IDH1-mutated transgenic zebrafish lines: An in-vivo model for drug screening and functional analysis

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Abstract

Introduction

The gene encoding isocitrate dehydrogenase 1 (IDH1) is frequently mutated in several tumor types including gliomas. The most prevalent mutation in gliomas is a missense mutation leading to a substitution of arginine with histidine at the residue 132 (R132H). Wild type IDH1 catalyzes oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG) whereas mutant IDH1 converts α-KG into D2-hydroxyglutarate (D2HG). Unfortunately, there are few in vivo model systems for IDH-mutated tumors to study the effects of IDH1 mutations in tumor development. We have therefore created transgenic zebrafish lines that express various IDH1 mutants.

Materials and methods

IDH1 mutations (IDH1R132H, IDH1R132C and loss-of-function mutation IDH1G70D), IDH1wildtype or eGFP were cloned into constructs with several brain-specific promoters (Nestin, Gfap or Gata2). These constructs were injected into fertilized zebrafish eggs at the one-cell stage.

Results

In total more than ten transgenic zebrafish lines expressing various brain-specific IDH1 mutations were created. A significant increase in the level of D2HG was observed in all transgenic lines expressing IDH1R132C or IDH1R132H, but not in any of the lines expressing IDH1wildtype, IDH1G70D or eGFP. No differences in 5-hydroxymethyl cytosine and mature collagen IV levels were observed between wildtype and mutant IDH1 transgenic fish. To our surprise, we failed to identify any strong phenotype, despite increased levels of the oncometabolite D2HG. No tumors were observed, even when backcrossing with tp53-mutant fish which suggests that additional transforming events are required for tumor formation. Elevated D2HG levels could be lowered by treatment of the transgenic zebrafish with an inhibitor of mutant IDH1 activity.
Conclusions

We have generated a transgenic zebrafish model system for mutations in *IDH1* that can be used for functional analysis and drug screening. Our model systems help understand the biology of *IDH1* mutations and its role in tumor formation.

Introduction

Somatic missense mutations in the gene encoding isocitrate dehydrogenase 1 (*IDH1*) or *IDH2* are frequently identified in various malignancies including gliomas, acute myeloid leukemia, cholangiocarcinoma, chondrosarcoma and sporadically in various other cancer types [1–8]. In gliomas, *IDH1* mutations are one of the earliest genetic changes identified, preceding other common genetic aberrations such as 1p19q co-deletion, and are therefore present in virtually all tumor cells [9–11]. *IDH1* and *IDH2* mutations are almost always mutually exclusive. For glioma patients, presence of IDH mutations is of clinical relevance as patients harboring *IDH* mutated gliomas have a better survival compared to those with wildtype *IDH*. The prognostic significance of *IDH* mutations has led to its incorporation in the WHO 2016 update to classify gliomas [12]. Mutations in *IDH1* are almost always heterozygous point mutations affecting the arginine at position 132 (R132). Approximately 90% of these mutations in gliomas are *IDH1* R132H.

Wildtype IDH1 is a cytoplasmic enzyme that catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate (αKG) and uses NADP⁺ as a co-factor [13, 14]. The mutant enzyme, however, uses αKG as a substrate to produce D-2-hydroxyglutarate (D2HG) with concomitant consumption of NADPH [15]. The resulting accumulation of D2HG then competitively inhibits a spectrum of αKG-dependent enzymes including TET2, JMJD2 and various prolyl hydroxylases [16–18]. This inhibition ultimately facilitates carcinogenesis by retaining cells in an undifferentiated and stem-like state. Because of the oncogenic role of mutant IDH1, several groups have developed compounds that specifically inhibit the activity of the mutant enzyme [19, 20]. These inhibitors are currently being tested in clinical trials.

Several *IDH1* R132H conditional knock-in (KI) mouse models were recently generated to further study the role of the mutant enzyme in an *in vivo* model system. Unfortunately most mice in which IDH1 mutations were conditionally expressed either died before birth or rapidly after induction of expression of the mutant enzyme [21]. Nevertheless, expression of mutant IDH1 results in a retention of cells in an undifferentiated state or induces pre-cancerous lesions in cartilage or the SVZ [22–24]. Despite these signs of early tumor formation, no gliomas in any of the three mouse models were thus-far identified, also not when backcrossing into a *Tp53* -mutant background [23, 25].

To further study the effects of IDH1 mutations in tumor development, we have generated transgenic zebrafish that express IDH1 mutants under the control of various CNS-specific promoters.

Materials and methods

Cloning

Human *pEGFP-IDH1*wildtype and *pEGFP-IDH1*R132H constructs were described as previous [26]. *IDH1*R132C and *IDH1*G70D mutations were cloned by in-fusion PCR with two sets of primers, 5’ − CTATCATCATAAGTTGCATGGTTATTGATGGGATCAATAC−3’ and 5’ − CCATAAGCATGCAACCTATGATGTAAGTGTGTAC−3’ for *IDH1*R132C; 5’ − AGAAGCATATGTGTGACGT
CAAATGTGCCAC-3’ and 5’-GTGGCACATTTG ACGTCAACATTATGCTTCT-3’ for IDH1<sup>G70D</sup>. The whole construct was linearized and inserted into a miniTol2 vector (Addgene, plasmid #31829).

**Generation of transgenic zebrafish**

All experiments with zebrafish (Tupfel long fin or TL) were conducted according to the protocols approved by the Animal Experimentation Committee of the Erasmus Medical center and EU guidelines. To generate transgenic zebrafish lines expressing GFP-IDH1<sup>wildtype</sup> and GFP-IDH1<sup>R132H</sup> driven by different promoters (Nestin, Gata2 and Gfap), we injected various constructs into the cells of fertilized zebrafish eggs at the one-cell stage. Embryos that showed GFP expression at 1 day post fertilization (dpf) were collected and raised to adulthood (3 months, F0) and then individually crossed with non-transgenic wildtype TL (F1). GFP expression in the F1 fish indicated that the constructs were integrated into the fish genome. The GFP-positive F1s were separately raised to adults and then interbred to generate homozygous F2. Although we did not actually test for homozygosity of our transgenic lines, we inferred this by the observation that all F2 inbred offspring expressed GFP. All F2 progenies were further inbred. The experiments were performed mainly on likely homozygous F4 zebrafish. tp53 mutant fish (tp53<sup>M214K</sup>) were described by Berghmans et al and obtained from ZFIN (ZFIN.org) [27].

**Histology and Immunohistochemistry**

Zebrafish embryos were fixed overnight in 4% paraformaldehyde (PFA) at 4˚C and then embedded in paraffin for further histological analysis. Paraffin sections (6 μm) were stained with hematoxylin and eosin (HE). For immunohistochemistry (IHC), paraffin sections were dewaxed and hydrated followed by boiling in 10 mM sodium citrate for eight minutes and 2 times 3 minutes of boiling in a microwave oven. Prior to immunostaining, the endogenous peroxidase activity was blocked by 30% hydrogen peroxide and 12.5% sodiumazide in PBS for 30 minutes. The slides were washed in PBS and PBS+ which contained 0.5% g/ml protifar and 0.15% g/ml glycine and then incubated with primary antibody overnight at 4˚C. The primary antibodies used were anti-GFP (1:2000) monoclonal antibody (Roche, Woerden, the Netherlands), 5hmC (1: 200, Active Motif, La Hulpe, Belgium) and anti-human IDH1<sup>R132H</sup> (1:200) monoclonal antibody (Dianova, clone DIA H09, Huissen, the Netherlands), diluted in PBS+. The sections were then washed in PBS+ and incubated with BrightVision Poly-HRP-Anti IgG (immunologic) for 60 minutes at room temperature (RT). The slides were washed in PBS+ and PBS and then treated with 1:50 diluted DAB-substrate (DAKO Liquid DAB substrate-chromogen system) for 4–8 minutes, followed by counterstaining with haematoxylin and mounted in entellan. Histological images were captured using an Olympus BX40 camera.

**Real-time PCR**

To examine the expression of IDH1 and GFP during envelopment, total RNA was extracted by dissolving embryos in 500 μl TRIzol<sup>®</sup> (Life technologies, Carlsbad, USA) and 100 μl chloroform followed by centrifugation at 12.000 g for 15 min at 4˚C. RNA in the aqueous phase was precipitated with 250 μl isopropanol and collected at 12 000 g for 10 minutes at 4˚C. The pellet was washed twice in 250 μl 75% ethanol, centrifuged at 12 000 g for 5 minutes at 4˚C, dried and dissolved in 10 μl nuclease free water (Ambion, Thermo Scientific, Rochester, USA). For cDNA synthesis, each reaction contained 1000 ng RNA, 1 μl hexamers, 1 μl 10 mM dNTP’s and milliQ water to 13 μl and was heated to 65˚C for 5 minutes and left on ice for at least 1 minute. The RNA was then treated with 4 μl 5x Firststrand buffer, 1 μl 0.1M DTT, 0.5 μl
RNaseOUT and 0.5 μl DNase. The samples were heated to 37˚C for 40 minutes and further heated to 65˚C for 10 minutes. The RNAs were then reverse-transcribed by adding 1 μl Superscript III (Invitrogen, Breda, the Netherlands) and 0.5 μl RNase OUT followed by incubation at 25˚C for 5 minutes and 42˚C for one hour. 1 μl cDNA was used in a 15 μl reaction containing 7.5 μl Syber Select Mastermix (Life technologies), 1mM primers and MilliQ. The primer sequences are described in S1 Table. The reactions were performed in triplicate using a CFX96 Real-Time PCR System (Bio-Rad). The threshold cycle (Cq) for each reaction was obtained and the values were averaged. The standard deviation (SD) had to be below 0.2. The relative expression levels, of different time points in zebrafish life, were calculated. First the ΔCq of a sample was calculated; ΔCq = IDH1 Cq mean - β-actin Cq mean. Then one time point was set as a reference (= 1.00) and the ΔΔCq was calculated as ΔΔCq = ΔCq reference—ΔCq unknown sample. To calculate the relative expression levels the formula $2^{\Delta\Delta Cq}$ was used.

**Protein extraction and immunoblotting**

Zebrafish embryos were lysed in 500 μl HEPES-buffer containing 1x protease inhibitor (cOmplete, Thermo Scientific) and 3 μM DTT followed by homogenization by a PRO 200 homogenizer and incubated on ice for 30 minutes. 50 μg of protein was separated by loading onto a precast SDS gel (Thermo Scientific) and electrophoresis at 150 V till loading buffer reached the bottom of the gel. Protein was then transferred to a nitrocellulose membrane in transfer buffer at 100 V, 380 mA, for 1 hour. The membrane was blocked with blocking buffer containing 1% BSA in PBS for 1 hour at room temperature and incubated with primary antibody overnight at 4˚C with agitation. Primary antibodies used were anti-Collagen Type IV (1:1000, Abcam, Hilversum, The Netherlands) and 5hmC (1: 1000).

**Microinjection of wildtype zebrafish embryos with Gfap constructs**

20 μl of injection solution containing 350 ng of Gfap constructs, 30 ng/μl Tol2 transposase RNA and 0.1% pheno-red was freshly prepared before injection. 4.2 nl of injection solution was injected into the cell of 1-cell stage embryos using a Pneumatic PicoPump (PV820, WPI). For each construct, injection was performed on 100 eggs in three independent experiments. The fertilization rate was calculated based on 30 uninjected embryos collected on the same day. The number of GFP⁺, GFP⁻, healthy and abnormal embryos were counted on 1dpf.

**5hmC assay**

Total DNA was extracted using whole fish embryos. A nitrocellulose membrane was pre-soaked in 20X SSC for 1 hour. 250 ng DNA was diluted in 150μl H2O and 150μl 20X SSC. The membrane and two layers of thick filter papers were placed on a manifold (manifold II slot-blot manifold, Cole-Parmer, Wertheim, Germany) and equilibrated with 10X SSC. DNA samples were then loaded and fixed on the membrane using a vacuum pump for 5 minutes. The membrane was air-dried and processed as described above in the immunoblotting section. Blots were stained using the 5hmC antibody (1:1000) and analyzed using ECL.

**IDH1 mutant inhibitor test**

Five transgenic zebrafish embryos from each line were screened for GFP expression at 1 or 2 dpf and removed from the chorion and raised in 2 ml tap water for 48 hours with either 10μM AGI-5198 (Xcess Biosciences, Inc.) in 0.1% DMSO or 0.1% DMSO. Zebrafish embryos were collected at 3 dpf in 25 μl of HBSS buffer for 2HG measurement.
Quantification of D/L2HG in zebrafish

To quantify the level of D- and L2HG in the zebrafish, five embryos were collected at 1, 2, 3, 5 and 6 dpf in HBSS buffer (5μl per embryo). The embryos were homogenized with a PRO 200 homogenizer and lysed with sonication before LC-MS/MS. The D and L forms of 2HG were separately measured and quantified as described previously [28].

Statistics

Differences in D- and L- 2HG levels between experimental conditions were evaluated using the students t-test. A Fisher’s exact test was used to compare differences between frequencies.

Results

Generation and characterization of transgenic zebrafish lines

We firstly generated transgenic lines for two constructs, eGFP-IDH1wildtype and eGF-P-IDH1R132H, in which the transgene was expressed under control of a Nestin promoter. These constructs are referred to as Nes-IDH1wt and Nes-R132H. At least two independent lines per construct were generated to control for integration-site dependent effects.

Transgene expression was detected in the brain and spinal cord on 1, 3 and 6 days post fertilization (dpf) by fluorescent imaging (Fig 1A and S1A Fig) and by immunohistochemistry (Fig 1B) using anti-GFP antibodies. Expression of Nes-R132H was confirmed using an IDH1R132H mutant specific antibody (S2 Fig). As expected, this antibody did not show staining in the Nes-IDH1wt fish. Expression of transgenes on 1, 2, 3 and 6 dpf was also detected on the RNA level by RT-QPCR (Fig 1C). We then measured D2HG levels to monitor the activity of the neomorphic enzyme. Consistent with RNA and protein expression, the D2HG level in Nes-R132H mutant transgenic fish was elevated compared to controls (non-transgenic and Nes-IDH1wt) on 1–5 dpf (Fig 1D). The increase in D2HG was virtually identical when using macro-dissected embryos (head region) compared to whole fish (S3 Fig). L2HG levels in all the transgenic lines were similar to the non-transgenic controls (Fig 1E); indicating expression of IDH1R132H only affects D2HG levels. D2HG levels returned to normal by 21 dpf. These experiments demonstrate CNS-specific expression of IDH1wt or IDH1R132H in our transgenic zebrafish lines during development. This temporal expression pattern in the CNS is consistent with the Nestin promoter activity [29–31].

It has been reported that accumulation of D2HG results in DNA hypermethylation by inhibition of TET enzymes [32]. In our transgenic fish, DNA methylation as determined by 5hmC antibody staining was however not affected (S4 Fig). We next screened for collagen maturation defects, as these were observed in an IDH1R132H, KI mouse model [21]. However, western blot analysis failed to detect the presence of immature isoforms of collagen in our transgenic fish lines (S5 Fig). In summary, despite expression of the transgene (and the elevated levels of D2HG in lines expressing Nes-R132H), all of the zebrafish lines remained healthy without presenting any identifiable developmental abnormalities (S6 Fig).

Given the short temporal expression of IDH1 constructs driven by the Nestin promoter, we cloned constructs under the control of a brain specific Gata2 promoter. This promoter was previously used for constructing a transgenic zebrafish model for neurodegeneration [33]. Three transgenic lines were generated, pGata2-GFP-IDH1wt, pGata2-GFP-IDH1R132H and pGata2-GFP. These constructs are referred to as Gata-IDH1wt, Gata-R132H and Gata-GFP. Unfortunately, we failed to observe any transgene expression in the developing (or adult) CNS in any of the lines generated. We did however observe expression in the notochord from 1 for up to 5 dpf (S7 Fig), but, despite expression of a D2HG-producing IDH1 mutant, all fish were viable, developed normally and produced offspring. Similar to the Nes-IDH1wt and Nes-R132H fish, no
gross abnormalities or (pre-) neoplastic lesions were observed. As we failed to observe expression in the CNS we did not further investigate these lines.

Because of the temporal limitations of the Nestin promoter and the lack of expression in the CNS using the Gata2 promoter, we generated six additional lines, one for eGFP-IDH1wildtype, eGFP-IDH1R132H, eGFP-IDH1G70D and eGFP, and two for eGFP-IDH1R132C, in which the transgene was expressed under control of a Gfap promoter. Constructs used for these lines are

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Fig 1. Characterization of NesIDH1 zebrafish lines. Expression of the transgene was detected in the central nervous system (CNS) of zebrafish embryos using fluorescent imaging (A: non-transgenic wildtype zebrafish showing background auto-fluorescence staining, mainly in the yolk sac; A': NesR132H show expression of the transgene in the CNS of 3dpf embryos). Expression was confirmed by immunohistochemistry staining using an anti-GFP antibody (B) and Q-PCR (C). D2HG only accumulated in NesR132H zebrafish (D, non-transgenic vs NesIDH1wt, p = 0.754, non-transgenic vs NesR132H, p = 0.003, student’s t-test). L2HG levels in the transgenic lines showed no such increase (E). For Q-PCR experiments, we used a pool of five fish per time-point; D2HG and L2HG measurements were averages of two replicates using 5 fish per replicate. Scale bar: 200 μm.
referred to as GfapIDH1wt, GfapR132H, GfapR132C, GfapG70D and GfapGFP. The expression vector (gfp:GFP) has been demonstrated to have glial-specific expression in the zebrafish CNS, detectable during the embryonic stage [34]. The IDH1G70D mutation is an enzymatic null mutation that was included to serve as a non-D2HG-producing control [15, 35]. The IDH1R132C mutation was generated to study potential differences between IDH1R132H and IDH1R132C, and is a mutant with reportedly higher neomorphic enzymatic activity [36].

Expression of the transgenes was confirmed using fluorescent imaging on 1, 3 and 5 dpf (Fig 2A and S1B Fig). GFP was observed in the brain and spinal cord in all the Gfap transgenic zebrafish lines. CNS-specific expression of the transgene was further confirmed by immunohistochemistry using anti-GFP antibodies (Fig 2B). Expression of the transgene on the RNA level was confirmed by RT-QPCR till at least 20 dpf (Fig 2C). There were no obvious differences in results from these assays between IDH1R132H and IDH1R132C transgenic zebrafish. The D2HG levels were markedly elevated in both GfapR132C zebrafish lines (line 84 and 85) on 3 dpf, which are about 8 and 18 times higher compared to the GfapGFP and GfapIDH1wt lines (line 73 and 92, Fig 2D). D2HG levels in GfapG70D zebrafish remained similar as in GfapGFP and GfapIDH1wt lines. L2HG levels of all the IDH1 transgenic lines were similar to the GfapGFP control, confirming that expression of transgene only affects the D2HG level (Fig 2E). When crossing GfapR132C line 85, an average of 21.1% of generated embryos showed abnormal tail development on 1dpf, which was higher than in the GfapGFP (3%) and GfapIDH1wt (0%) control lines (Fig 2F and 2G). The tail defects may be explained by the fact that the first detectable expression of Gfap is at 10 h post fertilization in the developing tail bud [34]. However, no clear (pre-) cancerous lesion was observed in any of the zebrafish lines studies.

It is possible that IDH1R132H/R132C induced a pathologic phenotype due to the site of integration of our transgene. To correct for potential integration site artifacts, we directly injected fertilized zebrafish eggs at the one-cell stage with various constructs and monitored zebrafish development. We specifically monitored tail development in our transgenic fish. In three independent experiments, we injected fertilized eggs with GfapGFP, GfapIDH1wt or GfapR132C constructs. While most embryos injected with GfapGFP remained healthy (n = 105/129, S8A and S8D Fig), most embryos injected with the GfapR132C construct were not (n = 75/85, P < 0.001, Fisher’s exact test). In line with our transgenic lines, many showed an abnormal development of the tail on 1dpf. However, zebrafish embryos injected with GfapIDH1wt constructs also sometimes had abnormal tail development, though the frequency was significantly lower than that of GfapR132C (n = 13/24, P < 0.001).

**tp53 deficient Transgenic zebrafish crossed with IDH1 mutant fish**

TP53 mutations often co-occur in IDH1-mutated astrocytomas. To determine whether GfapR132C affects tumor formation, we crossed the homozygous tp53M214K mutant transgenic zebrafish with our transgenic zebrafish lines. It was previously reported that homozygous tp53 mutant zebrafish developed tumors (Schwannomas) at ~8 months post fertilization with an incidence of 28% [27]. Although we find that heterozygous tp53 mutant fish developed tumors (Table 1 and S9 Fig, incidence = 15%, n = 2/13) with an average age of onset ~1 year post fertilization, this incidence was not increased when the fish were crossed into a pGfap: GFP-IDH1R132H (or wt) background, with incidence between 6 to 14.3% regardless of the IDH1 variants or GFP controls (P > 0.3 for all comparisons, Fisher’s exact test). The non-CNS tumors we observe in our transgenic lines are most likely Schwannomas, as previously described [27]. They are mainly in the abdominal cavity, an area where we do not see expression of our transgene. Our results therefore demonstrate that expression of mutant IDH1 does not promote tumor formation in tp53 mutant zebrafish.
Fig 2. Characterization of Gfap zebrafish lines. Expression of transgene was detected using fluorescent imaging (A), immunohistochemistry with an anti-GFP antibody (B) and QPCR (C). Elevated levels of D2HG were only detected in Gfap(R132C) lines (D). L2HG levels in the transgenic A transgenic zebrafish model for mutations in IDH1.
IDH1 mutated transgenic zebrafish as an in vivo model for drug screening

AGI-5198 is a specific inhibitor for the IDH1 mutant enzymatic activity [19]. To determine whether this inhibitor also affects D2HG production in vivo, we applied it to our transgenic zebrafish lines harboring different IDH1 variants under control of the Nestin and Gfap promoters at 1dpf for 48 hours. Dose-response analysis indicates maximal inhibition at 10 μM AGI-5198 on the Nes\textsuperscript{IDH1} transgenic zebrafish (S10 Fig). The inhibitor did not show any overt toxicity, even after prolonged (2 days) treatment. The accumulated D2HG in our Nes\textsuperscript{R132H} transgenic zebrafish was decreased by 10 μM AGI-5198 to 41% of the levels prior to treatment whereas D2HG levels in the non-transgenic and Nes\textsuperscript{IDH1wt} transgenic zebrafish were not affected (Fig 3A). The D2HG levels were also markedly reduced in the Gfap\textsuperscript{R132C} zebrafish line from 34.86 reduced to 8.77 pmol (25% of the D2HG level in the untreated fish (Fig 3B)). Levels of D2HG in control transgenic lines remained low and were not affected by the inhibitor. The L2HG level in all of the treated transgenic lines was not altered (Fig 3C and 3D). These data demonstrate that our transgenic zebrafish lines can be used to screen the efficacy and toxicity of drugs that inhibit IDH1 mutant enzyme activity.

Discussion

Studying IDH1 mutations in gliomas has been hampered by the difficulty in generating appropriate model systems. For example, IDH-mutated gliomas are notoriously difficult to propagate in vitro [37] and mouse models for IDH1 mutations often have a lethal phenotype when IDH\textsubscript{1R132H} is expressed at early stages of development [21, 23, 25]. Mice can survive when the mutant protein is expressed at later stages, but often with severe phenotypes (e.g. hydrocephalus). A Drosophila model with UAS-Idh-R195H resulted in defects in wing expansion [38]. Here we report on transgenic zebrafish model systems for IDH1 mutations, and show that expression of IDH1 mutations and subsequent accumulation of D2HG does not overtly affect zebrafish development in the majority of offspring. However, tail development defects were observed in a subset of offspring in one line and also following direct injection of mutant constructs in wildtype zebrafish embryos (S8 Fig). Expression of mutant, but not wildtype IDH1 may therefore affect the cells required for normal tail development.

In contrast to the Nestin or GFAP-R132H KI mouse models, we did not observe any overt phenotype in any of the Nes\textsuperscript{R132H} transgenic zebrafish and the majority of Gfap\textsuperscript{wt} transgenic fish. In mice, the brain hemorrhage phenotype is caused by collagen maturation defects (caused by inhibition of prolyl hydroxylases by D2HG) [21]. Alternatively, brain hemorrhages may be secondary to D2HG mediated coagulation defects [39]. In our transgenic lines, we failed to detect

| # of generated fish | # of fish with tumor (over 1 year post fertilization) | Incidence of non-CNS-tumors (%) | Incidence of CNS-tumors (%) |
|--------------------|-----------------------------------------------|--------------------------------|-----------------------------|
| Gfap\textsuperscript{GFP} | 35 | 3 | 8.6 | 0 |
| Gfap\textsuperscript{R132C} | 30 | 3 | 10 | 0 |
| Gfap\textsuperscript{G70D} | 28 | 4 | 14.3 | 0 |
| Gfap\textsuperscript{wt} | 15 | 1 | 6 | 0 |
| Heterozygous Tp53 mutant | 13 | 2 | 15 | 0 |
signs of collagen maturation defects or brain hemorrhage, which may provide an explanation why our fish are able to survive into adulthood. The absence of collagen maturation defects in our fish may be related to the level of D2HG accumulation in our model system, the expression level of IDH1\textsuperscript{R132H} in our transgenic fish, and/or to the more limited temporal expression of our constructs. D2HG accumulation may also be limited as it is likely able to diffuse out of the fish into the water of the tank. This may explain that only a modest increase in the D2HG level was detected in the IDH1-mutated fish. We failed to detect changes in 5hmc levels in the IDH1-mutated embryos which may also be caused by insufficient accumulation of local D2HG within the fish. In addition, any potential effects on fish ‘fitness’ is selected against in the process of generating transgenic lines: only healthy fish (despite elevated D2HG levels) are used to generate stable lines.

We were unable to detect CNS-specific tumors in our transgenic zebrafish lines. This is in line with data from mouse models in which brain tumors were thus far not detected. This supports the notion that IDH1 mutation alone may be insufficient to promote tumor formation and other genetic alterations are required [40]. In most astrocytomas, TP53 and IDH mutations often co-occur [41]. However, the combination of IDH1 and tp53 mutations did not induce gliomas in our zebrafish model. Similarly, IDH1 mutations also did not increase tumor incidence in Tp53 mutant mouse model, despite the observation that IDH1 mutations induce pre-cancerous lesions such as proliferative subventricular nodules in one mouse model, but interestingly not in an almost identical other model [23, 25]. Similarly, mutations in IDH1/2 in combination with Tp53 deficiency were insufficient to induce gliomagenesis in RCAS/tva mouse models [40]. Since mutations in IDH and TP53 are the most common genetic alterations...
in astrocytomas, it remains unclear which additional mutations are required to induce glioma formation in our zebrafish model. Candidate genes should include oncogenic drivers ATRX and/or PDGFRA as they are present at significant frequency in lower grade gliomas. Of note, zebrafish has been appreciated as a valid model to study tumorigenesis, for example, tp53-mutant fish develop schwannomas and gliomas can also be generated in zebrafish by activating akt1 alone [42]. Our data also show that D2HG can be present at high levels throughout the development of zebrafish without any overt signs of pathology (although a minority of our transgenic fish did show defects in tail development). These data are in line with the observation that some D2HG aciduria patients, which have high levels of D2HG due to mutations in IDH2 or D2HGDH, do not have any overt phenotype. Moreover, D2HG aciduria patients do not have an increased incidence of brain tumors [43].

In conclusion, we developed various transgenic zebrafish models with CNS-specific expression of $IDH1$ mutation. We identified tail defects in a subset of IDH1-mutant fish, but we thus-far failed to identify tumors. Nevertheless, our transgenic zebrafish are a suitable model to functionally study the $IDH1$ mutation in vivo or to use as a drug screening model.

Supporting information

S1 Fig. Fluorescent imaging showed expression of transgene in the central nervous system of Nestin (A) and Gfap (B) transgenic zebrafish lines on 1, 3 and 5 dpf. White arrow head: CNS-specific GFP. Yellow arrow head: auto fluorescence in the yolk sac. (TIF)

S2 Fig. Immunohistochemistry using anti-$IDH1^{R132H}$ antibody demonstrated expression of $IDH1^{R132H}$ specific expression in Nestin zebrafish but not in $IDH1^{wt}$ transgenic fish. (TIF)

S3 Fig. D2HG measurement in Nes$^{IDH1^{wt}}$ and Nes$^{R132H}$ transgenic fish. No differences in D2HG levels between macro-dissected and whole embryos were observed. (TIF)

S4 Fig. 5hmC levels was not affected by high levels of D2HG in transgenic Nes$^{R132H}$ mutant zebrafish. A. 5hmC levels in Nes$^{IDH1^{wt}}$, Nes$^{R132H}$ and non-transgenic zebrafish embryos were measured using slot blot stained with an 5hmC antibody (quantification of bands on the right panel). Similar results were obtained in three independent experiments one of which is shown below. B. Representative images showing 5-hmC immunostaining in Nes$^{IDH1^{wt}}$, Nes$^{R132H}$ transgenic and non-transgenic zebrafish embryo slices at 3dpf. NT: non-transgenic zebrafish. (TIF)

S5 Fig. Collagen maturation was not affected in Nes$^{R132H}$ mutant zebrafish. Top half of the blot was stained for type IV Collagen, bottom half was stained for Tubulin (as loading control). Similar data were obtained in three independent experiments. H: head of zebrafish embryos. W: whole embryo. (TIF)

S6 Fig. Nes$^{R132H}$ transgenic zebrafish with CNS-accumulation of D2HG showed no gross histological abnormalities on 3dpf on H&E staining. (TIF)

S7 Fig. Gata2GFP transgenic zebrafish shows expression in the notochord of zebrafish. Yellow arrow: In Gata2IDHwt transgenic fish the transgene is expressed in non-CNS regions (yellow arrow) whereas GfapIDH1wt transgenic fish show CNS-specific expression of
transgene (white arrow). The blue arrow shows an absence of GFP signal in non-transgenic fish.

(TIF)

S8 Fig. Direct injection of fertilized zebrafish embryos with Gfap constructs showed mutant-specific tail defects. Fluorescent imaging showed CNS-specific expression of injected construct Gfap\textsubscript{GFP} (A), Gfap\textsubscript{IDH1\textsubscript{wt}} (B) and Gfap\textsubscript{R132C} (C) and the corresponding bright-field images (A'-C'). D: the percentage of GFP-positive embryos with tail defects per construct. The ratio of injected embryos with tail defect were calculated based on results of three independent experiments (~100 eggs/construct/experiment). n.s: non-significant. Scale bar: 500μm.

(TIF)

S9 Fig. An example of a schwannoma in tp53 deficient transgenic zebrafish crossed with IDH1 transgenic fish. Euthanized 1-year old fish with a distended abdominal cavity (A). Fish were fixed in paraffin blocks (B) and sectioned slides were stained with hematoxylin/eosin for histological examination (C). D and E: enlarged images of sections in C, histological feature of tumors were consistent with the schwannomas as previously demonstrated (36).

(TIF)

S10 Fig. Dose-response analysis of AGI-5198 on Nestin transgenic zebrafish. Maximal inhibition is reached at 10μM.

(TIF)

S1 Table. Primers used for the examination of IDH1 expression levels in zebrafish by QPCR.

(PDF)

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Author Contributions

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References

1. Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, et al. IDH1 and IDH2 mutations in gliomas. N Engl J Med. 2009; 360(8):765 –73. Epub 2009/02/21. 360/8/765 [pii] https://doi.org/10.1056/NEJMoa080710 PMID: 19228619; PubMed Central PMCID: PMC2820383.

2. Baiss J, Meyer J, Mueller W, Korshunov A, Hartmann C, von Deimling A. Analysis of the IDH1 codon 132 mutation in brain tumors. Acta Neuropathol. 2008; 116(6):597 –602. https://doi.org/10.1007/s00401-008-0455-2 PMID: 18985363.

3. Hartmann C, Meyer J, Baiss J, Capper D, Mueller W, Christians A, et al. Type and frequency of IDH1 and IDH2 mutations are related to astrocytic and oligodendrogial differentiation and age: a study of 1,010 diffuse gliomas. Acta Neuropathol. 2009; 118(4):469 –74. https://doi.org/10.1007/s00401-009-0561-9 PMID: 19554337.

4. Cancer Genome Atlas Research N. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med. 2013; 368(22):2059–74. Epub 2013/05/03. https://doi.org/10.1056/NEJMoa1301689 PMID: 23634996; PubMed Central PMCID: PMC3767041.

5. Meyer SC, Levine RL. Translational implications of somatic genomics in acute myeloid leukaemia. Lancet Oncol. 2014; 15(9):e382–94. Epub 2014/08/01. S1470-2045(14)70008-7 [pii] https://doi.org/10.1016/S1470-2045(14)70008-7 PMID: 25079101.

6. Amary MF, Bacsí K, Maggiani F, Damato S, Halai D, Berisha F, et al. IDH1 and IDH2 mutations are frequent events in central chondrosarcoma and central and perosteal chondromas but not in other mesenchymal tumours. J Pathol. 2011; 224(3):334 –43. Epub 2011/05/21. https://doi.org/10.1002/path.2913 PMID: 21598255.

7. Amary MF, Damato S, Halai D, Eskandarpour M, Berisha F, Bonar F, et al. Ollier disease and Maffucci syndrome are caused by somatic mosaic mutations of IDH1 and IDH2. Nat Genet. 2011; 43(12):1262–5. Epub 2011/05/21. https://doi.org/10.1038/ng.994 PMID: 22057236.

8. Pansuriya TC, van Eijk R, d’Adamo P, van Ruler MA, Kuijjer ML, Oosting J, et al. Somatic mosaic IDH1 and IDH2 mutations are associated with enchondroma and spindle cell hemangioma in Ollier disease and Maffucci syndrome. J Pathol. 2009; 174(4):1149–53. Epub 2009/02/28. S0002-9440(10)60974-1 [pii] https://doi.org/10.1002/path.2913 PMID: 19246647; PubMed Central PMCID: PMC2671348.

9. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al. An integrated genomic analysis of human glioblastoma multiforme. Science. 2008; 321(5897) :1807–12. Epub 2008/09/06. https://doi.org/10.1126/science.1164382 PMID: 18772396; PubMed Central PMCID: PMC2820389.

10. Reitman ZJ, Yan H. Isocitrate dehydrogenase 1 and 2 mutations in cancer: alterations at a crossroads of cellular metabolism. J Natl Cancer Inst. 2010; 102(13):932–41. Epub 2010/06/02. dq187 [pii] https://doi.org/10.1093/jnci/djq187 PMID: 20513808; PubMed Central PMCID: PMC2978787.

11. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Brangé D, Cavenee WK, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. Acta Neuropathol. 2016; 131(6):803–20. Epub 2016/05/10. doi: 10.1007/s00401-016-1545-1. PMID: 27157931.

12. Gabriel JL, Zervos PR, Plaut GW. Activity of purified NAD-specific isocitrate dehydrogenase at modulator and substrate concentrations approximating conditions in mitochondria. Metabolism. 1986; 35 (7):661–7. Epub 1986/07/01. 0026-0495(86)90175-7 [pii]; PMID: 3724458.

13. Koh HJ, Lee SM, Son BG, Lee SH, Ryoo ZY, Chang KT, et al. Cytosolic NADP+-dependent isocitrate dehydrogenase plays a key role in lipid metabolism. J Biol Chem. 2004; 279(38):39968–74. https://doi.org/10.1074/jbc.M402260200 PMID: 15254034.

14. Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature. 2009; 462(7274):739 –44. Epub 2009/11/26. https://doi.org/10.1038/nature08617 PMID: 19935646; PubMed Central PMCID: PMC2818760.

15. Zhu J, Zuo J, Xu Q, Wang X, Wang Z, Zhou D. Isocitrate dehydrogenase mutations may be a protective mechanism in glioma patients. Med Hypotheses. 2011; 76(4):602–3. Epub 2011/02/03. S0306-9877 (11)00012-0 [pii] https://doi.org/10.1016/j.mehy.2011.01.011 PMID: 21284999.
17. Tefferi A. Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1. Leukemia. 2010; 24(6):1128–38. doi: 10.1038/leu.2010.69. PMID: 20428194

18. Prensner JR, Chinnaiyan AM. Metabolism unhinged: IDH mutations in cancer. Nat Med. 2011; 17(3):291–3. doi: 10.1038/nm0311-291. PMID: 21383741

19. Rohle D, Popovic-Muller J, Palaskas N, Turcan S, Grommes C, Campos C, et al. An inhibitor of mutant IDH1 delays growth and promotes differentiation of glioma cells. Science. 2013; 340(6132):626–30. Epub 2013/04/06. science.1236062 [pii] https://doi.org/10.1126/science.1236062 PMID: 23558169; PubMed Central PMCID: PMC3985613.

20. Brooks E, Wu X, Hanel A, Nguyen S, Wang J, Zhang JH, et al. Identification and Characterization of Small-Molecule Inhibitors of the R132H/R132H Mutant Isocitrate Dehydrogenase 1 Homodimer and R132H/Wild-Type Heterodimer. J Biomol Screen. 2014; 19(8):1193–200. Epub 2014/07/02. 1087057114541148 [pii] https://doi.org/10.1177/1087057114541148 PMID: 24980596.

21. Sasaki M, Knobbe CB, Itsurni M, Elia AJ, Harris IS, Chio, II, et al. D-2-hydroxyglutarate produced by mutant IDH1 perturbs collagen maturation and basement membrane function. Genes Dev. 2012; 26(18):2038–49. Epub 2012/08/29. gadv.198200.112 [pii] https://doi.org/10.1101/gad.198200.112 PMID: 22925884; PubMed Central PMCID: PMC3444730.

22. Hirata M, Sasaki M, Cairns RA, Inoue S, Puviindran V, Li WY, et al. Mutant IDH is sufficient to initiate encephalomyelitis in mice. P Natl Acad Sci USA. 2015; 112(9):2829–34. doi: 10.1073/pnas.1424401112. WOS:000350224900061. PMID: 25730874

23. Bardella C, Al-Dala mh O, Krell D, Brazauska s P, Al-Qahtan i K, Tomkova M, et al. Expression of Idh1R132H in the Murine Subventricular Zone Stem Cell Niche Recapitulates Features of Early Gliomagenesis. Cancer Cell. 2016; 30(4):578–94. Epub 2016/10/04. S1535-6106(16)30402-0 [pii] https://doi.org/10.1016/j.ccell.2016.08.017 PMID: 27695047; PubMed Central PMCID: PMC5064912.

24. Sasaki M, Knobbe CB, Munger JC, Lind EF, Brenner D, Brustle A, et al. IDH1(R132H) mutation increases murine haematopoietic progenitors and alters epigenetics. Nature. 2012; 488(7413):656–9. Epub 2012/07/06. nature12132 [pii] https://doi.org/10.1038/nature12132 PMID: 22763442; PubMed Central PMCID: PMC4005896.

25. Pirozzi CJ, Carpenter AB, WaItkus MS, Wang CY, Zhu H, Hansen LJ, et al. Mutant IDH1 Disrupts the Mouse Subventricular Zone and Alters Brain Tumor Progression. Mol Cancer Res. 2017; 15(5):507–20. https://doi.org/10.1158/1541-7786.MCR-16-0485 [pii] https://doi.org/10.1158/1541-7786.MCR-16-0485 PMID: 28148827; PubMed Central PMCID: PMC5415422.

26. Bratlan LB, Kloosterhof NK, Balvers R, Sacchetti A, Lapre L, Lamfers M, et al. IDH1 R132H decreases proliferation of glioma cell lines in vitro and in vivo. Ann Neurol. 2011; 69(3):455–63. Epub 2011/03/30. https://doi.org/10.1002/ana.22390 PMID: 21446021.

27. Berghmans S, Murphey RD, Wienholds E, Neuberg D, Kutok JL, Fletcher CD, et al. tp53 mutant zebrafish develop malignant peripheral nerve sheath tumors. Proc Natl Acad Sci U S A. 2005; 102(2):507–20. Epub 2007/02/06. 1541-7786.MCR-16-0485 [pii] https://doi.org/10.1073/pnas.0406252102 [pii] https://doi.org/10.1073/pnas.0406252102 PMID: 15630097; PubMed Central PMCID: PMC444293.

28. Struys EA, Jansen EE, Verhoeven NM, Jakobs C. Measurement of urinary D- and L-2-hydroxyglutarate enantiomers by stable-isotope-dilution liquid chromatography-tandem mass spectrometry after derivatization with diacetyl-L-tartaric anhydride. Clin Chem. 2004; 50(8):1391–5. Epub 2004/05/29. https://doi.org/10.1373/clinchem.2004.033399 clinchem.2004.033399 [pii]. PMID: 15166110.

29. Lendahl U, Zimmerman LB, McKay RD. CNS stem cells express a new class of intermediate filament protein. Cell. 1990; 60(4):585–95. Epub 1990/12/23. 0022-8674(90)90662-X [pii]. PMID: 21698217.

30. About I, Laurent-Maquin D, Lendahl U, Mitsiades TA. Nestin expression in embryonic and adult human teeth under normal and pathological conditions. Am J Pathol. 2000; 157(1):287–95. Epub 2000/07/06. S0002-9440(00)04539-7 [pii] https://doi.org/10.1016/S0002-9440(00)04539-7 PMID: 10880398; PubMed Central PMCID: PMC1850197.

31. Mahler J, Driever W. Expression of the zebrafish intermediate neurofilament Nestin in the developing nervous system and in neural proliferation zones at postembryonic stages. BMC Dev Biol. 2007; 7:89. Epub 2007/07/27. 1471-213X-7-89 [pii] https://doi.org/10.1186/1471-213X-7-89 PMID: 17651502; PubMed Central PMCID: PMC1950091.

32. Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer Cell. 2010; 18(6):553–67. Epub 2010/12/07. S1535-6108(10)00483-6 [pii] https://doi.org/10.1016/j.ccr.2010.11.015 PMID: 21130701; PubMed Central PMCID: PMC4105845.

33. Tomasiwicz HG, Flaherty DB, Soria JP, Wood JG. Transgenic zebrafish model of neurodegeneration. J Neurosci Res. 2002; 70(6):734–45. Epub 2002/11/22. https://doi.org/10.1002/jnr.10451 PMID: 12444595.
34. Bernardo RL, Raymond PA. GFAP transgenic zebrafish. Gene Expression Patterns. 2006; 6(8):1007–13. https://doi.org/10.1016/j.modgep.2006.04.006 PMID: 16765104.

35. Hemerly JP, Bastos AU, Cerutti JM. Identification of several novel non-p.R132 IDH1 variants in thyroid carcinomas. Eur J Endocrinol. 2010; 163(5):747–55. Epub 2010/08/13. EJE-10-0473 [pii] https://doi.org/10.1530/EJE-10-0473 PMID: 20702649.

36. Pusch S, Schweizer L, Beck AC, Lehmler JM, Weisss S, Balss J, et al. D-2-Hydroxyglutarate producing neo-enzymatic activity inversely correlates with frequency of the type of isocitrate dehydrogenase 1 mutations found in glioma. Acta Neuropathol Commun. 2014; 2:19. Epub 2014/02/18. 2051-5960-2-19 [pii] https://doi.org/10.1186/2051-5960-2-19 PMID: 24529257; PubMed Central PMCID: PMC3937031.

37. Balvers RK, Kleijn A, Kloezeeman JJ, French PJ, Kremer A, van den Bent MJ, et al. Serum-free culture success of glial tumors is related to specific molecular profiles and expression of extracellular matrix-associated gene modules. Neuro Oncol. 2013; 15(12):1684–95. Epub 2013/09/21. not116 [pii] https://doi.org/10.1093/neuonc/not116 PMID: 24046260; PubMed Central PMCID: PMC3829587.

38. Reitman ZJ, Sinenko SA, Spana EP, Yan H. Genetic dissection of leukemia-associated IDH1 and IDH2 mutants and D-2-hydroxyglutarate in Drosophila. Blood. 2015; 125(2):336–45. Epub 2014/11/16. blood-2014-05-577940 [pii] https://doi.org/10.1182/blood-2014-05-577940 PMID: 25398939; PubMed Central PMCID: PMC4287640.

39. Unruh D, Schwarze SR, Khoury L, Thomas C, Wu M, Chen L, et al. Mutant IDH1 and thrombosis in gliomas. Acta Neuropathol. 2016; 132(6):917–30. Epub 2016/09/25. https://doi.org/10.1007/s00401-016-1620-7 PMID: 27664011; PubMed Central PMCID: PMC5640980.

40. Amankulor NM, Kim Y, Arora S, Kargl J, Szulzewsky F, Hanke M, et al. Mutant IDH1 regulates the tumor-associated immune system in gliomas. Genes Dev. 2017; 31(8):774–86. Epub 2017/05/04. gad.294991.116 [pii] https://doi.org/10.1101/gad.294991.116 PMID: 28465358; PubMed Central PMCID: PMC5435890.

41. Draaisma K, Wijnenga MM, Weening B, Gao Y, Smid M, Robe P, et al. PI3 kinase mutations and mutational load as poor prognostic markers in diffuse glioma patients. Acta Neuropathol Commun. 2015; 3:88. Epub 2015/12/25. https://doi.org/10.1186/s40478-015-0265-4 PMID: 26699864; PubMed Central PMCID: PMC4690424.

42. Jung IH, Leem GL, Jung DE, Kim MH, Kim EY, Kim SH, et al. Glioma is formed by active Akt1 alone and promoted by active Rac1 in transgenic zebrafish. Neuro Oncol. 2013; 15(3):290–304. Epub 2013/01/18. nos387 [pii] https://doi.org/10.1093/neuonc/nos387 PMID: 23325864; PubMed Central PMCID: PMC3578497.

43. Kranendijk M, Struys EA, Salomons GS, Van der Knaap MS, Jakobs C. Progress in understanding 2-hydroxyglutaric acidurias. J Inherit Metab Dis. 2012; 35(4):571–87. Epub 2012/03/07. https://doi.org/10.1007/s10545-012-9462-5 PMID: 22391998; PubMed Central PMCID: PMC3388262.