DNA Mismatch Repair, Genome Instability and Cancer in Zebrafish

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Cover: close-up of zebrafish skin

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DNA Mismatch Repair, Genome Instability and Cancer in Zebrafish

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en kanker in de zebravis
(met een samenvatting in het Nederlands)

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Introduction: Zebrafish as a cancer model

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ABSTRACT

The zebrafish has developed into an important model organism for biomedical research over the last decades. Although the main focus of zebrafish research has traditionally been on developmental biology, keeping and observing zebrafish in the lab led to the identification of diseases similar to humans, such as cancer, which subsequently became subject to study. As a result, about 50 papers have been published since 2000 in which zebrafish were used as a cancer model. Strategies employed include carcinogenic treatments, transplantation of mammalian cancer cells, forward genetic screens for proliferation or genomic instability, reverse genetic target-selected mutagenesis in order to inactivate known tumour suppressor genes, and the generation of transgenics to express human oncogenes. Zebrafish have been found to develop almost any tumour type known from human, with similar morphology and, according to gene expression array studies, comparable signalling pathways. However, tumour incidences are relatively low, albeit highly comparable between different mutants, and tumours develop late in life. Also, tumour spectra are sometimes different when compared to mice and humans. Nevertheless, the zebrafish model has created its own niche in cancer research, complementing existing models with its specific experimental advantages and characteristics. Examples of these are imaging of tumour progression in living fish using fluorescence, treatment with chemical compounds, and screening possibilities, not only for chemical modifiers, but also for genetic enhancers and suppressors. This review aims to provide a comprehensive overview of the state of the art of zebrafish as a model in cancer research.

1. CANCER RESEARCH IN ZEBRAFISH

Zebrafish has been used as laboratory animal for a few decades now. Originally, the main focus was on developmental biology, because of the clear advantages of zebrafish such as large clutch size, transparent embryos and ex utero development of the embryo. While keeping zebrafish in the laboratory environment, however, researchers observed different diseases in adults, including cancer. Studies on the latter revealed that zebrafish spontaneously develop almost any type of tumour (1-4). The most common target tissues for spontaneous neoplasia are the testis, gut, thyroid, liver, peripheral nerve, connective tissue, and ultimobranchial gland. Less common target tissues include blood vessels, brain, gill, nasal epithelium and the lymphomyeloid system (2). In the first part of this

review the currently used approaches to induce cancer in zebrafish will be discussed. An overview of these and their main advantages and disadvantages are given in Table 1. Additionally, cancer results of the forward and reverse genetic and transgenic mutant models are summarized in Table 2.

Treatment with mutagens

Historically, researchers appreciated the relative ease of treating fish with carcinogens, as the chemicals can be dissolved or suspended in water and the animals can be exposed for longer time periods. When exposing zebrafish or guppies to different compounds, e.g. 7,12-dimethylbenz[a]anthracene (DMBA), N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (DEN), mainly liver and intestinal tumours were observed (5-7). More recently, similar but more extensive studies showed that DEN primarily induces liver and pancreas carcinomas (8), and NDMA only liver tumours (9). DMBA induces the broadest tumour spectrum, including liver neoplasms, epithelial tumours in intestine, pancreas, thyroid and testis, and mesenchymal tumours in cartilage, blood vessels, muscles, connective and lymphoid tissue, as well as neural tumours (10). N-methyl-N-nitro-N-nitrosoguanidine (MNNG) can also induce different tumour types, mainly liver and testicular neoplasms, but also haemangio(sarco)mas and others (11). Some of these carcinogenic treatments have been applied to mutants with a genetic predisposition to cancer but a low spontaneous tumour incidence, to show an increased sensitivity of mutants compared to treated wild-type animals (12-14). Not surprisingly, the alkylating mutagen N-ethyl-N-nitroso-urea (ENU), which introduces point mutations and is commonly used in forward and reverse genetic screens, has also been found to induce cancer. Following a group of animals from a mutagenesis screen, Beckwith *et al.* found that all fish developed skin papillomas over time, but not invasive skin cancers (15).

Transplantation of mammalian cancer cells into zebrafish

Different groups have been experimenting with transplantation of mammalian cancer cells into zebrafish embryos. This creates an *in vivo* system in which the advantages of cultured human cancer cells are combined with those of transparent zebrafish embryos in which development can be followed. Lee *et al.* transplanted fluorescently labelled human metastatic melanoma cells into zebrafish blastula-stage embryos and showed that these cells survive, migrate and divide, and are still present in adults, but do not cause cancer or metastases (16). Another study showed that aggressive human melanoma

cells are able to induce a secondary axis or an abnormal head when transplanted into 3 hours-old zebrafish embryos, which was shown to be due to Nodal signalling from the tumour cells (17, 18). In contrast to the above studies where no cancer development was observed, similar human melanoma cells as well as a colorectal and a pancreatic cancer cell line were found to induce tumour-like cell masses when transplanted into two-day-old zebrafish embryos (19).

Transplantation studies can be specifically effective in the study of vasculature remodelling, cancer invasion and metastasis. Currently, no good *in vivo* model is available in which such dynamic process can be followed in real time. When transplanted into two-day-old zebrafish embryos, human and murine tumour cell lines expressing FGF or VEGF induced rapid neovascularization inside the tumour graft, and this could be inhibited by treatment with antiangiogenic chemicals (20). In another elegant study, researchers used intraperitoneal injection of fluorescently labelled human breast cancer cells in 1-month-old zebrafish in combination with 3D modelling to show how the human cells interact with vessels and invade in tissues (Fig. 1C). They showed that expression of VEGF induces openings in vessel walls that can be used for invasion, which in turn is stimulated by RhoC expression (21).

Mutants from forward genetic screens

The largest impetus for zebrafish to become an important animal model was its suitability for forward genetic screens. Since the first mutagenesis experiments in the Nusslein-Volhard lab (22), screens have been carried out for almost any type of phenotype, including phenotypes related to cancer (Tab. 2). Amsterdam *et al.* rather coincidentally ran into a set of genes that cause cancer when mutated. Using retroviral insertions they carried out a screen for embryonic lethality (23), but while keeping the heterozygous founders they noticed that twelve of their lines displayed an elevated tumour incidence (24). Eleven of those lines carried insertions in ribosomal genes, for which there was no strong relation with cancer at that point. The other line was mutated in one of the two zebrafish homologs of the *neurofibromatosis 2* gene, a known tumour suppressor in humans.

The Zon lab performed a screen for proliferation defects, taking advantage of the fact that many oncogenes and tumour suppressors are actually essential for development. Homozygous mutant embryos are therefore lethal, and heterozygotes are, due to haploinsufficiency or loss-of-heterozygosity (LOH), predisposed to cancer later in life.

Two mutants from this screen have been reported so far, a loss-of-function mutation in *bmyb* (14) and a loss-of-function mutation in *separase* (13), both genes that had not previously been unequivocally implicated in cancer. Homozygous mutant embryos of both displayed mitotic defects and genomic instability, while heterozygous adults showed a marginal increase (2-2.5-fold) in cancer incidence when treated with MNNG.
An elegant screen for genomic instability used LOH of the *golden* pigmentation gene as a readout (25). When in *golden* heterozygous embryos cells of the retinal pigment epithelium of the eye acquire a mutation in the wild-type allele, this results in unpigmented patches that are easily scorable in large batches of embryos (Fig. 1A). The twelve mutant lines with embryonic genomic instability that were obtained all showed some or more sensitivity to cancer in heterozygous adults, confirming the strong connection between genomic instability and cancer. However, the underlying mutations have not been mapped or cloned yet.

Reverse genetics: target-selected inactivation of tumour suppressor genes
Since knockout technology in zebrafish became available by means of target-selected mutagenesis (26), several mutants for known tumour suppressor genes have been generated (Tab. 2). For *tp53*, the most frequently mutated gene in human cancers, two zebrafish mutants were isolated, one with a missense mutation in the DNA-binding domain ($tp53^{M214K}$) and one with a missense mutation that affects protein structure in a heat-sensitive manner ($tp53^{N168K}$). Homozygotes of both lines were developmentally normal, but showed suppressed apoptosis upon irradiation. 28% of $tp53^{M214K}$ homozygotes developed tumours at an average age of 14 months, which, except for one melanoma, were all diagnosed as malignant peripheral nerve sheath tumours (MPNSTs) (27).
The second most frequently mutated tumour suppressor in human cancers, *pten*, has undergone a gene duplication in zebrafish. Faucherre *et al.* isolated nonsense mutants for both *ptena* and *ptenb*. Single homozygous mutants of either allele did not display a developmental phenotype, but mutants lacking both *ptena* and *ptenb* died at day five post fertilization from pleiotrophic defects due to enhanced proliferation and cell survival, indicating redundant functions. However, adult fish lacking only *ptenb* developed ocular tumours with an incidence of 33% at 18 months, while no neoplasms were observed in adult *ptena* mutants. Tumours were not further classified, but their appearance and the fact that they occur only intraocular suggests that they are not MPNSTs (28).
Mutations in the adenomatous polyposis coli gene *APC* cause the vast majority of

human sporadic and inherited colorectal cancers by constitutively activating the Wnt-signalling pathway (29). A nonsense mutation in zebrafish *apc* was found to result in lethality when homozygous (30). Less than 30% of heterozygous fish spontaneously developed liver and intestinal tumours from 15 months of age onwards. Treatment of *apc* heterozygotes with DMBA enhanced tumorigenesis, resulting in intestinal, hepatic and pancreatic tumours with frequencies 3- to 4-fold higher than treated wild types. The tumours displayed activated Wnt-signalling, indicating that the genetic pathway is conserved (12).

TABLE 1. Comparison of techniques to induce zebrafish cancers

technique	advantages	disadvantages
chemical treatment	• easy applicable • large numbers of fish • long-term treatment	• unspecific • predominance of liver tumours • potential hazard for researcher
transplantation of mammalian cells	• rapid onset • study in transparent embryos • use of human cancers • use of fluorescence	• can not be propagated as a line • low penetrance of tumours
forward genetic screens	• identification of multiple genes at once • embryonic phenotype as readout • combination with drugs, mutants or transgenics	• laborious • screens for adult phenotypes require space and time • stochasticity of cancer makes it difficult as readout
reverse genetic knockouts	• inactivation of one specific gene • human-like cancer mutations	• background mutations • late onset and low penetrance • gene duplications • different tumour spectrum than in humans
expression of transgenes	• easy generation by injection • rapid onset • use of human genes • use of fluorescence • conditional and tissue-specific expression	• laborious if no transgenic line • lack of specific promoters

The first DNA repair genes that have been mutated in zebrafish are the mismatch repair genes *mlh1*, *msh2* and *msh6*, involved in the repair of small replication errors such as base mismatches and insertion/deletion loops. Homozygous mutants were genomic instable, as shown by the occurrence of variation in lengths of microsatellite sequences in their DNA. Taking the data for the three mutants together, on average 33% of homozygotes developed tumours at an average age of 17 months. Mainly MPNSTs were found at different places in the body, but also other tumour types were observed (H. Feitsma, R.V. Kuiper, J. Korving, I.J. Nijman, E. Cuppen, in press).

Targeted knockout strategies such as those used for making mouse knockouts are not available in zebrafish. The current strategy makes use of random ENU mutagenesis combined with targeted selection of mutations in the gene of interest, which means that the researcher is dependent on the random point mutations that are induced. However, the positive aspect of this is that the generated point mutations can be more similar to the type of spontaneous mutations that occur in human cancer patients than the large gene deletions or insertions in mouse knockouts. Indeed, two of the published zebrafish mutants do exactly mimic human cancer mutations: the $tp53^{M214K}$ point mutation in zebrafish *tp53* is frequently found in human cancers (27), and the splice site mutation in the zebrafish *msh2* gene results in an in-frame loss of exon 5 (H. Feitsma, R.V. Kuiper, J. Korving, I.J. Nijman, E. Cuppen, in press), which is one of the most frequent familial mutations in hereditary nonpolyposis colorectal cancer (HNPCC), the human syndrome that is caused by defective mismatch repair.
One of the disadvantages of the use of random mutagenesis in the knockout procedure is that each fish that is retrieved will, besides the mutation of interest, contain several background mutations. Those additional mutations are heterozygous and will probably not have a large impact on developmental phenotypes, but they may of course be of influence on the process of mutation accumulation that is necessary for tumour development. Therefore, ENU-induced mutants should be crossed out for several generations to exclude confounding effects of unknown background mutations.

Transgenic zebrafish expressing mammalian oncogenes

The largest number of studies on cancer development in zebrafish so far comes from transgenic zebrafish expressing mammalian oncogenes (Tab. 2). This approach makes use of another advantage of zebrafish as a laboratory animal: the convenience of

introducing foreign DNA into zebrafish cells and getting it expressed by injection into 1-cell embryos. Many of the models concern lymphomas and leukaemias, cancers that rarely occur spontaneously in zebrafish, but for which the transgenic model may be of great aid in searching for new treatments. This is especially important for this class of diseases, when considering that human patients usually need therapies severely affecting quality of life.

Frequently, haematological malignancies arise because of genetic rearrangements resulting in misexpression of certain oncogenes, for example by fusion to lymphoid-expressed genes. Although many of the genes and even chromosome regions known to be involved have been conserved in zebrafish (31), researchers have mostly limited their experiments to expressing human fusion constructs. For example, injection of a human RUNX1-CBF2T1 fusion, which is frequent in acute myeloid leukaemia, caused circulation defects, haemorrhages, abnormal vascular development and defective haematopoiesis, but not leukaemia in zebrafish embryos (32). The human TEL-AML1 fusion is responsible for 25% of childhood pre-B acute lymphoblastic leukaemia cases. When expressing a TEL-AML1 fusion construct in zebrafish, acute lymphoblastic leukaemia (ALL) developed in 3% of fish that expressed the transgene ubiquitously, but not when expression was restricted to lymphoid cells using the *rag2* promotor (33). In an attempt to model a molecular rearrangement associated with human lymphoblastic and myeloid leukaemias, Onnebo *et al.* injected embryos with a zebrafish tel-jak2 fusion under control of the myeloid *spi1* promotor, which caused severe perturbation of haematopoiesis but also high mortality, making it impossible to study adults (34).

Langenau *et al.* have put an enormous effort into generating optimal zebrafish models for leukaemia. They developed zebrafish expressing mouse *c-myc* under the zebrafish *rag2* promotor, to restrict the expression to lymphoid cells. MYC is known to play an important role in human and mouse lymphoid malignancies. 6% of injected fish developed infiltrative T-cell leukaemia (Fig. 1B) with a latency of 30-131 days (35). A germ line-transmitting transgenic line of these fish displayed 100% cancer incidence with a mean latency of 80 days (36), but because disease onset mostly precedes reproductive age, the line can only be propagated via *in vitro* fertilization, which makes experiments with this model labour-intensive. However, by creating a conditional transgene in which a *dsRED* gene flanked by loxP sites was put in between the *rag2* promotor and the mouse *c-myc* (*mMyc*) gene (*rag2-loxP-dsRED-loxP-EGFP-mMyc*), and using this transgenic line in combination with CRE injection, leukaemia development was made inducible.

TABLE 2. Cancer mutants in zebrafish

model	cancer	type	incidence	onset (months)	ref.
forward genetic mutants					
ribosomal protein	+	MPNST	35%	8-24	(24)
separase	induced	liver, intestinal	10%	3-12	(13)
bmyb	induced	testis, vascular	7%	3-12	(14)
genomic instability	+	MPNST and others	up to 48%	30-36	(25)
reverse genetic mutants					
tp53	+	MPNST	28%	14	(27)
ptenb	+	ocular	33%	7-18	(28)
apc	+	liver, intestinal	29%	15	(12)
mlh1, msh2, msh6	+	MPNST and others	33%	17	
transgenic mutants					
RUNX1-CBF2T1	-				(32)
TEL-AML1	+	leukaemia	3%	8-12	(33)
zebrafish tel-jak2	-				(34)
mouse c-myc	+	leukaemia	6%	1-4	(35)
mouse c-myc (line)	+	leukaemia	100%	2	(36)
mouse c-myc (conditional)	+	leukaemia	81%	4	(37)
zebrafish bcl2	-				(38)
NOTCH	+	leukaemia	40%	5-11	(39)
kRASG12D	+	rhabdomyosarcoma	47%	2	(40)
kRASG12D (conditional)	+	rhabdomyosarcoma and others	100% of survivors	1	(41)
BRAF-V600E	in $tp53^{-/-}$	melanoma	6%	4	(42)
MYCN	+	neuroendocrine	2%	5	(43)

Unfortunately, only partial recombination was obtained, resulting in leukaemia in only 12 out of 186 CRE-injected animals (36). A second improvement was made by crossing the conditional line to a transgenic line expressing CRE under a heatshock promotor (37). Upon heatshock, double transgenic fish developed T-lymphoblastic lymphoma that rapidly progressed to T-cell acute lymphoblastic leukaemia. The best heatshock treatment gave an induction of cancer in 81% of fish, with a mean latency of 120 days, but in the next generations of the transgenic line the cancer latency was higher (37). The same lab also developed a transgenic line overexpressing the zebrafish *b-cell leukaemia 2 (bcl2)* gene under the *rag2* promotor, in which lymphoid apoptosis is blocked (38). Irradiation treatment of fish that were transplanted with leukaemic cells from *mMyc* transgenic fish (36) ablated cancer in wild-type fish but not in the *bcl2*-overexpressing fish (38). In another leukaemia model, the knowledge was used that activating mutations in NOTCH1 are found in around 60% of T-cell acute lymphoblastic leukaemia. Zebrafish expressing constitutively active human *Notch* under the *rag2* promotor developed T-cell leukaemia at 5-11 months in about 40% of the cases (39). When crossing these fish with the *bcl2*-overexpressing line (38), disease onset was accelerated and the leukaemias were not radiation sensitive anymore, as apoptosis was inhibited.

Several transgenic models for solid tumours have also been generated. Langenau *et al.* observed that the zebrafish *rag2* promotor that they frequently used showed ectopic expression in undifferentiated cells of the muscle, making it possible to express human activated RAS (kRASG12D), a common mutation in human oncogenesis, in these cells, resulting in the development of rhabdomyosarcoma in 47% of the cases by 80 days of age, and in only one lymphoid hyperplasia (40). Tumorigenesis was accelerated when these experiments were performed on the *tp53* mutant genetic background. A conditional version of the same activated human RAS under a zebrafish *B-actin* promoter with a floxed EGFP gene inserted in-between, was combined with the previously mentioned heatshock-inducible CRE. This resulted in overall reduced viability in heat-shocked as well as non-heat-shocked animals. The latter is most likely due to leakiness of the heatshock promotor of the CRE line. Surviving fish were found to develop four types of tumours at around 35 days: rhabdomyosarcomas, myeloproliferative disorders, intestinal epithelial hyperplasms, and two cases of MPNST (41).
To test the role of activated BRAF in melanoma development, transgenic zebrafish expressing activated human BRAF-V600E under the zebrafish melanocyte *mitfa*

promotor were generated. These fish developed pigmented nevi in 10% of the cases, but not melanoma (42). Although *tp53* mutations are rare in human melanoma, in *tp53* mutant background four out of 66 transgenic fish developed highly invasive and transplantable melanoma.

Finally, human *MYCN* under the zebrafish *myod* promotor is expressed in neural tissue and pancreas, and in combination with a human core enhancer also in muscles. Only five out of 250 fish injected with this construct developed tumours around five months of age; one cranial and four abdominal, but all neuroendocrine (43).

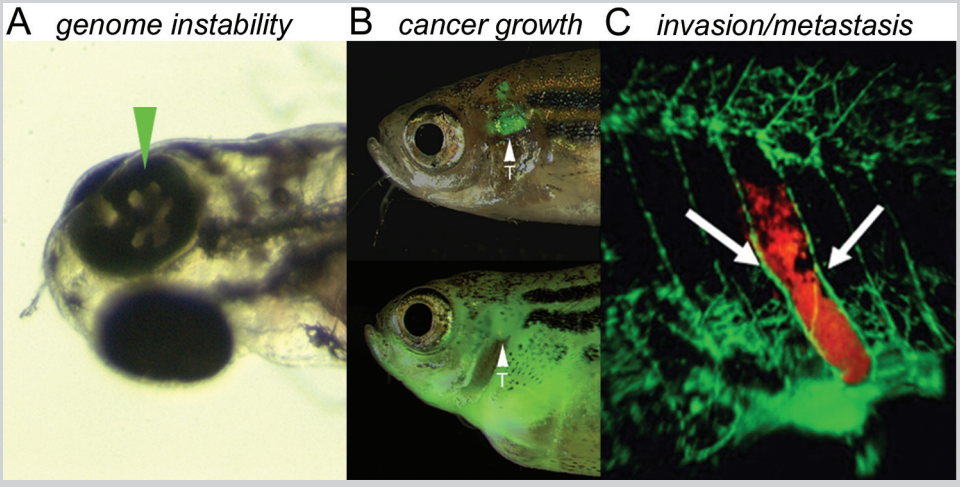

Figure 1. The unique possibilities of zebrafish can be employed to study different stages of cancer development. (A) Genome instability will lead to mutation accumulation that can result in cancer. Here, somatic loss-of-heterozygosity of the *albino* gene is shown in a patch of cells (arrow) in the pigment layer of the eye of an *msh6* mutant zebrafish embryo (H. Feitsma, unpublished results). (B) Cancer growth can be followed in living adult zebrafish. Here, the growth of a GFP-labelled T cell acute lymphoblastic leukaemia in stable transgenic fish is shown. Upper panel, *rag2-EGFP* control fish; lower panel, *rag2-EGFP-mMyc* fish with massive leukaemia; T, thymus (reprinted with permission from (36)). (C) Invasion of cancer cells in blood vessels is the first step of metastasis. Here, transplanted DsRed-labelled human cancer cells penetrate GFP-labelled blood vessels (arrows) in a zebrafish (reprinted with permission from (21)).

2. ZEBRAFISH CANCERS COMPARED TO MAMMALS

As mentioned previously, zebrafish can develop almost any type of cancer (2). Moreover, a tremendous asset of zebrafish as a cancer model is that many tumours histologically resemble human tumours (Fig. 2) (44). Also, more general cancer characteristics such as genomic instability, invasiveness, transplantability and the existence of cancer stem cells (40) apply to zebrafish tumours as well (Fig. 1), and many tumour suppressor genes and oncogenes have been conserved. Taken together, these studies validate zebrafish as a *bona fide* cancer model. However, many details are still unknown and some important differences with regard to human tumorigenesis have also become clear, which will be discussed below.

Cancer incidence and onset

The tumour suppressor genes that have been mutated in zebrafish have so far been limited to the few well-studied classical examples, for which mouse knockouts have been generated as well, and human hereditary diseases are known. Mouse *apc(Δ716)* knockouts are embryonic lethal in homozygous state, similar to what was found in zebrafish. However, all heterozygous mice develop intestinal polyps by 7 weeks of age (45). *Pten* homozygous mutant mice are also early embryonic lethal, and heterozygotes die before 3.5 months of age from malignant tumours (46). Likewise, 74% of *p53* homozygous knockout mice develop cancer before 6 months of age (47). The *msh2* and *mlh1* mouse knockouts were found to have a 50% cancer incidence at 6 months. For the *msh6* knockout this was less severe, as *msh6* is partially redundant with *msh3* (48). The conditions caused by mutations in the above genes in humans are Li-Fraumeni syndrome for *TP53* (49), familial adenomatous polyposis (FAP) for *APC* (50), Cowden disease and Bannayan-Riley-Ruvalcaba syndrome for *PTEN* (51), and hereditary nonpolyposis colorectal cancer (HNPCC) for mismatch repair genes (52). These human diseases are considered early-onset syndromes, becoming clinically relevant around the age of 40-45. Tumour incidences are probably high but mostly unknown, as unaffected carriers of disease mutations are usually not noticed.

Overall, the tumour incidences in zebrafish are generally lower and the onset is later as compared to the orthologous mouse models. Although animal numbers of the initial studies are limited, it turns out that the cancer incidence is quite similar for all zebrafish mutants, close to 30% (12, 27, 28) (H. Feitsma, R.V. Kuiper, J. Korving, I.J. Nijman,

E. Cuppen, in press). In addition, the average tumour frequency in the ribosomal gene mutant lines of the retroviral insertion screen was also around 35% (24). This high similarity in frequencies is remarkable, and suggests that this is the maximum frequency of cancer that spontaneously develops in genetically predisposed zebrafish. Furthermore, zebrafish develop tumours generally in the second year of life (12, 27, 28) (H. Feitsma, R.V. Kuiper, J. Korving, I.J. Nijman, E. Cuppen, in press), while the comparable mouse mutants develop cancer within the first six months of life. Since the overall life span is similar for mice and fish, the difference could be due to the lower number of cells in fish, which simply decreases the chance that one body cell will acquire all oncogene activations and tumour suppressor truncations necessary for tumour development. However, considering the zebrafish bodyweight is about 100-fold lower than that of mouse (0.3 g vs. 30 g) and the number of cells will be more or less accordingly, a time to disease onset that is four times larger (2 years vs. 6 months) and a disease incidence that is three times smaller (30% vs. 100%) are actually relatively small differences. In addition, the lower zebrafish disease incidence as compared to mice is experimentally not a major problem, as generating and keeping a three-fold larger number of fish is practically easy. In any case the frequency of cancer in mutant fish is much higher than in wild types, which spontaneously develop tumours in the order of 0% (27) to 11% (24) of the population. Another long term study of spontaneous neoplasms determined that wild-type fish have 1% cancer incidence in the first year, which increases in the second year (10). However, most groups agree that more data need to be generated on tumour incidence in wild-type fish to improve the model.
Carcinogen treatments on zebrafish give more robust induction of cancer, and are considerably easier to perform as compared to the mouse system. Although early studies of transgenic fish expressing oncogenes yielded highly variable tumour incidences, it is exciting and promising to see that technical improvements boosted cancer incidences to 80-100% (36).

A recurrent problem in zebrafish is the presence of duplicated genes resulting from a recent partial or complete genome duplication in teleosts (53), which may influence the role of oncogenes and tumour suppressors in carcinogenesis. An example is the presence of two forms of *pten* in the zebrafish genome, which were found to be functionally redundant in development but not in oncogenesis: no loss of *ptena* was observed in tumours of *ptenb* homozygous mutants (28). Also, although LOH is expected to be

the frequent mechanism in humans to explain the cancer predisposition in individuals with heterozygous tumour suppressor mutations (54), it was hardly ever observed in the comparable zebrafish mutants: not in *apc* (12), not in all mutant lines of the retroviral insertion screen (24), only once in the whole set of genomic instability mutants (25), twice in the *separase* mutant (interestingly, these particular mutants did not show a polyploidy phenotype) (13) and never in the *bmyb* mutant (14). An interesting study in this respect is the NDMA induction of liver tumours in triploid and diploid zebrafish (9). When assuming that loss-of-function of tumour suppressor genes is an important step in cancer development, one would expect that in case of a triploid fish three alleles need to be hit to lose the function of the gene, consequently taking longer for tumours to develop. Indeed, triploid fish had a slightly later onset of cancer, but concomitantly these fish showed a higher incidence of tumours, which indicates that activation of oncogenes is equally important.

Tumour spectrum

Most tumour classifications in zebrafish are still rather broad, lacking the more sophisticated identification marker stainings available for mammals. Unfortunately, many commonly used assays for human and mouse do not work on zebrafish material, despite repeated efforts from probably almost many zebrafish tumorigenesis labs. The Cheng lab is working on a systematic zebrafish tumour histology database (personal communication with K. Cheng), which is a very valuable initiative to standardize tumour classification. Nevertheless, the need for zebrafish specific antibodies is evident for this and many other research areas.

The most common target tissues for spontaneous tumours in wild-type fish are testis and liver (2). In contrast, genetic mutant lines most commonly develop MPNSTs, as observed in the *p53*, mismatch repair, ribosomal protein and genomic instability mutants (24, 25, 27) (H. Feitsma, R.V. Kuiper, J. Korving, I.J. Nijman, E. Cuppen, in press). Likewise, the transgenic line overexpressing human *MYCN* develops MPNSTs (43). MPNSTs form a large group of tumours that includes for example neurofibromas, the tumour type that occurs in human neurofibromatosis conditions (Fig. 2). In the *nf2* mutant fish line from the retroviral screen, this type of tumours can therefore certainly be expected (24). As already mentioned, defective mismatch repair in humans is linked to HNPCC, which is a colorectal cancer. However, this concerns patients that are heterozygous for the mismatch repair gene mutation. Interestingly, the rare patients with biallelic inactivation

of mismatch repair genes have been reported to develop a more neurofibromatosis type 1-like syndrome with skin neurofibromas and brain tumours. Mouse mismatch repair knockouts mainly develop lymphomas (48), indicating that the zebrafish models important aspects of the human disease that are not observed in mice (Tab. 3). *P53* mutants in human and mouse do not develop MPNSTs but rather osteosarcomas, rhabdomyosarcomas, breast cancer, brain tumours and leukaemias in human (49), and lymphomas and sarcomas in mouse (Tab. 3) (47).

The intestinal and liver tumours in zebrafish *apc* mutants (12) are comparable to those in mouse mutants and human patients, also regarding constitutive activation of the Wnt-pathway in tumours (Tab. 3; Fig. 2) (45, 50). Since the zebrafish gut is much smaller than that of mouse and human, the absolute chances for developing gut cancer are probably proportionally smaller, possibly explaining the observed difference in penetrance. Surprisingly, in zebrafish *ptenb* mutants only one type of tumours inside the eye was found (28), while mouse and human *pten* mutants show a broad spectrum of cancers (Tab. 3) (46, 51).

TABLE 3. Cancer types in tumour suppressor mutants in zebrafish, mouse and human

gene	zebrafish	mouse	human
TP53	MPNSTs	lymphomas and sarcomas	sarcomas, breast cancers, brain tumours, leukaemias (Li-Fraumeni)
PTEN	intraocular tumours	broad spectrum	broad spectrum
APC	liver and intestinal tumours	intestinal polyps	colorectal cancer (FAP)
MMR	MPNSTs and others	lymphomas and gastrointestinal tumours	heterozygous: colorectal cancer (HNPCC) homozygous: brain tumours and neurofibromas, lymphomas and leukaemias

To get a more detailed view on similarities of fish and human cancers, gene expression profiles of zebrafish and human tumours have been compared. For the *bmyb* mutant, gene expression profiles of homozygous embryos showed significant correlation with human cancers (14), which is noteworthy, as a developmental zebrafish phenotype was compared with a human cancer. A more extensive expression array study was performed on DMBA- and DBP-induced liver tumours in zebrafish, showing significant similarities with human liver tumours, but not with other human tumour types (55-57). The two human types of rhabdomyosarcoma, embryonic rhabdomyosarcoma (ERMS) and alveolar rhabdomyosarcoma (ARMS), have a distinct molecular signature. Microarray analysis on rhabdomyosarcomas of activated RAS transgenic fish showed that these have a signature similar to human ERMS but not to ARMS. The profile also showed similarities to other RAS-induced tumours such as human pancreatic adenocarcinoma and mouse lung adenocarcinoma, in addition to a more specific ERMS signature of muscles genes (40). These studies collectively indicate that the genetic pathways involved in cancer development are conserved between fish and mammals.

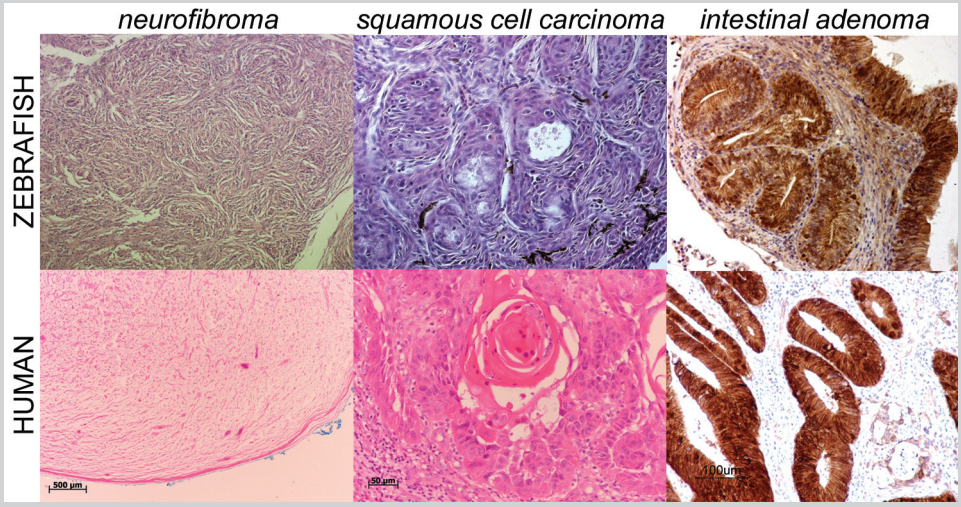

FIGURE 2. Zebrafish tumours resemble various types of human tumours at the histological level. Comparison of histology of neurofibroma (left column; stained with hematoxylin/eosin), squamous cell carcinoma (middle column; stained with hematoxylin/eosin) and intestinal adenoma (right column; immuno-stained for ß-*catenin*) in zebrafish (upper row) and human (lower row).

3. SPECIFIC ADVANTAGES OF ZEBRAFISH

The differences of zebrafish cancer compared to mammals do not compromise the organism as a cancer model, but should rather help focusing on the specific strengths of zebrafish to unravel mechanisms in carcinogenesis, complementary to other models.

Screens

The suitability of zebrafish for genetic screens has long been recognized, also in cancer research. But although very elegant strategies were chosen for the first screens for cancer genes that were performed in zebrafish, the results were promising but not completely convincing. Two new genes for which a relation to cancer was unknown or unclear were identified in the proliferation screen, but the cancer predisposition in heterozygous mutants is marginal and only visible when carcinogen treatment was used (13, 14). In relation to this, no mutations in *separase*, one of the identified genes, were found in 82 human tumour cell lines. Also, although the ribosomal protein mutants from the retroviral insertion screen were clearly cancer-prone, a clear connection of this important and large group of genes to cancer had never been observed in other organisms (24). Certainly, further research is necessary to qualify these genes as real 'cancer genes'. The mutants from the genomic instability all were clearly predisposed to cancer (25), but since the embryonic phenotype is a matter of chance and not fully penetrant, mapping the mutants is labour-intensive and time-consuming. Nevertheless, there are many options for advanced novel screens by performing mutagenesis in a cancer-prone background or crossing mutants to a cancer-prone line, in order to identify genetic modifiers of specific cancers. Additionally, making use of transgenic lines with fluorescent reporters and/or chemical compound libraries (see below) will certainly result in new steps in cancer research.

Imaging

The lauded advantage of zebrafish embryos being transparent does not, except for some transplantation studies, apply to most cancer studies in zebrafish that involve adult animals. However, a relatively transparent adult zebrafish line that lacks all types of pigment has been generated by Znomics (58). Additionally, Goessling *et al.* have successfully applied high-resolution microscopic ultrasound to follow tumour development and regression by treatment in living adult fish (59). Other existing imaging techniques that should in

principle be possible in zebrafish are tomography and MRI (58).

Most effective, however, and specific for zebrafish, is the use of fluorescence, as even adult zebrafish are small enough to be able to visualize fluorescent organs or tumours inside the living body. Tumour development and cancer spreading can therefore be followed over time in a living fish. Indeed, transplantation studies described above often used fluorescently labelled mammalian cells, facilitating monitoring of behaviour inside the host (16, 17, 19). Furthermore, by combining transgenic zebrafish lines expressing fluorophores in the vasculature with transplantation of differentially labelled mammalian cancer cells, the dynamics of vascular remodelling within the tumour can be visualized in a detailed fashion over time in a living model (20, 21), which is a unique opportunity for this research field (Fig. 1C).

Another example of the versatility of fluorescence in zebrafish is the generation of transgenic zebrafish lines expressing mammalian oncogenes, where the expression cassettes are usually provided with fluorescence markers to visualize the carcinogenesis process (Fig. 1B) (33, 35, 39, 41, 43). For example, Langenau *et al.* used fluorescence of labelled leukaemic cells to show that radiation treatment of wild-type fish transplanted with leukaemia cells resulted in disappearance of the cancer, while in *bcl2*-overexpressing, apoptosis-deficient fish the cancer remained present (38). Fluorescence was also very elegantly used for tracking recombination events in *cre*/*lox*-regulated systems in whole fish. A loxed *dsRED* gene in between the *rag2* promotor and *EGFP-mMYC* transgene resulted in dsRED expression in thymocytes, but upon recombination in the presence of CRE in a switch to EGFP expression in the same cells (36, 37). In the rhabdomyosarcoma model mentioned above, different fluorescent markers were successfully used to FACS sort four distinguishable cell types in order to identify cancer stem cells that have the capacity to induce new cancers in transplanted fish (40).

The above examples are pioneering studies, but they already show the enormous possibilities of using fluorescence in zebrafish cancer research, being indicative of a proportional number of opportunities for new studies. For example, some of the transgenic lines will be very well suited for performing genetic or chemical screens to identify modifiers of carcinogenesis, potentially in automated high-content screening setups.

Chemical treatments in search for drugs

As already mentioned, chemical treatment of both embryos and adult animals is relatively easy for zebrafish. In this respect, zebrafish can be a versatile model in the search for cancer therapeutics. Some proof of principle comes from the effective use of known angiogenesis inhibitors in transplantation-induced vascular remodelling. The neovascularization inside mammalian tumour grafts in zebrafish embryos could be inhibited by treatment with antiangiogenic drugs, while development of the normal vasculature in these embryos was not influenced (20). One of those compounds was also shown to block the angiogenic response to human tumour cell-secreted VEGF in zebrafish embryos (21). Another type of search for therapeutics concerns that of radioprotective agents, which are of great clinical relevance considering the importance of radiotherapy in human cancer treatment. As an example, the nanoparticle DF-1 was shown to reduce ionizing radiation damage in zebrafish embryos (60).

The state of the art would be to screen chemical compound libraries to identify novel drugs that inhibit certain aspects of cancer development. For this purpose, mutants that are embryonic lethal in homozygous state and cancer-prone in heterozygous state, such as those from the retroviral insertion and proliferation screens or the *apc* mutant (12-14, 24, 30), can be very useful, because the early embryonic phenotype can be used as a readout, while the obtained compound may very well be applicable to the adult cancer phenotype. In a nice and successful example of this, the *bmyb* mutant was used for a small-molecule screen in which the novel compound persynthamide was found to rescue its embryonic phenotype (61, 62). Unfortunately, the effectiveness of this compound in adults has not yet been reported. A similarly nice small-molecule screen identified the compound 4-bromo-3-nitropropiophenone as a radiation sensitizer specifically for cancer cells. In zebrafish embryos transplanted with human cancer cells and treated with this compound, tumour growth was inhibited by irradiation while there was no effect on embryonic development (63).

Outlook

Although the area of cancer research in zebrafish is still relatively young, from this overview it becomes clear that enormous progress has been made since the year 2000. Versatile tools and infrastructure for studying various aspects of carcinogenesis have become available and have been validated to various degrees. However, new possibilities may be contained in technologies that have been successfully employed in other cancer

models. For example in transgenesis, elegant systems for spatial and temporal control of gene expression, such as those driven by tetracycline or using GAL4-upstream activating sequences (UAS), are available in fruitfly and mouse (64, 65). Tetracycline-responsive systems (Tet-On and Tet-Off) allow for on and off switching of gene expression, which, when combined with oncogenes or tumour suppressors, would give the opportunity to follow tumour progression and regression in zebrafish. The GAL4-UAS system makes use of endogenous enhancers to target expression. Since the currently available tissue-specific promotors in zebrafish are limited and sometimes unspecific, generating a collection of specific enhancer-trap lines, such as successfully done by the Becker lab (66), would be valuable in strategies to target cancer gene expression. Nevertheless, while more universal standards are being developed, many opportunities are now on hand for exciting cancer-related studies in this small but multipurpose vertebrate model. As a result, the zebrafish can be expected to contribute to novel insights in tumour biology and cancer drug development in the near future.

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2

Mlh1 deficiency in zebrafish results in male sterility and aneuploid as well as triploid progeny in females

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ABSTRACT

In most eukaryotes, recombination of homologous chromosomes during meiosis is necessary for proper chromosome pairing and subsequent segregation. The molecular mechanisms of meiosis are still relatively unknown, but numerous genes are known to be involved, among which many mismatch repair genes. One of them, *mlh1*, colocalizes with presumptive sites of crossing-over, but its exact action remains unclear. We studied meiotic processes in a knockout line for *mlh1* in zebrafish. Male *mlh1* mutants are sterile and display an arrest in spermatogenesis at metaphase I, resulting in increased testis weight due to accumulation of prophase I spermatocytes. In contrast, females are fully fertile, but their progeny shows high rates of dysmorphology and mortality within the first days of development. SNP-based chromosome analysis shows that this is caused by aneuploidy, resulting from meiosis I chromosomal missegregation. Surprisingly, the small percentage of progeny that develops normally has a complete triploid genome, consisting of both sets of maternal and one set of paternal chromosomes. As adults, these triploid fish are infertile males with wild-type appearance. The frequency of triploid progeny of *mlh1* mutant females is much higher than could be expected for random chromosome segregation. Together, these results show that multiple solutions exist for meiotic cross-over/segregation problems.

INTRODUCTION

Meiosis is the cell division that produces haploid cells from diploid stem cells. This process consists of two division steps, from which the first, meiosis I, is the reduction division. The two homologues of each chromosome separate in this step. In most eukaryotic organisms, during the prophase of meiosis I recombination occurs between homologues, which is essential for the formation of stable bivalents. This in turn is necessary for proper alignment and spindle attachment in metaphase I and subsequent cell division.
In prophase I five different stages are recognized: leptotene, zygotene, pachytene, diplotene and diakinesis (1, 2). In leptotene, a large number of double strand breaks is generated in each chromosome (1). The homologues are still apart, but begin to search for homology. Synapsis, close association of homologues by binding of synaptonemal complex (SC) proteins, starts in zygotene and is completed in pachytene. During synapsis the double strand breaks are being repaired. Only a fraction, on average one or two breaks per pair, is repaired via homologous recombination with a nonsister chromatid of

the homologue. These crossing-over sites become visible in diplotene, when desynapsis takes place. Finally, in diakinesis, crossing-overs are stabilized as chiasmata, which are then the only sites that keep the chromosome pair together.

The involvement of mismatch repair (MMR) genes in meiosis first became noticed in the mouse, knockouts for several of them displaying fertility problems (3). The MMR proteins function in similar types of mutS and mutL heterodimeric complexes as they do in repair of replication errors in mitotic cells (4). However, the mutS complex that acts in recombination, mutS-gamma, consists of *msh4* and *msh5*, genes that do not appear to play any role in mismatch repair (3). MutS-gamma binds in foci together with replication protein A (RPA) to the DNA during synapsis in zygotene and pachytene (1, 5). The frequency of these foci in mammals is five- to ten-fold higher than the eventual number of crossing-over sites (6). MSH4 and MSH5 promote synapsis, as the mouse mutants display synapsis defects (6-8). As a result of that, both sexes do not have gametes and are sterile. This is much alike the phenotype of the knockout for one of the synaptonemal complex proteins, SYCP1 (5). In contrast, C. elegans *msh4* and *msh5* do not act before pachytene stage and are essential for crossing-over, a function more like *mlh1* in mammals (9, 10).

The mutL complex involved in meiosis is also different from the MMR mutL complex and consists of MLH1 and MLH3, although a minor role for MLH3 in MMR has been reported (11, 12). The MLH1/MLH3 complex is present in distinct foci on the synapsed elements during pachytene, at a later stage than the MSH4/MSH5 complex. After MLH3 binding MLH1 is recruited (4, 13). The MLH1/MLH3 foci coincide in timing, number and position with the presumptive sites of crossing-over (14, 15). The idea is therefore that the complex stabilizes a limited number of recombination sites for repair by crossing-over, while the other sites will be repaired via different mechanisms. *Mlh1* and *mlh3* knockout mice have very similar phenotypes in respect to meiosis (13, 16-19). Both sexes are sterile, but where males lack spermatozoa completely, females do have oocytes, which however hardly ever finish meiosis (13, 17, 19). The meiosis defect in these animals also occurs at a later time point than in *msh4* and *msh5* knockout mice. Synapsis is normal, but the frequency of crossing-overs is dramatically reduced (16, 20), and chromosomes are mostly present as univalents instead of bivalents in metaphase I (16, 19).

Zebrafish as a vertebrate model organism has been very important for the study of early embryonic development, due to its frequently mentioned advantages of external

fertilization, high numbers of progeny and easy traceable development of transparent embryos. These advantages similarly apply to meiosis research, but this direction has hardly been followed so far in zebrafish. We used our recently developed efficient reverse genetic procedure to generate a knockout for *mlh1* in the fish (21, 22). We show here that the necessity of MLH1 for recombination during meiosis in zebrafish is similar to that in mammals, but that its absence in female meiosis results in a different bypass of meiotic problems.

MATERIALS AND METHODS

Generation of mlh1 mutant fish

Two amplicons, covering exons 2-4 and 8-10 of the zebrafish *mlh1* gene respectively, were used for target-selected mutagenesis (21, 22). The obtained mutant fish in TL background was outcrossed with an AB fish, and heterozygous offspring was subsequently incrossed. Genotyping was done by amplification and resequencing, using exon 10 specific forward (5'-AGTGAAGGGCTTCATCTCC-3') and reverse (5'-AAGTAGTGCATCTATTGAAAATG-3') primers.

Western blot was performed using standard procedures with a monoclonal anti-human MLH1 antibody (BD Biosciences Pharmingen, Franklin Lake, N.J.) and an anti-alpha-actin antibody (Abcam, Cambridge, UK). Human Jurkat control lysate was provided with the anti-MLH1 antibody.

Histology and morphometry

Testes for histological and morphometric evaluation were dissected, fixed in 4% glutaraldehyde and subsequently weighed. They were embedded in 2-hydroxyethyl methacrylate, and 4 μm sections were stained with toluidine blue.

For morphometric analysis, the weights of various testicular tissue components were determined by light microscopy using a 441-intersection grid placed in the ocular of the light microscope. Fifteen fields chosen randomly (6,615 points) were scored for each animal at 400x magnification. Staining or cutting artefacts were rarely seen and were not considered in the total number of points utilized to obtain weights. Points were classified as one of the following: spermatogonia; primary spermatocytes; secondary spermatocytes; spermatids; spermatozoa; empty spaces; abnormal meiotic figures; apoptosis and others (somatic cells, blood and lymphatic vessels and connective tissue).

The weight (mg) of each testis component was determined as the product of the volume density (%) and the testis weight. To obtain a more precise measure of testis weight the testis capsule, the efferent ductules and connective tissue associated with testes were excluded from the testis weight using image analysis. For that, six longitudinal testis sections were made and evaluated (approximately 80 μm apart from each other) for each animal, considered to represent the entire testes. All data are presented as the mean ± SEM and analyzed via ANOVA (Newman-Keuls test). The significance level in comparisons was considered to be $p<0.05$.

TUNEL labelling was performed on tissue fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm thickness. A commercial in situ cell death detection kit (In Situ Cell Death Detection Kit, POD, Roche Diagnostics GmbH, Mannheim, Germany) was used. After labelling, the slides were counterstained with Mayer haematoxylin and mounted.

Spermatocyte spreads and immunocytology

Spermatocyte spreads were performed as described previously (14). Testes were dissected and suspended in 50 μl phosphate-buffered saline (PBS). 1 μl of cell suspension was added to 30 μl of 1:2 PBS:mQ water on each well of a multiwell slide. After settling for 20 min, the cells were fixed for 3 min in 2% paraformaldehyde (PFA) + 0.03% SDS, for another 3 min in 2% PFA at pH 7.5-8, and washed in 0.4% Photo-Flo 200 (Kodak, Rochester, N.Y.) or Agepon (AGFA, Mortsel, Belgium) and dried.

For immunocytology, the slides were blocked in 10% antibody dilution buffer and incubated in primary antibodies in antibody dilution buffer (10% goat serum, 3% bovine serum albumin, 0.05% Triton X-100 in PBS) for 2-3 h or overnight. After washing, the cells were treated with secondary antibodies for 1 h at 37 °C, washed and dried, and mounted in ProLong Antifade mounting agent (Molecular Probes, Carlsbad, CA) or DAPI vectashield (Vector Labs, Burlingame, CA). Primary antibodies for SYCP1, SYCP3 and SMC3 for visualization of SCs, for MLH1, and CREST serum to detect centromers were described previously (14).

Synapsis was visualized by silver staining, by putting a few drops of 40% silvernitrate on the slide and covering it with a piece of fine nylon mesh. Slides were stained in a 60 °C oven for 30-60 min until turning brown.

SNP sequencing

SNPs for detection of aneuploidy were selected from a large set of amplicons previously used for SNP verification (23). A nearly genome-wide set of 96 SNPs was compiled on two criteria: 1) being polymorphic between a homozygous mutant female and a wild-type male 2) showing a quantitative relationship to artificial mixtures of parental DNA in 1:1 and 1:2 proportions. This set covered 22 of 25 zebrafish chromosomes in the Zv4 genome assembly of Ensembl. SNP database numbers are provided in Supplemental Table 2. Embryonic DNA was amplified and sequenced for these SNPs. Peak area proportions were determined visually. They were partly confirmed by calculating sequence trace peak areas electronically (V. Guryev, personal communication; h094.niob.knaw.nl/peakarea).

Chromosome spreads

24 hpf embryos were dechorionated and incubated for 90 min in colchicine to arrest cells in metaphase. After hypotonic treatment in 1.1% sodiumcitrate and fixation in 3:1 methanol:HAc, cells were suspended in 50% HAc and spread onto glass slides. Slides were mounted and stained with DAPI vectashield (Vector Labs, Burlingame, CA).

RESULTS

Generation of *mlh1* knockout zebrafish

The *mlh1* gene in zebrafish was annotated based on homology with other species and consists of 19 coding exons. Using an ENU-driven target-selected mutagenesis screen, we isolated an individual that has a C to T transition in exon 10, thereby introducing a stop codon at position 265 (Fig. 1A, B). This individual was crossed out, and the heterozygous offspring subsequently crossed in to generate homozygous mutant zebrafish. Homozygous mutants were obtained with the expected frequency of 25%, and they had normal appearance. The sex ratio in the homozygote population was not significantly different from the other genotypes. The knockout phenotype was confirmed at the protein level by Western blotting, where no full-length MLH1 protein was detectable in testes of a homozygous mutant male (Fig. 1C). Homozygous mutant fish develop tumours from around six months of age, which will be subject of a next study.

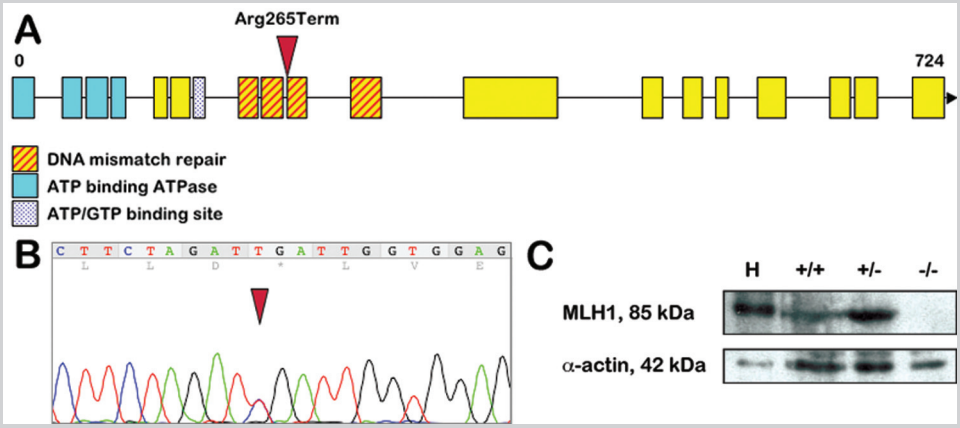


FIGURE 1. The generation of *mlh1* knockout zebrafish. (A) Genomic organization of the zebrafish *mlh1* gene. The position of the ENU-induced point mutation introducing a premature stop codon is marked by an arrow. (B) Sequence trace showing the C to T transition in the heterozygous founder fish. (C) Western blot of testes proteins and human Jurkat control lysate (H) stained with antibodies for MLH1 (85 kDa) and alpha-actin (42 kDa). MLH1 protein is completely absent in *mlh1*$^{-/-}$ testes.

Male mutant phenotype

Male *mlh1* mutant zebrafish showed normal mating behaviour and could induce egg lays from females, but none of the eggs became fertilized. Histological analysis of the testes revealed that post-meiosis I stages of spermatogenesis, that is secondary spermatocytes, spermatids and spermatozoa, as visible in the wild type (Fig. 2A) were completely absent in the mutant (Fig. 2B). In metaphase of meiosis I, wild-type primary spermatocytes showed strongly condensed chromosomes perfectly aligned on the metaphase plate (Fig. 2A, inset). In contrast, in mutant primary spermatocytes, chromosomes were dispersed throughout the nucleus (Fig. 2B, inset). Also, in mutant testis groups of cells with strongly condensed nuclei could be observed, which are cells in apoptosis (Fig. 2B). The high incidence of apoptotic spermatocytes in mutants was confirmed by TUNEL staining (Fig. 2D), while wild-type testis showed very low levels of apoptosis (Fig. 2C). Quantitative morphometric analysis of testis tissue components (6 males for each group) showed that apoptotic germ cells were indeed more prominent in the mutant (Fig. 2E; Suppl. Tab. 1), and a high incidence of abnormal meiotic figures was observed. More strikingly, we observed a strong accumulation (~ 150%) of primary spermatocytes in mutant testis, whereas the number of spermatogonia did not increase significantly

(Fig. 2E; Suppl. Tab. 1). As a result, absolute and relative (expressed as percent of body weight) testis weight was higher in mutants. For all parameters, no difference between wild-type and heterozygous animals was observed (data not shown).

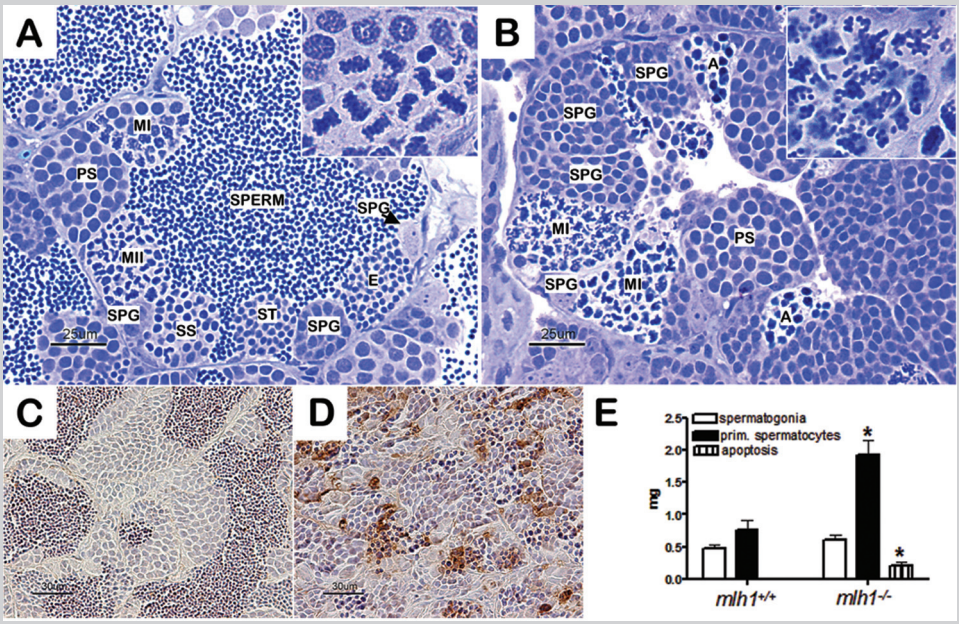


FIGURE 2. Testis histology of *mlh1*$^{-/-}$ zebrafish. (A, B) Cross section of seminiferous tubule in *mlh1*$^{+/+}$ (A) and *mlh1*$^{-/-}$ (B) zebrafish, showing spermatogenic cysts with different types of germ cells, spermatogonia at different mitotic divisions (SPG); primary spermatocytes (PS); first meiotic division (MI); secondary spermatocytes (SS); second meiotic division (MII); spermatids (ST); spermatozoa (SPERM); and apoptotic spermatocytes (A). Note the absence of post-meiosis I stages and the presence of apoptotic spermatocytes with strongly condensed nuclei in *mlh1*$^{-/-}$ testis. Inset shows a magnified view of the first meiotic division. In the wild type, chromosomes are aligned at the cell equator just prior to division. In the mutant, chromosomes are randomly distributed throughout the nucleus. (C, D) TUNEL staining of *mlh1*$^{+/+}$ (C) and *mlh1*$^{-/-}$ fish (D). High numbers of apoptotic spermatocytes can be seen in mutant testis. Wild-type testis shows a very low incidence of apoptosis. (E) Morphometric analysis of testes sections. The mutant shows a large increase in amounts of spermatocytes and apoptotic cells, but not of spermatogonia.

Synapsis and crossovers

To analyze further the progress of prophase I in *mlh1* mutant males, we performed immunocytology experiments on meiotic chromosome spreads of spermatocytes to follow synapsis of homologous chromosomes. The formation of the synaptonemal complex (SC), which starts from the chromosome ends in zygotene and is completed in

early pachytene (Fig. 3A), was normal in mutants (Fig. 3B). MLH1 foci, which are at the positions of crossing-over, can clearly be seen in wild-type spermatocytes (Fig. 3A). Their number and distribution per spermatocyte was similar to previous findings (14), where one or occasionally two foci per synapsed pair were seen, with in total 147 foci on 140 SCs analyzed. As expected, foci were completely absent in the mutant (Fig. 3B).
In diplotene, synaptonemal complex proteins have dissociated from the chromosomes. In wild-type situation, bivalents that are held together by one or two chiasmata per chromosome pair could be seen (Fig. 3C), but in the mutant most chromosomes were present as univalents (Fig. 3D). This indicates a failure in the stabilization of crossovers.

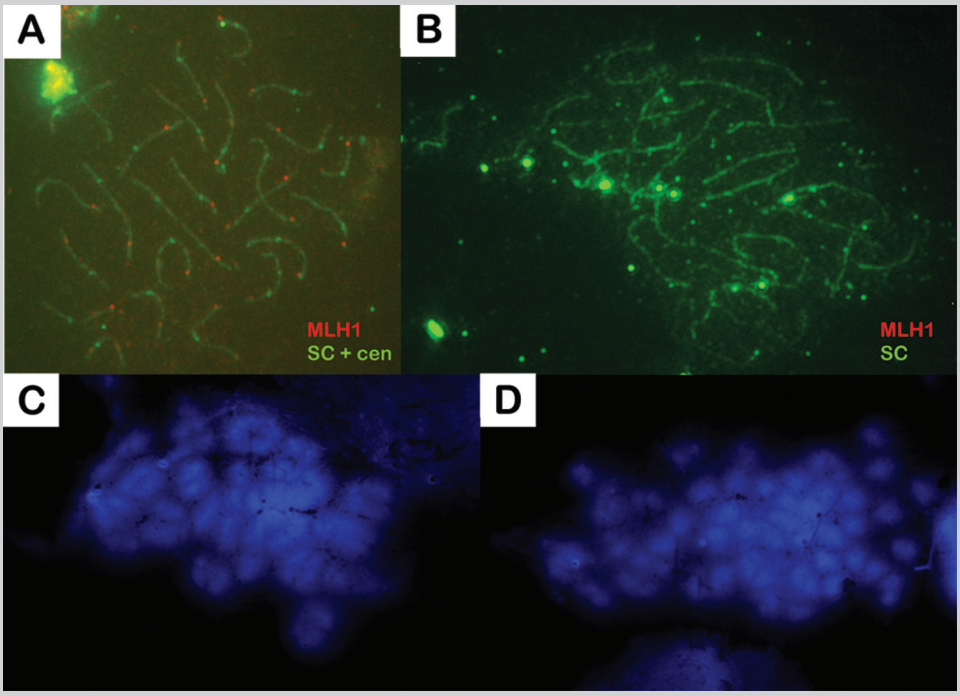


FIGURE 3. Chiasmata formation but not synapsis is defective in *mlh1*$^{-/-}$ spermatocytes. (A, B) 25 synapsed pairs of chromosomes are visible in pachytene spermatocytes of both wild-type (A) and mutant males (B). SCs and centromers (cen) are stained with green fluorescence, labelled with FITC. MLH1 is fluorescent red with rhodamine. One or two distinct MLH1 foci per synapsed pair can be seen in wild-type cells, but are absent in mutants. (C, D) DAPI-stained metaphase spermatocytes have bivalents in wild types (C), and mostly univalents in *mlh1*$^{-/-}$ mutants (D).

Female mutant phenotype

Mlh1$^{-/-}$ female zebrafish also showed normal mating behaviour. They had average size clutches that were normally fertilized. Ovary histology revealed no differences from wild type, including no increased apoptosis (data not shown). Fertilized eggs started to develop normally, but embryos showed high rates of dysmorphology and lethality within the first days post-fertilization (dpf) (Fig. 4A). Only 1-20% per cross was still alive and healthy at 7 dpf.

We crossed a mutant female with a wild-type male and genotyped 44 of their progeny at 24 hours post-fertilization (hpf) for the point mutation in the mlh1 gene. Heterozygosity at this position for all embryos was expected, but in contrast to that we observed that a large fraction has two or no copies of the maternal allele, as quantified by the peak height in the sequence trace (Fig. 4B), indicating chromosomal missegregation. To extend this observation to other chromosomes, we selected a set of SNPs that were distributed throughout the genome (23) and homozygously different between the parental *mlh1*$^{-/-}$ female and the wild-type male. This set covered 22 of 25 zebrafish chromosomes. Quantitative SNP analysis on sixteen 4-day-old embryos of a cross of these two animals revealed that all 16 deviated severely from the normal diploid chromosome number (Tab. 1), showing missegregation of on average 14 out of 22 chromosomes. This effect could always be assigned to the maternal contribution of chromosomes, as in all cases only a single paternal copy was present for each chromosome. As a comparison, only three cases out of 36 progeny from heterozygous females were aneuploid and no aneuploids were found in 30 progeny from wild-type females (Tab. 1). A second category of SNPs, being heterozygous in the mutant female, was used to be able to distinguish the two maternal homologues of a chromosome. We found that in trisomies always both maternal alleles were present, indicating a meiosis I defect and not meiosis II (data not shown).

Surprisingly, four out of the 16 mutant progeny had two maternal copies of each chromosome tested, together with one set of paternal chromosomes, which strongly suggests a complete triploid genome (Tab. 1). To determine the effect of triploidy on embryonic survival, we assigned categories of embryos with increasing survival rates and typed embryos from five pair crosses for two SNPs on two different chromosomes (Fig. 4C). Category A (n = 407) contained all embryos that died before day 4 or were unable to hatch. Category B (n = 63) embryos had hatched at 4 dpf but were clearly malformed and/or did not develop a swim bladder. Finally, category C (n = 65) contained

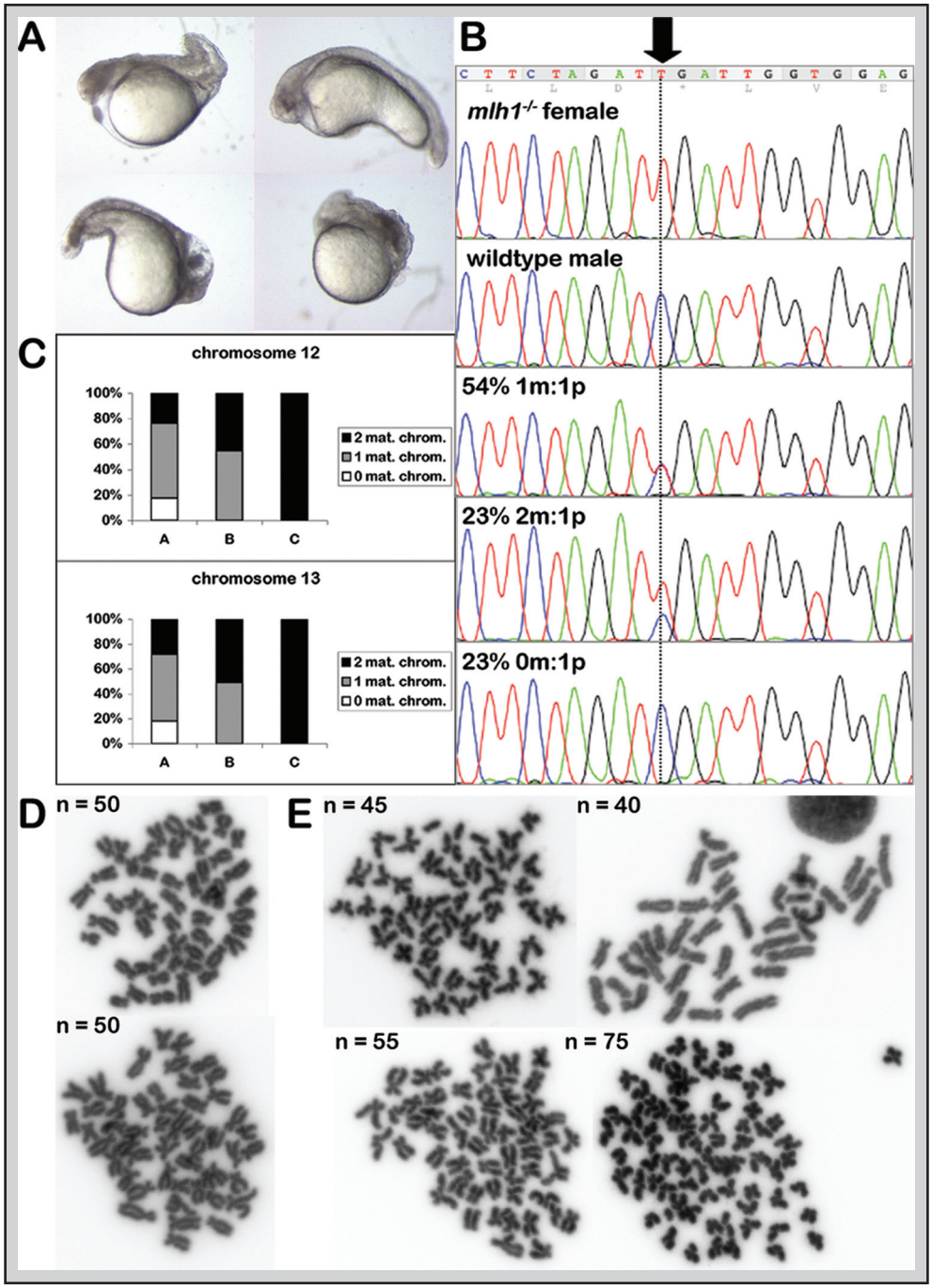
only healthy embryos at 4 dpf. Embryos from category A had no or two maternal copies of the analyzed chromosomes in approximately 50% of the cases. For a single embryo this would mean that it has no or two maternal copies of around half of its chromosomes. Embryos that survived longer in general had more chromosomes. Category B embryos had one or two maternal chromosomes and embryos from C had two maternal copies of both chromosomes in 100% of the cases. The latter will thus most likely have two maternal copies of all chromosomes and will therefore be the triploid embryos. Indeed, the fraction of embryos in category C is similar to the fraction of triploids found in the more extensively genotyped set.

To confirm the abnormal chromosome numbers, we performed chromosome counts in cells of 24 hpf embryos of similar crosses. Offspring from wild-type females had the normal diploid number of 50 chromosomes per cell (Fig. 4D). In mutant female progeny different cells of one embryo had the same chromosome number, but this number varied largely between embryos (Fig. 4E). We also observed cases of 75 chromosomes per cell, confirming the frequent existence of fully triploid embryos.

TABLE 1. Aneuploidy and triploidy in progeny of *mlh1*$^{-/-}$ females

maternal genotype (no. of embryos)	% euploid	% aneuploid	% triploid
-/- (16)	0	75	25
+/- (36)	92	8	0
+/+ (30)	100	0	0

FIGURE 4. Progeny from *mlh1*$^{-/-}$ females is severely aneuploid. (A) At 24 hpf, embryos from an *mlh1*$^{-/-}$ female are strongly malformed and show necrosis and apoptosis. (B) Quantitative genotyping of a single nucleotide polymorphism that has different alleles in a mutant female (upper panel) and a wild-type male (second panel) and its progeny. Only 54% of progeny is heterozygous at this position (third panel). 46% has either no or two maternal chromosomes (lower two panels). m, maternal; p, paternal. (C) Quantitative SNP typing of two chromosomes in embryos from four crosses that were assigned to different categories. Category A (n = 407) contained all embryos that died before day 4 or were unable to hatch. Category B (n = 63) embryos had hatched at 4 dpf but were clearly malformed and/or did not develop a swim bladder. Category C (n = 65) contained only healthy embryos at 4 dpf. The frequency of 0, 1 or 2 maternal chromosomes is plotted for each category. (D, E) Chromosome spreads of cells of 24 hpf embryos. Embryos from wild-type females (D) have the normal diploid number of 50 chromosomes per cell. Embryos from mutant females (E) have abnormal chromosome numbers, up to 75, which is equal to triploidy.


A
B
C T T C T A G A T T G A T T G G T G G A G
mlh1-/- female
wildtype male
54% 1m:1p
23% 2m:1p
23% 0m:1p
C
chromosome 12
100%
80%
60%
40%
20%
0%
A
B
C
2 mat. chrom.
1 mat. chrom.
0 mat. chrom.
chromosome 13
D
n = 50
n = 50
E
n = 45
n = 40
n = 55
n = 75

Triploid progeny

From a new cross of an *mlh1*$^{-/-}$ female with a wild-type male, we followed the embryos of the triploid category over a longer period. From 20 healthy embryos at 7 dpf, 17 survived until adulthood with normal appearance, and all were males. Partial SNP typing confirmed triploidy in 16 of these animals. These 16 males had strongly reduced fertility. Some were completely infertile, others were able to fertilize at maximum 3% of eggs, and all eggs that were fertilized developed dysmorphologically and died before 7 dpf.

Morphological analysis of testes showed that mitotic proliferation of germ cells appeared normal in the triploid males. However, although some germ cells were able to go through both meiotic divisions and in some animals a few mature sperm were found, overall only very low counts of spermatids and spermatozoa were observed (Fig. 5A, B). Furthermore, abundant apoptosis of primary spermatocytes was seen (Fig. 5A, B), as well as an unusual cellular composition of cysts during the second meiotic division, in which cells had lost synchrony and were in different developmental stages (Fig. 5A, inset). Silver stained spermatocyte spreads revealed that synapsis was strongly disorganized in triploid animals (Fig. 5D) as compared to wild type (Fig. 5C). Obviously, more than 25 chromosomal structures are present in the triploid, and irregular synapsis figures can be seen.

Discussion

To obtain better insight in the role of MMR proteins in meiotic processes, we have generated zebrafish that are deficient in MLH1 protein. We found that male *mlh1* mutants are sterile because spermatogenesis is arrested at the primary spermatocyte stage. A defect in chiasmata formation causes chromosomes to be unpaired at metaphase I, and therefore no proper chromosome alignment, spindle attachment and subsequent cell division are possible. This indicates that the function of MLH1 in zebrafish is similar as in mammals and is associated with stabilization of recombination sites that are repaired via crossing-over. The time point of arrest in mutant males is also similar to what was reported in mice (18). The defect in formation of crossing-overs occurs already in late pachytene, but it is only recognized as a problem by the cell in metaphase, when the high number of univalents does not allow cell division to progress. Apparently, after synapsis, spindle assembly is the next checkpoint for meiotic division.

We observed a large accumulation of primary spermatocytes in mutant testes. This was

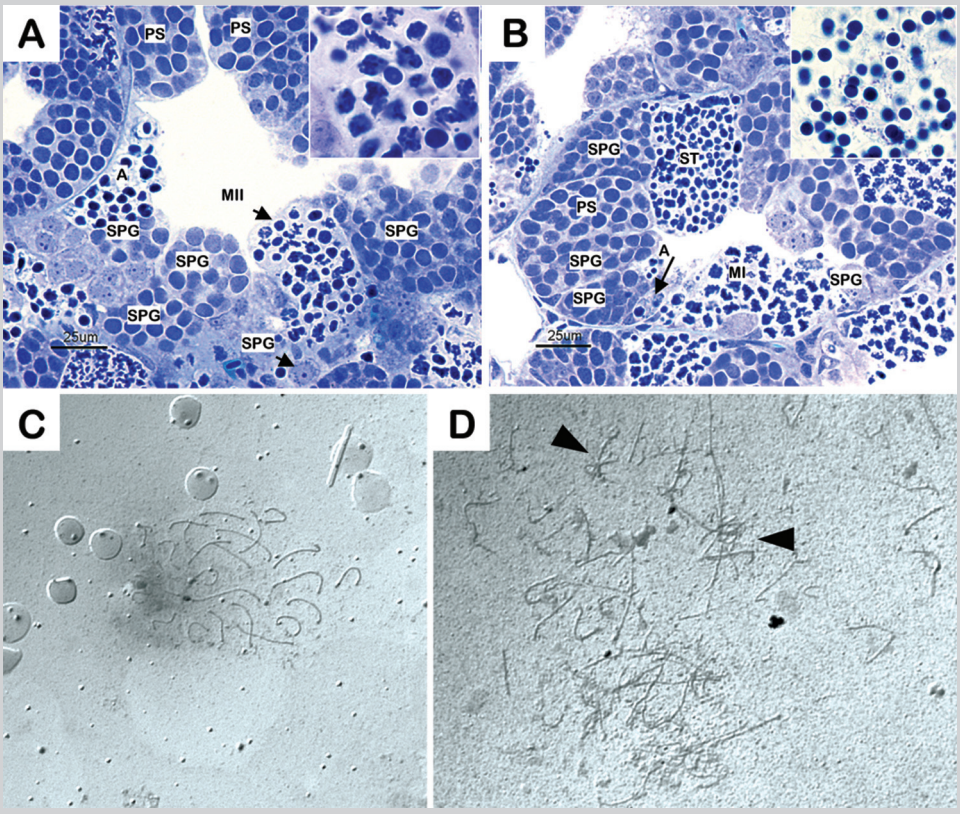


FIGURE 5. Triploid males have low fertility due to synapsis problems. (A, B) Cross sections of seminiferous tubules in infertile (A) and fertile (B) triploid zebrafish, with spermatogonia (SPG); primary spermatocytes (PS); second meiotic division (MII); and apoptotic germ cells (A). Inset in A: magnification of second meiotic division; note the concomitant presence of secondary spermatocytes, cells in second meiotic division and spermatids. Inset in B: magnification of spermatozoa in the efferent ductules. (C, D) synapsis in triploid spermatocytes (D) is strongly disorganized as compared to wild type (C). Irregular synapsis figures are indicated by arrows.

also reported for mouse, albeit not in a quantitative manner (18). The accumulation reflects a prolonged period during which mutant cells are arrested, eventually leading to apoptosis. We did not observe a significant difference in the amount of spermatogonia, indicating that the mitotic stages of spermatogenesis proceed normally. Even though the fate of spermatocytes in zebrafish resembles that in mouse *mlh1* loss-of-function mutants, a difference was observed with respect to the mutant's testis weight, which increased in

zebrafish but decreased in mice (13, 17). A possible explanation for this difference may be found in a different relative capacity of Sertoli cells to remove dead germ cells, which could be lower in zebrafish. On the other hand, there was very little cellular debris in the tubular lumen of zebrafish mutant testes, indicating that the Sertoli cells' capacity was not oversaturated.

In females, the situation in zebrafish is markedly different from mammals, as female *mlh1* mutant zebrafish show normal fertility. This seems, however, rather a result of zebrafish biology than of the existence of different mechanisms in zebrafish. Mouse female mlh1 mutants do have a normal ovary and fully developed oocytes. A small part of these oocytes is able to ovulate and extrude the first polar body, which means in fact that they can finish meiosis I, despite the lack of chiasmata (17, 19). The result is an aneuploid oocyte, which, upon fertilization, is hardly able to finish the second meiotic division and subsequent mitotic divisions (19). Oocytes from zebrafish *mlh1* mutants show the same error-prone finishing of meiosis I resulting in aneuploid eggs. The difference in this model is that after fertilization the embryo makes little use of its own genome initially, but instead develops on maternally supplied mRNAs and proteins from the yolk. Transcription from the zygotic genome does not commence before ca. 3 hpf (24) and maternal supplies are active until much later (25). In this respect, severe aneuploidy would be tolerable during the first hours of development. Only after the embryonic genome "starts up", problems caused by abnormal chromosomal content will become manifest. This has also been seen in other cases, such as for the futile cycle mutant, in which oocytes fail to undergo nuclear division but continue cell division, resulting in many anucleate cells (26).

The difference between sexes in this case is more striking, but this has been reported for many meiotic mutants (reviewed in (2)). Male meiosis is an ongoing, regulated process that can be highly selective on gametes as they are produced in large numbers in successive generations during the male's reproductive life. Female meiosis is much more programmed, producing only one generation of oocytes during development, which is stored in dictyotene until ovulation. In order to reproduce, the female needs oocytes of this pool to finish meiosis, but that comes with a cost of higher error rates. Recent studies on zebrafish female meiosis suggest that also in zebrafish, oogenesis takes place predominantly in young females (N. Kochakpour, personal communication). The differences in female fertility phenotypes in the different MMR mutants can also partly be explained as a difference in timing of the defects. In *mlh1* mutants, for both sexes

the problem occurs in pachytene, but the defect only becomes noticed in metaphase. In oogenesis however, dictyotene arrest is in diplotene, so just prior to the problem. The first meiotic division is only finished at ovulation. This explains why female mutants still have adult germ cells, and males have not. It contrasts with msh4 and msh5 mouse mutants, where the defect already occurs in zygotene and early pachytene, so well before diplotene, and where female mutants do not have oocytes. We predict that female msh4 mutants in zebrafish would also be fully sterile.

We found that progeny from *mlh1*$^{-/-}$ females had high levels of aneuploidy. The overall frequency of missegregation of chromosomes was close to 50%, which suggests random chromosome segregation. That is consistent with the observed unpairing of homologues in male zebrafish and male and female mice, due to the absence of chiasmata. As a result, chromosomes are dispersed throughout the nucleus in metaphase of MLH1 deficient spermatocytes. In the subsequent division each chromosome might randomly go to either side, which would result in an average missegregation frequency of 50%. However, the chance then for all chromosomes to go to one side, which would result in triploid embryos, would be $(0.5)^{50} = < 10^{-15}$. We find triploids at a much higher frequency of up to 25%. Several processes could be proposed to explain the high frequency of triploids. Possibly, mechanisms exist to circumvent meiotic problems and one of them could be complete skipping of the first meiotic division when it is strongly delayed due to for example spindle defects. In this case, however, we do also see many cases where embryos are close to triploidy but miss one or more chromosomes. Another explanation could be that chromosomes have a tendency to go to one spindle pole. An important characteristic of female meiosis is that both divisions have the spatially unequal result of an oocyte of original size and a small polar body. If chromosomes are not aligned at the metaphase plate but are randomly spaced, they might not be pulled properly to the polar body pole. Then it would be spatially more likely that most chromosomes stay within the oocyte. In line with this, human embryos resulting from maternal meiosis I missegregation commonly had extra chromosomes rather than missing chromosomes (27). In addition to this, monopolar spindles are frequent in female gametes (19), but absent in males. More generally, one could also speculate that it could be an advantage to keep most chromosomes in the oocyte and less in the polar body, since the latter will be degraded anyway. In our study we did however also see embryos with less than 25 chromosomes from the mother, which indicates that this does not hold true for all cases.

Interestingly, triploid but not aneuploid zebrafish embryos were found to develop normally. The fact that they were all males is striking, but the numbers are too small to draw conclusions, as sex ratios in zebrafish are known to vary highly between crosses. Breeding of more triploid zebrafish is necessary to verify if this is a fully penetrant phenotype. Polyploid individuals of many lower animal species and plants are viable (28-30), but in mammals they generally are not. However, in humans, where triploidy is often due to double sperm fertilizations, triploid embryos reach further developmental stages than most aneuploid ones, and rare cases of shortly surviving triploid humans have been reported (31).

Triploidy in fish can be induced by applying early pressure or heat-shock of oocytes to block the second meiotic division (28, 32). In zebrafish, gametogenesis has not been studied before in triploids, but in rainbow trout (33) and sea bass (34) it was shown to result in compromised fertility. As expected, we now also see in zebrafish that spermatogenesis is impaired due to disorganized synapsis, which is a logical consequence of the odd chromosome number. Apparently, spermatogenesis is not completely blocked, but the few resulting spermatozoa are aneuploid, resulting in non-viable embryos upon fertilization.

Taken together, we illustrate the versatility of zebrafish as a model for studying meiotic processes and defects. Our results on meiotic segregation problems in zebrafish show interesting similarities to mechanisms underlying human miscarriages, especially with respect to the female origin of aneuploidies and the substantial fraction of triploids within those.

Acknowledgments

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SUPPLEMENTARY DATA

SUPPLEMENTARY TABLE 1. Biometric and morphometric data in adult male wild-type (+/+) and *mlh1* mutant (-/-) zebrafish (mean ± SEM; n = 6 for each group)

Parameter	+/+	-/-
Body length (cm)	3.47 ± 0.08[a]	3.50 ± 0.09[a]
Body weight (g)	0.39 ± 0.02[a]	0.39 ± 0.02[a]
Testis weight (mg)[1]	2.49 ± 0.36[a]	3.63 ± 0.41[b]
Gonadosomatic index (%)	0.65 ± 0.11[a]	0.93 ± 0.05[b]
Spermatogonia (mg)	0.47 ± 0.07[a]	0.61 ± 0.07[a]
Primary spermatocytes (mg)	0.76 ± 0.15[a]	1.92 ± 0.22[b]
Secondary spermatocytes (mg)	0.03 ± 0.01	not detectable
Spermatids (mg)	0.21 ± 0.06	not detectable
Spermatozoa (mg)	0.65 ± 0.22	not detectable
Abnormal meiotic figures (mg)	not detectable	0.46 ± 0.06
Apoptosis (mg)	0.01 ± 0.01[a]	0.22 ± 0.04[b]

[1]Excluding tunica albuginea, efferent ductules and connective tissue associated with testis.
[a,b]Different superscripts for the same parameter indicate significant differences ($p < 0.05$).

SUPPLEMENTARY TABLE 2. dbSNP IDs for 96 zebrafish SNPs

ss49787988	ss49807212	ss49818810	ss49826172
ss49788005	ss49807351	ss49818965	ss49826206
ss49788813	ss49808109	ss49819166	ss49826336
ss49789703	ss49808269	ss49819470	ss49826605
ss49789898	ss49808552	ss49820108	ss49828320
ss49790820	ss49808867	ss49820331	ss49829291
ss49792334	ss49808926	ss49820534	ss49829468

ss49792589	ss49809247	ss49820912	ss49829494
ss49794901	ss49809820	ss49820933	ss49829949
ss49794971	ss49810474	ss49821714	ss49830686
ss49795128	ss49810899	ss49821748	ss49830719
ss49795323	ss49811442	ss49822129	ss49832080
ss49795599	ss49811861	ss49822678	ss49832554
ss49796354	ss49812054	ss49822730	ss49833744
ss49798024	ss49812622	ss49822853	ss49834191
ss49798468	ss49813761	ss49823042	ss49834276
ss49799120	ss49813802	ss49823213	ss49834451
ss49799476	ss49814819	ss49823313	ss49834906
ss49799854	ss49815004	ss49823410	ss49835061
ss49802487	ss49815237	ss49825127	ss49835737
ss49802668	ss49816223	ss49825486	ss49836407
ss49802849	ss49816773	ss49825806	ss49836987
ss49803052	ss49816987	ss49825811	ss49837083
ss49804030	ss49817716	ss49826138	ss49837121

3

Completion of meiosis in male zebrafish despite lack of DNA mismatch repair gene mlh1

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ABSTRACT

MLH1 is a member of DNA mismatch repair (MMR) machinery and is also essential for stabilization of crossovers during the first meiotic division. Recently, we showed that zebrafish *mlh1* mutant males are completely infertile due to a block in metaphase I, while females are fertile but have aneuploid progeny. When studying fertility in males in a twofold more inbred background however, we observed low numbers of fertilized eggs (approximately 0.4%). Histological examination of the testis revealed that all spermatogenic stages prior to spermatids (spermatogonia, primary spermatocytes and secondary spermatocytes) were significantly increased in the mutant while the total weight of spermatids and spermatozoa were highly decreased (1.8 mg in wild-type vs. 0.1 mg in mutants). This is clearly different from the outbred males of a previous study, where secondary spermatocytes or postmeiotic cells were absent. This indicates that there is a delay of both meiotic divisions rather than a complete arrest during meiosis I in these males. Eggs fertilized with mutant sperm develop as malformed embryos and were aneuploid, which makes this male phenotype much more similar to that previously described in mutant females. This indicates that crossovers are still essential for a proper meiosis, but that meiotic cell divisions can progress without it, suggesting that this mutant is a suitable model to study the cellular mechanisms of completing meiosis without crossover stabilization.

INTRODUCTION

The mismatch repair machinery corrects errors in newly synthesized DNA. This system is composed of many members, MLH1 being one of the key proteins (1-3). Several mismatch repair genes also play a role in meiosis (4), and the MLH1 protein is believed to stabilize crossover sites, which are essential for keeping the homologous chromosomes in a paired conformation during the first meiotic division. In prophase of meiosis I a number of double strand breaks are generated that are potential recombination sites. Only a fraction of those, one or two per chromosome pair, will be repaired by crossing over (5). During pachytene, MLH1 forms distinct foci on the synapsed chromosomes, which coincide with the sites of crossover in timing, number and position (6).

Both female and male *mlh1* knockout mice are sterile (7-10). However, examination of gonad histology revealed a sex difference, males lacking spermatozoa completely while females do have oocytes, which are however hardly able to finish meiosis (8, 10). Synapsis

proceeds normally in these mice, but the frequency of crossovers is dramatically reduced and chromosomes are mostly present as univalents instead of bivalents in metaphase I (7, 10), which impairs the subsequent cell division.

Zebrafish *mlh1* knockouts show sex-dependent meiotic problems very similar to mice. Male mutants are sterile, but females are fertile, giving aneuploid or triploid progeny. In males it was shown that spermatogenesis is blocked at metaphase I, because most chromosomes are in a univalent state. This is associated with an accumulation of primary spermatocytes, while secondary spermatocytes as well as the postmeiotic spermatids and spermatozoa are absent. In females meiotic divisions apparently can progress, although the unpaired chromosomes cause severe missegregation. However, during the first cell divisions after fertilization this does not give problems, as the zygotic aberrant genomic content is rescued by maternally provided mRNAs and proteins (11).

We have now crossed the original *mlh1* mutant founder back to wild-type fish of its own line twice, and show here, for the first time in vertebrates, that homozygous mutant males in this background are able to finish meiosis, albeit with strongly reduced numbers of spermatozoa, that also result in aneuploid embryos. This phenotype highly resembles that of female fish, but was never observed in mice. It shows that MLH1 is still essential for a proper meiosis, but that the cell divisions can progress without functional MLH1.

Materials and methods

Zebrafish lines and Western blot

To obtain *mlh1* mutant zebrafish in a more homogenous background, the founder fish with the original point mutation in *mlh1* (*hu1919*) was crossed out two times to wild-type fish of the TL line, which is the line the founder was generated in. Two independent incrosses of heterozygous progeny were then performed, from which homozygous mutant males were used for this study. Genotyping was performed as described previously (11). The albino females used for crosses originate from the b4 line that carries a 4 kb insertion in exon 6 of the zebrafish *SLC45A2* gene, which is located on chromosome 21.

Western blot was run on protein extracts of testes of wild-type, heterozygous and homozygous mutant males. Testis tissue was homogenized in 10 mM Tris buffer, pH 7.4, containing 1% SDS and 1 mM orthovanadate. Proteins were separated on 8% SDS-PAGE. For MLH1 detection a mouse monoclonal anti-human MLH1 antibody (BD Biosciences Pharmingen, Franklin Lake, NJ) was used. Tubulin, used as loading control,

was detected by a mouse monoclonal antibody against acetylated alpha-tubulin (Sigma-Aldrich, St. Louis, MO). Mouse antibodies were visualized with anti-mouse antibody conjugated to HRP and an ECL-detection kit (Amersham Biosciences, Freiburg, Germany).

Biometry, histology and morphometry

For biometrical analysis, 6 wild-type and 6 mutant males from incross 1 and 12 wild-type and 11 mutant males from incross 2 were anesthetized, weighed and sacrificed. Testes were dissected out, fixed in 4% glutaraldehyde, weighed, and embedded in 2-hydroxyethyl methacrylate. Four µm sections were stained with toluidine blue and used for histological and morphometric evaluation.

For morphometric analysis of testis tissue from the inbred TL line (12 wild-type and 11 mutant males from incross 2), the weights of the different testicular tissue components were determined using a 441 points intersection grid placed in the ocular of the light microscope. Fifteen fields chosen randomly (6,615 points) were scored for each animal at 400x magnification. Staining or sectioning artefacts were rarely seen and were not considered in the total number of points utilized to obtain weights. Intersection points were classified as being over one of the following components: spermatogonia; primary spermatocytes; secondary spermatocytes; spermatids/spermatozoa; apoptotic cells, and finally others (somatic cells, blood and lymphatic vessels, connective tissue and empty spaces). The scores were first expressed as volume fraction (%) per testis tissue component. The weight (mg) of each testis component was then determined as the product of the volume fraction (%) and the total testis weight, as described in Feitsma *et al.* (11). All data are presented as the mean ± SEM and analyzed via ANOVA (Student-Newman-Keuls test). The significance level in comparisons was considered to be $p<0.05$.

TUNEL

Detection of apoptotic cells was performed in five-micrometer Bouin-fixed, paraffin-embedded testis sections (n = 3 per genotype) that were incubated for 5 min in 10 mM citric buffer (pH 6.0) at 98 °C and then allowed to cool down to room temperature. Endogenous peroxidase was blocked with 3% H_2O_2 in MilliQ (MQ) for 5 min. Sections were washed three times with PBS before incubation for 1 hr in 5-triphosphate-biotin nick end labelling (TUNEL) mix at 37 °C. The TUNEL mix consisted of 0.3 U/µl calf thymus terminal deoxynucleotidyl transferase (Amersham Biosciences), 6.66 µM/

µl biotin dUTP (Roche, Basel, Switzerland) in terminal transferase buffer (Amersham Biosciences). The reaction was stopped by incubating the sections in 300 mM NaCl, 30 mM sodium citrate in MQ for 15 min at room temperature. After washing with PBS, non-specific staining was blocked by incubation with 2% BSA (Sigma) in PBS at room temperature for 10 min, before the sections were treated for 30 min at 37 °C in a moist chamber with a 1:20 dilution of ExtrAvidin peroxidase antibody. After three washes in PBS, detection was performed with DAB+ (Dako, Glostrup, Denmark). Sections were counterstained with Mayer hematoxylin, dehydrated, and mounted with Pertex (Cellpath Ltd., Hemel Hempstead, U.K.).

Histone H3 immunodetection

Proliferation of spermatogonia and primary spermatocytes was assessed by phosphorylated histone H3 (pH3) immunodetection. Histone H3 is a chromosomal protein component involved in the condensation of mitotic and meiotic chromosomes and becomes phosphorylated during late G2 phase, being present until the metaphase in the cell cycle (12, 13), i.e. it is detectable in cells preparing to divide. For this purpose, testes were fixed in Bouin (n = 6 per genotype), embedded in paraffin, and the sections were mounted on glass slides coated with 3-aminopropyl triethoxysilane (TESPA, Sigma, St. Louis, MO), and dried over night at 37 °C. The sections were deparaffinized and rehydrated before incubation in a plastic chamber filled with 1mM EDTA solution containing 0.05% Tween 20, pH 8.0 (Merck-Schuchardt, Hohenbrunn, Germany). For epitope retrieval, the glass chamber was transferred to a boiling water-bath for 20 minutes and then left to cool down to room temperature (RT). Non-specific protein binding sites were blocked with 5% goat serum (Vector Laboratories, Burlingame, CA) + 1% BSA (Sigma) in PBS for 30 min, followed by an incubation with a polyclonal rabbit anti-human phospho-histone H3 IgG preparation (Upstate, Charlottesville, USA; 1:200 dilution in 1% BSA PBS, 1 hr, RT). After being rinsed in PBS, sections were immersed in 0.35% hydrogen peroxide in PBS for 10 min to quench endogenous peroxidase activity. The subsequent incubation with biotinylated goat anti-rabbit IgG (1:100, Vector Laboratories) in 1% BSA in PBS lasted 30 min at RT, after which slides were incubated with ABC (Avidin-Biotin Complex; Vector Laboratories) during 1 hr, according to the manufacturer's protocol. DAB (Dako, Glostrup, Denmark) substrate development was done for 30 sec. Nuclei were counterstained and mounted as described above. For negative control, the primary antibody was replaced by the same concentration of normal rabbit IgG (Santa Cruz

Biotechnology, Santa Cruz, California, USA).

SNP sequencing and chromosome spreads

Embryonic DNA was amplified and resequenced, using specific forward (5'-AGTGAAGGGCTTCATCTCC-3') and reverse (5'-AAGTAGTGCATCTATTGAA-AATG-3') primers for the polymorphism in the *mlh1* gene, which is located on chromosome 13. Peak area proportions were determined visually. Chromosome spreads were performed as described previously (11).

RESULTS

Male mutant phenotype

Thirty eight homozygous mutant males were used for repeated crosses. To our surprise they turned out to be not completely infertile, although only very few eggs became fertilized. In total, from 118 crosses 28 contained fertilized eggs, with 86 out of 22266 eggs fertilized (average fertilization = 0.4 %). Histological analysis of the testes revealed that post-meiosis II stages of spermatogenesis, spermatids and spermatozoa, were easily noticed in wild-type testes in the seminiferous tubules (Fig. 1A) and, as regards spermatozoa, in the efferent ducts (Fig. 1A, inset). In mutant testes, however, the weight of post-meiotic cells was strongly reduced (Fig. 1B, C). Nevertheless, and in accordance with the (limited) fertility described above, some spermatozoa were visible in the efferent ducts of all mutant animals (Fig. 1B, inset). This shows that some germ cells are able to complete both meiotic divisions and spermiogenesis also in the mutant testis. Further, in mutant testes secondary spermatocytes were released into the lumen of the seminiferous tubules, indicating that precocious release of germ cells from the germinal epithelium can occur (Fig. 1B). Moreover, a higher number of apoptotic figures was observed in the mutants.

The body weight was similar in wild-type and mutant males (data not shown). However, the testis weight was significantly higher in mutants (5.3 vs. 4.0 mg; $p<0.05$), resulting also in a significantly ($p<0.05$) higher gonadosomatic index (GSI – the ratio between the testes weight and the body weight), being 1.3 and 1.1 %, in mutant and wild-type males, respectively.

Quantitative morphometrical analysis of testis tissue components showed that the mass of spermatogonia and spermatocytes – mainly primary but also secondary – was

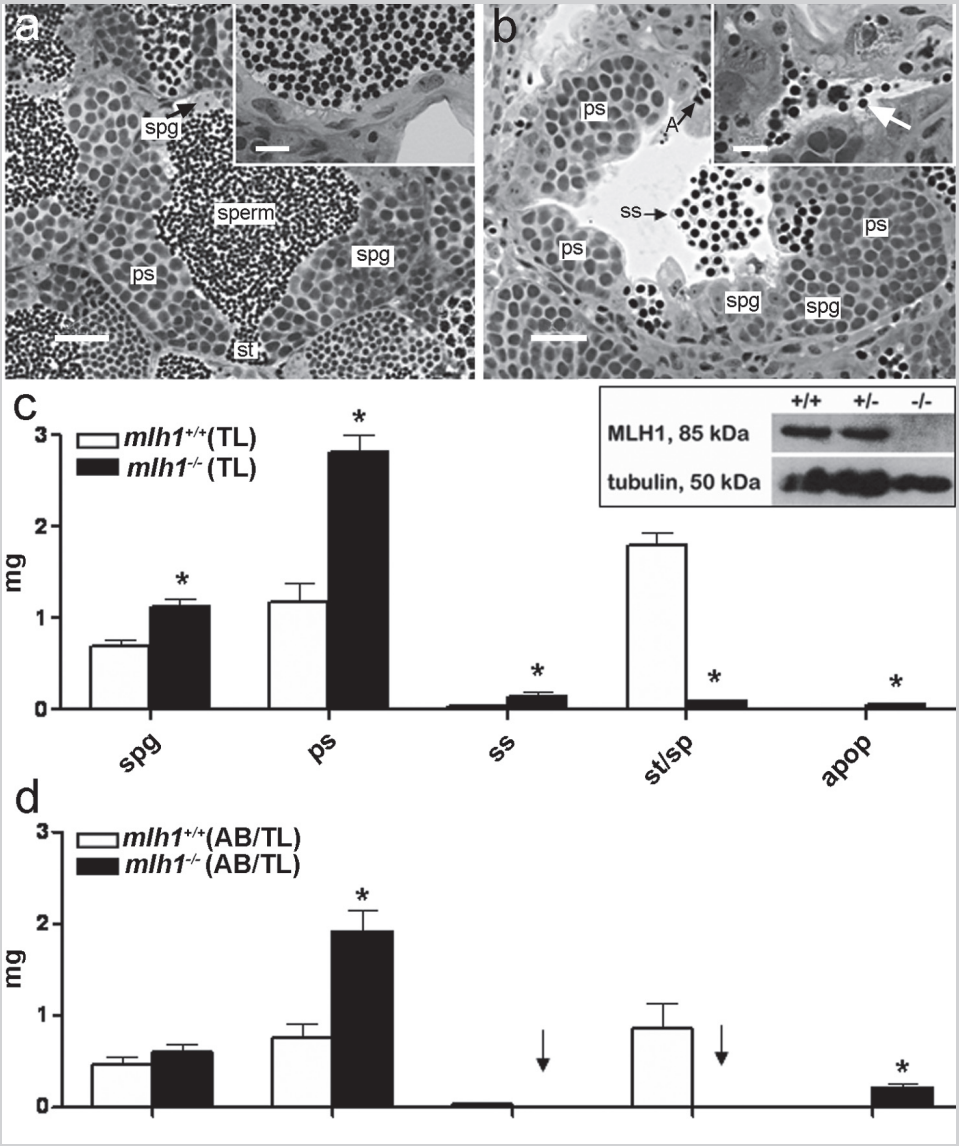

FIGURE 1. Testis histology of *mlh1*$^{-/-}$ zebrafish. (a, b) Cross section of seminiferous tubule in *mlh1*$^{+/+}$ (a) and *mlh1*$^{-/-}$ (b) zebrafish, showing spermatogenic cysts with different types of germ cells: different types of spermatogonia (SPG); primary spermatocytes (PS); secondary spermatocytes (SS); spermatids (ST); spermatozoa (SPERM); and apoptotic cells (A). Note the abnormal spermatogenesis in the mutant with many secondary spermatocytes released in the lumen and very few sperm produced (arrow – inset b),

while numerous spermatozoa are visible in the tubular lumen and efferent ducts of wild-type males (inset a). (c) Morphometric analysis of testis sections in TL background. The mutant shows significant increases in the amounts of spermatogonia (spg), primary spermatocytes (ps), secondary spermatocytes (ss) and of apoptotic cells (apop). On the other hand, spermatids and spermatozoa (st/sp) are significantly reduced. Inset shows a Western blot of testes proteins stained with antibodies for MLH1 (85 kDa) and tubulin (50 kDa). MLH1 protein is completely absent in *mlh1*$^{-/-}$ testes but detectable in wild-type (+/+) and heterozygous (+/-) males. (d) Morphometric analysis of testis sections from wild-type and *mlh1*$^{-/-}$ (n = 6 for both genotypes) mutant zebrafish in AB/TL background. The mutant shows an increased mass of spermatogonia (not significant), primary spermatocytes, and apoptotic cells. Secondary spermatocytes, spermatids, and spermatozoa were completely absent (arrows). Asterisks (*) indicate significant difference between wild-type and mutant genotypes ($p<0.05$). Scale bars in are 25 μm (a and b), or 10 μm (insets to a and b).

significantly higher in mutant than in wild-type testes (Fig. 1B, C). We recorded an increase in the mass of apoptotic cells as well, but the absolute mass changes were small compared to those recorded for spermatogonia and spermatocytes. In addition, tissue components summarized as "others" (somatic cells, blood and lymphatic vessels, connective tissue and empty space) were higher ($p<0.05$) in the mutants as compared with wild-type fish (1.13 vs 0.34 mg, respectively), mainly representing the empty space in the lumen of seminiferous tubules that was occupied by spermatozoa in wild-type males (Fig. 1A, B). Hence, spermatids/spermatozoa were almost absent (0.1 mg or ~ 1.7% of the testis parenchyma) as compared to wild-type animals (1.8 mg or ~ 45% of the testis parenchyma) (Fig. 1A-C). The weight increase of spermatogonia and spermatocytes exceeded slightly the loss of post-meiotic cells in the mutant, which, together with the probably fluid-filled empty space, explains the overall weight gain of mutant testes. Finally, in mutant testis groups of cells with strongly condensed nuclei could be observed, which are cells in apoptosis (Fig. 1B), while wild-type testis showed very low levels of apoptosis in toluidine blue stained sections (Fig. 1A). The elevated incidence of apoptosis – mainly primary and secondary spermatocytes – in mutant testis and the very low number of apoptotic cells in wild-type males was confirmed by TUNEL-based staining of DNA fragments (Fig. 2A, B).

Comparing the morphometric data of the inbred TL line (Fig. 1C) with data from completely infertile animals carrying the same mutation in a different genetic background (AB/TL; n = 6 for wild-type as well as *mlh1*$^{-/-}$ genotype; (11)) revealed a major difference as regards secondary spermatocytes and postmeiotic germ cells (Fig. 1D). These more advanced stages were not found in AB/TL outbred mutant males. In the TL inbred

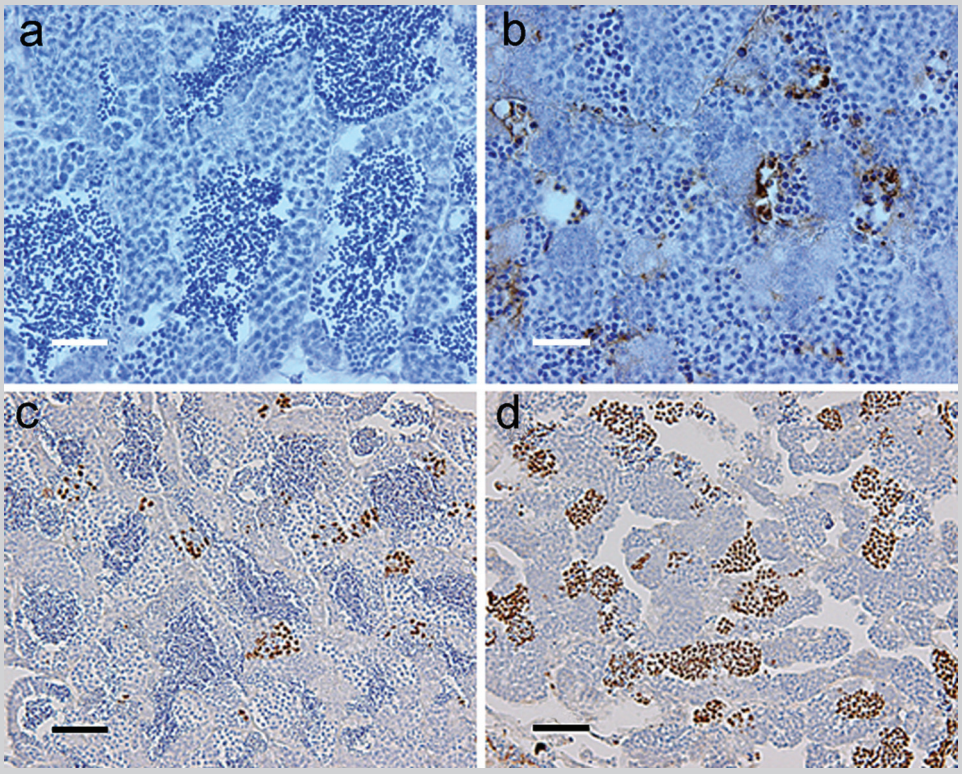

FIGURE 2. Testis immunohistochemistry of *mlh*$^{-/-}$ zebrafish. (a, b) TUNEL staining of *mlh1*$^{+/+}$ (a) and *mlh1*$^{-/-}$ (b) fish. High numbers of apoptotic cells can be seen in mutant testis (brown staining). Wild-type testis shows a very low incidence of apoptosis. (c, d) Histone H3 (cells in metaphase) staining of *mlh1*$^{+/+}$ (c) and *mlh1*$^{-/-}$ (d) fish. Note the higher incidence of stained cells in mutant testis indicating a higher number of mitotic and meiotic cells in metaphase. Scale bars are 25 μm (a and b), or 50 μm (c and d).

mutant males, on the contrary, the weight of secondary spermatocytes increased above wild-type levels, and postmeiotic cells were found in all samples, albeit at greatly reduced levels compared to wild-type animals.

Histone H3 staining of testis sections indicated a clear increase in the number of immunopositive cells in mutant compared to wild-type testis (Fig. 2C, D), demonstrating an increase of the proportion of both spermatogonia and spermatocytes in the metaphase stage of the cell cycle.

Progeny

Most of the fertilized eggs developed into abnormal embryos that died early in development (Fig. 3A), suggesting that they are aneuploid due to meiotic missegregation similar to the progeny from *mlh1* mutant females. Additionally, mutant males were crossed with females homozygous mutant for the *albino* gene, located on chromosome 21. This would normally result in *albino* heterozygous and thus wild-type pigmented progeny, but in this case some unpigmented progeny was observed (Fig. 3B), indicative of loss of the wild-type paternal copy of the *albino* gene by some form of genomic instability. Sequencing the *mlh1* DNA polymorphism on chromosome 13 in 24 hours old progeny of mutant males showed that in 4 out of 10 cases they had no or two copies of the paternal chromosome 13 (Fig. 3C). Finally, chromosome numbers of similar progeny deviated from the normal number of 50 (Fig. 3D), with a distribution of chromosome numbers around 50 and close to 75 (Fig. 3E) similar to the distribution in progeny from mutant females (11), although in the current set no complete triploid embryos were found. Altogether, this indicates that embryos fertilized by *mlh1* mutant males are aneuploid, and that the meiotic missegregation resembles that in mutant females.

DISCUSSION

Previously we reported that male zebrafish missing Mlh1 protein are infertile, because spermatogenesis arrests at the metaphase of meiosis I and the cells die by apoptosis, so that later germ cell stages were absent (11). This was explained by the absence of chiasmata, which normally hold together homologous chromosomes. In *mlh1*$^{-/-}$ mutants, the primary spermatocytes' chromosomes were therefore present as univalents, which impaired chromosome alignment, spindle formation, and thus cell division. In contrast, in *mlh1*$^{-/-}$ females lack of chiasmata did not block cell division, but caused random chromosome segregation resulting in aneuploid and triploid progeny. Sex differences have also been seen in several mouse knockouts for meiotic genes (14). These sex differences in outcomes of meiotic problems were related to general differences in male and female meiosis. In mammals, male gametogenesis is a continuous, tightly regulated process. In contrast, females have a single, programmed wave of gametogenesis with a limited number of oocytes. In fish, however, oogonia are present in the adult ovary (15, 16) allowing for the high female fertility typical of many teleost species.

Here we show that *mlh1*$^{-/-}$ zebrafish males that are twofold more inbred are partially

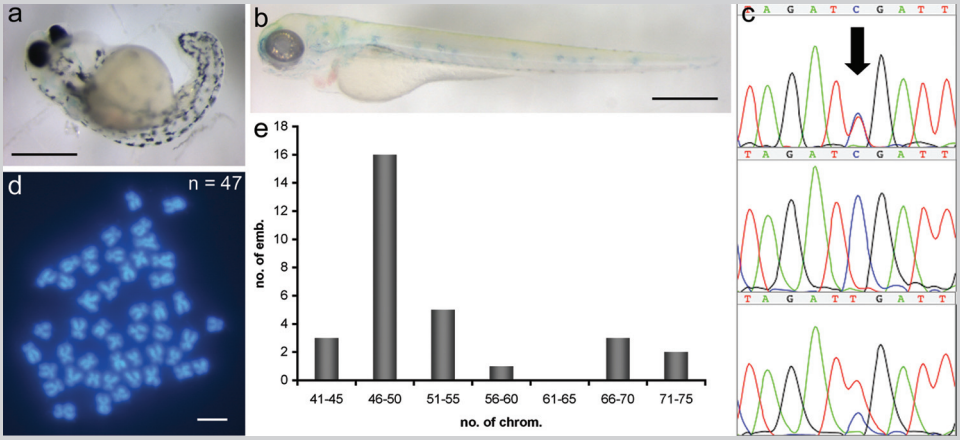

FIGURE 3. Progeny of *mlh1*$^{-/-}$ males. (a) Severely malformed three-day-old embryo from a cross of a *mlh1*$^{-/-}$ male with a wild-type female. (b) Unpigmented three-day-old embryo from a cross of a *mlh1*$^{-/-}$ male with an *albino* female, showing that the wild-type paternal allele of the *albino* gene has been lost in this embryo. The weak blue staining is caused by methylene blue containing medium. (c) Sequence traces of the polymorphism in *mlh1* (arrow). The paternal allele (mutant) is a T, the maternal allele (wild type) is a C. The sequence peak areas show quantitatively that some progeny of mutant males have one chromosomal copy of each parent (upper panel), while others have only the maternal allele (second panel) or two copies of the paternal allele (lower panel). (d) Embryos have chromosome numbers different from the normal number of 50, such as this example of 47 chromosomes. (e) Distribution of chromosome numbers in embryos from *mlh1*$^{-/-}$ males, 30 embryos in total. Scale bars are 500 µm (a and b), or 1 µm (d).

fertile. Different from the outbred (AB/TL) males carrying the same mutation but being completely infertile with a general arrest in metaphase I (11), males of the inbred TL line do not show a complete block at metaphase I but rather an accumulation of all pre-meiotic and meiotic stages as well as apoptotic cells and, as a consequence, very low levels of post-meiotic cells, associated with low fertilization rates. The observation of secondary spermatocyte weight being higher than in wild-type testis indicates that these cells are not escapers of a meiosis I checkpoint, but rather that both meiotic divisions are delayed. The meiotic phenotype of these males is therefore markedly different from that of the outbred males from our previous study. Additionally, we have shown that the low number of mutant germ cells that completed meiosis and spermiogenesis, were aneuploid spermatozoa, because they result in aneuploid progeny upon fertilization of wild-type eggs. This was shown via three different ways: 1) fertilization of eggs of an albino female sometimes results in unpigmented embryos due to loss of the paternal

allele, 2) sequencing of the *mlh1* polymorphism in progeny shows loss or duplication of the paternal allele, 3) counting chromosomes of embryonic cells reveals numbers different from 50. Altogether, this indicates that, in the absence of MLH1, spermatogenesis still can be completed by a limited number of germ cells, but is certainly abnormal. The problems during the first meiotic division are likely due to the presence of univalent chromosomes as previously described (11), the problems during meiosis II are possibly related to the cells being aneuploid already at this stage. This is consistent with our observation of high numbers of histone H3 positive primary and secondary spermatocytes in the mutant testis, which indicates that most cells are delayed at metaphase.

The *mlh1*$^{-/-}$ females of these crosses behaved similar to the outbred females in terms of fertilization and numbers of phenotypically abnormal embryos (results not shown). In the specific line described here, female and male responses to meiotic problems are therefore highly comparable and different from higher vertebrates. The determination of such low fertilization may be experimentally only feasible in fish models, such as zebrafish and medaka (*Oryzias latipes*) as a result of the high numbers of eggs produced. However, to our knowledge, no spermatozoa or other post-meiosis I stages have ever been seen in mouse *mlh1* knockout males and also oocytes are hardly able to finish meiosis (8, 10). This suggests that specifically in zebrafish, or maybe more general in lower vertebrates, meiotic divisions are less restricted. As we have shown previously, also triploid male zebrafish are able to complete meiosis (11).

The difference in male fertility between the two fish lines that we used is so far unexplained. One possible explanation is that the lines have a genetic modifier background that for example decreases or increases the restrictiveness of meiotic checkpoints. Alternatively, sequence divergence between homologous chromosomes might result in a smaller capacity to finish meiosis. In yeast it has been shown that high levels of polymorphisms decrease the frequency of recombination (17). Together with a severe reduction in recombination by the absence of MLH1, this might pass a certain threshold below which meiosis I is completely blocked. In this respect it might be interesting to cross this *mlh1* mutant with mutants for mismatch repair genes that are not essential for crossover formation, but are involved in repair of heteroduplex sequences that arise during meiotic recombination, such as *msh2* and *msh6* (18, 19). Experimental evidence for both explanations would need a long-term experiment of in- and outcrosses with different lines, which would certainly be feasible in zebrafish, but is beyond the scope of this study. Whatever explanation turns out to be true, both sexes of this mutant line seem suitable models for investigations on

the cellular mechanisms allowing meiosis to be completed in the absence of MLH1 protein in part of the mutant germ cells.

Acknowledgments

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4

Zebrafish with mutations in mismatch repair genes develop neurofibromas and other tumours

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ABSTRACT

Defective mismatch repair (MMR) in humans causes hereditary nonpolyposis colorectal cancer (HNPCC). This genetic predisposition to colon cancer is linked to heterozygous familial mutations, and loss-of-heterozygosity is necessary for tumour development. In contrast, the rare cases with biallelic MMR mutations are juvenile patients with brain tumours, skin neurofibromas and café-au-lait spots, resembling the neurofibromatosis syndrome. Many of them also display lymphomas and leukaemias, which phenotypically resembles the frequent lymphoma development in mouse MMR knockouts. Here we describe the identification and characterization of novel knockout mutants of the three major MMR genes *mlh1*, *msh2* and *msh6* in zebrafish and show that they develop tumours at low frequencies. Predominantly neurofibromas/malignant peripheral nerve sheath tumours were observed, but also a range of other tumour types. Our findings indicate that zebrafish mimic distinct features of the human disease and are complementary to mouse models.

INTRODUCTION

Mismatch repair corrects small replication errors that are caused by the DNA polymerase and skipped by its proofreading capacity (1). In humans, defective mismatch repair is associated with early-onset colorectal cancer in the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome (2). According to the second-hit hypothesis (3), only one extra mutation, one that inactivates the wild type allele of the mismatch repair gene, is necessary to create a genomic instable environment that strongly enhances cancer development. Perhaps due to the high cell turnover in the colon, this organ is most prone to the loss-of-heterozygosity followed by tumorigenesis, but several other types of tumours have also been found in HNPCC patients (4).

In the years following discovery of HNPCC, continuous monitoring of HNPCC families has revealed several cases of humans with innate inactivating mutations in both alleles of a MMR gene, resulting from inbreeding or coincidence (5-19). These patients lack the MMR protein from the beginning and therefore develop cancer at extremely early ages, generally before age 10. From a summary of the tumours that occurred in this group of patients (Tab. 1) it becomes clear that the tumour spectrum is markedly different from HNPCC patients. Most striking is the high frequency of brain tumours, such as glioblastoma, medulloblastoma, oligodendroglioma, primitive neuroectodermal tumour

TABLE 1. Tumours in human patients with biallelic inactivation of MMR genes

gene	N	cancer (%)	brain tumour[1]	lymphoma	leukaemia	GI[2] tumour	skin tumour	other	CALS[3]	ref.
human										
MSH2	3		1	1	1				1	5, 6
MLH1	11		4	2	2	2	4[4]	2	10	7-11
MSH6	10		8	4	1	4			9	10-14
PMS2	27		17	5	4	10		3	20	11, 15-19
total (%)	51		30 (59)	12 (24)	8 (16)	16 (31)	4 (8)	5 (10)	40 (78)	
mouse										
Msh2	207	204 (99)	4	161	10	60	12	18		22-26
Mlh1	76	69 (91)		41		40	8			27-29
Msh6	35	35 (100)		20	2	8	7	8		30-32
Pms2	45	34 (76)		31				6		28, 29, 33
total (%)	363	342 (94)	4 (1)[5]	253 (74)[5]	12 (4)[5]	108 (32)[5]	27 (8)[5]	32 (9)[5]		

[1]In humans: glioma, glioblastoma, medulloblastoma, oligodendroglioma, PNET, supratentorial PNET (SPNET) and astrocytoma; in mouse: not further specified
[2]Gastrointestinal
[3]Cafe-au-lait spots
[4]Neurofibromas
[5]Percentage of mice with this type of tumour of the total number of mice that developed cancer

(PNET) and astrocytoma. In combination with a high incidence of neurofibromas in the skin and café-au-lait spots, this resembles the neurofibromatosis type I syndrome (20). Second most frequent are lymphomas and leukaemias (Tab. 1). Gastrointestinal tumours do occur (Tab. 1), but at much lower rate than in HNPCC patients and at a later age than the other tumour types. Apparently, constant genomic instability in all cells causes different cancers, which prompted some authors to call this the mismatch repair deficiency syndrome (MMR-D) (13) or childhood cancer syndrome (CCS) (19), while others consider it a variant of Turcot's syndrome (14, 15, 21). A number of cases were found for the four most important MMR genes MSH2, MLH1, MSH6 and PMS2. Striking is that biallelic inactivation of PMS2 leads to juvenile cancer, while heterozygous mutated PMS2 has not frequently been detected in cases of HNPCC (2).

In order to obtain animal models for HNPCC, mouse knockouts for all MMR genes have been generated (22-33). Homozygous mutants predominantly die of lymphomas (Tab. 1). Intestinal tumours occur at much lower frequencies than in HNPCC, which lead some researchers to conclude that these mouse mutants were not properly modelling HNPCC. However, when comparing the tumour spectra of these homozygous mouse models to the spectrum of humans with biallelic mutations (Tab. 1), it becomes clear that they are similar in terms of frequent lymphoma development and relatively low abundance of gastrointestinal tumours. A difference is that brain tumours are very rare, and neurofibromas and café-au-lait spots absent in mouse MMR knockouts (Tab. 1).

Here we describe the isolation of zebrafish mutants for the MMR genes *mlh1*, *msh2* and *msh6* and show that homozygous mutants of all three lines develop neoplasms, with a frequency of 6–45%. Most frequently, neurofibromas/malignant peripheral nerve sheath tumours (MPNSTs) in the eye and abdomen were observed, but also other tumour types occurred, such as PNET in the brain, and hemangiosarcoma on the head.

MATERIALS AND METHODS

PCR

The sequences of all oligonucleotides that were used are given in Suppl. Table 1.

Generation of mutant zebrafish lines

All animal experiments were approved by the Animal Care Committee of the Royal Dutch Academy of Science according to the Dutch legal ethical guidelines. For

obtaining *msh6* and *msh2* mutant fish amplicons that covered most of the large exon 4 of the zebrafish *msh6* gene and exon 5 and 6 of the zebrafish *msh2* gene, respectively, were used in an ENU-driven target-selected mutagenesis screen (34). The obtained mutant fish were outcrossed with wild type fish, and heterozygous offspring was subsequently incrossed. Genotyping was done either by PCR amplification and resequencing or by using KASPAR genotyping technology (Kbioscience, Hoddesdon, UK). The *mlh1 (hu1919)* mutants were generated as described previously (35).

RT-PCR and quantitative PCR

RNA was isolated from tail tissue of adult zebrafish and wild type embryos using FastRNA Pro Green kit (Q-biogene, Irvine, CA) and reverse transcribed by using the RETROscript kit (Ambion, Austin, TX). For *msh2* RT-PCR, cDNA specific primers on the boundary of exon 3 and 4 and within exon 6 were used, resulting in a 354 bp fragment in the wild type. For *msh6* quantitative PCR, cDNA specific primers within exon 6 and within exon 8 were used. As a control, the *msh2* primers mentioned above were used. The reactions contained iQ™ SYBR® Green Supermix (Biorad, Hercules, CA) and were run and analyzed with the MyIQ single colour Real-Time PCR detection system and software (Biorad). A dilution series of wild type cDNA was used as a standard. All genotypes were run in five-fold. The cDNA concentrations were calculated in arbitrary units compared to the wild type average, and expressed as the ratio msh6:msh2.

Microsatellite instability

Two mononucleotide (A_{22} and A_{25}) and two dinucleotide markers (CA_{17} and CA_{22}) were PCR amplified using embryonic DNA as a template by using one fluorescently labeled and one unlabelled primer per marker. The appropriate volume of PCR product was mixed with 0.5 µl GeneScan 500 LIZ size standard (Applied Biosystems, Foster City, CA) in 5 µl mQ, denatured for 5 minutes at 95 °C and subsequently run on an ABI3730XL capillary DNA analyzer (Applied Biosystems). Product lengths were analyzed using Genemapper software (Applied Biosystems).

Cancer development

Homozygous mutant fish and heterozygous siblings of all three mutant lines were monitored weekly. Animals with signs of disease or apparent cell masses were sacrificed. Fish were fixed in 4% paraformaldehyde at 4 °C for 4 days, decalcified in 0.25 M EDTA

pH 8 at room temperature for 2 days and embedded in paraffin. 6 µm sections were taken at different positions of the body and were stained with haematoxylin/eosin for histological characterization. All surviving fish were sacrificed at 24 months of age and analyzed similarly. Tumour incidences were analyzed statistically using one-tailed Fisher exact tests.

RESULTS

Generation of MMR mutants in zebrafish

Using ENU-driven target-selected mutagenesis we isolated three zebrafish mutants with putative loss-of-function mutations in the major MMR genes *msh6*, *msh2* and *mlh1*.

The *msh6* mutant *(hu1811)* carries a point mutation causing a premature stop codon in the large fourth exon (Fig. 1A). This exon is well conserved in comparison with the similarly large fourth exon of the human *MSH6* gene. According to the database of the International society for gastrointestinal tumours (www.insight-group.org), among HNPCC families there are several alleles known of point mutations causing premature translation termination in this exon, increasing the likelihood that our zebrafish mutation will be pathogenic. We could not confirm the knockout phenotype at the protein level in the zebrafish mutant, as we were not able to find an anti-MSH6 antibody that recognizes the zebrafish protein. However, we could show by quantitative PCR that the levels of *msh6* transcript are decreased to about 44% of the wild type level in heterozygous and 15% in homozygous mutants, presumably due to nonsense-mediated decay of the premature stopcodon-containing transcript (Fig. 1B).

The mutation in *msh2 (hu1886)* changes the G of the splice donor site of exon 5 to an A (Fig. 1A). Using RT-PCR we could show that it results in a 150 bp shorter mRNA (Fig. 1C). Sequencing of this product showed that this is the result of an in-frame deletion of exon 5 (Fig. 1C). The genomic organization of the *msh2* gene is highly homologous from human to fish, including complete conservation of the exon-intron boundaries of exon 5 (Suppl. Fig. 1). Interestingly, in humans, in-frame deletion of exon 5 of *MSH2* is the most common mutation in HNPCC families, accounting for 11% of all known pathogenic *MSH2* mutations (36). It results in a truncated protein (37), and carriers have a high risk of developing cancers (38), which generally are microsatellite instable (39). This suggests that the splice site mutation of *msh2* in zebrafish results in loss of function of the gene, and provides a translational animal model for *MSH2*-driven disease

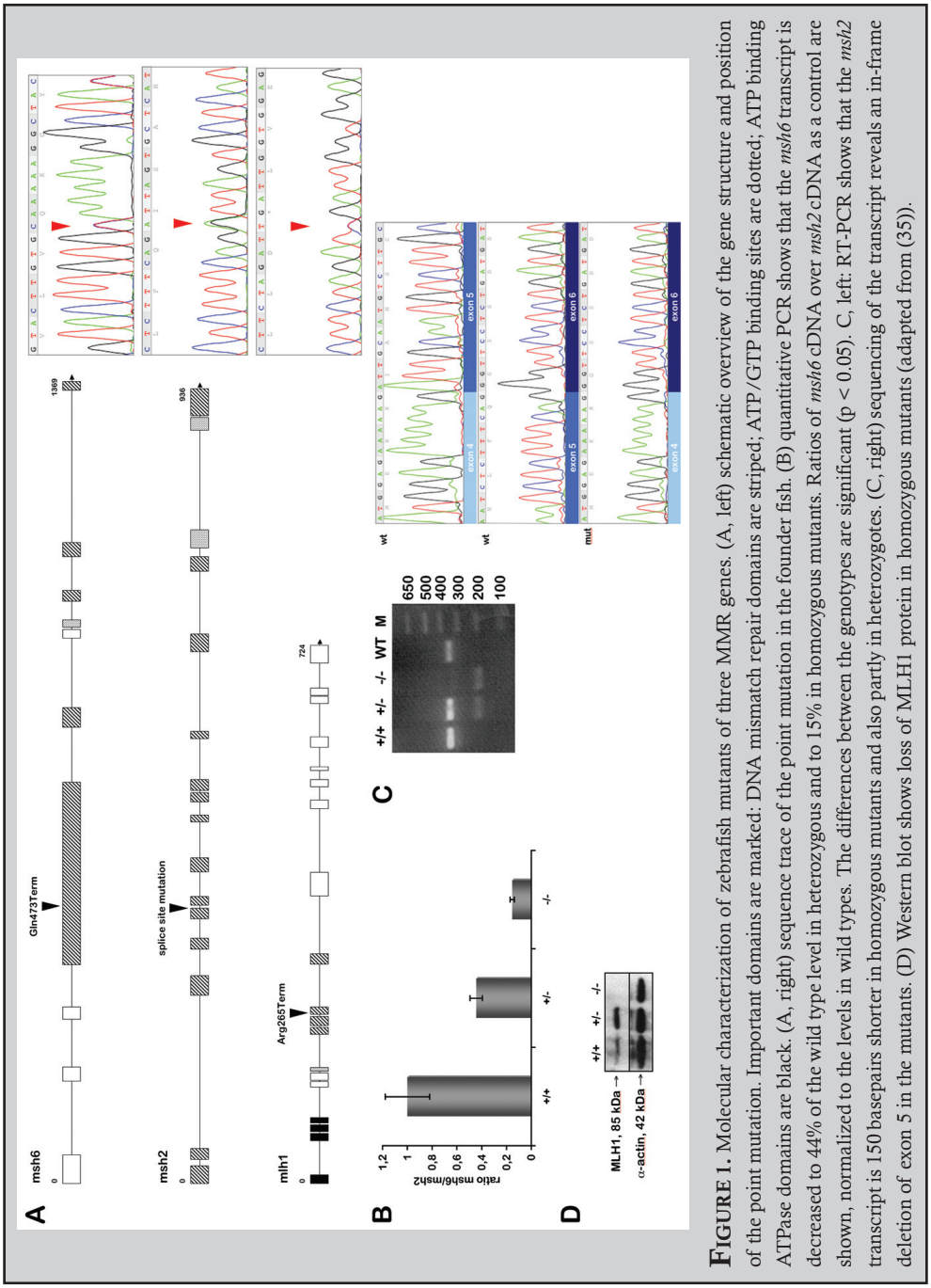

FIGURE 1. Molecular characterization of zebrafish mutants of three MMR genes. (A, left) schematic overview of the gene structure and position of the point mutation. Important domains are marked: DNA mismatch repair domains are striped; ATP/GTP binding sites are dotted; ATP binding ATPase domains are black. (A, right) sequence trace of the point mutation in the founder fish. (B) quantitative PCR shows that the *msh6* transcript is decreased to 44% of the wild type level in heterozygous and to 15% in homozygous mutants. Ratios of *msh6* cDNA over *msh2* cDNA as a control are shown, normalized to the levels in wild types. The differences between the genotypes are significant ($p < 0.05$). C, left: RT-PCR shows that the *msh2* transcript is 150 basepairs shorter in homozygous mutants and also partly in heterozygotes. (C, right) sequencing of the transcript reveals an in-frame deletion of exon 5 in the mutants. (D) Western blot shows loss of MLH1 protein in homozygous mutants (adapted from (35)).

in humans.
The third MMR mutant that is used in this study contains a premature stop mutation in *mlh1 (hu1919)* (Fig. 1A, D). We recently showed that this mutation results in full loss-of-function of MLH1 and causes a strong meiotic phenotype (35).

MMR deficient zebrafish show microsatellite instability
One of the hallmarks of defective mismatch repair is instability of microsatellite sequences (2). We studied MSI within two mononucleotide and two dinucleotide repeat loci in the germ line of *msh2* and *msh6* mutants - *mlh1* mutant males are sterile (35) and could therefore not be included in this assay. Homozygous mutant males were crossed with wild type females and microsatellites were typed in the progeny. As the latter are heterozygous and thus MMR proficient, any observed clonal instability should have occurred in the germ line of the male founder (Fig. 2). 3% of offspring of *msh2*$^{-/-}$ males have altered microsatellite lengths, both in mono- and dinucleotide repeat sequences. *Msh6*$^{-/-}$ progeny predominantly shows instability of mononucleotide markers, in 4% of the cases. No MSI was detected in progeny from wild type or heterozygous males (Tab. 2).

TABLE 2. Microsatellite instability in MMR mutant zebrafish

	mononucleotide			dinucleotide		
genotype male	**A22**	**A25**	**frequency[1]**	**CA17**	**CA22**	**frequency[1]**
msh2+/-	0/95	0/95	0%	0/95	0/95	0%
msh2-/-	4/244	9/273	3%	2/272	12/272	3%
msh6+/-	0/42	0/42	0%	0/42	0/42	0%
msh6-/-	5/219	14/222	4%	0/221	1/223	0%
wild type	0/156	0/151	0%	0/147	0/154	0%

[1]Average percentage of embryos that is instable for this type of markers

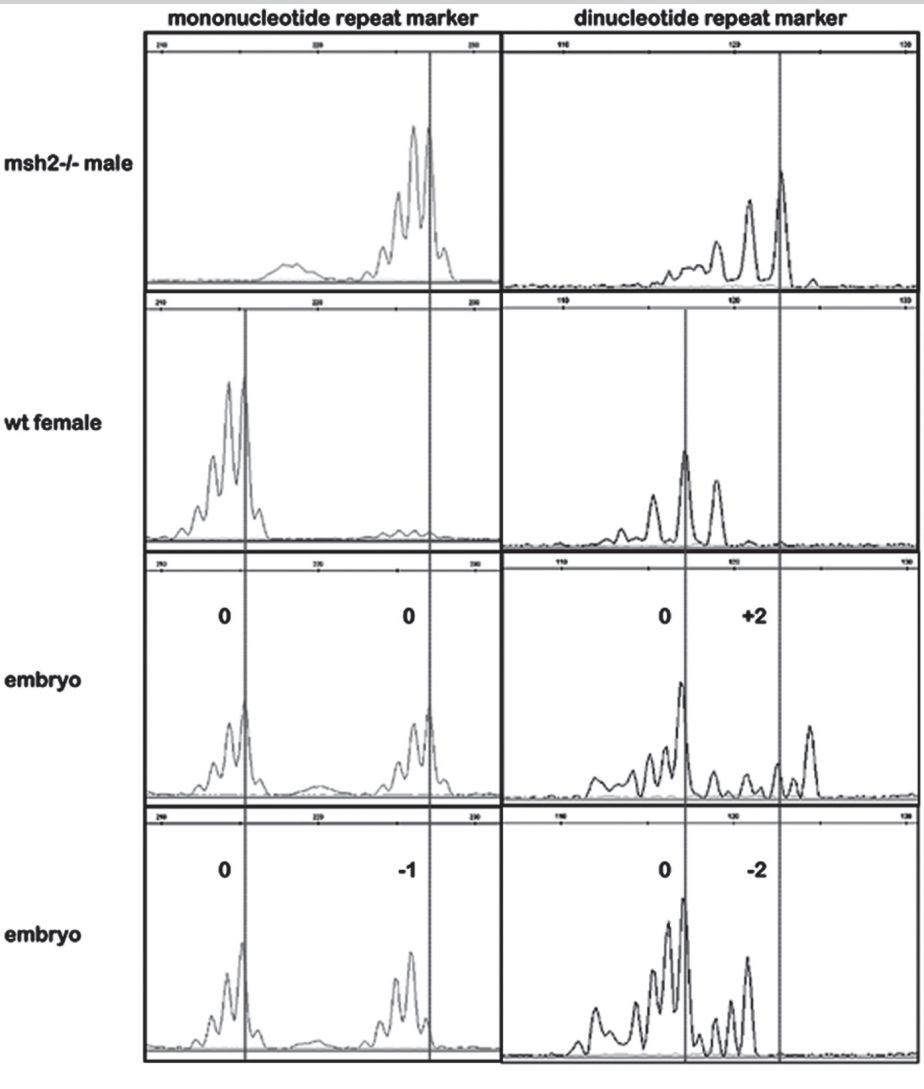

Figure 2. Microsatellite instability in MMR mutant zebrafish. Examples of instability in a mononucleotide (left) and a dinucleotide (right) marker are shown. The upper row shows the allele lengths of a *msh2*$^{-/-}$ male, the second row those of a wild type female. Embryos of a cross of those two show changes in the paternal allele length, as shown in the two bottom rows.

TABLE 3. Tumour development in MMR mutant zebrafish

genotype	N	cancer (%)	av. age cancer	ocular nf.[1]	abdominal nf.[1]	other nf.[1]	other neural[2]	cranial non-neural[3]
mlh1-/-	29	13 (45)	14 mnt	1	8	1	2	1
mlh1+/-	30	0 (0)						
msh2-/-	16	1 (6)	22 mnt	1				
msh2+/-	15	0 (0)						
msh6-/-	31	11 (35)	20 mnt	2	5	1		3
msh6+/-	12	0 (0)						
total -/- (%)	76	25 (33)	17 mnt	4 (16)[4]	13 (52)[4]	2 (8)[4]	2 (8)[4]	4 (16)[4]
total +/- (%)	57	0 (0)						

[1]nf. = neurofibroma
[2]primitive neuroectodermal tumour and olfactory neuroblastoma
[3]haemangioma, hemangiosarcoma, thyroid carcinoma and squamous cell carcinoma
[4]Percentage of zebrafish with this type of tumour of the total number of fish that developed cancer.

MMR deficient zebrafish develop tumours

From six months of age MMR mutant zebrafish started developing cancer. Tumours were only found in homozygous mutants and mostly developed in the second year of life (Tab. 3). The cancer incidence was relatively low, but much higher than generally found in wild types, 33% on average for all three lines together. In total, 25 tumours were detected, of which 13 in *mlh1*$^{-/-}$, 11 in *msh6*$^{-/-}$ and one in *msh2*$^{-/-}$ fish (Tab. 3).

The tumour frequency in homozygous mutants was significantly higher than in heterozygous siblings for the *mlh1* (N = 29 for homozygotes, N = 30 for heterozygotes; $p = 2*10^{-5}$) and *msh6* (N = 31 for homozygotes, N = 12 for heterozygotes; p = 0.01) mutants. This is not the case for the *msh2* mutant (N = 16 for homozygotes, N = 15 for heterozygotes; p = 0.5), as the number of animals and tumours is low. This is most likely caused by the reduced general fitness of the line. For all three mutants together, the difference in tumour frequency is highly significant (N = 76 for homozygotes, N = 57 for heterozygotes; $p = 1*10^{-7}$).

Tumours are characterized as neurofibromas/malignant peripheral nerve sheath tumours and other types

Frequently, tumours were poorly differentiated, but a high proportion (76%) showed features consistent with piscine neural crest origin tumours, such as unencapsulated growth of spindle shaped neoplastic cells in interconnected fascicles and scattered presence of pigmentary cells (iridophores and melanocytes). The neoplastic growth invaded surrounding structures including bone and cartilage of the skull, connective tissue and sclera, and striated muscle. As clearly distinct Antoni A and B patterns were not recognized, palissading was generally absent, and whorls of tumour cells resembling Pacinian-like corpuscles were more frequently observed, the diagnosis neurofibrosarcoma was favoured to malignant Schwannoma for these tumours. However, due to the lack of reliable immunohistochemical markers for these lesions in zebrafish, the distinction between these two types of MPNSTs cannot be made definitely, so that we use both characterizations together. Neurofibromas/MPNSTs were most frequent in the eye (Fig. 3A) and in the abdomen (not shown). Most tumours in the trunk and one extra-ocular cranial tumour were located dorsally, extending into the renal tissue and skeletal muscle surrounding skull and vertebral column, or invading bones and cartilage of the skull. Therefore, a meningothelial component cannot be excluded, which could explain the histological similarity between intra-ocular, cranial and abdominally located neoplasms.

Furthermore, two distinct cases of cranial neural crest tumours were observed. In one case, a mass protruding from the rostral aspect of the cranium and invasively growing between the eyes histologically consisted of clusters and nests of small polygonal to cuboid cells. These cells frequently had apical cilia and were forming rosettes and tubules, separated by areas where cells were more stellate with abundant myxoid intercellular material (Fig. 3B). Based on the histological appearance and localization, this tumour was diagnosed as olfactory neuroblastoma. A third type of neural crest tumour was found inside the cranium of one fish, extending from the brain through cartilage and bones of the skull into the surrounding tissues including the eye. Histologically, the neoplasm consisted of poorly differentiated small polymorphic cells with limited amounts of basophilic cytoplasm and distinct cellular borders, growing in solid sheets (Fig. 3C). This morphology was consistent with PNET diagnosis.

Tumours other than neuroectodermal of origin included a cavernous haemangioma (not shown) and a hemangiosarcoma (Fig. 3D), both in the head. Histologically, these

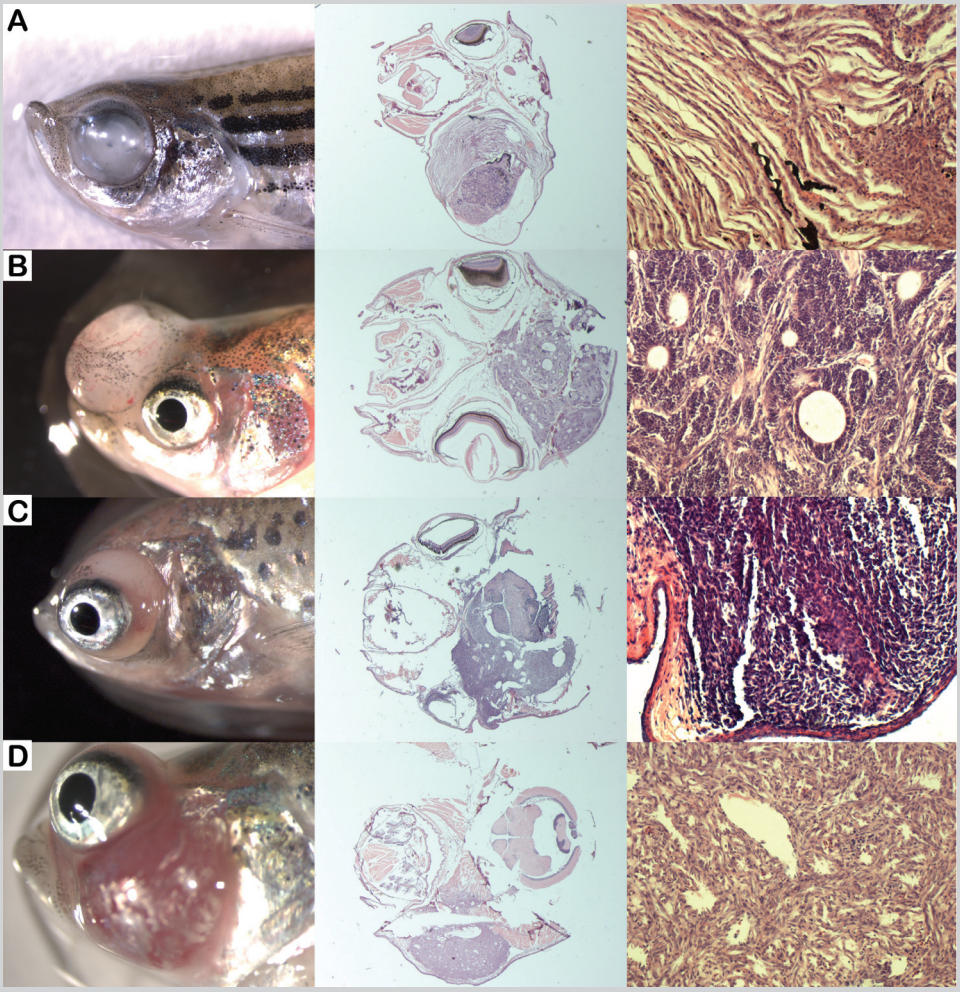

FIGURE 3. Tumour development in MMR mutant zebrafish. First column: whole mount; second column: cross section; third column: higher magnification. (A) Eighteen months old *msh6*$^{-/-}$ fish with an ocular neurofibroma. (B) Eight months old *mlh1*$^{-/-}$ fish with an olfactory neuroblastoma. (C) Six months old *mlh1*$^{-/-}$ fish with a primitive neuroectodermal tumour in the brain. (D) Seventeen months old *msh6*$^{-/-}$ fish with a hemangiosarcoma.

lesions were characterized by plump fusiform cells growing in a cribriform pattern, with intercellular spaces frequently containing erythrocytes. Hyperplastic epithelial lesions with formation of irregular dermal islets of dysplastic epithelial cells without connection to the epidermis were observed in the head of one fish, consistent with squamous cell carcinoma (not shown). One animal showed a poorly differentiated densely cellular neoplasm located ventrally between the branchial arches, invading the tissues surrounding the ventral aorta, consisting of small cuboidal to polygonal cells with indistinct borders growing in solid sheets, with occasional palissading of tumour cells and rare formation of rosettes or follicles containing small amounts of brightly eosinophilic material. Consistent with the location of the teleost thyroid, this tumour was diagnosed as a solid thyroid carcinoma (not shown).

Although the above neoplasms showed marked invasion of the surrounding tissue, mitotic figures were infrequent in all tumours and no indications for metastasis were observed. The tumour classification was done solely on morphological characteristics, since the necessary immunohistochemical stainings (for glial fibrillary acidic protein (GFAP), synaptophysin, epithelial membrane antigen (EMA), neuronal nucleï (NeuN) and s100) to confirm these tumour types did not work on zebrafish. Some exemplary tumours (N = 5) were stained with Ziehl-Neelsen to exclude that cell masses were the result of infections, and all were indeed negative (results not shown).

Discussion

We generated the first MMR-deficient models in zebrafish by knocking out the major MMR genes *mlh1*, *msh2* and *msh6*, respectively. Homozygous mutants for *msh2* and *msh6* show MSI, which is a hallmark of defective MMR. The frequency at which we find instability (3-4%) is lower than generally observed in mice (15-40%) (22, 29, 33, 40), but this can be explained by the fact that we studied markers in the germ line, where mutations rates are usually lower than in somatic cells that were used in the mouse studies. The per generation mutation rate for a large set of repeat markers in wild type zebrafish was found to be $1.5*10^{-4}$ (41), which means that it is increased around 200-fold in our mutants. In mouse somatic cells the microsatellite mutation frequency was on average 50-fold higher in the mutant than in wild types (40), which is a comparable increase. The observed MSI in both mono- and dinucleotide repeats in *msh2* mutants and in mononucleotide repeats only in *msh6* mutants is consistent with the respective

functions of MSH2 and MSH6 in repair of different types of mutations (1), although dinucleotide repeat instability has been observed in tumours of mouse *msh6* knockouts (32).

Homozygous mutants of all three fish lines are prone to tumour development at low but significant incidence, and primarily develop neurofibromas/MPNSTs in the eye and abdomen, but also a PNET, an olfactory neuroblastoma, a haemangioma and hemangiosarcoma, a squamous cell carcinoma, and a thyroid carcinoma were observed. Although the number of tumours is low, these data suggest that the zebrafish model resembles an important part of the phenotype of human patients, where biallelic MMR inactivation causes a neurofibromatosis type I-like phenotype, a phenotype that is not seen in mouse MMR knockouts. Similar to mouse mutants, tumours were only found in homozygous mutant fish.

Since lack of MMR results in a specific form of genomic instability in repeat sequences, we tested the hypothesis that the observed differences in tumour types in different species may be caused by the species-specific presence of repeat sequences in the coding sequence of selected tumour suppressor genes. We performed an *in silico* analysis of human genes involved in neural, gastrointestinal or haematological cancers or generally in many types of cancers and their mouse and zebrafish homologues, but found no significant differences in numbers of repeats between the three species (Suppl. Text, Suppl. Tab. 2 and Suppl. Fig. 2 and 3). Even more, targeted analysis of *neurofibromatosis I*, a strong candidate gene for neural tumours, did not show a correlation between the number of repeat stretches and the development of neural tumours in human, mouse and zebrafish. Nonetheless, it could still be interesting to sequence the genes containing large repeats in tumour samples of different species, to obtain information about their inactivation rates, although the presence of frameshift mutations does not necessarily mean that inactivation of the gene was essential for tumour development. Importantly, types of mutations are known to depend on selection pressure in tumour development and are thus hard to predict. A good example of this was given by Smits *et al*, who showed that, in *msh2* deficient background, *apc* mutations in intestinal tumours were mostly located in small dinucleotide repeats in mice wild type in *apc,* where both alleles needed inactivation. In contrast, they were point mutations more upstream in the gene in *apc* heterozygous mice, where loss of heterozygosity had to take place (25). Here, it is therefore more likely that the organism-specific tumour spectra are the result of physiological and anatomical differences.

Only very recently, zebrafish has become a model in cancer research. Three tumour suppressor mutants in zebrafish have been generated by reverse genetics: *tp53*, *pten* and *apc* (42-44), all of which spontaneously develop tumours. Homozygous *tp53* mutants develop MPNSTs in the eye and abdomen (42), similar to the MMR mutant fish. MPNSTs have been reported in several fish species, but these are certainly not the only type of neoplasm in fish. For example, *tp53* knockout medaka develop a broader range of tumours (45). Also, the ocular tumours that were found in *pten* knockout fish seem of a different type, although they have not been characterized in detail (43), and *apc* mutant fish develop intestinal and liver neoplasms (44). This, as well as the occurrence of other tumours besides neurofibromas in the MMR mutants, indicates that different genetic predispositions lead to different types of cancer, similar to the situation in humans.

Loss of MMR results in high and intermediate frequencies of haematological defects in mice and humans, respectively. Lymphomas and leukaemias generally arise due to genomic rearrangements that result in misexpression of certain oncogenes (46). The number of known tumour suppressor genes is low, decreasing the chance that MSI will be causal to lymphoma development. The high occurrence of misrecombination in immune cells is likely the result of the large number of immunoglobin gene rearrangements that have to be made during VDJ junction and class switch recombination in order to create a functional immune system. Since MMR genes *mlh1*, *msh2* and *msh6* are known to play a role in class switch recombination (47-50), it is tempting to speculate that their deficiency can directly lead to immune malignancies, without the need for creating a genomic instable situation that mutates other tumour suppressors. The fact that some patients with biallelic MMR inactivation were reported to be IgA-deficient supports this idea (11). Also, MMR-deficient mice show a reduction in isotype switching (47, 48, 50). Zebrafish have three classes of immunoglobulin heavy chains, which are genomically organized by VDJ junction or alternative splicing. Class switch recombination does not occur (51), which may explain why MMR deficiency does not lead to lymphoma development in zebrafish, even though lymphomas and leukaemias have been observed in zebrafish (e.g. (52)).
The question why mice are more prone to lymphoma development than humans remains unanswered.

Homozygous MMR-deficient patients that have been cured from primary cancers such

as brain tumours and lymphomas, sometimes develop intestinal cancers at later age. In homozygous knockout mice, tumours in the intestine also occur later in life, but in the zebrafish MMR mutants we did not observe any gastrointestinal cancers. Colon cancer is among the most frequent cancers in humans. The large cell number and the high cell turnover make the organ sensitive for tumour development. In HNPCC patients this is apparently the most likely place for loss of heterozygosity to occur. In zebrafish on the other hand, the intestine is in proportion a much smaller organ, decreasing the chance for tumours to develop there. However, *apc* heterozygous fish do develop intestinal tract neoplasms (44), indicating that basic biological mechanisms in the gut are similar.
As mentioned, humans with heterozygous MMR mutations develop HNPCC. Although this type of cancer is considered early-onset, it develops around the age of 45 (2), which is obviously much later than the lifespan of mice and zebrafish. This may explain why heterozygous zebrafish and mice are generally not much more cancer prone than wild types, although we have no idea how the physiologic ages of the species do relate.

From our *msh2* line we did not observe many fish dying from cancer. This is partly because homozygous mutants generally had a weak appearance and died of cancer-unrelated causes (results not shown). In addition, nearly all homozygous mutants were males and had significantly lower bodyweights than siblings. Although we can not exclude that these phenotypes result from background mutations, it is striking that in humans also very few homozygous *msh2* cases were encountered while heterozygous patients are common, which could suggest that homozygous *msh2* inactivation reduces viability in general. It should be mentioned that such mechanism or phenotype has not been reported for *msh2* knockout mice.

Taken together, we have shown that mutants for MMR genes in zebrafish display MSI and are at increased risk for tumour development. This is similar to mice and humans, although the tumour spectra are different. From this and other recent publications it can be concluded that zebrafish proves to be a useful cancer model that provides new insights and experimental possibilities, and complements studies in mouse and human.

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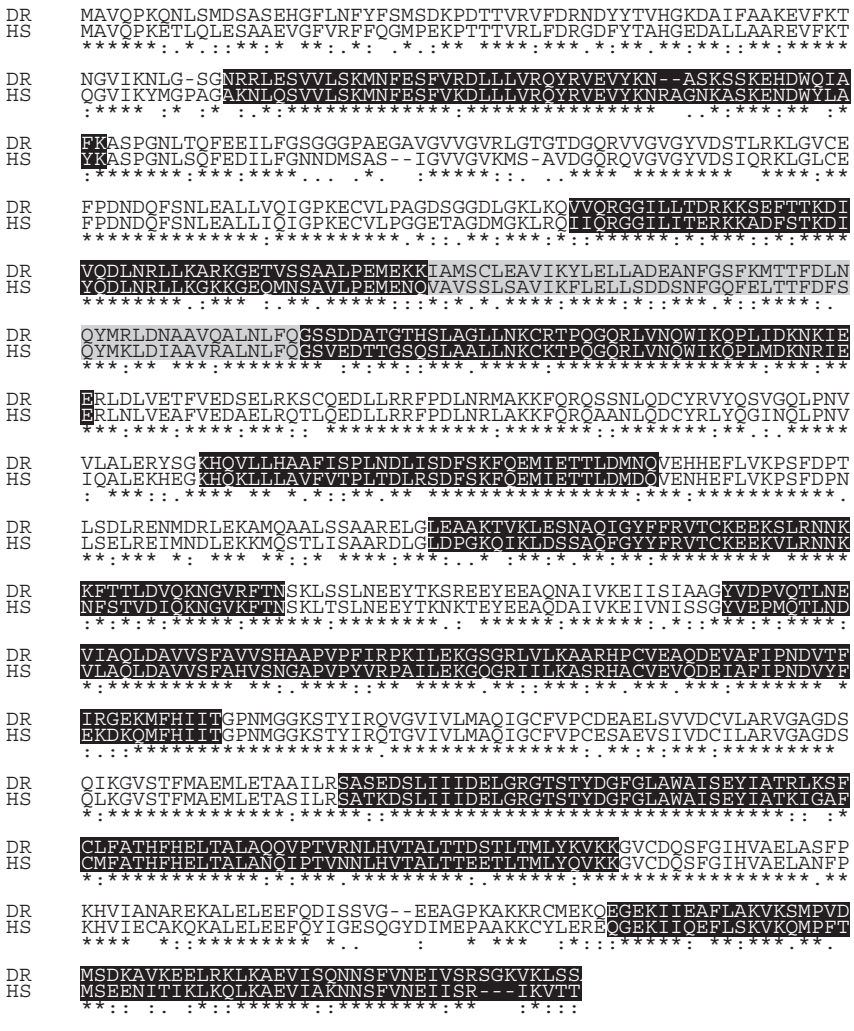
SUPPLEMENTARY DATA



SUPPLEMENTARY FIGURE 1. Homology of human and zebrafish MSH2. ClustalW alignment of zebrafish (DR) and human (HS) MSH2 protein, with consecutive exons in alternating black and white and exon 5 in light grey.

SUPPLEMENTARY TABLE 1. Sequences of primers that were used for genotyping by resequencing or KASPAR analysis, for Q-PCR and for analysis of microsatellite markers

locus	assay	name	sequence
msh6	resequencing	forward	5'-GCTGGTGGCAACTTAAATC-3'
msh6	resequencing	reverse	5'-GCTCAACAGATACTTGCTTTG-3'
msh2	resequencing	forward	5'-TTTCAGATTGCAATGTCTTG-3'
msh2	resequencing	reverse	5'-GTGGAGTCCGACATTTGTTC-3'
msh6	KASPAR	wild type	5'-GAAGGTGACCAAGTTCATGCTCGCTTCTCAG-ACGTACTTGTGC-3'
msh6	KASPAR	mutant	5'-GAAGGTCGGAGTCAACGGATTCCGCTTCTCA-GACGTACTTGTGT-3'
msh6	KASPAR	common	5'-CAGTCTGTTCTACGCGAGCTACTTT-3'
msh2	KASPAR	wild type	5'-GAAGGTGACCAAGTTCATGCTATTTCTCGTT-TTTAAATGAGCACTAAC-3'
msh2	KASPAR	mutant	5'-GAAGGTCGGAGTCAACGGATTCCTATTTCTC-GTTTTTAAATGAGCACTAAT-3'
msh2	KASPAR	common	5'-CTGCTGTACAGGCTCTCAATCTCTT-3'
mlh1	KASPAR	wild type	5'-GAAGGTGACCAAGTTCATGCTAGGCACTAG-ACTCCACCAATCG-3'
mlh1	KASPAR	mutant	5'-GAAGGTCGGAGTCAACGGATTAAGGCACTA-GACTCCACCAATCA-3'
mlh1	KASPAR	common	5'-GATAAGCACATCTTTTTAATCACCTTGTTG-3'
msh2	RT-PCR	forward	5'-AAACTTAAACAGGTGGTGCAG-3'
msh2	RT-PCR	reverse	5'- AAGTCCAGCCAGAGAGTGAG-3'
msh6	Q-PCR	forward	5'-TCAGTTGGGTTGTTATGTGC-3'
msh6	Q-PCR	reverse	5'-CACTAGTGAGTGGTAGTGTGTGG-3'
A22	MSI	forward	6FAM 5'-CATTGCACAAATTCAGTGTG-3'
A22	MSI	reverse	5'-TGGCCAGAAGAGACGAATAC-3'
A25	MSI	forward	NED 5'-AGTTAGGGACGGTGTAGACG-3'
A25	MSI	reverse	5'-ATTTGACGTGCGAATGAAG-3'
CA17 (Z9158)	MSI	forward	NED 5'-TAGATCTCTCGCTCCACCGT-3'
CA17 (Z9158)	MSI	reverse	5'-CCGCATGACTGTACTGTA-3'
CA22 (Z1234)	MSI	forward	NED 5'-CATTTAGATTAGGCCTTCCGG-3'
CA22 (Z1234)	MSI	reverse	5'-TATTACTGTTTACTTTCAGTGTGGCC-3'

Supplementary text

The tumour types that predominantly occur in different models can be the result of differences in animal physiology and anatomy. However, there could also be a genetic difference in terms of genes that are the most probable candidates to be hit by mutations. In a completely genomic instable organism, mutations are in principle occurring randomly, which means that the hit chance increases with gene size. Additionally, in the case of defective MMR there is a strong bias for instability in repeat sequences, due to uncorrected slippage errors during replication. Although repeat sequences occur mostly in non-coding DNA, it has been shown previously that genes with small repeats in translated sequences are frequently inactivated by frameshifts in MMR deficient tumours (1-4). To test the hypothesis that the observed differences in tumour types in different species may be caused by the species-specific presence of repeat sequences in the coding sequence of selected tumour suppressor genes, we compared human, mouse and zebrafish tumour suppressor genes for coding repeats.

We assembled a list of human tumour suppressor genes known to be involved in either gastrointestinal, neural, or haematological cancers or in a broad range of cancer types (Suppl. Tab. 2). These genes were checked for mono- and dinucleotide repeat sequences of six repeat units and longer in the protein coding regions in human, mouse and zebrafish. The cut-off at six repeats units was chosen because it was the lowest repeat length that was found mutated in humans (1), and it is just below the limit that we calculated from a small analysis of the relation between mononucleotide repeat length and mutation frequency in MMR deficient background in zebrafish, mouse, worm and human (Suppl. Fig. 2). However, this is just an estimation and only calculated for mononucleotide repeats. It will certainly not be absolute limit below which no instability will occur for all types of repeats. For example, when typing *apc* mutations in intestinal tumours of *msh2* knockout mice, the most frequent mutations were in dinucleotide repeat stretches of only four or five repeat units (5).

The number of coding basepairs in all gene categories was significantly lower in the zebrafish (Suppl. Fig. 3A), which may result from incomplete annotation of many genes in the current version of the zebrafish genome assembly. In general, either calculated per gene or per basepair of coding sequence, the number of repeats is significantly higher in humans compared to mouse and fish (Suppl. Fig. 3A), and genes from the general tumour suppressor category contain more repeats than the other categories. However, we did not observe strong differences between the three organisms within categories (Suppl. Fig. 3B).

A gene frequently mentioned as a candidate for mutational inactivation in patients with biallelic MMR inactivation (3, 4) is *neurofibromatosis 1* (*nf1*), because of the neurofibromatosis-like symptoms of brain tumours, skin neurofibromas and café-au-lait spots. *Nf1* is a large gene containing three $mono_7$ and seven $mono_6$ repeats, making it sensitive for mutations. However, while mouse MMR knockouts hardly develop brain cancer, the mouse *nf1* gene is equally large and is still rich in repeats, two $mono_7$ and four $mono_6$ stretches. Additionally, the mouse *neurofibromatosis 2* (*nf2*) gene contains a 14 nucleotide-long dinucleotide repeat and a $mono_7$ repeat, while human *nf2* is devoid of repeat stretches longer than 5 nucleotides. Zebrafish *nf1* has one $mono_7$ and three $mono_6$ sequences; the *nf2* gene is much shorter than its mammalian orthologues, and contains only one T_6 stretch. This example indicates that a potential highly mutable gene cannot account on its own for the development of a specific tumour type.

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SUPPLEMENTARY TABLE 2. Human tumour suppressor genes and mouse and zebrafish homologues

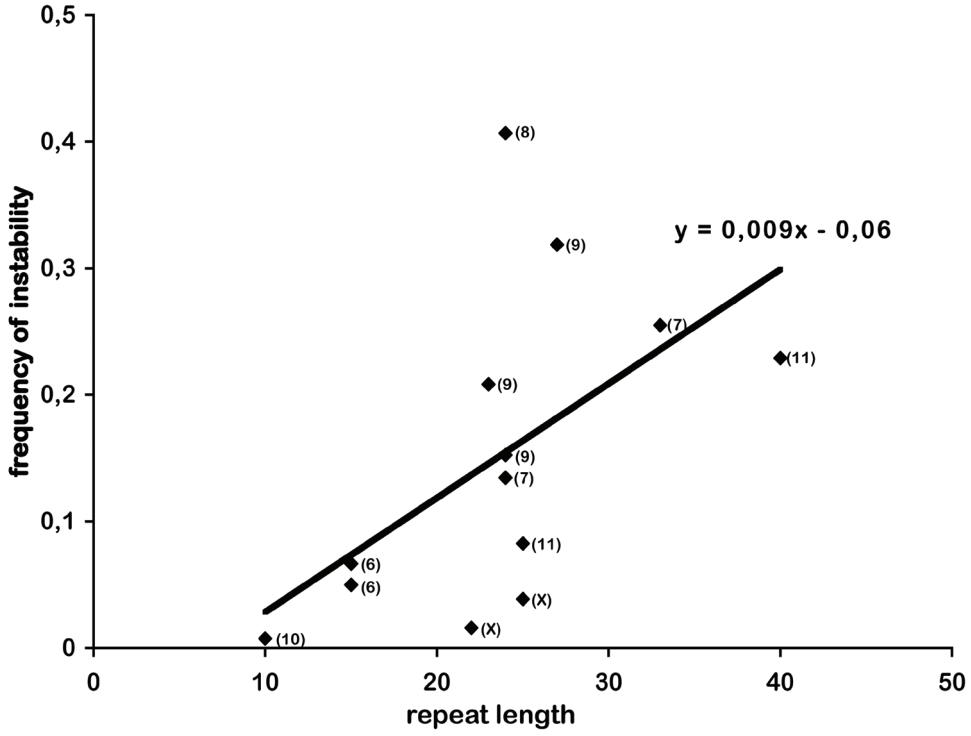
Candidate tumour suppressor genes were searched from the Online Mendelian Inheritance in Man (OMIM) database from NCBI, and mouse and zebrafish orthologues were assigned from Ensembl NCBI m36 and Zv6 assemblies, respectively. For zebrafish, putative homologues were discarded when absent in the pre-release of Zv7 (July 2007), and genes for which more than two orthologues were found were not used in the analysis (1 gene). If genes had more than one transcript, the one with the coding size that was closest to the human transcript was used.

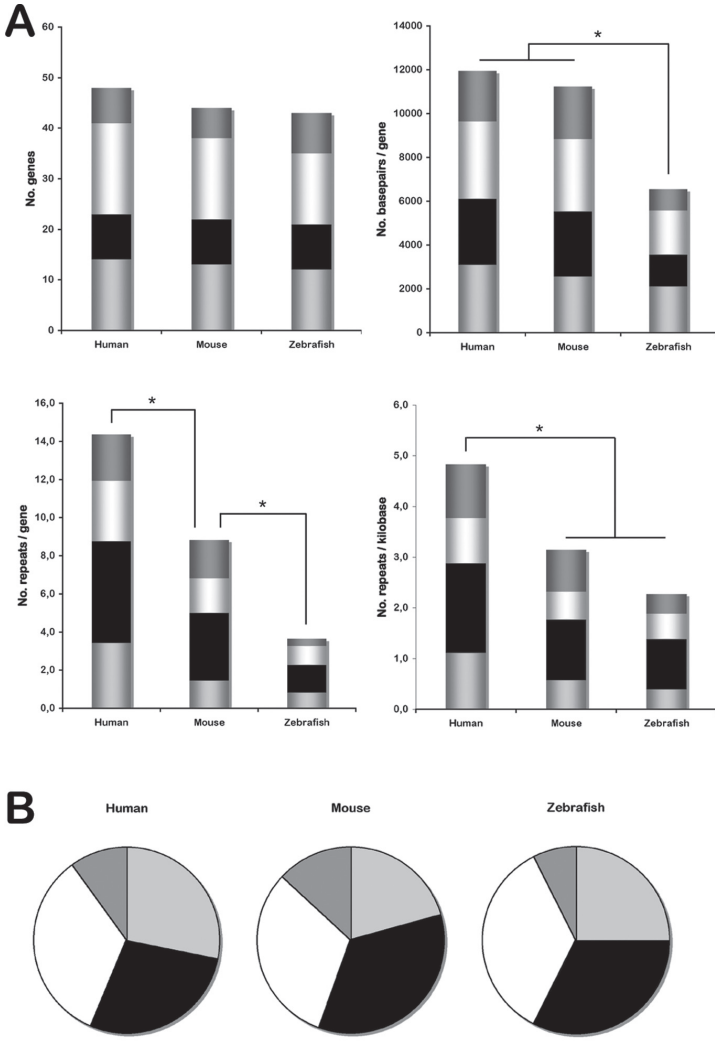
gene	human	mouse	zebrafish
general			
BAX	ENSG00000087088	ENSMUSG00000003873	ENSDARG00000020623
APC	ENSG00000134982	ENSMUSG00000005871	ENSDARG00000004425
BRCA2	ENSG00000139618	ENSMUSG00000041147	ENSDARG00000063720
RB1	ENSG00000139687	ENSMUSG00000022105	ENSDARG00000006782
TP53	ENSG00000141510	ENSMUSG00000059552	ENSDARG00000035559
CDKN2B	ENSG00000147883	ENSMUSG00000073802	ENSDARG00000037262
CDKN2A	ENSG00000147889	ENSMUSG00000044303	
PTEN	ENSG00000171862	ENSMUSG00000013663	ENSDARG00000056623
			ENSDARG00000057001
CHEK2	ENSG00000183765	ENSMUSG00000029521	ENSDARG00000025820
neural			
GLTSCR1	ENSG00000063169		
EPB41L3	ENSG00000082397	ENSMUSG00000024044	
SMARCB1	ENSG00000099956	ENSMUSG00000000902	ENSDARG00000011594
			ENSDARG00000033647

AP1B1	ENSG00000100280	ENSMUSG00000009090	ENSDARG00000020621
GLTSCR2	ENSG00000105373	ENSMUSG00000041560	ENSDARG00000013489
SUFU	ENSG00000107882	ENSMUSG00000025231	ENSDARG00000056801
ING4	ENSG00000111653	ENSMUSG00000030330	ENSDARG00000030716
MXI1	ENSG00000119950	ENSMUSG00000025025	ENSDARG00000040884
EPHB2	ENSG00000133216	ENSMUSG00000028664	
ABR	ENSG00000159842	ENSMUSG00000017631	ENSDARG00000059587
MN1	ENSG00000169184	ENSMUSG00000070576	ENSDARG00000059512
NF2	ENSG00000186575	ENSMUSG00000009073	ENSDARG00000020204
DMBT1	ENSG00000187908	ENSMUSG00000047517	
NF1	ENSG00000196712	ENSMUSG00000020716	ENSDARG00000004184
			ENSDARG00000012982
gastrointestinal			
PTPN3	ENSG00000070159		
EP300	ENSG00000100393	ENSMUSG00000055024	
SFRP1	ENSG00000104332	ENSMUSG00000031548	ENSDARG00000035521
			ENSDARG00000057678
BMPR1A	ENSG00000107779	ENSMUSG00000021796	ENSDARG00000019728
			ENSDARG00000045097
FBXW7	ENSG00000109670	ENSMUSG00000028086	ENSDARG00000060994
PRDM2	ENSG00000116731	ENSMUSG00000057637	
STK11	ENSG00000118046	ENSMUSG00000003068	ENSDARG00000046074
SLC5A8	ENSG00000139357	ENSMUSG00000020062	ENSDARG00000003697
SMAD4	ENSG00000141646	ENSMUSG00000024515	ENSDARG00000023527
			ENSDARG00000045094
PTPRF	ENSG00000142949	ENSMUSG00000033295	
PTPRG	ENSG00000144724	ENSMUSG00000021745	ENSDARG00000045006
PTPN14	ENSG00000152104	ENSMUSG00000026604	
FLCN	ENSG00000154803	ENSMUSG00000032633	ENSDARG00000062385
TGFBR2	ENSG00000163513	ENSMUSG00000032440	ENSDARG00000034541
PTPN13	ENSG00000163629		
AXIN2	ENSG00000168646	ENSMUSG00000000142	ENSDARG00000014147
PTPRT	ENSG00000196090	ENSMUSG00000053141	
IGF2R	ENSG00000197081	ENSMUSG00000023830	ENSDARG00000006094

haematological			
CASP10	ENSG00000003400		ENSDARG00000058325
			ENSDARG00000058341
BLNK	ENSG00000095585	ENSMUSG00000061132	ENSDARG00000042722
IRF1	ENSG00000125347	ENSMUSG00000018899	ENSDARG00000032768
BCL10	ENSG00000142867	ENSMUSG00000028191	ENSDARG00000063493
ATM	ENSG00000149311	ENSMUSG00000034218	ENSDARG00000002385
			ENSDARG00000063465
ARL11	ENSG00000152213	ENSMUSG00000043157	ENSDARG00000036637
PRF1	ENSG00000180644	ENSMUSG00000037202	

SUPPLEMENTARY FIGURE 2. Relation between length of mononucleotide repeat and frequency of instability in MMR deficient background, using zebrafish data from this paper (X) and human, mouse and C. elegans data from the indicated references (6-11). According to the trendline, instability would be zero at a repeat length of 6.8 units.





SUPPLEMENTARY FIGURE 3. Repeat sequences in coding regions of tumour suppressor genes
Four categories of genes were evaluated: haematological (dark grey), gastrointestinal (white), general (black) and neural (light grey) tumour suppressors. The translatable sequences of these genes for all three animal species were screened for mono- and dinucleotide repeat stretches of at least 6 repeat units (6 nucleotides and longer for mononucleotide repeats, and 12 nucleotides and bigger for dinucleotide repeats). Statistical analysis was performed using paired t-tests and Chi-square tests and $p < 0.05$ as the significance level; significant differences are indicated by an asterisk. (A) Numbers of genes, gene size, average number of repeats per gene and average number of repeats per kilobasepair for each organism. (B) Distribution of repeats over the four categories per organism. No significant differences between organisms were observed.

5

Alkylation damage causes MMR-dependent chromosomal instability in vertebrate embryos

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ABSTRACT

S_N1-type alkylating agents such as N-methyl-N-nitrosourea (MNU) and N-ethyl-N-nitrosourea (ENU) are potent mutagens. They act by transferring their alkyl-group to DNA bases, which, upon mispairing during replication, can cause single basepair mutations in the next replication cycle. Exposure to alkylating agents frequently gives rise to O^6-alkylguanine, a modified base that is recognized by DNA mismatch repair (MMR) proteins but is not repairable, resulting in replication fork stalling and cell death. Alkylating drugs have therefore been usefully employed as anticancer chemotherapy, although the mechanism of action has so far mostly been studied *in vitro*. We took advantage of the experimental accessibility of zebrafish in combination with an *in vivo* assay for mutation detection to study the *in vivo* effect of alkylation damage on lethality and mutation frequency in developing embryos. Consistent with the damage-sensing role of the MMR system, mutant embryos lacking the MMR enzyme MSH6 displayed lower lethality than wild types after exposure to ENU and MNU, although for MNU only at low concentration. In line with this, alkylation-induced somatic mutation frequencies were found to be much higher in wild type embryos than in the *msh6* loss-of-function mutants. These mutations were found to be chromosomal aberrations, likely caused by chromosomal breaks that in turn result from stalled replication forks. As these chromosomal breaks arise at replication, they are not expected to be repaired by non-homologous end-joining. Indeed, *Ku70* loss-of-function mutants were found to be equally sensitive to ENU as wild type embryos. Taken together, our results suggest that *in vivo* alkylation damage predominantly results in chromosomal instability and cell death due to aberrantly processed MMR-induced stalled replication forks.

INTRODUCTION

The S_N1-type alkylating agents N-methyl-N-nitrosourea (MNU) and N-ethyl-N-nitrosourea (ENU) are strong chemical mutagens. They cause DNA damage by transferring their methyl- or ethylgroup to bases, mainly to their oxygen and nitrogen atoms (1, 2). These base adducts are not mutations by themselves, but during replication they tend to mispair, which in the following round of replication can cause a full mutation (1). The recently described structures of normal and O^6-methylated guanine (O^6-meG) paired with either cytosine or thymine in the replication complex, provided

more detail why alkylated bases form mispairs so frequently (3). The relative replication efficiency of unmethylated G with C exceeds that of G with T around 100,000-fold, but for O^6-meG the replication efficiency for a pair with a T is 10-fold higher than for pairing with a C (3). Additionally, the O^6-meG pairs with C and T are indistinguishable for the proofreading activity of the polymerase, so that the mismatch can persist.

As DNA alkylation does occur naturally, cells have developed repair mechanisms to prevent mutagenesis. The molecular principles have been studied most extensively for methylation, and those will be discussed here. Different types of methylation adducts are repaired by different repair pathways, such as methylpurine DNA glycosylase and base excision repair (2). The methyl adduct of O^6-meG is specifically removed by the enzyme O^6-meG methyltransferase (Mgmt) (4-6). Additionally, when O^6-meG pairs with thymine or cytosine during replication, this is recognized as a mispair by mismatch repair enzymes (2, 4, 7-9). DNA mismatch repair (MMR) is the machinery that corrects small replication errors such as base-base mismatches and insertion/deletion loops (IDLs). In mammals, there are five major MMR genes that operate in three different heterodimers. The MutS heterodimer is the first one to recognize the replication error. There are two forms: MutS-alpha, consisting of the MMR proteins MSH2 and MSH6, mainly binds one-basepair mismatches and small IDLs, whereas MutS-beta, a heterodimer of MSH2 and MSH3, is primarily involved in the recognition of larger IDLs. Subsequent to MutS binding, a MutL heterodimer is recruited. MutL-alpha, the predominant form functioning in MMR, consists of MLH1 and PMS2 (10). Besides replication errors, MMR proteins recognize other forms of DNA damage, such as modified basepairs. Upon recognition of O^6-meG pairs, the MMR system will induce strand excision towards the methylated site in order to repair the damage. However, since the methylated base is in the template strand of the replicated DNA, it can not be replaced. Instead, it will continuously be paired with C or T by the polymerase, followed by MMR recognition and excision. This 'futile cycle' repair principle will finally result in stalling of the replication fork. Upon activation of checkpoints it may lead to cell cycle arrest and apoptosis. MMR-mediated DNA damage checkpoint signalling has been shown to go via ATR and phosphorylation of Chk1 (11-13), and ATM plays a non-essential role (13, 14). The apoptotic response was shown to be independent of P53 (7).

The O^6-meG-induced stalled replication forks may collapse when a direct DNA damage response is absent, resulting in double strand breaks (DSBs) (13, 15). A mechanism for this last step was revealed recently by showing that MMR-dependent futile cycling

results in persistent small stretches of single-stranded DNA at the first replication round, which result in DSBs in the second cycle of replication (16). The DSBs are in principle repaired by homologous recombination (HR), resulting in sister chromatid exchanges (2, 5, 14, 17, 18). The latter is supported by the fact that HR-deficient yeast strains are more sensitive to alkylation damage (19).

Altogether, this makes O^6-meG the most toxic lesion induced by methylation, to which MMR mutants are resistant (4, 9, 15, 20, 21). The basic molecular response may be similar for ethylation, although affinities of the polymerase, Mgmt and MMR will probably be different for O^6-ethyl-guanine. Claij *et al* indeed found a higher survival of *msh2* mutant cells compared to wild-type cells after exposure to ENU, albeit less pronounced than after exposure to MNNG (21). Also, the same homozygous mutant cells showed a selective growth advantage over heterozygous cells when treated with ENU (20). However, in two other studies on human and hamster cells, no differences were observed between MMR-deficient cells and their wild-type counterparts in response to ENU treatment (22, 23).

When alkylated mispairs in MMR-deficient cells do not induce arrest but are allowed to persist, single basepair mutations could accumulate, resulting in increased genomic instability in MMR mutants. A higher mutation frequency in MMR deficient cell lines has indeed been reported for methylating agents (4, 18) and it was also shown for ethylating agents, by treating embryonic stem cells of *msh2* knockout mice with ENU (21).

In zebrafish, ENU mutagenesis has a long history of efficient use in both forward and reverse genetic screens (24-27), where it mainly is applied to adult fish to introduce basepair changes in germ cells. We now applied ENU and MNU treatment to early embryos in order to study the effect of alkylation-induced damage in fast-dividing somatic cells *in vivo*. We found that wild type zebrafish embryos show decreased survival and a high number of mutations after alkylation treatment, resulting from chromosomal instability. This is dependent on MMR activity, as the effect is strongly reduced in embryos deficient in the MMR-component MSH6.

MATERIAL AND METHODS

Zebrafish lines

Msh6 mutant fish *(hu1811)* were obtained by target-selected mutagenesis, and the initial characterization was described elsewhere (H. Feitsma, in press). Genotyping was done by PCR amplification and resequencing, using exon 10

specific forward (5'-GCTGGTGGCAACTTAAATC-3') and reverse (5'-GCTCAACAGATACTTGCTTTG-3') primers or using KASPAR genotyping technology (KBioscience, Hoddesdon, UK) and forward primers for wild type (5'-GAAGGTGACCAAGTTCATGCTCGCTTCTCAGACGTACTTGTGC-3') and mutant (5'-GAAGGTCGGAGTCAACGGATTCCGCTTCTCAGACGTACTTGTGT-3') and the common reverse primer (5'-CAGTCTGTTCTACGCGAGCTACTTT-3'). The wild type embryos that are used as controls in all experiments have a similar genetic background as the *msh6*$^{-/-}$ fish, as they are derived from wild type siblings of the *msh6*$^{-/-}$ fish that produced the mutant embryos.

To generate *msh6*, *albino* double mutant fish, the original *msh6* heterozygous founder was crossed with an albino line that carries a point mutation in exon 6 of the zebrafish *SLC45A2* gene. This mutation causes a glycine to arginine change at position 461, and although this line has not been documented elsewhere, the phenotype is indistinguishable from other albino lines. The allele is identified as alb^{hu1844} (*hu1844*), but for clarity it will be called '*alb*' in the report. Double heterozygous fish (*hu1874*) were subsequently incrossed to generate both *msh6*$^{-/-}$*,alb/alb* and *msh6*$^{+/+}$*,alb/alb* fish.

The *Ku70* line (*hu2485*) also originates from target-selected mutagenesis and contains a thymine to adenine mutation in exon 10 resulting in a premature stop codon. This line has not been reported yet. Genotyping of the *Ku70* allele was done by resequencing similar as for the *msh6* line, with forward (5'-ATGACATACGCACTGTGGAC-3') and reverse (5'-AATCAGGAGGATAGACCAAATC-3') primers.

The *tp53* line (*zdf1*) with mutation M214K has been described elsewhere (28) and will be identified as *tp53* throughout the study. Genotyping was done using KASPAR forward primers for wild type (5'-GAAGGTGACCAAGTTCATGCTGAGGATGGGCCTGCGGTTCA-3') and mutant (5'-GAAGGTCGGAGTCAACGGATTGAGGATGGGCCTGCGGTTCT-3') and the reverse primer (5'-CAACTGTGCTACTAAACTACATGTGCAAT-3').

Somatic mutation frequency

Pair crosses of *msh6, albino* double mutants were performed to assay the somatic mutation frequency. At three days post-fertilization (dpf), both eyes of all embryos were inspected for pigmentation. Patches of unpigmented cells were considered the result of a somatic mutation in the *albino* locus. For detailed analysis of these patches, embryos were fixed in 4% PFA, embedded in plastic and sectioned.

Chemical and ionizing radiation treatment of embryos

Embryos were treated at 5-6 hours post fertilization (hpf) for 1 hour with ENU or MNU in 10 mM $NaPO_4$ buffer pH 6.6. After treatment they were rinsed once with $NaPO_4$ buffer and once with embryo medium before they were put back to embryo medium at 28.5 °C. Embryos were irradiated at 5-6 hpf in a small volume of embryo medium in a Gammacell 1000 (Gammaster, Ede, the Netherlands) and then moved to 28.5 °C in fresh embryo medium.

The survival of the embryos was monitored during the first days post-treatment, with final scoring at 3 dpf. Embryos that died, were unhatched and/or had severe phenotypes, e.g. curled bodies, oedemas and reduced body size, were considered not surviving. Albino cells in the eye were also scored at day three, as described above.

All experiments were done with multiple independent crosses, and were repeated at least two times. Data were analyzed using ANOVA and significance levels $p < 0.05$, and are represented as mean +/- standard error of the mean (SEM).

Expression arrays

RNA from 10 embryos coming from five different crosses treated with 0.4 mM ENU and from 10 untreated embryos from five different crosses was used to balance the potential effects of genetic variation. Total RNA was isolated with the *mir*Vana miRNA isolation kit (Ambion, Austin, TX). cDNA synthesis and labelling were done using the low RNA input linear amplification kit (Agilent, Santa Clara, CA) and Cyanine 3-CTP and 5-CTP (Agilent) and subsequent purification by using the RNeasy Mini kit (Qiagen, Hilden, Germany). Couples of samples were run in dye-swap. Labelled samples were hybridized overnight to 44K zebrafish expression arrays (Agilent), washed, scanned with scanner (Agilent), and analyzed using Feature Extraction and Array-Assist software (Agilent), all according to standard procedures and instructions of the suppliers.

Metaphase spreads

24 hpf embryos were dechorionated and incubated for 90 min in colchicine to arrest cells in metaphase. After hypotonic treatment in 1.1% sodiumcitrate and fixation in 3:1 methanol:HAc, cells were suspended in 50% HAc and spread onto glass slides. Slides were mounted and stained with DAPI vectashield (Vector Labs, Burlingame, CA).

Results

Msh6$^{-/-}$ embryos have an increased spontaneous somatic mutation frequency

First, an assay was set up for the detection of somatic mutations *in vivo* in zebrafish embryos, by using loss of heterozygosity of the *albino* gene in pigmented cells of the eye. Pigmentation of zebrafish embryos is visible from day two of development as individual cells on the trunk and in a uniformly pigmented layer of cells in the eye. It is totally absent in albino mutants. To be able to score every inactivating mutation, the assay requires that only one functional allele of the *albino* gene is present. To this end, *msh6*$^{-/-}$ zebrafish were crossed with *msh6*$^{-/-}$*,alb/alb* animals to obtain *msh6* mutant embryos that are *albino* heterozygous, and *msh6*$^{+/+}$ with *msh6*$^{+/+}$*,alb/alb* animals to obtain embryos that are wild type for *msh6* in an *albino* heterozygous background. Inactivation of the *albino* wild-type allele by a mutation in these embryos will result in a cell that lacks pigmentation. If this cell subsequently divides, a patch of unpigmented cells will arise, which can easily be scored in the pigment layer of the embryonic eye (Fig. 1A, B). In order to validate the assay, the spontaneous somatic mutation frequency in *msh6*$^{-/-}$ and *msh6*$^{+/+}$ embryos was determined. The frequency of embryos with albino patches was 17-fold increased in the *msh6* mutant background (Tab. 1). In progeny from *msh6*$^{-/-}$ females crossed with *msh6*$^{+/+}$ males the mutation frequency was similar to the frequency in *msh6* mutant embryos, while in progeny from *msh6*$^{+/+}$ females crossed with *msh6*$^{-/-}$ males the frequency was as in wild type embryos, no matter which of the two parents was albino (Tab. 1). The fact that the maternal genotype determines the mutator phenotype shows that the mutations arise early in development, before midblastula transition when zygotic transcription is switched on (29). In this period, the embryonic phenotype reflects the transcripts and proteins that are provided by the mother in the yolk; e.g. if the mother is mutant for *msh6*, her progeny will also be devoid of the enzyme. To more closely investigate the number of mutant cells, some eyes with albino patches were sectioned. In most cases, not a single unpigmented patch but multiple smaller patches were seen (Fig. 1C), which is likely due to intensive cell migration during eye development (30), as the overall mutation frequency, with most eyes without any patches, indicates that all patches in one eye are caused by a single mutation. Altogether, the *albino* loss-of-heterozygosity assay in zebrafish proves very powerful for *in vivo* somatic mutation detection.

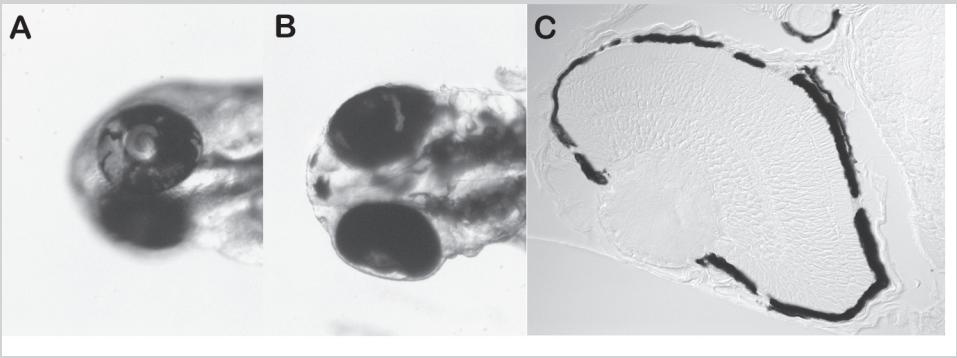

FIGURE 1. Loss of heterozygosity at the *albino* locus in zebrafish embryos. (A,B) Examples of unpigmented patches of cells in the pigment layer of the eye caused by mutations in the wild-type allele of the *albino* gene. (C) Example of a cross-section of the eye to show that one eye usually contains multiple small patches.

TABLE 1. Spontaneous non-pigmented patches in the eyes of heterozygous *albino* zebrafish in wild type and MMR-deficient backgrounds

cross	no. embryos scored	no. with patches	frequency
msh6$^{+/+}$F X msh6$^{+/+}$M	2068	2	0.00097
msh6$^{-/-}$F X msh6$^{-/-}$M	1434	24	0.017
msh6$^{+/+}$F X msh6$^{-/-}$M	1043	1	0.00096
msh6$^{-/-}$F X msh6$^{+/+}$M	1612	32	0.020

F, female; M, male

Msh6$^{-/-}$ embryos are more resistant to ENU and MNU

Next, the sensitivity of MMR-deficient zebrafish embryos to alkylating agents was tested. In the developing zebrafish embryo the first ten cell divisions occur synchronously and rapidly after each other, without time for checkpoints. After midblastula transition, cell cycles become longer with more extensive G1 and G2 phases, which supplies time for checkpoint signalling, cell cycle arrest and DNA repair (29). We chose to treat embryos of midblastula stage, around five hours post-fertilization (hpf). First, groups of embryos were exposed to increasing concentrations of ENU, as ENU is the most efficient mutagen in zebrafish (24, 25). *Msh6*$^{-/-}$ embryos displayed increased survival at high

concentrations (Fig. 2A), consistent with other systems. Secondly, to compare the effects of ethylation to methylation, MNU was used, which also resulted in increased survival of mutant compared to wild-type embryos. To our surprise this difference was only, but reproducibly, present at the low concentration of 0.2 mM MNU, due to extremely high lethality of wild-type embryos (Fig. 2B). At higher concentrations, mutants and wild types showed similar survival rates. The survival of wild types at this specific dose of 0.2 mM was also significantly lower than at 0.5 mM (Fig. 2B). In addition to this, we observed that embryonic phenotypes were much more severe at 0.2 mM MNU as compared to 0.5 and 1.0 mM (not shown).

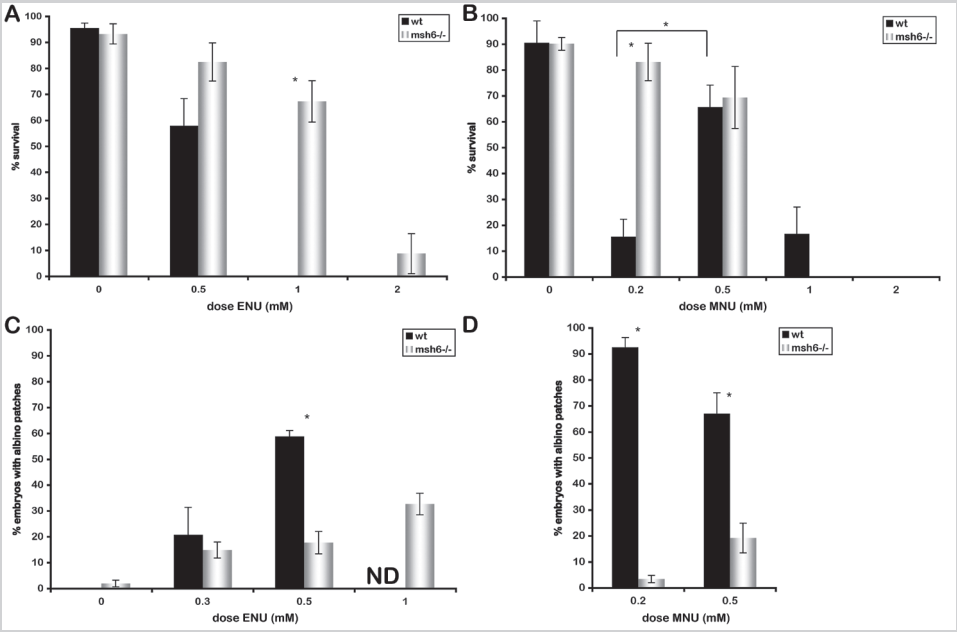

FIGURE 2. *Msh6*$^{-/-}$ embryos are more resistant to alkylation damage and show reduced mutation induction. (A,B) Survival of wild-type (black) and *msh6*$^{-/-}$ (white) embryos at increasing doses of ENU (A) and MNU (B). (C,D) Mutation frequency measured by the albino loss-of-heterozygosity assay in wild-type (black) and *msh6*$^{-/-}$ (white) embryos induced by increasing doses of ENU (C) and MNU (D). Asterisks indicate significant differences. ND, not done.

The ENU- and MNU-induced mutation frequency is reduced in *msh6*$^{-/-}$ embryos

Low doses of ENU treatment were used to study the chemical-induced mutation

frequency, by applying the albino loss-of-heterozygosity assay. The mutation frequencies induced by alkylating drugs were found to be around one order of magnitude higher than the frequency in untreated embryos in absence of MMR. The mutation frequency was found to be highly increased in wild type embryos as compared to mutants (Fig. 2C), opposite to what was expected. The same experiment was done for MNU, resulting in an even higher increase in mutation load in the wild type relative to the mutant (Fig. 2D). This indicates that the absence of MMR recognition of alkylation damage does not result in increased accumulation of point mutations. However, comparison of the graphs for survival and mutation frequency for ENU as well as MNU suggested a correlation between mutation load and killing. Therefore, these mutations might be other types of lesions than point mutations, presumably resulting from MMR-dependent replication fork stalling following O^6-alkylguanine (O^6-alkG) recognition.

ENU-induced lethality is the result of MMR-dependent chromosomal aberrations

MMR-mediated stalled replication forks may turn into DSBs, which can cause chromosomal aberrations when they are not properly repaired. To investigate if aberrant chromosomes were present in embryos treated with ENU and whether those could be the source of the observed high frequency of mutations, metaphase spreads on embryonic cells were performed. Since it takes two rounds of replication for the alkylation damage to corrupt replication forks, treated embryos were allowed to develop until 24 hpf before chromosomal analysis (cells in the developing embryo undergo on average two to three cell divisions between 6 hpf and 24 hpf; (31). Different kinds of aberrant chromosomes, such as fragments and fused and asymmetric chromosomes, which are hardly ever present in untreated cells (Fig. 3A), were observed very frequently in ENU-treated wild type cells (Fig. 3B). When quantified, the levels of chromosomal instability were very high in treated wild types as compared to untreated embryos, while treated mutants showed a very mild increase (Fig. 3C). Altogether, these results strongly indicate that chromosomal instability is causal to the MMR-dependent induction of both mutations and lethality by alkylation.

To get a more systematical insight in which signalling and repair pathways are triggered by these chromosomal aberrations *in vivo*, we looked for transcriptome expression changes in low-dose ENU-treated embryos compared to untreated embryos in a microarray experiment. A more than threefold upregulation of TP53 was observed in treated wild-type embryos, which was not seen in mutants. However, except for this, no significant

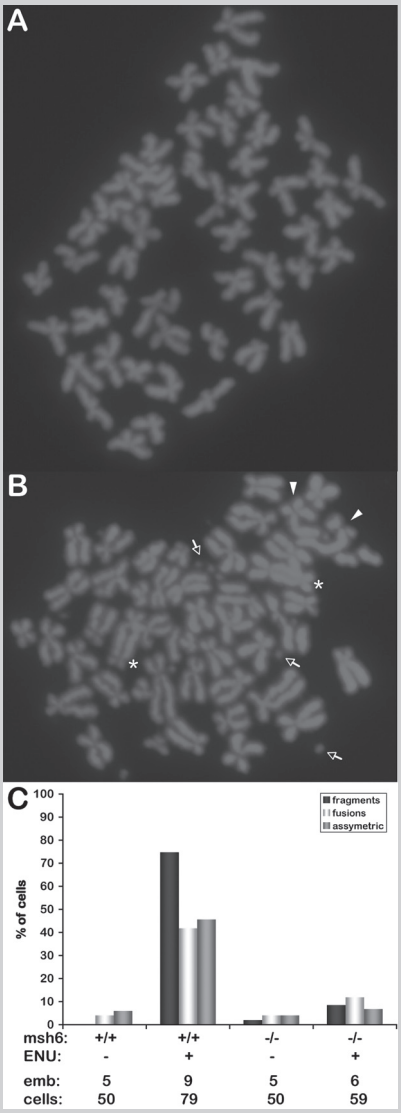

Figure 3. (A) Metaphase spread of an untreated embryo showing 50 regular chromosomes of one cell. (B) Metaphase spread of a cell of a wild-type embryo treated with 1 mM ENU, showing chromosome fragments (arrows), fused chromosomes (asterixes) and asymmetric chromosomes (arrowheads). (C) Quantification of fraction of cells that shows any of the three different types of aberrant chromosomes mentioned in B. Below the graph are indicated the *msh6* genotype, untreated or treated with 1 mM ENU, number of embryos scored and total number of cells scored.

changes in DNA metabolizing or signal transduction genes were found, including ATR and ATM pathway genes and factors downstream of TP53 (results not shown).

ENU-induced chromosomal instability arises at replication

To investigate whether DSBs that are formed independent of alkylation and replication also give a different response in wild types and MMR mutants, increasing doses of ionizing radiation (IR) were applied to 5 hpf wild-type and *msh6* mutant embryos. Ionizing radiation induces random DSBs, independent of cell cycle phase. No difference in survival between mutants and wild types was observed for any of the doses given (Fig. 4A).

DSBs can be repaired via two different mechanisms that act in different phases of the cell cycle. Homologous recombination (HR) is active during S and G2 phase when sister chromatids are available, and non-homologous end-joining (NHEJ) predominantly repairs during G1 phase. For testing which of these processes are important for repair of alkylation damage *in vivo*, a zebrafish mutant that has a premature stop mutation in the NHEJ factor *Ku70* was used. First, to confirm that it is a loss-of-function mutant, a dose range of IR was applied to *Ku70* homozygous mutant embryos. A small but significant decrease in survival was seen (Fig. 4B), indicating that the mutant is a functional *Ku70* knockout. In addition, these results confirm that NHEJ is active in developing embryos. The observed phenotype is analogous to morpholino knockdown experiments of *Ku70* and *Ku80*, which have previously been shown to result in increased apoptosis in response to irradiation (32, 33). As a control, *tp53* mutant embryos were exposed to similar doses of IR. Those mutants have been shown to display reduced apoptosis upon irradiation (28). Here, *tp53* mutant embryos indeed displayed increased survival at the highest IR dose (Fig. 4B).

Subsequently, *Ku70* mutant embryos were treated with ENU to study the involvement of NHEJ in the repair of alkylation-induced DSBs. This was only done in wild-type and not in *msh6* mutant background, as ENU-induced damage only results in stalling of replication forks and DSBs in the presence of functional MMR. No difference in sensitivity to ENU was seen in the *Ku70* knockouts as compared to wild types (Fig. 4C), indicating that HR and not NHEJ is the most likely candidate to repair ENU-induced DSBs. Together, the irradiation and *Ku70* experiments indicate that MMR-dependent ENU-induced DNA damage arises during replication and not during interphase.

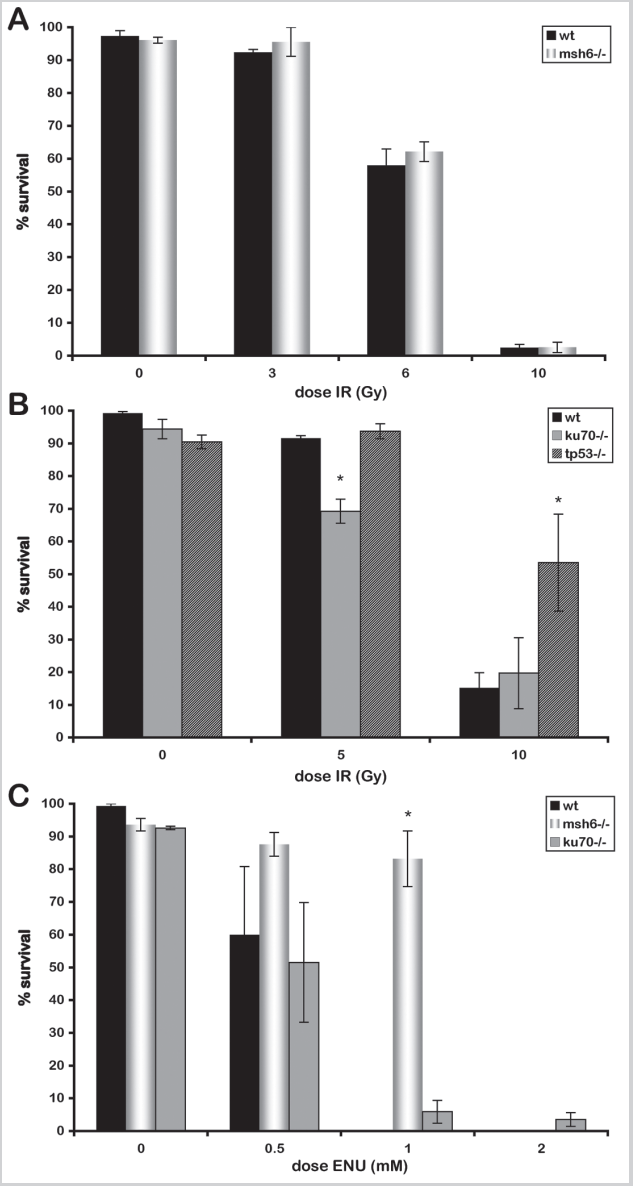

FIGURE 4. MMR-dependent ENU-induced chromosomal instability arises at replication. (A) Induction of DSBs by IR does not result in MMR-dependent differences in survival. (B) *Ku70* mutants are more sensitive to IR, whereas *tp53* mutants are more resistant than wild types. (C) *Ku70* mutant embryos are equally sensitive to ENU treatment as wild types. Asterisks indicate significant differences.

DISCUSSION

Although the effects of alkylating agents *in vitro* have been studied extensively, *in vivo* studies in vertebrate species are still lacking. Here, the effect of alkylation-induced damage was studied for the first time *in vivo,* in both MMR-proficient and -deficient zebrafish embryos. For this purpose *msh6* mutant zebrafish were used, which had previously been shown to display microsatellite instability and a cancer predisposition (H. Feitsma et al., in press), illustrating that they are MMR-deficient. To study somatic mutation accumulation *in vivo*, an assay was set up for the detection of loss-of-heterozygosity at the *albino* locus in pigmented cells of the embryonic eye. Using this assay, the background somatic mutation frequency in the *msh6* mutant was found to be 17-fold increased. This is consistent with studies in other organisms, where increases in the mutation frequency of 10-100-fold were found in MMR-deficient backgrounds (21, 34-36). Since the zebrafish *albino* gene does not contain clear repeat sequences, the underlying mutations are expected to be point mutations, although their nature has not been determined experimentally.

Next, mutant and wild type embryos were treated with S_N1 alkylating agents. Previous studies using cell culture experiments did not give conclusive evidence whether ethylation lethality is also dependent on MMR. Some of these studies show increased resistance in MMR mutants (20, 21) and others reported no differences with wild type (22, 23). Here, we show that *msh6* mutants survive ENU treatment much better than wild type fish. Surprisingly, MNU induced severe MMR-dependent killing only at the lowest concentration of 0.2 mM, which was reproducibly found in multiple experiments. It is hard to explain why a certain compound is more lethal at lower than at higher concentrations. We speculate that this may be related to the fact that DNA methylation itself is a natural regulation mechanism, so that thresholds need to exist above which it triggers the DNA damage response and repair pathway. Lower dose methylation damage, however, could still deregulate cellular processes and result in cell death. In that respect, this type of *in vivo* studies may be clinically very significant, as not only alkylating agents but also DNA demethylating agents are currently used in chemotherapy, because epigenetic changes are frequent in cancers (37). According to this study, the alkylation damage response in zebrafish is similar as in cells of other organisms. However, more detailed studies will be needed to firmly establish zebrafish as a general model for studying human DNA repair processes. For example, it is not known whether zebrafish has a functional *mgmt* homologue. The Ensembl database (www.ensembl.org) gives

an orthologue (ENSDARG00000043275), but data on how and when it functions and whether it is maternally provided are lacking.

As discussed, O^6-alkG-induced stalled replication forks can turn into DSBs. These DSBs arise MMR-dependently, which was confirmed by showing that DSBs induced by IR did not result in reduced lethality in *msh6* mutant embryos. Additionally, DSBs occurring during replication are normally repaired by HR and not by NHEJ (5, 14, 17, 18). *Ku70* mutant zebrafish, which were shown to be NHEJ-deficient, were equally sensitive to ENU as wild types, indicating that NHEJ does not repair ethylation-induced DSBs. Previously, it was shown that hamster cells mutated in *Ku80* were also unable to repair IR-damage but not MNNG-damage (38). These data confirm that DSBs resulting from MMR-recognized O^6-alkG arise at replication.

The observed lower ENU- and MNU-mutation rate in *msh6* mutants can now be explained by the fact that the albino loss-of-heterozygosity mutations are large chromosomal lesions. Chromosomal aberrations have also been reported after MNU treatment of human, mouse and hamster cells (14, 22, 23). They occur when DSBs caused by O^6-alkG are not or aberrantly repaired, which subsequently results in cell death.

Although DNA checkpoint signalling induced by O^6-alkG-mediated stalled replication forks was shown to depend on ATR/Chk1 signalling (11-13), secondary lesions may very well induce a different DNA damage response. This can explain why we found TP53 to be upregulated in embryos with high levels of chromosomal instability, while MMR-dependent alkylation DNA damage signalling was reported to be independent of TP53 (7). Similarly, it explains the involvement of ATM-signalling upon alkylation damage (14). In our *in vivo* zebrafish experiments the alkylation response is apparently mostly directed to chromosome instability. It should be mentioned that while DNA damage checkpoints are absent during the first cell divisions, the ability to undergo checkpoint-induced apoptosis was found to start even a few hours later in zebrafish (39), which may explain why the O^6-alkG damage is able to cause high levels of chromosomal aberrations.

MMR deficient embryos were found to die from alkylation treatment in a similar fashion as MMR proficient embryos, although only at much higher concentrations. This is probably due to other alkylation-induced DNA damage such as N-alkylation products, which can also induce apoptosis, presumably due to incomplete base excision repair of alkylated bases resulting in abasic sites (2). The potential effects of alkylation treatment are schematically summarized in Figure 5. The three different outcomes of alkylation

damage, 1) cell death, 2) point mutation, and 3) repair, can be reached via different repair and signalling routes. This indicates that absence of one function, such as MMR, will shift the balance of these three outcomes rather than result in one specific outcome.
According to the observed mutation load, the induction of DSBs by alkylation is exceeding the effect of point mutations by far in developing wild type embryos. Considering that ENU and MNU are primarily used as inducers of point mutations, this suggests that their effect in embryos may be different from that in the germ line. Nevertheless,

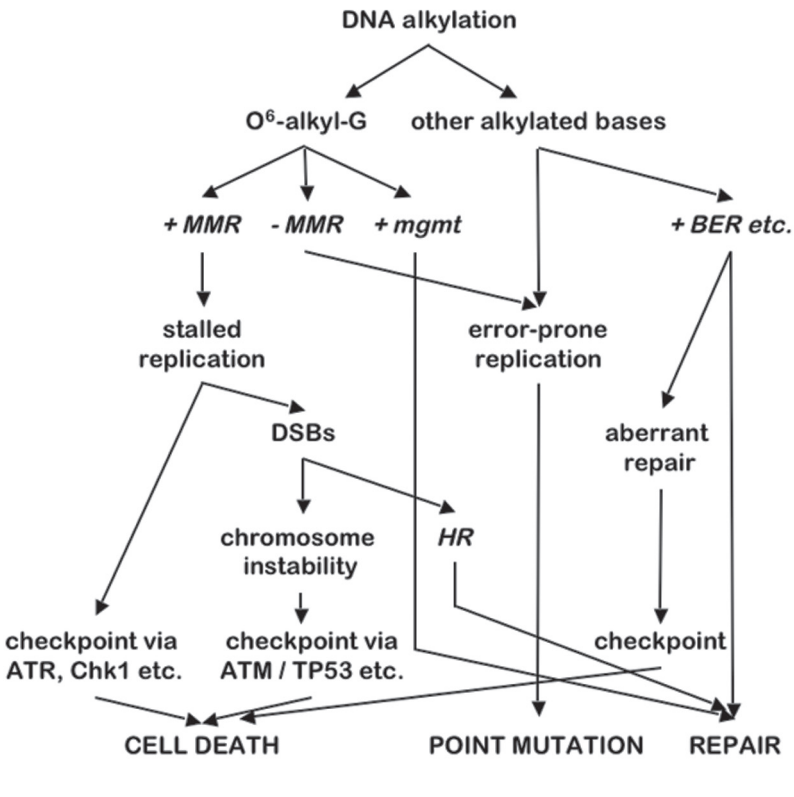

FIGURE 5. Schematic overview of the responses to alkylation damage and the three possible outcomes 'cell death', 'point mutation' and 'repair' (capitals) via routes involving different types of DNA repair (italics). Absence of functions such as MMR will shift the balance of these three outcomes rather than result in one specific outcome. MMR, mismatch repair; mgmt, O^6-methylguanine methyltransferase; BER, base excision repair; DSBs, double strand breaks; HR, homologous recombination.

DSB-related mutations have been observed in several mutagenesis studies aiming for point mutations, indicating that this process also occurs in the germ line. In zebrafish mutagenesis treatment of post-meiotic germ cells, two out of six obtained mutations were found to be dominant lethal in embryos, suggesting that they represent larger lesions rather than point mutations (40). For five mutants from a similar mutagenesis experiment the mutation was found to be a translocation in one line and a large deletion in the four others (41). ENU and MNU germ line mutagenesis in mice also caused large lesions in many cases (42).

Taken together, this study shows that alkylation damage induces high levels of chromosomal aberrations in early zebrafish embryos in an MMR-dependent fashion, which result in reduced embryonic survival. Overall, the data indicate that the basic responses to alkylation damage in zebrafish are similar to other organisms. However, the frequent induction of aberrant chromosomes rather than point mutations in these embryos is clearly different from germ line mutagenesis and also *in vitro* experiments. These experiments show that *in vivo* and in somatic cells chromosomal instability is a more relevant outcome of alkylation damage than point mutations. These findings are highly relevant for alkylating events underlying carcinogenesis processes as well as alkylation chemotherapy.

Acknowledgments

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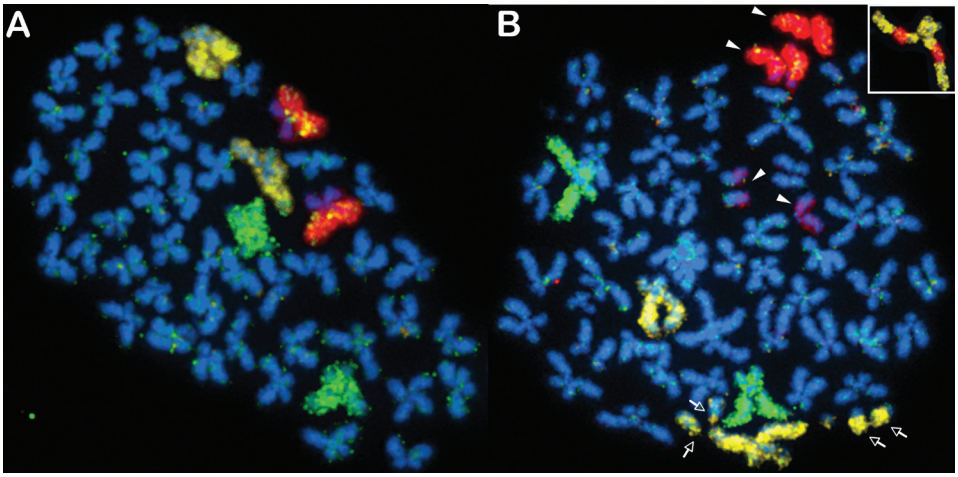

SUPPLEMENTARY FIGURE 1. Chromosomal aberrations stained with chromosome paints

A) Metaphase spread of an untreated embryo with chromosome paints for chromosome 4 (yellow), 5 (green) and 7 (red), showing two of each of those per cell. B) Metaphase spread of an exemplary cell of a wild-type embryo treated with 1 mM ENU with chromosome paints for chromosome 4 (yellow), 5 (green) and 7 (red), showing fragments of chromosome 4 (arrows) and both chromosomes 7 having broken into halves (arrowheads). (Note that the short arms of chromosome 7 show weaker staining than the long arms also in A.) Inset in B) Example of a rearranged chromosome 4 with fragments of chromosome 7 from a similar embryo as in B.

6

Mismatch repair deficiency does not improve ENU mutagenesis in the zebrafish germ line

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ABSTRACT

S_N1-type alkylating agents such as *N*-ethyl-*N*-nitrosourea (ENU) are very potent mutagens. They act by transferring their alkylgroup to DNA bases, which, upon mispairing during replication, can cause single basepair mutations in the next replication cycle. As DNA mismatch repair (MMR) proteins are involved in the recognition of alkylation damage, we hypothesized that ENU-induced mutation rates could be increased in a MMR-deficient background, which would be beneficial for mutagenesis approaches. We applied a standard ENU mutagenesis protocol to adult zebrafish deficient in the MMR gene *msh6* and heterozygous controls to study the effect of MMR on ENU-induced DNA damage. Dose-dependent lethality was found to be similar for homozygous and heterozygous mutants, indicating that there is no difference in ENU resistance. Mutation discovery by high-throughput dideoxy resequencing of genomic targets in outcrossed progeny of the mutagenized fish did also not reveal any differences in germ line mutation frequency. These results may indicate that the maximum mutation load for zebrafish has been reached with the currently used, highly optimized mutagenesis protocol. Alternatively, the MMR system in the zebrafish germ line may be saturated very rapidly, thereby having a limited effect on high-dose ENU mutagenesis.

INTRODUCTION

N-ethyl-N-nitrosourea (ENU) is one of the strongest chemical mutagens known. In zebrafish this was recognized a long time ago, and since then ENU mutagenesis has effectively been used in numerous forward genetic screens (1-4). In addition to that, target-selected ENU mutagenesis is currently the only available reverse genetics technology to create gene knockouts in both zebrafish and rat, indicating its high efficiency (5-8).

ENU acts by transferring its ethylgroup to DNA bases, mainly to their oxygen and nitrogen atoms. These DNA adducts are not mutations by themselves, but during replication they can mispair, which in the following round of replication can cause a full mutation (9). Since alkylation also occurs naturally, cells have developed repair mechanisms to remove this type of DNA damage. The molecular mechanisms have been studied most extensively for methylation, which will therefore be discussed first. Different types of methylation adducts are repaired by different repair pathways, such as methylpurine DNA glycosylase and base excision repair (10). The methyl adduct of O^6-methyl-guanine

(O^6-meG) is specifically removed by the enzyme O^6-meG methyltransferase (*mgmt*) (11, 12). Additionally, when O^6-meG pairs with thymine or cytosine during replication, this is recognized as a mispair by mismatch repair (MMR) enzymes (10, 13-16). Those will induce strand excision of the newly synthesized strand towards the site of methylation in an attempt to repair the mismatch. However, since the methylated base is in the template strand of the replicated DNA, it cannot be replaced. Instead, it will continuously be paired with C or T by the polymerase, followed by MMR recognition and excision. This 'futile cycle' repair principle will finally result in replication fork stalling and the formation of double strand breaks (DSBs) (17, 18). It leads to apoptosis in MMR-proficient cells and organisms, while MMR mutants are resistant to alkylation-induced death (16-21).

The basic molecular response for ethylation-induced DNA damage by ENU may be analogous, although different studies have resulted in controversial data. Claij *et al* found that mouse embryonic stem cells lacking MSH2, a major component of the MMR system, were more resistant to ENU compared to wild-type cells, albeit this difference in survival was less pronounced than for methylation by MNNG (20). These same homozygous mutant cells also displayed a selective growth advantage over heterozygous cells when treated with ENU (19). In contrast to these two reports, no differences were observed between MMR-deficient cells and their wild-type counterparts in response to ENU treatment in two other studies, on human and hamster cells, respectively (22, 23). However, when treating zebrafish embryos with ENU in a separate experiment, we found increased tolerance of embryos deficient in *msh6* (H. Feitsma, A. Akay and E. Cuppen, submitted). MSH6 is the MMR component that dimerizes with MSH2 to form a complex that specifically recognizes single base mismatches and small insertion-deletion loops. Our preliminary results indicate that MMR is very likely to play a role in the recognition of ENU-induced DNA damage *in vivo* in zebrafish.

Unrecognized alkylated mispairs in MMR-deficient cells that persist in a genome may result in the accumulation of mutations. A higher mutation frequency in MMR deficient cell lines has indeed been reported for methylation (21) and it was also shown for ethylation, by ENU treatment of mouse embryonic stem cells mutated in *msh2* (20).

We used zebrafish mutants deficient in *msh6* to test whether a standard zebrafish ENU mutagenesis protocol could result in a higher mutation load in a MMR deficient background. We observed that neither mutagen-induced lethality nor germ line mutation frequency was different in the *msh6* mutants compared to heterozygous siblings.

MATERIAL AND METHODS

Zebrafish lines

Msh6 mutant fish *(hu1811)* were obtained by target-selected mutagenesis, and the initial characterization was described elsewhere (H. Feitsma, R.V. Kuiper, J. Korving, I.J. Nijman and E. Cuppen, in press). Genotyping was done by PCR amplification and resequencing, using exon 10 specific forward (5'-GCTGGTGGCAACTTAAATC-3') and reverse (5'-GCTCAACAGATACTTGCTTTG-3') primers. For the crosses to determine germ line mutation frequency, albino females of the b4 line were used. Those are homozygous for a 4 kb insertion in exon 6 of the zebrafish *SLC45A2* gene (E. Wienholds, unpublished).

ENU mutagenesis

ENU mutagenesis was carried out as described in detail in a "Methods in Molecular Biology" zebrafish volume (E. de Bruijn, E. Cuppen and H. Feitsma, submitted). Briefly, fish were treated six times at weekly intervals by bathing them one hour in ENU in $NaPO_4$ buffer pH 6.6, followed by a short wash and a recovery phase over night in a dark and quiet room under slight sedation. The next day they were placed back in their homecages. After four treatments, two females were put in each cage to stimulate spermatogenesis. Animals that became moribund during or after the treatment were sacrificed. The appropriate ENU concentrations were carefully prepared using OD measurement at 238 nm (extinction coefficient = 5830 $M^{-1}cm^{-1}$) of the ENU stock solution.

Determination of ENU mutation induction

Clutches fertilized by mutagenized founders were collected and the fertilization rates recorded, after which embryos developed at 28.5 °C. Healthy embryos were collected at five days post-fertilization (dpf) and DNA was isolated using standard procedures. For both genotypes, four 96-well plates with embryos from four different founders were used for mutation detection. In total, 33 randomly chosen genomic loci were resequenced. The sequencing procedure was as described previously (6), using a nested PCR and subsequent sequencing reaction. These were ethanol-purified and directly run on a 3730XL sequencer (Applied Biosystems, Foster City, CA). Mutation detection was done semi-manually using PolyPhred software and automatically using in-house-developed Perl scripts. Basepair-countings were also done automatically with Phred scores of 20 as

cut-off. All identified mutations were confirmed in independent PCR reactions.

Results

Spontaneous germ line mutation frequency

It was shown previously that zebrafish msh6 knockouts display microsatellite instability (H. Feitsma, R.V. Kuiper, J. Korving, I.J. Nijman and E. Cuppen, in press), which is the characteristic form of genomic instability associated with defects in MMR. However, the level of point mutations has also been shown to be increased in MMR deficient backgrounds (20, 24), and since this type of mutations has a higher chance of inactivating genes, it is of more relevance to germ line mutagenesis. Therefore, the germ line mutation frequency in male *msh6* mutant fish was studied at a specific locus: the *albino* gene. *Msh6*$^{-/-}$ or wild type control males were crossed with albino females, and at three dpf the progeny was scored for lack of pigmentation. Completely unpigmented embryos (Fig. 1) were considered the result of a germ line mutation in the *albino* locus, and since the zebrafish albino gene does not contain significant repeats, these most likely represent point mutations. Although we screened considerable amounts of progeny, the number of scored albinos is relatively low. Three albino embryos were found in more than 13,000 progeny from mutants and none in 17,000 progeny from wild types, suggesting an increased mutation frequency in *msh6*$^{-/-}$ fish (Tab. 1). More importantly, the spontaneous mutation frequency is around 15-fold lower than what is regularly obtained after ENU mutagenesis of wild types (Tab. 1) (8).

Table 1. Single locus mutation frequency in the germ line of *msh6*$^{-/-}$ and wild-type zebrafish

father	embryos scored	albinos	mutation frequency
wild type	17,133	0	<< 0.00006
msh6$^{-/-}$	13,667	3	0.0002
wild type +ENU (8)	3,427	11	0.003

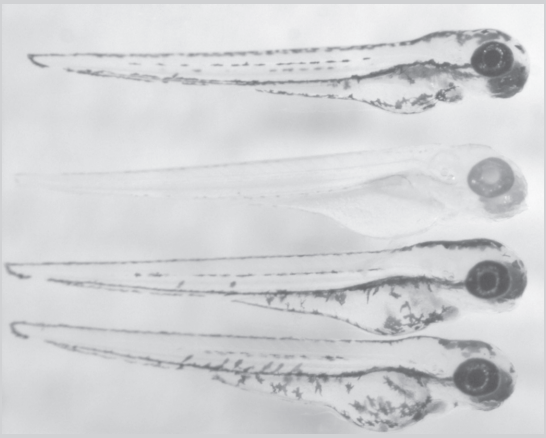

FIGURE 1. The germ line mutation frequency of *msh6* mutant male zebrafish was determined at the *albino* locus. Completely unpigmented embryos (second from top) resulting from crosses of *msh6*$^{-/-}$ or wild type males with albino females were scored. Those are considered the result of a mutation in the *albino* gene in the male germ line.

ENU mutagenesis

Homozygous and heterozygous male *msh6* mutant animals of three months of age with proven fertility were selected for the mutagenesis experiment. In addition to the standard ENU concentration of 3.3 mM, groups of fish were treated with 2.2 mM and 4.4 mM ENU to test for changes in tolerance. A fourth group consisted of untreated controls. Six animals of each genotype were used per treatment group. During the treatment period some animals were lost, especially among animals treated with 4.4 mM ENU. In general, no difference in survival between homozygotes and heterozygotes was observed, or if any, the mutants seemed slightly more sensitive, but this was not significant due to small group size (Fig. 2).

From the first week post-treatment, males were crossed with wild-type females. 4.4 mM-treated animals were in bad condition and were, except for one small clutch from one heterozygous animal, not able to induce egglays. Otherwise, no significant differences in fertility between genotypes or treatments were observed (Fig. 3). The first egglay was always discarded because of potential mosaicism and residual ethylation due to mutagenesis of post-meiotic germ cells (2), which was also visible by malformations in the embryos. From the second clutches onwards, embryos were collected for DNA isolation.

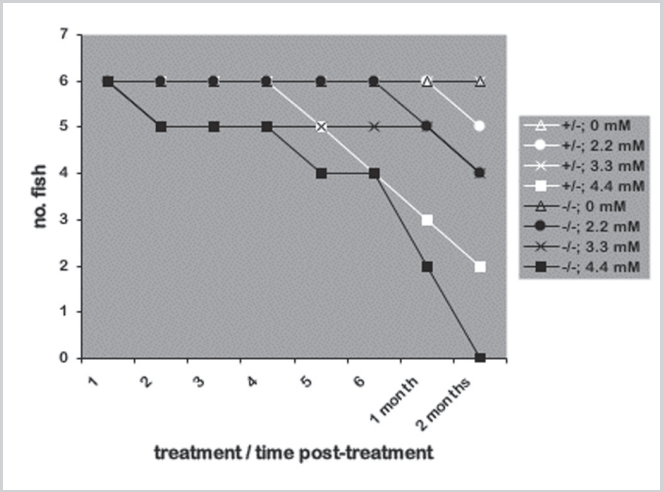

FIGURE 2. The survival of ENU-treated males was recorded after each mutagenesis treatment as well as one and two months after the last treatment. Many curves from different treatment groups are overlapping, as no lethality was observed.

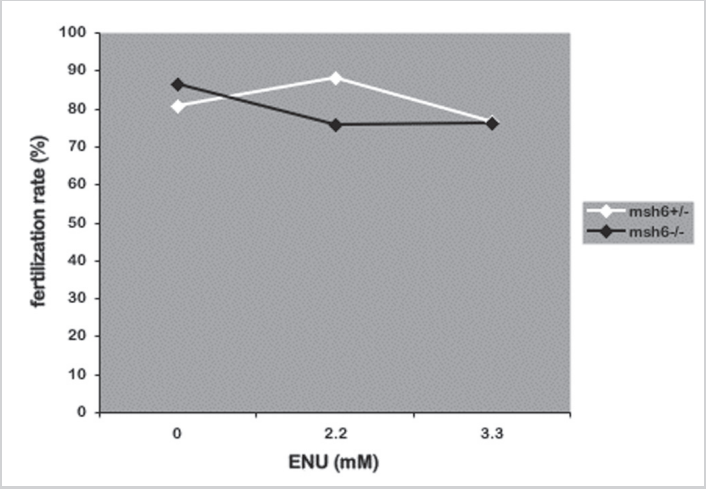

FIGURE 3. Fertilization rates of *msh6*$^{+/-}$ and *msh6*$^{-/-}$ males treated with different concentrations of ENU. Percentages of fertilized eggs were measured and averages calculated for all clutches obtained. The 4.4 mM ENU treatment groups are excluded, because only one small clutch was obtained for this concentration.

ENU-induced germ line mutation frequency

We chose to score mutations in progeny of the 3.3 mM treatment groups only, to be able to obtain a high number of mutations for calculating an appropriate and comparable mutation frequency. Almost 4 million high-quality DNA bases were evaluated and in total 26 mutations were identified for both *msh6*$^{-/-}$ and *msh6*$^{+/-}$ animals (Tab. 2). The mutation frequency was therefore similar for MMR deficient and control fish, and was found to be around one mutation in 150,000 basepairs. Since young embryos were used for the frequency determination, it was possible that the frequency was higher than what could be obtained in adults, as embryos with a high hit rate might die during development. To investigate this possibility, F1 adults of a target-selected mutagenesis library obtained from mutagenized wild-type males were resequenced for mutations in a subset of genomic loci. Although these fish were generated in a different experiment, the mutation frequency was found to be virtually identical to that obtained in embryos (Tab. 2).

When comparing the spectrum of all basepair changes between the different genotype groups, the number of GC to AT mutations tend to be decreased in the germ line of homozygous mutants, which is compensated by an increase of AT to TA mutations (Fig. 4). However, these differences were not significant due to the limited number of mutations.

TABLE 2. ENU-induced point mutation frequency in the germ line of *msh6*$^{+/-}$, *msh6*$^{-/-}$ and wild-type zebrafish

genotype male	mutations	basepairs sequenced	mutation frequency
msh6$^{+/-}$ +ENU	26	$3.93 * 10^{6}$	$6.6 * 10^{-6}$
msh6$^{-/-}$ +ENU	26	$3.74 * 10^{6}$	$6.9 * 10^{-6}$
wt (F1 library) +ENU	43	$6.47 * 10^{6}$	$6.6 * 10^{-6}$

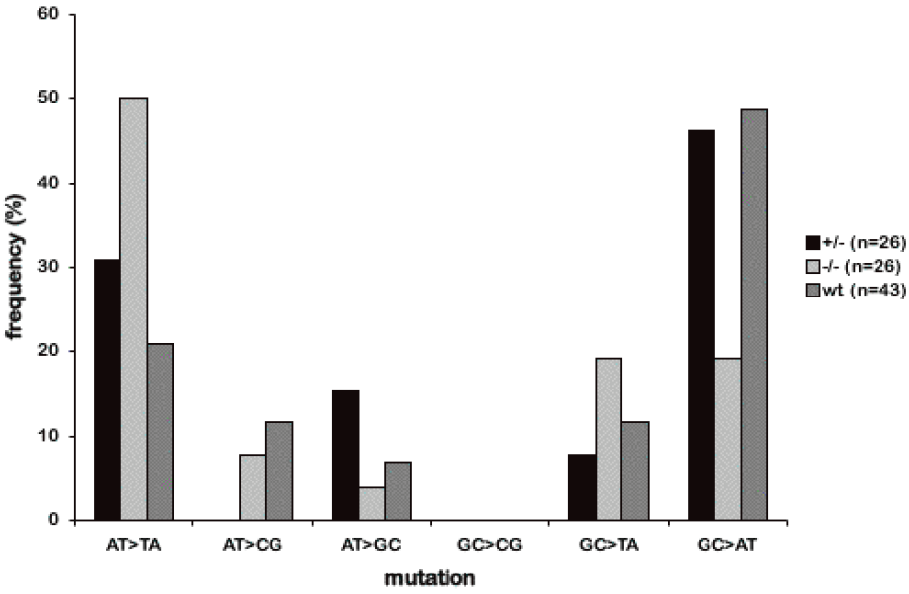

Figure 4. ENU-induced germ line mutation spectra of *msh6* heterozygous and homozygous mutant and wild-type males. The relative frequency of mutation types among all scored mutations in progeny of these males is depicted.

Discussion

We tested the mutagenic activity of ENU in the germ line of male zebrafish deficient in the MMR gene *msh6*. First, the spontaneous germ line mutation frequency was studied in these mutants using the *albino* single locus test. Although the number of albinos was small, the mutation frequency was found to be higher than in wild types. However, the observed frequency of one in 5,000 embryos in the mutant background is in the same order as the frequency previously reported in wild type fish (2, 25), being around one albino in 10,000 embryos. In any case, the ENU-induced germ line mutation frequency has frequently been found to be around one albino in 300 embryos for a good mutagenesis (2, 3, 8), indicating that the background mutation frequency due to MMR deficiency is one order of magnitude lower.

Previously it was reported that MMR deficient cells are more resistant to alkylation damage. However, the results for ENU are not conclusive, some studies showing increased

tolerance in MMR mutants (19, 20), while others reported no differences with wild types (22, 23). Although alkylation damage recognition mechanisms may be similar for methylation and ethylation, affinities of the polymerase, Mgmt and MMR will probably be different for O^6-ethyl-guanine than for O^6-methyl-guanine. Another difference is that the level of GC to AT transitions induced by ENU is, at least in mice, relatively low and the level of AT to TA transversions high (26-28), indicating that O^6-ethyl-G is not the primary lesion. In zebrafish, however, GC to AT and AT to TA mutations were found to be equally frequent in a set of positionally cloned mutations from forward screens (4), and GC to AT changes were the most abundant in a relatively unbiased set of sequenced mutations from a reverse genetic screen (7) as well as in samples from wild types and heterozygotes in this study. This indicates that O^6-ethyl-G dependent killing will be relevant in zebrafish. Indeed, we observed increased survival of *msh6* deficient zebrafish embryos compared to wild types after treatment with ENU (H. Feitsma, A. Akay and E. Cuppen, submitted), indicating that ethylation damage is processed in an MMR-dependent way *in vivo* in zebrafish. To be able to detect a decreased sensitivity towards ENU, we applied a higher dose of 4.4 mM ENU in addition to the standard 3.3 mM. No differences were observed in survival between heterozygous and homozygous adult mutants. The 4.4 mM mutagenesis concentration was clearly too harsh for the fish. Although some animals survived, only one clutch of eggs was obtained, and most animals became sick during or after the treatments. The lack of difference in survival between the genotypes can be explained by assuming that the level of DNA damage in adults is relatively low as compared to other cytotoxic effects of ENU, such as ethylation of RNA and toxicity from degradation products (9). The DNA damage caused by alkylation is dependent on replication, and although zebrafish keep proliferating throughout life, the proliferation levels will be much lower *in vivo* in adult animals than *in vitro*. The replication-independent toxicity will then be more important for killing, and this is also unrelated to MMR. An alternative explanation is that the high mutation load is the cause of death of these adult animals. To our opinion this is not very likely, as increased levels of mutations would, differently from its effect in F1 progeny, cause cancer rather than direct lethality. This was not observed, except for one animal that developed a tumour some time after the treatments.

Considering O^6-ethyl-G to be the only ENU-induced damage that is recognized by MMR, one could primarily expect an increase in GC to AT transitions in a MMR-deficient environment. However, in our experiment a tendency of the opposite effect was

observed - a decrease in GC to AT transitions, and an increase in AT to TA mutations - although numbers are small and differences not significant. Also in *msh2*-deficient mouse cells treated with ENU, mutations at AT sites were most frequent (20), which would suggest that O^6-ethyl-G is not the main target for MMR. But also in the latter study the number of identified mutations was low.

A higher ENU-induced point mutation frequency has been reported previously for MMR-deficient mouse cells (20). Ethylated base mispairs that do not undergo MMR-dependent apoptosis may result in mutations, and thus result in an increased mutation frequency. In our study we obtained no indications for an increased mutation frequency in the germ line of *msh6* mutant male fish. The observed frequency was one mutation in 150,000 basepairs in embryos of both homozygous mutants and heterozygous controls, and this was identical to the mutation frequency in adult progeny from treated wild types. The absence of a difference can be due to the fact that the mutation frequency that is obtained with the highly optimized mutagenesis protocol is the maximum that can be reached in the zebrafish germ line. In favour of this explanation is the fact that highly similar mutation levels are routinely achieved in different experiments in different labs. A mutation frequency of one per 100,000 to 200,000 basepairs could be the maximum rate that is compatible with a viable genome in general, because the maximum mutation frequency that was obtained in *C. elegans* using EMS mutagenesis is one per 109,000 basepairs (29). Above a certain mutation load the number of deleterious mutations becomes incompatible with life. In our study, the mutation frequency could be maximized by cell death at two different levels. First, spermatogonial stem cells could be eliminated from the testis, which would result in infertility of males. However, no difference in fertility between the genotypes was observed, and there was no obvious decrease of fertility with increasing doses of ENU. The inability of 4.4 mM-treated animals to induce egglays was presumably more due to sickness than to infertility, as we know from a previous study that sterile male zebrafish can still induce egglays without fertilizing them (30). Secondly, development of fertilized oocytes could be inhibited or arrested due to high numbers of mutations. However, no difference in survival of embryos was found, and the mutation frequency in embryos was similar to that in adult progeny from wild-type mutagenized animals, indicating that loss of progeny during development due to a high mutation load is not likely.

An alternative explanation for the absence of a difference in mutation frequency in MMR deficient and proficient backgrounds is that the MMR system in the zebrafish

germ line is saturated very rapidly, so that the effect on high-dose ENU mutagenesis is limited. Clearly, for the reported data on mouse *msh2* mutant cells, the ENU-induced mutation frequency is in the same order of magnitude as the increase of mutations by loss of MMR alone (ca. 200 per 10^6 cells vs. ca. 80 per 10^6 cells) (20). In fact, almost half of the increase in mutations in ENU-treated mutant cells compared to ENU-treated wild type cells is attributable to the absence of *msh2*. This would mean that the levels of ENU-induced damage and of MMR activity are more or less in proportion. We did not determine the frequency of spontaneous point mutations in the germ line of *msh6* mutant fish, but according to the *albino* locus test this is expected to be around 15-fold lower than the frequency that can be induced by ENU. These observations strongly suggest that the capacity of MMR in the zebrafish germ line is negligible compared to the large number of mispairs induced by ENU. However, to firmly establish the role of MMR in zebrafish in this process, more detailed studies on other DNA repair pathways and enzymes are necessary. An example is *mgmt*, which is highly important in the repair of alkylation damage. Although the Ensembl database (www.ensembl.org) gives a zebrafish orthologue for *mgmt*, there is no data available on whether it is functional and in which tissues. This will be an important direction for future research.

Taken together, survival and germ line mutation frequency of ENU-treated adult male zebrafish are not affected by the absence of MSH6. In contrast to observations in cell culture systems, we did not find increased survival nor an increased mutation frequency *in vivo*. As a result, MMR deficient backgrounds will not be useful for increasing the efficiency of forward genetic screens or target-selected mutagenesis screens for making gene knockouts in zebrafish.

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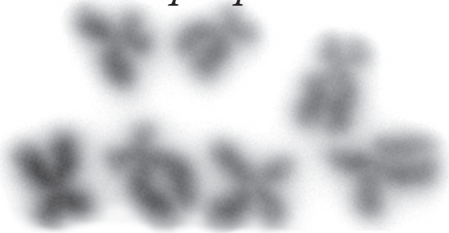
7

Future perspectives



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In this thesis it was shown that the functions of mismatch repair (MMR) genes in zebrafish are similar to those in mouse and human, which validates the zebrafish as a model for studying DNA repair, genome instability and cancer. Now, further studies should be taken that exploit the versatile properties of zebrafish as an animal model, to gain novel insights in the mechanisms of genome integrity and cancer.

Much is still to be learned in meiosis research. So far it has been difficult to study vertebrate meiosis in detail and in reasonable numbers, since gametes are not easily accessible and because malformed fertilized eggs are usually resorbed *in utero* without notice. Basic studies have therefore been carried out in yeast, fruitfly and worm, but meiosis in these organisms differs from vertebrate meiosis. Zebrafish may have many advantages in this respect. First, the number of progeny that can be generated in a single cross (>100) is sufficient for generating statistically valid data. Second, embryos with abnormal genomic content, for example resulting from meiotic missegregation, will undergo the first couple of cell divisions normally, as long as the maternally provided set of proteins and mRNAs is adequate. This allows studying the genetic outcome of meiosis. Third, the opportunities to inactivate male or female chromosome sets prior to fertilization and to block certain cell divisions after fertilization are unique for zebrafish (1). When treating eggs or sperm with UV-radiation and applying *in vitro* fertilization, the resulting embryo will have a haploid genome content, which is also sufficient for a few days viability. This means that a specific outcome of meiotic divisions can be studied, without interference with the other parents' germ cell DNA. Additionally, the application of timely pressure treatment on fertilized eggs blocks the second meiotic or first mitotic divisions. When combined with UV-inactivated germ cells, one can generate homozygous progeny with the meiotic results of one parent. The high levels of polymorphisms within and between different zebrafish strains (2) make it relatively simple to distinguish chromosomes. In addition, with the successful application of fluorescence *in situ* hybridization as a tool for completing the zebrafish genome sequence (3), a large set of probes has become available that can be used for tracking specific chromosome portions. Altogether, this offers many opportunities for studying crossover events and other elements of meiosis. A small drawback is that zebrafish do not have sex chromosomes (4), which makes its meiosis probably mechanistically somewhat different from mammals. Also, currently not many meiotic mutants are available, but this may change rapidly. In fact, a number may have been generated and partially phenotyped already in maternal effect screens

that have been performed (5,6).

We have shown that the cancers of zebrafish MMR mutants are similar to those in human patients, but also that there are clearly some differences. Although this may limit the possibilities for using zebrafish directly as a translational model, an advantage is that the generation of new models provides the opportunity to compare cancer development in different organisms, which could help revealing underlying biological mechanisms. Secondly, the availability of zebrafish models with known frequencies and spectra of tumours enables screens for mutants or compounds that influence these parameters. Most useful for this may be transgenic cancer models that express fluorescent proteins in their tumours (e.g. (7)), thus making it possible to precisely monitor cancer progression and regression. However, in genetic models such as our MMR mutants, tumour growth can also be relatively obvious and thus observable while the fish is still alive. A limitation of this category of cancer models is the late onset and relatively low incidence of tumorigenesis, which would complicate modifier screens. To bypass this, one could make use of the fact that some of the mutants display a clear embryonic phenotype that can serve as readout. Genome instability or alkylation tolerance are illustrative examples of this principle that can be used for screens in the mutants described in this thesis.

We show that the pigmentation loss-of-heterozygosity assay in zebrafish embryonic eyes is a very useful readout for somatic genome instability. Not just point mutations, but all types of lesions that inactivate a gene are scored in this assay. Until now, this type of large scale mutation detection was only possible in cell culture or in lower organisms such as yeast and worm that are not capable of developing cancer. The strength of the *in vivo* approach in zebrafish was shown by a forward genetic screen (8), from which all identified genome instability mutants were prone to tumour development, confirming the strong relation between genome instability and cancer. Unfortunately, underlying mutations have not been positionally cloned yet, because the phenotype is not completely penetrant, and mapping therefore laborious. Identification of new cancer genes is of course important, but also chemical screens would be feasible using the genome instability assay. It is quantitative to a certain extent, which makes comparison of substances possible. Many carcinogens, which increase genome instability, are known, but it would also be of interest to screen for compounds that protect against genome instability.

In accordance with the theoretical rationale that ENU-induced DNA damage does not lead to cell killing in absence of MMR enzymes, and with experimental evidence (9), we hypothesized that MMR deficiency could increase the ENU-induced germ line mutation frequency in zebrafish, but found this not to be true. However, there are reasons to believe that the maximum-tolerable mutation frequency was not reached yet, as neither sterility of males nor high levels of lethality of embryos were observed. Therefore, other approaches to increase the germ line mutation frequency could be considered. One possibility would be to knock out the O^6-alkylguanine dealkylating enzyme Mgmt. It probably exists in zebrafish, as the Ensembl genome database lists a zebrafish orthologue. An Mgmt mutant could be crossed to MMR mutants to reduce alkylation-induced killing (10). Since cancer development is usually late in zebrafish, there will likely be sufficient time to generate a mutant library before the founders die. Alternatively, eggs of Mgmt/MMR double mutant females could be fertilized with wild type sperm exposed to ENU. Due to the absence of maternally contributed repair enzymes, most damage induced by ENU will not be repaired during the first cell divisions, establishing many mutations in a mosaic fashion. As soon as the embryonic genome becomes active, however, the embryo is genetically heterozygous for both repair genes, so that any viability-reducing effects of the lack of either are compensated. Nevertheless, it is debatable whether an increase in mutation frequency is desirable. The currently used, efficient protocol results in an extremely high mutation frequency, and any increase in that would proportionally boost the load of background mutations with unknown influence.

To conclude, this thesis is one of many examples of the virtually unlimited possibilities of zebrafish as a laboratory animal. The model's popularity will certainly keep increasing, but efforts to develop technologies and reagentia have to be taken in parallel. For example, there is obviously great demand for zebrafish-specific antibodies, and, with the zebrafish genome sequence becoming complete, many DNA repair pathways need to be confirmed. This will require studies similar to the ones described in this thesis that, although at first sight suffering from lack of scientific novelty, are the necessary basis for future discoveries.

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Summary

Damage in DNA accumulates slowly during a human life, leading to aging. However, when by coincidence several injuries in one cell inactivate necessary protection mechanisms, this can lead to unimpaired division of the cell, thereby evolving into a tumour. DNA damage is normally neutralized as much as possible by numerous repair systems. If one of those is missing, the cancer process can be accelerated. This is known as a hereditary predisposition for cancer.
Counterintuitively, the traditional treatment of cancer uses the induction of high levels of DNA damage by irradiation or chemotherapy. This is effective because large amounts of DNA damage mainly impair the cell division, causing cell death. Frequently dividing cancer cells are therefore extremely sensitive, while normal cells, which in a mature human body divide quite rarely, stay alive.

In this thesis the zebrafish is used as a laboratory animal. Until now, the zebrafish has mostly been used for studying the earliest development of an animal. The reason for this is that it has the big advantage of its progeny being laid as transparent eggs and thus growing up outside the mother. Therefore, development can be followed from the first cell division until a full-grown embryo. While studying the fish in the lab for this purpose, one discovered that also adult animals resemble humans in many perspectives, and hence can serve as a model. The objective of this study was to find out whether the zebrafish can be an appropriate model for studying DNA repair and cancer. For this purpose I have used three fish lines that lack components of an important mechanism for the repair of small DNA damage, the so-called mismatch repair. They are therefore hereditary predisposed to develop cancer. I show that the action of this repair system with regard to the formation of germ cells, the growth of tumours and the response to chemotherapeutic agents is similar to that in human and mouse. This means that the zebrafish can be considered a useful model. However, within all these areas there are clear differences. In this case those are equally important, as they enable us to study new aspects of cancer - research that is not possible in mouse and human.

Chapter 1 gives an overview of the current research on cancer in the zebrafish. Although almost all publications about this subject are not older than the year 2000, there are more than 50 already, which reflects the strong growth of the research field. Specific for the zebrafish is the ease of treatment with cancer-inducing or cancer-inhibiting agents, by simply dissolving them in the aquarium water. This can be of great value in the search

for novel drugs.

Chapter 2 describes the role of a specific mismatch repair component in meiosis, the type of cell division leading to germ cells with only half of the normal number of chromosomes. I show that the absence of this component in female fish causes progeny with abnormal chromosome numbers, which are malformed and usually die within a few days. Studying this in mammals is hardly possible, because erroneous oocytes are typically eliminated directly in the uterus.

Chapter 3 is a follow-up on chapter 2. Male relatives of the females in chapter 2 are completely infertile, but in a different genetic background they do possess some full-grown spermatozoa. Also in these the chromosomes have been distributed incorrectly, resulting in malformed offspring.

Chapter 4 shows that the three different fish families with defects in mismatch repair are indeed more sensitive to the development of cancer. The tumour frequency is reasonably low, and the cancer usually develops not before the end of the fish life, but this is quite normal for fish. Different types of tumours do develop, of which some resemble the types of cancers of people with the same familial predisposition.

Chapter 5 studies the cellular reaction on treatment with two compounds that are both cancer-inducing and used for chemotherapy. In normal fish embryos this leads to breaks in their chromosomes, but not in embryos that lack mismatch repair. It is the first time this can be studied in living organisms and not just in cells.

In **chapter 6**, finally, we use adult fish and one of the substances from chapter 5 for a technical application. When the chemical-induced, unrepaired DNA-damage also occurs in the germ cells of a fish, this could lead to inactivation of genes in progeny. This could be an efficient way to create fish lacking a specific mechanism, in order to study for example other diseases.

Samenvatting

Beschadingen in het DNA hopen zich langzaam op gedurende een mensenleven, waardoor veroudering optreedt. Wanneer echter bij toeval meerdere beschadigingen in één cel noodzakelijke beschermingsmechanismen uitschakelen, kan dit leiden tot ongeremde deling van deze cel, waardoor een tumor ontstaat. Normaalgesproken wordt DNA-schade zoveel mogelijk tegengegaan door allerlei reparatiesystemen. Als een daarvan ontbreekt, kan dat het kankerproces versnellen. Dan is er sprake van erfelijke aanleg voor kanker.
De gangbare behandeling van kanker maakt gek genoeg juist gebruik van het aanbrengen van zoveel mogelijk DNA-schade door bestraling of chemotherapie. Dit werkt omdat grote hoeveelheden DNA-schade vooral de celdeling afremmen, waardoor de cel doodgaat. Zich vaak delende kankercellen zijn hiervoor dus erg gevoelig, terwijl normale cellen, die zich in een volwassen mens relatief weinig delen, gewoon blijven leven.

In dit proefschrift wordt gebruik gemaakt van de zebravis als laboratoriumdier. De zebravis werd voorheen vooral gebruikt om de vroegste ontwikkeling van een dier te bestuderen. Het heeft namelijk als groot voordeel dat de nakomelingen als doorzichtige eitjes gelegd worden en dus buiten de moeder opgroeien. Daardoor is de ontwikkeling vanaf de allereerste celdeling tot aan een volgroeid embryo te volgen. Terwijl de vis op deze manier in het lab bestudeerd werd, ontdekte men dat ook de volwassen beesten in veel opzichten op de mens lijken, en dus model kunnen staan. Het doel van dit onderzoek was om te kijken of de zebravis geschikt kan zijn voor het bestuderen van DNA-reparatie en kanker. Daartoe heb ik gebruik gemaakt van drie vissenstammen die componenten missen van een belangrijk mechanisme voor de reparatie van kleine DNA-schade, de zogenaamde mismatch-reparatie. Ze hebben hierdoor erfelijke aanleg voor het ontwikkelen van kanker. Ik laat zien dat de werking van mismatch-reparatie met betrekking tot de vorming van geslachtscellen, het ontstaan van tumoren en de reactie op chemotherapeutische stoffen vergelijkbaar is met die in de mens en de muis. Dit betekent dat de zebravis als bruikbaar model beschouwd mag worden. Er zijn echter op al deze gebieden ook duidelijke verschillen. In dit geval zijn die minstens zo belangrijk, omdat ze ons in staat stellen nieuwe aspecten van kanker te bestuderen - onderzoek dat in de muis en de mens niet mogelijk is.

Hoofdstuk 1 geeft een overzicht van het huidige onderzoek naar kanker in de zebravis. Hoewel vrijwel alle publicaties hierover van na het jaar 2000 stammen, zijn het er

inmiddels al meer dan 50, wat de sterke groei van het onderzoeksveld goed weergeeft. Specifiek voor de zebravis is de eenvoud van behandeling met kankerverwekkende of kankerremmende stoffen, door deze simpelweg in het aquariumwater op te lossen. Dit kan van grote waarde zijn in de zoektocht naar nieuwe medicijnen.

Hoofdstuk 2 behandelt de rol van een specifieke mismatch-reparatie component in meiose, de celdeling die leidt tot geslachtscellen met maar de helft van het normale aantal chromosomen. Ik laat zien dat de afwezigheid van deze component in vrouwtjesvissen zorgt voor nakomelingen met afwijkende aantallen chromosomen, die misvormd zijn en vaak binnen enkele dagen sterven. Dit is in zoogdieren nauwelijks te bestuderen, omdat mislukte eicellen meestal meteen in de baarmoeder opgeruimd worden.

Hoofdstuk 3 is een vervolg op hoofdstuk 2. Mannelijke familieleden van de vrouwtjes in hoofdstuk 2 zijn compleet onvruchtbaar, maar in een andere genetische achtergrond hebben ze toch enkele volgroeide zaadcellen. Ook in deze cellen zijn de chromosomen echter verkeerd verdeeld, met misvormde nakomelingen als gevolg.

Hoofdstuk 4 laat zien dat de drie verschillende vissenstammen met defecte mismatch-reparatie inderdaad gevoeliger zijn voor de ontwikkeling van kanker. De tumorfrequentie is vrij laag, en de kanker ontwikkelt zich vaak pas helemaal aan het einde van het vissenleven, maar dat is voor vissen vrij normaal. Er ontwikkelen zich verschillende types tumoren, waarvan sommige lijken op de kankers die mensen met dezelfde erfelijke afwijking krijgen.

Hoofdstuk 5 kijkt naar de cellulaire reactie op de behandeling met twee stoffen die kankerverwekkend zijn maar ook voor chemotherapie gebruikt worden. In normale vissenembryo's leidt dit tot breuken in hun chromosomen, maar in embryo's die mismatch-reparatie missen niet. Dit is voor het eerst dat dit in een levend organisme en niet alleen in losse cellen bestudeerd kan worden.

In **hoofdstuk 6**, tot slot, gebruiken we volwassen vissen en een van de stoffen uit hoofdstuk 5 voor een technische toepassing. Wanneer de chemisch-geïnduceerde, niet-gerepareerde DNA-schade ook in de geslachtscellen van een vis optreedt, zou dit kunnen leiden tot de uitschakeling van genen in nakomelingen. Zo kunnen op een efficiënte manier vissen gecreëerd worden die een bepaald mechanisme missen, waardoor bijvoorbeeld andere ziektes bestudeerd kunnen worden.

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Harma

List of publications

Feitsma H, Kuiper RV, Korving J, Nijman IJ, Cuppen E. Zebrafish with mutations in mismatch repair genes develop neurofibromas and other tumours.
Cancer Research; in press

Feitsma H, de Bruijn E, van de Belt J, Nijman IJ, Cuppen E. Mismatch repair deficiency does not enhance ENU mutagenesis in the zebrafish germ line.
Mutagenesis; in press

Feitsma H, Cuppen E. Zebrafish as a cancer model.
Molecular Cancer Research; in press

Leal MC*, **Feitsma H***, Cuppen E, França LR, Schulz RW. Completion of meiosis in male zebrafish (Danio rerio) despite lack of DNA mismatch repair gene mlh1.
*equal contribution
Cell and Tissue Research; 2008 Apr; 332(1):133-9

Blaschke RJ, Hahurij ND, Kuijper S, Just S, Wisse LJ, Deissler K, Maxelon T, Anastassiadis K, Spitzer J, Hardt SE, Schöler H, **Feitsma H**, Rottbauer W, Blum M, Meijlink F, Rappold G, Gittenberger-de Groot AC. Targeted mutation reveals essential functions of the homeodomain transcription factor Shox2 in sinoatrial and pacemaking development.
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Kuijper S, **Feitsma H**, Sheth R, Korving J, Reijnen M, Meijlink F. Function and regulation of Alx4 in limb development: complex genetic interactions with Gli3 and Shh.
Developmental Biology; 2005 Sep 15; 285(2):533-44

Submitted publications

Feitsma H, Akay A, Cuppen E. Alkylation damage causes MMR-dependent chromosomal instability in vertebrate embryos. *Submitted*

de Bruijn E, Cuppen E, **Feitsma H.** Highly efficient ENU mutagenesis in zebrafish.
Invited chapter in Methods in Molecular Biology; Submitted

Wansleeben C, **Feitsma H**, Tertoolen L, Guryev V, Cuppen E, Meijlink F. A novel mutant allele of Ncx1: A single amino acid substitution leads to both placental and cardiac dysfunction. *Submitted*

Curriculum vitae

Harma Feitsma werd geboren in Veenwouden op 23 augustus 1979. Haar VWO-opleiding voltooide ze in 1997 aan het Lauwerscollege in Buitenpost. Ze ging vervolgens Bioprocestechnologie studeren aan Wageningen Universiteit, waarbij ze onderzoeksstages deed in de vakgroep Virologie van Wageningen Universiteit onder begeleiding van Dr. Richard Kormelink en Prof. Dr. Rob Goldbach, aan het Hubrecht Instituut onder begeleiding van Dr. Sanne Kuijper en Dr. Frits Meijlink, en in Zwitserland aan het Instituut voor Cel Biologie van de ETH in Zürich onder begeleiding van Dr. Isabelle Weiss en Prof. Dr. Isabelle Mansuy. Ze studeerde af in 2003 en begon aansluitend aan het in dit proefschrift beschreven promotieonderzoek bij Prof. Dr. Edwin Cuppen aan het Hubrecht Instituut.

Harma Feitsma was born in Veenwouden on August 23rd 1979. She completed her secondary education in 1997 at the Lauwerscollege in Buitenpost. Next, she started studying Bioprocesstechnology at Wageningen University, for which she performed research projects at the Department of Virology of Wageningen University under supervision of Dr. Richard Kormelink and Prof. Dr. Rob Goldbach, at the Hubrecht Institute under supervision of Dr. Sanne Kuijper and Dr. Frits Meijlink, and in Switzerland at the Institute of Cell Biology of the ETH in Zurich under supervision of Dr. Isabelle Weiss and Prof. Dr. Isabelle Mansuy. She graduated in 2003 and subsequently started the PhD research that is described in this thesis with Prof. Dr. Edwin Cuppen at the Hubrecht Institute.

De vis als ziektemodel

Komt een vis bij de dokter, zegt de dokter: "Ik zie het al, uit de kom!"