgenesis cassette. A single BstAPI digest followed by ligation will remove both the loxP and the lox2272 sites and will maintain the correct orientation of the inserts	
2. MCS I (to clone in promoter controlling rtTA expression)	1,046–1,085
AscI, AgeI, SbfI, SacII, EcoRV, BclI	
3. Inducible expression cassette	1,086–3,350
a. rtTA-TRE elements	
i. Tet-ON 3G (3rd generation of rtTA inducible activator)	1,099–1845
ii. SV40 polyA	1860–2,315
iii. TRE3G (3rd generation of tet-responsive element)	2,962–3,337
b. Insulator sequences (protect ORF from transgenic promoter)	
i. HS4 core (see Note 1.b.)	2,316–2,547
ii. HS4 3′ 400 bp	2,548–2,961
c. SfiI sites (remove inducible expression cassette)	1,086–1,098,3,338–3,350
Note: This pair of SfiI sites (GGCCnnnn\nGGCC) surrounds the inducible expression cassette allowing a single digest/ligation to be used to remove it or introduce a different cassette and maintain correct insert orientation	
4. Mouse β-globin 5′UTR leader sequence (enhance ORF expression)	3,351–3,402
5. MCS II (to add N-terminal tags to ORF)	3,403–3,427
BspEI, AfeI, SalI, Bsu36I	
Note: These sites do not occur within CreER-T2, enabling cloning of CreER-T2 driven by a ubiquitous promoter followed by a tissue-specific promoter controlling “brainbow” for fate mapping	
6. Gateway cloning cassette (site of ORF insertion)	3,428–5,132
a. *attR1*	3,428–3,552
b. CmR: Chloramphenicol resistance for counter selection	3,661–4,320
c. ccdB: Toxin targeting *E. coli* DNA gyrase	4,662–4,967
d. *attR2*	5,008–5,132
7. MCS IIIa (to add STOP or replace C-terminal tag)	5,134–5,152
BbvCI, BmgBI, ClaI	
Note: Works in conjunction with MCS IIIb (see 8.b.)	

(Continues)

TABLE 2 (Continued)

8. C-terminal tag cassette (cells expressing ORF have red nuclei)	5,155–7,194
a. T2A peptide (EGRGSLLTCGDVEENPGP)	5,155–5,208
Note 1: T2A will be in-frame with the STOP-less ORF following the LR clonase reaction	
Note 2: The T2A peptide links the ORF to the following fluorescent protein. During translation this linker is cleaved between the 17th and 18th amino acid, such that the ORF ends up with a 17aa long C-terminal tag (commercial antibodies against T2A tags are available) and the 18th aa becomes the first aa of a separate but concurrently translated peptide. Thus, equivalent amounts both peptides are produced, regulated by the same promoter	
b. MCS IIIb (see 7. Annotation)	5,209–5,232
ClaI, FseI, NotI	
c. mCherry	5,233–5,940
Note 1: This monomeric mCherry is in frame with the preceding ORF and T2A peptide and fluorescently marks all cells in which the ORF is expressed. This mCherry lacks a STOP codon for fusion with ensuing *Xenopus* histone H2B	
Note 2: Red was chosen as it is less likely than green to be used in further analysis of transgenic animals and will not interfere with DAPI as a nuclear counterstain	
d. *X. laevis* histone H2B (to target mCherry to the nucleus)	5,947–6,327
e. SV40 polyA signal	6,328–6,548
f. Insulator elements (to protect transgenesis marker from promoter)	
i. cHS4 core (see Note 1.b.)	6,549–6,780
ii. cHS4 3′ 400 bp	6,781–7,194
9. MCS IVa (to enable exchange of transgenic reporter cassette)	7,195–7,224
PacI, SgrAI, EcoRI, SwaI	
Note: Works in conjunction with MCS IVb (below)	
10. Transgenesis reporter cassette (to detect transgenic animals)	7,225–8,479
a. γ-Crystallin minimal promoter: Drives expression in the eye	7,225–7,523
b. mCherry: Monomeric red fluorescent protein variant	7,548–8,258
c. SV40 polyA signal	8,259–8,479
11. MCS IVb (see MCS IVa annotation)	8,480–8,507
AsiSI, AvrII, PmeI, BglII	
12. 3′ transgenesis cassette	8,508–9,447
a. Restriction sites (see 1.c. annotation)	
i. BstAPI	8,508–8,518, 8,553–8,563
ii. BsmBI	8,564–8,574, 8,623–8,633
b. Insulator sequences (to reduce insertion site variegation)	
i. cHS4 core (see Note 1.b.)	8,634–8,865
ii. cHS4 3′ 400 bp	8,866–9,279
c. Transgenic features	
i. lox2272 site	8,519–8,552
ii. FRT3 site	8,575–8,622
iii. tol2 3′ sequence (150 bp minimal sequence)	9,280–9,429
iv. I-SceI 3′ site (ATTACCCTG\TTAT/CCCTA)	9,430–9,447
13. pGEM-T vector backbone	9,448–21
a. AatII and MluI (cloning sites in pGEM-T MCS)	16–21, 9,448–9,453
b. SP6 RNA polymerase promoter	9,473–9,492
c. pUC/M13 reverse sequencing primer binding site	9,510–9,526
d. β-Lactamase coding region (ampicillin resistance)	10,671–11,531
e. pUC/M13 forward sequencing primer binding site	12,290–12,306
f. T7 RNA polymerase promoter (−17 to +3)	12,333–3

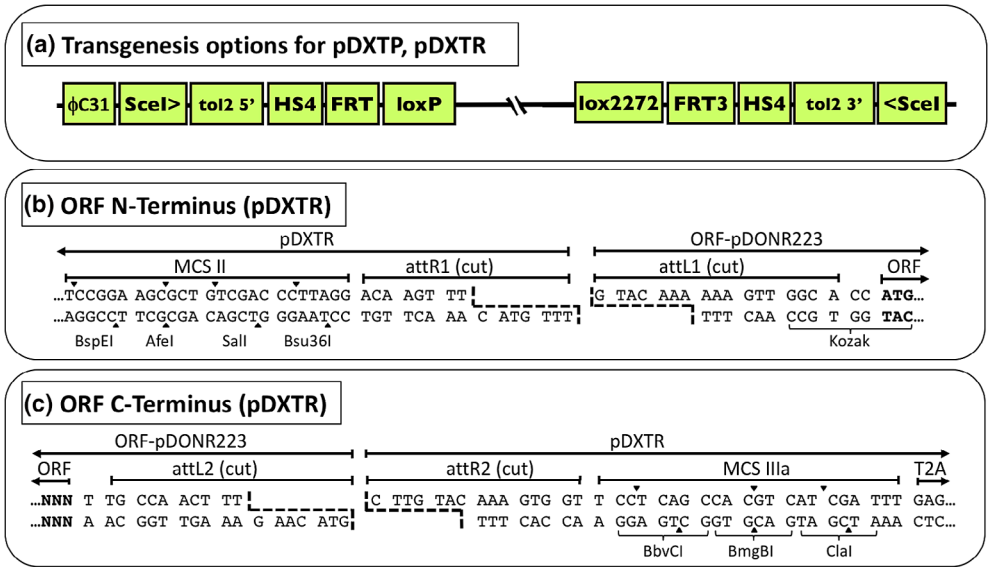


FIGURE 2 Diagrams of transgenesis cassettes and ORF N- and C-termini. (a) the transgenesis cassettes in pDXTP and pDXTR provide the options for phiC31 integrase, SceI meganuclease, and tol2 transposase methods of transgenesis. The chicken HS4 insulator sequence reduces the influence an integration site may have on the integrated transgenic DNA. The loxP/lox2272 and FRT/FRT3 recombination sequences allow for transgenic animals with these sequences to serve as target sites for future integration of donor plasmids with matching lox and FRT sites using CRE and FLP recombinases. (b) Just upstream of the 5′ gateway recombination site (*att*R1) where ORFs enter pDXTR are restriction enzymes sites available for addition of N-terminal tags. (c) The 3′ gateway recombination site (*att*R2) maintains the reading frame of the ORF with the T2A-mCherry-histone H2B C-terminal tag. Restriction enzyme sites 3′ of the ORF enable replacing the built-in C-terminal tag with a STOP codon or a custom tag

enzyme cloning. If desired, pDXTR can be modified prior to Gateway cloning to make a common destination vector for ORFs requiring the same tag and/or same transgenesis marker.

pDXTR has the capacity to enable tissue-specific inducible transgene expression in a single plasmid via the rtTA-TRE binary expression system (but see below). The ORF would be cloned into pDXTR using Gateway, and a promoter would be cloned in using restriction enzymes upstream of the rtTA-TRE cassette. Previous experience with rtTA-TRE indicated effective inducibility and low background expression can be obtained using this binary system for control of transgene expression in *Xenopus* (Das & Brown, 2004). pDXTR contains the third generation of rtTA, namely Tet-On 3G, a tetratcycline-activated transcription factor, and the third-generation tetracycline responsive element TRE3G (Clontech). If inducible expression is not desired, the rtTA and TRE portions of pDXTR can be removed via SfiI restriction enzyme digestion resulting in control of ORF expression by the custom promoter. Transgenic animals harboring pDXTR with promoter and ORF together are cost-effective in the short run because of the difficulty and time investment of making and characterizing a transgenic line (Buchholz, 2012; Rankin et al., 2011). However, it is important to note that a "fully loaded" pDXTR plasmid with promoter of interest and ORF including the capacity for inducible expression may cause difficulty due to its large size (Yang & Yang, 2012). Also, "single-use" transgenic lines would lose the benefit of future combinatorial experiments. A profitable solution is to use separate plasmids to produce separate transgenic lines to constitute the binary ORF expression system. In the long run, it is advisable to produce such separate promoter and ORF lines that could be used combinatorially to support a large number of projects off the shelf as a significant benefit to the *Xenopus* community.

To allow the possibility of producing separate promoter and ORF transgenic lines, we designed pDXTP based on pDXTR to serve as the promoter part of the rtTA-TRE binary system, and pDXTR would serve as the ORF part of the binary system. To convert pDXTR into pDXTP, the transgenesis marker CRY:mCherry of pDXTR was replaced with CRY:GFP, the Gateway cloning cassette was inserted upstream of the rtTA coding sequence, and the TRE and C-terminal tag sequences were removed. Thus, pDXTP can receive promoters via Gateway cloning to control rtTA expression and has green lens fluorescence as a transgenesis marker. pDXTR, with red lens fluorescence as a transgenesis marker, would then only receive an ORF (and not a promoter). A major convenience of pDXTP is the availability of the set of Gateway-compatible pTransgenesis plasmids containing promoters (Table 3). ORFs from the *Xenopus* ORFeome cannot be used in the pTransgenesis system because the ORFs, which have *att*L1-*att*L2 sites, would be cloned into the wrong position of the pTransgenesis system, which uses Multisite Gateway cloning. Conversely, all pTransgenesis p2 plasmids (Table 3) and any future promoters cloned into pENTR vectors with attL1-attL2 sites can use Gateway cloning to insert a promoter into pDXTP.

Functional testing of the features of pDXTR began with cloning GFP into the Gateway cloning site and injection into an established rtTA-expressing transgenic line, pCAR-TRDN (inducible; Brown et al., 2005; Figure 3a). Successful production of pDXTR-GFP (see Section 3) verified the functionality of Gateway cloning. We then, without cloning in a promoter, injected pDXTR-GFP using the SceI meganuclease transgenesis method into the pCAR-TRDN (inducible) line. This pCAR line

TABLE 3 pTransgenesis p2 promoter plasmids

Plasmid name	Expression domain
p2 CAG	Widespread
p2 CMV	Widespread
p2 Ef1α	Widespread
p2 ubiquitinC	Widespread
p2 ROSA26	Widespread
p2 hsp70	Heat shock-inducible
p2 14x UAS	GAL4 inducible
p2 CarA	All muscle types
p2 NBT	Differentiated neurons
p2 pax6	Brain, eye, spinal cord
p2 HB9	Motor neurons
p2 flk1	Vasculature
p2 nectin-2	Superficial neuroepithelium
p2 pax3	Neural ectoderm
p2 sox3	Ectoderm/neural progenitors
p2 vimentin	Neural progenitors/glia
p2 Xlurp	Myeloid cells
p2 foxi1 short	Ionocytes
p2 foxi1 long	Ionocytes
p2 lmo2 short	Blood
p2 lmo2 long	Blood
p2 bra	Mesoderm

Note: Reference Love et al. (2011).

expresses rtTA under control of the muscle-specific promoter pCAR. This line also harbors a doxycycline-inducible dominant negative form of thyroid hormone receptor but does not affect the results reported here. Upon examination of the resulting transgenic animals treated with and without the inducing chemical doxycycline, we found mosaic GFP expression localized to muscle cells in some of the Sce-injected tadpoles after addition of doxycycline (Figure 3b). In addition, each muscle cell expressing GFP also expressed mCherry in its nucleus (Figure 3c). These results confirm function of the SceI transgenesis sites, TRE promoter, C-terminal tag, and the CRY:mCherry transgenesis marker.

To directly test the ability of pDXTP and pDXTR to express an ORF in transgenic animals, we chose a promoter and ORF expected to give an obvious embryonic phenotype. Using Gateway cloning, we cloned the *Ef1α* promoter from the pTrangenesis system into pDXTP to make pDXTP-Ef1α, and we cloned *bmp4* from the *Xenopus* ORFeome into pDXTR to make pDXTR-bmp4. Then, we injected pDXTP-Ef1α and pDXTR-bmp4 singly and together using Sce meganuclease into 1- to 2-cell stage embryos and treated them with and without 50 μM doxycycline continuously starting at 4 hr after fertilization for 5–8 days. Overexpression of bmp4 prior to gastrulation causes reduction in anterior structure development (Hartley, Hardcastle, Friday, Amaya, & Papalopulu, 2001). We observed ~30–60% of doxycycline-treated, doubly injected animals had increased incidence of reduced head structures (Figure 4; Table 4). The variation in phenotype incidence and severity likely reflect mosaicism and variation in transgene insertion site, which may alter the kinetics and magnitude of transgene induction. These head phenotypes represent a 2- to 6-fold increase in doxycycline-treated, doubly injected animals compared to controls across the three experiments. Not all animals injected with one or both constructs exhibited eye fluorescence reflecting the less than 100% efficiency of the transgenesis technique. Specifically, green eye fluorescence ranged from 20 to 60%, and red eye fluorescence was less than 10%. In animals treated with doxycycline, an assessment of eye fluorescence is not expected to be a reliable indicator of successful transgensis with this transgene because anterior structures including eyes are expected to be absent or reduced. Importantly, about half of the doubly injected animals treated with doxycycline that did not have an anterior defect phenotype had green or green and red eye fluorescence, indicating that, similar to eye fluorescence, phenotype induction is not 100% efficient in founders.

In conclusion, we have produced two Gateway destination vectors, pDXTP and pDXTR, for the specific purpose of facilitating production of separate transgenic lines to express rtTA and express an ORF. These two constructs have different transgenesis reporters, conforming to our suggested convention that rtTA lines have green eyes and ORF lines have red eyes. Despite the great potential for combinatorial multiplication of experiments, few characterized transgenic lines suitable for study of disease processes are currently available (Table 5). We envision that as few as 5–10 rtTA and ORF lines that could be used combinatorially would immediately support a large number of projects. Furthermore, the set of binary transgenic lines may grow because if only an rtTA or ORF line were available for a particular project, it would be cost-effective for individual investigators to make a single rtTA or ORF line corresponding to their project. A drawback to transgenic lines is that animal housing typical of *Xenopus* researchers does not allow for keeping numerous transgenic lines separate. However, use of visible implant elastomer tags (https://tropicalis.berkeley.edu/husbandry/tags/E-ANTs.html) or photographs of dorsal patterning (Reed, 2005) may allow researchers to avoid any changes to current husbandry practices.

3 | MATERIAL AND METHODS

3.1 | Plasmid construction

The pGEM-T vector was used as the backbone plasmid into which four gene-synthesized DNA fragments were sequentially cloned using conventional restriction enzyme-based cloning to produce pDXTR (GenBank accession MN044710). pDXTR was modified using conventional and PCR-based cloning to produce pDXTP (GenBank accession MN044709). An extensive annotated feature list gives details and rationale for features included in pDXTR (Tables 1 and 2). The backbone vector and gene synthesized fragments were sequence-verified by the providers, and we verified the sequence of all portions of plasmids involving PCR or Klenow during cloning. Gateway cloning was then used to insert *bmp4* from the *Xenopus* ORFeome into pDXTR to make pDXTR-bmp4. Also, Gateway cloning was used to insert *Ef1α* (elongation factor alpha) and *tubb2* (neural beta tubulin) promoters

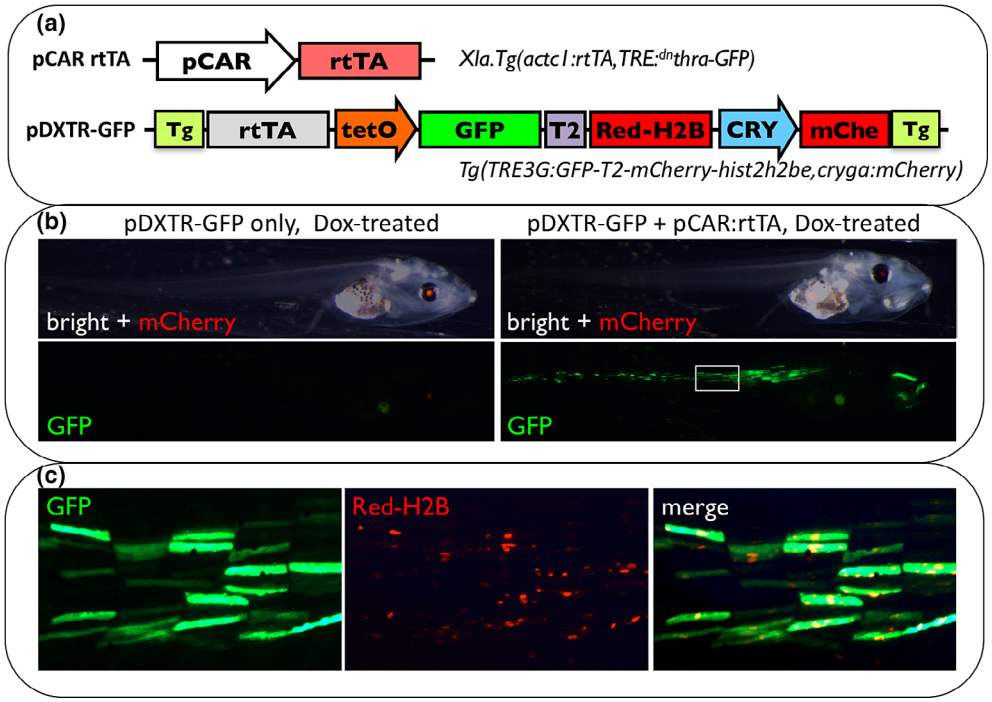
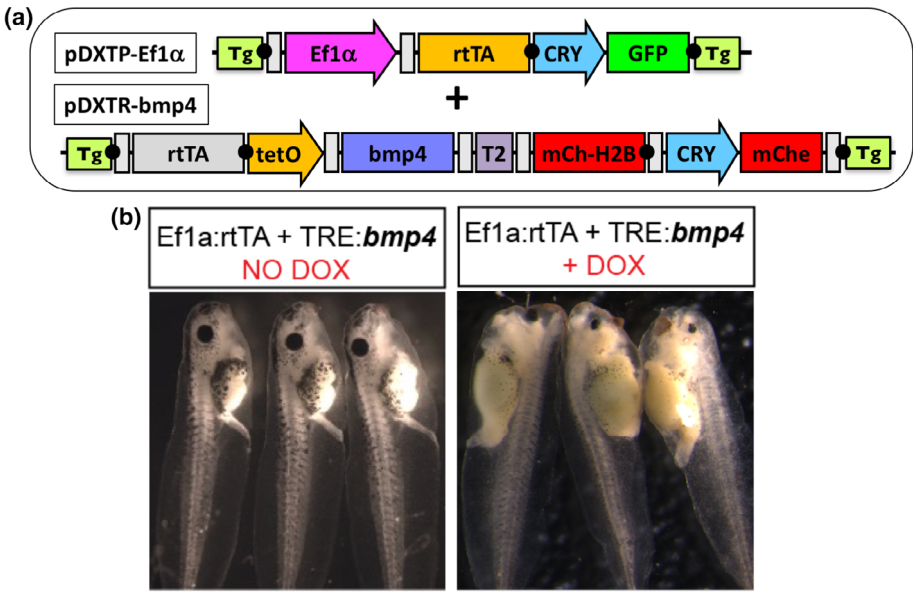


FIGURE 3 Functional analysis of the cloning and transgenesis features in pDXTR. (a) Diagrams of transgenesis plasmids pCAR:TRDN-GFP (inducible) and pDXTR-GFP. (b) pDXTR-GFP was cloned and injected into eggs from a wild-type female crossed to a male transgenic for the pCAR-TRDN construct. Due to absence of transgenesis markers, the presence of the pCAR:rtTA transgene can be readily detected only by dox-induction of TRDN-GFP (data not shown). Both tadpoles were treated with 50 μg/mL doxycycline for 24 hr. The tadpole on the left is singly transgenic for pDXTR-GFP as evidenced by red eye fluorescence and no GFP expression in muscle from TRDN-GFP. The tadpole on the right has both constructs as evidenced by red eye fluorescence and strong green fluorescence in the cytoplasm. Top images represent merged bright field and red fluorescence, and bottom images represent fluorescence with green filter. (c) Higher magnification of the muscle cells from the boxed region in (b) shows green, red, merged fluorescence images. Each green rod represents a single, multinucleated muscle cell with nuclei labeled with mCherry-histone H2B

FIGURE 4 Functional analysis of gateway-cloned promoter and ORF in pDXTP and pDXTR. (a) Using gateway cloning, the Ef1α promoter from pTransgenesis was cloned into pDXTP, and bmp4 from the *Xenopus* ORFeome was cloned into pDXTR. (b) Exemplar embryos co-injected with both constructs show normal phenotype in the absence of Dox and show reduced and absent head structures when treated continuously with 50 nM Dox starting at early cleavage stages



from the pTransgenesis plasmid collection into pDXTP to make pDXTP-Ef1α and pDXTP-NβT. Further, pENTRD-GFPnoTAA, a Gateway-based GFP entry vector, was produced using PCR cloning to insert GFP minus the STOP codon into a pENTR vector, and then Gateway cloning was used to insert GFP from pENTRD-GFPnoTAA into pDXTR to make pDXTR-GFP. Remaking pDXTP and pDXTR prior to Gateway cloning requires ccdB-resistant bacteria, but standard bacterial strains can be used after Gateway recombination. Importantly,

pDXTP and pDXTR have the potential to undergo "spontaneous" recombination during bacterial culturing, even in recA-bacterial strains, resulting in contamination with smaller vectors or complete loss of the full-size plasmid. A simple precaution to prevent this instability is to carry out bacterial plating and overnight cultures at 30 °C rather than 37 °C, and each new batch of plasmid can be easily checked with a BamHI test digestion. The empty pDXTP and pDXTR plasmids are available from *Xenopus* stock centers, and all derived plasmids used here are available from the authors upon request.

3.2 | Animals and transgenesis

X. laevis adults were housed in XR4 and XR5 recirculating systems (Pentair Aquatic EcoSystems) at 22 °C with a 12-hr photoperiod and fed frog brittle (Nasco). pCAR:TRDN-GFP transgenic *X. laevis* were a gift from Dr. Don Brown (Brown et al., 2005). Two groups of transgenesis experiments were performed, pDXTR-GFP injected into embryos from pCAR:TRDN-GFP crossed to wild-type (one clutch) and pDXTP-Eflα and pDXTR-bmp4 co-injected into wild-type embryos (three clutches). Transgenesis was carried out using the SceI meganuclease procedure as described (Ogino et al., 2006; Pan, Chen, Loeber, Henningfeld, & Pieler, 2006; Rankin, Hasebe, Zorn, & Buchholz, 2009). Briefly, the SceI reaction (400 ng of DNA, 2.0 μL SceI buffer, 2.0 μL of bovine serum albumin, 2 μL of SceI in 20 μL total reaction volume) was incubated at 37 °C for 40 min. Then stored on ice until injected into dejellied 1- to 2-cell stage embryos (10 nL per embryo) at room temperature using a Picospritzer III (Parker Hannifin Corp.). Embryos were cultured in petri dishes with 1× Marc's modified ringers at 18 °C and sorted at the 4- to 128-cell stage and daily thereafter to remove dead and dying embryos as described (Wlizla, McNamara, & Horb, 2018) and to replace doxycycline treatments (see below).

TABLE 4 Embryo treatments and head phenotype frequency

Promoter	ORF	pg/plasmid	Dox	Head phenotype
Ef1α	bmp4	100	–	12% (6/52)
Ef1α	bmp4	100	+	37% (15/41)
Ef1α	bmp4	200	–	9% (3/32)
Ef1α	bmp4	200	+	60% (18/30)
–	–	–	+	12% (14/114)
Ef1α	–	200	–	0% (0/45)
Ef1α	–	200	+	14% (17/119)
–	bmp4	200	–	3% (1/32)
–	bmp4	200	+	10% (7/67)
Ef1α	bmp4	200	–	2% (2/90)
Ef1α	bmp4	200	+	32% (47/147)
–	–	–	–	23% (8/25)
–	–	–	+	18% (7/38)
Ef1α	bmp4	200	–	47% (27/59)
Ef1α	bmp4	200	+	64% (41/64)

3.3 | Embryo treatments and morphological analysis

Injected embryos and uninjected control embryos were haphazardly assigned to treatments with and without 50 μg/mL doxycycline continuously beginning ~3–5 hr after fertilization at a density of ~100 embryos per petri dish. When the uninjected embryos achieved Nieuwkoop and Faber stage 40–42 (NF40-42, approx. 7 days after fertilization; Nieuwkoop & Faber, 1994), all surviving embryos were anesthetized with 0.1% buffered MS-222 to stop swimming. Tadpoles from the pCAR:TRDN-GFP clutch injected or not with pDXTR-GFP were scanned for fluorescent protein expression in the eyes and body using a Leica fluorescence dissecting microscope with GFP2 and RFP filter sets, and images were taken with a Leica DFC420 digital camera from 1 to 2 tadpoles treated with and without doxycycline of each genotype, as determined by eye color and the weak GFP expression from TRDN-GFP transgene (not shown; Brown et al., 2005). Tadpoles

TABLE 5 Available rtTA and TRE lines in *X. laevis* (Grant et al., 2015; Horb et al., 2019)

Tg construct	Official name	Description	Reporter
rtTA lines			
pDPCrtTA-TREG-HS4	Xla.Tg(CMV:RTTA, TRE:eGFP,cryga:GFP3)Buchz	Ubiquitous rtTA expression, induces GFP upon addition of Dox	GFP expression in eye
pDPcol2.3rtTA-TRECre-HS4	Xla.Tg(col2a1:RTTA, TRE:Cre, cryga:GFP3)Buchz	Expression of rtTA, CRE, and GFP in new cartilage	GFP expression in eye
Inducible CAR:TRDN-GFP	Xla.Tg(actc1:dnthra-GFP)Brown	Muscle-specific rtTA expression, induces TRDN upon addition of dox	None
Inducible NBT:TRDN-GFP	Xla.Tg(tubb2b:dnthra-GFP)Brown	Neuron-specific rtTA expression, induces TRDN upon addition of dox	None
Inducible collagen:TRDN-GFP	Xla.Tg(Mmu.col1a2:dnthra-GFP)Brown	Ubiquitous rtTA expression, induces TRDN upon addition of Dox	None
TRE lines			
pDRTREG-HS4	Xla.Tg(TRE:eGFP, cryga:dsRed)Buchz	Dox-inducible GFP in presence of rtTA	DsRed expression in eye
pDRTREdpTR-HS4	Xla.Tg(TRE:dpthra, cryga:dsRed)Buchz	Dox-inducible dpTR in presence of rtTA	DsRed expression in eye

co-injected with pDXTP-Eflα and pDXTR-bmp4 with the resulting samples sizes (Table 4) were scored one by one for anterior and dorsal defects (Hartley et al., 2001), and for fluorescent protein expression in the eyes using a Leica fluorescence dissecting microscope.

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