NOVEL GENETIC MANIPULATION APPROACHES TO INVESTIGATE GENOMIC REARRANGEMENT IN OXYTRICHA TRIFALLAX

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Abstract

The dual nuclear structure of ciliates, and the derivation of the somatic nucleus from the germline nucleus makes ciliates great model system for studying genomic rearrangement. *Oxytricha trifallax* is a ciliate with a complex genomic rearrangement process, converting megabase sized conventional chromosomes into 16,000 kilobase sized unique chromosomes. While *Oxytricha trifallax* is an excellent model organism for studying complex programmed genomic rearrangement, the unusual nature of its dual nuclear structure makes conventional genetic manipulation approaches challenging or inaccessible in *Oxytricha*. In this thesis, I describe various approaches for genetic manipulation I have developed and their implications to *Oxytricha* biology.

In my thesis, I show that injecting synthetic DNA molecules homologous to native somatic chromosomes into *Oxytricha* at different stages in its life cycle result in divergent outcomes. Injections of synthetic chromosomes during its genomic rearrangement programs the deletion of those somatic chromosomes from the next generation. This phenomenon is robust across a variety of different somatic chromosomes. In addition, by backcrossing the programmed chromosome deletion lines to parental wild type, the programmed deletion can be inherited, implying an unidentified mechanism of elimination from soma through epigenetic inheritance in *Oxytricha*.

While injections of artificial chromosomes during genomic rearrangement cause deletion, injections during regular growth incorporate the construct into the somatic nucleus. These introduced artificial chromosomes are stably maintained, transcribed, and successfully translated. In addition, they can be used for various applications including characterizing the functional role of *Oxytricha* genes with an example of such applications shown here. In essence, these phenomena provide biological insights and are exploitable as means to investigate in previously unavailable ways.
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Chapter 1: Introduction

The diversity of life has resulted in a vast variety of genomic architectures for storage of genetic information and methods to produce functional elements from it. This variety of architectures includes circular or linear DNA and various levels of chromosome copy numbers. The clade of ciliates has intriguingly separated the dual nuclear roles of information storage and transcription of the genes. In ciliates, these single-celled organisms have two genomes in different nuclei, the “encrypted” germline the “active” somatic genomes. Along with being regionally separated, the genomes are also structurally and functionally distinct. The germline genome, located in the micronucleus, has a more conventional chromosome architecture with megabase, centromere-containing, diploid chromosomes (reviewed in Prescott 1994). The conventional structure of the germline genome belies the unconventional transcriptional inactivity of the genome. While the germline nucleus is transcriptionally silent, the somatic genome is the source of the cell’s RNA expression. The somatic genome, located within the macronucleus, is comprised of smaller chromosomes which range in size from a few kilobases in length in certain species to hundreds of kilobases (reviewed in Yerlici and Landweber, 2014). In addition to having shorter chromosomes, the somatic genome has a higher ploidy than the germline.

One fascinating feature of ciliates is the interaction and interdependence of the two genomes. In nominal growth conditions, the cells divide in a manner akin to binary fission where the micronucleus undergoes mitosis while the macronucleus, depending on genomic structure, may performed an amitotic division process instead (reviewed in Prescott 1994). Under stress conditions, such as food deprivation, ciliates will undergo their sexual cycle. During the sexual cycle, the compatible mating types will pair, forming a cytoplasmic bridge between the two cells, meiotically divide their micronucleus and cross-fertilize their germline with the paired cell. This
new zygotic micronucleus then mitotically divides and specializes, as one copy become the next micronucleus, and the other develops into the macronucleus.

In the ciliate *Oxytricha trifallax*, the cell must convert its conventional genomic structure of the ~100 micronuclear chromosomes into ~30 million chromosomes of the macronucleus (Swart *et al.* 2013, Chen *et al.* 2014). During this conversion, 90% of the germline genome is deleted, the remaining 10% is then rearranged and amplified to a high ploidy (Greslin and Prescott, 1989, Chen *et al.* 2014). In order to complete this rearrangement, the information of the sequences and arrangement of the somatic genome are epigenetic transfer from the parental macronucleus to the developing one.

Ciliates eliminate DNA during their genomic rearrangement through a variety of mechanisms. In *Tetrahymena* and *Paramecium*, a series of small RNAs are produced from the macronucleus and micronucleus. The small RNAs from the micronucleus that match with RNAs produced from the macronucleus are loss, leaving only a series of small RNAs that match specifically to the micronucleus (reviewed in Bracht *et al.* 2013). These micronuclear small RNAs, known as scan RNAs, associate with Piwi proteins and label the micronuclear limited sequences to eliminate them from the developing macronucleus.

In *Oxytricha*, two primary RNA based mechanisms have been identified for the epigenetic inheritance. Prior to the generation of the subsequent macronucleus, the somatic chromosomes from the parental macronucleus are fully transcribed, producing full-length RNA copies of the chromosomes along with small piwi-associated RNAs (piRNAs) (Nowacki *et al.* 2008, Fang *et al.* 2012). In *Oxytricha*, the Piwi associated small RNAs label sequences to be retained instead of deleted, as shown by introduction of synthetic piRNAs into *Oxytricha* during early sexual development against micronuclear limited sequences results in their retention in the next generation macronucleus (Fang *et al.* 2012). In addition to these piRNAs, the RNA copies of chromosomes, known as RNA templates, guide the genomic rearrangement process along with piRNAs. Introduction of an altered synthetic RNA chromosome can reprogram the
rearrangement somatic chromosome in the next generation macronucleus, while inhibition of the RNA templates results in improper and failed rearrangement (Nowacki et al. 2008).

*Oxytricha*s unusual genomic rearrangement, along with its other features, makes the organism an interesting model system for study. These features that make the system interesting also make it unamenable to many convention approaches, especially in genetic manipulation. The methods that available, as described in previously published research, were limited to transient knockdown by RNAi or antisense oligonucleotides and somatic insertion mutants by exploiting the epigenetic RNA pathways to retain germline limited sequence in the soma (Nowacki et al. 2008, Nowacki et al. 2009, Fang et al. 2012, Khurana et al. 2018).

In this thesis, I will present approaches to delete DNA from and introduce DNA to *Oxytricha*’s somatic genome. Through the process of microinjection of artificial chromosomes into *Oxytricha* at various points in its life cycle, DNA can be incorporated into the macronuclear genome or eliminated from it. This greatly increases the applications and avenues of research within *Oxytricha trifallax*.

In chapter 2, I will discuss programming deletion of somatic chromosomes via introduction of DNA early in genomic rearrangement. Previous work in *Oxytricha* has shown injected DNA to be able reprogram/alter the arrangement structure of sequences in next generation macronucleus (Nowacki et al. 2008). Following these type experiments, I show that by using a slightly modified approach, the injection does not result in the programmed alteration of the sequence, but in programmed deletion of the entire somatic chromosome. This deletion is apparently specific to the targeted chromosome and is generalizable to a variety of somatic chromosomes.

In addition to being a powerful technique to develop genetic knockouts in *Oxytricha*, I have found evidence for epigenetic inheritance of the deletions, providing unexpected insights into the genomic rearrangement process. Specifically, that deletions can be epigenetically inherited even when backcrossed to wild type cells. As the identified pathways to guide
rearrangement in *Oxytricha* are additive, the subtractive nature of the dominant inheritance of deletion implies an unidentified subtractive pathway in *Oxytricha*’s genomic rearrangement.

Chapter 3 of the thesis details the stable transformation of artificial chromosomes in *Oxytricha*. The ability to generate stable, transcriptionally active constructs by transformation enables a variety of genetic manipulation approaches. In ciliates, the divergence of the macronucleus from the micronucleus, along with the ploidy increase in the somatic genome creates certain challenges to transformation. Various techniques to transform the macronucleus of ciliates have been developed. In *Paramecium* and *Tetrahymena*, electroporation-based approaches have been used (Boileau *et al.* 1999, Gaertig and Gorovsky, 1992), while for *Oxytricha* and other Spirotrichs, the conventional genetic manipulation approach is through microinjections. Stable maintenance of DNA chromosomes has been reported in the Spirotrichs, *Stylonychia* and *Euplotes* along with evidence of transcriptional activity (Bender *et al.* 1999, Erbeznik *et al.*, 1999, & Skovorodkin *et al.* 1999). In both of these species, while successfully transformed and shown that the construct was transcriptionally active, though no direct evidence of translation of the constructs was shown.

In this chapter I show stable transformation of *Oxytricha trifallax* along transcriptional and direct evidence for translational activity of the artificial chromosomes. Along with displaying that transformed constructs are translated into functional proteins, I show applications of the techniques including tagging a protein of interest to investigate its associated proteins and RNA, verifying its association with various mRNAs and mRNA binding proteins. In addition to applications shown, somatic transformation opens new routes to investigate epigenetic inheritance.

These approaches enable us to have a better understanding *Oxytricha*’s biology along with additional methods of investigation for numerous lines of inquiry of this unusual model system.
Chapter 2: Programmed chromosome deletion in the ciliate

*Oxytricha trifallax*

Summary:

The ciliate *Oxytricha trifallax* contains two nuclei: a germline micronucleus and a somatic macronucleus. These two nuclei diverge significantly in genomic structure. The micronucleus contains approximately 100 chromosomes of megabase scale, while the macronucleus contains 16,000 gene-sized, high ploidy “nanochromosomes.” During its sexual cycle, a copy of the zygotic germline micronucleus develops into a somatic macronucleus via DNA excision and rearrangement. The rearrangement process is guided by multiple RNA-based pathways that program the epigenetic inheritance of sequences in the parental macronucleus of the subsequent generation. Here, we show that the introduction of synthetic DNA molecules homologous to a complete native nanochromosome during the rearrangement process results in either loss or heavy copy number reduction of the targeted nanochromosome in the macronucleus of the subsequent generation. This phenomenon was tested on a variety of nanochromosomes with different micronuclear structures, with deletions resulting in all cases. Deletion of the targeted nanochromosome results in the loss of expression of the targeted genes, including gene knockout phenotypes that were phenocopied using alternative knockdown approaches. Further investigation of the chromosome deletion showed that, although the full length nanochromosome was lost, remnants of the targeted chromosome remain. We were also able to detect the presence of telomeres on these remnants. The chromosome deletions and remnants are epigenetically inherited when backcrossed to wild type strains, suggesting that an undiscovered mechanism programs DNA elimination and cytoplasmically transfers to both daughter cells during conjugation. Programmed deletion of targeted chromosomes provides a novel approach to investigate genome rearrangement and expands the available strategies for gene knockout in *Oxytricha trifallax.*
Introduction:

The dual nuclear structure of the ciliate *Oxytricha* trifallax partitions the germline and somatic functional elements into two distinct nuclei, the micronucleus (MIC) and macronucleus (MAC). The somatic macronucleus is comprised of approximately 16,000 gene-sized chromosomes while the germline micronucleus contains an estimated 100 megabase-sized chromosomes (Swart et al. 2013). Under normal growth conditions, like other ciliates, *Oxytricha* reproduces asexually and its macronuclear nanochromosomes are maintained through amitotic division at an average of 2,000 copies per cell (Prescott 1994). Under stressful conditions, the cell can undergo a non-reproductive sexual cycle. During *Oxytricha*’s sexual development, cells of different mating types pair and transfer a single post-meiotic haploid micronucleus. The exchanged haploid nuclei fuse with their non-exchanged counterpart to form a new diploid micronucleus. The new macronucleus develops from the newly formed micronucleus through elimination of more than 90% of the DNA sequence (Chen et al. 2014) and rearrangement of the remaining sequences generates nanochromosomes in the new macronucleus (reviewed in Yerlici and Landweber 2014).

Ciliates have specific mechanisms to identify sequences for retention or deletion from the rearranging nucleus. In the distantly related ciliates *Tetrahymena* and *Paramecium*, the differentiation of germline-limited internally eliminated sequences (IESs) from macronuclear destined sequences (MDSs) is programmed by scan RNAs (scnRNA) (reviewed in Allen and Nowacki et al. 2017). These small RNAs are produced from the parental micronucleus during genomic rearrangement and are selectively enriched by alignment to macronuclear sequences. Small RNAs that do not match sequences in the parental macronucleus proceed to mark sequences in the developing macronucleus for excision and elimination.

In *Paramecium* species, there is evidence of an interaction between the scnRNA and RNA interference (RNAi) pathways that can reprogram MDS deletion in subsequent generation. Experimental introduction of truncated genes into the vegetative macronucleus leads to the
production of siRNA against the targeted gene and the subsequent silencing of both the native and introduced copies of the gene (Meyer 1992, Ruiz et al. 1998, Galvani and Sperling 2001, Götz et al. 2016). Upon undergoing conjugation, this RNAi silencing can trigger, in the subsequent generation, deletion of the genomic regions, flanked by a “TA” dinucleotide, which is the cut site for PiggyMAC transposases (Klobutcher and Herrick 1995; Baundry et al. 2009). Further evidence of this interaction came from the observation that injection of RNAi against specific MDS regions resulted in the deletion of these MDSs from the next generation’s macronucleus (Garnier et al. 2004).

In contrast to Paramecium and Tetrahymena, the RNA pathways identified so far in Oxytricha mark sequences for retention rather than deletion. Early in Oxytricha’s genomic rearrangement process, the parental macronuclear genome appears to be completely transcribed, generating long RNA templates (Nowacki et al. 2008; Lindblad et al. 2017) together with the production of 27 nucleotide piRNAs (Fang et al. 2012; Zahler et al. 2012). Collectively, these template RNAs and piRNAs mark retained sequences and program the necessary rearrangements to convert a germline into the somatic nucleus. Experimentally, injection of synthetic piRNAs can result in the retention of normally deleted IESs in the macronucleus. In Oxytricha, there is no simple consensus dinucleotide “TA” cut site, instead adjacent MDS ends in the macronucleus contain microhomologous sequences that vary and can be much longer (Chen et al. 2014). Only one copy of the microhomologous sequence is retained in the macronucleus after MDSs fuse during rearrangement. The general presence of these microhomologous repeats at the ends of MDSs has long suggested that they play an important role in facilitating Oxytricha’s genomic rearrangement (Prescott 1994).

Introduction of artificial DNA or RNA templates early in genomic rearrangement reprograms rearrangement of the next generation’s macronucleus (Nowacki et al. 2008, Fang et al. 2012). Here, we show that introduction of synthetic copies of a full length nanochromosome early in genomic rearrangement can reprogram deletion of that chromosome from the subsequent
generation’s macronucleus. Furthermore, we exploited this surprising observation to develop a tool for targeted chromosomal deletion to facilitate functional genetic manipulation of *Oxytricha trifallax*.

**Results:**

*DNA injection can trigger chromosome deletion:*

Previous research demonstrated that injection of synthetic DNA or RNA copies of chromosomes during nuclear development can specifically influence DNA rearrangement in the subsequent generation (Nowacki *et al.* 2008; Nowacki *et al.* 2011; Bracht *et al.* 2017). To further investigate the influence of exposure to a synthetic DNA molecule during conjugation, we injected a copy of wild type Contig16116.0, the nanochromosome encoding the Otiwi1 gene, into *O. trifallax* cells. Surprisingly, some (approximately 10%) of the resulting progeny displayed significant copy number reduction of the endogenous chromosome (Figure 2.1). In addition, we injected a modified version of Contig16116.0, containing a 28 nucleotide deletion flanked by 7 base pair endogenous repeats (see figure 2.2), anticipating that this DNA template could program deletion of the 28 nt sequence in the next generation’s somatic genome (Nowacki *et al.* 2008). Instead, we observed deletion of the endogenous chromosome, suggesting robustness of this effect to modest deletions in the injected template (Figure 2.1).
Figure 2.1: DNA injections result in the deletion of nanochromosomes from the subsequent generation

A) PCR amplification from genomic DNA harvested from screened and established lines shows loss of the targeted nanochromosome. The top gel section of each panel shows the deleted chromosome as labeled above, with the name of deletion lines (marked Δ) established and assayed in this experiment. The bottom gel section of each panel shows PCR amplification of the TEBP-β gene as input loading control. Deletion line shown in comparison to the parental lines (strains JRB310 and JRB510, marked simply 310 or 510) or the uninjected F1 population derived from mating the two parental lines. In the ΔOtiwi1 panels, deletion lines 1 and 2 were generated through injection of a construct containing a 28 bp deletion while line 3 was generated using a full length Otiwi1 chromosome construct. B) Diagram of the nanochromosomes in the PCRs in panel A with the following features labeled: telomeres (red), the locations of genes (green), and the location of the primers used in panel A (pink).
Figure 2.2: Injected construct designs
List and schematic illustrations of the various constructs used in injections in this article. The constructs were built by PCR of the native nanochromosome using JRB310 genomic DNA. The inclusion or absence of double stranded telomeres, along with the presence or absence of a synthetic deletion, are included with the name. Constructs are identical to the full length native nanochromosome except for the absence of telomere overhangs and indicated deletions.
Diagrams of the constructs shown to the right depict various features of the sequences, including the location of MDSs (gold) and genes (green) on the native nanochromosomes. In addition, the locations of the double stranded telomeres (red) and the synthetic deletions (black) are depicted.
In order to determine the extent of this phenomenon; five additional chromosomes were tested. Contig20822.0 was also injected as a full-length DNA chromosome containing a 37 nucleotide deletion, whereas the remaining four cases were injected as full-length wild type DNA versions (Figure 2.1 and figure 2.2). qPCR analysis and whole genome sequencing demonstrated severe copy number reduction of the target chromosome within the established cell lines, with some variation in DNA copy level at regions across the chromosomes (Figure 2.3 and figure 2.4).
Figure 2.3: Sequence coverage for a deleted chromosome is decreased in deletion lines
Normalized read depth from Illumina whole genome sequencing mapped to targeted chromosomes shows a strong decrease in the deletion lines relative to the uninjected F1 lines derived from JRB310xJRB510 matings. The first deletion line is labeled in red and second line depicted in blue.
Figure 2.4: Validation of chromosome deletion via qPCR
A) Diagram of the Otiwi1 (contig16116.0) nanochromosome with the locations of the Otiwi1 gene (green), the telomeres (red), MDSs (gold), and locations of the qPCR primers labeled (pink, and labeled with the qPCR they belong to in panel B). Validation of the Otiwi1 chromosome deletion by quantitative PCR on genomic DNA using primers at various positions along the nanochromosome contig. C) Diagram of the contig18510.0 nanochromosome with the locations of telomeres (red), MDSs (gold), and qPCR primers labeled as above. D) Quantitative PCR on the contig18510.0 chromosome in the contig18510.0 deletion lines along with respective controls. Relative copy level was determined by ΔΔCT with 310x510 F1 levels normalized to a value of 1. Error bars represent standard deviation.
Some cases of chromosome deletion result in maintenance of DNA fragments:

To compare copy number variation across different locations on the same chromosome, including the 5’ and 3’ ends of Contig20822.0 that encodes an Alba domain gene (hence known as Alba-like 1 or AL1), a Southern blot was performed on these deletion lines, together with the control parental strains, against a probe for the 3’ end of the chromosome (Figure 2.5C). This also indicated a significant reduction in the full-length AL1 chromosome levels. Furthermore, in agreement with the qPCR results (Figure 2.5B), deletion lines 1 and 2 (Figure 2.5 panels B and C) appear to contain a less abundant, shorter 3’ chromosome fragment. Whole genome sequencing reads were used to search for the presence of short chromosome remnants in other deletion lines.
Figure 2.5: Remnants of deleted chromosomes are detected in some of the deletion lines
A) A diagram of the AL1 nanochromosome with various features labeled, including the locations of the telomeres (red), the AL1 gene (green), the MDSs (gold), the qPCR primers (pink, labeled with which qPCR they belong to in panel B), and hybridization region of the Southern probe (blue). B) Quantification of the 3’ and 5’ end of the chromosome via qPCR and the relative copy versus the JRB310xJRB510 F1 genomic DNA. Error bars represent the standard deviation. C) Southern analysis of the first 3 contig20822.0 (AL1) deletion lines (#1, #2, and #3) using a probe against the 3’ end of the nanochromosome. D) The same membrane was then stripped and re-probed with TEBP-beta for loading control. E) A depiction of the contig14335.0 nanochromosome with MDSs (gold), telomeres (red), and the gene specific primers for the two rounds of telomeric PCR (pink, labeled with which PCR round they are involved in) labeled. F) Second round of telomeric PCR for the 3’ end of the Contig14335.0 chromosome in contig14335.0 deletion line #2.
The most abundant set of remnants was identified in Contig14335.0 deletion line 2, which has high Illumina read coverage exclusively in the center of the nanochromosome, and no sequence coverage at the ends (Figure 2.3). Moreover, telomeric reads mapping to the central portion of Contig14335.0 were identified in the deletion lines but not the wild type controls. In order to confirm the presence of telomeres on the remnants, PCR using a telomeric primer and a gene specific primer was performed as in (Chang et al. 2004) on Contig14335.0, deletion line 2. This telomeric PCR confirmed the presence of an internal telomere addition site in the deletion line (Figure 2.5E). In the case of Contig16116.0, an aberrant product was the dominant product in the conventional PCR screening of deletion line 1 (see figure 2.6). Sanger sequencing of this product revealed that it contained incorrect MDS fusion sites at a series of microhomologous repeats.

Figure 2.6: Aberrant rearrangement remnant detected in contig16116.0 (Otiwi1) deletion lines
A) PCR amplification with 20 ng of input genomic DNA on contig16116.0 (Otiwi1) deletion lines, along with wild type controls. B) Annotation of the Sanger sequencing from the cloned aberrant 1 kb product associated with the contig16116.0 deletion #1 in (A). Germline MDS segments are labeled by their sequence in wild type cells. Deletions internal to an MDS and abnormal rearrangement junctions at novel, or cryptic, pointers with sequence microhomology are indicated in cyan.
Chromosome deletion eliminates expression of genes on the nanochromosome:

The ostensible loss or severe copy number reduction of the targeted nanochromosomes could lead to a phenotype since the genes present on these nanochromosomes are heavily reduced in DNA copy number or completely absent from the somatic macronucleus. Indeed, we observed the same conjugation specific lethality in the Otiwi1 (Contig16116.0) deletion lines that phenocopy knockdowns of Otiwi1 gene (Fang et al. 2012). In addition, RT-PCR and qPCR analysis of the AL1 (Contig20822.0) deletion lines showed a massive reduction in the expression of the AL1 gene (Figure 2.7). Thus, programmed chromosome deletion provides a novel mechanism for generating somatic mutant strains in O. trifallax. Furthermore, deletion of the AL1 gene results in a lethal phenotype when the deletion lines are mated to each other, with no cells surviving beyond 30hrs post-mixing. This suggests the AL1 gene is essential during sexual development, and provides proof of principle for the use of chromosome deletion to test gene essentiality.

Figure 2.7: Deletion of the AL1 chromosome disrupts AL1 gene expression
Quantitative PCR assay of cDNA generated from RNA harvested from ΔAL1xΔAL1 (lines #4 and #5) mating at 12 hours post mixing. ΔΔCT quantification was normalized to matched 12 hours post mixing wild type mating. Otiwi1 and TEBP-α mRNA are quantified for additional controls. Error bars represent the standard deviation.
**Epigenetics of chromosome deletion:**

Given our knowledge that macronuclear chromosome architecture relies on the transfer of parental epigenetic information across sexual generations (Nowacki *et al.* 2008; Fang *et al.* 2012; Bracht *et al.* 2017), we examined the influence across generations of loss or copy number reduction of targeted nanochromosomes. Since the deletion lines for Contigs 17155.0 and 9679.0 target genes under further investigation (V.T.Y., unpublished) and Contigs 20822.0 and 16116.0 encode genes essential for conjugation, we tested the ability of the deletion lines for Contigs 14335.0 and 18510.0 to generate sexual progeny, since both of these nanochromosomes contain no predicted genes.

Cell lines containing the deletion of Contig14335.0 were mated to each other or backcrossed to the parental strain JRB510. The Contig14335.0 deletion lines mated to each other had a low survival rate, while the backcrosses showed no apparent survival defect. Both the deletion lines crossed to each other (Figure 2.8 panels C and D) and Contig14335.0 deletion line 1 backcrossed to JRB510 (Figure 2.8 panels F and G) showed no recovery of the deleted chromosome. DNA from backcrosses of Contig14335.0 deletion line 2 mated to JRB510 could amplify the full-length version of the deleted chromosome on a population level (Figure 2.8I).
C) 

<table>
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<th>Ladder</th>
<th>F1's #1</th>
<th>F1's #2</th>
<th>F1's #3</th>
<th>Δ #1</th>
<th>Δ #2</th>
<th>310</th>
<th>510</th>
<th>310x510</th>
<th>No DNA</th>
</tr>
</thead>
</table>

Ctg14335

TEBP-β

D) 

Relative copy level (3p10s10 set to 1)

E) 

Contig14335 deletion generation

JRB310 x JRB510

DNA injection

Contig14335 deletion lines

(Δ#1 & Δ#2)

Contig14335 deletion Δ#1 backcrosses

Δ#1 x JRB510

F1’s x JRB510

F2’s

(#1, #2, & #3)

Wild type cross

JRB310 x JRB510

“310x510” F1’s

I) 

<table>
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<tr>
<th>Ladder</th>
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<th>F1’s isolate #2</th>
<th>F1’s isolate #3</th>
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<th>310</th>
<th>510</th>
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Ctg14335

TEBP-β
Figure 2.8: Deletions and remnants are epigenetically inherited
A) An illustration of the contig14335.0 with primer locations depicted (purple for the detection PCR shown in panels C and E, pink for the qPCR primers, labeled with the corresponding qPCR they belong to in panels C, E, G). In addition, the locations of the MDSs (gold) and telomeres (red) on the nanochromosome are shown. B) Diagram of the various crosses involved generating the progeny involved in panels C and D. The colors in the diagram correspond to the colors in panel D. C) PCR amplification of genomic DNA harvested from populations of the progeny of ctg14335.0 deletion line 1 mated to deletion line 2, with parental lines and grandparental lines, along with uninjected 310x510 as controls. Lower gel section shows PCR amplification of TEBP-β as loading control. D) Quantitative PCR on the F1 from ctg14335.0 deletion line 1 mated to deletion line 2 surveyed across various regions of the contig14335.0 nanochromosome. E) Diagram of the various crosses involved generating the progeny involved in panels F and G. The colors from the diagram correspond to the colors in the following panel G. F) PCR amplification from genomic DNA harvested from populations of ctg14335.0 deletion line 1 backcrossed to wt strain JRB510 for up to 2 generations (labeled F1 and F2) with parental lines and grandparental lines along with uninjected 310x510 as controls. Lower gel section shows PCR amplification of TEBP-β as loading control. G) Quantitative PCR on genomic DNA used in panel A across multiple locations of ctg14335.0, together with an unrelated locus, TEBP-α. Arrows represent undetectable levels at the respective loci, with arrow color corresponding to the legend. Relative copy levels were determined by setting the levels of JRB310xJRB510 F1 to 1. H) Diagram of the various crosses involved generating the progeny in panels I and J. The colors from the diagram correspond to the colors in the following panel J. I) PCR amplification of genomic DNA harvested from clonal isolates and populations (labeled respectively) of the progeny of ctg14335.0 deletion line 2 backcrossed to JRB510, with parental and grandparental lines, along with uninjected JRB310xJRB510 as controls. Lower gel section shows PCR amplification of TEBP-β as loading control. J) Quantitative PCR on F1 genomic DNA from ctg14335.0 deletion line 2 across various regions of contig14335.0.
Contig18510.0 deletion lines can be successfully backcrossed to the parental strain JRB510. Their progeny, however, do display recovery of the deleted chromosome via conventional PCR (see figure 2.9C).

Figure 2.9: Reversion of chromosome loss in contig18510.0 deletion lines
A) An illustration of the contig14335.0 chromosome with primer locations depicted (purple for the PCR shown in panel C, pink for the qPCR primers, labeled with the qPCR they belong to in panel D). In addition, the locations of the MDSs (gold) and telomeres (red) on the nanochromosome are shown. B) Diagram of the various crosses involved in generating the progeny in panels C and D. The colors from this diagram correspond to the colors in panel D. C) PCR amplification on genomic DNA harvested from populations of ctg18510.0 deletion lines backcrossed to JRB510 with parental lines and grandparental lines along with uninjected 310x510 as controls. Lower gel section shows PCR amplification of TEBP-β as loading control. D) Quantitative PCR on genomic DNA used in panel A on multiple locations of ctg18510.0, along with an unrelated locus, TEBP-α. Relative copy levels were determined by setting the levels of 310x510 F1 to 1.
To assess how common recovery was in the backcrossed progeny of Contig14335.0 deletion line 2 to JRB510, clonal lines were singled out. Nine such cell lines (out of 9) displayed no recovery of the full-length chromosome (Figure 2.8I), suggesting that recovery was infrequent. Quantitative PCR analysis of the backcrossed cells revealed an abundant center fragment derived from the deleted chromosome, with copy number at near wild type levels, as also seen in the parental Contig14335.0 deletion line 2 (Figure 2.8J). To test whether the internal telomere addition site in the parental deletion is inherited in the offspring, telomeric PCR and Sanger sequencing were performed (Figure 2.10). This confirmed that the highly abundant remnant in the F1 progeny has the same telomeric addition sites as those identified in the parental line, demonstrating that deletion of Contig14335.0 epigenetically transfers with incomplete penetrance to the subsequent generation, including the inheritance of a truncated telomere-containing chromosome fragment.

**Figure 2.10: Telomere sequence on remnant in contig14335.0 deletion line inherited in F1 progeny**
Sanger sequencing on TOPO cloned telomeric PCR products from parental deletion line # 2 and two lines generated from line #2 backcrossed to 510. The portions of the Sanger sequence that contains the telomeric repeat (C4A4) and sequence from the contig14335.0 MDS #3 are labeled. The vector backbone and further sequence into the Contig14335.0 MDS are trimmed from the alignment.
Previous experiments involving programmed alterations to macronuclear chromosome structure demonstrated the gradual reversion to the wild type genotype, i.e. loss of the programmed alteration, after multiple sexual generations (Fang et al. 2012, Nowacki et al. 2008). We tested the recovery of the deleted chromosome after an additional sexual generation by mating the progeny of the Contig14335.0 deletion line 1 that had been backcrossed to JRB510 in a second backcross to JRB510. The resulting F2 cells still show no recovery of the targeted chromosome, as assessed by conventional PCR and qPCR (Figure 2.8 panels F and G). Inheritance of the Contig14335.0 deletion across multiple generations suggests a strong penetrance and dominant influence of the chromosome deletion in *Oxytricha*.

**Discussion:**

*Injection of synthetic chromosomes into mating cells results in deletion*

Microinjection of a DNA template into *Oxytricha* cells during genome rearrangement can result in the deletion of the homologous sequence in the next generation macronucleus. This effect has been observed in many cases, implying that it is a general phenomenon. Strong sequence similarity is essential to target chromosome deletion, but the effect is robust to minor sequence variation, such as small deletions. Contig16116.0 and Contig20822.0 chromosome constructs containing small deletions, respectively 28 and 37 nucleotides, were still able to induce chromosome deletion. Surprisingly, whole genome sequencing revealed no significant off-target effects on nanochromosomes containing regions with high similarity to the deleted nanochromosomes (Table 2.1). This suggests that target recognition occurs at a level above local similarity. We have not determined the precise level of similarity that is sufficient to cause the deletion effect in the subsequent generation.
Table 2.1: DNA copy number of highly similar nanochromosomes in deletion lines

<table>
<thead>
<tr>
<th>Deleted chromosome</th>
<th>Blast hit</th>
<th>Lowest E-value (# of &lt;10^4 hits)</th>
<th>% of WT in deletion lines (RPKM in WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contig20822.0 (AL1)</td>
<td>Contig4598.0</td>
<td>$8 \times 10^{-19}$ (1)</td>
<td>107% (63.47)</td>
</tr>
<tr>
<td>Contig14335.0</td>
<td>Contig17852.0</td>
<td>$4 \times 10^{-75}$ (5)</td>
<td>102% (35.62)</td>
</tr>
<tr>
<td>Contig9679.0</td>
<td>Contig1707.0*</td>
<td>$4 \times 10^{-124}$ (6)</td>
<td>120% (2.44)</td>
</tr>
<tr>
<td></td>
<td>Contig12344.0*</td>
<td>$4 \times 10^{-124}$ (6)</td>
<td>75.2% (30.98)</td>
</tr>
<tr>
<td></td>
<td>Contig12028.0†</td>
<td>$2 \times 10^{-88}$ (8)</td>
<td>74.3% (5.21)</td>
</tr>
<tr>
<td></td>
<td>Contig15190.0†</td>
<td>$2 \times 10^{-56}$ (8)</td>
<td>54.8% (1.80)</td>
</tr>
<tr>
<td></td>
<td>Contig11881.0†</td>
<td>$4 \times 10^{-83}$ (7)</td>
<td>88.3% (9.01)</td>
</tr>
</tbody>
</table>

* Paralogous chromosomes (99% identical sequence)
† Paralogous chromosomes (92% to 98% identical sequence)

Feeding *Oxytricha* cells *E. coli* expressing RNAi from a plasmid containing a nanochromosome fragment previously resulted in failed rearrangement of the targeted chromosome (Nowacki *et al.* (2008). It is possible that the injected DNA in the current experiments may be acting in the same pathway as the previous RNAi feeding experiments. However, there is one striking difference. In Nowacki *et al.* (2008) and (2010), injection at 1 to 3 hours post-pairing (slightly earlier than the 3 to 5 hour injection timepoint in the present study) of an entire synthetic nanochromosome, including telomere overhangs, resulted not in chromosome deletion but instead in amplification of the chromosome copy number in the population of cells examined. The difference in these prior results from the current study may be due to subtleties in the timing of injection, or to differences between average effects on a population of cells (Nowacki et al. 2010) vs. individually isolated and cloned cells. Hence, the deletion phenomenon may have been overlooked in Nowacki *et al.* (2008, 2010) because we observe deletions at a rate of ~10% of injected cells, which would be missed in populations.

Comparison to other DNA deletion phenomena in ciliates
While programmed DNA deletion is now observed in both Oxytricha and Paramecium, the apparent substrate requirements are different. On a structural level, the deletion in Paramecium depends on the presence of a truncated gene product in order to generate RNAi against the gene. This is not the case in Oxytricha as the injection of a full length nanochromosome results in its deletion from the next generation. Additional evidence for the case that Oxytricha deletion is programmed via a separate mechanism is the ability to delete essential early conjugation genes (Otiwi1 and AL1), given that silencing in injected cells would cause lethality immediately after injection. The apparent mechanistic differences between deletion in Paramecium and Oxytricha implicate an alternative model for deletion in Oxytricha.

In addition, the ciliate Tetrahymena thermophila has recently been reported to have endogenous programmed deletion of macronuclear chromosomes. 50 minichromosomes are transiently maintained and then lost after six generations of vegetative growth post-sexual development (Lin et al. 2016, Feng et al. 2017). Interestingly, these minichromosomes encode post-conjugation development specific genes, suggesting the possibility of programmed elimination from the macronucleus as a mechanism for gene regulation. While the phenomenon of macronuclear chromosome deletion that we report in Oxytricha is induced via the injection of an artificial chromosome, it bears similarity to the observed elimination of whole chromosomes in Tetrahymena as a mechanism to restrict gene expression to early macronuclear development.

**Implications of epigenetic inheritance of the deletions**

The epigenetic inheritance shows different effects in the two cases examined, which seem to correlate with the fold-reduction in the parental deletion. In the case of Contig14335.0, deletion has a dominant effect, with the deletion being propagated to the subsequent generation after parental backcross. In case of the Contig18510.0 deletion, the nanochromosome was recovered in the subsequent generation after parental backcross. One of the apparent variables
between the two chromosome deletions is efficiency. The established Contig14335.0 deletion lines have strong copy number reduction, based on qPCR and genomic sequencing, whereas the Contig18510.0 deletion lines display only moderate reduction in copy number (~6-fold decrease) based on qPCR results compared to wild type cells. The dominance of the deletion phenotype could be dependent on the strength in the parental deletion line. This dominant deletion inheritance is similar to the dominant inheritance of IES retention (Fang et al 2012), where a normally deleted sequence is retained by progeny even when crossed to wild type cells.

The dominant inheritance of the chromosome deletion is surprising in the case of contig14335.0, based on the current model of early genome rearrangement. In *Oxytricha*, the piRNAs in association with Otiwi1 guide retention, while RNA templates help program rearrangement. Neither of these systems can easily explain the dominant inheritance of the chromosome deletion of contig14335.0. These results suggest that in addition to these known guiding RNA molecules, there are epigenetic elements that guide deletions. Hence, the deletion lines provide novel insight into the genome rearrangement process and can be used to further investigate the subtractive mechanisms at play.

**Model for the deletion phenomenon**

We show that the injected template has an influence on the subsequent macronuclear generation, likely by interacting with one of the *Oxytricha* RNA pathways that guide rearrangement, and perhaps transcription of the injected templates could interfere with this pathway. We did not investigate transcription of the injected DNA, however, due to limited amount of material and the relative low efficiency of deletion (~10% of injected cell lines result in chromosome deletion). As an alternative hypothesis to transcription of the injected product triggering DNA deletion, the injected DNA might inhibit or interfere directly with *Oxytricha*'s genome rearrangement pathway. In this second view, the injected DNA could inhibit the
marking of native sequences for retention in the newly developing macronucleus, perhaps via soaking up of piRNA or RNA templates similar to the targeted sequence. In this model, the injected DNA would act as a sponge for RNA templates or piRNAs, leaving an inadequate level of RNA to target the native sequence for retention.

Relevance of chromosome deletion

Targeted deletion of chromosomes has been previously demonstrated in other model systems through the use of various genetic tools (Matsumura et al. 2007, Li et al. 2012). Recently, targeted chromosome deletion has been generated in mice and human cell lines (Adikusuma et al. 2017, Zuo et al. 2017) through use of CRISPR/Cas9 and induction of targeted double stranded breaks. Chromosome deletion in Oxytricha offers an alternative demonstration of programmed chromosome deletion and emphasizes the diversity of genome rearrangement pathways in Oxytricha.

The heavy reduction in copy number of the targeted chromosome and their encoded gene(s) opens up new avenues for generating somatic knockdowns or knockout strains. Moreover, despite the low rate of chromosome loss, it is still a viable method for generating mutant strains, because several dozen injected cells can be screened (even one 24-well plate can be reasonably expected to yield two deletion lines), and the resulting strains can be stably maintained and propagated for use in future experiments. Gene knockdown by IES retention (Fang et al. 2012; Khurana et al. 2018) suffers from a variety of sequence constraints, in particular requiring the IES to be near the amino-terminus of the target encoded protein in order to disrupt its open reading frame. The chromosome deletion strategy that we describe here is free of this limitation, thus allowing the functional analysis of a wider range of target genes. In addition, the chromosomal deletion approach offers a simple and more potent way to knock out target genes, compared to programmed IES retention.
Methods:

*Generation of DNA constructs for microinjection*

To generate full length DNA templates including small (30 to 50 bp) deletions, overlap extension PCR was performed using JRB310 genomic DNA as template and Phusion DNA Polymerase (NEB). PCR Primers were synthesized by IDT with standard desalting conditions. Wild type DNA constructs were produced via conventional PCR with JRB310 genomic DNA as template. The PCR products were gel purified using Qiagen MinElute columns according to manufacturer’s instructions. PCR products were singly A-tailed with Taq polymerase (Roche) and TA-TOPO cloned (Invitrogen). TOPO cloned plasmids were transformed into TOP10 One shot chemically competent cells (Invitrogen) according to manufacturer’s instructions. Plasmid DNA was isolated from individual clones using the QIAprep Spin Miniprep kit (Qiagen). TOPO plasmids were verified via Sanger sequencing through Genewiz.

Validated plasmids were used as templates for PCR to generate approximately 100 μg of PCR product. Quality of the PCR products was verified by gel electrophoresis. PCR products were phenol:chloroform extracted and concentrated by ethanol precipitation. DNA pellets were resuspended in nuclease-free water (Ambion) and run through an ultra-free MC column (Millipore) according to manufacturer’s instructions. DNA was brought to a final concentration of 1 to 3 mg/mL for microinjection.

*Oxytricha culturing*

*Oxytricha trifallax* cells were grown in Pringsheim media (0.11 mM Na$_2$HPO$_4$, 0.08mM MgSO$_4$, 0.85 mM Ca(NO$_3$)$_2$, 0.35 mM KCl, pH 7.0), fed *Chlamydomonas reinhardtii* and supplemented with *Klebsiella* for improved growth. Cells were cleansed of debris and algae by filtering through cheesecloth and concentrated via centrifugation at 80g for 1 minute for harvesting (Khurana *et al*, 2014).
**DNA template injections and screening for deletion lines**

*Oxytricha* cells were mated approximately 1 to 4 weeks post-excystment by mixing 3 mL of each mating type, JRB310 and JRB510, along with 6 mL of fresh 1X Pringsheim. At 3 to 5 hours post mixing, pairs were isolated and placed in Volvic water with 0.2% bovine serum albumin according to previously published methods (Fang et al. 2012). DNA molecules were injected at 3 to 5 hours post-mixing into the macronuclei of the paired cells as previously described except for the timing (Nowacki et al. 2008). After injection cells were pooled in Volvic water to improve survival rates. At 60 to 72 hours post mixing, the pooled cells were singled out to grow clonal injected cell lines. As clonal population size grew, lines were transferred to 10 cm petri dishes and grown in 1X Pringsheim media.

*Oxytricha* clonal lines were screened for deletions using cells or purified genomic DNA in PCR. Genomic DNA was harvested via the NucleoSpin Tissue kit (Macharey-Nagel). Cells were concentrated via centrifugation for 1 minute at 80g, and the supernatant was aspirated.

**Mating of deletion lines**

Using the same method for setting up mating as above, pairs were collected at 6 to 12 hours post mixing and pooled. Clonal lines were isolated at 60 to 72 hours post mixing and cultured according to the protocol described above.

**RNA isolation and cDNA synthesis**

RNA was collected from pools of isolated pairs (approximately 50 pairs) at 12 hours post-mixing via the mirVANA miRNA Isolation Kit (Ambion) using the total RNA isolation protocol according to manufacturer’s instructions. RNA was DNase-treated with Turbo DNase (Ambion) according to the manufacturer’s instructions. SuperScript III First-Strand Synthesis (Thermofisher Scientific) kit was used according to manufacturer’s instructions to generate cDNA for qPCR analysis.
Quantitative PCR for genomic DNA and cDNA

Quantitative PCR was done with Sybr green power mix (ABI) according to the manufacturer's instructions using either ABI 7900 (ABI) or CFX384 (BioRad) qPCR machines. Standards were generated via conventional PCR and purified by MinElute PCR purification (Qiagen). Purified standards were quantified by Qubit High Sensitivity DNA Assay Kit following the standard protocol (Thermofisher Scientific). Results from qPCR were quantitated via ΔΔCT and standard curves.

Southern Hybridization

200 ng of genomic DNA was loaded onto a 1.0% agarose gel and Southern transfer and hybridization were performed according to previously published protocols (Bracht et al. 2017) with minor alterations.

Illumina sequencing of deletion lines

Genomic DNA from deletion and wild type cell lines were used for preparing TruSeq Illumina libraries according to manufacturer's instructions and sequenced in single-end mode on the Illumina HiSeq2500 platform. Raw reads (average length 150nt) were processed using Galaxy on the Princeton University webserver (galaxy.princeton.edu) (Goecks et al., 2010). Reads were trimmed using Trim Galore version 0.4.3 to remove low quality ends (<Q20) and adapter sequences (maximum allowed error rat: 0.1, minimum read lenght: 20nt) (Felix Krueger, Babraham Institute https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Duplicate reads were collapsed using FASTX-toolkit (Assaf Gordon, http://hannonlab.cshl.edu/fastx_toolkit/).

Reads were then mapped using BWA-mem with default parameters (Li and Durbin, 2009) onto the Oxytricha JRB310 MAC genome assembly (Lindblad, 2018). SAM files were
processed to remove alignments with a low mapping score and non-primary alignments using SAMtools view (parameters: -bSq 5 and -F 256) (Li et al., 2009). The mapped reads from each library were then subsampled using seqtk (https://github.com/lh3/seqtk) according to the number of reads mapping to the macronuclear genome to normalize for sequencing depth and converted into bedgraph format using BEDtools genomecov (Aaron et al., 2010). Coverage tracks were generated using Bioconductor software Sushi package (Phanstiel, 2015) (R version 3.4.1).
Chapter 3: Transformation with artificial chromosomes in

*Oxytricha trifallax* and their applications

Summary

*Oxytricha trifallax*, like other ciliates, has separate germline and somatic nuclei. The diploid germline genome in the micronucleus is composed of long conventional chromosomes, the macronucleus, contains a polyploid somatic genome which is naturally fragmented into thousands of kilobase-sized chromosomes. Here, we develop a method to stably incorporate artificial chromosomes into the macronucleus. We report two cases of successful transformation and demonstrate the use of somatic transformation to investigate gene regulation and gene function in *Oxytricha*. We show that the transformed artificial chromosomes are maintained through multiple asexual divisions. Furthermore, they support the transcriptional regulation of the native chromosome from which they were derived and are translated to produce functional proteins. To test whether transformed chromosomes are amenable to practical applications, we generated a tagged version of a representative gene (AL1) and used it to co-precipitate associated proteins. This revealed an association with nucleic acid binding proteins, specifically RNA-binding proteins, and RNA immunoprecipitation of AL1 revealed its association with multiple RNAs. The use of artificial chromosomes in *Oxytricha* enables an array of genetic and molecular biological assays, as well as new avenues of inquiry into the epigenetic programming of the macronuclear development and genome rearrangement.

Introduction

The dual nuclear structure of ciliates provides an unusual challenge in the genetic manipulation of these model systems. In the hypotrich *Oxytricha trifallax*, the transcriptionally active macronucleus (MAC) is comprised of thousands of gene-sized nanochromosomes, on average 3kb long (Swart *et al.*, 2013), maintained at high ploidy (reviewed in Prescott, 1994;
Yerlici and Landweber, 2014). Though the ploidy of each gene-sized chromosome varies, transcription is only modestly correlated with gene copy number (Swart et al. 2013). This implies that the sequence of the nanochromosome, rather than copy number, may play a role in dictating the level of transcription. However, Oxytricha’s nanochromosomes contain small noncoding regions (on average 107 bp upstream and 103 bp downstream of the gene) with potential regulatory sequences (Swart et al. 2013). Even with these short noncoding regions, evidence for regulation within them has been described for the Hsp70 nanochromosome in the related species Oxytricha nova by showing that upstream transcriptional heat shock elements are conserved (Anderson et al. 1996). This conventional sequence specific control of gene expression offers the opportunity for genetic manipulation, especially as only a single gene is typically present on a chromosome.

In essence, Oxytricha’s tiny chromosomes are fully functional units of a size amenable to in vitro synthesis (Beh et al. 2018). These kilobase sized chromosomes provides a potential avenue to artificially produce them and introduce them into macronucleus. In Euplotes and Stylonychia, linear constructs have been introduced into the vegetative macronucleus through microinjection and lipid transfection. These constructs were stably maintained at high copy levels and transcriptionally active (Bender et al. 1999, Erbeznik et al., 1999, & Skovorodkin et al. 1999). In Euplotes crassus, nanochromosomes containing the TATA-binding protein gene replaced with a neomycin resistance gene, and the rDNA chromosome with an anisomycin resistance mutation have been successfully transformed and confer antibiotic resistance to the transformed cells (Bender et al. 1999, Erbeznik et al. 1999). In Stylonychia lemnæ, the α-Tubulin minichromosome has been successfully transformed into vegetative cells and the authors demonstrated the importance of telomeric sequences for the transformant to be stably maintained (Skovorodkin et al. 1999, Skovorodkin et al. 2001). In addition to proof of concept, further studies transformed multiple variations of the α-Tubulin minichromosome to identify promoter elements upstream of the α-Tubulin gene (Skovorodkin et al. 2007).
The genomic resources in *Oxytricha* are well established (Swart *et al.* 2013, Chen *et al.* 2014), as well as use of *Oxytricha* as a model system for studies of RNA biology and epigenetic inheritance (Nowacki *et al.* 2008, Fang *et al.* 2012). However, to date there have been no studies reporting transformation in *Oxytricha*. Here we describe the successful transformation of vegetative *Oxytricha trifallax* cells, along with the stable maintenance of constructs that are both transcriptionally and translationally active. In addition, we show successful applications of chromosome transformation to investigate biologically relevant questions in this model system.

**Results**

*Injected constructs are stably maintained during asexual growth*

To determine if an artificial PCR construct could be maintained in the vegetative macronucleus, we microinjected a copy of the AL1 nanochromosome (ctg20822.0) with a carboxy-terminal 6x histidine tag into the macronucleus of a clonal line in which the AL1 chromosome had been deleted (Clay *et al.* submitted). We found that the tagged chromosome was indeed present in the macronuclear genome and continued to be present after multiple asexual generations, as well as post encystment, storage at -80°C, and excystment. Other artificial constructs were similarly maintained (Table 3.1). To further characterize transformations, the histidine-tagged AL1 and Hsp70-GFP transformants were further investigated. To quantify the copy number of the transformed construct, conventional and quantitative PCR were performed. This revealed that the constructs were maintained at a copy number within 2-fold of the native chromosome in wild type cells (Figure 3.1).
Figure 3.1: Detection and quantification of transformed nanochromosome in cell lines

PCR amplification of genomic DNA collected from cell lines shows the successful transformation of the artificial chromosome AL1 with a C-terminal histidine-tag (A) and the chromosome containing GFP in place of the Hsp70 gene (B). The AL1 construct was injected into the F1 progeny of JRB310 (labeled 310 in the figure) cells mated to JRB510 (510). These cells also contained a programmed deletion of the AL1 chromosome. The GFP construct, on the other hand, was introduced into WT JRB310 cells. PCR of the native TEBP-β locus provided a loading control. Quantitative PCR shows the abundance of the transformed chromosomes of histidine-tagged (His tag) AL1 (C) and GFP (D). TEBP-α and the wild type HSP70 chromosome were quantified as controls. Error bars for the quantitative PCR results represent standard deviation.
Table 3.1: List of transformants generated

<table>
<thead>
<tr>
<th>Transformed construct</th>
<th>Background strain</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-terminus 6x histidine-tagged ctg20822.0.g90 (AL1)*</td>
<td>AL1 chromosome deletion line #5 (see chapter II)</td>
<td>C-terminus 6x histidine tag with linker (GSGGSG) after AL1 protein</td>
</tr>
<tr>
<td>C-terminus 10x histidine-tagged AL1</td>
<td>AL1 chromosome deletion line #4 (see chapter II)</td>
<td>C-terminus 10x histidine tag with no linker after AL1 protein</td>
</tr>
<tr>
<td>N-terminus 6x histidine-tagged AL1</td>
<td>AL1 chromosome deletion line #5 (see chapter II)</td>
<td>N-terminus 6x histidine tag with linker (GSGGSG) prior to AL1 protein</td>
</tr>
<tr>
<td>C-terminus MYC-tagged ctg15169.0.g18 (AL2)</td>
<td>JRB310</td>
<td>C-terminus MYC tag with linker (8x glycine) after AL2 protein</td>
</tr>
<tr>
<td>GFP in ctg18685.0 (Hsp70) nanochromosome*</td>
<td>JRB310</td>
<td>Ciliate codon-corrected GFP replacement of the Hsp70 gene on the Hsp70 nanochromosome</td>
</tr>
<tr>
<td>Mango RNA aptamer in ctg4739.0</td>
<td>JRB310</td>
<td>Mango RNA replacement of the ctg4739.0 gene on the ctg4739.0 nanochromosome</td>
</tr>
</tbody>
</table>

* Transformants further characterized in this paper

While the artificial constructs are maintained after numerous generations, this does not exclude the possibility of end erosion. To test if any erosion was occurring on the introduced chromosomes, we performed telomeric PCR (Chang et al. 2004) and Sanger sequencing on genomic DNA harvested post encystment/excystment, representing 40 days of vegetative growth (approximately 40 generations) post-transformation. The resulting sequencing showed that the majority of GFP constructs had not had any erosion of sequence at the ends of the initial construct (Figure 3.2).
Figure 3.2: Sanger sequencing of artificial nanochromosomes shows no changes in telomere location

A) Diagram of the Hsp70-GFP construct transformed along with GFP specific primers (dark green triangles) used in the first or nested, second round of telomeric PCR. Telomeres are marked schematically as red rectangles, the GFP open reading frame highlighted in green, and the non-coding regions from the Hsp70 nanochromosome in which GFP replaced the Hsp70 gene are shown in yellow. B) Agarose gel of second round telomeric PCR from the GFP transformants with both 3' and 5' telomeric PCR products. Ladder is 1 KB+ (Thermofisher Scientific). Product sizes are 388 bp (3’) and 514 bp (5’). C) Sanger sequencing of the telomeric PCR products in panel B after gel extraction. Sequencing is aligned with respect to the GFP construct depicted in panel A. Grey bars at the bottom represent the Sanger sequenced region (along with details of the PCR from which they were generated on the left). The sequenced regions perfectly matched the designed construct in both cases, indicating correct retention of telomere addition sites.

Transformed chromosomes are expressed similarly to native chromosomes

The stable transformation of the artificial chromosomes suggests that they have been functionally incorporated into the cell. To validate the artificial chromosomes’ functionality, we assayed their transcriptional activity. To investigate the tagged AL1, RNA was collected at
various time points during the *Oxytricha* mating cycle. When backcrossed to the parental line JRB510, the levels of the tagged mRNA increased 100-fold during the early sexual cycle, mirroring endogenous AL1 expression (see figure 3.3A). To test the transcriptional activity of the Hsp70-GFP transformed constructs, the transformed line was assayed under heat shock conditions and also showed similar induction of expression after heat shock to that of the wild type Hsp70 (Figure 3.3B).
Figure 3.3: Quantitation of expression patterns of transformant and relevant native genes
A) Quantitative PCR on cDNA collected from JRB310 or the GFP transformed cells (JRB310 cells transformed with the GFP construct) shows the levels of GFP, HSP70, and TEBP-α expression after exposure to either heat shock for 10 minutes (10’ HS) or control, room temperature for 10 minutes (10’ RT). B) Quantitative PCR on cDNA collected from JRB310 x JRB510 mated cells or from the AL1+His-tag transformant mated to JRB510 at 0, 10, and 20 hours post mixing. Error bars represent the standard deviation of the three biological replicates.

Transformed chromosomes are successfully translated into expected protein products

It has been previously reported that transformed constructs in *Euplotes* and *Stylonychia* are transcribed, and indirect evidence of translation in *Euplotes crassus* was provided through screening for transformants via neomycin selection (Bender *et al*. 1999). Using our tagged protein and GFP construct, we set out to validate if the genes present on the transformed
chromosomes are successfully translated into functional proteins directly. In the case of the Hsp70-GFP transformation, heat shock resulted in detectable fluorescence within the transformed cells, while no increase in fluorescence was detected in wild type cells after heat shock (Figure 3.4). To verify translation of the tagged AL1 gene, a Western blot was performed on mating cells at 18 hours post mixing using an Anti-histidine tag antibody, which produced a strong signal at around 70 kilodaltons (expected size of 65 kD) in the histidine-tagged AL1 transformed line and no detectable product in the wild type cells (Figure 3.5).

Figure 3.4: Detection of translated protein from the transformant constructs
Fluorescence microscopy of live cells immobilized under mineral oil, for the GFP transformant cell line and JRB310. Images were taken 2 hours after incubation either at room temperature or at 37°C for 20 minutes (heat shock), as labeled.
Figure 3.5: Validation of successful translation of the histidine tagged AL1 protein
Western blot with Anti-rabbit Anti-histidine tag antibody (RM146) against cell lysate for JRB310 x JRB510 (wild type) and AL1 His-tag transformant x JRB510 mating at 18 hours post mixing. The band shown is approximately 70 kilodaltons in size (expected size of the tagged product is 65 kD).

Application of chromosome transformation to characterizing the function of AL1 protein

Having verified the translation of the AL1 histidine tagged protein, we proceeded to use the poly-histidine tag to immunoprecipitate the AL1 protein and associated proteins (Figure 3.6). Mass spectrometry on the immunoprecipitated elutes validated the pulldown of the targeted protein in the tagged samples, while no peptides of the AL1 protein were identified in the untagged samples. In tagged samples, a set of associated proteins was identified. Domain analysis of the co-precipitated proteins showed that the majority contained nucleic acid associated functions, with RNA binding the second most common GO term associated with the precipitates (nucleic acid binding was first). Domains identified among the abundant coprecipitates included other Alba domain proteins, PolyA binding, HMG box, and DEAD/DEAH helicase domains (Figure 3.7). To further investigate the property of RNA binding, RNA immunoprecipitation of the tagged protein showed enrichment for various *Oxytricha* mRNAs and RNA from the micronuclear limited 170 bp repeats, while showing a depletion of mitochondrial mRNA (Figure 3.8).
Figure 3.6: Gel electrophoresis quality control of the histidine tag immunoprecipitation
SDS-PAGE gel of the Anti-histidine tag immunoprecipitation with the AL1 band, the heavy chain and light chain of the antibody labeled along with bands on the ladder. Lanes contain various fractions (as labeled) from the immunoprecipitation on the cell lysates from 18 hours post mixing of AL1-histidine tag mating (AL1-HT transformant x JRB510) and wild type mating (JRB310 x JRB510).
Figure 3.7: Application of the transformed AL1 tagged protein for immunoprecipitation and identification of associated proteins
Spectrum counts of protein domains from the domains identified in the top 20 co-immunoprecipitated proteins (exclusive to the tagged sample) (greater than 35 spectra counts across the three replicates) from AL1 pulldown by mass spectrometry.

Figure 3.8: Tagged AL1 associates with numerous RNAs
Quantitation via qPCR of RNA from immunoprecipitation with an Anti-histidine tag antibody (RM146) of the histidine tagged AL1 mating (AL1-His tag x JRB510) normalized to levels in the immunoprecipitation of a wild type mating (JRB310 x JRB510). Targets quantified are RNA from the micronuclear-limited 170bp repeats, various macronuclear genes (AL2, Otiwi1, and TEBP-α), and the mitochondrial Cox1 gene (Mito Cox1). Error bars represent standard deviation of the technical replicates.
Discussion

Transformed chromosomes are incorporated into the macronucleus

Here we showed that microinjected constructs are stably maintained at high copy number in Oxytricha cell lines, even after encystment and excystment. To our knowledge, this is first demonstration of the use and maintenance of artificial chromosomes in Oxytricha, and at a ploidy level similar to the native wild type chromosomes from which the constructs derive. The copy number maintenance may derive from an RNA-regulated mechanism for internal copy number regulation (Khurana et al. 2018) but selection during the PCR screen for transformants may have also introduced a bias for high copy number chromosomes. In addition to being replicated and maintained like their native counterparts, the GFP-Hsp70 synthetic chromosome shows no obvious signs of erosion at either end of the construct. This suggests that telomerase, which in Oxytricha is expressed during vegetative growth, is able to add the telomeric overhangs, preventing erosion during replication (Swart et al. 2013). Telomeric erosion depends on the length of the telomeric overhangs, suggesting a low erosion rate in Oxytricha due its 16 base overhangs (Klobutcher et al. 1981, Huffman et al. 2000). In Trypanosoma brucei, the telomeric overhangs are reported to be less than 30 bases, and telomerase knockouts in T. brucei have an erosion rate of 3 to 6 base pairs per generation (Dreesen et al. 2005). By contrast, after approximately 40 generations, the Oxytricha Hsp70-GFP construct shows no change in the telomeric addition site, as also seen with the native chromosomes.

Injected synthetic chromosomes are expressed at similar levels to their native counterparts.

Along with stable maintenance, the expression patterns of the transformed constructs, while not identical, are close to that of the wild type gene that the constructs replace. Transcription levels of the AL1 histidine tagged construct follow the conventional AL1 gene’s expression, with over a hundred-fold increase in expression from the 0 hour and 10 hour time points, followed by a 20-fold decline by 20 hours. Due to the high sequence identity of the AL1 tagged construct compared to the native chromosome, this recapitulation of expression suggest
that transcriptional regulation is at least partly driven by DNA sequence. Expression of the GFP-Hsp70 chromosome further supports the conclusion that DNA sequence is the defining feature for transcriptional regulation in these cases, and that these features specifically reside outside of the open reading frame, as the entire Hsp70 gene has been replaced in this case, yet the GFP transcriptional activity mimics that of the native Hsp70 gene.

**Tagged proteins generated from transformed constructs provide in vivo tools for studying gene function**

The tagged AL1 protein provides opportunities to investigate the protein in vivo that previously required custom antibodies and other resources, whereas the ability to tag a protein of interest expands the repertoire of techniques available to investigate gene function in *Oxytricha*. Due to the evolutionary distance (Parfrey et al. 2011) of *Oxytricha* from other model organisms, antibodies developed to target proteins in other systems are not often useful for targeting the homologous proteins in *Oxytricha*. Tagging proteins also lowers the cost and time investment for developing resources in *Oxytricha*. Furthermore, the ability to label proteins of interest may enable other tool development, such as labeling of subcellular compartments.

**Maintained macronuclear constructs and epigenetic inheritance in ciliates**

One of the defining features of the dual nuclear structure of ciliates is that the somatic macronucleus is replaced during sexual development by a rearranged copy of the zygotic micronucleus. To program the conversion of the micronucleus into a new macronucleus, various RNA based pathways have been identified that transfer the informational content of the old macronucleus to the developing nucleus (reviewed in Bracht et al. 2013). The ability to generate synthetic constructs that differ from existing somatic chromosomes permits reproducible and scalable studies of the influence of the new or modified chromosome on genomic rearrangement.

As previously identified in *Oxytricha*, the retention of germline limited sequences along with alterations in sequence ordering in the soma are epigenetically inherited in the next
Transformation will permit further investigation of the parameters of somatic epigenetic transfer, using artificial somatic chromosomes to test their ability to program novel rearrangements, similar to the DNA injections in Nowacki et al. (2008), but by mating transformed lines to wild type cells, obviating the need for direct manipulation of cells during early development. Transformation also permits the ability to alter defined strains, whereas template or piRNA injection (Nowacki et al. 2008, Fang et al. 2012) produces the initial alteration in the F1 progeny of the injected mating cells (typically a cross between strains JRB310 x JRB510).

Conclusions: Transformation in Oxytricha

The development of somatic transformations in Oxytricha opens many opportunities to study the general biology of Oxytricha as well as the process of genome rearrangement. The successful incorporation of artificial chromosomes into the macronuclear genome, with no degradation, as demonstrated here, contributes to the expanding toolkit for this model system.

Methods

Generation of DNA constructs for microinjection

To generate artificial chromosomes, overlap extension PCR was performed on Oxytricha genomic DNA from the JRB310 strain or plasmid with a ciliate codon corrected enhanced GFP as template (Nowacki et al. 2005) using Phusion high-fidelity polymerase (NEB) and then purified with MinElute columns using the PCR purification instructions (Qiagen). Purified constructs were A-tailed with Taq polymerase (Roche) and then TOPO-TA cloned (Invitrogen) according to manufacturer’s instructions. TOPO plasmids were transformed into TOP10 One shot chemically competent cells (Invitrogen) following manufacturer’s instructions. Plasmid DNA was harvested from clones using the QIAprep Spin Miniprep kit (Qiagen). Plasmids were verified by Sanger sequencing through Genewiz.
The plasmids with verified insert sequence were used as template for PCR to produce 100 ug of synthetic chromosome with 20 base pair double stranded telomeres. The PCR products were then ethanol precipitated, resuspended in nuclease free water (Ambion) and put through ultra-free MC column (Millipore) according to manufacturer’s instructions to remove impurities. DNA constructs were quantified by QUBIT High Sensitivity DNA Assay kit (Thermofisher Scientific) for a final concentration of 1 to 3 mg/mL.

**Oxytricha culturing**

*Oxytricha trifallax* cells were cultured in Pringsheim media (0.11 mM Na$_2$HPO$_4$, 0.08 mM MgSO$_4$, 0.85 Ca(NO$_3$)$_2$, 0.35 mM KCl, pH7.0) and fed with *Chlamydomonas reinhardtii* and *Klebsiella* according to previous published methods (Khurana et al. 2014). Cells of two compatible mating types (strains JRB310 and JRB510) were starved 12 hours to induce mating. Mating was initiated by mixing approximately equal numbers of starved cells from each type. Pringsheim was added to dilute the mating cells to a final concentration of 5,000 cells/mL.

Cells were encysted by filtering cells with cheesecloth, and then concentration by centrifugation (100g for 1 minute). The cells were then resuspended in fresh Pringsheim media and left for three day in a petri dish without food. Cysts from the starved culture were concentrated by placing the culture into a graduated cylinder and allowing cysts to settle at the bottom of the cylinder. Liquid was aspirated off from the top and DMSO was added to a final concentration of 10% to the remaining cyst containing liquid. Cysts in 10% DMSO were stored at -80° C. For excystment, cysts were thawed, washed three times with Pringsheim, and then fed with *Chlamydomonas reinhardtii* and *Klebsiella*.

**DNA transformation through injection**

Vegetative *Oxytricha* cells were isolated and placed in Volvic brand mineral water with 0.2% BSA by mass. DNA constructs were injected into the macronuclei of the individual cells in the method described previously for paired cells (Nowacki et al. 2008). After injection, single cells were isolated into 1 mL of Volvic brand mineral water in individual wells on 24 well plates
and were treated according to standard cell culturing methods mentioned above. As clonal population size grew, lines were transferred to 10 cm petri dishes and then grown in 1X Pringsheim media.

**Validation of transformation**

*Oxytricha* lines were screened for successful transformation by using cells or purified genomic DNA as template in a conventional PCR. Telomeric PCR on the Hsp70-GFP transformant were performed using a telomeric primer with linker sequence (AP12-C4A4; 15 cycles for each round with a 1:1000 dilution between rounds) and GFP specific primer for the first round followed by a second round PCR using a linker primer (AP2) and nested GFP primers. Second round PCR products were gel extracted via MinElute gel extraction protocol (Qiagen) and sent for Sanger sequencing through Genewiz.

**Nucleic acid extraction and cDNA generation**

Genomic DNA was harvested from *Oxytricha* through concentrating the cells with centrifugation for 1 minute at 80g and then using the NucleoSpin Tissue kit (Macharey-Nagel) according to manufacturer’s instructions on the cell pellet.

RNA from cells was collected using the mirVANA miRNA Isolation Kit (Ambion) using the total RNA isolation protocol according to the manufacturer’s instructions. Isolated RNA was then DNAse-treated with Turbo DNase (Ambion) following manufacturer’s instructions. cDNA was generated from RNA by using the SuperScript III First-Strand Synthesis kit (Thermofisher Scientific). Isolation of RNA for heat shock experiment was done by concentrating 3 mL of cells via centrifugation (80g for 1 minute), aspirating off media and resuspending in previous volume using temperature appropriate 1X Pringsheim (room temperature or 37° C) and placed in a well of a 6 well plate. Cells were then incubated for 10 minutes at 37° C or room temperature for 10 minutes, followed by RNA harvesting protocol above.

**Quantitative PCR on genomic DNA and cDNA**
DNA and cDNA quantitation were done using SYBR Green power mix (ABI) according to manufacturer’s instructions on a CFX384 (Bio-Rad) qPCR machine. Standards for qPCR were generated from conventional PCR purified by MinElute PCR purification (Qiagen). Standards were quantified by Qubit High Sensitivity DNA Assay Kit (Thermofisher Scientific) and diluted with nuclease free water (Ambion) to appropriate concentration for the standard curves. Results from qPCR assays were calibrated using the standard curves.

**Live cell imaging**

Live *Oxytricha* cells were isolated on a glass coverslip and immobilized using the same protocol as for microinjections listed above. Cells were imaged by phase-contrast inverted microscopy (Zeiss, Axiovert 200) with a mercury vapor lamp source (LEJ, EBQ 100).

**Transformation protein detection and isolation**

To collect cleared cell lysate, matings (AL1-histidine tag transformant X JRB510 and JRB310 X JRB510) at 18 hours post mixing were concentrated by 10-micron Nylon Mesh (Small Parts) and further concentrated by centrifugation (100g for 1 minute). Cleared cell lysate was generated from cell pellets after the aspiration of the supernatant. Cells were lysed with lysis buffer (lysis buffer: 10% glycerol, 1x protease inhibitor cocktail (Sigma), 1 mM PMSF, 1x Halt phosphatase inhibitor cocktail (Thermofisher Scientific), and 1 mM DTT in stock lysis solution) (stock lysis solution: 20 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% NP-40) with 1 mL of buffer per 625,000 cells. Cell lysate was rotated at 4° C for 30 minutes. Lysate was then spun down at 16,000g for 30 minutes. Supernatant was collected and flash frozen with liquid nitrogen prior to storage at -80° C.

Western Blotting was performed on the cell lysates using the RM146 Anti-histidine tag antibody (Abcam) 1:2,000 with 1:3000 of Goat Anti-Rabbit IgG (H + L)-HRP Conjugate (#170-6515, Bio-Rad). Chemiluminescence was perform with Amersham ECL Western Blotting detection reagent (GE Healthcare) according to manufacturer’s instructions and imaged on silver film with a Kodak X-Omat film processor.
Co-immunoprecipitation and mass spectrometry of histidine-tagged AL1 protein

Dynabead G (Invitrogen) were prepared with RM146 Anti-histidine tag antibody following manufacturer’s instructions. Cleared cell lysate was incubated with Dynabead G for 3 to 4 hours at 4°C rotating before being washed 3X with IP wash buffer (lysis buffer without NP-40 detergent) and then 3X with volatile wash buffer (130 mM KCl, 50 mM NH₄HCO₃). Protein was eluted with NH₄OH prior to being flash frozen with liquid nitrogen. For quality control, 20% of elution was collected and dried via speed vac before being resuspended in 1x SDS-PAGE buffer for gel analysis with Krypton staining (Thermo Fisher Scientific). A Typhoon scanner (GE) was used for imaging to ensure quality control. Mass spectrometry was done through the Princeton Mass Spectrometry Core facility.

RNA-immunoprecipitation of histidine-tagged AL1 protein

18 hours post mixing cells were concentrated with 10-micron Nylon Mesh (Small Parts) to a final volume of 36 mL. To the concentrated cells, 1 mL of formaldehyde (37%) to fix the sample by shaking the samples at room temperature for 10 minutes. To quench the fixation, glycine was added to result in a final concentration of 125 mM and shaken for 5 minutes at room temperature. The fixed cells were pelleted by centrifugation (500g for 1 minute) and then washed twice with ice cold TBS. The pellet was then resuspended in RNA-IP lysis buffer (0.1% sodium deoxycholate, 1x Protease inhibitor, 1 mM PMSF, 1 mM DTT, and 0.1% RNAseOUT inhibitor in stock lysis solution), vortexted thoroughly, and then flash frozen with liquid nitrogen for storage at -80°C.

Lysates were then sonicated (LE220 Covaris) for 5 minutes (450 W peak incident power, 10% duty cycle, 200 cycles per burst) for immunoprecipitation. Sonicated samples were centrifuged for 10 minutes at 16,000g to collect the supernatant with 10% (200 μL) being collected as input and flash frozen. To pull down the RNA, Dynabead G (Invitrogen) were prepared with RM146 Anti-histidine tag antibody following manufacturer’s instructions with the modification of 0.1% of RNAseOUT (Thermofisher Scientific) added to the PBST. Lysate was
incubated with bead antibody conjugated beads overnight. Beads were washed twice at room temperature with each of the following buffers: buffer A (20 mM Tris pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS), buffer B (20 mM Tris pH 8.0, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS), buffer C (10 mM Tris pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate), buffer D (10 mM Tris pH 8.0, 1 mM EDTA). Proteinase K treatment was performed by adding 200 μl of 2x proteinase K buffer (200 mM Tris pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% SDS) and 1 μl of 5 M NaCl to both the beads and the previously collected input samples. Crosslinking was reversed by incubating the samples at 65° C for 2 hours. Samples were then treated with proteinase K (200 μg, Macharey-Nagel) for one hour at 60° C. Afterwards, RNA was acid phenol:chloroform (Ambion) extracted, ethanol precipitated, and resuspended in nuclease-free water (Ambion).
Appendix
Primer list for Chapter 2

Chromosome creation primers:

**AL1:**
5’ end Fw:
ATG AGA GTT TGT GAA AAA TTA AGT TTG TTG AGG TTA CTT TTG ATT GGT TAA TTA G
3’ end Rev:
TAT ATT AAA TAT CAA GAA AAA GTA AAA AGA CAG TAA AAA TAT ATA TAT ATT AAA TGC ATG

**Deletion generation overlap extension Fw:**
CTA CGC CCG TCC TTG TTC TCA AAA TCT CTC TCA TGG GTG GTC TGT TTT CTC TGT TTA TG

**Deletion generation overlap extension Rev:**
CAT AAA CAG AGA AAA CAG ACC ACC CAT GAG AGA GAT TTT GAG AAC AAG GAC GGG CG
5’ telomere addition Fw:
CCC CAA AAC CCC AAA ACC CCA TGA GAG TTT GTG AAA AAT TAA GTT TG
3’ telomere addition Rev:
CCC CAA AAC CCC AAA ACC CCT ATA TTA AAT ATC AAG AAA AAG TAA AAA GAC AG

**Otiwi1:**
5’ end Fw:
ATG AGT ATA ATT TGA ATT GTT GTA AAG AGA GTT TCC ATT TGA TTG
3’ end Rev:
GAT TAA TGG ATG GAA GAG ATA GAA GTA AAT TAG AGA TGA TTA ATT TAA TAT AAT TAT AAA

**Deletion generation overlap extension Fw:**
GGA ATC CGC TTT GAC ACC AAG CTT TCC CAA GGA CAA GAG CTC AAG TTG TTC TCC

**Deletion generation overlap extension Rev:**
GGA GAA CAA CTT GAG CTC TTG TTC GGA AAG CTT GGT GTC AAA ACG GAT TCC
5’ telomere addition Fw:
CCC CAA AAC CCC AAA ACC CCA TGA GTA TAA TTT GAA TTG TTG TAA AGA GAG TTT CC
3’ telomere addition Rev:
CCC CAA AAC CCC AAA ACC CCT TCC TGT ATA TTT CGA ATT TAA TCA G

**Contig14335.0:**
5’ telomere Fw:
CCC CAA AAC CCC AAA ACC CCA TTA ATT ATA TTG TTT TTG ACA TTG TAA TAG
3’ telomere Rev:
CCC CAA AAC CCC AAA ACC CCT TCC TGT ATA TTT CGA ATT TAA TCA G

**Contig18510.0:**
5’ telomere Fw:
CCC CAA AAC CCC AAA ACC CCA TTA ATT ATA TTG TTG ACA TTG TAA AAT T
3’ telomere Rev:
CCC CAA AAC CCC AAA ACC CCA AGG AGT GTT TCA GAA ATA GAA AAA TTC
Contig17155.0:
5’ telomere Fw:
CCC CAA AAC CCC AAA ACC CCA TTA GAG ATA TAA CCA GAA TAT TTT ATG GG
3’ telomere Rev:
CCC CAA AAC CCC AAA ACC CCA TTA GAG ATA TAA CCA GAA TAT TTT ATG GG

Contig9679.0:
5’ telomere Fw:
CCC CAA AAC CCC AAA ACC CCA AGA ATT AAT TGA AGT TGA TAA GTA AA
3’ telomere Rev:
CCC CAA AAC CCC AAA ACC CC T AAG TCT ATA TAA AAG TAT TGT TTT AAA AGT A

Detection primers:

AL1:
Fw:
ATG AGA GTT TGT GAA AAA TTA AGT TTG TTG AGG TTA CTT TTG ATT GGT TAA TTA G
Rev:
GAG AGC ACA ACT ACA CTT TGC CTC ACA CTC

Otiwi1:
Same as Otiwi1 5’ end Fw and 3’ end Rev primers

Contig14335.0:
Fw:
GAT AGA ATT GAA AGT AAA TTA GCT GTA AGG
Rev:
GAT CAA GCG ATC TAG CAA GGA G

Contig18510.0:
Fw:
TAA CAT AGT CCA GCA TTA CTA AAA TGA TG
Rev:
CTT AAA TGA GTT AAT ATA GAT CCA ACT CG

Contig17155.0:
Fw:
AAC TTA GCG GAA AAG TAA AAG CCA TAA TG
Rev:
TGA ACA TTA TCT TTA AGC TGT TAC AAA AC

Contig9679.0:
Fw:
ATT GTA GTT ACT GTA TGC TGC TGG
Rev:
TGT CTT CTG TCC ATA CCT TGA AGG
TEBP-β (Contig22260.0):
Fw:
GAG CAA ATC ACA ACA AGT TCA ACA ACA AAG CG
Rev:
GCT CAT TTC TTG GTT GAT CTC TTT GAG GCC

Telomere detection:

AP12C4A4 (1st round):
GTA ATA CGA CTC ACT ATA GGG CAC GCG TGG TCG ACG GCC CGG GCT GGT CCC CAA AAC CCC AAA ACC CCA AAA
Contig14335.0 telomeric fragment Rev (1st round):
CAC TTA TGA TAA GCA TAT CAA TCG GG G CTC
AP2 (2nd round):
ACT ATA GGG CAC GCG TGG T
Contig14335.0 telomeric fragment nested Rev (2nd round):
GGA TTA CTG GTA CTG TAG AAA GAA GGA ATG

Southern primers
AL1 3' Southern probe:
Fw:
ATG AGA CCA TCA ACA GAG ACT ATC CCT AAC
Rev:
CTC TTG TCT CTC CTT CTT GAA TCG AGG GTG

TEBP-β:
Same as TEBP-β detection chromosome

qPCR primers:

Mito Cox1:
qPCR Fw:
GCC GTG TTT ACG CTT ATT TAC A
qPCR Rev:
CGT CTA GGC ATA CCA GCA TAT C

AL1 (Contig20822.0):
5' qPCR Fw:
AAA GTC TGG TTC TCG TGG C
5' qPCR Rev:
TCT GAA GCT TGT CCT TGG ATG
3' qPCR Fw:
GAC AAG AGA CTA GCA GAC ACC
3' qPCR Rev:
ACT ACA CTT TGC CTC ACA CTC

Otiwi1 (Contig16116.0):
5' qPCR Fw:
TTT AAC AAG AAC AGA AAA GCA TTC AAG
5' qPCR Rev:
TGG ATG GTG AAT CAA ACT CGA G
Mid qPCR Fw:
CCT GAG ATT TGA GAC CAT CGG
Mid qPCR Rev:
AGT CTA GCA TCA AAT CCA GGC
3’ qPCR Fw:
GAG ACC AAG GAG CCA ACC
3’ qPCR Rev:
TCC AAT TAG TAG TTG CCA TGA GAG

Contig14335.0:
5’ qPCR Fw:
TCT CTC ATG AAC GGT TAG AGT TTA G
5’ qPCR Rev:
GGA TGA GTT TAT CGA TTC TGC ATT AG
Mid qPCR Fw:
CCA ATA CTC GAA GTC CTC CTT G
Mid qPCR Rev:
GGT AAT GGA TTA CTG GTA CTG TAG AA
3’ qPCR Fw:
GCT CTC GAG CTC ATC TGA TTC
3’ qPCR Rev:
TCC AAT TAG TAG TTG CCA TGA GAG

Contig18510.0:
5’ qPCR Fw:
CTG TCA TAT TAA CAT AGT CCA GCA TTA C
5’ qPCR Rev:
ATC CGT ATT CCT GTC TTC CAT TAG
Mid qPCR Fw:
AGT ACT TCT CTC AAA CAG GAT AGA AC
Mid qPCR Rev:
AGA TAT TGG AGT CTC TCT TTG TGT T
3’ qPCR Fw:
TTC ACA ACA GCC TAA GAA GTA TCT
3’ qPCR Rev:
CAT TTG TAT CAT TCA ATG GCA CTC T

TEBP-α (Contig22209.0):
qPCR Fw:
CCA CAG AGC CAC CAT CAG ACT TTA
qPCR Rev:
GAG AAT GGT ACG AGA TCG CTA GTA GC
Primer list for Chapter 3

Chromosome creation primers

**AL1 C-terminus His tag:**
5' telomere addition Fw:
CCC CCA AAC CCC AAA ACC CCA TGA GAG TTT GTG AAA AAT TAA GTT TG
3' telomere addition Rev:
CCC CAA AAC CCC AAA ACC CCT ATA TTA AAT ATC AAG AAA AAG TAA AAA GAC AG
**AL1/Hisidine tag overlap primer Fw:**
GGT GAT GGT GAT GAC CAC TTC CAC CTG ATC CTT CTT GAT TGG TCA AGT CTA CGG TGA TGG
**AL1/Hisidine tag overlap primer Rev:**
AGG TGG AAG TGG TCA TCA CCA TCA CCA TCA CGT AGA CCA TCA ACA GAG ACT ATC CCT AAC

**Hsp70-GFP:**
5' telomere addition Fw:
CCC CCA AAC CCC CAA ACC CCT GTA GTA TCA AAT ATG TAA GA
3' telomere addition Rev:
CCC CAA AAC CCC AAA ACC CCA TGA TAA ATA ATT TGA AGT AC
5' Hsp70 UTR/GFP overlap primer Fw:
ATT CAT AAT TTT AGT GTT TAG TGT TTA ATT AAA AGT TAT AAT TAT TAC ATC ATT AAT CGC
5' Hsp70 UTR/GFP overlap primer Rev:
CTT TTA ATT AAA CAC TAA ACA CTA AAA TTA TGA ATT CTA GAG GAG AAG AAC TTT TCA CTG
3' Hsp70 UTR/GFP overlap primer Rev:
CAC TGA TTA GCT CAA TCT CCA GCA TGT TGG GCC ATA TG
3' Hsp70 UTR/GFP overlap primer Fw:
CTG GAG ATT GAG CTA ATC AGT GAA AGC AAT CTC AGC TAA ATA TTC TTT ATG

**Chromosome detection primers**

**AL1-Hisidine tag detection:**
**AL1 c-terminus primer Fw:**
GAC AAG GAT CAG AAG ATC AAC AAC AGT C
**AL1 c-terminus primer Rev:**
GTT AGG GAT AGT CTC TGT TGA TGG TCT C

**GFP detection:**
5' Hsp70 UTR/GFP overlap primer Fw & 3' Hsp70 UTR/GFP overlap primer Rev as listed above

**TEBP-β (Contig22260.0):**
**TEBP-β Fw:**
GAG CAA ATC ACA ACA AGT TCA ACA ACA AAG CG
**TEBP-β Rev:**
GCT CAT TTC TTG GTT GAT CTC TTT GAG GCC
qPCR primers

Mito Cox1:
qPCR Fw: GCC GTG TTT ACG CTT ATT TAC A
qPCR Rev: CGT CTA GGC ATA CCA GCA TAT C

AL1 (Contig20822.0):
AL1 qPCR Fw: AAA GTC TGG TTC TCG TGG C
AL1 qPCR Fw: TCT GAA GCT TGT CCT TGG ATG

AL1-Histidine tag qPCR Fw:
GAG GCC ATC CAA CAA GAT CAC
AL1-Histidine tag qPCR Rev:
ATG ACC ACT TCC ACC TGA TCC

Hsp70 (Contig18685.0):
Hsp70 qPCR Fw: TCC CCA ACG ATC AAG GAA AC
Hsp70 qPCR Rev: ATA AGT CTC TTG GCG TCG AAG

GFP:
GFP qPCR Fw: TGC CAT GCC AGA AGG ATA TG
GFP qPCR Rev: ACT TCA GCT CTT GTC TTG TAG TT

Otiwi1 (Contig16116.0):
Otiwi1 qPCR Fw: CCT GAG ATT TGA GAC CAT CGG
Otiwi1 qPCR Rev: AGT CTA GCA TCA AAT CCA GGC

AL2 (Contig15169.0):
AL2 Fw: GAA TGG ACC TGT GAC TGA GAA G
AL2 Rev: GAT ACG ATT GCT GAA CTT GCT

TEBP-α (Contig22209.0):
qPCR Fw: CCA CAG AGC CAC CAT CAG ACT TTA
qPCR Rev: GAG AAT GGT ACG AGA TCG CTA GTA GC
170 Repeat:
170 Repeat qPCR Fw:
TGA CCA TCA CTT GAA AAT GCA TG
170 Repeat qPCR Rev:
CCA CGT ATC TGG TGA GTT GAG

Telomeric PCR

AP12C4A4 (1st round):
GTA ATA CGA CTC ACT ATA GGG CAC GCG TGG TCG ACG GCC CGG GCT GGT CCC
CAA AAC CCC AAA ACC CCA AAA
GFP-specific primers (1st round):
Same as 3’ Hsp70 UTR/GFP Rev & 5’ Hsp70 UTR/GFP Fw from Hsp70-GFP creation primer set
AP2 (2nd round):
ACT ATA GGG CAC GCG TGG T
GFP-specific Fw primer (2nd round):
CTG CTG CTG GAA TTA CAC ATG GCA TGG ATG
GFP-specific Rev primer (2nd round):
TAA GTT GCA TCA CCT TCA CCC TCA CCA GAG
Bibliography


