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Fen1 does not control somatic hypermutability of the $(CTG)_n \cdot (CAG)_n$ repeat in a knock-in mouse model for DM1

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Abstract The mechanism of trinucleotide repeat expansion, an important cause of neuromuscular and neurodegenerative diseases, is poorly understood. We report here on the study of the role of flap endonuclease 1 (Fen1), a structure-specific nuclease with both 5′ flap endonuclease and 5′-3′ exonuclease activity, in the somatic hypermutability of the $(CTG)_n \cdot (CAG)_n$ repeat of the *DMPK* gene in a mouse model for myotonic dystrophy type 1 (DM1). By intercrossing mice with *Fen1* deficiency with transgenics with a DM1 $(CTG)_n \cdot (CAG)_n$ repeat (where $104 \leqslant n \leqslant 110$), we demonstrate that Fen1 is not essential for faithful maintenance of this repeat in early embryonic cleavage divisions until the blastocyst stage. Additionally, we found that the frequency of somatic DM1 $(CTG)_n \cdot (CAG)_n$ repeat instability was essentially unaltered in mice with Fen1 haploinsufficiency up to 1.5 years of age. Based on these findings, we propose that Fen1, despite its role in DNA repair and replication, is not primarily involved in maintaining stability at the DM1 locus.

Keywords: Trinucleotide repeat; Somatic instability; Myotonic dystrophy; Microsatellite; Flap endonuclease 1; DNA repair and replication

1. Introduction

Maintenance of genome stability is of critical importance for normal growth, viability, and functional homeostasis of cells. While DNA replication and repair are becoming increasingly better understood, the molecular events that determine stability across long tracts of simple repetitive sequences remain relatively obscure. Microsatellite instability of mono- or multinucleotide repeats is a distinguishing feature of certain forms of cancer and expansion of trinucleotide repeat (TNR) sequences beyond an unaffected range is the direct basis of a series of hereditary neurodegenerative disorders, including Huntington's disease (HD), several spinocerebellar ataxias (SCAs) and myotonic dystrophy (DM).

Myotonic dystrophy type 1 (DM1) is characterized by muscular dystrophy and myotonia in combination with a highly variable manifestation of features like cataract, heart conduction defects, insulin insensitivity and cognitive impairment. DM1 is caused by the expansion of a $(CTG)_n$ repeat in the 3′ UTR of the *DMPK* gene on chromosome 19. Once DM1 alleles are in the disease-associated length range (>50 CTGs), repeat tracts become dramatically unstable. Intergenerational mutation rates may be almost 100% per generation, with a strong tendency towards further repeat gains [1–3]. The DM1 repeat is also somatically unstable and extensive instability has been reported in a wide range of human tissues [1,4]. This somatic expansion is mediated by multiple small length changes in a highly deterministic process which is clearly age dependent, with longer average DM1 repeat lengths and broader ranges of variability observed in older patients [3,5]. We know that somatic expansions accumulate in both proliferating and post-mitotic tissues, suggesting that expansion is cell-division independent [4,6]. Furthermore, the rate of instability of the $(CTG)_n \cdot (CAG)_n$ repeat in DM1 is correlated to repeat length and is probably due to an increased ability of the repeat sequence to form aberrant DNA structures, like slipped strand hairpins or cruciforms with single-stranded regions [7]. Failure to process these structures by mismatch repair proteins – e.g., MSH2, MSH3, PMS2 or MLH1 – and/or aberrant activity of machinery involved in DNA break repair, may underlie mutagenic instability [8–11]. *Cis*-acting factors such as nearby presence of replication origins or proximity of CpG islands may also influence instability [12,13].

Our group has developed a knock-in mouse model in which somatic expansion of the DM1 $(CTG)_n \cdot (CAG)_n$ tract is faithfully reproduced. In this model, a human chromosomal segment spanning the exon 13–15 region of the *DMPK* gene of a DM1 patient with a $(CTG)_{84}$ repeat replaces the cognate endogenous segment of the mouse *DMPK* gene [10]. Hence, this places chromatin embedding of the repeat element and production of repeat containing *DMPK* mRNA under "natural" host control. Study of this model revealed that activity of the Msh2/Msh3 protein complex is essential for expansion [10]. We report here on the use of this mouse model to study the involvement of another DNA processing enzyme, Fen1, a structure-specific nuclease with both 5′ flap endonuclease and 5′ to 3′ exonuclease activity [14]. On replicating DNA, Fen1

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Abbreviations: DM1, myotonic dystrophy type 1; Fen1, flap endonuclease 1; TNR, trinucleotide repeat; HD, Huntington's disease; SCA, spinocerebellar ataxia

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serves to remove displaced RNA–DNA primers during discontinuous lagging strand synthesis, promoting normal replisome and DNA fork progression. Fen1 possesses also a specific gap endonuclease activity critical for progress of stalled replication forks. Its activity has furthermore been implicated in repair processing of DNA in G0-arrested cells, in long patch base or nucleotide excision and in non-homologous end joining and recombination [14].

Based on theoretical considerations, a sequence expansion model has been proposed to explain a role for this enzyme in destabilization and tendency towards length increase of $(CTG)_n \cdot (CAG)_n$ repeat tracts [15]. *In vitro* experiments and genetic assays in yeast model systems with long $(CTG)_n \cdot (CAG)_n$ repeats or other types of triplet repeats showed that presence of the Fen1 homologue Rad27p restrains both the expansion behavior and the fragility of these DNA repeat motifs, presumably via its flap degrading ability [16–18]. These studies showed that *Rad27* mutation induces both contractions and expansions, but does not affect the length-threshold for repeat expansion [19]. Experimental verification for the role of Fen1 in higher eukaryotes appeared difficult and has resulted in contrasting findings regarding its involvement in $(CAG)_n \cdot (CTG)_n$ triplet repeat expansion in HD [20,21]. In recent work on SCA7 $(CAG)_n \cdot (CTG)_n$ repeat expansion in Drosophila no effects of Fen1 absence were observed [22].

We here show that Fen1 absence does not affect faithful maintenance of the DM1 $(CTG)_{110}$ repeat in early embryonic divisions up to the blastocyst stage. Additionally, we found that the rate of somatic hypermutability was essentially unaltered in mice with *Fen1* haploinsufficiency up to 1.5 years of age. We propose that *Fen1* is not essential for trinucleotide stability at the DM1 locus.

2. Materials and methods

2.1. Targeting vector and mouse generation

Fen1 deficient mice were generated via targeted mutagenesis in ES cells derived from mouse strain 129/Ola, essentially as described [23]. A ~7 kbp genomic fragment spanning the *Fen1* gene was amplified from mouse 129/Ola DNA by PCR with Herculase polymerase (Stratagene) and primers 5′-CGAAGCTGGGAACGATACTGAAAGAACG-3′ and 5′-GGTAAGGCACTTCCTATCCAAGTTCCGATC-3′ and cloned in a pBS vector. In several cloning steps, a 543 bp *Cla*I–*Sbf*I internal segment was replaced by a 1178 bp Neo cassette and a HSV-TK cassette was placed downstream (Fig. 1A). The targeting vector was linearized by *Kpn*I and introduced into ES cells by electroporation. Screening of G418/FIAU resistant clones was performed by Southern blotting of genomic DNA, after digestion with *Bam*HI. The 5′ probe from the upstream flanking genomic segment was obtained by PCR using primers 5′-GTTAACTTCCATTGTCACTTTC-3′ and 5′-CATAGTGGGCGTTCTTTCTG-3′; the 3′ probe downstream of the region of homology was obtained by PCR using primers 5′-CAGGATGTGGTAACAGCATT-3′ and 5′-CATGTCACAAACAGGAAGGTT-3′. One ES cell clone with a correctly disrupted *Fen1* allele and correct karyotype was identified and injected into blastocysts to generate germline chimeras. Transmitting males were mated with C57BL/6 females and F1 pups were screened by tail biopsy to identify $Fen1^{+/-}$ mice. $Fen1^{+/-}$ mice were crossed with $(CTG)_n/(CTG)_n$ mice [10] to obtain $Fen1^{+/-}$//wt/$(CTG)_n$ mice. These mutants were bred to generate $Fen1^{+/-}$//$(CTG)_n$/$(CTG)_n$ mice, which were then crossed with $Fen1^{+/-}$ mice to get pregnant females for isolation of morulae and blastocysts carrying one $(CTG)_n$ allele and either no, one or two mutated *Fen1* alleles.

2.2. PCR genotyping and analysis of the $(CTG)_n \cdot (CAG)_n$ repeat

Mouse tail DNA was used for PCR genotyping using diagnostic *Fen1* primers 5′-TACCATGGGAATTCACGGC-3′ and 5′-GCACAGATCCACAAACTG-3′ (32 cycles). Length typing of the $(CTG)_n \cdot (CAG)_n$-containing segment was done by PCR using primers 5′-GAAGGGTCCTTGTAGCCGGGAA-3′ and 5′-GGAGGATGGAACACGGACGG-3′ (32 cycles) followed by analysis in the ALF DNA sequencing system (Amersham Pharmacia Biotech, Uppsala, Sweden), as described [10]. Each $(CTG)_n \cdot (CAG)_n$ repeat length profile was normalized to its highest peak to be able to optimally compare the relative contribution of fragments with expanded repeats between different profiles.

Individual morulae and blastocysts were washed in HEPES-buffered medium and finally PBS and lysed in 20 μl 1 × Herculase buffer (Stratagene) with 60 μg/ml proteinase K for 3 h at 55 °C. Proteinase K was inactivated for 10 min at 99°C and 6 μl of the lysis mixture was used as template in the *Fen1* PCR (38 cycles).

2.3. Western blotting

Wt and $Fen1^{+/-}$ ES cells were washed in PBS and lysed in Laemmli sample buffer. Protein lysates were separated by SDS–PAGE and transferred to PVDF membrane. The blot was incubated with polyvalent

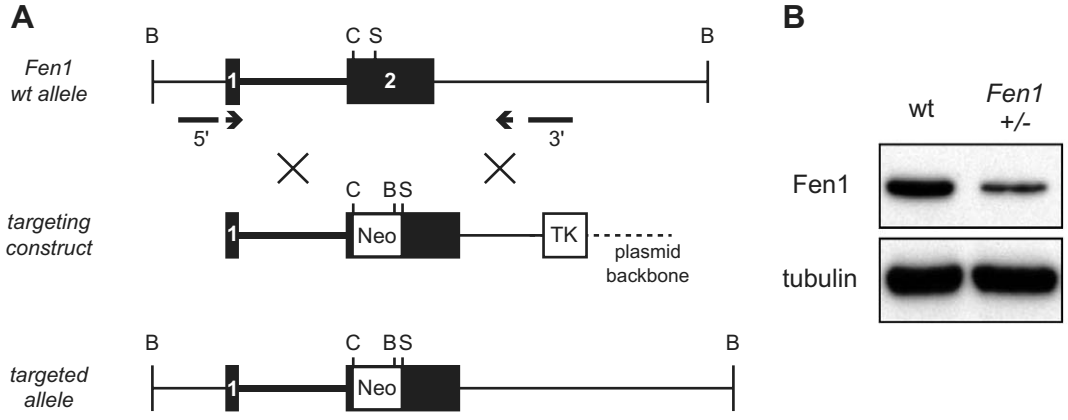

Fig. 1. Targeted mutation of the *Fen1* gene. (A) Strategy for the replacement of part of exon 2 of the *Fen1* gene. The entire ORF is contained within exon 2, with the ATG initiation codon right at the beginning, just 5′ of a *Cla*I site. Schemes depict the exon–intron arrangement in the *Fen1* gene, the structure of the targeting vector with the Neo and HSV-TK cassettes, and the structure of the targeted allele. Arrows indicate locations of primers used to amplify the genomic fragment for constructing the targeting vector. Positions of relevant *Cla*I, *BamH*I and *Sbf*I sites are indicated. Bars – labeled 5′ and 3′ – indicate positioning of genomic segments flanking the *Fen1* gene, which were used as probes for Southern analysis of correct targeting events. (B) Western blot probed with a Fen1 antibody to show Fen1 protein (~42 kDa) levels in whole cell lysates of wt and $FEN^{+/-}$ mutant ES cells. Tubulin antibody staining was used to verify protein loading. Note that the signal representing Fen1 protein level in the correctly targeted ES cell clone, is ~50% reduced.

rabbit Fen1 antibody (homemade) or monoclonal anti-tubulin (E7), and presence of protein was revealed by ECL detection using standard protocols.

2.4. Immunohistological analysis of morulae and blastocysts

Oviducts (at 2.5 dpc) or uteri (at 3.5 dpc) of pregnant mice were flushed with HTF/HEPES (Cambrex BE02-022F), 0.5% BSA solution (Sigma A-4503) to obtain mouse morulae or blastocysts. The zona pellucida was disrupted with acidic tyrode solution [24] incubation for a few min. Embryos were caught in a fibrin clot by activating fibrinogen with thrombin [25]. Fixation was performed with 2% (w/v) formaldehyde, 0.15% Triton X-100 in PBS for 30 min. After 1 h at 37 °C with blocking solution including 10% normal goat serum, overnight incubation with a Fen1 antibody [26] was done at 4 °C. Then, coverslips were incubated for 2 h with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes), washed, stained with DAPI and embedded. Images were obtained with a Bio-Rad MRC1024 confocal laser-scanning microscope equipped with an argon/krypton laser, using a 60× 1.4 NA oil objective and LaserSharp2000 acquisition software. Images were further processed with Adobe Photoshop 7.0. Afterwards, stained embryos were collected and processed for *Fen1* PCR analysis as described above. The experiment was performed in triplicate; altogether, 72 embryos were examined.

3. Results

3.1. Targeted mutation of the Fen1 gene

A segment of the *Fen1* open reading frame was removed in mouse ES cells via targeted replacement (Fig. 1A). Fen1 protein levels in targeted ES cells were reduced to ~50% of normal (Fig. 1B), indicating that no compensatory adaptation of expression of the wt allele had occurred. $Fen1^{+/-}$ mice were overtly normal during development, adulthood and ageing, with a normal fertility profile. In contrast, breeding of $Fen1^{+/-}$ mice never yielded homozygous offspring, indicating prenatal lethality (Table 1). Genotyping of preimplantation embryos suggested that loss already occurred during the morula and blastocyst stage of gestation, although these findings did not reach statistical significance (Table 1).

Table 1
Genotyping offspring from $Fen1^{+/-} \times Fen1^{+/-}$ crossings

Genotype	Morulae (%)[a]	Blastocysts (%)[a]	Newborn pups (%)[b]
wt	5 (45)	27 (33)	34 (35)
+/−	5 (45)	41 (50)	64 (65)
−/−	1 (10)	14 (17)	0 (0)[*]
Total	11	82	98

[a]From $Fen1^{+/-} \times Fen1^{+/-}$//$(CTG)_n$/$(CTG)_n$ and $Fen1^{+/-}$//wt/$(CTG)_n \times Fen1^{+/-}$//$(CTG)_n$/$(CTG)_n$ crossings, where $104 \leqslant n \leqslant 110$.
[b]From $Fen1^{+/-} \times Fen1^{+/-}$ crossings.
[*]$P < 0.001$, Chi-square test.

3.2. Haploinsufficiency of Fen1 has no effect on somatic $(CTG)_n \cdot (CAG)_n$ repeat instability

Earlier, using established procedures for repeat length typing in different tissues of our $(CTG)_n \cdot (CAG)_n$ knock-in model, we observed extensive somatic instability [10]. Hypermutability of the repeat was dependent on the mouse background strain, age and tissue type, with a general tendency to length increase. To assess the role of *Fen1* in this process, we compared repeat profiles in liver, kidney and skeletal muscle of $Fen1^{+/-}$//wt/$(CTG)_n$ and wt/$(CTG)_n$ mice at 6, 12 or 18 months of age (Fig. 2). Although we did encounter small differences in repeat length profiles between individual animals and also sometimes the fraction of cells involved in a given tissue was different, we observed no consistent changes in average allele length or length spreading between animals in the wt and $Fen1^{+/-}$ cohorts (nine mouse pairs examined). Collectively, our data indicate that *Fen1* haploinsufficiency has neither an effect on the rate of repeat length mutation nor on the tendency to length increase of the $(CTG)_n \cdot (CAG)_n$ transgene.

At this stage, we cannot exclude an effect of *Fen1* haploinsufficiency on $(CTG)_n \cdot (CAG)_n$ repeat instability in the germ line, simply because intergenerational instability occurs at too low frequency in our lineage [10], and we presumably need

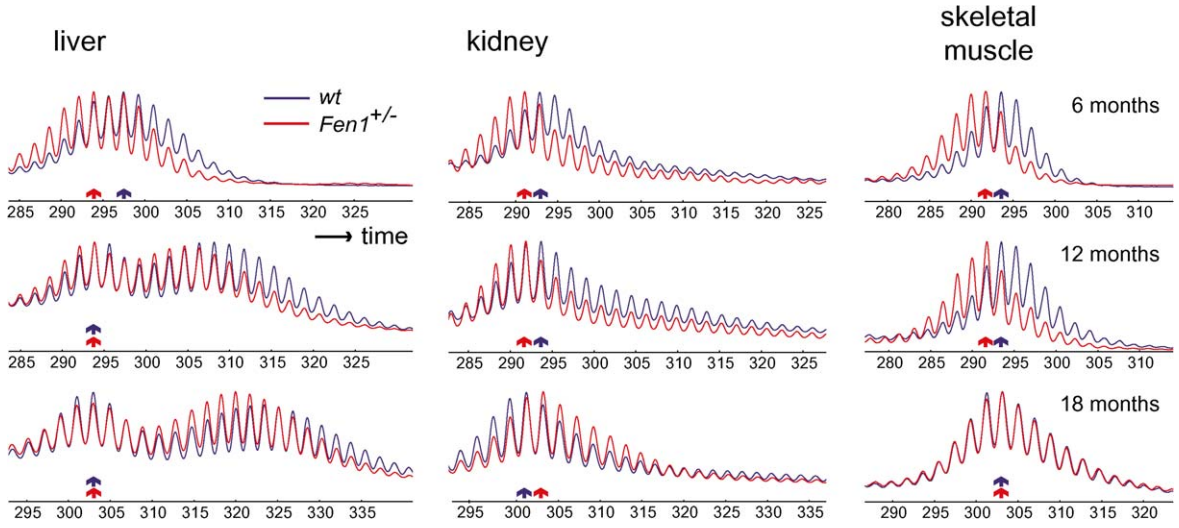

Fig. 2. Somatic instability of the $(CTG)_n \cdot (CAG)_n$ repeat in wt and $Fen1^{+/-}$ mice. Representative profiles of $(CTG)_n \cdot (CAG)_n$ repeat containing PCR products of genomic DNAs from liver, kidney and skeletal muscle of 6-, 12- and 18-month-old wt/$(CTG)_n$ (blue) and $Fen1^{+/-}$//wt/$(CTG)_n$ (red) mice. Arrows indicate the position of the predominant progenitor allele length in profiles of tail DNAs determined at 3 weeks of age. Progenitor allele lengths varied between individual mice due to intergenerational instability and ranged between $(CTG)_{104}$ and $(CTG)_{110}$ in the cohort used. Note that somatic expansion (visible as a shift towards the right) increases with age in all three tissues shown, but is most prominent in liver. $(CTG)_n \cdot (CAG)_n$ length profiles were pairwise compared between age-matched wt and $Fen1^{+/-}$ animals on one gel, but different gels were used for different age classes.

to follow allele lengths over more than 10 generations before significant data would become apparent. What we do know, however, is that loss of 50% of Fen1 in the germ line is not associated with an overt increase in intergenerational instability (data not shown).

3.3. Early effects of Fen1 deficiency?

Given the role of Fen1 in replication and repair, we wished to determine whether absence of Fen1 had any effect on the accuracy of replication of the $(CTG)_n \cdot (CAG)_n$ tract in early embryos. To this end, $Fen1^{+/-}$//$(CTG)_n$/$(CTG)_n$ mice were crossed with $Fen1^{+/-}$ or $Fen1^{+/-}$//wt/$(CTG)_n$ mice and morulae and blastocysts were collected. Some of the $Fen1^{-/-}$ embryos had an anomalous appearance, suggesting that they would have been lost before gastrulation. This observation and the presumed underrepresentation of $Fen1^{-/-}$ embryos (Table 1) are in line with findings obtained with another *Fen1* knock out model [27]. Larsen et al. suggested that *Fen1* knock out embryos may survive initial cell divisions and develop normally until maternally supplied Fen1 protein is exhausted [27]. If this assumption is true, it would be difficult to study $(CTG)_n \cdot (CAG)_n$ repeat behavior in absence of Fen1, due to presence of maternal Fen1 in early embryos and embryonic lethality later on.

To examine this in more detail, we studied Fen1 expression in early embryogenesis by subjecting morulae and blastocysts to immunohistological analysis with a Fen1-specific antiserum. Comparison of signals in $Fen1^{-/-}$ and wt blastocysts revealed absence of staining in nuclei of $Fen1^{-/-}$ blastocysts (Fig. 3), whereas nuclear immunofluorescence was consistently observed in $Fen1^{+/-}$ blastocysts (data not shown). To us this suggests that the maternal contribution of Fen1 at this stage is low, if at all existing. However, we must be cautious with this interpretation as our analyses were hampered by a high cytoplasmic background staining, independent of the genotype. This phenomenon was seen with three independent Fen1 antibodies and may be an effect of epitope sharing with other proteins (data not shown).

Analysis of $(CTG)_n \cdot (CAG)_n$ repeat length in all embryos that could be genotyped showed a rather narrow distribution of product lengths, with characteristic sloping at lower and higher repeat lengths in the profile (Fig. 4). The profiles observed were similar to that seen in tail biopsies of the parental animals at three weeks of age. No differences in mean repeat length were observed between wt, $Fen1^{+/-}$ and $Fen1^{-/-}$ embryos. Assuming that maternal Fen1 is not a factor in our analyses, our findings indicate that Fen1 is not necessary for faithful replication of the DM1 $(CTG)_n \cdot (CAG)_n$ repeat during the first 4–6 rounds of replication during early embryogenesis.

4. Discussion

The propensity of simple $(CAG)_n \cdot (CTG)_n$, $(CCG)_n \cdot (CGG)_n$ or $(GAA)_n \cdot (TTC)_n$ TNRs to form highly mutagenic structures and undergo frequent length expansions has been studied intensively after the recognition that these DNA elements cause human neurodegenerative diseases, now more than 15 years

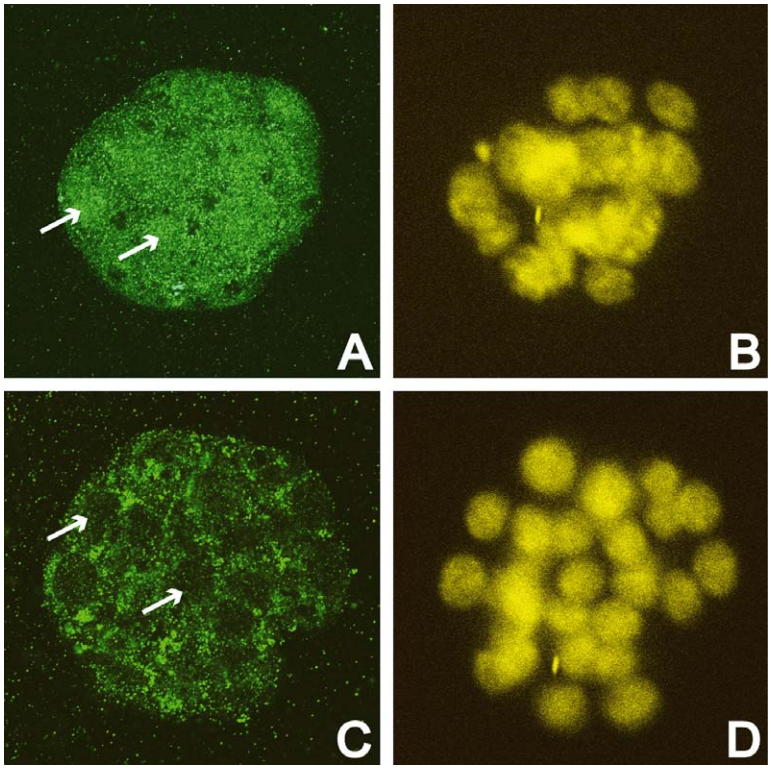

Fig. 3. Fen1 is absent in nuclei of cells in early $Fen1^{-/-}$ embryos. Wild type (A, B) and $Fen1^{-/-}$ (C, D) embryos were isolated and processed for immunohistological detection with a Fen1 antiserum (A, C; one optical section each) [26]. DNA was counterstained with DAPI (B, D; whole embryos). A clear Fen1 nuclear staining observed in wt embryos was absent in $Fen1^{-/-}$ embryos (see arrows). A cytoplasmic background staining was observed independent of the genotype, which may be caused by binding of the Fen1 antiserum to cross-reactive proteins. Genotyping was done after image recording.

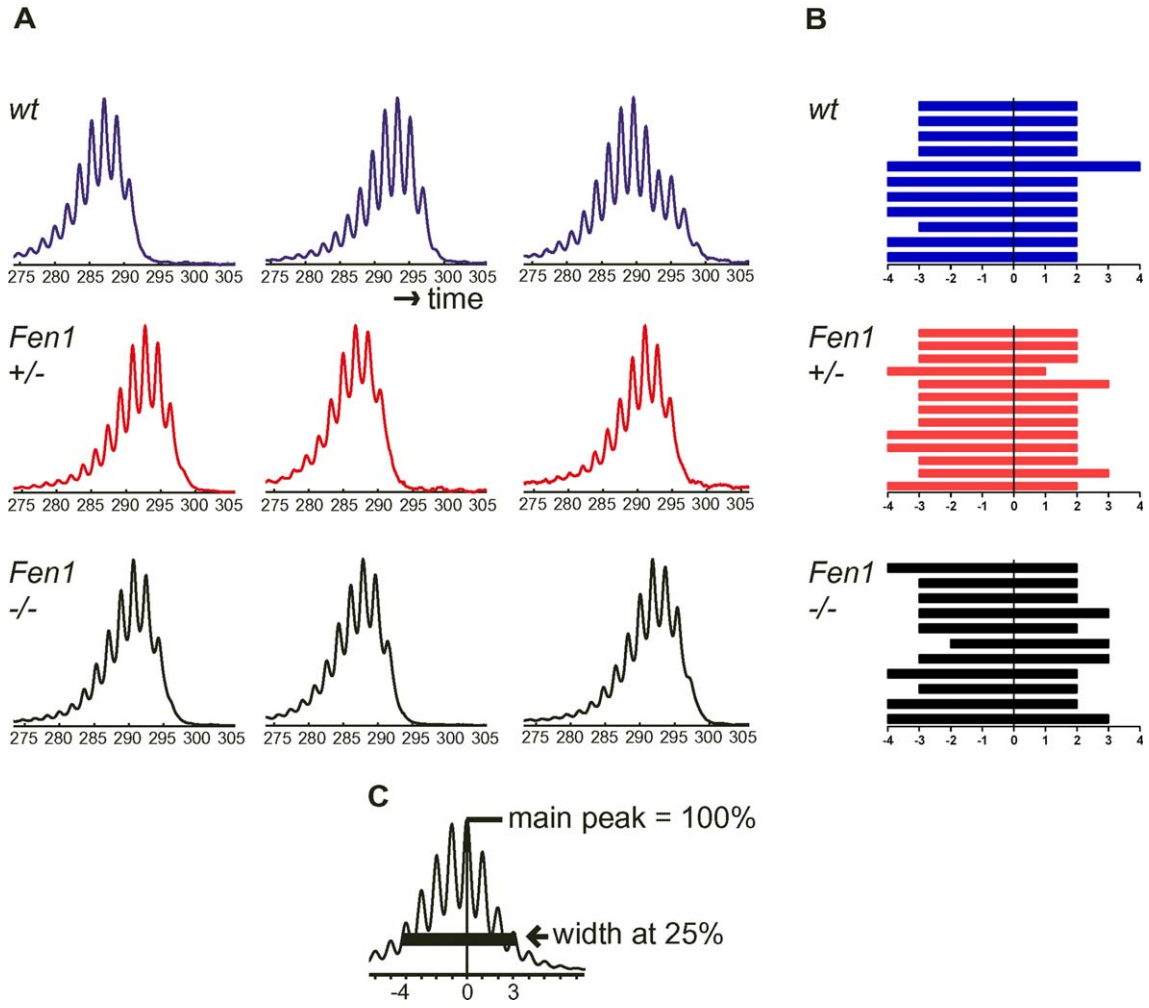

Fig. 4. Somatic hypermutability of the $(CTG)_n \cdot (CAG)_n$ repeat in early embryos. (A) $(CTG)_n \cdot (CAG)_n$ length profiles were collected from morulae and blastocysts of different genotypes. Three examples are shown for each genotype: wt, $FEN^{+/-}$, and $FEN^{-/-}$. (B) Intercomparison between the width of allelic lengths in profiles for embryos of different genotypes. (see panel (C) for the method used). From top to bottom profile widths of wt ($n = 11$), $Fen1^{+/-}$ ($n = 13$) and $Fen1^{-/-}$ ($n = 11$) embryos are shown. (C) Illustration of method used to quantify somatic hypermutability in early embryos. The width of the profile, including all peaks with 25% or more of the signal intensity of the main peak, was determined (horizontal bar). Peak variation, resulting from strand slippage during the amplification reaction or representing true somatic instability ranges in this example from −4 (contractions) to +3 (expansions). To facilitate interembryonic comparison and exclude effects of small gains or losses in progenitor allele length, the vertical line indicating the position of the main peak (= 100%), was placed at position 0 for each embryo.

ago [7]. Despite these efforts, the mechanisms behind TNR expansion are still not clearly understood. Current models fall into two categories, based on molecular events involved in (i) DNA replication or (ii) DNA repair and recombination [28]. Background knowledge implicated in these models mainly comes from studies of the biophysical properties of abnormal DNA structures formed by TNRs in vitro (in the test tube and in cell extracts), and from studies in vivo in *Escherichia coli* and *Saccharomyces cerevisiae*, which are continuously active in DNA replication followed by cell division.

Here, we have analyzed how somatic TNR instability in the $(CTG)_n \cdot (CAG)_n$ repeat associated with DM1 is modulated by absence or presence of *Fen1*. Fen1 is a multifunctional enzyme with a proposed critical role in RNA primer removal and joining of Okazaki fragments during lagging strand DNA synthesis, processing of stalled DNA replication forks, long-patch base excision repair, and recombination and resolution of misaligned hairpin and bubble structures in di- and trinucleotide repeats [7]. Based on these functions, *Fen1* has long been considered a good candidate for being involved in $(CTG)_n \cdot (CAG)_n$ repeat instability in DM1, HD or SCA7 [15,22]. In yeast model systems, absence of *Rad27* renders repeat elements profoundly unstable [16–19,29]. However, findings in yeast should not be simply extrapolated for prediction of TNR maintenance in mammals. Yeast cells may not be ideal models as TNRs do not appear as natural elements in their DNA and mechanisms for repeat maintenance have not been evolutionarily tested in context in this species. Moreover, genetic assays in mutant yeasts are virtually always done under conditions of active growth. For this reason, *Rad27* mutants cannot be regarded as faithful models to reproduce the situation in G0-arrested cells in mature somatic tissues of mammals. For these and obvious other reasons, we have tested *Fen1*'s involvement in embryos and adult animals of a mouse knock-in strain with a DMPK gene carrying a humanized 3′ segment with expanded $(CTG)_n \cdot (CAG)_n$ repeat. Originally, the $(CTG)_n \cdot$

$(CAG)_n$ length in this DM1 mouse model was 84 triplets, but as repeat length has slowly but steadily increased over 20 successive generations, the repeat length was 104–110 CTGs at the time of intercrossing for this study. Previously, we have already reported that somatic hypermutability occurred at high frequency in our model, with doubling of allele length occurring mainly in adulthood and at old age in tissues like kidney, stomach and liver [10].

Although slight variations in genetic background (mixed C3H-129/Ola-C57BL/6) are not accounted for in our analyses, we conclude that the normal frequency of somatic instability in our model has not changed in a background of *Fen1* haploinsufficiency. Also no effects were seen in the timing of onset of repeat expansion, which undergoes a strong boost after three to four months of age (data not shown). Thus, we tentatively conclude, that maintenance of proper Fen1 protein levels is not critical for suppression of $(CTG)_n \cdot (CAG)_n$ repeat instability. Obviously, also no other large-scale repair effects on anomalously paired tracts in the repeat area have been superimposed, as could theoretically have been expected for endogenously induced long-patch base excision repair or other repair events on repeat DNA structures for which normal Fen1 levels would have been required. Indirectly, these observations argue against a prominent role for *Fen1* in somatic repeat instability. Our findings corroborate observations in a HD mouse model with an expanded $(CAG)_n \cdot (CTG)_n$ repeat [21]. Studies about *Fen1* effects on intergenerational TNR repeat expansion are obviously more controversial. Earlier, based on the analysis of mutations or polymorphisms within the *Fen1* gene, *Fen1* was excluded as candidate gene implicated in intergenerational $(CAG)_n$ repeat expansion in families with HD [20]. However, because only normal *Fen1* genes were observed, the actual role of *Fen1* in $(CTG)_n \cdot (CAG)_n$ maintenance was not correctly addressed in this study. In contrast, minor effects on instability in progeny, limited to the developing germ cells, have been reported for the HD mouse model [21], but also in this study effects were only marginally significant.

To avoid further confusion about the role of *Fen1* in TNR hypermutability, we deliberately confined a large part of our study to the analysis of repeat fate in adult somatic tissues. Our findings should be interpreted with caution, however, as study of repeat behavior in complete absence of Fen1 is not possible at this stage of life, because *Fen1* deficiency is early lethal in mammals [27,30]. With reduction of *Fen1* expression to 50% of normal levels, the pathogenic threshold for faithful DNA replication may simply not be reached, but further lowering of Fen1 level in the whole animal context is technologically much more demanding if not entirely impossible. Apparently, other enzymes that can handle complex DNA structures with unpaired strand regions – e.g., DNA2, EXO1 or SRS2 – cannot subserve Fen1 in correcting the loss of replication/repair/recombination function in early embryos. What then explains *Fen1* significance in preventing the extent and timing of cell death in the early mouse embryos? *Fen1* appeared not essential for DNA maintenance and replication in vertebrate DT40 cells [31]. Knockout in yeast also did not cause lethality [29]. More study will thus be necessary to precisely pinpoint what renders preimplantation embryos at the morula/blastocyst stage so highly vulnerable in the absence of Fen1.

Larsen and co-workers suggested an involvement of S-phase entry block and surmised that presence of a maternal Fen1 pool from oocytes may help embryo survival through the first cell cleavages [27]. Our immunohistological observations with polyvalent antibodies against Fen1 argue against this possibility, as Fen1 protein was undetectable in nuclei in embryos with a homozygous gene deletion. In contrast, Fen1 staining was visible in the cell nuclei of heterozygous and wild type embryos. One difficulty with this analysis was that we observed a high cytosolic background staining in cells in all embryos. This makes interpretation somewhat difficult as residual maternal Fen1 may hide under the cytosolic background staining. Qiu et al. [32] have reported that Fen1 shuttles between cytosol and nucleus, and that nuclear localization is cell-cycle and DNA-state dependent. We consider it unlikely, however, that cytosolic translocation would completely and synchronously hide Fen1 signal in all cells in our $Fen1^{-/-}$ embryos, as this was never observed in embryos with at least one intact *Fen1* allele. Taken together, a more plausible explanation is that the bulk of protein in morulae/blastocysts is embryonically expressed and not of maternal origin. This interpretation is supported by our observation that Fen1 protein is prominently expressed in ES cells – i.e., a stage corresponding to those of inner mass cells of early embryos. Furthermore, also large-scale analysis of the mouse transcriptome indicates that *Fen1* expression strongly increases during early embryogenesis [33].

Our repeat length analysis did not reveal any differences in profiles between wt, $Fen1^{+/-}$ or $Fen1^{-/-}$ embryos. Assuming that the lack of nuclear Fen1 staining in $Fen1^{-/-}$ embryos indeed reflects complete absence of Fen1 protein during the entire initial state of embryogenesis, this suggests that Fen1's role is completely dispensable, or at least that the enzyme is not very frequently used for DNA flap processing, hairpin processing or maintenance of replication fork progression across the DM1 repeat tract during the initial cell divisions in early embryos. Our findings concerning the role of *Fen1* in early embryos and aged tissues in mature animals are thus congruent. Further analyses are necessary to reveal if replication and repair complexes and topology of the DM1 $(CTG)_n \cdot (CAG)_n$ repeat are indeed of similar nature in early embryonic and terminally differentiated cells. The use of conditional knockout strategies to ablate *Fen1* completely from specific somatic cells late in development should therefore be considered. More detailed study is also imperative to allow further comparison of effects of exonuclease and endonuclease activities of *Rad27/Rth1*, and cell state and growth effects, between yeast and murine models containing $(CTG)_n \cdot (CAG)_n$ repeats [14,16–19,29].

In summary, we provide here genetic evidence that *Fen1* activity is not a primary factor involved in somatic TNR instability in a DM1 mouse model.

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References

[1] Lavedan, C., Hofmann-Radvanyi, H., Shelbourne, P., Rabes, J.P., Duros, C., Savoy, D., Dehaupas, I., Luce, S., Johnson, K. and Junien, C. (1993) Myotonic dystrophy: size- and sex-dependent dynamics of CTG meiotic instability, and somatic mosaicism. Am. J. Hum. Genet. 52, 875–883.

[2] Brunner, H.G., Bruggenwirth, H.T., Nillesen, W., Jansen, G., Hamel, B.C.J., Hoppe, R.L.E., de Die, C.E.M., Howeler, C.J., van Oost, B.A., Wieringa, B., Ropers, H.H. and Smeets, H.J.M. (1993) Influence of sex of the transmitting parent as well as of parental allele size on the CTG expansion in myotonic dystrophy (DM). Am. J. Hum. Genet. 53, 1016–1023.

[3] Monckton, D.G., Wong, L.J., Ashizawa, T. and Caskey, C.T. (1995) Somatic mosaicism, germline expansions, germline reversions and intergenerational reductions in myotonic dystrophy males: small pool PCR analyses. Hum. Mol. Genet. 4, 1–8.

[4] Jansen, G., Willems, P., Coerwinkel, M., Nillesen, W., Smeets, H., Vits, L., Howeler, C., Brunner, H. and Wieringa, B. (1994) Gonosomal mosaicism in myotonic dystrophy patients: involvement of mitotic events in $(CTG)_n$ repeat variation and selection against extreme expansion in sperm. Am. J. Hum. Genet. 54, 575–585.

[5] Wong, L.J., Ashizawa, T., Monckton, D.G., Caskey, C.T. and Richards, C.S. (1995) Somatic heterogeneity of the CTG repeat in myotonic dystrophy is age and size dependent. Am. J. Hum. Genet. 56, 114–122.

[6] Anvret, M., Ahlberg, G., Grandell, U., Hedberg, B., Johnson, K. and Edstrom, L. (1993) Larger expansions of the CTG repeat in muscle compared to lymphocytes from patients with myotonic dystrophy. Hum. Mol. Genet. 2, 1397–1400.

[7] Sinden, R.R., Potaman, V.N., Oussatcheva, E.A., Pearson, C.E., Lyubchenko, Y.L. and Shlyakhtenko, L.S. (2002) Triplet repeat DNA structures and human genetic disease: dynamic mutations from dynamic DNA. J. Biosci. 27, 53–65.

[8] Baker, S.M., Plug, A.W., Prolla, T.A., Bronner, C.E., Harris, A.C., Yao, X., Christie, D.M., Monell, C., Arnheim, N., Bradley, A., Ashley, T. and Liskay, R.M. (1996) Involvement of mouse Mlh1 in DNA mismatch repair and meiotic crossing over. Nat. Genet. 13, 336–342.

[9] Manley, K., Shirley, T.L., Flaherty, L. and Messer, A. (1999) Msh2 deficiency prevents in vivo somatic instability of the CAG repeat in Huntington disease transgenic mice. Nat. Genet. 23, 471–473.

[10] van den Broek, W.J., Nelen, M.R., Wansink, D.G., Coerwinkel, M.M., te Riele, H., Groenen, P.J. and Wieringa, B. (2002) Somatic expansion behaviour of the $(CTG)_n$ repeat in myotonic dystrophy knock-in mice is differentially affected by Msh3 and Msh6 mismatch-repair proteins. Hum. Mol. Genet. 11, 191–198.

[11] Gomes-Pereira, M., Fortune, M.T., Ingram, L., McAbney, J.P. and Monckton, D.G. (2004) Pms2 is a genetic enhancer of trinucleotide CAG · CTG repeat somatic mosaicism: implications for the mechanism of triplet repeat expansion. Hum. Mol. Genet. 13, 1815–1825.

[12] Nichol Edamura, K., Leonard, M.R. and Pearson, C.E. (2005) Role of replication and CpG methylation in fragile X syndrome CGG deletions in primate cells. Am. J. Hum. Genet. 76, 302–311.

[13] Brock, G.J., Anderson, N.H. and Monckton, D.G. (1999) *Cis*-acting modifiers of expanded CAG/CTG triplet repeat expandability: associations with flanking GC content and proximity to CpG islands. Hum. Mol. Genet. 8, 1061–1067.

[14] Shen, B., Singh, P., Liu, R., Qiu, J., Zheng, L., Finger, L.D. and Alas, S. (2005) Multiple but dissectible functions of FEN-1 nucleases in nucleic acid processing, genome stability and diseases. Bioessays 27, 717–729.

[15] Gordenin, D.A., Kunkel, T.A. and Resnick, M.A. (1997) Repeat expansion – all in a flap? Nat. Genet. 16, 116–118.

[16] Callahan, J.L., Andrews, K.J., Zakian, V.A. and Freudenreich, C.H. (2003) Mutations in yeast replication proteins that increase CAG/CTG expansions also increase repeat fragility. Mol. Cell. Biol. 23, 7849–7860.

[17] Liu, Y. and Bambara, R.A. (2003) Analysis of human flap endonuclease 1 mutants reveals a mechanism to prevent triplet repeat expansion. J. Biol. Chem. 278, 13728–13739.

[18] Henricksen, L.A., Tom, S., Liu, Y. and Bambara, R.A. (2000) Inhibition of flap endonuclease 1 by flap secondary structure and relevance to repeat sequence expansion. J. Biol. Chem. 275, 16420–16427.

[19] Rolfsmeier, M.L., Dixon, M.J., Pessoa-Brandao, L., Pelletier, R., Miret, J.J. and Lahue, R.S. (2001) *Cis*-elements governing trinucleotide repeat instability in *Saccharomyces cerevisiae*. Genetics 157, 1569–1579.

[20] Otto, C.J., Almqvist, E., Hayden, M.R. and Andrew, S.E. (2001) The "flap" endonuclease gene FEN1 is excluded as a candidate gene implicated in the CAG repeat expansion underlying Huntington disease. Clin. Genet. 59, 122–127.

[21] Spiro, C. and McMurray, C.T. (2003) Nuclease-deficient FEN-1 blocks Rad51/BRCA1-mediated repair and causes trinucleotide repeat instability. Mol. Cell. Biol. 23, 6063–6074.

[22] Jackson, S.M., Whitworth, A.J., Greene, J.C., Libby, R.T., Baccam, S.L., Pallanck, L.J. and La Spada, A.R. (2005) A SCA7 CAG/CTG repeat expansion is stable in Drosophila melanogaster despite modulation of genomic context and gene dosage. Gene 347, 35–41.

[23] van Deursen, J. and Wieringa, B. (1992) Targeting of the creatine kinase M gene in embryonic stem cells using isogenic and nonisogenic vectors. Nucleic Acids Res. 20, 3815–3820.

[24] Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[25] van der Heijden, G.W., Dieker, J.W., Derijck, A.A., Muller, S., Berden, J.H., Braat, D.D., van der Vlag, J. and de Boer, P. (2005) Asymmetry in histone H3 variants and lysine methylation between paternal and maternal chromatin of the early mouse zygote. Mech. Dev. 122, 1008–1022.

[26] Warbrick, E., Coates, P.J. and Hall, P.A. (1998) Fen1 expression: a novel marker for cell proliferation. J. Pathol. 186, 319–324.

[27] Larsen, E., Gran, C., Saether, B.E., Seeberg, E. and Klungland, A. (2003) Proliferation failure and gamma radiation sensitivity of Fen1 null mutant mice at the blastocyst stage. Mol. Cell. Biol. 23, 5346–5353.

[28] Pearson, C.E., Nichol Edamura, K. and Cleary, J.D. (2005) Repeat instability: mechanisms of dynamic mutations. Nat. Rev. Genet. 6, 729–742.

[29] Freudenreich, C.H., Kantrow, S.M. and Zakian, V.A. (1998) Expansion and length-dependent fragility of CTG repeats in yeast. Science 279, 853–856.

[30] Kucherlapati, M., Yang, K., Kuraguchi, M., Zhao, J., Lia, M., Heyer, J., Kane, M.F., Fan, K., Russell, R., Brown, A.M.C., Kneitz, B., Edelmann, W., Kolodner, R.D., Lipkin, M. and Kucherlapati, R. (2002) Haploinsufficiency of flap endonuclease (Fen1) leads to rapid tumor progression. Proc. Natl. Acad. Sci. USA 99, 9924–9929.

[31] Matsuzaki, Y., Adachi, N. and Koyama, H. (2002) Vertebrate cells lacking FEN-1 endonuclease are viable but hypersensitive to methylating agents and H_2O_2. Nucleic Acids Res. 30, 3273–3277.

[32] Qiu, J., Li, X., Frank, G. and Shen, B. (2001) Cell cycle-dependent and DNA damage-inducible nuclear localization of FEN-1 nuclease is consistent with its dual functions in DNA replication and repair. J. Biol. Chem. 276, 4901–4908.

[33] Su, A.I., Cooke, M.P., Ching, K.A., Hakak, Y., Walker, J.R., Wiltshire, T., Orth, A.P., Vega, R.G., Sapinoso, L.M., Moqrich, A., Patapoutian, A., Hampton, G.M., Schultz, P.G. and Hogenesch, J.B. (2002) Large-scale analysis of the human and mouse transcriptomes. Proc. Natl. Acad. Sci. USA 99, 4465–4470.