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A direct molecular link between the autism candidate gene RORa and the schizophrenia candidate MIR137

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Retinoic acid-related orphan receptor alpha gene (RORa) and the microRNA MIR137 have both recently been identified as novel candidate genes for neuropsychiatric disorders. RORa encodes a ligand-dependent orphan nuclear receptor that acts as a transcriptional regulator and miR-137 is a brain enriched small non-coding RNA that interacts with gene transcripts to control protein levels. Given the mounting evidence for RORa in autism spectrum disorders (ASD) and MIR137 in schizophrenia and ASD, we investigated if there was a functional biological relationship between these two genes. Herein, we demonstrate that miR-137 targets the 3'UTR of RORa in a site specific manner. We also provide further support for MIR137 as an autism candidate by showing that a large number of previously implicated autism genes are also putatively targeted by miR-137. This work supports the role of MIR137 as an ASD candidate and demonstrates a direct biological link between these previously unrelated autism candidate genes.

The retinoic acid-related orphan receptor alpha (RORa) gene encodes for a ligand-dependent orphan nuclear receptor that acts as a transcriptional regulator and has been identified as a novel candidate gene for autism spectrum disorders (ASD)[1]. Evidence implicating RORa in autism first came from the finding that RORa levels were reduced in samples taken from autistic patients. In both lymphoblast cell lines derived from affected individuals[2] and in the prefrontal cortex and cerebellum of autistic brains[1] RORa protein levels were reduced compared to matched controls. Furthermore, transcriptional targets of RORa are involved in the pathways impaired in ASD and some directly regulated genes such as *NLGN1* and *NTRK2* have been independently implicated in ASD in association studies[3–5].

MicroRNAs (miRNAs) are a class of small non-coding RNAs that interact via complimentary base pairing to the 3′ untranslated region (3'UTR) of gene transcripts to control the levels of proteins in cells. Mature miRNAs are 21–25 nucleotides in length and recognise a core motif of 7–8 base pairs in the transcripts of multiple target genes, resulting in either degradation of the transcript or suppression of translation[6]. A single miRNA can regulate the expression of thousands of targets. Mounting evidence has implicated miRNAs in the molecular genetics of a range of neurological and neuropsychiatric disorders. A number of miRNAs have shown differing expression levels in samples from autistic individuals compared to matched controls[7–9], suggesting that dysregulation of miRNAs could be related to the molecular causes or consequences of autism.

The increasing importance of RORa in ASD led us to investigate which miRNAs regulate its expression. Herein, we demonstrate a direct molecular link between RORa and miR-137, demonstrating that this brain enriched microRNA targets the 3'UTR of RORa in a site specific manner. We also show that a large number of previously implicated autism genes are putatively regulated by miR-137. This work supports the role of MIR137 as an ASD candidate and demonstrates a direct biological link between these previously unrelated autism candidate genes.

Results

In the 3'UTR of the RORa transcript we identified several brain enriched miRNAs predicted to target the RORa transcript including miR-19ab, miR-34ac and miR-137 using the TargetScan 6.2 algorithm[10]. It was striking that there were multiple (5) separate binding sites predicted for miR-137 in the *RORa* 3'UTR, highly indicative of this being a true target. MiR-137 is highly expressed in the brain, is important for neuronal maturation and synapse development and has shown repeated association with other cognitive disorders such as schizophrenia and intellectual disability[11,12]. Furthermore, MIR137 has itself recently been implicated in ASD via a meta-analysis

that considered single nucleotide polymorphism (SNP) data across five disorders; autism spectrum disorder, attention deficit-hyperactivity disorder, bipolar disorder, major depressive disorder, and schizophrenia and using a best fit model found association of the mir-137 SNP with both schizophrenia and ASD[13]. Although miR-137 expression is enriched in the brain, in the cerebellum where the highest RORa expression can be found, miR-137 expression is very low[12]. Taken together, these data suggested a putative link between these two autism candidate genes and in this study we use functional assays to demonstrate direct regulation of RORa by miR-137.

We identified five predicted binding sites for miR-137 (M137-BS 1–5) that all showed high conservation (Figure 1a–b). To test if these were functionally active we cloned three regions containing the five putative miR-137 binding sites to act as a 3'UTR of the Firefly luciferase reporter gene (Figure 1c). To confirm the activity of endogenous and transfected mir-137, and assess the sensitivity of the system we also generated a positive control reporter (M137 Rep) that had multiple consensus miR-137 binding sites downstream of the luciferase reporter gene. Co-transfection of mir-137 with this positive control resulted in strong repression of reporter gene activity (Figure 2a). When we introduced each of the RORa 3'UTR regions into the reporter system in neuron like cells we saw that reporter gene activity decreased, both in the presence of endogenous or co-transfected mir-137 expression. Region 1 showed only a mild repression compared to control (lacking the 3'UTR region) and this effect was not significant (Figure 2b). Regions 2 and 3 led to significant reductions in reporter activity compared to controls (Figure 2c–d) and showed the strongest effects when mir-137 was overexpressed alongside the reporter assay (Figure 2c–d). Interestingly, region 2 showed similar decreases in activity with or without miR-137 co-expression, suggesting that this region is efficiently targeted by endogenous levels of miR-137. These data suggest that miR-137 interacts with the binding sites in the 3'UTR of RORa to regulate expression.

To independently validate our results, we tested these same constructs in another human cell line; HEK293 cells. These cells are a commonly used model for testing the activity of proteins and miRNAs and have the added benefit of expressing no endogenous mir-137 (data not shown). Thus we can confirm that the regulation of the reporter fragments is due to the presence of miR-137 (via co-transfection with the mir-137 expression plasmid). We began by testing regulation of our positive control reporter with and without mir-137 transgene expression. Without mir-137 co-transfection the positive control reporter showed no activity differences compared to the 'scrambled' reporter (SCR Rep - which is designed to be unresponsive to miR-137), reflecting the lack of endogenous miR-137 in HEK293 cells. When mir-137 was co-transfected, the positive control reporter activity was significantly reduced (by ~90%), demonstrating that the transfected miR-137 was capable of strongly regulating this transcript (Figure 3a). All three RORa 3'UTR regions were repressed in this cell model when miR-137 was co-expressed. Regions 2 and 3 only showed effects when mir-137 was co-transfected (Figure 3c–d). By contrast, region 1 was similarly repressed by endogenous miRs and by transgenic miR-137 (Figure 3b), hinting that the repression observed when this region is present may not be due to miR-137 but caused by other endogenous miRs found in these cells.

To determine if regulatory effects were due to a specific interaction between miR-137 and the predicted binding sites in the RORa 3'UTR, we created deletion constructs that retained the 3'UTR regions, but lacked the miR-137 binding site(s). Region 1 activity was not rescued by deletion of the miR-137 binding site (M137-BS 1; Figure 4a), confirming that the effect is likely due to other miRs interacting with region 1 - such as miR-19ab which has been shown to regulate RORa via interaction with binding sites in region 1[14] or miR-34ac for which putative sites are also found in this region. When region 2 was present, reporter activity was significantly reduced. This effect was completely abolished by deletion of the miR-137 binding site (Figure 4b), demonstrating the specific interaction of miR-137 with M137-BS 2. Region 3 contained three separate miR-137 binding sites (M137-BS 3–5) and was the most strongly repressed 3'UTR region. To determine which of these sites was biologically active we created a series of constructs containing every combination of binding site deletions. The pattern of reporter gene expression across these different constructs suggests that all three binding sites are functionally active and contribute equally to the ability of miR-137 to regulate RORa (Figure 4c). Taken together these results show that miR-137 specifically interacts with 4 binding sites (M137-BS 2–5) within the RORa 3'UTR in order to modulate its expression. The
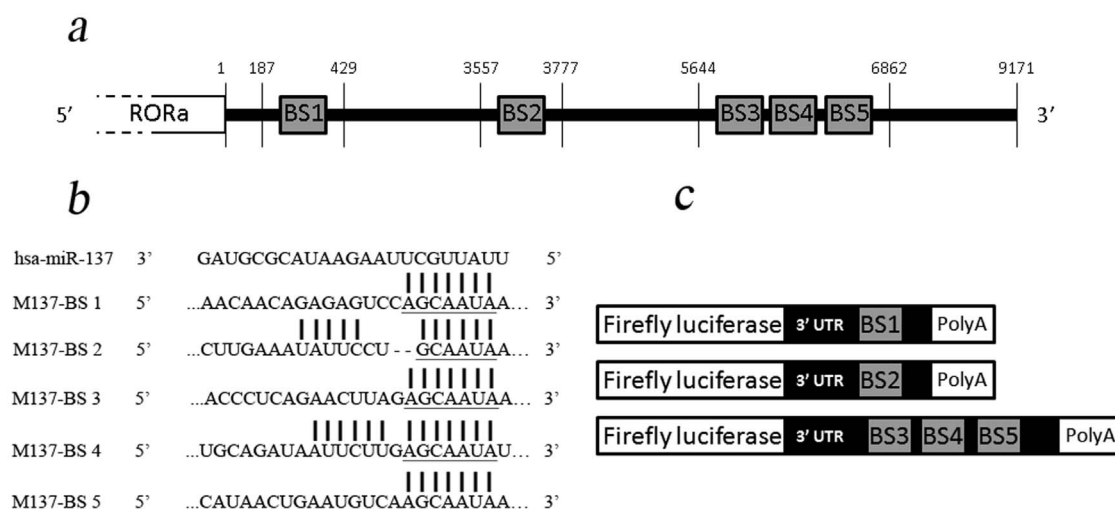

Figure 1 | The RORa 3' untranslated region (3'UTR) contains multiple mir-137 binding sites. (a) Five consensus binding sites for mir-137 (BS1-5) were identified within the 3'UTR of RORa using the TargetScan 6.2 algorithm. (b) The mature mir-137 sequence is given, aligned to the complimentary sequence of the five predicted binding sites in the RORa 3'UTR. Complimentary base pairing is indicated by vertical lines. For each binding site the core recognition sequence is underlined. (c) To test if they were functionally active we cloned three regions containing the binding sites to act as a 3'UTR of the Firefly luciferase reporter gene (in the pGL4.23 vector, Promega). Regions 1 and 2 contained M137-BS 1 and 2 respectively, and region 3 contained M137-BS 3–5 (coordinates for the cloned fragments are shown in part a). Deletion constructs contained the same fragment however the core recognition sequence (underlined in part b) has been deleted.
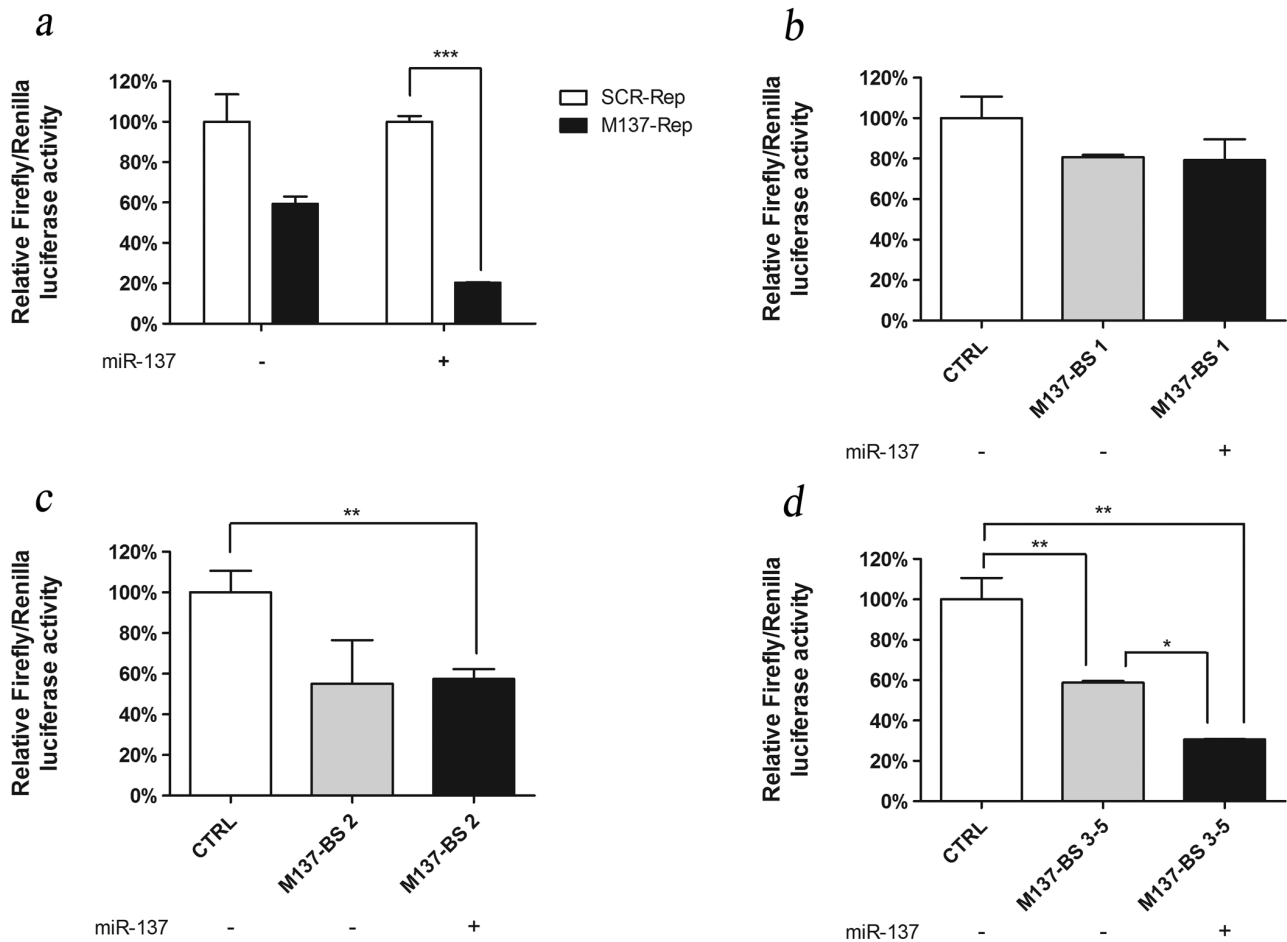
Figure 2 | Regions of the RORa 3'UTR are regulated by mir-137 in human neuron-like cells. Luciferase reporter assays were performed in a human neuron-like cell line (SHSY5Y) to measure the ability of mir-137 to regulate regions of the RORa 3'UTR. SHSY5Y cells express endogenous mir-137 and assays were performed with or without co-transfection of transgenic mir-137 (a) Positive control reporter construct (M137 Rep) activity was subtly reduced by endogenous mir-137 levels, but activity was very strongly and significantly reduced when mir-137 was co-expressed with the reporter. The scrambled control (SCR Rep) was unaffected by mir-137 expression. Significance was calculated using a students t-test where *** $p < 0.001$. (b) Reporter gene activity was not significantly reduced when Region 1 (M137-BS 1) was present. No difference in activity was observed when mir-137 was strongly co-expressed. (c) When Region 2 (M137-BS 2) was introduced, activity was more strongly reduced with or without mir-137 coexpression, however a significant reduction in activity was only observed when mir-137 was overexpressed. (d) Region 3 (M137-BS 3–5) was the most strongly repressed region. This construct was significantly repressed by endogenous mir-137 levels and overexpression of mir-137 resulted in significantly stronger repression of activity compared to all conditions. Significant differences between groups in parts b–d were calculated using an ANOVA test followed by post-hoc Tukey calculation. Significance is indicated by *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. All results are reported as the average $+/-$ standard deviation of 2 biological replicates.

presence of multiple functional target sites in the 3'UTR suggests an important role for miR-137 in regulating RORa.

To further investigate the role of MIR137 as an autism candidate we determined if genes targeted by miR-137 were themselves implicated in autism. From the Autism Knowledgebase (AutismKB)[15] we extracted genes implicated in syndromic and non-syndromic autism (N = 3067). Using the TargetScan 6.2 algorithm[10] we identified predicted targets of miR-137 (N = 1144). When comparing these two gene lists we observed extensive overlap (N = 263) that was calculated to be highly significant (p < 7.130e-23)(Supplementary Table S1). The overlapping gene list showed a significant over-representation for GO categories including synaptic transmission (adjP = 4.58e-07) and voltage gated cation channel activity (adjP = 7.36e-06) and was enriched for proteins localised to the synapse (adjP = 8.53e-07) and axonal compartment (adjP = 4.25e-06) (See Supplementary Table S2 for full GO results). This overlapping gene list also included some of the most well validated autism candidates such as *NRXN1*, *SHANK2*, *SCN2A* and *CACNA1C*. Thus in addition

to regulating RORa, miR-137 may be responsible for controlling the levels of a number of targets acting in autism gene networks.

Discussion

ASDs are a group of developmental disabilities characterized by impaired social interaction and communication, repetitive stereotypical behaviours and restricted interests. ASDs demonstrate a strong genetic component however the genetic underpinnings are complex and many genes have been associated with ASD susceptibility thus far[15,16]. Reflecting the genetic complexity underlying ASD, neuroanatomical and functional studies have shown impairment in many different biological functions such as neuronal differentiation and adhesion, neural connectivity, synaptogenesis, synaptic transmission and plasticity.

A number of brain regions have been implicated in the neuropathology of ASDs, however the cerebellum represents one of the most strongly supported regions affected in autism. Anatomical abnormalities in the cerebellum have been frequently observed via
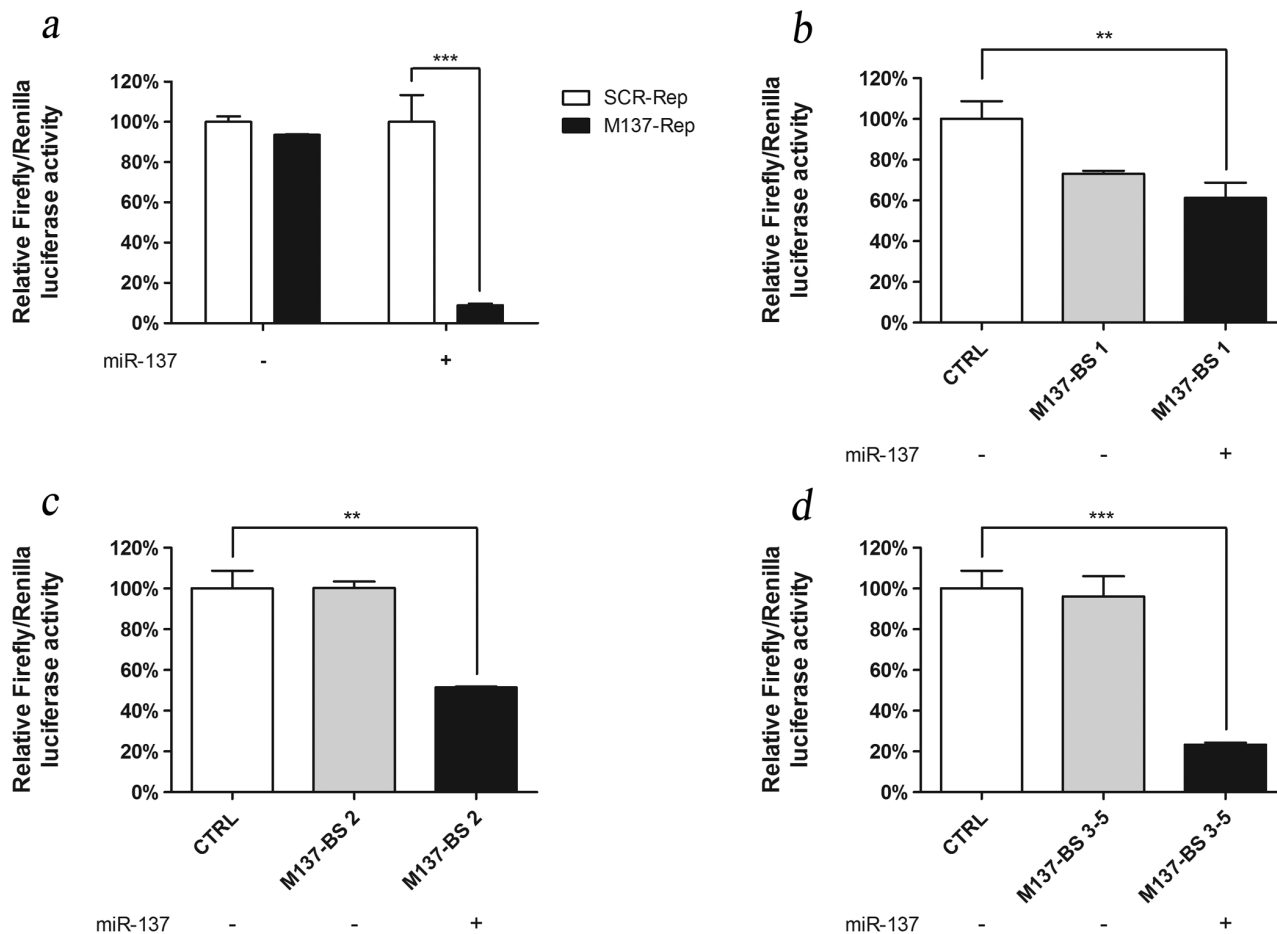
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Figure 3 | Mir-137 expression is required for specific regulation of RORa 3'UTR. Luciferase assays were carried out in an independent human cell line, derived from kidney tissue (HEK293) that is a widely used model for the activity of human genes and microRNAs, but that have no detectable endogenous mir-137 expression. (a) The positive control reporter construct activity was not affected by endogenous miR levels, but activity was very strongly and significantly reduced when mir-137 was co-expressed. Significance was calculated using a students t-test where *** $p < 0.001$. (b) Reporter gene activity was slightly, but not significantly reduced when Region 1 (M137-BS 1) was present. Co-expression of mir-137 produced a further minimal reduction of reporter activity. (c) The region 2 reporter construct (M137-BS 2) was significantly reduced only when mir-137 was overexpressed. (d) Region 3 (M137-BS 3–5) was unaffected by endogenous miR levels, but strong and significant repression was observed when mir-137 was co-expressed. Significant differences between groups in parts b–d was calculated using an ANOVA test followed by post-hoc Tukey calculation. Significance is indicated by *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. All results are reported as the average +/− standard deviation of 2 biological replicates.

magnetic resonance (MRI) and histological studies in autistic patients[17–19]. Cerebellar activation measured via functional MRI (fMRI) is also affected in autistic individuals during motor tasks[20,21]. Purkinje cells (PCs) in the cerebellum are reduced in number in autistic brains (as reviewed by Palmen et al[22]), and morphological changes have been observed in neurons of the deep cerebellar nuclei[23]. Autistic patients commonly display dyspraxia and other motor control phenotypes, but the cerebellum has also been implicated in non-motor processing related to autistic endophenotypes such as language and higher order social or emotional processing[17]. Furthermore, many of the genes that are strongly related to ASDs (such as *SHANK3*, *EN2* and *MET*) play important roles in cerebellar development and maintenance.

Molecular analysis of gene expression and epigenetic markers linked to ASD affected status have implicated RORa as a novel autism candidate gene. RORa is a transcriptional regulator and its target genes have also been linked to susceptibility to ASD and have roles in pathways associated with ASD[3]. Interestingly RORa is very highly expressed in the cerebellum and complete loss of RORa in mouse models leads to massive cerebellar atrophy and an ataxic phenotype[24,25]. Conditional transgenic mice that lost RORa expression in

Purkinje cells at postnatal stages (>P10) revealed an important role in the continued survival of PCs and maintenance of the mature differentiation state[26]. Motor abilities were strongly impaired in these mice, due to the dramatic loss of Purkinje cells and the reduced foliation and spine density of the dendritic trees in the surviving PCs[26]. Reduced RORa expression in the cortex and cerebelllum has been observed in autistic brains. Given the role of miR-137 in targeting and downregulating RORa, it is possible that ectopic or inappropriate expression of mir-137 and subsequent RORa mysregulation, for example in the cerebellum, could contribute to ASD.

miR-137, a brain enriched miRNA, has been shown to be important in neuronal maturation, migration and connectivity by regulating genes such as *KLF4* - a transcription factor involved in axon growth[12]. Several studies including a GWAS meta-analysis have linked MIR137 with cognitive disorders such as schizophrenia and most recently ASD[27]. Overlapping deletions of the MIR137 locus have also been identified in intellectual disability patients[12]. A large scale GWAS study identified strong association between a SNP close to MIR137 and schizophrenia[27] and subsequent follow up suggested that the SNP identified was associated with reduced mir-137 expression levels in post-mortem patient samples[28]. Interestingly, other
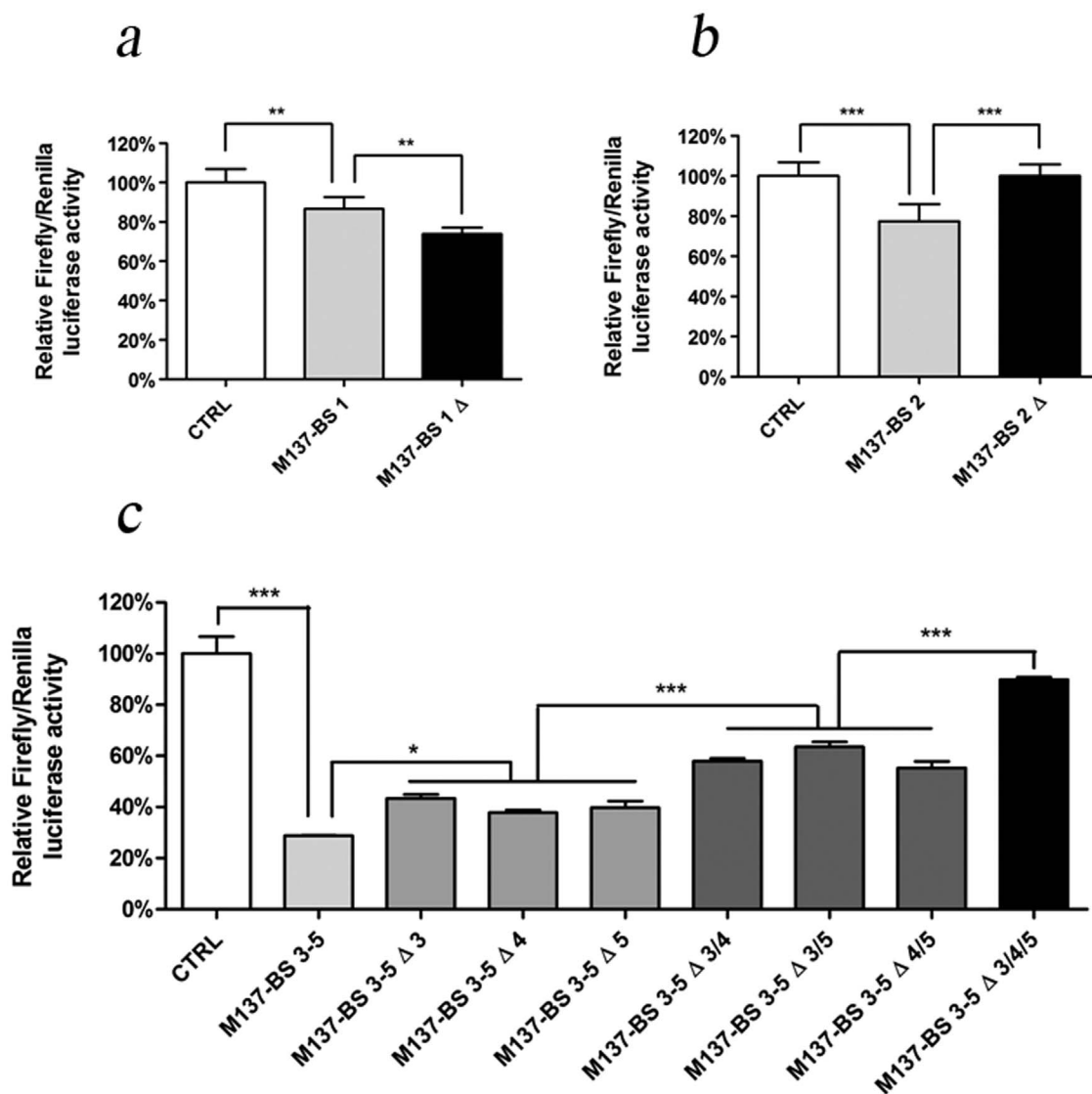
Figure 4 | Mir-137 regulates the 3'UTR of RORa in a site specific fashion. Relative activity of reporter constructs carrying the RORa 3'UTR regions were compared to deletion constructs or a vector without a 3'UTR sequence as a control. (a) A subtle reduction of luciferase activity was observed when region 1 is present (M137-BS 1) however this was not rescued by deletion of the mir-137 binding site (M137-BS 1 Δ), suggesting that mir-137 is not responsible for this effect. (b) Reporter activity was significantly reduced (by ~20%) when region 2 was present (M137-BS 2), and this effect was completely abolished when the mir-137 binding site was deleted (M137-BS 2 Δ) (c) Region 3 (M137-BS 3–5) contains three predicted binding sites and was the most dramatically affected construct. (~80% reduction of control luciferase activity). To determine which of the sites was functional, we created a series of deletion constructs containing every combination of binding site deletions. Individual deletion of the binding sites increased activity by ~10% in each case (M137-BS 3–5 Δ3, M137-BS 3–5 Δ4, M137-BS 3–5 Δ5), and deleting any combination of 2 of the 3 sites increased activity by a further ~20% (M137-BS 3–5 Δ3/4, M137-BS 3–5 Δ3/5, M137-BS 3–5 Δ4/5). Deleting all three binding sites expression levels returned to ~90% of the control luciferase activity (M137-BS 3–5 Δ3/4/5). Significant differences between groups was calculated using an ANOVA test followed by post-hoc Tukey calculation. Significance is indicated by $*p < 0.05$, $** p < 0.01$ and $*** p < 0.001$. Note that in part c the significance of the difference between the region 3 construct (M137-BS 3–5) and the effect of deleting individual binding sites was as follows: M137-BS 3–5 Δ3 ($p < 0.05$), M137-BS 3–5 Δ4 ($p < 0.01$), M137-BS 3–5 Δ5 ($p < 0.001$). We have summarised this in the figure as all three being $p < 0.05$ for clarity. Results are representative of at least two independent experiments and are reported as the average +/− standard deviation of 3 biological replicates.

Table 1	Cloning of RORa 3'UTR regions		
Region	3'UTR coordinates	Forward primer (5′-3′)	Reverse primer (5′-3′)
1	187–429	TGAATTCTAGACAACAGGAGGAGGGTACTAAAGTC	TGAATGGCCGGCCGCTATGGACCCTTTTCATGCC
2	3557–3777	TGAATTCTAGACTTGTCTGGACCACTTTAGAG	TGAATGGCCGGCCCCAAAGACAGATAGACAAATTCC
3	5644–6862	TGAATTCTAGAGTCTGTCTGTCTGTTTGTGTAGGTG	TGAATGGCCGGCCGACTCAAAAGCAGGGACAGG

Table 2 | Site directed mutagenesis primers used to create deletion constructs

Binding site	Forward primer (5′-3′)	Reverse primer (5′-3′)
M137-BS 1	TAACAACAGAGAGTCCTCGGTGACTGGTGTGC	GCACACCAGTCACCGAGGACTCTCTGTTGTTA
M137-BS 2	CCCTTCTATAAATATTTGATGGCACTTGAAATATTAAAATGTGATTTGTGTAAAGAAAAAAGAT	ATCTTTTTTTCTTTACACAAATCACATTTTAATATTTCAAGTGCCATCAAATATTTATAGAAGGG
M137-BS 3	GTTCAACCAAACCCTCAGAACTTAGAATACATTCAGTCATTTATTTATAA	TTATAAATAAATGACTGAATGTATTCTAAGTTCTGAGGGTTTGGTTGAAC
M137-BS 4	ATTGCATATGTAAATGCAGATAATTCTTGTAGTGAAAATGATTTCACAAAAAAAATCC	GGATTTTTTTTGTGAAATCATTTTCACTACAAGAATTATCTGCATTTACATATGCAAT
M137-BS 5	AATCTAGCCATAACTGAATGTCAAAACAAATGACTTTTGTGCATAG	CTATGCACAAAAGTCATTTGTTTTGACATTCAGTTATGGCTAGATT

genes displaying association with schizophrenia in this GWAS study were shown to be targeted by miR-137[29,30], suggesting that convergent pathways may contribute to this complex neuropsychiatric disorder.

Although clinically distinct, schizophrenia and autism seem to share some neurological, cellular and/or genetic factors. For example, changes in dendritic spine density have been implicated in both disorders, with dendritic spine loss observed in schizophrenia, but increased spine numbers in autistic samples[31,32]. High levels of miR-137 have been shown to be present at the synapse, which play a role in directly controlling dendritic spine maturation and density[33]. Interestingly, herein we identified a significant enrichment of synaptically localised genes that were both autism candidate genes and miR-137 predicted targets, including *SHANK3* – a gene important for synapse formation and function. Similarly, the miR-137 target gene *NRXN1* is a risk factor for both autism and schizophrenia and a SNP in this gene has been associated with changes in frontal white matter volume - an endophenotype common to both disorders[34].

Herein, we demonstrate a direct molecular link between two genes previously associated with autism, RORa and MIR137. RORa reduction is linked to autistic phenotypes and miR-137 acts to repress RORa, thus it is possible that duplications or mutations that produce overexpression of miR-137 in patients, particularly in the cerebellum, could result in similar effects. The previously reported associations coupled to our functional data and the over-representation of autism candidate genes targeted by this miR provides support for MIR137 as an ASD candidate. Direct testing of mir-137 variants and expression levels in autistic patients may shed light on the contribution of this microRNA to the complex phenotype of ASD and reveal shared genetic pathways that underlie complex neuropsychological disorders like autism and schizophrenia.

Methods

Cloning. A mir-137 expression construct was generated by amplifying the region encoding the primary transcript from human genomic DNA using the following primers: Forward- 5′-TTACTACCGGTGAGCAGCAAGAGTTCTGGTG-3′ and Reverse- 5′-AGTTACGAATTCGAAAGTGCTACCTTGGCAACCAC-3′. The amplified fragment was cloned into the pLKO.1 vector using AgeI and EcoRI and direct sequencing was used to confirm presence of the desired sequence. Expression of the precursor and mature miR-137 was confirmed via qPCR (data not shown).

A positive control reporter construct (M137 Rep) containing 4 sequential miR-137 binding sites directly downstream of the Firefly luciferase reporter in the pGL4.23 expression vector (Promega) was created to verify the sensitivity of the assay. The construct was generated according to previously published protocols[35]. Briefly, oligo duplexes were designed to contain two high-sensitivity miR-137 binding sites separated by a 6 nucleotide spacer. When annealed, the duplexes form overhangs compatible with the KflI restriction endonuclease to enable directional insertion into the destination vector. Since the original pGL4.23 does not contain any KflI restriction sites we engineered this vector adding a single KflI restriction site into the 3′UTR of the Firefly luciferase gene. The following complimentary oligos were used: Sense: 5′-GACCCCTACGCGTATTAAAGCAATAAATATTCCTACGCGTATTAAAGCAATAAGG-3′, Antisense: 5′-GTCCCTTATTGCTTTAATACGCGTAGGAATATTTATTGCTTTAATACGCGTAGGG-3′. Annealed oligos were ligated into the KflI site and because of the small fragment size and non-palindromic sequence, multiple copies of the probe (representing 2 binding sites each) can be inserted into the vector. We screened a number of the resulting plasmids via direct sequencing and selected a construct that contained four sequential miR-137 binding sites for use as our positive

control construct (M137 Rep). Using the same technique, a negative control "scrambled" binding site reporter construct (SCR Rep) was created using the following oligos: Sense: 5′-GACCCTCCTCTGTCACTACATCTTAGAATATTCTCCTCTGTCACTACATCTTAGAGG-3′, antisense: 5′-GTCCCTCTAAGATGTAGTGACAGAGGAGAATATTCTAAGATGTAGTGACAGAGGAGG-3′. This construct contains very similar base composition as the positive control however the bases have been shuffled to ensure that no miR-137 binding sites remain.

RORa 3′UTR regions were cloned into the pGL4.23 expression vector (Promega) downstream of the Firefly luciferase reporter gene using the primers from Table 1 and XbaI/FseI restriction sites. Deletion constructs were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene) as per manufacturer's instructions with the primers detailed in Table 2. Direct sequencing was used to confirm presence of the desired deletions.

Cell culture and transfection. HEK293 cells were grown in Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen) and SH-SY5Y cells in DMEM:F12 media (Invitrogen). Media was supplemented with 10% Foetal Calf Serum (Sigma), 2 mM L-glutamine (Sigma) and 2 mM Penicillin/Streptomycin (Sigma). Cells were grown at 37°C in the presence of 5% CO_2. Transfections of SHSY5Y and HEK293 cells were performed using GeneJuice® (Novagen), according to manufacturers' instructions.

Luciferase assay. Cells were seeded at a density of 2.5×10^4 cells per cm^2 (60–70% confluency), 24 hours prior to transfection. Reporter constructs were co-transfected into human cells (SHSY5Y or HEK293) along with the Renilla reporter gene (pRL-TK) for internal normalization. 48 hours post-transfection firefly luciferase and Renilla luciferase activities were measured as per manufacturer's instructions (Dual Luciferase reporter assay system, Promega). Luciferase experiments were performed in the presence or absence of co-transfection with a miR-137 overexpression construct.

Gene Ontology analysis. Gene Ontology (GO) analysis was performed using the Webgestalt program (http://bioinfo.vanderbilt.edu/webgestalt/). Over representation of gene ontology categories was determined via hypergeometric testing using Benjamini & Hochberg multiple testing correction[36].

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Author contributions

P.D. conceived the study design, carried out the experimental and analytical work and drafted the manuscript. S.C.V. conceived the study design, performed analytical work and drafted the manuscript.

Additional information

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