Neoplasia and Neoplasm Associated Lesions in Laboratory Colonies of Zebrafish Emphasizing Key Influences of Diet and Aquaculture System Design

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Abstract

During the past decade the zebrafish has emerged as a leading model for mechanistic cancer research due to its sophisticated genetic and genomic resources, its tractability for tissue targeting of transgene expression, its efficiency for forward genetic approaches to cancer model development, and its cost-effectiveness for enhancer and suppressor screens once a cancer model is established. However, in contrast to other laboratory animal species widely used as cancer models, much basic cancer biology information is lacking in zebrafish. As yet data are not published regarding dietary influences on neoplasm incidences in zebrafish. Little information is available regarding spontaneous tumor incidences or histologic types in wild-type (wt) lines of zebrafish. So far a comprehensive database documenting the full spectrum of neoplasia in various organ systems and tissues in not available for zebrafish as it is for other intensely studied laboratory animal species. This manuscript confirms that as in other species diet and husbandry can profoundly influence tumor incidences and histologic spectra in zebrafish. We show that in many laboratory colonies wt lines of zebrafish exhibit elevated neoplasm incidences and neoplasm associated lesions such as heptocyte megalocytosis. We present experimental evidence showing that certain diet and water management regimens can result in high incidences of neoplasia and neoplasm associated lesions. We document the wide array of benign and malignant neoplasms affecting nearly every organ, tissue and cell type in zebrafish, in some cases as a spontaneous aging change, and in other cases due to carcinogen treatment or genetic manipulation.

Key Words: *Danio rerio*; diet; hepatocyte megalocytosis; husbandry; neoplasia; naturally occurring carcinogen; non-protocol induced variation; zebrafish

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Introduction

Zebrafish have emerged as a premier vertebrate model system for understanding genes and signaling pathways controlling development and mechanisms of disease affecting nearly every organ system (Dahme et al. 2009; Ingham 2009; Zhu and Zon 2002). Mutant lines of zebrafish produced worldwide have helped to clarify normal and abnormal development and have provided models for understanding human diseases from polycystic kidney disease to hereditary anemias to cancer. Optimization of tools for inducible tissue-specific expression of transgenes and recently developed techniques for efficient targeted mutagenesis such as zinc finger nucleases and transcription activator-like effector nucleases (TALENS) now allow production of

"custom made" zebrafish models for precise histologic types of cancer affecting specific organs (Amatruda and Patton 2008, Foley et al., 2009; Koh et al. 2010; Mione and Trede 2010; Moore et al., 2012). Xenografts of human tumors into zebrafish embryos and zebrafish cancer models allowing tumor induction very early in life provide a cost-effective, high-throughput system for drug discovery (Lally et al. 2007; Mandrekar and Thakur 2009; Marques et al. 2009; Taylor et al. 2010; Yeh et al. 2008). Despite the high level of sophistication of genetic and genomic tools available for the zebrafish model, basic pathology data for this species still lag far behind the data available for most mammalian laboratory and domestic animal species. While we understand the genetics controlling induction of specific cancer types in zebrafish, little basic information is published regarding spontaneous tumor incidences or histologic types in commonly used wt or mutant lines (Smolowitz et al. 2002 ; Spitsbergen et al. 2009; Spitsbergen and Kent 2003). Because of a strong primary focus on cancer genetics, many of the recent reports of neoplasia in transgenic or mutant lines of zebrafish do not provide data regarding the spontaneous tumor incidences or histologic spectrum of neoplasia in the genetic lines of fish used in the research and do not report the incidences or morphologic diagnoses of all tumor types in mutant or carcinogen-treated fish. Additionally, data regarding dietary influences on neoplasia in zebrafish are not yet published.

As early as 1940 scientists recognized that dietary restriction could influence cancer incidence in animals (Tannenbaum 1940). Extensive data over the past 3 decades from carcinogenesis studies in rodents as well as human epidemiology clearly document strong influences of dietary components such as lipid and protein as well as caloric intake on cancer incidence (Abo and Kari 1996; Campbell 2007; Fontana et al. 2006; Hursting and Kari 1999; Kari et al. 1999; Li et al. 1999; Prentice et al. 2009; Wei et al. 2008). A wide variety of natural

carcinogens and anticarcinogens in plants and other dietary factors are now well characterized in research focused on optimization of healthy aging (Aiyer et al. 2008; Akhtar et al. 2009). Recently dietary factors such as trace contamination by arsenic and other metals (Kozul et al. 2008) and estrogenic plant components in practical laboratory animal diets (Adlercreutz 2007; Adlercreutz et al. 2004; Cross et al. 2004; Green and Kelly 2008; Ziegler et al. 2004) have been recognized as confounding factors in toxicology and carcinogenesis studies.

To minimize such confounding effects of variable dietary components in practical diets in carcinogenesis studies using rainbow trout and aquarium fish, scientists at Oregon State University (OSU) developed a semi-purified diet, Oregon Test Diet (OTD), with gelatin and casein serving as the protein sources (Lee et al. 1991). This diet has ensured consistency and reproducibility in studies utilizing fish as cancer research models conducted over the past 30 years (Bailey et al. 1996, 2009; Spitsbergen et al. 2000a,b; Reddy et al. 1999).

As fish pathologists from OSU began providing diagnostic pathology expertise to zebrafish research laboratories from around the world as a service through the Zebrafish International Resource Center (ZIRC) we observed very different patterns of incidences and histologic types of neoplasia in untreated control fish from many laboratory colonies compared to our colony at OSU in which fish were fed OTD in a flow-through system receiving well water. Therefore we undertook a two-pronged approach to clarify the factors that might be contributing to these perplexing patterns of spontaneous tumors in many well-managed colonies. We conducted prospective studies of neoplasm incidences in replicate groups of AB wt strain that were fed either commercial flake diet or OTD and raised in either a flow-through or a recirculating water system. We also examined retired broodstock from various flow-through and recirculating water systems over a period of two years. This manuscript will discuss the variety

of neoplasia observed in zebrafish submitted to the ZIRC diagnostic service, neoplasia and related lesions in sentinel fish from selected colonies, results of our prospective tumors studies with wt and mutant lines, our studies of neoplasia in retired broodstock from various colonies, and the diversity of neoplasia documented in carcinogen and genetic research using zebrafish. Wt lines of zebrafish fed semi-purified diets and raised in flow-through water systems had low incidences of neoplasia at one or two years of age and showed a limited variety of neoplasm types. Zebrafish fed commercial diets containing fish meal and reared in certain recirculating water systems showed far higher tumor incidences and a much wider variety of histologic types of neoplasia. Both diet and water system had strong influences on tumor incidences and a significant interaction occurred between diet and water system in determining tumor incidences. These studies highlight the need for careful consideration of diet and husbandry in order to ensure valid and reproducible data in research using the zebrafish model. Carcinogenesis studies with various lines as well as genetic studies to create zebrafish models for specific types of neoplasia have demonstrated convincingly that zebrafish can develop similar histologic types of neoplasia as those affecting humans as long as zebrafish have a similar tissue analog.

Experimental Methods and Results

Experimental methods and data from experimental studies investigating causes of neoplasia and carcinogen-induced neoplasia are reported as supplementary information (Supplemental Methods and Data). We present here in print illustrations highlighting the dramatic influence of diet and water systems on total neoplasm incidence (Fig. 1) and examples of key neoplasm types and neoplasm associated lesions occurring in zebrafish from these studies (Figs. 2 and 3). Detailed

data on tumor incidence in specific treatment groups and tissue-specific tumor incidence are provided for the diet and water system studies (Tables S1 and S2). Table S3 summarizes relative incidence data for neoplasms of specific organ systems and tissues from studies of diagnostic cases, sentinels, retired broodstock, carcinogenesis studies and mutant tumor models. Table S4 reports tumor incidence and morphologic diagnoses for wt lines fed OTD in flow-through systems. Table S5 outlines target organs or tissues in wt and mutant lines from carcinogenesis studies. Figure S1, S2 and S3 illustrate the diversity of neoplasia occurring in studies of carcinogenesis and mutant tumor models. Figure S4 illustrates the typical location of small cell carcinoma of intestine near the ampulla of Vater in the anterior intestine and shows the paradoxical lack of high cell proliferation in this common site of tumor formation. S4 also shows that certain cytochrome P450 enzymes critical to carcinogen metabolism are highly expressed in the segment of intestine most prone to neoplasia.

Role of Infectious Agents in Neoplasia

Pseudocapillaria tomentosa is a nematode parasite infecting the gut of zebrafish. This parasite causes moderate to severe multifocal to diffuse hyperplasia and dysplasia in intestine. Elevated incidences of intestinal neoplasia occur in colonies infected with this parasite, and gut neoplasms often occur in close proximity to profiles of nematodes (Kent et al. 2002). In dietary studies with DMBA at OSU, zebrafish were more likely to develop intestinal neoplasia if infected with the nematode parasite (Spitsbergen et al. 2000b). We have recently shown that the specific protocol for infection of zebrafish with *P. tomentosa* is critical for optimal tumor induction and promotion in carcinogen studies. Bath treatment of 3 wk old fry of the sensitive *uma*^{s2068} mutant line with

DMBA followed by infection with nematodes induced no more than a 10% incidence of intestinal neoplasia. However, natural early life infection in a colony endemically infected with *P. tomentosa* acted as a potent tumor promoter when infected fry were given bath treatments with DMBA at 3 and 5 wk. Incidences of intestinal neoplasia in this study were greater than 50% by 1 yr following carcinogen treatment. Higher incidences of myelodysplastic syndrome also occurred in the *uma*^{s2068} line infected with nematodes compared to uninfected fish (Spitsbergen et al. 2008; Figure S1).

Certain other infectious agents that often cause profound hyperplasia in zebrafish tissues, such as *Piscinoodinium pillulare* in the gill have not acted as a tumor promoter in any carcinogen experiments that we have conducted. The strain of mycobacterium seems critical in determining whether this infectious agent will act as a tumor promoter in carcinogen experiments with zebrafish. The mycobacterial strain which most often infects zebrafish colonies in Oregon is *Mycobacterium chelonae*, a relatively nonpathogenic agent which typically causes mild focal lesions in and around the gas bladder (Kent et al. 2004; Murray 2012; Whipps et al 2008). This strain does not appear to increase the incidence of neoplasia in zebrafish colonies with or without carcinogen treatment. In contrast the more pathogenic strain *Mycobacterium haemophilum* occurring in zebrafish colonies in Singapore causes severe diffuse inflammation throughout most visceral organs (Whipps et al. 2007). This greater inflammation acts as a tumor promoter in carcinogen studies and neoplasms often arise in the center of inflammatory lesions in tissues such as liver or intestine. We have not yet investigated any possible role of enteric bacterial flora such as *Helicobacter* in spontaneous intestinal neoplasia in zebrafish.

To date, no pathogenic viruses have been isolated from zebrafish. Ultrastructural studies of a variety of histologic types of neoplasia from zebrafish including seminomas, neuroblastoma of

brain, esthesioneuroblastoma of nose, spindle cell sarcoma of skeletal muscle, malignant peripheral nerve sheath neoplasia, benign and malignant vascular tumors, and enlarged spleens with myelodysplastic syndrome have not revealed viral agents in the tissues. Likewise tumor transmission trials in which whole live cells from neoplasms were injected intraperitoneally into zebrafish fry have failed to yield evidence of any transmissible neoplasms. Since the wt Nadia (NA) line was recently introduced to the laboratory from field conditions in India, we considered this line most likely to be harboring possible latent pathogenic viruses. We used large seminomas from 2–year-old NA zebrafish to inject zebrafish fry of the TL and AB lines. A year following injection, the fish were free of neoplasia grossly and histologically. We are anxious to obtain live zebrafish with skin or fin papillomas as we believe that these papillomas are the best neoplasm candidates for harboring a tumorigenic virus of zebrafish.

The Need for an Immunohistochemistry Panel for Better Identification of Specific Types of Neoplasia in Diagnostic Pathology and Research

Investigators worldwide have extensively used immunohistochemical analysis of teleost fish tissues as a research technique for the past 35 years. Varied antibodies, chromogens, antigen retrieval and blocking procedures have been used to answer specific questions regarding cellular and tissue composition as well as gene and protein expression. However to date little effort has focused on creation and utilization of specific antibodies for general application in fish diagnostic and toxicologic pathology We currently lack standardized, validated immunohistochemical protocols for formalin-fixed and paraffin-embedded tissues for a comprehensive panel of antibodies to be used to characterize cell and tissue types in fish

neoplasms. Custom made antibodies directed against zebrafish-specific target antigens are available if the antigen amino acid sequence is known. Current application of common basic antibody panels, familiar to human and veterinary pathologists, can be generated using the amino acid sequence of known zebrafish protein antigens (National Center for Biotechnology Information GenBank; www.ncbi.nlm.nih.gov) for epithelial, mesenchymal, neural and endocrine neoplasia that include cytokeratin, vimentin, desmin, smooth muscle actin, myoglobin, glial fibrillary acidic protein, S-100, thyroglobulin and insulin (Ramos-Vara 2005).

One of the best examples of the utility of immunohistochemistry in pathology is cytokeratin filament expression profiling of suspected or questionable carcinoma, which frequently allows definitive determination of the neoplastic cell's epithelial origin. Cytokeratins are thought to be highly conserved across vertebrate species and in teleost fish they have been previously characterized, demonstrating similar molecular weights and isoelectric points among different genera (Garcia et al. 2005). Attempts to examine cytokeratin expression profiles in the medaka and common carp, a close relative of zebrafish, met with limited success as a wide spectrum of tissues showed non-specific immunopositive reactivity. Although many of the epithelial tissues, such as epidermis, branchial, biliary, intestinal and renal epithelium stained cytokeratin positive using mammalian AE1/AE3 antibody, several tissues other than those of ectodermal origin stained positive including fibroblasts, chondrocytes, testicular myoid cells, vascular adventitia, skeletal muscle and glial cells (Bunton 1993, 1994; Groff et al. 1997). Similar to cytokeratins, other mammalian antibodies have been used to identify or confirm the histotype and mitotic activity of certain teleostean fish tumors such as peripheral nerve sheath tumors, intestinal adenocarcinoma, gonadal tumors such as seminoma and perineoplastic stromal cells that includes calretinin, S-100, PCNA (proliferating cell nuclear antigen), vimentin, placental alkaline

phosphatase, alpha fetal protein, neuron-specific enolase, c-KIT, estrogen receptor, actin and desmin with variable success (Bunton, 1994; Bunton 1995; Faro et al. 2009, Marino et al. 2007, Sirri et al. 2010). The issue of what constitutes appropriate antigen and control tissues as a means of validating the immunohistochemical reactivity remains problematic as long as mammalian antibodies are used.

Discussion and Conclusions

Neoplasia in Liver of Zebrafsh and Other Species

Considering spontaneous tumors in diagnostic cases and retired broodstock as well as carcinogenesis bioassays and mutant tumor models, we have observed neoplasia of a wide variety of histologic types affecting nearly every organ and most cell types. Liver is the most common target organ for nearly all of the carcinogens studied in all wt and mutant lines of zebrafish (Table S4). This targeting of liver by most carcinogens is similar to the data regarding rainbow trout (Bailey et al. 1996) and other small aquarium fish such as medaka and guppy (Bunton 1996). Liver is more often targeted in neoplasia in fish than in mammals, most likely because fish liver grows throughout life, whereas, adult mammal liver is quiescent unless damaged. Compared to mammals, zebrafish and trout more often show mixed hepatic neoplasms comprised of biliary and hepatic components (Bailey et al. 1996; Hendricks 1996; Spitsbergen et al. 2000b; Tsai 1996).

Species Differences in Target Tissues and Histologic Types of Neoplasia

The range of tumor types and affected tissues that we have observed in zebrafish, differ from those seen in mammals and in other well-studied fish species such as rainbow trout. Regardless of the class of carcinogen, rainbow trout show 3 primary target organs: liver, stomach, and gas bladder, developing almost exclusively epithelial neoplasia (Bailey et al. 1996). In contrast zebrafish and other small aquarium fish such as medaka and guppy show a much wider range of target organs, and a broader range of histologic types of neoplasia, including epithelial, mesenchymal, neural and neural crest tumors. Zebrafish are agastric, so no stomach neoplasia occurs. Neoplasia of gas bladder is quite rare in zebrafish, with or without carcinogen treatment. However, as we have examined large numbers of fish from a variety of fish strains and treatment regimens, we have now observed several benign as well as malignant neoplasms of gas bladder in zebrafish (Spitsbergen et al. 2000a; Zhan et al. 2010).

Neural Neoplasia in Zebrafish and Other Species

Neural neoplasms affecting brain, eye and spinal cord are relatively common spontaneous tumors in diagnostic cases and retired broodstock from systems with recirculating biofilters in which fish are fed commercial diets. Neural tissues are also common targets for several carcinogens including DMBA, MNNG and MAMA (Table 5). In adult humans, dogs and cats, gliomas are the most frequent primary brain tumor (Behin et al. 2003; Koestner et al. 1999). In contrast, in wt zebrafish most spontaneous or induced primary neurogenic neoplasms of the central nervous system (CNS) are poorly differentiated, highly embryonal neuroblastomas or primitive neuroectodermal tumors (PNETS). Brain of adult zebrafish is histologically quite distinct from that of mammals, with a much greater component of highly cellular areas comprised of deeply basophilic embryonal cells surrounding the ventricular system of forebrain,

diencephalon and myelencephalon (Kizil et al. 2012; Kroehne et al. 2011). These abundant embryonal periventricular cells may predispose zebrafish to develop more embryonal neoplasms of CNS resembling those seen in pediatric cases in humans. Until recent collaborations with investigators creating transgenic tumor models (Ju et al. 2010) we had not documented a glioma of brain or spinal cord in our studies of spontaneous or carcinogen induced neoplasia in zebrafish. Now we have observed low grade astrocytomas as well as glioblastomas in zebrafish CNS. In comparison to mammals, zebrafish are unusually predisposed to develop neoplasia of nerve sheath of peripheral and cranial nerves. Many of the zebrafish models with inactivating mutation in tumor suppressor genes including *tp53*, *mlh1*, *msh2*, *msh6*, and ribosomal genes show high incidences of malignant peripheral nerve sheath tumors when the human or rodent cancer spectrum from inactivating mutations in the orthologous tumor suppressor genes cause a much wider range of neoplasms in mesenchymal, epithelial or lymphomyeloid tissues (Amsterdam et al. 2004; Berghmans et al. 2005; Feitsma et al. 2008; Parant et al. 2010).

Enteric Neoplasia in Zebrafish and Other Species

Like liver tumors occurring in zebrafish, gastrointestinal tumors (GI) of zebrafish are more likely to be pluripotential neoplasms comprised of multiple cell lineages than the spontaneous or carcinogen-induced GI tumors of mammals. Mixed malignant intestinal neoplasia comprised of malignant smooth muscle cells and malignant mucosal epithelial cells is a relatively common lesion induced by DMBA in zebrafish (Spitsbergen et al. 2000b). Such carcinosarcomas of the GI tract are rare in mammals (Riddell et al 2003; Whiteley 1996). Interestingly, most of the spontaneous GI neoplasia occurring in zebrafish diagnostic cases and retired broodstock is strictly epithelial, principally small cell carcinomas or mucosal adenocarcinomas.

Renal Neoplasia in Zebrafish and Other Species

Kidney is a common target of carcinogens including DMBA, MNNG and MAMA in rainbow trout. When treatment with MNNG occurs early in life, up to 50% of rainbow trout develop nephroblastomas (Bailey et al. 1996). In contrast to rainbow trout, kidney is rarely a target for carcinogens in zebrafish. We have seen a single renal adenoma and one renal carcinoma following early life stage exposure to MAMA and MNNG, respectively, in the 5-D Florida wt line. We have observed nephroblastoma primarily in the TL line, and then only in diagnostic cases or retired broodstock from systems with recirculating biofilters and/or feeding commercial diets (Figure S3). We have not yet observed nephroblastoma in TL or other lines of zebrafish intentionally treated with carcinogens.

Pigment Neoplasia in Zebrafish and Other Species

Melanomas are common skin tumors in mammals (Goldschmidt et al. 1998), and occur at high incidences spontaneously or after carcinogen exposure in certain genetic lines of *Xiphophorus* (Kazianis and Walter 2002; Walter and Kazianis 2001). We have observed a single case of malignant melanoma in a diagnostic case (Table S3) and a single benign melanocytoma of optic nerve in retired broodstock. We have not yet found a carcinogen treatment regimen that yields increased numbers of melanomas. However in recent years several laboratories have developed genetic protocols to induce high incidences of melanoma in zebrafish (Patton et al. 2005; Santoriello et al. 2010).

Vascular Neoplasia in Zebrafish

Vascular neoplasia has occurred in many tissues throughout the body of zebrafish treated with carcinogens. However, most vascular neoplasms occur in the rete of the choroid gland of the eye or in the gill (Figures S1 and S3). Perhaps this tissue tropism reflects the high density of small blood vessels in these two sites.

Epithelial Skin Neoplasia in Zebrafish and Other Species

In our studies epithelial skin neoplasia was exceedingly rare in zebrafish spontaneously or following carcinogen treatment. We were unable to induce papillomas of skin or fin by bath treatment of fry of the TL, TU or KOLN lines with the maximum tolerated dose of ENU. Various factors might explain our findings compared to the 100% incidence of cutaneous papillomas occurring in Florida wt zebrafish treated as adults with ENU (Beckwith et al. 2000). Only Florida wt skin may respond to ENU. Although it seems unlikely to us, adult exposure to ENU may be required to induce papillomas. Typically early life stages of fish are more responsive to all carcinogens than adults, so long as the fish have reached a stage of development at which they metabolize carcinogens requiring metabolic activation. ENU is a direct-acting carcinogen and does not require metabolic activation for effect. Epithelial skin neoplasms in a variety of fish species are associated with oncogenic viruses (McAllister et al. 1985; Yoshimizu et al. 1995). Pennsylvania State College of Medicine zebrafish may have carried a virus, which was activated following treatment with ENU (see Supplemental Methods and Data, Section on Carcinogen-Induced Neoplasia). This hypothesis that the colony at Pennsylvania State College of Medicine has some unique factor predisposing it to epithelial neoplasia is supported by the finding that papillomas have not been observed on the skin or fins in several large zebrafish colonies including those at the University of Oregon, Cornell University (Paul Bowser, personal

communication) and Harvard University (Leonard Zon, personal communication) in which adult males have been mutagenized using ENU by protocols similar that used by Beckwith et al.

Soft Tissue Sarcoma in Zebrafish and Other Species

Liposarcoma is the most common soft tissue sarcoma in humans (Dei Tos, 2000). We have not yet documented a single liposarcoma or lipoma in zebrafish from diagnostic cases, retired broodstock, or carcinogen studies. We find no reports of liposarcoma in zebrafish in the literature. The reasons that zebrafish adipocytes do not act as targets in tumorigenesis are unclear. Lipomas do occur rarely in other fish species (Bruno et al., 1991; Chen et al., 1996).

Neoplasia of Gonad in Zebrafish and Other Species

One factor influencing tissue tropism of carcinogens is the rate of cell proliferation in the tissue. Cell division is required for activity of most mutagens (Winn et al. 2000). Yet cell proliferation rates alone do not adequately explain the disparity between spontaneous and carcinogen-induced neoplasia in zebrafish testis in comparison to ovary. We have observed just 2 spontaneous, no carcinogen-induced, and a few genetically influenced (morphant or mutant fish) ovarian neoplasms in zebrafish. In contrast seminomas in males are the most common spontaneous and one of the common carcinogen-induced neoplasms in zebrafish. Most of the ovarian neoplasms in zebrafish were carcinomas, fewer adenomas, and one dysgerminoma. In contrast to zebrafish, in medaka, spontaneous seminomas occur in females as well as in males (Hawkins et al. 1996) and dysgerminomas are a common finding in control fish in some studies (Reddy et al, 1999b). In female zebrafish with normal functional adult ovaries, we have seen sperm producing testicular tissue in pancreas of wt lines. In wt fish treated with DMBA we have also seen

occasional spermatocytic seminomas in pancreas of female fish. Ectopic germ cells which develop outside of the gonad in zebrafish and medaka differentiate into testis in female fish.

Lymphomyeloid Neoplasia in Zebrafish and Other Species

Hemopoietic tissues of fish have a relatively high proliferation rate, but except for lymphoma, spontaneous or carcinogen-induced hemopoietic neoplasia is extremely rare in wt lines of zebrafish. Acute or chronic myeloblastic leukemia are important cancers in humans and other mammals (Iovino and Camacho 2003; Wertheim et al. 2002), yet we had not documented granulocytic leukemia until we examined mutant lines of zebrafish. Now that we have extensively studied some of the mutant lines of zebrafish predisposed to lymphomyeloid neoplasia, we have documented numerous cases of myelodysplastic syndrome and neoplasia in erythroid and granulocytic lineages.

Ultimobranchial Neoplasia in Zebrafish and the Role of Diet in this Neoplasm

Ultimobranchial neoplasia is among the most common histologic tumor types in diagnostic cases, retired broodstock, and carcinogenesis studies, regardless of diet and husbandry. In recent years we have evaluated in our carcinogenesis bioassays the usefulness of Aquatox (Ziegler), a commercial diet formulated to have low nitrosamine levels, and have typically found low tumor incidences in most tissues in control fish, however in some experiments hyperplasia of ultimobranchial glands occurred that was associated with elevated carcinogen-induced neoplasia in these lines of fish. In our assays of tissue-specific cell proliferation rates, we have found PCNA expression in ultimobranchial to be among the highest in any tissue (Figure S4). Diet analyses indicated that the batch of Aquatox used in these experiments contained 2% calcium on a dry weight basis compared to the 1% calcium present in OTD. More controlled experiments are

needed in order to define the optimal calcium levels in zebrafish diets (Watts et al, 2012), but we speculate that as with bulls showing medullary thyroid neoplasia when fed diets high in calcium designed for lactating dairy cows (Geelhoed 1996), elevated dietary calcium may cause hyperplasia of the ultimobranchial gland and predispose zebrafish to elevated neoplasm levels. In contrast to zebrafish, in medaka, ultimobranchial neoplasia does not occur with or without carcinogen treatment (Bunton et al. 1996; Masahito et al 1989). One might speculate that medaka evolved in an environment with high calcium levels, so that high dietary calcium does not cause ultimobranchial hyperplasia as in zebrafish. Much more information is needed regarding tissue-specific carcinogen metabolism, DNA repair and cell turnover rates to begin to understand tissue tropism of carcinogens in zebrafish (Law 2001).

A single diet is not likely to be optimal for all research applications with zebrafish (Watts et al, 2012). We did not rigorously compare OTD with Aquatox in carcinogenesis or spontaneous tumor studies, however, we conducted selected carcinogenesis and aging experiments using Aquatox to determine its influence on tumor incidences. We observed rather profound senescence of aging zebrafish over 2 years old of most genetic lines when fed OTD. Although these fish were free of infectious diseases, they showed reduced appetite and became cachexic. We found that these same lines reared under similar conditions in our flow-through systems but fed Aquatox maintained good appetites and showed healthier aging, in some cases living over 4 years while maintaining normal weight. We have not carefully compared the composition of Aquatox versus OTD, but visually Aquatox contains much more carotenoids which may act as antioxidants that promote healthy aging. OTD was optimized for tumor bioassays, not for healthy aging.

Critical Role of Genetic Background as an Influence on Neoplasia in Zebrafish and Other Species

A factor that has received little attention in zebrafish research until the past 5 years is the critical role of genetic background in determining tumor incidences as well as other physiological parameters such as disease resistance, immune responses and other endpoints in response to toxicant exposure. Extensive data from rodents and other laboratory animal species confirm that genetic background as well as specific mutations and transgenes are critical in determining both spontaneous and carcinogen induced neoplasm incidences, target organs and the histologic spectrum of tumors which occur (Ward and Devor-Henneman 2004). Observations regarding hyperplasia of bile ducts in the TL line of zebrafish illustrate the importance of genetic background in determining tumor phenotype in fish. The TL line from most laboratories shows moderate to severe hyperplasia of bile ducts in liver as well as about 10% incidences of biliary neoplasia by 1-1.5 years of age. This trait is not linked to the *long fin*^{dt2} (*lof* ^{dt2}) gene because siblings of *lof*^{dt2} fish with wt fin length show similar biliary hyperplasia and neoplasia. This biliary trait acts as a dominant genetic factor in crosses to other wt strains. Surprisingly older adult fish of the TL line from certain laboratories do not show bile duct hyperplasia, biliary neoplasia or myelodysplastic syndrome seen in the TL line from most laboratories, so these traits seem likely to be determined by the genetic background of the commonly occurring TL lines.

Role of Diet and Water Systems in Neoplasia in Zebrafish

A great advantage of small aquarium fish for cancer bioassays has been their low background tumor incidences in comparison to mammals (Hawkins et al. 1985, 2003; Spitsbergen et al. 2000a,b). Recently we have found that water system design and diet exert profound effects on spontaneous tumor incidences in zebrafish. One of the most urgent issues in the rapidly growing

field of cancer research using the zebrafish model is the need to optimize aquaculture systems and diets to eliminate or minimize the natural carcinogens and possible tumor promoters that currently confound research in many recirculating systems feeding commercial diets. Many gastrointestinal, pancreas, ultimobranchial, thyroid, nerve sheath, brain and eye tumors seen in diagnostic cases and retired broodstock probably are caused by natural carcinogens and/or tumor promoters in water systems or diets. These neoplasms are rare, even in 2 year old fish of most wt and certain mutant lines when born and raised in flow-through systems and fed a semi-purified diet. When spontaneous seminomas and other neoplasms occur in older fish in these flow-through systems, these tumors are typically quite small (1-4 mm) rather than 10-14 mm as are many seminomas from recirculating systems feeding commercial diets. The finding of elevated age-specific tumor incidences, remarkably large spontaneous tumors, and hepatocyte megalocytosis in a high percentage of intensive zebrafish aquaculture facilities from around the world has profound implications for many institutes at the National Institutes of Health, not just those funding cancer research. Now that the zebrafish genome is sequenced (Bowen et al. 2012; Leshchiner et al. 2012), and our knowledge of genetics, genomics, and molecular and cellular development mechanisms has become very sophisticated, interest has grown regarding use of zebrafish as models for understanding the genetic mechanisms underlying a wide variety of human diseases. Episodic exposure of zebrafish colonies to potent naturally occurring mutagens and carcinogens from recirculating systems, and continuous exposure to possible carcinogens and/or tumor promoters in commercial diets, jeopardizes the integrity of many types of research using fish more than a few weeks old. Physiology and histology of these fish will not be normal, in addition to problems with elevated liver lesions and neoplasia in various tissues. Perhaps some of the genetic polymorphism seen in certain colonies is more a reflection of husbandry protocols

than actual baseline polymorphism. We suspect that there are multiple carcinogens and/or tumor promoters in recirculating aquaculture systems, which vary in predominance over time, because some cohorts of a given line of fish at a particular facility show just hepatic megalocytosis, without elevated age-specific tumor incidences, some lots show liver tumors only, and other groups show predominately gastrointestinal neoplasia, with or without hepatic megalocytosis. There are many possible sources of natural carcinogens in aquaculture systems including biofilters, microbial and algal biofilms in water distribution systems and fish tanks, *Paramecium* cultures, and leechates from system components. Nitrosamines and nitrosamides are the most plausible natural carcinogens, which might form in recirculating aquaculture systems. These carcinogens are known to form in natural waters and sediments in microenvironments in which organic matter is high and pH is low. Such naturally formed carcinogens in sediments in waterways can occur at concentrations that induce cancer in fish (Alexander and Tate 1975; Ayanaba and Alexander 1974; Mills and Alexander 1976; Yordy and Alexander 1981; Spitsbergen and Wolfe 1995). Clearly some recirculating aquaculture systems house zebrafish colonies that are free from hepatocyte megalocytosis and elevated age-specific tumor incidences. Recirculating aquaculture systems have been used for decades and we have studied a variety of species of fish housed in conventional recirculating systems in which the biofilter material consists of polyurethane foam, plastic cylinders or beads, gravel, crushed oyster shell, or activated carbon. So far we have observed hepatocyte megalocytosis and elevated tumor incidences only in certain systems with fluidized sand biofilters. However some systems with fluidized sand biofilters used for small aquarium fish are free of these problems (Dr. Gary Marty, personal communication).

Many commercial fish foods contain detectable levels of nitrosamines principally from formation of these agents during the manufacture of fish meals used in the diets. To minimize the risk of elevated spontaneous tumor incidences in their carcinogenesis bioassays, scientists at the Gulf Coast Research Laboratory, Ocean Springs, MS, arrange for pretesting of fish meal to ensure acceptable levels of nitrosamines. They have found that a tolerance level of 100 ppb for the most commonly occurring nitrosamine in fish meal, *N*-nitrosodimethylamine (DMN), ensures low background tumor incidences in medaka and guppy carcinogen bioassays (Dr. William Hawkins, personal communication; Hawkins et al. 2003). Aquatox Flake, a diet manufactured by Ziegler Brothers (Gardners, PA) and also supplied in small batches through Aquatic Ecosystems, Inc. (Apopka, FL) is available pretested for nitrosamine levels in fish meal.

Summary

Over the past 15 years our knowledge of zebrafish carcinogenesis and tumor biology has advanced greatly and the number of laboratories studying transgenic and mutant zebrafish models for cancer has grown rapidly. Yet much potential remains for applying this highly sophisticated and facile model to clarify mechanisms occurring at each stage in the carcinogenesis process and to better understand interactions of the complex array of oncogenes and tumor suppressor genes in oncogenesis. Models for several tumor types of humans are now available, yet many neoplasm types remain to be studied in detail using this model system. The immune system of zebrafish is fundamentally similar to that of humans, however the roles of innate and adaptive immunity in all stages of the tumorigenesis process have not yet been addressed in zebrafish. The zebrafish model can play a unique role in discovery of novel contrast agents for tumor imaging (Canaple et al, 2008; Ullmann et al, 2011; Spitsbergen et al,

2007; Zheng et al, 2011) as well as in development of innovative anticancer drugs and more effective delivery methods such as use of nanoparticles to deliver drug combinations in a tissue targeted fashion (Harfouche et al, 2009).

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Figure Legends

Figure 1 Influences of diet and husbandry regimen on total neoplasia incidences in AB wild-type fish at 22 months of age. Replicate tanks of fish fed each of 2 diets, OTD (semi-purified Oregon Test Diet) or COM (mixture of commercial diets containing fish meal) and reared at each of 3 sites, FT-A (flow-through system design, location A), FT-B (flow-through system design, location B), or RC-C (recirculating system with fluidized sand biofiler). Tumor incidences were significantly higher in the recirculating system compared to flow through systems, regardless of diet (P=0.0000, chi-square with Yates' correction; red asterisk). Also, diet did not significantly influence tumor incidences at the flow-through sites (P>0.24 for FT-A and P>0.7 for FT-B), but did significantly influence tumor incidences in the recirculating system (P<0.0001; blue asterisk).

Figure 2 Gross and microscopic lesions observed in zebrafish (*Danio rerio*) from the prospective study of diet and water system effects on tumors in AB wt fish. (a) and (b) Hepatocellular carcinoma and hepatocyte megalocytosis. Gross and histologic appearance of soft tan mass of hepatocellular carcinoma in liver of fish from system RC-C fed COM diet. Long arrows point to hepatocellular carcinoma. Short arrows point to enlarged nuclei characteristic of hepatocyte megalocytosis present in nonneoplastic liver adjacent to carcinoma. Inset illustrates cellular pleomorphism and nuclear atypia with prominent nucleoli characteristic of hepatocellular carcinoma. (c) and (d) Acinar cell carcinoma of exocrine pancreas. Gross and histologic appearance of soft tan mass of acinar cell carcinoma occurring in fish from Lot 17 system FT-A fed OTD. (e) and (f) Spermatocytic seminoma. Gross and microscopic appearance of soft white lobulated mass of spermatocytic seminoma in testis of fish from system RC-C fed COM diet. (g) and (h) Rhabdomyosarcoma in skeletal muscle of trunk. Gross and microscopic appearance of firm mass protruding from skeletal muscle of caudal trunk in fish from system RC-C fed COM diet. Inset in h shows striations (arrow) in some of the neoplastic myocytes viewed under Nomarsky differential interference microscopy.

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).

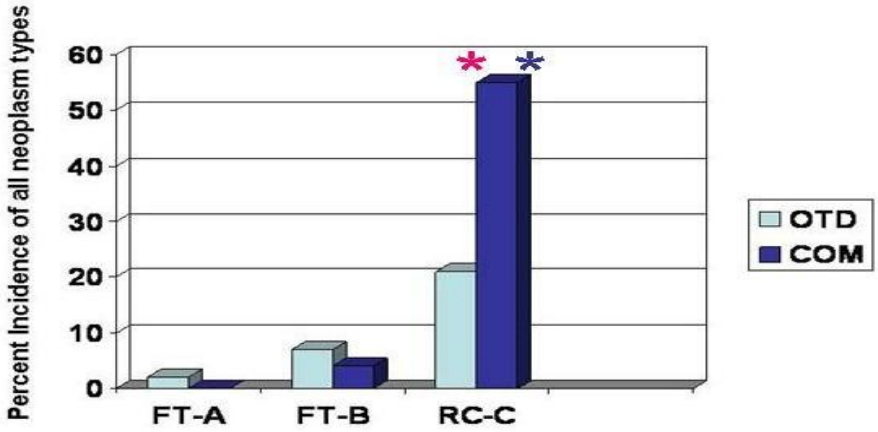

Figure 1.—Influences of diet and husbandry regimen on total neoplasia incidences in AB wild-type fish at 22 months of age. Replicate tanks of fish fed each of 2 diets, OTD (semi-purified Oregon Test Diet) or COM (mixure of commercial diets containing fish meal) and reared at each of 3 sites, FT-A (flow-through system design, location A), FT-B (flow-through system design, location B), or RC-C (recirculating system with fluidized sand biofiler). Tumor incidences were significantly higher in the recirculating system compared to flow through systems, regardless of diet (P=0.0000, chi-square with Yates' correction; red asterisk). Also, diet did not significantly influence tumor incidences at the flow-through sites (P>0.24 for FT-A and P>0.7 for FT-B), but did significantly influence tumor incidences in the recirculating system (P<0.0001; blue asterisk).

Figure 2; Spitsbergen et al

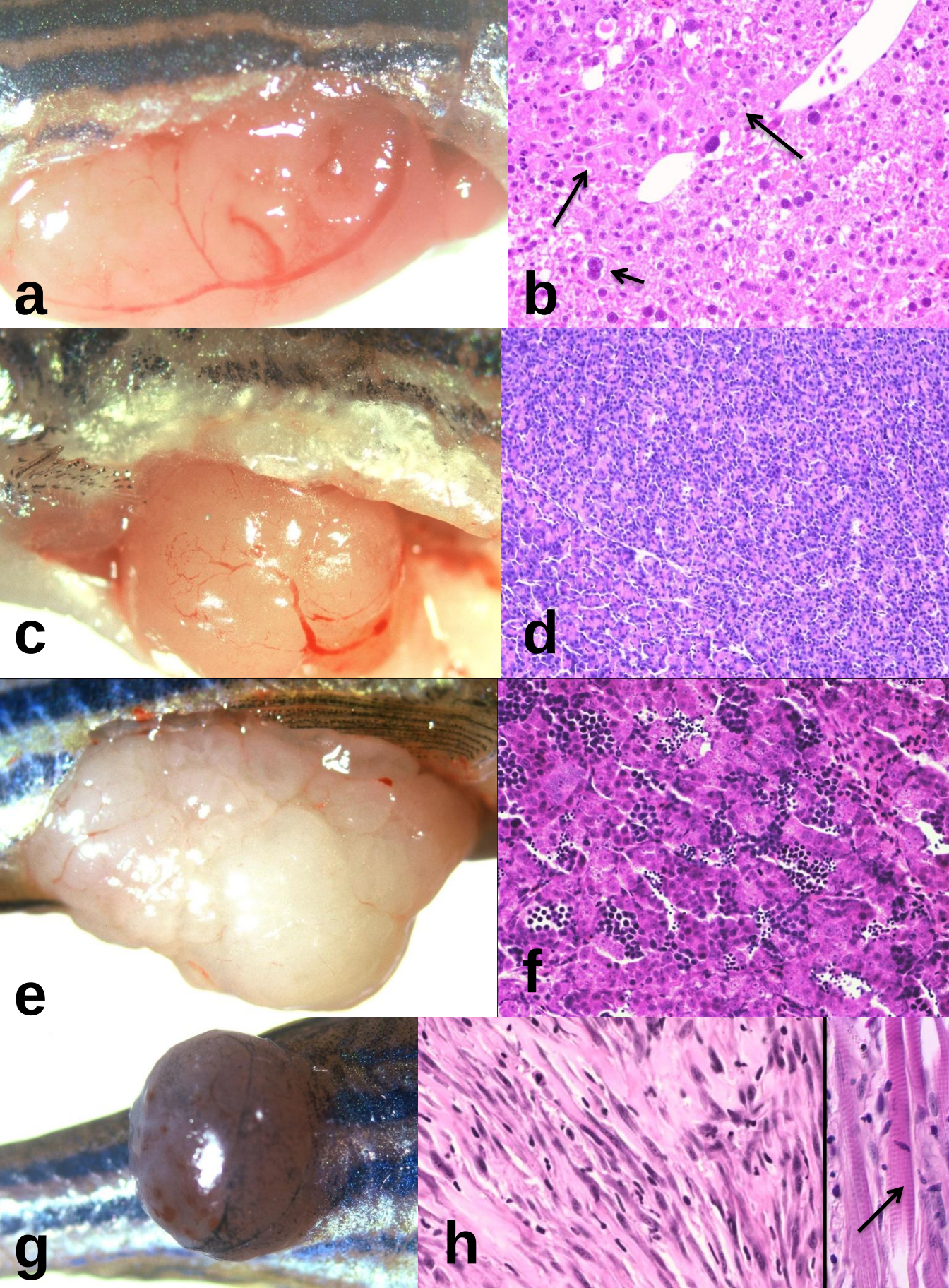
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Figure S1; Spitsbergen et al

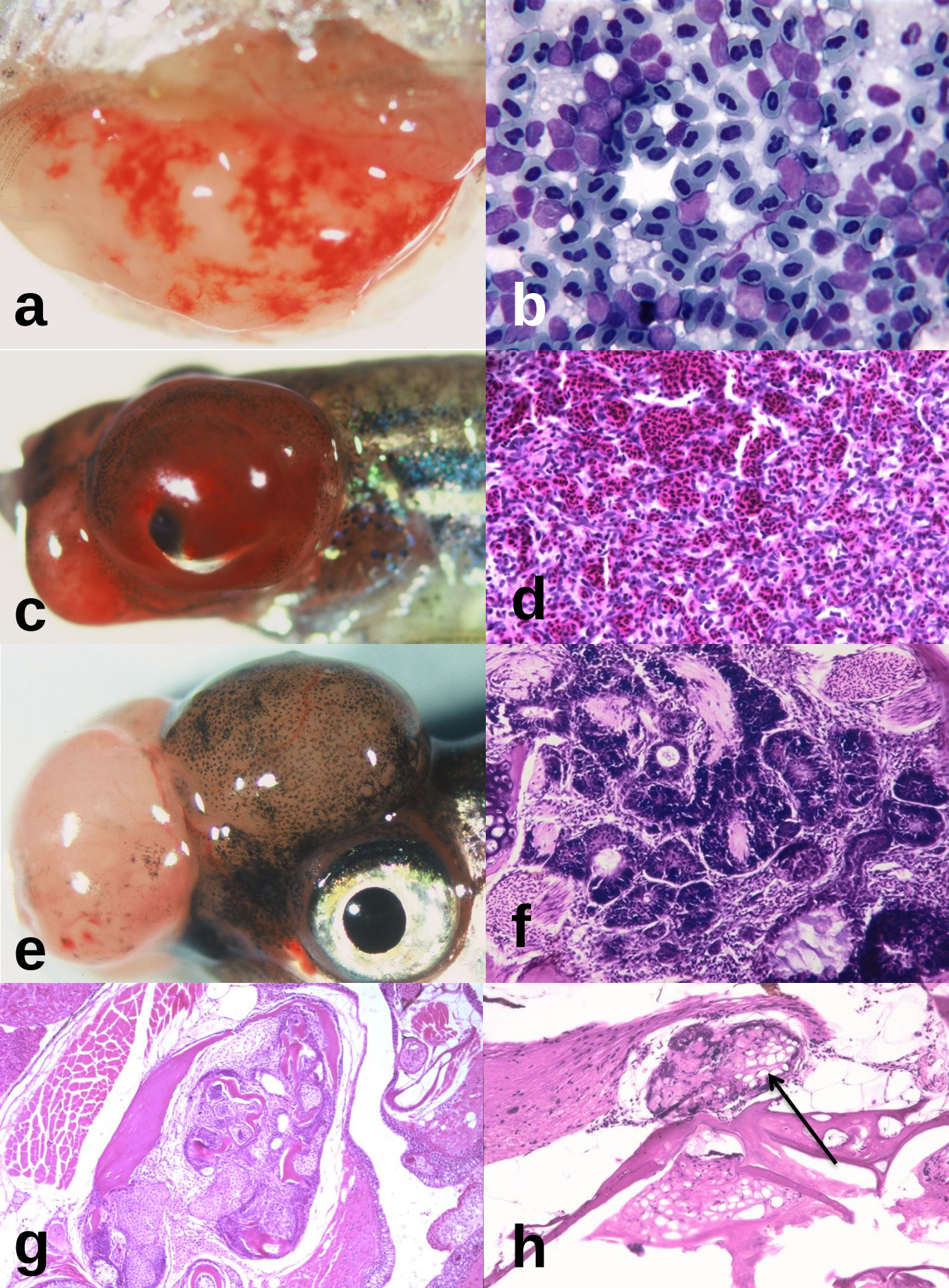
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Figure S2; Spitsbergen et al

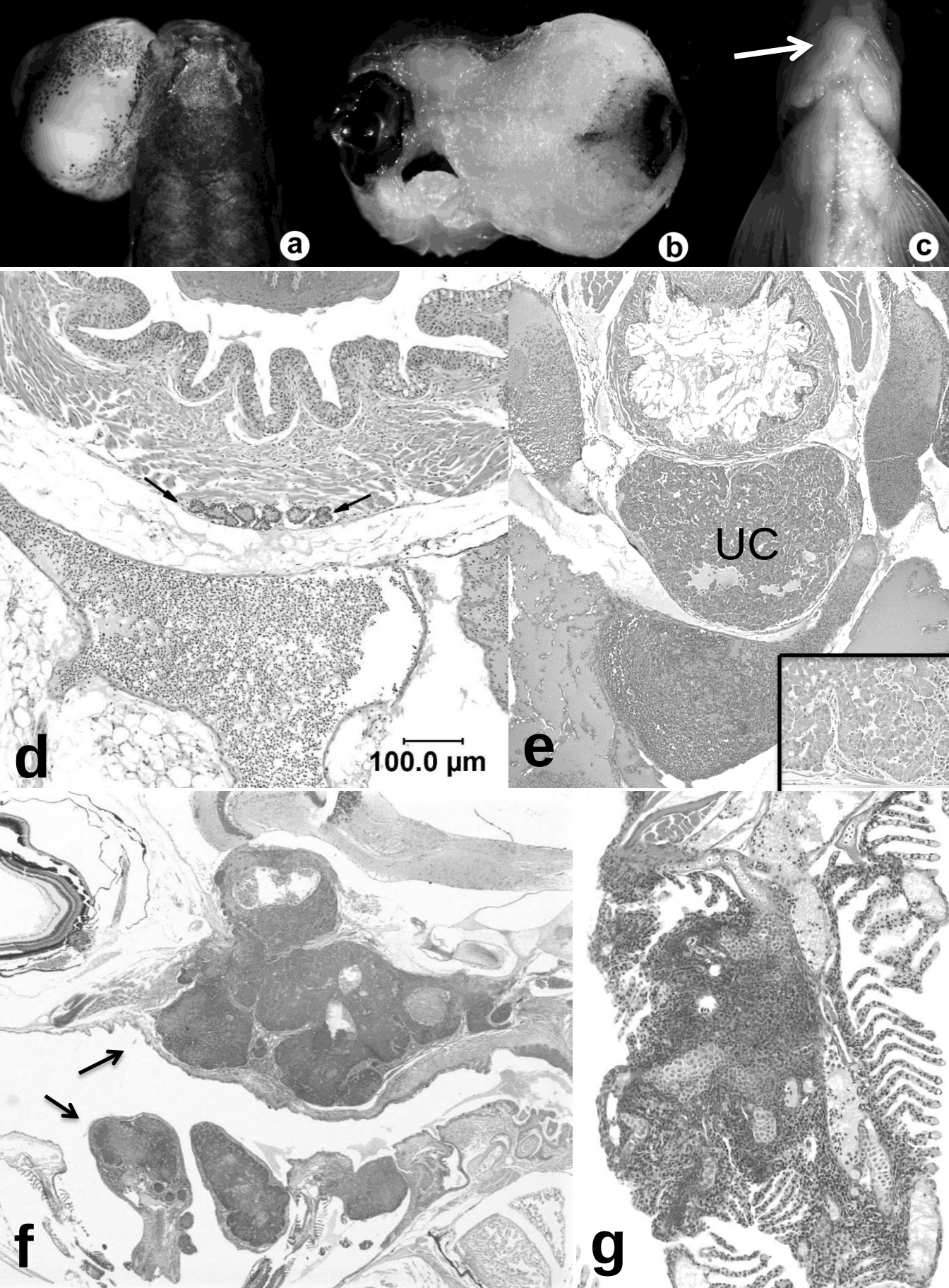
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Fig S3; Spitsbergen et al

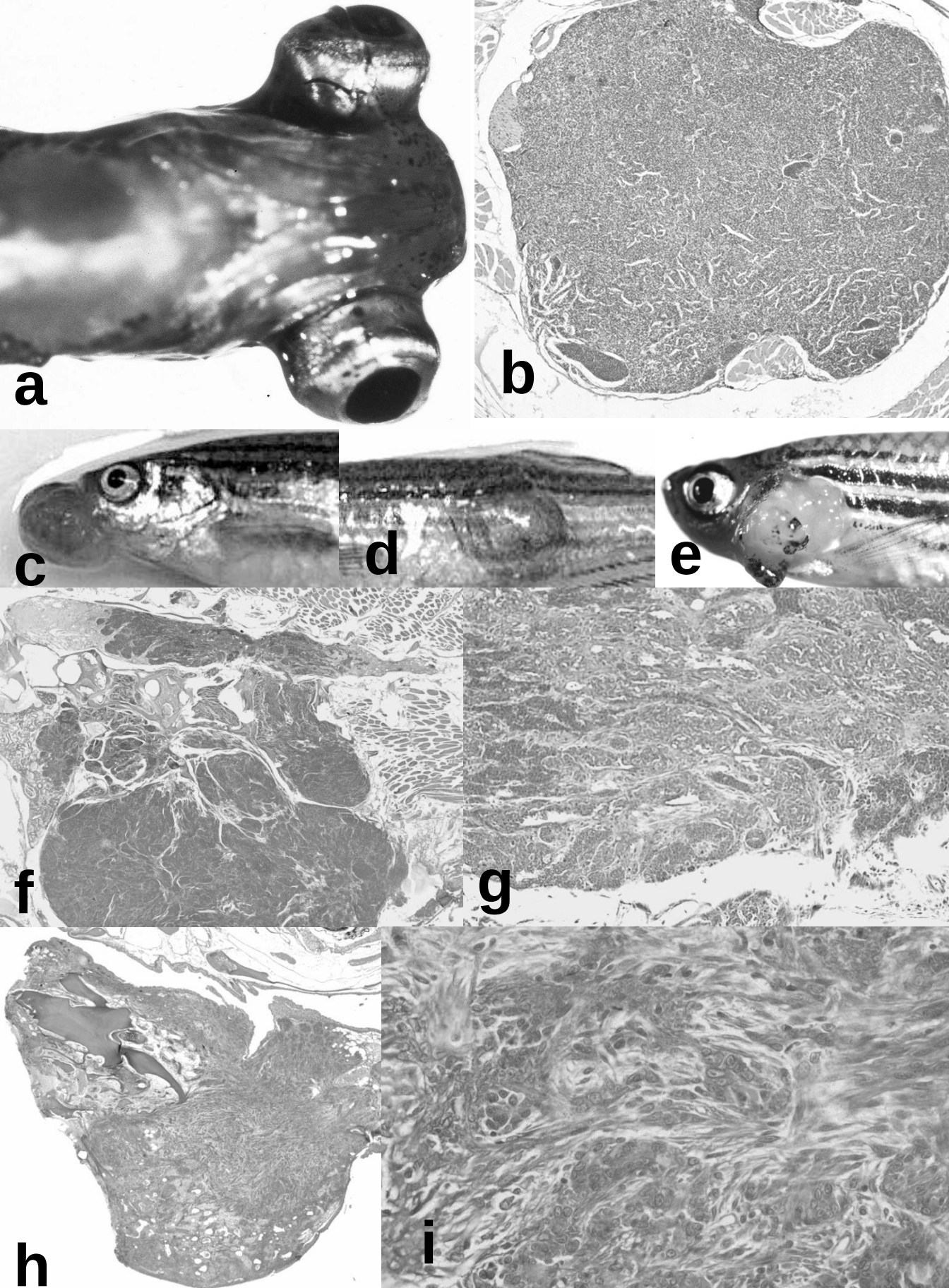
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Table S1 Analysis of overall neoplasm incidences, mortality and hepatocyte megalocytosis in various treatment groups with different diet and husbandry regimens.

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

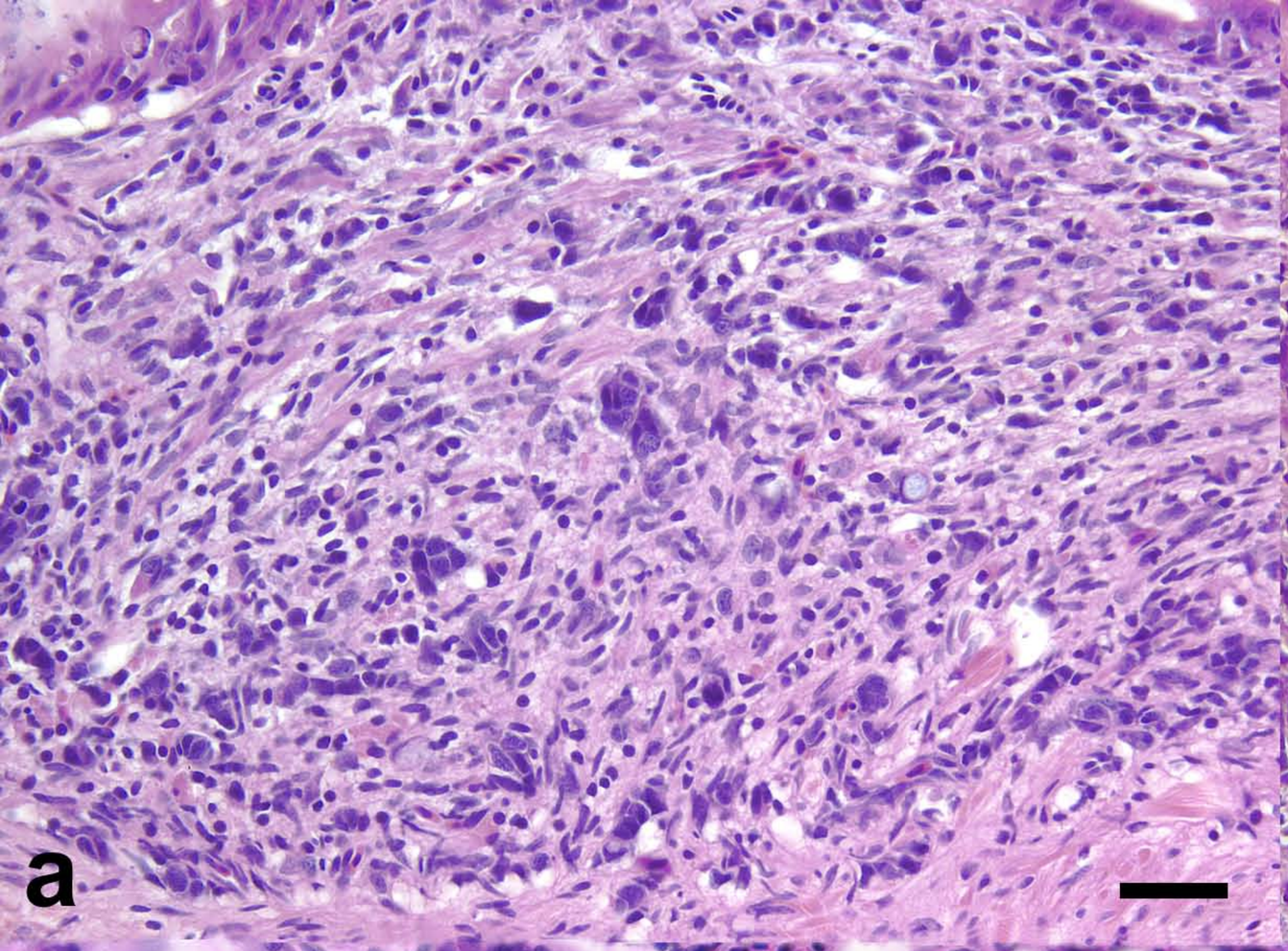
[f] Spitsbergen *et al*., 2000a

[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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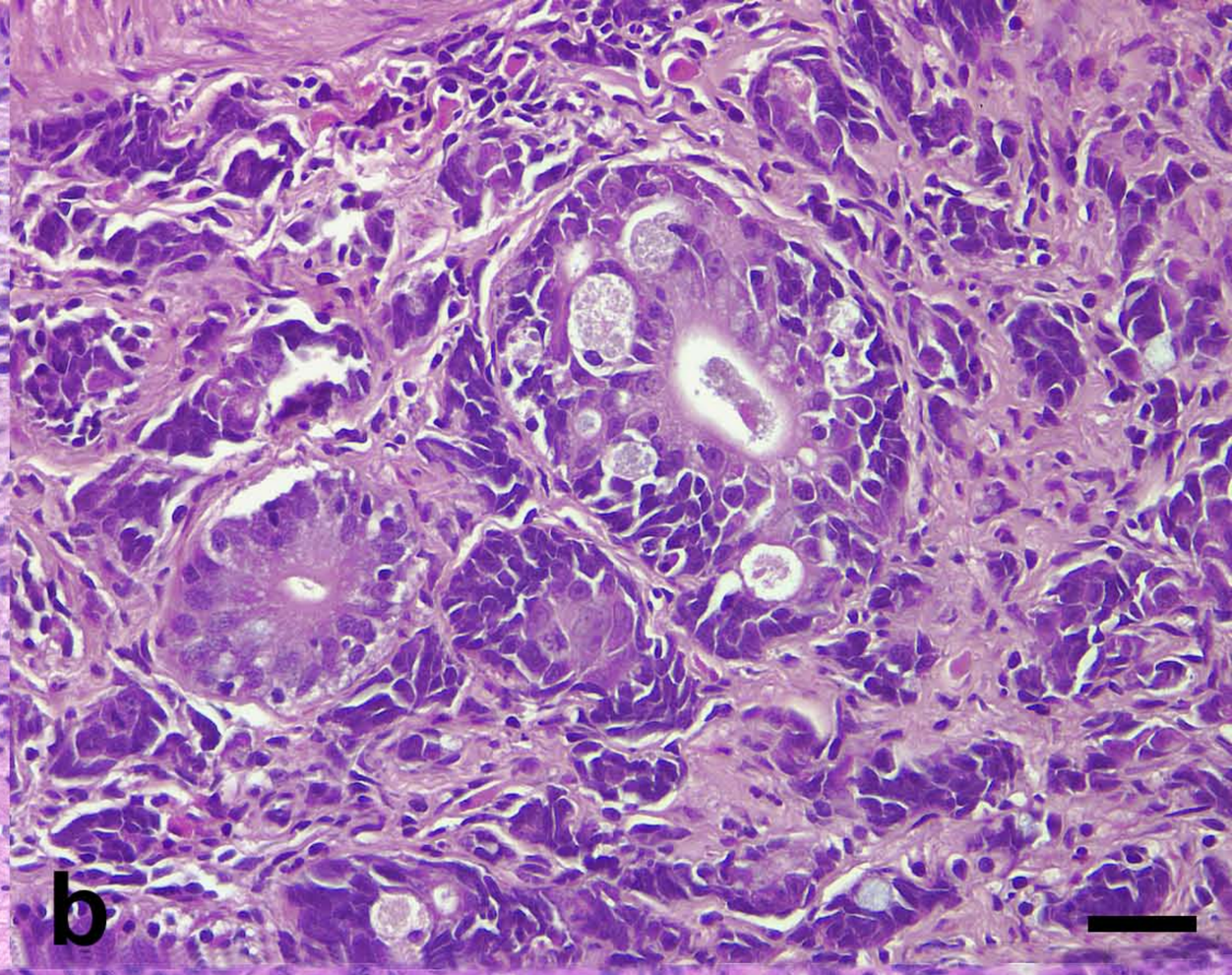
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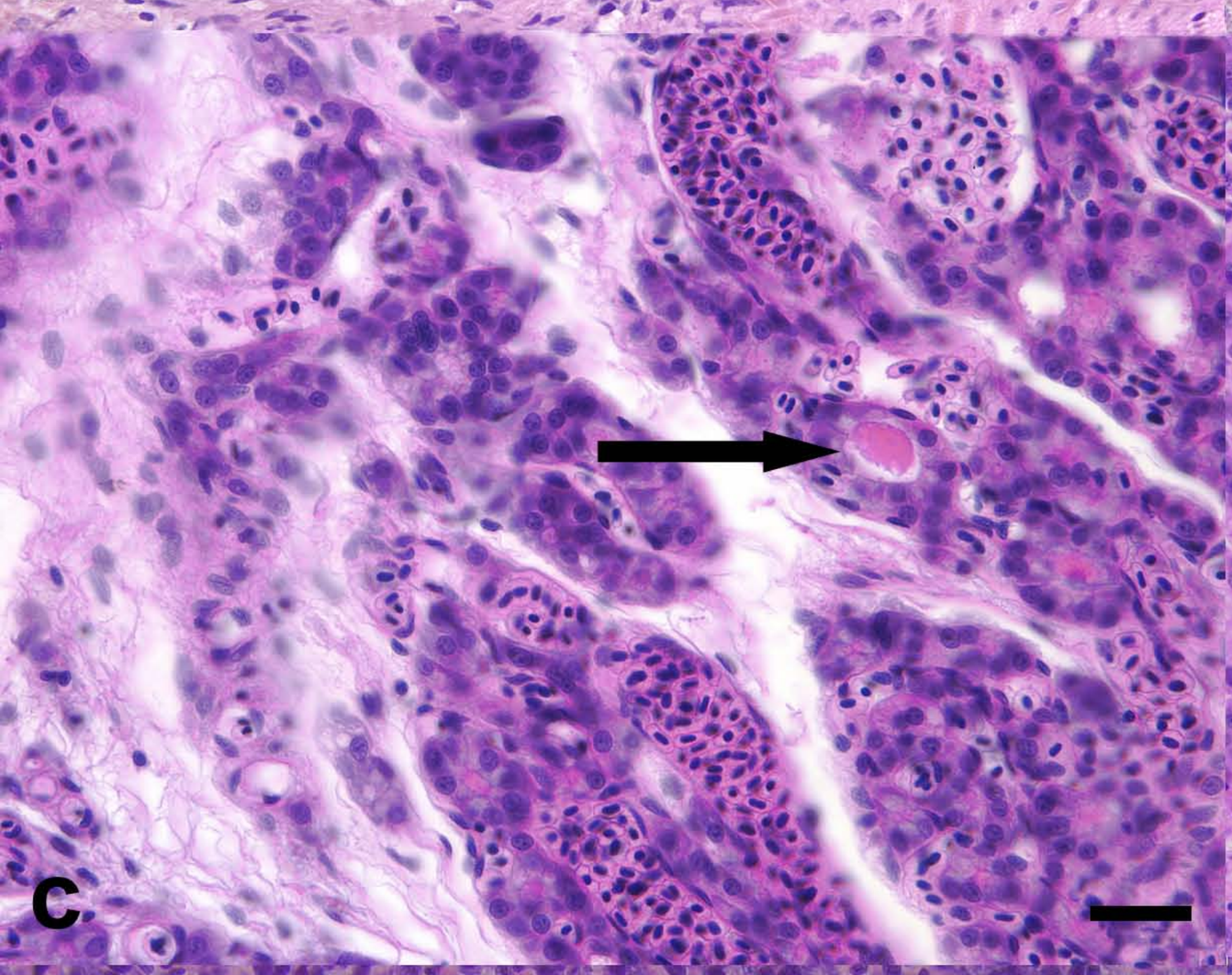
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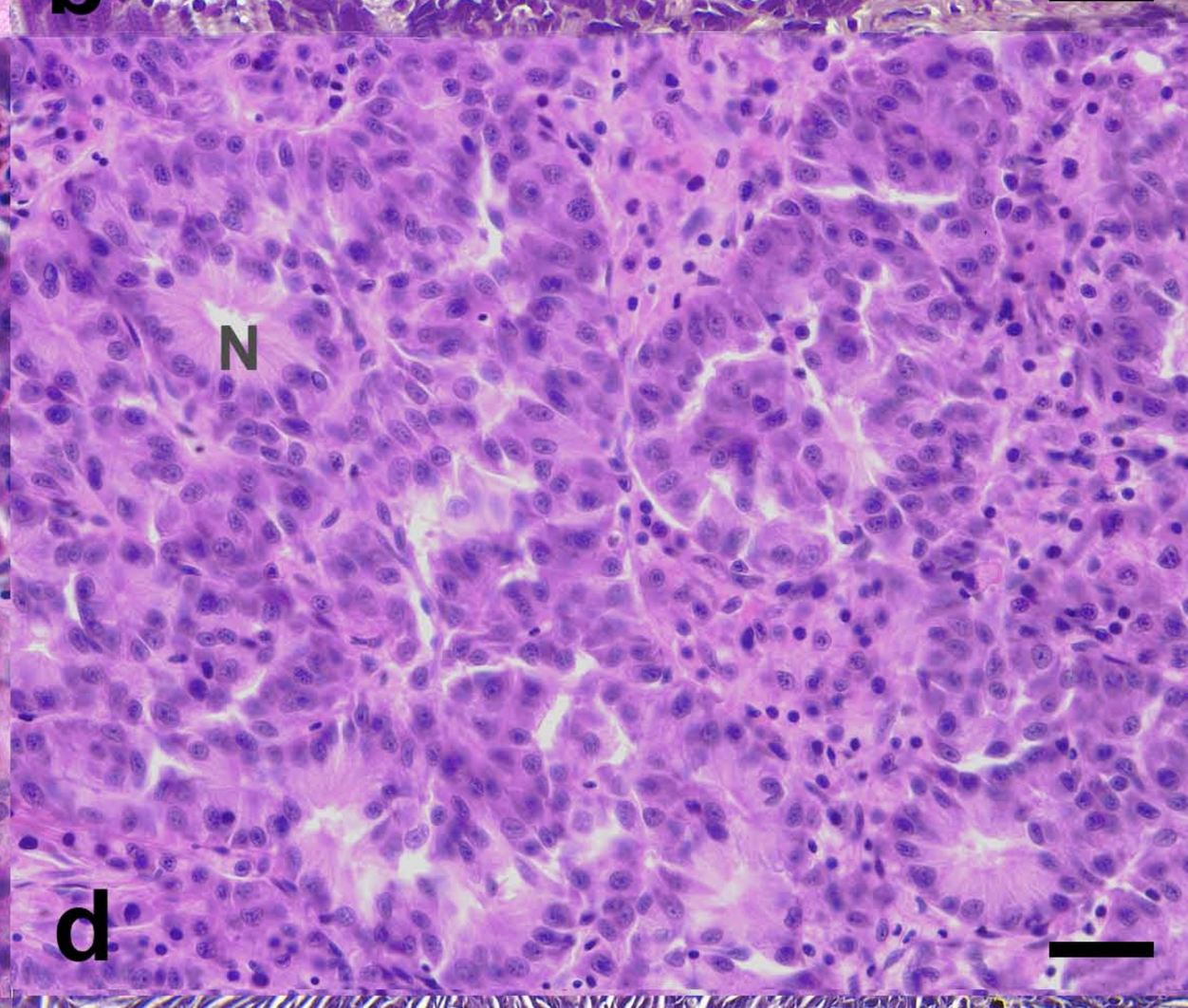
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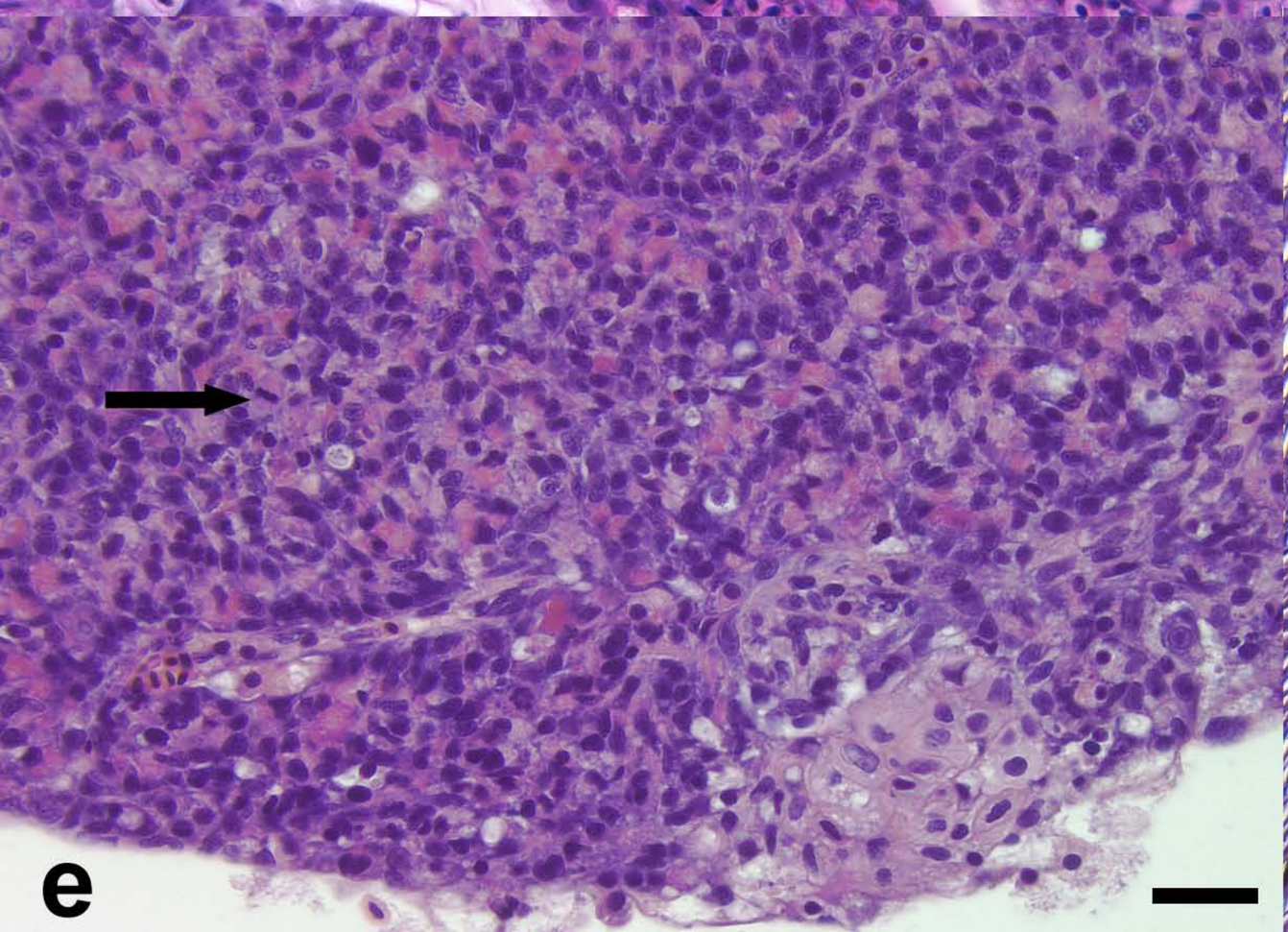
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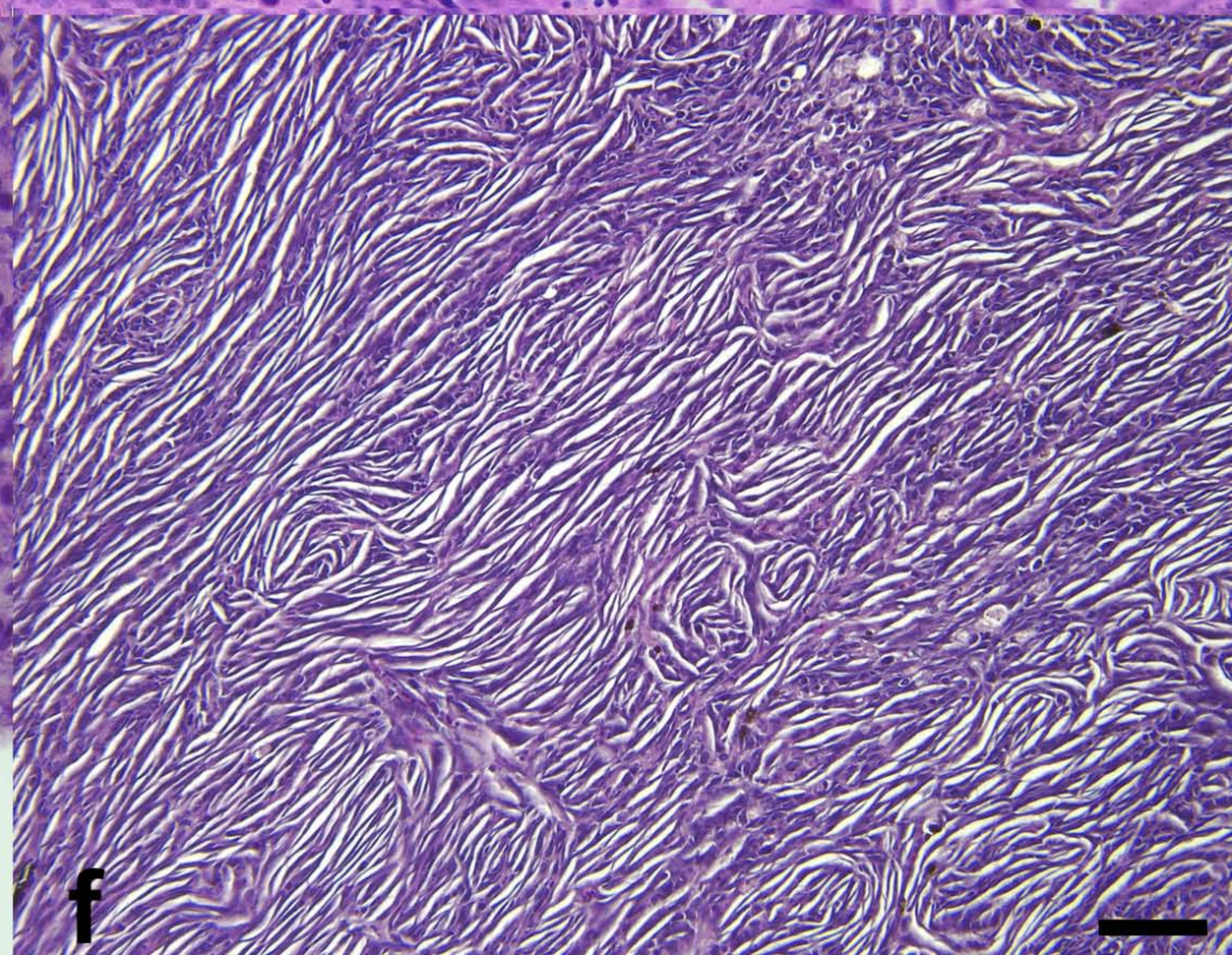
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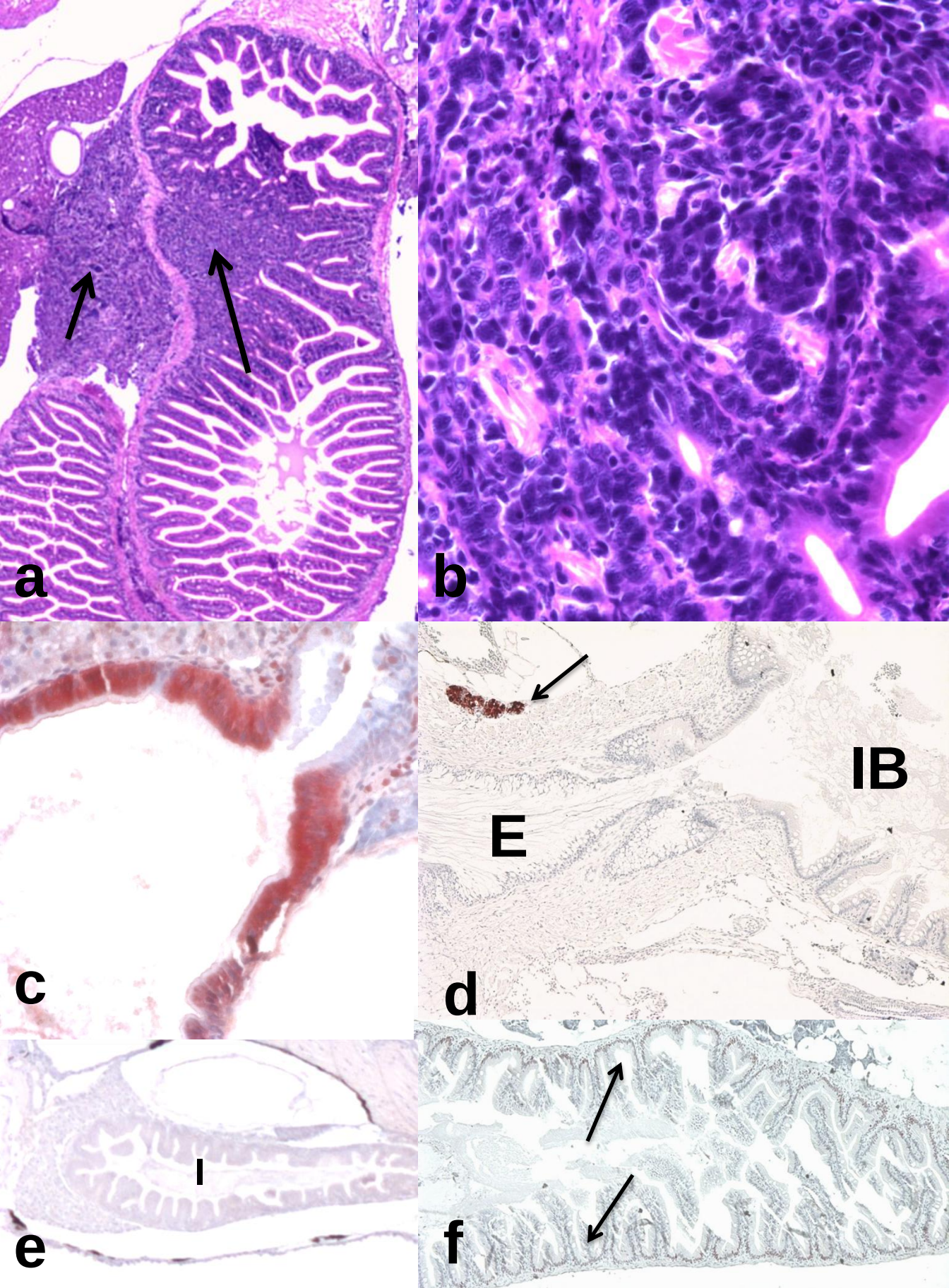
Fig S4; Spitsbergen et al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