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FAN1 protects against repeat expansions in a Fragile X mouse model.

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Abstract

The Fragile X-related disorders (FXDs) are members of a large group of human neurological or neurodevelopmental conditions known as the Repeat Expansion Diseases. The mutation responsible for all of these diseases is an expansion in the size of a disease-specific tandem repeat tract. However, the underlying cause of this unusual mutation is unknown. Genome-wide association studies have identified single nucleotide polymorphisms (SNPs) in the vicinity of the *FAN1* (MIM 613534) gene that are associated with variations in the age at onset of a number of Repeat Expansion Diseases. FAN1 is a nuclease that has both 5'-3' exonuclease and 5' flap endonuclease activities. Here we show in a model for the FXDs that $Fan1^{-/-}$ mice have expansions that, in some tissues including brain, are 2-3 times as extensive as they are in $Fan1^{+/+}$ mice. However, no effect of the loss of FAN1 was apparent for germ line expansions. Thus, FAN1 plays an important role in protecting against somatic expansions but is either not involved in protecting against intergenerational repeat expansions or is redundant with other related enzymes. However, since loss of FAN1 results in increased expansions in brain and other somatic tissue, FAN1 polymorphisms may be important disease modifiers in those Repeat Expansion Diseases in which somatic expansion contributes to age at onset or disease severity.

Keywords

FMR1-related disorders (*FMR1* disorders); FX-associated tremor and ataxia syndrome (FXTAS); FX-associated primary ovarian insufficiency (FXPOI); Fragile X syndrome (FXS); Repeat Expansion; Mismatch repair; 5' flap endonuclease activity; 5'-3' exonuclease activity

1. Introduction

The Fragile X related disorders (FXDs) result from expansion of a CGG-repeat tract located at the 5' end of the transcript of the *FMR1* gene. These disorders, which include Fragile X

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

syndrome (MIM# 300624), Fragile X-associated tremor/ataxia syndrome (MIM# 300623) and Fragile X-associated primary ovarian insufficiency (MIM# 311360), belong to a larger group of genetic disorders known as the Repeat Expansion Diseases, that includes Huntington Disease (HD) and many of the Spinocerebellar ataxias (SCAs). The mechanism of expansion in these disorders is not well understood nor are the factors that protect against such expansions.

Genome-wide association studies (GWAS) have identified SNPs in the vicinity of FANCD2 and FANCI Associated Nuclease 1 (*FAN1*) gene (MIM* 613534) as being associated with variation in the age at onset (AAO) of a number of the CAG-repeat expansion diseases [1-3]. FAN1 is an evolutionarily conserved nuclease whose biological roles are not fully understood. It has a C-terminal virus-type replication repair nuclease (VVR_NUC) domain and, in complex eukaryotes, an N-terminal ubiquitin-binding zinc finger (UBZ) domain. The UBZ domain is important for interaction with components of the Fanconi anemia (FA) pathway, while the VRR_NUC domain contains a catalytic PD-(D/E)XK nuclease motif characteristic of many endonucleases. FAN1 cleaves 5' flaps endonucleolytically and acts exonucleolytically on both 5' and 3' flaps, as well as double-stranded DNA substrates [4]. FAN1 was first identified based on its ability to repair interstrand crosslinks (ICLs) [5]. However, despite its name, FAN1 is not epistatic with the Fanconi anemia (FA) pathway [6-8] and a loss of FAN1 is associated with Karyomegalic Interstitial Nephritis (MIM# 614817) in humans [9] and mice [10-12] rather than FA. In addition to a role in the repair of ICLs, FAN1 is also recruited to stalled replication forks independently of ICLs [8, 13]. FAN1 also interacts with the mismatch repair protein MLH1 [14, 15] and is required for efficient homologous recombination but not for double-strand break resection [15].

Here we show that FAN1 also protects against repeat expansion in a FXD mouse model. Our previous data suggests that expansion occurs via a replication-independent process triggered by oxidative damage [16], that involves the interaction between the base excision repair (BER) machinery [17] and components of the mismatch repair (MMR) machinery [18-20]. Thus, the effects of FAN1 on repeat expansion suggests yet another way that this protein contributes to the maintenance of genome integrity.

2. Materials and methods

2.1 Reagents and services

All reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Primers were from Life Technologies (Grand Island, NY). Capillary electrophoresis of fluorescently labeled PCR products was carried out by the Roy J Carver Biotechnology Center, University of Illinois (Urbana, IL).

2.2 Mouse breeding and maintenance

The generation of the FXD mice was described previously [21]. *Fan1* mice were produced from cells generated by the National Institutes of Health (NIH) Knock-Out Mouse Program (KOMP) at University of California, Davis, and generously provided to us by Dr. Rannar Airik of the University of Pittsburgh [10]. All mice were on a C57BL/6 background. Mice

were maintained in accordance with the guidelines of the NIDDK Animal Care and Use Committee, who approved this research (ASP-K021-LMCB-15) and consistent with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996).

2.3 DNA isolation

DNA from mouse tails at 3-week-old was extracted for genotyping using KAPA Mouse Genotyping Kit (KAPA Biosystems, Wilmington, MA). DNA was isolated from different organs of 3-month and 6-month old mice using a Maxwell®16 Mouse Tail DNA Purification kit (Promega, Madison, WI) according to the manufacturer's instructions.

2.4 Genotyping and analysis of repeat number

Fan1 genotyping was carried out as previously described [10]. *Fmr1* PM allele genotyping and repeat size analysis was carried out as described previously [20]. The PCR products were resolved by capillary electrophoresis on an ABI Genetic Analyzer [22]. The resultant fsa file was then displayed using a custom R script [23] that is available on request. This allows the repeat number to be determined in the tail DNA taken at weaning from the progeny of $Fan1^{+/+}$ and $Fan1^{-/-}$ parents and compared to the tail DNA taken at weaning of the parents. The extent of somatic expansion was assessed in two ways. The number of repeats added to the original allele was determined by comparison of the repeat number in each organ, as determined by capillary electrophoresis, to the repeat number present in the allele in heart. The heart shows little/no postnatal expansion and thus reflects the number of repeats present in the original allele. The previously described Somatic Instability Index (SII) was also determined as previously described [24] with minor modifications [17]. Fisher's exact test and Student's t test were carried out using the GraphPad QuickCalcs website (http://www.graphpad.com/quickcalcs). Mann-Whitney U tests were carried out using the Vassarstats website (vassarstats.net). The t test and U test were always concordant and so for simplicity sake only the t test p values are reported.

2.5 *Fan1* mRNA quantitation

Mouse tissues were homogenized using Precellys lysing kits (Bertin Technologies, Rockville, MD). Total RNA was isolated using Maxwell® 16 LEV simplyRNA tissue Kits (Promega) according to the manufacturer's instructions. The RNA was quantitated using a Nanodrop Spectrophotometer (Denovix). Reverse transcription of RNA was done using a SuperScript™ VILO™ cDNA synthesis kit (Life Technologies). Real-time PCR was done in triplicate using TaqMan® Fast universal PCR master mix and the Taqman probe-primer pairs Mm00625959_m1 (Life Technologies). For comparison of mRNA expression in different tissue, equal amounts of RNA were used for each determination since there is no good endogenous control for different tissues. Normalization was carried out by comparing the Ct value for the mRNA in different organs to the Ct value obtained from heart.

3. Results

3.1 FAN1 protects against repeat expansion in somatic tissues of the FXD mouse.

Comparison of the repeat PCR profiles in the tissues of 3-month old and 6-month old male *Fan1*$^{+/+}$ and *Fan1*$^{-/-}$ mice shows a larger increase in the extent of expansion in the livers and brains of the *Fan1*$^{-/-}$ mice at both ages (Fig. 1). In particular, the repeat PCR profiles from the liver of the 3-month old *Fan1*$^{-/-}$ mouse already shows a bimodal distribution of repeat sizes that is only beginning to become apparent in the 6-month old *Fan1*$^{+/+}$ mouse. A bimodal profile arises in tissues like liver where some cell types expand whilst others do not. Over time, as repeat tract continues to increase in size in the expansion-prone cells, there is a decrease in the extent of overlap between the PCR products from the expansion-prone cell population and those from the cells that do not expand. At 3 months of age expanded alleles in the livers of *Fan1*$^{-/-}$ mice show the addition of an average of 11.3 repeats compared to just 2 in *Fan1*$^{+/+}$ mice (top panel of Fig. 2), while at 6 months of age, the expanded alleles seen in the livers of *Fan1*$^{+/+}$ mice are still smaller than the alleles seen in 3-month old *Fan1*$^{-/-}$ mice. In brain which shows less expansion than liver in wildtype animals, a unimodal distribution is seen for the PCR products in both *Fan1*$^{+/+}$ and *Fan1*$^{-/-}$ mice even at 6 months of age. However, the average repeat number added in *Fan1*$^{-/-}$ mice was about twice that of *Fan1*$^{+/+}$ mice at both 3 and 6 months of age. While these are the only tissues that show a significantly larger increase in the number of repeats added, a more sensitive measure of the extent of expansion, the somatic instability index (SII), showed that expansions were also more extensive in tails and kidneys of *Fan1*$^{-/-}$ mice than *Fan1*$^{+/+}$ mice in both 3 and 6-month old cohorts (bottom panel of Fig. 2). Thus, FAN1 shows a significant protective effect against expansion in a number of somatic tissues in this mouse model.

3.2 Loss of FAN1 does not increase intergenerational repeat expansions.

Three-month old *Fan1*$^{-/-}$ mice showed a slightly larger increase in the numbers of repeats added in testes relative to age-matched *Fan1*$^{+/+}$ mice (Fig. 1). However, this did not reach statistical significance. This trend was also seen when the SII was used, but again this did not reach statistical significance (Fig. 2). At 6 months of age, the testes repeat PCR profiles, the repeat number added and SII in *Fan1*$^{+/+}$ and *Fan1*$^{-/-}$ mice were indistinguishable. Since gametes are the most abundant cells in the testis and are the only cell type to expand [22], any effect on expansion in this cell type should have been apparent.

The proportion of expansions were also similar in the progeny of male *Fan1*$^{+/+}$ and age-matched *Fan1*$^{-/-}$ fathers with similar repeat numbers (Fig. 3). Furthermore, distribution of transmitted allele sizes was similar in *Fan1*$^{+/+}$ and *Fan1*$^{-/-}$ mice. When the repeat size was monitored on maternal transmission of the PM allele, the proportion of the progeny of *Fan1*$^{+/+}$ mice with expanded alleles was also not significantly different from the proportion seen in the progeny of *Fan1*$^{-/-}$ mice, nor was the distribution of transmitted allele sizes (Fig. 3). Thus, the loss of FAN1 does not have a significant effect on intergenerational expansions.

3.3 The protective effect of FAN1 in different organs does not correlate with Fan1 gene expression.

FAN1 protein expression in humans is high in kidney and gonads but relatively low in liver [9]. Since no comparable data is available for mice and a number of commercially available anti-mouse FAN1 antibodies produce no FAN1-specific bands on western blots (data not shown), we quantified the levels of *Fan1* mRNA in different mouse tissues using quantitative real-time PCR. As with FAN1 protein levels in humans, *Fan1* mRNA was high in mouse kidney and testis, but relatively low in liver (Fig. 4). High levels of *Fan1* mRNA were also seen in brain. Therefore, there does not seem to be a good correlation between the tissues that show a significant protective effect of FAN1 and the level of FAN1 expression. This may reflect the contribution of other FAN1-like enzymes that are able to compensate for the loss of FAN1 in some tissues.

4. Discussion

FAN1 protects the genome against expansion of the CGG-repeat tract in the *Fmr1* gene of our FXD model as evidenced by an increase in the extent of expansion that was seen in *Fan1*$^{-/-}$ mice (Fig. 1 and 2). However, while this effect was seen in a number of somatic cells tested including those of the brain and liver, *Fan1*$^{-/-}$ mice did not show significantly more extensive expansions in testis, an organ that is primarily comprised of germ line cells (Fig. 1 and 2). Furthermore, no effect of the loss of FAN1 was seen on intergenerational transmission from either males or females (Fig. 3). Thus, our data suggest that FAN1 protects against somatic but either does not protect against intergenerational expansions or is redundant with other enzymes that may act in a similar way.

FAN1 expression did not correlate well with those tissues that show the greatest protective effect. For example, high levels of expression were seen in testis, an organ that does not show a significant effect of the loss of FAN1 on repeat expansion (Fig. 4). In contrast, low levels of FAN1 expression were seen in liver, despite the significant protective effect of FAN1 that was seen. This suggests that it is not the absolute level of the protein that determines whether its loss is felt in a given tissue. Rather, its protective effect may depend on the relative levels of the proteins that promote expansions or the expression of one of the many other 5'-3' exonucleases/5' Flap endonucleases that may have a similar protective effect. For example, we have shown that EXO1 (MIM* 606063), another enzyme with 5'-3' exonuclease and 5' flap nuclease activity, also protects against expansion and does so primarily in the germ line (Zhao and Usdin, manuscript in revision). EXO1 is also highly expressed in testis [25] and it may be that the EXO1 activity in the testis of *Fan1*$^{-/-}$ mice is able to compensate for the loss of FAN1.

EXO1 plays an important role in MMR by excising the strand nicked by the MMR protein, MutLα, and it has been suggested that FAN1 may contribute to some of the EXO1-independent MMR that is seen in mammalian cells [26]. Since FAN1, like EXO1 is part of the MutLα interactome [14] and EXO1 can also participate in ICL removal [27], the two nucleases may act similarly to prevent expansions. One current model for repeat expansion involves strand-slippage and strand-displacement during repair synthesis by Polβ (MIM* 17460) [17, 28]. The loop-outs formed by strand-slippage in turn recruit MutSβ [20, 29-31]

and sometimes MutSα [20, 32]. FAN1 may act like EXO1 to repair the repeat tract in an error-free way via a MMR-like process. Since both EXO1 and FAN1 also cleave 5' flaps, they may both also protect against repeat expansion via the removal of the 5' flap generated by strand-displacement in a manner analogous to the effect of the yeast FEN1 homolog, rad27p in yeast models of repeat expansion [33, 34]. Since FAN1's flap cleavage activity is higher than its exonuclease activity, at least *in vitro* [15], this may be the major way that FAN1 protects against repeat expansion. While no effect of the loss of FEN1 (MIM* 600393) was seen in early embryos of a mouse model of a CTG-expansion disorder, myotonic dystrophy type 1 (MIM# 160900) [35], it may be that FEN1 is redundant with EXO1 and/or FAN1.

Suppression of somatic expansion delays the onset of pathophysiology in a mouse model of HD [36], suggesting that in some Repeat Expansion Diseases modulation of the extent of somatic expansion could also affect the AAO. Since the brain is an organ in which FAN1's protective effect is seen in mice, FAN1 polymorphisms could be clinically relevant in those Repeat Expansion Diseases in which somatic expansion is an important contributor to AAO and disease severity.

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Highlights

- FAN1 protects against repeat expansions in a mouse model of the FXDs
- Protection is seen in somatic tissues, including brain, but is not seen for intergenerational expansions
- As GWAS suggest, FAN1 mutations may affect the age at onset of some Repeat Expansion Diseases.

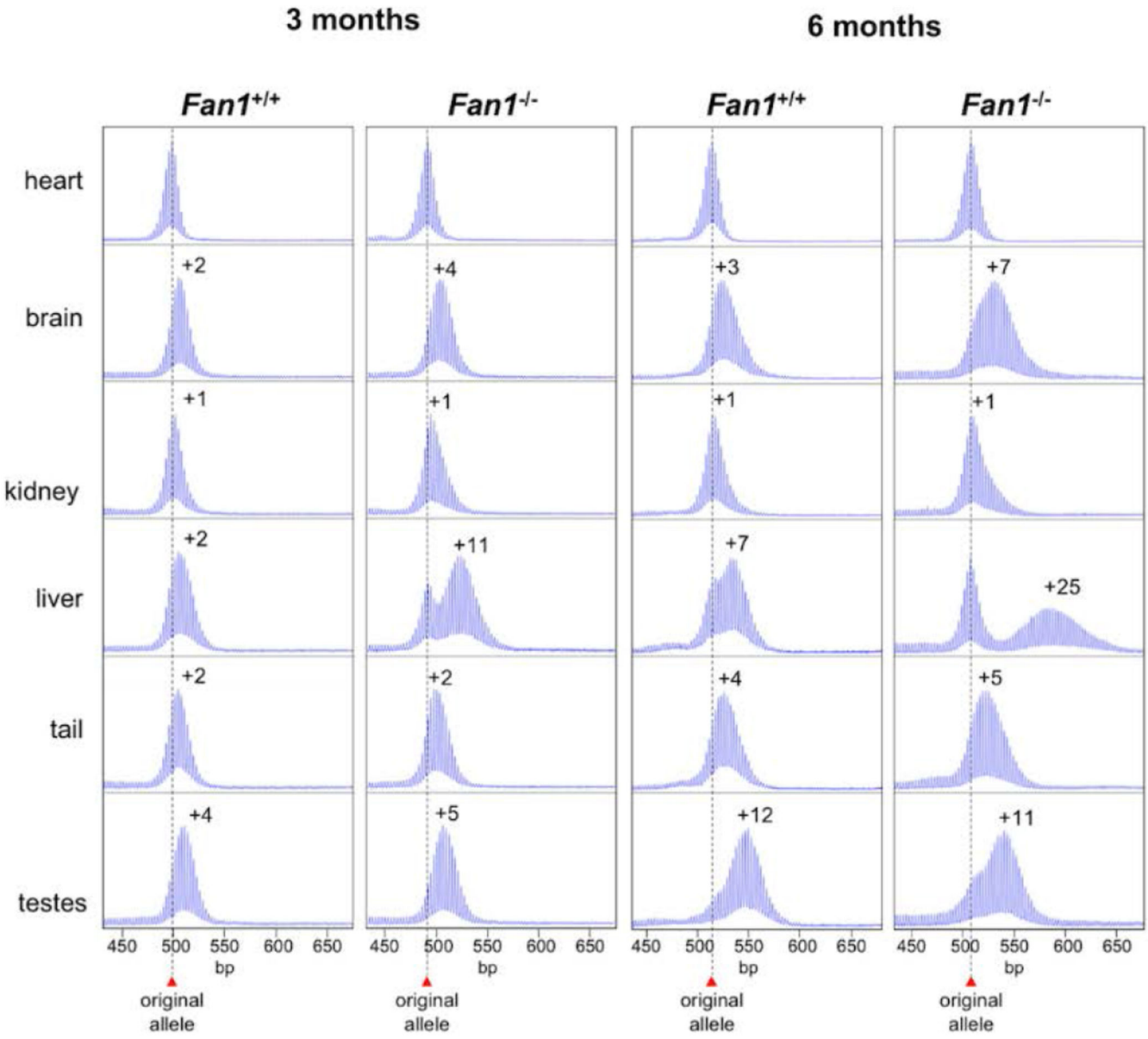

Fig. 1. The effect of the loss of FAN1 on expansion in the FXD mouse model.

Representative repeat PCR profiles from different organs of *Fan1*$^{+/+}$ and *Fan1*$^{-/-}$ males at 3-months (A) and 6-month of age (B). The number shown beside each profile is the difference in the number of repeats present on expanded alleles relative to the repeat number on the original allele, i.e., the number of repeats added. The dotted line marks the size of the original allele as assessed by the allele size in heart, an organ that shows little, if any, postnatal expansion [22]. The original allele sizes are 150 and 148 for the 3-month old *Fan1*$^{+/+}$ and *Fan1*$^{-/-}$ males and 155 and 153 repeats for the 6-month old *Fan1*$^{+/+}$ and *Fan1*$^{-/-}$ males respectively.

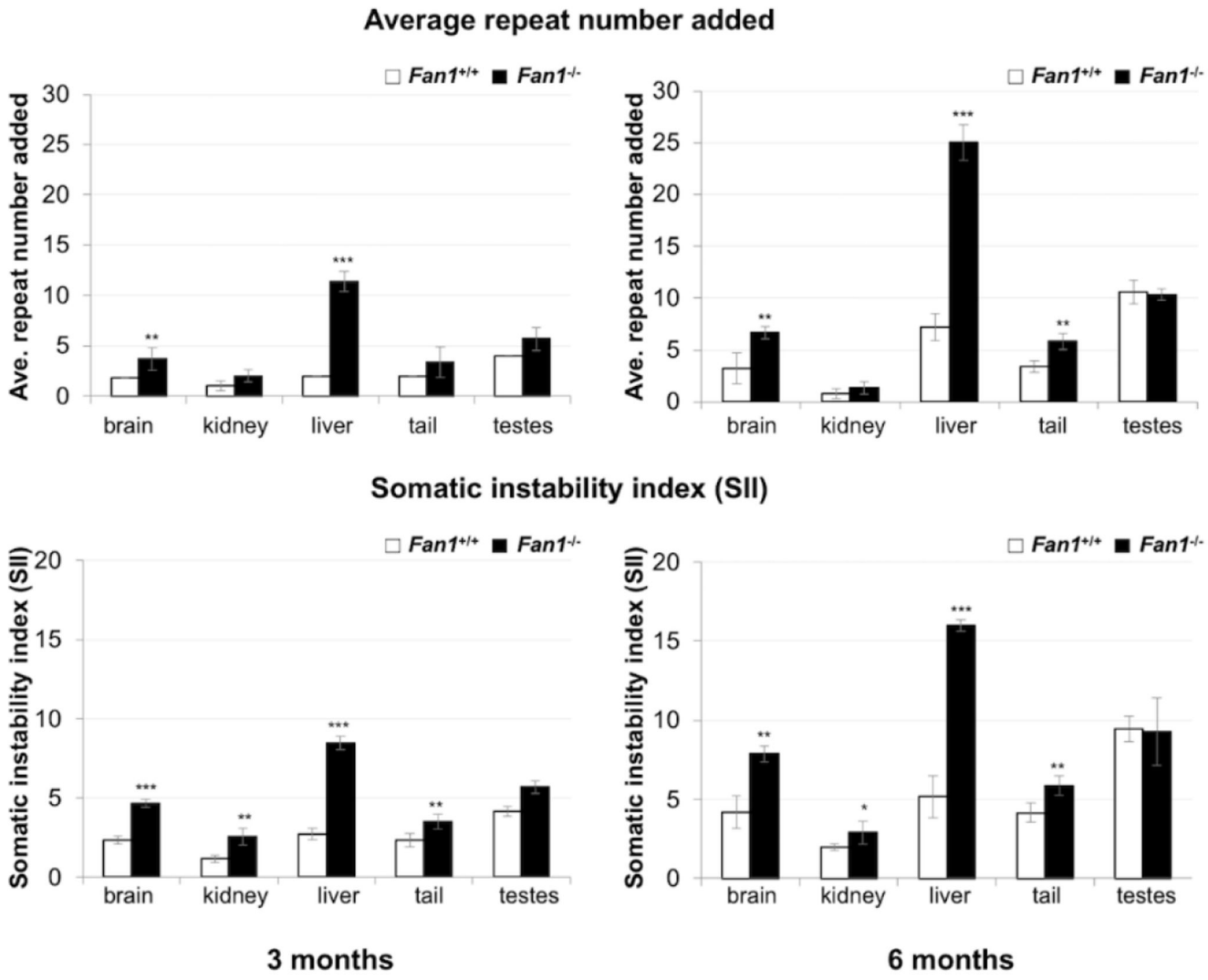

Fig. 2. Quantification of the effect of FAN1 on repeat expansion.

Top panels: The average of the repeats added to expanded alleles in different tissues of 3 *Fan1*$^{+/+}$ and 3 *Fan1*$^{-/-}$ mice at 3 months of age and 5 *Fan1*$^{+/+}$ and 3 *Fan1*$^{-/-}$ male mice at 6 months of age. Bottom panel: Somatic instability index (SII) from the animals shown in the top panels. Both the 3-month old *Fan1*$^{+/+}$ mice and the 3-month old *Fan1*$^{-/-}$ mice had 148-150 repeats. The 6-month old *Fan1*$^{+/+}$ mice had 152-159 repeats with an average of 156.2. The 6-month old *Fan1*$^{-/-}$ mice had repeats of 153-154 repeats with an average of 153.3 repeats. *: $p<0.05$; **: $p<0.01$ ***: $p<0.001$ (t test). Error bars represent the standard deviation.

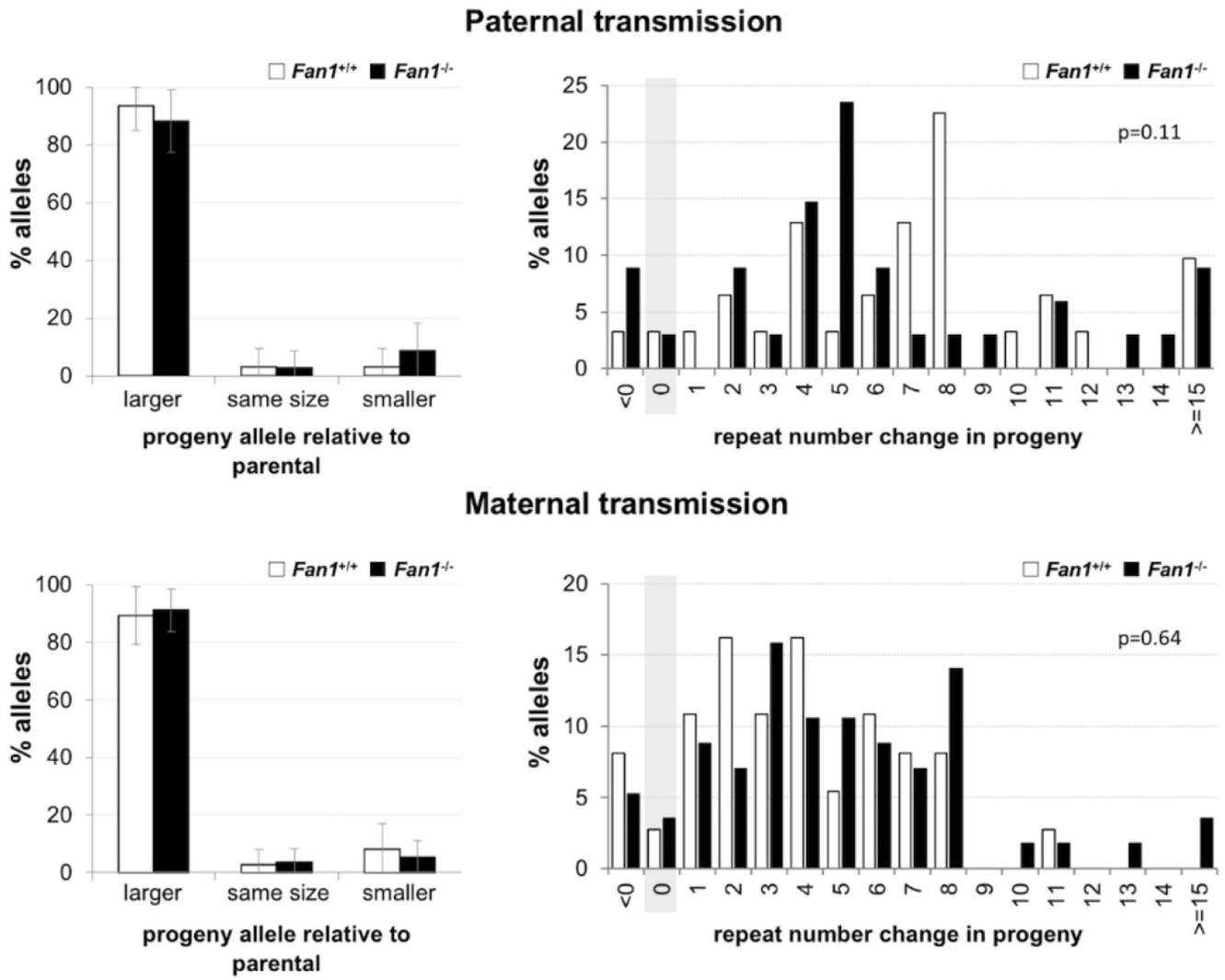

Fig. 3. The effect of the loss of FAN1 on the frequency of intergenerational expansions.

Left panel: the number of alleles bigger than, smaller than and the same size as the parental allele seen on paternal (top) and maternal (bottom) transmission from *Fan1*$^{+/+}$ and *Fan1*$^{-/-}$ parents that were 2-6 months old. At least 5 breeding pairs were used for each condition. For paternal transmission, 31 pups from *Fan1*$^{+/+}$ parents and 34 pups from *Fan1*$^{-/-}$ parents were tested. For maternal transmissions, 37 pups from *Fan1*$^{+/+}$ parents and 57 pups from *Fan1*$^{-/-}$ parents were tested. Right panel: The distribution of allele sizes in the progeny of *Fan1*$^{+/+}$ and *Fan1*$^{-/-}$ male (top) and female (bottom) mice. The p values are from the t test of the distribution of repeat numbers added. While some repeat change classes show large differences between *Fan1*$^{+/+}$ and *Fan1*$^{-/-}$ mice, these are likely statistical anomalies that do not affect the distribution of the repeat changes observed. Error bars represent the 95% confidence interval.

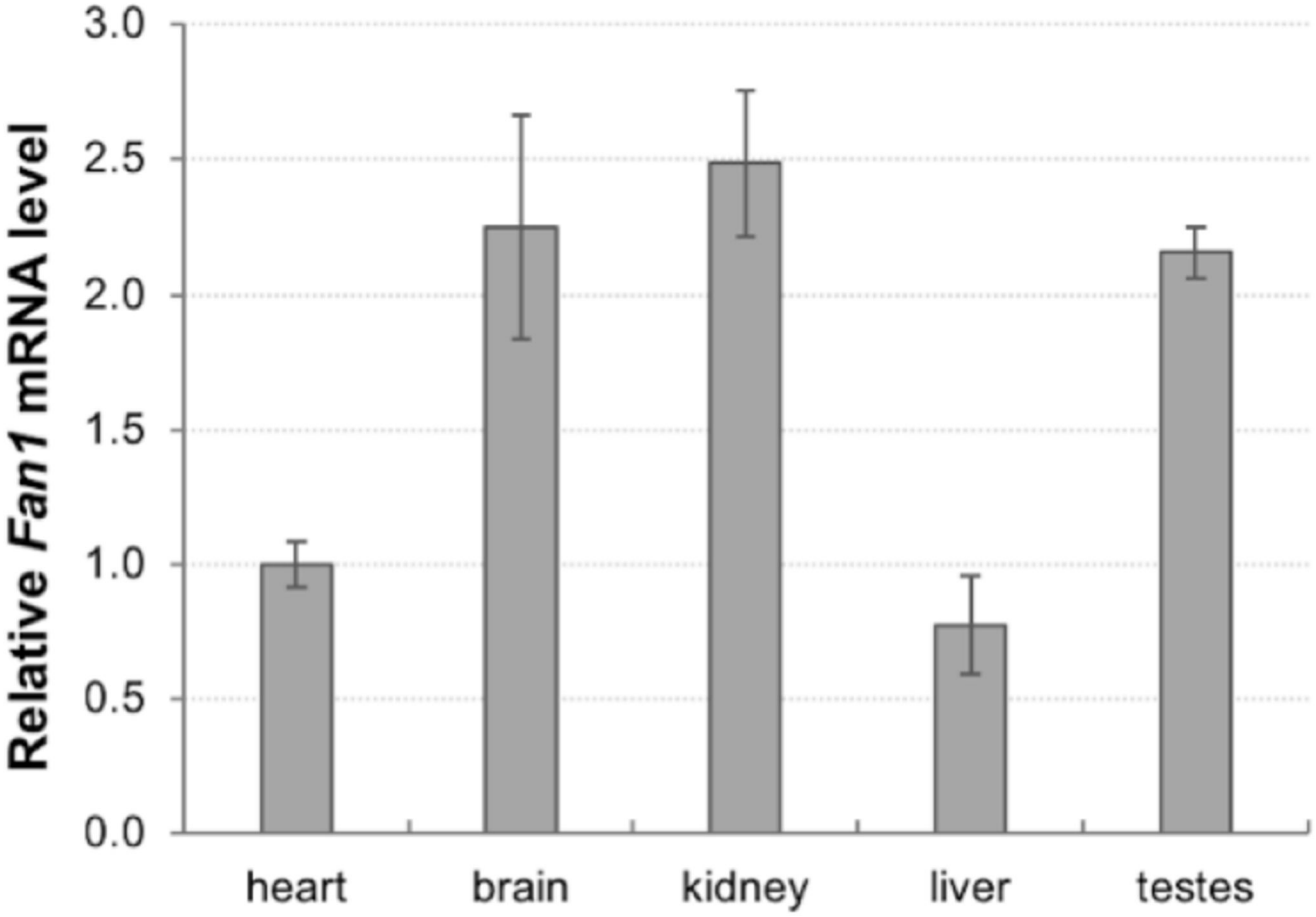

Fig. 4. *Fan1* mRNA expression in different mouse tissues.

The amount of *Fan1* mRNA in different tissues of three 3 month old male mice was evaluated by real-time qPCR as a function of total RNA and the values expressed relative to the levels of *Fan1* transcript in heart. Error bars represent the standard deviation.