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Epigenetic mechanism of FMR1 inactivation in Fragile X syndrome

MERAV HECHT[1], AMALIA TABIB[1], TAMAR KAHAN[1], SHARI ORLANSKI[1], MICHAL GROPP[3], YUVAL TABACH[1], OFRA YANUKA[2], NISSIM BENVENISTY[2], ILANA KESHET[1] and HOWARD CEDAR*[,1]

[1] Department of Developmental Biology and Cancer Research, [2] Department of Genetics, Hebrew University, Jerusalem and [3] The Sydney and Judy Swartz Human Embryonic Stem Cell Research Center, Hadassah-Hebrew University Medical Center Jerusalem, Israel

ABSTRACT Fragile X syndrome is the most frequent cause of inherited intellectual disability. The primary molecular defect in this disease is the expansion of a CGG repeat in the 5' region of the *fragile X mental retardation1* (*FMR1)* gene, leading to *de novo* methylation of the promoter and inactivation of this otherwise normal gene, but little is known about how these epigenetic changes occur during development. In order to gain insight into the nature of this process, we have used cell fusion technology to recapitulate the events that occur during early embryogenesis. These experiments suggest that the naturally occurring Fragile X*FMR1* 5' region undergoes inactivation post implantation in a Dicer/Ago-dependent targeted process which involves local SUV39H-mediated tri-methylation of histone H3K9. It thus appears that Fragile X syndrome may come about through inadvertent siRNA-mediated heterochromatinization.

KEY WORDS: *DNA methylation, chromatin, RNAi, histone modification*

Introduction

The primary defect in Fragile X syndrome is a triplet repeat expansion in the 5' UTR of the *FMR1* gene on chromosome X that probably takes place during oogenesis or in the very early embryo (Jin and Warren, 2000). Once the expansion attains a critical size, it induces de novo methylation of the repeat region as well as the nearby CpG island promoter, and it is this embryonic event that brings about the epigenetic repression of *FMR1* and results in a spectrum of phenotypes including intellectual disability and autism (Jin and Warren, 2000). The mechanism and developmental timing of this de novo methylation has not yet been deciphered. One possibility is that this occurs as part of the massive genome-wide wave of de novo methylation that takes place at the time of implantation (Kafri *et al.*, 1992). While CpG islands are normally protected from modification by virtue of inherent common sequence elements (Brandeis *et al.*, 1994), the triplet expansion might disrupt these sequence motifs, thus allowing promoter modification. Previous studies demonstrated that embryonic cells (F9 or ES) are able to mimic implantation embryos by constitutively carrying out global de novo methylation while protecting CpG islands. Although the precise mechanism for this process is not known, it has been demonstrated that pre-methylated CpG islands actually undergo demethylation after being inserted into these cells (Bhutani *et al.*, 2010, Frank *et al.*, 1991), and this is exactly what happens to the Frax *FMR1* upstream region when it is transferred from patient somatic tissues to a mouse embryonic environment by cell fusion (Wohrle *et al.*, 2001). In confirmation of this idea, it has also been shown that this locus is hypomethylated in ES cells from human Frax embryos (Avitzour *et al.*, 2014, Eiges *et al.*, 2007). These data strongly suggest that in patients, the Frax *FMR1* gene promoter is actually recognized and protected like other CpG islands, and must therefore only become de novo methylated following the implantation stage of development in parallel with other well-known embryonic sequences.

X chromosome inactivation in female cells represents a good example of this post-implantation phenomenon (Heard, 2004). The initial stages of X inactivation take place in the late blastocyst, but de novo methylation of CpG island sequences actually occurs several days later in the mouse (Lock *et al.*, 1987), and this appears to be

Abbreviations used in this paper: FMR, Fragile X mental retardation; Frax, Fragile X; hES, human embryonic stem (cell).

***Address correspondence to:** Howard Cedar. Dept. Developmental Biology and Cancer Research, Hebrew University Medical School, P.O.B. 12272, Jerusalem, Israel 91120. Tel: 972-26758167. E-mail: cedar@mail.huji.ac.il http://orcid.org/0000-0001-6472-9640

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a targeted process directed by Xist RNA that is accompanied by the Ezh2 polycomb complex-mediated tri-methylation of histone H3 at lysine 27 (Plath *et al.*, 2003, Silva *et al.*, 2003). In a similar manner, *Oct-3/4* and other pluripotency genes are initially active and unmethylated in the early embryo, but then undergo targeted repression a few days after implantation in the mouse (Epsztejn-Litman *et al.*, 2008, Feldman *et al.*, 2006, Gidekel and Bergman, 2002). This process involves an initial stage of direct transcriptional repression followed by heterochromatinization through G9a-mediated tri-methylation of H3K9 and subsequent binding of HP1 at the promoter. G9a itself is also responsible for recruiting Dnmt3a & 3b that then bring about local de novo methylation. Once inactivated, these genes remain permanently packaged with nucleosomes containing H3K9me3 throughout the life of the organism (Epsztejn-Litman *et al.*, 2008, Feldman *et al.*, 2006). In this paper, we have used a sophisticated new developmental-mimicking system to demonstrate that in Fragile X syndrome, the FMR1 gene undergoes a similar process of heterochromatinization and DNA methylation that appears to be directed by a Dicer-mediated siRNA mechanism.

Results

In order to gain some insight into how and when *FMR1* silencing takes place in vivo, we set up our own embryonic cell system for reprogramming a somatic Frax *FMR1* to its unmethylated state and then attempted to recapitulate gene silencing by inducing differentiation. To this end, we fused Fragile X human fibroblasts with *Hprt* – mouse A9 cells and then used microcell fusion to insert this same human chromosome (which carries the *HPRT* gene) into mouse *Hprt* – OTF9 cells in culture.

This was done using selection for the HPRT$^+$ phenotype and picking individual colonies. Each clone was then expanded and its DNA analyzed to confirm that the *FMR1* promoter is indeed unmethylated, as has been previously reported (Wohrle *et al.*, 2001).

We measured methylation by employing a methylcytosine (mC)-specific antibody to specifically immunoprecipitate methylated DNA (Keshet *et al.*, 2006, Weber *et al.*, 2005). The input and bound fractions are then subjected to site specific real-time PCR and normalized by comparison to negative (*Aprt*) and positive (*Cryaa*) controls. This method has been shown to be extremely accurate as determined by independent bisulfite analysis (Straussman *et al.*, 2009). Using this assay, we found that while the defective *FMR1* 5' region is highly methylated in the parent somatic cells, it becomes demethylated (Fig. 1) and reactivated when placed in the new embryonic cell environment, and this has been verified by bisulfite analysis (legend to Fig. 1). Upon retinoic acid treatment for 10 days, however, the Frax *FMR1* gene underwent clearcut de novo methylation (Fig. 1) accompanied by transcriptional repression, indicating that this system can be used to recapitulate the process of inactivation as it probably occurs in vivo.

In light of previous studies, it seemed likely that the Fragile X *FMR1* gene might undergo targeted methylation in a process that is directed by SET-domain enzymes responsible for local methylation of histone H3 (Cedar and Bergman, 2009, Kumari and Usdin, 2010). Although it was not possible to follow the process of *FMR1* de novo methylation as it occurs in Fragile X patients in vivo, we reasoned that the mechanisms involved in this event could be deduced by determining whether this defective gene promoter is marked by histone H3 tri-methylation in adult cells, in the same manner that this modification serves as an "ontogenic footprint" of *Oct-3/4* and other genes that undergo embryonic repression in the mouse (Epsztejn-Litman *et al.*, 2008, Feldman *et al.*, 2006).

As a first step, we carried out ChIP analysis on normal and Fragile X lymphoblasts using antibodies against H3K9me3. This experiment clearly demonstrated that the Fragile X *FMR1* 5' region is highly enriched for this modification. In contrast, this identical region in the normal *FMR1* gene is packaged with histone H3 that remains unmethylated on this specific lysine residue (Fig. 2). H3K9me3 is known to bind heterochromatin protein 1 (HP1) through its chromodomain (Taverna *et al.*, 2007). In order to test whether this occurs on the Fragile X *FMR1* gene, we carried out ChIP using an antibody specific to HP1β. Strikingly, this protein was found to be enriched on the defective, but not the normal, *FMR1* gene (Fig. 2), strengthening the idea that Fragile X *FMR1* has a unique chromatin structure that was probably formed initially during post implantation development.

During development, the genomic DNA methylation pattern is maintained through a semi-conservative copying process that is mediated by Dnmt1 located in the replication complex (Leonhardt *et al.*, 1992). In addition to this general mechanism, sequences that undergo de novo methylation following implantation, such as pericentric heterochromatin (Chen *et al.*, 2004, Lehnertz *et al.*, 2003), and the *Oct3/4* promoter (Feldman *et al.*, 2006), apparently preserve their ability to recruit Dnmts in a targeted manner in somatic cells.

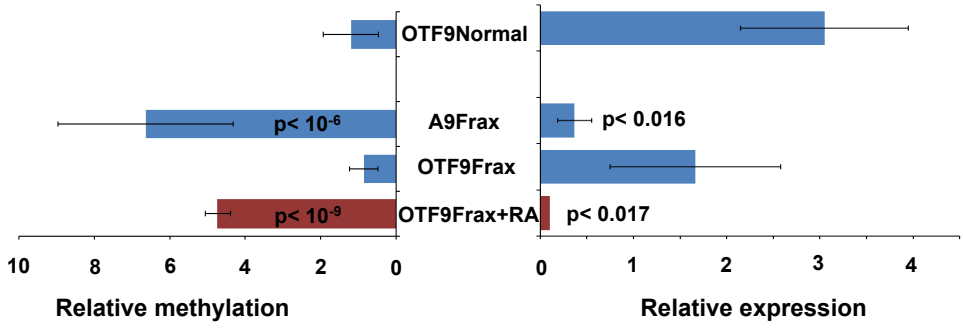

Fig. 1. DNA methylation and transcription analysis. *A9 fibroblasts carrying a Fragile X chromosome were fused to Hprt$^-$ OTF9 cells and individual colonies were isolated. DNA samples from the original A9 cells as well as the OTF9 fusants were subjected to methyl-DNA ImmunoPrecipitation (mDIP) (left panel) at the human FMR1 5' upstream region. For all of the OTF9/Frax fusants, analysis was carried out in undifferentiated cells (blue), and in some, following differentiation (red) by treatment with 0.5 μM retinoic acid for 10 days in the presence of HAT (Materials and Methods). The degree of enrichment is relative to the Aprt negative control (set at 1) and the total sample set was normalized by comparison to Cryaa. Bisulfite analysis of DNA from OTF9/Frax cells showed that the 3 CpG sites closest to the FMR1 promoter (see Fig. S1) were, on average, 7% methylated in undifferentiated cells, and became 92% methylated after differentiation. RNA samples (right panels) were analysed for human FMR1 by RT-PCR. A control PCR for human HPRT showed that this gene did not undergo inactivation when OTF9 Frax cells were treated with RA. All results (±SD) are based on at least 3 independent analyses and were compared to the OTF9 Frax sample.*

Consistent with this concept, we find that DNMT3A is also recruited to the Fragile X *FMR1* gene in patient lymphoblasts (Fig. 2).

In light of the observation that H3K9 modification is maintained in cycling cells, it seemed likely that the enzymatic machinery for carrying out this reaction must be constantly present at the gene promoter. A number of different SET-domain proteins are known to be capable of carrying out this histone methylation reaction. One potential candidate is SUV39H, a key protein that plays a role in directing heterochromatinization of both telomeres (Garcia-Cao *et al.*, 2004) and pericentric satellite sequences (Lehnertz *et al.*, 2003). Indeed, ChIP analysis on human lymphoblasts using an antibody specific for SUV39H1 showed that this protein is specifically bound to the Fragile X *FMR1* 5' region (Fig. 2), even though it is not detected in the normal gene. This factor appears to be present at about the same level as that seen for human β-satellite sequences, a known target for this enzyme (Lehnertz *et al.*, 2003). These studies provide strong support for the idea that SUV39H may be involved both in the initiation and maintenance of Fragile X *FMR1* inactivation.

Since the silent Fragile X *FMR1* gene appears to undergo epigenetic resetting when introduced into embryonic cells but can then become inactivated upon differentiation, we reasoned that this dynamic system could be used to test genetically whether SUV39H is indeed responsible for heterochromatinization at this locus. To this end, we took advantage of an established mouse ES cell line that is knocked-out for both *Suv39h1* and *Suv39h2* (Peters *et al.*, 2001). These cells lack H3K9me3 in their DAPI-rich heterochromatin regions, and are depleted for major satellite DNA methylation (Lehnertz *et al.*, 2003). In order to use them as recipients for fusion experiments, we first generated an *Hprt*$^-$ variant (Materials and Methods). In addition, it was necessary to transfect these cells with shRNA against human *SUV39H*, since one of these gene copies (*SUV39H1*) is actually located on the X chromosome in man (UCSC).

In order to test the effect of Suv39h on the epigenetic state of the Fragile X *FMR1* promoter region, we attempted to fuse patient lymphoblasts directly with mouse wild-type or mutant *Hprt*$^-$ ES cells and then selected for the HPRT$^+$ phenotype, and this was best carried out by electrofusion rather than the microcell fusion technique employed for OTF9 cells. Resulting colonies initially grow with a stem-cell morphology, but after about 2 weeks in culture, they appear to undergo spontaneous differentiation and stop dividing. In light of our previous experiments, we assume that the Fragile X *FMR1* gene initially becomes structurally reactivated, and then undergoes silencing as a function of differentiation (see Fig. 1). In keeping with this, ChIP analysis of pooled colonies indeed shows that the FMR1 expanded-repeat region is enriched for H3K9me3 in normal ES cell fusants. In contrast, this local heterochromatin marker is dramatically lower in fused ES cells that are knocked-down (see Materials and Methods) for *Suv39h* (Fig. 3A), despite the fact that they are still capable of undergoing normal differentiation (Fig. S1A). This indicates that while the H3K9me3 must have been removed following fusion, it could not be reestablished upon spontaneous differentiation in the mutant.

Methyl DNA Immunoprecipitation (mDIP) analysis of fused colonies from individual experiments demonstrated that the Frax locus had also become undermethylated and remained this way in differentiated *Suv39h*$^-$ mutant cells (Fig. 3B) and bisulfite sequencing confirmed that this occurs mainly in the promoter region (Fig. S2). Furthermore, ChIP analysis showed that this may be partially caused by the lack of Dnmt3a recruitment in cells deleted for *Suv39h* (Fig. 3A). This is in striking contrast to the *Oct-3/4* promoter which still undergoes normal inactivation in these same cells (Fig. S1A). Taken together, these studies provide strong genetic evidence that SUV39H may be responsible for the establishment of heterochromatinization and methylation of the Fragile X *FMR1* gene during development. Real-time RT-PCR analysis showed that despite the relatively open epigenetic state observed in the *Suv39h*$^-$ embryonic cells, the defective *FMR1* gene still remained transcriptionally inactive (Fig. 3B), suggesting that there may be additional underlying mechanisms that play a role in repressing this gene (Colak *et al.*, 2014), in a manner very similar to events that bring about inactivation of *Oct-3/4* and other pluripotency genes during early development (Epsztejn-Litman *et al.*, 2008, Feldman *et al.*, 2006).

Studies in yeast have shown that heterochromatinization of tandemly repeated sequences is initiated by the generation of complementary dsRNA that is processed by Dicer to form a self-targeting repression complex, and there is some suggestion that this may also be true in animal cells (Fukagawa *et al.*, 2004, Kanellopoulou *et al.*, 2005, Sugiyama *et al.*, 2005). In order to determine whether a mechanism of this nature may also play a role in the early developmental inactivation of *FMR1* in Fragile X syndrome, we used an shRNA transfection vector to make *Hprt*$^-$ ES cells with a specific knockdown of the mouse and human *Dicer* genes. One ES cell clone that was verified as having less than 10% the normal level of Dicer mRNA (see Materials and Methods) was then selected to serve as the recipient for electro-cell fusion with lymphoblasts from a Fragile X patient, and resulting Hprt$^+$ colonies were then analyzed for their epigenetic structure over the *FMR1* 5' gene region.

In contrast to the heterochromatin-like structure observed when

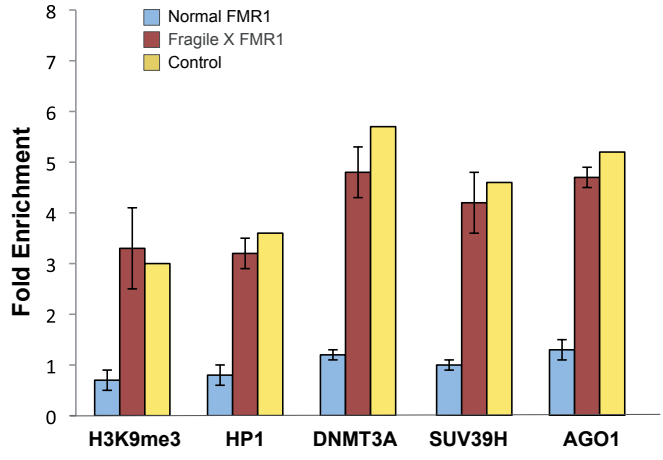

Fig. 2. Epigenetic structure of Fragile X mental retardation1 (FMR1) in normal and Fragile X cells. *Chromatin from normal (blue) or Fragile X (red) lymphoblasts was subjected to ChIP analysis at the 5' region of FMR1 using the indicated specific antibodies. In each case, the results (± SEM) were first normalized to a negative control (APRT) that was set at 1 with the normal FMR1 then being compared to the Frax FMR1 by correcting for the level of a positive control in each sample. The human β-satellite sequence was used as a positive control (yellow) for all ChIPs except for AGO1 that was compared to PGR (Huang* et al.*, 2013, Janowski* et al.*, 2006). Each ChIP experiment was repeated at least 3 times ($P < 0.001$) for all markers.*

the Fragile X *FMR1* gene is inserted into wild type ES cells, this sequence was found to lose its H3K9 modification (Fig. 3A) and become undermethylated after being introduced into ES cells that lack sufficient Dicer activity, as demonstrated both by mDIP (Fig. 3B) and bisulfite analysis (Fig. S2). Furthermore, RT-PCR demonstrated that this defective *FMR1* gene actually becomes transcriptionally activated, with RNA present at levels expected of a normal *FMR1* gene in these cells (Fig. 3B). This lack of repression was not due to a lack of Suv39h (Fig. S1C) nor to a general deficiency in the ability of these Dicer knockdown ES cells to undergo differentiation, as shown by the fact that they still adopt the expected morphology for this stage and carry out normal silencing of the *Oct-3/4* gene as well as other standard developmental markers (Fig. S1A). It

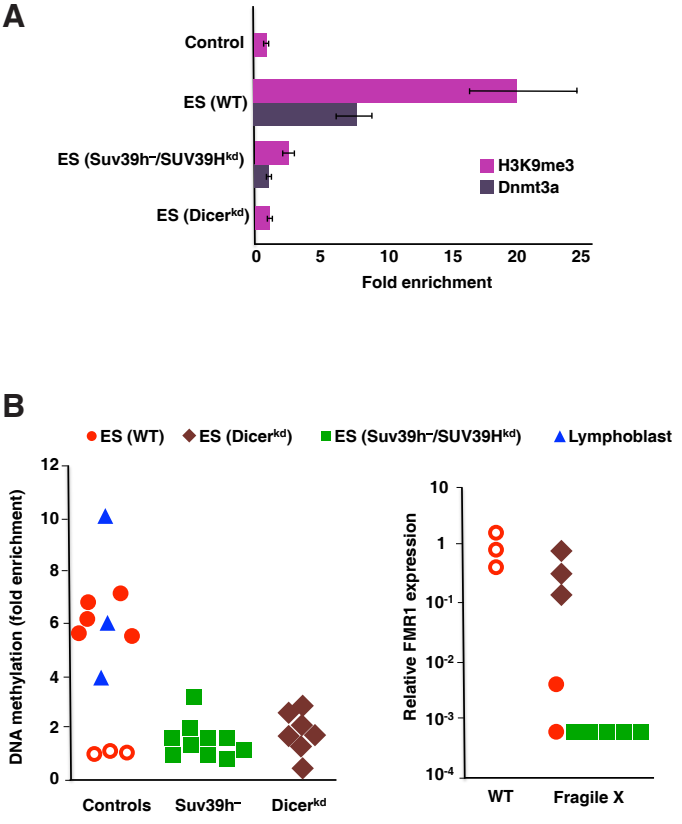

Fig. 3. Factors involved in Fragile X mental retardation1 (FMR1) inactivation. *The Fragile X FMR1 was inserted into WT or genetically modified ES cells by electrofusion with patient lymphoblasts and fused cells then isolated by selection with HAT. In all cases, these cells had a differentiated phenotype (Fig. S2).* **(A)** *H3K9me3 or Dnmt3a ChIP analysis of the FMR1 5′ region in pools of fused cells. An FMR1 gene from normal lymphoblasts fused with WT ES cells was also analyzed (control). Enrichment levels (± SEM) were adjusted by normalizing to Pou5F1 (Oct3/4) in the case of ES cells containing SUV39H or Dicer shRNA. All ChIP experiments were carried out at least 3 times.* **(B)** *DNA methylation (left panel) of the 5′ FMR1 region was analyzed in pooled colonies from individual fusion experiments by mDIP and normalized to a positive (methylated) endogenous control (Cryaa). FMR1 RNA from individual fusions was measured by real-time RT-PCR (right panel). The WT FMR1 from normal lymphoblasts fused to ES cells is shown for comparison as a positive control. Filled symbols indicate fusion with the Fragile X chromosome, while open symbols represent fusion with an X chromosome from normal cells. Blue triangles represent analysis of FMR1 in non-fused Fragile X lymphoblasts.*

has been reported that the complete absence of Dicer can bring about an indirect decrease in Dnmt3a/3b levels, causing a general reduction of DNA methylation (Benetti *et al.*, 2008, Sinkkonen *et al.*, 2008). This does not seem to be the case for the partial *Dicer* knockdown used in our experiments, as indicated by the observation that *Oct-3/4* is highly methylated in these differentiated cells (Fig. S1B).

In yeast, Dicer is known to play a direct role in the generation of heterochromatin by processing local naturally-occurring double-strand RNA molecules into sequence specific siRNA of a defined size (Buhler and Moazed, 2007). In order to determine whether inactivation of the *FMR1* locus may also involve a similar mechanism, we reasoned that appropriate siRNAs would most likely be abundantly present only at the time when the Frax *FMR1* gene is still fully active prior to its DNA methylation in vivo. To this end, we carried out Northern blot analysis on ES cells isolated from a human Fragile X embryo (Eiges *et al.*, 2007) using a CGG repeat sequence probe (Fig. 4A). This revealed a specific band of ~21 nt (arrow) that was barely detectable in normal ES cells. As confirmation of this, RNA-Seq analysis of small RNA from these same cells showed the presence of many highly specific (see legend to Fig. 4B) 19-23 nt CGG repeat molecules in Fragile X with 2-3 fold lower levels in normal cells. In contrast, RNAs of this nature could not be easily detected in somatic cells from a Fragile X or normal patient (Fig. S3). These data suggest that at the approximate stage in development when the Fragile X *FMR1* gene undergoes inactivation and de novo methylation *in vivo* CGG repeat siRNAs are specifically available for carrying out the inactivation process. Companion experiments in mouse ES cells indicate that these are probably generated through a Dicer-dependent process (Fig. S3).

In the yeast heterochromatin sequence model, once the double-strand RNA is processed by Dicer, the resulting siRNA molecules are incorporated into a RITS (RNA-induced Initiation of Transcriptional gene Silencing) complex that is then recruited by sequence recognition to the targeted region of the genome (Buhler and Moazed, 2007). One of the main components of this system is Ago1, a protein known to be involved in both the establishment and maintenance of siRNA-mediated transcriptional repression (Janowski *et al.*, 2006, Partridge *et al.*, 2007). Using ChIP analysis with antibodies to AGO1, we have been able to show that AGO1 indeed binds differentially to the Frax *FMR1* promoter region in somatic cells, but is not recruited to a normal *FMR1* gene (Fig. 2). Furthermore, immunoprecipitation experiments indicated that the AGO1 complex actually contains small CGG repeat RNAs (Fig. 4C), thus filling in all the links required by this model. Taken together, these experiments suggest that a Dicer-dependent gene-targeting system may play a role in the inactivation of Fragile X *FMR1*, working upstream of H3K9me3 heterochromatinization and DNA methylation (Kanellopoulou *et al.*, 2005).

Discussion

Although a great deal has been learned about the stable epigenetic structure of the *FMR1* gene in somatic cells of Fragile X patients (Coffee *et al.*, 2002, Kumari and Usdin, 2010, Pietrobono *et al.*, 2005), it has proven difficult to actually characterize the molecular events that generate this repressive state in the early embryo, mainly because there are no animal or cell models that accurately mimic this process. Recently, for example, ES cells

from Fragile X patients differentiated in culture were used to characterize a proposed RNA-directed, Dicer-independent *FMR1* transcriptional-repression mechanism (Colak *et al.*, 2014), but it is unlikely that this alone can explain long-term inactivation. Previous experiments have already shown that these human ES cells may not be capable of actually carrying out proper DNA methylation of the triplet repeat (Eiges *et al.*, 2007), the hallmark of Fragile X syndrome (Jin and Warren, 2000), and even X chromosome inactivation is not always carried out properly in these cells (Lessing *et al.*, 2013). In this study, we have succeeded in using a mouse embryonic cell-fusion system to recapitulate the developmental dynamics of these epigenetic mechanisms on a naturally occurring expanded-repeat *FMR1* gene derived from patient cells. On the basis of biochemical and genetic data, it is now possible to suggest a molecular model for this inactivation process.

Previous results clearly demonstrated that the Fragile X *FMR1* gene is still unmethylated and probably transcriptionally active in very early embryonic cells both *in vivo* (Willemsen *et al.*, 2002) and *in vitro* (Colak *et al.*, 2014, Eiges *et al.*, 2007). According to ES-cell models, repression in the embryo itself appears to take place during post-implantation differentiation, although the Frax-*FMR1* gene may still remain active in extraembryonic tissues (Luo *et al.*, 1993, Willemsen *et al.*, 2002). This occurs at about the same time that embryonic genes such as *Oct-3/4* or *Nanog* get turned off (Epsztejn-Litman *et al.*, 2008, Feldman *et al.*, 2006) and in parallel with the process of X chromosome inactivation (Mlynarczyk-Evans *et al.*, 2006). All of these events appear to be associated with targeted heterochromatinization involving histone H3 methylation at lysine 9 or lysine 27 by SET domain enzymes and subsequent DNA methylation.

As opposed to models suggesting that expanded CGG repeat secondary structures in the DNA are directly responsible for de novo methylation (Chen *et al.*, 1995, Chen *et al.*, 1998), we propose that inactivation of the defective *FMR1* gene in Fragile X patients actually initiates in the early post-implantation embryo with transcription of CGG repeat sequences located either at the *FMR1* locus itself (Ladd *et al.*, 2007) or other sites in the genome (see Materials and Materials). Long CGG repeats can apparently form hairpin structures which have been shown to be good substrates for Dicer, yielding an approximate 21-22 nt long RNA (Krol *et al.*, 2007). Since these repeats are not programmed for specific cleavage like miRNA molecules, Dicer may cut them in a non-canonical manner, yielding a variety of different products (Fig. 4). Alternatively, R-loops found in the unmethylated *FMR1* CGG repeat region (Groh *et al.*, 2014, Loomis *et al.*, 2014) may induce antisense transcription which in turn leads to the generation of double-stranded RNA and the recruitment of Dicer (Skourti-Stathaki *et al.*, 2014).

In support of this model, we have demonstrated that in Fragile X embryonic cells an ~21 nt Dicer-dependent RNA is actually present in higher concentration at the exact same stage that has the ability to initiate the inactivation process. Following Dicer-mediated cleavage, a RITS-type complex containing Ago could be targeted to the *FMR1* sequence locus in a process that is initially triggered by post-implantation differentiation, but this key step apparently can occur only on the defective Frax *FMR1* locus, perhaps because stable binding requires a high concentration of both the RNA (Fig. 4) as well as the target repeat sequence (Krol *et al.*, 2007) or because of its unusual secondary structure (Weisman-Shomer *et al.*, 2000).

Although the exact details are not yet known, this event apparently causes gene silencing in a 2-pronged manner (see (Feldman *et al.*, 2006)), first by directly inhibiting transcription (see (Murchison *et al.*, 2005)) and then by independently recruiting SUV39H (Sugiyama *et al.*, 2005), thereby inducing stable heterochromatinization and binding of DNMT3A (Fig. 2), which is part of a complex that is probably involved in bringing about local DNA methylation. In keeping with this model, a number of independent studies have already demonstrated that animal cells must contain the molecu-

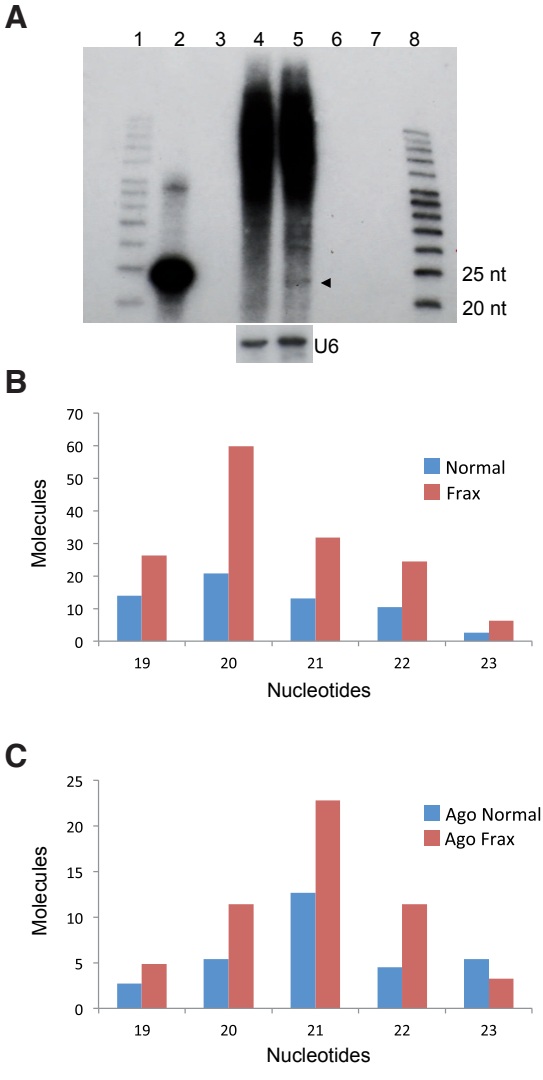

Fig. 4. Analysis of small RNA in human embryonic stem (hES) cells. **(A)** *Small RNA was isolated from normal (lane 4) and Frax (lane 5) hES cells, subjected to gel electrophoresis and hybridized with a P^{32}-labelled $(CCG)_7$ probe. Also shown are an RNA size ladder (lanes 1 and 8) and a specific $(CGG)_7$ RNA marker (lane 2). No hybridization was observed using a complementary $(CGG)_7$ probe. The gel was also hybridized with a U6 probe (bottom) as a loading control.* **(B)** *Small RNA from WT and Frax hES cells was subjected to RNA-Seq and the number of molecules (per 10^7 reads) containing full or truncated pure CGG repeats in all frames is shown. As a control, we found that 3 different miRNAs were expressed at relatively equal levels in both cell types.* **(C)** *RNA bound to Ago was isolated and subjected to RNA-Seq as in B. It should be noted that no CCG triplet repeats in this size range were detected either in the Northern analyses (A) or the RNA-Seq analysis (B,C).*

lar machinery for targeting exogenous siRNA molecules to their complementary sequences in the genome where they bring about the formation of a repressive chromatin structure (Morris *et al.*, 2004, Ting *et al.*, 2005) and, in some cases, this has been shown to be mediated by Ago proteins (Hawkins *et al.*, 2009, Kim *et al.*, 2006, Schmitz *et al.*, 2010). A similar mechanism has also been observed for endogenous RNA, as well (Zamudio *et al.*, 2014).

While this overall pathway has been deduced from studies on embryonic cells in vitro, the unique protein-DNA structure (Fig. 2) resulting from this process is stably maintained in somatic cells, strongly suggesting that this is indeed what occurs during development in Fragile X patients. It thus appears that the defective *FMR1* gene in Fragile X syndrome is targeted for inactivation mainly because CGG triple-repeat sequences within the genome undergo transcription, yielding duplex RNA molecules that are inadvertently processed by the Dicer system to produce a site-specific repression complex. This non-programmed pathological event may represent the first example of an endogenous Dicer-mediated small RNA transcriptional repression mechanism in animal cells.

Materials and Methods

Immunoprecipitation

For methyl DNA immunoprecipitation (mDIP), purified sonicated (500 – 2,000 bp average size) DNA (10 μg) was denatured by heating and immunoprecipitated with 20 μl of cell supernatant containing an anti-5-methylcytidine monoclonal antibody (Mayer *et al.*, 2000, Reynaud *et al.*, 1992). This antibody is commercially distributed by EMD Biosciences (U.S.A.), Serotec (U.K.) and Eurogentech (Belgium). Input and bound fractions were separated using protein A Sepharose beads (Sigma) and SpinX columns (Corning NY) (Maruyama *et al.*, 2002) and extracted by phenol-chloroform and ethanol precipitation. The DNA was then resuspended in 100 μl double distilled water, dialyzed and subjected to real-time PCR from specific gene regions. Since we usually precipitated < 1% of the DNA, PCR of the bound fraction was compared to 1/100 dilutions of the input DNA (Keshet *et al.*, 2006). Enrichment is calculated as bound/input as compared to a control sequence (*Cryaa*).

For chromatin immunoprecipitation (ChIP), cells were cross-linked and chromatin extracted and then sonicated to an average size of 500-2,000 bp. Immunoprecipitation was carried out using the ChIP assay kit as recommended by the manufacturer (Millipore,Upstate Biotechnology, Lake Placid N.Y.). Antibodies (about 5 μg/10-30 μg DNA) were directed against H3K9me3, SUV39H1 or AGO1 (Millipore, Upstate Biotechnology), HP1 (Serotec) and DNMT3A (Imgenex). Incubation with the various antibodies was followed by Salmon Sperm DNA/Protein A Agarose (60 μl/10 μg DNA) (Millipore, Upstate) precipitation in order to isolate the bound fraction. Because we usually precipitated < 1% of the chromatin, PCR analysis of the bound (B) fraction was compared to a 1:100 dilution of the input (I) DNA. Amplification was carried out by real time PCR or, in the case of human satellite DNA, by semiquantitative PCR using two different concentrations of DNA in the linear range, and B/I enrichment values were then normalized by setting the negative control (ACTB or APRT) to 1. Multiple assays of the same sample or the same gene sequence in separate IPs from a given chromatin preparation showed an average coefficient of variance of ~17%. Primer sequences are shown in Table S1.

For RNA immunoprecipitation (RIP), $2X10^8$ human ES (Fragile X and normal) cells were cross-linked, sonicated and immunoprecipitated, as described (Gilbert and Svejstrup, 2006) with 30 μl AGO1 antibodies (Upstate Biotechnology). This was then incubated with Salmon Sperm DNA/Protein A Agarose (Upstate) in order to isolate the bound fraction. Small RNAs were prepared using the Mirvana Kit (Ambion) and analysed by RNA-Seq.

Cells

Mouse A9 and OTF9 cells were grown in DMEM supplemented with 10% FCS, P/S and Glutamine. Fragile X and normal lymphoblast cell lines were grown in RPMI, supplemented with 20% FCS, P/S, Glutamine, non-essential amino acids, Na Pyruvate and 2-mercaptoethanol. ES cell lines were grown in DMEM with the same supplements plus leukemia inhibitory factor (LIF). In order to generate $Hprt^-$ derivative cell lines, mouse A9 fibroblasts, OTF9 and wt, $G9a^{-/-}$ or $Suv39h1/h2^{-/-}$ ES cells were grown on 30 μM 6 thioguanine (Sigma) for several weeks, and individual clones were then isolated. For all mouse ES cells, this procedure was carried out by growing on a feeder layer composed of 6 thioguanine-resistant MEFs.

Human SUV39H1 (SH2502-A-1) and SUV39H2 (SH2558-G-7) or mouse Dicer1 (V2LMM_30829) and human DICER1 (V2LHS_99123) shRNAs (Open Biosystems) were co-transfected into $Hprt^-$ ES cells (wt or $Suv39h1/h2^{-/-}$) using FuGene (Roche) and subjected to puromycin selection after 48 h in order to derive individual colonies. Fused $Suv39h1/h2^{-/-}$ ES cells carrying SH2502-A-1 and SH2558-G-7 shRNAs were shown to have < 20% of the level of SUV39H1 mRNA than fused cells without these shRNAs as determined by RT-PCR using Actb as a normalization control. Fused mouse ES cells carrying Dicer1 shRNA were shown by RT-PCR to have < 10% Dicer1 RNA.

Human ES cells were grown on human fibroblast feeder cells treated with mitomycin (10 μg/ml) in knock-out DMEM media (GIBCO #10829-018) supplemented with knock-out serum replacement (GIBCO #10828-028), P/S, Glutamine, non-essential amino acids, 2-mercaptoethanol and bFGF, GIBCO #13256-029 (or equivalent), at 4ng/ml final concentration. Cells where passaged using Collagenase Type IV, (GIBCO #17104-019). Before crosslinking for RNA immunoprecipitation, cells were also treated by a short incubation with collagenase to enable the separation of human ES from the more resistant feeder layer.

Cell fusions

Primary human embryonic fibroblasts containing a Fragile X FMR1 gene were isolated through amniocentesis. The FMR1 gene was found to have a CGG expansion of ~570 repeats, and the promoter region was fully methylated as shown by Southern analysis. FMR1 was expressed at about 2% of normal levels as measured by RT-PCR using HPRT as a normalization control. These cells were then fused into mouse $Hprt^-$ A9 fibroblast cells using PEG 1500 (Kugoh *et al.*, 1999). One clone, validated to have acquired the human X chromosome with an intact HPRT gene, as well as the methylated Fragile X FMR1 gene was isolated and used as the donor for microcell fusions (Wohrle *et al.*, 2001) with $Hprt^-$ OTF9. Cells were plated in 25 ml flasks, and 0.05 μg/ml colcemid (Sigma) was added when the culture reached 70-80% confluency. The medium was changed after 48 h and flasks filled to the neck with DMEM without serum, supplemented with 10 μg/ml cytochalasin B (Sigma) for an additional 2 h. Flasks were centrifuged at 8,000 rpm for 1 h at 32°C, and the microcell pellets then resuspended in 2 ml DMEM with 200 μg/ml Phytohemaglutinin-P (Sigma). Recipient cells (OTF9) were seeded in order to reach 80-90% confluency after 24 h. Prior to fusion, these cells were washed 3 times with DMEM without serum and microcells were then added for 20 min at room temperature. Following attachment, the medium was removed and 1 ml of prewarmed PEG 1500 was added. After 1 min the fused cells were rinsed by washing 5 times with serum-free DMEM and then incubated overnight in DMEM + 10% FCS, split and allowed to grow for 3 days before adding HAT. Individual colonies were isolated after 3 weeks, and some of these were induced to differentiate by treatment with 0.5 μM retinoic acid for 10 days. Differentiation was verified by detecting a decreased level (at least 20 fold) of Oct-3/4 mRNA by RT-PCR.

Lymphoblasts (Chiurazzi *et al.*, 1999, Pietrobono *et al.*, 2002) from a Fragile X patient were obtained from B. Oostra. This patient has a CGG expansion containing ~970 repeats, as determined by Southern blotting. The promoter region was shown to be fully methylated by restriction enzyme digestion and bisulfite sequencing (Pietrobono *et al.*, 2002), and the level

of transcription was determined by RT-PCR to be < 0.01% of that seen in normal lymphoblasts. We were unable to carry out microcell fusion on ES cells in culture. Instead, we found that it was possible to introduce the Fragile X chromosome from patient lymphoblasts directly into mouse ES cells by employing electrocell fusion (Huang *et al.*, 2013, Kimura *et al.*, 2002). Mouse Hprt^{-} ES cells and human Fragile X or normal lymphoblasts were mixed at a ratio of 1:5, washed 3 times in PBS, resuspended in 0.3 M Manitol at a concentration of 1 X 10^6 cells/ml and placed in an Electro Cell Manipulator BTX 2000 using a 1 mm electrode gap. Two sequential steps of electric pulses were applied, first using alternating current at 10 V for 99 sec and then employing direct current at 270 V for 30 μsec. Cells were then transferred to 6-well plates containing an MEF feeder layer and LIF, subjected to HAT selection after 24 h and transferred to 10 mm dishes after culturing for 2 weeks. In all cases, fused HAT resistant cells grew as individual colonies that stopped dividing after 2-3 weeks and adopted a differentiated morphology characterized by decreased levels of Oct-3/4 (Pou5f1), Sox2, Nr0b1 and Zfp42 mRNA as determined by RT-PCR. In this regard it should be noted that Oct3/4 has been shown to undergo normal inactivation, heterochromatinization and DNA methylation in retinoic acid induced differentiating Suv39h1/h1$^{-/-}$ (Feldman *et al.*, 2006), as well as Dicer$^{-/-}$ (data not shown) ES cells.

Small RNA analysis

miRNA isolated (mirVana, Ambion) from $2X10^7$ human ES cells or lymphoblast cells was denatured and run on a 7 M Urea 12% polyacrylamide gel together with single-strand RNA size markers, transferred to a membrane and hybridized using an endlabeled probe containing 7 CGG repeats in Amersham Rapid Hybridization Buffer (RPN 1635). This RNA was also subjected to RNA-Seq (Illumina HiSeq 2000) to yield 10-20 million single-end reads per sample. Analysis was limited to small RNAs between 18-25 bp in length, which along with adaptor and quality trimming were performed using cutadapt (Martin, 2011) with parameters cutadapt -a TGGAATTCTCGG -O 1 --match-read-wildcards -q 28 -m 18 -M 25.

Read counts in the 19-23 net size range were determined using custom scripts and normalized based on control miRNA sequences and non-CGG repeats per million reads. It should be noted that this cutoff represents only a fraction of the small RNA molecules that contain CGG repeats with one or two mismatches that would probably be detected by Northern blot analysis. By BLAT analysis we found over 350 loci containing CGG repeats (> 7) in the human genome and over 100 of these are transcribed to yield RNA molecules containing CGG repeats (http://genome.ucsc.edu/cgibin/hgBlat?command=start).

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