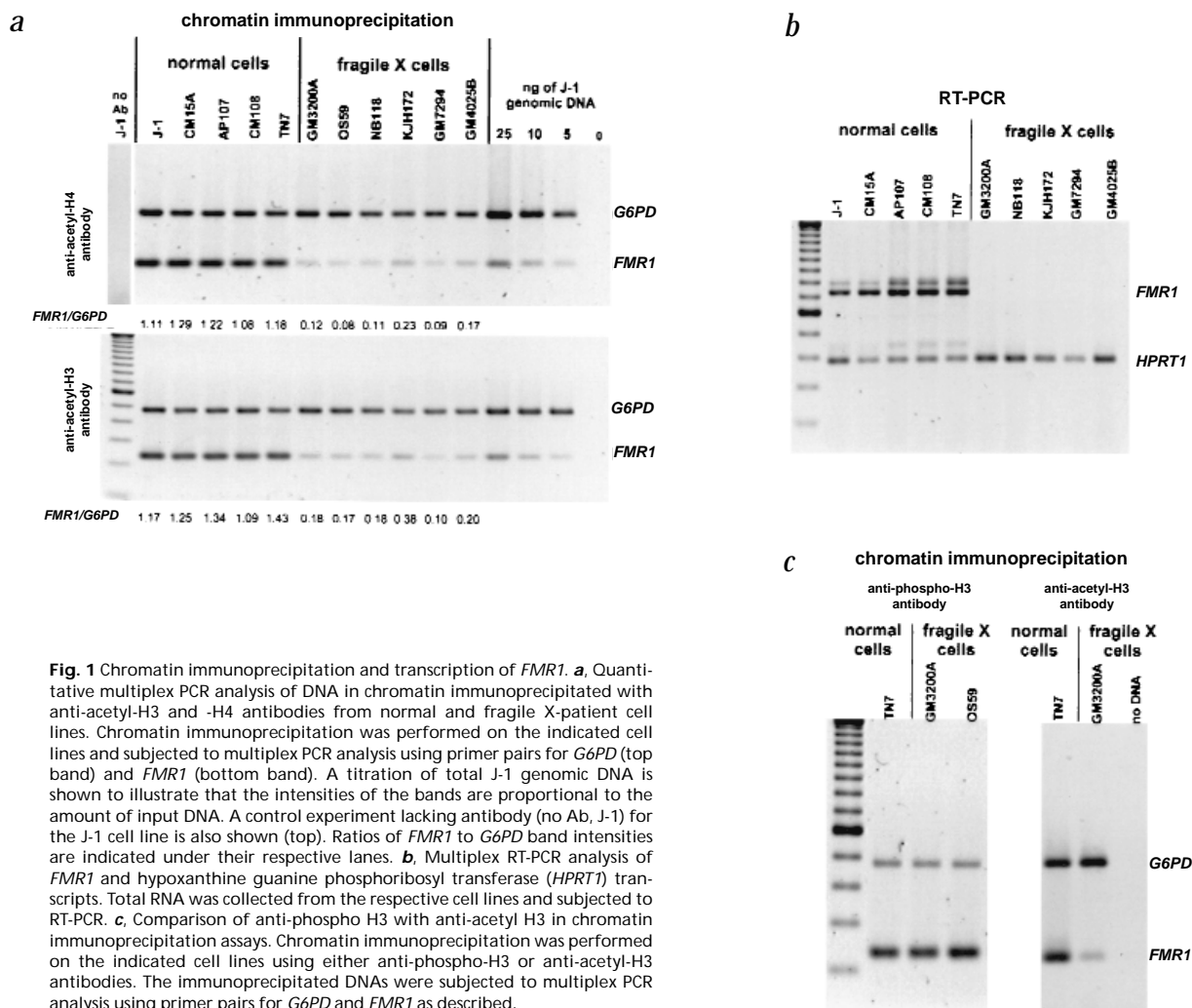


# Acetylated histones are associated with *FMR1* in normal but not fragile X-syndrome cells

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Mutation of *FMR1* results in fragile X mental retardation<sup>1</sup>. The most common *FMR1* mutation is expansion of a CGG repeat tract at the 5' end of *FMR1* (refs 2–4), which leads to cytosine methylation and transcriptional silencing<sup>5,6</sup>. Both DNA methylation and histone deacetylation have been associated with transcriptional inactivity<sup>7–9</sup>. The finding that the methyl cytosine-binding protein MeCP2 binds to histone deacetylases and represses transcription *in vivo*<sup>10,11</sup> supports a model in which MeCP2 recruits histone deacetylases to methylated DNA, resulting in histone deacetylation, chromatin condensation and transcriptional silencing<sup>12</sup>. Here we demonstrate that the 5' end of

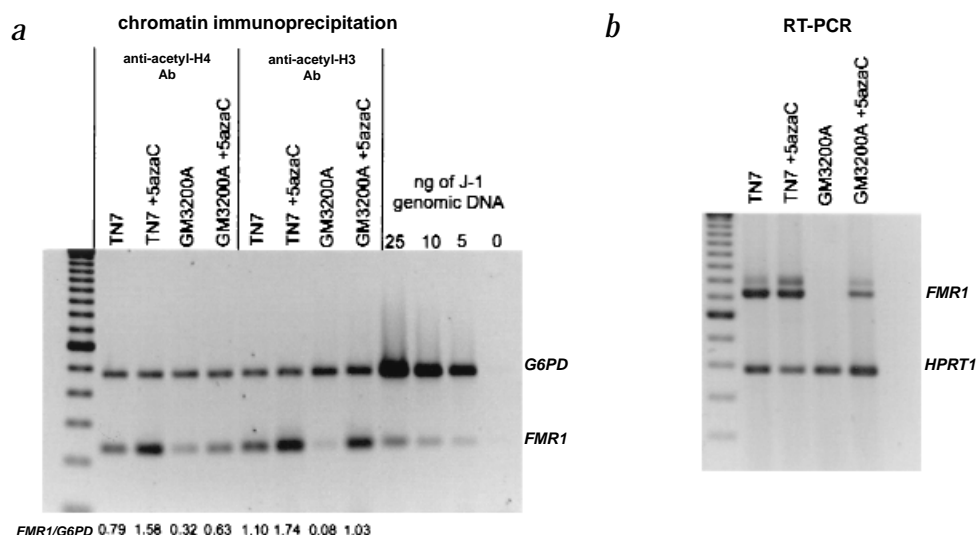
*FMR1* is associated with acetylated histones H3 and H4 in cells from normal individuals, but acetylation is reduced in cells from fragile X patients. Treatment of fragile X cells with 5-aza-2'-deoxycytidine (5-aza-dC) resulted in reassociation of acetylated histones H3 and H4 with *FMR1* and transcriptional reactivation, whereas treatment with trichostatin A (TSA) led to almost complete acetylated histone H4 and little acetylated histone H3 reassociation with *FMR1*, as well as no detectable transcription. Our results represent the first description of loss of histone acetylation at a specific locus in human disease, and advance understanding of the mechanism of *FMR1* transcriptional silencing.



**Fig. 1** Chromatin immunoprecipitation and transcription of *FMR1*. **a**, Quantitative multiplex PCR analysis of DNA in chromatin immunoprecipitated with anti-acetyl-H3 and -H4 antibodies from normal and fragile X-patient cell lines. Chromatin immunoprecipitation was performed on the indicated cell lines and subjected to multiplex PCR analysis using primer pairs for *G6PD* (top band) and *FMR1* (bottom band). A titration of total J-1 genomic DNA is shown to illustrate that the intensities of the bands are proportional to the amount of input DNA. A control experiment lacking antibody (no Ab, J-1) for the J-1 cell line is also shown (top). Ratios of *FMR1* to *G6PD* band intensities are indicated under their respective lanes. **b**, Multiplex RT-PCR analysis of *FMR1* and hypoxanthine guanine phosphoribosyl transferase (*HPRT1*) transcripts. Total RNA was collected from the respective cell lines and subjected to RT-PCR. **c**, Comparison of anti-phospho H3 with anti-acetyl H3 in chromatin immunoprecipitation assays. Chromatin immunoprecipitation was performed on the indicated cell lines using either anti-phospho-H3 or anti-acetyl-H3 antibodies. The immunoprecipitated DNAs were subjected to multiplex PCR analysis using primer pairs for *G6PD* and *FMR1* as described.

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**Fig. 2** Chromatin immunoprecipitation and transcription of *FMR1* after 5-aza-dC treatment. **a**, Quantitative multiplex PCR analysis of DNA in chromatin immunoprecipitated from a normal cell line (TN7) and a fragile X-patient cell line (GM3200A) treated with 5-aza-2'-deoxycytidine for 10 d using anti-acetyl-H4 and anti-acetyl-H3 antibodies. Multiplex PCR analysis was carried out as described in Fig. 1. **b**, Multiplex RT-PCR analysis of total RNA prepared from the same cell lines used above. Primer pairs are as described.



To determine if the silencing of hypermethylated *FMR1* observed in fragile X syndrome is consistent with a model in which methylation is coupled with histone-acetylation state, we immunoprecipitated chromatin<sup>13</sup> from normal and fragile X-syndrome lymphoblastoid cells. Proteins were cross-linked to DNA *in situ* followed by sonication, which randomly fragmented the DNA. DNA associated with acetylated histone H4 or acetylated histone H3 was immunoprecipitated by antibodies against acetylated H4 or acetylated H3 peptides. The immunoprecipitated DNA was analysed by PCR with primers specific for the 5' end of *FMR1* (240 bp downstream from the CGG repeats), or a nearby gene on the X chromosome that encodes glucose-6 phosphate dehydrogenase<sup>14</sup> (*G6PD*).

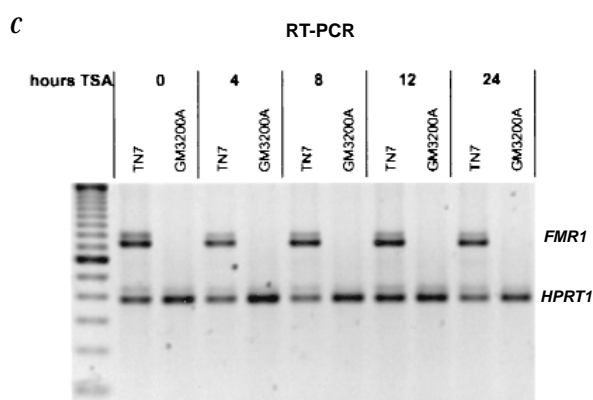
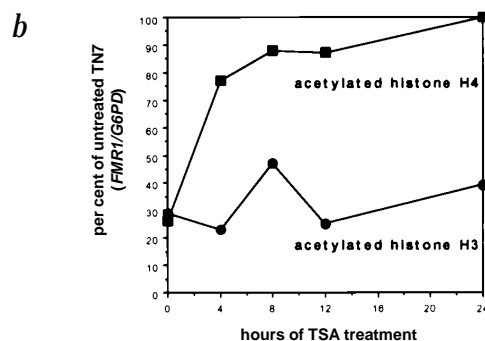
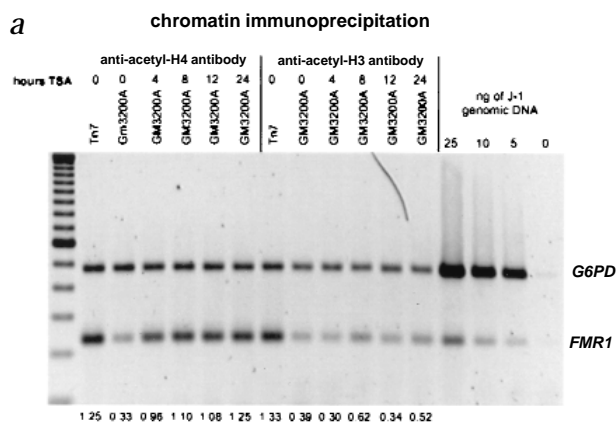
We recovered *G6PD* DNA equally well from normal or patient cell lines using anti-acetyl-H4 or anti-acetyl-H3 peptide antibodies. Conversely, *FMR1* DNA levels were reduced in immunoprecipitations from six unrelated fragile X-patient (male) cell lines compared with five normal (male) cell lines (Fig. 1a). With anti-acetyl-H4 antibody, the ratio of *FMR1* to *G6PD* DNA averaged  $1.17 \pm 0.08$  in 5 normal cell lines, whereas the ratio in 6 fragile X-patient cell lines was only 11% of control ( $0.13 \pm 0.06$ ). We obtained a similar result with anti-acetyl-H3 antibody (*FMR1*:*G6PD*,  $1.26 \pm 0.13$  and  $0.20 \pm 0.09$  in normal and fragile X cells, respectively). Obtaining PCR products was dependent on the addition of antiserum (Fig. 1a). Chromatin immunoprecipitation with anti-phospho-H3 peptide antibodies, in contrast to the anti-acetyl-H3 antibodies used above, yielded similar amounts of *FMR1* DNA from patient and normal cells (Fig. 1c). Hence, *FMR1* in fragile X patients appears to be associated with unacetylated histones. RT-PCR analysis of total RNA from five normal and five fragile X cell lines showed that *FMR1* was transcriptionally active in the former and transcriptionally silent in the latter (Fig. 1b). These results show a direct correlation between loss of *FMR1* expression and the reduced acetylation of histones at the 5' end of *FMR1*.

Cells treated with the DNA methyltransferase inhibitor 5-aza-dC show reduced DNA methylation and increased transcription of target genes, including *FMR1* (refs 15–17). If MeCP2 is responsible for *FMR1* silencing by recruiting histone deacetylases specifically to the methylated gene, then demethylation with 5-aza-dC should result in increased acetylation of histones H3 and H4. We treated the fragile X cell line, GM3200A, and a normal cell line, TN7, with 5-aza-dC, and

immunoprecipitated chromatin with anti-acetyl-H4 or anti-acetyl-H3 antibodies. The association of acetylated H3 and H4 with *FMR1* in patient cells increased to nearly control levels (Fig. 2a). Treatment with 5-aza-dC also increased the amount of both histones associated with wild-type *FMR1*. Whether this reflects a basal level of methylation present at unexpanded *FMR1* alleles or is independent of methylation is unclear. RT-PCR analysis confirmed that transcription was reactivated in 5-aza-dC-treated fragile X cells (Fig. 2b) and Southern-blot analysis of 5-aza-dC-treated, fragile X-cell DNA showed partial demethylation of *FMR1* promoter DNA (data not shown). Presumably, renewed transcription results from an increase of acetylated histones at *FMR1*, which in turn follows the reduction in DNA methylation. As the length of the CGG repeat tract is unchanged in 5-aza-dC-treated cells, these data also demonstrate that association with acetylated histones depends on the methylation state of DNA and not the length of the CGG repeat tract.

Inhibition of histone deacetylases with TSA increases the acetylation level of histones and in some cases activates gene transcription<sup>18–20</sup>. Treatment of fragile X cells with TSA (100 ng/ml) resulted in a gradual increase in the amount of *FMR1* DNA co-immunoprecipitated by anti-acetyl H4, reaching a level comparable with normal cells by 24 hours (Fig. 3a,b). In contrast, the level of acetylated H3 associated with *FMR1* increased slightly, if at all (Fig. 3a,b). Restoring acetylated H4 to *FMR1* under these conditions was insufficient to reactivate transcription (Fig. 3c). Transcription remained undetectable even after 96 hours of treatment (data not shown). The inability to fully restore acetylated histones to *FMR1* or reactivate transcription after TSA treatment may be due to TSA-resistant histone deacetylases operating at *FMR1*. Indeed, differential sensitivities to TSA have been documented for purified yeast histone deacetylases A and B (ref. 21). Alternatively, methylation-dependent silencing of transcription may have a component independent of deacetylase activity.

We have shown that there is a loss of acetylation of histones H3 and H4 associated with the 5' end of *FMR1* that is specific to fragile X-patient cell lines containing a CGG repeat expansion. It has been previously shown by nuclease sensitivity that the chromatin structure at the 5' end of *FMR1* is altered in fragile X cells<sup>22</sup>. In addition, *in vivo* footprinting studies showed that protein-DNA interactions in the *FMR1* promoter in normal cells are absent in cells derived from fragile X



**Fig. 3** Chromatin immunoprecipitation and transcription of *FMR1* after TSA treatment. **a**, Quantitative multiplex PCR analysis of DNA precipitated from chromatin prepared from a normal cell line (TN7) and a fragile X-patient cell line (GM3200A). Cells were treated for the indicated times with TSA (100 ng/ml) and subjected to immunoprecipitation using anti-acetyl-H3 and anti-acetyl-H4 antisera. Multiplex PCR analysis was performed as described in Fig. 1. **b**, Quantitation of *FMR1* and *G6PD* DNA from chromatin immunoprecipitated with anti-acetyl-H3 (circles) and anti-acetyl-H4 (squares). Signals from PCR reactions in (a) were normalized to levels obtained from untreated TN7 cells (100%). **c**, Multiplex RT-PCR analysis of total RNA from the TN7 and GM3200A cell lines treated with TSA.

patients whose *FMR1* gene is transcriptionally silent<sup>23,24</sup>. These results are consistent with a model in which CGG-repeat expansion and methylation of *FMR1* result in the recruitment of transcriptional silencing machinery to the gene, followed by loss of transcription. The inability to reactivate transcription with TSA is in accordance with other reports showing that drug-induced increases in histone acetylation are ineffective in activating transcription from some promoters such as the P1 promoter of *c-myc* (ref. 25), the herpes simplex virus thymidine kinase promoter<sup>26</sup> and certain methylated genes in tumour cells<sup>27</sup>. A survey of human genes by differential display indicated that TSA treatment alters expression of only a small (2%) fraction of those examined<sup>18</sup>. We conclude that methylation-dependent silencing of *FMR1* is refractory to the inhibition of deacetylase activity by TSA and the resulting increase in association with acetylated H4. It is possible that a critical threshold of acetylated histone reassociation has not been achieved by TSA treatment, particularly with acetylated H3. Dual treatment of cells with TSA and a subthreshold dose of 5-aza-dC might reactivate patient *FMR1*, as described recently for a number of hypermethylated genes silenced in cancer<sup>27</sup>. Although other factors involved in regulating *FMR1* activity will need to be considered, including the poor translational competency of *FMR1* mRNA with lengthy repeats<sup>28</sup>, our data reveal details of the mechanism of *FMR1* transcriptional silencing. Further work will be required to learn if MeCP2 (or another methyl-cytosine binding protein) is involved in *FMR1* silencing and which histone acetylases and deacetylases act on *FMR1*-associated nucleosomes.

## Methods

**Cell culture and drug treatments.** EBV-transformed lymphoblastoid cell lines were derived from normal males or males with the typical clinical phenotype of fragile X syndrome. In normal cells, the *FMR1* repeat is of normal length and methylation status, whereas those cells derived from patients exhibited repeat lengths in excess of 300 triplets and were hypermethylated. Cells were cultured in RPMI1640 media supplemented with 10% fetal calf serum, L-glutamine (2 mM) and 1×penicillin/streptomycin at 37 °C in a 5% CO<sub>2</sub> atmosphere. We added TSA (100 ng/ml; Sigma) to cells for the indicated times before collecting them. For 5-aza-dC (Sigma) treatments, we synchronized lymphoblastoid cells with thymidine<sup>17</sup> (1 mM) for two 8-h blocks. Cells were seeded in two separate flasks and cultured for 10 d, with one flask containing 5-aza-dC (1 μM) and the other containing no drug. We changed the media (±5-aza-dC) every 48 h.

**Chromatin immunoprecipitation and quantitative multiplex PCR.** We carried out chromatin immunoprecipitation as recommended by the supplier of the anti-acetyl-H3 and anti-acetyl-H4 antibodies (Upstate Biotech). These antibodies were generated against chemically synthesized peptides corresponding to aa 2–19 of *Tetrahymena thermophila* H4 and 1–21 of *T. thermophila* H3 with acetyl groups on lysines 4,7,11 and 15, and 9 and 14, respectively<sup>29</sup>. Anti-phospho-H3 (serine-10) peptide antisera was a gift from D. Allis<sup>30</sup>. After chromatin immunoprecipitation, DNA was purified by gel exclusion (P6, BioRad) equilibrated with Tris-HCl (pH 8.0). Immunoprecipitated DNA (but not J-1 genomic samples) was digested with *Xho*I before PCR to separate the CGG repeat from the PCR-amplification target site. This digestion improved the efficiency of amplification of *FMR1* DNA to nearly the same level as *G6PD*, presumably due to the separation of the CGG repeat from the PCR target. We used primers 5′-GCTCGGCGGGATGTTGTTGGGAGGGAAGGA-3′ (forward) and 5′-GGGAATAA-GCCATCGCCGCTACTTAGCGCCGAT TTC-3′ (reverse) to amplify *FMR1* (+436 to +671 relative to the transcription start site) and primers 5′-TAGGGCCGCATCCCGCTCCGA-GAGAAGTCT-3′ (forward) and 5′-CTGCCATA-CCCGCTGCCGCT-GCTCTGCATC-3′ (reverse) to amplify *G6PD* (–278 to +202) from immunoprecipitated DNA. We performed amplification (32 cycles) at 94, 65 and 72 °C for 30, 30 and 60 s, respectively. PCR products were electrophoresed on 2% agarose gels, ethidium-bromide stained, imaged with a Stratagene EagleEye imaging system and quantitated with Molecular Dynamics ImageQuant software.

**RT-PCR.** Total RNA was collected from the cells with Trizol (Gibco) following the manufacturer's instructions. RT-PCR was carried out using the GeneAmp RNA PCR kit (Perkin Elmer) following the manufacturer's protocol. PCR products were electrophoresed and imaged as described above. Primers for *HPRT1* amplification were 5'-CGTGGGGTCCCTTTTACCAGCAAG-3' (forward, spanning exons 2 and 3) and 5'-AATTATGGACAGGACT-GAACGTC-3' (reverse, located in exon 7). Primers for *FMR1* were 5'-CACTTTCCGGAGTCTGCGCAC-3' (forward, located in exon 7) and 5'-TAGCTCCAATCTGTGCGCAACTGC-3' (forward, located in exon 14).

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1. Imbert, G., Feng, Y., Nelson, D., Warren, S.T. & Mandel, J.-L. In *Genetic Instabilities and Hereditary Neurological Disorders* (eds Wells, R.D. & Warren, S.T.) 27-53 (Academic Press, San Diego, 1998).
2. Verkerk, A.J. *et al.* Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting a length variation in fragile X syndrome. *Cell* **65**, 905-914 (1991).
3. Rousseau, F. *et al.* Direct diagnosis of fragile X syndrome of mental retardation. *N. Engl. J. Med.* **325**, 1673-1681 (1991).
4. Yu, S. *et al.* Fragile X genotype characterized by an unstable repeat region of DNA. *Science* **252**, 1179-1181 (1991).
5. Sutcliffe, J.S. *et al.* DNA methylation represses *FMR1* transcription in fragile X syndrome. *Hum. Mol. Genet.* **1**, 397-400 (1992).
6. Pieretti, M. *et al.* Absence of expression of the *FMR1* gene in fragile X syndrome. *Cell* **66**, 817-822 (1991).
7. Razin, A. & Riggs, A.D. DNA methylation and gene function. *Science* **210**, 604-610 (1980).
8. Allfrey, V.G., Faulkner, R. & Mirsky, A.E. Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. *Proc. Natl Acad. Sci. USA* **51**, 786-794 (1964).
9. Turner, B.M. Decoding the nucleosome. *Cell* **75**, 5-8 (1993).
10. Nan, X. *et al.* Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393**, 386-389 (1998).
11. Jones, P.L. *et al.* Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nature Genet.* **19**, 187-191 (1998).
12. Razin, A. CpG methylation, chromatin structure and gene silencing—a three way connection. *EMBO J.* **17**, 4905-4908 (1998).
13. Braunstein, M., Rose, A.B., Holmes, S.G., Allis, C.D. & Broach, J.R. Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. *Genes Dev.* **7**, 592-604 (1993).
14. Chen, E.Y. *et al.* Sequence of human glucose-6-phosphate dehydrogenase cloned in plasmids and a yeast artificial chromosome. *Genomics* **10**, 792-780 (1991).
15. Sasaki, T., Hansen, R.S. & Gartler, S.M. Hemimethylation and hypersensitivity are early events in transcriptional reactivation of human inactive X-linked genes in a hamster X human somatic cell hybrid. *Mol. Cell. Biol.* **12**, 3819-3826 (1992).
16. Ammerpohl, O., Schmitz, A., Steinmuller, L. & Renkawitz, R. Repression of the mouse *M*-lysozyme gene involves both hindrance of enhancer factor binding to the methylated enhancer and histone deacetylation. *Nucleic Acids Res.* **26**, 5256-5260 (1998).
17. Chirurazzi, P., Pomponi, M.G., Willemsen, R., Oostra, B.A. & Neri, G. *In vitro* reactivation of the *FMR1* gene involved in fragile X syndrome. *Hum. Mol. Genet.* **7**, 109-113 (1998).
18. Van Lint, C., Emiliani, S. & Verdin, E. The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. *Gene Expr.* **5**, 245-253 (1996).
19. Arts, J., Lansink, M., Grimbergen, J., Toet, K.H. & Kooistra, T. Stimulation of tissue-type plasminogen activator gene expression by sodium butyrate and trichostatin A in human endothelial cells involves histone acetylation. *Biochem. J.* **310**, 171-176 (1995).
20. Van Lint, C., Emiliani, S., Ott, M. & Verdin, E. Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. *EMBO J.* **15**, 1112-1120 (1996).
21. Rundlett, S.E. *et al.* HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. *Proc. Natl Acad. Sci. USA* **93**, 14503-14058 (1996).
22. Eberhart, D.E. & Warren, S.T. Nuclease sensitivity of permeabilized cells confirms altered chromatin conformation at the fragile X locus. *Somat. Cell Mol. Genet.* **22**, 435-441 (1996).
23. Drouin, R. *et al.* Structural and functional characterization of the human *FMR1* promoter reveals similarities with the hnRNP-A2 promoter region. *Hum. Mol. Genet.* **6**, 2051-2060 (1997).
24. Schwemmler, S. *et al.* Characterization of *FMR1* promoter elements by *in vivo* footprinting analysis. *Am. J. Hum. Genet.* **60**, 1354-1362 (1997).
25. Madisen, L., Krumm, A., Hebbes, T. & Groudine, M. The immunoglobulin heavy chain locus control region increases histone acetylation along linked *c-myc* genes. *Mol. Cell. Biol.* **18**, 6281-6292 (1998).
26. Luo, R., Postigo, A. & Dean, D. Rb interacts with histone deacetylase to repress transcription. *Cell* **92**, 463-473 (1998).
27. Cameron, E. *et al.* Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nature Genet.* **21**, 103-107 (1999).
28. Feng, Y. *et al.* Translational suppression by trinucleotide repeat expansion at *FMR1*. *Science* **268**, 731-734 (1995).
29. Lin, R., Leone, J., Cook, R. & Allis, C.D. Antibodies specific to acetylated histones document the existence of deposition- and transcription-related histone acetylation in *Tetrahymena*. *J. Cell Biol.* **108**, 1577-1588 (1989).
30. Hendzel, M. *et al.* Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106**, 348-360 (1997).

