Zebrafish *Hagoromo* Mutants Up-Regulate *fgf8* Postembryonically and Develop Neuroblastoma

Adam Amsterdam,1 Kevin Lai,1 Anna Z. Komisarczuk,2 Thomas S. Becker,2 Roderick T. Bronson,3 Nancy Hopkins,1 and Jacqueline A. Lees1

1David H. Koch Institute for Integrative Cancer Research at MIT, Cambridge, Massachusetts, 2Sars Centre for Marine Molecular Biology, University of Bergen, Bergen, Norway, and 3Tufts Cummings School of Veterinary Medicine, North Grafton, Massachusetts

Abstract

We screened an existing collection of zebrafish insertional mutants for cancer susceptibility by histologic examination of heterozygotes at 2 years of age. As most mutants had no altered cancer predisposition, this provided the first comprehensive description of spontaneous tumor spectrum and frequency in adult zebrafish. Moreover, the screen identified four lines, each carrying a different dominant mutant allele of *Hagoromo* previously linked to adult pigmentation defects, which develop tumors with high penetrance and that histologically resemble neuroblastoma. These tumors are clearly neural in origin, although they do not express catecholaminergic neuronal markers characteristic of human neuroblastoma. The zebrafish tumors result from inappropriate maintenance of a cell population within the cranial ganglia that are likely neural precursors. These neoplasias typically remain small but they can become highly aggressive, initially traveling along cranial nerves, and ultimately filling the head. The developmental origin of these tumors is highly reminiscent of human neuroblastoma. The four mutant *Hagoromo* alleles all contain viral insertions in the *fbxw4* gene, which encodes an F-box WD40 domain–containing protein. However, although one allele clearly reduced the levels of *fbxw4* mRNA, the other three insertions had no detectable effect on *fbxw4* expression. Instead, we showed that all four mutations result in the postembryonic up-regulation of the neighboring gene, *fibroblast growth factor 8* (*fgf8*). Moreover, *fgf8* is highly expressed in the tumorigenic lesions. Although *fgf8* overexpression is known to be associated with breast and prostate cancer in mammals, this study provides the first evidence that *fgf8* misregulation can lead to neural tumors. (Mol Cancer Res 2009;7(6):841–50)

Introduction

The zebrafish is an increasingly popular model organism in which to study cancer (1). Mutation of known tumor suppressors such as *nf2*, *p53*, *apc*, and *mlh1* (2–5) result in cancer susceptibility, and tumorigenesis can also be driven by the expression of a number of oncogenes such as *c-myc* (6), mutant *Kras* (7), or mutant *braf* (8). Additionally, forward genetic screens in zebrafish can identify novel mutations that lead to cancer susceptibility. For example, by screening initially for mutations that affect the cell cycle in homozygous embryos, mutations in *bmyb* and *separable* were found to modestly increase the rate of carcinogen-induced cancer in heterozygous adults (9, 10). In another study, by conducting a pilot screen of an existing collection of heterozygous carriers of embryonic lethal insertional mutations for the development of externally visible spontaneous tumors, we established that the haploinsufficiency of 17 different ribosomal protein genes is highly tumorigenic (2, 11). This validates the use of zebrafish to identify novel cancer genes.

The mutant collection used for this pilot screen includes over 500 mutations in more than 370 genes (12). These mutations were made by retroviral insertion, allowing rapid identification of the mutated gene in almost all cases by cloning the genomic DNA flanking the mutagenic insertion (13). These mutations were recovered in a large-scale screen designed to find recessive mutations with embryonic phenotypes (14), and nearly all of the mutants are embryonic or larval lethal in their homozygous state. In the course of the screen, we also recovered a few dominant mutations with viable adult phenotypes affecting either pigmentation or fin growth. None of these dominant mutations had any visible phenotypes during embryogenesis as either heterozygotes or homozygotes.

We maintain all of the zebrafish mutant lines as stocks of 15 to 25 heterozygotes until 2 years of age, thereby allowing for an assessment of adult phenotypes in the heterozygous state. In this study, we extended our analysis of the full mutant collection by screening for spontaneous tumor development by histologic examination as opposed to just externally visible tumors. This revealed that all four alleles of the dominant mutation *Hagoromo* (14, 15) were highly predisposed to the development of neuroblastoma–like tumors arising in the cranial ganglia.
Results

Cancer Screen of Insertional Mutants Identifies Hagoromo

We had previously discovered that heterozygous mutation of many ribosomal protein (rp) genes, as well as the nf2a gene (one of two paralogs of the mammalian tumor suppressor NF2) predisposed zebrafish to the development of cancer, primarily malignant peripheral nerve sheath tumors (2). Notably, these mutants developed tumors spontaneously, in the absence of chemical carcinogens. Whereas the ribosomal protein mutants generally developed large, externally visible tumors, the nf2a heterozygotes usually developed much smaller tumors that were only evident upon histologic examination of the fish. Thus, it is unlikely that we would have discovered that these fish were tumor-prone had we not examined sections of a cohort of apparently healthy fish. Reasoning that there might be other mutations which similarly predisposed fish to tumors that might not be externally visible, we set about systematically examining heterozygotes from all of the insertional mutant lines in our collection by histology. Initially, we examined at least 15 fish for each line at ∼2 years of age. In most cases, two longitudinal sections near either side of the midline were examined, although in some cases, six sections were screened. For any families that had a tumor frequency in the first cohort greater than background (see below), we collected additional 2-year-old fish to determine if this was a true tumor predisposition or just a sampling artifact.

All of the analyzed fish, with the exception of those from lines that we determined to be tumor-prone, served as a control set. Analysis of these 10,000 fish from 437 lines (including mutant lines that we determined to be tumor-prone, served as a control group) indicated that the frequency of tumorigenic lesions, including preadenoma bile duct hyperplasia, was ∼5% at 2 years of age (473 of 9,988). The most common tumor types were seminomas and bile duct lesions (hyperplasia, adenoma, and adenocarcinoma), followed by pancreatic islet cell adenoma and leukemia/lymphoma (Fig. 1). None of these tumor types appeared in >2% of the fish and we did not find any families in which these tumors were significantly overrepresented. As far as we are aware, this is the first comprehensive analysis of spontaneous tumor incidence and spectrum in zebrafish.

Of the 342 loci screened in this manner, 339 were recessive embryonic lethal mutations. Beyond the previously noted rp and nf2a genes, none of the heterozygous carriers of recessive lethal mutations had a spontaneous cancer frequency significantly above background. This included mutations in two genes, c-myb and separase, in which heterozygous mutation has been shown to increase cancer frequency in carcinogen-treated fish (9, 10). In addition to these recessive mutations, the collection contains dominant mutations in three loci that have no embryonic phenotype (as homozygotes or heterozygotes) but rather have visible adult phenotypes either in fin growth or stripe patterning. One of these loci, for which we have four mutant alleles, is Hagoromo (Hag). Hag mutants, named for “the dress of a goddess”, are characterized by disorganized stripes in the adult pigment pattern that are first apparent during the reorganization of iridophores and melanophores during metamorphosis at 3 to 4 weeks of age (15). Neither heterozygotes nor homozygotes have any embryonic phenotype; homozygotes are viable and have, on average, more severe stripe defects than heterozygotes, sometimes resulting in a spotty appearance in the anterior flank. All four Hag alleles carry insertions in the fbxw4 gene, which encodes an F-box containing the WD40 repeat protein (15). Our screen revealed a significant tumor predisposition in these four Hag alleles. In each of these lines, 25% to 50% of 2-year-old heterozygotes had tumors that histologically resembled neuroblastoma (Table 1; Fig. 2). Importantly, these neuroblastoma-like tumors were not observed in any of the ∼10,000 screened fish that lacked insertions in the fbxw4 gene, including 150 noncarrier siblings from the Hag families. Thus, we conclude that fish without insertions in the Hag locus spontaneously develop this tumor type with a frequency of <1 in 10,000. This, coupled with the observation that four different insertions in this locus lead to this tumor type, indicates that the insertions at the Hag locus are unquestionably responsible for these cancers.

Hagoromo Tumors Resemble Neuroblastoma and Are Preceded by the Inappropriate Maintenance of a Putative Neural Precursor Cell Population

To better understand the nature and origin of these tumors, we conducted a careful analysis of the Hag fish. H&E staining

Table 1. Incidence of Neuroblastoma in 2-y-Old Hag Heterozygotes and their Wild-Type Siblings

<table>
<thead>
<tr>
<th>Allele</th>
<th>Wild-type</th>
<th>Heterozygote (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hiD1</td>
<td>0/34</td>
<td>16/34 (47)</td>
</tr>
<tr>
<td></td>
<td>Small neoplasias</td>
<td>0/34 (7/34 (21))</td>
</tr>
<tr>
<td></td>
<td>Advanced tumors</td>
<td>0/34 (9/34 (26))</td>
</tr>
<tr>
<td>hiD2</td>
<td>0/56</td>
<td>19/77 (25)</td>
</tr>
<tr>
<td></td>
<td>Small neoplasias</td>
<td>0/56 (19/77 (25))</td>
</tr>
<tr>
<td></td>
<td>Advanced tumors</td>
<td>0/56 (0/77)</td>
</tr>
<tr>
<td>hiD2038</td>
<td>0/39</td>
<td>17/38 (45)</td>
</tr>
<tr>
<td></td>
<td>Small neoplasias</td>
<td>0/39 (13/38 (34))</td>
</tr>
<tr>
<td></td>
<td>Advanced tumors</td>
<td>0/39 (4/38 (11))</td>
</tr>
<tr>
<td></td>
<td>Total tumors</td>
<td>0/24 (18/59 (30))</td>
</tr>
<tr>
<td>hiD4000</td>
<td>0/24</td>
<td>9/59 (15)</td>
</tr>
<tr>
<td></td>
<td>Small neoplasias</td>
<td>0/24 (9/59 (15))</td>
</tr>
<tr>
<td></td>
<td>Advanced tumors</td>
<td>0/24 (9/59 (15))</td>
</tr>
</tbody>
</table>


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indicated that the tumors in *Hag* fish were made up of small, densely packed, oval cells with very little cytoplasm (Fig. 2). Although in some tumors, cells were arranged in “rosettes” (Fig. 2B) commonly seen in partly differentiated human neuroblastoma (16), more frequently, the cells were simply present as sheets with little organization (Fig. 2C), as is seen in undifferentiated human neuroblastoma (16). The tumors were found in a wide range of sizes, from small neoplasias (20-30 cells per planar section) to large tumors that occupied nearly the entire head, pushing aside (although never invading) the brain and pushing into the musculature of the cheeks and dorsal body wall. The highly aggressive tumors, such as that shown in Fig. 2A, were least common, and we rarely observed fish with any external signs of tumor growth, such as a bump on the dorsal surface or bulging of an eye. Small neoplasias were most common (Table 1) and always arose in cranial ganglia, either just below the midbrain (D) or posterior of the ear (E; original magnification, ×400). F. This tumor can be seen growing along the nerve running behind the eye as well as another below the skull (original magnification, ×100); H&E (G), anti-HuC (H), and anti-TH (I) of the same tumor (original magnification, ×200). Inset in I, TH staining of adrenal cells in the kidney on the same slide as a positive control for the antibody.

Although in some tumors, cells were arranged in “rosettes” (Fig. 2B) commonly seen in partly differentiated human neuroblastoma (16), more frequently, the cells were simply present as sheets with little organization (Fig. 2C), as is seen in undifferentiated human neuroblastoma (16). The tumors were found in a wide range of sizes, from small neoplasias (20-30 cells per planar section) to large tumors that occupied nearly the entire head, pushing aside (although never invading) the brain and pushing into the musculature of the cheeks and dorsal body wall. The highly aggressive tumors, such as that shown in Fig. 2A, were least common, and we rarely observed fish with any external signs of tumor growth, such as a bump on the dorsal surface or bulging of an eye. Small neoplasias were most common (Table 1) and always arose in cranial ganglia, either just below the midbrain but still within the skull (Fig. 2D), or in ganglia projecting posteriorly from behind the ear (Fig. 2E). As these tumors grew larger, they clearly overtook the entire ganglia and tracked along nerves, for example, those leaving the skull and projecting towards the ear or behind the eye (Fig. 2F). These tumors did not seem to be ganglioneuromas as they did not produce ganglion cells as they grew. Thus, based on the origin of these tumors in ganglia, as well as their histologic appearance, we suspected that they were neuroblastomas. By staining with the pan-neural marker HuC, we confirmed that these tumors were of neural origin (Fig. 2G and H). However, the tumors failed to stain for tyrosine hydroxylase (Fig. 2I). This marker is expressed in the catecholaminergic neurons of the peripheral sympathetic nervous system that are most often the cell of origin for human neuroblastoma (17, 18). Therefore, although these tumors were unquestionably neural and histologically resemble human neuroblastoma, they did not seem to arise from the same population of neurons that contributes to most human neuroblastoma. However, they may share other characteristics with the human disease.

Neuroblastoma is usually a neonatal or childhood cancer in humans. Tumors of this class typically originate from a precursor/progenitor population that persists inappropriately because of a failure to instigate the normal differentiation and/or apoptotic program (18, 19). In order to determine the origin of the tumors in *Hag* zebrafish, we conducted a time course analysis of the progeny of *Hag* heterozygous crosses (including wild-type, heterozygous, and homozygous sibs) by analyzing a
HuC mRNA by small precursor-like cells (Fig. 3C and D; Table 2). These cells were neural as they strongly express HuC (Fig. 3E-H). Taken together, these data suggest that the presence of this cell population in the wild-type zebrafish denotes a stage of neuronal development that is normally completed before 3 months of age. The simplest explanation for this finding is that these cells represent a neural precursor population. We believe that the persistence of these cells in 3-month-old Hag mutants is an early event in the tumorigenic process. Notably, these small neoplasias occurred at a similar frequency in both heterozygous and homozygous Hag mutants.

By as early as 5 months, these neoplasias began to grow into advanced tumors in a small proportion of Hag fish, spreading throughout entire ganglia and following nerve bundles away from their site of origin (Fig. 3I and J). These advanced tumors emerged at a slightly higher rate in homozygotes than in heterozygotes (Table 2). However, our ability to track tumor development in homozygotes was compromised by an additional phenotype of the homozygous fish; with variable penetrance, they developed severe inflammation in the wall of the posterior esophagus and anterior gut, leading to wasting and eventual mortality, possibly due to starvation (Table 2; Fig. S1). Taken as a whole, this time course experiment indicates that heterozygotes and homozygotes have a similar propensity to inappropriately maintain this presumed precursor cell population, but these neoplasias develop into more advanced tumors at a somewhat faster rate in homozygous versus heterozygous Hag mutants.

**Hag Mutations Up-Regulate fgf8 Expression Rather Than Affect tbxw4**

We wished to determine how the Hag mutant alleles contribute to the tumorigenic state. The insertional mutants used in this screen were made with Moloney murine leukemia virus (MoMLV)–based vectors. In mammals, MoMLV insertions can cause either gene activation through enhancer activity of the viral long terminal repeats (LTR) or inactivation by disruption of splicing or coding sequences (20, 21). Most of the mutants we recovered in our screen were recessive (491 of 497), and we isolated only six dominant mutations that represent insertions in three different loci (12, 14). Molecular analysis shows that in all of the recessive mutations that we examined, expression of the gene in which the insertion lies is reduced or abrogated (11, 13, 22). In contrast, the cause of the dominant mutations is less clear; they could be loss-of-function alleles of haploinsufficient loci or gain-of-function alleles.

To understand why the Hag mutants are tumor-prone, we first examined if expression of the tbxw4 gene was affected, as all of the insertions lie within this gene, hiD2058 at the splice donor of the first exon and hiD1, hiD2, and hiD4000 all in the fifth intron (Fig. 4A). We chose to analyze gene expression in homozygotes as this would facilitate the detection of gene expression changes in cis to the insertion, especially if the effect was down-regulation. We used real-time reverse transcriptase PCR (RT-PCR) on RNA isolated from whole fish.
We examined the expression of *fbxw4* in wild-type fish and homozygotes for three of the lines at two developmental stages: the end of embryogenesis (5 days) and after juvenile morphogenesis (6 weeks), when the pigment phenotype that is the defining characteristic of the *Hag* mutants is clearly visible (Fig. 4B). For *hiD2058*, the mutant with an insertion at the splice donor of the first exon, expression was reduced to ∼1% of wild-type levels (Fig. 4B). Additional analysis indicated that this insertion interferes with the splicing of the message, using a cryptic splice site in the first exon upstream of the initiation codon (data not shown). Thus, for *hiD2058*, the level of *fbxw4* is greatly reduced and is also incapable of producing full-length protein, if any protein at all. However, for two of the lines with insertions in the fifth intron (*hiD1* and *hiD4000*), there was no change in level of *fbxw4* mRNA at either of these time points (Fig. 4B). Because all of the alleles have the same phenotype (both pigment and tumor phenotypes) but at least two of the three alleles with insertions in the large intron did not affect *fbxw4* expression (expression in *hiD2* was not examined), it is unlikely that the loss of *fbxw4*, even in *hiD2058*, was responsible for any of the observed phenotypes.

The gene immediately upstream of *fbxw4* is *fgf8* (Fig. 4A), and this juxtaposition is conserved between fish and mammals. *Fgf8* is a common site for mouse mammary tumor virus insertions in murine mammary cancer, in which viral insertions lead to *fgf8* overexpression (23-25). There are even some reported cases in which mouse mammary tumor virus had inserted in the murine *fbxw4* gene to cause activation of *fgf8* (25). Although we have not previously seen the MoMLV virus activate genes in zebrafish, given the mouse results, we thought it would be prudent to examine the effects of these insertions on *fgf8* expression in a manner identical to that used for *fbxw4*. During embryogenesis, *fgf8* expression is very dynamic and its function is especially important, as both loss-of-function mutants and mutations that lead to *fgf8* overexpression have early embryonic phenotypes (26, 27). In wild-type fish, *fgf8* expression declines after embryogenesis (Fig. 4C), although it is known to remain expressed in some tissues (28, 29). During embryogenesis, we did not see any differences in *fgf8* mRNA levels between whole wild-type fish and *Hag* homozygotes (Fig. 4C). However, at 6 weeks of age, *fgf8* mRNA levels were 6 to 12 times higher in *Hag* homozygotes than in wild-type controls for all three lines tested (Fig. 4B). To further explore this deregulation, we examined *fgf8* mRNA levels in wild-type and homozygous fish over a number of intervening time points for one allele (*hiD4000*; Fig. 4C). In wild-type fish, the overall level of *fgf8* mRNA began to decline 1 to 2 weeks after embryogenesis was complete, and *fgf8* mRNA dropped to very low levels by 5 to 6 weeks of age. In contrast, the amount of *fgf8* mRNA was maintained at near-embryonic levels in *Hag* homozygotes throughout the time course. Notably, there was an excellent correlation between the loss or maintenance of

### Table 2. Incidence of Neural Neoplasias and Advanced Neuroblastoma in *Hag* Heterozygotes, Homozygotes, and Wild-type Siblings Over Time

<table>
<thead>
<tr>
<th>Allele</th>
<th>Age (wk)</th>
<th>Wild-type</th>
<th>Heterozygotes (%)</th>
<th>Homozygotes (%)</th>
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<tbody>
<tr>
<td>hiD4000</td>
<td>12</td>
<td>Small neoplasias</td>
<td>0/17</td>
<td>28/30 (93)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Advanced tumors</td>
<td>0/17</td>
<td>0/30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dead*</td>
<td>0/17</td>
<td>0/30</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>Small neoplasias</td>
<td>0/10</td>
<td>20/28 (71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Advanced tumors</td>
<td>0/10</td>
<td>1/28 (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dead*</td>
<td>0/10</td>
<td>0/28</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>Small neoplasias</td>
<td>0/16</td>
<td>18/28 (64)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Advanced tumors</td>
<td>0/16</td>
<td>0/28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dead*</td>
<td>0/16</td>
<td>0/28</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>Small neoplasias</td>
<td>N.D.</td>
<td>6/14 (43)</td>
</tr>
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<td></td>
<td></td>
<td>Advanced tumors</td>
<td>N.D.</td>
<td>2/14 (14)</td>
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<td></td>
<td></td>
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<td>N.D.</td>
<td>0/14</td>
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<tr>
<td>hiD1</td>
<td>17</td>
<td>Small neoplasias</td>
<td>0/10</td>
<td>22/26 (85)</td>
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<tr>
<td></td>
<td></td>
<td>Advanced tumors</td>
<td>0/10</td>
<td>0/26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dead*</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>Small neoplasias</td>
<td>0/21</td>
<td>37/52 (71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Advanced tumors</td>
<td>0/21</td>
<td>6/52 (11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dead*</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>Small neoplasias</td>
<td>N.D.</td>
<td>3/10 (30)</td>
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<td></td>
<td>Advanced tumors</td>
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<td>3/10 (30)</td>
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<td>0/10</td>
</tr>
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<td>hiD2058</td>
<td>17</td>
<td>Small neoplasias</td>
<td>0/16</td>
<td>14/17 (82)</td>
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<td></td>
<td></td>
<td>Advanced tumors</td>
<td>0/16</td>
<td>0/17</td>
</tr>
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<td>Dead*</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>Small neoplasias</td>
<td>0/15</td>
<td>29/37 (78)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Advanced tumors</td>
<td>0/15</td>
<td>0/37</td>
</tr>
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<td></td>
<td></td>
<td>Dead*</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>Small neoplasias</td>
<td>0/6</td>
<td>5/11 (45)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Advanced tumors</td>
<td>0/6</td>
<td>4/11 (36)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dead*</td>
<td>0/6</td>
<td>0/11</td>
</tr>
<tr>
<td>hiD2</td>
<td>24</td>
<td>Small neoplasias</td>
<td>0/12</td>
<td>33/38 (87)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Advanced tumors</td>
<td>0/12</td>
<td>0/38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dead*</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
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</table>

Abbreviation: N.D., not determined.

*These fish died from wasting, presumably subsequent to severe esophageal/gut inflammation, not from tumors.
fgf8 mRNA in the wild-type and Hag mutant fish and the loss or maintenance of the presumed neural precursor population. Importantly, the timeframe of fgf8 down-regulation precedes the loss of putative precursor cells in wild-type fish, suggesting that high fgf8 somehow contributes to the maintenance of this population in the Hag mutants. As expression was measured from RNA prepared from whole fish, it is not clear if this difference is the result of higher levels of fgf8 in cells that normally express it or ectopic expression of fgf8. Nonetheless, it is clear that fgf8 is dramatically up-regulated in Hag mutants and that it is likely fgf8, not fbxw4, which is the gene responsible for the Hagoromo phenotypes. Consistent with this hypothesis, we note that an enhancer trap screen using another MoMLV-based retrovirus (30) yielded two insertions, CLGY1030 and CLGY508 (31), which lie in intergenic sequences upstream and downstream of fgf8, respectively (Fig. 4A). Analysis of fgf8 expression in these mutants revealed both increased fgf8 expression in the midbrain-hindbrain boundary at 3 day old and ectopic fgf8 expression in the spinal region of 35 day old fish, and these two mutants develop a striped phenotype that is highly reminiscent of our Hagoromo mutants.4 Histological analysis of these lines show that they also develop neuroblastoma tumors (Fig. S2). Taken together, these data strongly suggest that the tumor predisposition in these six lines result from a failure to down-regulate fgf8 subsequent to embryogen-

4 A. Komisarczuk and T.S. Becker, unpublished data.
esis and the consequent persistence of a putative neuronal pre-
cursor population.

As a further test of this hypothesis, we wished to determine
if increased fgf8 expression occurs in the region of the cranial
ganglia from which the tumors originate. Thus, we analyzed
fgf8 expression by both semiquantitative RT-PCR and real-time
RT-PCR on mRNA from several parts of the head of wild-type
and both heterozygous and homozygous Hag fish at 3 months
of age, the time at which the clusters of neural precursors are
first completely absent in wild-type fish but maintained in the
Hag mutants. The tissues analyzed included the brain, the skull,
the soft tissue ventral to the skull (which included the cranial
ganglia), and the jaws and skin. Of these tissues, the brain had
the highest overall fgf8 expression in wild-type fish, and fgf8
expression seemed slightly higher in the brain of Hag mutants.
The other three tissues, in which fgf8 was expressed at a low
level in wild-type fish, showed dramatically higher levels of
fgf8 in Hag mutants (Fig. 5A). Thus, we conclude that elevated
fgf8 expression is not restricted to the cranial ganglia but is
clearly present in this region of the Hag mutants. Most impor-
tantly, in situ hybridization with antisense fgf8 probe on sec-
ctions cut from fish with tumors shows robust expression of
fgf8 in most, although not all, cells in the tumor (Fig. 5B and
C). Thus, we conclude that the neuroblastomas themselves ex-
press fgf8 at a very high level.

Discussion

Through the unbiased histologic screening of a collection of
zebrafish insertional mutants, we determined that four indepen-
dent lines carrying mutations in the Hagoromo locus develop
neuroblastoma-like tumors at high penetrance. In some regards,
these tumors are quite different from human neuroblastoma,
which arises from neural crest–derived neural precursors in
the sympathetic nervous system and grow predominantly in
the abdomen or neck (17, 18). Tumors in Hagoromo fish arise
in cranial ganglia and grow in the head. Although they are
clearly of neural origin, as judged by their expression of

![FIGURE 5. Hag mutants overexpress fgf8 in the head region of
young adults and in tumors. A, fgf8 mRNA in multiple parts of the head of
3-mo-old wild-type, hiD2058 heterozygote and hiD2058 homozygote
fish were measured by real-time and semiquantitative RT-PCR;
gapdh was used as a normalization control. Samples were isolated from three
(brain) or four wild-type fish and two each of Hag hiD2058 heterozy-
gotes and homozygotes. Ethidium bromide–stained gels from semi-
quanitative RT-PCR (left); quantifi-
cation of real-time PCR (right). Quantification is normalized within
the data set for each tissue to the av-
erage wild-type sample of that tis-
sue; error bars show the SD of
three technical replicates. B and C,
(original magnification, ×20) H&E
(B) and fgf8 in situ hybridization
(C) on a very advanced tumor in a
2-y-old hiD2058 homozygote.

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HuC, these zebrafish tumors do not express markers of catecholaminergic neurons, indicating that they are derived from a different neural lineage than human neuroblastoma. Despite this difference, the Hagoromo tumors seem to arise through a similar mechanism as the human disease. Specifically, human neuroblastoma typically arises in infants and is thought to be a tumor of embryonic origin, i.e., derived from neural precursors present during fetal development (18, 19). Our analysis shows that wild-type zebrafish have a population of cells which exists in the cranial ganglia of juvenile fish but is lost as the fish grow to adulthood. We hypothesize that these cells represent neural precursors, and their disappearance, presumably through differentiation or apoptosis, reflects the normal developmental program. These cells are inappropriately maintained in Hagoromo mutants and they are the originating cell of the Hagoromo mutant tumors. Although in many cases, these clusters of cells fail to grow past the size of small neoplasias, in 10% to 20% of the mutants, they eventually grow into advanced tumors beginning as early as 5 months. This stochastic event likely reflects the need to acquire additional mutations within these cells. The Hagoromo mutation seems to act by prolonging the existence, and possibly driving the expansion, of this target cell population.

The four Hagoromo mutant lines each carry viral insertions in the fbxw4 gene. However, our data show that they do not have a shared effect on fbxw4 expression: one allele, in which the insertion lies at the splice donor of the first exon, diminished fbxw4 mRNA levels, but the other insertions, which lie in the fifth intron, had no detectable effect on fbxw4. Instead, our data suggests that the key consequence of the Hagoromo insertions is to maintain high levels of fgf8 expression after embryogenesis. Specifically, we find that fgf8 mRNA is dramatically up-regulated in Hagoromo mutants after the completion of embryonic development, including expression in the head region where the inappropriately maintained precursors are first observed. In addition, very high fgf8 expression is seen in the tumors themselves. Finally, insertional mutants have been recovered with a related virus from a different screen with phenotypes including both the stripe phenotype and the neuroblastoma phenotype in which the insertions lie outside of fbxw4, either between fgf8 and fbxw4 or upstream of fgf8, distal to fbxw4.

The mechanism by which these insertions activate fgf8 expression is unclear. That the gene containing the viral insertions is not truly the affected gene should serve as a cautionary example for the analysis of other insertional mutants. In this case, although we cannot rule out direct viral activation, our previous studies suggest that the MoMLV LTR does not activate transcription in zebrafish cells; viruses relying on the LTR to drive the expression of the lacZ gene produced no β-galactosidase activity, whereas viruses with an internal promoter driving lacZ were able to do so (32). Additionally, in all other cases in which the expression of genes at the site of MoMLV insertions in zebrafish had been examined, gene expression had either decreased or was unaffected (11, 13, 22, 33). Thus, we instead favor the notion that the virus disrupts the transcriptional control of fgf8 by distal cis-acting elements. This hypothesis is supported by several lines of evidence showing that fgf8 expression is controlled by a number of enhancers upstream, within, and downstream of the gene, including elements within the fbxw4 gene (31, 34, 35). Additionally, retroviral insertions in the Fgf8/Fbxw4 locus in the mouse (36, 37) or genomic duplications near FGF8 in humans (38, 39) cause a limb outgrowth phenotype (dactylaplasia or split hand foot malformation, respectively). The mechanism of these mouse and human mutations is not understood. However, the mouse mutants have been shown to have a defect in the maintenance of the apical ectodermal ridge of the developing limb, and Fgf8 expression is normal at the beginning of limb development but is quickly lost (36). Although it has been suggested that this altered Fgf8 expression is a secondary consequence of apical ectodermal ridge loss, it also seems possible that the insertions in the mouse dactylaplasia mutants affect fgf8 expression in a direct, but complex, way that somehow accounts for the observed down-regulation after limb development begins. Although the molecular and phenotypic consequences of insertions and chromosomal rearrangements of the Fgf8/Fbxw4 locus clearly differ between mammals and fish, it seems plausible that the resulting phenotypes in each organism (limb outgrowth defects in mammals, maintenance of neural precursor cells and stripe disruption in zebrafish) can be accounted for by differential effects on fgf8 expression.

Regardless of the mechanism by which the zebrafish insertions cause continued high levels of fgf8 expression after embryogenesis, it clearly leads to the inappropriate maintenance and expansion of a putative neural precursor population that ultimately become tumors. We note that the increase in fgf8 expression in the head region of Hagoromo fish occurs at a time when this cell population diminishes in wild-type fish. This suggests that fgf8 is capable of contributing to the maintenance of these cells, either by blocking differentiation or by promoting survival. It would seem paradoxical that fgf8 should prevent the differentiation of these precursor cells as many studies have associated fgf8 with neural differentiation. A requirement for fgf8 in the development of neurons in various areas of both the central and peripheral nervous system, including the epibranchial placodes that give rise to the cranial ganglia, has been shown both in zebrafish (40-42) and in mice (43). Furthermore, Fgf8 cooperates with retinoic acid to force mouse embryonic carcinoma P19 cells to differentiate into neurons, whereas inhibition of fgf signaling in this cell culture system attenuates neural differentiation (44). However, this prodifferentiation role for fgf8 might be restricted to specific developmental stages such as embryogenesis. For example, in some settings, fgf8 signaling can promote the proliferation of neural precursors at the expense of differentiation (45). The other possibility, that fgf8 acts as a prosurvival signal for these cells, is supported by the observation that several fgfs, including fgf8, have antiapoptotic functions in both neural crest and ectoderm-derived neurons (43, 46). The effects of fgf8 on differentiation and survival may depend on timing and context, such that early in development, neural differentiation is the favored response, but sustained fgf8 expression past the time when such differentiation normally occurs may result in inappropriate survival and/or proliferation of the few remaining neural precursor cells.

Thus, fgf8 seems to be an oncogene, in that its overexpression predisposes zebrafish to tumorigenesis. However, the tumor phenotype in Hagoromo mutants is not fully penetrant.
Notably, it seems that fgf8 overexpression in Hagoromo mutants is always sufficient to disrupt the orderly alignment of pigment cells (which are moving into their stripe pattern at the time when we first see fgf8 overexpression in the mutants), as well as the inappropriate maintenance of putative neural precursor cells in the cranial ganglia. However, these only progress to tumors in a subset of cases, suggesting that additional mutations are required. It is possible that there is only a limited window of opportunity for the precursor cell population to acquire these changes. This model is supported by our finding that small neoplasias exist in nearly all of the mutants at 3 to 6 months of age (and we might miss some due to sampling only three sections per fish) but are detected in fewer than half of the fish at 2 years of age. Thus, we speculate that fgf8 overexpression allows for the persistence of a cell population that is then a target for additional mutations, and there may be a limited time in which such mutations can contribute to tumorigenesis. We do not currently know what these additional mutagenic events are, and this is an important question for future studies. However, we believe it is unlikely that mutation of the tumor suppressor gene p53 is one such key event, as fish mutant for both Hagoromo and p53 (3) do not have a higher rate of neuroblastoma formation by 1 year of age compared with fish mutant for Hagoromo alone (data not shown).

Fgf8 has also been implicated in tumorigenesis in other tumor types in mammals. Overexpression of FGF8 has been observed in a number of human tumors, especially prostate and breast cancer (47-49). Furthermore, whole genome association studies indicate that specific polymorphisms in the fgf receptor FGFR2 correlate with an increased frequency of breast cancer (50, 51). Direct evidence for the oncogenic properties of Fgf8 in these tissues is even clearer in mice. The Fgf8 locus is a common insertion site for mouse mammary tumor virus in retrovirus-induced mammary tumors (23-25), implying that its activation in mammary tissue can lead to tumorigenesis. Additionally, transgenic tissue-specific overexpression of Fgf8 cooperates with Pten loss in mouse models of prostate cancer (52). Thus, FGF8 seems to function as an oncogene in numerous settings, including prostate and breast cancer in mammals, and now neuroblastoma in zebrafish.

**Materials and Methods**

**Fixation and Histology**

Adult fish were euthanized in 500 mg/L tricaine and fixed in either Bouin’s fixative or 10% neutral buffered formalin (in cases in which we wished to retain the option of immunohistochemistry or in situ hybridization). Embedding in paraffin and sectioning were done as previously described (2, 53). Prior to fixation, a piece of tail tissue was retained for the isolation of genomic DNA and either Southern analysis or PCR was conducted to determine the genotype of fish from the Hag heterozygous crosses (54).

**Antibody Staining**

Slides from fish fixed in 4% paraformaldehyde/PBS and paraffin embedded were stained as previously described (55). Primary antibodies used were anti-TH (1:100; Pel-Freez) and anti-HuC (1:250; Molecular Probes).

**RNA Hybridization**

Dig-labeled riboprobes were produced for both sense and antisense strands of the coding regions of the zebrafish fgf8 and HuC genes using a dig-labeling kit (Roche). Slides from fish fixed in 4% paraformaldehyde/PBS and paraffin embedded were dewaxed (55), rinsed with PBS, treated with proteinase K (40 μg/mL) for 7 min, rinsed with PBS, refixed in 4% paraformaldehyde/PBS for 20 min, rinsed with PBS, and rinsed twice in 2× SSC. They were then prehybridized in hybridization solution (50% formamide, 5× SSC, 0.1% Tween, 1 mg/mL RNA, and 50 μg/mL heparin) for 3 h at 70°C, and hybridized overnight at 70°C in a humid box with 100 μL of hybridization solution plus probe (1 μg/mL) under a coverslip. Slides were washed at 70°C for 10 min with 75% hyb/25% 2× SSC, 50% hyb/50/2× SSC, 25% hyb/75% 2× SSC, 2× SSC, and for 30 min with 0.2× SSC. Slides were equilibrated in MAB + 0.1% Tween for 30 min, blocked in MAB + 0.1% Tween + 10% lamb serum + 2% blocking reagent (Roche), then alkaline-phosphatase-conjugated anti-DIG Fab fragments were applied (1:1,000; Roche), overnight at 4°C. Antibody was washed five times for 30 min in MAB + 0.1% Tween, and alkaline phosphatase activity was detected with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as recommended by the manufacturer (Roche). Figures show results with antisense probes; no blue stain was observed with sense strand probes of simultaneously processed adjacent level slides.

**Quantitative RNA Analysis**

RNA was prepared from embryos, adult fish tissues, or whole juvenile fish using Trizol reagent (Invitrogen); in the case of whole fish, 17 dpf or older, fish were flash-frozen in liquid nitrogen and mashed in a mortar and pestle prior to homogenization in Trizol. First-strand cDNA was prepared using Superscript III reverse transcriptase (Invitrogen) and gene expression levels were determined using real-time PCR with Sybr Green Master Mix (ABI). Standard curves were established for each primer set with cDNA dilutions and all samples were run in triplicate and normalized with primers for gapdh. All primer sequences are available upon request.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Molecular Cancer Research

Zebrafish *Hagoromo* Mutants Up-Regulate *fgf8* Postembryonically and Develop Neuroblastoma

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