



# Generation of a *Xenopus laevis* F1 albino J strain by genome editing and oocyte host-transfer



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## ARTICLE INFO

### Article history:

Received 11 December 2015  
Received in revised form  
27 February 2016  
Accepted 2 March 2016  
Available online 15 March 2016

### Keywords:

*Xenopus laevis*  
TALENs  
J strain  
Tyrosinase  
Oocyte host-transfer  
Genome editing

## ABSTRACT

Completion of the *Xenopus laevis* genome sequence from inbred J strain animals has facilitated the generation of germline mutant *X. laevis* using targeted genome editing. In the last few years, numerous reports have demonstrated that TALENs are able to induce mutations in FO *Xenopus* embryos, but none has demonstrated germline transmission of such mutations in *X. laevis*. In this report we used the oocyte host-transfer method to generate mutations in both *tyrosinase* homeologs and found highly-penetrant germline mutations; in contrast, embryonic injections yielded few germline mutations. We also compared the distribution of mutations in several FO somatic tissues and germ cells and found that the majority of mutations in each tissue were different. These results establish that *X. laevis* J strain animals are very useful for generating germline mutations and that the oocyte host-transfer method is an efficient technique for generating mutations in both homeologs.

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## 1. Introduction

*Xenopus laevis* has a long history as a vertebrate model system in biomedical research, and has made important contributions to many fields, including cell cycle, somatic cell nuclear transfer, embryonic development, and cell fate specification. Due to its allotetraploid genome, the use of genetically heterogeneous animals, and its long generation time, *X. laevis* has not been utilized as a genetic model. In recent years, an inbred J strain has become more widely available through the National *Xenopus* Resource (NXR) and European *Xenopus* Resource Centre (EXRC), and this inbred strain was used in sequencing the *X. laevis* genome (Gantress et al., 2003; Pearl et al., 2012; Tochinai and Katagiri, 1975). As an allotetraploid, *X. laevis* is composed of two separate diploid genomes ( $2n=36$ ) that segregate independently; there are nine pairs of homeologous chromosomes named Xla1S and Xla1L through Xla9S and Xla9L, where S (short) and L (long) refer to their relative lengths (Krylov and Tlapakova, 2015; Matsuda et al., 2015). With the recent sequencing of the *X. laevis* genome (Session et al., 2016), combined with advances made in genome editing, *X. laevis* now offers additional benefits as a genetic model system.

The development of genome editing technologies (CRISPR/Cas and TALENs) has greatly facilitated the ability of investigators to

create loss-of-function mutant alleles in virtually any model organism, including *Xenopus* (Harrison et al., 2014; Peng et al., 2014). Transcription activator-like effector nucleases (TALENs) are comprised of two modular arrays of DNA-binding domains coupled to opposing catalytic subunits of the heterodimeric FokI nuclease, and can be designed to target almost any region in the genome (Joung and Sander, 2013; Kim and Kim, 2014). Simple micro-injection of TALEN mRNAs into *X. laevis* embryos is sufficient to induce site-specific lesions and can be designed to target either one or both alleles (Nakade et al., 2015; Sakane et al., 2013; Suzuki et al., 2013). Although these studies demonstrated that TALENs are capable of inducing mutations in both homeologous genes, injections into embryos were not sufficient to generate a null phenotype. This result probably reflects the time required for the TALEN mRNAs to be translated during the rapid cell divisions of early *Xenopus* development.

A complementary approach that would provide more time for TALEN proteins to act on genomic DNA is to inject TALEN mRNAs into oocytes, rather than embryos. This well-established technique is known as the oocyte host-transfer (OHT) method, and has been commonly used to knock down maternally stored mRNAs to examine their function in early embryogenesis (Heasman et al., 1991; Olson et al., 2012). In the OHT method, the ovary is isolated and stage VI oocytes are defolliculated manually; they can then be injected and cultured for 24–48 h *in vitro*, after which maturation is induced by addition of progesterone to the media. The next day, matured oocytes are implanted into an ovulating host female and the eggs are collected and fertilized *in vitro* the same day. Although

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laborious, in comparison to embryo injections, it is very effective in producing F0 TALEN-mediated gene knock-out in *Xenopus laevis*, with mutations present in both alleles (Miyamoto et al., 2015; Nakajima and Yaoita, 2015a). However, the TALEN-induced mutations were mosaic, with several different mutations identified in each allele. The distribution of mutations in different tissues was not described and it was not clear whether mutations were present in the germ cells.

With increased use of J strain animals, our goal was to develop an albino J strain *X. laevis* line that would benefit the wider *Xenopus* community. In this paper, we examined the efficiency of germline transmission of TALEN-induced mutations in F0 adult *X. laevis* J strain animals produced by oocyte host-transfer, and determined whether the same germ cell mutations were induced in somatic tissues. Tyrosinase is an essential component of melanin biosynthesis, the disruption of which produces an albino phenotype in *Xenopus*, allowing for easy phenotypic identification of a knockout phenotype (Blitz et al., 2013; Guo et al., 2014; Ishibashi et al., 2012; Nakajima et al., 2012; Nakajima and Yaoita, 2015b; Nakayama et al., 2013; Suzuki et al., 2013). We designed TALENs to target both *tyrosinase* homeologs present on the 2S and 2L chromosomes and examined the efficiency of germline transmission to the F1 generation, and genotyped five different tissues in the adult female F0 frog.

## 2. Materials and methods

### 2.1. TALEN production

A single pair of TALENs were designed using the TAL Effector Targeter 2.0 (<https://tale-nt.cac.cornell.edu/>) to target the 3' end of the first exons of both homeologs of *X. laevis tyrosinase* located on the 2S and 2L chromosomes based on the *X. laevis* genome data (Session et al., 2016) available from Xenbase, <http://www.xenbase.org/>, RRID: SCR\_003280 (James-Zorn et al., 2015). The forward TALEN was designed to target GCCCATGAAGCTCCAG, and the reverse TALEN was designed to target CCGTACTTCTTGCTGC. The TALEN constructs were created using the Golden Gate and TAL Effector Kit 2.0 (Addgene) (Cermak et al., 2011) and cloned into the destination vectors pCS2-TAL3DDD and pCS2-TAL3RRR (provided Dr. David Grunwald) (Hu et al., 2013). The final constructs were confirmed by Sanger sequencing, and mRNAs were *in vitro* transcribed from the linearized constructs with a mMACHINE SP6 Transcription kit (Life Technologies, Carlsbad, CA).

### 2.2. Oocyte host-transfer

700 pg of each TALEN mRNA were co-injected into manually-defolliculated, stage VI oocytes from a J strain female, and the oocytes were cultured in OCM (70% Leibovitz L-15 media, 0.4% BSA, 1X Pen-Strep, 0.5 µg/mL gentamicin, pH 7.8) for 24 h. The oocytes were then matured by overnight application (16 h) of 2 µM progesterone in OCM (Sigma, St Louis, MO); an albino host wild type female was induced to ovulate by injection with 500 IUs of human chorionic gonadotropin (HCG). The following day, matured J strain oocytes were transplanted into the wild type host female body cavity through a 16-gauge needle, and eggs were collected in 1X MMR and then fertilized *in vitro* with J strain sperm. The donor injected-embryos were pigmented and easily identifiable from the albino host eggs. For embryo injections, the same amount of each TALEN mRNA was injected into one-cell embryos that had been produced by *in vitro* fertilization with J strain sperm.

### 2.3. Husbandry

All animals were housed and handled in the National *Xenopus* Resource in accordance with animal care protocol 15-02B approved by the Marine Biology Laboratory IACUC. The *Xenopus* J strain animals were bred in house at the NXR from parents obtained from Jacques Robert (University of Rochester). Female frogs were primed with 50U PMSG 3–4 days prior to induction of ovulation. Ovulation was induced by injecting females with either 400 IU HCG or with 150 µg ovine luteinizing hormone (LH) (National Hormone and Peptide Program, Torrance, California). LH dose used was equivalent to 2 µg/g body weight. These albino J strain F0 frogs are deposited in the NXR (<http://www.mbl.edu/xenopus/>, RRID:SCR\_013731) and designated *Xla.tyr<sup>tm1NXR</sup>* (RRID:NXR\_0.0041).

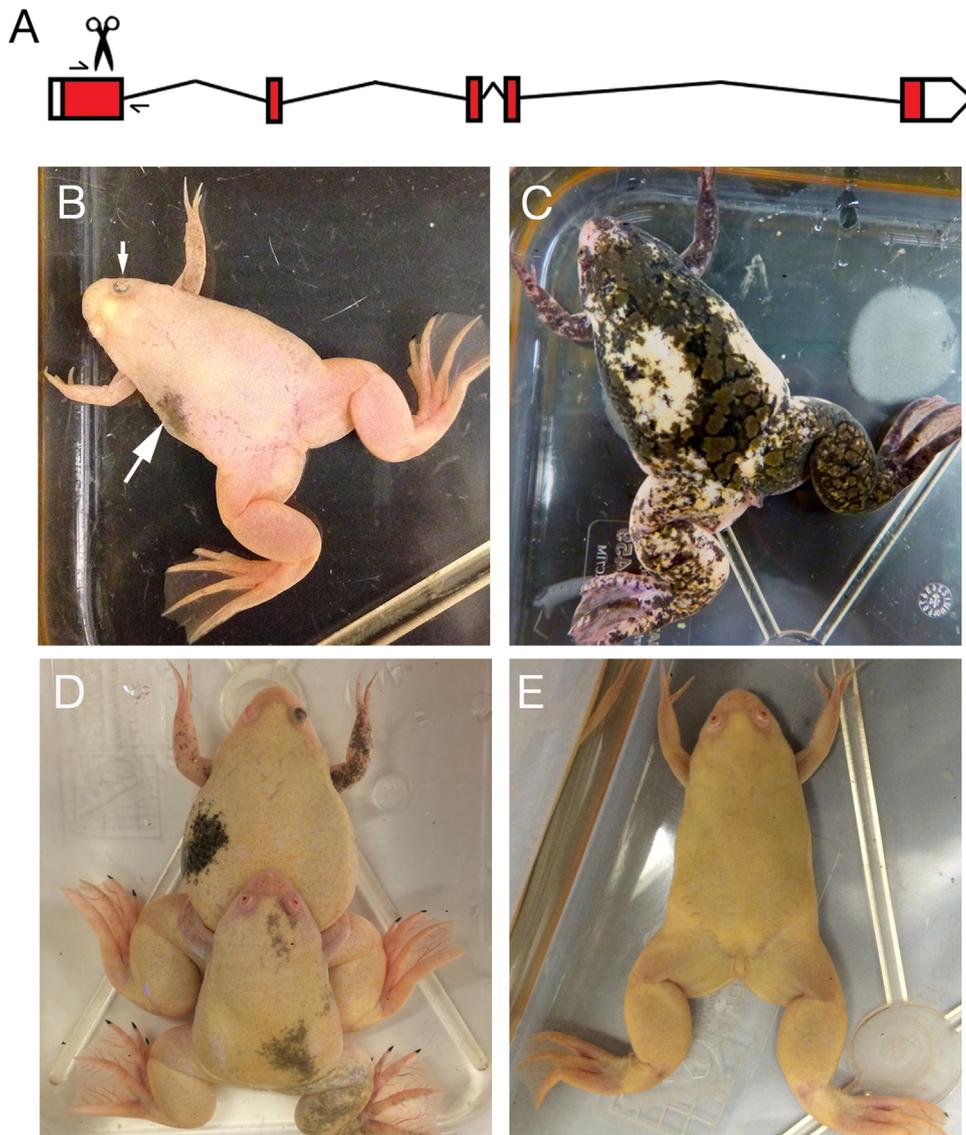
### 2.4. Genotyping

Genomic DNA was isolated from F0 tissue biopsies from the single OHT female and individual F1 embryos that were produced by backcrossing the F0 OHT frogs with wild type J strain using DNazol reagent (Thermo Fisher, Waltham, MA) and DNeasy Blood and Tissue kits (Qiagen, Hilden, Germany). Homeolog-specific PCR of genomic DNA was performed using Taq polymerase with Thermopol buffer (New England Biolabs, Ipswich, MA) and the following primers: *tyr.L* fwd GTTTGCAATGGCCAGTTTCC, *tyr.L* rev TCATCCCACCACGAGCAA, *tyr.S* fwd GCTTGAATGGCCAGTTCCC, *tyr.S* rev CCATCCCACCACCACCAC. PCR products were then cloned into pCR2.1 (Life Technologies, Carlsbad, CA).

## 3. Results

Recent sequencing of the *X. laevis* genome has shown that the homeologous *tyrosinase* (*tyr*) genes are located on the 2S and 2L chromosomes (Xenbase). Previous reports showed that injection of TALENs targeting both *tyr* homeologs (*tyr.L* and *tyr.S*) into one-cell stage *X. laevis* embryos results in mosaic pigmentation defects (Suzuki et al., 2013), whereas injection into oocytes produces more complete albinism (Miyamoto et al., 2015; Nakajima and Yaoita, 2015a). None of these studies however, examined the distribution of mutations within different adult organs. To determine how mosaic the mutations are in different adult organs, we injected one pair of TALEN mRNAs targeting the 3' end of the first exon of both *tyr.L* and *tyr.S* into stage VI oocytes (Fig. 1A). After transferring the oocytes into a donor female, we obtained 16 viable embryos. At stage 45, 10 tadpoles were completely albino, two tadpoles showed partial albinism and four tadpoles had normal pigmentation (data not shown). We raised the resulting albino tadpoles to adulthood, and three survived past metamorphosis (two males and one female); all three were found to be primarily albino, except for a few patches of pigmentation (Fig. 1B). In contrast, embryos injected with the same TALEN mRNAs showed a higher level of mosaic pigmentation (Fig. 1C). Once these frogs reached sexual maturity we mated them to each other as well as to wild type J strain partners to determine the genotype and phenotype of F1 offspring and to create a line of J strain albino frogs with a defined genetic mutation in each allele. Mating the OHT female to OHT male #1 resulted in ~75% F1 albino tadpoles, whereas mating with OHT male #2 resulted in ~50% albino tadpoles (Fig. 1D,E and data not shown).

To determine the exact mutations present in germ cells, we crossed them with wild type J strain frogs, and genotyped both homeologs in at least 17 F1 embryos from each cross; to identify homeolog-specific mutations, we used PCR primers specific to *tyr.S* or *tyr.L*. The number of different mutations inherited from a single founder F0 frog ranged from 2 to 5 (Fig. 2A). Analysis of genomic



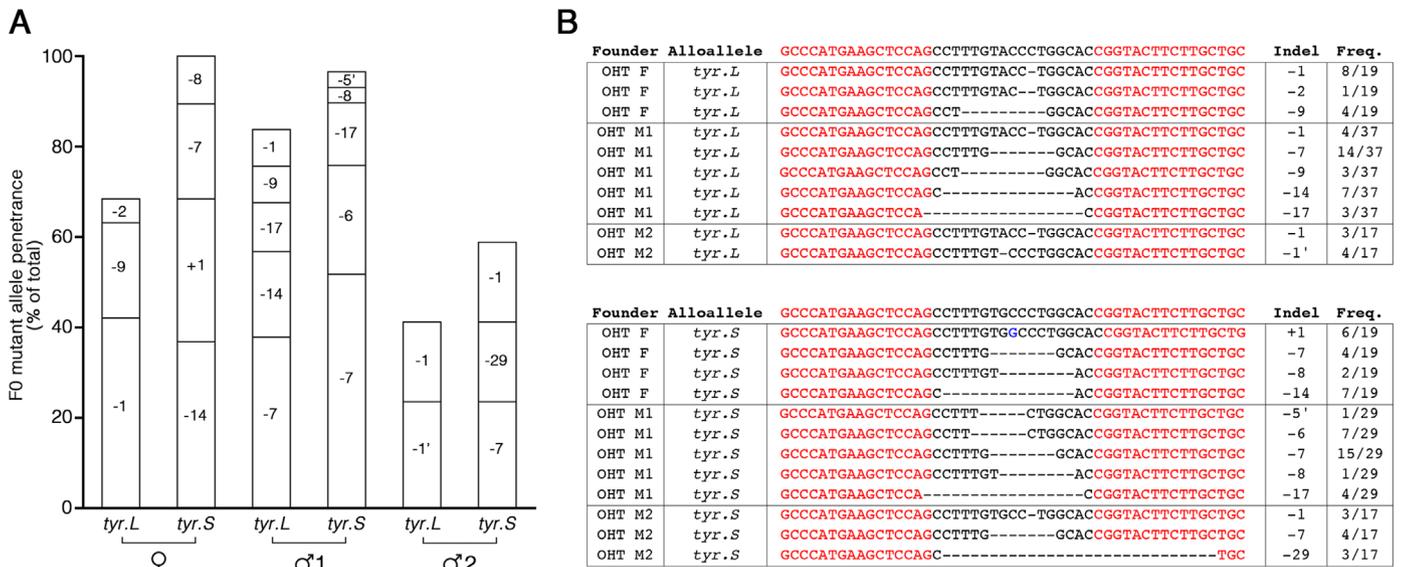
**Fig. 1.** Examples of F0 and F1 *X. laevis tyr* mutants. (A) Schematic diagram of tyrosinase locus, showing the five exons. The scissors show where the TALENs were designed to target the 3' end of the first exon, and the arrows show the location of primers used for genotyping. (B) Picture of an 8-month-old *tyr* mutant F0 J strain frog generated by oocyte host transfer. Notice small patch of pigmentation in the body and eye (arrows). (C) Picture of an 8-month-old F0 *tyr* mutant frog generated by injection of TALEN mRNAs into one-cell stage embryos. (D) Picture of natural mating of two *tyr* mutant F0 frogs (OHT female and OHT male #1) at 14 months. (E) Picture of an F1 *tyr* mutant frog from mating of OHT female and OHT male #1 at 6 months of age.

DNA from single F1 progeny confirmed a high level of germ line transmission of mutant *tyr* alleles: the female founder transmitted mutant alleles to ~85% of progeny, one male founder transmitted mutant alleles to ~90% of his progeny, and the other male founder transmitted mutant alleles to ~50% of his progeny (Fig. 2A). Both homeologs were similarly affected, and several identical deletions were observed independently on both chromosomes in separate founders (Fig. 2B). This demonstrates there are several different germline mutations in a single F0 adult, consistent with the fact that there are four presumptive primordial germ cells at the 32-cell stage (Houston et al., 1998; Resson and Dixon, 1988). In contrast, we found almost no germline mutations in F1 embryos derived from F0 adult frogs that had been injected with *tyr* TALEN mRNAs at the one-cell stage (data not shown). Overall, these results demonstrate that the oocyte host-transfer method is an effective strategy for generating germ line mutations.

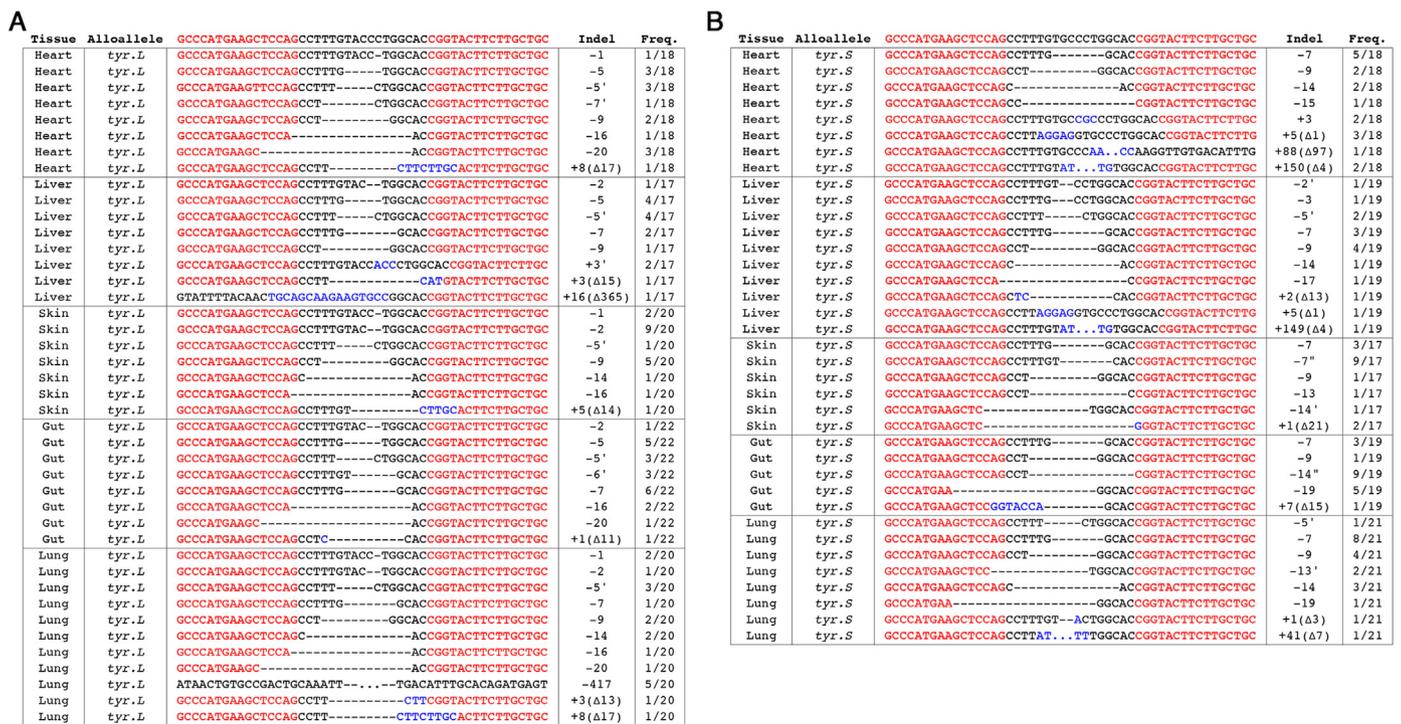
We anticipated that injecting TALENs into the oocyte would lead to the induction of mutations at an early stage, since the TALEN proteins would be present before fertilization. However, the

presence of more than two mutations (and some wild type alleles) in the germ cells suggests that the TALENs were acting later in development. We wanted to determine if the same mutations found in the germ cells were also present in other tissues. If the TALENs induced mutations early in development then we expected to find identical mutations in somatic tissues as well. We therefore isolated genomic DNA from five different organs of the female OHT frog representing each germ layer, heart (mesoderm), skin (ectoderm), liver, lung and gut (endoderm), and genotyped the *tyr.L* and *tyr.S* loci.

Unexpectedly, we identified many additional mutations in each somatic tissue. For example, in F1 embryos derived from the F0 OHT female, we identified four different *tyr.L* mutations, -1, -2, -9, and wild type (Fig. 2A), yet in the F0 somatic tissues we recovered an additional sixteen unique mutant alleles (Fig. 3A). Furthermore, the most prevalent *tyr.L* germline mutation, -1, which was found in ~40% of F1 embryos (Fig. 2B), was only found in three of the five somatic tissues sampled (heart, skin and liver) and at much lower frequencies of 5–10% (Fig. 3A). Similar



**Fig. 2.** Genotyping of F1 tyr mutant embryos. (A) Bar graph illustrating the frequency of tyr.L and tyr.S mutations in F1 embryos generated by mating FO OHT frog with wild type J strain. Wild type alleles make up the remainder of each column. (B) Sequence of mutations identified in F1 embryos. Frequency refers to the number of F1 embryos that contained the mutation. Red font indicates TALEN target sites.



**Fig. 3.** Genotyping of FO somatic tissue of female OHT tyr mutant frog. (A) Sequence of tyr.L mutations identified in the heart, liver, skin, gut and lung of FO female OHT frog. (B) Sequence of tyr.S mutations identified in heart, liver, skin, gut and lung of FO female OHT frog. Frequency refers to the number of clones that contained the mutation. Red font indicates TALEN target sites.

discrepancies were observed between FO somatic tissues and F1 embryos for tyr.S mutations. The most prevalent germline mutations in the OHT FO female, +1 and -14, which accounted for nearly 70% of all alleles transmitted to F1 progeny (Fig. 2B), were either entirely absent or found at a low frequency in only a subset of the somatic tissues (Fig. 3B). For both homeologs we found unique mutations in each somatic tissue that were not present in the other organs; many more mutations in somatic tissues that were not found in the germline, and drastically different frequencies for those mutations that occurred in both germline and somatic tissues. These results demonstrate that expressing TALENs

in oocytes followed by host-transfer can lead to efficient bi-allelic mutagenesis in *X. laevis*, but that the mutations are still induced after fertilization, which results in mosaic tissues in the FO frogs. The -9 mutation was identified in all five FO somatic tissues at similar penetrance (19% vs 24% in germ cells), whereas the -1 mutation was only found in the skin (10%), which was much less than that observed in F1 embryos (47%). A similar discrepancy was observed between FO somatic tissues and F1 embryos for the tyr.L-2 mutation; the -2 mutation was found in 6% of F1 embryos (Fig. 2), but present only in FO skin in 66% of clones (Fig. 3). For the tyr.S alloallele there were five genotypes in F1 embryos, +1, -7,

–8, –14 and wild type (Fig. 2), yet only the –7 mutation was found in each F0 somatic tissue (Fig. 3). Much like the *tyr.L* allele, we identified twenty two additional *tyr.S* mutations in F0 somatic tissues that were not present in F1 embryos (Fig. 3B).

#### 4. Discussion

Here we report the successful generation of highly-penetrant, germline-transmitted mutant alleles of both homeologs of the *X. laevis* tyrosinase (*tyr*) gene by injection of TALEN mRNAs into oocytes. Our results demonstrate that the oocyte host-transfer technique is a more effective method to generate mutations in *X. laevis* in both homeologs. Injection the *tyrosinase* TALEN mRNAs into oocytes results in almost complete albinism in F0 animals, whereas injection into embryos produced only patches of albinism. In raising these animals to adulthood, we show, for the first time in *X. laevis*, highly-efficient germline transmission of TALEN-induced mutations to the F1 generation. Although germline transmission of mutations have been demonstrated in *X. tropicalis* this is the first instance of efficient germline transmission of mutations in *X. laevis* (Guo et al., 2014; Ishibashi et al., 2012; Lei et al., 2012; Nakajima et al., 2012). These results demonstrate that germline mutants can now be produced in *X. laevis*, and opens up the experimental toolkit for *X. laevis* researchers. The development of this new J strain *X. laevis* line, designated *Xla.tyr<sup>tm1NXR</sup>*, will be of benefit to the wider *Xenopus* community.

Our injection and culture of oocytes with TALEN mRNAs followed by host-transfer aimed to effect the disruption of coding sequence as early in development as possible, either by induction of indels in the oocyte prior to fertilization, or by allowing sufficient time for translation of TALEN mRNAs prior to the rapid cell divisions during early embryogenesis. Although we did find that injection of TALEN mRNAs into oocytes produced highly-penetrant mutant alleles, the mosaicism observed in somatic and germ line tissues within the founders suggests that editing of genomic DNA occurred after fertilization, which is in agreement with previous reports (Miyamoto et al., 2015; Nakajima et al., 2012; Nakajima and Yaoita, 2015a). The disparate mutations observed in this study between somatic tissues of different germ-layer origins and those found in the F1 progeny suggests care should be taken in extrapolating genotype results from a single F0 embryo. This is in agreement with results obtained with zinc-finger nucleases targeting the *tyrosinase* locus in *X. tropicalis*, where they found mutations in different regions of the skin of adult frogs that were not present in sperm (Nakajima et al., 2012). The presence of unique mutations in different organs illustrates the difficulty of deciphering the genotype of specific cells in the F0 generation. Notably, the fact that we found wild type alleles in some F0 organs, derived from tadpoles that were completely albino, suggests that genotyping early F0 embryos will not reveal all of the mutations present in that animal.

Our results are in agreement with two recent papers that also expressed TALENs prior to fertilization to demonstrate efficient F0 knockout of *tyr* and *pax6* in *X. laevis* (Miyamoto et al., 2015; Nakajima and Yaoita, 2015a). The fact that we found unique mutations in each somatic organ that differed from those found in F1 embryos, suggests that mutations were induced after the 16-cell stage, consistent with recent results where the timecourse of induction of mutations was examined in OHT F0 embryos (Nakajima and Yaoita, 2015a). They found 50% mutagenesis of the *tyr* loci at the 8-cell stage, rising to 75% at the 32-cell stage, and only reaching 100% at stage 8.

The identification of unique mutations in three somatic tissues is consistent with *Xenopus* fate maps that show that heart and liver both arise from the D2.1 dorso-vegetal blastomere in the 16-

cell embryo (Moody, 1987a). By the 32-cell stage the heart and liver lineages have started to separate, with heart mesoderm coming from dorsal C blastomeres, and liver coming from the dorsal D blastomeres (Dale and Slack, 1987; Moody, 1987b). In contrast, the skin lineage is already separated from heart and liver at the 8-cell stage; at this stage trunk epidermis (skin) arises from the V1 blastomere, whereas heart and liver derive from D1 and D2 (Moody and Kline, 1990). In contrast to the large number of mutations found in somatic tissues, we only found 3–5 mutations in F1 embryos, consistent with the fact that presumptive primordial germ cells arise from approximately four cells at the blastula stage (Houston et al., 1998; Ressom and Dixon, 1988). Such tissue-specific mutations suggests that care should be taken in extrapolating results based on the genotyping an entire F0 embryo, since it will not be clear if the mutations detected are in the specific tissue being studied.

#### Acknowledgments

We wish to thank Sean McNamara, Brian Suh, Lynn Ware and Alyssa Arseneau for their care of the frogs and help in the breeding and to Esther Pearl for her help throughout this project. This work was supported by grants from the NIH (OD010997 and HD084409). We also thank Dr. Doug Houston for showing us the oocyte host-transfer technique.

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