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Zebrafish Germ Cell Tumors

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Introduction

Germ cell tumors (GCTs) are malignant cancers that arise from embryonic precursors known as Primordial Germ Cells. GCTs occur in neonates, children, adolescents and young adults and can occur in the testis, the ovary or extragonadal sites. Because GCTs arise from pluripotent cells, the tumors can exhibit a wide range of different histologies. Current cisplatin-based combination therapies cures most patients, however at the cost of significant toxicity to normal tissues. While GWAS studies and genomic analysis of human GCTs have uncovered somatic mutations and loci that might confer tumor susceptibility, little is still known about the exact mechanisms that drive tumor development, and animal models that faithfully recapitulate all the different GCT subtypes are lacking. Here we summarize current understanding of germline development in humans and zebrafish, describe the biology of human germ cell tumors, and discuss progress and prospects for zebrafish GCT models that may contribute to better understanding of human GCTs.

Germline development in fish, mouse and human

Primordial Germ Cell (PGC) Specification

The earliest cells of the germline lineage are known as Primordial Germ Cells (PGCs). PGC specification follows two distinct mechanisms, either preformation or induction, depending on the organism. Shortly after fertilization, zebrafish embryos, similar to *C. elegans*, *Drosophila* and *Xenopus*, contain preformed maternal RNA and proteins that are prepackaged in a cytoplasmic nuage, known as a germ plasm [1-4]. The germ plasm selectively and transcriptionally silences the expression of somatic genes while keeping the PGCs in a primitive pluripotent state. In flies and worms, RNA binding proteins including

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Pumilio and Nanos contribute to this repression of somatic fate [5, 6]. In zebrafish, *nanos* is essential for the development of PGCs [5].

The identification of the zebrafish *vasa* ortholog greatly facilitated efforts to understand the development of the zebrafish germline [7-10]. Vasa is an RNA helicase that was previously shown to be a component of the germline in nematodes, flies and frogs [11-14]. In zebrafish, maternally produced *vasa* mRNA is localized to electron-dense material at the cleavage planes of embryos at the 4-cell stage. By the 4000-cell stage, four *vasa*-positive PGCs can be identified. These will undergo several rounds of proliferation to produce a population of 25-50 migrating PGCs that will form the gonad [7, 8, 10, 15].

In contrast to organisms with preformed germline components, in mice and humans PGCs are produced in the epiblast through a process known as induction. In response to signals produced in the extraembryonic ectoderm and visceral endoderm, including Bone Morphogenetic Proteins 2, 4 and 8b [16-19], some of the epiblast cells begin to express the marker *Fragilis* (also known as *Mil-1/Ifitm3*), signaling their competence to become germ cells [18, 20-26]. A few of these *Fragilis*-expressing cells will begin to express the transcriptional repressor *Prdm1* (PR-domain-containing protein 1), also known as *Blimp1* (B lymphocyte-induced maturation protein 1) [27]. *Blimp1*, along with *Blimp14*, acts to repress expression of somatic genes and maintain expression of pluripotency genes such as *Stella*, *Oct3/4* and *Nanog* [20, 28-36].

PGC Migration

In nearly all metazoans, PGCs arise at sites distant from the future gonad and must migrate during embryogenesis to reach the proper location for eventual gonadogenesis [37-40]. In contrast to other organisms, zebrafish PGCs arise in four random locations with respect to the developmental axis of the embryo. The PGCs migrate to a series of intermediate targets, converging into bilateral clusters in the presomitic mesoderm, where the gonad will form [3, 10, 15, 41]. This guided migration is orchestrated by an attractant gradient established by somatic expression of Stromal-derived factor 1 (*sdf1a*), also known as CXCL12 [42, 43]. The *sdf1a* ligand interacts with the chemokine receptor *cxc4b* expressed on the surface of PGCs [42, 43]. This homing signal is further fine-tuned by the *cxc7* receptor on somatic cells, which acts as a repulsive guidance cue [44].

In mice and other mammals, the newly specified PGCs must travel from the primitive streak and localize to the endoderm around stage E7.5 [45, 46]. From E8 to E9.5 the PGCs migrate through the dorsal mesentery of the hindgut. Upon reaching the level of the gonadal ridges, the PGCs exit the hindgut in bilateral streams to populate the gonadal mesoderm [37, 46]. The c-Kit receptor and its ligand, KitL, are critical for the migration and survival of PGCs in mice [47-50].

Germ cell differentiation

Irrespective of the future sex of the animal, germ cells in zebrafish initially begin to differentiate into oocytes and are in a state known as juvenile hermaphroditism [51]. Upon the start of male germ cell differentiation, oocytes in male zebrafish undergo apoptosis as the mature male germline emerges [52]. Characterizations of male sex determination factors

have identified several genes that are specific to the male gonads, such as anti-Mullerian hormone (*amh*) and *sox9a* [53-55]. Furthermore, *sox9a* was shown to control juvenile hermaphroditism and regulate the transition to male fate producing mature sperm [56].

During gonadogenesis, the number and persistence of germ cells makes important contributions to sex determination [57, 58]. Several studies have revealed that lower PGC numbers promote male differentiation in zebrafish. Loss of *dead end (dnd)*, an RNA-binding protein that is essential for PGC survival [59], leads to loss of all germ cells and male phenotypic differentiation [57, 60, 61]. Similarly, loss of function *ziwi*, the zebrafish *piwi* homolog, leads to reduced numbers of germ cells and male phenotypic development [62]. Remarkably, Dranow and Draper found that adult female zebrafish depleted of germ cells due to loss-of-function mutations in *nanos3* convert to a male phenotype [58]. They concluded that a germ cell-derived signal acts on the somatic gonad to promote female development directly or indirectly by repressing male-specific gene expression.

In mice and other mammals, licensing is the initial step in which germ cells begin to differentiate into their respective sex cells dependent on the sex chromosomes present in the organism. This licensing event is initiated by the RNA-binding protein known as Deleted in azoospermia-like (DAZL); *Dazl* mutants no longer express sex specific markers and do not begin meiosis [63, 64]. During this transition, the germ cells are released from their transcriptional repression [65]. In prenatal males, presence of the SRY gene on the Y chromosome regulates expression of Sox9 and is necessary for proper male gonadal development and spermatogenesis [66-68]. Humans with a heterozygous mutation in *SOX9* develop a bone disorder known as campomelic dysplasia that is accompanied by gonadal sex reversal, while XY mice with homozygous mutations exhibit ovarian development [69-71]. These studies emphasized the importance of proper Sox9 expression in testis development. To maintain male identity prenatally, Stra8 (Stimulated by retinoic acid gene 8), an enzyme required for meiosis initiation, must be persistently repressed by Cyp26B1 [72-74]. Postnatally, repression of Cyp26B1 releases repression of Stra8 and allows germ cells to respond to retinoic acid, which initiates meiosis [72-74]. The germ cells are now able to exit G1/G0 and can then proceed to make mature sperm.

As in the fish, gonadal sex in mammals appears not to be completely fixed. The transcription factor DMRT1 is required in both somatic cells and germ cells for normal male gonadal differentiation [75, 76]. Loss of *Dmrt1* in adult Sertoli cells of the testis results in transformation of the cells to granulosa cells and transition of the gonad to a more ovarianlike phenotype [77]. Furthermore, forced expression of DMRT1 in the ovary reprograms granulosa cells to Sertoli cells, leading to the development of structures resembling seminiferous tubules [78].

Human Germ Cell Tumors

In humans, GCTs occur in infants, children and young adults [79] (Figure 1). Both childhood and adolescent/adult GCTs are thought to originate from Primordial Germ Cells (PGCs). Owing to the pluripotent nature of these cells, GCTs can take on a variety of different histologic fates [80]. GCTs in which the PGCs retain pluripotency and do not

differentiate are known as seminomas (also called 'dysgerminomas' in females and 'germinomas' when occurring in the CNS). In contrast, GCTs in which the cells take on a variety of differentiation states are designated non-seminomas, of which embryonal carcinoma is thought to represent the stem cell component. GCTs differentiated to somatic cell lineages (endoderm, mesoderm, and ectoderm) are known as teratomas. Finally, GCTs may take on extraembryonic differentiation resembling the fetal yolk sac (yolk sac tumors) or the placenta (choriocarcinoma).

The histology of GCTs is similar in both males and females, whether occurring in the testis, ovary or extragonadal sites, implying origin from a common precursor cell [79]. However, there are some epidemiologic differences in the incidence of different types of GCT. In males there are two peaks of testicular GCT incidence, one in early childhood at age 3-4, and a second, much larger peak that begins at puberty and is maximal at around age 30. In females, there is an early peak from age 0-2 representing the incidence of sacrococcygeal teratoma, an extragonadal GCT, in newborns and infants. Beginning at age 5-6, the incidence of ovarian GCT increases with age, becoming maximal at age 20-25 [81].

While the overall incidence of GCT (about 12,500 cases/year in the USA) is lower than that of common epithelial cancers such as lung, breast and prostate cancer, testicular GCT is the most common cancer and the leading cause of cancer death in young men [79]. The incidence of GCT is increasing around the world, for unknown reasons [81]. There are several known risk factors that increase the risk of developing testicular germ cell tumors. They include but are not limited to disorders of sexual development (DSD), gonadal dysgenesis, cryptorchidism (undescended testis), familial background, environmental exposure, and genetic association. Familial risk can increase the risk of developing GCT 4-fold in a male with a father who had GCT or up to 9-fold in a male whose brother had GCTs. The incidence of GCT varies widely in different geographic regions [82, 83], leading to the idea that the environment may strongly influence risk for testicular GCT.

There are also important age-dependent differences in GCT histologic spectrum. Type I GCTs occur in infants and children and consist of teratomas and yolk sac tumors (YSTs). Type II tumors in adolescents and adults have more diverse histology, and include seminomas, non-seminomas or mixed tumors containing both seminomatous and nonseminomatous elements. Based on differing epidemiology, clinical outcome and histologic spectrum, GCTs in young children may be biologically distinct from GCTs occurring in older (post-pubertal) populations.

This idea is increasingly supported by molecular evidence. Whereas Type I tumors show variable Loss of Imprinting (LOI) at loci such as IGF2 and H19, adult-type GCTs tend to show complete erasure of imprinting [84, 85]. This result is interesting, because it implies that Type I GCTs may arise at an earlier stage of PGC development, since PGCs undergo erasure of imprinting during early development, and this erasure is largely completed by the end of PGC migration. Cytogenetic data consistently show loss of Chromosome 1p and 6q in Type I tumors, while Type II tumors also commonly exhibit amplification of Chromosome 12p. More recently, studies directly comparing the gene expression patterns of pediatric and adult GCTs have demonstrated distinct transcriptional profiles in tumors of similar histology

arising in different age groups (for example, in yolk sac tumors of children vs. yolk sac tumors of adolescent/adults) [86]. Taken together, these studies support the notion that different biological mechanisms may drive childhood and adolescent/adult germ cell tumorigenesis.

Current cisplatin-based therapy for GCTs cures most patients [81, 87, 88], but major challenges remain. 15% of patients have cisplatin-resistant tumors and will eventually die of their disease [89]. Furthermore, there is significant therapy-related toxicity in patients treated for GCTs. Late effects in patients treated with cisplatin, etoposide and bleomycin (the current first-line regimen for GCT) include pulmonary fibrosis [90], renal insufficiency and saltwasting [91-93], infertility and hormonal changes [94-98], hyperlipidemia [99, 100], Raynaud's phenomenon [91, 98, 101, 102], obesity [99, 100, 103-105] and neuropathy [106]. Ototoxicity results in significant hearing loss, as assessed by audiogram, in nearly 80% of patients [91, 97]. Survivors of GCT treatment have twice the risk of early onset cardiovascular disease [96] and second malignancies [107, 108].

Compared to many other solid malignancies, relatively few somatic mutations have been described in GCTs. This lack of knowledge inhibits the development of targeted therapy that could provide an alternative or adjunct to standard chemotherapy. Amplification of Chromosome 12p is a pathognomonic feature of adolescent/adult GCTs, but no genes in this region have definitively been linked to germ cell tumorigenesis [109]. The most commonly reported mutated gene is *KIT*, a tyrosine kinase growth factor receptor that plays important roles in germ cell development [110-115]. Mutations have also been reported in *NRAS* and *KRAS*, signaling components of the MAP kinase pathway that act downstream of *KIT* [116-119]. Central Nervous System GCTs exhibit *KIT* and *RAS* mutations [120]. Somatic mutations in *BRAF*, another MAP kinase pathway member, have been associated with cisplatin resistance in adult TGCTs [121]. More recently, exome sequencing of TGCT identified somatic mutations in several genes including *CIITA*, *NEB*, *PDGFRA*, *WHSC1* and *SUPT6H* [122]. A larger study of 42 adult TGCTs identified somatic mutations in *CDC27* and mutations in *XRCC2* associated with cisplatin resistance [123]. To date, the mutation spectrum of Type I GCTs has not been reported.

A number of genome-wide association studies (GWAS) have been conducted in men with TGCT, resulting in the identification of a large number of novel germ cell tumor susceptibility loci. In 2009, two groups reported strong linkage of GCT susceptibility to loci on chromosomes 5, 6 and 12, suggesting roles for *SPRY4* and *KITLG*, both components of receptor tyrosine kinase signaling, as well as for the pro-apoptotic *BAK1* gene [124, 125]. A follow-up study by Turnbull and co-workers identified additional susceptibility loci near the *DMRT1*, *TERT* and *ATF7IP* genes [126]. The studies have subsequently been replicated in other patient populations [127-129]. More recently, further GWAS studies have identified new candidate GCT susceptibility loci, including genes with potential roles in telomere regulation, such as *PITX1*, or germ cell development, such as *TEX14*, *RAD51C*, *PRDM14* and *DAZZL* [130, 131].

Because of the many unresolved issues concerning the causes and biological mechanisms of GCTs, and the urgent need to develop and test new treatments, animal models of GCT are

badly needed. However, to date relatively few models have been available. The 129Sv inbred mouse strain was found more than 50 years ago to be susceptible to testicular teratoma formation, with spontaneous tumors forming in 1-5% of animals [132]. Mutations in several genes have been shown to increase the teratoma incidence in 129Sv mice, including *Dead end* [133] and *Dmrt1*. In fact, *Dmrt1*-mutant 129Sv mice develop testicular teratomas at nearly 100% incidence [134, 135]. The teratomas that develop in the 129Sv background strongly resemble the Type I testicular teratomas occurring in young boys. However, mouse models of seminomas or of non-seminomas other than Type I teratomas have not been described. Recent progress suggests that zebrafish may be a useful addition to GCT models, at least for seminomas.

Zebrafish Germ Cell Tumor Models

During the past several years, several fish models have been described with abnormal germline development that recapitulates certain aspects of human germ cell tumors. Three of these models were identified using the zebrafish, while one was identified in Medaka. All of these models demonstrate an inability to undergo proper germ cell differentiation and as a result exhibit immature, enlarged testes. Three of the models were identified through forward genetic N-ethyl-N-nitrosourea (ENU)-based mutagenesis screens while one is a transgenic line. These models can serve to provide further insight into human seminomas with the possibility to contribute to our understanding of the human disease.

The first fish model described was identified in Medaka, following an ENU mutagenesis screen to uncover genes that are important for germ cell and gonadal development [136]. Morinaga et al identified the *hotei* “hot” mutant fish, which was initially characterized as having extreme gonadal hypertrophy. The gonads were further examined and found to have increased numbers of germ cells that resided in disrupted testicular architecture, as well as decreased ability to produce mature sperm as compared with their wild-type siblings. The loss of function mutation was mapped, by positional cloning, to reside in the anti-Mullerian hormone receptor II (*amhrII*). This receptor belongs to the TGF- β /BMP superfamily and in humans is known to play a role in Mullerian duct regression in males. The mutation, in exon 9 of *amhrII*, changes a tyrosine residue in the highly conserved kinase domain to cysteine.

Neumann and co-workers used ENU mutagenesis and high-throughput histologic screening to identify a line of zebrafish that developed testicular germ cell tumors (GCTs) with high penetrance (>80% incidence in homozygotes by 4 months of age) (Figure 2). They showed that the mutation is dominantly inherited, and causes the development of tumors of primitive, undifferentiated testicular GCTs resembling human seminoma (Figure 3). Like human seminomas, the zebrafish seminomas were sensitive to radiation therapy and underwent apoptosis in response to DNA damage [137]. To identify the molecular nature of the mutation, these investigators developed a haplotype-mapping panel and, using this technique coupled with further high-resolution meiotic mapping, identified a mutation in the Type IB BMP receptor, *alk6b/bmpr1bb*, as the cause of the zebrafish GCTs [138]. BMPs (Bone Morphogenetic Proteins), members of the TGF- β superfamily, play important roles in development and differentiation, but had not been linked to GCTs. Neumann et al. showed that the mutation causes loss of BMP signal transduction, and that BMP signaling is required

for germ cell differentiation. This work represents the first identification through positional cloning of a cancer predisposition mutation in the zebrafish.

To understand the relevance of BMP signaling to human GCTs, Fustino *et al.* profiled the state of the BMP pathway in clinical GCT specimens and showed that undifferentiated human GCTs (seminomas and embryonal carcinomas) also lack BMP signaling [139]. This work also identified the miR-200 family of microRNAs as modulators of the BMP pathway, providing an explanation for differential BMP signaling activity in seminomas vs. yolk sac tumors. Collectively these studies identified BMP signaling as a key node in GCT differentiation and a promising target for novel therapies.

The first and only transgenic model of GCTs was identified by Gill *et al.* In this model, the Simian Virus 40 (SV40) T-antigen (TAg) is driven by the lymphocyte specific tyrosine kinase (lck) promoter from *Fugu*. Originally intended as a T-cell leukemia model, these fish did not manifest thymic malignancies, but rather testicular GCT. By 36 months, about 20% of the offspring developed tumors [140]. When comparing the tumors with wild type testis, the GCTs were clearly enlarged and lacked organized seminiferous tubules. Furthermore, the testicular GCTs were predominantly composed of undifferentiated spermatogonial-like germ cells determined by both sorting the cells by size (FACs) and histological analysis (Figure 3) [140]. Expression patterns of human seminoma specific genes were unchanged as compared to zebrafish wildtype testis. Various RAS genes were also sequenced in the zebrafish to identify any de novo mutations that could explain the TGCTs in the fish but none were found.

Finally, the most recent GCT model was identified through a forward genetic ENU screen. The causative mutation is a nonsense mutation in the leucine-rich repeat (LRR)-containing protein *lrrc50* [141]. Initially, this forward genetic screen was performed to identify genes involved in ciliary motility. *lrrc50* mutants develop a phenotype resembling that of human primary ciliary dyskinesia (PCD). Early in development, the *lrrc50* homozygous mutants develop a curved body axis, dilated pronephric ducts, kidney cysts and severe edema, which ultimately results in embryonic death at 8 days post-fertilization (dpf) [141]. Interestingly, heterozygous *lrrc50* mutants display increased incidence of testicular GCT, suggesting a novel tumor suppressor role for *lrrc50*.

lrrc50 heterozygous mutants display a 90% GCT penetrance in their 2nd and 3rd years of life [142]. Histological analysis of the tumors found that there were severely reduced numbers of differentiated germ cells, and most of the cells morphologically resembled early spermatogonial cells (Figure 3). These cells also stained positive for phospho-histone H3 in single cells unlike the wild type testis, which showed reduced staining in clusters. 44.4% of the tumors examined showed a loss of the *lrrc50* wildtype allele, consistent with a role for *lrrc50* as a tumor suppressor.

This model is, to date, the only one that found mutations in the respective gene in human seminomas [142]. Basten *et al.* identified *LRRc50* nonsense mutations in two pedigrees with family history of seminomas. In a separate analysis of 38 patients with sporadic seminomas, 5 demonstrated heterozygosity for different germline *LRRc50* mutations. The

mutations were shown to be functional nulls by their inability to complement the lethal larval phenotype of *lrrc50* mutants. These results establish *LRRC50* as the first gene specifically linked to seminoma predisposition in humans. These results prompted an investigation of the possible roles of cilia in germ cell development. In a novel finding, the investigators demonstrated the presence of primary cilia in normal spermatogonia and showed that *LRRC50* colocalizes with the axoneme in spermatogonial stem cells, that its expression is cell cycle-regulated and that it colocalizes with condensed chromosomes [142]. These results shed new light on the possible importance of ciliary signaling in germ cell development and germ cell tumorigenesis.

Summary

Germ cell tumors are among the most common cancers of young people, and the leading cause of cancer death in young men. The wide age range of incidence and histologic diversity of the tumors creates challenges in GCT biology and treatment. While some genomic data are now beginning to point to possible molecular origins of these tumors, the paucity of representative animal models is an impediment to further functional genomic and translational studies.

To date the zebrafish has proved to be a powerful model for understanding the biology of PGC development and migration. While there are important differences between human and fish germ cells, a number of conserved mechanisms have been identified, suggesting that studies in zebrafish can contribute importantly to our understanding of human germ cell biology.

Zebrafish models of human germ cell tumors have also begun to make important contributions. The studies described above have identified several signaling pathways, such as the TGF- β /BMP pathway and cilia-mediated signaling, that are directly relevant to human seminomas. The development of seminoma-like tumors in fish transgenic for the known oncogene SV40 large T, and the response of GCTs to DNA-damaging agents, suggests that these models recapitulate important aspects of the oncogenic phenotype of human GCTs.

There are, however, several important caveats that apply to the current models. There is no evidence that these models of GCTs become invasive or malignant, as do some examples of human seminomas. To date, true zebrafish models of ovarian GCT (as opposed to models of impaired maturation of post-meiotic oocytes) have not been described. Reverse genetic models that use gain or loss-of-function approaches and germ cell-specific promoters to test the role of specific GCT candidate genes, at specific developmental stages, remain to be developed. This is a particularly significant gap in current approaches, because it is likely that the ability of germ cells to develop along somatic lineages, as seen in non-seminomas, is limited to specific stages of development in which germ cells retain some pluripotency. In humans, non-seminomas are more resistant to treatment and more likely to metastasize than are seminomas, and are responsible for the majority of deaths from GCT, emphasizing the need for such models. However, there is reason to be optimistic. The great recent progress in the field, with the availability of new promoters, new genome editing techniques and increasing information about the genomic landscape of human GCT, means that the next few

years promise to see great advances in our understanding of GCT, and better options for GCT patients.

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Figure 1. Mammalian spermatogenesis and human germ cell tumor (GCT) histological classification

The transition of primordial germ cells from embryonic stem cells to gonocytes is facilitated by increased expression of germ cell specific genes and decreased expression of embryonic pluripotency genes. In humans, sex chromosomes dictate the fate of a gonocyte; in the testis self-renewing spermatogonial stem cells give rise to progeny that undergo meiosis to produce mature sperm. Germ cell tumors arise from primordial germ cells (PGCs). Type I GCTs are generally seen in neonates and young children and include Teratomas and Yolk Sac Tumors. Type II GCTs occur in adolescents and adults and may proceed through a carcinoma *in situ* (CIS) precursor (also known as Intratubular Germ Cell Neoplasia). CIS cells can give rise to both Seminoma and Non-Seminoma, the stem cell component of which is Embryonal Carcinoma (EC). EC may undergo somatic differentiation into Teratomas, containing derivatives of ectoderm, mesoderm and endoderm. Alternatively EC cells may differentiate into GCTs resembling extraembryonic tissues, such as Yolk Sac Tumor and Choriocarcinoma. Mixed tumors containing both seminoma and non-seminoma components also occur.

Figure 2. Testicular GCT in *bmp1bb* mutant zebrafish

Mutants develop GCTs because of impaired germ-cell differentiation. Compared with adult wild-type males (a and c) (arrowhead: normal testis), adult *bmp1bb* males display abdominal distension and marked testicular enlargement (b and d) (arrow: testicular tumor). Modified with permission from Neumann *et al.*, 2011

Figure 3. Zebrafish germ cell tumor models exhibit histologic similarity

a, normal testis. The testis consists of cysts or lobules of spermatogenic cells surrounded by a basement membrane and somatic cells. Small clusters of spermatogonia are seen adjacent to the basement membrane (arrowheads). Successive stages of differentiation are also evident, including primary and secondary spermatocytes, spermatids and mature spermatozoa. b, Germ cell tumor in a *bmpr1bb* mutant testis, showing loss of differentiation and an excess of primitive, spermatogonial-like cells. c, Testicular germ cell tumor from the *Tg(flck:TA)* T24 line. Tu: tumor. d, testicular GCT in a *lrrc50^{H255h}* fish. Two magnifications of the same tumor shown (largest in insert), with loss of differentiated germ cells in the tumor. Scale bars: 50 microns. (a,b: modified with permission from Neumann *et al.*, 2011. c, modified with permission from Gill *et al.*, 2010. d, modified from Basten *et al.*, 2013.)