Figure 3 Histomorphologic patterns and features of relatively common types of neoplasia in adult zebrafish. Images are from AB wt (a-e) and *tp53*^{zdf1} null (f) zebrafish. (a) Small cell carcinoma of the anterior intestine. Small clusters and packets of 3-8 basophilic polygonal cells infiltrating the lamina propria, embedded within a dense fibrous stroma and interspersed chronic inflammatory cells. It is common in zebrafish for small cell carcinoma to invade into the coelomic cavity and line the serosal surfaces of adjacent organs (carcinomatosis). (b) Adenocarcinoma of the anterior to mid-intestine. Irregularly shaped, disorganized acinar structures lined by hyper- and dysplastic epithelial cells and nests of neoplastic cells within the lamina propria, surrounded by dense schirrous matrix intermingled with chronic inflammatory cells. (c) Thyroid gland carcinoma. Cords and nests of basophilic neoplastic cells within an edematous fibrovascular matrix; rare follicular structures contain intraluminal colloid (arrow). (d) Ultimobranchial gland carcinoma. Nests, cords and ribbons of amphophilic polygonal cells surrounded by fibrovascular tissue; occasional "normal" acinar structures (N) can be observed. (e) Pancreatic carcinoma. Sheets of densely packed neoplastic acinar cells completely efface normal pancreas architecture; mitotic figures (arrow) are common and some of the neoplastic cells retain eosinophilic zymogen granules. (f) Malignant peripheral nerve sheath tumor. Dense, streaming and interlacing fascicles of basophilic spindle cells with interfascicular clefts and prominent whorls; there was extensive local invasion and extension of this tumor. (a-e); bar = 25 microns; (f); bar = 50 microns

Figure S1 Gross and microscopic lesions in adult fish from retired broodstock and carcinogenesis experiments. (a) Myelodysplastic syndrome in untreated *uma*^{s2068} mutant fish. Spleen enlarged 100X normal size, meaty in texture and mottled red and white in color. (b) Myelodysplastic syndrome. Impression smear of spleen from *uma* ^{s2068} mutant fish. Myelocytic lineage shows a high proportion of blast cells. Erythrocytic lineage shows asymmetric and radial mitoses and micronuclei. (c) and (d) Hemangiosarcoma. Gross and microscopic appearance of mass which has extensively invaded head of zebrafish of *alf*^{ty86d} line treated by fry bath with DMBA. (e) and (f) Esthesioneuroblastoma of nose. Gross

and microscopic appearance of soft white mass on head of a *koi*^{tl226d} mutant fish with TL genetic background. Flexner-Wintersteiner neuroepithelial rosettes are evident in the histologic sections. (g) Complex odontoma in pharyngeal tooth. Histologic appearance of neoplasm in Singapore strain of zebrafish treated by fry bath with DMBA. (h) Chordoma of caudal spine compressing terminal spinal cord. Histologic appearance of small ovoid mass in zebrafish fed DBP for 2 mo as juvenile.

Figure S2 Examples of some common types of neoplasia in adult zebrafish. (a)-(c) Gross lesions. Images are from *tp53*^{zdf1} null (a and b) and AB wt (c) zebrafish. (a) Malignant peripheral nerve sheath tumor of the left eye; marked exophthalmia with the large protruding mass destroying the eye. (b) Malignant peripheral nerve sheath tumor; transverse section proximal to the optic chiasm shows a bulbous, well-demarcated expansile mass originating from the optic nerve that is completely obliterating the eye and surrounding local tissues as well as significantly compressing the left aspect of the oropharyngeal cavity. (c) Thyroid gland neoplasia (arrow); ventral aspect of the fish shows multiple discrete to coalescing, variably-sized smooth nodular masses elevating and laterally displacing the operculae and branchiostegal membranes. (d) Normal ultimobranchial gland. Low magnification photograph of histologic section of ultimobranchial (arrows) showing characteristic cluster of acini lined by tall columnar epithelial cells with basally oriented nuclei. Ultimobranchial gland is located between the esophagus and the heart. (e) Ultimobranchial carcinoma. Low magnification photograph of histologic section of ovoid mass with higher magnification inset. Epithelial cells of ultimobranchial gland are pleomorphic and have lost their normal acinar arrangement as well as the basal orientation of nuclei. (f) and (g) Branchioblastoma present in gill of Singapore strain of zebrafish given fry bath treatment with DMBA. (f) Low magnification photograph of histologic section of multilobulated mass (arrows) in pharyngeal cavity. Mass is comprised primarily of highly embryonal blastema and is invading into meninges of the brain. This is the most poorly differentiated and invasive branchioblastoma that we have observed in our tumor studies in zebrafish. (g) Higher magnification photograph of a more differentiated region of branchioblastoma forming a distorted caricature of gill with mixture of blastema, epithelium, cartilage and blood vessels.

Figure S3 Gross and microscopic lesions in adult fish from carcinogenesis and transgenesis experiments. (a) and (b) Bilateral retrobulbar hemangiomas of choroid glands of eyes in TU line zebrafish following fry bath treatment with ENU. (a) Bilateral exophthalmos due to the neoplasms. (b) Histologic appearance of hemangioma of choroid gland. Mass is comprised of uniform well differentiated small capillaries. (c) Squamous cell carcinoma of lower jaw. Gross photograph of firm spherical mass protruding from jaw. Incidental finding in Singapore strain fish from transgenesis experiments targeting other tissues. (d) Nephroblastoma. Gross appearance of irregular ovoid mass protruding through the body wall of the lateral aspect of the mid-region of trunk. Incidental finding in Singapore strain fish from transgenesis experiments targeting other tissues. (e) Branchioblastoma. Multilobulated firm white mass protruding from beneath operculum of an alf^{ty86d} mutant fish treated by fry bath with DMBA. Most branchioblastomas are evident only microscopically even in carcinogen studies with sensitive mutant lines. (f) Nephroblastoma. Histologic section of mass shown in (d). Mass has invaded extensively into abdominal cavity, spine and spinal cord. (g) Higher magnification photograph of histologic section of nephroblastoma showing disorganized admixture of abortive tubular structures, distorted glomerular capillaries, and irregular clusters of blastemal cells. (h) Squamous cell carcinoma. Histologic section of mass shown in (c). Extensive invasion throughout bone, skeletal muscle and skin of jaw. (i) Photomicrograph of higher magnification of squamous cell carcinoma showing irregular sheets and clusters of anaplastic polygonal to round epithelial cells set in abundant stroma.

Figure S4 Small cell carcinoma of anterior intestine in adult zebrafish and immunohistochemistry studies investigating parameters that might influence sensitivity of anterior gut to neoplasia. (a) Low magnification photomicrograph to illustrate the location of this mass (arrows) in the vicinity of the ampulla of Vater, the site at which bile and pancreatic ducts enter the intestine. This neoplasm invades through the wall of the intestine into the surrounding tissue (b) High magnification photograph of small cell carcinoma of intestine showing clusters and sheets of embryonal small round cells with prominent nucleoli and scant cytoplasm. (c) Histologic section of immunohistochemical stain for Cyp3a27 showing

intense staining of mucosal epithelium of intestinal bulb in untreated 3-wk-old fry. Primary antibody was polyclonal rabbit raised against rainbow trout Cyp3a27. Dako Envision Plus horseradish peroxidase kit utilized with 3-amino-9-ethylcarbazole (AEC) as chromogen. (d) Immunohistochemical stain of distal esophagus and anterior intestine for PCNA in tissues from 6-mo-old wt zebrafish. Primary antibody mouse monoclonal PC10 (Dako). Dako Envision Plus horseradish peroxidase kit utilized with AEC as chromogen. Mucosal epithelium of esophagus (E) and intestinal bulb (IB) are negative for staining for PCNA, whereas the adjacent ultimobranchial gland (arrow) stains intensely. (e) Low magnification photograph of intestine (I) of 3-wk-old fry. Immunohistochemistry assay using nonimmune rabbit serum as the primary antibody shows low background staining. (f) Immunohistochemical stain of posterior intestine for PCNA showing moderate diffuse staining of nuclei of mucosal epithelial cells (arrows)

Supplemental Methods and Data

Experimental Methods

Fish Husbandry

At Oregon State University's (OSU's) Food Toxicology and Nutrition Laboratory (FTNL, designated site FT-A in experiments) zebrafish were spawned and reared in a temperature controlled room at 27 ± 2° C with a 14-hour light/10-hour dark cycle. Conditioned water (CW) for fish rearing and maintenance was produced by passing well water through an ultraviolet sterilization unit, degassing column, and sand and activated carbon filters. This treated water then was buffered to pH 7.2-7.4 with phosphate buffer. Fastidious lines of fish including AB [from the University of Oregon (UO)], Tuebingen (TU from the Tuebingen Stock Center), and mutant lines maintained in those backgrounds were raised from fertilization up to 6 weeks of age in embryo rearing solution (ERS; Westerfield 1995) prepared using water purified by reverse osmosis. Other lines of fish including Florida wt (5-D Tropical), Tuebingen or Tupfel *long fin* ^{dt2} [*cx41.8*^{t1}(*leopard*);*long fin* ^{dt2}] [*cx41.8*^{t1} (*leo*^{tl});*lof*^{dt2}](TL), Cologne (KOLN), TU X AB, and TU X TL showed acceptable survival when raised from fertilization in CW. The Florida wt strain used for carcinogenesis studies at OSU was a *Pseudoloma*-free closed colony obtained from 5-D Tropical Fish, Plant City Florida. At 6 weeks of age, fish were placed into fish tanks receiving CW. Fish groups up to 30 were housed in 40 liter glass tanks. Larger groups were housed in 80-110 liter tanks. Water flow to tanks was intermittent and controlled by a timer activating water flow several times per day to ensure at least 30% replacement of tank volume daily. Airstones aerated each tank. Larvae were initially fed equal parts of Microfeast (Salt Creek, Inc., Salt Lake City, UT), a powdered complete diet, and Encapsulon (Argent Laboratories, Redmond, WA), a microencapsulated larval fish diet 3-5X daily. At 2 weeks of age, Microfeast was discontinued

and brine shrimp nauplii (Silver or Gold Label Argentemia, Argent Laboratories) were added to the diet. At 6 weeks of age, Encapsulon was discontinued, and fish were fed Oregon Test Diet (OTD; Lee et al. 1991) twice daily *ad libitum* and brine shrimp once daily. For fish raised at the Salmon Disease Laboratory (SDL, site FT-B) at OSU, larvae were reared at the FTNL. At the SDL, husbandry and diet were similar to those at the FTNL. Well water passed through a degassing column. Since the fish room was not heated to optimal zebrafish temperatures, water was warmed initially in a holding tank with a stainless steel immersion heater. For more precise temperature control and safety, each fish tank contained an immersion heater.

Recirculating systems (RC-C) from which we obtained retired broodstock, and at which we conducted prospective tumor studies utilized city water purified by reverse osmosis. System water was buffered to pH 7.5 with calcium carbonate from aragonite sand and conductivity was adjusted to 500 uS using a stock salt solution of 14 kg NaCl, 5 kg $MgCl_2$, 0.8 kg $CaCl_2$, and 0.4 kg KCl. A fluidized sand biofilter was utilized for purification of the system water, with 10% water renewal daily. Fry were initially fed *Paramecium* cultures, which were supplemented with brine shrimp nauplii when fish reached 9 days of age. Juveniles and adults were fed a mixture of commercial diets [Tetra Staple Flake (multiple suppliers), Omega 1 Color Flake (multiple suppliers), Omega 1 First Flake, Golden Pearl (Aquatic Ecosystems, Apopka, FL), Hikari Micro Pellet (Aquatic Ecosystems), Cyclop-eeze (Argent Laboratories)] twice daily *ad libitum* and brine shrimp once daily. In the recirculating systems, fry were fed brine shrimp from San Francisco Bay Brand, Inc. (Newark, CA) and adults were fed brine shrimp from INVE Aquaculture Inc. (Grantsville, UT). Fish rooms were maintained at 28.5 ± 1° C with a light cycle of 14-hour light/10-hour dark. Quarantine rooms for the recirculating systems used the same dietary regimens, but had flow-through water distribution systems supplied with either reverse-

osmosis purified water or water dechlorinated by passage through activated charcoal. Eggs from fish in quarantine rooms were disinfected by immersion for 2 min in 0.5% sodium hypochlorite (Westerfield 2007) prior to entry into the nursery for the main colonies. Retired broodstock were also obtained from another recirculating system (RC-D) for which husbandry procedures were similar to those described above except the conductivity of the system was maintained at 1000 uS, the salt solution for adjusting conductivity contained 35 mg KI in addition to the previously described salts, temperature was 27 ± 0.5° C, and the diet mixture for juvenile and adult fish contained Tetramin Flakes (Foster and Smith Aquatics, Rhinelander, WI), BioDiet Grower pellets (Bio-Oregon, Inc., Warrington, OR), and Silver Cup 3 Pigment diet (Nelson and Sons, Inc., Murray, UT).

Prospective Tumor Study with AB Wt Line

Eggs for all treatment groups were obtained from 30 breeding pairs of AB genetic background maintained in a recirculating system (RC-C described above). Fish were spawned in water from the recirculating system and eggs were maintained in that water for the first 48 hours post-fertilization. At this time, eggs were sorted and unfertilized, dead, and abnormal eggs were discarded. Eggs from all breeding pairs were mixed together. Groups of 80 normal eggs were assigned to each treatment group. Treatment groups 1-8 were transported in coolers to flow-through site A (FT-A) and were raised for the first 6 wk of life at this site. Thus fry in treatment groups 1-8 were not fed *Paramecium* cultures as were fry in groups 9-22. For fish raised at flow-through site B (FT-B), fry were reared at FT-A. At FT-B husbandry and diet were similar to those at FT-A. This experiment was conducted before staff at FT-A had developed extensive experience rearing highly fastidious lines like AB and TU, so that we did not yet have a reverse

osmosis (RO) unit for water purification. This explains why the mortality rate of fry in groups 1-8 was unusually high. Once an RO unit was installed and used to prepare ERS for fastidious fish lines, survival of TU and AB lines at FT-A was good (greater than 50% from hatch to 6 wk). At FT-B more tank overflows occurred than at the other facilities so that more fish from this site were lost as juveniles and adults. At the facility with a recirculating system design (RC-C), eggs were initially reared in plastic petri dishes, and fry were transferred to glass beakers. At 2 weeks of age fry were placed into small custom-made flow-though fry chambers. At 3 wk of age juvenile fish were either placed into 80 L glass fish tanks equipped with individual air stones at RC-C (treatment groups 9-12 and 21-22) or were transported in plastic bags held in coolers to sites FT-A (groups 17-20) of FT-B (groups 13-16). Designated replicate treatment groups were fed either OTD or a mixture of commercial diets (COM) twice daily *ad libitum* and brine shrimp (INVE Aquaculture Inc., Grantsville, UT) once daily. During the experiment, any moribund fish or fish with grossly evident lesions were sampled for histology. We planned to sample fish from all treatment groups at 24 month of age, however, the groups at RC-C began to show a significant incidence of grossly visible lesions and elevated mortality by 22 months, so that we chose to necropsy fish from all treatment groups at that time.

Carcinogen Exposures

Carcinogens including *N*-nitrosodimethylamine (DMN), methylazoxymethanol acetate (MAMA), aflatoxin B1 (AFB1), *N*-ethylnitrosoureas (ENU) were obtained from Sigma Chemical Co. (St. Louis, MO), 7,12-dimethylbenz[*a*]anthracene (DMBA) and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) from Aldrich Chemical Co. (Milwaukee, WI), dibenzo[*a,l*]pyrene (DBP, also called dibenzo[*def,p*]chrysene) from Chemsyn Laboratories

(Lenexa, KS) and *N*-nitrosodiethylamine (DEN) from Fluka Chemical Corp. (Ronkonkoma, NY). Static embryo and fry immersion exposures were conducted in 50 ml or 100 ml dosing solution, respectively, in glass beakers. Typically we used treatment groups of 100-150 eggs or fry. For TU and AB lines, dosing solutions were prepared in ERS made with water purified by reverse osmosis. For other lines, CW was utilized. DMSO at a final concentration of 1% was used as the carrier for most exposures. For ENU, stock solutions were prepared in 11 mM citrate buffer, pH 6. These stock solutions were diluted 1/10 in CW or ERS to prepare dosing solutions. Depending on the carcinogen, exposures lasted 1-24 hr. When exposures were completed, fish were rinsed in 3 changes of CW or ERS, and placed into polypropylene tubs for rearing until 6 weeks of age when they were placed into fish tanks.

For dietary exposures, hydrophilic carcinogens were dispersed into the aqueous component of the OTD mix and hydrophobic agents were combined with the fish oil, using DMSO as a carrier. Most carcinogen-containing diets were fed for 3 or 4 months beginning at 2 months of age. Because of its anticipated greater potency, DBP was fed for just 1 month. Fish treated with carcinogens were typically sampled for histology 6-12 months following the onset of carcinogen exposure. Some of the highly responsive mutant lines of zebrafish have required sampling as early as 3 months post-treatment due to the rapid development of large neoplasms.

Experimental design and procedures conducted at all study sites were approved by each institution's Institutional Animal Care and Use Committee and were consistent with the most recent *Guide for the Care and Use of Laboratory Animals* from the Institute of Laboratory Animal Resources, National Research Council.

Histology Procedures

In carcinogenesis studies with the Florida wt line, fish were anesthetized in tricaine methanesulfonate (MS 222; Argent Laboratories) pH 7.4 in phosphate buffer, the tail was removed, and the belly slit from heart to anus. Fish were fixed in Bouin's fixative for 24 hr. Fish were dehydrated in a graded series of ethanol solutions, then embedded in paraffin. Sagittal step sections were cut from the left side. Three 4-6 micron sections were saved and placed onto a single glass slide, one section through the lens of the left eye, one just medial to the left eye and 1 from midline. Sections were stained with hematoxylin and eosin (H & E). In our recent carcinogenesis bioassays, we have found more optimal detection of neoplasia when both halves of the fish are sectioned for histology. Also, since we are interested in fin tumors, we section the caudal peduncle and caudal fin. We now fix the fish in buffered zinc formalin for 24 hr, decalcify for 48 hr in Cal X II (formic acid/formalin; Fisher Scientific) and save 9 step sections cut between the middle of the lens of the left eye and the middle of the lens of the right eye. Three sections are placed onto each of 3 slides and stained routinely with H & E. This protocol was also used for retired broodstock. For diagnostic cases submitted to the Zebrafish International Resource Center at UO (ZIRC), fish were routinely fixed in Dietrich's fixative, decalcified overnight in 5% trichloroacetic acid in Dietrich's fixative. Fish were bisected for embedding by cutting transversely, just to the left of midline, using a razor blade. The two halves were placed into a single cassette. Detailed histology protocols are available on the ZFIN web site (http://zebrafish.org/zirc/health/diseaseManual.php). Several serial sections were cut and placed onto 2 or more slides as appropriate if special stains for infectious agents were anticipated. If necessary, fish were embedded in dorsal recumbency to best evaluate lesions of spine or opercula.

Statistical Analysis

Body weight and mortality were analyzed using the Generalized Linear Modeling (GENMOD) procedure using SAS software (SAS Institute, SAS OnlineDoc version 9.2, Cary, NC). Patterns of neoplasm incidence were evaluated by logistic regression. In general little evidence of tank effect on endpoints was evident, so fish-level binomial models were fit to the data to evaluate factors and their interactions including location, diet and gender. Comparisons between neoplasm and other lesion incidence in specific treatment groups were analyzed by chi-square or Fisher exact tests as were comparisons of mortality between specific treatment groups (Fleiss et al. 2003). Incidence of total neoplasia, as well as incidence of specific histologic types of neoplasia were analyzed. The influence of sex on odds ratios for neoplasia was evaluated using the Mantel-Haenszel test (Matthews and Farewell 1996). The level of significance for statistical analyses was typically set at alpha = 0.05. In a few cases, we considered alpha = 0.1 as an indication of a significant trend showing a need for follow-up studies with larger numbers of animals.

Experimental Results and Observations from Diagnostic Cases and Retired Broodstock

Influence of Water Systems and Diet on Spontaneous Neoplasia

Diagnostic cases submitted to ZIRC from around the world, retired broodstock from various sources in the U.S., and prospective studies of tumor incidences in 2-year-old zebrafish raised under various husbandry and diet protocols clearly showed that both age-specific tumor incidences and the histologic spectrum of neoplasia seen in zebrafish were strongly influenced by water system and diet. Zebrafish raised in the flow-through aquaculture system at OSU's FTNL, where they were fed a semi-purified diet--Oregon Test Diet (OTD)--used for over 30

years in carcinogenesis studies in fish (Lee et al. 1991), showed much lower age-specific tumor incidences than zebrafish fed commercial diets and/or raised in recirculating aquaculture systems. Only certain recirculating systems were associated with elevated tumor incidences and these systems all had fluidized sand biofilters. In those systems where elevated tumor incidences occurred in zebrafish, these incidences were highly variable over time within a given line such as AB wt. In addition to neoplasia, commercial diets and recirculating aquaculture systems were associated with hepatic megalocytosis, a lesion indicative of toxicant damage to DNA or the mitotic apparatus (Haschek and Rousseaux 1998; Spitsbergen and Kent 2003). Approximately 50% of tanks of retired broodstock from recirculating systems feeding commercial diets showed hepatic megalocytosis. In megalocytic lesions, hepatocyte cytoplasmic volumes and nuclear volumes were 5-50x normal (Figure 2). Between 3 and 100% of fish in affected lots of broodstock exhibited hepatic megalocytosis, with severity of the lesion in particular fish varying from mild to severe. We have not yet identified the design characteristics of recirculating systems that are associated with the toxicity causing hepatic megalocytosis and elevated tumor incidences, but we have seen these problems only in systems with fluidized sand biofilters. It is clear that toxicity events in these systems are episodic, with some cohorts of wt lines of fish showing no hepatic megalocytosis and low age-specific tumor incidences, while other cohorts born a few days later show high incidences of hepatic megalocytosis and elevated tumor incidences. We also do not know what factors trigger the spikes in toxicants that are observed in recirculating systems. We have not seen hepatic megalocytosis in any untreated control zebrafish of any wt line up to 4 years of age, which were born at the FTNL or SDL, raised in a flow-through system, and fed OTD.

To help clarify the relative roles of diet and water systems in determining spontaneous tumor incidences, we conducted 2-year prospective tumor studies using AB wt fish at 3 sites, feeding replicate fish tanks either OTD or a mixture of commercial diets. Analysis of variance based on body weights of individual fish in treatment groups or average body weight in each group did not indicate significant differences between groups. Average body weights in treatment groups varied from 0.39-0.47 g at 22 months of age. The incidence of total neoplasia at 22 months of age was 13% or less (Table S1) in fish fed either OTD or commercial diet in flow-through systems (A or B). In fish fed OTD in a recirculating system (C), the incidence of total neoplasia was 20-23%, and in fish fed commercial diet in system C, the incidence of total neoplasia was greater than 50%. Only seminomas and a benign neoplasm of the digestive tract (adenoma of pneumatic duct) occurred in fish in systems A or B when fish were raised at these sites from 48 hours of age (Table S2). In fish raised for the first 2 weeks at RC-C then reared at FT-A or FT-B, a few more histologic tumor types occurred including 1 hepatocellular adenoma, 1 malignant peripheral nerve sheath tumor, and one acinar cell carcinoma of exocrine pancreas (Figure2). In systems A or B, tumor-bearing fish had just one tumor each, and neoplasms were small in size (1-4 mm). A 48 hr exposure of eggs to system C was sufficient to cause megalocytosis in fish then reared in system A or B, with megalocytosis being greater in incidence and severity in fish raised for the first 2 weeks in system C (Table S1). In system C, fish showed a wide variety of histologic types of neoplasia including many malignant neoplasms, with several fish having tumors affecting 2 or more separate organ systems. Many of the tumors in system C were large, up to 10 mm in diameter (Table S2; Figure 2). Hepatocyte megalocytosis occurred at a higher incidence and greater severity in fish from system C, particularly those fed COM diet (Table S1).

Spontaneous Neoplasia in Diagnostic Cases and Retired Broodstock

Fish pathologists working with ZIRC have examined diagnostic cases from research laboratories worldwide since 1999. Over 4,000 fish have been evaluated from zebrafish of a wide variety of wt and mutant lines that were moribund, had grossly visible lesions, or were submitted as sentinels for colony health surveillance. The most common tissues showing neoplasia in diagnostic cases were testis, gastrointestinal tract, ultimobranchial gland, and peripheral nerve (Kent et al. 2007; Murray et al. 2012 this issue). Table S3 summarizes the organs affected and the histologic types of neoplasia documented in diagnostic cases. Figures 2, 3, and S1-S3 illustrate some of the neoplasm types occurring in diagnostic cases and retired broodstock. Some remarkable findings among diagnostic cases include hepatocellular carcinomas, large, highly invasive malignant peripheral nerve sheath neoplasia, and a large carcinoma of ultimobranchial gland (20X normal size) invading the sinus venosus occurring in fish of the wt AB line younger than one year of age, when raised in a recirculating aquaculture system and fed commercial diet. One group of 10 sentinel AB line fish showed a 50% incidence of intestinal neoplasia. These fish were over one year of age and were housed in a tank collecting effluent from all fish tanks in a recirculating system. This colony was free of intestinal nematode parasites. We have not yet induced intestinal neoplasia at an incidence of over 16 % in our studies with carcinogens in any line of zebrafish raised at the FTNL.

We have examined over 2000 retired broodstock of various wt and mutant lines from 6-41 months of age from flow-through and recirculating systems. Most of the fish were raised in systems in which they were fed *Paramecium* cultures in the nursery and fed commercial diets as juveniles and adults. Fewer cohorts of fish in this sample were fed sempurified diets. Except for strains such as TL that are unusually susceptible to unique histologic types of neoplasia, the

influence of genetic strain on neoplasia has been confounded by the potent but episodic effects of natural carcinogens in the recirculating water systems. To distinguish environmental effects from genetic influences on spontaneous tumors in aquaculture systems in which spikes of natural carcinogens occur intermittently, one would need to always have a paired wt control of identical genetic background born and raised at the same time under identical conditions with any mutant line. In fish raised in recirculating systems, the incidences of total neoplasia in cohorts of retired broodstock varied widely in both wt and mutant lines, from 0-67%. The majority of tanks of retired broodstock from recirculating systems (40/53; 75%) showed neoplasia of at least one histologic type. About half of the tanks of fish showing neoplasia also showed hepatocyte megalocytosis. Consistent with our hypothesis of exposure of fish to episodic occurrences of spikes of carcinogens in recirculating systems is our observation that within particular systems that we studied, as specific wt or mutant lines aged, neither hepatocyte megalocytosis nor the incidence of total neoplasia increased in a predictable fashion. Also the histologic types of neoplasia occurring in specific wt or mutant lines in a certain recirculating system varied from cohort to cohort, unrelated to the age at evaluation. For example, in AB wt, in some cohorts, liver neoplasia predominated, but in other cohorts, intestinal neoplasia occurred at higher incidences. This suggests that the putative mixture of natural toxicants causing hepatocyte megalocytosis and elevated neoplasm incidences is variable in composition over time within a given system, with some episodes causing primarily hepatocyte megalocytosis, some episodes causing primarily liver neoplasia, some causing principally intestinal neoplasia, and some causing all of these lesions in addition to other types of neoplasia. The oldest fish of any line were not more likely to have either hepatocyte megalocytosis or high incidences of any type of neoplasia. We have not been able to pinpoint the factors that predict when spikes of carcinogens will occur in specific

systems. Neither hepatocyte megalocytosis nor elevated tumor incidences occured more commonly in fish born on certain days of the week. The most common neoplasm occurring in retired broodstock, regardless of age, was seminoma. Up to 100% of males over 1.5 year of age showed seminomas, with much variation from tank to tank in seminoma incidences within a given genetic line. These seminomas were among the largest neoplasms we studied, some being 14 mm in diameter and weighing half of the body weight of the affected fish. Neoplasms of liver and intestine occurred in about half as many tanks of retired broodstock as seminomas, and generally at lower incidences per tank. The majority of liver neoplasms were hepatocellular adenomas, and most intestinal neoplasms were small cell carcinomas with fewer adenocarcinomas. Neoplasms of ultimobranchial gland were the fourth most common neoplasms in tanks of retired broodstock. As in diagnostic cases, a wide variety of histologic types of neoplasia occurred in various organs at low incidences in retired broodstock. Histologic types of neoplasia seen in retired broodstock but not in diagnostic cases included 3 papillomas of vent in eggbound females, 1 myxoma of peritoneum near caudal ovary, 1 hemangioma of spleen, 1 osteochondroma of lower jaw, 6 islet cell carcinomas of endocrine pancreas, 1 benign melanocytoma of optic nerve. Some indications that certain strains of fish might be prone to certain tumor types were evident, but additional experiments would be necessary to prove this association. For example, most of the benign and malignant neoplasms of endocrine pancreas (6/10) occurred in wt fish of WIK background or in crosses to this strain. Also an unusually high incidence of seminomas occurred in all cohorts of the *after eight* (*dld*^{tr233}) line (4/4 and 7/9 males from tanks of 17 mo fish, ½ males 26 mo). Table S3 summarizes the types of neoplasia occurring in diagnostic cases and retired broodstock.

We hypothesized that zebrafish lines with fin overgrowth would be more sensitive to spontaneous neoplasia of fins. Studies with the TL line up to 2.7 years of age (1000 controls 6-14 mo from 20 separate carcinogen experiments, 200 broodstock 12-34 mo from FT systems with fish fed OTD and 100 broodstock 19-24 mo from RC systems fed COM) and with the *another long fin* (*alf*^{ty86d}) line up to 4 years of age (400 *alf*^{ty86d} control fish 6-14 mo and 12 alf^{ty86d} broodstock each of 24 mo and 42-49 mo from FT systems with fish fed OTD, 94 *alf*^{ty86d} fish 15-19 mo from RC fed COM) have not indicated increases in spontaneous tumors affecting fins. Incidences of spontaneous skin and fin neoplasia in all lines of fish that we have studied to date are exceeding low—we have not seen a single epithelial skin or fin neoplasm in diagnostic cases, retired broodstock, or control fish from carcinogen experiments except for a few cases of papilloma of the vent. The papillomas of vent occurred exclusively in eggbound females with increased abdominal pressure that caused partial prolapse of the terminal intestine. The protruding vents in these old females become chronically traumatized and show severe hyperplasia or frank papillomas. Close observation of large groups of broodstock over time indicated that the eggbound condition precedes hyperplasia of the vent and vent papillomas. We have not observed a papilloma of the vent in a fish not eggbound.

Spontaneous Neoplasia in Fish Raised in a Flow-Through Aquaculture System and Fed a Semi-Purified Diet

All of the wt lines that we have studied so far have shown a consistently low incidence of spontaneous neoplasia by 14 months of age. Because our initial large-scale carcinogenesis studies were done with the 5-D Florida wt line, the most substantial sample of control fish of any line is available for Florida wt. The spontaneous rate of neoplasia in this line at 6-14 months of

age was 1%, based on 3,000 untreated controls. In these studies the most common spontaneous neoplasms were seminoma, hepatocellular adenoma, and adenoma of exocrine pancreas, with intestinal adenocarcinoma less common. Table S4 shows the numbers of control fish (vehicle and sham control numbers combined) examined histologically from several wt strains at 7 or 13-14 months of age. We observed no neoplasia in any of these control fish.

We have conducted prospective studies to determine the spontaneous tumor rate at 2 years of age with the AB and KOLN wt lines. Our data regarding the AB line are reported in Tables S1 and S2. We raised 4 replicate tanks of 150 KOLN fish for study of spontaneous tumors. Most of the tumors occurring in this line at 2 years of age were hepatic. Although very few of the KOLN fish showed bile duct hyperplasia when sampled at 7-14 months of age, most fish of this line showed mild to moderate locally extensive to multifocal hyperplasia of bile ducts by 2 years of age. This spontaneous bile duct hyperplasia which acts as a tumor promoter probably explains the elevation in spontaneous liver neoplasia in this line at 2 years. The incidence of hepatic neoplasia in the 2-year-old KOLN fish was 20% (80/398), with neoplasms exclusively affecting biliary tissue. Most neoplasms were cholangiocellular adenomas, fewer carcinomas. These hepatic neoplasms were not large enough to observe grossly at necropsy. The incidence of seminomas in KOLN fish was (3/398) 1% in the 398 fish evaluated. Interestingly these seminomas occurred all in one of the 4 tanks studied. These seminomas were 2-8 mm in diameter. Two of these fish with seminomas 6 and 8 mm in diameter required early necropsy at 20 months of age due to distended abdomens.

We evaluated neoplasia in 26 month old AB/TU wt fish raised at the FTNL but reared for the first 2 weeks in system RC-C where they were fed *Paramecium* cultures. Mild to moderate bile duct hyperplasia was evident in the liver of 10/29 (34%) of these fish. However this hyperplasia

was not associated with hepatic neoplasia. The incidence of total neoplasia in this cohort was 10/29 (34%), with 1 adenoma and 1 carcinoma of ducts of exocrine pancreas occurring. The remainder of the neoplasia was comprised of seminomas in 8 of 20 males. These seminomas varied from 1-3 mm in diameter.

Spontaneous Neoplasia in the Tupfel *leopard;long fin* [*cx41.8*^{t1} (*leo*^{tl})*;lof*^{dt2}](TL) Line

In diagnostic cases from laboratories with recirculating aquaculture systems and/or feeding commercial diets, we have observed unique patterns of neoplasia in the TL line. In fish just 9-10 months old we have observed highly anaplastic thyroid masses or ultimobranchial adenomas 50X normal gland size. Among our diagnostic cases, nearly all of the thyroid neoplasia, including several widely disseminated malignant follicular thyroid adenocarcinomas have occurred in the TL line. Nearly all of the nephroblastomas that we have seen in any studies have occurred in the TL line or in lines containing TL in their genetic background. In a sample of 20 retired TL broodstock 27 months of age from a recirculating system, we observed 2 nephroblastomas. Interestingly, to date, we have not yet observed nephroblastoma in any line, including the TL line, treated with carcinogens. Sensory neural neoplasms of nose (esthesioneuroepithelioma or esthesioneurblastoma) observed in diagnostic cases occurred primarily in fish of the TL line or with TL in the genetic background. A large hemangioma of choroid gland and retrobulbar benign peripheral nerve sheath neoplasia occurred in diagnostic cases of fish just 1-1.5 years of age of the TL line. In the TL line raised in a flow-through system and fed commercial diet in 6 separate tanks, the incidence of seminomas in males at 1.5 year of age was 9/33 (27%). Spontaneous hyperplasia of bile ducts occurs in the TL line from most laboratory stocks (see Discussion section), regardless of diet and water system, with 100%

of the fish showing mild to severe multifocal to diffuse lesions by 1 year of age in affected stocks (Spitsbergen and Kent 2003). This bile duct hyperplasia predisposes the TL line to an elevated incidence of spontaneous liver neoplasia. Approximately 10% of fish show biliary neoplasia by 1.5 year of age.

We have conducted prospective studies of spontaneous tumor incidences at 2 years of age with TL line fish in a flow-through aquaculture system feeding OTD. Among 215 fish sampled, 8 showed seminomas, with seminoma incidences in males varying from 0% to 29% in particular tanks (0/63, 1/21, 5/17, 2/25). Seminomas did not exceed 4 mm in size. An ovoid white 3mm mass near the first gill arch in a fish sampled early at 22 months of age was a thymic lymphoma, a neoplasm that we have not before observed in untreated zebrafish of wt lines or in younger fish of the TL line housed in flow-through systems and fed OTD. The incidence of liver neoplasia in 2-year-old TL line fish was 28/215 (13%), consisting primarily of cholangioma and cholangiocarcinoma, with occasional hepatocellular adenoma. Intestinal neoplasia, mucosal adenocarcinoma, occurred at an incidence of 2/215 (1%). Ultimobranchial adenoma occurred by 13 months of age in a fish sampled early due to ascites. In this case the spleen was greatly enlarged and showed cystic degeneration due to passive congestion caused by restriction of venous return by the large neoplasm. The incidence of ultimobranchial neoplasia was 2/215 (1%). Other neoplasm types occurred rarely including chordoma (4/215; 2% incidence) and fibroma of skull (2/215; 1% incidence).

Carcinogen-Induced Neoplasia

Zebrafish were the first fish species in which laboratory experiments conducted in the 1960's confirmed that carcinogens active in mammals cause neoplasia in fish (Stanton, 1965; Stanton,

1966). Yet, until the past 15 yr little additional carcinogenesis research utilized the zebrafish (Khudoley 1984; Pliss and Khudoley 1975; Pliss et al. 1982). Recent studies conducted at OSU exposing 5-D Florida wt zebrafish to a panel of structurally diverse carcinogens including DMBA, MNNG, DEN, DMN, MAMA, and AFB1 by bath exposure as eggs or 2-3 week old fry, and by dietary exposure beginning at 2 months of age showed that zebrafish are quite responsive to most carcinogens when treated as eggs or fry (Hendricks 1996; Tsai 1996; Spitsbergen et al. 1997). Like other small aquarium fish species treated with carcinogens, zebrafish show a wide variety of target organs and develop a diversity of histologic types of neoplasia following carcinogen exposure, including epithelial, mesenchymal, neural and neural crest tumors. Zebrafish are unusually resistant to carcinogenic effects of AFB1 when treated as eggs, fry or 2-month-old juveniles. OSU scientists conducted dietary studies in 5-D Florida wt zebrafish with DBP, the most potent polycyclic aromatic hydrocarbon carcinogen in mammals and rainbow trout. In these dietary studies with DBP, carcinogen-treated zebrafish showed a tumor rate barely above that of controls, with only a small number of very unusual neoplasms occurring including nasal esthesioneuroblastoma, ganglioglioma of optic nerve, and chordoma of the spine (Reddy et al. 1999b). In the 5-D Florida wt line, the greatest diversity of histologic types of neoplasia occurred with DMBA and MAMA, with 27 and 23 histologic types of neoplasia observed, respectively. Recent studies by Keith Cheng's group at Pennsylvania State College of Medicine report a 100% incidence of cutaneous papillomas occurring in 18 zebrafish of the Florida wt line within 1 year following 3 adult bath exposures to 2.5-3 mM ENU (Beckwith et al. 2000).

Our recent carcinogenesis studies at OSU focused on identification of mutant lines of zebrafish highly sensitive to carcinogens. We also compared the responses of various wt and mutant lines of zebrafish to 2 carcinogens, AFB1 and DBP, to which the 5-D Florida wt line was

relatively resistant. One of our goals was to develop lines of zebrafish that are efficient models for sensitive carcinogenesis bioassays shorter than the standard lifetime studies currently utilized by the National Toxicology Program. Ideally, we would like lines with low background tumor incidences by 6 months of age, but which develop relatively high incidences of neoplasia in response to a panel of structurally diverse carcinogens by 6 months post-treatment. Another goal was to clarify the factors that control strain-specific variations in response to certain carcinogens. Toward this end, we obtained antibodies to new cytochrome P450 (CYP) enzymes from zebrafish, and investigated the activities of these CYP enzymes in early life stages and adult zebrafish. The wt lines that we have tested so far are less responsive to DBP than to DMBA, typically showing much lower incidences of liver neoplasia and other histologic types of neoplasia at 6-12 months following fry bath exposure to DBP. These findings are surprising in light of the fact that DBP is a more potent carcinogen than DMBA in mammals (Higginbotham et al. 1993) and rainbow trout (Reddy et al. 1999b; Williams et al. 2003). To date, our immunohistochemistry studies of CYP expression in various tissues of fry of different wt and mutant zebrafish strains has not indicated significant strain-specific differences in expression of these enzymes in untreated fish, in fish treated with the inducer beta naphthoflavone, or in fish treated with carcinogens. Table S5 summarizes the target organs and tissues that we have documented at OSU in carcinogen studies with wt and selected mutant lines of zebrafish.

We identified two mutant lines of zebrafish showing unusually high incidences of hepatic neoplasia compared to wt lines following treatment with DMBA (Spitsbergen et al. 2004). One of these lines, *uma*^{s2068} shows 100% incidence of liver neoplasia at 1 year following fry bath treatment to DMBA and is also quite responsive to DBP, showing 50-70% incidences of liver neoplasia by 1 year following fry bath treatment with 0.6-1.25 ppm. This line develops a

relatively high incidence of spontaneous myelodysplastic syndrome compared to its TL genetic background strain. This sensitive line also shows large neuroblastomas affecting brain and eye, as well as large, grossly visible liver, ultimobranchial and vascular neoplasia by 3 months post-treatment when given bath exposure to DMBA at 3 weeks of age. The *another long fin* (*alf*^{ty86d}) in AB/TU genetic background shows a high incidence of myelodysplastic syndrome, but only following treatment with relatively high doses of DMBA. This second line is much less responsive to DBP than to DMBA.

We hypothesized that zebrafish lines with fin overgrowth would be more sensitive to carcinogen-induced neoplasia of fins. No evidence of carcinogen-induced fin tumors has been observed in the TL line. In the *alf*^{ty86d} line, we have seen an upward trend in tumors of fins with early life stage exposure to MNNG, DMBA or DBP. We observed a teratoma 1 year post-treatment at the base of the caudal fin in 1/10 zebrafish given bath exposure to 2.5 ppm MNNG at 3 weeks of age. We observed a hemangioma of dorsal fin in 1/37 zebrafish 1 year following immersion treatment with DMBA at doses from 0.6-5 ppm. Among control *alf*^{ty86d} fish in this experiment 0/30 showed fin tumors, so although a trend toward elevation in tumors is observed with MNNG and DMBA, these results are not significant using chi-square or Fisher's exact tests with Type I error set at 0.05. One year following bath treatment with 2.5 ppm DBP at 3 weeks of age 5/70 fish had vascular neoplasms on the caudal fin or at the base of the anal fin, while 0/37 control fish had fin neoplasms. This difference in fin tumor incidences between treated and control fish is significant using the chi-square test if Type I error is set at 0.1 (P=0.096). To try to induce skin papillomas like those described by Beckwith et al. (2000), we exposed the TL line and 2 wt lines to the maximum tolerated dose of ENU. Following bath treatment of early life stages of the TL (1 treatment at 3 weeks of age), TU (3 treatments at 3, 5 and 7 weeks of age) or

Cologne (1 treatment at 3 weeks of age) lines to 2.5 mM ENU, epithelial skin or fin neoplasms were not observed at 1 year post-treatment in the TL or Cologne lines, or at 1 and 2 years post-treatment in the TU line. However, this regimen of early life stage exposure to ENU was clearly carcinogenic to TL, Cologne and TU lines, with hepatic, neural, and/or vascular neoplasia occurring in ENU-treated fish of these lines, but not in control fish (Table S5).

We investigated the pathogenesis of neoplasms of the zebrafish intestine in our immunohistochemistry studies. Zebrafish intestinal neoplasia differs from that of humans and other mammals in that most neoplasia of zebrafish, whether spontanteous or induced with experimental carcinogen treatment, occurs near the anterior end of the intestine in the transition zone from distal esophagus to the ~~intestineal~~intestinal bulb, in the intestinal bulb, or in the region of the ampulla of Vater where bile and pancreatic ducts enter the intestine just distal to the intestinal bulb. In contrast, in mammals, neoplasia occurs throughout the intestine, including the distal colon and rectum (Riddell et al 2003; Whiteley et al 1996). However, the ampulla of Vater in humans is the most common location for the occurrence of carcinomas in the small intestine. Albores-Saavedra et al (2000) speculate that such regions of transition between various histologic types of epithelium are inherently more unstable and prone to neoplasia than other sites. So we evaluated rates of cell proliferation in different regions of zebrafish intestine to see whether high rates of cell proliferation occur in those areas most prone to neoplasm development. Tissues with high cell proliferation are often highly sensitive to carcinogen-induced neoplasia because cell proliferation acts to fix mutations in the genome and cell proliferation acts as a tumor promoter (Pan et al. 2011). Using proliferating cell nuclear antigen (PCNA) as a marker of cells actively moving through the cell cycle, we showed that almost no cell proliferation occurred in those areas of anterior gut that are most prone to neoplasm

development in zebrafish (Figure 6). However, our studies of expression of certain CYP enzymes in the tissues of 3 wk old zebrafish indicated that the areas of anterior intestine that are most susceptible to neoplasm development also express much higher levels of expression of certain key CYP proteins such as Cyp3a27 than other regions of the gastrointestinal tract (Corley-Smith, et al. 2006; Taylor 2005; Wang-Buhler et al. 2005a and b).

Figure S1; Spitsbergen et al

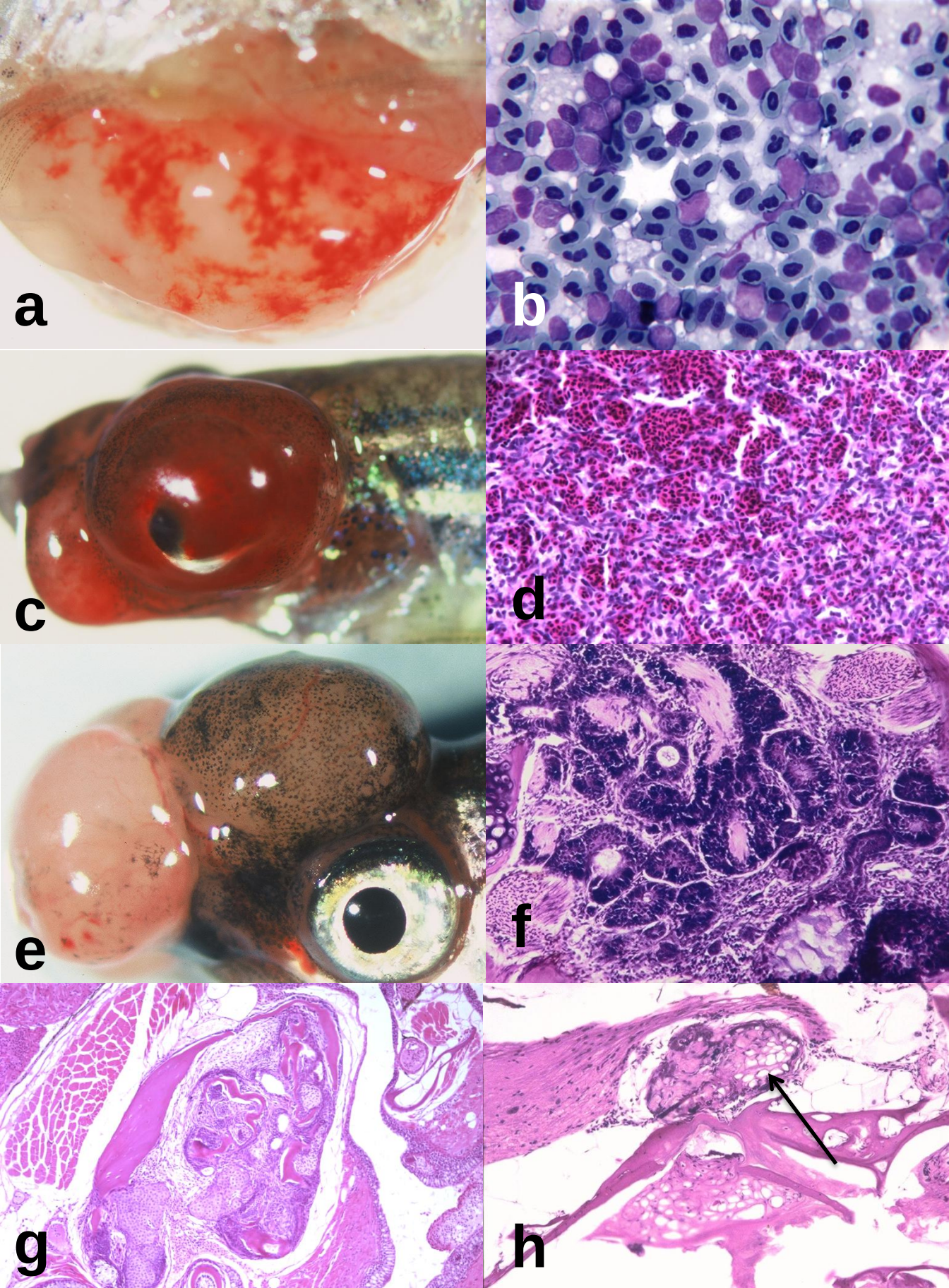
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Figure S2; Spitsbergen et al

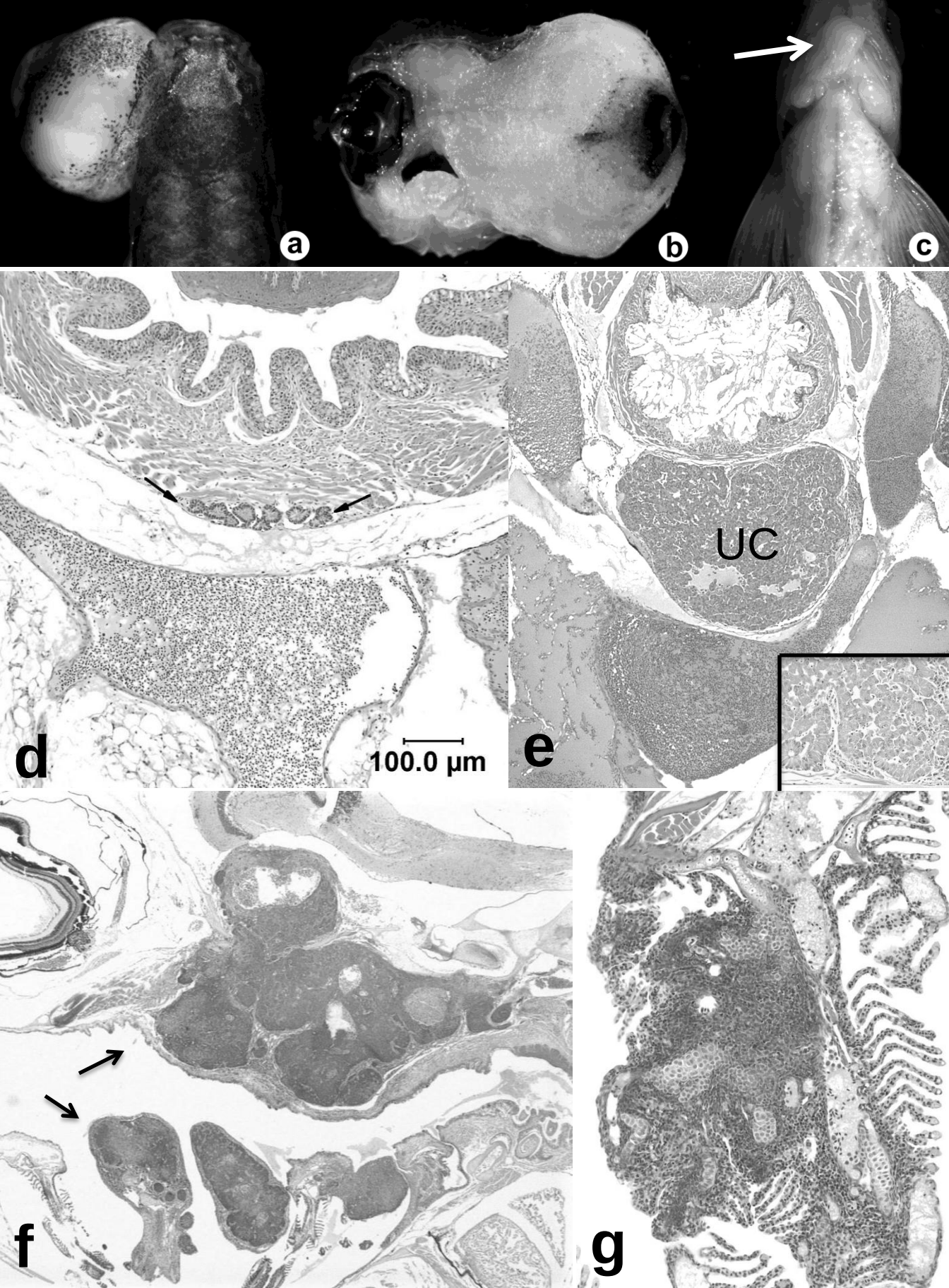
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Fig S3; Spitsbergen et al

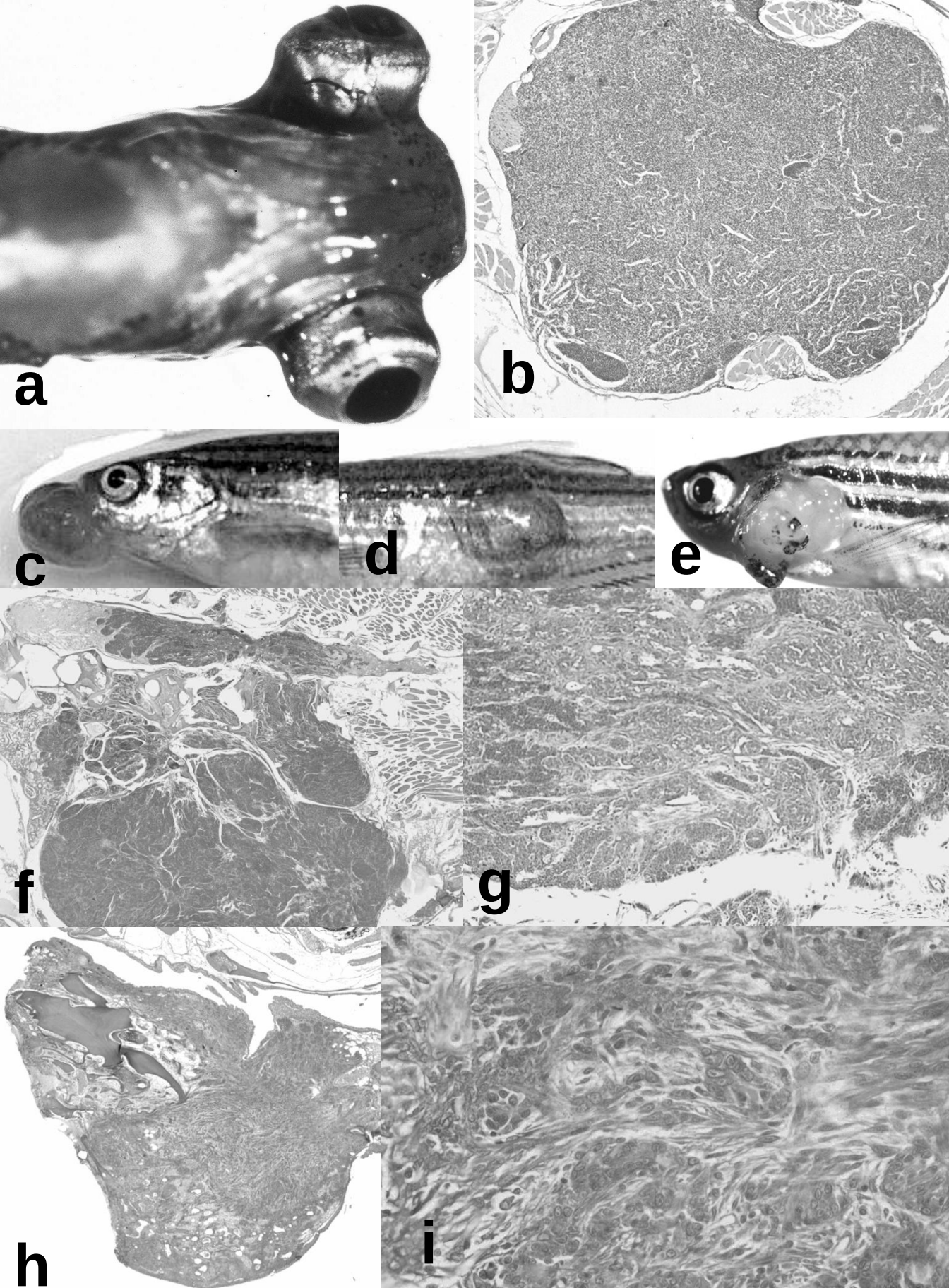
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Table S1 Analysis of overall neoplasm incidences, mortality and hepatocyte megalocytosis in various treatment groups with different diet and husbandry regimens.

Lot #	Age at sampling (mo)	Diet and husbandry regimen	Total neoplasia (all histologic types; %)	Fish with >1 histologic type of neoplasia	Fish with 2 histologic types of neoplasia	Fish with 3 or more histologic types of neoplasia	Mortality (%)	Sex Ratio M/F (% M)	Hepatocyte megalocytosis (%;severity)
1+2	22	OTD[a]; FT-A[b]	1/24 (4%)	0/24			85	17/7 (71%)	4/24 (17%;1+)[c]
3+4	22	COM;FT-A	0/24 (0%)	0/24			85	14/10 (58%)	3/24 (13%;1+)
5+6	22	OTD;FT-B	1/10 (10%)	0/10			94	7/3 (70%)	2/10 (20%;1+)
7+8	22	COM;FT-B	1/8 (13%)	0/8			95	7/1 (89%)	4/8 (50%;1+)
9	22	OTD;RC-C	11/48 (23%)	3/48 (6%)*	2/48 (4%)	1/48 (2%)	40	34/14 (71%)	41/48 (85%;1-3+)[d]
10	22	OTD;RC-C	9/46 (20%)	0/46 (%)*			43	25/21 (54%)	43/46 (93%;1-3+)
11	22	COM;RC-C	34/59 (58%)	11/59 (19%)*	9/59 (15%)	2/59 (3%)	26	32/27 (54%)	59/59 (100%;1-3+)[e]

12	22	COM;RC-C	25/49 (51%)	7/49 (14%)*****	5/49 (10%)	2/49 (4%)	39	33/16 (67%)	49/49 (100%;1-3+)
13	22	OTD;FT-B	4/33 (12%)	0/33			59	14/19 (42%)	20/33 (61%;1+)[†]
14	22	OTD;FT-B	1/39 (3%)	0/39			51	20/19 (51%)	20/39 (51%;1-2+)
15	22	COM;FT-B	1/33 (3%)	0/33			59	22/11 (67%)	27/33 (82%;1-2+)
16	22	COM;FT-B	2/35 (6%)	0/35			56	18/17 (51%)	21/35 (60%;1+)
17	22	OTD;FT-A	1/56 (2%)	0/56			30	32/24 (57%)	35/56 (63%;1-2+)
18	22	OTD;FT-A	1/60 (2%)	0/60			25	26/34 (43%)	36/60 (60%;1-2+)
19	22	COM;FT-A	0/65 (0%)	0/65			19	32/33 (49%)	45/65 (69%;1-2+)
20	22	COM;FT-A	0/53 (0%)	0/53			34	21/53 (40%)	34/53 (64%;1+)
21	24	COM;RC-C	28/45 (62%)	5/45 (11%)*****	3/45 (7%)	2/45 (4%)	44	29/16 (64%)	45/45 (100%;1-3+)[g]
22	24	COM;RC-C	6/15 (40%)	3/15 (20%)*****	2/15 (13%)	1/15 (7%)	81	10/5 (67%)	15/15 (100%;1-3+)

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[a] Diet: OTD = Oregon Test Diet; COM = mixture of commercial flake and pellet diets.

[b] Husbandry system: FT-A = flow-through design site A; FT-B = flow-through design site B; RC-C = recirculating design site C.

[c,d,e,f,g] Hepatocye megalocytosis incidences were significantly different when comparing lots 1-8 with lots 13-20 (chi-square with Yates' correction; P=0.0000) and when comparing all lots from flow-through systems (1-8 and 13-20) with lots 9-12 and 21, 22 from the recirculating system. Hepatocyte megalocytosis incidences were higher in fish fed COM compared to OTD at site C (chi-square with Yates' correction; P=0016). Severity of hepatocyte megalocytosis: 1+=mild, 2+=moderate, 3+=severe

***** Numbers of fish with greater than 1 histologic type of neoplasm were significantly increased in RC-C, regardless of diet, in comparison to FT-A and FT-B (chi-square test, P=0.0000 with Yates' correction).

Table S2 Tissue-specific incidences of neoplasia, morphologic diagnoses, and neoplasm sizes.

Lot #	Liver (L) [a] Neoplasia;MDX[b]	Intestinal (I) Neoplasia;MDX	Ultimobranchial; MDX	Seminoma (SM[c]; Fraction of Males)	Pancreas;MDX	Other Neoplasia	Size of Neoplasms
1+2	0/24 (0%)[d]	0/24 (0%)	0/24 (0%)	0/17 (0%)	0/24 (0%)	1/24 pneumatic duct: adenoma	Pneumatic duct: AD <1mm
3+4	0/24 (0%)	0/24 (0%)	0/24 (0%)	0/14 (0%)	0/24 (0%)		
5+6	0/10 (0%)	0/10 (0%)	0/10 (0%)	1/7 (14%)	0/10		T: SM 4 X 3 mm
7+8	0/8 (0%)	0/8 (0%)	0/8 (0%)	1/7 (14%)	0/8		T: SM 2 mm
9	3/48 (6%);2HA; 2HA;several HA, HC, HB	1/48 (2%); SMCC bulb to amp	0/48 (0%)	5/34 (15%)	1/48 (2%); Exo pan:ACC	1/48 spine: chordoma invading intestine; 1/48 vent: papilloma	Exo pan: ACC 1 mm; I: SMCC 2mm; L: HA 1/4-2 mm, HC 2 mm; HB 1/2 mm; Spine: chordoma 2mm; T:

							SM 2-6 mm; Vent: papilloma 1 mm
10	3/46 (7%); HA;HC;HA	2/46 (4%);1 SMCC bulb;1 SMCC midgut	1/46 (2%); ULAD	2/25 (8%)	0/46	1/46 distal esophagus: SCC	Dist esoph: SCC 1 mm; I: SMCC 1-2 mm; L: HA 1mm, HC 1/2 mm; T: SM 2-3 mm; UL gland: ULAD 1.5 mm
11	21/59 (36%);18 HA;5HC	6/59 (10%);2 AC (amp;);4 SMCC (3 amp;1 midgut)	1/59 (2%); ULAD	4/32 (13%)	7/59 (12%); Exo pan:5 ACC; 1 Pan ductal AD; 1 Pan ductal CA	1/59 distal esophagus: AC; 1/59 ventricle of heart: rhabdomyoma; 1/59 multicentric lymphoma; 1/59 lymphomyeloid system: erythroleukemia	Dist esoph: SCC 1 mm; Exo pan: ACC up to 10 mm; Pan ductal AD 1.5 mm; Pan ductal CA 1/2 mm; ; Ht: rhabdomyoma 1mm; L: HA up to 6 mm; HC up to 3 mm; I: SMCC up to 2 mm; I: AC up to 1/2 mm; T:SM 4-10 mm; UL

							gland: ULAD 0.5 mm
12	14/49 (29%);11 HA;3 HC;1 HB; 1 BC	8/49 (16%);5 SMCC (3 bulb;1 amp, 1 midgut);3 AC (2 amp, 1 midgut)	2/49 (4%); 2 ULAD	5/33 (15%)	0/49 (0%)	1/49 upper jaw: fibroma; 1/49 heart, pericardium of bulbus: hemangioma; 1/49 ventricle of heart: rhabdomyoma; 1/49 distal esophagus SCC	Ht: rhabdomyoma 1mm; I: SMCC up to 3 mm; L: HA up to 4 mm; BC 6 mm; T:SM 2-5 mm
13	1/33 (3%);1HA	0/33 (0%)	0/33 (0%)	3/14 (21%)	0/33		L: HA 3/4 mm; T: SM 2 mm
14	0/39 (0%)	0/39 (0%)	0/39 (0%)	1/20 (5%)	0/39 (0%)		T: SM 1mm
15	0/33 (0%)	0/33 (0%)	0/33 (0%)	1/22 (5%)	0/33		T: SM 3mm
16	0/35 (0%)	0/35 (0%)	0/35 (0%)	1/18 (6%)	0/35	1/35 abdominal viscera: MPNST	Abdominal viscera: MPNST 7mm; T: SM

							1mm
17	0/56 (0%)	0/56 (0%)	0/56 (0%)	0/32	1/56 (2%); Exo pan:ACC		Exo pan: ACC 4 mm
18	0/60 (0%)	0/60 (0%)	1/60 (2%); ULAD	0/26	0/60		UL gland: ULAD 1 mm
19	0/65 (0%)	0/65 (0%)	0/65 (0%)	0/32	0/65 (0%)		
20	0/53 (0%)	0/53 (0%)	0/53 (0%)	0/21	0/53 (0%)		
21	16/45 (36%);10 HA;9 HC;2 HB	2/45 (4%);1 SMCC amp;1 AC bulb to amp	0/45 (0%)	8/29(28%)	4/45 (9%); Exo pan: 4 ACC	1/45 gut/liver: granulocytic sarcoma; 1/45 ventricle of heart: rhabdomyoma; 1/45 ovary: myxoma ; 1/45	Exo pan: ACC up to 8 mm; Gut/liver: granulocytic sarcoma 6 mm; Ht: rhabdomyoma up to 1.5 mm; I: AC 2 mm;

						lower jaw: chondroma	L: HA up to 5 mm; HC up to 8 mm; HB 1 mm; Lower jaw chondroma 2 mm; Ovary: myxoma 2 mm; T: SM 2-8 mm
22	5/15 (33%);4 HA;3 HC;1 HB	0/15 (0%)	1/15 (7%); ULAD	0/10 (0%)	1/15 (7%); Exo pan: ACC	1 skel m., trunk: RMSA	Exo pan: ACC 4 mm; L:HA up to 4 mm; HC up to 8 mm; HB up to 8 mm; RMSA 4 mm

[a] Abbreviations of organ, region or tissue of tumor location: I=intestine; bulb=intestinal bulb just distal to junction of intestine and esophagus (zebrafish are agastric); amp=ampulla of Vater distal to bulb; L=liver; Exo pan=exocrine pancreas; skel m.=skeletal muscle; UL=ultimobranchial gland

[b] MDX=morphologic diagnosis

[c] Abbreviations of morphologic diagnoses of tumor types: HA=hepatocellular adenoma; HC=hepatocellular carcinoma; HB=hepatoblastoma; SMCC=small cell carcinoma of intestine; AC= adenocarcinoma; ULAD=adenoma of ultimobranchial gland; ACC=acinar cell carcinoma of exocrine pancreas; MPNST=malignant peripheral nerve sheath tumor; Pan ductal AD=adenoma of duct of pancreas; Pan ductal CA=carcinoma of duct of pancreas; RMSA= rhabdomyosarcoma; SM=seminoma; SCC=squamous cell carcinoma. In treatment groups having less than 5 neoplasms, the morphologic diagnosis is listed for each tumor. In groups of fish having more neoplasms, the total numbers of each histologic type are listed.

[d] Statistical analyses of tissue-specific tumor incidences. The incidence of liver neoplasia in fish from flow-through systems (FT-A, FT-B) was much less than that in RC-C ($p<0.0001$). Within the RC-C raised fish, there was evidence of both additive diet effects ($p<0.0001$, with incidence when fed OTD less than when fed COM) and gender effects ($p=0.004$ with incidence in males less than in females) with no evidence of nonadditivity of theses effects ($p=0.35$ for gender-by-diet interaction). Intestinal neoplasia occurred only in the RC-C system. Within fish in the RC-C system, there was no evidence of either consistent (additive) diet or gender effects on intestinal neoplasia($p=0.06$ for both factors). Ultimobranchial neoplasia was rare and primarily found in the RC-C system (4 of 5). These low numbers provide only suggestive evidence of a difference between the three husbandry systems ($p=0.096$ exact p-value). No evidence of consistent differences between diets or genders in incidences of ultimobranchial neoplasia was evident within the RC-C system ($p>0.4$ all effects). Seminoma incidences differed significantly at the 3 husbandry locations ($p<0.0005$). Compared pairwise, both FT-B and RC-C had higher incidences than FT-A ($p=0.0036$ and $p<0.0001$, respectively). Seminoma incidences at FT-B and RC-C did not differ significantly ($p=0.36$). Neoplasia of exocrine pancreas did not show consistent differences between treatment groups in lots 9-20.

Table S3 Relative incidence of specific histologic types of neoplasia in various wild-type and mutant lines of zebrafish

Organ System, Tissue, and Morphologic Diagnosis of Neoplasm				Spontaneous neoplasia in diagnostic cases, broodstock, sentinels or prospective tumor studies				Carcinogen-induced neoplasia		Mutant model
				Diet/Husbandry FT-OTD[1]		Diet/Husbandry RC-COM[2]				Transgene or tumor suppressor deletion or inactivation
Organ system	Organ	Tissue or cell type	Morphologic diagnosis	Wt lines	Mutant lines	Wt lines	Mutant lines	Wt lines	Mutant lines	
Skin and subcutis	Skin	Keratinocyte	Papilloma (exophytic)			R[2,3]	R[3]	R[4] - Florida wt ENU		
			Inverted papilloma					R- Florida wt DEN		
			Squamous cell carcinoma						R	
		Fibroblast	Fibroma					R	R	
			Fibrosarcoma					RC-DEN, DMBA, MAMA, MNNG	RC-DBP, DMBA, MNNG	
		Pluripotential mesenchymal cell	Spindle cell sarcoma			R	R	R-ENU	R	
		Blood vessel, subcutis	Hemangioma		R-*alf*	R	R	R-ENU	RC- *alf* DMBA	
	Fin	Keratinocyte	Papilloma (exophytic)					R[4] - Florida wt ENU		
		Blastema	Teratoma					R	R	
		Blood vessel, subcutis	Hemangioma		R-*alf*				RC- *alf* DMBA	
			Hemangiosarcoma					R-MAMA		
Gastro-	Oro-	Blood vessel,	Hemangioma					R	R	

Table S3 Relative incidence of specific histologic types of neoplasia in various wild-type and mutant lines of zebrafish

Organ System, Tissue, and Morphologic Diagnosis of Neoplasm				Spontaneous neoplasia in diagnostic cases, broodstock, sentinels or prospective tumor studies				Carcinogen-induced neoplasia		Mutant model
				Diet/Husbandry FT-OTD[1]		Diet/Husbandry RC-COM[2]				Transgene or tumor suppressor deletion or inactivation
intestinal	pharynx	propria-submucosa								
		Pharyngeal tooth	Complex odontoma						R-DMBA	
	Esophagus	Mucosal epithelium	Squamous cell carcinoma			R			R	
			Adenoma			R				
			Adenocarcinoma			R			R	
		Smooth muscle	Leiomyoma	R				R-DMBA		
			Leiomyosarcoma	R		R			R-DMBA	
		Pluripotential stem cell	Mixed malignant						R-DMBA	
		Smooth muscle	Leiomyoma					R	R	
	Intestine	Mucosal epithelium	Adenoma			R	R	RC	RC	
			Adenocarcinoma	R		R	R	RC	RC	
			Small cell carcinoma			RC	RC			
		Smooth muscle	Leiomyoma					R-DMBA	R-DMBA	
			Leiomyosarcoma					R-DMBA, MAMA, MNNG	RC-DMBA	
		Gut stem cell	Mixed malignant			R		R	RC-DMBA	
	Vent	Skin epidermis	Papilloma			R	R			
	Gas	Mucosal	Adenoma					R-MNNG		

Table S3 Relative incidence of specific histologic types of neoplasia in various wild-type and mutant lines of zebrafish

Organ System, Tissue, and Morphologic Diagnosis of Neoplasm				Spontaneous neoplasia in diagnostic cases, broodstock, sentinels or prospective tumor studies				Carcinogen-induced neoplasia		Mutant model
				Diet/Husbandry FT-OTD[1]		Diet/Husbandry RC-COM[2]				Transgene or tumor suppressor deletion or inactivation
	bladder	epithelium								
			Papillary adenoma					R	R	
			Papillary adenocarcinoma					R-DMBA		
		Smooth muscle	Leiomyoma						R-DMBA	
	Pneumatic duct	Mucosal epithelium	Adenoma or papillary adenoma	R				ER-DMBA		
	Liver	Hepatocyte	Hepatocellular adenoma	R	R	RC	RC	C	C	C several transgenes
			Hepatocellular carcinoma			RC	RC	RC	RC	C several transgenes
		Cholangiocyte	Cholangiocellular adenoma		R	R	R	RC	RC	
			Cholangiocellular carcinoma		R	R	R	RC	RC	
		Stem cell	Mixed cholangio-cellular/hepato-cellular adenoma					R	R	
			Mixed cholangio-cellular/hepato-cellular carcinoma					RC	RC	C several transgenes
		Embryonal stem cell	Hepatoblastoma					R	C-TL, *alf* DMBA, DBP	
		Pericyte	Hemangiopericytoma					ER-MAMA		
	Pancreas	Acinar cell	Adenoma			R	R	R-DMBA	R-DMBA	
			Carcinoma	R		RC	R	R	R	C certain transgenes
		Duct	Adenoma	R				R	R	

Table S3 Relative incidence of specific histologic types of neoplasia in various wild-type and mutant lines of zebrafish

Organ System, Tissue, and Morphologic Diagnosis of Neoplasm				Spontaneous neoplasia in diagnostic cases, broodstock, sentinels or prospective tumor studies				Carcinogen-induced neoplasia		Mutant model
				Diet/Husbandry FT-OTD[1]		Diet/Husbandry RC-COM[2]				Transgene or tumor suppressor deletion or inactivation
			Carcinoma			R	R	R	R	
			Leiomyosarcoma						R-DMBA	
		Ectopic germ cell in females	Seminoma					R-DMBA		
Cardio-vascular	Heart	Bulbus arteriosus	Hemangioma						R	
		Ventricle	Rhabdomyoma			R		R-MAMA	R-DMBA	
			Hemangiosarcoma						R	
		Blood vessel	Hemangioma			RC	RC	C	C	
			Hemangiosarcoma					C	C	
Musculo-skeletal	Skeletal muscle	Myocyte	Rhabdomyoma							R
			Rhabdomyosarcoma				R	R-MAMA, MNNG	R	C certain transgenes
		Fibroblast	Fibrosarcoma			R		R	R	
	Axial skeleton	Notochord	Chordoma		R	R	R	R	R	
		Vertebra	Osteoma	ER						
		Spine primitive mesenchymal cell	Myxoma						R-*alf, uma* DMBA	
	Appen-dicular skeleton	Fin	Chondrosarcoma					R-MAMA		
	Skull	Bone	Osteoma							
			Osteochondroma	R						
		Periosteal fibroblast	Fibroma					R		
			Fibrosarcoma					R		
Urinary	Kidney	Renal tubule	Adenoma					R-MAMA	R	

Table S3 Relative incidence of specific histologic types of neoplasia in various wild-type and mutant lines of zebrafish

Organ System, Tissue, and Morphologic Diagnosis of Neoplasm				Spontaneous neoplasia in diagnostic cases, broodstock, sentinels or prospective tumor studies				Carcinogen-induced neoplasia		Mutant model
				Diet/Husbandry FT-OTD[1]		Diet/Husbandry RC-COM[2]				Transgene or tumor suppressor deletion or inactivation
			Carcinoma					R-MNNG	R	
		Stem cell	Nephroblastoma			R	RC-TL			R
	Meso-nephric duct		Adenoma					R	R	
Repro-ductive	Ovary	Epithelium	Papillary adenoma							R
			Papillary adeno-carcinoma			R	R		R	R
		Smooth muscle	Leiomyosarcoma				R			
		Mesenchymal cell	Myxoma		R-*koi* in TL	R				
		Stem cell	Mixed malignant				R			
		Germ cell	Dysgerminoma							R
	Testis	Germ cell	Seminoma	C	C	C	C	C-DMBA, MAMA, MNNG, 4-amino-biphenyl	C	C- *brca2*
		Interstitial cell	Interstitial cell tumor							C-*brca2*
Lympho-hemo-poietic	Kidney	Lymphocyte	Disseminated or multicentric lymphoma	R	R	R	R	R-DMBA	R	
		Erythroid stem cell	Erythroleukemia (acute myelocytic leukemia erythroid lineage)		C- *uma* in TL			R-4 amino-biphenyl		
		B lymphocyte	B cell acute lymphocytic leukemia							C transgene
		Granulocytic stem	Granulocytic leukemia		C-*uma*					C transgene

Table S3 Relative incidence of specific histologic types of neoplasia in various wild-type and mutant lines of zebrafish

Organ System, Tissue, and Morphologic Diagnosis of Neoplasm				Spontaneous neoplasia in diagnostic cases, broodstock, sentinels or prospective tumor studies				Carcinogen-induced neoplasia		Mutant model
				Diet/Husbandry FT-OTD[1]		Diet/Husbandry RC-COM[2]				Transgene or tumor suppressor deletion or inactivation
		cell	(acute myelocytic leukemia granulocytic lineage)		in TL					
	Thymus	T lymphocyte	Lymphoma	R	R			R-DMBA		C transgene
			T cell acute lymphocytic leukemia							C transgene
	Spleen	Hemopoietic stem cell	Myelodysplastic syndrome		R		R			
		Lymphocyte	Lymphoma	R	R	R	R			
		Erythroid stem cell	Erythroleukemia		C- *uma* in TL					
		Granulocytic stem cell	Granulocytic leukemia		C-*uma* in TL					C transgene
Central nervous system	Brain	Neuron	Neuroblastoma					R-DMBA	RC- *uma* in TL DMBA	C transgene
		Embryonal neuroectodermal cells	Primitive neuro-ectodermal tumor			R	R			
			Medulloepithelioma							R
		Glial cell	Glioma							C several transgenes
		Glial cell	Glioblastoma							RC several transgenes
	Spinal cord	Neuron	Ganglioglioma					R	R	
		Ependyma	Medulloblastoma					R-TL ENU		
	Optic nerve	Neuron/glia	Ganglioglioma					R-DBP		
		Astrocyte	Glioma							C certain transgenes

Table S3 Relative incidence of specific histologic types of neoplasia in various wild-type and mutant lines of zebrafish

Organ System, Tissue, and Morphologic Diagnosis of Neoplasm				Spontaneous neoplasia in diagnostic cases, broodstock, sentinels or prospective tumor studies				Carcinogen-induced neoplasia		Mutant model
				Diet/Husbandry FT-OTD[1]		Diet/Husbandry RC-COM[2]				Transgene or tumor suppressor deletion or inactivation
		Pineal	Pineoblastoma					R		
Peripheral nervous system	Peripheral nerve	Schwann cell	Benign nerve sheath neoplasm			R	RC	R	R	
			Malignant nerve sheath neoplasm	R	R	R	RC	R	R	C-*tp53* deficient, mutant ribosomal genes
	Spinal ganglia		Ganglioneuroma					R		
Pigment		Melanocyte	Benign melanoma			ER	ER			C several transgenes
			Malignant melanoma				ER			C several transgenes
Sensory organs	Eye	Ciliary body or retinal neuro-epithelium	Medulloepithelioma					R-DBP		C certain transgenes
			Retinoblastoma							C certain transgenes
		Primitive neuroectodermal cells	Primitive neuro-ectodermal tumor			RC	RC			C certain transgenes
		Iris	Glioma				R-*erb3b* (*pic*)			
		Sclera	Chondroma					R-DMBA	R-DMBA	
			Chondrosarcoma					R-DMBA		
			Osteochondroma					R-MAMA		
		Choroid vascular plexus	Hemangioma			R	R	RC	RC	

Table S3 Relative incidence of specific histologic types of neoplasia in various wild-type and mutant lines of zebrafish

Organ System, Tissue, and Morphologic Diagnosis of Neoplasm				Spontaneous neoplasia in diagnostic cases, broodstock, sentinels or prospective tumor studies				Carcinogen-induced neoplasia		Mutant model
				Diet/Husbandry FT-OTD[1]		Diet/Husbandry RC-COM[2]				Transgene or tumor suppressor deletion or inactivation
			Hemangiosarcoma			R		RC	RC	
	Nose	Sensory neuro-epithelium	Esthesio-neuroepithelioma				R			
			Esthesio-neuroblastoma		R-TL		R	R	RC-*alf* MNNG	
Endocrine	Ultimo-branchial	Neuroendocrine cell	Adenoma	R	R	RC	RC	RC	RC	
			Carcinoma			RC	RC	R	R	
	Thyroid	Follicular epithelium	Adenoma			R	R			
			Carcinoma				RC-TL	R	R	
	Endocrine Pancreas	Islet Cell	Adenoma			R-WIK				
			Carcinoma		R	RC-WIK				C certain transgenes
	Pituitary Gland	Adenohypophysis cells	Adenoma							RC certain mutants and morphants
Respiratory	Gill	Stem cell	Branchioblastoma		R		R	C-DMBA, DBP	C- DMBA, DBP	
		Cartilage	Chondroma					RC-DMBA, DBP, MNNG		
			Chondrosarcoma					RC-DMBA		
			Osteochondroma					R-MNNG		
		Blood Vessel	Hemangioma					C-DMBA,	C-DMBA, DBP	

Table S3 Relative incidence of specific histologic types of neoplasia in various wild-type and mutant lines of zebrafish

Organ System, Tissue, and Morphologic Diagnosis of Neoplasm				Spontaneous neoplasia in diagnostic cases, broodstock, sentinels or prospective tumor studies				Carcinogen-induced neoplasia		Mutant model
				Diet/Husbandry FT-OTD[1]		Diet/Husbandry RC-COM[2]				Transgene or tumor suppressor deletion or inactivation
								DBP, MAMA, MNNG		
			Hemangiosarcoma					C	C	
		Bone	Osteoma					R		
			Osteosarcoma					ER-AFB, MAMA		
	Pseudo-branch	Stem cell	Hamartoma					ER		
		Stem cell	Branchioblastoma				R-*alf*			R
Peritoneum		Mesothelium	Mesothelioma					ER	ER	

[1]Diet/Husbandry System
FT-OTD: Flow-through system with fish fed semipurified diet
RC-COM: Recirculating system with fish fed commercial diet

[2]Frequency of Occurrence of Histologic Types of Neoplasia
Common (C)
Relatively common (RC)
Rare (R)
Exceedingly rare (ER)
Not reported (NR)
Indication of a genetic strain denotes predisposition to that neoplasm or that only that strain is reported to show the neoplasia to date.

[3]Cutaneous papillomas in zebrafish not treated with carcinogen have been limited to the vent region in eggbound females with partial prolapse of the distal intestine.

[4]Reported in a single instance in which a viral agent may have been present (Beckwith et al., 2000).

Table S4 Numbers of Control Wild-Type Fish Raised in Flow-Through Aquaculture Systems and Fed a Semi-Purified Diet then Examined Histologically for Neoplasia at 7-14 Months of Age

Wild-Type Line	Fraction of Fish with Any Neoplasm at 7 Months of Age	Fraction of Fish with Any Neoplasm at 13-14 Months of Age
AB	0/70	0/161
TU	0/110	0/137
TU X AB		0/81
Cologne (KOLN)	0/168	0/273

Table S5 Neoplasia in Zebrafish from Carcinogenesis Studies Conducted at Oregon State University

Genetic background	Mutant line	Mutant gene	Carcinogen	Exposure age	Exposure route	Dosage	Target tissues										References
							Liver	Gill	GI[a]	Gonad	Eye	CV[b]	Neural	NC[c]	LH[d]	Other	
Florida wt			DMBA	Embryo	Bath	0.25-1 ppm x 24 hr	X						X				e
				Fry	Bath	1.25-5 ppm x 24 hr	X	X	X		X	X		X	X	Thyroid, skeletal muscle	e

				Juvenile	Diet	100-1000 ppm x 12 wk		X	X							Pancreas	e
Florida wt			MNNG	Embryo	Bath	1-10 ppm x 1 hr	X	X	X	X						Pancreas, ultimo-branchial gland	f
				Fry	Bath	0.5-1.5 ppm x 24 hr	X	X		X		X				Cartilage, bone, kidney, ultimo-branchial gland	f

				Juvenile	Diet	500-2000 ppm x 12 wk										None	f
Florida wt			MAMA	Embryo	Bath	10-50 ppm x 12 hr	X	X		X	X	X	X	X		Heart, kidney, cartilage, bone, pancreas	g
				Fry	Bath	6.25-100 ppm x 2 hr	X	X		X		X				Fin	g

				Juvenile	Diet	500-2000 ppm X 12 wk	X		X								g
Florida wt			DEN	Embryo	Bath	1000-3000 ppm x 24 hr	X	X	X							Notochord, skin, ultimo-branchial gland	g
				Fry	Bath	500-2000 ppm x 24 hr	X										g

				Juvenile	Diet	500-2000 ppm x 12 wk										None	g
Florida wt			AFB1	Embryo	Bath	0.25-1 ppm x 1 hr	X		X							Bone	g
				Fry	Bath	0.5-1 ppm x 1 hr	X										g
				Juvenile	Diet	100 ppm x 9 mo	X		X								g

Florida wt			DBP	Juvenile	Diet	225 ppm x 4 wk							X			Notochord	h
AB wt			DMBA	Fry	Bath	0.6-5 ppm x 24 hr	X	X		X		X					i
AB wt			DBP	Fry	Bath	1.25-5 ppm x 24 hr				X	X	X					j
TU X AB wt			DMBA	Fry	Bath	0.6-5 ppm x 24 hr	X	X		X							i

			MNNG	Fry	Bath	2.5 ppm x 24 hr										Sensory neural tissue of nose	i
TL	*Leo^{tl}; Iof^{dt2}*	Genes not yet identified	DMBA	Fry	Bath	0.6-1.25 ppm x 24 hr	X			X		X		X		Skeletal muscle	i
			ENU	Fry	Bath	0.6-2.5 mM x 1 hr	X						X			Pancreas	i
TU			ENU	Fry	Bath	2.5 mM x 1 hr	X				X	X					i

TU			DBP	Fry	Bath	1.25-5 ppm x 24 hr	X	X	X							Thyroid	j
KOLN			DBP	Fry	Bath	0.6-5 ppm x 24 hr	X	X									j

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[a] Target Tissue. GI=gastrointestinal

[b] Target Tissue. CV=cardiovascular

[c] Target Tissue. NC=neural crest

[d] Target Tissue. LH=lymphohemopoietic

[e] Spitsbergen *et al*., 2000b

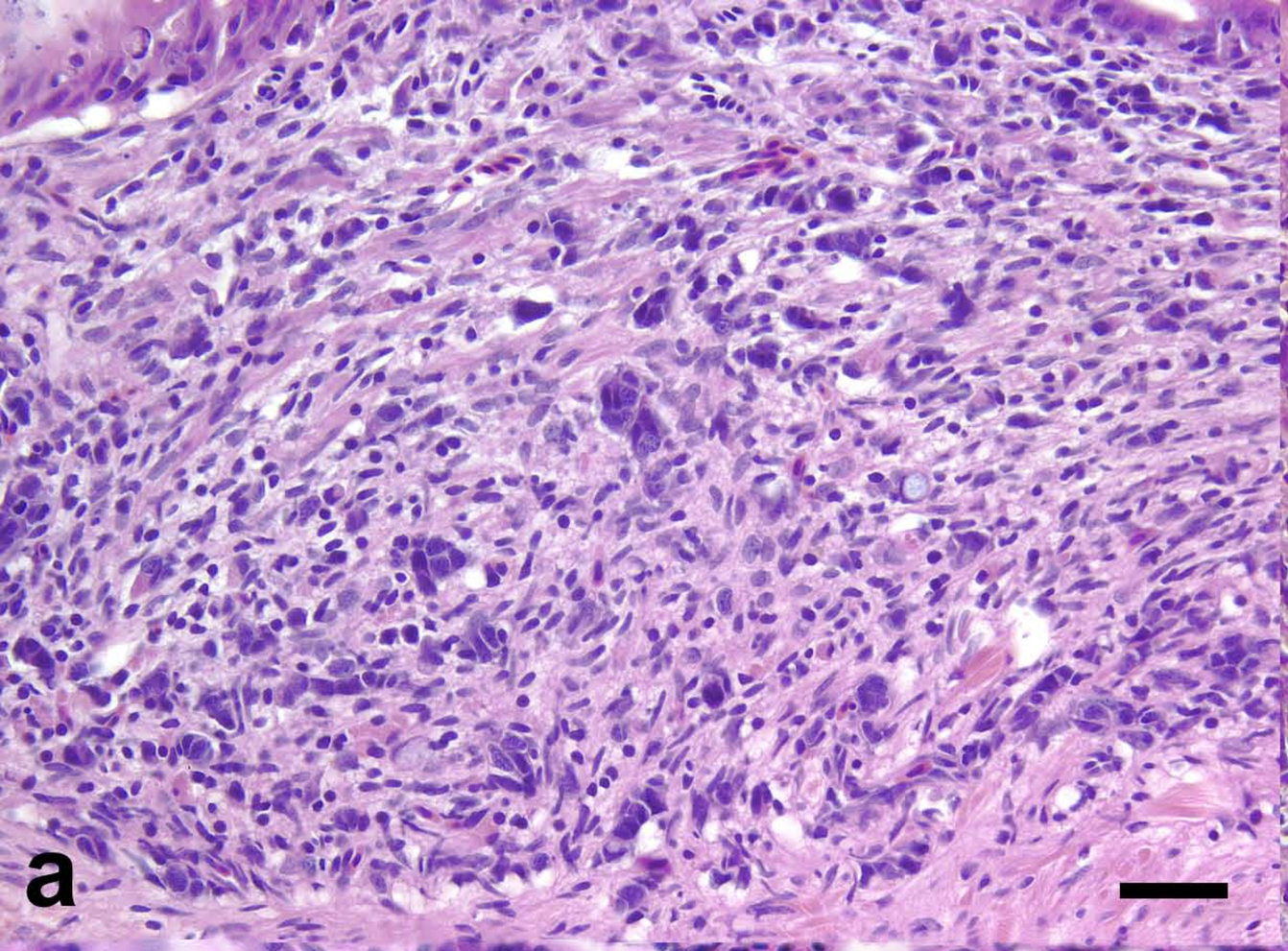
[f] Spitsbergen *et al*., 2000a

[g] Hendricks, 1996; Tsai, 1996; Spitsbergen *et al*., 1997

[h] Reddy *et al*., 1997a

[i] Spitsbergen and Kent, unpublished

[j] Spitsbergen and Buhler, unpublished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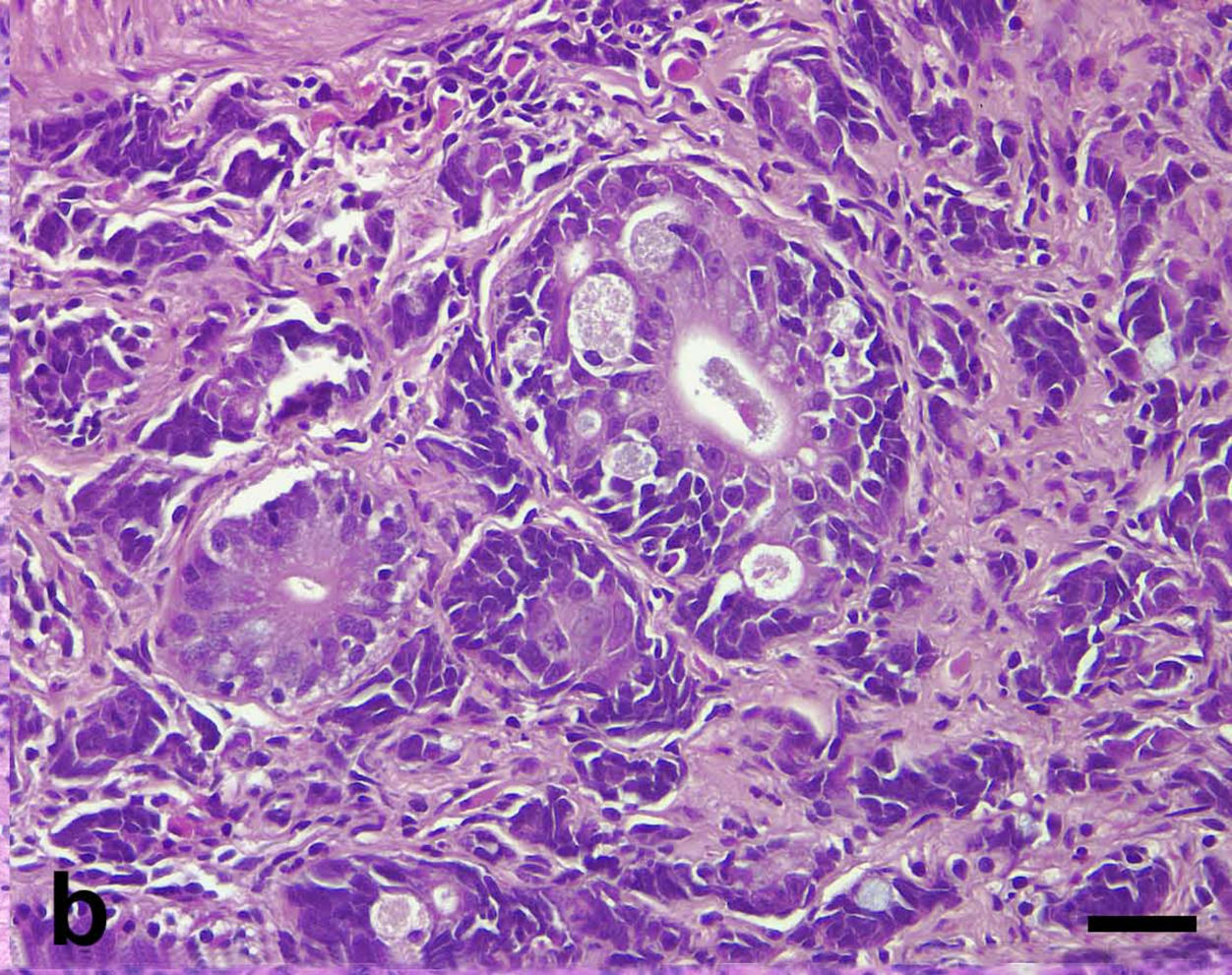
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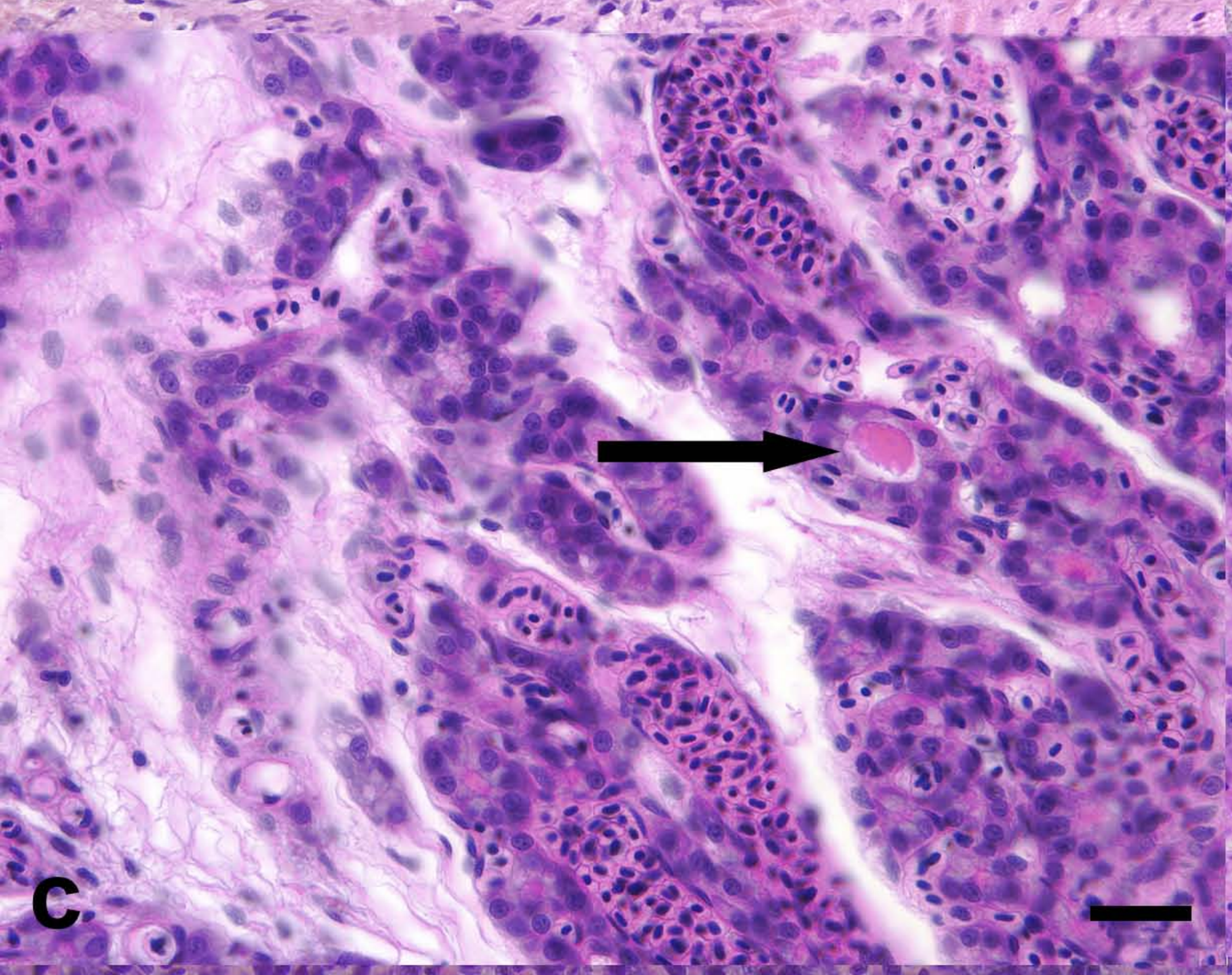
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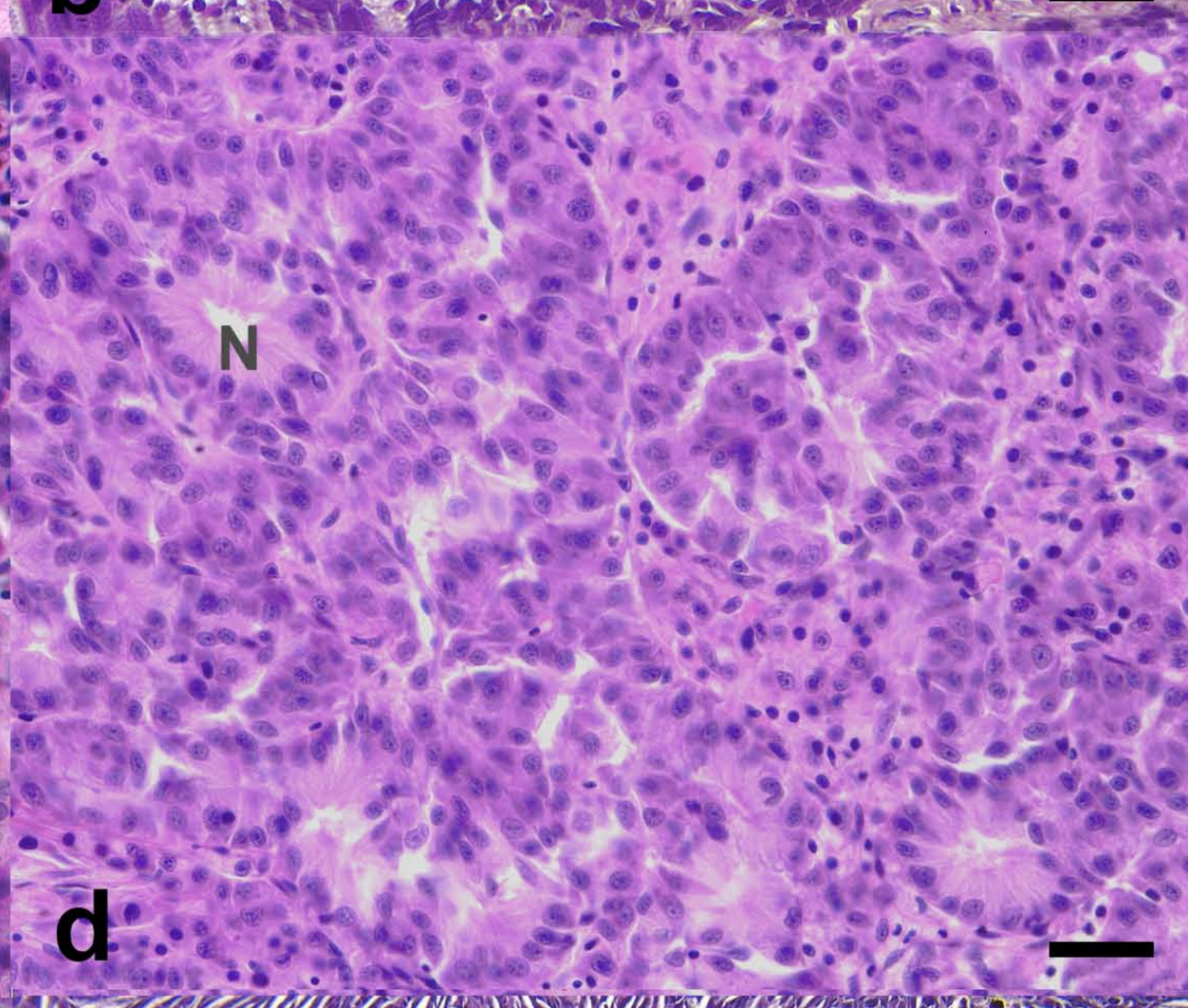
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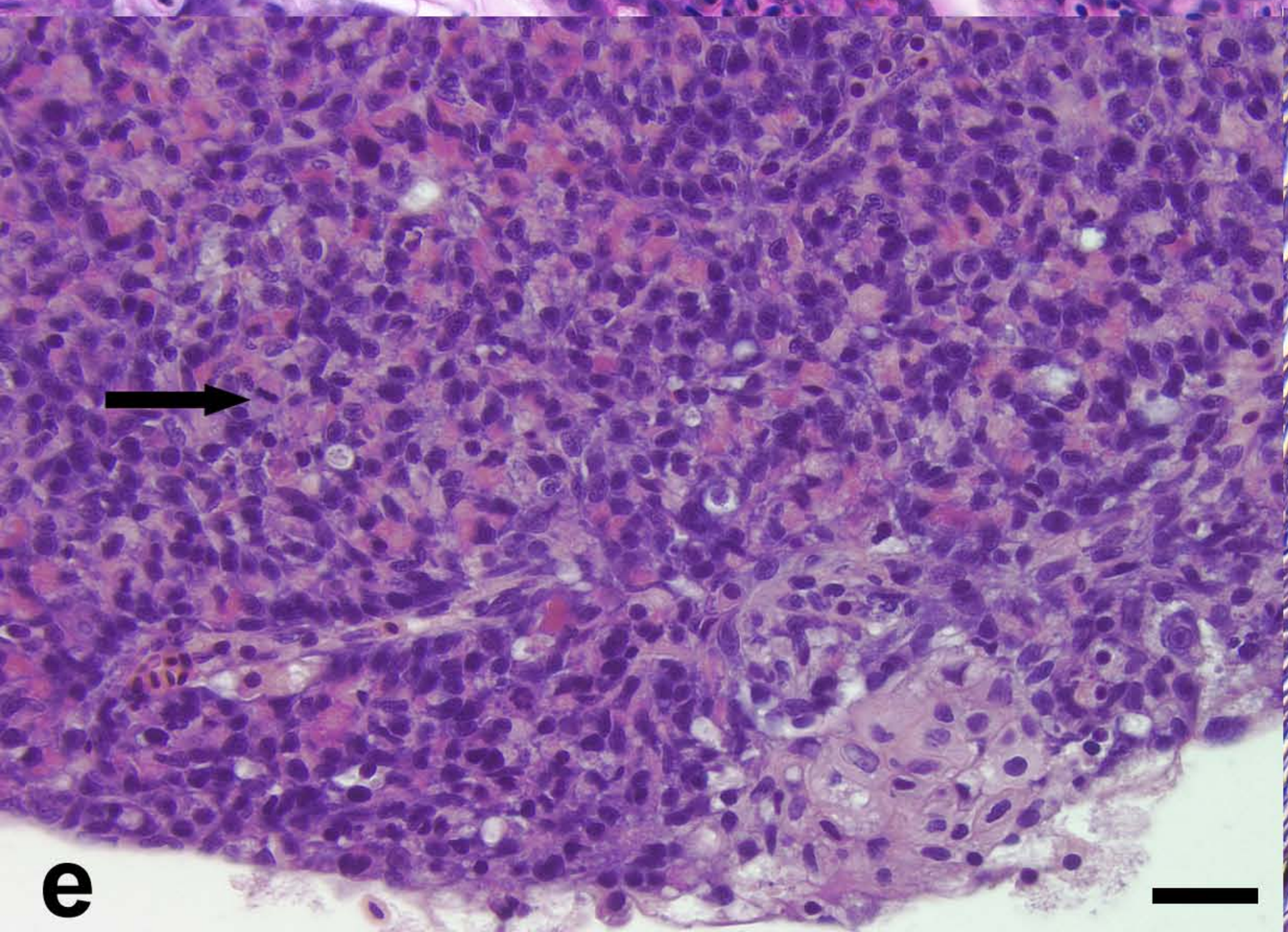
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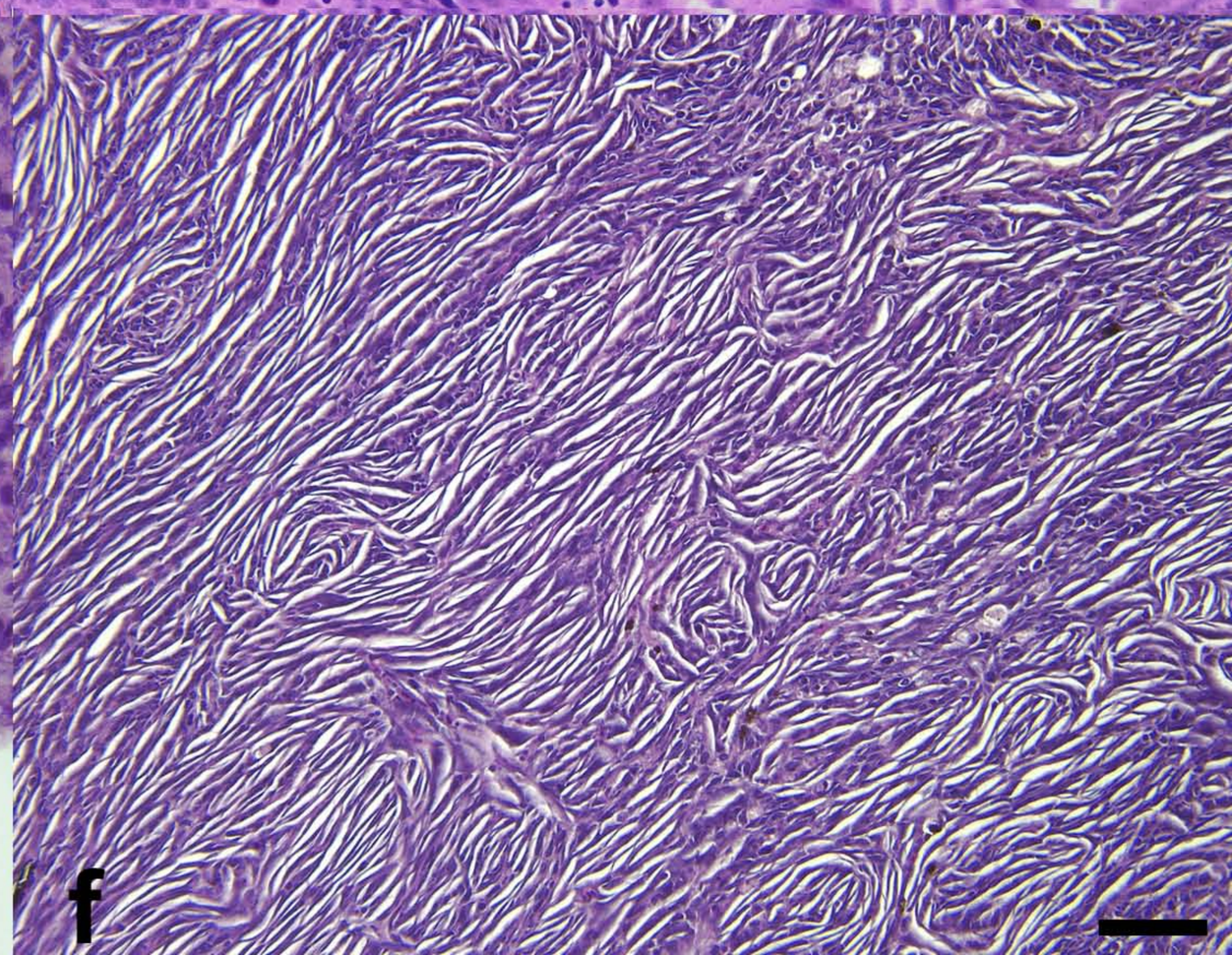
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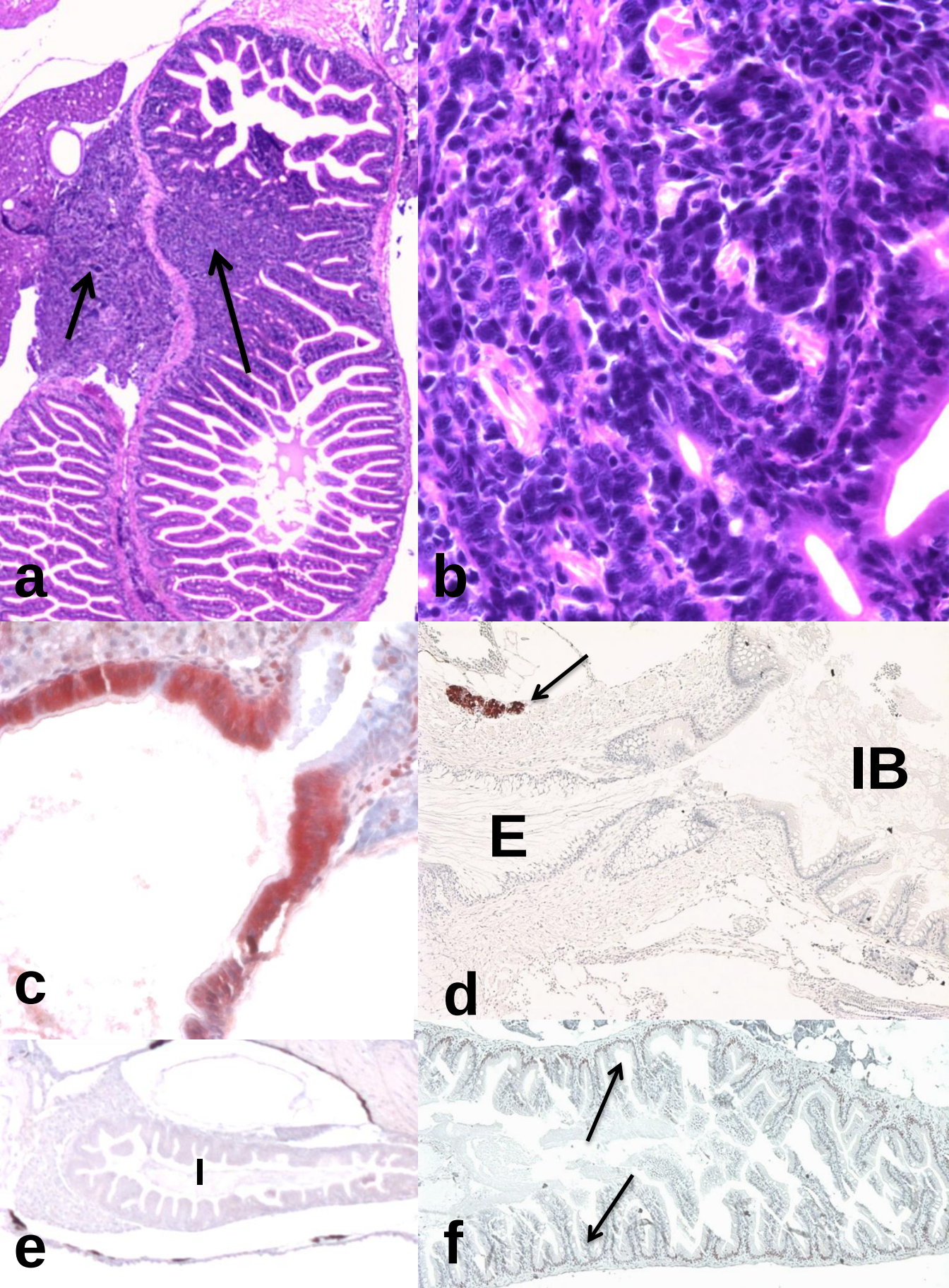
Fig S4; Spitsbergen et al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