Harnessing zebrafish xenograft models for ocular melanoma treatment discovery

Yin, J.

Chapter 2

Ortho- and ectopic zebrafish xeno-engraftment of ocular melanoma to recapitulate primary tumor and experimental metastasis development

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Summary

Here, we present a protocol to establish both orthotopic and ectopic zebrafish xenograft models for ocular melanoma, where we subsequently use these models to assess the growth kinetics of the primary tumor, dissemination, extravasation, and distant, peri-vascular metastasis formation and the effect of chemical inhibition thereon.

Abstract

There are currently no animal models for metastatic ocular melanoma. The lack of metastatic disease models has greatly hampered the research and development of novel strategies for the treatment of metastatic ocular melanoma. In this protocol we delineate a quick and efficient way to generate models for both the primary and disseminated stage of ocular melanoma, using retro-orbital orthotopic and intravascular ectopic cell engraftment, respectively. Combining these two different engraftment strategies we can recapitulate the etiology of cancer in its totality, progressing from primary, localized tumor growth under the eye to a peri-vascular metastasis formation in the tail. These models allow us to quickly and easily modify the cancer cells prior to implantation with specific labeling, genetic or chemical interference; and to treat the engrafted hosts with small molecular inhibitors to attenuate tumor development.

Here we describe the generation and quantification of both orthotopic and ectopic engraftment of ocular melanoma (conjunctival melanoma) using fluorescent stable cell lines. This protocol is also applicable for engraftment of primary cells derived from patient biopsy and patient-derived material [1]. Within hours post engraftment cell migration and proliferation can be visualized and quantified. Both tumor foci are readily available for imaging with both epifluorescence microscopy and confocal microscopy. Using these models, we can confirm or refute the activity of either chemical or genetic inhibition strategies within as little as 8 days after the onset of the experiment, allowing not only highly efficient screening on stable cell lines but also enabling patient-directed screening for precision medicine approaches.

Introduction

Metastatic dissemination is considered the main cause of death of ocular melanoma, and currently, there is no viable treatment regime for disseminated ocular melanoma [2,3]. Furthermore, there are no animal models available for ocular melanoma that recreate the metastatic disease. To bridge this gap, we generated two distinct zebrafish models that recapitulate either primary tumor formation or the early stages of

metastatic dissemination, thus readily allowing the study of these normally difficult-to-study processes [4]. We developed micro-metastasis models that allow the analysis of the last phases of metastatic spread, including homing, colonization, and extravasation. Genetic or chemical interventions at this stage and beyond could potentially provide a powerful handhold in the treatment of metastatic ocular melanoma.

The use of the zebrafish larvae as a recipient xeno- and allograft model is supported by the intrinsic strengths of using this species as an animal model, such as its optical transparency at the early stages of development (or its entire life cycle for Casper mutants [5]), high fecundity and ex utero fertilization [6]. High transcriptional homology in vertebrates ensures the retention of core signaling mechanisms between the zebrafish and humans and therefore high potential translatability of results [7], although genetic approaches are sometimes marred or complicated due to the teleost genome duplication [8]. Recent developments have underscored the importance of zebrafish xenograft models as pre-clinical "avatars" of human disease [9], effectively yielding a multitude of personalized cancer therapy models for the pre-clinical evaluation of treatment strategies from a single zebrafish experiment [10].

Considering the lack of animal models and the concordant lack of treatment options for metastatic ocular melanoma, our models provide a quick and easy translational platform to screen both genetic alterations (cancer cell-intrinsic) or develop chemical intervention strategies in a pre-clinical setting. Within the same model, we can visualize and measure cancer cell growth kinetics, engraftment rate/metastatic potential, and cell homing on a whole animal level using low-level magnification in a stereo fluorescent microscope, and make similar measurements using medium or high magnification confocal microscopic analysis, thus being able to focus to single cell and subcellular levels [11].

Here we describe comprehensive and detailed protocols from the generation of fluorescently labelled cancer cells, using highly optimized lentiviral transduction protocols [12] for fluorescent labelling of cells, subsequent engraftment both ectopic and orthotopic into 2 days post fertilization (dpf) zebrafish larvae and the following data acquisition and analysis. These methods although comprehensive for the applications described herein can be modified to engraft cells in the hindbrain cavity, liver, and perivitelline space when required (solely by changing the injection site, or time of injection) [13,14].

As a proof-of-concept, we replicated the findings of Pontes *et al.* 2018, where we showed a dose and cell intrinsic mutation-specific response of conjunctival melanoma cell lines in the zebrafish model [15]. We elaborated upon these findings by showing

the efficacy of $BRAF^{V600E}$ mutation-specific inhibitor vemurafenib in both metastatic and primary conjunctival melanoma models.

Methods

1. Agarose Dish Preparation:

When using dishes that have been stored for a long time make sure to add a small volume of egg water to the dishes before starting injection (this will prevent the fish from drying out too fast). Prepare 1.5% (w/v) agarose-coated dishes (agarose dissolved in egg water). Use immediately, or store at 4°C in an inverted position.

2. Needles Preparation:

Make sure that the capillaries have been calibrated on the filament you are using, when you switch either filament or capillary you should determine the ramp value of the capillaries on the filament you are using (see needle puller manual).

2.1. One glass capillary will yield two micro-injection needles. Before making needles check the structural integrity of the filament (2.5 mm box filament, Science product, Hofheim, Germany) of the needle puller (P97 or P1000 Micropipette puller, Sutter, Novato, U.S.A).
2.2. Make sure that both filament and capillary are calibrated to get the corresponding ramp value. When the filament's structural integrity is compromised i.e. (uneven, holes, molten, etc.) change the filament.
2.3. Use the following program (Needle #99, Heat=ramp+15, pull=95, velocity=60, time=90). Store the needles in a designated Petri dish (containing either clay or tape to stick the needles).

3. Generation of lentiviral particles:

To prevent a waste of time and resources a quick tumorigenicity check can be performed prior to lentiviral transduction. This is done to ensure that the cell line to be used is actually sufficiently tumorigenic in the zebrafish model, to this end the cells can be stained with a CM-Dil (or analogous tracer) as described in Liverani *et al.* [16].

3.1. Plate HEK 293T (ATCC, USA) cells one day prior to transfection to achieve a confluency of approximately 70% (routinely done by splitting a full flask to the same volume culture flask at a dilution 1:3 one day prior).
3.2. On the day of transfection, the required packaging plasmids psPAX2 and pMD2.G viral envelope expressing plasmid (both psPAX2 and pMD2.G were gifted by Didier Trono (Addgene plasmid #12260 and #12259

respectively) are co-transfected along with either a GFP (Plasmid #106172) or tdTomato (Plasmid #106173) encoding transfer plasmid.

3.3. Mix all plasmids together in 500 µL serum-free medium, to allow complete mixing of all plasmids. Add 32 µL LipoD293 reagent to 500 µL serum-free DMEM, and vortex to mix completely. Mix both volumes together thoroughly. Allow the plasmids and the lipoD293 to complex for 20 min.

3.4. Add dropwise to a 75 cm^2 cell culture flask containing 70% confluent HEK 293T cells containing 9 mL of complete culture medium, and add the transfection mixture directly to the cell layer using a serological pipette (flask in horizontal orientation).

3.5. Replace medium with 20 mL fresh complete DMEM 16 hours post-transfection. Harvest supernatant after 72 hours post-transfection. Aliquot viral supernatant in 1 mL aliquots and store at -80°C. lentiviral supernatant is stable at -80°C for at least 1 year.

4. Lentiviral transduction:

4.1. Prior to lentiviral transduction a kill curve has to be established when using a selectable lentiviral construct.

4.2. For the kill curve, plate the cell line to be transduced in a 12-well plate (confluence app 10-20%), add a dose curve of the selectant (approximate concentrations for kill curves: puromycine 0.5-10 µg/mL, blasticidin 1-20 µg/mL, geneticin (G418) 100-2000 µg/mL, hygromycin 100-2000 µg/mL (all Gibco, Thermo scientific, Bleiswijk, the Netherlands).

4.3. Medium should be changed every three days to ensure a stable concentration of the chosen selectant.

4.4. 1 mL of lentiviral supernatant is added to 9 mL culture medium, containing a final concentration of 8 µg/mL polybrene (Sigma) on 20-40% confluent cells, after changing the medium (volumes can be scaled down while maintaining this ratio of supernatant/medium).

4.5. 16-24 hours post-transduction the medium is exchanged and when required the former step can be repeated to enhance phenotype penetrance (check fluorescence to decide if another transduction is required).

4.6. 48 hours post-transduction the cells can be selected using the antibiotic corresponding to the resistance marker incorporated into the lentiviral cassette. The concentration to use for the selection of your transduced cell population should kill the wild-type population within 7 days after application of the selectant (*i.e.*, allowing the transduced cells to outgrow the wild-type population).

4.7. Viral supernatant should be applied in different multiplicities of infection (MOI's) to ensure that the transduction and the genetic lesions incurred by the cellular genome do not negatively affect cell viability or tumorigenicity.

5. Breeding zebrafish:

5.1. On day 0, 2 days prior to engraftment of cancer cells, adult zebrafish are mated in a "family cross" fashion at room temperature (as shown in **Figure 1**).

5.2. The tank of zebrafish is removed from the housing system (maintained at 28.5°C). The fish are separated into small breeding clusters at a 1:1 ratio male to female, with 10 fish per cluster. The fish are placed in small breeding tanks, in water drawn from the housing system, above a slanted grate (slanted, to mimic the shallows wherein zebrafish would naturally spawn).

5.3. Induced by the decline in temperature from 28.5°C to room temperature (25°C) and the entrance into the next light phase of the dark/light cycle the fish will spawn. Subsequently, the adults are removed and transferred into their housing tank.

5.4. Eggs are subsequently collected and washed with egg water using a strainer, eggs are divided into approximately 75-100 per dish and are maintained at 28.5°C. Approximately 6 hours post-collection the dishes are cleaned of dead or malformed embryos. The next morning the egg water is exchanged and the dishes are again cleaned of dead embryos.

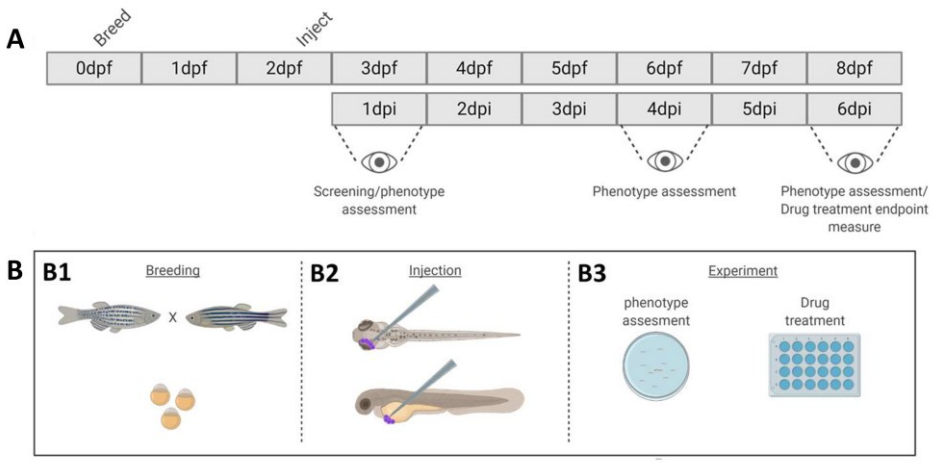

Figure 1. Schematical representation of our zebrafish engraftment system. **A)** The timeline describing the kinetics of our approach, with breeding the zebrafish at day 0 (**B1**), here the fish are harvested in the morning after crossing the fish (day 1). After 48-54 hours the fish have largely hatched (shedding their chorion) and the fish are injected (retro-orbitally or

systemically, **B2**) after cleaning the water of the chorion debris (day 2), the larvae are subsequently screened using a stereo fluorescent microscope and all larvae displaying unwanted phenotypes are discarded (day 3). Depending on the goal of the experiment either the larvae are imaged over time (**B3**, engraftment kinetics, imaged at 1, 4, and 6 days post injection(dpi)) or the fish are randomized and entered into experimental groups, treated with drugs and compared to vehicle control (drug screening, imaged at 6 dpi).

6. Harvesting cells:

The proper cell preparation is key to the implantation procedure, using a superfluous number of cells allows for easier downstream processing. The third centrifugation step is critical, as this will leave you with only the cell pellet, the remaining PBS stuck on the sides of the centrifuge tube greatly exceeds the final resuspension volume.

6.1. All media and solutions used in cell culture are pre-warmed in a 37°C water bath before use.

6.2. Add 2 mL TryplE/75 cm^2 culture flask or 1 mL 25 cm^2 flask and incubate until all cells are rounded, for most cell lines 2-5 min should be sufficient. For highly epithelial cells or fibroblastic cells, 5-10 min should allow for proper detachment (insufficient trypsinization will hinder downstream processes, and facilitate cell aggregation during implantation). Gently tap the side of the flask to dislodge the remaining cells.

6.3. Add up to the original culture volume of the complete medium. Pipette up and down gently but thoroughly with a serological pipette to shear cell clumps into single-cell suspension (do not generate foam during this process as foam is indicative of mechanical shearing of the cells).

6.4. Transfer into a sterile 15 mL tube and centrifuge for 5 min at 200 x *g* at room temperature. Aspirate supernatant and add 1 mL sterile Phosphate-buffered saline (PBS). Carefully and thoroughly resuspend the cells using a sterile 1000 µL tip.

6.5. Remove 20 µL cell suspension for counting and transfer the remaining cell suspension to the centrifuge. Centrifuge for 4 min at 200 x *g* at room temperature.

6.6. Remove all PBS, Centrifuge for 30 sec at 200 x *g* at room temperature, and remove the remaining PBS.

6.7. Dilute the cells to 250 cells/nL in 2% polyvinylpyrrolidon 40 (PVP_{40}) as follows: Cell concentration x * 10^6 / (250 cells/nL) *1000 = required amount of PVP40 in µL. Thoroughly resuspend the cells, while preventing the formation of air bubbles (cells can be kept for at least 2 hours in PBS without loss of tumorigenic potential).

7. Xenograft modeling:

All experiments should be performed in compliance with local animal welfare regulations. Depending on the application two main variations in experimental design are classified as a phenotype assessment 7.1 the pre-screening stage 7.2 a screen where either the cells have been modified prior to engraftment or 7.3 where the embryos are treated with a chemical inhibitor.

7.1. Pre-screening and determination of tumorigenic potential. Zebrafish larvae are engrafted at 2 dpf with a varying number of fluorescent cells (*i.e.*, 200, 400, 600 ±100).

7.1.1. Larvae are screened 16-24 hours after injection to remove outliers (extremely high or low cell numbers in circulation) and wrongly engrafted fish are removed. Indicate nr of larvae per experimental group for group analysis vs kinetic analysis of the same larvae.

7.1.2. The zebrafish larvae are monitored at regular intervals (1, 2, 4, and 6 days post injection (dpi)) and 20 individuals imaged (as described in Method 9 and 10), out of a pool of ±50 larvae.

7.1.3. General phenotype and disease progressions are monitored and subsequently quantified with ImageJ (measuring integrated density of the fluorophore signal in the cancer cells).

7.1.4. This data is subsequently plotted to visualize the cancer cell growth kinetics within the zebrafish.

7.2. Cells are modified a priori (knock down or knock out of a gene of interest) and engrafted into zebrafish.

7.2.1. Fish are engrafted, and all unwanted phenotypes are removed (per condition).

7.2.2. The individuals are imaged at 1 dpi (20 larvae per group).

7.2.3. Individuals can be imaged at set intervals (1, 2, 4, and 6 dpi).

7.2.4. At 6 dpi after imaging the fish are euthanized by overdosing with tricaine (10-fold overdosing at 0.4 mg/mL) and are discarded on absorbent paper lining a funnel.

7.3. Fish are treated with drugs after engraftment.

7.3.1. Prior to drug application on engrafted zebrafish, determine the maximum tolerated dose (MTD) on zebrafish (titrate down from 20-10 μM- 0.150 nM, using the highest volume of solvent as a negative control) we have set the MTD as the concentration where > 80% of individuals survive the entire treatment.

7.3.2. One day post injection the unwanted phenotypes are removed.

7.3.3. Fish are randomly divided into groups (36-48 individuals/condition) and maintained in a 24-well plate with 6 larvae per well in 1 mL of egg water.

7.3.4. Drugs are applied 24 hours after engraftment, as a control use the same amount of solvent (DMSO, EtOH, etc.) at the highest volume applied for an experimental group.

7.3.5. Start drug treatment at the maximum tolerated dose, change the egg water containing drug every other day. Removing egg water and dead larvae as completely as possible during every change.

8. Injection:

Use a pneumatic pulse controller (Pico pump, World Precision Instruments) coupled to a compressed air line, supplying pressure in surplus of 100Psi, this allows for enough pressure to both inject (≈20Psi) and to eject possible cell aggregates (≈100Psi), starting pressure and time should be approximately 200ms at 20 Psi.

8.1. Carefully remove a capillary needle from its container. Break the needle to form an opening of app. ø20µm, using fine forceps.

8.2. Carefully and thoroughly resuspend the cells using a 20 µL pipette tip. Pipette cell suspension into the open glass capillary needle using a long (microloader) tip. Load the needle into the micromanipulator.

8.3. Place app. 20-40 larvae anesthetized in 0.04 mg/mL tricaine on an agarose dish using a transfer pipette. Remove excess moisture to immobilize the larvae using a transfer pipette. The larvae will mostly be oriented in a lateral fashion due to the presence of a still relatively large yolk sac.

8.4. Inject the larvae with approximately 200, 400, and 600 cells via the Duct of Cuvier (doC) for the ectopic model. Similarly, the larvae are injected retro-orbitally (RO), to yield the orthotopic model (injecting 100±50 cells) modifying pneumatic pulse length on the Pico pump (Start at app. 20 Psi, 200 ms and adjust accordingly). During injection ensure that the larvae do not dry out, and make sure that all (or most) larvae are injected.

8.5. Injected larvae are flushed off with fresh egg water and transferred to a labelled clean Petri dish (pooling up to 150 individuals per dish). This process is repeated until sufficient larvae are injected.

8.6. After engraftment the fish are maintained at 34°C in a humidified incubator, where 34°C is the highest temperature readily tolerated by zebrafish and allows for efficient engraftment of mammalian cancer cells.

8.7. In general, with injection of single cell lines in both doC and RO we have observed an approximate death due to mechanical damage of < 5% (mechanical damage kills the larvae between 1-16 hours post injection).

9. Screening:

9.1. Using a stereo-fluorescence microscope the fish are screened for the appropriate phenotype 1 hour post implantation when comparing cells modified a priori (or 1 day post implantation, when screening drugs, before the random assignment into treatment groups).

9.2. Larvae implanted through the doC should have cells in the tail between 1 hour and 16 hours post implantation, all other fish, including fish that display abnormality are removed from the injected pool.

9.3. Larvae implanted retro-orbitally should have cells only in the interstitium behind the eye, larvae that have cells spread throughout the head or body are removed from the pool.

9.4. Positively screened larvae are cleaned and randomly assigned to experimental groups.

9.5. After engraftment the fish are maintained at 34°C in a humidified incubator and monitored daily. Hematogenous dissemination of cells implanted through the doC is almost instantaneous, whereas metastatic spread of cells implanted in the RO cavity will spread after 2-4 days.

10. Inflorescent imaging of zebrafish larvae:

10.1. Zebrafish larvae are anaesthetized with 0.2 mg/mL tricaine, either by adding tricaine to the water of the fish or by moving a sub-population of fish from the maintenance dish to a dish containing 0.2 mg/mL tricaine. Zebrafish are kept in a dish with tricaine until they remain stationary, until stimulation of the lateral line does not induce flight behaviour.

10.2. Fish are transferred to an agarose-covered Petri dish. Approximately 10 per dish. The majority of the water is removed by gently raising one end of the dish (allowing the water to gently pool in the lower end of the Petri dish). If done carefully all fish will align, tails facing downwards. All fish are imaged from the top of the dish to the bottom, after which the fish are washed off with egg water into a dish without tricaine.

10.3. This method is repeated until enough individuals are imaged. The larvae are either transferred back to the 34°C or culled (at 6 dpi) through overdosing with tricaine (*i.e.*, 0.5 mg/mL, incubating for 10 min, prior to discarding on absorbent paper lining a funnel).

11. Confocal imaging of (engrafted) zebrafish larvae:

11.1. Zebrafish are anaesthetized with 0.2 mg/mL tricaine as described previously.
11.2. Place a glass bottom confocal dish under a stereo microscope, and focus on the bottom of the dish. Transfer 5-10 larvae to a glass bottom confocal dish. Remove as much water as possible.
11.3. Cover the larvae with 42°C, 1% low melting agarose dissolved in egg water (important: make sure that the agarose has cooled down to at least 42°C before use, higher temperatures might harm or kill the larvae). Using the stereo microscope, quickly but gently orient the larvae pushing it down, using a trimmed-down micro loader tip. If a ventral orientation is required the larvae can be held in place with the tongs of a watchmaker's forceps (without touching the embryo).
11.4. While the agarose sets make fine adjustments to the orientation of the larvae. Allow the larvae to set completely before transferring to the confocal microscope.

12. Setting the confocal microscope:

12.1. Switch on the green (488 nm) and red (564 nm) excitation laser lines. Place the confocal dish in the holder of the confocal microscope. Using the epifluorescence, move the light bundle to coalesce with the first fish (setting x and y). Through the ocular set the focus coincides with the center of the larvae (setting z).
12.2. Set 700 gain on both fluorescent channels, 1-5% laser power. Increase laser power and decrease offset to approximate full dynamic range. Do not oversaturate the signal, but enhance the signal to merely show a few saturated pixels.
12.3. When capturing a stitch, set the start and end of the larvae along one axis (either x or y), if set along one axis a whole embryo can be imaged in 1 x 4 segments and can be post processed into one image using ImageJ.
12.4. After imaging the larvae can be removed from the agarose by gently tearing it around the embedded larvae using watchmaker's forceps. Otherwise, the larvae can be euthanized with overdosing with undiluted tricaine, covering the agarose with a layer of tricaine, and incubating 10 min.

13. Data analysis:

13.1. Open the individual data sets in ImageJ/Fiji (*i.e.*, control, drug A, drug B, drug A+B) separately, starting with vehicle control.

13.2. Open the analysis macro (annotated script available) (http://doi.org/10.5281/zenodo.4290225).
13.3. In brief the macro analysis does the following:
  13.3.1. Concatenates all open images (one condition).
  13.3.2. Splits the images into the separate channels comprising the image.
  13.3.3. Closes all accessory channels, (leaving the cancer cell channel).
  13.3.4. Runs a thresholding algorithm, on the entire concatenated sequence.
  13.3.5. Measures integrated density of each individual image.
  13.3.6. Saves the measures as an Excel sheet in the root folder.
13.4. The macro analysis is run on all conditions.
13.5. Measurements are combined (in general at least n=2*20), outliers are removed (Q-test in Graph pad Prism 8).
13.6. Measurements are normalized either to solvent control or to day 1 (dependent on the type of experiment, the former for a drug inhibition experiment and the latter for a growth kinetics experiment), measurements are expressed as normalized cancer cell burden (y axis) over time or condition (x axis) as shown in **Figure 3** and **4** respectively.

Reagents:

- Egg water: 0.6 mg/L final concentration sea salt (Instant Ocean, Blacksburg, U.S.A).
- Tricaine 25x stock, 5 mg/mL: 5g tricaine (Ethyl 3-aminobenzoate methanesulfonate or MS-222) powder, 900 mL demineralized water add 21 mL 1 M Tris (pH=9) and adjust to pH=7 and fill up to 1L. Tricaine can be stored at 4°C for a short term (up to six months), or can be stored at room temperature for a month at room temperature when protected from sunlight. (Sigma, Zwijndrecht, the Netherlands).
- Agarose: 1.5% (w/v) in egg water. 1.5 g in 100 mL DPBS, microwave to dissolve.
- Low-melting agarose: 1% (w/v) in egg water 1.5 g in 100 mL DPBS, microwave to dissolve.
- PVP40 stock: PVP_{40} 2% (w/v) in DPBS, 1 g PVP_{40} in 50 mL DPBS. Vortex and incubate at 37°C to facilitate dissolving. Store at room temperature.
- DMSO: Often used as a solvent in drug treatments, should be stored at 2-8°C the dark.
- TryplE: Synthetic trypsine replacement, less damaging to the cells and allows for the gentle dispersion of strongly adherent cells. (Thermo Fischer Scientific, Bleiswijk, Netherlands).

DPBS: Dulbecco's phosphate buffered saline, without Mg^{2+} and Ca^{2+} for washing the cells, lack of Ca^{2+} impairs cell-cell adhesion through cadherins.

Materials:

- Micro loader tips: Microloader tips for loading micro injection needles, trimmed down tips can be used for fine manipulation of zebrafish larvae (Eppendorf, Nijmegen, the Netherlands).
- Capillaries: borosilicate glass outer diameter (O.D) 1.0 mm, inner diameter (I.D) 0.8 mm (Science product, Hofheim, Germany).
- Nr.5 watchmaker's forceps.
- 3 mL transfer pipettes: for transferring and selection of zebrafish embryos.
- Needle puller: P-97 or P-1000, Sutter instrument, Novato, U.S.A.
- 2.5 mm box filament, Science product, Hofheim, Germany.
- Picopump: Pneumatic microinjector, World precision instruments, Sarasota, U.S.A.
- Micro manipulator, with weighted base plate and M10 magnetic stand: (World precision instruments).
- Differential interference contrast (DIC) stereo microscope: (Leica, Amsterdam, the Netherlands).
- Leica sp8 confocal equipped with 405, 488, 532 and 633 nm lasers, with 10 and 20x objectives (Leica microsystems, Amsterdam, the Netherlands).

Results

We have provided step by step instructions for a fast and easy approach to progress from a novel cell line to its analysis. Starting with the over expression of a fluorescent tracer using a lentiviral overexpression cassette (Method 3 and 4). Followed by cell preparation to ensure the least possible dead volume while injecting, allowing to inject high cell numbers into both doC and retro-orbital space (Method 6 and 7). Subsequent semi-high throughput data acquisition using stereo-fluorescent microscopy and higher magnification confocal microscopy for qualitative analysis of whole-body cancer cell dissemination (see in **Figure 2A**, Method 10, 11 and 12). Care has to be taken when acquiring data, as to ensure the reproducibility for both stereo and confocal microscopic imaging, the generic settings and standardization are delineated (Method 11 and 12). Data analysis is discussed (using ImageJ/Fiji) [17], along with standardization using ImageJ macros (Method 13).

In Method 3 we mention the transient labelling of (cancer) cells to perform a quick pre-screen to assess the tumorigenic potential of a new cancer cell line, one important caveat is that although easy to use and long living, the transient stain described herein has the possibility to form artefacts e.g. care has to be taken to ensure that cell fragments can be distinguished from whole cells as was performed extensively by Fior and colleagues [10]. In our experience the formation of these artefacts is directly linked to the extreme stability of the stain and the brightness (even after cell death), where cell fragments are dispersed and taken up by immune cells, which could subsequently be falsely concluded to derive from active metastasis.

In both of the described models, both the systemic engraftment through the doC and the localized engraftment in the retro-orbital space, thorough screening of the larvae one day after injection is of paramount importance. As shown in **Figure 2B** and **C**, all larvae that display mechanical displacement of the engrafted cells into the head area (beyond the retro-orbital site) in the retro-orbital model and cells in the yolk sac, or displaying an edema in the doC injected pool should be removed. All negatively selected phenotypes are displayed as high-resolution confocal stitches in **Figure 2**, but can be readily seen and removed through stereo microscopical observation.

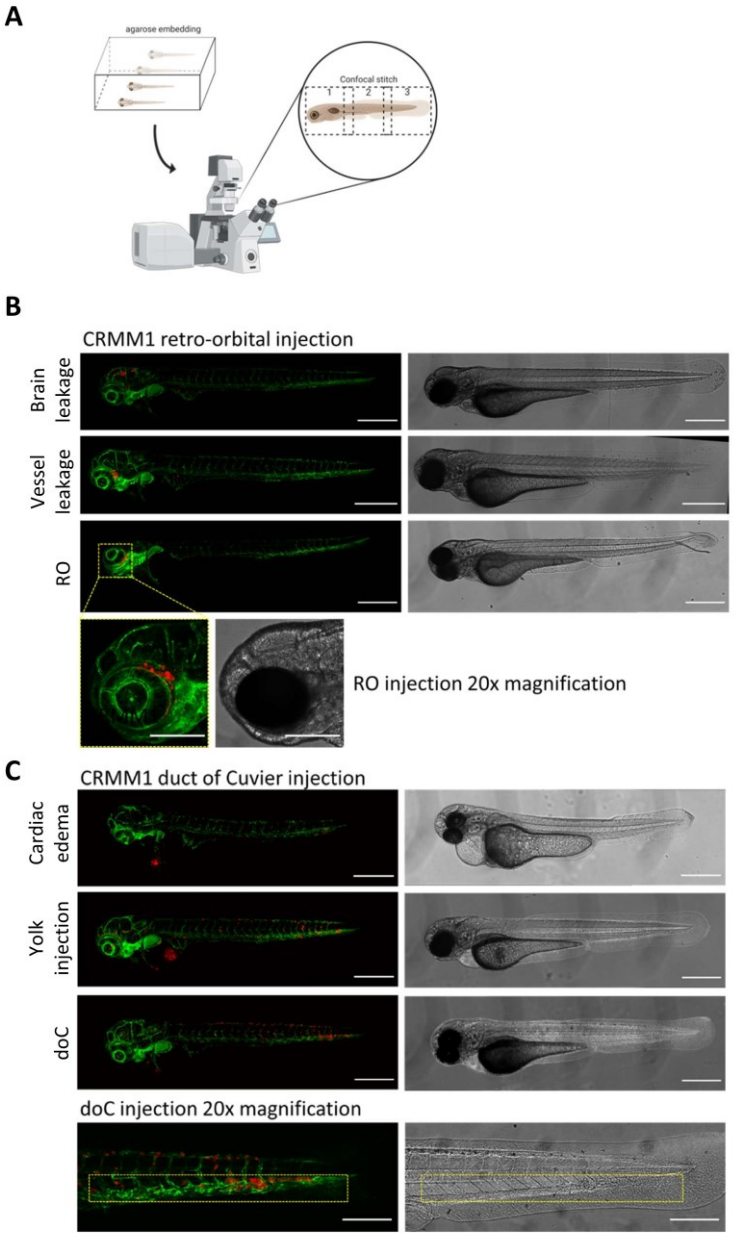

Figure 2. Phenotypic assessment and screening after injection. A) Schematical depiction of zebrafish xenograft confocal stitch generation, yielding seamless, high-resolution images after integration of subsequent confocal projection. Here zebrafish xenografts are embedded in 1% low melting agarose and mounted on a glass bottom confocal dish. **B)** All possible outcomes of retro-orbital and duct of Cuvier engraftment are displayed injected in green fluorescent blood vessel reporter zebrafish (Tg(*fli1*:GFP), with cells stained through lentiviral overexpression of tdTomato), here denote the correct engraftment at 1 dpi (RO panel) and the

unwanted phenotypes (both brain leakage and blood vessel leakage), the latter two population have to be removed to ensure they do not confound downstream experimental findings. **C)** The unwanted phenotypes for the hematogenous engraftment through the duct of Cuvier (doC) are outlines where cardiac edematous embryos (Cardiac edema) and embryos with cells leaking into the yolk sac (Yolk injection) have to be removed to prevent interference with downstream measurements. (all images acquired at 1 dpi, using a Leica sp8 confocal, scale bars 200 μm, yellow boxes indicate metastatic sites for both RO and doC engraftments, head region, and caudal hematopoietic tissue respectively).

Over time cells will both migrate and proliferate, for the retro-orbital model we observed infiltration into neighbouring tissues for CRMM1 and for CRMM2 we observed less proliferation. We strikingly did observe distant metastasis arising between 2-4 dpi in some individuals (20%), where we see a significant difference at 6 dpi, as shown in **Figure 4**. For both cell lines we tested the proliferative potential when injected in both sites, for CRMM1 there was a significant ($p<0.0001$) increase in cancer cell number for injection sites, when displayed as normalized tumor cell burden, normalizing to day one for each model (7.8-fold increase, ±3.2 for the RO model and an increase of 15-fold ±8,8 for the doC model). CRMM2 did not display significant growth when normalized to day one for each individual model (2.4-fold increase, ±1.9- and 2.3-fold increase, ±1.14 for the RO and doC). CRMM1 was found to readily proliferate in both retro-orbital tissue and the caudal hematopoietic tissue after engraftment. Cell line CRMM2 was less proliferative in both models, but interestingly was found to be capable of distant metastasis when injected in the retro-orbital space as show in **Figure 3B** and **C**.

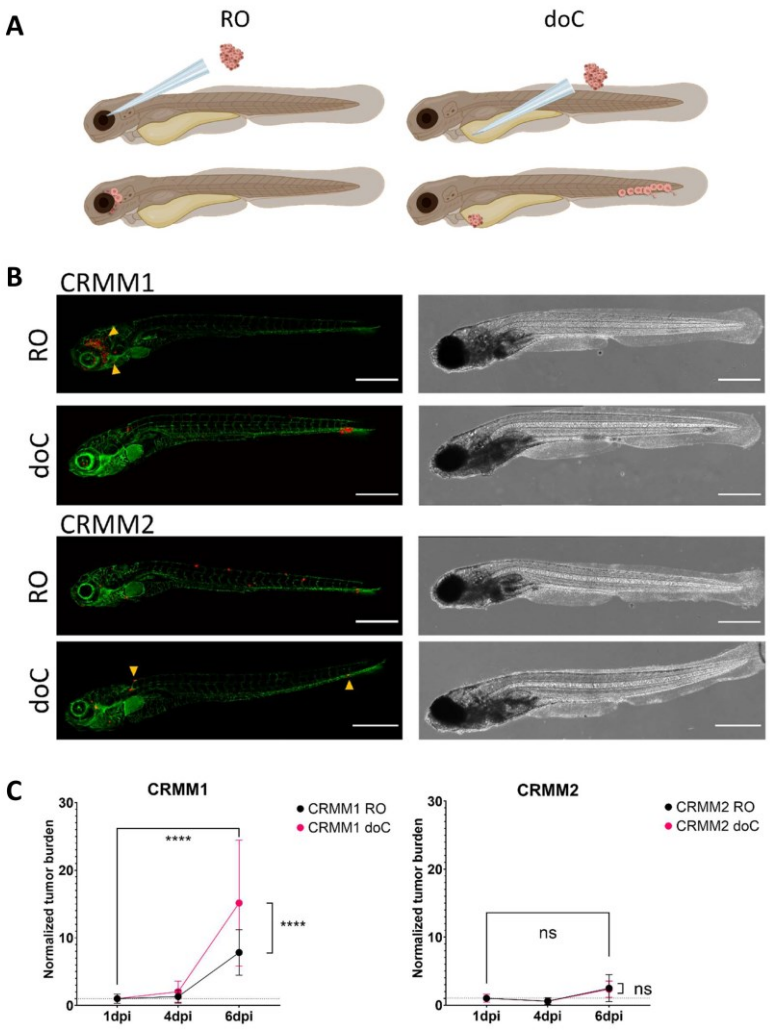

Figure 3. **Comparative analysis of conjunctival melanoma cell lines CRMM1 and CRMM2 show differential metastatic and growth capacity. A)** Schematic representation of injection models, retro-orbital model (RO) and hematogenous engraftment model (doC) the fish used is Tg(fli1: GFP) green blood vessel reporters, with cells overexpressing dTomato shown in red. **B)** Representative phenotypes of fish engrafted with CRMM1 and CRMM2, CRMM1 displays efficient engraftment (both RO and doC) and small-scale invasion into the tissue surrounding the RO engraftment site (RO, yellow arrowheads). CRMM2 shows a remarkably lower engraftment efficiency for both engraftment models but shows distant metastasis when injected retro-orbitally (as shown in RO, denoted by the arrowheads) (all images acquired at 1 dpi, scale bars 200 μm, yellow arrowheads indicate metastatic sites for both RO and doC engraftments, head region, and caudal hematopoietic tissue respectively). **C)** Kinetic engraftment plots for both CRMM1 and CRMM2, comparing both engraftment models to day 1 (normalizing to day 1), there is a significant ($p<0,0001$) increase in normalized

tumor burden for cell line CRMM1(between 1 dpi and 6 dpi) where there is a (non-significant) upward trend for CRMM2. CRMM1 shows a significant difference between RO and doC growth, whereas the doC model shows a higher tumor expansion rate (approximately 2-fold higher for the doC engrafted embryos). Graphs show the mean and standard error of the mean (SEM), All groups were normalized to 1 dpi for each individual condition.

After screening the injected larvae at 1 dpi and randomly assigning the individuals to either treatment or control groups the fish are treated for 6 days, changing the water containing Vemurafenib (this inhibitor can readily be interchanged for any other titrated antitumor compound). We chose to elaborate upon the previously published hematogenous conjunctival melanoma dissemination model engrafting CRMM1, by testing vemurafenib's efficacy on orthotopically engrafted CRMM1. CRMM1 showed a strong significant reduction of the vemurafenib treated ectopically engrafted group and a stunted yet significant response for the orthotopically engrafted model as shown in **Figure 4**.

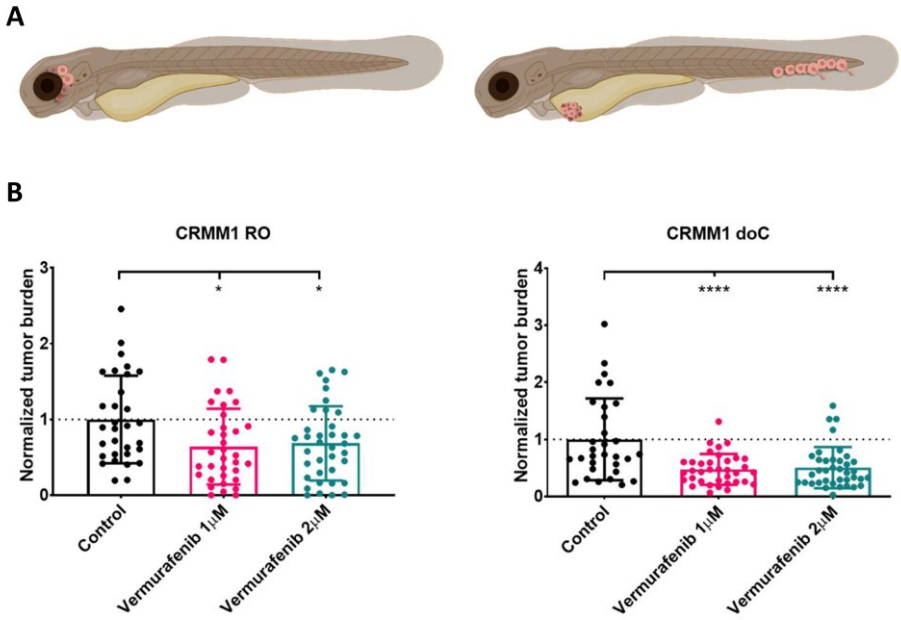

Figure 4. BRAF^{V600E} inhibitor Vermurafenib significantly inhibits both RO and doC conjunctival melanoma engrafted zebrafish embryos. A) Schematical representation of zebrafish RO and doC models. **B)** Both retro-orbitally and doC engrafted larvae, injected with conjunctival melanoma cell line CRMM1 show a significant reduction of normalized tumor burden. Graphs show the mean and standard error of the mean (SEM), All groups were normalized to 1 dpi for each individual condition.

Discussion

Here we have defined a meticulous approach to model primary and metastatic ocular melanoma in zebrafish xenografts. By combining both a localized, orthotopic injection and a systemic, ectopic injection model we have recapitulated the etiology of carcinogenesis for a cancer where no animal models were previously available. The inherent transparency of the early zebrafish larva allows the tracking of fluorescently labeled cancer cells on a whole animal level, ensuring the easy visualization of potential metastatic sites [5]. Moreover, high magnification confocal microscopical analysis allows us to track cells to subcellular resolution [11].

Using these models, we simulated primary tumor development by physically confining the engrafted cells within the retro-orbital interstice. Subsequent thorough screening at 1-day post engraftment ensures that cells found at distant sites later in the experiment have actively metastasized (intravasated and disseminated, ultimately to extravasate at the metastatic niche). Engraftment through the doC, the embryonic common cardinal vein, allows for easy and highly reproducible implantation of large quantities of cells (at a surplus of 600 cells when properly concentrated) effectively circumventing the primary stages of the metastatic cascade (intravasation) and allowing us to focus on the later stages of the metastatic cascade (adhesion, extravasation, and outgrowth). Although powerful tools when used properly, both models should be monitored extensively during the first day post-engraftment to ensure that no false positive conclusions are drawn during the later stages of the experiment.

In line with previous publications, we have shown that conjunctival melanoma lines readily form metastatic colonies within the zebrafish blood circulation [15]. Here we report the expanding of the engraftment repertoire with the retro-orbital engraftment as an orthotopic model, and the subsequent active metastasis to the caudal hematopoietic tissue of the cell line CRMM2. Subsequently, we report the efficacy of $BRAF^{V600E}$ specific inhibitor Vemurafenib also on the primary form of conjunctival melanoma when modeled in zebrafish larvae.

Using the aforementioned methods, a skilled researcher is capable of generating in excess of hundreds of engrafted larvae per day (approximately 200 per hour) of either model proposed. In a timescale of two weeks, a drug can be both titrated for the maximum tolerated dose and screened on established cell lines. From start to finish, using a non-transduced cell line, to having a drug sensitivity profile in the zebrafish model can be achieved within a month (given that the injected cell line is tumorigenic within the zebrafish model). In our hands, as little as 20 larvae per experiment and two biological repeats have reproducibly yielded robust drug inhibition, when two

individual experiments conflict (or do not yield statistically significant growth inhibition) a third biological repeat can be introduced.

Through minor adjustments, these models have allowed us to quickly adapt these implantation strategies for glioblastoma (hindbrain cavity injection), breast cancer (doC injection), and osteosarcoma (doC) among others [18-21]. These models can subsequently be used for both basic research and pre-clinical screening of both single drugs and combinatorial drug strategies.

Ethics statement

All animal experiments were approved by animal Experimental Committee (*Dier Experiment Commissie*, D.E.C.) under license AVD1060020172410. All animal were maintained in accordance with local guidelines using standard protocol (www.ZFIN.org).

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Disclosures

No authors declare a conflict of interest.

References

1. Yin, J.; Zhao, G.Y.; Kalirai, H.; Coupland, S.E.; Jochemsen, A.G.; Forn-Cuni, G.; Wierenga, A.P.A.; Jager, M.J.; Snaar-Jagalska, B.E.; Groenewoud, A. Zebrafish Patient-Derived Xenograft Model as a Preclinical Platform for Uveal Melanoma Drug Discovery. *Pharmaceuticals-Base* **2023**, *16,* 183.
2. Yang, J.; Manson, D.K.; Marr, B.P.; Carvajal, R.D. Treatment of uveal melanoma: where are we now? *Ther Adv Med Oncol* **2018**, *10,* 1758834018757175.
3. Wong, J.R.; Nanji, A.A.; Galor, A.; Karp, C.L. Management of conjunctival malignant melanoma: a review and update. *Expert Rev Ophthalmo* **2014**, *9*, 185-204.
4. Nguyen, D.X.; Bos, P.D.; Massague, J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer* **2009**, *9*, 274-U265.
5. White, R.M.; Sessa, A.; Burke, C.; Bowman, T.; LeBlanc, J.; Ceol, C.; Bourque, C.; Dovey, M.; Goessling, W.; Burns, C.E.; et al. Transparent adult

zebrafish as a tool for in vivo transplantation analysis. *Cell Stem Cell* **2008**, *2*, 183-189.
6. Zon, L.I.; Peterson, R.T. In vivo drug discovery in the zebrafish. *Nat Rev Drug Discov* **2005**, *4*, 35-44.
7. Howe, K.; Clark, M.D.; Torroja, C.F.; Torrance, J.; Berthelot, C.; Muffato, M.; Collins, J.E.; Humphray, S.; McLaren, K.; Matthews, L.; et al. The zebrafish reference genome sequence and its relationship to the human genome. *Nature* **2013**, *496*, 498-503.
8. Palmblad, M.; Henkel, C.V.; Dirks, R.P.; Meijer, A.H.; Deelder, A.M.; Spaink, H.P. Parallel deep transcriptome and proteome analysis of zebrafish larvae. *BMC research notes* **2013**, *6*, 1-8.
9. Yan, C.; Brunson, D.C.; Tang, Q.; Do, D.; Iftimia, N.A.; Moore, J.C.; Hayes, M.N.; Welker, A.M.; Garcia, E.G.; Dubash, T.D.; et al. Visualizing Engrafted Human Cancer and Therapy Responses in Immunodeficient Zebrafish. *Cell* **2019**, *177*, 1903-1914.
10. Fior, R.; Povoa, V.; Mendes, R.V.; Carvalho, T.; Gomes, A.; Figueiredo, N.; Ferreira, M.G. Single-cell functional and chemosensitive profiling of combinatorial colorectal therapy in zebrafish xenografts. *P Natl Acad Sci USA* **2017**, *114*, E8234-E8243.
11. Campbell, P.D.; Chao, J.A.; Singer, R.H.; Marlow, F.L. Dynamic visualization of transcription and RNA subcellular localization in zebrafish. *Development* **2015**, *142*, 1368-1374.
12. Campeau, E.; Ruhl, V.E.; Rodier, F.; Smith, C.L.; Rahmberg, B.L.; Fuss, J.O.; Campisi, J.; Yaswen, P.; Cooper, P.K.; Kaufmann, P.D. A Versatile Viral System for Expression and Depletion of Proteins in Mammalian Cells. *Plos One* **2009**, *4,* e6529.
13. van der Helm, D.; Groenewoud, A.; de Jonge-Muller, E.S.M.; Barnhoorn, M.C.; Schoonderwoerd, M.J.A.; Coenraad, M.J.; Hawinkels, L.J.A.C.; Snaar-Jagalska, B.E.; van Hoek, B.; Verspaget, H.W. Mesenchymal stromal cells prevent progression of liver fibrosis in a novel zebrafish embryo model. *Sci Rep-Uk* **2018**, *8,* 16005.
14. Chen, Q.C.; Ramu, V.; Aydar, Y.; Groenewoud, A.; Zhou, X.Q.; Jager, M.J.; Cole, H.; Cameron, C.G.; McFarland, S.A.; Bonnet, S.; et al. TLD1433 Photosensitizer Inhibits Conjunctival Melanoma Cells in Zebrafish Ectopic and Orthotopic Tumour Models. *Cancers* **2020**, *12,* 587.
15. Pontes, K.C.D.; Groenewoud, A.; Cao, J.F.; Ataide, L.M.S.; Snaar-Jagalska, E.; Jager, M.J. Evaluation of (flI:GFP) Casper Zebrafish Embryos as a Model for Human Conjunctival Melanoma. *Invest Ophth Vis Sci* **2017**, *58*, 6065-6071.
16. Liverani, C.; La Manna, F.; Groenewoud, A.; Mercatali, L.; Van der Pluijm, G.; Pieri, F.; Cavaliere, D.; De Vita, A.; Spadazzi, C.; Miserocchi, G.; et al. Innovative approaches to establish and characterize primary cultures: an ex vivo 3D system and the zebrafish model (vol 6, pg 133, 2017). *Biol Open* **2017**, *6*, 309-309.
17. Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; et al. Fiji: an

open-source platform for biological-image analysis. *Nat Methods* **2012**, *9*, 676-682.
18. Mercatali, L.; La Manna, F.; Groenewoud, A.; Casadei, R.; Recine, F.; Miserocchi, G.; Pieri, F.; Liverani, C.; Bongiovanni, A.; Spadazzi, C.; et al. Development of a Patient-Derived Xenograft (PDX) of Breast Cancer Bone Metastasis in a Zebrafish Model. *Int J Mol Sci* **2016**, *17,* 1375.
19. Tulotta, C.; He, S.; van der Ent, W.; Chen, L.; Groenewoud, A.; Spaink, H.P.; Snaar-Jagalska, B.E. Imaging Cancer Angiogenesis and Metastasis in a Zebrafish Embryo Model. *Adv Exp Med Biol* **2016**, *916*, 239-263.
20. Paauwe, M.; Schoonderwoerd, M.J.A.; Helderman, R.F.C.P.; Harryvan, T.J.; Groenewoud, A.; van Pelt, G.W.; Bor, R.; Hemmer, D.M.; Versteeg, H.H.; Snaar-Jagalska, B.E.; et al. Endoglin Expression on Cancer-Associated Fibroblasts Regulates Invasion and Stimulates Colorectal Cancer Metastasis. *Clin Cancer Res* **2018**, *24*, 6331-6344.
21. Cao, J.F.; Pontes, K.C.S.; Heijkants, R.C.; Brouwer, N.J.; Groenewoud, A.; Jordanova, E.S.; Marinkovic, M.; van Duinen, S.; Teunisse, A.F.A.S.; Verdijk, R.M.; et al. Overexpression of EZH2 in conjunctival melanoma offers a new therapeutic target. *J Pathol* **2018**, *245*, 433-444.