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ARTICLE

GluD1 is a common altered player in neuronal differentiation from both *MECP2*-mutated and *CDKL5*-mutated iPS cells

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Rett syndrome is a monogenic disease due to *de novo* mutations in either *MECP2* or *CDKL5* genes. In spite of their involvement in the same disease, a functional interaction between the two genes has not been proven. MeCP2 is a transcriptional regulator; *CDKL5* encodes for a kinase protein that might be involved in the regulation of gene expression. Therefore, we hypothesized that mutations affecting the two genes may lead to similar phenotypes by dysregulating the expression of common genes. To test this hypothesis we used induced pluripotent stem (iPS) cells derived from fibroblasts of one Rett patient with a *MECP2* mutation (p.Arg306Cys) and two patients with mutations in *CDKL5* (p.Gln347Ter and p.Thr288Ile). Expression profiling was performed in *CDKL5*-mutated cells and genes of interest were confirmed by real-time RT-PCR in both *CDKL5*- and *MECP2*-mutated cells. The only major change in gene expression common to *MECP2*- and *CDKL5*-mutated cells was for *GRID1*, encoding for glutamate D1 receptor (GluD1), a member of the δ -family of ionotropic glutamate receptors. GluD1 does not form AMPA or NMDA glutamate receptors. It acts like an adhesion molecule by linking the postsynaptic and presynaptic compartments, preferentially inducing the inhibitory presynaptic differentiation of cortical neurons. Our results demonstrate that *GRID1* expression is downregulated in both *MECP2*- and *CDKL5*-mutated iPS cells and upregulated in neuronal precursors and mature neurons. These data provide novel insights into disease pathophysiology and identify possible new targets for therapeutic treatment of Rett syndrome.

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INTRODUCTION

Rett syndrome (RTT, OMIM #312750) is a monogenic disorder that affects normal brain development during early childhood with an incidence of 1 in 10 000–15 000 live female births.^{1,2} Patients with the classic form of RTT show apparently normal development until 6–18 months after birth when clinical signs of a mental regression start to become apparent. These include loss of speech and purposeful hand use, stereotypic hand movements, appearance of postnatal microcephaly, autistic features, ataxia, and hand apraxia.^{2,3} Other clinical hallmarks include spasticity, respiratory abnormalities, and autonomic dysfunctions.⁴ Depending on disease severity, some variants such as Z-RTT (previously known as Preserved Speech Variant (PSV)), ‘*forme fruste*’, congenital form, and early-onset seizures variant have been described in addition to classic RTT.^{2,5}

Mutations in *MECP2* gene, located in Xq28, are responsible for ~90% of classical RTT cases and a lower percentage of variant patients.^{6,7} *MECP2* encodes for a transcriptional regulator and epigenetic modifier. MeCP2 protein is widely expressed and its relatively higher expression in brain is spatially and developmentally regulated: its levels are low during embryogenesis and increase during postnatal neuronal maturation.^{8,9} A very small number of MeCP2

target genes have been identified so far and the several transcriptional profiling studies performed on various tissues (brain from mouse models, fibroblasts and lymphocytes derived from mutated patients, and autaptic brain tissue) resulted in sets of de-regulated genes that differed for each study.^{10–12} Notably, analysis of postmortem brain tissue from patients carrying *MECP2* mutations showed reduction in the mRNA and protein levels of many synaptic markers and a decreased number of dendritic spines in distinct cortical areas.^{13,14} A significant alteration of NMDA, AMPA, and GABA receptors was demonstrated in the cortex and basal ganglia of patients, indicating that RTT is associated with abnormalities in the expression of molecules crucial for both excitatory and inhibitory synaptic transmission.^{15,16}

Mutations in another X-linked gene, *CDKL5* (*cyclin dependent kinase like 5*), are responsible for the early-onset seizures variant of RTT.^{17,18} Cdkl5 is a ubiquitous protein kinase particularly expressed in the brain.^{19,20} It shuttles between the cytoplasm and the nucleus;²¹ in neurons, the shuttling is regulated by the activation of extrasynaptic NMDA receptors that induce protein relocalization from nucleus to cytoplasm, and also regulate its degradation by the proteasome.²² Moreover, neuronal Cdkl5 is enriched on the

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postsynaptic site of excitatory synapses where it seems to be involved in the establishment of correct spine morphology and function.²³ A direct interaction of Cdkl5 with Dnmt1 has been reported, suggesting that it might also be involved in the regulation of DNA methylation and thus gene expression.²⁴ Transcriptome analysis has been recently performed on fibroblasts from *CDKL5*-mutant patients revealing alterations in a few genes involved in differentiation and oxidative stress.²⁵

The overlapping phenotype of *MECP2*- and *CDKL5*-mutated patients, the similar expression pattern during postnatal mouse brain development, and the interaction of both proteins with Dnmt1 suggest that Cdkl5 and MeCP2 might participate in a common neuronal pathway.²⁴ Studies investigating the possibility of a direct functional interaction between the two proteins gave conflicting results. Mari *et al*²⁶ suggested that: (1) *CDKL5* binds and phosphorylates MeCP2 *in vitro* and *in vivo*; (2) *CDKL5* is able to autophosphorylate itself and to phosphorylate MeCP2 *in vitro*; and (3) this capability is lost in mutant MeCP2 proteins.²⁷ Opposite results were obtained by Lin *et al*²⁰ who showed that MeCP2 binds *CDKL5* but is not a direct target of its phosphorylation. More recently, a role for MeCP2 in the regulation of *CDKL5* expression levels in brain has been demonstrated.²⁸

A revolutionary approach for the creation of patient-specific human cellular models for neurologic and neurodevelopmental disorders has been developed in 2007 when Yamanaka and colleagues²⁹ demonstrated that adult somatic cells can be reverted to induced pluripotent stem (iPS) cells, an immature status analogous to embryonic stem cells. The iPS cells are similar to human embryonic stem cells (hESCs) for gene expression, morphology, proliferation, and pluripotency.^{29,30} Thanks to the capacity of these cells to differentiate into neurons, many neurodegenerative (amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), Huntington's disease (HD), familial dysautonomia (FD), Parkinson's disease (PD), Alzheimer's disease (AD)) and neurodevelopmental diseases (Fragile X syndrome (FXS), schizophrenia (SCZD), Prader-Willi/Angelman's syndrome (PW/AS)) have been modeled with patient-specific iPS cells.^{31–33} This technology has also been employed for the study of RTT. In particular, different groups generated *MECP2*-mutated iPS lines and characterized the resulting neurons reporting morphological and electrophysiological defects corresponding to those found in both mouse brain and patient autoptic material.^{34–36} In 2010, we generated iPS lines from two patients with *CDKL5* mutations, one female and one male.³⁷ For the female patient, isogenic clones expressing either the wild-type or the mutant *CDKL5* allele were obtained. In order to test whether *CDKL5* could be involved in the regulation of gene expression and to define the molecular mechanisms involved in *CDKL5*-associated disease, we now searched for alterations in global gene expression profiles in *CDKL5*-mutant iPS cells. This analysis identified six dysregulated genes of which *GRID1* was dysregulated in both *MECP2*-mutated and *CDKL5*-mutated iPS cells.

MATERIALS AND METHODS

See Supplementary Online Data for full methods.

iPS clones

Previously characterized fully reprogrammed iPS clones derived from one male and one female *CDKL5*-mutated patient were used.³⁷ The female patient harbors a truncating mutation c.1039C>T (p.(Gln347Ter)), whereas the male patient has a missense change c.863C>T (p.(Thr288Ile)). For the female patient, three iPS clones differing for *CDKL5* allelic expression were used: clone 20 that exclusively expresses the wild-type *CDKL5* allele and clone 46 and clone

33 with exclusive expression of the mutated *CDKL5* allele. For the male patient, one clone was used (clone 58) and a clone derived from normal male fibroblasts (BJ) was used as control. One *MECP2* mutant clone c.916C>T (p.(Arg306Cys)) and BJ control clone were obtained from James Ellis laboratory (Toronto, ON, Canada).³⁸ Mutation numbering is based on the following reference sequences: NM_004992.3 for *MECP2* and NM_003159.2 for *CDKL5*.

Clones maintenance and neuronal differentiation

The iPS cells were maintained and differentiated as previously described (see Supplementary Data).³⁷ At day 18, neural precursor cells (NPCs) were isolated using Anti-PSA-NCAM MicroBeads (30-092-966, Miltenyi Biotec, Bologna, Italy) according to the manufacturer's protocol. Sorted cells were fluorescently stained with an anti-IgM antibody conjugated with PE dye (130-095-908; Miltenyi Biotec) and analysed by flow cytometry using a GUAVA EasyCyte 6-2L instrument (Merk-Millipore group, Billerica, MA, USA) to assess purity of the recovered population. Unlabelled cells were used as negative control. Cultures were harvested at day 70 for analysis of mature neurons. To obtain a more pure population of neurons for qRT-PCR, mature cultures were sorted using a CD24 MicroBead Kit (130-095-951, Miltenyi Biotec).³⁹ Sorted neurons were plated on poly-ornithine and laminin-coated slides and allowed to attach for 48 h before being stained for β -III-Tubulin (Chemicon; Merk-Millipore group) to confirm their identity.

RNA isolation

Total RNA was extracted from iPS clones with RNeasy mini kit (Qiagen, Milano, Italy). For clones derived from the female patient, the exclusive expression of only one *CDKL5* allele was confirmed as previously reported.³⁷

Microarray analysis

Agilent Two-Color Microarray-Based gene expression analysis (Quick Amp Labeling, Agilent Technologies, Torino, Italy) protocol was used for global gene expression analysis. For each clone, four technical replicates were performed to control technical bias. Microarray raw data were analysed by Agilent Feature Extraction Software v9.5 for quality assessment. Gene Spring GX software v11.5 (Agilent Technologies) was used for data processing and analysis. Data for each mutant/control pair were analysed independently. Significantly modulated genes were defined as those with absolute fold change (FC) of > 1.5. The lists of deregulated genes obtained for the two mutant/control pairs were compared in order to identify common entries. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2223.

Real-time qRT-PCR

Quantitation was performed using commercial TaqMan probes from Applied Biosystem (Life Technologies, Monza, Italy) (*GRID1* assay id: Hs00324946_m1; *LST1* assay id: Hs00938298_g1; *RESPI8* assay id: Hs01563187_m1; *ODF4* assay id: Hs00537806_m1). The *Cyclophilin* (*CYCLO*) gene was used as a reference (assay id: Hs01565700_g1). Student's *t*-test with a significance level of 95% was used for the identification of statistically significant differences in expression levels among different clones.

Immunoblotting

Proteins from neuronal cell pellets were extracted with a 10-fold excess of RIPA buffer, cleared with an ultracentrifugation step, and analysed by western blot.

Immunoprecipitation

Proteins were immunoprecipitated from mouse brain lysate by incubation with Protein A-covered beads and the specific antibody, before being washed three times and being analysed in western blot.⁴⁰

Chromatin immunoprecipitation (ChIP)

Chromatin was prepared from human SH-SY5Y neuroblastoma cells as described previously.⁴¹ ChIP-isolated chromatin was subjected to quantitative PCR with primers targeting the *GRID1* promoter region (*GRID1*_For: 5'-ATG

AGCCAGCAAGGTGACTT-3'; *GRID1*_Rev: 5'-GGGTGGGTCAGGTTTCACTA-3'). PCR was performed in triplicate. Statistical evaluation was performed using a two-tailed *t*-test, assuming unequal variance.

RESULTS

Microarray analysis of iPS cells

Expression profiling was performed on iPS clones from two patients (one female and one male) with *CDKL5* mutations.³⁷ For the female patient (*CDKL5* mutation c.1039C>T, p.(Gln347Ter)) we compared one clone expressing the mutant *CDKL5* allele (46) to another clone from the same patient that expresses the wild-type allele (20). Maintenance of X-chromosome inactivation and the resulting monoallelic expression of *CDKL5* were confirmed by androgen receptor assay and direct sequencing of *CDKL5* mRNA. The clone derived from the male patient (58; *CDKL5* mutation c.863C>T, p.(Thr288Ile)) was compared with a clone derived from a normal newborn male (BJ).³⁸ For each mutant/control pair, four technical replicates were performed for a total of eight chip hybridizations. Differences in gene expression between wild-type and mutant clones for each patient were calculated using the FC cutoff of 1.5 and $P < 0.05$. From the first comparison (46 *versus* 20) we obtained 137 dysregulated genes, whereas for the second comparison (58 *versus* BJ) 190 genes with altered expression were identified (Supplementary Tables 1 and 2). None of these genes passed the Benjamini and Hochberg correction for FDR, probably because of the very low number of genes with altered expression. We decided, however, to compare the two gene lists to check whether common genes were present and we identified six common genes; four genes were consistently dysregulated in the same direction in both cases, one with decreased levels and the others with increased expression (Table 1). To confirm the dysregulation, real-time RT-PCR analysis was performed on an independent preparation of RNA from the same clones. RNA derived from a second clone from the female patient expressing mutant *CDKL5* allele (clone 33) and from an *MECP2*-mutated iPS clone (15; MeCP2 mutation c.916C>T, p.(Arg306Cys)) were also included. A downregulation (~50%) of *GRID1* gene was confirmed in the *CDKL5*-mutated clones; intriguingly, a similar reduction was also observed in the clone with *MECP2* mutation (Figure 1). *GRID1* expression in clone 20 (expressing wild-type *CDKL5* allele) was comparable to that observed in BJ control (Figure 1), strongly suggesting that the reduction in expression is a specific consequence of *CDKL5* or *MECP2* mutation.

GRID1 expression in NPCs and neurons

GRID1 encodes GluD1, a member of the orphan δ -glutamate ionotropic receptor family. We thus decided to validate our results on NPCs and mature neurons. To reduce the potentially confounding effect of genetic background, for *CDKL5* mutations only the clones derived from the female patient were used. The iPSCs were

differentiated toward a neuronal fate and NPCs were isolated from differentiating cultures using a MicroBeads-conjugated antibody that recognizes PSA-NCAM⁴² (Figure 2a). Flow cytometry analysis on purified NPCs confirmed the efficiency of enrichment (Figure 2b). *GRID1* expression in NPCs appears more variable with significantly different expression between BJ control and clone 20 (Figure 2c). Surprisingly, a significant overexpression was observed in NPCs from both clone15 (*MECP2* mutation) and clones 46 and 33 (*CDKL5* mutation c.1039C>T, p.(Gln347Ter)). Neurons were isolated from mature cultures using a MicroBeads-conjugated anti-CD24 antibody³⁹ (Figure 2a). Immunofluorescence staining with β -III-Tubulin confirmed the neuronal identity of the sorted cells (Supplementary Figure 1). Analysis of mature neurons confirmed *GRID1* overexpression, although the difference did not reach statistical significance for the *MECP2*-mutated clone (Figure 2d). To verify whether the increased mRNA levels were accompanied by alterations in protein levels, GluD1 protein was quantitated in extracts from mature neurons differentiated with the same protocol. Because of the difficulty of isolating sufficient amounts of protein from sorted neurons, the analysis was performed on nonsorted cultures. The analysis confirmed a significant increase in protein levels in extracts obtained from both *CDKL5*-mutated and *MECP2*-mutated clones (Figure 3).

Considering the role of MeCP2 as a transcriptional regulator, we wondered whether the alteration in *GRID1* expression in the *MECP2*-mutated clone might indicate that MeCP2 protein directly binds to the *GRID1* promoter to regulate its expression. To answer this question, ChIP experiments with an anti-MeCP2 antibody were performed in SHSY-5Y neurons. The analysis indeed demonstrated a modest but statistically significant binding of MeCP2 protein to the *GRID1* promoter (Figure 4).

DISCUSSION

RTT is a monogenic disease due to *de novo* mutations in either *MECP2* (classic and Zappella variant) or *CDKL5* (early-onset seizures variant) genes on the X chromosome. The overlapping phenotype observed in patients with mutations in the two genes suggests that they might act, at least in part, on common pathways. MeCP2 is a transcriptional regulator involved in both activation and repression of gene expression.⁴³ *CDKL5* encodes for a kinase protein that exerts multiple functions inside the cell.^{23,24,44,45} It directly interacts with Dnmt1 and mediates its phosphorylation and it is involved in the structural organization and dynamics of nuclear speckles, suggesting that, like MeCP2, it might be implicated in the regulation of gene expression.^{24,45} We thus hypothesized that mutations in the two genes may lead to similar phenotypes by dysregulating the expression of common genes. To test this hypothesis, we used iPS cells derived from patients mutated in *MECP2* and *CDKL5*. Expression profiling in *CDKL5*-mutated iPS cells from two patients with different mutations

Table 1 Common deregulated genes

Gene symbol	Gene name	Accession number	Fold change 58/BJ	Fold change 46/20
<i>GRID1</i>	Glutamate receptor, ionotropic, δ -1	NM_017551	-2.1036077	-1.6889609
<i>LST1</i>	Leukocyte-specific transcript 1	NM_007161	3.537233	1.5218107
<i>SLC23A3</i>	Solute carrier family 23 (nucleobase transporters), member 3	AK055730	2.0275977	3.1108685
<i>RESP18</i>	Regulated endocrine-specific protein 18	NM_001007089	2.1038992	2.5666316
<i>HLA-DQA2^a</i>	Major histocompatibility complex, class II, DQ α 2	NM_020056	-1.5860842	1.5345135
<i>GUSBP3^a</i>	Glucuronidase, β pseudogene 3	NR_027386	-2.231126	1.6598059

^aThese genes have been excluded from RT-PCR validation because of the opposite values of fold change observed in the two sample pairs.

resulted in six common genes and a larger number of clone-specific alterations. None of these genes is present among those recently reported by Nectoux *et al*⁴⁶ in fibroblasts from three patients with

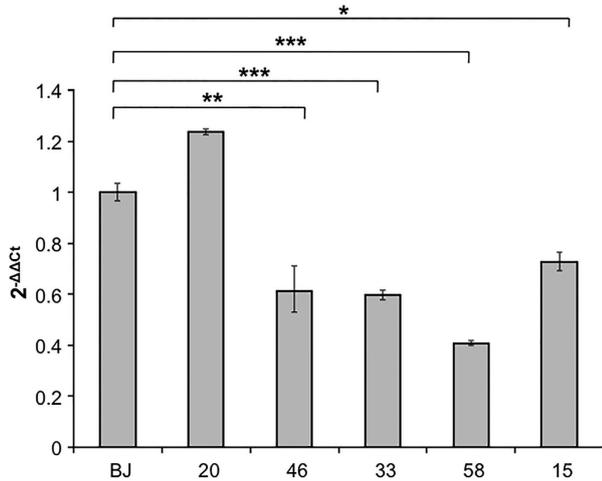


Figure 1 Real-time RT-PCR analysis of *GRID1* mRNA levels in iPS clones. *CDKL5* (33, 46, and 58)- and *MECP2* (15)-mutated clones were analysed. BJ is a control clone derived from a healthy newborn male and clone 20 is a clone harbouring the *CDKL5* mutation in one X chromosome but expressing the wild-type allele from the other X chromosome because of X-chromosome inactivation. The y axis shows $2^{-\Delta\Delta Ct}$ values (\pm SD). * $P \leq 0.01$; ** $P \leq 0.05$; *** $P \leq 0.001$.

frameshift *CDKL5* mutations. This is not an unexpected finding, considering the different cell types analysed (iPS *versus* fibroblasts). Moreover, very limited overlap is usually present among dysregulated genes resulting from different expression profiling analyses, as widely documented from studies on *MECP2*.^{11,14,47} Validation of the six common genes demonstrated that *GRID1* was consistently downregulated in both *CDKL5*-mutated and *MECP2*-mutated iPS cells (Figure 1). Analysis of neuronal precursors derived from these clones demonstrated that the absence of a functional MeCP2 or *CDKL5* protein resulted in a significant upregulation of *GRID1* mRNA in these cells (Figure 2). The upregulation persists in mature neurons.

GRID1 encodes the orphan δ -glutamate receptor subunit-1 (GluD1) and, together with *GRID2*, constitutes the δ -family of ionotropic glutamate receptors.⁴⁸ However, no channel function has been presently demonstrated either alone or in combination with other receptor subunits, and available data point to a role in synapse development rather than functioning.^{49–51} In particular, the postsynaptic GluD1 binds to presynaptic neurexin through cerebellin (Cbln) to induce presynaptic differentiation. Depending on the specific brain region, this can result in the formation of glutamatergic (cerebellum and hippocampus) or GABAergic (cortex and hippocampus) synapses, although GABAergic differentiation seems to be the prevalent outcome.^{51–53} Our experiments show that GluD1 is overexpressed in mature neurons derived from both *MECP2*- and *CDKL5*-mutated iPS cells. ChIP analysis in SHSY-5Y neurons indicate that MeCP2 binds to the *GRID1* promoter, suggesting that the increased expression might arise from the

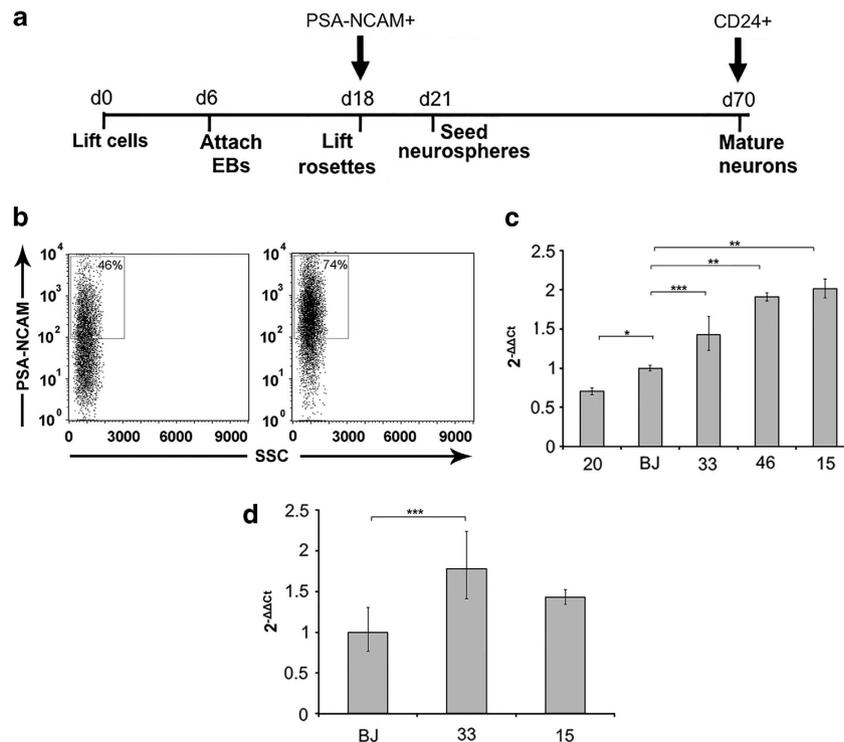


Figure 2 (a) Overview of the neuronal differentiation protocol showing the timing of specific passages expressed in days respect to day 0 (d0) defined as the first day of the protocol. The arrows indicate the day at which neuronal precursors (PSA-NCAM+) or neurons (CD24+) were sorted for expression analysis. (b) FACS analysis of differentiating cultures before sorting (left) and after sorting with anti-PSA-NCAM microbeads (right) showing a significant enrichment in positive cells. The x axis shows SSC (side scatter), and the y axis shows fluorescence intensity. (c) *GRID1* expression analysis in PSA-NCAM+ NPCs (day 18) mRNA derived from neuronal differentiation of *CDKL5* (20, 46, and 33)- and *MECP2* (15)-mutated clones (for clone identification see Figure 1 legend). (d) Expression analysis in mature neurons at day 70 of differentiation protocol sorted with anti-CD24 microbeads. The y axis in (c) and (d) shows $2^{-\Delta\Delta Ct}$ values (\pm SD). * $P \leq 0.01$; ** $P \leq 0.0001$; *** $P \leq 0.05$.

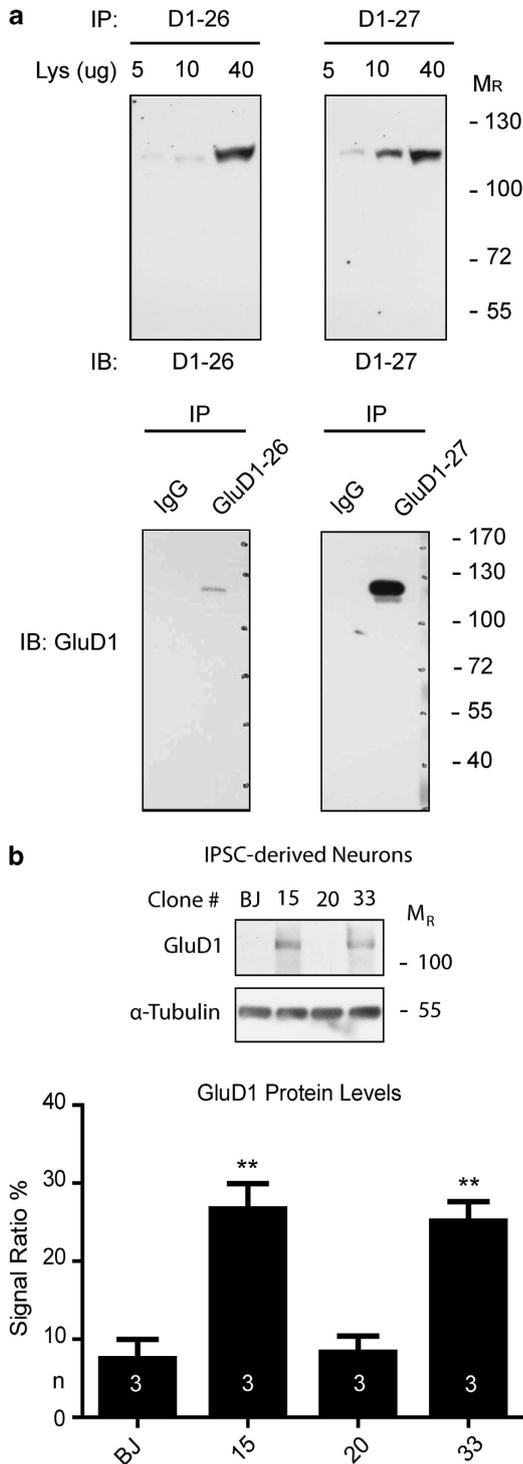


Figure 3 (a) Anti-GluD1 antibody characterization. Different amounts of mouse brain lysate as well as immunoprecipitates (with mouse nonspecific IgG as control) were loaded and probed for two different antibody batches (26 and 27) to double-proof the identity of the recognized epitope. (b) GluD1 expression in iPSC-derived neurons. Each lane was loaded with 30 μ g of total protein. Experiments were performed in triplicate ($n=3$). Immunosignals were detected using autoradiographic films, and glutamate- δ -1 receptor levels were quantified using Photoshop CS3 software (Adobe Systems, San Jose, CA, USA) and normalized against α -tubulin levels (** $P<0.01$, Student's t -test).

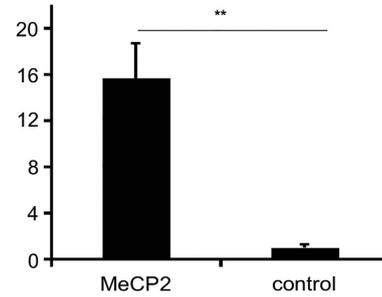


Figure 4 Chromatin immunoprecipitation with anti-MeCP2 and control antibodies shows MeCP2 binding to the *GRID1* promoter in SHSY-5Y neuroblastoma cells. The graph shows fold enrichment of target DNA isolated by ChIP using MeCP2 NH2 antibody normalized to a control preimmune antibody. Error bars show SEM. ** $P=0.0048$ by two-tailed t -test, assuming unequal variance.

absence of a direct repression by MeCP2. In *Mecp2*-mutant mice, a significant reduction in dendritic spine number has been reported, suggesting decreased excitatory synapse capacity.^{54,55} Accordingly, *MECP2*-null neurons have a significant reduction in VGLUT/PSD-95 puncta and excitatory postsynaptic current (EPSC) amplitude, whereas MeCP2 overexpression results in the opposite effects, suggesting that MeCP2 might regulate the excitation/inhibition balance in the CNS by controlling the number of excitatory synapses.^{56,57} A similar impairment has been reported in both *CDKL5*-silenced mouse hippocampal neurons and patient iPSC-derived neurons.²³ Considering the ability of GluD1 to induce the maturation of GABAergic synapses in telencephalic neurons, it can be hypothesized that its overexpression in the absence of both MeCP2 and CDKL5 might contribute to the reduction in excitatory synapses number by causing a shift toward differentiation of inhibitory synapses. Although no such alteration has been evidenced in the above-mentioned studies, analyses on *Mecp2* mouse models have demonstrated extensive region-specific variation in synaptic defects.^{51,58–60} Additional studies will thus be necessary to verify whether an increased GABAergic differentiation is indeed present in our culture conditions and to possibly characterize the identity of the neuronal population involved.

Although available data point to a role for *GRID1* in neuronal maturation, we already detected alteration of *GRID1* expression in NPCs. No data are presently available on what could be the function of GluD1 in these cells. However, both glutamate and GABA ionotropic receptor subunits and functional receptor channels are expressed very early during brain development in proliferating neuroepithelial cells and are considered important for events such as precursor proliferation, migration, differentiation, and survival.^{61–63} It is thus possible that an alteration of GluD1 levels in precursor cells might influence one of these processes and result in alteration of subsequent brain development.

Apart from the six common dysregulated genes, independent analysis of expression profiling results for the two *CDKL5*-mutated clones identified more than 300 clone-specific genes. The easiest explanation for this very limited overlap is the presence of different *CDKL5* mutations that might have different consequences on protein functionality: the female patient has a truncating mutation (p.Gln347Ter) that leads to a protein lacking the domains responsible for the interaction with MeCP2 and DNMT1 and the nuclear export signal, whereas the male patient has a missense change (p.Thr288Ile) in an highly conserved amino acid inside the catalytic kinase domain

that might result in a protein with altered kinase activity.³⁷ This scenario is consistent with the phenotypic differences observed between the two patients. In fact, the female patient has been diagnosed as having an early-onset seizures variant of RTT, whereas the male has X-linked epileptic encephalopathy. Although we are conscious that comparing cells from patients with different phenotypes might reduce the number of common genes identified, we selected this strategy as it might lead to the identification of those genes that represent the basal targets of *CDKL5*. In support of our hypothesis, a similar mutation-specific effect has also been reported by Nectoux et al⁴⁶ in *CDKL5*-mutated fibroblasts. Finally, we cannot exclude that some of the clone-specific alterations might be a nonspecific consequence of the integration site of reprogramming virus rather than a consequence of *CDKL5* absence.

The finding that GluD1 expression is altered in the presence of a mutation in *MECP2* or *CDKL5* appears particularly intriguing as mutations in GluD1 presynaptic partner are associated with Pitt-Hopkins-like syndrome 1 (OMIM #610042) that shares phenotypic features with RTT.^{64,65} The other gene mutated in this syndrome is *CNTNAP2* (also known as *NRXN4*), another member of Neurexin gene family. Data recently obtained from our group indicate that *CNTNAP2* might be involved in the modulation of the phenotype arising from mutations in *MECP2*.⁶⁶ In light of these results, it seems likely that all these genes act on the regulation of common processes and thus the alteration of one or more of them results in overlapping phenotypic outcomes. If this is the case, we would expect that alterations in *GRID1* might also cause a neurological phenotype. Indeed, linkage and association studies have established *GRID1* as a strong candidate gene for schizophrenia, major depressive disorder, and autism spectrum disorders (ASD).^{67–70} Moreover, *GRID1*-null mice present aberrant social and emotional behaviours, further stressing the importance of this gene for normal cognition.⁷¹

In conclusion, our results demonstrate that *GRID1* gene expression is downregulated in both *MECP2*- and *CDKL5*-mutant iPSC cells and upregulated in neuronal precursors and mature neurons. Our data provide the first potential functional link between *MECP2* and *CDKL5* genes. These data provide novel insights into disease pathophysiology and identify possible new targets for therapeutic treatments of RTT.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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