A novel chemical screening strategy in zebrafish identifies common pathways in embryogenesis and rhabdomyosarcoma development

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SUMMARY
The zebrafish is a powerful genetic model that has only recently been used to dissect developmental pathways involved in oncogenesis. We hypothesized that operative pathways during embryogenesis would also be used for oncogenesis. In an effort to define RAS target genes during embryogenesis, gene expression was evaluated in embryos during drug treatment. Dusp6 was activated by RAS, and this was used as the basis for a chemical genetic screen to identify small molecules that interfere with RAS signaling during embryogenesis. A KRASG12D-induced zebrafish embryonal rhabdomyosarcoma was then used to assess the therapeutic effects of the small molecules. Two of these inhibitors, PD98059 and TPCK, had anti-tumor activity as single agents in both zebrafish embryonal rhabdomyosarcoma and a human cell line of rhabdomyosarcoma that harbored activated mutations in Nras. PD98059 inhibited MEK1 whereas TPCK suppressed S6K1 activity; however, the combined treatment completely suppressed eIF4B phosphorylation and decreased translation initiation. Our work demonstrates that the activated pathways in RAS induction during embryogenesis are also important in oncogenesis and that inhibition of these pathways suppresses tumor growth.

KEY WORDS: RAS, Embryogenesis, Rhabdomyosarcoma, Translational control, Zebrafish

INTRODUCTION
The zebrafish was first established as a powerful vertebrate model organism for large-scale developmental genetic screens (Gaiano et al., 1996; Haffter et al., 1996). Because of the ease and low cost of raising large numbers of fish, and the highly conserved genetic and biochemical pathways between zebrafish and mammals, the zebrafish has also been utilized for chemical screens in vitro (North et al., 2007). In the last decade, a variety of zebrafish cancer models have been developed ranging in complexity from carcinogen-induced tumors to transgenic zebrafish models of human disease, and thus can be implemented in different steps of novel anti-cancer agent development.

The RAS pathway is a key developmental pathway during embryogenesis. In developing zebrafish embryos, the FGF/RAS pathway plays an antagonistic role with the BMP pathway in dorsal-ventral patterning (Schier, 2001). The RAS genes encode a family of GTPases, which function as binary molecular switches that transduce extracellular growth factor signaling to control intracellular pathways to modulate diverse cellular responses including proliferation, differentiation and survival (Malumbres and Barbacid, 2003). RAS activates various mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK). Activated ERK regulates diverse gene expression programs through transcription factors and the translation machinery (McCubrey et al., 2007). RAS also binds directly to PI3K and initiates the AKT/mTOR signal transduction cascade (Rodriguez-Viciana et al., 1994). The best-characterized downstream targets of mTOR are ribosomal protein S6 kinase 1 (S6K1; also known as p70S6K or RPS6KB1) and eukaryotic translation initiation factor 4E-binding protein 1 (eIF4E-BP1), both of which are crucial to the regulation of protein synthesis (Anjum and Blenis, 2008). Thus, RAS signaling regulates translation through diverse mechanisms.

RAS family members are the most commonly mutated oncogenes in human cancers (Bos, 1989). Point mutations in RAS genes constitutively activate the above-mentioned pathways to drive cell overproliferation. Given the prevalence of RAS signaling activation in human cancers, a significant effort has been dedicated to developing both inhibitors of RAS activation and its downstream signaling pathways, such as MEK and mTOR (Easton and Houghton, 2006; Sebolt-Leopold et al., 1999). Although experimental agents are under development, there are as yet no clinically available drugs directly targeting the RAS pathway. Understanding RAS signaling in tumors and targeting RAS pathways to treat tumors remain a challenge.

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In the last decade, multiple zebrafish cancer models have been generated with RAS mutations, including KRAS-induced myeloproliferative disease, rhabdomyosarcoma, colon adenoma and hepatocellular carcinoma, and KRAS-induced melanoma (Dovey et al., 2009; Langenau et al., 2007; Le et al., 2007). The short time to tumor onset and ease of fluorescent labeling of tumors, together with the feasibility of creating large numbers of animals, make zebrafish an advantageous model for chemical genetic studies to aid our understanding of RAS oncology.

Here, a two-step screening approach was undertaken to examine whether RAS activation in zebrafish embryogenesis illuminates possible mechanisms operative in tumors: first, we used zebrafish embryos to dissect RAS pathways and identified anti-RAS chemicals in vivo; second, we evaluated the therapeutic value of each hit in a zebrafish model of KRAS-induced ERMS. Two compounds (PD98059 and TPCK) demonstrated efficacy in suppressing fish tumor growth. Combined treatment resulted in enhanced therapeutic efficacy. Finally, we investigated the mechanism of combined treatment with PD98059 and TPCK, and revealed the importance of suppression of translation initiation in RAS-activated tumor cells. Our studies utilized common pathways activated by oncogenic RAS during embryogenesis and rhabdomyosarcoma development, and established that developing zebrafish embryos can provide a productive platform for the identification of anticancer agents.

MATERIALS AND METHODS

Animals and stable transgenic lines

Zebrafish (Danio rerio) were maintained in accordance with Animal Research Guidelines at Children’s Hospital Boston. The Tg(hsp70-HRAS\textsuperscript{G12V}) stable transgenic line (http://zf.in.org/action/genotype/genotype-detail?zdbID=ZDB-GENO-100723-7) was first described by Lee et al. (Lee et al., 2009). The Tg(dusp6-d2EGFP) transgenic line (http://zf.in.org/action/fish/fish-detail/ZDB-GENO-071017-5,ZDB-GENO-110131-12,ZDB-GENO-120807-2) was generously provided by Professor Michael Tang (University of Pittsburgh).

Microarray analysis

Heterogeneous Tg(hsp70-HRAS\textsuperscript{G12V}) embryos were obtained by mating male homozygous transgenic fish to wild-type females. They were raised to 24 hours post-fertilization (hpf) and received heat shock at 37°C in a waterbath for 1 hour, and were then kept at 28.5°C until 30 hpf for RNA extraction by Trizol reagent (Invitrogen). Wild-type embryos (AB strain) receiving the same heat shock treatment were used as controls. Each microarray sample was prepared by pooling 50 embryos. Biological duplicates were obtained in both transgenic groups and controls. NimbleGen array chips were used. The probe-level data were normalized and translated to gene-level with custom Python scripts, then analyzed with Goldenspike (http://www2.ccr.buffalo.edu/halfon/spike/). The R Goldenspike package background-correts, normalizes, multiple-tests, and computes the sample statistics per chip. The annotation of the zebrafish probe sets was completed using the Zon Laboratory/Children’s Hospital Zebrafish Gene Transcription Collection (ZGTG: http://zf.imaps.ich.harvard.edu/zgmap/ZGTG.c.htm). The dataset was deposited at Gene Expression Omnibus under accession number GSE 44364.

Quantitative RT-PCR

RNA was isolated from whole zebrafish embryos at 30 hpf using RNAlater and RNeasy Kits (Qiagen). RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and quantitative PCR was performed using SYBR Green (Invitrogen). PCR primers are described in supplemental material Table S1.

Chemical library

A collection of 2896 compounds was screened in zebrafish embryos for suppressors of oncogenic RAS. Among these, 2460 compounds were from JCCB-Longwood Harvard Medical School, including: Biomol JCCB known bioactives [480 compounds, 5 mg/ml (~13 μM) as stock, 33.3 μg/ml final concentration], NINDS (1040 compounds, 10 mM as stock, 66 μM final concentration), Prestwick (1120 compounds, 2 mg/ml as stock, 13.3 μM final concentration). 256 compounds were from HSCI/CHB hEScing (1-5 μg/ml as stock, 6.7-33.3 μg/ml final concentration). Each chemical was screened at a single concentration (1/150 of stock concentration).

Embryonic RAS screen

A chemical screen (see Fig. 2A) was designed using heat shock-inducible Tg(hsp70-HRAS\textsuperscript{G12V}) embryos. Pathway activation was read out using dusp6 mRNA expression. Heterozygous Tg(hsp70-HRAS\textsuperscript{G12V}) embryos were raised at 28°C until 22 hpf and then transferred to a 48-well tissue culture plate (8-15 embryos per well), where each well contained a test chemical dissolved in embryo water. After 2 hours of chemical treatment, heat shock was applied by incubating the 48-well plates at 37°C in a water bath for 1 hour. Then, 24-hpf embryos were returned to a 28°C incubator and fixed at 30 hpf. dusp6 expression levels were evaluated by in situ hybridization and classified as: (1) complete suppression of dusp6 in all embryos; (2) partial/complete suppression with more than two-thirds of embryos having suppression of dusp6 expression; (3) no effect; and (4) enhancement of dusp6 expression in more than two-thirds of embryos. In each 48-well plate, two wells received no chemical treatment and two wells did not receive heat shock.

Therapeutic evaluation using a zebrafish model of RAS-induced ERMS

One-cell stage AB strain zebrafish were injected with rag2-KRAS\textsuperscript{G12D} and rag2-DsRed. Injected animals were screened under a fluorescence microscope at 7 days post-fertilization (dpf) to identify DsRed-positive tumor-bearing fish. All the tumor-bearing fish were numbered, raised in isolated tanks, and randomized into two groups. One group received chemical treatment at the maximum tolerated dose (MTD) while the other received vehicle treatment. MTD was determined by incubating 7-dpf wild-type larval fish in the compound for 5 days and was experimentally defined as the dose at which 75% of fish survived treatment. Each group received two consecutive days of treatment, at day 7 and day 8, of either compound or vehicle control. At 9 dpf, animals were fed with paramecium and fresh water added to the wells to allow recovery and growth of fish over this time. From 10-11 dpf, animals were again bathed in compound or DMSO vehicle. At day 12, animals received feeding and fresh water as at day 9. At days 7, 10 and 13, animals were photographed using a defined exposure time, magnification and gain. Tumor size was measured by quantifying the total number of pixels within the fluorescent area. The relative tumor growth was defined as total pixel numbers at day 10 or day 13 normalized by the total pixel number at day 7 (see Fig. 3B-D). Researchers were blinded as to which animals received treatment or control vehicle until completion of imaging on day 13.

Human cell culture, shRNA knockdown and reporter assay

The human rhabdomyosarcoma RD cell line and the mouse embryonic fibroblast (MEF) cell line were generously provided by Professor Amy Wagers (Harvard Medical School). Cells were maintained in DMEM (Roche) with 10% FBS and 1% penicillin/streptomycin. Cells were plated and proliferation was measured (by MTT Assay Kit, Cayman Chemical) as described in the results. Apoptotic levels were measured by TdT assay (TiterTACS Kit, Trevigen).

For S6K1 knockdown, lentiviral vectors were purchased from Open Biosystems (TRCN0000003158 and TRCN0000003159), and lentiviral particles were produced by cotransfection of HEK 293T cells with plKO.1 constructs and packaging plasmids pMD.G and pCMV8.91 (A.J.W. lab.). Transfections were carried out with FuGENE 6 (Promega), and virus was harvested 48 hours after transfection and frozen. To test the efficacy of shS6K1, RD cells were incubated with lentiviral supernatants in the presence of 8 μg/ml Polybrene (American Bioanalytical) for 24 hours, and infected cells were selected with 10 μg/ml puromycin. After 48 hours of selection, cells were evaluated by MTT assay on day 3, 5 and 7 for proliferation. To test the synergistic effect of S6K1 with PD98059, RD cells...
were incubated with lentiviral supernatant (TRCN0000003158) (shS6K1-58) in the presence of 8 mg/ml Polybrene, and infected cells were selected with 10 mg/ml puromycin. After 48 hours of selection, cells were washed and cultured in medium containing chemicals or vehicle controls. Medium containing chemicals or vehicle controls was changed on day 7 and day 9 for continuous chemical exposure and selection. Cell proliferation was evaluated by MTT assay on day 5, 7 and 9.

The bicistronic reporter SV40-Renilla-IRES-Firefly was provided by Dr John Blenis (Harvard Medical School). The plasmid was transfected into RD cell lines using FuGENE 6 (Promega) and kept in complete medium. At 24 hours post-transfection, cells were starved for 12 hours, and then treated with chemicals or controls. Thirty minutes after chemical exposure, serum (20%) was added to cells to stimulate translation. Luciferase activities were measured 4 hours after serum stimulation (Dual-Luciferase Assay System, Promega), and the Renilla/firefly luciferase light unit ratio was calculated.

**Western blotting**

Anti-phospho-Erk1/2, anti-total Erk1/2, anti-phospho-AKT (Ser473) and anti-phospho-S6 ribosomal protein (Ser240/244) were purchased from Cell Signaling. Anti-actin antibody was from MP Biomedicals. The use of secondary antibodies, dilution of primary antibodies and blocking were performed according to the manufacturer’s recommendations. Zebrafish embryos were collected and manually homogenized in 1× SDS sample buffer and subjected to SDS-PAGE followed by blotting onto nitrocellulose membrane. RD cells were harvested in 1× sample buffer and subjected to SDS-PAGE followed by blotting onto nitrocellulose membrane. ECL detection reagents were used (GE Healthcare).

**RESULTS**

**Establishing a chemical screen platform using inducible RAS zebrafish embryos to dissect tumorigenesis pathways**

The activation of RAS during embryogenesis may recapitulate the activation of its pathways during tumorigenesis. We sought to evaluate the effects of increased RAS activity on embryos. Injection of oncogenic RAS mRNA into zebrafish results in early embryonic death. To bypass the RAS-induced lethality, we used an inducible transgenic zebrafish line that expresses the human HRAS<sup>G12V</sup> gene under the heat shock promoter, Tg(hsp70-HRAS<sup>G12V</sup>) (Lee et al., 2009), and induced RAS expression after gastrulation.

A microarray analysis was performed by comparing the transcription profiles of Tg(hsp70-HRAS<sup>G12V</sup>) and wild-type embryos subjected to heat shock. Both groups of embryos received heat shock at 37°C for 1 hour at 24 hpf, and were kept at 28.5°C until 30 hpf for RNA extraction. Three different fold change cutoffs were utilized in defining the upregulated gene lists to verify that differences were reproducibly related to RAS activation and not due to arbitrary assignment of gene lists. Using a false discovery rate (FDR) of zero and log fold change cutoffs greater than 1, 0.7 and 0.5 (with the correlating absolute fold change greater than 2, 1.6245 and 1.4142), three gene lists encompassing 2423, 3540 and 4129 genes were defined as upregulated (supplemental material Table S2; downregulated genes are listed in Table S3). Ingenuity pathway analysis (IPA) was performed using the three lists. In all three analyses, ‘cancer’ was the top ‘diseases and disorders’, with a known transcription target of the FGF/MAPK pathway and a negative regulator of Erk1/2 (Mapk3/1) (Ekerot et al., 2009), was robustly upregulated in 100% of the heat shocked embryos, demonstrating strong staining by in situ hybridization (ISH). Such activation was also confirmed in the Tg(dusp6-d2EGFP) reporter line (supplementary material Fig. S1). Furthermore, dusp6 was concordantly upregulated in reg2-KRAS<sup>G12D</sup>-induced zebrafish ERMS as detected by ISH on the tumor sections, confirming dusp6 as a target of oncogenic RAS in tumors (supplementary material Fig. S2).

**Identification of suppressors of RAS signaling through a zebrafish embryonic chemical screen**

The chemical screen was further optimized by utilizing RT-PCR and Tg(dusp6-d2EGFP) reporter line transgenic fish to determine peak expression of dusp6. Both approaches demonstrated that, at 6 hours post-heat shock, embryos exhibited peak expression of dusp6 (Fig. 2A, dashed line; supplemental material Figs S1, S4). Therefore, 6 hours post-heat shock was chosen as the time for readout of RAS activity.

A collection of 2896 bioactive small molecules was screened for compounds that suppress dusp6 expression following heat shock in Tg(hsp70-HRAS<sup>G12V</sup>) embryos (Fig. 2; also see Materials and methods). This collection includes many classes of well-characterized compounds such as ion channel blockers, nuclear receptor ligands, protease inhibitors, gene regulation agents and lipid biosynthesis inhibitors, and covers more than 46% of Food and Drug Administration-approved drugs. Each compound was
screened at a single concentration with 5-10 embryos. Thirty-one of the 2896 compounds screened demonstrated complete suppression of *dusp6* expression and another 67 exhibited partial suppression (Table 2). The 31 compounds with the most potent suppression were purchased and individually retested using the same experimental conditions and concentration. Twenty-five (80.6%) of the 31 retested chemicals were validated to have potent suppressive effects on *dusp6*, indicating that the primary screen could robustly identify suppressors of the RAS signaling pathway.

Heat shock was applied to Tg(hsp70-HRASG12V) embryos after 2 hours of chemical exposure; thus, our experimental design would be likely to identify drugs that suppress both RAS activity and heat shock responses. To select drugs that specifically affect RAS pathway activation, Tg(hsp70-Cre) fish were assessed for Cre expression following heat shock and subsequent drug treatment (Le et al., 2007). Chemicals that suppressed Cre mRNA expression were eliminated from further study, as these compounds were likely to regulate hsp70 promoter expression and/or the heat shock response rather than RAS activity (supplementary material Fig. S5). Using this approach, 18 compounds were confirmed as RAS pathway inhibitors (supplementary material Table S4).

Identification of chemical inhibitors of RAS-induced embryonal rhabdomyosarcoma
To evaluate the effects of the 18 RAS signaling pathway inhibitors in cancer, each chemical was tested as a single agent for suppressing...
zebrafish KRASG12D-induced RMS using a randomized trial design. Co-injection of rag2-KRASG12D and rag2-DsRed DNA leads to externally visible zebrafish ERMS by 10 days (Langenau et al., 2008). As early as 7 dpf, tumor-bearing fish can be identified by visualizing DsRed fluorescence in muscle fibers, with 100% of DsRed-positive animals developing ERMS (n=11). ERMS growth can be followed within individual animals by serial imaging over several days based on the fluorescent tumor area. The therapeutic effect of each compound can be quantified by determining relative tumor growth as compared with that in non-treated animals (Fig. 3A-F; see also Materials and methods).

Two compounds significantly delayed tumor growth at their maximum tolerated dose (MTD). The MEK inhibitor PD98059 (supplementary material Fig. S6A; MTD 15.2 μM) inhibited relative tumor growth (1.259±0.171, n=13 at day 10; 1.373±0.285, n=8 at day 13) as compared with vehicle-treated sibling fish (1.778±0.906, n=10 at day 10; 2.034±1.621, n=10 at day 13; P<0.05, ANOVA; Fig. 3G). The chymotrypsin-like serine protease inhibitor tosyl

Table 1. The 17 genes tested to identify a robust readout for the chemical screen

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene in maximally contributing group</th>
<th>RT-PCR (whole embryos)</th>
<th>NimbleGen ID</th>
<th>Fold change in microarray</th>
<th>q value (FDR)</th>
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<tr>
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<td>OTTDA0000026849</td>
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hERMS, human embryonal rhabdomyosarcoma; HMECs, human mammary epithelial cells.

Fig. 2. A small-molecule screen in Tg(hsp70-HRASG12V) zebrafish embryos. (A) Scheme of the chemical screen. Heterozygous Tg(hsp70-HRASG12V) embryos were placed in a 48-well plate for chemical treatment starting at 22 hpf and heat shocked from 24-25 hpf in a 37°C waterbath to activate RAS signaling (dashed line). At 30 hpf, embryos were fixed and the dusp6 expression level was evaluated by ISH. The solid line represents the dynamic changes in dusp6 RNA level, as confirmed by RT-PCR; the dashed line represents the predicted activation of RAS based on hsp70 promoter dynamics (Le et al., 2007). (B) ISH of dusp6 on embryos treated with PD98059, TPCK, Lovastatin, Tyrphostin A9, Valinomycin and Catechin.
phenylalanyl chloromethyl ketone (TPCK; supplementary material Fig. S6B; MTD 0.3 μM) also inhibited relative tumor growth as a single agent (1.056±0.163, n=15 at day 10; 1.058±0.293, n=6 at day 13) as compared with vehicle-treated sibling fish (P<0.05, ANOVA; Fig. 3H). The gross morphology of the fish was not affected by chemical treatment. Their swimming and eating behaviors were also normal. To ensure that chemical effects were specific to tumors and did not affect the growth of the entire fish, the overall length of each fish was recorded under brightfield illumination at 7, 10 and 13 dpf. Neither TPCK nor PD98059 significantly altered overall fish growth at the MTD when compared with vehicle-treated fish (supplementary material Fig. S7A,B). No statistically significant difference in survival was detected among chemical-treated groups versus controls (P=0.53, ANOVA). The impaired survival in the treatment trials was likely to be due to reduced feeding, repeated anesthesia for imaging, and mechanical manipulation during larval development.

Taken together, we have developed a two-step screening system for the oncogenic RAS pathway: we first identified 18 chemical suppressors of RAS signaling pathways during zebrafish embryogenesis, and then found that two of them – PD98059 and TPCK – have effects in suppressing tumor growth in a genetically engineered zebrafish model of ERMS.

PD98059 and TPCK suppress different downstream RAS signaling targets

We measured the activity levels of selected RAS targets to understand how the two hits affect RAS signaling pathways. PD98059 is a known MEK1 (MAP2K1) inhibitor and has been previously shown to inhibit MEK activity in zebrafish (Pozios et al., 2001). PD98059 suppressed dusp6 expression in zebrafish embryos in a dose-dependent manner (supplementary material Fig. S8). Western blot analysis (Fig. 4) showed that PD98059 (18.7 μM) suppressed phospho (p-) Erk1/2 levels in zebrafish embryos, but not levels of p-Akt or p-p38 (Mapk14 – Zebrafish Information Network) (data not shown), demonstrating that PD98059 indeed inhibits the MAPK pathway in zebrafish. TPCK was originally designed as a chymotrypsin-like serine protease inhibitor; however, it has subsequently been shown to be a potent inhibitor of S6K1. In the Tg(hsp70-HRASG12V) embryos, TPCK suppressed dusp6 expression in a dose-dependent manner (supplementary material Fig. S8). Western analysis indicated that the levels of p-Rps6, which is a target of S6k1, were greatly suppressed in zebrafish embryos treated with TPCK (1 μM), indicating that TPCK suppressed S6k1 activity in zebrafish embryos. TPCK did not alter the levels of p-Erk1/2 or p-Akt in zebrafish (Fig. 4).

These results suggest that PD98059 suppresses the MAPK pathway of RAS signaling, whereas TPCK specifically suppresses
the S6K1 pathway without significantly suppressing the MAPK or AKT pathways in zebrafish.

To assess whether PD98059 and TPCK also have anti-tumor effects in human ERMS, each was assessed for growth and apoptotic effects in the human RD cell line that has activated RAS signaling through mutation of \textit{NRAS} \textit{(NRAS}\textit{Q61H}) \cite{Stratton1990}. By MTT assay in RD cells, both PD98059 (10 \(\mu\)M), TPCK (1 \(\mu\)M) or DMSO control from 22 hpf, heat shocked from 24-25 hpf at 37°C and whole embryos were homogenized in 1x SDS sample buffer at 28 hpf.

**PD98059 and TPCK synergistically suppress tumor progression in zebrafish rhabdomyosarcoma and human RD cells**

The above data indicate that PD98059 and TPCK act on independent signaling modules downstream of activated RAS; thus, we speculated that combined treatment with both compounds would result in an improved therapeutic effect. ERMS-bearing fish were treated with one-third of the MTD of each compound alone or in combination. A cohort of tumor-bearing fish was randomized into four groups at 7 dpf: (1) vehicle control [0.28% (v/v) DMSO], (2) PD98059 alone (5.2 \(\mu\)M), (3) TPCK alone (0.1 \(\mu\)M) and (4) PD98059 (5.2 \(\mu\)M) with TPCK (0.1 \(\mu\)M). Drug treatment, recovery and tumor measurement were carried out as described above. During the 6-day treatment regimen, single drug treatment at the lower concentration did not delay tumor progression (PD98059: \(1.463\pm0.416, n=13\); TPCK: \(1.377\pm0.353, n=7\); DMSO: \(1.412\pm0.348, n=12\); at day 10). Strikingly, combined treatment with TPCK and PD98059 achieved significant suppression of tumor growth compared with each single drug treatment or vehicle control (1.069\(\pm0.221, n=12\); \(P=0.0009\,\text{ANOVA}\); Fig. 6A,C). Overall fish growth was unaffected by drug treatment in all groups, suggesting that combined drug treatment elicited only anti-tumor effects on larval fish (supplementary material Fig. S7C). A similar synergistic anti-proliferative effect was observed in the human ERMS (RD) cell line when treated simultaneously with PD98059 and TPCK (Fig. 6B); however, drug combinations had no effect on apoptosis (supplementary material Fig. S10).

We have shown that TPCK suppresses the activity of S6K1. Because TPCK has been reported to have different effects on various signaling pathways, we next sought to demonstrate that TPCK treatment results in reduced proliferation through inhibition of the S6K pathway. Using lentivirus-mediated RNA interference, we tested whether knockdown of S6K1 could mimic the synergistic effect of TPCK on cell proliferation when combined with PD98059. An shRNA for S6K1 (shS6K1) was constructed and its effect confirmed in a cell proliferation assay. The suppressive effect of S6K1 knockdown combined with PD98059 (10 \(\mu\)M) or TPCK (2.5 \(\mu\)M) was compared with controls (scrambled shRNA) with either chemical alone or in combination (Fig. 6D). The relative growth of cells treated with shS6K1 and TPCK (1.582\(\pm0.074\) at day 7, 1.663\(\pm0.05\) at day 9) was not

![Fig. 4. PD98059 and TPCK selectively suppress different downstream RAS signaling pathways in zebrafish embryos. Western blot analysis was performed using \textit{Tg} (\textit{hsp70-}HRAS\textit{G12V}) embryos to study the phosphorylation status of Erk1/2 (T202/Y204), Akt (S473) and Rps6 (S240). The embryos were treated with PD98059 (18.7 \(\mu\)M), TPCK (1 \(\mu\)M) or DMSO control from 22 hpf, heat shocked from 24-25 hpf at 37°C and whole embryos were homogenized in 1x SDS sample buffer at 28 hpf.](image)

![Fig. 5. PD98059 and TPCK suppress cell proliferation in the human RD cell line. Cells were plated in 96-well tissue culture plates at day 1. Cells were treated with a range of concentrations of (A) PD98059 (10-40 \(\mu\)M) and (B) TPCK (1-10 \(\mu\)M) starting at day 0 and continuing throughout the 6-day treatment. Medium/chemicals were changed on days 0, 2 and 4 to ensure chemical activity and adequate nutrients for cell growth. Cell proliferation was measured by MTT assay at days 2, 4 and 6. \(y\)-axis represents absolute OD from the MTT assay. Error bars indicate s.e.m.](image)
significantly different from cells treated with TPCK alone (1.621±0.032 at day 7, 2.036±0.076 at day 9; \( P = 0.13 \), ANOVA), suggesting that shS6K1 and TPCK are acting redundantly in the same pathway. By contrast, treatment with shS6K1 and PD98059 (1.069±0.063 at day 7, 1.151±0.048 at day 9; \( P = 0.01 \), ANOVA), demonstrating a synergistic effect. This synergistic suppression by shS6K1 plus PD98059 was similar to that of TPCK plus PD98059 (1.201±0.070 at day 7, 1.357±0.089 at day 9; \( P = 0.22 \), ANOVA). These data showed that S6K1 knockdown or pharmacological treatment with TPCK exhibited a similar synergistic effect when combined with PD98059, supporting the conclusion that TPCK suppresses cell proliferation through inhibiting the S6K1 pathway.

**PD98059 and TPCK converge on translation initiation to suppress tumor proliferation**

We next focused on understanding the mechanism of how TPCK and PD98059 synergistically suppress tumor cell growth. Studies from other groups suggest that the activated RAS/MAPK and AKT/S6K1 pathways both independently increase protein synthesis by optimizing cap-dependent translation initiation. An important component of this translation initiation complex is eukaryotic translation initiation factor 4B (eIF4B) (Gingras et al., 2001). A phosphorylation site at Ser422 of eIF4B was demonstrated to be partially responsive to mTOR/S6K1 and partially responsive to MEK/ERK/RSK (Holz et al., 2005; Shahbazian et al., 2006); thus, we hypothesized that dual inhibition of the MAPK and S6K1 pathways leads to complete suppression of eIF4B phosphorylation, whereas single pathway inhibition still allows eIF4B activation...
through the other pathway. Western blotting of eIF4B to detect phosphorylation of Ser422 was performed in RD cells treated with PD98059 and TPCK individually or in combination. As expected based on our zebrafish studies, p-ERK1/2 levels were suppressed by PD98059 and p-RPS6 levels were suppressed by TPCK; p-eIF4B levels were partially suppressed by PD98059 or TPCK single drug treatment, and were completely suppressed by combined treatment (Fig. 7A). These results suggest that PD98059 and TPCK synergistically downregulate the levels of p-eIF4B.

To demonstrate that suppression of p-eIF4B leads to suppression of cap-dependent translation activity, a bicistronic reporter was used to determine whether combined treatment with PD98059 and TPCK individually or in combination. As expected, based on our zebrafish studies, p-ERK1/2 levels were suppressed by PD98059 and p-RPS6 levels were suppressed by TPCK; p-eIF4B levels were partially suppressed by PD98059 or TPCK single drug treatment, and were completely suppressed by combined treatment (Fig. 7A). These results suggest that PD98059 and TPCK synergistically downregulate the levels of p-eIF4B.

DISCUSSION

A common concept is that cancer cells often acquire embryonic character through activation of developmental pathways (Abbott et al., 2007; Dreesen and Brivanlou, 2007). Developmental biology has uncovered a number of signaling pathways involved in cancers; for example, the Hippo pathway was identified in Drosophila and shown to be crucial in cancer cell apoptosis (Saucedo and Edgar, 2007). Few studies have utilized zebrafish to directly compare the processes of embryogenesis and oncogenesis. Our work describes a new strategy to utilize zebrafish embryos to screen for pathways that participate in cancer development. We first demonstrated that conditional activation of oncogenic HRASG12V in developing zebrafish embryos mimics RAS pathway activation during tumorigenesis. We then tested the therapeutic potential of these compounds on tumor progression in a zebrafish model of RAS-induced ERMS. This rhabdomyosarcoma model allows the study of tumors during larval development by 13 dpf, chemical exposure and direct imaging of tumors, demonstrating this tumor model as a powerful tool for cancer research. This two-step screening approach identified chemicals with anti-RAS activity that could be assessed in human cell lines that harbored RAS mutations, and identified pan-RAS inhibitors that modulate the function of all three RAS family members – HRASG12V in our embryonic screen, KRASt12D in zebrafish ERMS, and NRASQ61H in the human RD cell line.

Among the hits in the screen, known inhibitors of both RAS activation and RAS downstream pathways were identified. For example, Lovastain is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), a known inhibitor of RAS, and acts by suppressing the recruitment of RAS to the cell membrane (Issat et al., 2007). PD98059 was also identified in our
screen and is an inhibitor of MEK1, a major component of the MAPK cascade. The identification of these two groups of known inhibitors verified the design of our screen and demonstrated that the zebrafish embryo can be used to dissect specific signaling pathways in vivo.

TPCK was identified in our larval screen to suppress RAS pathway activation and also showed potent inhibition of ERMS growth as a single agent. TPCK was originally synthesized as a protease inhibitor (Schoellmann and Shaw, 1963). TPCK has been shown to be a potent in vivo inhibitor of S6K1, PDK1 and other related kinases with a conserved domain (known as AGC kinases), although an in vitro kinase assay showed that S6K1 is not the direct molecular target of TPCK (Ballif et al., 2001; Grammer and Blenis, 1996). TPCK has also been described as inhibiting the endoprotease responsible for cleaving the C-terminal AAX sequence on RAS (Porter et al., 2007). Alternative mechanisms have been proposed including recent work demonstrating that TPCK blocks specific cysteine residues on IkappaB kinase (IKK) and p65/RelA (Ha et al., 2009). TPCK was first found, 40 years ago, to potently inhibit tumorigenesis initiated in mouse skin lesions induced by DMBA (Troll et al., 1970), but the mechanism of how TPCK suppresses tumor growth remained unclear. In our experiments, TPCK completely abolished the RPS6 phosphorylation that is dependent on S6K1, without affecting p-ERK or p-AKT levels. Although α-actin was used as loading control for AKT and RPS6, our data from zebrafish embryos and cell culture argue against the possibility that TPCK inhibits the RAS CAAX modification as other RAS downstream pathways were relatively intact, but are instead consistent with the finding that TPCK is a suppressor of the S6K1 pathway. We further validated that both knockdown of S6K1 by lentivirus-mediated RNA interference and pharmacological treatment with TPCK demonstrated a similar synergistic effect with PD98059, suggesting that TPCK suppresses RD cell proliferation through inhibiting the S6K1 pathway. The toxicity of TPCK, however, has been a major concern (Lewis, 2004). To circumvent toxicity, we tested the efficacy of a combination treatment regimen using both PD98059 and TPCK at lower concentrations. PD98059 and TPCK delayed tumor progression individually but showed a greater effect in impeding tumor progression when the two were combined, even at a significantly lower dose.

Translational control in eukaryotic cells is crucial for gene regulation to rapidly adjust protein production in conditions of nutrient deprivation and stress (Sonenberg and Hinnebusch, 2009). Aberrant function of components of the translation machinery underlies a variety of human diseases including certain cancers and metabolic disorders. The MAPK and PI3K pathways regulate the translation machinery, especially at the translation initiation complex, which binds to the cap region of mRNA to initiate translation (Parsa and Holland, 2004). eIF4B plays a crucial role in recruiting the 40S ribosomal subunit to the mRNA (Ma and Blenis, 2009). In response to growth factors, eIF4B is phosphorylated on Ser422 by both RSK and S6K increases the interaction of eIF4B with eIF3 (Holz et al., 2005; Shahbazian et al., 2006). The recruitment of the translation initiation complex increases the mRNA binding and processivity of the activated helicase complex, potentially enhancing translation rates. We showed that PD98059 or TPCK partially inhibits, whereas a combination of the two completely abolishes, eIF4B Ser422 phosphorylation. We further demonstrated that in vivo cap-dependent translation was significantly decreased after combination treatment, whereas it was only partially decreased by single agent treatments. We propose that the blockade of two major signaling pathways downstream of RAS – MAPK/ERK and AKT/S6K1 – results in effective suppression of eIF4B phosphorylation and the inhibition of translation initiation in proliferating tumor cells.

Our study interfaces embryogenesis and oncogenesis in a vertebrate model. We demonstrate successful use of zebrafish embryos and cancer models for anticancer chemical screens in a shared pathway, and proved the feasibility of performing chemical screens directly in tumor-bearing animals. By inhibiting both the MAPK/ERK and AKT/S6K1 pathways we showed that inhibition of translation initiation suppresses tumor growth, thereby demonstrating the translational initiation complex to be a drug target for cancer therapy.

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Competing interests statement
L.I. is a founder and stock holder of Fate, Inc. and a scientific advisor for Stemgent.

Supplementary material
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