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DISRUPTION OF THE MIR-137 PRIMARY TRANSCRIPT RESULTS IN EARLY EMBRYONIC LETHALITY IN MOUSE

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To the editors:

Multiple lines of evidence support a role for microRNA 137 (miR-137) in the etiology of schizophrenia (1–4) and fundamental neuronal processes (5–7). In the largest genome-wide association meta-analysis for schizophrenia,(8) the second most significant association is in *MIR137HG*, the gene encoding miR-137 (rs1702294, $P=3.4\times10^{-19}$). Prior reports indicated that genes with predicted miR-137 target sites were enriched for smaller GWAS *P*-values (2), raising the possibility that miR-137 regulates a gene network involved in schizophrenia. To understand more about the role of miR-137, we describe here our characterization of embryonic development, behavior, and gene expression in mice with targeted disruption of the *Mir137* transcript (supplementary detailed information can be found at: http://crowley.web.unc.edu/files/2014/03/mir137.ko_.supplement.pdf).

Mir137^{tm1Mtm} mice were created in a project to generate conditional, reporter-tagged targeted mutations in 162 miRNAs (9). These mice were created using a “knockout-first” strategy (10) to produce a knockout at the RNA processing level. We purchased heterozygous (+/−) breeder mice (www.mmrrc.org/catalog/sds.php?mmrrc_id=36301). We

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Author Contributions
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Conflicts of Interest
The authors report no conflicts.

confirmed genetic background (~75% C57BL/6J) and backcrossed twice to C57BL/6J to create animals of >95% C57BL/6J ancestry.

To our knowledge, these *Mir137* mutant mice have not been characterized previously. Seven heterozygous × heterozygous matings produced 35 living offspring, none of which were homozygous (−/−) for the targeted *Mir137* allele (Figure 1A, $P < 0.001$), suggesting complete embryonic lethality in the presence of knockout of *Mir137*.

To confirm embryonic lethality and to determine when lethality occurred, we conducted timed matings (six heterozygous × heterozygous and four heterozygous × wildtype). On embryonic day 11.5 (E11.5), all embryos were collected (Figure 1A). Again, no homozygous (−/−) embryos were identified ($P < 0.001$). We observed significantly more resorbed embryos from heterozygous × heterozygous matings ($P < 0.0001$), indicating that embryonic lethality must occur after implantation (~E4.5) but before E11.5. Efforts to identify a *Mir137* −/− mouse by genotyping resorbed embryos at E11.5 or collecting flushed embryos at E3.5 were unsuccessful, likely due to insufficient and/or impure DNA preparations from small amounts of tissue.

We did not observe reduced viability of heterozygous mice (+/−) after genotyping 256 offspring (P=0.14) collected from birth to three weeks of age, indicating that one copy of *Mir137* is sufficient for survival (Figure 1A). We subjected wild-type and heterozygous animals to a battery of behavioral tests (e.g., basic sensorium, activity, social behavior, learning/memory, etc.), and observed no consistent and interpretable differences. These results also suggest compensation from the remaining allele.

We next examined the expression of *Mir137* and the levels of mature miR-137 in the brains of heterozygous and wildtype animals (−/− embryos died too early for collection of sufficient tissue). Using allele-specific reverse transcriptase PCR, we confirmed that the targeted allele leads to complete loss of *Mir137* expression downstream of the polyA sequence (Figure 1B). However, mature miR-137 levels were not significantly different between heterozygous and wild-type animals (Figure 1C), suggesting that heterozygous mice have compensatory upregulation or decreased degradation of miR-137. These expression data are consistent with the lack of a viability or behavioral phenotype in the heterozygous mice.

To circumvent the embryonic lethality of *Mir137* −/− mice, we attempted to make conditional knockouts (9), such that ablation of *Mir137* could be limited to a particular tissue. The construct contains a combination of FRT and loxP sites intended to allow conditional mutagenesis by crossing to germline deleter Flp mice (expected to restore the wild-type allele) and then crossing to tissue-specific Cre transgenic mice (expected to generate adult brain-specific knockouts). Crosses between *Mir137* +/− mice and an efficient Flp line led to several successfully recombined mice, heterozygous for the rescued allele. Repeated intercrosses of these recombined mice, however, failed to yield any homozygous offspring. Therefore, we were unable to rescue embryonic lethality.

We reasoned that there were two major possibilities for the rescue failure. First, the targeting construct could have inserted into the wrong genomic position or could have sequence

errors. We have likely excluded this possibility by confirming the location of the integration site and re-sequencing >95% of the construct without identifying an error. Second, Flp may have been unable to restore proper gene function due to issues inherent with the gene construct (e.g., FRT or LoxP sites in critical part of the gene). We favor this possibility because we were unable to detect any reporter gene (beta-galactosidase) activity in *Mir137* +/− embryos or adult tissues, suggesting that the endogenous *Mir137* promoter was inadvertently inactivated. Furthermore, bioinformatic analysis of the region (using additional data that became available after we were well into this project), suggests that the exogenous gene targeting elements may indeed interfere with endogenous functional elements, and act to prevent conditional mutagenesis (Figure 1D). The LoxP site upstream of *Mir137* is in a putative splice donor sequence and the FRT site lies between highly conserved DNase hypersensitivity sites that are active in embryonic brain.

In conclusion, these results suggest that at least one functional copy of *Mir137* is essential for embryonic development. These data are consistent with miR-137 playing important roles in development and perhaps also in neurodevelopmental disorders like schizophrenia. It appears that miR-137's biological pathway is capable of homeostatic compensation and, while we do not yet understand the functional impact of *Mir137* schizophrenia risk variants, it is conceivable that this homeostatic capacity could be affected in some way. It is clear, however, that this genomic region is more complex than previously thought, and the design of targeted mutations in this region should incorporate all available genomic data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Mating type	Time-point	Offspring genotypes (N)			Resorptions (N)	P-value	
		+/+	+/-	-/-		genotype	resorption
+/- x +/-	birth	11	24	0	N/A	<0.001	N/A
	E11.5	6	21	0	8	<0.001	<0.0001
+/- x +/+	E11.5	9	7	N/A	1	0.45	
	birth	140	116	N/A	N/A	0.14	N/A

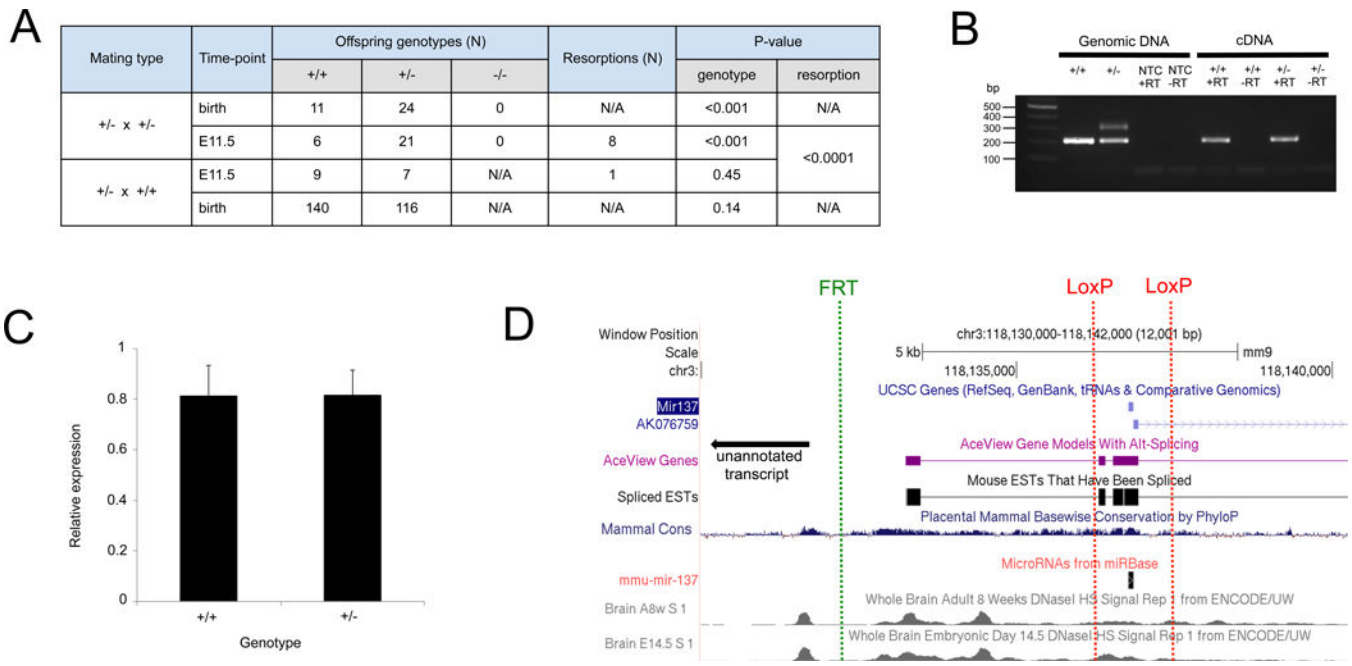

Figure 1.

Mir137 knockout mice (−/−) are embryonic lethal. (**A**) Heterozygous × heterozygous matings failed to yield a live born (−/−) mouse or embryo, and timed matings revealed an excess of resorbed embryos at E11.5. Heterozygous mice did not show a reduction in viability. (**B**) Verification that the mutant allele does not express *Mir137* by allele-specific expression. Representative reverse transcriptase PCR results from adult mouse brain using primers flanking a LoxP insertion site in the *Mir137* primary transcript. Amplification from genomic DNA is shown as a positive control for amplification of the mutant and wildtype alleles. The negative control reactions (−RT) confirm the absence of genomic DNA contamination. The absence of an upper band in the (+/−) +RT sample indicates lack of expression from the mutant allele. NTC = no template control. RT = reverse transcriptase. (**C**) Quantitative PCR analysis of mature miR-137 expression from wildtype and heterozygous brain tissue revealed no significant difference, consistent with the viability and behavioral data. (**D**) Exogenous gene targeting elements may interfere with endogenous functional elements, preventing conditional mutagenesis. Shown are the positions of the non-native sequences (FRT and LoxP sites that would remain after crossing to Flp) relative to known and predicted transcripts, DNase hypersensitivity sites, and conservation data.