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Dnmt1 Deficiency Promotes CAG Repeat Expansion in the Mouse Germline

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Abstract

Expanded CAG repeat tracts are the cause of at least a dozen neurodegenerative disorders. In humans, long CAG repeats tend to expand during transmissions from parent to offspring, leading to an earlier age of disease onset and more severe symptoms in subsequent generations. Here, we show that the maintenance DNA methyltransferase Dnmt1, which preserves the patterns of CpG methylation, plays a key role in CAG repeat instability in human cells and in the male and female mouse germlines. SiRNA knockdown of Dnmt1 in human cells destabilized CAG triplet repeats, and Dnmt1 deficiency in mice promoted intergenerational expansion of CAG repeats at the murine spinocerebellar ataxia type 1 (*Sca1*) locus. Importantly, *Dnmt1*^{+/-} SCA1 mice, unlike their *Dnmt1*^{+/+} SCA1 counterparts, closely reproduced the intergenerational instability patterns observed in human SCA1 patients. In addition, we found aberrant DNA and histone methylation at sites within the CpG island that abuts the expanded repeat tract in Dnmt1-deficient mice. These studies suggest that local chromatin structure may play a role in triplet repeat instability. These results are consistent with normal epigenetic changes during germline development contributing to intergenerational instability of CAG repeats in mice and in humans.

INTRODUCTION

CAG repeat expansion is the underlying cause of at least 12 neurodegenerative diseases (1, 2). In humans, repeat tracts tend to expand from one generation to the next, causing more severe phenotypes in the progeny, a phenomenon called anticipation (2). The extent of expansion bias in parent-to-offspring transmissions often depends on the sex of the transmitting parent. For example, in patients with spinocerebellar ataxia type 1 (SCA1), transmissions through the paternal germline are sharply biased toward expansions, while maternal transmissions are only slightly biased toward expansions (3–5). The mechanistic bases for these parent-of-origin differences are not known. The processes of replication, recombination, DNA repair, and transcription—acting separately or in combination—have been proposed to contribute to these differences, perhaps in conjunction with key events in male and female germline development (2). Here we explore the potential contribution of the epigenetic effects of CpG methylation on the stability of CAG repeat tracts during intergenerational transmissions.

Extensive repeat tract instability has been documented in both early embryogenesis and germline development (2). These two periods of repeat tract instability coincide with the two

major cycles of epigenetic reprogramming that occur during mammalian development. Immediately after fertilization, CpG methylation is largely removed from the genome and then restored in a tissue-appropriate manner (6). A second wave of demethylation occurs during germline development, when CpG methylation is again removed and then re-established (6). The similar timing of CpG methylation changes and repeat tract instability raises the possibility that these two processes may be mechanistically linked (2, 7).

A connection between DNA methylation and repeat instability has been noted for the expanded CGG repeats in the 5' end of the *FMR1* gene, which are responsible for Fragile X syndrome (8, 9). When an *FMR1* CGG repeat reaches about 200 units in humans, it usually becomes methylated. In somatic tissues, methylated CGG repeat tracts at the *FMR1* locus were found to be much more stable than unmethylated ones of comparable length (10, 11). Similarly, CGG expansions in the germline were observed only in male patients that carried unmethylated repeats (12). In contrast to humans, CGG repeat tracts introduced into the mouse *FMR1* locus do not become methylated, even when they exceed 200 repeats (13, 14). This unmethylated locus displays an ongoing instability in intergenerational transmissions (13, 14). Finally, when vectors carrying CGG repeats were introduced into primate cells, or into bacteria, methylated repeats proved to be more stable than unmethylated ones (15).

Although these observations indicate that CpG methylation influences CGG repeat stability, they provide little mechanistic insight into how methylation might prevent such tract-length changes. For the human and mouse studies, there is a strong correlation between repeat methylation and *FMR1* transcription: unmethylated repeats are transcribed; methylated repeats are not (16). Recent studies with CAG repeats in human cells (17, 18) and in *Drosophila* (19) indicate that transcription through repeat tracts can trigger repeat instability, presumably by promoting formation of secondary structures in the repeat tracts that are then resolved by various DNA repair processes (17–19). Thus, a plausible hypothesis is that CpG methylation of CGG repeats stabilizes them by preventing transcription through the repeat tract.

It is less obvious what effect DNA methylation might have on CAG repeats, which are themselves not targets for DNA methylation since they contain no CG dinucleotides. Nevertheless, two observations suggest that there is also a connection between DNA methylation and CAG repeat stability. First, in bacteria, CAG repeats in plasmids that were methylated at CpG sites were mildly stabilized relative to repeats in unmethylated plasmids (15). Second, treatment of mammalian cells with 5-aza-2'-deoxycytosine (5-aza-CdR), which leads to passive depletion of DNA methylation (20) and destruction of Dnmt1 (21), or with hydralazine, which induces demethylation by inhibiting expression of Dnmt1 (22, 23), both dramatically increased CAG repeat instability (7). Again, neither of these studies provides a mechanistic link between DNA methylation and repeat stability; however, they reinforce the idea that epigenetic changes to the DNA (or to the chromatin) contribute to the stability of CAG repeats.

Studies in humans and mice have demonstrated that epigenetic changes do occur in the vicinity of long CAG repeat tracts. The most extensive studies have been carried out for the *DMPK* gene, in which expanded CTG repeats in the 3' untranslated region cause myotonic dystrophy (DM1). Whereas normal individuals have a DNase hypersensitive site immediately downstream of the repeat tract, in DM1 patients with several thousand repeats, the hypersensitive site is missing (24). Absence of this hypersensitive site correlates with a change in transcription at both the *DMPK* gene and the downstream *SIX5* gene (25–29). Aberrant transcription of the *SIX5* gene apparently arises due to spreading of DNA methylation into its promoter region (29). The CTG expansion and flanking sequences in DM1 patients are also associated with changes in histone modifications—deacetylation of

histone H3 and hypermethylation of histone H3 at lysine 9—changes that are indicative of heterochromatin (30). In transgenic mice carrying tandem arrays of a reporter gene linked to an adjacent 192 CTG repeat, the reporter gene is consistently silenced (31). Taken together, these results suggest that long CTG repeats can trigger the formation and spreading of heterochromatin, which causes aberrant transcription and ultimately contributes to the disease phenotype (32, 33). These kinds of epigenetic changes have not been demonstrated for the shorter repeats that characterize many CAG repeat diseases, which are typically less than 100 repeats in length.

Collectively, these observations at the *FMR1* and *DMPK* loci suggest that repeat instability may be linked to the epigenetic changes that occur to DNA (and to the overlying chromatin), perhaps via associated changes in transcription through these genes. In this study, we investigate these potential connections, using a mouse model for spinocerebellar ataxia type 1 (SCA1), which carries 143 CAG repeats at the endogenous mouse *Sca1* locus (34). We sought to perturb DNA methylation patterns in these mice by making them heterozygous for a null allele of *Dnmt1*, the gene for the major maintenance DNA methyltransferase (35). By comparing *Dnmt1*^{+/-} SCA1 mice to *Dnmt1*^{+/+} SCA1 mice, we address four questions. Does *Dnmt1* deficiency affect the stability of CAG repeats during intergenerational transmission? Do epigenetic changes to the DNA, or to the chromatin, occur in the vicinity of the repeat? Is transcription through the repeat altered? Is the effect of *Dnmt1* deficiency the same in somatic tissues as it is in the germline?

RESULTS

Effects of *Dnmt1* knockdown on CAG repeat tract stability in human cells

Initially, we used a well-characterized selection assay for CAG contractions in human cells (17, 18) to test whether *Dnmt1* depletion could alter the instability of long CAG repeat tracts (Fig. S1). These modified HT1080 cells carry an integrated copy of a human *HPRT* minigene, whose function is blocked by a (CAG)₉₅ repeat tract inserted into the intron. Contraction of this long repeat tract to less than 39 units restores the function of the *HPRT* gene and allows the cells to survive in selective medium (17). Treatment with either of two *Dnmt1* siRNAs, each of which reduced *Dnmt1* expression by about 50%, doubled the frequency of large CAG contractions ($P < 0.001$) (Fig. S1). These results suggested that a reduced level of *Dnmt1* might increase CAG repeat instability *in vivo*.

Dnmt1 deficiency promotes repeat expansion during germline transmission in mice

We investigated the effect of *Dnmt1* CAG repeat instability *in vivo* by assaying germline transmission in mice. We took advantage of a null allele of *Dnmt1*, which was constructed to lack the conserved catalytic residues essential for methyltransferase activity, but which expresses no stable transcript or detectable protein (35). Because *Dnmt1* homozygous null mice die early during embryogenesis (35), we examined repeat instability in heterozygous *Dnmt1*^{+/-} mice, which do not differ from wild type mice in appearance or lifespan (36).

We crossed wild type mice to *Dnmt1*^{+/-} SCA1 and *Dnmt1*^{+/+} SCA1 mice, which carry a long CAG tract in one allele at the *Sca1* locus (34). We then compared the length of the repeat tract in each SCA1 donor parent to that of its progeny at weaning (Fig. 1). These data were gathered over a single generation. Our data with *Dnmt1*^{+/+} SCA1 mice corroborate those of a previous study (37): both paternal and maternal transmissions showed a bias toward contractions, with more extensive contractions generated in crosses with female donors (Fig. 1A). In addition, the frequency of stable transmissions—those with no change in repeat tract length—was higher among the progeny of male donors than it was among the progeny of female donors, as previously observed (37). This agreement with published

results suggests that strain background does not contribute to the significant differences observed between *Dnmt1^{+/+}* SCA1 mice and their *Dnmt1^{+/-}* SCA1 littermates.

In sharp contrast to the results with *Dnmt1^{+/+}* SCA1 mice, transmissions from *Dnmt1^{+/-}* SCA1 mice yielded substantially increased proportions of expansions in both paternal and maternal transmissions (15% to 56% in males, $P < 0.0001$, and 6% to 22% in females, $P = 0.0009$), with an overall bias toward expansions during paternal transmissions (Fig. 1). Furthermore, the maximum expansion length increased from +1 to +6 in males and from +2 to +7 in females, which is similar to the range of CAG expansions seen in human SCA1 patients (Fig. S2). Dnmt1 deficiency decreased the percentage of stable transmissions among the progeny of male donors (from 43% to 29%), but increased their frequency during maternal transmission (2% to 22%). We conclude that Dnmt1 deficiency alters the stability of CAG repeat tracts during intergenerational transmission and increases the bias toward expansions in both male and female donors.

Age dependence of repeat expansions during germline transmission in *Dnmt1^{+/-}* SCA1 mice

Previous characterizations of the SCA1 knock-in mouse model and of a transgenic SCA1 mouse model carrying a shorter repeat tract demonstrated that larger CAG repeat contractions occurred with advancing age of the mother (37, 38). To examine this issue for germline transmission from *Dnmt1^{+/-}* SCA1 mothers, we re-plotted the data in Figure 1 after grouping the donor mice by age (Fig. 2). For female *Dnmt1^{+/+}* SCA1 donors, the dominant process is repeat contraction, with the average contraction size increasing from -2.8 to -5.3 to -7.7 with age, consistent with previous studies (37, 38). For female *Dnmt1^{+/-}* SCA1 donors, the situation is more complicated. There is a similar trend in contractions, with the average contraction size increasing from -1.5 to -2.4 to -8.3 with age. Expansions, however, are observed only in the young and middle-aged donors. Thus, in females the effect of Dnmt1 deficiency on repeat expansion is apparent at early times, but is eventually overwhelmed by whatever age-dependent process generates contractions in oocytes. The confounding effects of contractions in the female germline make it difficult to determine whether expansions in *Dnmt1^{+/-}* SCA1 female donors are age dependent. As a result, the distributions of repeat lengths in the progeny from young and middle-aged *Dnmt1^{+/-}* SCA1 female donors are significantly different from those of *Dnmt1^{+/+}* SCA1 female donors ($P = 0.001$ and $P = 0.0001$, respectively), but the distributions in >25-week old *Dnmt1^{+/+}* SCA1 and *Dnmt1^{+/-}* SCA1 female donors are indistinguishable ($P = 0.1$).

Previous studies in SCA1 mice did not detect age-dependence repeat instability in paternal germline transmissions (37, 39). In our experiments, as well, the distribution of repeat lengths in the progeny of *Dnmt1^{+/+}* SCA1 fathers is too narrow to reliably discern an age-dependent trend, if it exists. For male *Dnmt1^{+/-}* SCA1 donors, however, we observe a gradual shift in the average size of expansions from 1.4 in young donors, to 1.7 in the middle-aged donors, to 2.5 in older donors, with a corresponding increase in maximum expansion size from 3 to 4 to 6 repeats (Fig. 2). The distribution of repeat lengths at >25 weeks is marginally different from that at 5–15 weeks ($P = 0.04$), consistent with a small age-dependent effect on tract-length changes in the male germline of *Dnmt1^{+/-}* SCA1 mice.

Expansions in *Dnmt1^{+/-}* SCA1 donor mice depend only the genotypes of the parental mice

A strong dependence of expansions on parental age during male and female transmissions would have suggested that the changes occur in the donors themselves rather than in the first few divisions of the embryo, which is the other identified time for CAG repeat instability (2). To address these alternatives more directly, we analyzed the data in Figure 1B for effects due to the progeny genotypes. If the track-length expansions arose because of the

Dnmt1 deficiency early during embryogenesis, they would be expected to be dependent on the genotype—*Dnmt1*^{+/-} SCA1 or *Dnmt1*^{+/+} SCA1—of the progeny embryo. By contrast, if expansions arose due to Dnmt1 deficiency during germline development or maturation in the parents, they would be expected to depend solely on the parental genotype. Analysis of the distribution of tract-length changes in *Dnmt1*^{+/-} SCA1 and *Dnmt1*^{+/+} SCA1 progeny mice (Fig. 3) showed that there was no significant difference for male donors ($P = 0.8$). For female donors, we analyzed progeny that arose from donors up to 25 weeks in age, where expansions were present among the progeny (Fig. 2). The progeny from older mice (>25 wks) were specifically excluded from this analysis to avoid potential bias because they display only contractions, which are known to arise in the germline (38). Once again, there was no significant difference between *Dnmt1*^{+/-} SCA1 and *Dnmt1*^{+/+} SCA1 progeny mice ($P = 0.5$). These data indicate that the observed expansions arose in the donor mice, rather than in the progeny embryos.

As one gauge of the reproducibility of these measurements, we selected *Dnmt1*^{+/-} SCA1 progeny from the first-generation experiments above, bred them to wild type mice, and analyzed the repeat lengths in their progeny. Progeny of these second-generation donors replicated the data for *Dnmt1*^{+/-} SCA1 mice shown in Figures 1 and 2: a similar bias toward expansion in both male and female donors, and fewer stable transmissions in males and more stable transmissions in females (data not shown).

CpG methylation in testes and ovaries from *Dnmt1*^{+/+} SCA1 and *Dnmt1*^{+/-} SCA1 mice

The normal appearance and lifespan (36) of *Dnmt1*^{+/-} SCA1 mice suggest that major changes in CpG methylation are unlikely. Subtle differences, however, have been detected by the coat-color readout of the methylation-sensitive *A^{iap}y* allele (40). We analyzed DNA from testes and ovaries of *Dnmt1*^{+/+} SCA1 and *Dnmt1*^{+/-} SCA1 mice for differences in global CpG methylation, differences adjacent to the repeat tract, and nonstandard CpA and CpT DNA methylation within the repeat tract itself.

We used methylation-sensitive restriction enzymes and quantitative bisulfite sequencing of repetitive intracisternal A-type particles to assess DNA methylation genome-wide (41) (Fig. S3). We found that testes are hypomethylated relative to ovaries, but we detected no genome-wide differences in CpG methylation between *Dnmt1*^{+/+} SCA1 and *Dnmt1*^{+/-} SCA1 gonads (Fig. S3A & B). Moreover, digestion of genomic DNA from testes by methylation sensitive enzymes did not uncover differences in genome-wide DNA methylation (Fig. S3C). Similarly, we found no differences in nonstandard cytosine methylation in CAG repeat DNA from testes and ovaries. Analysis of at least 350 CAG repeats from gonads of each genotype revealed about 1% apparent cytosine methylation, which was indistinguishable from the background levels found in control reactions with unmethylated *Sca1* repeat DNA that was generated by PCR and cloning into a plasmid. Thus, altered methylation of the *Sca1* CAG repeat itself does not account for the increased frequency of expansions found in *Dnmt1*^{+/-} SCA1 mice.

To assess DNA methylation adjacent to the repeat tract on the expanded *Sca1* allele, we selectively analyzed CpG sites 17 and 20 bp upstream of the expanded CAG tract and a CpG site 32 bp downstream (Fig. 4A). No differences were apparent at the downstream site; however, at the upstream sites we found small, but significant, differences between *Dnmt1*^{+/-} SCA1 mice and *Dnmt1*^{+/+} SCA1 mice in both testes ($P = 0.03$ and 0.02 for sites 1 and 2, respectively) and ovaries ($P = 0.04$ and 0.06 for sites 1 and 2, respectively) (Fig. 4B & C). Thus, Dnmt1 deficiency has opposite effects on local *Sca1* DNA methylation in testes and ovaries, with elevated levels in testes and reduced levels in ovaries ($P = 0.002$) (Fig. 4). These observations on DNA methylation echo the opposite effects of Dnmt1 deficiency on the percentage of stable germline transmission in *Dnmt1*^{+/-} SCA1 mice, which decreased in

male donors, but increased in female donors (Fig. 1). We find that high local DNA methylation correlates with low frequencies of stable transmissions ($P=0.01$). This correlation implies that local DNA methylation may contribute to the propensity of a repeat tract to become unstable.

Histone modification at the *Sca1* locus in testes from *Dnmt1*^{+/+} SCA1 and *Dnmt1*^{+/-} SCA1 mice

One of the most striking features of the DNA methylation data at the expanded *Sca1* allele is the very broad range of methylation levels in both testes and ovaries of *Dnmt1*^{+/-} SCA1 mice as compared to *Dnmt1*^{+/+} SCA1 mice. This extreme variability between mice (but not in replicate tests of the same mouse) is reminiscent of position-effect variegation, a process caused by stochastic spreading of heterochromatin to adjacent chromatin domains. Indeed, transgene arrays containing trinucleotide repeats have been shown to induce such variegation (31). As indicators of chromatin alteration, we examined specific histone modifications in the region immediately upstream and downstream of the CAG repeat at the *Sca1* locus. We chose to examine acetylated histone H3 (H3Ac) and dimethylated histone H3 at lysine 9 (H3K9me2) because *Dnmt1* is known to interact with the histone deacetylases HDAC1 and HDAC2 (42, 43), and with the histone methyltransferase G9a, which adds two methyl groups to H3K9 (44).

We immunoprecipitated chromatin-bound H3Ac and H3K9me2 from testes of 40-week old *Dnmt1*^{+/+} *Sca1*^{143Q/2Q} and *Dnmt1*^{+/-} *Sca1*^{143Q/2Q} mice (SCA1 mice) and from 40-week old *Dnmt1*^{+/+} *Sca1*^{2Q/2Q} and *Dnmt1*^{+/-} *Sca1*^{2Q/2Q} mice (“wildtype” mice). We then quantified the precipitated DNA on either side of the CAG repeat (Fig. 5). We found that chromatin immunoprecipitation (ChIP) by H3Ac-specific antibodies did not enrich for DNA sequences around the site of the repeat tract, relative to the sequences precipitated by nonspecific antibodies, for any of the 33 mice that were tested. By contrast, H3K9me2-specific ChIP identified 1 mouse out of the 33 mice tested, in which DNA sequences were dramatically enriched relative to ChIP by the nonspecific antibody. This *Dnmt1*^{+/-} SCA1 mouse was enriched 68 fold for upstream sequences; it yielded high enrichment values in three independent immunoprecipitations. Although these results do not rise to the level of statistical significance, they are consistent with the idea that the repeat tract itself may trigger stochastic formation of heterochromatin. These results, along with similar findings of DNA methylation changes, raise the possibility that changes in DNA methylation and altered chromatin structure in the vicinity of the repeat tract may alter repeat tract stability.

Effects of *Dnmt1* deficiency on transcription at the *Sca1* locus in testes

We showed previously that increased transcription through a CAG tract in human cells destabilizes the repeats (17, 18), and transcription has recently been linked to repeat instability in the *Drosophila* germline (19). Given the inverse correlation between promoter methylation levels and gene expression (45), *Dnmt1* deficiency could increase transcription through the CAG tract in the *Sca1* gene, thereby destabilizing the repeats. To test this possibility, we used quantitative RT-PCR to measure the levels of *Sca1* mRNA in testes from *Dnmt1*^{+/-} SCA1 and *Dnmt1*^{+/+} SCA1 mice. Testicular levels of *Sca1* transcripts were not affected by *Dnmt1* genotype (Fig. S4), implying that transcription-altering changes in DNA methylation in the *Sca1* promoter region are not the source of the observed increase in expansion frequencies. Consistent with this conclusion, we observed no difference in H3Ac and H3K9me2 levels in the *Sca1* promoter region (~300kb upstream of the repeat tract) in *Dnmt1*^{+/+} SCA1 and *Dnmt1*^{+/-} SCA1 mice (Fig. S5).

Evidence against NHEJ as a source of instability

Previously, we used a mammalian cell culture assay to determine whether long CAG repeat tracts (98 and 183 repeats) stimulated either homologous recombination or gene rearrangements (46). We found that repeat tracts in this size range did not stimulate homologous recombination above spontaneous levels, but they did increase gene rearrangements more than 50-fold—presumably via nonhomologous end joining (NHEJ) of double-strand breaks. Some of the rearrangement junctions had deletions that extended beyond the repeat tract, whereas others had extra sequences inserted at the rejoined ends, as is typically found at 10–20% of end-joining junctions (see, for example (47, 48)).

If most of the CAG repeat instability at the *Sca1* locus resulted from breaks that were repaired by NHEJ, we would expect to find similar kinds of deletions and insertions at the repeat tract. We examined all the sequences gathered to analyze intergenerational triplet repeat instability, both donors and progeny, for a total of 109 *Dnmt1*^{+/+} SCA1 and 153 *Dnmt1*^{+/-} SCA1 mice. In no instance did we find a deletion that extended outside the repeat tract or an insertion of extra nucleotides within the repeat tract. In addition, there were no mutations that altered either the sequence of the repeat or of any nucleotides in the 60 bp upstream of the repeat. The absence of these telltale signatures suggests that NHEJ is not responsible for the observed instability.

Dnmt1 deficiency does not substantially affect somatic instability of SCA1 repeats

Next, we asked whether Dnmt1-deficiency increases expansions specifically in the germline, or also increases expansions in somatic tissues. We analyzed DNA methylation and repeat length variations in cerebellum, liver, and striatum, which display low, moderate, and high levels of instability, respectively (37). In contrast to what we observed for IAPs in oocytes and testes, where we saw no effect of Dnmt1 deficiency, we found that DNA methylation of IAPs was slightly lower in all three tissues in *Dnmt1*^{+/-} SCA1 mice compared to *Dnmt1*^{+/+} SCA1 mice ($P = 0.002$). We found that DNA methylation was also slightly lower for the two CpG sites upstream of the repeat tract at the *Sca1* locus ($P = 0.02$) (Figure S6 and Figure S7), similar to our observations in oocytes.

To determine the effects of Dnmt1 deficiency on the SCA1 repeat, we carried out small-pool PCR analyses of repeat tract lengths in cerebellum, liver, and striatum in *Dnmt1*^{+/-} SCA1 mice and *Dnmt1*^{+/+} SCA1 mice (Figure S8). These results, which are summarized in Table 1, indicate that repeat expansions are not enhanced in these three tissues in *Dnmt1*^{+/-} SCA1 mice relative to those from *Dnmt1*^{+/+} SCA1 mice. It should be noted that the expansions we observe in liver and striatum are generally larger than those we observe in the germline, but the distributions of lengths are comparable in *Dnmt1*^{+/-} and *Dnmt1*^{+/+} SCA1 mice. These results indicate that the deficiency of Dnmt1 in these somatic tissues is not sufficient to affect expansion frequencies. Thus, it appears that the effects of Dnmt1 deficiency on CAG repeat tract stability are specific for the germline.

DISCUSSION

Here we have investigated the potential links between epigenetics and CAG repeat stability at the *Sca1* locus in mice. This study stems from three observations. First, the two identified periods of enhanced repeat instability during intergenerational transmission—during early embryogenesis and in germline development—overlap with the two major cycles of epigenetic reprogramming, when DNA methylation patterns are erased and re-established (6). Second, observations at the *FMR1* and *DMPK* loci in humans and mice show that long repeat tracts are associated with changes in DNA methylation (for CGG repeats), histone modification, chromatin structure, and transcription (8–11, 16, 25–30, 49, 50). Third,

treatments that lead to genome-wide demethylation dramatically stimulate CAG repeat instability in a cell-culture assay (7).

After confirming that siRNA knockdown of Dnmt1 in human cells altered CAG repeat stability in a cell culture assay, we sought to perturb DNA methylation in mice by making them heterozygous for a null allele of *Dnmt1*. We showed that Dnmt1 deficiency leads to a dramatic increase in intergenerational expansions at the *Sca1* locus in both male and female mice; however, it causes no apparent increase in expansions in liver, cerebellum, and striatum. These results suggest that Dnmt1 deficiency specifically affects expansion in the germline. The dependence of the expansions on the genotype of the donating parent (and not on that of the progeny) in both males and females indicates that the critical events leading to expansions in Dnmt1-deficient mice occur during germline development. The enhanced instability of CAG repeats that is caused by genetically altering Dnmt1 levels is consistent with the notion that normal epigenetic changes during germline development may play a role in the intergenerational instability of repeat tracts at the *Sca1* locus—and potentially at other repeat loci—in mice and human.

Progeny from both male and female *Dnmt1*^{+/-} SCA1 donors display a clear increase in expansions—relative to progeny from *Dnmt1*^{+/+} SCA1 donors—at the earliest donor ages (5–15 weeks). Subsequently, the progeny from male donors show a small increase in expansions with donor age, while the progeny from female donors ultimately show only contractions at late donor age (>25 weeks). These data suggest that two distinct processes may operate during germline development. An expansion-biased process appears to function early on in the germles of both male and female *Dnmt1*^{+/-} SCA1 donors. It will be crucial to know the timing of this process more precisely, but the present data are consistent with the idea that it overlaps the period of hypomethylation in normal germline development. The second process operates later on and differs significantly in the male and female germles. In male donors, a slight expansion bias continues with age. In female donors a contraction-biased process predominates at later times in both *Dnmt1*^{+/+} SCA1 and *Dnmt1*^{+/-} SCA1 mice. This contraction-biased process is thought to operate in arrested oocytes and has been attributed to oocyte-specific DNA repair (38).

Because long repeat tracts at both the *FMR1* and *DMPK* loci are associated with epigenetic changes, we examined the region around the CAG repeat tracts at the *Sca1* locus to determine whether any changes in DNA methylation, or in the overlying chromatin, were present. To try to capture data that is relevant to germline transmission, we analyzed testes and ovaries. We found that the methylation changes in these tissues were in opposite directions in *Dnmt1*^{+/-} SCA1 mice relative to *Dnmt1*^{+/+} SCA1 mice, with elevated levels in testes and reduced levels in ovaries. Although we had expected Dnmt1 deficiency to lead to reduced DNA methylation, as we observed in oocytes and somatic tissues, the paradoxical local increase in testes has precedent in other systems. For instance, cancer cells with decreased Dnmt1 levels are generally hypomethylated genome-wide, but local hypermethylation is common (51). In the region just upstream of the repeat tract in testes, we also found increased levels of histone H3K9me2, which is indicative of heterochromatin. These results suggest that Dnmt1 deficiency can induce epigenetic changes at the CAG repeat tract at the *Sca1* locus, similar to those found at the *FMR1* and *DMPK* loci in humans.

At the *Sca1* locus the changes in DNA methylation and in histone modification were found only at sites within the CpG island that abuts the upstream side of the CAG repeat tract. This CpG island is conserved in humans and mice. A strong association between unstable repeats and nearby CpG islands has been noted for several triplet-repeat diseases (52, 53), but no mechanistic connection has been established. At the *Sca1* locus, as well, we do not know

whether the epigenetic changes cause the germline expansions we observe, or are merely associated with them. These epigenetic changes in the adjacent CpG island cannot be the entire story, however, because similar changes in somatic tissues are not associated with increased repeat expansions. Thus, if these epigenetic changes are causative, they must interact with germline specific factors to bring about repeat expansions. Additional experiments in SCA1 mice in which specific CpG sites have been eliminated, or the island itself removed, will be required to distinguish cause from association.

Although we have shown that Dnmt1 deficiency leads to expansions in both the male and female germline, and we have identified local epigenetic changes, we have not identified the mechanistic link between Dnmt1 deficiency and repeat expansion. Our study eliminates several potential sources of triplet repeat instability caused by Dnmt1 deficiency, thereby providing useful mechanistic information. First, we found no DNA methylation within the CAG repeat tract itself in DNA isolated from testes or ovaries, showing that CAG repeat tracts do not serve as a nidus for abnormal CpA methylation in either *Dnmt1*^{+/+} SCA1 mice or in *Dnmt1*^{+/-} SCA1 mice. Second, we showed that neither histone acetylation at the *Sca1* promoter nor transcription through the *Sca1* locus is altered in testes from *Dnmt1*^{+/-} SCA1 mice relative to those from *Dnmt1*^{+/+} SCA1 mice. These results argue against the possibility that increased transcription through the *Sca1* locus causes repeat expansion during germline transmission in *Dnmt1*^{+/-} SCA1 mice, an attractive hypothesis based on the link between transcription and repeat instability established recently in human cells and *Drosophila* germline (17–19). It is important to note that our studies with whole testes would have missed a dependence on transcription if the critical events occurred in a small proportion of the population. Third, while we cannot rule out the possibility that DNA methylation changes alter transcription of other genes, which then act on the repeat tract in *trans*, we analyzed mRNA levels of several genes that have been identified to participate in triplet repeat instability: *Xpa* (18), *Xpg* (17), *Msh2* (18, 54–56), and *Fen-1* (57). We found no differences in the abundance of their mRNA in testes from *Dnmt1*^{+/+} SCA1 mice and *Dnmt1*^{+/-} SCA1 mice (data not shown). Thus, it is unlikely that changes in the levels of those particular genes are the cause of the increase in expansion in *Dnmt1*^{+/-} SCA1 mice. Fourth, analysis of the repeat tracts transmitted through the germline showed none of the signature features of NHEJ, suggesting that changes in the lengths of the repeat tracts did not arise by nearby DSBs that were rejoined by the typical mechanism of NHEJ (47, 48, 58). Finally, we have recently found that 5-aza-CdR-induced genome-wide demethylation leads to instability by a mechanism that is independent of homologous recombination (7, 46, 59)

In addition, although we have demonstrated subtle changes in DNA methylation and local chromatin structure, it is possible that Dnmt1-deficiency has its primary effect on repeat stability via a mechanism that is unrelated to its enzymatic function. Dnmt1 is a component of many different complexes involved in various aspects of DNA metabolism, including chromatin structure (42–44, 60–63), DNA replication (64, 65), DNA damage response (66–68), and mismatch repair (69–72). Each of these processes has been shown to impinge on the stability of repeat tracts (2). If the deficiency of Dnmt1 in *Dnmt1*^{+/-} SCA1 mice compromised the function of any of these complexes, repeat tracts might be destabilized.

One important feature of Dnmt1-deficient SCA1 mice is that they closely resemble the intergenerational instability pattern seen in SCA1 patients, which has been difficult to demonstrate for most models of repeat diseases (73). The knock-in SCA1 mouse model used in our studies is no exception. As described initially (37) and confirmed here, transmission in both male and female *Dnmt1*^{+/+} SCA1 mice is strongly biased toward contraction, so that the distributions of tract-length changes do not match those of human patients ($P < 0.0001$ for males and females). By contrast, the distributions in Dnmt1-deficient mice are much more similar to the patient distributions ($P = 0.08$ for males, and $P = 0.6$ for female donors

less than 25 weeks old) (Fig. S2). Thus, *Dnmt1*^{+/-} SCA1 mice provide the most accurate model to date for studying intergenerational repeat instability in polyglutamine diseases. Why a deficiency of Dnmt1 should bring mouse intergenerational transmission data more in line with that for humans is not yet clear. The answer to that question will likely provide insights into the human disease.

Finally, the high variability in the adjacent epigenetic marks indicates that the expanded repeat tract at the *Sca1* locus creates a metastable epiallele. Typically, metastable epialleles are susceptible to environmental influences such as diet (74). If the stability of the CAG repeat tract at the *Sca1* locus proves to be determined by these epigenetic changes, it may ultimately prove possible to control repeat stability through diet, a possibility that extends not only to SCA1 disease, but also potentially to other repeat-associated neurodegenerative diseases.

MATERIALS AND METHODS

Mice

Mice were handled in accordance with an approved protocol from the Animal Research Committee of Baylor College of Medicine. C57BL/6 mice were purchased from Harlan Sprague Dawley. The *Dnmt1* mice were in a pure 129/Sv background (35). The null allele used, *Dnmt1*^c, has a deletion of the catalytic domain of Dnmt1, but expresses no stable transcript or detectable protein (35). We refer to this allele as *Dnmt1*⁻ for simplicity. Males carrying the knock-in allele of *Sca1* with the expanded repeat tract (which we refer to as SCA1 mice) had been backcrossed to C57BL/6 females for 8 to 10 generations (34) before we bred them to *Dnmt1*^{+/-} mice. The litter sizes were normal, the genetic ratios of *Dnmt1*^{+/-} SCA1 progeny were as expected, and the Dnmt1 deficiency did not affect body weight of the *Dnmt1*^{+/-} SCA1 mice. The male and female *Dnmt1*^{+/-} SCA1 and *Dnmt1*^{+/+} SCA1 progeny from these crosses were then crossed to C57BL/6 mice to measure intergenerational repeat stability. Thus, the data reported in Figures 1, 2, and 3 were gathered over a single generation. The repeat tract lengths in the parents and offspring were determined by sequencing tail DNA at weaning. The repeat tract lengths in *Dnmt1*^{+/+} SCA1 male donors (n=5) ranged from 142 to 145; in *Dnmt1*^{+/+} SCA1 female donors (n=10), from 142 to 150; in *Dnmt1*^{+/-} SCA1 male donors (n=11), from 141 to 146; and in *Dnmt1*^{+/-} SCA1 female donors (n=7), from 142 to 147. We did not observe any correlation in these ranges between the repeat-tract length in the donor and the extent of the intergenerational instability.

PCR primers and sequencing

The *Dnmt1* locus was genotyped as described (75): the pair of PCR primers specific for the *Dnmt1*⁺ allele amplifies an exon deleted in the *Dnmt1*⁻ allele; the primer pair specific for the *Dnmt1*⁻ allele amplifies sequences unique to the null allele. Genotyping primers were oVIN-24F (5'-aac atg ggc agt ctg agc cag) and oVIN-24R (5'-agc cct gct gag gtg ctg ctg) for the *Sca1* locus. To determine repeat size at weaning, we amplified the CAG repeat with oVIN-106F (5'-cgt gta ccc tcc tcc tca gt) and oVIN-24R and sequenced the PCR products directly using oVIN-95F (5'-ggc cac cac tcc atc aca gc). Sequencing was performed by the Baylor College of Medicine Sequencing Core. To determine whether this sequencing approach was reliable, we independently amplified and sequenced 11 samples 4 to 5 times each (Table S1). Thirty-eight of the 46 runs (83%) were in agreement. The remaining 8 runs (17%) gave a repeat number that was either one CAG repeat higher or lower than the most commonly observed tract length. This low frequency of potential one-repeat errors does not significantly alter the data presented in this study, which were derived primarily from single sequencing runs.

Bisulfite Sequencing

To determine whether *Dnmt1*^{+/-} SCA1 mice showed decreased levels of DNA methylation, we isolated DNA from ovaries and testes of 6, 12, 24, and 40 week-old *Dnmt1*^{+/-} SCA1 and *Dnmt1*^{+/+} SCA1 mice. We could not obtain enough DNA from gametes for our methylation analysis at the expanded *Sca1* locus and thus we opted for gonadal DNA. Methylation patterns in testis and sperm are identical (76). The differences in DNA methylation levels between wild type and heterozygous testes were not due to an altered number or morphology of somatic cells in the *Dnmt1*^{+/-} SCA1 testes (data not shown). Bisulfite modification and PCR amplification were performed as described (77). The amplicons were sequenced using ³³P-labelled ddNTPs (GE Healthcare) and the products were separated by electrophoresis on a polyacrylamide denaturing gel. Intensities of bands at methylation sites were quantified in a blinded fashion by phosphoimaging (77). CpG methylation was calculated as: % methylation = 100 x [$C_{\text{volume}} / (C_{\text{volume}} + T_{\text{volume}})$]. For IAP sequences, bisulfite-treated DNA was amplified using primers IAPLTRF2 and IAPLTRR1 (77). Methylation on the expanded allele at the *Sca1* locus was analyzed as follows: before the bisulfite treatment, 10 µg of genomic DNA was digested with *Ban*II (NEB), which cuts only the wild type allele, at a position 22 bp upstream of the two CAGs. PCR of the intact, expanded allele was then performed using oVIN-147F (5' - ttt ttt tta gtt gat ttt ttt att agg taa) and oVIN-61R (5' - tat aaa aaa cta aaa ata taa ata tac taa ttc tac), which prime 160 bp upstream and 57 bp downstream of the repeat tract, respectively. The amplification products were sequenced using oVIN-57F (5' - ttt att ttg ttg gtt aat atg gg), which primes 57 bp upstream of the CAG tract. We used oVIN-151F (5' - tag tag tat ttt agt agg gtt gta gga tta gtt aat t), which primes immediately adjacent to the third CpG site, to sequence it.

Chromatin immunoprecipitation

Chromatin immunoprecipitation experiments were performed as described (78), using rabbit polyclonal antibody against H3Ac (Upstate) or H3K9me2 (Upstate). We used rabbit IgG (Sigma-Aldrich) for nonspecific immunoprecipitations. We performed quantitative PCR on the immunoprecipitated DNA, using oVIN-106F (5' -cgt gta ccc tcc tcc tca gt) and oVIN-186R (5' -ctg cag ctg gga gcg) for the region upstream of the CAG repeat tract, and oVIN-200F (5' - cac cca gca gaa gta ca) and oVIN-200R (5' - gat ggg tgg ggg aga tgt) for the downstream region. For each mouse analyzed, we made two or three independent chromatin preparations. Each chromatin preparation was immunoprecipitated using nonspecific antibodies and antibodies specific for either H3Ac or H3K9me2. The DNA in each precipitation was measured by quantitative PCR using primers upstream and downstream of the repeat. Amplification values were normalized for the amount of DNA in the original chromatin preparation, which was also measured by quantitative PCR. Enrichment ratios were calculated by dividing the quantity of normalized DNA for the specific antibody by that for the nonspecific antibody. The values obtained from each chromatin preparation were averaged for each mouse, and the average of these means was calculated for all mice of the same genotype.

Small-pool PCR

DNA isolated from different mouse tissues was digested with *Ban*II to fragment the DNA. DNA was quantified by spectrophotometry and then small-pool PCR (SP-PCR) reactions were set up at DNA concentrations of 100 pg, 50 pg, 25 pg, and 12.5 pg per reaction. An initial set of seven SP-PCRs were run to determine the optimum dilution to use for each tissue sample, so that the reactions yielded approximately 2–3 amplifiable genomes per reaction. The PCR primers were oVIN-25F (5' - gtc acc agt gca gta gcc tca g), which primes 111 bp upstream of the repeat, and oVIN-25R (5' - atg tac tgg ttc tgc tgg gtt), which primes 51 bp downstream of the repeat. We used ChromaTaq (Denville Scientific) in accordance to the supplier's instructions, with the addition of DMSO to a final concentration of 5% in 10

μ l reactions. The PCR program was 94°C for 2 min for initial denaturation, followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min. The PCR products were separated by electrophoresis on a 2% agarose TAE gel at 80 V for 16 hours, transferred to a nylon membrane, and hybridized with a (CAG)₁₀ probe that had been labelled at the 5' end with γ -³²P-ATP and polynucleotide kinase (Invitrogen). The resulting blots were analyzed by phosphorimaging. Tail DNA from the same mouse as the tissue sample was included on either side of each set of reactions. Lines bracketing the top and bottom of the tail-DNA bands were drawn, and sample bands were judged against those lines. Sample bands that were more than $\frac{3}{4}$ of the way above or below the lines were classified as expansions or contractions, respectively. Reconstruction experiments using repeats of defined length indicated that according to this criterion repeats that were more than 5 CAG units longer than the tail DNA would be classified as expansions, and those that were more than 5 CAG units shorter would be classified as contractions. The total number of alleles examined was calculated by multiplying the total number of individual samples analyzed by the average number of alleles per sample, which was estimated from the Poisson distribution by counting the number of samples that had 0 alleles.

Statistics

Distributions of tract-length changes were compared using the non-parametric Mann-Whitney test, which does not assume a normal distribution. Data on site-specific CpG methylation at IAPs and at the *Scal* locus were analyzed by repeated-measures analysis of variance (SAS PROC MIXED), which acknowledges the intercorrelation among multiple CpG sites within each individual. SP-PCR data was analyzed using a Chi-squared test with 2 degrees of freedom.

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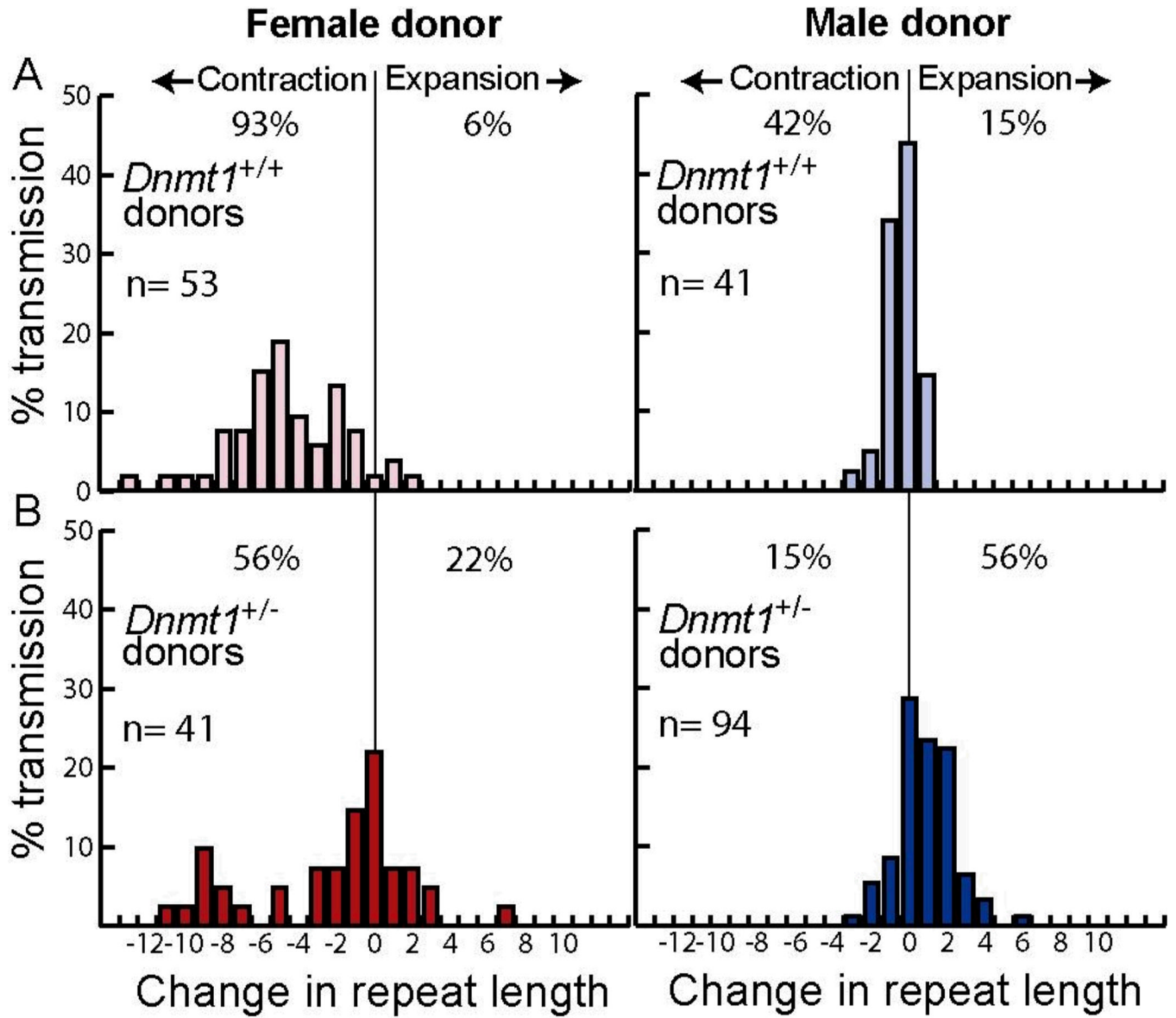


Figure 1. Intergenerational changes in CAG repeat tract lengths in SCA1 progeny mice. Percent transmission is the number of alleles with a given change in repeat length divided by the total number of alleles (n) multiplied by 100%. Change in repeat length is defined as the number of repeats in a progeny mouse minus the number of repeats in its donor parent. A) *Dnmt1*^{+/+} SCA1 mouse donors. B) *Dnmt1*^{+/-} SCA1 mouse donors. The progeny from the male and female *Dnmt1*^{+/-} SCA1 donors includes *Dnmt1*^{+/+} SCA1 and *Dnmt1*^{+/-} SCA1 mice.

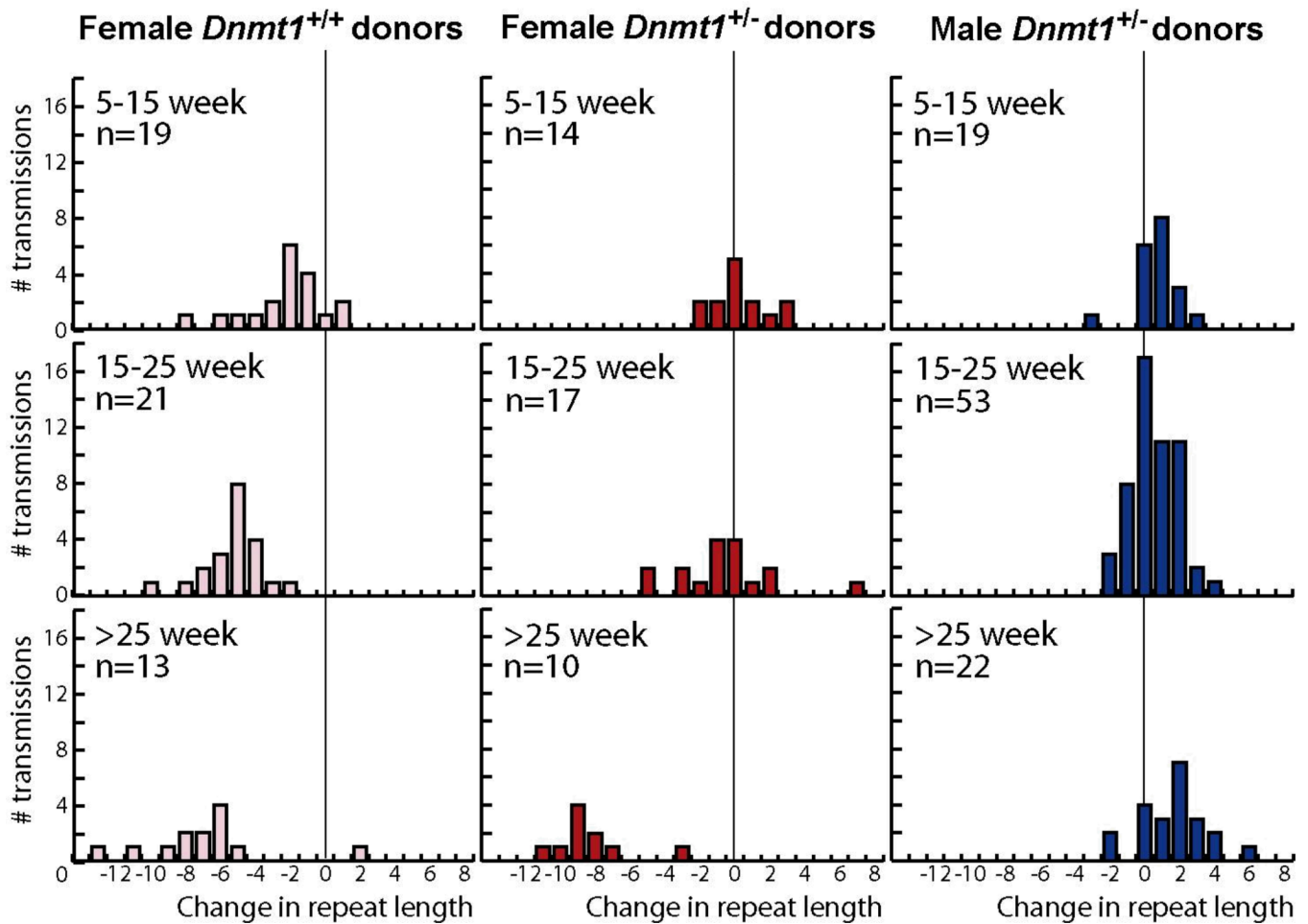


Figure 2.

Age association of track-length changes in progeny of *Dnmt1*^{+/+} SCA1 and *Dnmt1*^{+/-} SAC1 donor mice. The number of transmissions taken from figure 1A&B were plotted as a function of age of the donor parent at conception. Top row: 5–15 week-old mouse donors. Middle row: 15 week and 1 day to 25 week-old donors. Bottom row: donors older than 25 weeks.

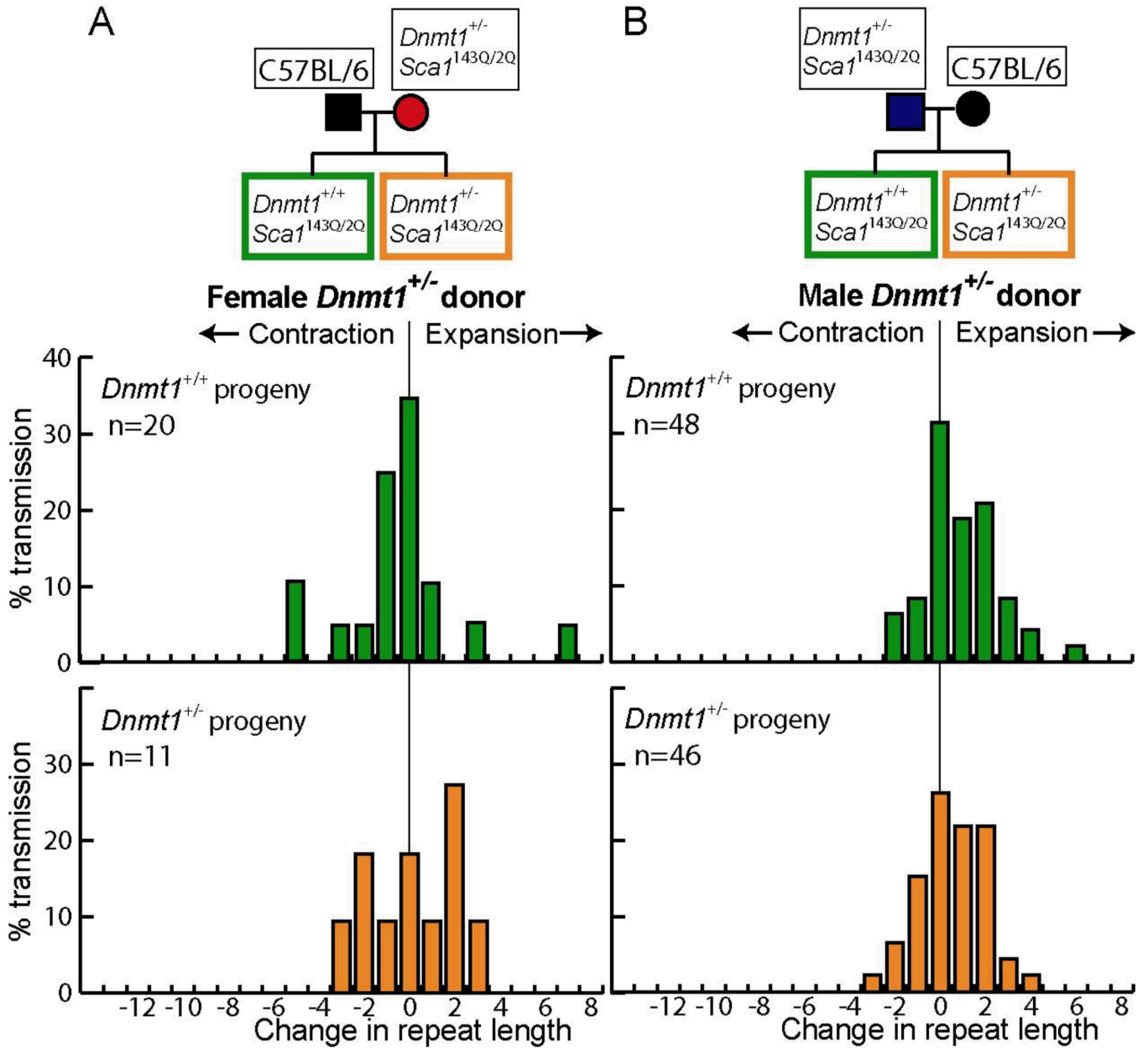


Figure 3. Progeny genotype does not alter CAG tract-length changes in *Dnmt1*^{+/-} SCA1 mouse donors. The breeding schemes are shown at the top. The *Dnmt1*^{+/+} SCA1 and *Dnmt1*^{+/-} SCA1 progeny are labelled in green and orange, respectively. A) Progeny from female *Dnmt1*^{+/-} SCA1 donors. Only the progeny from donors that were 25 weeks of age or younger are shown. B) Progeny from male *Dnmt1*^{+/-} SCA1 donors.

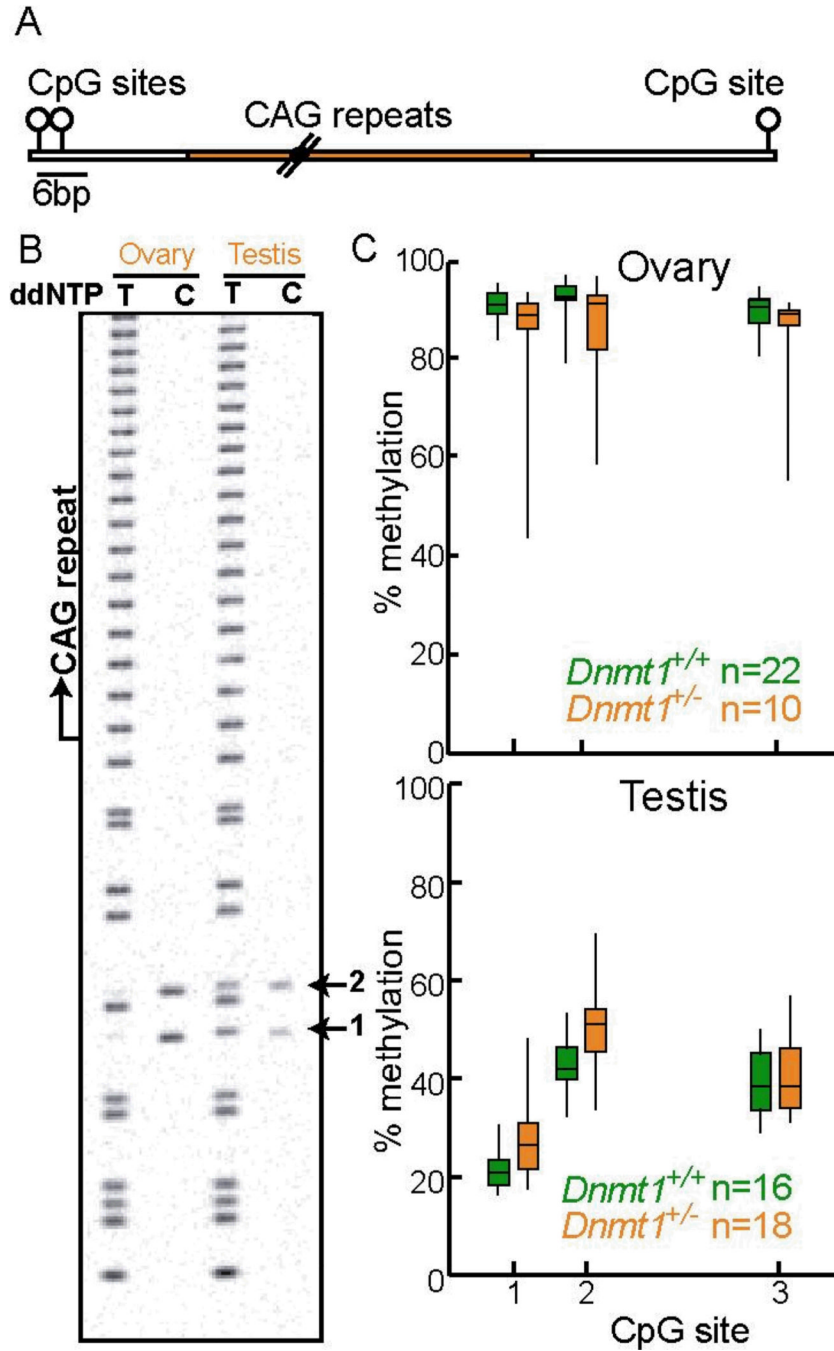


Figure 4. DNA methylation analysis of the *Sca1* repeat region. A) Map of the CpG sites for the expanded allele at the *Sca1* locus. The two upstream CpG sites are 17 bp and 20 bp in front of the CAG repeat tract, the third site is 32 bp downstream of the repeat. B) Representative bisulfite sequencing gel of the expanded allele of the *Sca1* locus. The ovary and the testis are from *Dnmt1*^{+/-} SCA1 mice. Arrows indicate the CpG sites analyzed. C) Box plot of DNA methylation for each CpG site shown in A in ovaries and testes from *Dnmt1*^{+/+} SCA1 (green) and *Dnmt1*^{+/-} SCA1 (orange) mice. Boxes represent the middle 50% of all data points. The median is shown as a horizontal bar. Vertical lines represent the 95th percentile.

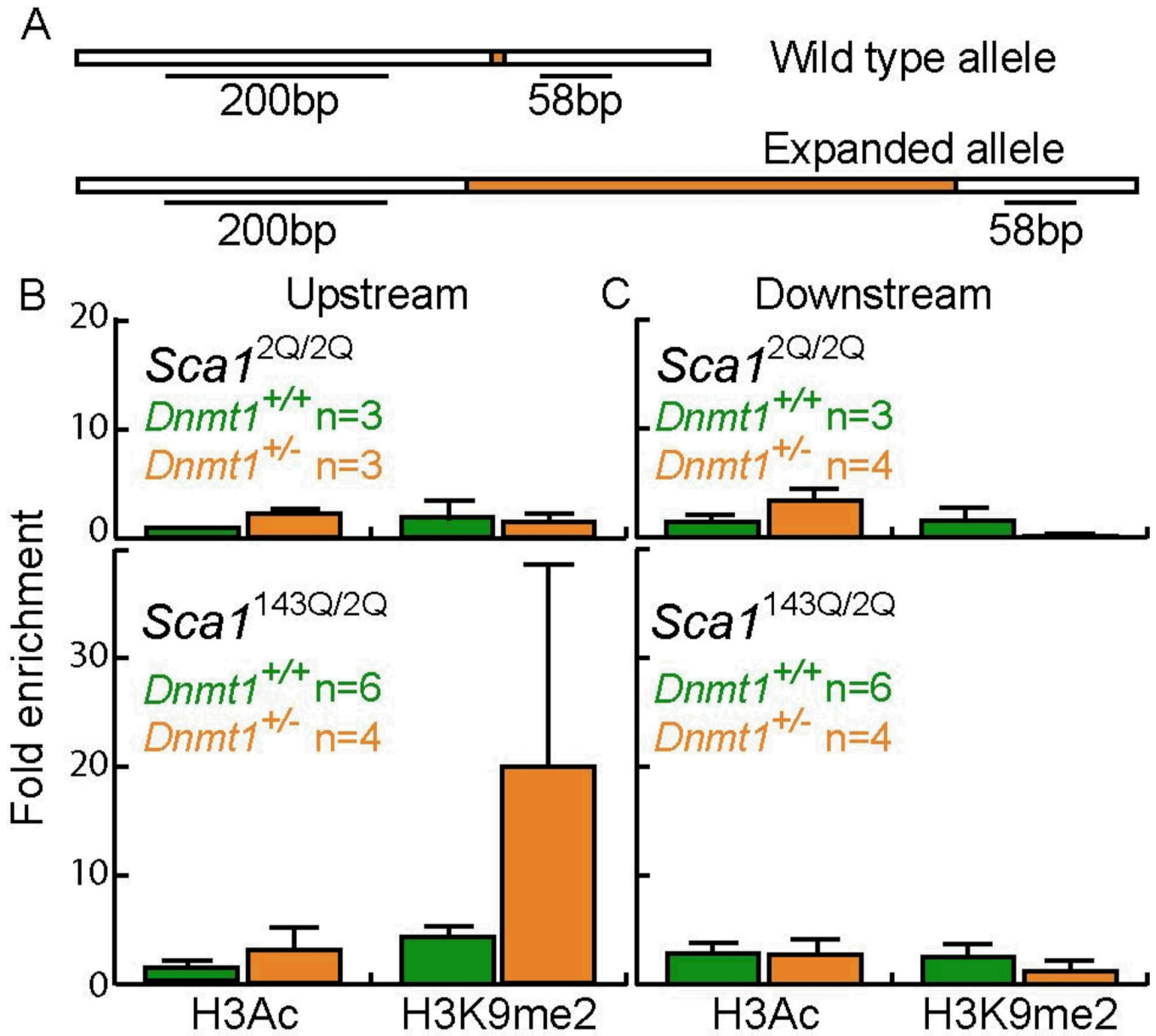


Figure 5. Chromatin immunoprecipitation of the *Sca1* repeat region. A) Map of the *Sca1* locus around the repeat tract showing the regions analyzed by ChIP. H3Ac and H3K9me2 levels upstream (B) and downstream (C) of the *Sca1* repeat tracts in 40-week old testes. Error bars represent one standard error.

Table 1

Somatic instability of CAG repeats in *Dmmt1*^{+/+} SCA1 and *Dmmt1*^{+/-} SCA1 mice

Tissue	Age ^d (weeks)	<i>Dmmt1</i> genotype	Mice tested	Contractions (%)	Stable ^b (%)	Expansions (%)	Alleles ^c (total)	P-value ^d
Cerebellum	6	+/+	2	0	100	0	145	1
	6	+/-	2	0	100	0	203	
	40	+/+	2	1.1	89.8	9.1	614	0.001
	40	+/-	2	1.7	95.4	2.9	346	
Liver	6	+/+	2	12.5	86.1	1.4	313	0.11
	6	+/-	2	4.9	93.3	1.8	238	
	40	+/+	2	4	32.2	63.8	348	0.09
	40	+/-	2	2.9	40.8	56.3	128	
Striatum	6	+/+	2	4.2	95.4	0.4	262	0.34
	6	+/-	2	3	95.7	1.3	368	
	24	+/+	2	2.8	58.5	38.7	145	0.09
	24	+/-	2	8.1	48.7	43.2	99	

^a Ages of mice to be analyzed in depth were chosen based on a preliminary screen to find times when there was very little variation (6 weeks) and times when the variation was pronounced, but not excessive.

^b Stable alleles are defined as being within 5 CAG repeats from the size of the repeat tract in tail at weaning. We note that most of our intergenerational instability was within the resolution that we could reliably achieve in our SP-PCR (± 5 CAGs).

^c Total alleles is the average number of alleles per reaction (as determined by the Poisson distribution) multiplied the number of reactions set up for a given sample.

^d P-value was determined by comparing *Dmmt1*^{+/+} SCA1 mice and *Dmmt1*^{+/-} SCA1 mice of the same tissue and at the same age, using a Chi-squared test with 2 degrees of freedom.