Modeling human hereditary syndromes using CRISPR/Cas9 mediated genome editing in *Xenopus tropicalis*

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Fig. 2. Phylogenetic relationships, estimated divergence dates and timing of paleotetraploidization events for the principal vertebrate animal models. The branching order of seven model species is shown. Colored horizontal panels represent geologic periods. The divergence times are given in million years ago and represent the mean of time estimates from different studies (see main text for details). Blue arrowheads indicate estimated times of the paleotetraploidization events in the lineages leading to *X. laevis* and zebrafish, respectively. Abbreviations: C, Carboniferous; Cm, Cambrian; CZ, Cenozoic; D, Devonian; J, Jurassic; K, Cretaceous; O, Ordovician; P, Permian; Pg, Paleogene; S, Silurian; Tr, Triassic.
Xenopus – main advantages

- Vertebrate – tetrapod (aquatic)
- High number offspring (> 1000 eggs)
- **External** embryonic development
- embryos have a large size
- embryogenesis proceeds **rapidly**

- *Xenopus tropicalis* is true **diploid**
- High **synteny** of genome
- **Targeted** injections are possible

= X. laevis and zebrafish

<< X. laevis and zebrafish

- **They don’t smell** (> <the mouse)
Xenopus – targeted injections

ectoderm
- cement gland
- epidermis (head)
- epidermis (trunk)
- lens
- olfactory placode
- otocyst

neurectoderm
- brain
- brain (dorsal ventral)
- hindbrain
- cranial ganglia
- neural crest
- retina
- spinal chord
- spinal chord (dorsal ventral)

see http://www.xenbase.org
Targeted injections
Increased tissue restrictiveness
Reduced toxicity and potential embryo-lethality
Injections Cas9 and CRISPR guide RNAs (2–8 cell stage)

Xenopus – injections CRISPR/Cas9

Phenotypic Readout
Design gRNA (CRISPRscan)

Primer ligation + direct T7 RNA synthesis (cloning free)

Inject gRNA + Cas9 protein

Assess efficiency (NGS – MiSeq) Mostly > 70%

Raise or Adjust protocol

Analysis F0 mosaic (e.g. cancer models)

F0

F1 phenotyping

> 80 genes targeted
Gene KO by Non-Homologous End Joining (NHEJ)

F0 mosaic

A

B

F1 offspring

A

B

C

Amaya lab

CTGTTCTTCTTCTTCCATGTTPCGAGGGCAGTTCCTCTCTCAAGGCATGTAGCACGG
F1-1
CTGTTCTTCTTCTTCCATGTTPCGAGGGCAGTTCCTCTCTCAAGGCATGTAGCACGG  WT  4/4
CTGTTCTTCTTCTTCCATGTTPCGAGGGCAGTTCCTCTCTCAAGGCATGTAGCACGG
F1-2
CTGTTCTTCTTCTTCCATGTTPCGAGGGCAGTTCCTCTCTCAAGGCATGTAGCACGG  WT  1/4
CTGTTCTTCTTCTTCCATGTTPCGAGGGCAGTTCCTCTCTCAAGGCATGTAGCACGG  Δ2  3/4
CTGTTCTTCTTCTTCCATGTTPCGAGGGCAGTTCCTCTCTCAAGGCATGTAGCACGG
F1-3
CTGTTCTTCTTCTTCCATGTTPCGAGGGCAGTTCCTCTCTCAAGGCATGTAGCACGG  WT  1/4
CTGTTCTTCTTCTTCCATGTTPCGAGGGCAGTTCCTCTCTCAAGGCATGTAGCACGG  Δ2  3/4
CTGTTCTTCTTCTTCCATGTTPCGAGG--CCAGTTCTCTCTCAAGGCATGTAGCACGG
F1-a
CTGTTCTTCTTCTTCCATGTTPCGAGG--CCAGTTCTCTCTCAAGGCATGTAGCACGG  Δ1  8/8
**Methods for genotyping**

**F0 mosaics**
- Heteroduplex Mobility Assay (HMA)
- TA-cloning and Sanger sequences
- NGS + Batch-GE analysis

**F1 offspring**
- High Resolution Melting Analysis (HRMA)
- Sanger Sequencing - Tracking of Indels by DEcomposition (TIDE) analysis

https://tide-calculator.nki.nl/
- Heteroduplex Mobility Assay (HMA) (for F0)

- TA-cloning and Sanger sequences
CRISPR/Cas efficiency analysis by NGS

PCR

NGS (MiSeq)

Data analysis

Speed
High throughput
Availability
Decreasing cost

Slides courtesy of Annekatrien Boel (CMGG)
CRISPR/Cas efficiency analysis by NGS

Speed
High throughput
Availability
Decreasing cost

PCR
NGS (MiSeq)
Data analysis

BATCH-GE
BATCH-GE

Singleplex PCRs → Raw equimolar pooling Library preparation → Illumina sequencing

Experiment 1
S1
S2
S3
S4
Sn

Experiment 2
S1
S2
S3
S4
Sn

Experiment n
S1
S2
S3
S4
Sn
Methods for genotyping

**F0 mosaics**

- Heteroduplex Mobility Assay (HMA)
- TA-cloning and Sanger sequences
- NGS + Batch-GE analysis

**F1 offspring**

- High Resolution Melting Analysis (HRMA)
- Sanger Sequencing - Tracking of Indels by DEcomposition (TIDE) analysis

https://tide-calculator.nki.nl/
• High Resolution Melting Analysis (HRMA) (for F1)
Genotyping work flow for analysis of F1-offspring

1. Cut tails
2. 96-well PCR plate
3. Perform HRMA assay
4. Recover animals with desired genotype
5. Lyse DNA in thermocycler
6. Perform PCR according to TIDE instructions and Sanger sequence
7. Genome editing site
8. TIDE analysis
9. Data analysis
Modeling human (mono)genetic disease
Pseudoxanthoma Elasticum  (collab. O. Vanakker)

Abcc6 (full KO)
Inherited Retinal Dystrophy (collab. E. De Baere)

**Rcbtb1** (mosaic KO) – Unilateral injection

![Images of retinal sections with labels](Images)

**Left eye**
- IPL
- INL
- OPL
- ONL

**Right eye**
- IPL
- INL
- OPL
- ONL

**RCBTB1-mosaic**
- GCL
- IPL
- INL
- OPL
- ONL
- PRL

**Caption:**
- Normal Vision
- Retinitis Pigmentosa
Limb defects

Ptch1 (mosaic KO)
Targeting Anterior-Posterior polarity – Shh

Regulation $Shh$ expression (limb enhancer (ZRS) in $LMBR1$ intron)

ZRS = ZPA (Zone of Polarizing Activity) Regulation Sequences

Visel et al., Nature 2009
Targeting Anterior-Posterior polarity – Shh

Regulation *Shh* expression (limb enhancer (ZRS) in *LMBR1* intron)
Targeting Anterior-Posterior polarity – Shh

Targeting shh limb enhancer (ZRS) in lmbr1 intron

- Point mutations in mouse, human and cat ZRS
  - Ectopic Shh
  - Shh

- PPD

- Preaxial polydactyly type 2
  - * * 2 3 4 5

- Deletion of the MFCS1
  - No Shh expression in the limb bud

- No hand plate
  - One small digit
Modeling human cancer

• APC and Familial Adenomatous Polyposis
Targeting APC MCR in X. tropicalis (TALEN)

- 15 AA repeat (β-catenin)
- 20 AA repeat (β-catenin)
- SAMP repeat (Axin)

Apc

**MCR**

- MT binding
- EB1 binding

**Arm**

TCTTCAGTACACCATAACGGACTAAAAAACAAACAGACTTCAAACATCAAA (∆1) x2
TCTTCAGTACACCATAACGGACT-AAAAACACAGACTTCAAACATCAAA (∆1) x2
TCTTCAGTACACCATAACGGACT-ACACGACTTCAAACATCAAA (∆13) x2
TCTTCAGTACACCATAACGGACT-TCAAACATCAAA (∆14)
Familial Adenomatous Polyposis in *Xenopus* (targeting APC tumor suppressor gene)

Van Nieuwenhuysen et al., Oncoscience 2015
Familial Adenomatosis Polyposis in *Xenopus* (targeting APC tumor suppressor gene)

- Extra-colonic manifestations:
  - Congenital Hypertrophy of Retinal Pigment Epithelium (CHRPE) ✓
  - Desmoid tumors ✓
  - Brain tumors (medulloblastoma) ✓
  - Abnormal dentition
  - Osteomas
  - Epidermal cysts
Modeling human cancer

- Retinoblastoma (Rb1 + Rbl1)
A Xenopus tropicalis retinoblastoma model
H&E of Rb1/Rbl1 model

A *Xenopus tropicalis* retinoblastoma model

H&E of Rb1/Rbl1 model

A Xenopus tropicalis retinoblastoma model

H&E of Rb1/Rbl1 model

cancer models under development?

- Medulloblastoma (Wnt, Shh, other) √
- Neurofibromatosis type 1 (NF1) /
- Neurofibromatosis type 2 (NF2) (√)
- Bladder cancer (NF1, PTEN) √
- Leukemia (Notch1, FBXW7, PTEN) √

Add. KOs: p53, Rag2
Modeling human cancer

- T-ALL (Notch1* + PTEN)
WT froglet

Notch1* + PTEN mosaic
Well you have tumors in your frogs .... So what??
application 1:
Desmoid tumor preclinical compound testing

BC-2059: inhibitor of TBL1
dependent β-catenin signaling
application 3:
Desmoid tumor therapeutic target identification via gRNA multiplexing workflow

Genome modification of \(APC\) and gene \(x\) → Dissect tumor → Isolate DNA → Sequence Gene X

Gene X disrupted in tumor? → YES → Gene X not important for tumorigenesis

Gene X disrupted in tumor? → NEVER → Gene X REQUIRED for tumorigenesis

Gene X = therapeutic target
Midkine (MDK)

bi-allelic mutations in MDK detected in desmoid tumors (7);

*MDK* is not essential for *in vivo* desmoid tumor initiation

Naert et al. unpublished
Identification of genes essential for desmoid tumorigenesis

Dissect tumor
Targeted deep sequencing of \textit{apc} and \textit{ptt} loci

\textit{apc + ptt} CRISPR/Cas9

Percentage of tumors exhibiting bi-allelic out-of-frame mutations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>lox</td>
<td>n=3</td>
</tr>
<tr>
<td>adam-12</td>
<td>n=12</td>
</tr>
<tr>
<td>MDK</td>
<td>n=6</td>
</tr>
<tr>
<td>hmr</td>
<td>n=3</td>
</tr>
<tr>
<td>wisp-1</td>
<td>n=5</td>
</tr>
<tr>
<td>NUAK1</td>
<td>n=9</td>
</tr>
<tr>
<td>FAP-\alpha</td>
<td>n=6</td>
</tr>
<tr>
<td>PCLAF</td>
<td>n=22</td>
</tr>
<tr>
<td>abc</td>
<td>9</td>
</tr>
<tr>
<td>def</td>
<td>12</td>
</tr>
</tbody>
</table>
User CRISPR for Domain Mapping

Negative selection to small in frames within functional domain reveals this domain as essential for tumorigenesis

**Domain 1** does not drive negative selection and **can tolerate in-frame mutations**

Raw targeted deep sequencing data

In frames removed
**User CRISPR for Domain Mapping**

Negative selection to small in frames within functional domain reveals this domain as essential for tumorigenesis

**Domain 5** targeting results in desmoid tumors that **retain one fully wild type allele**

![Diagram of domain targeting with percentage of mutant sequencing reads](chart.png)
Why do this in *Xenopus tropicalis*?

- Extremely simple, cheap and fast (>< mouse)
- Diploidy required (>< zebrafish)
- Targeted injections are great benefit (>< zebrafish)
• So far primarily KO experiments

• Can we do KI ?
  – HDR with repair template – using oocyte transfer
  – Microhomology Mediated Endjoining (PITCH)
TECHNIQUES AND RESOURCES

High-efficiency non-mosaic CRISPR-mediated knock-in and indel mutation in F0 Xenopus

Yetki Aslan, Emmanuel Tadjuidje, Aaron M. Zorn and Sang-Wook Cha*
A. Host transfer method using CRISPR

- Remove ovary & defolliculate

- Microinjection of CRISPR components

- HOST TRANSFER

- EMBRYO

B. Embryo injection using CRISPR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Host Transfer</th>
<th>Embryo Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tadpoles /% of heterozygotes (# of oocytes /total embryos)</td>
<td># of different mutations</td>
</tr>
<tr>
<td>ctnnb1</td>
<td>23/200 83% (19/23)</td>
<td>1</td>
</tr>
<tr>
<td>smad1</td>
<td>37/400 94% (34/36)</td>
<td>1</td>
</tr>
<tr>
<td>vangl2S</td>
<td>45/300 80% (33/41)</td>
<td>1</td>
</tr>
<tr>
<td>wnt7b.L</td>
<td>26/200 92% (23/24)</td>
<td>1</td>
</tr>
<tr>
<td>average</td>
<td>131/1100 87.90% (109/124)</td>
<td>1</td>
</tr>
</tbody>
</table>

11.9% recovery rates

F0: +/- wildtype

F1: +/- heterozygote

-/- mutants
Generation *Xenopus* knock-in models

**Generation KO**

- Generation KO
- Injection
- F0
- WT/KO
- F1

**Generation KI using host transfer**

- Generation KI
- Injection of CRISPR components
- Oocyte isolation
- Maturation and staining
- Host transfer
- Fertilization
- Host
- WT/KO
- WT/WT
- WT/KO
- KI/KO
- KO

*Slide courtesy of Marjolein Carron*
Microhomology-mediated end-joining-dependent integration of donor DNA in cells and animals using TALENs and CRISPR/Cas9

Shota Nakade, Takuya Tsubota, Yuto Sakane, Satoshi Kume, Naoki Sakamoto, Masanobu Obara, Takaaki Daimon, Hideki Sezutsu, Takashi Yamamoto, Tetsushi Sakuma, and Ken-ichi T. Suzuki

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