Investigating Subfunctionalization of *Xenopus Laevis* AHR1 Paralogs in a Tadpole Cell Line

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Kenyon College Summer Science 2015

Abstract

The aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor, mediates an exceptional range of biological effects, from embryonic development and immune response to xenobiotic toxicity. However, the molecular mechanisms by which AHR affects this diverse set of processes are not well understood. The African clawed frog (*X. laevis*) serves as a novel model organism in which to study AHR functions due to its possession of paralogous AHR genes. While humans and most mammals possess only a single gene that codes for AHR, *X. laevis* possesses two paralogous AHR genes, AHR1α and AHR1β, that arose from a whole genome duplication event ~40 million years ago. The retention of duplicated genes commonly results from functional or regulatory divergence between the two copies of the progenitor gene, and we predict that AHR1α and AHR1β possess distinct, yet overlapping functions. To develop a route for investigating the functions of each AHR paralog, we employed transcription activator-like effector nucleases (TALENs) to generate AHR-paralog-specific knockouts in XTC-2 cells, a cell line derived from *X. laevis* tadpole tissue. We have generated multiple cell lines possessing 21, 11, and 9 base pair deletions in the AHR1β gene and currently seek cell lines possessing mutations in the AHR1α gene and both AHR genes simultaneously. Once we generate cell lines homozygous for paralog-specific AHR knockouts, we will employ qPCR to measure the consequential transcriptional response of each paralog knockout to determine the potential functions of AHR1α and AHR1β.

Background

-AHR1α and AHR1β share 86% amino acid identity.

-AHR1α and AHR1β are expressed at different levels between distinct organs and cells. (Lavine 2005)

-AHR is ligand activated to modulate the classical target gene, cytochrome P450 1A6 (CYP1A6). (Figure 1)

-Characterization of paralog-specific AHR knockouts in the XLK-WG cell line (*X. Laevis* kidney) revealed that both gene products contribute to CYP1A6 induction by 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD), a xenobiotic agonist. (SHF, Figure 2)

-Paralog-specific AHR activity in the XTC-2 cell line derived from X. laevis tadpole tail cells may prove a more relevant developmental model.

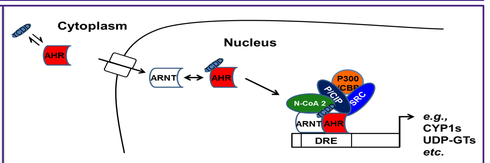
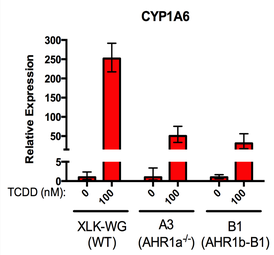


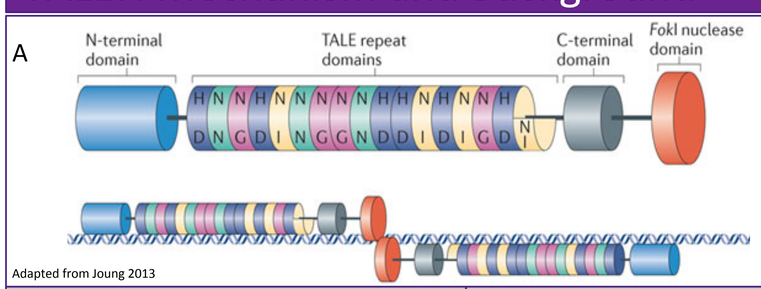
Figure 1: Classical AHR pathway. Inactivated cytoplasmic AHR is bound by a ligand and transported into the nucleus where active AHR heterodimerizes with ARNT. The AHR-ARNT complex binds dioxin response elements and modulates the transcription of AHR-dependent genes.



Scott Freeburg Unpublished Data

Figure 2: Mutant Characterization. Relative expression of CYP1A6 in XLK-WG cells, the A3 cell line (XLK-WG, AHR1α$^{-/-}$) and the B1 cell line (XLK-WG, AHR1β$^{-/-}$) treated with 100 nm of TCDD

TALEN Mechanism and Background



Adapted from Joung 2013

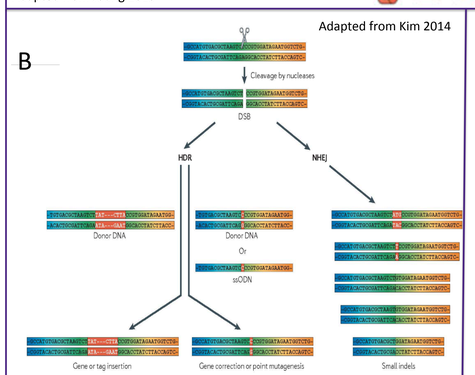


Figure 3: A) A representation of the TALEN mechanism is shown. TALENs bind specific DNA sequences and the C-terminal FokI nuclease cleaves the DNA generating a double stand break. **B)** The repair of the double strand break through non-homologous end joining can generate mutations.

-Transcription activator-like effector nucleases (TALENs) have been employed to modify targeted endogenous genes with minimal off target effects in a diverse range of animals and cultured cells.

-TALENs provide the gene targeting specificity necessary to generate mutations in only a single AHR paralog despite 85% shared nucleotide identity between the two paralogs.

-We employed TALENs to generate indels in AHR paralogs in the hopes of generating a frameshift mutation leading to a functionally inactive gene product protein.

TALEN Target sites

```
xl ahr1a ATGAACACGAACATCATGTACGCCAGCAGGAAGAGGAGAAAACCCGTCCAGAAAACAATT
xl ahr1b ATGAACACGAACATCATATACGCCGGCAGGAAGAGGAGAAAACCCGTCCAGAAAATAATT
         ********** ****** ****************************** ****

xl ahr1a AAGCCAACCCAGTCTGAAGGTGTCAAGTCCAACCCGTCAAAGAGGCACAGAGATCGGCTC
xl ahr1b AAGCCAACACAGGCTGAAAGTGTCAAGTCCAACCCATCTAAGAGGCACAGAGATCGCCTG
         ******** *** ***** ********** ** ********** **

xl ahr1a AACACTGAGTTGGACAGATTGGCAAGCCTGCTTCCATTCTCAGACGAGATCATATCAAAA
xl ahr1b AACACTGAGCTGGAAAGATTGGCAAGCCTGCTTCCATTCCAAGAGGAGATAATATCAAAA
         ********* **** ************************  *** ***** *********

xl ahr1a CTTGACAAGCTTTCAGTGCTTAGACTCAGTGTTAGTTATTTGAGGGCCAAGGGTTTTTTT
xl ahr1b CTTGACAAGCTTTCCGTGCTTAGACTCAGTGTTAGCTATTTAAGGGCCAAGGGTTTCTTT
         ************** ******************** ***** ************** ***
```

Figure 4: TALEN constructs. Three TALEN pairs have been employed in an attempt to edit the *X. laevis* genome: a TALEN pair to generate AHR1α knockout, a TALEN pair to generate AHR1β knockout, and and a TALEN pair knockout both genes. TALEN constructs were designed by Cellectis Bioresearch (Paris, France) to target the desired AHR paralog early in exon 2.

Red = AHR1α TALEN target site

Purple = AHR1β TALEN target site

Green = AHR1α + AHR1β TALEN target site

Transfection Workflow

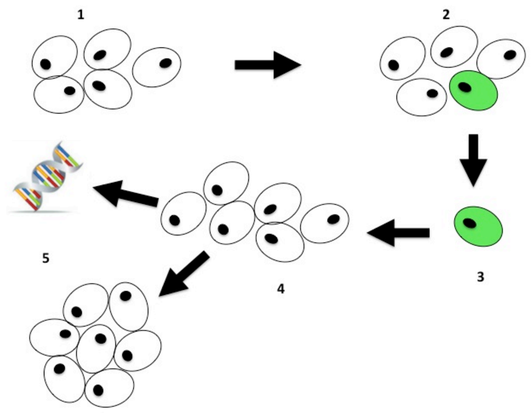


Figure 5: Role of green fluorescent protein reporter gene. 1. XTC-2 cells are trypsinized and brought into a suspension. 2. XTC-2 cells are transfected with TALEN plasmids and GFP by electroporation by the nucleofector 4D (Lonza) 3. Cells are clonally isolated, and GFP positive cells are identified using fluorescent microscopy (Olympus IX-70) 4. Fluorescing cell are allowed to grow. 5. Clonal cell lines are split. Some cells are isolated and prepared for DNA isolation. DNA is then sequenced (Retrogen) and analyzed for potential mutations. Remaining cells are left to grow to preserve the potential knockout cell line.

Transfection Optimization and Efficiency

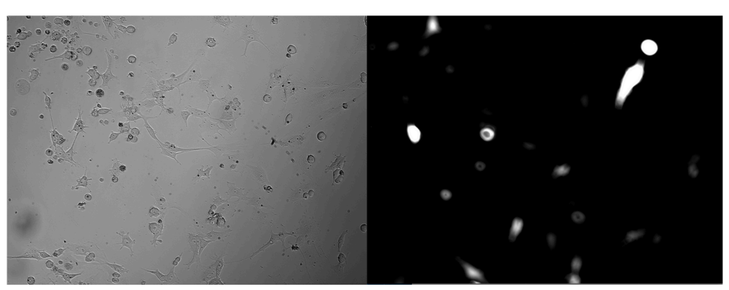


Figure 6: XTC-2 transfection screen. XTC-2 cell were examined under brightfield (left) and fluorescent light (right) to assess transfection efficiency. The transfection efficiency was calculated by taking the ratio of cells fluorescing with our reporter gene to the total number of cells in the frame.

-No protocol to transfect XTC-2 cells with the 4D-nucleofector previously existed.

-XTC-2 cells were transfected with 16 distinct electroporation shocks in 3 unique transfection solutions.

-Transfected cells were counted to calculate the ratio of cells fluorescing with the GFP reporter gene to the number of total cells. An estimate of cell death was also calculated.

-Optimized transfection procedure ensures that TALENs can pass into XTC-2 cells while minimizing cell death and damage.

Candidate AHR1B Knockout Cell Lines

Potential mutant 1B1.5

```
1B1.5.1          60 CCTGAACACTGAGCTGGAA----------CCTGCTTCCATTCCAAGAGGAGATAATATC 108
1B1.5.4          60 CCTGAACACTGAGCTGGAA----------CCTGCTTCCATTCCAAGAGGAGATAATATC 109
AHR1b exon 2     60 CCTGAACACTGAGCTGGAAAGATTGGCAAGCCTGCTTCCATTCCAAGAGGAGATAATA 117
```

Potential mutant 1B1.6

```
1B1.6.1          60 CCTGAACACTGAGCTGGAAAGATTGGCAAGCCTGCTTCCATTCCAAGAGGAGATAATA 117
1B1.6.3          60 CCTGAACACTGAGCTGGAAAGATTGGCAAGCCTGCTTCCATTCCAAGAGGAGATAATA 118
1B1.6.4          60 CCTGAACACTGAGCTGGAAAGATTGGCAAGCCTGCTTCCATTCCAAGAGGAGATAATA 117
1B1.6.5          60 CCTGAACACTGAGCTGGAAAGATTGGCAAGCCTGCTTCCATTCCAAGAGGAGATAATA 117
1B1.6.7          60 CCTGAACACTGAGCT----------TCCATTCCAAGAGGAGATAATATC  98
1B1.6.8          60 CCTGAACACTGAGCTGGAA----------CCTGCTTCCATTANAAGAGGAGATAATATC 108
AHR1b exon 2     60 CCTGAACACTGAGCTGGAAAGATTGGCAAGCCTGCTTCCATTCCAAGAGGAGATAATA 117
```

Potential mutant 1B1.7

```
1B1.7.1          60 CCTGAACACTGAG----------CTTCCATTCCAAGAGGAGATAATATC  98
1B1.7.2          60 CCTGAACACTGAG----------CTTCCATTCCAAGAGGAGATAATATC  98
1B1.7.3          60 CCTGAACACTGAG----------CTTCCATTCCAAGAGGAGATAATATC  99
1B1.7.4          60 CCTGAACACTGAG----------CTTCCATTCCAAGAGGAGATAATATC  98
AHR1b exon 2     60 CCTGAACACTGAGCTGGAAAGATTGGCAAGCCTGCTTCCATTCCAAGAGGAGATAATA 117
```

1B1.5 Protein Alignment

```
1B15.1 1 IKPTQAESVKSNPSKRHRDRLNTE---------LEPASIPRGDNIKTQVFRAYTHCLFGG 51
1B15.2 1 IKPTQAESVKSNPSKRHRDRLNTE---------LEPASIPRGDNIKTQAFRA-TQCLFKG 50
```

1B1.6 Protein Alignment

```
1B1.6.1          1 IKPTQAESVKSNPSKRHRDRLNTELERLASLLPFQEEIISKLDKLSVLRLSV---SYLRA 57
1B1.6.3          1 IKPTQAESVKSNPSKRHRDRLNTELERLASLLPFQEEIISKLDKLSVLRLSV---SYLRA 57
1B1.6.4          1 IKPTQAESVKSNPSKRHRDRLNTELERLASLLPFQEEIISKLDKLSVLRLSV---SYLRA 57
1B1.6.5          1 IKPTQAESVKSNPSKRHRDRLNTELERLASLLPFQEEIISKLDKLSVLRLSV---SYLRA 57
1B1.6.7          1 IKPTQAERVKPNPSNRPTDRLNTE-------LPFQEEIISELDKLSELXLSG---XYLRA 50
1B1.6.8          1 IKPTQAESVKSNPSKRHRDRLNTE---------LEPASIXRGDNIKTQVLRAXTHCLFRG 51
```

1B1.7 Protein Alignment

```
1B1.7.1          1 IKPTQAESVKSNPSKRHRDRLNTE-------LPFQEEIISXLDKLSVLILGV---SYLRA 50
1B1.7.3          1 IKPTQAESVKSNPSKRHIDRLNTE-------LPFQEEIISKLDKLSVLRLSV---SYLRA 50
1B1.7.4          1 IKPTQAESVKSNPSKRHRDRLNTE-------LPFQEEIISKLDKLSVLRLSV---SYLRA 50
1B1.7.7          1 IKPTQAESVKSNPSKRHRDRLNTE-------LPFQEEIISKLDKLSVLRLSV---SYLRA 50
```

Figure 7: Candidate AHR1β mutant cell lines. The cell lines 1B1.5, 1B1.6, and 1B1.7 generated from wild type XTC-2 cells are promising for the possession of a homozygous AHR1β knockout. 1B1.6 contains some wild type cell contamination that may be resolved by a clonal dilution of the cell line. However, 1B1.6 may be possess a heterozygous mutation and require further gene-editing.

Results Summary and Future Work

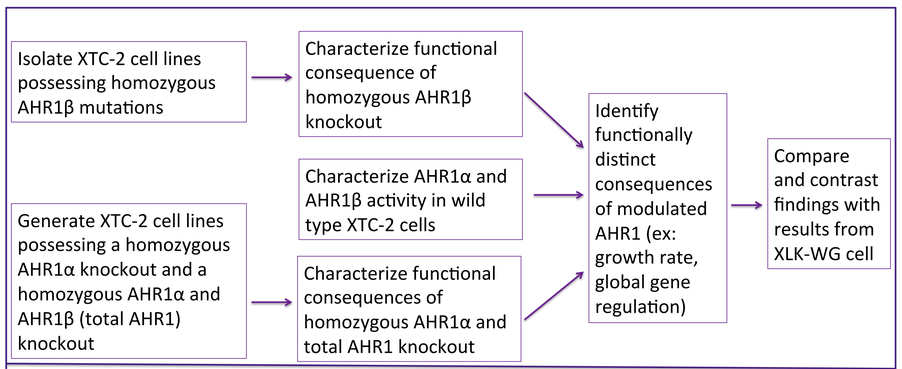


Figure 8: Summary of current standing and workflow of future work. While potential AHR1β knockouts have been generated, further sequencing analysis must be performed to confirm homozygosity of the mutation. Homozygous complete AHR1 knockout and homozygous AHR1α knockout cells lines will also be generated. The transcriptional consequence of each mutations will be characterized. Results will be analyzed for functional distinctions between different mutations and between the mutations and the wild type. The findings will also be compared to characterization results from XLK-WG cells to determine the presence of distinct activity between cell types.

References

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Acknowledgements

This work was funded by NIH R15 ES011130 to WHP and by the Kenyon College Summer Science Scholars program.