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RAPID COMMUNICATION

Atm deficient zebrafish model reveals conservation of the tumour suppressor function and a role in fertility

Biallelic loss-of-function variants in *ATM* (Ataxia Telangiectasia Mutated) cause Ataxia Telangiectasia (AT), a rare disorder associated with cerebellar degeneration and ataxia, cancer predisposition, infertility, growth retardation, etc. ATM is a phosphoinositide 3-kinase-related kinase (PIKK) with a role in DNA repair and maintenance of genome stability. Studying a multisystem genetic disease like AT requires animal models to ascertain its pathogenesis at the level of tissues, organs and the organism. Due to its small size, cheap maintenance, large progeny, rapid development and initial transparency, zebrafish (*Danio rerio*) is an increasingly popular vertebrate model organism, suitable for genetic modifications and large-scale *in vivo* therapeutic screens as embryos are chemically permeable to small compounds. Currently, no zebrafish model for AT exists.[1] We generated *atm* knock-outs through CRIPSR-Cas9 mutagenesis. We show that *atm* conserved its function as a tumour suppressor gene and is involved in gametogenesis and fertility. Therefore, this mutant is of great value for further studies investigating the role of *atm* in reproduction and tumorigenesis.

Zebrafish *atm* evolved to a single copy after the teleost genome duplication, which appears distinctive for DNA damage repair genes.[2] Human *ATM* and zebrafish *atm* both comprise 62 coding exons with high amino acid conservation in functional domains (Fig. 1A and Table S1). Therefore, ATM functions may be conserved in zebrafish and *atm* deficiency might recapitulate human disease.

Through CRISPR/Cas9 mutagenesis we generated two heterozygous *atm* knock-out mutant zebrafish lines, with premature stop codons in exon 4 (atm^{cmg34}) and 9 (atm^{cmg33}) (Fig. 1A; Fig. S1 and Table S2). For all experiments, these lines were in-crossed to obtain compound heterozygous mutants ($atm^{cmg33/cmg34}$, "*atm*-mutants"), avoiding potential homozygosity of CRISPR-induced off-target mutations. Atm deficiency is not embryonically lethal: offspring of $atm^{cmg33/+}$x$atm^{cmg34/+}$ ($n = 77$) displayed 27% wild types, 51% heterozygotes and 22% mutants (Fig. 1B), in agreement with Mendelian inheritance ($Chi^2 = 0{,}80$). Equal rates of males:females were found for wild types and heterozygotes; however, $atm^{cmg33/cmg34}$ fish developed exclusively as males. This all-male phenotype was found over multiple crosses and generations of *atm*-mutants. *Atm*-mutant males were able to induce egg laying in wild-type females, but eggs remained unfertilized (Fig. S2). H&E (hematoxylin and eosin) stained histological sections of gonads displayed male testes in all *atm*-mutants (Fig. 1C) but lacked spermatids and adult spermatozoa (Fig. 1D), while wild-type testes contained cells at all stages of differentiation during spermatogenesis. Additionally, testes of mutants displayed large groups of spermatocytes in the bouquet phase of meiosis and some spermatocytes appeared pyknotic, suggesting apoptosis. Apoptosis was confirmed by Caspase-3 immunostaining and TUNEL labelling (Fig. 1E; Fig. S3) and indicates that zebrafish *atm* is essential in spermatogenesis.

Zebrafish lack identifiable heteromorphic sex chromosomes. The current model hypothesizes that zebrafish sex is partially determined by the number of primordial germ cells (PGC), with reduced numbers causing zebrafish to become males. The Fanconi anaemia (FA) pathway is required for PGC to survive and thus allow female sex to occur.[2] ATM is attributed multiple roles in homologous recombination (HR), essential for repair of crosslinks through the FA pathway. However, ATM is also implicated in control of meiotic double strand break (DSB) formation. Absence of functional ATM could lead to over-activation of SPO11-mediated DSBs, ultimately leading to apoptosis.[3] The all-male phenotype suggests that PGC of juvenile zebrafish require *atm* for survival. Next, we investigated if apoptosis of PGC causes the all-male phenotype in *atm*-

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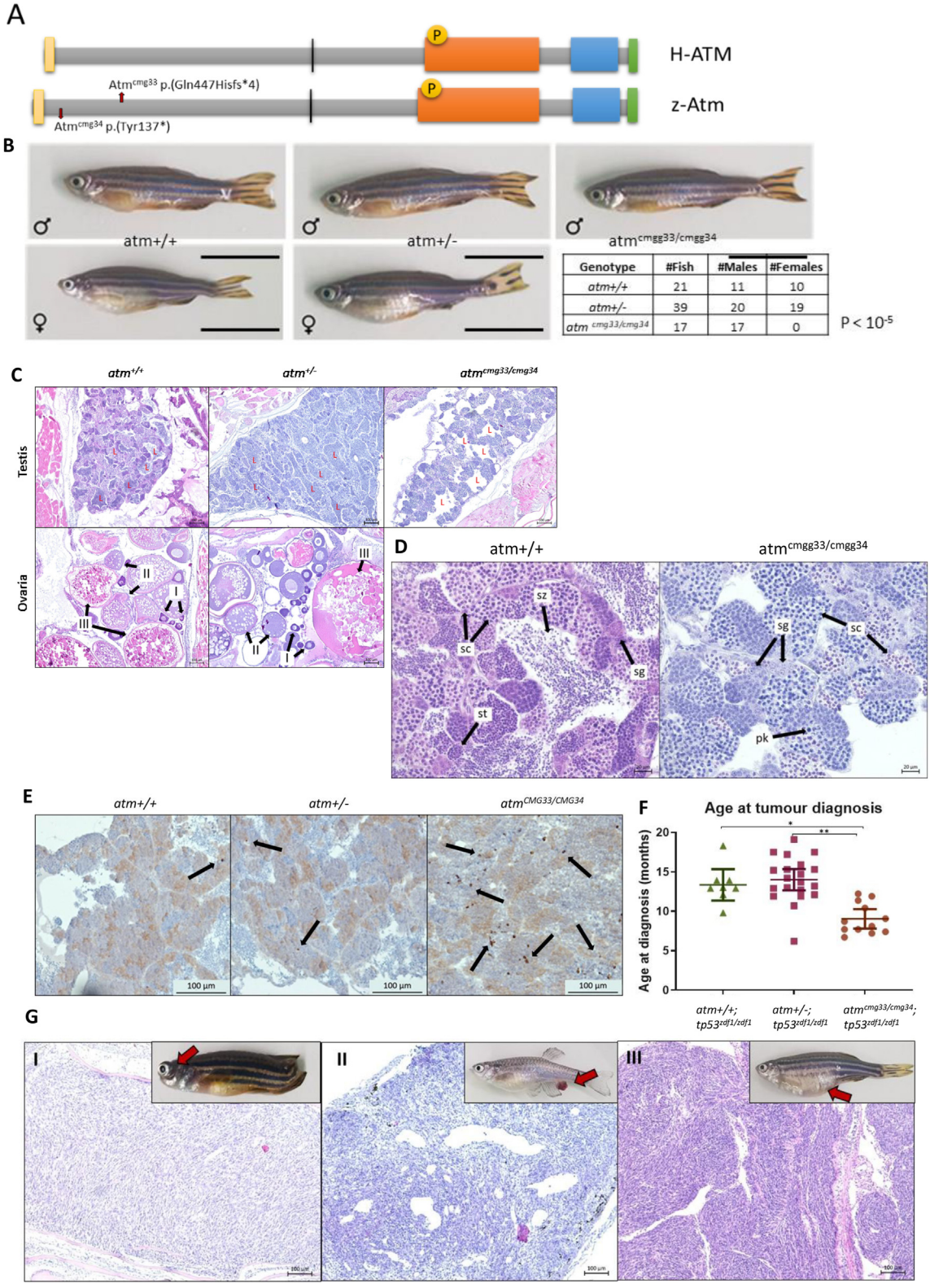

Figure 1 Characteristics of Atm deficient zebrafish. **(A)** Zebrafish and human Atm/ATM structure. *atm* mutations introduced by CRISPR-Cas9 mutagenesis are indicated. Protein domains of human ATM (3056 aa) and zebrafish Atm (3091 aa) are highly conserved. The encoded proteins display an overall AA sequence identity of 54% with a higher conservation for the important functional protein domains (yellow = *TAN* (Tel1/ATM N-terminal motif: LxxxKxxE/DRxxxL); black stripe = *c-ABL*; orange = *FAT* (FRAP-ATM-TRRAP) domain; blue = *PI3K* (phosphoinositide 3-kinase-like) domain; green = *FATC* (FAT C-terminal). These domains display a homology of 77, 78, 60, 82 and 94% respectively, with also Ser1981, an important autophosphorylated site, being preserved at AA position 2012 in zebrafish **(B)** atm deficient zebrafish developed exclusively as males ($P < 10^{-5}$, Fisher's exact test). Mendelian

mutants by disabling p53-mediated apoptosis. Hereto we generated a double knock-out *atm*$^{cmg33/cmg34}$;*tp53*$^{zdf1/zdf1}$ model. *tp53*$^{zdf1/zdf1}$ mutants displayed equal rates of males:females, while *atm*-mutants with a functional *tp53* allele developed exclusively as males (Table S3). However, *atm*$^{cmg33/cmg34}$;*tp53*$^{zdf1/zdf1}$ males could not fertilize eggs of wild-type females (Fig. S4A). Histology confirmed that spermatids and spermatozoa were absent in these zebrafish, similar to *atm*$^{cmg33/cmg34}$; *tp53*$^{+/+}$ *zebra*fish (Fig. S4B). *atm*$^{cmg33/cmg34}$;*tp53*$^{zdf1/zdf1}$ females produced some fertilized eggs, but oocytes likely acquired genomic aberrations, as all embryos died within 1 day post fertilization (Fig. S4C). Histology of the ovaries appeared normal (Fig. S4D). As *atm*$^{+/+}$;*tp53*$^{zdf1/zdf1}$ and *atm*$^{+/-}$;*tp53*$^{zdf1/zdf1}$ females were able to produce healthy offspring, we conclude that *atm* is essential for the viability of zebrafish oocytes. Therefore, *atm* plays a role in zebrafish sex development and fertility. Infertility has been reported in AT individuals and multiple Atm-deficient animal models.

Another key characteristic of AT patients is their increased cancer risk (30%–40% lifetime risk). We sectioned 12 atm-mutants of 16 months old but could not find tumours/neoplasia. This is not surprising, since knocking out tumour suppressors in zebrafish does not cause the same increased cancer risk as in humans: *TP53* and *BRCA2* germline mutations confer a 70%–100% cancer risk in humans. In zebrafish, *tp53* deficiency caused a 40% cancer risk,[4] and only 30% of *brca2*-deficient zebrafish displayed testicular neoplasia at 16 months.[5] To accelerate tumour formation, we investigated *atm*-deficiency in a *tp53*-mutated background. *atm*$^{cmg33/cmg34}$;*tp53*$^{zdf1/zdf1}$ zebrafish developed tumours much faster (average: 9.0 months) compared to *atm*$^{+/-}$;*tp53*$^{zdf1/zdf1}$ (average: 14,1 months, $p < 10^{-4}$) or *atm*$^{+/+}$;*tp53*$^{zdf1/zdf1}$ zebrafish (average: 13,4 months, $p < 1\ 0^{-3}$) (Fig. 1F and Table S4), suggesting that *atm* conserved its tumour suppressor role and that *atm* deficiency accelerated tumorigenesis in *tp53*-mutant zebrafish. Malignant peripheral nerve sheath tumours (MPNST), undifferentiated spindle cell sarcomas (US) and seminomas were found (Fig. 1G), which are standard for *tp53*^{zdf1}. AT patients regularly develop leukaemia, which we did not observe in *atm*-mutants. Similarly, *brca2*$^{-/-}$ zebrafish do not present with haematological tumours while Fanconi anaemia patients develop such malignancies. Studying leukaemia in *atm*-mutants may require transgenic lines containing oncogenic drivers or another type of *tp53* variant: *tp53*^{zdf1} contains a missense variant and MPNSTs are typically found, while *tp53*$^{del/del}$ harbours a deletion and showed a high proportion of leukaemia.[4]

The ataxia-phenotype of AT patients was not reproduced in zebrafish *atm*-mutants, based on swim movements in a rotating chamber at the age of 6 or 12 months (Fig. S5A). This is in contrast to some murine, rat and porcine models. We cannot rule out that degenerative phenotypes only manifest at later ages. Alternatively, *atm*-deficient zebrafish may display a subtle phenotype, requiring more sophisticated methods, like studying the optokinetic response, as AT patients have trouble reading due to impaired coordination of the eye movement. The cerebellum of AT patients displays neurodegeneration, hallmarked by loss of Purkinje cells. Histological analysis of the cerebellum in aged *atm*-mutants revealed a normal Purkinje cell count and molecular layer thickness (Fig. S6A). Also in murine models, histology was unable to show abnormal cerebellar architecture. A higher complexity of the brain may be needed for *atm* to play a major role in neurodegeneration. To come to definite conclusions additional tests with larger sample sizes and zebrafish of different ages may be required.

In contrast to human AT patients, growth retardation was not displayed in adult (12 months old) *atm*-deficient zebrafish compared to age-matched wild-type control males (Fig. S7). Generally, zebrafish with loss of DNA damage response (*DDR*) genes lack this phenotype. Zebrafish may suffer less from dysphagia, which in children leads to reduced caloric intake and compromises growth. Other explanations could be a role of modifier genes, or a lack of environmental challenges in laboratory settings. We cannot fully exclude residual functionality of the mutant protein as suitable Atm antibodies to check for residual protein were not available.

In conclusion, despite the absence of a clear neurodegenerative phenotype, we show that zebrafish *atm* is required for sex-determination and fertility and acts as a tumour suppressor gene. The model could be useful to discern the role of this kinase in genome stability maintenance and tumour suppression and may be used to discover novel compounds targeting ATM deficiency.

inheritance laws rule out embryonic lethality. Black line = 1 cm. **(C)** Histology of zebrafish gonads. Zebrafish *atm* wild types and heterozygotes have fully functional gonads. Oocytes in all stages of maturation are present, while testes have adult sperm in their lumen. Zebrafish *atm* mutants do not develop ovaria. Zebrafish *atm* testis are devoid of spermatids and spermatozoa. Red L = *lumen*, I = *Stage I oocytes*, II = *Stage II oocytes*, III = *Stage III oocytes*. **(D)** Wild-type testis has spermatogonia, spermatocytes, spermatids and spermatozoa. In contrast, *atm*-mutants lack spermatids and spermatozoa in their lumen. Spermatocytes are arrested in the bouquet phase of meiosis, and some spermatocytes appear pyknotic. sg: spermatogonia, sc: spermatocytes, st: spermatids, sz: spermatozoa, pk: pyknosis. **(E)** Caspase-3 immunostaining of testis clearly shows cleaved caspase-3 positive apoptotic cells in *atm*-mutants. Black arrows indicate cells undergoing apoptosis. *atm*$^{cmg33/cmg34}$;*tp53*$^{zdf1/zdf1}$ mutant zebrafish are prone to develop tumours. **(F)** Age of tumour onset is significantly lower in *atm*$^{cmg33/cmg34}$;*tp53*$^{zdf1/zdf1}$ zebrafish compared to *atm*$^{+/-}$;*tp53*$^{zdf1/zdf1}$ and *atm*$^{+/+}$;*tp53*$^{zdf1/zdf1}$ zebrafish. *: $P < 10^{-3}$, **: $P < 10^{-4}$. Unpaired *t*-test was used. Error bars indicate 95% Confidence Interval (CI). In **GI-III** fish with protruding tumours are shown (inset: tumour location indicated with red arrow; main figure: showing histopathological image of tumour **(G.I)** Malignant peripheral nerve sheath tumour (MPNST) in the eye of a double mutant zebrafish. The tail of this zebrafish was deformed since early development **(G.II)** undifferentiated spindle cell sarcoma (US) growing in the tail **(G.III)** MPNST in the coelom invading the intestinal lining.

Conflict of interests

Authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.04.023.

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