Novel Variants in *ZNF34* and Other Brain-Expressed Transcription Factors Are Shared Among Early-Onset MDD Relatives

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Manuscript Received: 24 February 2015; Manuscript Accepted: 17 November 2015

There are no known genetic variants with large effects on susceptibility to major depressive disorder (MDD). Although one proposed study approach is to increase sensitivity by increasing sample sizes, another is to focus on families with multiple affected individuals to identify genes with rare or novel variants with strong effects. Choosing the family-based approach, we performed whole-exome analysis on affected individuals (n = 12) across five MDD families, each with at least five affected individuals, early onset, and prepubertal diagnoses. We identified 67 genes where novel deleterious variants were shared among affected relatives. Gene ontology analysis shows that of these 67 genes, 18 encode transcriptional regulators, eight of which are expressed in the human brain, including four KRAB-A box-containing Zn²⁺ finger repressors. One of these, ZNF34, has been reported as being associated with bipolar disorder and as differentially expressed in bipolar disorder patients compared to healthy controls. We found a novel variant-encoding a nonconservative P17R substitution in the conserved repressor domain of ZNF34 protein-segregating completely with MDD in all available individuals in the family in which it was discovered. Further analysis showed a common ZNF34 coding indel segregating with MDD in a separate family, possibly indicating the presence of an unobserved, linked, rare variant in that particular family. Our results indicate that genes encoding transcription factors expressed in the brain might be an important group of MDD candidate genes and that rare variants in ZNF34 might contribute to susceptibility to MDD and perhaps other affective disorders. © 2016 Wiley Periodicals, Inc.

Key words: major depressive disorder; whole-exome analysis; psychiatric disorders; transcription factors; gene ontology

INTRODUCTION

Major depressive disorder (MDD) is among the most common psychiatric disorders and the leading cause of disability in the

How to Cite this Article: Subaran RL, Odgerel Z, Swaminathan R, Glatt CE, Weissman MM. 2016. Novel Variants in *ZNF34* and Other Brain-Expressed Transcription Factors Are Shared Among Early-Onset MDD Relatives.

Am J Med Genet Part B 9999:1-9.

United States [Stewart et al., 2003]. Globally, depression is the third leading contributor to disease burden and is associated with serious comorbidity including increased rates of substance abuse, mortality, and numerous medical complications [Ferrari et al., 2013; Whiteford et al., 2013]. The heritability of MDD has been estimated to be as high as 70% [Kendler et al., 1993] with relative risk to first-degree relatives of probands with MDD at least two-to threefold higher than relatives of controls. This risk is further increased in relatives of probands with earlier onset of MDD,

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Conflicts of interest: The authors have no conflicts of interest to report. Grant sponsor: Brain and Behavior Research Foundation (formerly NARSAD) Young Investigator Award 17832; Grant sponsor: NIH; Grant numbers: R01 MH036197, P50 MH090966, U01 MH099225, R01 NS 061829-04, T32-MH65213; Grant sponsor: Sackler Institute for Developmental Psychobiology; Grant sponsor: Nationwide Children's Hospital, Columbus, OH.

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Article first published online in Wiley Online Library (wileyonlinelibrary.com): 00 Month 2015 DOI 10.1002/ajmg.b.32408

especially for relatives of children diagnosed with prepubertal MDD [Puig-Antich et al., 1989]. Therefore, samples enriched with early-onset cases yield higher power to detect genetic effects [Holmans et al., 2007]. Where exactly to draw the age cutoff, and/ or whether or not age should be modeled as a continuous variable, is unclear. It is, however, quite clear from epidemiologic and family studies that prepubertal onset of MDD is uncommon suggesting that, similar to many Mendelian disorders, rare and private genetic variants play a significant role. But, although studies of some psychiatric disorders, such as schizophrenia and autism, have led to the discovery of important candidate disease variants [Rodriguez-Murillo et al., 2012; Jeste and Geschwind, 2014], genetic studies of MDD have yet to reproducibly find any variants with strong genetic effects [Flint and Kendler, 2014; Levinson et al., 2014].

The lack of reproducible genetic findings for MDD can be interpreted a number of ways. The lack of strongly associated variants (e.g., via genome-wide association studies) could mean either that the majority of MDD genetic susceptibility is not due to common variants, or that the number of common variants required to reach the MDD threshold is too large to be detected by the sample sizes reported [Flint and Kendler, 2014]. Furthermore, the presence of multiple MDD subtypes might cause substantial genetic heterogeneity between studies that affect reproducibility. Therefore, it might be helpful to apply stricter phenotypic constraints, such as requiring early onset or multiple affected relatives. We hypothesize that such an approach, when combined with a variant filtering scheme designed to enrich for putative disease-causing variants, can allow us to identify genes with strong effects on MDD susceptibility.

Here, we perform whole-exome analysis of MDD families, with early onset and multiple affected individuals, to identify novel deleterious mutations shared among affected relatives. We sampled at least two individuals from five MDD families. Each family was required to segregate MDD with high (observed) penetrance and contain at least one prepubertal MDD case, as families of such individuals show increased MDD heritability [Puig-Antich et al., 1989; Holmans et al., 2007]. All individuals were phenotypically confirmed by multiple clinical assessments, a measure that has been shown to maximize observed heritability [Kendler et al., 1993]. In addition to choosing a sample in which effect sizes are predicted to be strong, we also designed our variant filtering scheme to enrich for variants that might affect disease. It has been proposed and observed that novel non-synonymous coding variants can strongly impact disease susceptibility [Gilissen et al., 2012; Ku et al., 2013] making them high-priority candidates for being causal alleles. In this study, we focus on novel coding mutations predicted to be deleterious and consider only variants shared among affected individuals in MDD families allowing us to, additionally, use the powerful tool of cosegregation/variantsharing analysis. We hypothesize that, given our focus on families where genetic effects are predicted to be strong and on mutations predicted to confer strong phenotypic effects, we have reasonable power to detect strong genetic effects, even with a small sample.

RESULTS Sample Selection

Drawing from a longitudinal study of multigenerational MDD families, in which individuals have undergone repeated and independent clinical assessments up to six times over 30 years, with final diagnosis made blind to any genotype data or clinical status of other family members, by a psychiatrist or psychologist [Warner et al., 1999; Weissman et al., 2005, 2006], we selected five families (Fig. 1) from which to sample affected relatives for variant discovery analysis. To focus on families in which the genetic effects might be particularly large, each of these families had a minimum of five affected individuals. In addition, each family had relatively low median ages of onset-that is, younger than 20 years old and ranging from 12 to 19.8 and an average median of 16.9-and at least one individual with a prepubertal MDD diagnosis (onset before age 12). We chose two individuals for whom DNA was available from each of these five families for whole-exome sequencing, with the exceptions of the large Families II and IV, where we chose to sample three affected individuals (Fig. 1).

Shared Novel Deleterious Variants

Whole exome sequencing revealed a total of 622,029 exome-wide variants across our sample, 234,425 of which were distinct. Figure 2A summarizes our subsequent filtering scheme. Filtering out all variants that were either intronic, synonymous, or predicted to be benign (see Methods section), left a total of 10,907 "deleterious," which include variants encoding amino acid substitutions predicted to adversely affect protein function or change total protein length and/or composition (i.e., indels, frameshifts, etc.) (Table I). Novel mutations have been hypothesized and observed to have an increased chance of affecting disease phenotypes [Gilissen et al., 2012; Ku et al., 2013]. Therefore, of the 10,907 deleterious variants, we focused only on the 1,776 variants in this set not found in any examined databases (see Methods section). Of these novel deleterious mutations, only 67 were shared among affected relatives within all families where they were found (while meeting variant quality control requirements [see Methods section]). This observed sharing indicates that these novel variants are not sequencing artifacts (as the chances the same error occurred twice in a family at the same position are exceeding low) and implicates them as potentially being involved in MDD. We, therefore, refer to the genes where these mutations were found as putative MDD candidate genes (Table II). Gene ontology analysis shows that 18 (27%) of these genes encode transcription factors and 32 (48%) are expressed in the human brain (Table II). Joint analysis of the 18 transcription factorencoding genes and the 32 brain-expressed genes shows that 8 of the 67 putative MDD genes encode transcription factors expressed in the brain (Fig. 2B). Compared to the number of transcription factors expressed in the brain (as designated using gene ontology [GO] analysis outlined in Methods section) this corresponds to an EASE score P-value of 0.007 suggesting significant enrichment. However, the true null distribution for transcription factors is not the list of all GO transcription factors, but rather the list of all transcription factors which would have



FIG. 1. The complete pedigrees of each of the five families from our study: half shaded shapes represent individuals with an MDD diagnosis at age 12 or below. The individuals chosen for initial exome sequencing are denoted with an asterisk and bold numbers.

variants that made it through our filtering scheme by chance from our exome data, which is more difficult to estimate. Therefore, this *P*-value can only be a, perhaps conservative, approximation. For the eight transcription factor encoding-genes expressed in the brain in our dataset, we checked whether variants of any frequency were found shared among affected relatives in our sample, as these variants might co-segregate with rare variants not captured in our exome sequencing. This was true for only two genes: *ZNF34* and the X-linked *TCEAL2*. The *ZNF34* gene (also called *KOX32*) is expressed in the developing



FIG. 2. A: Variants identified by whole-exome sequencing from five families were filtered to yield a list of 67 novel putative MDD genes. B: Among the putative novel MDD genes, many are transcriptions factors, and transcription factors expressed in the human brain [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmgb].

brain [Lorenz et al., 2010] and is a putative bipolar disorder (BPD) candidate gene, as it (i) is differentially expressed in BPD patients relative to healthy controls [Zhao et al., 2015], (ii) contains common variants that show gene-based association with BPD [Lee et al., 2013; Zhao et al., 2015], and (iii) is found in a region of chromosome 8q24 shown by multiple studies to be linked to BPD [Cichon et al., 2001; McInnis et al., 2003].

MDD ZNF34 Alleles

The novel *ZNF34* mutation we discovered was found shared in both (affected) exome-sequenced individuals in Family III. This mutation encodes a non-conservative P17R substitution in the ZNF34 protein Krüppel-associated box-A (KRAB-A) domain, an evolutionarily conserved motif required for the activity of Zn^{2+} finger transcrip-

tional repressors. This substitution has a CADD (combined annotation dependent depletion) C-score of 11.07 indicating that this variant is predicted to be more deleterious than over 90% of all possible substitutions in the human genome [Kircher et al., 2014]. In the 1,617 bp of coding sequence for the *ZNF34* gene, the exome variant server (http://evs.gs.washington.edu/EVS/) lists only two missense mutations reported more than once in the European American population, and even these variants are rare (rs149062206 = 17/8211 alleles, rs371047009 = 2/8596 alleles). We confirmed the presence of the P17R mutation in the two affected Family III relatives by Sanger sequencing. We, then, Sanger sequenced the locus in all Family III individuals for whom DNA was available (Supplemental Fig. S1) and found that all five affected members were carriers of the novel *ZNF34*P17R mutation and that the single available unaffected relative was not (Fig. 2). We calculated the exact probability of

TABLE I. Filtering Cascade of Variants in Each of the Five MDD Families

Filter	Family I	Family II	Family III	Family IV	Family V
Total number of unique variants	119,967	147,031	120,158	124,920	121,048
Exonic nonsynonymous variants	11,485	15,042	11,492	13,121	11,476
Deleterious variants	3,915	5,142	3,860	4,448	3,940
Novel variants	382	438	319	464	333
Shared heterozygous variants	18	2	18	14	15

observing the pattern of rare allele sharing among affected individuals (n = 5) in Family III for *ZNF34* P17R using the RVsharing algorithm [Bureau et al., 2014], and found the presence of this rare allele among affected relatives to be statistically significant (*P*-value = 0.026). Furthermore, the *ZNF34* P17R mutation showed complete segregation with MDD in Family III, as it was not observed in the unaffected individual examined. These data provide evidence that this rare variant, in a brain-expressed, putative BPD gene, might be involved in determining MDD susceptibility in, at least, this single family.

When removing the requirement that coding mutations be novel, the only other ZNF34 variant that remained in our dataset was the common indel rs3830702 (TTT/-) found in both exomesequenced (affected) individuals of Family I. We Sanger sequenced this variant in all members of Family I for whom DNA was available and observed complete segregation of the deletion (minus) allele with MDD (Fig. 1). It is improbable that this common variant (or some common variant in strong linkage disequilibrium) plays a role in MDD per se, as it would have likely already been detected by GWAS. However, it is possible that rs3830702 might be linked to some unobserved (perhaps non-coding) MDD variant in this particular family, a hypothesis that bears further investigation. Additionally, it can also not be ruled out that both the novel ZNF34 P17R-encoding variant in Family III and the common ZNF34 rs3830702 variant in Family I show complete segregation with MDD because of some unobserved (perhaps structural and/or non-exonic) variant nearby. Nonetheless, in summary, our results from the P17R-encoding variant in Family III and rs3830702 in Family I show two ZNF34 variants in our study segregating completely with MDD and with full, observed, penetrance in all individuals for whom DNA was available.

DISCUSSION

The identification of new candidate genes using longitudinally followed multigenerational families affected with MDD may be useful because the heritability is higher in these families than in population or twin samples. The inclusion of families studied with repeated measures may also increase the likelihood of identifying causative variants as evidence for heritability is increased with multiple phenotypic assessments [Kendler et al., 1993] (this may be due to increased accuracy of the diagnosis with repeated assessments and also having assessments obtained as the subject lives through the full age of risk.). Furthermore, families with prepubertal MDD cases have an increased chance of having other MDD relatives [Puig-Antich et al., 1989; Holmans et al., 2007]. We hypothesized that taking these considerations into account in our study design could afford us increased power to detect medium to strong genetic effects. Although large families have been studied in several psychiatric disorders, the current ability to rapidly sequence entire exomes allows us to identify rare and novel variants that might have been missed in previous studies. Through wholeexome sequencing of a relatively small family-based sample and selecting for families in which genetic effect sizes are hypothesized to be large, we were able to generate a list of novel putative MDD candidate genes/variants. Although the variants we report are novel and are predicted to introduce non-conservative changes in aminoacid sequence, their ability to affect disease phenotypes is particularly strong compared to other classes of variants [Gilissen et al., 2012; Ku et al., 2013]. Furthermore, since we required variants to be shared among affected MDD relatives, they are candidates for influencing the MDD phenotype. We found that many of the novel MDD candidate genes encode brain-expressed transcription factors and we validated extended segregation of a variant in one of these genes, the putative bipolar disorder (BPD) gene ZNF34.

ZNF34 and Transcription

By regulating the expression of many downstream effectors, transcription factors can influence a vast array of cellular events. Although candidate genes for neuropsychiatric disorders have focused on genes known to biologically affect, perhaps intuitive, disease-related processes-for example, neurotransmitter and ion channels-encoding genes-it is not surprising that variation in transcription factors can contribute strongly to complex disease phenotypes. In humans many of the C2H2 Zn²⁺ finger transcription factors are genomically encoded in large gene clusters and many of their biological functions remain unknown. The ZNF34 and ZNF418 (another brain-expressed transcription factor gene in found in our dataset [Table II]) gene products are closely related. They share 41% amino acid identity and each contains an N-terminal KRAB-A box and multiple C-terminal Zn²⁺ fingers. Both ZNF34 and ZNF418 show existing evidence for influencing affective disorder susceptibility. The Psychiatric Genetics Consortium (PGC) MDD GWAS mega-analysis found a P-value of 5×10^{-4} at the rs8102308 marker found ~25 kb downstream of ZNF418 in a chromosome 19 "ZNF "gene cluster [Lee et al., 2013]. Although this P-value fails to meet genome-wide significance after correcting for multiple tests, it likely does reach significance when correcting only for the number markers in brain-expressed transcription factors loci post hoc, an approach that our findings suggest is justifiable.

TABLE II. Exome-Wide Novel Deleterious Mutation Shared Between Affected MDD Relatives in This Study

1 7.89	CHR	BP	Class	Gene	TR	Familu
1 15,212,121 Nonspongnous SW KAZN V 1 38,079,553 Nonspongnous SW K5701 V 1 38,079,553 Nonspongnous SW K5701 V 1 226,005,573 Nonspongnous SW K7972 I 3 124,272,447 Nonspongnous SW K7471 I 4 189,526,17.0 Nonspongnous SW K7651 II 4 189,526,17.0 Nonspongnous SW LEMI1 II 4 189,526,17.0 Nonspongnous SW LEMI1 III 4 169,526,17.0 Nonspongnous SW LEMI1 III 4 169,514,4384 Nonspongnous SW LEMI1 V 5 101,100,752 Nonspongnous SW LEMI1 V 6 139,416,875 Nonspongnous SW LEMI1 V 8 19,416,874 Nonspongnous SW LPR II 8 19,414,8344 Nonspongnous SW LPR V 8 19,414,414 <td>1</td> <td>7.887.201</td> <td>Nonsunonumous SNV</td> <td>PER3</td> <td>+</td> <td>·</td>	1	7.887.201	Nonsunonumous SNV	PER3	+	·
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4 165,641,433 Nonsynonymous SNV <i>CPE</i> I 4 185,941,483 Nonsynonymous SNV <i>KHE</i> + V 5 101,100,762 Nonsynonymous SNV <i>KHE</i> V 7 66,39,250 Nonsynonymous SNV <i>LPL</i> V 8 19,816,875 Nonsynonymous SNV <i>LPL</i> V 8 19,816,875 Nonsynonymous SNV <i>LPL</i> V 8 134,446,974 Nonsynonymous SNV <i>LPL</i> V 8 146,4003,870 Nonsynonymous SNV <i>SVPI</i> V 9 139,273,619 Nonsynonymous SNV <i>SVPI</i> V 9 139,273,619 Nonsynonymous SNV <i>SVPI</i> V 10 16,96,633 Frameshift deletion <i>MAL01</i> III 10 75,886,642 Nonsynonymous SNV <i>ZSMMB</i> III 11 75,886,642 Nonsynonymous SNV <i>KK67</i> V 12 113,327,71 Nonsynonymous SNV <i>KK67</i> V 11 75,886,642 Nonsynonymous SNV <i>KK67</i> V <	4	120,190,897	Nonsynonymous SNV	USP53		111
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6 39,545,925 Nnssynonymous SNV <i>KVF6</i> V 7 6,639,520 Nnssynonymous SNV <i>C7arl26</i> 1 8 13,916,975 Nnssynonymous SNV <i>LPL</i> NV 8 23,915,950 Nnssynonymous SNV <i>LPL</i> NV 8 134,146,974 Nonsynonymous SNV <i>LPL</i> NV 8 134,146,974 Nonsynonymous SNV <i>ZN34</i> + III 9 133,127,17 Nonsynonymous SNV <i>SVFP1</i> V 9 139,273,619 Nonsynonymous SNV <i>SVMPC4</i> + III 10 69,961,657 Nonsynonymous SNV <i>MVPN</i> I I 10 75,896,452 Nonsynonymous SNV <i>AP3M1</i> III III 11 75,896,518 Nonsynonymous SNV <i>AV2M1</i> III III 12 11,332,27,191 Nonsynonymous SNV <i>MVPN</i> III III 10 75,896,318 Nonsynonymous SNV <i>AV2M1</i> IIII IIII	4	185,941,483	Nonsynonymous SNV	HELT	+	V
5 111107,762 Nonsynonymous SNV ASEC3 + V 7 6,639,520 Nonsynonymous SNV <i>LPL</i> I 8 19,316,875 Nonsynonymous SNV <i>LPL</i> IV 8 29,195,960 Nonsynonymous SNV <i>DUSPA</i> I 8 134,146,974 Nonsynonymous SNV <i>DUSPA</i> I 8 134,146,974 Nonsynonymous SNV <i>TG</i> V 9 133,273,619 Nonsynonymous SNV <i>SVEP1</i> - III 10 69,961,657 Nonsynonymous SNV <i>SVEP4</i> + III 10 75,552,350 Nonsynonymous SNV <i>AVINH</i> III 10 12,93,03,139 Nonsynonymous SNV <i>SUN420H</i> + I 11 67,926,194 Nonsynonymous SNV <i>SUN420H</i> + I 12 113,386,812 Nonsynonymous SNV <i>SUN420H</i> + I 12 113,386,812 Nonsynonymous SNV <i>SUN420H</i> I I	6	39,545,925	Nonsynonymous SNV	KIF6		V
2 6,639,520 Nonsynonymous SNV <i>LPL</i> I 8 19,816,875 Nonsynonymous SNV <i>LPL</i> IV 8 29,159,560 Nonsynonymous SNV <i>DLSFA</i> I 8 153,145,674 Nonsynonymous SNV <i>T6</i> V 8 146,003,870 Nonsynonymous SNV <i>ZMF3A</i> + III 9 13,13,717 Nonsynonymous SNV <i>SMFCA</i> + III 10 19,876,539 Frameshift deletion <i>MALROL</i> III III 10 75,596,642 Nonsynonymous SNV <i>ZSNIMB</i> III III 10 72,896,642 Nonsynonymous SNV <i>ZSNIMB</i> III III 11 67,926,194 Nonsynonymous SNV <i>SUM</i> 2DH1 + I 12 11,339,274 Nonsynonymous SNV <i>SUM</i> 2DH1 + I 12 11,348,58,11 Nonsynonymous SNV <i>SUM</i> 2DH1 + I 13 24,200,859 Nonsynonymous SNV <i>SUM</i> 2DH1	6	101,100,762	Nonsynonymous SNV	ASCC3	+	V
8 19.816.875 Norsynonymous SNV LPL NV 8 29.195.960 Nonsynonymous SNV DUSP4 I 8 13.41.46.974 Nonsynonymous SNV IE V 8 13.41.46.974 Nonsynonymous SNV IE V 9 13.92.73.619 Nonsynonymous SNV SKAFC4 + III 10 6.9.961.657 Nonsynonymous SNV SKAFC4 + III 10 6.9.961.657 Nonsynonymous SNV ZSIWIAB III III 10 75.552.350 Nonsynonymous SNV AZWIAB III III 10 12.9.903.139 Nonsynonymous SNV AZWIAB III III 11 67.326.194 Nonsynonymous SNV MCI67 V V 12 11.338.224 Nonsynonymous SNV MCI67 III 12 12.3.654.672 Nonsynonymous SNV MAPKAFK5 II 13 24.2.00.859 Nonsynonymous SNV MAPKAFK5 II 14 </td <td>7</td> <td>6,639,520</td> <td>Nonsynonymous SNV</td> <td>C7orf26</td> <td></td> <td>I</td>	7	6,639,520	Nonsynonymous SNV	C7orf26		I
8 25,195,00 Nonsynampuous SNV DUSPA I 8 134,146,974 Nonsynampuous SNV T6 V 9 113,13,717 Nonsynampuous SNV ZVF34 + III 9 133,273,619 Nonsynampuous SNV SVEP1 V 9 139,273,619 Nonsynampuous SNV SVEP1 III 10 19,676,539 Frameshift deletion MARD01 III 10 75,552,550 Nonsynampuous SNV ZSWMB IIII 10 75,562,614 Nonsynampuous SNV MYPN IIII 11 67,966,452 Nonsynampuous SNV MKR7 V 11 67,966,452 Nonsynampuous SNV UCP2 V 12 11,339,224 Nonsynampuous SNV UCP2 V 12 112,323,791 Nonsynampuous SNV MAFKAPK5 II 12 112,3694,672 Nonsynampuous SNV MAFKAPK5 IV 13 24,200,859 Nonsynampuous SNV MAFKAPK5 IV 14 12,3846,672 Nonsynampuous SNV MAFKAPK5	8	19,816,875	Nonsynonymous SNV	LPL		IV
8 55,493,637 Nonframeshift isertion BHLHE22 + II 8 134,146,974 Nonsynonymous SNV 76 V 9 113,137,717 Nonsynonymous SNV SVF1 V 9 139,273,619 Nonsynonymous SNV SVF1 VI 10 19,676,539 Frameshift deletion MALROI III 10 69,961,657 Nonsynonymous SNV ZSWMR III 10 75,582,350 Nonsynonymous SNV ARAND III 11 67,926,194 Nonsynonymous SNV ARADD III 11 67,926,194 Nonsynonymous SNV BUR2P2 V 12 113,332,24 Nonsynonymous SNV BUR2P2 V 12 113,332,791 Nonsynonymous SNV MAPARAF5 III 13 24,200,859 Nonsynonymous SNV MRED1 V 14 12,354,677 Nonsynonymous SNV MRED3 III 13 26,170,680 Nonsynonymous SNV MRED3 II	8	29,195,960	Nonsynonymous SNV	DUSP4		I
8 134,146,974 Nonsynonymous SNV 76 V 8 144,003,874 Nonsynonymous SNV ZVF34 + III 9 133,27,17 Nonsynonymous SNV SVVF1 V 9 139,273,619 Nonsynonymous SNV SVVF24 + III 10 19,676,539 Frameshift deletion MLD01 III 10 75,552,350 Nonsynonymous SNV ZSWNR III 10 75,552,350 Nonsynonymous SNV ZSWNR III 10 75,956,452 Nonsynonymous SNV ZSWNR III 11 67,926,194 Nonsynonymous SNV KUR27 V 12 11,339,224 Nonsynonymous SNV ILCP2 V 12 11,339,224 Nonsynonymous SNV KDR24 III 12 11,339,224 Nonsynonymous SNV KDR24 III 12 11,339,224 Nonsynonymous SNV KDR24 III 13 24,200,843,372 Nonsynonymous SNV KDR44	8	65,493,637	Nonframeshift insertion	BHLHE22	+	11
8146,003,870Nonsynonymous SNVSNF34+III9113,137,717Nonsynonymous SNVSVEP1V9139,273,619Nonsynonymous SNVSMAPC4+III1019,676,539Frameshift deletionMALRD1III1069,661,657Nonsynonymous SNVZSWIM8III1075,959,452Nonsynonymous SNVAP3M1III1167,926,194Nonsynonymous SNVZSWIM8III1211,339,224Nonsynonymous SNVUCP2V1211,339,224Nonsynonymous SNVUCP2V12113,365,612Nonsynonymous SNVMARAKSIII12120,984,379Nonfameshift deletionRNF10V1321,375,026Nonsynonymous SNVMP04I1321,375,026Nonsynonymous SNVMP04III1325,840,377Nonsynonymous SNVMP05FH9V1560,298,061Nonsynonymous SNVMRR6III1667,397,493Nonsynonymous SNVFDXB1+V1751,18,314Nonsynonymous SNVFDXB1+V1667,397,493Nonsynonymous SNVFDXB1+I1751,18,261Nonsynonymous SNVFDXB1+V1844,398,314Nonsynonymous SNVFDXB1+I1932,63,434Nonsynonymous SNVFDXB1+I1774,60,933Frameshift deletion <td< td=""><td>8</td><td>134,146,974</td><td>Nonsynonymous SNV</td><td>TG</td><td></td><td>V</td></td<>	8	134,146,974	Nonsynonymous SNV	TG		V
9 113,132,712 Nonsynonymous SNV SVEP1 V 9 139,273,619 Nonsynonymous SNV SVAPC4 + III 10 19,675,539 Frameshift deletion MALRD1 III 10 75,552,350 Nonsynonymous SNV MYPN III 10 75,552,350 Nonsynonymous SNV AP3M1 III 10 129,903,139 Nonsynonymous SNV AP3M1 III 10 129,903,139 Nonsynonymous SNV SU420H1 + I 11 67,926,194 Nonsynonymous SNV SU4224 V 12 113,39,224 Nonsynonymous SNV MAPKAPK5 I 12 113,865,812 Nonsynonymous SNV SDSL II 12 120,894,677 Nonsynonymous SNV MP100 IV 13 24,200,859 Nonsynonymous SNV MP103PH9 V 13 24,200,859 Nonsynonymous SNV MP103PH9 V 13 24,200,372 Nonsynonymous SNV	8	146,003,870	Nonsynonymous SNV	ZNF34	+	
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10 19,676,539 Frameshift deletion $MALRD1$ III 10 69,961,657 Nonsynonymous SNV MPN I 10 75,522,510 Nonsynonymous SNV $ZSWIM8$ III 10 75,896,452 Nonsynonymous SNV $ZSWIM8$ III 10 129,903,139 Nonsynonymous SNV $MKI67$ V 11 67,326,194 Nonsynonymous SNV $SUV42DH1$ + I 12 11,339,224 Nonsynonymous SNV $MZP2$ V 12 113,865,812 Nonsynonymous SNV $MSLA$ III 12 123,694,677 Nonsynonymous SNV $MPACPSF19$ V 13 24,200,659 Nonsynonymous SNV $MPAGS$ III 13 24,840,377 Nonsynonymous SNV $MRE6$ III 15 60,298,061 Nonsynonymous SNV $MRE6$ III 15 62,170,843 Nonsynonymous SNV $FDRB1$ + V 16 67,386,141 Nonsynonymous SNV $FDRB1$ + V 15 62,170,843	9	139,273,619	Nonsynonymous SNV	SNAPC4	+	Ш
10 69,961,657 Nonsynonymous SNV MYPN I 10 75,552,350 Nonsynonymous SNV ZSWNR III 10 75,856,452 Nonsynonymous SNV AP3M1 III 10 128,903,139 Nonsynonymous SNV AP3M1 III 11 67,926,194 Nonsynonymous SNV UCP2 V 12 11,339,224 Nonsynonymous SNV MAPAR/S I 12 113,39,224 Nonsynonymous SNV MAPAR/SER42 IV 12 113,39,224 Nonsynonymous SNV MSER42 III 12 123,694,677 Nonsynonymous SNV MSER4 II 13 21,375,026 Nonsynonymous SNV MFID IV 13 24,200,859 Nonsynonymous SNV MRFSF19 V 13 25,840,377 Nonsynonymous SNV MRB6 III 13 76,179,899 Nonsynonymous SNV MRB7 V 15 60,238,011 Nonsynonymous SNV FOKB1 + V 16 67,318,314 Nonsynonymous SNV FOKB1 + </td <td>10</td> <td>19.676.539</td> <td>Frameshift deletion</td> <td>MALRD1</td> <td></td> <td>Ш</td>	10	19.676.539	Frameshift deletion	MALRD1		Ш
10 75,552,350 Nonsynonymous SNV ZSWIM8 III 10 75,836,452 Nonsynonymous SNV AP3M1 III 11 67,926,194 Nonsynonymous SNV SUV420H1 + I 11 67,926,194 Nonsynonymous SNV SUV420H1 + I 11 73,688,018 Nonsynonymous SNV UCP2 V 12 112,323,791 Nonsynonymous SNV MAFK4PK5 I 12 112,323,791 Nonsynonymous SNV MAFK4PK5 I 12 123,694,677 Nonsynonymous SNV MAFK4PK5 IV 13 24,270,0859 Nonsynonymous SNV MPIOSPH9 V 13 24,200,859 Nonsynonymous SNV MFK10 III 14 375,026 Nonsynonymous SNV MIMR6 III 15 60,298,061 Nonsynonymous SNV IV V 15 60,298,061 Nonsynonymous SNV FDXB1 + V 16 67,286,149 Nonsynonymous SNV FDXB1 + I 16 67,286,149 No	10	69,961,657	Nonsynonymous SNV	MYPN		I
10 75,896,452 Nonsynonymous SNV AP3M1 III 10 129,903,139 Nonsynonymous SNV MKI67 V 11 67,926,194 Nonsynonymous SNV SUV420H1 + I 11 73,688,018 Nonsynonymous SNV UCP2 V 12 11,339,224 Nonsynonymous SNV MAZRAPK I 12 113,865,812 Nonsynonymous SNV MAPKAPKS III 12 120,984,379 Nonframeshift deletion RNF10 IV 13 21,375,026 Nonsynonymous SNV MPROSPH9 V 13 25,840,377 Nonsynonymous SNV MRRS III 13 26,840,377 Nonsynonymous SNV MMRS III 14 39,861 Nonsynonymous SNV IV IX 15 60,298,061 Nonsynonymous SNV FX IX 15 62,170,843 Nonsynonymous SNV FX IX 16 67,236,149 Nonsynonymous SNV FX IX </td <td>10</td> <td>75,552,350</td> <td>Nonsynonymous SNV</td> <td>ZSWIM8</td> <td></td> <td>Ш</td>	10	75,552,350	Nonsynonymous SNV	ZSWIM8		Ш
10 129,903,139 Nonsynonymous SNV MKI67 V 11 67,926,194 Nonsynonymous SNV UV420H1 + I 11 73,680,018 Nonsynonymous SNV UCP2 V 12 11,339,224 Nonsynonymous SNV TAS2R42 IV 12 113,855,812 Nonsynonymous SNV MAPKAPK5 I 12 120,984,379 Nonframeshift deletion RNF10 IV 13 21,356,94,677 Nonsynonymous SNV MPH0SPH9 I 13 24,200,859 Nonsynonymous SNV MPH0SPH9 I 13 25,840,377 Nonsynonymous SNV MIRR6 III 13 76,179,899 Nonsynonymous SNV MIRR6 III 14 33,681 Nonsynonymous SNV WCHJ3 I 15 60,298,061 Nonsynonymous SNV WD80 IV 15 62,170,843 Nonsynonymous SNV FAM192A I 16 67,381,484 Frameshift deletion PRH7 + I 17 5,118,261 Nonsynonymous SNV L	10	75,896,452	Nonsynonymous SNV	AP3M1		Ш
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11 73,688,018 Nonsynonymous SNV UCP2 V 12 11,339,224 Nonsynonymous SNV TAS2R42 IV 12 112,323,791 Nonsynonymous SNV SDSL III 12 113,865,812 Nonsynonymous SNV SDSL III 12 120,984,379 Nonfynonymous SNV SDSL III 12 123,694,677 Nonsynonymous SNV MPH0SPH9 IV 13 24,200,859 Nonsynonymous SNV MPH0SPH9 III 13 25,840,377 Nonsynonymous SNV MIMR6 III 13 76,179,899 Nonsynonymous SNV UCHL3 I 15 62,170,843 Nonsynonymous SNV INR80 IV 15 62,170,843 Nonsynonymous SNV VS13C V 16 67,38,144 Nonsynonymous SNV FAM192A I III 16 67,397,493 Nonsynonymous SNV ELM03 III I 16 67,397,493 Nonsynonymous SNV ELM03 III I 16 68,381,548 Frameshift delet	11	67,926,194	Nonsynonymous SNV	SUV420H1	+	I
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	19	44,223,770	Nonsynonymous SNV	IRGC		IV

TABLE II. (Continued)						
CHR 19	BP 49,416,392	Class Nonsynonymous SNV	Gene NUCB1	TR	Family IV	
19	53,740,407	Frameshift insertion	ZNF677	+	III	
19	58,438,626	Nonsynonymous SNV	ZNF418	+	III	
20	52,199,337	Nonsynonymous SNV	ZNF217	+	V	
21	45,212,597	Nonsynonymous SNV	RRP1		IV	
22	20,920,847	Nonframeshift deletion	MED15	+	IV	
Х	53,595,767	Nonsynonymous SNV	HUWE1		IV	
Х	101,381,945	Nonsynonymous SNV	TCEAL2	+	I	
Х	129,150,059	Nonsynonymous SNV	BCORL1	+	IV	
Transcriptional r	egulators (TR) expressed in the brain are	e highlighted and in bold.				

Analysis of the PCG BPD data showed ZNF34 to yield a genebased association P-value of 0.018 in a study [Zhao et al., 2015] that also showed the ZNF34 gene to be differentially expressed between BPD patients and healthy controls. Furthermore, ZNF34 is found in a region of chr8 showing linkage to BPD in multiple studies [Cichon et al., 2001; McInnis et al., 2003]. Our validation that novel coding mutations and common variants in ZNF34 can be found co-segregating with MDD supports a hypothesis that transcription factors are plausible candidates for genetic studies of MDD. That ZNF34 is expressed in the developing brain [Lorenz et al., 2010] points to a possible developmental window through which the circuitry influencing MDD is particularly susceptible. As a transcriptional repressor, the ZNF34 gene product might be responsible for maintaining a gene-expression program in the developing brain that is protective against MDD later in life, and that the P17R variant is compromised for such a function. In addition, the potential involvement of ZNF34 in BPD may represent a kind of variable expressivity and genetic overlap between MDD and BPD, a topic of much discussion in the field of affective disorders. It is worth noting that while the complete observed segregation and penetrance of the ZNF34P17R-encoding variant suggests a monogenic model of early-onset MDD in Family III, it is possible that other genetic and/ or environmental factors also contribute to the phenotype.

Modeling MDD as a Genetic Disease

The study designs, sample sizes and resulting findings from previous MDD studies, subjects the possible genetic models for MDD susceptibility to several constraints [Flint and Kendler, 2014], including the minimum number of the genes involved, their frequencies, and effect sizes. By introducing a list of novel candidates with potentially large effect sizes, our study helps generate testable hypotheses that can further constrain these parameters to develop a more tractable model of MDD genetics than that which currently exists. Being able to estimate better the number of rare MDD variants/genes with strong effect sizes of common MDD variants and allows us to test and model phenotypic interactions between combinations of rare and common alleles across the genome.

One proposed explanation for the lack of reproducibility for positive findings in the MDD literature is that there are dozens or hundreds of common MDD alleles—certain combinations of which exceed some threshold for MDD expression—and thus, extremely large sample sizes are required to detect their effects. However, it is plausible to assume that both common and rare variants affect most complex disorders. Flint and Kendler [2014] discussed a model in which rare variants with large effect sizes in MDD genes might be detected by focusing on "more homogeneous heritable phenotypic groupings." Our ability to identify *ZNF34* in our study using multiple-affected, early onset families and focusing on novel variants is potential evidence for bearing this out. Another strength of our approach is our ability to assess co-segregation, which helps make up for the relatively small sample size in our study. For example, the linkage studies that discovered many causative variants for highly penetrant disorders often relied on single families to map loci of strong effect sizes.

Whether or not brain-expressed transcription factors play a more generalizable role in more common forms of depression (i.e., later onset and from families with fewer affected individuals) is yet to be determined. Nonetheless, the new candidate genes we identify here, including the putative bipolar disorder gene *ZNF34*, which we validate, may point to, as yet unknown, molecular substrates for MDD. Therefore, understanding the biology and function of these genes may yield useful insight that might help in the prediction and treatment of MDD and related psychiatric disorders.

METHODS

Subjects, Sequencing, and Genotyping

All subjects were recruited from the longitudinal study of extensively phenotyped mutil-generational families, the methods are fully described in [Weissman et al., 2006]. The purpose of the study was to determine the transmission of MDD and other disorders in offspring and then grandchildren in high versus low risk families with risk defined as depression in Generation 1 of the identified proband. Probands (Generation 1) were identified with moderate to severe major depression and were attending a medication clinic for treatment. The controls (not depressed probands) were selected from a population-based study in the same community and were matched on gender and age. All subjects reported Caucasian ancestry. All offspring and then grandchildren were interviewed by clinically trained personnel blind to the clinical status of the probands or previous interviews. Extensive assessments included refined research methods developed for this sample and have been fully described [Weissman et al., 2005, 2006]. The data were then reviewed by a clinical psychiatrist or psychologist and a best estimate diagnosis was made blind to the status of the proband in Generation 1. The subjects were interviewed up to six times over 30 years. DNA was collected in the fifth and sixth wave which was the 25 and 30 year follow-ups.

We performed exome sequencing on all 12 individuals from our discovery phase (see Results section). Capture, sequencing, alignment, mapping, and variant calling were performed by Axeq Technologies (Rockville, MD). Capture was performed using Agilent SureSelect V4+UTR, which targets 71 Mb from 20,965 genes and the resulting libraries sequenced using an Illumina HiSeq 2000. We targeted to obtain $100 \times$ raw data in order to achieve $50 \times$ on target depth. BWA algorithm was then used to align the sequenced reads to the UCSC hg19 reference genome and variants detected using SAMTOOLS. The large list of variants was then subject to multiple rounds of filtering to narrow it down to the most probable candidate set of causative variants. The average Phred (Q) score across all individuals was 119.3, corresponding to an average 1.2×10^{12} probability of erroneous variant calling, and the average total read depth was 57.4. The ZNF34 variants found in our discovery phase were confirmed and further interrogated in family members by Sanger sequencing.

Variant Selection and Statistical Analysis

The variant lists, output by SAMTOOLS, were subject to a two way filtering process. The first filtering process was to produce a list of shared, novel, deleterious mutations. In order to get this list, we filtered out all nonsynonynous and splice variants from the 12 individuals in the discovery sample. We, then, chose only variants designated as predicted disease causing variants by either SIFT, LRT, or MutationTaster. Novel variants from each dataset were then selected by excluding all variants present in either 1000 Genomes, The Exome Sequencing Project, Complete Genomics, dbSNP, COS-MIC, NCI databases. All variants that met the novel and deleteriousness criteria also had to be shared among all affected members within a family. Finally, for novel mutations that were shared, we removed variants with a read depth <10 in all individuals for whom the variant was found. Deleteriousness of the ZNF34 P17R-encoding mutation was assessed by combined annotation dependent depletion (CADD), which scores the effect of individual substitutions relative to all possible substitutions in the human genome [Kircher et al., 2014]. Functional annotation of the genes harboring the novel, deleterious variants (obtained in the above step) was retrieved using DAVID [Huang da et al., 2009a, 2009b] online gene ontology function (http://david.abcc.ncifcrf.gov), and the reported EASE score P-values were used to determine significance [Hosack et al., 2003]. Used in gene enrichment analysis, the EASE score is a version of a Fisher exact test modified to be conservative, but not as conservative as implementing Boferroni or Benjamini-Hochberg FDR multiple test correction techniques, which can compromise sensitivity in gene enrichment applications [Huang da et al., 2009a]. For calculation of the EASE score P-value for the enrichment of brain-expressed transcription factors the background value for this group in the genome was determined using two steps. All genes annotated with the gene ontology term "transcription"

(GO:0006351) in Uniprot (http://www.uniprot.org) were retrieved (n = 2,500). This list was then used as input for DAVID which reported the number expressed in the brain to be n = 1009.

ACKNOWLEDGMENTS

This work was supported by Brain and Behavior Research Foundation (formerly NARSAD) Young Investigator Award 17832, NIH grants R01 MH036197; P50 MH090966; U01 MH099225; and R01 NS 061829-04; T32-MH65213, The Sackler Institute for Developmental Psychobiology, and by Nationwide Children's Hospital, Columbus, OH. We thank David A. Greenberg for computational and other resources, Iulinana Ionita-Laza and Esther N. Drill for insightful input and members of the Greenberg lab for editorial assistance.

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