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piRNAs warrant investigation in Rett Syndrome: An omics perspective

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Abstract. Mutations in the *MECP2* gene are found in a large proportion of girls with Rett Syndrome. Despite extensive research, the principal role of MeCP2 protein remains elusive. Is MeCP2 a regulator of genes, acting in concert with co-activators and co-repressors, predominantly as an activator of target genes or is it a methyl CpG binding protein acting globally to change the chromatin state and to supress transcription from repeat elements? If MeCP2 has no specific targets in the genome, what causes the differential expression of specific genes in the *Mecp2* knockout mouse brain? We discuss the discrepancies in current data and propose a hypothesis to reconcile some differences in the two viewpoints. Since transcripts from repeat elements contribute to piRNA biogenesis, we propose that piRNA levels may be higher in the absence of MeCP2 and that increased piRNA levels may contribute to the mis-regulation of some genes seen in the *Mecp2* knockout mouse brain. We provide preliminary data showing an increase in piRNAs in the *Mecp2* knockout mouse cerebellum. Our investigation suggests that global piRNA levels may be elevated in the *Mecp2* knockout mouse cerebellum and strongly supports further investigation of piRNAs in Rett syndrome.

Keywords: Rett Syndrome, MeCP2, piRNAs, LINE 1, short RNAs

Rett Syndrome (RTT), a severe neurodevelopmental disorder, leads to intellectual disability in girls. After a normal prenatal and postnatal period, patients usually present with developmental delay between 6 and 18 months of age, followed by the development of stereotypic hand movements and loss of acquired skills including voluntary hand use, language and communication. This regression is characteristic of Rett Syndrome which, in 97% of clinically diagnosed classic cases and 70% of atypical cases, is caused by mutations in the methyl CpG binding protein 2 gene, (*MECP2*) [1]. In some patients with atypical Rett Syndrome, where some but not all clinical features are seen, mutations in *CDKL5* [2] or *FOXG1* [3] are found, albeit infrequently. Diagnosis of Rett Syndrome is based on clinical criteria [4] and confirmed upon detection of a mutation in *MECP2*, *CDKL5* or *FOXG1*. However, in approximately 20% of girls clinically diagnosed with classic or atypical Rett syndrome, mutations cannot be detected in either of these genes.

MECP2 gene undergoes X chromosome inactivation (XCI) [5], which means that in any cell with two X chromosomes, RNA transcripts arise only from the *MECP2* gene on the active X chromosome. This is because the *MECP2* gene on the inactive X chromosome has been silenced. Chaumeil et al. demonstrated, through in-situ hybridization in mouse ES cells, that the *Mecp2* gene moves inside the silencing compartment of *Xist* on the 4th day after differentiation [6]. Although some genes are known to escape X inactivation in humans and mouse [7–10], Carrel et al. showed using rodent/human somatic hybrid cell lines that in humans MECP2 transcripts are not expressed from the inactive X chromosome [7]. Due to the random nature of X inactivation, part of the clinical variability in RTT is attributed to the differences in the X inactivation status of patients [11–13], however more recent data suggest X inactivation status may not adequately explain the phenotypic variations [14].

MECP2 gene is composed of 4 exons and generates two transcripts which encode two nearly identical protein isoforms [15,16]. MeCP2_e1, which commences translation from exon 1, is encoded from a transcript encompassing exons 1, 3 and 4; and MeCP2_e2, which starts translation from the end of exon 2, is generated from a transcript arising from exons 1, 2, 3 and 4, where exon 1 and most of exon 2 form the 5'UTR [15,

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16]. Although *MECP2* is expressed in all tissues, semi-quantitative PCR analysis has shown that *Mecp2_e1* may have a higher expression level over *Mecp2_e2* in the brain [16]. Mutations in exons 3 and 4 affect both protein isoforms and are frequently found in Rett patients. Mutations in exon 1 can cause Rett syndrome despite the fact that mutations in exon 1 do not affect the coding region of the MeCP2_e2 protein. Interestingly, mutations in exon 2 of the gene, which have the potential to affect the MeCP2_e2 isoform alone, have so far not been found in patients. While earlier work on an Australian patient with a recurrent deletion in exon 1 of *MECP2* gene demonstrated the absence of MeCP2_e2 protein correlated with X inactivation status, suggesting translational interference from the mutation [17], a recent publication found no evidence of loss of MeCP2_e2 protein in a Canadian patient with a similar mutation indicating that some patients may present with clinical features of Rett syndrome even in the presence of a fully functional MeCP2_e2 isoform [18]. Interestingly, this data also suggests that despite high sequence similarities there is no functional redundancy between the two protein isoforms.

Due to its property to bind methylated DNA with high affinity and its association with repressor complexes consisting of HDAC1/2 and Sin3A, MeCP2 was believed to function as a transcriptional repressor [19, 20]. The MeCP2-DNA interaction was shown to result in chromatin compaction, which is also correlated with silencing of chromatin [21]. Subsequent studies revealed that MeCP2 had binding affinity to methylated DNA as well as non-methylated DNA [22]. Absence of MeCP2 in mouse brains also results in an increase in H3Ac levels, suggesting a role for MeCP2 in chromatin modification [23,24]. Recent data suggest that MeCP2 protein may be a regulator of transcription, acting in concert with activators as well as repressors to regulate gene expression. Yasui et al. first reported that promoter occupancy by MeCP2 may not result in gene silencing [25]. Using a custom tiling array of selected chromosomal regions totalling 26.3 Mb, they performed ChIP-chip analysis on SH-SY5Y cells with antibodies against MeCP2 and RNA polymerase II. The data revealed co-occupancy of MeCP2 and RNA Polymerase II at selected promoters suggesting that MeCP2 binding may not be correlated to gene repression [25]. Using ChIP-chip assays for 24,275 promoters, they demonstrated that only 2600–4300 promoters were occupied by MeCP2, of which 1534 promoters showed strongest enrichment. Comparison with gene expression arrays in the same cell lines revealed that almost 63% of the "strongest" promoters were expressed in SH-SY5Y cells. Subsequent MeDIP-ChIP analysis revealed that just 2.2% of methylated promoters were occupied by MeCP2 [25]. These data were supported in part by Chahrour et al. who used microarrays to determine differentially expressed genes in the hypothalamus of 6 week old *Mecp2* knockout (KO) mouse and in the hypothalamus of a mouse model that overexpressed *Mecp2* under its endogenous promoter (Tg) [26]. Combining their data from the KO and Tg models, they identified 2561 genes as direct targets of MeCP2, of which ∼85% were activated by MeCP2 and ∼ 15% were repressed by MeCP2 [26]. Using mass spectrometry on proteins co-immunoprecipitated with an anti-MeCP2 antibody, they identified CREB1 as a co-activator associated with MeCP2 and demonstrated co-occupancy of the two proteins at an activated target *Sst [26]*. Together, these data established MeCP2 as an activator of transcription [25,26]. Thus transcriptional mis-regulation is believed to underlie the phenotype seen in patients with mutations in the *MECP2* gene. In view of the fact that FOXG1 is a member of the forkhead family of transcription regulators, it is likely that in patients carrying mutations in *FOXG1,* mis-regulation of genes may contribute to the phenotypic features. The molecular pathology leading to the clinical phenotype of Rett Syndrome in mutation negative patients remains unknown. While much has been reported on the mis-regulation of specific genes after MeCP2 knockdown (KD) or KO, such studies have not yet been reported for FOXG1. Other studies reveal subtle changes in the expression levels of specific genes after MeCP2 KD or in the *Mecp2* KO mouse brain rather than genome wide transcriptional mis-regulation [27–29].

However, a recent report suggests that the absence of a functional MeCP2 may result widespread mis-regulation of repeat elements. Skene et al. investigated MeCP2 binding on selected loci in the mature mouse brain using ChIP-qPCR and demonstrated that MeCP2 was enriched all across the loci, but the enrichment was reduced over CpG islands, which are generally methylation free [24]. With bisulfite modification and sequencing of selected loci they demonstrated the recovery of predominantly methylated chromatin from the MeCP2 ChIP, re-emphasizing the role of MeCP2 as a methyl CpG binding protein [24]. Based on their investigation of the histone acetylation status by Western blotting and H3Ac ChIP-qPCR at 100 loci, they concluded that the association of MeCP2 with chromatin causes a genome-wide decrease in histone acetylation [24]. To investigate the binding sites of MeCP2

genome wide, Skene et al. performed MeCP2-ChIP-sequencing on the whole brain. Despite deep sequencing, they did not find peaks of MeCP2 occupancy, but found reads which coincided with methylated regions of the genome. Since they did not uncover specific binding targets of MeCP2 in the genome, they hypothesized that MeCP2 may act at a global level, most likely to supress transcription from the repeat regions of the genome [24]. Using qPCR, they demonstrated a 1.6 fold increase in transcripts arising from repeat sequences such as LINE-1, intra-cisternal A particles (IAPs) and major satellite DNA in the nuclear fraction of the *Mecp2* KO mouse brain. Based on their data they proposed that MeCP2 functions to repress spurious transcription of repeat elements [24] rather than to activate specific gene targets. An earlier investigation into the association between MeCP2 and LINE-1 and Alus had revealed that MeCP2 repressed LINE-1 expression and transposition, but activated Alu expression [30]. The role of MeCP2 in repressing transcription and transposition of LINE-1 elements was also corroborated by independent studies from the Gage Lab that showed that LINE-1 is over expressed in neuron progenitor cells after KD of MeCP2 and in neurons derived from Rett patients [31]. The data from Yasui et al. suggests that MeCP2 displays limited binding to methylated sites [25] and from Chahrour et al. proposes that MeCP2 acts as a transcriptional activator of specific targets [26]. In contrast, the data from Skene et al. suggests that MeCP2 binds methylated DNA, is a modulator of global chromatin state and may not have specific gene targets [24]. We note that some of these studies were conducted using microarrays or custom tiling arrays, which are generally limited to gene specific probes and exclude repeat sequences. Despite contradictory inferences on MeCP2 function, the fact remains that specific genes are mis-expressed and repeat elements are over-expressed in *Mecp2* KO mice. To reconcile the two opposing views, an alternative model would suggest that the key role of MeCP2 is to silence LINEs and similar repeat elements globally and that the observed mis-expression of genes is a downstream consequence of mis-expressed repeat elements.

Several recent reports suggest that non-coding RNAs play a role in the regulation of transcription through epigenetic modifications, [for a review see [32]]. Repeat elements, particularly LINEs, are known to participate in the silencing of genes on the X chromosome [33]. Expression of LINEs in the vicinity of genes is instrumental for their inclusion into the Xist silencing compartment [33]. It is not yet known whether transcripts from LINE elements are associated with chromatin remodelling complexes to mediate epigenetic changes and fine-tune gene transcription, but we note that the elevated repeat elements were found in the nuclear compartment of the *Mecp2* KO mouse brain cells [24] and there is emerging evidence of enrichment of LINEs in nuclear and chromatin fraction of cells [34]. A recent report using a retrotransposon capture sequencing technique (RC-seq) reveals that somatic transposition of LINE-1 (L1) in the hippocampus results in insertions, predominantly in exons and introns of protein coding genes [35]. Comparing microarray data with their RC-seq data, Baillie et al. reported that intronic L1 insertions are likely to cause overexpression of such genes in the brain, suggesting a regulatory role for L1 [35]. It is not clear if the increase in retrotransposon expression in the *Mecp2* KO brain leads to their active transposition even in post mitotic neurons. Random integration of transposons is suppressed in differentiated somatic cells by transcriptional [36] and post-transcriptional mechanisms [37]. It would be interesting to investigate if somatic retrotransposition is increased in the *Mecp2* KO brain and whether overexpressed genes identified in *Mecp2* KO mouse show novel intronic L1 insertion events.

Retrotransposons such as LINEs can be further processed into short 21–24 nucleotide double stranded siRNAs [38] or into single stranded 24–31 nucleotide long piRNAs [39,40]. Watanabe et al. described in mouse oocytes dicer dependent double stranded endogenous siRNAs mapping exclusively to retrotransposons or expressed mRNA transcripts [38]. While the presence of endogenous siRNAs has not been demonstrated in the mouse brain, given that such short RNAs are shown to regulate the expression levels of specific genes and specific retrotransposons [38,41], and that dysfunctional MeCP2 may result in the overexpression of LINE-1 [24,31], it would be interesting to investigate the presence of endogenous siRNAs in the MeCP2 KO mouse brain.

piRNAs are germ line specific short RNAs of size 24 to 31 nucleotides generated through dicer independent processing of long single strand RNA transcripts. piRNAs interact with the PIWI proteins (MILI, MIWI and MIWI2 in mouse) [39,40] and their function, though not fully understood, appears to relate to silencing of transposons especially LINE-1 [39,40,42] intracisternal A particles [39,40] and specific genes through DNA hypermethylation [43]. In mouse testis, 17% of piRNAs bound to the MIWI protein map to repeats including LINEs, SINEs and LTRs [40]. In addition, through

a unique ping-pong cycle, piRNAs are amplified from existing retrotransposon transcripts, mostly LINE-1 elements [44,45]. This amplification cycle also results in the depletion of LINE-1 in germ line cells and is believed to deplete the levels of retrotransposon transcripts after differentiation [42]. Thus piRNAs regulate expression of LINEs and Intracisternal A particles both transcriptionally and post transcriptionally. Interestingly, piRNAs have recently been shown to regulate expression of a single imprinted gene in an imprinted locus in mouse spermatogonia via DNA methylation by piRNA targeting of a non coding RNA (pitRNA) arising from the locus [43]. It is as yet not known if such specific targeting by piRNAs is a widespread phenomenon, nevertheless it highlights a mechanism through which piRNAs may regulate expression of specific genes.

Until recently, piRNAs and their associated proteins were presumed to be germ line specific in mouse, but a report published last year confirmed the presence of MIWI and its associated piRNAs in the mouse hippocampus through sequencing, RIP-qPCR, northern blots, western blots and in situ hybridization studies [46]. Through bioinformatics analysis, Lee at al. identified specific piRNAs expressed in the brain and showed through piRNA inhibition studies, that one piRNA in the brain, DQ541777, may play a role in regulating the size of dendritic spines [46]. It appears plausible that in the absence of MeCP2, over expression of repeat elements, particularly LINE-1 may result in an increase in piRNA amplification from transposons. It would be interesting to investigate whether akin to germ line cells, in brain also, the increase in piRNAs result in depletion of retrotransposon transcript levels through transcriptional and post transcriptional silencing. The mechanism of transcriptional silencing by piRNAs through DNA methylation may require recruitment of repressor complexes by proteins that bind methylated DNA, including MeCP2, thus highlighting a feedback loop for MeCP2 requirement.

To investigate our hypothesis that piRNAs may be overexpressed in the *Mecp2* KO mouse brain, we analysed a short RNA library made from mouse cerebellum [47]. To identify miRNAs differentially expressed in the cerebellum, Wu et al. performed short RNA sequencing of pooled 6 week old pre-symptomatic wildtype and *Mecp2* KO cerebellum ($n = 4$ in each pool) using the SOLiD version 2 sequencer [47]. We downloaded the pooled libraries from the DDBJ database (DDBJ accession number SRP005132). ncRNAs were downloaded from NONCODE version 3 [48] and a total of 75,814 mouse piRNAs were extracted from this database. As the SOLiD reads correspond to the 5' ends of small RNAs, we directly mapped the respective reads from the pooled WT and KO libraries, using SHRiMP version 2.2.2 [49] with the default parameters, to the mouse piRNAs. After mapping we corrected tag numbers for reads multi-mapping to more than one piRNA, so that if a read mapped equally well to 2 or more individual piRNA sequences, the tag numbers were divided by the number of times it multi mapped, followed by equal assignment to all the piRNAs. For expression analysis we did not take into consideration multi mapped reads that mapped to 5 or more piRNAs and also filtered out reads that had less than 5 tags in the KO samples. The tag numbers were normalized by tags per million before comparison between wildtype and KO samples. Our very preliminary analysis of piRNAs in the cerebellum reveals 357 piRNAs in the cerebellum libraries (Supplementary Table 1). While 81% (287) of the individual piRNAs found in the cerebellum have a higher expression in KO, 59% (208) piRNAs show an expression change of over 1.5 fold in the KO cerebellum compared with the wildtype (Fig. 1B and supplementary Table 1). Overall, we found a striking 1.9 fold increase in the total piRNAs in the KO cerebellum in comparison with the wildtype cerebellum (Table 1 and Fig. 1A) suggesting a global increase in piRNAs in the *Mecp2* KO sample. We next investigated whether the 20 most abundant piRNAs identified by Lee et al. in the mouse hippocampus were represented in the mouse cerebellum [46]. We found 19 out of the 20 piRNAs reported by Lee et al. in the cerebellum libraries (Table 2) including DQ 541777 (the piRNA implicated in regulating the size of dendritic spines) and of these, 12 piRNAs (60%) revealed a fold change of over 1.5 in the KO cerebellum (Table 2). Interestingly, DQ541777 is the 5^{th} most abundant piRNA in the cerebellum libraries, the two most highly abundant piRNAs in the cerebellum libraries map to rRNA loci, which were incidentally excluded in the hippocampal analysis [46].

Based on our preliminary findings, we suggest a model for Rett Syndrome where, in the absence of a functional MeCP2, the over-expressed repeat elements lead to an increase in the total piRNAs. The over-represented piRNAs may function, not only to deplete the load of repeat transcripts in cells, but also to fine-tune the expression level of specific genes. Thus piRNA mis-regulation may contribute to some of the differences in gene expression seen in the *Mecp2* KO mouse brain.

While our analyses provide preliminary evidence of genome wide piRNA over-expression in the *Mecp2*

Table 1
piRNA analysis in the short RNA libraries made from 6 week old pooled cerebellum from the wildtype mouse and the *Mecp2* knockout mouse [38] (DDBJ accession number SRP005132)

	WT cerebellum	KO cerebellum
Read ID	SRR089647	SRR089648
Total sRNA reads	3660124	2789136
Total reads mapped to piRNAs (no filter)	362089	522238
Reads mapped to piRNAs (filter out < 5 reads in KO)	356283	518589
piRNA mapped tags normalized by TPM	97341.74762	185931.8441
Fold change (tpm normalized piRNAs over WT)	1	1.910093548

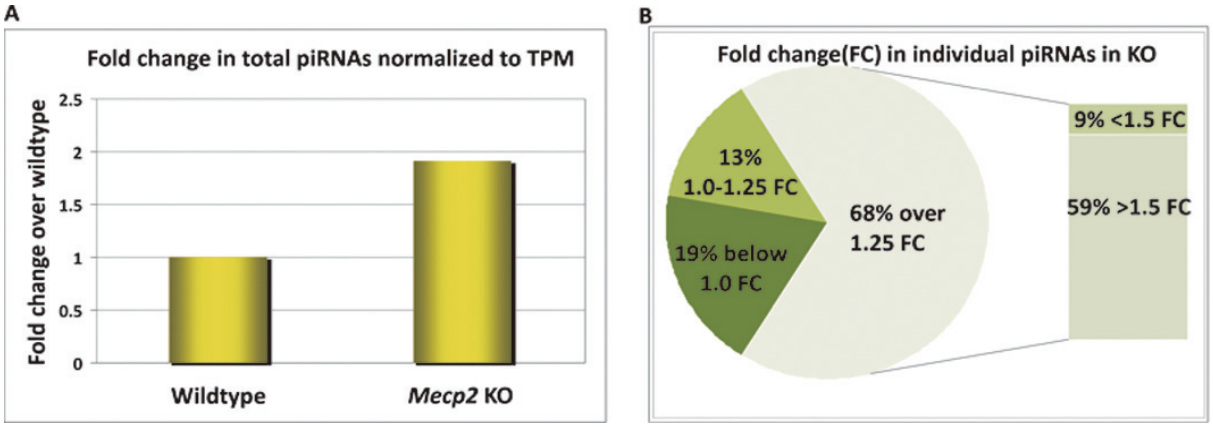

Fig. 1. piRNA levels are elevated in *Mecp2* KO cerebellum. Read numbers for individual piRNAs found in the wildtype (WT) and *Mecp2* knockout (KO) samples were normalized to tags per million as described in the text. After filtering out the piRNAs with less than 5 reads in the KO sample, the total piRNA reads were summed up. The histogram in panel A shows that the total numbers of piRNAs are almost doubled (1.9 fold) in the KO sample suggesting a global rise in piRNAs. The fold change relative to WT was calculated for each individual piRNA in KO. The pie chart in panel B reveals that 81% of piRNAs show a higher expression level in the KO sample. Of these, 59% have a fold change of over 1.5 in the KO sample (see supplementary Table 1). (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-2012-0932)

KO cerebellum, this data was generated from libraries without replicates. Thus additional detailed investigations are warranted to affirm the over-representation of piRNAs and gain insights into the extent of their contribution to the gene mis-regulation seen in the Rett mouse model. We did not venture into the identification of gene targets of mis-regulated piRNAs and their intersection with the known mis-regulated genes or repeats. Such data may provide insights into the mis-regulation of some genes and the biogenesis of the over-represented piRNAs. Notably, in humans and mouse, an absence of MeCP2 results in fewer dendritic spines when compared with wildtype neurons [50,51]. While inhibition of DQ541777 was reported to cause a decrease in spine density, whether the overexpression of piRNAs, including DQ541777, can cause such morphological changes in the brain is not yet known. Further, recent reports have demonstrated that unlike mRNAs, miRNAs are stable in extracellular environments including blood serum [52,53]. Although such analyses have not been conducted for piRNAs, if piRNAs are found to be stable in extracellular fluids, differentially expressed piRNAs may potentially represent clinical biomarkers for the diagnosis and prognosis of Rett Syndrome.

There is overwhelming complexity in unravelling the molecular pathogenesis of the phenotype seen in Rett syndrome. Although the miRNA repertoire of *Mecp2* KO cerebellum has been investigated using next generation sequencing approaches, the field would benefit in re-identifying mis-regulated transcripts through deep, long and short (to identify sRNAs other than miRNAs), RNA sequencing of specific brain regions. Additionally, RC-seq conducted to identify somatic integration events would reveal whether the overexpressed genes are correlated with intronic LINE-1 insertion events. And while it has been established for the *Mecp2* KO mouse model, that investigating a specific brain region is more fruitful than investigating the whole brain [26], analysis of neuronal subtypes would be even more insightful. Until now, the isolation of neuronal subtypes from adult mouse brain using high throughput techniques such as FACS sorting was challenging, yielding few nuclei and poor quality RNA. A recently pub-

Table 2
Comparison of the top twenty piRNAs reported in the hippocampus [38] with the piRNAs found in the cerebellum

Top 20 piRNAs in hippocampus	WT hippocampus tag numbers	WT cerebellum tag numbers	KOcerebellum tag numbers	WT cerebellum TPM	KO cerebellum TPM	FC KO/WT
DQ541777	16130	1995.50	2411.00	545.20	864.43	1.59
DQ705026	6257	154.00	377.00	42.08	135.17	3.21
DQ555094	3439	202.00	140.00	55.19	50.19	0.91
DQ719597	2459	168.00	306.00	45.90	109.71	2.39
DQ689086	1514	65.00	78.00	17.76	27.97	1.57
DQ540285	1433	457.40	548.23	124.97	196.56	1.57
DQ540981	1360	126.50	124.00	34.56	44.46	1.29
DQ720186	849	336.00	251.00	91.80	89.99	0.98
DQ555093	775	189.50	129.50	51.77	46.43	0.90
DQ540862	639	21.50	33.50	5.87	12.01	2.04
DQ540284	635	456.90	548.23	124.83	196.56	1.57
DQ541506	580	523.90	627.40	143.14	224.94	1.57
DQ539915	304	35.50	28.00	9.70	10.04	1.04
DQ540861	252	20.50	31.50	5.60	11.29	2.02
DQ715526	207	20.00	20.00	5.46	7.17	1.31
DQ543676	182	438.90	518.70	119.91	185.97	1.55
DQ722288	175	2.00	13.00	0.55	4.66	8.53
DQ551351	168	Not found	Not found	Not found	Not found	Not found
DQ550765	118	10.75	9.50	2.94	3.41	1.16
DQ708131	115	3.00	6.00	0.82	2.15	2.62

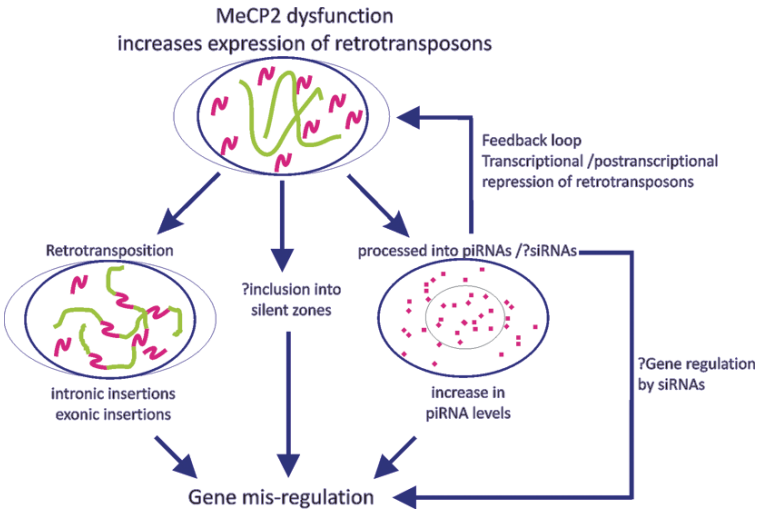

Fig. 2. Schematic of the proposed model showing that changes in the expression level of some genes may be a consequence of the increase in expression of retrotransposons. DNA is depicted in green, retrotransposon transcripts in pink and piRNAs as pink dots. See text for details. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-2012-0932)

lished trehalose enhanced technique for FACS sorting individual neuronal subtypes could help isolate high quality RNA from *Mecp2* null neuronal subtypes for transcriptome sequencing [54].

In conclusion, we propose that overexpression of LINE-1 may contribute to the mis-regulation of some genes in Rett syndrome, mediated through insertional events or by an increase in piRNAs (Fig. 2). Our preliminary data suggests that piRNA expression levels may be altered globally in the absence of MeCP2. Application of next generation sequencing technologies may resolve some key questions regarding MeCP2 function and the downstream consequences of a dysfunctional MeCP2.

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References

[1] Amir, R.E., et al., Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nat Genet, 1999. **23**(2): 185-8.
[2] Weaving, L.S., et al., Mutations of CDKL5 cause a severe neurodevelopmental disorder with infantile spasms and mental retardation. Am J Hum Genet, 2004. **75**(6): 1079-93.
[3] Ariani, F., et al., FOXG1 is responsible for the congenital variant of Rett syndrome. Am J Hum Genet, 2008. **83**(1): 89-93.
[4] Neul, J.L., et al., Rett syndrome: revised diagnostic criteria and nomenclature. Ann Neurol, 2010. **68**(6): 944-50.
[5] Adler, D.A., et al., The X-linked methylated DNA binding protein, Mecp2, is subject to X inactivation in the mouse. Mamm Genome, 1995. **6**(8): p. 491-2.
[6] Chaumeil, J., et al., A novel role for Xist RNA in the formation of a repressive nuclear compartment into which genes are recruited when silenced. Genes Dev, 2006. **20**(16): 2223-37.
[7] Carrel, L. and H.F. Willard, X-inactivation profile reveals extensive variability in X-linked gene expression in females. Nature, 2005. **434**(7031): 400-4.
[8] Lingenfelter, P.A., et al., Escape from X inactivation of Smcx is preceded by silencing during mouse development. Nat Genet, 1998. **18**(3): 212-3.
[9] Xu, J., X. Deng and C.M. Disteche, Sex-specific expression of the X-linked histone demethylase gene Jarid1c in brain. PLoS One, 2008. **3**(7): e2553.
[10] Yang, F., et al., Global survey of escape from X inactivation by RNA-sequencing in mouse. Genome Res, 2010. **20**(5): 614-22.
[11] Hoffbuhr, K.C., et al., Associations between MeCP2 mutations, X-chromosome inactivation, and phenotype. Ment Retard Dev Disabil Res Rev, 2002. **8**(2): 99-105.
[12] Weaving, L.S., et al., Effects of MECP2 mutation type, location and X-inactivation in modulating Rett syndrome phenotype. Am J Med Genet A, 2003. **118A**(2): 103-14.
[13] Huppke, P., et al., Very mild cases of Rett syndrome with skewed X inactivation. J Med Genet, 2006. **43**(10): 814-6.
[14] Xinhua, B., et al., X chromosome inactivation in Rett Syndrome and its correlations with MECP2 mutations and phenotype. J Child Neurol, 2008. **23**(1): 22-5.
[15] Kriaucionis, S. and A. Bird, The major form of MeCP2 has a novel N-terminus generated by alternative splicing. Nucleic Acids Res, 2004. **32**(5): 1818-23.
[16] Mnatzakanian, G.N., et al., A previously unidentified MECP2 open reading frame defines a new protein isoform relevant to Rett syndrome. Nat Genet, 2004. **36**(4): 339-41.
[17] Saxena, A., et al., Lost in translation: translational interference from a recurrent mutation in exon 1 of MECP2. J Med Genet, 2006. **43**(6): 470-7.
[18] Gianakopoulos, P.J., et al., Mutations in MECP2 exon 1 in classical Rett patients disrupt MECP2_e1 transcription, but not transcription of MECP2_e2. Am J Med Genet B Neuropsychiatr Genet, 2012. **159B**(2): 210-6.
[19] Nan, X., et al., Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature, 1998. **393**(6683): 386-9.
[20] Jones, P.L., et al., Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet, 1998. **19**(2): 187-91.
[21] Georgel, P.T., et al., Chromatin compaction by human MeCP2. Assembly of novel secondary chromatin structures in the absence of DNA methylation. J Biol Chem, 2003. **278**(34): 32181-8.
[22] Nikitina, T., et al., Multiple modes of interaction between the methylated DNA binding protein MeCP2 and chromatin. Mol Cell Biol, 2007. **27**(3): 864-77.
[23] Shahbazian, M., et al., Mice with truncated MeCP2 recapitulate many Rett syndrome features and display hyperacetylation of histone H3. Neuron, 2002. **35**(2): 243-54.
[24] Skene, P.J., et al., Neuronal MeCP2 is expressed at near histone-octamer levels and globally alters the chromatin state. Mol Cell, 2010. **37**(4): 457-68.
[25] Yasui, D.H., et al., Integrated epigenomic analyses of neuronal MeCP2 reveal a role for long-range interaction with active genes. Proc Natl Acad Sci U S A, 2007. **104**(49): 19416-21.
[26] Chahrour, M., et al., MeCP2, a key contributor to neurological disease, activates and represses transcription. Science, 2008. **320**(5880): 1224-9.
[27] Urdinguio, R.G., et al., Mecp2-null mice provide new neuronal targets for Rett syndrome. PLoS One, 2008. **3**(11): e3669.
[28] Yakabe, S., et al., MeCP2 knockdown reveals DNA methylation-independent gene repression of target genes in living cells and a bias in the cellular location of target gene products. Genes Genet Syst, 2008. **83**(2): 199-208.
[29] Smrt, R.D., et al., Mecp2 deficiency leads to delayed maturation and altered gene expression in hippocampal neurons. Neurobiol Dis, 2007. **27**(1): 77-89.
[30] Yu, F., et al., Methyl-CpG-binding protein 2 represses LINE-1 expression and retrotransposition but not Alu transcription. Nucleic Acids Res, 2001. **29**(21): 4493-501.
[31] Muotri, A.R., et al., L1 retrotransposition in neurons is modulated by MeCP2. Nature, 2010. **468**(7322): 443-6.
[32] Saxena, A. and P. Carninci, Long non-coding RNA modifies chromatin: epigenetic silencing by long non-coding RNAs. Bioessays, 2011. **33**(11): 830-9.
[33] Chow, J.C., et al., LINE-1 activity in facultative heterochromatin formation during X chromosome inactivation. Cell, 2010. **141**(6): 956-69.
[34] Djebali S, et al., Landscape of transcription in human cells. Nature, 2012. doi:10.1038/nature11233.
[35] Baillie, J.K., et al., Somatic retrotransposition alters the genetic landscape of the human brain. Nature, 2011. **479**(7374): 534-7.
[36] Slotkin, R.K. and R. Martienssen, Transposable elements and the epigenetic regulation of the genome. Nat Rev Genet, 2007. **8**(4): 272-85.
[37] Yang, N. and H.H. Kazazian, Jr., L1 retrotransposition is suppressed by endogenously encoded small interfering RNAs in human cultured cells. Nat Struct Mol Biol, 2006. **13**(9): 763-71.
[38] Watanabe, T., et al., Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. Nature, 2008. **453**(7194): 539-43.
[39] Aravin, A., et al., A novel class of small RNAs bind to MILI protein in mouse testes. Nature, 2006. **442**(7099): 203-7.
[40] Girard, A., et al., A germline-specific class of small RNAs

binds mammalian Piwi proteins. Nature, 2006. **442** (7099): 199-202.

[41] Chen, L., et al., Naturally occurring endo-siRNA silences LINE-1 retrotransposons in human cells through DNA methylation. Epigenetics, 2012. **7**(7): 758-71.

[42] Reuter, M., et al., Miwi catalysis is required for piRNA amplification-independent LINE1 transposon silencing. Nature, 2011. **480**(7376): 264-7.

[43] Watanabe, T., et al., Role for piRNAs and noncoding RNA in de novo DNA methylation of the imprinted mouse Rasgrf1 locus. Science, 2011. **332**(6031): 848-52.

[44] Brennecke, J., et al., Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. Cell, 2007. **128**(6): 1089-103.

[45] Gunawardane, L.S., et al., A slicer-mediated mechanism for repeat-associated siRNA 5' end formation in Drosophila. Science, 2007. **315**(5818): 1587-90.

[46] Lee, E.J., et al., Identification of piRNAs in the central nervous system. RNA, 2011. **17**(6): 1090-9.

[47] Wu, H., et al., Genome-wide analysis reveals methyl-CpG-binding protein 2-dependent regulation of microRNAs in a mouse model of Rett syndrome. Proc Natl Acad Sci U S A, 2010. **107**(42): 18161-6.

[48] Bu, D., et al., NONCODE v3.0: integrative annotation of long noncoding RNAs. Nucleic Acids Res, 2012. **40** (Database issue): D210-5.

[49] David, M., et al., SHRiMP2: sensitive yet practical SHort Read Mapping. Bioinformatics, 2011. **27**(7): 1011-2.

[50] Belichenko, P.V., et al., Widespread changes in dendritic and axonal morphology in Mecp2-mutant mouse models of Rett syndrome: evidence for disruption of neuronal networks. J Comp Neurol, 2009. **514**(3): 240-58.

[51] Chapleau, C.A., et al., Dendritic spine pathologies in hippocampal pyramidal neurons from Rett syndrome brain and after expression of Rett-associated MECP2 mutations. Neurobiol Dis, 2009. **35**(2): 219-33.

[52] Wang, K., et al., Export of microRNAs and microRNA-protective protein by mammalian cells. Nucleic Acids Res, 2010. **38**(20): 7248-59.

[53] Turchinovich, A., et al., Characterization of extracellular circulating microRNA. Nucleic Acids Res, 2011. **39**(16): 7223-33.

[54] Saxena, A., et al., Trehalose-enhanced isolation of neuronal sub-types from adult mouse brain. Biotechniques, 2012. **52**(6): 381-5.