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A genetic screen for mutations affecting embryonic development in medaka fish (*Oryzias latipes*)

Felix Loosli[1,2], Reinhard W. Köster[1,3], Matthias Carl[2], Ronald Kühnlein[4], Thorsten Henrich[2], Manuela Mücke, Annette Krone[2], Joachim Wittbrodt[2,*]

SFB 271 Junior Group, c/o Max-Planck Institute for Biophysical Chemistry, Am Fassberg, 37077 Göttingen Germany

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Abstract

In a pilot screen, we assayed the efficiency of ethylnitrosourea (ENU) as a chemical mutagen to induce mutations that lead to early embryonic and larval lethal phenotypes in the Japanese medaka fish, *Oryzias latipes*. ENU acts as a very efficient mutagen inducing mutations at high rates in germ cells. Three repeated treatments of male fish in 3 mM ENU for 1 h results in locus specific mutation rates of 1.1–1.95 $\times 10^{-3}$. Mutagenized males were outcrossed to wild type females and the F1 offspring was used to establish F2 families. F2 siblings were intercrossed and the F3 progeny was scored 24, 48 and 72 h after fertilization for morphological alterations affecting eye development. The presented mutant phenotypes were identified using morphological criteria and occur during early developmental stages of medaka. They are stably inherited in a Mendelian fashion. The high efficiency of ENU to induce mutations in this pilot screen indicates that chemical mutagenesis and screening for morphologically visible phenotypes in medaka fish allows the genetic analysis of specific aspects of vertebrate development complementing the screens performed in other vertebrate model systems.

Keywords: Medaka fish; Oryzias latipes; Genetic screen; Embryonic development; Eye development

1. Introduction

The detailed current understanding of plant and animal development is largely due to systematic mutagenesis screens. Large scale genetic screens in *Arabidopsis* (Mayer et al., 1991), *C. elegans* (Brenner, 1974) and *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980) have led to the identification of complex genetic pathways required for embryonic development. These screens were based on the identification of morphologically visible alterations in mutant embryos. Recently, large-scale mutagenesis screens were carried out in a vertebrate model system, the zebrafish (Driever et al., 1996; Haffter et al., 1996). The transparent, externally fertilized embryos allowed to systematically screen for mutations that affect embryonic and early larval development. A pilot mutagenesis screen in mouse has recently been reported (Kasarskis et al., 1998).

The chemical mutagen used in these vertebrate screens was N-ethyl-N-nitrosourea (ENU), which has been shown to efficiently induce mutations in mouse and zebrafish (Mullins et al., 1994; Russell et al., 1979; Solnica-Krezel et al., 1994). The specific locus rate for ENU is similar in both organisms and was shown to be in the range of 3.9×10^{-3} to 0.5×10^{-3} (Driever et al., 1996; Hitotsumachi et al., 1985). ENU predominantly induces point mutations and thus results in mutations that are limited to single genes.

Although many structural and developmental features are conserved between vertebrate species, they exhibit a diverged morphology and embryonic development. To identify the basic molecular mechanisms underlying development it is necessary to study different (not closely related) vertebrate species. Medaka (*Oryzias latipes*), a well established genetic vertebrate model system (Yamamoto, 1975) is a small fresh water fish that, similar to the zebrafish,

* Corresponding author. EMBL, Developmental Biology Programme, Meyerhofstrasse 1, 69117 Heidelberg, Germany. Tel.: +49-6221-387-576; fax: +49-6221-387-166.

E-mail address: jochen.wittbrodt@embl-heidelberg.de (J. Wittbrodt).

[1] These authors contributed equally to the work.

[2] Present address: European Molecular Biology Laboratory, Developmental Biology Programme, Meyerhofstrasse 1, 69117 Heidelberg, Germany.

[3] Present address: California Institute of Technology, Beckman Institute (139-74), Pasadena, CA 91125, USA.

[4] Present address: Max-Planck-Institute for Biophysical Chemistry, Department for Molecular Developmental Biology, Am Fassberg, 37077 Göttingen, Germany.

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combines several advantages that allow to systematically screen for mutations resulting in morphological alterations.

The transparent eggs are fertilized externally. Synchronous embryonic development takes 7 days and the generation time is 8 weeks. The hatched fry is autotrophic. A comparison of different mutagens showed that the highest mutagenesis rates are obtained using ENU with a gene specific mutation rate of 1×10^{-3} (Shima and Shimada, 1991) and ENU induced mutants have been reported (Ishikawa, 2000).

Here we describe the pilot three-generation screen (Fig. 1) to isolate mutations that lead to a morphologically visible phenotype in the developing eye. The pilot screen resulted in the isolation of 48 recessive mutations that affect embryonic development by morphological criteria. Of 33 mutations that affect eye development, 28 also resulted in defects in the brain, whereas the morphologically visible phenotypes of the remaining 5 mutations were restricted to the eye. These results show that chemical mutagenesis in medaka is a practical means of identifying genes involved in specific processes of vertebrate development.

2. Results

The aim of our pilot screen was to establish the conditions for a systematic screen for mutations that affect eye development in the teleost medaka (*Oryzias latipes*). Test crosses of mutagenized males to homozygous mutant albino and *AA2* females (Shima and Shimada, 1991), respectively

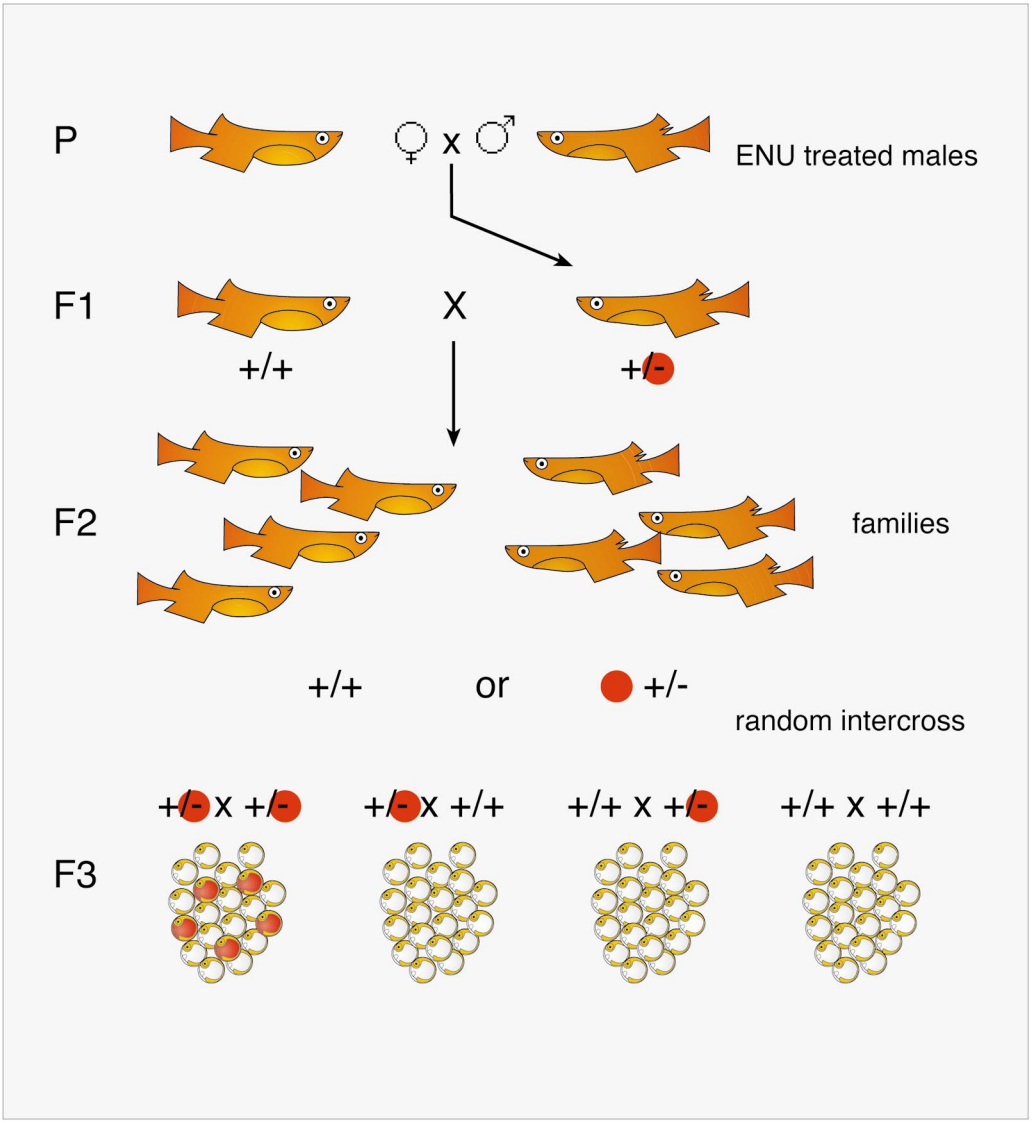

Fig. 1. Mutagenesis scheme. Males were mutagenized with ENU and crossed to wild-type females four weeks after the treatment. From each individual male 10–20 F1 offspring fish were used to establish F2 families by either crossing them to wild type fish or by F1 intercrosses. Mutations induced by the treatment are eventually uncovered in the F3 generation after random intercrosses within the F2 families. F3 siblings of clutches with phenotypes segregating in a Mendelian fashion were kept for the establishment of mutant lines.

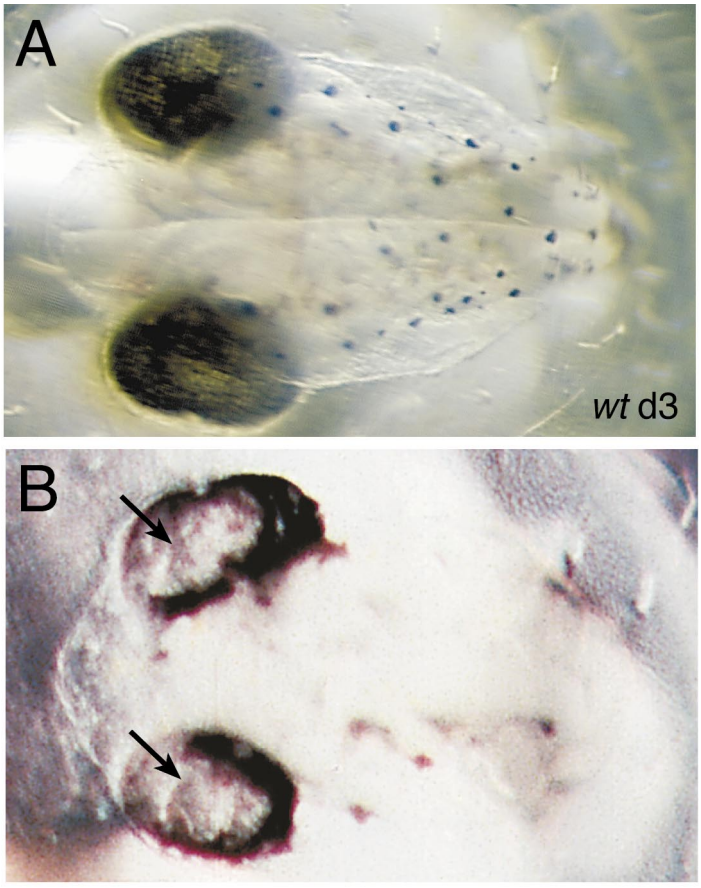

Fig. 2. Mosaicism due to heteroduplexes in sperms (A,B) Three-day-old embryos obtained from a cross of a mutagenized male to a homozygous albino female. (B) Embryo mosaic for an ENU induced mutation in the albino locus shows patches of mutant tissue (arrows) in the pigmented retinal epithelium.

resulted in embryos mosaic for the phenotype in some cases (Fig. 2). The occurrence of mosaic offspring decreased in the following 2 weeks and was no longer observed after 4 weeks of continuous mating. Similar results have been reported in test crosses of ENU mutagenized zebrafish and mice (Mullins and Nüsslein-Volhard, 1993; Solnica-Krezel et al., 1994). These observations have been explained by ENU induced single strand modifications in post replicative sperm cells, leading to mosaicism in the offspring (Russell et al., 1988). DNA modifications, on the other hand, that occurred premitotically can become fixed as mutations during subsequent DNA divisions.

To assess the frequency of mutations induced in the ENU treatment a simple complementation assay was used. Mutagenized males were crossed to females of the tester strain *AA2*(Shima and Shimada, 1991). This triple mutant strain is homozygous for viable mutations in *b* (amelanotic melanophores), *lf* (no leucophores) and *gu* (no guanophores) respectively, resulting in pigmentation phenotypes which can be easily scored. A mutation in one of these genes induced in the germline of the G0 fish will lead to a non-complementation and to an F1 fish exhibiting the respective phenotype. Non-complementation was assayed in the progeny of the ENU-treated males mated to the tester females for 8 weeks starting 4 weeks after the ENU treatment. The ENU treatment applied in our pilot screen resulted in an average rate of one specific mutation in 700 screened genomes (Table 1). Thus, the locus specific mutagenesis test in our screen revealed a rate that is comparable to those reported for zebrafish and mouse.

A three generation screen was employed, similar to those used in the zebrafish mutagenesis screens (Fig. 1; Driever et al., 1996; Haffter et al., 1996). This crossing scheme allows to test the segregation of recessive mutations and to identify heterozygous carrier pairs in the F2 generation. We screened the F3 offspring of 213 F2 families from a total number of 340 F2 families established. Due to the use of F2 families resulting from F1 sibling crosses (cxc and axc crosses, see Section 4), the number of screened haploid genomes is 313. A total of 142 mutations with Mendelian

Table 1
Locus specific mutation rates

Locus	Genomes screened	Recovered alleles	Specific locus rate
b	1820	2	1.10×10^{-3}
lf	1536	3	1.95×10^{-3}
gu	1536	2	1.30×10^{-3}

Table 2
Classes of identified mutants

Mutants	Number	%
Total identified	142	–
Mutants kept	48	100
Eyeless	1	2
Cyclopia	2	4
Abnormal lens development	3	6
Retina and lens affected	3	6
Abnormal shape and size of eye	20	42
Pigmentation of retinal epithelium	3	6
Necrotic eye	1	2
Others	15	31

segregation were identified (Table 2). Ninety-four mutations showed general abnormalities of development, e.g. lethality prior to gastrulation, general necrosis or retarded development. Of 48 mutations resulting in a specific morphological alteration, 28 exhibited a morphologically visible phenotype in the developing eye and brain. In addition five mutations specifically affected eye development but not other parts of the developing embryo. The remaining 15 mutants showed specific defects in other body parts. Two mutations were novel alleles of previously isolated medaka mutations. In *Da* mutant embryos the dorsal trunk region is ventralized, resulting in a mirror image duplication of the ventral trunk region (Fig. 3I). This mutation did not complement a previously isolated mutation leading to the same

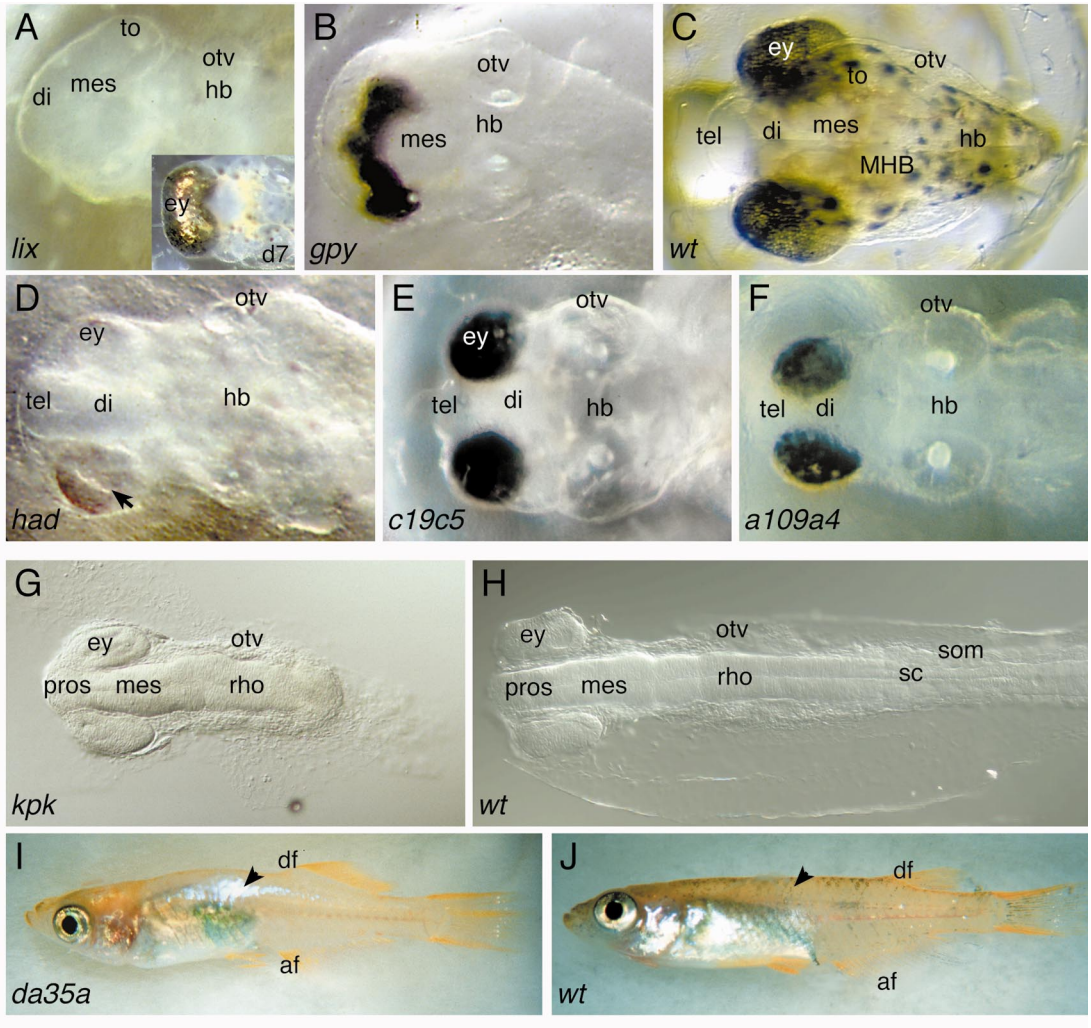

Fig. 3. Examples of phenotypes isolated in the pilot screen. Embryos at day 1 (G,H) and day 3 (A–F) of development as well as adult fish (I,J) with wild-type (C,H,J) and mutant morphology (A,B,D–G,I). (A) *Lix* mutant embryos show strong cyclopia, no obvious eyes form at day 3. Inset: embryo with single cyclopic eye at day 7 (B) In *gpy* mutants pigmented retinal epithelium extends into the optic stalk, the mesencephalon is reduced and a morphologically visible MHB does not form. (D) *had* mutant embryos form slit like cavities in the eyecup (arrow), lack lenses and exhibit severe brain defects. (E,F) Examples of late phenotypes affecting eye morphology as well as brain morphology. (G) *koepke* mutant embryos do not form trunk and tail tissue posterior to the hindbrain. (I) *da* mutants show a dorsal mirror image duplication of the ventral body side. Note the shiny pigmentation on the back of the mutant (arrowhead) and the huge dorsal fin exhibiting anal fin morphology. Abbreviations: af, anal fin; df, dorsal fin; di, diencephalon; ey, eye; hb, hindbrain; mes, mesencephalon; MHB, mid-hindbrain boundary; otv, otic vesicle; pros, prosencephalon; rho, rhombencephalon; sc, spinal cord; som, somites; tel, telencephalon; to, optic tectum.

phenotype (Ishikawa, 1990). *Heino* mutant embryos exhibit albinism. This mutation was non-complementing with the previously isolated albino mutation *i3* (Hong and Schartl, personal communication).

2.1. Mutations affecting eye development

The identified mutations affecting eye development can be assigned to three groups on the basis of the phenotype and its manifestation during eye development. The first group comprises three mutations that result in a phenotype, which is visible already at neurula stages. In one of these mutants the formation and subsequent differentiation of anterior neuroectoderm derivatives including the optic cups are affected resulting in an eyeless phenotype (*c38c8*). The other two mutations of this group result in a cyclopia phenotype (Fig. 3A, *Lix*; *Pom,* not shown). Mutations leading to cyclopia have been described in various vertebrate species and it has been shown that the splitting of the single retinal anlage into two retinal primordia under the influence of midline signaling does not occur (Gritsman et al., 1999; Varga et al., 1999). Thus, it is likely that this process is affected in these medaka cyclopia mutants.

The second group comprises six mutations that affect lens development, in one case the lens is completely lacking. In three of these mutations, retina development is affected as well (Fig. 3B, *guppy* (*gpy*)-*c22c2*; Fig. 3D, *hammerhead* (*had*), *C3c3*; *zorro* - *c18C1*; *a39a1*; *c29C2*, not shown). Studies in amphibian embryos have shown that inductive interactions between the developing retina and lens are required for the proper development of the respective structures (Grainger, 1996). Therefore the phenotype of these three mutants suggests that these inductive events are affected.

The third and largest group encompasses 23 mutations with normal early eye development (Fig. 3E,F). However, subsequent growth is affected and in three cases pigmentation defects become apparent. In several of these mutations also the development of the brain is affected. Therefore it is possible that in these cases neurogenesis rather than specifically retina development is perturbed. In good agreement with this assumption, many genes shown to play a role in eye development also function in specific processes of brain development (reviewed in Jean et al., 1998; Rubenstein et al., 1998).

Of the remaining 15 mutations that affect other body parts one phenotype affecting axis formation was especially striking. In *koepke* (*kpk*) mutant embryos trunk and tail structures are completely lacking, while the head forms normally (Fig. 3G,H). A phenocopy has been generated in Xenopus embryos by the overexpression of a dominant negative FGF-receptor (Amaya et al., 1991).

3. Discussion

We carried out a pilot mutagenesis screen in the Japanese medaka (*Oryzias latipes*) to establish the conditions for a systematic mutagenesis screen and to identify genes that are required for eye development. Our results demonstrate that chemical mutagenesis by an ENU treatment of adult males results in a gene specific mutagenesis rate that is comparable to that reported for mouse and zebrafish.

The viability and fertility of the treated males was such that 40 males were sufficient to carry out all test crosses and to establish over 300 F2 families. Mosaicism in the F1 progeny of mutagenized males was not observed after 4 weeks of continuous mating to a triple mutant tester strain and to an albino strain respectively. Thus, by this time most of the post-meiotically mutagenized sperm cells have been utilized. Thereafter, the majority of the sperms carry fixed mutations, i.e. DNA single strand modifications are complemented during the subsequent DNA replication thus resulting in a fixed mutation. Our observation correlates well with the reported duration of spermatogenesis in *Oryzias latipes* (Egami and Hyodo-Taguchi, 1967). A similar decrease in mosaicism during subsequent matings has also been reported in the zebrafish (Mullins and Nüsslein-Volhard, 1993; Solnica-Krezel et al., 1994).

The morphological criteria applied to screen for phenotypes in the F3 generation were restricted to eye and brain as well as general phenotypes. Nevertheless phenotypes were detected in almost every second genome screened (0.45/genome). The frequencies of phenotypes affecting eye and brain development obtained in the pilot screen presented here (0.11/genome) are slightly higher than those reported for the corresponding class of zebrafish phenotypes (0.06/genome; Driever et al., 1996; Haffter et al., 1996). The overall efficiencies in gene specific mutagenesis rates do not differ significantly between the vertebrate species looked at, indicating that the different phenotype frequencies observed largely depend on the screening criteria.

Screening the F3 progeny of 213 F2 families for morphologically visible phenotypes resulted in the identification of 48 mutations that affect specific processes of embryonic development. In two cases, novel alleles of known mutations were isolated. Of the remaining 46 mutations, 33 affected eye development. The high number of mutations isolated in this pilot screen indicates that chemical mutagenesis and screening for morphologically visible phenotypes in medaka fish provides a useful system for a detailed analysis of specific aspects of vertebrate development. The comparison of the respective mutations in medaka, zebrafish and mouse will provide important information to unravel the evolution of vertebrates and allow to identify evolutionary conserved molecular mechanisms controlling vertebrate development.

4. Material and methods

4.1. Fish strains and fish keeping

A medaka strain (*Cab*) was originally obtained from

Carolina Biological Supply Company (NC) and has been kept in the laboratory as a closed stock under conditions of successive brother sister matings for more than 30 generations. The AA2 strain was obtained from A. Shima (Tokyo). This strain is homozygous for three recessive pigmentation mutations (*b*: amelanotic melanophores, *gu*: no guanophores, *lf*: no leucophores) resulting in the absence of melanin in melanophores and the lack of guanophores and leucophores respectively (Shima and Shimada, 1991). Albino (*i3*) and *Da* mutant fish have been obtained from M. Schartl (Würzburg).

Medaka fish were kept in a system with recirculating water (tap water) under 14 h light/10 h dark cycles at 26°C. Embryos were kept in 1× hatching medium (0.1% NaCl, 0.003% KCl, 0.004% $CaCl_2$, 0.016% $MgSO_4$, 0.0001% Methylen blue) until hatching. The hatchlings were then reared in 1× hatching medium diluted 1:1 with tapwater for the first week and subsequently kept in system water.

4.2. ENU mutagenesis

Solutions containing ENU and contaminated accessories were handled as described elsewhere (Solnica-Krezel et al., 1994). Forty-two adult *Cab* males, tested for fertility, were treated in groups of seven fish in 300 ml of 3 mM ENU (N-ethyl-N-nitrosourea, N-3385, Sigma) with 0.03% sea salt solution adjusted to pH 6.3 with 1 mM phosphate buffer for 1 h at 26°C and subsequently allowed to recover for 2 h in recovery solution (tap water, 0.0001% Methylene blue) with an exchange of the solution after 1 h. This treatment was repeated after 2 and 3 days, respectively. The males were allowed to recover for four days before they were mated in single crosses with *Cab* or albino females for 4 weeks. High embryonic lethality was observed in the first week in F1 offspring of these crosses that gradually declined. Surviving embryos of these initial crosses were not used to establish F1 families in order to prevent mosaicism in the germline of F1 families (see below and also Egami and Hyodo-Taguchi, 1967).

Four weeks after the ENU treatment, individual mutagenized males were bred with *Cab* females and AA2 females respectively to establish F1 families and to determine the locus specific mutagenesis rate (Table 1). Five hundred and twenty-six offspring obtained from crosses of mutagenized males to *Cab* females (c individuals) and 463 offspring from crosses of mutagenized males to *AA2* females (a individuals) were raised to adulthood. The F1 progeny was then crossed to *Cab* fish in single crosses (cxC and axC, respectively). To increase the number of screened genomes, some F1 progeny was crossed inter se in single crosses (cxc and axa). Three hundred and forty F2 families were thus established. Of each family, at least 60 individuals were raised to adulthood.

4.3. Screening procedure

For each F2 family up to eight single pair crosses were set up in small breeding tanks. The eggs of successful matings were collected on three successive days. Rolling clutches in the petri dish individualized eggs by tearing the attachment filaments off the chorion. Separated eggs were kept in 6-well tissue culture plates at 28°C in 1× hatching medium. On average, the offspring of 4.5 single pair crosses per F2 family were analyzed. We screened at least 30 eggs per single pair cross.

Embryos were scored on three successive days for specific phenotypes. After 24 h (approximately stage 18; Iwamatsu, 1994), the neurula stage embryos were inspected for abnormalities in the brain and outbudding optic vesicles. After 48 h (approximately stage 25), the early organogenesis stage embryos were examined for formation of the lens, optic cup and compartmentalization of the brain. After 72 h of development (approximately stage 30), the late organogenesis stage embryos were checked for pigmentation of the body and optic cups, for lens differentiation, cornea formation and size and shape of the optic cups.

F3 offspring of single pair crosses in which a mutation had been identified were raised to adulthood. Carrier fish were identified by random crosses of the F3 siblings. Stocks were kept as outcrossed lines and sperm of carrier males was frozen for long-term storage (Krone and Wittbrodt, 1997).

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