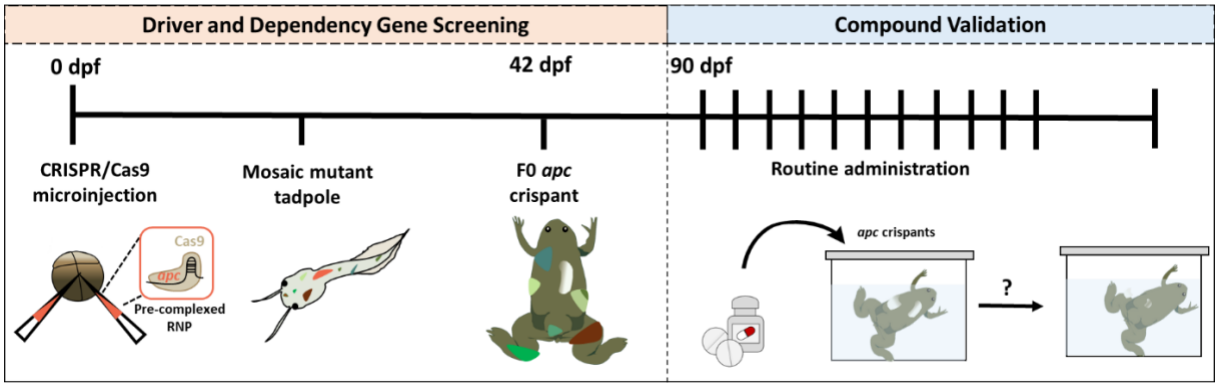
CRISPR/Cas9-mediated generation of mosaic mutant *X. tropicalis* animals harboring desmoid tumors

General outline

A desmoid tumor model with high penetrance and short latency can be obtained in *Xenopus tropicalis,* an aquatic frog species with a diploid genome. Early embryos are injected with CRISPR/Cas9 reagents targeting the *apc* tumor suppressor gene. By using low amounts of the reagents, the majority of the cells in the tadpoles and froglets will be wild type or heterozygous mutant for the *apc* gene. Only those progenitor cells that acquire a bi-allelic frame-shift mutation in *apc* acquire tumorigenic properties. The mosaic mutant animals can be used for dependency mapping or compound validation as described in Naert et al., 2021.



Detailed protocol

The protocol below is based on our publication Naert et al., 2021, but can be adapted using established methods running in your laboratory.

gRNA design and generation

The *apc* sgRNA targets the 5'-TGTATGGTGTACTGAAGAAC-3' sequence in the *Xenopus tropicalis* genome. To obtain the sgRNA, a 5'-gaattaatacgactcactataggGTTCTTCAGTACACCATACAgttttagagctagaaatagc-3' oligo along with the complementary reverse oligo 5'-aaaagcaccgactcggtgccacttttttcaagttgataacggactagccttattttaacttgctatttctagctctaaaac-3' were purchased (Integrated DNA Technologies, Coralville, IA, USA)[(1)]. The oligos are hybridized and extended via a standard PCR amplification reaction. Amplicons are purified using a Quick PCR purification kit (InvitrogenTM Thermo ScientificTM) and nucleic acid concentration is measured using a NanoDrop 2000 spectrophotometer (ThermoFisherTM). Next, in vitro transcription of sgRNAs is performed using a HiScribe T7 polymerase kit (New England Biolabs). RNA is isolated via a phenol/chloroform extraction with UltrapureTM Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v). The concentration of the purified RNA product is determined through fluorometric quantification using a Qubit 1.0 fluorometer (InvitrogenTM). The purity of the sgRNAs can be visually confirmed via RNA gel electrophoresis (1X MOPS, 1.16% formaldehyde). To prevent repeated freeze-thaw cycles potentially mediating sgRNA degradation, aliquots of 1.5 µL need to be stored at -80°C until further use.

Priming and boosting of *Xenopus* frogs to stimulate mating behavior

Mating behavior of WT male and female frogs is stimulated by injecting a priming dose (10 and 20 IU, respectively) of human chorionic gonadotropin (hCG) hormone (e.g. Chorulon) the day prior to mating, followed by a boosting dose (100 IU and 150 IU, respectively) on the morning of injection. Fertilized eggs are collected from the water and stripped from their jelly coat with a 2% (w/v) cysteine solution

(adjusted to pH 8.0) and washed with 0.1x MMR buffer (from 10x Marc's Modified Ringers – 1 M NaCl, 20 mM KCl, 10 mM MgSO4, 20 mM CaCl2, 50 mM HEPES (pH 7.4-7.8). Fertilization and following cell-division(s) are monitored under the stereomicroscope and at the desired embryonic stage, embryos are placed on an injection grid in 6% Ficoll (in 0.1X MMR buffer).

Microinjection of sgRNAs/Cas9 complexes

The *apc* sgRNA is precomplexed (30 seconds at 37°C) with recombinant Streptococcus pyogenes Cas9 protein (a Cas9 variant with a nucleic localization signal (NLS) on both N and C terminal) (e.g. GenScript, IDT, …), prior to loading into a glass capillary injection needle. We use ribonucleoprotein mixes with a concentration of 100 pg/µl NLS-Cas9-NLS and 40 pg/µl sgRNA for efficient genome editing[(2)]. A volume of 0.5 nL is injected into the vegetal-dorsal blastomeres[(3)] of a 4- or 8-cell embryo. Injected embryos are collected in a 6% Ficoll solution (in 0.1x MMR) to promote healing of potential puncture scars generated by the microinjection. Approximately one hour post microinjection, embryos are washed with 0.1x MMR to wash away excess Ficoll solution, and incubated overnight at 25°C. The wash step is repeated twice, one day post injection. Embryos should initially be kept in 0.1x MMR, on the second day post fertilization a third of the buffer can be replaced with frog water, and further with half a volume of water on the third day. Tadpoles are initially fed from day 5 post fertilization with SeraMicron (Green Algae). Late tadpole/froglet feeding and care is performed according to Tran et al., 2014.

Monitoring desmoid tumor formation

The first desmoid tumors become visible on the back of pre-metamorphic tadpoles and remain detectable on the back and belly of the froglets (note that the skin becomes less transparent at that stage).

Footnotes:

1. Commercial ready-made sgRNA templates can also be purchased.

2. Disruption of the *apc* gene activates the Wnt pathway, which is centrally involved in early development. Therefore, to prevent developmental abnormalities, only low levels for sgRNAs/Cas9 are injected. Since concentrations of the sgRNAs are not always easy to measure accurately, it may be advisable to titrate down a starting concentration until developmental phenotypes are no longer observed. Once this is determined, subsequent experiments can then use this concentration from the aliquoted sgRNA stock.

3. Targeting of a vegetal-dorsal blastomere mediates the highest frequency of desmoid tumors in absence of other neoplasms (e.g. in the limbs) and developmental abnormalities.

<u>References:</u>

Naert et al., CRISPR-SID: Identifying EZH2 as a druggable target for desmoid tumors via in vivo dependency mapping. Proc Natl Acad Sci USA. 118(47):e2115116118 (2021).

Tran et al., Design and use of transgenic reporter strains for detecting activity of signaling pathways in *Xenopus*. Methods. 66(3):422-32 (2014).