Elevated 5-hydroxymethylcytosine in the Engrailed-2 (EN-2) promoter is associated with increased gene expression and decreased MeCP2 binding in autism cerebellum

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INTRODUCTION

Engrailed-2 (EN-2) is a developmentally regulated homebox gene that is essential for Purkinje cell maturation and normal cerebellar development and patterning.1 We recently reported for the first time that alterations in DNA and histone lysine methylation within the EN-2 promoter region were associated with EN-2 overexpression in the post-mortem autism cerebellum.2 These findings were unusual in that an increase in EN-2 gene expression and protein level was associated with promoter DNA hypermethylation that could be partially explained by a significant decrease in repressive histone H3 lysine 27 trimethylation (H3K27) in the same region. Previous studies have considered EN-2 to be an autism-susceptibility gene based on family linkage studies indicating an increased transmission of EN-2 polymorphic variants from parents to affected children3 and additionally on similarities between autism cerebellar abnormalities and cerebellar abnormalities in EN-2 transgenic rodent models.4,5 In mice, EN-2 is highly expressed in Purkinje cells during fetal and early postnatal development acting primarily as a transcriptional repressor until it is downregulated during the perinatal period.6 EN-2 is also expressed in hindbrain nuclei involved in the development of serotonin (raphe nucleus) and norepinephrine (locus coeruleus) neurotransmitter systems that have been implicated in autism.7,8 It is important to note that the programmed timing of Purkinje cell maturation and cerebellar patterning is critically dependent on perinatal EN-2 downregulation,6,9 which is obviated by sustained EN-2 overexpression.9,10 Our finding of sustained EN-2 gene overexpression in the postnatal autism cerebellum suggests that a critical developmental window for normal downregulation may have been missed in some individuals with autism.

DNA methylation as 5-methylcytosine (5-mC) has long been considered a highly stable epigenetic mark that is established during embryogenesis and early fetal development and serves to define tissue-specific cell identity by dense DNA methylation and silencing of functionally irrelevant genes. This initial notion has been modified by reports of dynamic gene-specific and genomewide loss of 5-mC (DNA hypomethylation) and altered gene expression in response to endogenous or exogenous environmental signals during postnatal development, aging and carcinogenesis.11-13 However, the elucidation of the molecular mechanisms underlying DNA demethylation has proved to be a challenge. The recent discovery that 5-hydroxymethylcytosine (5-hmC) is an intermediate in the oxidative demethylation of 5-mC via ten-eleven-translocase (TET) family of dioxygenases has added...
a new dimension to the epigenetic repertoire and cell-specific regulation of gene expression.\textsuperscript{14,15}

Whereas 5-hmC is present in genomic DNA of all cells, it is most highly concentrated in mammalian neurons relative to other cell types with one of the highest levels in cerebellar Purkinje cells where 5-hmC was first isolated.\textsuperscript{16} Genome-wide 5-hmC profiling has revealed that 5-hmC distribution is highly variable in the brain and can be concentrated in 5′ enhancer regions, gene bodies or CpG island shores depending on cell type, gene activation and/or differentiation stage.\textsuperscript{17,19} For example, 5-hmC content is 0.6% in Purkinje cells but only 0.2% in granule cells underscoring the cell type specificity of 5-hmC distribution.\textsuperscript{16} Genomic mapping of 5-hmC during mouse neureogenesis found that it is enriched in the body of genes with the high CpG density, whereas genes with moderate or low CpG density accumulate 5-hmC in distal and proximal promoter regions.\textsuperscript{18} Nonetheless, reports of the stable presence of 5-hmC in the postnatal cerebellum and hippocampus\textsuperscript{17,20} suggest that this modified base may have additional roles beyond that of a transient intermediate during TET-mediated 5-mC demethylation. Thus, it is highly likely that 5-hmC, as the sixth base in DNA, functions as an independent epigenetic mechanism in chromatin remodeling and gene expression regulation, although much more research will be required before definitive conclusions are possible.

The sequence-specific accumulation of 5-hmC has been associated with gene activation during neuronal neurogenesis, differentiation and aging.\textsuperscript{7,15,20} Interestingly, several reports have shown that a stable increase in 5-hmC can occur without substantial reduction of 5-mC content (demethylation).\textsuperscript{21-23} The possibility of 5-mC to 5-hmC conversion under conditions of oxidative stress has received some theoretical support.\textsuperscript{24-26} For example, hydroxyl radical generation with Fenton reaction chemistry resulted in the conversion of 5-mC to 5-hmC in liver microsomes;\textsuperscript{24} however, similar demonstration in brain DNA has not been reported. Further, 5-mC and other oxidatively modified DNA bases have been shown to decrease MeCP2-binding affinity. An in vitro study using the electrorophoretic mobility assay with an oligonucleotide duplex containing an MeCP2 recognition sequence demonstrated that the replacement of 5-mC with 5-hmC or 8-oxo-dG on one or both strands of the duplex significantly reduced MeCP2 binding, suggesting that oxidatively damaged bases could alter the epigenomic landscape and gene expression.\textsuperscript{25} However, conflicting evidence was recently reported using a similar in vitro oligonucleotide electrophoretic mobility assay and a synthetic N-terminal fragment of human MeCP2 and found that MeCP2 binds with equal affinity to both 5 mC and 5 hmc.\textsuperscript{27} It is likely that the in vivo binding affinity of MeCP2 to 5-hmC may vary depending on its interaction with the chromatin environment and/or the specific region and DNA sequence; however, this possibility will require much more research using neuronal cell populations rather than in vitro oligonucleotide binding.

It is important to note that previous methods for quantifying CpG methylation in DNA, including bisulfite sequencing and methylation-sensitive restriction methodologies, are not capable of distinguishing 5-hmC from 5-mC.\textsuperscript{28} Therefore, published studies of DNA methylation density may need to be repeated with the newer methodology capable of independently measuring 5-hmC and 5-mC content in DNA. In the present report, we refine and extend our previous investigation of EN-2 gene overexpression in the autism cerebellum by quantifying both 5-mC and 5-hmC within EN-2 gene promoter and gene body. One includes 5-hmC methylation was also measured by the McrBC assay as described previously.\textsuperscript{29,30}
Chromatin immunoprecipitation assay

The level of MeCP2 at the EN-2 promoter region was determined by chromatin immunoprecipitation assay using primary anti-MeCP2 antibody (Active Motif, Carlsbad, CA, USA). Purified immunoprecipitated DNA and input DNA were subsequently co-amplified with qPCR using the same primers utilized for hMeDIP assay described above. All assays were run in triplicate and data expressed as the mean (± s.e.) percent input DNA after adjusting for total input DNA: 100×2^(ΔCt – ΔΔCt).

Statistical analyses
All statistical analyses were performed using Graphpad Prism software (La Jolla, CA, USA). Normal distribution of the data was determined using the Kolmogorov–Smirnov test. Results are expressed as means ± s.d. with statistical significance set at 0.05. The mean differences between case and control groups were evaluated using the Student’s t-test for data that were normally distributed and the nonparametric Mann–Whitney U-test was used for data that were not normally distributed. Relationships between variables were determined using linear regression analysis within the Graphpad Prism software.

RESULTS

Cerebellar tissue sample matching and selection

Careful case–control matching resulted in no mean differences in age, gender, race or post-mortem interval between the autism and control groups (P > 0.05). The mean age was 15.5 ± 9.5 years in the autism group (eight children under 18 years and five adults) and 15.8 ± 8.6 years (nine children under 18 years and four adults) in the control group. In both groups, 69% (9/13) of individuals were male. The mean post-mortem interval was 18.4 ± 5.7 h for the autism samples and 15.7 ± 6.2 h in the control samples. Age, gender, race and post-mortem interval were the primary variables matched between groups. An attempt was made to match the cause of death as closely as possible but, given limited sample availability, we made the best matches possible. For example, asphyxia, smoke inhalation or aspiration in cases were matched to asthma and drowning in controls. Head trauma was matched with brain bruising and gastrointestinal (GI) bleeding matched with multiple injuries.

Figure 1. Global alterations in cerebellar levels of 5-mC (a), 5-hmC (b), DNA methyltransferases DNMT1, DNMT3A and DNMT3B (c), TET1, TET2, TET3 (d) and 8-oxo-dG (e) in 13 autism (ASD) and 13 matched control cerebellar samples. Results are expressed as the mean and s.d.’s with associated P-values.

Chromatin immunoprecipitation assay

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Global 5-hmC, 5-mC, expression of DNA methyltransferases, TET dioxygenases and 8-oxo-dG in autism and the control cerebellum. It is well accepted that standard bisulfite and enzymatic assays previously used to quantify levels of 5-mC in DNA cannot distinguish 5-mC from 5-hmC. As 5-hmC is now emerging as a significant and independent epigenetic mark, it becomes imperative to redefine previous reports of global and gene-specific alterations in DNA methylation and associated alterations in gene expression in terms of the independent contributions of both 5-mC and 5-hmC. We previously reported global and EN-2 gene-specific elevation in 5-mC that was associated with increased gene expression using standard methodology that does not distinguish the contribution of 5-hmC. To refine our determination of global DNA cytosine methylation, we separated and quantified global 5-mC and 5-hmC bases in the same cerebellar DNA using LC/MS/MS and expressed the results as percent 5-mC and 5-hmC/total cytosine content. As shown in Figure 1a and b, we observed a significant increase in both 5-mC and 5-hmC in the autism cerebellum relative to the control samples. The level of 5-mC was 5.9 ± 1.2% in 13 autism cerebellum compared with 4.7 ± 1.1% in 13 control samples (P = 0.005) and the level of 5-hmC was 0.57 ± 0.14% in the autism samples and 0.37 ± 0.10% in control samples (P = 0.004). We then measured the expression of DNMT1, DNMT3A and DNMT3B as possible reasons for unexpected parallel increase in both 5-mC and 5-hmC. As shown in Figure 1c, DNMT3A and DNMT3B expression levels were significantly increased in the autism cerebellum relative to control samples, suggesting a compensatory increase in de novo DNA methylation in the autism cerebellum. In contrast, there was no significant difference in DNMT1 expression. We next evaluated the expression of the TET family of dioxygenases (TET1-3) to determine whether the elevation in 5-hmC content could be related to an increase in TET-mediated conversion of 5-mC to 5-hmC. As shown in Figure 1d, the results indicated a significant increase in both TET1 and TET3 expression with no significant difference in TET2 in case compared with control cerebellum. Thus, it is possible that a sustained increase in TET1 and TET3 expression contributed to the increase in 5-hmC in the EN-2 promoter. However, if this were the case, one might expect a concomitant decrease in 5-mC via the TET-mediated 5-mC demethylation pathway instead of the increase in 5-mC observed. Finally, given previous reports of possible conversion of 5-mC to 5-hmC under conditions of oxidative stress, we assessed the relative pro-oxidant environment in autism and control cerebellum by quantifying 8-oxo-dG in DNA, a well-established marker of oxidative DNA damage and oxidative stress. Figure 1e confirms a significant increase in 8-oxo-dG content in the autism cerebellum relative to control (P = 0.001). Figure 2 shows a strong positive correlation (P = 0.002) between 5-mC and 5-hmC in the autism cerebellum (Figure 2b) that was not significant in control samples (Figure 2a). A marginally significant correlation (P = 0.08) was observed between 5-hmC and 8-oxo-dG in autism (Figure 2d) but not control cerebellar samples (Figure 2c).

5-hmC level within the upstream EN-2 CpG island promoter and gene body

In our initial epigenetic evaluation of the developmentally regulated EN-2 gene, we reported promoter hypermethylation that was unexpectedly associated with an increase in gene expression and level of EN-2 protein. Genomic mapping reports of 5-hmC distribution during rodent and human neurodevelopment have linked elevated 5-hmC content in the promoter and gene body with gene activation and overexpression. Therefore, it was of considerable interest to quantify 5-hmC content and determine its association with 5-mC content within the overexpressed EN-2 gene. Figures 3a and b show the level of 5-hmC in the upstream EN-2 promoter region using two independent assays (Epimark and 5-hMeDIP, respectively). Both assays confirmed a significant increase in 5-hmC content within the upstream EN-2 promoter region. In contrast, both assays found no significant difference in 5-hmC content within the proximal promoter in control samples (data not shown). Within the gene body (+1861 to +1996), the level of 5-hmC was significantly elevated (0.55 ± 0.26 in autism and 0.31 ± 0.33 in control samples).
$P = 0.03$), albeit at a fraction (3%) of 5-hmC content in the 5′ distal promoter CpG island. Within the 5′ promoter CpG island, there was a highly significant positive correlation between 5-mC and 5-hmC as shown in Figures 3c ($r = 0.77$, $P = 0.002$) similar to the positive relationship observed globally in the cerebellum (Figure 2b). The 5-hmC/5-mC ratio was 0.87 in autism and 0.42 in control samples ($P = 0.03$).

On the basis of recent reports of a positive association between actively transcribed neurodevelopmental genes and the abundance of 5-hmC in defined 5′ promoter regions and in gene bodies, $^{17,19,32}$ we interrogated the relationship between the level of 5-hmC in the EN-2 upstream CpG island promoter region and in the gene body with EN-2 gene expression. As shown in Figure 4a,
EN-2 gene expression was significantly increased in the autism cerebellum compared with control as previously reported. Further, a highly significant positive correlation \((r = 0.67, P = 0.001)\) was found between 5-hmC content and EN-2 gene expression in the 5' promoter CpG island in autism but not in control samples (Figures 4c and b, respectively). In contrast to previous reports, the level of 5-hmC in the EN-2 gene body was not correlated with EN-2 gene expression (data not shown).

MeCP2 binding to the EN-2 promoter and correlation between EN-2 gene expression and MeCP2 binding

When MeCP2 binding is reduced, the recruitment of chromatin-modifying enzymes that promote chromatin condensation and gene repression are concomitantly reduced. A reduction in MeCP2 binding, whether because of MeCP2 mutation as in Rett syndrome or reduced 5-hmC affinity, has been associated with aberrant increase in gene expression\(^{33,34}\) and could offer a partial explanation for the unexpected increase in EN-2 expression we previously observed despite the increase in 5-mC content (DNA hypermethylation). To address this possibility, we quantified the extent of MeCP2 binding to the EN-2 promoter region using a chromatin immunoprecipitation-based approach with the same primer sets employed for hMeDIP. Figure 5a reveals a highly significant \((P = 0.02)\) decrease in MeCP2 binding to the same 5' promoter region that contained elevated levels of 5-hmC (Figure 3b) in autism relative to control samples. Given the significant decrease in repressive MeCP2 binding in the EN-2 promoter, it was important to determine whether reduced binding would be correlated with increased EN-2 gene expression. Figure 5b shows the correlation between MeCP2 binding and EN-2 expression in the EN-2 promoter region in the autism and control cerebellum. The significant inverse relationship \((P = 0.05)\) suggests that reduced MeCP2-mediated gene repression may have contributed to persistent EN-2 gene overexpression in the autism samples. Recently, a novel association between MeCP2 binding and histone H3K27 trimethylation was reported, suggesting that histone marks may influence nucleosomal MeCP2 binding and that the two chromatin-modulating proteins may work in concert with DNA methylation to repress gene expression by maintaining chromatin in a closed inaccessible conformation.\(^{35-37}\) In our previous report, we showed that histone H3K27me3 binding was significantly reduced in the EN-2 promoter in autism but not control samples. Thus, an interactive decrease in both repressive marks may contribute to aberrant overexpression of EN-2 in the autism cerebellum.

**DISCUSSION**

Using an autism candidate gene approach, we define for the first time global and gene-specific 5-hmC content in the EN-2 gene and explore the relationship between the 5-hmC level and gene expression in a chromatin context and the potential role of oxidative stress in the generation of 5-hmC. Our results confirm an epigenetic role for 5-hmC that is distinct from 5-mC and suggest that the increase in 5-hmC content and decrease in both MeCP2 and histone H3K27me3 binding may alter local chromatin conformation to facilitate enhancer binding and the persistent upregulation of EN-2 gene expression in the postnatal autism cerebellum.

The significant positive correlation between 5-hmC and 5-mC both globally and within the EN-2 promoter was unexpected. If the increase in 5-hmC derived solely from TET enzyme-mediated oxidative demethylation of 5-mC, one might expect an inverse correlation between these two modified cytosines as previously observed.\(^{37,27,38}\) However, an increase in both epigenetic modifications simultaneously suggests that different cytosines were targeted by TET1/3 and DNMT3A/B. The increase, rather than decrease, in 5-mC plus the stable accumulation 5-hmC strongly suggest an independent epigenetic role for 5-hmC beyond 5-mC demethylation in the postnatal autism cerebellum. Indeed, the expression of EN-2 was highly correlated with 5-hmC levels in the upstream promoter CpG island (Figure 3c) suggesting that 5-hmC accumulation is mechanistically related to gene upregulation. A similar positive association between global levels of 5-mC and 5-hmC has been recently reported in the temporal cortex of Alzheimer's patients.\(^{21}\) Other studies using genome-wide mapping of 5-hmC distribution have also reported an increase in 5-hmC density associated with increased gene expression but, in contrast, find a stronger association with 5-mC in gene bodies.
than in promoters, whereas others have reported 5-hmC accumulation in promoters and upstream enhancer regions. These divergent results suggest that 5-hmC density and function appears to vary with cell type, differentiation stage and gene-specific function.

The significant increase in DNMT3A and DNMT3B expression together with an increase in TET1 and TET3 expression may offer clues to the unexpected positive relationship between 5-mC and 5-hmC. Although the increase in TET1/3 expression may contribute to the increase in 5-hmC in the EN-2 promoter, it was not associated with a decrease in total 5 mC content. We hypothesize that the upregulation of de novo methyltransferases DNMT3A and DNMT3B could be an abortive attempt to downregulate the persistent elevation in EN-2 expression during early cerebellar development. Although de novo DNMT3A normally functions to establish and maintain DNA methylation patterns, Hashimoto et al. have shown that DNMT3A but not DNMT1 can methylate hemihydroxylated 5-hmC and may provide a mechanism for the maintenance of 5-hmC levels during replication and DNA repair. Thus, the increase in de novo methyltransferases in the autism cerebellum may have a dual role in maintaining both 5-mC and 5-mC methylation within the EN-2 gene. Clearly, the simultaneous increase in both 5-mC and 5-hmC within the same EN-2 upstream promoter sequence indicates that different CpG sites were targeted for 5-mC and 5-hmC by DNMT3A/B and TET1/3, respectively, and may explain the parallel increase in both cytosine modifications.

During prenatal cerebellar development, EN-2 homeobox transcription factor is normally upregulated acting primarily as a repressor of gene expression and is then downregulated in the late prenatal and early postnatal period. The programed upregulation and downregulation of EN-2 expression during cerebellar development is reminiscent of other neurodevelopmental windows that open and close in a precisely programmed temporal sequence that is essential for normal brain development. Indeed, autism has been proposed to be a ‘critical period’ disorder. Early life is thought to be a period with increased sensitivity to epigenetic regulation of the dynamic alterations in gene expression during brain development when dysregulation can contribute to abnormal behavior phenotypes.

Inappropriate delay or failure to close a critical window can disrupt the hierarchal progression of downstream critical periods and negatively affect structural organization and neuronal synchronisation between regions. The persistent postnatal elevation in EN-2 expression suggests that this developmental window failed to close appropriately during early cerebellar development and in this way could contribute to the structural and neuronal abnormalities reported in the autism cerebellum.

The non-enzymatic oxidation of 5-mC to 5-hmC cannot be ruled out as a mechanism for 5-hmC accumulation in the autism cerebellum. In our previous publication, we reported a significant decrease in glutathione antioxidant/detoxification capacity (GSH/GSSG) and an increase in DNA and protein oxidative damage (8-oxo-dG and nitrotyrosine, respectively) using the same cerebellar sample. In the present study, other investigators have reported similar markers of oxidative stress in the autism cerebellum. Whereas precedent exists for a non-enzymatic oxidative conversion of 5-mC to 5-hmC in the production of 5-hmC in liver microsomes with exposure to hydroxyl radicals, this intriguing observation requires confirmation in cellular DNA. The DNA adduct 8-oxo-dG is an established marker of oxidative DNA damage generated by excessive hydroxyl ion exposure that exceeds repair capacity and confirms a chronic pro-oxidant microenvironment in the autism cerebellum. The positive correlation between 8-oxo-dG and 5-hmC provides indirect support for possible free radical oxidation of 5-mC to 5-hmC; however, this provocative alternative mechanism for 5-hmC generation will require further focused investigation. Finally, it should be noted that reduced binding of MeCP2 to the oxidized bases 5-hmC and 8-oxo-dG in vitro has been reported.

A decrease in MeCP2-binding affinity to 5-hmC compared with 5-mC has been reported by several investigators using genome-wide profiling or in vitro binding affinity assays. Our in vivo finding of a significant decrease in MeCP2 binding to the same promoter sequence that contained increased 5-hmC content in the autism cerebellum is consistent with these reports but inconsistent with a recent in vitro finding that MeCP2 has equal binding affinity for both 5-hmC and 5-mC. Using a similar candidate gene approach to explore MeCP2 binding and 5-hmC content in the autism cerebellum, Zhubi et al. examined GAD1 and RELN genes previously shown to be downregulated in this region. These investigators found an increase in 5-hmC and TET1 expression associated with a decrease in 5-mC and an unexpected increase in MeCP2 binding in the promoters of these downregulated genes. Although an increase in MeCP2 binding is consistent with repression of gene expression, it is not consistent with reports of gene activation and increased gene expression with elevated 5-hmC. The positive association between 5-hmC and gene expression in the present study are more consistent with gene upregulation as observed with the MeCP2 mutation in Rett syndrome. These contrasting results in the autism cerebellum emphasize the plurality of gene expression alterations associated with MeCP2 binding that appears to vary with developmental context, local microenvironment and gene activation and transcriptional status in vivo. Further, the results underscore the importance of re-examining previous reports of DNA hypermethylation associated with altered gene expression in the autism brain that use methodology that is incapable of distinguishing 5-hmC from 5-mC.

Highly relevant to the observed positive association between 5-hmC level and EN-2 expression shown in Figure 4b, Szulwach et al. demonstrated the presence of a developmentally regulated 5-hmC-enriched area (differential 5-hmC region, DHRM) in the mouse cerebellum directly upstream of the En-2 gene that overlapped with a Pax 2/5/8-regulated enhancer element. Supporting this observation, Song et al. confirmed the presence of several PAX-binding sites in the mouse upstream En-2 enhancer region that were essential for En-2 activation and, in addition, demonstrated strong evolutionary conservation in this region. Enhancers are transcriptional regulatory regions that have been shown to be modulated by epigenetic modifications during development. Recently, whole-genome analysis with base resolution of 5-hmC and 5-mC in the human brain reported increased 5-hmC at poised enhancer regions. Further, 5-hmC accumulation has been shown to be an early event in enhancer activation during neuronal differentiation in mouse P19 cells. On the basis of these reports, we searched the EN-2 upstream CpG island sequence for PAX5-binding sites using the JASPAR transcription factor database (jaspar.genereg.net/cgi-bin/jaspar_db.pl?m=Browse&db=core&tax_group=vertebrates). Our search yielded six PAX5-binding sites within the EN-2 upstream CpG island in the human cerebellum. In addition, we found a CCAAT enhancer-binding protein (C/EBP)-binding site at -2793 bases of our primer sequence. The presence of several enhancer-binding sites together with the significant increase in 5-hmC in this region suggests that the positive association of 5-hmC with gene expression may be because of epigenetic facilitation of enhancer activation. Taken together, our data also support the possibility that the increase in 5-hmC accompanied by reduced MeCP2 and histone H3K27me3 binding collaborate to promote a more open chromatin configuration that facilitates enhancer protein binding and increased EN-2 expression.

Our study was limited by methodology that does not provide base resolution, which would identify the exact location of the modified cytosines within the promoter and gene body regions. The Epimapk assay targets cytosines within the CCGG sequences.
only, whereas a monoclonal antibody to 5-hmC with hMeDIP quantifies the total 5-hmC content in defined regions. Nonetheless, both assays independently confirmed the increase in 5-hmC content and reduced 5-hm5-mC ratio in the EN-2 promoter. Another limitation was the report of TET gene expression rather than TET activity. An increase in 5-hmC could reflect and increase in TET2 activity independent of gene expression.

In sum, our results indicate that elevated 5-hmC in the EN-2 promoter is associated with a significant decrease in repressive MeCP2 and histone H3K27me3 that appear to over-ride 5-mC hypermethylation. These epigenetic alterations would repress EN-2 promoter is associated with a significant decrease in TET2 activity independent of gene expression.

In conclusion, our results indicate that elevated 5-hmC in the autism cerebellum regulates genes related to vesicle formation and transport in cerebellar Purkinje cells. Mol Cell Neurosci 2008; 38: 495–504.

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