Development of a series of genetically engineered NTRK fusion-driven pediatric-type high-grade glioma mouse models

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Abstract (300 words)

Infant-type and pediatric-type high-grade gliomas are genetically distinct from their adult counterparts and frequently harbor oncogenic gene fusions involving receptor tyrosine kinase genes, including the three neurotrophic tyrosine kinase receptor (NTRK) genes. In clinic trials, these tumors show high initial response rates to tyrosine kinase inhibition, however, ultimately recur due to the accumulation of additional resistance-conferring mutations, highlighting the need to further study the biology of these tumors, their response to treatment and the process of resistance. In this study, we developed a series of genetically engineered mouse models of NTRK1/2/3 fusion-driven gliomas using the RCAS/tv-a system for somatic cell gene transfer. We find that all eight tested NTRK fusions are oncogenic by themselves when intracranially expressed in Nestin-positive cells of neonatal mice. Both the type of gene fusion as well as the age of transgene expression can impact tumor aggressiveness and tumor histomorphology. The additional loss of tumor suppressors (*Cdkn2a*, *Pten*) greatly enhances tumor aggressiveness and allowed tumor formation also in adult mice. We then employed 3D spheroid cultures of NTRK fusion-driven cells to assess the response of different NTRK fusions to four tyrosine kinase inhibitors and the impact of resistance-associated kinase mutations on treatment efficacy in vitro. Lastly, we analyzed the ability of the first generation TRK kinase inhibitors Larotrectinib and Entrectinib to extend the survival of six NTRK fusion-driven glioma models. While we observed a significantly increased survival in all six mouse models, we found that all tumors eventually recurred due to incomplete tumor clearing, further highlighting the need for additional therapeutic approaches. In summary, our results highlight NTRK fusions as strong oncogenic drivers and the likely tumor-initiating events in infant-type and pediatric-type NTRK fusion-positive gliomas. Furthermore, we show that tyrosine kinase inhibition significantly increases the survival of tumor-bearing mice but fails to fully eradicate the disease.

Introduction

Although much less common than their adult counterparts, pediatric brain tumors are the most common solid tumors in children. Adult-type diffuse gliomas frequently harbor a highly aberrant genome with gains and losses of entire chromosomal regions, leading to the dysregulation of multiple redundant mitogenic pathways and rendering targeting therapy almost impossible. By contrast, the genomes of infant-type and pediatric-type diffuse gliomas are in turn oftentimes relatively more stable and oncogenic growth signaling is frequently mediated through other types of mutations, such as point mutations in histone genes (H3K27M or H3.3G34R/V) and signal transducers (BRAF-V600E) or gene fusions involving receptor tyrosine kinases (NTRK1-3, ALK, ROS1, FGFR1-3, and MET fusions), signal transducers (KIAA1549-BRAF), or transcription factors/transcriptional co-activators (ZFTA-RELA, YAP1 fusions) (1-7).

Tropomyosin receptor kinases (TRKA-C, encoded by the NTRK1-3 genes) belong to the family of receptor tyrosine kinases (RTKs), are activated by a series of ligands (NGF, BDNF and others), and are involved in neuronal development (8). All three of these NTRK genes have multiple splice variants. The canonical TRK receptor variants consist of an extracellular ligand binding domain, a transmembrane domain, and an intracellular cytoplasmic kinase domain. Under physiological conditions, the expression and activity of these receptors is tightly regulated. Ligand binding to the receptor causes the formation of receptor dimers and subsequent autophosphorylation of several tyrosine residues in the activation loop of the kinase domain. By contrast, in NTRK fusion proteins, a 5' upstream partner that frequently induces dimerization is fused to the TRK tyrosine kinase domain, leading to its cytoplasmic localization as well as constitutive and de-regulated activation independent of ligand binding (9).

NTRK gene fusions are estimated to occur in up to 1% of all solid tumors (9, 10). Large-scale next-generation sequencing efforts have identified recurrent gene fusions involving the three NTRK genes in subsets of infant-type and pediatric-type high-grade gliomas (1, 2, 4). The prevalence of NTRK fusions in pediatric gliomas ranges from 0.4 to 40%, depending on the exact tumor type (11). In these pediatric-type tumors, the NTRK fusions are frequently the only known oncogenic drivers and potentially the tumor-initiating events. In addition to being found in pediatric gliomas, NTRK fusions are also found in adult-type gliomas, albeit at a lower frequency. However, in adult-type gliomas these fusions frequently occur along with other common aberrations (such as gain of chromosome 7 and loss of chromosome 10), suggesting that they may not be the initiators or main oncogenic driver mutations in these tumors. Recurrent NTRK fusions have also been identified in a variety of peripheral cancers (including salivary-gland tumors, soft-tissue sarcomas, infantile fibrosarcoma, as well as thyroid, colon, skin, and lung cancers), both in pediatric and adult patients (12).

Clinical trials with the first-generation TRK-specific tyrosine kinase inhibitors (TKIs) Larotrectinib (LOXO-101) and Entrectinib (RXDX-101) have reported high overall response rates in NTRK fusion-positive solid tumors, including both peripheral tumors and brain tumors (both primary and metastatic) (10, 12-15). However, these responses are transient, and tumors ultimately progress due to the presence of residual persister cells that ultimately acquire additional primary mutations in the kinase domain of the NTRK fusion or secondary mutations in downstream pathway effectors such as Ras or Raf and eventually lead to tumor recurrence. Although novel

second generation TKIs, such as Seletrectinib (LOXO-195) and Repotrectinib (TPX-0005) can overcome some of these resistances, tumors frequently also become resistant to these inhibitors (16-18). The median duration of treatment with Larotrectinib for patients with glioma was 11.1 months, with around half of the patients discontinuing treatment due to progression of disease (10). The inability of current clinical inhibitors to elicit a long-term tumor regression highlights the necessity to better understand the biology of NTRK fusions and of fusion-positive tumors, as well as the cellular responses of these tumors to tyrosine kinase inhibition *in vivo*.

Although the genomic landscapes differ between adult-type and pediatric-type tumors, understanding what pathways are dysregulated by strong oncogenic drivers (such as gene fusions) in pediatric-type gliomas can be used to understand the overall biology of cancer and adult glioma. Several unanswered questions remain about the biology and oncogenic capacities of these fusions. Are these gene fusions sufficient to induce tumors experimentally in vivo and do the different fusions vary in their oncogenic potential? Do the different NTRK fusions respond to each TKI equally and does the therapeutic response seen in culture mimic that seen in vivo?

Genetically engineered mouse models (GEMMs) of human cancers are valuable tools for preclinical drug testing and for studying the underlying oncogenic drivers and activated molecular pathways in these tumors (19, 20). GEMMs have several advantages over traditional patient-derived xenograft models, including the use of immune-competent mice as well as the ability to introduce specific driver mutations in a defined genetic background. We have previously used the RCAS/tv-a system for somatic cell gene transfer system to study the functions of common genetic drivers in glioma (such as PDGF or loss of NF1) as well as gene fusions found in meningioma or ependymoma (6, 7, 21, 22). This system has specific advantages over germline GEMM models, such as the ability to rapidly modify or mutate the oncogenic driver or introduce additional drivers or tumor suppressor losses.

In this study, we addressed these questions by modeling eight different NTRK fusion-driven mouse gliomas using the RCAS/tv-a system. We found that all eight tested NTRK fusions were able to induce tumors when expressed in mice but observed significant differences in the oncogenic potentials of different fusions. The oncogenesis was significantly enhanced when introducing additional tumor suppressor losses, most importantly *Cdkn2a* loss. We then determined the therapeutic response of NTRK fusion-driven cell line models to TKIs in vitro and subsequently performed pre-clinical in vivo trials, treating mice harboring NTRK fusion-driven gliomas with the two first-generation TKIs Larotrectinib and Entrectinib. In vivo, we observed significantly increased survival rates for all fusion tumor types, however, all tumors ultimately recurred after treatment discontinuation due to the presence of treatment-resistant persister cells. In summary, our results suggest that NTRK gene fusions are strong oncogenic drivers and the likely tumor-initiating events in infant-type and pediatric-type NTRK fusion-positive gliomas. Tyrosine kinase inhibition is able to significantly extend survival, however, ultimately failed to completely eliminate tumors, eventually resulting in tumor recurrence.

Results:

Expression of NTRK1/2/3 fusions alone is sufficient for the formation of gliomas in mice.

To determine if the expression of different *NTRK1/2/3* gene fusions is sufficient to induce the formation of glioma-like tumors in mice, we cloned the human coding sequences of nine *NTRK* fusions frequently found in human infant-type and pediatric-type high-grade gliomas into the RCAS retroviral vector, including two *NTRK1* fusions (*TPM3-NTRK1* (*TN1*), *CHTOP-NTRK1* (*CN1*)), three *NTRK2* fusions (*GKAP1-NTRK2* (*GN2*), *NACC2-NTRK2* (*NN2*), and *QKI-NTRK2* (*QN2*)), and three *NTRK3* fusions (*ETV6-NTRK3* (*ETV6-N3*), *EML4-NTRK3* (*EML4-N3*), and *BTBD1-NTRK3* (*BN3*)) (Suppl. Figure S1A-B).

We used the RCAS/tv-a system for somatic cell gene transfer in combination with *Nestin*/tv-a (N/tv-a) mice (20), to intracranially express the different *NTRK* fusion constructs in Nestin-positive stem- and progenitor cells. While *NTRK* gene fusions are enriched in infantile/pediatric patients (1, 2, 4), they are also found in adult patients (23, 24); we therefore intracranially expressed the different *NTRK* fusion constructs in mice of different ages (neonatal mice postnatal day 0-2 (p0-p2) or 1-week-old mice (p7), as well as 5-7-week-old mice).

We observed that each of the analyzed *NTRK* gene fusions was able to induce the formation of tumors from Nestin-expressing cells in the brain on their own without the loss of additional tumor suppressors (Suppl. Figure S1C). Importantly, we observed prominent differences in tumor penetrance and aggressiveness depending on the age of transgene expression (Figure 1A-D, Suppl. Figure S1C-D). Tumors induced in neonatal mice were frequently of high penetrance and generally more aggressive, whereas tumors induced in 1-week-old mice also displayed a high penetrance but were generally slow-growing and remained non-symptomatic even 120 days post-injection (study endpoint). In rare instances when tumors grew to a larger size and became symptomatic, these were usually centered around the third ventricle (Suppl. Figure S1E), suggesting that Nestin-positive cells in that area are more susceptible to oncogenic transformation by *NTRK* fusions compared to cells near the lateral ventricles at that point in development. Of note, *NTRK* gene fusions have been observed in human pediatric diffuse midline gliomas (24). By contrast, we observed that upon expression in adult *Cdkn2a* wild type mice, only the expression of *CHTOP-NTRK1* was able to induce the formation of small non-symptomatic tumors (2 out of 3 mice), whereas the other tested *NTRK* fusions failed to do so. In these adult *Cdkn2a* wild type tumors, only a minority of cells in the tumor stained positive for the TRK fusion kinase domain.

Taken together, our data suggests that expression of *NTRK* fusions alone is sufficient to induce tumor formation without the loss of additional tumor suppressor and that these fusions are the likely tumor-initiating events in *NTRK* fusion-positive high-grade gliomas. The age of expression has significant influence on the aggressiveness of the resulting tumors.

Additional loss of tumor suppressors (*Cdkn2a*, *Pten*) increases aggressiveness of *NTRK* fusion-driven gliomas.

CDKN2A/B and *PTEN* loss are frequent events in adult-type IDH-wild type glioblastoma. Albeit at a lower frequency, deletions in *CDKN2A/B* and inactivating mutations in *PTEN* have also been reported in *NTRK1/2/3* fusion-positive infant-type and pediatric-type high-grade gliomas (1, 2, 4). To assess if the loss of *Cdkn2a* affects the growth behavior of *NTRK* fusion-driven

gliomas in our system, we transferred expression of the different *NTRK* fusions into the parenchyma of N/tv-a *Cdkn2a* null mice. In addition, we intracranially co-expressed *TN1* and *CN1* in combination with a short hairpin against *Pten* (22) in N/tv-a *Cdkn2a* wild type mice to model *Pten* loss.

We observed that loss of *Cdkn2a* significantly enhanced tumor growth and increased tumor histologic grade (Figure 1E-F, Suppl. Figure S1F-H). Similar to our findings with Cdkn2a wild type tumors, we again observed that the age of transgene expression had an impact on tumor latency, with earlier expression resulting in more aggressive tumors. Mice with tumors induced at p0 frequently became symptomatic before 21 days of age, however also caused hydrocephalus formation in a large percentage of mice, while tumors induced at p7 were slightly slower growing. By contrast, tumors induced in adult mice showed a significantly longer latency compared to either p0 or p7 tumors. We observed that adult-initiated tumors – regardless of the gene fusion – frequently developed an extra-axial and extra-cranial component, in addition to the intraparenchymal tumor component.

We observed significant differences in the latency of *Cdkn2a* null tumors derived from different *NTRK* fusions, in part even if they were derived from the same *NTRK1/2/3* variant (Figure 1E, Suppl. Figure S1F-H). Tumors induced by either *TN1*, *CN1*, *GN2*, *EML4-N3*, or *BN3* showed similar latencies (17-21 days and 26-28 days median survival for p7 and adult tumors, respectively), whereas tumors induced by either *QN2* or *NN2* showed a slightly longer latency (23-24 days and 41-42 days median survival for p7 and adult tumors, respectively). By contrast, tumors induced by *ETV6-N3* displayed the longest latency (38 days and 53 days median survival for p7 and adult tumors, respectively). This is surprising, since *ETV6-N3* is the most common *NTRK* gene fusion found in infant-type gliomas (1, 2). The *ETV6-N3* fusion sequence reported from glioma samples contains a breakpoint that generates a shorter kinase domain sequence compared to the other two *NTRK3* fusions in our cohort (Suppl Figure S1A-B). A variant of *ETV6-N3* with a longer kinase domain sequence – equal to *EML4-N3* and *BN3* – has been reported in other cancers (Suppl. Figure S1A-B) (25). To determine the influence of the length of the kinase domain sequence on the oncogenicity of *EVT6-N3* we also intracranially expressed this longer *ETV6-N3* variant (*ETV6-N3*-v2) in p7 N/tv-a *Cdkn2a* null mice. We observed a non-significant trend ($p = 0.09$) towards a longer latency of tumors induced by *ETV6-N3*-v2 (55 days versus 38 days median survival) (Suppl. Figure S1I). These results suggest that the longer latency of tumors induced by *ETV6-N3* compared to tumors induced by *EML4-N3* or *BN3* is not caused by the truncated *NTRK3* kinase domain present in *ETV6-N3*.

The majority of the *NTRK* gene fusion driven tumors (*TN1*, *CN1*, *GN2*, adult *NN2*, *ETV6-N3*, pediatric *QN2*) appeared histologically as diffuse astrocytic gliomas, demonstrating an infiltrating growth pattern and were of glial origin (Figure 1F, Suppl. Figure S1H, J). In general, these gliomas had variable mitotic activity and occasionally showed palisading necrosis. The astrocytic-appearing neoplastic cells in these tumors were characterized by enlarged, angulated, and hyperchromatic nuclei present on a fibrillary background. A couple of the *NTRK* fusion tumors (*EML4-N3*, pediatric *NN2*) appeared to have a more compact arrangement with the cells appearing more epithelioid, characterized by round cells with more defined cell borders, abundant eosinophilic cytoplasm, and variably enlarged and round nuclei with prominent nucleoli. One of the *NTRK* gene fusion tumors (adult *QN2*) had a predominantly

spindled cell component, that was almost sarcomatous-like, and the cells had a compact fascicular arrangement.

By contrast, loss of *Pten* expression had a more subtle impact on tumor growth compared to Cdkn2a loss (Figure 1G-H, Suppl. Figure S1K). There was no significant difference in the survival of *TN1* or *CN1* tumors upon loss of *Pten* expression compared to *Pten* wild type tumors, however, we observed that a subset of tumors originating from the lateral ventricles were of larger size and became symptomatic. Histologically, these tumors often demonstrated oligodendroglial-like perinuclear clearing, which differed from that of *Pten* wild type or *Cdkn2a* null tumors (26).

As described above, adult-initiated tumors frequently consisted of an intra-parenchymal and a connected extra-parenchymal component. The extra-parenchymal component frequently grew as an extra-axial tumor and also grew out through the needle tract as extra-cranial tumor tissue. Similar to the intra-parenchymal tumor component, tumor cells in the extra-parenchymal tumors were also Olig2-positive, indicating a glial tumor-lineage (Suppl. Figure S1L). We performed magnetic resonance imaging (MRI) (T1-weighted and T2-weighted, pre- and post-contrast) on mice harboring intracranial *TPM3-NTRK1* tumors that exhibited extra-cranial tumor growth. We observed that the extra-cranial and to a lesser degree also the extra-axial tumor components were contrast enhancing, whereas the intraparenchymal tumor component was not, suggesting that the extra-cranial and extra-axial tumor components are more permeable to the contrast agent and therefore potentially also for small molecule inhibitors (Suppl. Figure S1M).

Since *NTRK* gene fusions are also found in non-CNS cancers (including lung, liver, and colon cancers), we also expressed these gene fusions in the flank and abdominal cavity of N/tv-a *Cdkn2a* null mice and observed the formation of highly aggressive sarcomatous-like spindle cell neoplasms that appear to arise from the soft tissue and encase nearby organs such as colon and kidney (Suppl. Figure S1N-O).

Taken together, these results suggest that *NTRK* gene fusions are strong oncogenic drivers and that both the N-terminal fusion partner as well as the age of expression can influence tumor aggressiveness and latency. Additional loss of tumor suppressors substantially increases tumor aggressiveness.

Modeling of *NTRK* fusion-driven mouse gliomas harboring resistance-associated kinase domain point mutant variants.

Targeted sequencing of recurrent patient tumors that have developed resistance to first-generation TKIs (such as Larotrectinib or Entrectinib) has recurrently identified the presence of point mutations in the kinase domains of *NTRK* gene fusion, suggesting that these mutations may constitute an early escape mechanism of *NTRK* fusion-positive tumor cells to bypass TKIs (12). The most common mutation hotspots include the gatekeeper residue (F589L in NTRK1), the solvent front residue (G595R in NTRK1), and xDFG mutations in the activation loop (G667C in NTRK1). The gatekeeper and the solvent front mutations are also frequently observed in combination (9, 12).

In order to generate models that mimic treatment-experienced tumors, we intracranially expressed the different *TPM3-NTRK1* kinase point mutant variants in one-week-old N/tv-a *Cdkn2a* null and wild type mice (Suppl. Figure S1Q-T). Using this approach, we are able to generate genetically defined gliomas to analyze the efficacy of different first- and second-generation TKIs against different point mutant variants in vivo, without relying on the spontaneous occurrence of these additional mutations. We observed that similar to unmutated TPM3-NTRK1, all four mutant variants were able to rapidly induce tumor formation in a *Cdkn2a* null mice (Suppl. Figure S1R-S). The F589L, G595R, and F589L-G595R mutant variants displayed a latency similar to unmutated TPM3-NTRK1, whereas the G667C mutant version displayed a significantly longer latency (27 days versus 21 days, $p = 0.0048$).

We then tested the ability of the different TPM3-NTRK1 mutants to induce tumors in N/tv-a *Cdkn2a* wild type mice and observed that the F589L, G595R, and G667C mutant variants displayed a similar oncogenic potential compared to unmutated TPM3-NTRK1. We observed a high tumor penetrance, but tumors generally remained small and non-symptomatic even 120 days post-injection (Suppl. Figure S1P-Q), apart from a small number of symptomatic tumors originating from the third ventricle. By contrast, we observed that tumors induced by the expression of TPM3-NTRK1-F589L-G595R were significantly faster growing compared to tumors induced by unmutated TPM3-NTRK1 ($p = 0.026$), although the majority of tumors still remained non-symptomatic 120 days after induction. In addition, we observed the formation of large symptomatic tumors originating from the lateral ventricles in 2 out of 14 mice injected with TPM3-NTRK1-F589L-G595R (Suppl. Figure S1T), something not observed upon expression of any of the other constructs.

Taken together, our data suggests that by expressing *NTRK* fusion variants that harbor resistance-associated kinase point mutations, we can induce the formation of gliomas that mimic treatment-experienced tumors. Some of these kinase point mutations seem to influence tumor aggressiveness.

***NTRK* fusion-induced mouse gliomas show activation of the PI3K-AKT-S6 and the RAF-MEK-ERK pathways.**

In order to characterize these mouse gliomas in more detail, we performed immunohistochemistry (IHC) stainings for the TRK kinase domain, the glial markers Olig2 and GFAP, as well as the proliferative marker Ki67 (Figure 2, Suppl. Figure S2A-H). Tumors derived from the *TN1*, *CN1*, *GN2*, *NN2*, or *ETV6-N3* fusions were uniformly positive for the glial marker Olig2 regardless of the age of injection, whereas tumor cells frequently also stained positive for GFAP in pediatric tumors, but not in adult tumors. By contrast, tumors derived from *QN2*, *EML4-N3*, or *BN3* fusions predominantly stained negative for GFAP (both pediatric and adult), and only a subset of tumor cells stained positive for Olig2. All tumors showed a high abundance of Ki67-positive cells.

Two of the main mitogenic downstream pathways activated by receptor tyrosine kinases, including NTRK receptors, are the PI3K-AKT-S6 and RAF-MEK-ERK pathways. To analyze if these pathways are also activated in the different NTRK fusion-driven mouse gliomas, we

performed IHC stainings for phospho-ERK, phospho-AKT, and phospho-S6 and observed robust staining for all three epitopes in the different tumors, suggesting that both the PI3K-AKT-S6 and the RAF-MEK-ERK pathways are activated in these tumors (Figure 2, Suppl. Figure S2A-H).

Taken together, our results indicate that both the PI3K-AKT-S6 and the RAF-MEK-ERK pathways are activated in *NTRK* fusion-driven mouse gliomas.

Development of *NTRK* fusion cell line-based models to test the efficacy of various TKI against resistant-associated kinase point mutations in vitro.

In order to assess the efficacy of TKI treatment to inhibit the growth of *NTRK* fusion-driven cells in vitro, we established several *NTRK* fusion-driven cell line models, using both in vitro-transduced NIH-3T3-tv-a cells and primary mouse cell lines established from primary RCAS *NTRK* fusion-driven tumors. To this end, we transduced NIH-3T3-tv-a cells with RCAS viruses encoding for either GFP, TN1, GN2, or ETV6-N3 and subsequently performed 3D spheroid growth assays (Figure 3A, Suppl. Figure S3A-B). We and others have previously used this assay to test the oncogenic potential of different drivers and could show that in the absence of a strong oncogenic driver NIH-3T3 cells are unable to grow in 3D spheroid conditions, whereas oncogenic signaling can induce the growth of these cells under these conditions (6, 7, 27). While GFP-expressing cells were unable to overcome contact inhibition and did not grow in ultra-low attachment conditions as spheroids, spheroids derived from *NTRK* gene fusion-expressing cells continued to grow in size over several days (Figure 3B, Suppl Figure S3C). We then tested the efficacy of four different TKIs – Larotrectinib, Entrectinib, as well as the two second-generation inhibitors Repotrectinib (TPX-0005) and Selitrectinib (LOXO-195) – to inhibit the growth of these spheroids (Figure 3C-E, Suppl. Figure S3C-E). Treatment with any of the four TKIs did not influence the growth of GFP-expressing spheroids compared to DMSO. By contrast, the growth of *NTRK* fusion-driven spheroids was inhibited by the different TKIs in a dose-dependent manner, suggesting that in vitro all four TKIs were equally able to inhibit the oncogenic functions of the different *NTRK* fusions. We observed that Entrectinib treatment was more effective in *TN1*-expressing cells, compared to cells expressing *GN2* or *ETV6-N3*, but did otherwise not observe a difference in treatment efficacy of the different TKIs between the different *NTRK* fusions (Suppl. Figure S3E).

As mentioned earlier, resistance to therapy against first-generation TKIs frequently occurs due to the accumulation of *NTRK* kinase domain point mutations. To analyze how the different point mutations influence treatment response in vitro, we transduced NIH-3T3-tv-a cells with RCAS viruses encoding the different TPM3-NTRK1 mutants (F589L, G595R, G667C, F589L-G595R) and tested the efficacy of the different TKIs to inhibit the growth of these spheroids (Figure 3F-M. Suppl. Figure S3B,F-J). We observed that cells expressing the F589L gatekeeper residue mutation were resistant to Larotrectinib, but still susceptible to Entrectinib, Repotrectinib, and Selitrectinib (Figure 3F, Suppl. Figure S3J). Cells expressing the G595R solvent front mutation were resistant to both Larotrectinib and Entrectinib (Figure 3J, Suppl. Figure S3J). By contrast, cells expressing either the G667C xDFG mutation, or a combination of the F589L and G595R

mutations were also completely resistant to Selitrectinib and also showed a decreased sensitivity to Repotrectinib (Suppl. Figure S3F-G, J). Western Blots of TKI-treated cells showed a decrease in p-TRK, p-ERK, and p-AKT levels that reflected the susceptibilities of the different resistance point mutations to the different TKIs (Figure 3E,I,M; Suppl Figure S3F-G).

We then established cell lines from primary mouse flank tumors induced by the injection of RCAS viruses encoding either TPM3-NTRK1-F589L, TPM3-NTRK1-G595R, or TPM3-NTRK1-F589L-G595R into N/tv-a *Cdkn2a* null mice and again performed 3D spheroid assays with these cells. We again observed that TKI treatment was able to inhibit the growth of these spheroids in a resistance mutation-dependent manner that mirrored our findings from the NIH-3T3-tv-a cell line models (Figure 3G,K; Suppl. Figure S3H-I).

Taken together, *NTRK* fusion driven cell line models can predict the response of different kinase point mutants to first and second-generation TKIs.

In vivo TKI treatment leads to the regression of *NTRK* gene fusion-driven mouse gliomas.

Based on our in vitro results with *NTRK* fusion-driven cells, we then wanted to evaluate if TKIs can induce the regression of intracranial *NTRK* fusion-driven gliomas in vivo. We expressed an HA-tagged version of *EML4-N3* in N/tv-a *Cdkn2a* null mice and treated them with either Vehicle (two mice) or Entrectinib (60 mg/kg, two mice) twice-a-day for 24 hours (three injections in total). Mice were euthanized one hour after the third treatment, brains were formalin-fixed and subsequently analyzed by H&E and IHC.

We observed that Entrectinib treatment was able to induce the regression of the *EML4-N3*-driven mouse tumors after just 24 hours of treatment (Figure 4A-C). Entrectinib-treated tumors appeared smaller than Vehicle-treated tumors and displayed decreased levels of the proliferation marker phospho-Histone H3 and increased levels of apoptosis (Cleaved Caspase-3) (Figure A-B). Of note, Entrectinib treatment led to the decrease of total HA staining in a majority of tumor cells, suggesting that TKI treatment can induce the degradation of total NTRK fusion protein levels (Figure 4A). We observed a similar degradation of total TRK protein upon TKI treatment in vitro in both NIH-3T3 and primary mouse tumor cells (Figure 3E,I; Suppl. Figure S3D,I). Entrectinib-treated tumors also displayed a decrease in Olig2 staining in tumor cells. In addition, we observed a prominent reduction in both p-ERK and p-S6 staining in Entrectinib-treated tumors. However, while a large percentage of tumor cells showed a decrease in these markers (HA, Olig2, p-ERK, p-S6), a subset of cells did not, suggesting that they may be less affected by TKI treatment.

Taken together, our results suggest that TKI treatment can induce the regression of *NTRK* fusion-driven mouse gliomas in vivo within hours of treatment start.

TKI treatment significantly prolongs the survival of *NTRK* gene fusion-driven mouse gliomas, but tumors ultimately recur after treatment discontinuation.

Encouraged by these results, we then wanted to assess if the two first-generation TKIs Larotrectinib and Entrectinib can prolong the survival of *NTRK* fusion-driven mouse gliomas in vivo and if there is a difference in the response between tumors induced by different *NTRK* fusions. To this end, we selected six *NTRK* fusions and intracranially induced gliomas by RCAS viral injection into either neonatal (*ETV6-N3*, *EML4-N3*) or adult (*TN1*, *CN1*, *GN2*, *NN2*) N/tv-a *Cdkn2a* null mice and performed preclinical in vivo trials. To assess the initial tumor size before treatment and ensure a distribution of similarly sized tumors into each treatment group, a first T2w MRI was performed 15-25 days post tumor induction (depending on the *NTRK* fusion, see Suppl. Methods) and mice were then treated with either Vehicle (DMSO), Larotrectinib, or Entrectinib for 14 days. The day after the last treatment, the mice received a second T2w MRI and were subsequently observed for the onset of tumor-related symptoms. No MRIs were performed for *EML4-N3* mice, since these were too young at treatment start due to the aggressive growth of these tumors. We selected a dosing strategy of 100 mg/kg Larotrectinib and 30 mg/kg Entrectinib for these trials (for dosing and toxicity experiments see Suppl. Methods).

Treatment with either Larotrectinib or Entrectinib significantly extended survival compared to Vehicle treatment, however, treated tumors ultimately recurred upon treatment discontinuation in all mice due to the presence of residual disease and treatment-resistant persister cells (Figure 5A, Suppl. Figure S4A). The extent to which survival was prolonged differed between the different *NTRK* fusions and the two TKIs (Suppl. Figure S4B). The largest increase in survival was observed for *EML4-N3*-driven tumors treated with Larotrectinib (36 days versus 16 days (Vehicle) median survival, $p < 0.0001$) and *ETV6-N3*-driven tumors (57 days (Larotrectinib) and 53 days (Entrectinib) versus 41.5 days (Vehicle) median survival, $p < 0.0001$ and $p = 0.0001$ respectively). We observed that for some fusion-driven tumor types, a specific TKI was more effective. In *CN1*-driven tumors Entrectinib treatment led to a significant survival benefit over Larotrectinib treatment (48 versus 41 days median survival, respectively, $p = 0.0003$), whereas in *NN2* (56 versus 50 days median survival, respectively, $p = 0.001$), *ETV6-N3* (57 versus 53 days median survival, respectively, $p = 0.05$), and *EML4-N3*-driven tumors (36 versus 19 days median survival, respectively, $p < 0.0001$) Larotrectinib treatment led to a significantly better outcome compared to Entrectinib treatment. We did not observe a significant difference between both TKIs at the tested concentrations for *TN1* and *GN2* fusion-driven tumors.

We calculated the fold-change increase in tumor size based on pre- and post-treatment MRIs and observed that intra-parenchymal tumors were less susceptible to TKI treatment than extra-parenchymal tumors (Figure 5B). Extra-parenchymal (extra-axial and extracranial) tumors were almost uniformly present in Vehicle-treated mice, but frequently absent in TKI-treated mice (Suppl. Figure S4C-D). We observed that intracranial tumors in Larotrectinib and Entrectinib-treated mice were significantly smaller compared to Vehicle-treated mice for each of the *NTRK1/2/3* fusion-driven glioma models at the end of treatment (Figure 5B-C). However, we found that adult intracranial tumors (*TN1*, *CN1*, *GN2*, *NN2*) continued to grow while on treatment with Larotrectinib and Entrectinib and were between 3 to 6-fold larger compared to before treatment, albeit at a lower rate compared to Vehicle-treated tumors (10 to 22-fold larger compared to before treatment, depending on the tumor type). By contrast, pediatric *ETV6-N3* tumors remained stable or even shrank during TKI treatment; Larotrectinib and Entrectinib

treatment resulted in a regression or stagnation of intra-parenchymal tumor growth (66% and 100% average tumor volume, respectively, relative to before treatment start), whereas Vehicle-treated tumors significantly grew (651% average tumor volume relative to the first MRI) (Figure 5D, Suppl. Figure S4E). Interestingly, the differences in survival between Larotrectinib and Entrectinib (such as for *CHTOP-NTRK1*) were not reflected by significantly different tumor sizes as measured by MRI.

As described above, we observed that similar to human *NTRK* fusion-positive tumors, TKI treatment led to tumor regression/stable disease or at least slow tumor growth in our mouse models, but was unable to completely eradicate tumors, ultimately resulting in tumor recurrence once treatment was discontinued. Therefore, we sought to assess if an increased TKI dose would be sufficient to completely ablate all tumor cells in vivo. To this end, we treated *TN1*, *CN1*, and *GN2* tumor mice with 60 mg/kg Entrectinib b.i.d. (n = 2 per group) or Vehicle for 7 days. We had previously used a concentration of 30 mg/kg b.i.d. for the 14-day treatment since higher Entrectinib doses resulted in increased toxicity over longer treatment periods. We found that while tumors in Entrectinib-treated mice were significantly smaller compared to Vehicle-treated tumors, we still observed the presence of treatment-resistance persister cells even at this increased dose, suggesting that TKI treatment alone is unable to completely eradicate these tumors in vivo (Figure 5E, Suppl. Figure S4F).

Taken together, our results show that treatment with the two first generation TKIs Larotrectinib and Entrectinib can significantly prolong the survival of *NTRK* fusion-driven mouse gliomas in vivo. Tumors derived from different *NTRK* fusions do not respond equally to the two TKIs, e.g. at the tested doses Entrectinib was superior to Larotrectinib in *CHTOP-NTRK1*-driven tumors, whereas Larotrectinib was superior to Entrectinib in *NACC2-NTRK2*, *ETV6-NTRK3*, and *EML4-NTRK3*-driven tumors. However, ultimately all tumors recurred upon treatment discontinuation due to the presence of persister cells.

Discussion

Most adult IDH-wild type glioblastomas harbor a highly aberrant genome, including gains and losses of whole chromosomal arms as well as additional activating or inactivating point mutations, leading to multiple concurrent and redundant drivers that make targeting of specific pathways difficult. By contrast, infant-type and pediatric-type high-grade gliomas are enriched for gene fusions involving receptor tyrosine kinase genes (such as *NTRK1-3*, *ALK*, and *ROS1*) and harbor an otherwise relatively stable genome, making them ideal candidates for targeted therapy with tyrosine kinase inhibitors, such as Larotrectinib and Entrectinib for NTRK fusion-positive tumors. These inhibitors show high initial response rates, frequently leading to either tumor regression or stable disease; however, surviving therapy-resistant persister cells eventually accumulate additional mutations, allowing them to regrow the tumor even in the presence of the kinase inhibitors (10, 28). Resistance-associated mutations can be either on-target in the kinase domain of the oncogenic fusions, thereby directly impeding the binding of the kinase inhibitors, or off-target (such as MET, FGFR, RAF, or RAS), leading to the activation of oncogenic pathways independently of the oncogenic fusion protein, rendering the tumor cells

less reliant on the oncogenic signaling induced by the fusion (9, 18, 29). Second-generation kinases inhibitors can in part circumvent the on-target mutations, but tumors ultimately develop additional off-target mutations that also render these inhibitors ineffective (18). In addition, the blood-brain barrier limits drug availability in the brain and brain tumors, further impeding the efficacy of these inhibitors.

Current pre-clinical trials frequently utilize patient-derived xenograft transplantation models, that however have several drawbacks, such as that they can only be used in immune-compromised mice. Using the RCAS/tv-a system (20), we have developed a series of novel genetically engineered mouse models of infant-type and pediatric-type *NTRK* fusion gene-driven high-grade gliomas. These GEMMs have several advantages over conventional xenograft or syngraft/allograft models and even over germline GEMMs. Our RCAS/tv-a tumor models are caused by somatic cell gene transfer of defined oncogenes (such as *NTRK* gene fusions) into *Nestin*-expressing neural stem and progenitor cells. Since these tumors arise de novo from within the mouse's own cells, there is no potential graft versus host effect that might occur in allograft models, and in contrast to xenograft transplantation models these tumors can be induced in mice that harbor a completely intact immune system. An advantage over germline GEMMs is the flexibility of this system, that allows us to rapidly introduce additional mutations into the system to model for example treatment-experienced tumors, such as resistance-associated point mutations in the fusion protein kinase domain and test different *NTRK* fusion types. These models will help us to better understand the biology of treatment response and resistance to therapy of *NTRK* fusion-driven infant-type and pediatric-type gliomas in a genetically-defined system with tumors that are caused and driven by the actual *NTRK* gene fusion, and that therefore directly rely on its activity.

There is a plethora of different *NTRK* fusions and clinical trials (either with *NTRK* fusion-positive brain or peripheral tumors) show that not all tumors respond to TKI treatment equally, sometimes even if they harbor the same gene fusion (10, 12, 13). This might be in part due to the presence of additional oncogenic driver mutations or the presence of kinase domain point mutations, but it is also unclear if different *NTRK* fusions (and the tumors that harbor these fusions) are equally susceptible to TKI treatment. All of these individual fusions are rare in humans, making trials comparing drugs against tumors with specific fusions impractical. Therefore, mouse models with a defined genetic background of tumors driven by specific gene fusions and their resistant mutations are needed.

Using the RCAS system we determined that gene fusions to all of three NTRK genes tested were highly oncogenic in the brain, and in the absence of *Cdkn2a* expression resulted in uniform tumors that are ideal model systems for pre-clinical in vivo trials. Our data suggests that NTRK gene fusions indeed could be the initiating events in humans and is consistent with the relative lack of additional genetic rearrangement found in these tumors relative to adult gliomas. We also found that tumors were more aggressive when induced in younger mice (e.g. shortly after birth) compared to older mice, which may give an explanation why these gene fusions are enriched in tumors in infants and younger children but generally co-occur with additional hallmark aberrations (such as gain of chr 7 and loss of chr 10) in adult-type gliomas.

In addition, we observed that tumors induced by different NTRK fusions could vary significantly in aggressiveness. Tumors induced by *EML4-NTRK3*, *BTBD1-NTRK3*, *CHTOP-NTRK1*, *TPM3-NTRK1*, and *GKAP1-NTRK2* were generally the most aggressive, whereas tumors induced by *ETV6-NTRK3* displayed the longest latency. This is especially interesting, since 1) *ETV6-NTRK3*, *EML4-NTRK3* and *BTBD1-NTRK3* all harbor the same NTRK3 kinase domain, and 2) *ETV6-NTRK3* is one of the most common *NTRK* fusions in infant-type gliomas and other peripheral tumors (1, 12). It remains unclear why *ETV6-NTRK3* is less oncogenic compared to the other fusions. Since the predominant *ETV6-NTRK3* variant retains a shorter portion of the NTRK3 kinase domain compared to *BTBD1-NTRK3* and *EML4-NTRK3*, we also tested an *ETV6-NTRK3* fusion variant retaining a longer NTRK3 kinase domain but observed an even further reduction in oncogenicity. This suggests that the N-terminal fusion partner can influence the oncogenicity of the *NTRK* fusion, potentially through varying abilities to induce fusion dimerization necessary for autophosphorylation and activation (30).

All tested *NTRK* fusion-driven mouse gliomas responded to treatment with the two first-generation TKIs Larotrectinib and Entrectinib. We observed treatment responses within the first 24 hours of treatment, accompanied by the down-regulation of oncogenic PI3K-AKT-S6 and RAF-MEK-ERK signaling pathways in a majority of cells. However, similar to *NTRK* fusion-positive tumors in patients, we observed that tumor clearing was incomplete and surviving persister cells led to the regrowth of tumors after treatment discontinuation. The adult-induced tumors even continued to grow while on treatment, albeit at a slower rate than Vehicle-treated tumors. Limited drug penetration of the two first-generation inhibitors into the brain likely contributed to the incomplete responses of intra-parenchymal brain tumors, since extra-cranial and extra-axial tumors showed a more complete response, although these tumors also regrew in some cases, suggesting that there were persister cells in the extra-cranial/-axial tumors as well. Future characterization of these treatment-resistant persister cells will be necessary and important in order to develop effective combination therapies to improve the efficacy of TKIs.

Interestingly, we observed reproducible differences in the therapeutic responses to TKIs between the various *NTRK* gene fusion-driven tumors at the tested inhibitor concentrations. *CHTOP-NTRK1*-driven tumors responded significantly better to Entrectinib over Larotrectinib treatment, while in *NACC2-NTRK2*, *ETV6-NTRK3*, and *EML4-NTRK3*-driven tumors Larotrectinib treatment led to a significantly longer survival compared to treatment with Entrectinib. It is unclear whether this effect is related to fusion or tumor biology. In our in vitro systems we observed that Entrectinib was more potent against *TPM3-NTRK1*-expressing cells compared to *GKAP1-NTRK2* and *ETV6-NTRK3*-expressing cells. In general, Larotrectinib (100 mg/kg) was better tolerated than Entrectinib (30 mg/kg) by tumor-bearing mice, limiting the active concentration that could be administered.

In order to mimic on-target resistance-associated mutations, we used our system to directly introduce kinase domain point mutations into the sequence of *TPM3-NTRK1*, resulting in tumor cells that were resistant to first-generation TRK inhibitors in vitro. Most of these mutants demonstrated a similar oncogenic capacity compared to unmutated TPM3-NTRK1, however we observed that tumors generated by TPM3-NTRK1-F589L-G595R were significantly more aggressive. It will need to be assessed if this is also the case in human tumors carrying this mutations.

In summary, this study highlights the utility of genetically engineered mouse models of specific oncogenic drivers to optimize therapy of tumors driven by specific molecular mechanisms where clinical trials are not possible because of their rarity. These models will be valuable tools for the study of treatment response and the development of resistance to therapy in these tumors.

Materials & Methods

Generation of RCAS mouse tumors

All animal experiments were done in accordance with protocols approved by the Institutional Animal Care and Use Committees of Fred Hutchinson Cancer Center (protocol no. 50842) and followed National Institutes of Health guidelines for animal welfare. The RCAS/tv-a system used in this work has been described previously (7). *Nestin* (N)/tv-a;*Cdkn2a*-wild type and null mice were used for RCAS-mediated brain tumor formation in this study and have been described previously (7). DF1 cells (1×10^5) in a volume of 1 µL were injected near the ventricles into newborn (p0-p2) or one-week-old (p7) pup brains or into the striatum of 5-7-week-old adult mice as described previously (7, 31). For peripheral injections, DF1 cells (1×10^5) in a volume of 1-2 µL were injected intraperitoneally (i.p.). Mice were monitored until they developed symptoms of disease, such as visible tumors, lethargy, poor grooming, weight loss, dehydration, macrocephaly, seizures, jumping, or paralysis, or until a predetermined study end point.

In vivo TKI treatment

For in vivo treatment, inhibitors were resuspended in DMSO and diluted to a final buffer solution of 10% DMSO, 10% EtOH, 30% Solutol (Sigma Aldrich 42966), and 50% Tartaric acid buffer. Tartaric acid buffer stock was prepared by mixing 10 ml of 0.15 M DL-tartaric acid (Sigma Aldrich PHR1472) with 50 ml of 0.15 M tartrate sodium (Sigma Aldrich PHR1409). 10 ml of the Tartaric acid buffer stock were subsequently mixed with 40 ml of Saline Solution (0.9%) to create the final Tartaric acid buffer. Mice were treated with either Vehicle (10% DMSO), Larotrectinib (100 mg/kg), or Entrectinib (30 or 60 mg/kg) at 10 ul per gram i.p. injection twice-a-day (b.i.d.) for 14 days.

See also supplemental experimental procedures.

Author Contributions: Conceptualization SS, ZRR, DKK, REP, ECH, FS. Performed experiments SS, ZRR, ASY, MW, JW, DR, JZY, FS. Data analysis SS, ZRR, ASY, AGP, DCQ, BNG, NA, PJC, FS. Original manuscript writing SS, PJC, ECH, FS. Review and editing ECH, FS. Funding acquisition ECH. Supervision REP, ECH, FS. All authors read, reviewed, and approved the manuscript.

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Data and materials availability: The data that support the findings of this study are included with the manuscript and supplemental data files and are also available from the corresponding author upon reasonable request.

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Figure legends:

Figure 1: Expression of *NTRK* gene fusions in Nestin-positive cells induces the formation of experimental mouse gliomas. A) Aggressiveness of *NTRK* fusion-driven mouse gliomas is influenced by both the mouse age at the time of tumor induction (transgene expression) and the loss of additional tumor suppressor genes (such as *Cdkn2a* or *Pten*). B) Schematic depicting the locations of DF1/RCAS injection and tumor formation for neonatal and adult mice. C) Kaplan-Meier curve showing the symptom-free survival of Nestin/tv-a *Cdkn2a* wild type mice upon intracranial expression of the different *NTRK* gene fusion. D) Representative stainings (H&E and IHC for the TRK kinase domain) of mouse gliomas induced by the expression of *CHTOP-NTRK1* in N/tv-a *Cdkn2a* wild type mice of different ages (Newborn pups, one-week-old pups, adult mice). Tumor aggressiveness decreases with increased age of tumor induction. Additional stainings of gliomas from other *NTRK* fusions can be found in Suppl. Figure S1D. E) Kaplan-Meier curve showing the symptom-free survival of Nestin/tv-a *Cdkn2a* null mice upon intracranial expression of the different *NTRK* gene fusion. F) Representative stainings (H&E and IHC for the TRK kinase domain) of mouse gliomas induced by the expression of *CHTOP-NTRK1* in N/tv-a *Cdkn2a* null mice of different ages (Newborn pups, one-week-old pups, adult mice). Additional stainings of gliomas from other *NTRK* fusions can be found in Suppl. Figure S1H. G) Kaplan-Meier curve showing the symptom-free survival of *Nestin*/tv-a *Cdkn2a* wild type mice upon intracranial expression of *TPM3-NTRK1* and *CHTOP-NTRK1* gene fusions with and without additional *Pten* knockdown. H) Representative stainings (H&E and IHC for the TRK kinase domain) of a mouse glioma induced by the expression of *CHTOP-NTRK1* in N/tv-a *Cdkn2a* wild type mice with additional knockdown of *Pten*. Scale bar indicates 100 µm. Black dotted line indicates tumor boundaries (B,F,H).

Figure 2: *NTRK* fusion-driven experimental mouse gliomas show activation of the PI3K-AKT-S6 and RAF-MEK-ERK pathways. Representative IHC stainings of a *TPM3-NTRK1*-driven mouse glioma for TRK (TRK kinase domain), Olig2, GFAP, Ki-67, phospho-ERK1/2 (Thr202/Thr185), phospho-AKT (Ser473), and phospho-S6 (Ser240/244). Scale bar indicates 100 µm.

Figure 3: *NTRK* fusion-driven cells respond to TKI treatment in vitro. A) Schematic overview of the workflow of the 3D spheroid assay. B) Growth curve of a 3D spheroid assay with either untransduced NIH-3T3-tv-a cells (blue line) or cells expressing *TPM3-NTRK1* (red line). C) Growth curve of TPM3-NTRK1-expressing NIH-3T3-tv-a cell spheroids treated with either DMSO (0.1%) or TKIs (Larotrectinib, Entrectinib, Selitrectinib, Repotrectinib; 100 nM each). D) Dose response curve of TPM3-NTRK1-expressing NIH-3T3-tv-a cell spheroids treated with different TKI concentrations (1, 10, 100, 200, 500 nM, 1 µM, 1.5 µM, or 2 µM) relative to DMSO-treated cells. E) Representative Western Blot of TPM3-NTRK1-expressing NIH-3T3-tv-a

cells treated with 100 nM of indicated TKIs. F-G) Growth curve of TPM3-NTRK1-F589L-expressing NIH-3T3-tv-a (F) or primary mouse tumor (G) cell spheroids treated with either DMSO (0.1%) or TKIs (Larotrectinib, Entrectinib, Selitrectinib, Repotrectinib; 100 nM each). H) Dose response curve of TPM3-NTRK1-F589L-expressing NIH-3T3-tv-a cell spheroids treated with different TKI concentrations (same as D) relative to DMSO-treated cells. I) Representative Western Blot of TPM3-NTRK1-F589L-expressing NIH-3T3-tv-a cells treated with 100 nM of indicated TKIs. J-K) Growth curve of TPM3-NTRK1-G595R-expressing NIH-3T3-tv-a (J) or primary mouse tumor (K) cell spheroids treated with either DMSO (0.1%) or TKIs (Larotrectinib, Entrectinib, Selitrectinib, Repotrectinib; 100 nM each). L) Dose response curve of TPM3-NTRK1-G595R-expressing NIH-3T3-tv-a cell spheroids treated with different TKI concentrations (same as D) relative to DMSO-treated cells. M) Representative Western Blot of TPM3-NTRK1-G595R-expressing NIH-3T3-tv-a cells treated with 100 nM of indicated TKIs. Error bars show SEM. Analysis was done using ordinary Two-way ANOVA (B,C,F,G,J,K). (****) $P < 0.0001$.

Figure 4: In vivo TKI treatment leads to the regression of *NTRK* gene fusion-driven mouse gliomas. A) Representative H&E and IHC (HA-tag, Olig2) images of HA-EML4-NTRK3-driven mouse gliomas treated with either Vehicle or Entrectinib (60 mg/kg) for 24 hours. Black dotted line indicates tumor boundaries. B) Left: Representative images of IHC stainings for Cleaved Caspase 3 and phospho-Histone H3 of HA-EML4-NTRK3-driven mouse gliomas treated with either Vehicle or Entrectinib (60 mg/kg) for 24 hours. Right: Quantification of Cleaved Caspase 3 and phospho-Histone H3 stainings (n = 2 per condition). C) Representative IHC stainings for phospho-ERK and phospho-S6 of HA-EML4-NTRK3-driven mouse gliomas treated with either Vehicle or Entrectinib (60 mg/kg) for 24 hours. Scale bar indicates 100 µm. Error bars show SD. Analysis was done using two-tailed t-test (B). (**) $P < 0.01$.

Figure 5: TKI treatment significantly prolongs the survival of NTRK gene fusion-driven mouse gliomas. A) Survival graphs of mice harboring *TPM3-NTRK1*, *CHTOP-NTRK1*, *GKAP1-NTRK2*, *NACC2-NTRK2*, *ETV6-NTRK3*, and *EML4-NTRK3*-driven gliomas treated with either Vehicle, Larotrectinib (100 mg/kg), or Entrectinib (30 mg/kg) via i.p. injection twice-a-day (b.i.d.) for 14 days. After treatment discontinuation, mice were observed for the onset of tumor-related symptoms. B) Graphs depicting change of tumor-size during the 14-day period of treatment with Vehicle, Larotrectinib, or Entrectinib. An initial MRI was performed one day before treatment start and a second MRI one day after treatment end. C-D) Representative MRIs of mice harboring *TPM3-NTRK1* (C)- or *ETV6-NTRK3* (D)-driven tumor treated with either Vehicle or Larotrectinib. E) Representative IHC stainings (TRK kinase domain) of *TPM3-NTRK1*-mouse gliomas after 7 days of treatment (i.p.; b.i.d.) with either Vehicle or Entrectinib (60 mg/kg). Red or black dotted line indicates tumor boundaries. Analysis was done using Log-rank (Mantel-Cox) test (A) or ordinary one-way ANOVA (B). (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (****) $P < 0.0001$.

Figure 1

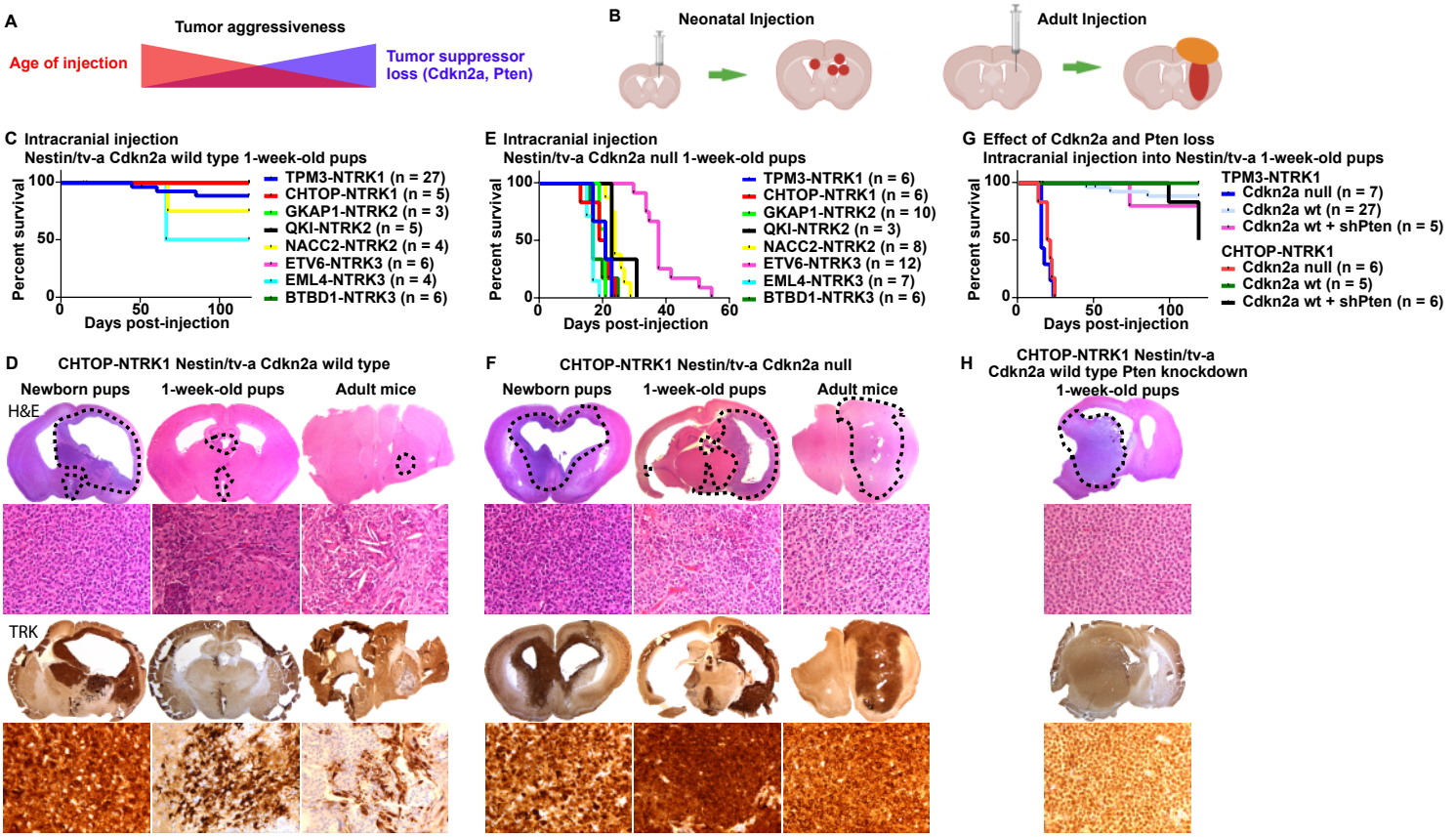

A
Tumor aggressiveness
Age of injection
Tumor suppressor loss (Cdkn2a, Pten)
B
Neonatal Injection
Adult Injection
C Intracranial injection
Nestin/tv-a Cdkn2a wild type 1-week-old pups
Percent survival
Days post-injection
TPM3-NTRK1 (n = 27)
CHTOP-NTRK1 (n = 5)
GKAP1-NTRK2 (n = 3)
QKI-NTRK2 (n = 5)
NACC2-NTRK2 (n = 4)
ETV6-NTRK3 (n = 6)
EML4-NTRK3 (n = 4)
BTBD1-NTRK3 (n = 6)
E Intracranial injection
Nestin/tv-a Cdkn2a null 1-week-old pups
TPM3-NTRK1 (n = 6)
CHTOP-NTRK1 (n = 6)
GKAP1-NTRK2 (n = 10)
QKI-NTRK2 (n = 3)
NACC2-NTRK2 (n = 8)
ETV6-NTRK3 (n = 12)
EML4-NTRK3 (n = 7)
BTBD1-NTRK3 (n = 6)
G Effect of Cdkn2a and Pten loss
Intracranial injection into Nestin/tv-a 1-week-old pups
TPM3-NTRK1
Cdkn2a null (n = 7)
Cdkn2a wt (n = 27)
Cdkn2a wt + shPten (n = 5)
CHTOP-NTRK1
Cdkn2a null (n = 6)
Cdkn2a wt (n = 5)
Cdkn2a wt + shPten (n = 6)
D CHTOP-NTRK1 Nestin/tv-a Cdkn2a wild type
Newborn pups
1-week-old pups
Adult mice
H&E
TRK
F CHTOP-NTRK1 Nestin/tv-a Cdkn2a null
Newborn pups
1-week-old pups
Adult mice
H CHTOP-NTRK1 Nestin/tv-a
Cdkn2a wild type Pten knockdown
1-week-old pups

Figure 2

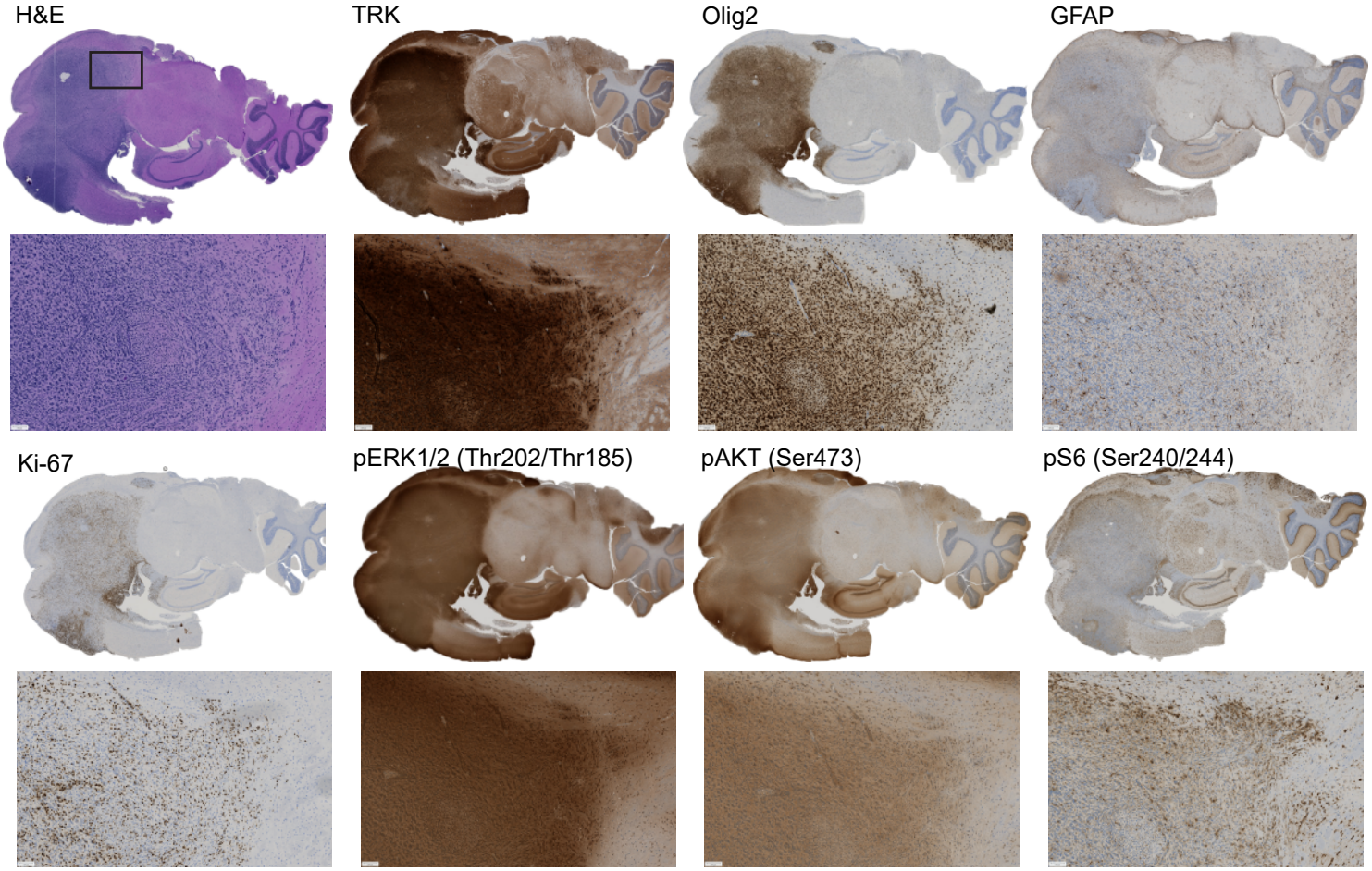

H&E
TRK
Olig2
GFAP
Ki-67
pERK1/2 (Thr202/Thr185)
pAKT (Ser473)
pS6 (Ser240/244)

Figure 3

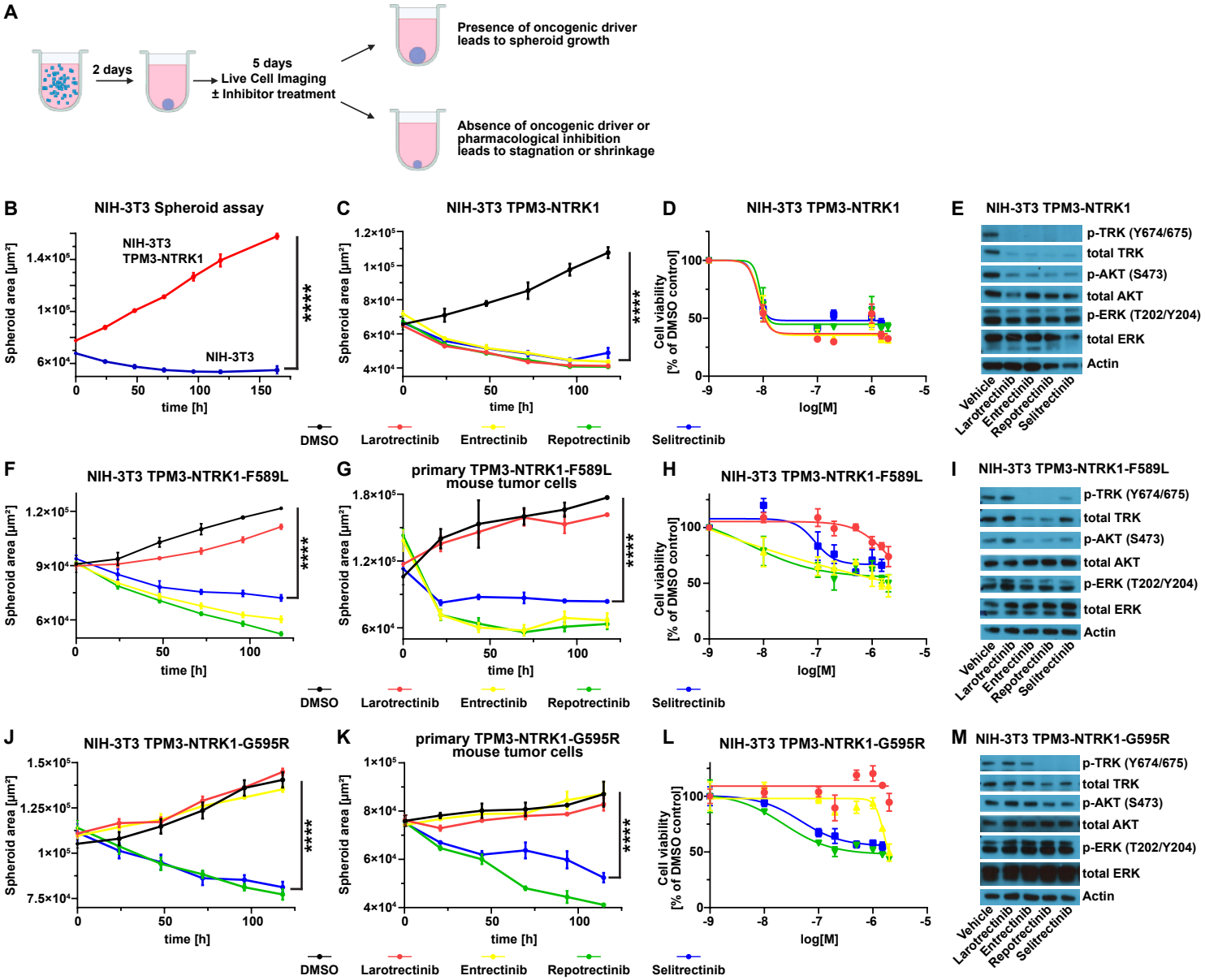

A
2 days
5 days
Live Cell Imaging
± Inhibitor treatment
Presence of oncogenic driver leads to spheroid growth
Absence of oncogenic driver or pharmacological inhibition leads to stagnation or shrinkage
B
NIH-3T3 Spheroid assay
NIH-3T3 TPM3-NTRK1
NIH-3T3
Spheroid area [µm²]
time [h]
****
C
NIH-3T3 TPM3-NTRK1
D
NIH-3T3 TPM3-NTRK1
Cell viability [% of DMSO control]
log[M]
E
NIH-3T3 TPM3-NTRK1
p-TRK (Y674/675)
total TRK
p-AKT (S473)
total AKT
p-ERK (T202/Y204)
total ERK
Actin
Vehicle
Larotrectinib
Entrectinib
Repotrectinib
Selitrectinib
DMSO
F
NIH-3T3 TPM3-NTRK1-F589L
G
primary TPM3-NTRK1-F589L mouse tumor cells
H
NIH-3T3 TPM3-NTRK1-F589L
I
NIH-3T3 TPM3-NTRK1-F589L
J
NIH-3T3 TPM3-NTRK1-G595R
K
primary TPM3-NTRK1-G595R mouse tumor cells
L
NIH-3T3 TPM3-NTRK1-G595R
M
NIH-3T3 TPM3-NTRK1-G595R

Figure 4

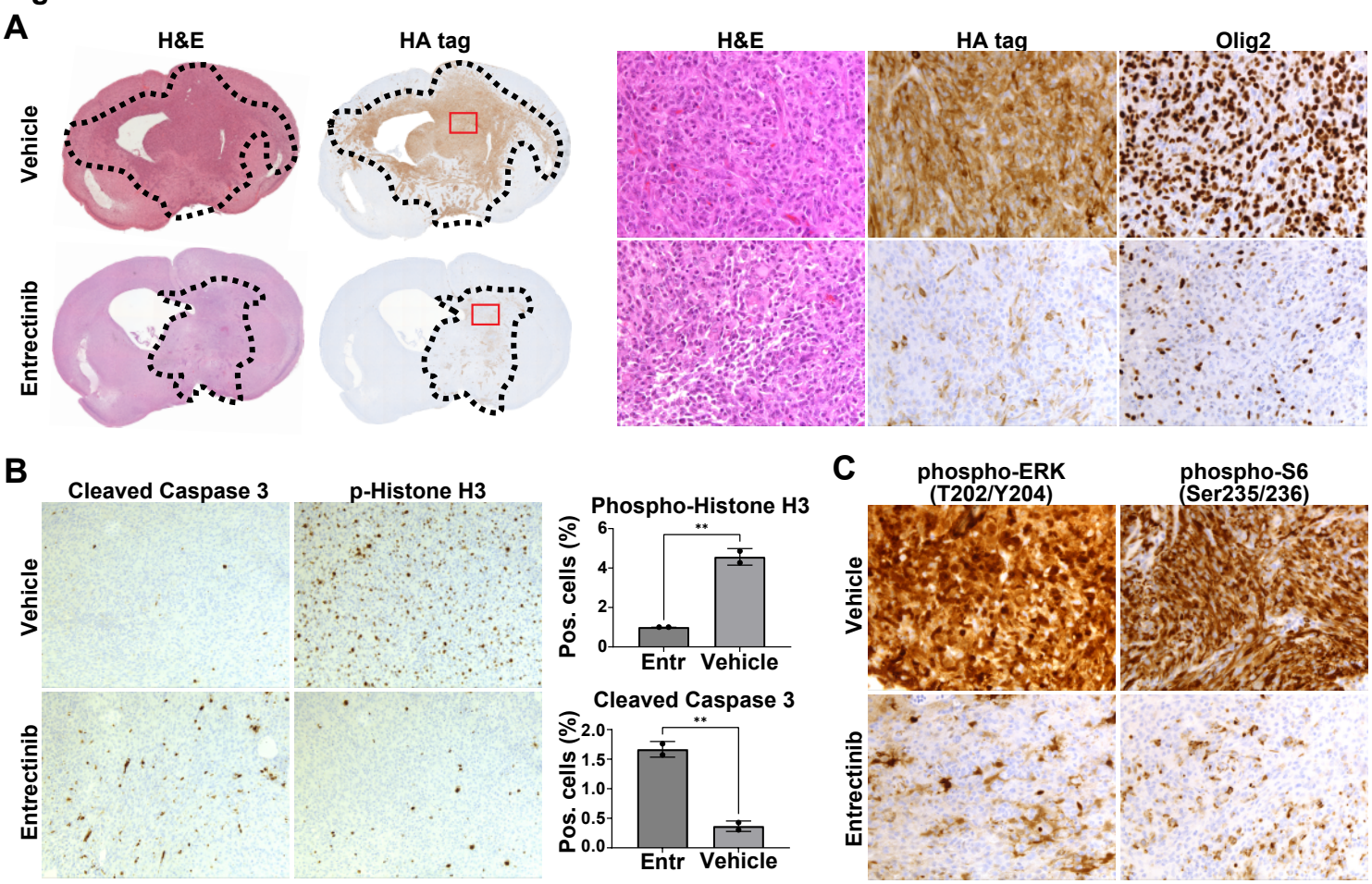
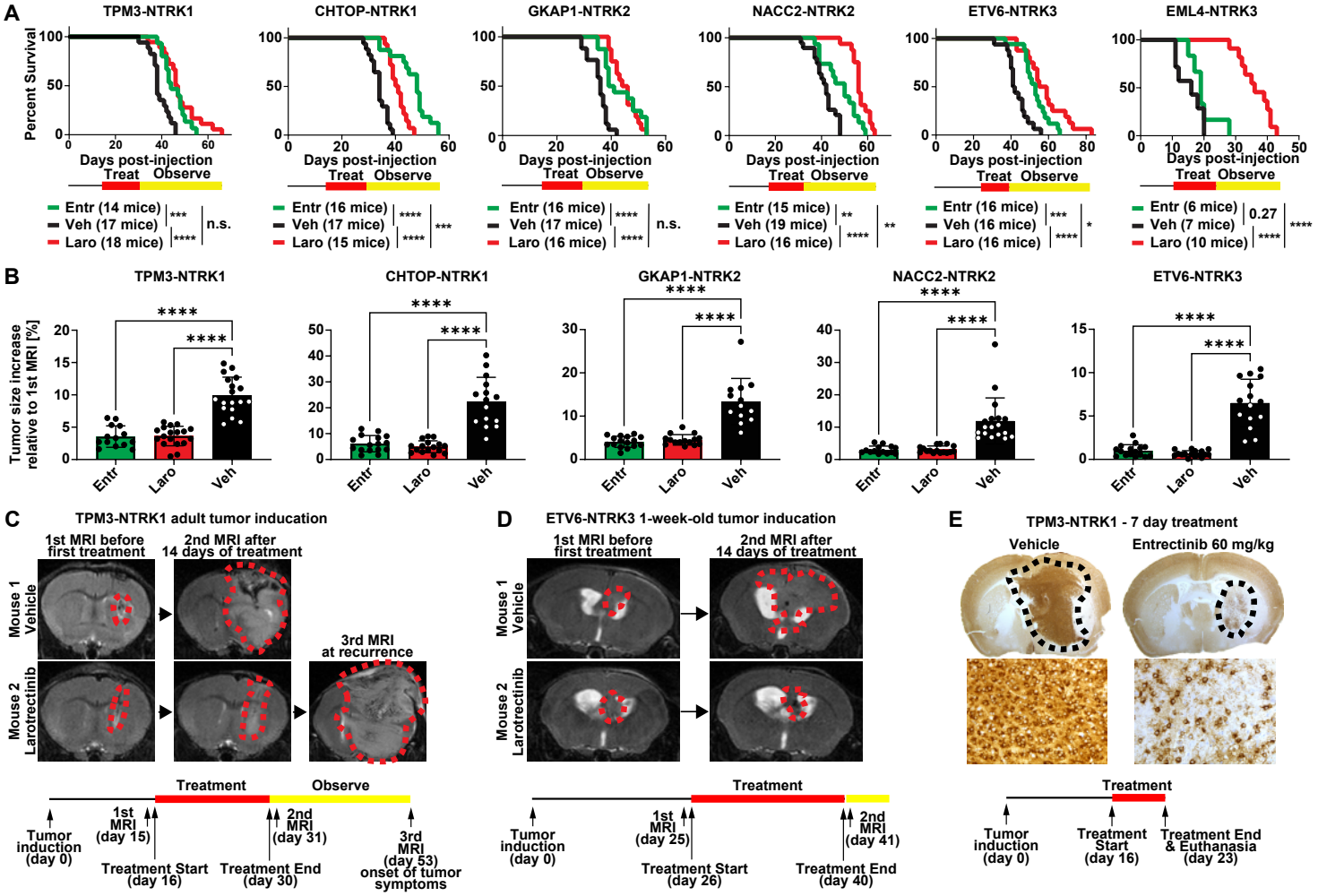

A
H&E
HA tag
Vehicle
Entrectinib
H&E
HA tag
Olig2
B
Cleaved Caspase 3
p-Histone H3
Vehicle
Entrectinib
Phospho-Histone H3
Pos. cells (%)
6
4
2
0
**
Entr
Vehicle
Cleaved Caspase 3
Pos. cells (%)
2.0
1.5
1.0
0.5
0.0
**
Entr
Vehicle
C
phospho-ERK
(T202/Y204)
phospho-S6
(Ser235/236)
Vehicle
Entrectinib

Figure 5


A
TPM3-NTRK1
CHTOP-NTRK1
GKAP1-NTRK2
NACC2-NTRK2
ETV6-NTRK3
EML4-NTRK3
Percent Survival
Days post-injection
Treat Observe
Entr (14 mice)
Veh (17 mice)
Laro (18 mice)
Entr (16 mice)
Veh (17 mice)
Laro (15 mice)
Entr (16 mice)
Veh (17 mice)
Laro (16 mice)
Entr (15 mice)
Veh (19 mice)
Laro (16 mice)
Entr (16 mice)
Veh (16 mice)
Laro (16 mice)
Entr (6 mice)
Veh (7 mice)
Laro (10 mice)
n.s.
0.27
B
Tumor size increase relative to 1st MRI [%]
Entr
Laro
Veh
C
TPM3-NTRK1 adult tumor induction
1st MRI before first treatment
2nd MRI after 14 days of treatment
3rd MRI at recurrence
Mouse 1 Vehicle
Mouse 2 Larotrectinib
Treatment
Observe
Tumor induction (day 0)
1st MRI (day 15)
Treatment Start (day 16)
Treatment End (day 30)
2nd MRI (day 31)
3rd MRI (day 53) onset of tumor symptoms
D
ETV6-NTRK3 1-week-old tumor induction
1st MRI before first treatment
2nd MRI after 14 days of treatment
Mouse 1 Vehicle
Mouse 2 Larotrectinib
Treatment
Tumor induction (day 0)
1st MRI (day 25)
Treatment Start (day 26)
Treatment End (day 40)
2nd MRI (day 41)
E
TPM3-NTRK1 - 7 day treatment
Vehicle
Entrectinib 60 mg/kg
Treatment
Tumor induction (day 0)
Treatment Start (day 16)
Treatment End & Euthanasia (day 23)