# *In vivo* targeted and deterministic single cell malignant transformation

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**How cancer arises from a single normal cell is still the subject of active debate, affecting intervention strategies. While many cells may harbor oncogenic mutations, only a few unpredictably end-up developing a full-blown tumour[1,2,3,4]. Various theories have been proposed to explain that transition[5,6,7], but none has been tested *in vivo* at the single cell level. Here using an optogenetic approach we permanently turn on an oncogene (KRASG12V) in a single cell of a zebrafish brain that, only in synergy with the transient co-activation of a reprogramming factor (VENTX/NANOG), undergoes a deterministic malignant transition and robustly and reproducibly develops within 6 days into a full-blown cancer. The controlled way in which a single cell can thus be manipulated to give rise to cancer lends support to the "ground state theory of cancer initiation[7]" through "short-range dispersal[8]" of the first malignant cells preceding tumour growth.**

A zebrafish transgenic line was engineered (Tg(*actin*:loxP-EOS-stop-loxP-KRASG12V-T2A-H2B-mTFP; *ubi*:Cre-ERT; *myl7*:EGFP) which allowed for activation[9], i.e. expression of the oncogene KRASG12V (and its nuclear fluorescent marker H2B-mTFP) upon incubation in caged cyclofen (cCyc) followed by UV (375nm) illumination[10] (Fig. S1). Consistent with an acquired refractory cell state and loss of oncogenic competence[11], whole body expression of the oncogene at 1dpf did not result in tumorigenesis.

To test for the possible impact of a reprogramming factor on the malignant transition, embryos from this transgenic line were injected at the one-cell stage with the mRNA of a construct consisting of a glucocorticoid receptor (GR) fused with a reprogramming factor (Ventx, Nanog or Oct4)[13,14] (Fig. S3). The resulting protein (e.g. Ventx-GR) is sequestered by cytoplasmic chaperones and transiently activated upon incubation of zebrafish in Dexamethasone (Dex) (Fig. S2A). Activation of the oncogene[9] at 1dpf, only if followed by transient activation of Ventx (or Nanog or Oct4) did yield reproductible hyperplasic outgrowths (Fig. S3B-E). Immuno-histo-chemistry detection of phospho-ERK activity (Fig. S4A), Hematoxylin & Eosin staining (Fig. S4B) and RT-qPCR of selected genes (Fig. S5A, B) all display features associated with tumorigenesis. None of the controls (activation of kRasG12V only, Ventx-GR only, incubation in cyclofen or dexamethasone, etc.) developed hyperplasia, but rather grew into normal zebrafish (Fig. S3).

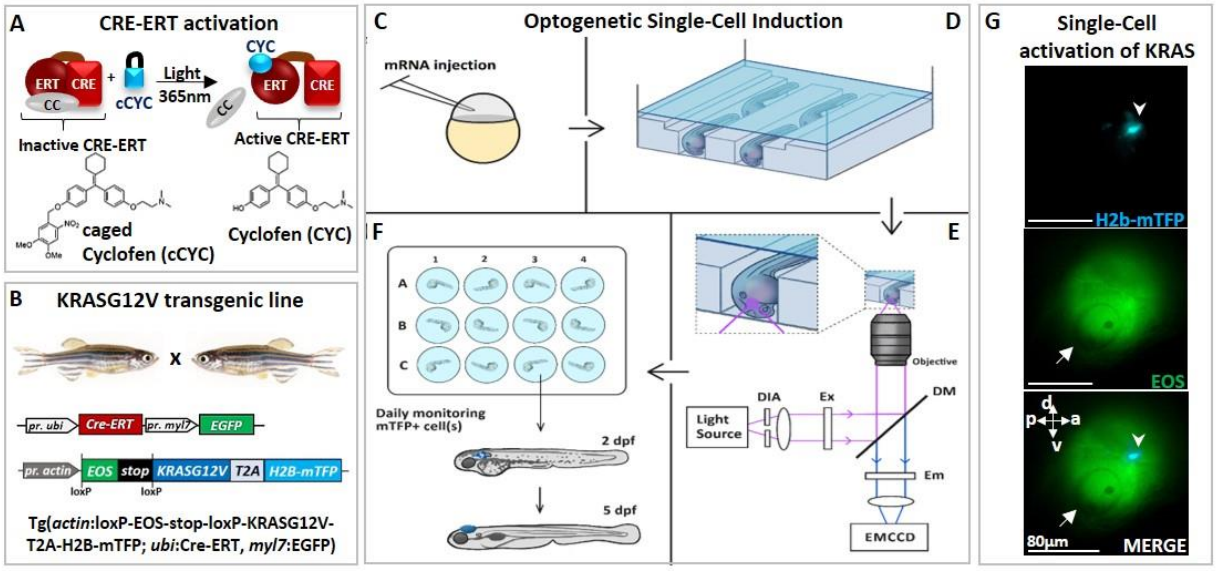


**FIGURE 1: Optogenetic set-up for single cell induction. (A)** Photo-control of a protein fused to an Estrogen Receptor (ERT) is achieved by releasing the protein from its complex with cytoplasmic chaperones (CC), upon uncaging of cCYC. **(B)** The transgenic zebrafish line engineered to express an oncogene (KRASG12V) upon photo-activation of a CRE-recombinase fused to ERT (CRE-ERT) (as shown in A). **(C)** The mRNAs of Ventx-GR and mRFP are injected at the one cell stage. **(D)** At 1dpf the embryos are mounted in channels in an agarose gel and incubated for 45 min in cCyc on a microscope stage. **(E)** They are washed and illuminated at 405nm on a microscope to uncage cCyc close to the otic vesicle. A diaphragm (DIA) defines an illumination zone of ~80mm diameter (see G). Excitation (EX), Dichroic mirrors (DM) and emission (EM) filters allow for visualization of Eos and mTFP. The cell in which KRASG12V has been induced is observed within 1h by the fluorescence of mTFP (see G). **(F)** The embryos are transferred into individual wells, incubated overnight in DEX, washed at 1dpi and monitored over the next 5 days. **(G)** Lateral view of zebrafish at <1h post-activation displays a single induced cell (top: blue spot shown by arrowhead in mTFP channel) in the illumination region (middle: Eos channel) in the vicinity of the otic vesicle (white arrow) and bottom: merger of both channels. Body axes (a: anterior; p: posterior; d; dorsal; v: ventral) are shown.

Since both kRasG12V and Ventx have been reported to play a role in brain cancer[12,13], we decided to activate the oncogene kRasG12V at 1dpf in a single normal cell of the brain of a transgenic zebrafish (injected with the mRNA of Ventx-GR at 1 cell stage). Activation of the oncogene in a single cell was achieved by illumination at 405nm to uncage cCyc in a small region (diameter ~80 µm) of the brain (in the vicinity of the otic vesicle) (Fig. 1). On average the oncogene is then activated in a single cell, identified within ~30 min by the blue fluorescence of its nuclear marker (H2B-mTFP), see Fig. 1. Notice that single-cell activation of the oncogene alone does not give rise to cancer (Fig. S7A). However, if following the local expression of the oncogene (and its fluorescent marker), Ventx-GR is transiently activated the cell divides and proliferates (Fig. 2). We observed that at 1-day post induction (dpi) 50% of the induced cells had divided and expanded clonally by a factor ~3 (Fig. 2B), while the other 50% had neither divided nor died. Surprisingly, at 3-5 dpi, we observed in all zebrafish larvae that the induced cell gave rise to progeny that display short-range dispersion (Fig. S6A, B) and then give rise to a tumour mass in the brain with local infiltration of malignant cells (Fig. 2C). Hematoxylin-Eosin (H&E) staining of the brain tissue (Fig. 2D, E) and metastases (Fig.3) further confirm the malignant state of the induced cell and its progeny.

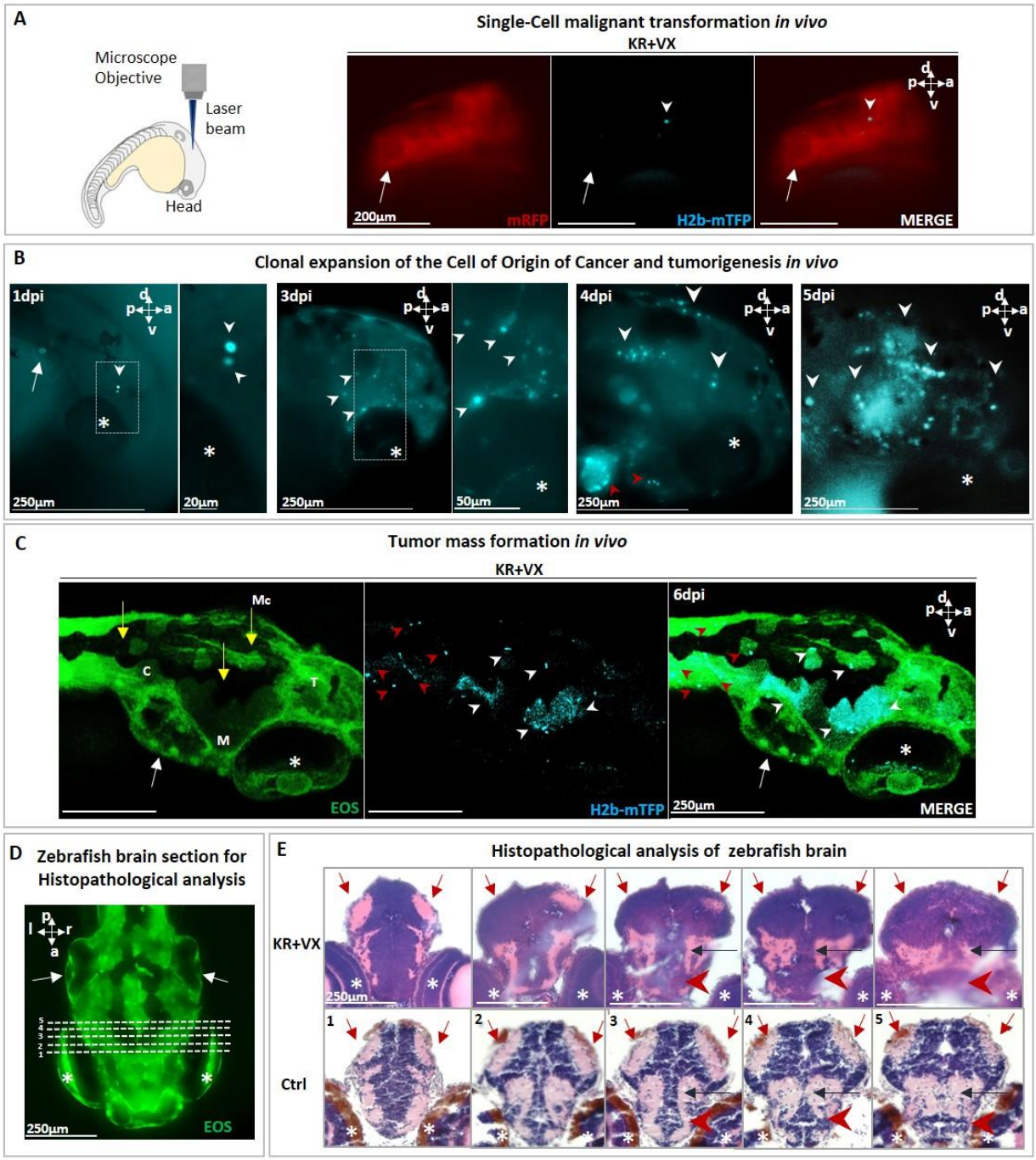


**FIGURE 2: Malignant transformation of a single cell triggering carcinogenesis *in vivo*. (A)** At 1dpf, a single cell in a zebrafish brain was photo-induced to express the oncogene KRASG12V, identified (white arrowhead) within ~30 min by the fluorescent H2B-mTFP. Membrane-bound mRFP is used as tracer. Transient (24h) DEX activation of Ventx is done following photoactivation. **(B)** At 1day post induction (1dpi), the activated cell has divided, giving rise to two cells (white arrowhead). After 3dpi the original cell expand clonally (white arrowheads) by short-range dispersal within the brain. At 4dpi the brain has been colonized (left panel) by the progeny of the activated cell (white arrowheads) that display tumour growth as well as dispersal in the head or entering into the cardiovascular system (red arrowhead). At 5dpi, a tumour mass is formed. **(C)** Confocal microscopy of a larval head displaying tumours (arrowheads) and dispersal in the trunk (red arrowheads). **(D)** Histopathological sections of larval brain (dorsal view). Dashed lines (1 to 5) indicates sections showed in **(E)** Hematoxylin & Eosin (H&E) staining of brain at 5dpi of KR+VX-induced larva (depigmented) is compared to normal brain (Ctrl, melanocytes in brown). At 5dpi, the optic tectum (red arrows), the tegmentum (black arrow) and hypothalamus (red arrowhead) are infiltrated by a dysplasic tumour progeny of the initially induced single cell. An asterisk (*) indicate the eye and a white arrow the otic vesicle. Scale bars and the body axes (a: anterior; p: posterior; d; dorsal; v: ventral) are shown. T=telencephalon; M=Mesencephalon; C= Cerebellum; Mc= Melanocytes.

In parallel with tumour mass formation in the brain (Fig. 2), we observed that some cells of the progeny re-localized to new loci far from the brain, such as the heart (Fig. 3A), the digestive tract (Fig. 3B, Fig. S6D) and the trunk (Fig. S6C). We therefore deduce that the transient activation of Ventx alters the state of a cell expressing a mutated oncogene (i.e. KRASG12V) and induces its malignant transformation *in vivo*, with tumorigenic potential and the capacity to generate invasive progeny.

Of the larvae in which a single cell was expressing the oncogene and in which Ventx was transiently activated (KR+VX cell), all (n=15) developed tumours within 5 dpi. The frequency of tumour development is therefore $F_1$=1. Conversely the probability for such a cell to not develop a tumour is $F_0$=0. Due to the finite size of the sample, we estimate the probability of tumour development from a single cell to be: $P_1 > 80\%$ ($\chi^2 = 3{,}75$; df=1).

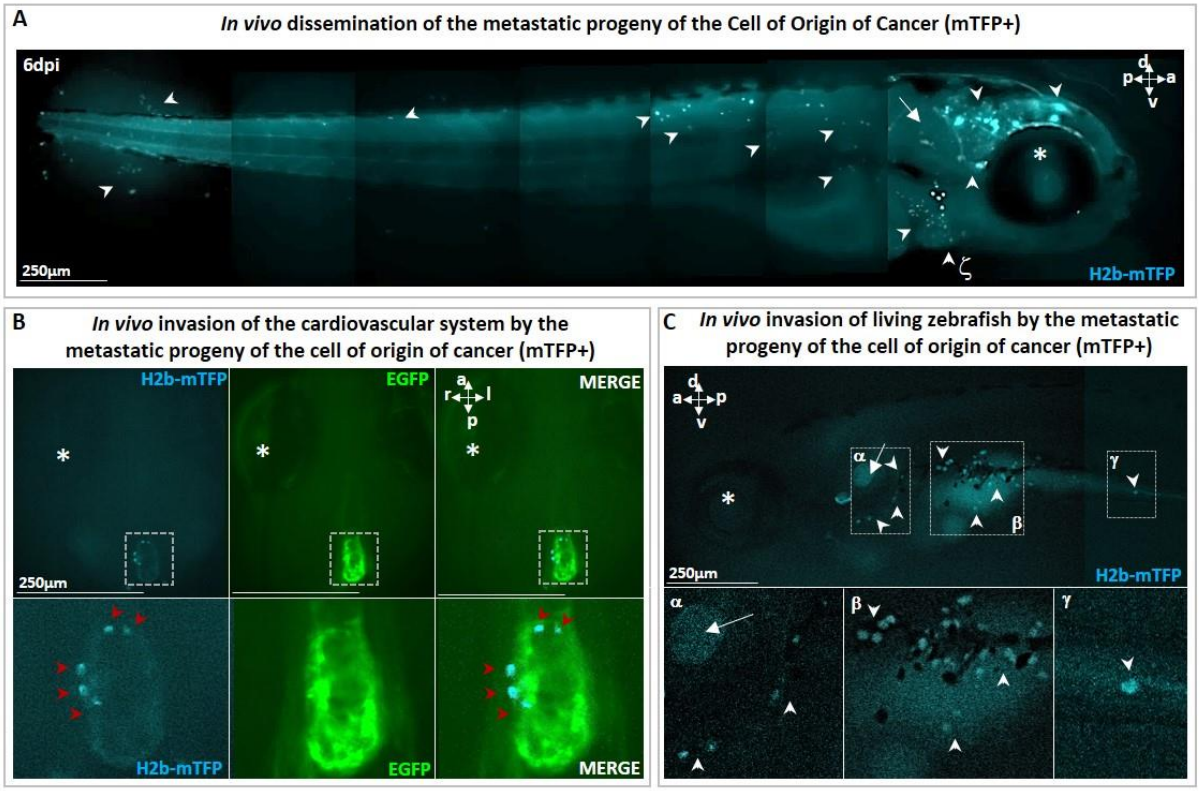


**FIGURE 3: Metastatic cells following single cell malignant transformation. (A)** A zebrafish larva, in which a single cell in the brain was photo-induced (at 1dpf) to express the oncogene KRASG12V by the blue fluorescence of the expression marker (H2b-mTFP) as in (Fig. 2), show that the progeny of the Cell of Origin of Cancer give rise to a tumour mass in the brain (white arrowhead), as well as to migrating metastatic-like cells that disseminate in the whole organism (white arrowhead), thus localizing in branchial arches (in proximity of arterial branchial arches, indicated by ζ white arrowhead), trunk and tail fin. **(B)** H2b-mTFP positive cells can migrate far from the site of induction (brain) and colonize ectopic tissues located in the heart and **(C)** the bottom of otic vesicle (in proximity of the primary head sinus, magnificent in α), the digestive tract (magnificent in β and γ) a feature characteristic of metastatic cancer cells. Note that no anaesthetics (e.g. tricaine) or mounting media (low melting point agarose or methylcellulose) were used to block live zebrafish, during both monitoring and imaging performed on live zebrafish. An asterisk (*) indicate the eye and a white arrow the otic vesicle. Scale bars and body axes (a: anterior; p: posterior; d; dorsal; v: ventral) are indicated.

To definitely confirm the carcinogenic nature of the KR+VX induced cells we isolated and injected a single cell from a hyperplasic tissue (identified by the blue fluorescence of its nucleus) into a naïve host zebrafish larva. This led to integration, migration and colonization of the host tissues by the progeny of the transplanted cell (Fig. 4A, B), with strong pERK activity

detected in tumour masses of the host (Fig. 4B). Out of 52 transplanted host zebrafish, 31 developed tumours, a probability of tumour development (60%) consistent with previously reported efficiency of tumour cells transplantation in zebrafish[14,15]. This result definitely confirms the cancerous nature of cells expressing a mutated oncogene and exposed to the transient activation of Ventx, with subsequent alteration of the homeostasis. Notice that injection into a naïve larva of an oncogene expressing cell (identified by its nuclear blue fluorescence) not experiencing transient activation of a reprogramming factor does not yield a tumour (Fig. S7B).

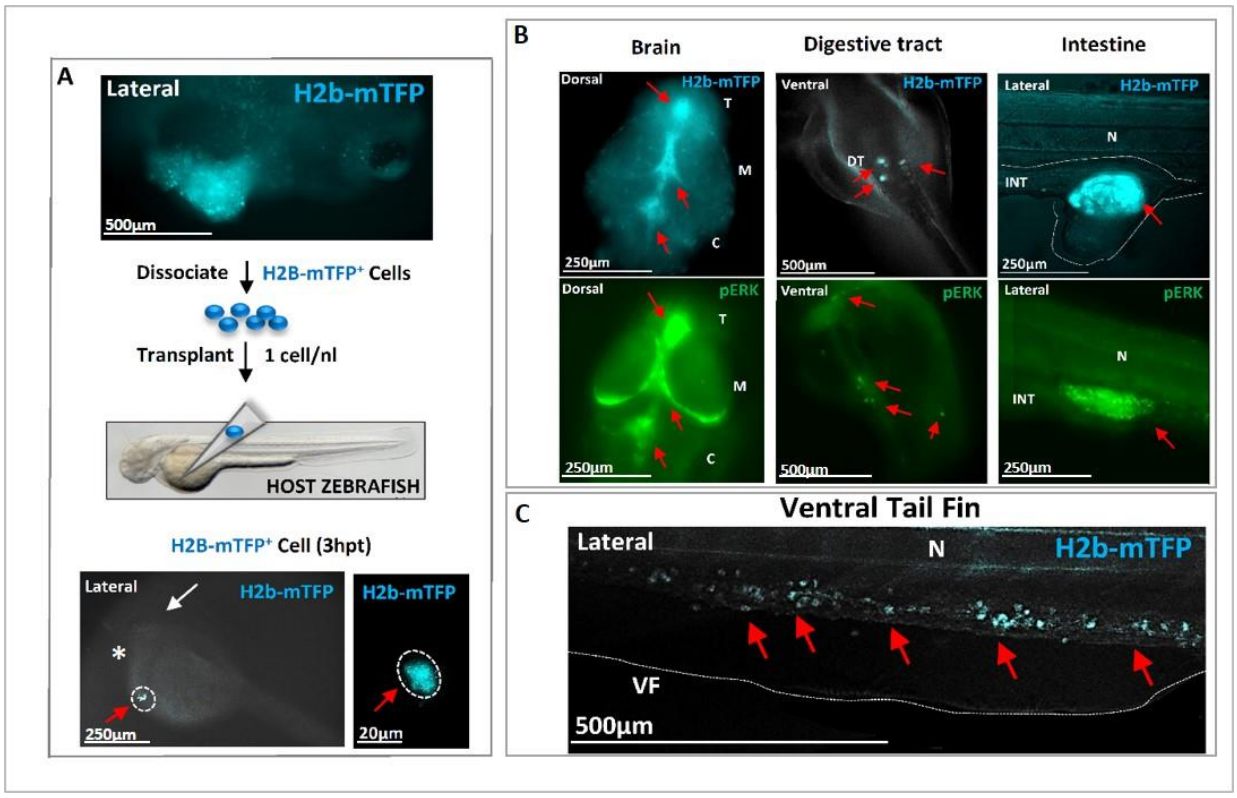


**FIGURE 4: Transplantation of single cell(s) from hyperplasic tissue reveals cancer-initiating potential. (A)** Following KRASG12V *plus* Ventx activation, hyperplasic tissues (blue fluorescent) are observed at 6dpf, top figure. The cells of the hyperplasia were dissociated and isolated blue (KRAS expressing) cells were transplanted (≈ 1 cell *per* host) at 2dpf in a *Nacre* (*mitf* -/-) zebrafish line for a better tracking of the transplanted cells. The transplanted H2b-mTFP positive (blue) cell (red arrow) can be visualized as early as 3 hours post transplantation (3hpt) in the yolk of the host, close to the duct of Cuvier. White arrow indicates the head/eye (lateral view). **(B)** At 3 dpt the blue cell(s) from the hyperplasic tissue of the donor have colonized the host *Nacre* zebrafish larvae. Tumours in the brain, digestive tract and intestine, are observed and characterized by the blue fluorescence of the donor KRAS expressing cells (red arrows; n=31 out of 52 host individuals). In the bottom, immunofluorescence (IF) analysis of representative host zebrafish larvae with specific high level of phosphorylated ERK activity (pERK, red arrows) in the brain, intestine and digestive tract. **(C)** A high number of exogenous blue fluorescent cells are here observed to migrate in the tail (red arrows). These observations indicate that the transplanted founder cell has both migratory, colonizing behavior, as well as survival growth advantage in the host to form tumours, and thus to re-initiate carcinogenesis. Scale bars and body axes (a: anterior; p: posterior; d; dorsal; v: ventral) are shown; T=telencephalon; M=Mesencephalon; C= Cerebellum; N=notochord; VF= ventral fin.

The results reported here are compatible with the recently proposed "ground state theory of cancer initiation[7]". According to that theory a malignant transformation may occur in a cell harboring an oncogenic mutation upon a change of its functional state (its "ground state"). Here the transient activation of a reprogramming factor (i.e. Ventx, Nanog or Oct4) possibly synergizes with the oncogene to alter the epigenetic and functional state of the cell allowing its transformation into a tumorigenic cell.

Importantly, our observation that the progeny of the cell(s) of origin of cancer display a short-range dispersal within the tissue during the earliest phases of tumorigenesis and prior to the effective appearance of tumour mass corroborates a recent theoretical model predicting such an early dispersal during carcinogenesis to explain heterogeneity, therapeutic resistance and tumour relapse[8].

Being the first experiments to predictably control the malignant transformation of a single cell in an unaltered microenvironment our approach opens a new vista on the study of "the cell of origin of cancer"[16], an acknowledged enigma of cancer research. As such our results raise many questions: what mechanisms are at work in the transition from the altered ground state to a tumorigenic state? Are reprogramming factors as efficient in triggering this transition in adults as they are in the young? Is the aberrant reactivation of reprogramming factors a key step in the initiation of carcinogenesis, independent of the tissue or the oncogenic mutations? Do drugs against these factors reduce the probability of carcinogenesis? Conversely, do some carcinogen act by reactivating those factors? By opening the way for a quantitative analysis of the initial stages of cancer growth, the optogenetic approach described here should be able to address many of these questions.

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**Authors contributions**: DB, BD and PS conceived the experiments and wrote the paper. BD, PS and BT performed the experiments, while HD and MD did the genomic analysis.

## MATERIALS&METHODS

**Fish lines and maintenance**. Zebrafish were raised and maintained in an approved Fish Facility (C75-05-32 at IBENS) on a 14–10 h light-dark diurnal cycle with standard culture methods[17]. Embryos collected from natural crosses were staged according to Kimmel[18]. The Tg(*actin*:loxP-EOS-stop-loxP-KRASG12V-T2A-H2B-mTFP) was generated by injecting the plasmid pT24-*actin*:loxP-EOS-stop-loxP-KRASG12V-T2A-H2B-mTFP, which contains the homologous cDNA sequence of KRASG12V from human, with tol2 mRNA transposase. Founder transgenic fish were identified by global expression of Eos. The Tg(*ubi*:Cre-ERT; *myl7*:EGFP) was previously described[19]. The double transgenic line Tg(*actin*:loxP-EOS-stop-loxP-KRASG12V-T2A-H2B-mTFP; *ubi*:Cre-ERT; *myl7*:EGFP) was created by crossing Tg(*actin*:loxP-EOS-stop-loxP-KRASG12V-T2A-H2B-mTFP) and Tg(*ubi*:Cre-ERT; *myl7*:EGFP). Founder double transgenic fish were selected by global expression of Eos and expression of EGFP in the developing heart. Zebrafish were imaged for phenotypic analysis throughout early development, from embryonic to larval stage, and then fixed (at 6dpf) with PAXgene Tissue Container Product (Qiagen) for RT-qPCR or with 4% PFA (Thermofisher) for histological (Hematoxylin & Eosine, H&E) analyses.

**Dexamethasone induction:** transgenic zebrafish were injected at the one cell stage with the mRNA of a gene-construct Ventx-GR (*Xenopus* ventx2, 450pg), Nanog-GR (mouse Nanog, 100pg) or Pou5/Oct4 (*Xenopus* pou5f3.1/oct91, 100pg) together with a red fluorescent expression marker mRFP (50 pg). The

protein products of these constructs are sequestered by cytoplasmic chaperones and released upon incubation of the embryos in a medium containing 10 µM Dexamethasone (DEX). In single cell activation experiments, zebrafish were selected from their strong intensity of fluorescent mRFP signal for local activation of kRasG12V via uncaging of 6µM cCYC (a gift of I.Aujard and L.Jullien).

**Reverse transcriptase quantitative PCR (RT-qPCR)**. Zebrafish larvae were fixed (as mentioned above) at 6 dpf. Total RNAs were extracted using the RNeasy micro kit (Qiagen) according to the manufacturer's protocols. Sample quantity and purity, reverse transcription, pre-amplification and High throughput qPCR were performed as in Zhang et al.[20]. Quantitative PCR was performed using the high throughput platform BioMark™ HD System and the 48.48 GE Dynamic Arrays (Fluidigm). RTqPCR measurements were done in triplicate on pooled zebrafish larvae and single zebrafish larva (for KR+VX condition), as indicated in the text. Normalization and quantification were obtained with the Δ(ΔCt) method using *rpl13a* as a reference gene.

**Immunohistochemistry (IHC)**. The zebrafish obtained in the various conditions and the stages mentioned in the text were fixed in 4% PFA overnight at 4 °C, followed by dehydration with 100% methanol at −20 °C for more than 1 day. After gradual rehydration of methanol and wash with PBS/Tween 0.1%, the embryos were incubated in a blocking solution: 1%Triton, 1% DMSO, 1% BSA and 10% sheep serum (Sigma) in PBS on a shaker for 1 h at room temperature, followed by incubation on a shaker overnight at +4°C with 1:500 anti-phospho-Erk antibody (phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® ). Rabbit mAb detects endogenous levels of p44 and p42 MAP Kinase (Erk1 and Erk2) when dually phosphorylated at Thr202 and Tyr204 of Erk1 (Thr185 and Tyr187 of Erk2), and singly phosphorylated at Thr202 - this antibody does not cross-react with the corresponding phosphorylated residues of either JNK/SAPK or p38 MAP kinases) (ref 4370S Cell Signalling). After washing with PBS/Tween, embryos were incubated overnight at +4°C with 1/1000 secondary fluorescent conjugated antibody, Donkey anti-Rabbit Alexa Fluor 488 (Thermo Fisher A-21206). To visualize the distribution of Ventx-GR in the fish, embryos were incubated with 1:500 anti-HA antibody, tagging the HA sequence fused to Ventx-GR, as the conjugated antibody (Anti-HA, mouse IgG1, clone 16B12 Alex Fluor™ 488 conjugated, Thermofisher Ref. A21287) on a shaker overnight at 4 °C. All images were taken on a Nikon Ti microscope equipped with a Hamamatsu Orca camera, except for confocal microscopy (Fig.2C, S4B) carried out on a Leica SP5 and Zeiss LSM800 microscope.

**Caged Cyclofen (cCYC) treatment and UV uncaging.** To induce kRASG12V expression, 24hpf embryos were directly incubated in 6µM caged cyclofen (cCYC) for 1 hour (in the dark), briefly rinsed in E3 medium followed by 5 min. photo-activation with a ~365 nm UV lamp. They were then transferred back to E3 medium + 10 µM DEX (to release Ventx-GR from its complex with cytoplasmic chaperones). The embryos at 1 dpi were washed 3x in E3 medium to remove DEX. For this experiment, we used a benchtop UV lamp (Fisher VL-6-L) which emits a peak wavelength at 365 nm with a FWHM (full width at half maximum) of 40 nm and delivers on the illuminated sample a typical photon flux of ~4.3 Einstein/($s \cdot m^2$).
Localized uncaging was performed by illumination for 7 min on a Nikon Ti microscope equipped with a light source peaking at 405 nm, Fig.1. The size of the uncaging region was controlled by an iris that defines a circular illumination of diameter ~ 80 µm. After 0,5-1 h following uncaging the illuminated region of each embryo was imaged to identify and count mTFP positive cell(s). Embryos were transferred to a 12-well plate with E3 + 10 µM DEX, incubated in total darkness overnight and washed 3x with E3 at 1 dpi (to remove DEX).

**Cell transplantation.** Zebrafish larvae were grown until 6dpi, dissociated in dissociation medium[21] (025% trypsin-EDTA *plus* 1/10 Collagenase 100mg/ml) 10min at 30°C, mechanically homogenized,

resuspended in DMEM-10% Fetal Bovine Serum (FBS), filtered through 70µm nylon mesh (Corning Cell Strainer Ref. 431751) and resuspended in DMEM-10% FBS at 1cell/1nl concentration by using LUNA-II cell counter (Logos BioSystem).

**Microscopy**. Fluorescent images were taken on a Nikon Ti microscope equipped with a Hamamatsu ORCA V2+ camera and a 10X plan fluo objective. Filter setting of CFP: excitation at 438 ± 24 nm, emission at 483 ± 32 nm; mEosFP and Alexa 488: excitation at 497 ± 16 nm, emission at 535 ± 22 nm. Image analysis was done using ImageJ software.

## REFERENCES


[1] Cairns, J. Mutation selection and the natural history of cancer, *Nature* **255**, 197-200 (1975)

[2] Riva, L. et al. The mutational signature profile of known and suspected human carcinogens in mice. *Nat. Genet.* **52**, 1189–1197 (2020).

[3] Adashek JJ, Kato S, Lippman SM, Kurzrock R. The paradox of cancer genes in non-malignant conditions: implications for precision medicine. *Genome Med.* **12,** 16 (2020)

[4] Hanahan, D. & Weinberg, R. A., The hallmarks of cancer, *Cell* **100**, 57-70 (2000).

[5] Soto, A. M. & Sonnenschein, C. The tissue organization field theory of cancer: a testable replacement for the somatic mutation theory. *BioEssays* **33**, 332–340 (2011).

[6] Tomasetti C, Vogelstein B, Cancer etiology. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. *Science* **347**, 78-81 (2015).

[7] Jassim A, Rahrmann E.P, Simons B.D. and Gilbertson R.J., Cancers make their own luck: theories of cancer origins, *Nat.Rev.Can*. **23**, 710-724 (2023)

[8] Waclaw B. *et al.*, A spatial model predicts that dispersal and cell turnover limit intratumour heterogeneity, *Nature* **25**, 261-264 (2015)

[9] Feng Z *et al*., Optical Control of Tumour Induction in the Zebrafish. *Sci.Rep.* **7,** 9195 (2017)

[10] Zhang W et al., Control of protein activity and gene expression by cyclofen-OH uncaging, *ChemBioChem* **19**,1-8 (2018).

[11] Baggiolini A et al., Developmental chromatin programs determine oncogenic competence in melanoma. *Science* **373**, 6559 (2021)

[12] Lam KHB et al., Topographic mapping of the glioblastoma proteome reveals a triple-axis model of intra-tumoral heterogeneity, *Nat Commun*, **13**, 116 (2022).

[13] Ducos B, Bensimon D, Scerbo P. Vertebrate Cell Differentiation, Evolution, and Diseases: The Vertebrate-Specific Developmental Potential Guardians *VENTX*/*NANOG* and *POU5*/*OCT4* Enter the Stage. *Cells* **11**, 2299 (2022)

[14] Scerbo P, Monsoro-Burq AH. The vertebrate-specific VENTX/NANOG gene empowers neural crest with ectomesenchyme potential. *Sci Adv.* **6**, 1469 (2020)

[15] Campbell NR, Cooperation between melanoma cell states promotes metastasis through heterotypic cluster formation. *Dev Cell.* **56**, 2808-2825 (2021)

[16] Visvader JE. Cells of origin in cancer. *Nature* **469**, 314-22 (2021)

[17] Westerfield, M. The Zebrafish Book. A Guide for The Laboratory Use of Zebrafish (Danio rerio) (2000).

[18] Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F. Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253–310 (1995)

[19] Mosiman, C. et al., Ubiquitous transgene expression and Cre-based recombination driven by the *ubiquitin* promoter in zebrafish, *Development* **138**, 169-177 (2011)

[20] W.Zhang, P.Scerbo, M.Delagrange, V.Candat, V.Mayr, S.Vriz, M.Distel, B.Ducos, and D.Bensimon, Fgf8 dynamics and critical slowing down may account for the temperature independence of somitogenesis, *Commun.Biol.* **5**, 113 (2022).

[21] Bresciani E, E.Broadbridge and P.P.Liu, An efficient dissociation protocol for generation of single cell suspension from zebrafish embryos and larvae., *MethodsX* **5**, 1287-1290 (2018).

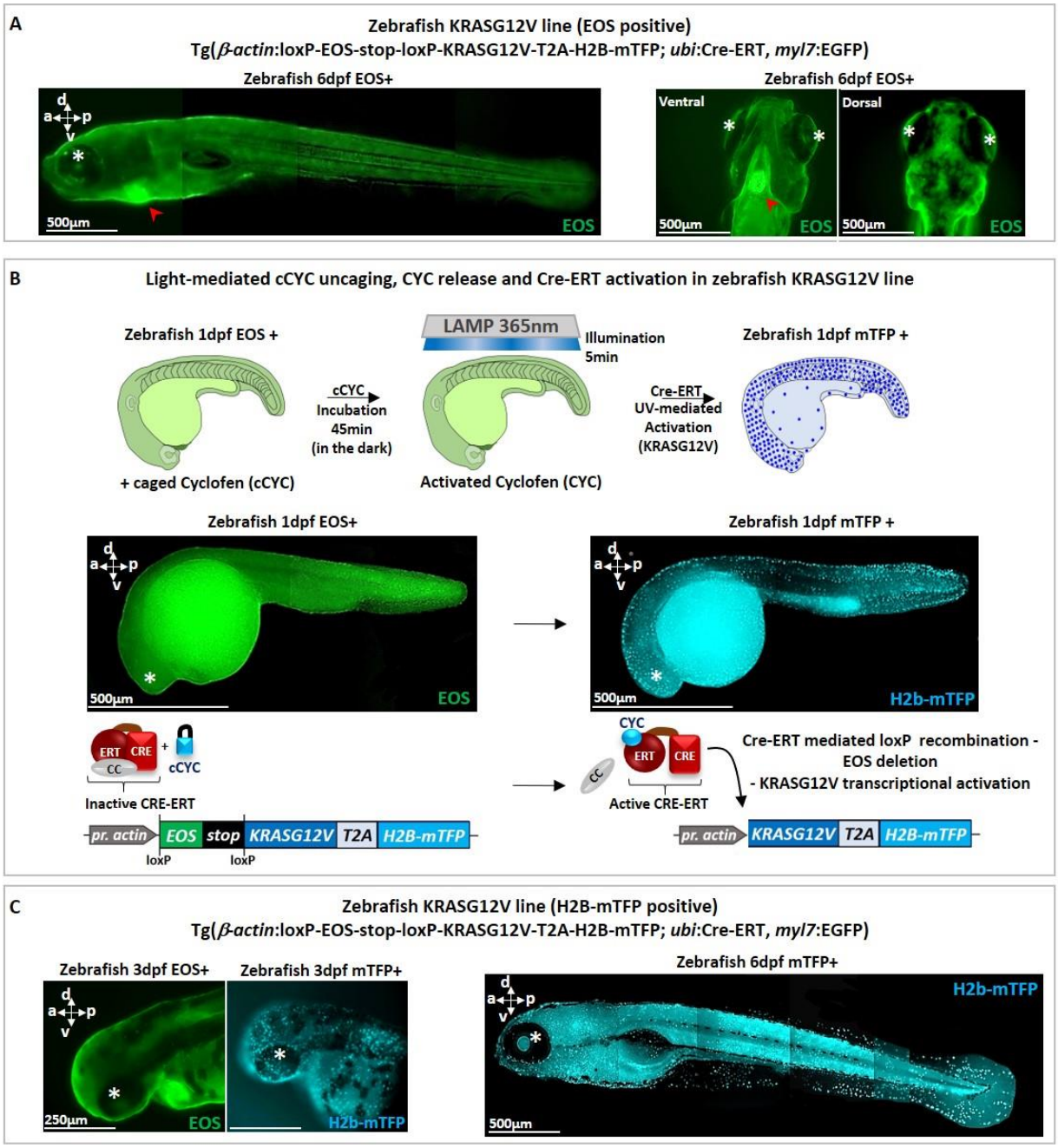


**FIGURE S1: Characterization of the double transgenic line Tg(β−*actin*:loxP-EOS-stop-loxP-KRASG12V-T2A-H2B-mTFP; *ubi*:Cre-ERT; *myl7*:EGFP). (A)** A transgenic fish at 6dpf displays green EOS fluorescence throughout the body and a strong green fluorescence in the heart due to the additive expression of EGFP under a heart specific (*myl7*) promotor, red arrowhead. **(B)** Upon global illumination (at 1dpf) with a ~365nm UV lamp in presence of cCYC, cyclofen is uncaged which releases CRE-ERT from its complex with cytoplasmic chaperones and results in floxing of EOS and ubiquitous expression of KRASG12V and H2B-mTFP. The embryos thus display blue fluorescence in the nuclei shown here at 1h post induction (hpi). (**C**) Stable and ubiquitous expression of the blue H2B-mTFP fluorescent protein in zebrafish late embryos (3dpf) and larvae (6dpf). Note that in all pictures the asterisk (*) indicate the eye. Scale bars and body axes (a: anterior; p: posterior; d; dorsal; v: ventral) are shown.

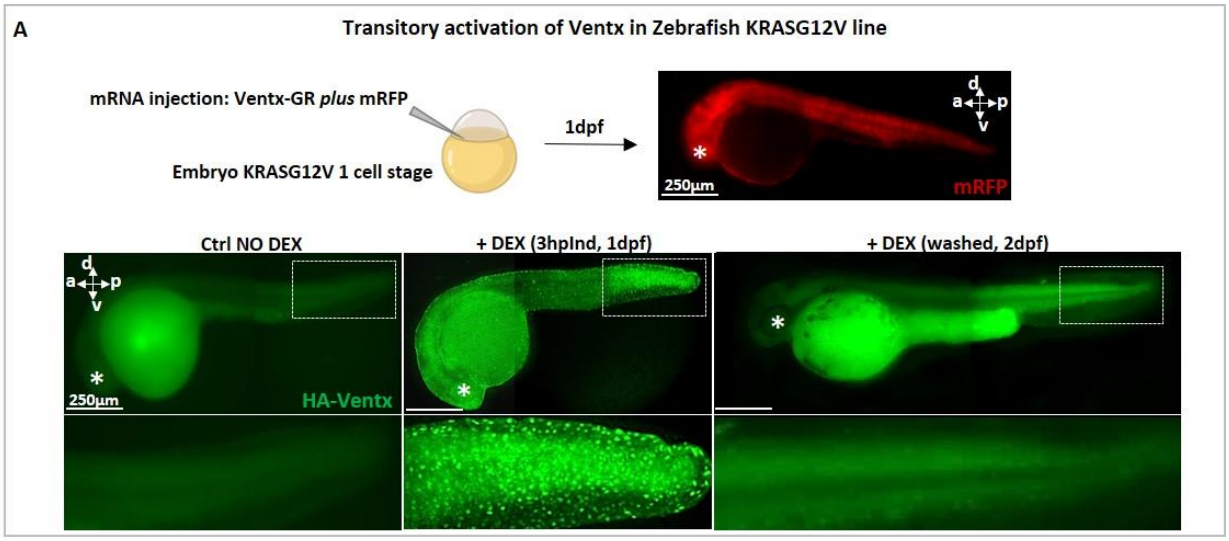


**FIGURE S2: Transient activation of Ventx reprogramming factor. (A) top:** At one cell stage Ventx-GR mRNA is injected together with mRFP mRNA (used as a tracer), which expression throughout the embryo (here shown at 1dpf) is a proxy for Ventx-GR expression. **Bottom:** Immunofluorescence analysis show that Ventx protein can be visualized in fixed embryos with the help of an antibody (Ab) directed against the HA tag linking Ventx and GR (and visualized by a second Ab linked to a green fluorescent marker). Upon addition of DEX at 1dpf embryos, Ventx is released from its complex with cytoplasmic chaperones and diffuses into the cell nucleus, resulting in a pointillist image of the nuclei (middle). At 2dpf, after washing out DEX, Ventx protein is no longer detected and the pointillist image is lost (right). Note that in all pictures the asterisk (*) indicate the eye. Scale bars and body axes (a: anterior; p: posterior; d; dorsal; v: ventral; l: left; r: right) are shown.

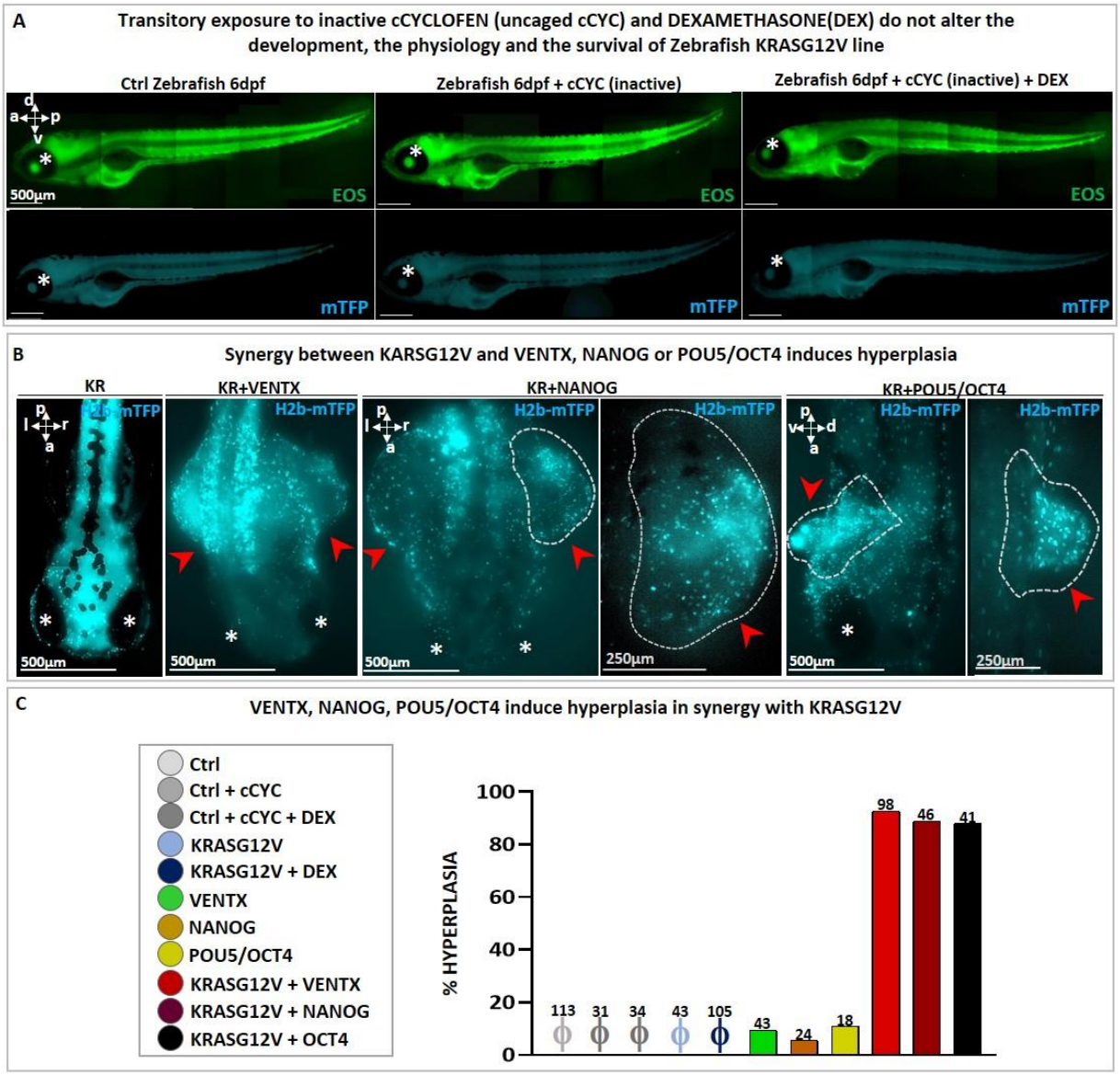


**FIGURE S3: Synergy between a reprogramming factor and KRASG12V oncogene efficiently induces tumours in zebrafish larvae. (A)** Images of control zebrafish larvae at 6dpf: without any treatment (left), with transient incubation in cCYC (without UV, middle) and with incubation in DEX + cCYC (without UV, right). Note that zebrafish does show neither morphological anomalies nor mortality. **(B)** Photoactivation (*via* cCYC uncaging and Cre-ERT activation) of KRASG12V (blue nuclei) in 1dpf zebrafish without (left) or with subsequent transient activation (right) of Ventx-GR by Dexamethasone (DEX) (described in Fig. S2). These larvae (labelled as KR+VX) develop hyperplasic tissues within 6-9 dpf (dorsal view, red arrows indicate hyperplasia). Photoactivation (*via* cCYC uncaging and Cre-ERT activation) of KRASG12V (blue nuclei) in 1dpf zebrafish with subsequent transient activation of NANOG-GR or POU5/OCT4-GR by Dexamethasone (DEX) (set-up described in A). These larvae (labelled as KR+NANOG or KR+POU5/OCT4) develop hyperplasic tissues within 6-9 dpf (dorsal view, red arrows indicate hyperplasia). **(C)** Quantification of zebrafish developing abnormal hyperplasia upon the indicated treatments. Note that only the synergy between reprogramming factors (i.e. VENTX, NANOG or POU5/OCT4) and the KRASG12V oncogene reproducibly and efficiently induce tumours in zebrafish larvae. The total number of zebrafish larvae analyzed are indicated above the bars. ϕ indicates no event (0%). Note that in all pictures the asterisk (*) indicate the eye. Scale bars and body axes (a: anterior; p: posterior; d; dorsal; v: ventral; l: left; r: right) are shown.

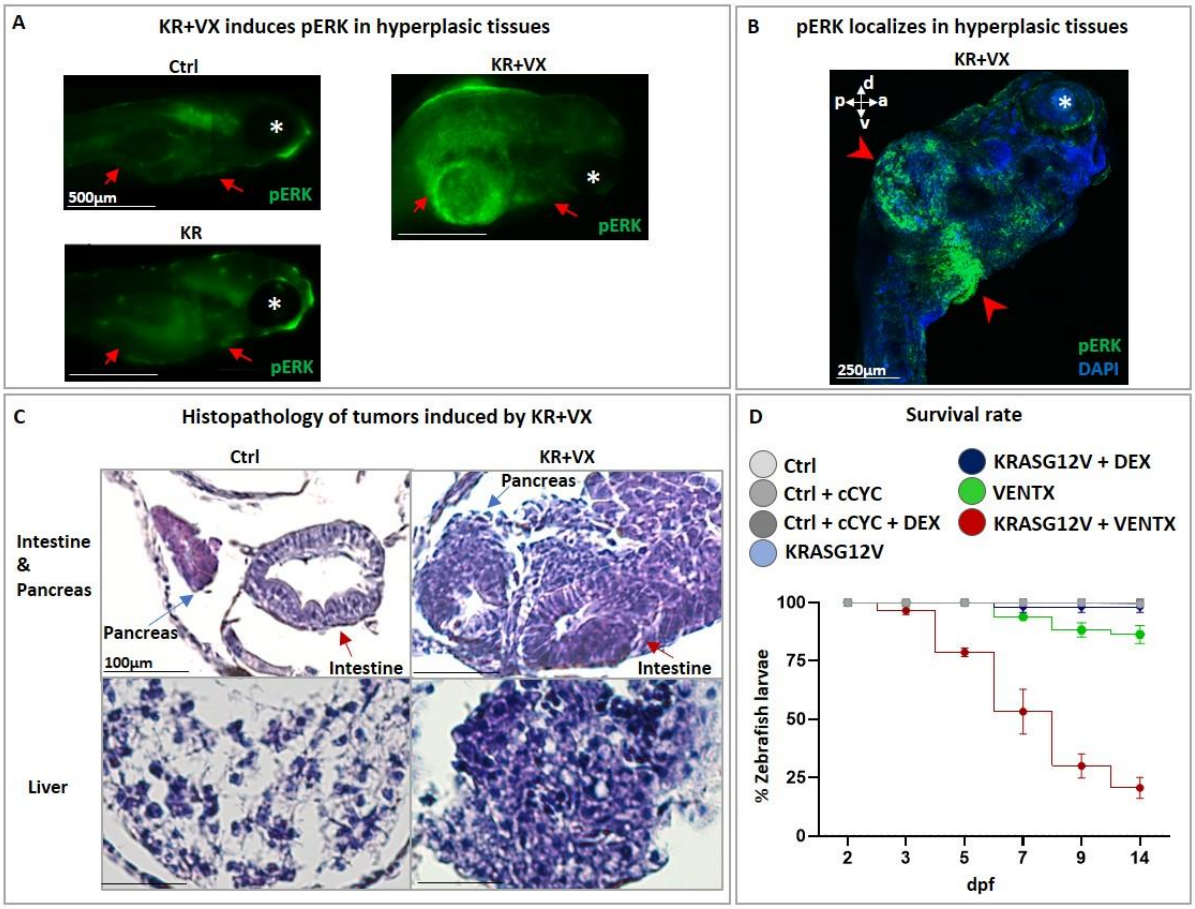


**FIGURE S4: Synergy between the Ventx reprogramming factor and the KRASG12V oncogene induces tumours in zebrafish larvae. (A)** Phosphorylated ERK (pERK) immunofluorescence at 6dpf in non-activated larvae (Ctrl), in larvea in which only KRASG12V was activated (KR) and in larvae in which both KRASG12V and VENTX were activated (KR+VX). Notice in the last one the strong pERK activation in the hyperplasic abdominal outgrowth. The digestive tract is shown between the red arrows. **(B)** Confocal microscopy of KR+VX zebrafish larva at 6dpf (ventro-lateral view) displays the immunofluorescence of active phosphorylated ERK (pERK, in green and indicated by red arrowheads) in the abdomen of a DAPI labelled zebrafish larva. **(C)** Histopathological analysis by Hematoxylin & Eosin (H&E) staining of hyperplasic tissues in zebrafish larvae (6dpf) upon activation of KRASG12V *and* Ventx (KR+VX) is compared with normal tissues (Ctrl). Larvae overexpressing KR+VX develop hyperplasic and dysplastic cancer-like features and tissue outgrowth in the gut/intestine (red arrow), pancreas (blue arrow) and liver. **(D)** Such cancer-like features specifically and exclusively reduce the survival rate to 20% two weeks after induction.

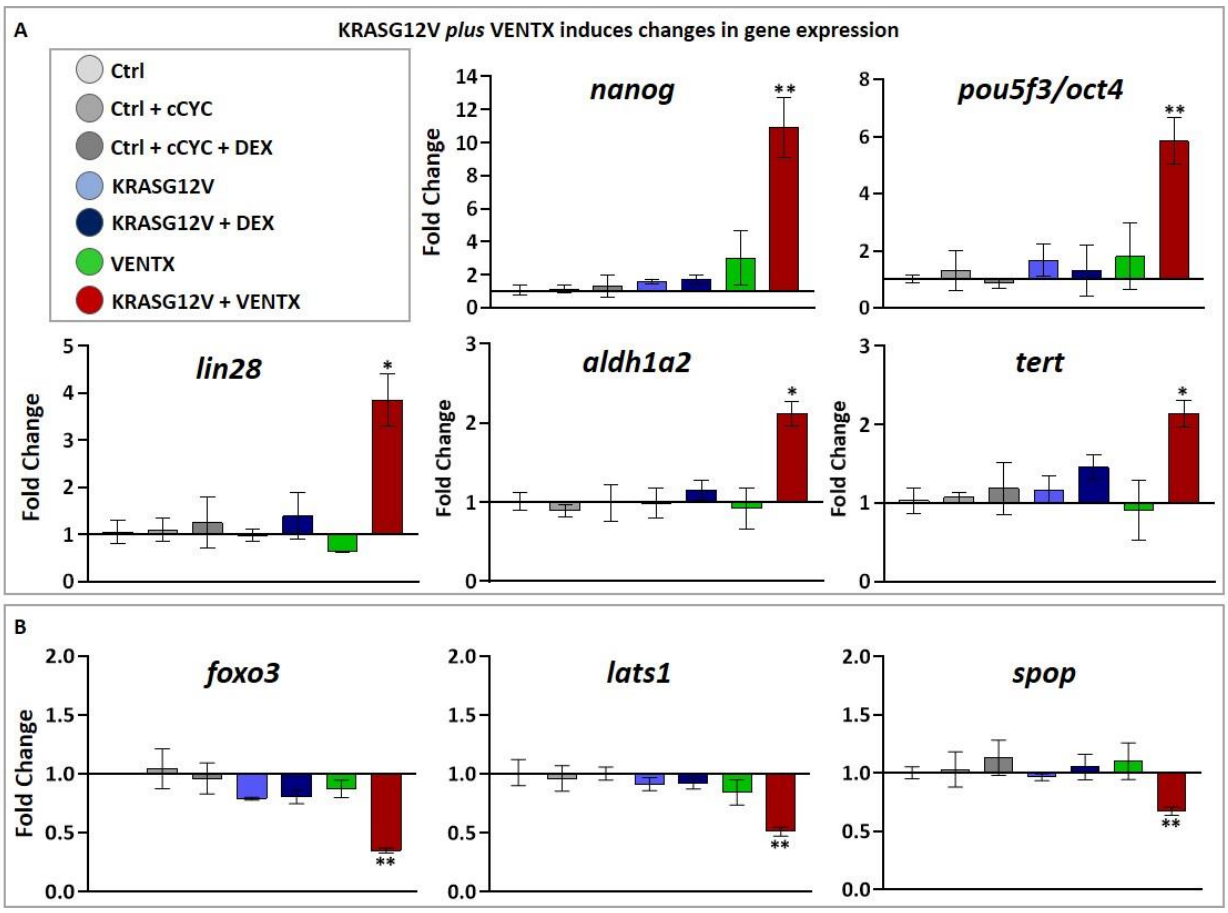


**FIGURE S5: KRASG12V *plus* Ventx induces a cancer-like gene expression signature.** Both KRASG12V *and* Ventx-GR (KR+VX) were activated in zebrafish at 1dpf. The larvae (n=24) were collected at 6 dpf and processed for RT-qPCR analysis. Compared to the controls (Ctrl in light grey; Ctrl+ cCyc in grey; Ctrl + cCYC + DEX in dark grey) and larvae in which only KRASG12V (KRASG12V in light blue; KRASG12V + DEX in blue) or Ventx (VENTX in green) were activated, the co-activation of KRASG12V + VENTX (in red) displayed significant changes in gene expression. **(A)** Pluripotency/reprogramming factors like *nanog*, *pou5f3*/*oct4* and *lin28*, as well as stemness markers like *aldh1a2* and *telomerase* (*tert*) are significantly up-regulated ($p<0.05$, compared to Ctrl) by KR+VX but not by KRASG12V (KR) or Ventx (VX) alone. **(B)** Conversely, onco-suppressors such as *foxo3*, *lats1* and *spop* are significantly down-regulated ($p<0.05$) by KR+VX but not by KRASG12V (KR) or Ventx (VX) alone when compared to control. For all qPCR graphs, error bars represent s.e.m. values. For statistical analyses, samples were compared with the respective control by Unpaired Student's t-test. *$p<0.05$, **$p<0.005$. ***$p<0.0001$.

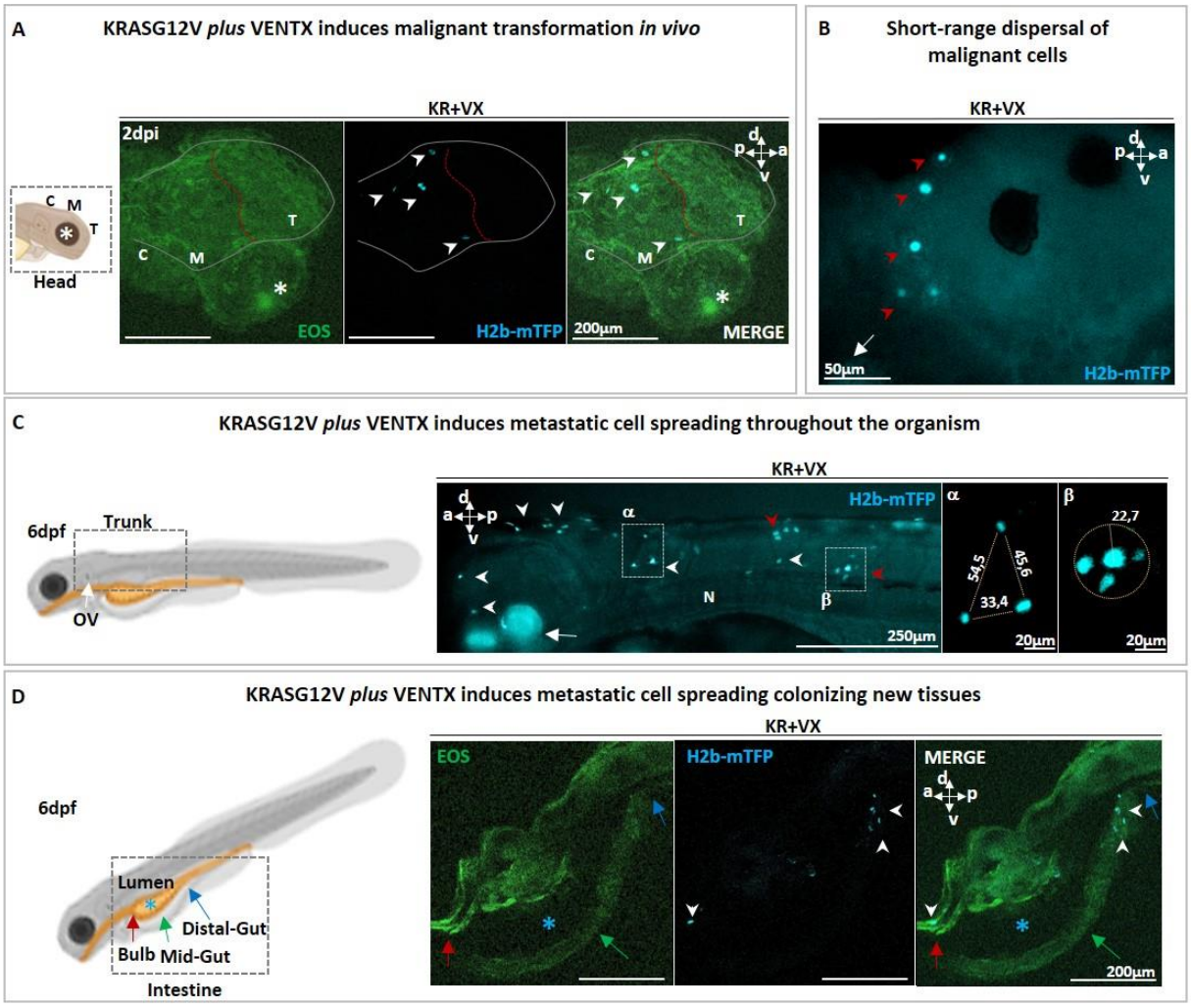


**FIGURE S6: Metastatic spreading following activation of KRASG12V and Ventx (KR+VX). (A)** Schematic representation and confocal microscopy of zebrafish head showing the early malignant cells (H2B-mTFP $^{+}$ blue cells; white arrowheads) in the brain. Green EOS fluorescence has been used to visualize the whole zebrafish head. **(B)** Short range dispersal in the brain of the early progeny of the initial malignant cell (H2B-mTFP $^{+}$ blue cells; red arrowheads). An asterisk (*) indicate the eye and a white arrow the otic vesicle. **(C)** Schematic representation of the zebrafish larva trunk (left, dotted square)**,** where H2B-mTFP$^{+}$ cells (white arrowheads in center panel) can localize in KR+VX larvae, both as isolated cells (as in the dotted square α , 44,5µm of mean distance) or as small clusters (as in the dotted square β , r=22.7µm). **(D)** Schematic representation of zebrafish larva digestive tract (left, dotted square), and confocal microscopy showing H2b-mTFP$^{+}$ cells (white arrowheads, center and right panel) localized in the gut. Green EOS fluorescence has been used to visualize the digestive tract. Red arrows indicate the bulb, the green arrows point to the mid-gut, the blue arrows the distal-gut and the blue asterisk (*) indicate to the gut lumen. Scale bars and body axes (a: anterior; p: posterior; d; dorsal; v: ventral) are shown.

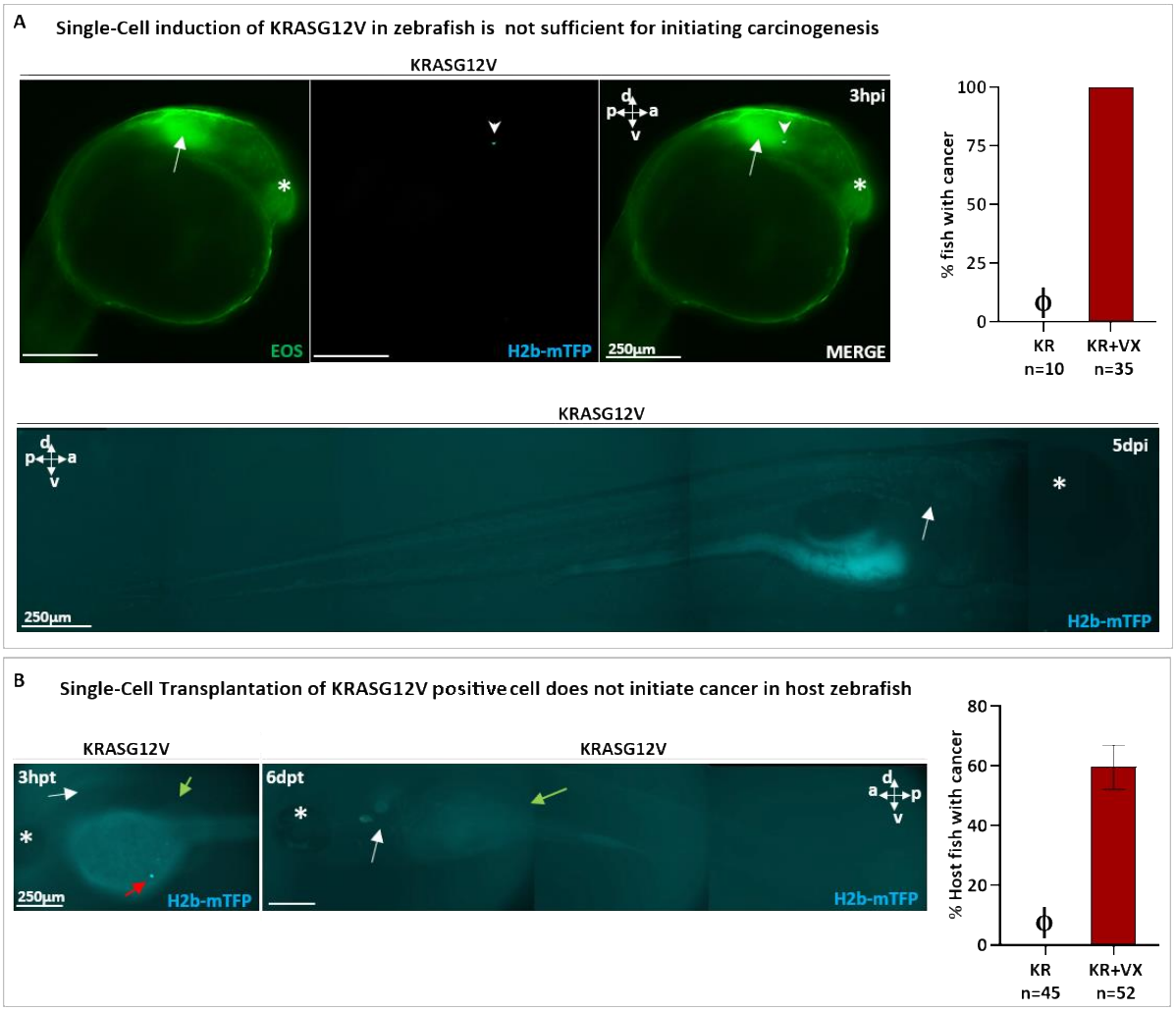


**FIGURE S7: Single-cell activation of the KRASG12V oncogene is not sufficient to initiate carcinogenesis. (A)** At 1dpf, a single cell in the brain was photo-induced to express the oncogene kRasG12V and identified (white arrowhead) within ~30 min by the blue fluorescence of the expression marker (H2b-mTFP). The otic vesicle (indicated by white arrow) is used as a spatial reference. At 5 days post induction (5 dpi), the activated cell has disappeared. Note in the graph that, whereas KRASG12V *plus* VENTX (KR+VX) efficiently induces cancer (100%), KRASG12V alone (KR) is not sufficient (0%, indicated by ϕ). **(B)** Expression of kRasG12V was activated in 1 dpf embryos (without subsequent activation of Ventx). The cells of the larvae were dissociated and isolated cells (kRas expressing, H2b-mTFP$^+$ blue cells) were transplanted (≈ 1 cell *per* host) at 2dpf in a *Nacre* (*mitf* -/-) zebrafish line for a better tracking of the transplanted cells. The transplanted H2b-mTFP$^+$ blue cell (red arrow) can be visualized as early as 3 hours post transplantation (3hpt) in the yolk of the host. At 6 dpt the blue cell has disappeared in host *Nacre* zebrafish larvae. Note in the graph that, whereas the transplanted cell experiencing KRASG12V *plus* VENTX (KR+VX) activation efficiently give rise to cancer (60%), KRASG12V alone (KR) is not sufficient to initiate cancer in host zebrafish (0%, indicated by ϕ). In all figures, the otic vesicle (white arrow) is indicated as well as the eye (white asterisk: *). Scale bars and body axes (a: anterior; p: posterior; d; dorsal; v: ventral; l: left; r: right) are shown.