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Deletion of TOP3β, a component of FMRP-containing mRNPs, contributes to neurodevelopmental disorders

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Abstract

Implicating particular genes in the generation of complex brain and behavior phenotypes requires multiple lines of evidence. The rarity of most high impact genetic variants typically precludes the possibility of accruing statistical evidence that they are associated with a given trait. We show here that the enrichment of a rare Chromosome 22q11.22 deletion in a recently expanded Northern Finnish sub-isolate enables the detection of association between *TOP3β* and both schizophrenia and cognitive impairment. Biochemical analysis of TOP3β revealed that this topoisomerase is a component of cytosolic messenger ribonucleoproteins (mRNPs) and is catalytically active on RNA. The recruitment of TOP3β to mRNPs was independent of RNA *cis*-elements and was coupled to the co-recruitment of FMRP, the disease gene product in fragile X mental retardation syndrome (FXS). Thus, we uncover a novel role for TOP3β in mRNA metabolism and provide several lines of evidence implicating it in neurodevelopmental disorders.

Introduction

Disruptions in messenger RNA (mRNA) metabolism play an important role in the etiology of human disease[1]. Genetic studies have identified disease-causing mutations in several known mRNA binding proteins, while molecular biology approaches have connected disease genes of unknown function to mRNA metabolism. Here, we combine both methods to show that deletion of the topoisomerase TOP3β is associated with neurodevelopmental disorders, and identify a function for this DNA-processing enzyme in mRNA metabolism.

The Finnish disease heritage (FDH) refers to rare Mendelian disorders (http://www.findis.org/) that are more prevalent in Finland than elsewhere in the world and that vary substantially in frequency between different parts of Finland. This variation reflects extreme genetic drift at the causative loci, due to multiple population bottlenecks that have generated sub-isolates within the overall Finnish isolate, in particular in Northeastern Finland[2, 3]. Drift can also dramatically elevate the frequencies, in such isolates, of rare alleles with a high impact on common traits[2]. It is unlikely that any single variant could substantially influence the overall population risk for specific disorders, such as schizophrenia. We hypothesized, however, that enrichment in Northern Finland for multiple high-impact variants could contribute to an increased frequency of particular classes of disorders in this region compared to other parts of country, acting along with environmental influences such as socioeconomic factors[4, 5].

The prevalence of several neurodevelopmental phenotypes in Finland follows a striking gradient from Northeast to Southwest (Fig. 1), as exemplified by schizophrenia, which is almost three times more prevalent in Northeastern sub-isolates than in Finland overall[4, 6, 7]. Considerable evidence supports the association of large deletions, both overall variant burden as well as specific variants, with a wide range of neurodevelopmental disorders, including schizophrenia and cognitive impairment[8, 9]. We previously showed an association between the burden of large deletions and neurodevelopmental phenotypes in a Northern Finnish population cohort, and established that several of these deletions represent founder mutations, carried on haplotypes shared identical by descent[10]. We hypothesized that some smaller deletions might be sufficiently increased in frequency in Northern Finland to enable the detection, by analyses of single-variants, using currently available study samples, of associations to schizophrenia and other neurodevelopmental phenotypes that are particularly frequent in this region. As we show here, the enrichment of a rare Chromosome 22q11.22 deletion in the Northern Finnish sub-isolates enabled analyses suggesting *TOP3β*, which is wholly contained within the deletion, as a risk gene for neurodevelopmental disorders.

TOP3β encodes the DNA topoisomerase III β. Our biochemical investigations of TOP3β revealed functions for this protein that could explain the phenotypic impact of its deletion. In particular, it was strikingly and unexpectedly associated with FMRP, an RNA binding protein, that normally inhibits the translation of neuronal mRNAs[11, 12]. The deregulated expression of these mRNAs, in the absence of this protein is the molecular defect underlying FXS[13] (OMIM#300624). The process through which FMRP-containing mRNPs form remains ill-defined; however, recent investigations of the functional impact of the highly pathogenic I304N *FMR1* missense mutation suggest that protein-protein interactions may contribute to the recruitment of FMRP to its target mRNAs[14].

Our studies have uncovered a mechanism for such a recruitment: The Tudor domain containing protein 3 (TDRD3), which itself binds to the exon junction complex (EJC)[15] deposited on newly spliced mRNAs, mediates the concomitant integration of TOP3ββ and FMRP into mRNPs. Our demonstration of TOP3β topoisomerase activity on RNA substrates suggests its involvement in the metabolism of FMRP-bound mRNAs.. Together with the results from genetic analyses of the Northern Finnish 22q11.22 deletion and from independent investigations of *de novo TOP3β* mutations[16-18], this biochemical evidence indicates the important role of this protein in neurodevelopment.

Results

Identification of deletions enriched in Northern Finland

Using genome-wide SNP arrays, we searched for deletions enriched in the Northeastern sub-isolates compared to the rest of Finland, in representative samples from each population (Sub-isolate Population Sample, N=173; Whole Finland Population Sample, N=1586)[19, 20], (see Online Materials and Methods and Table S1 for descriptions of all cohorts used in this study). To minimize false positives we considered only deletions of > 20 kb in length, detected with ≥10 probes. We identified, in the combined samples, 5,313 putative deletions (5,004 heterozygous, 309 homozygous) comprising 1,041 distinct genomic loci, with a mean size of 89.4 kb.

Two deletion loci, on chromosomes 4q12 and 22q11.22, displayed significantly higher frequencies in the Sub-isolate Population Sample compared to the Whole Finland Population Sample (empirical p-value < 0.001 after 10,000 permutations), were in Hardy-Weinberg Equilibrium, and were confirmed by manual inspection of intensity distribution plots (Fig. S1). The 4q12 deletion (181.7 kb) occurred in 6/173 individuals from the Sub-isolate Population Sample and was not observed in the Whole Finland Population Sample (p= 8.7×10^{-7}, Fisher's Exact Test) while the 22q11.22 deletion (243.9 kb) occurred in 18/173 individuals from the Sub-isolate Population Sample and only in 1/1586 individuals from the Whole Finland Population sample (p= 8.55×10^{-18}, Fisher's Exact Test).

Shared SNP haplotypes among deletion-carriers indicated a single ancestral origin for each deletion. Carriers of the 4q12 deletion shared a 2.248 Mb haplotype (248 kb proximal and 2.0 Mb distal to the deletion), while carriers of the 22q11.22 deletion shared a 289 kb haplotype (167 kb proximal and 122 kb distal to the deletion). The presence of the shared 4q and 22q haplotypes among Finnish non-carriers of the deletions (population frequencies of 1.1% and 13.0%, respectively, assuming full LD between their proximal and distal portions) indicate that both deletions arose relatively recently.

Neither the 4q12 deletion nor the shared haplotype in this region overlapped with known genes. The 240 kb 22q11.22 deletion lies within the 1.4 −2.1 Mb distal 22q11.2 microdeletion syndrome region[21]. Several studies have implicated this region, ~400kb telomeric to the region deleted in velocardiofacial syndrome (VCFS)[22], in both schizophrenia and developmental delay[9, 23, 24]. The deletion described here encompasses two possible genes; *TOP3β*, is a stable protein-coding gene predicted by all algorithms, and with an unambiguous genomic position, while *IGLV2-14* is a predicted transcript of unknown function, whose position has shifted between different genome builds, and which shares sequence homologies with immunoglobulin variants. Two complementary low copy repeats (chr22:20638870-20642866 and chr22:20904962-20908959) flank the 22q11.22 deletion and likely facilitated its formation (Fig. 2a).

Association of the 22q deletion with schizophrenia

To assess whether the increased frequencies of the 4q12 and 22q11.22 deletions in the Northeastern sub-isolate are related to risk for neurodevelopmental disorders, we compared previously genotyped samples; 185 individuals diagnosed with schizophrenia (Sub-isolate Schizophrenia Sample[25]) with 747 unscreened controls (Sub-isolate Control Sample). The 22q11.22 deletion showed significantly increased frequency in the Sub-isolate Schizophrenia Sample (OR = 1.84, p < 0.03), while the 4q12 deletion showed no such difference (p = 0.14) (Table 1).

We additionally assessed the association between the 22q11.22 deletion and schizophrenia in case-control samples from other parts of Finland and from elsewhere in Europe, using large samples given the rarity of the deletion outside of the sub-isolate (Whole Finland Schizophrenia Sample[23] and Whole Finland Control Sample, 467 cases and 11,124 controls; International Schizophrenia Consortium, ISC[23], and Swedish Schizophrenia Consortium, SSC[26], 9176 cases and 9529 controls). The deletion displayed nominal association with schizophrenia in the Whole Finland samples (OR = 2.63, p = 0.008, after controlling for

genomic inflation [λ = 1.20]) but not in the non-Finnish samples, although the effect was in the same direction (OR = 2.17, p= 0.12; Table 1). The combined estimate of significance was p=0.007 (OR = 1.84, 95%-CI: 1.18 - 2.87), with no heterogeneity of effect observed between the three samples.

Association of the 22q deletion with cognitive impairment

We analyzed the relationship between the 4q12 and 22q11.22 deletions and seven neurodevelopmental phenotypes evaluated previously with respect to large deletions in 4,872 genotyped members of the Northern Finland 1966 Birth Cohort (NFBC1966)[10], a geographically representative population cohort, drawn from the Northeastern sub-isolates as well as the surrounding, less genetically isolated regions; we excluded 59 individuals diagnosed with schizophrenia who were included in the schizophrenia case-control analyses. As with the larger deletions investigated previously in NFBC1966[10], carriers of the 22q11.22 deletion showed significantly higher frequencies of intellectual deficit compared to non-carriers (OR = 4.6, 95%-CI: 1.41-14.96, p = 0.03), and an even greater overrepresentation of milder learning difficulties (OR= 5.9, 95%-CI: 1.78-8.94, p=0.003) (Table 2). The deletion carriers, however, did not show a significantly increased risk for psychosis (excluding diagnosis of schizophrenia), although the effect was in the predicted direction (OR = 4.1, 95%-CI: 0.55-30.17, p = 0.2). No phenotypes were significantly overrepresented among 4q12 deletion carriers.

Cognitive function in schizophrenic deletion carriers

Schizophrenia is associated with cognitive impairments, and this association may reflect a common genetic basis for these phenotypes[27]. Given the observed association of the 22q11.22 deletion with both schizophrenia and cognitive impairment, we evaluated cognitive performance in relation to both diagnosis and deletion status in 566 schizophrenic subjects (19 deletion carriers and 547 non-carriers), each of whom is the proband in a family from the Finnish Schizophrenia Family Study (comprising the majority of the individuals in the Sub-isolate and Whole Finland Schizophrenia Samples). Deletion carriers performed worse than non-carriers in tests assessing verbal memory after short delay (B = −1.5, p =0.03) and showed a similar trend for other measures, although not reaching statistical significance (Table S2). Within the pedigrees of which the 19 deletion carriers are members, we observe no apparent segregation with neurodevelopmental phenotypes of the 22q11.22 deletion, in heterozygous form (Fig. S2), as expected given the magnitude of the effects observed in the association analyses.

Phenotypic impact of the 22q deletion, in recessive form

We identified, in NFBC1966 and the Sub-isolate Schizophrenia Sample, four homozygous carriers of the 22q11.22 deletion, three of whom descended from consanguineous matings (Fig. 2b). All four individuals displayed cognitive impairment, ranging from poor performance on tests of executive function and information processing to moderate mental retardation. Two of them also carried a diagnosis of schizophrenia.

TOP3β expression is disrupted among 22q deletion-carriers

Given the limited genomic information and lack of prior genetic evidence relating to *IGLV2-14*, we chose to focus on *TOP3β;* prior studies have suggested that *de novo TOP3β* mutations may contribute to neurodevelopmental disorders[16, 17], and we hypothesized that disruption of this gene could account for the observed phenotypic effects of the 22q11.22 deletion. Microarray analysis in 65 individuals (51 non carriers, 12 heterozygous carriers, and two homozygous carriers drawn from 18 families of the Finnish Schizophrenia Family Study) confirmed the simple dosage-dependent effect of the deletion on *TOP3β* expression (probe 4900386, Fig. 2c).

The deletion had no genome-wide significant effect on mRNA levels of other genes, either in 22q or elsewhere in the genome. However, 813 genes (858 of 9872 probes) showed nominal difference in mRNA levels between deletion carriers and non-carriers ($p<0.05$). From this set of 813 genes, we evaluated whether any functional gene category, plausibly affected by the down-regulation of TOP3β would be enriched among deletion carriers compared to non-carriers. Genes involved in two related categories, translation and ribosomal complexes displayed a 3.1 and a 2.8 -fold enrichment, respectively, ($p = 6.2 \times 10^{-8}$ and 2.8×10^{-10}, Table S3), suggesting the possibility that the 22q11.22 deletion exerts its phenotypic effect via disturbed translational regulation. We were unable to detect, in deletion carriers, an enrichment of differentially expressed FMRP target genes[12] (data not shown). For the majority of the FMRP target genes (458/843), expression levels in peripheral blood were below the threshold for detection. These 458 undetectable genes were, in contrast to the 385 detected genes, overrepresented in gene categories related to neuronal functioning, suggesting that this negative result may reflect our lack of the most biologically relevant tissues for this analysis.

A complex of TOP3β, TDRD3 and FMRP binds early mRNPs

To gain insight into the cellular function of TOP3β we generated a stable cell line allowing the expression of FLAG/HA-tagged TOP3β. Immunoprecipitations with anti-FLAG antibodies uncovered an interaction of TOP3β with TDRD3 and FMRP (Fig. 3a). These proteins co-purified in an RNAse insensitive manner, illustrating a direct rather than RNA-mediated interaction. TDRD3 is a multidomain protein that, in addition to its EJC-binding motif (EBM), contains an N-terminal OB-fold domain (NTD) of unknown function and a C-terminal FMRP-interacting motif (FIM)[16, 17]. TOP3β bound to the NTD (Fig. S3a-c), suggesting that TDRD3 is a bridging factor in a heterotrimeric TOP3β-TDRD3-FMRP (TTF) complex (Fig. S3d). Immunoprecipitation of TOP3β also resulted in the RNAse-sensitive co-purification of the EJC component MAGOH and the cytosolic poly(A)-binding protein PABPC1 (Fig. 3a), suggesting that TTF is associated with spliced mRNPs. TTF exists in mouse brain lysates and NSC-34 cells, indicating its presence in neuronal cells (Fig. 3b and Fig. S3e).

TOP3β is a stress granule component and binds RNA directly

FMRP and TDRD3 are nucleo-cytoplasmic shuttling proteins and localize predominantly to the cytosol under steady state conditions[14, 28]. The identification of TOP3β as an interactor of these proteins prompted us to analyze its subcellular distribution. Immunofluorescence

microscopy, expression of GFP-tagged fusion protein and subcellular fractionation all indicated that TOP3β also is mostly cytosolic (Fig. 3c and S4a, b). In addition, upon arsenite treatment or strong overexpression, TOP3β co-localized with TDRD3 and FMRP in cytosolic foci reminiscent of stress granules, storage sites of translationally silenced mRNAs[29] (Fig. 3c and S4c, d). Treatment of cells with the nuclear export-inhibitor leptomycin-B or deletion of the putative nuclear export signal shifted GFP-TOP3β to the nucleus (Fig. S4e-g). Thus, all components of the TTF complex exhibit similar intracellular localization and trafficking. To test whether TOP3β directly associates with mRNAs, we purified mRNA-bound proteins using oligo(dT)-cellulose, and captured TOP3β, along with FMRP and PABPC1, in an RNAse sensitive manner (Fig. 3d). In a vice versa experiment, we performed UV crosslinking and immunoprecipitation and found cellular RNAs efficiently crosslinked to FLAG/HA-TOP3β (Fig. 3e). These results indicated that TOP3β is a component of cytosolic mRNPs.

TOP3β is catalytically active on RNA

TOP3β is a type IA topoisomerase that catalyzes transient breakage of a DNA strand by the reversible transesterification between an active site (AS) tyrosyl and a DNA 5′-phopshoryl group[30]. Cleaved intermediates can be released by SDS-denaturation of the enzyme during its catalytic cycle (Fig. 4a). The ability of TOP3β to directly bind RNA (see Fig. 3e) encouraged us to test whether TOP3β might be active on RNA substrates. Indeed, the addition of RNA inhibited cleavage of 5′-labeled DNA by recombinant TOP3β (Fig. 4b), indicating that both molecules compete for the same active site (Fig. 4c). Also, incubation of TOP3β with 5′-labeled RNA resulted in a specific cleavage product (Fig. 4d). The formation of covalent complexes of TOP3β and 3′-labeled RNA was consistent with a 5′-phosphotyrosyl intermediate (Fig. 4e). Of note, we observed a high molecular weight smear in Western blot analysis of FLAG/HA-TOP3β oligo(dT)-purified from cellular extracts (Fig. 3d, lane 2). Reasoning that this smear might represent covalent complexes between FLAG/HA-TOP3β and RNA, we eluted oligo(dT)-purified FLAG/HA-TOP3β from the column and treated the eluate with RNAse (Fig. 4f). RNAse treatment eliminated the FLAG-positive high molecular weight species, suggesting that TOP3β is active on mRNA substrates *in vivo*.

TTF-containing mRNPs are associated with polyribosomes

To analyze whether TTF-bound mRNPs are associated with the translation machinery, we first purified translation factors from HeLa cells (Fig. 5a). As reported for FMRP[31], TDRD3 and TOP3β were present in crude ribosomal pellets but dissociated upon high-salt treatment (Fig. 5b), suggesting that they are components of translating mRNPs.

Western blot analysis of polysome gradient centrifugation from stable FLAG/HA-TDRD3 cell extracts showed that FLAG/HA-TDRD3 and endogenous TOP3β co-migrated with FMRP in polysomal fractions in an RNAse sensitive manner (Fig. 5c), further demonstrating that part of the TTF-bound mRNP pool is undergoing translation. This observation is noteworthy considering the binding of TDRD3 to the EJC[15], as the latter is thought to be removed from the mRNA during the first round of translation[32]. A hallmark of mRNPs that participate in this “pioneer round” of translation is their association with the nuclear cap

binding proteins CBP20 and CBP80 instead of eIF4E[32]. Immunoprecipitation of TTF-containing mRNPs via FLAG/HA-TDRD3 revealed their binding to CBP20 and CBP80, but not to eIF4E (Fig. 5d), suggesting that TTF is a component of mRNPs undergoing the pioneer round of translation.

TTF recruitment to mRNPs requires the TDRD3 Tudor domain

To dissect the mechanisms leading to TTF formation and integration into mRNPs, we generated TDRD3-mutants in which individual protein-protein interactions were disrupted (Fig. 6a). We established stable cell lines expressing FLAG/HA-tagged versions of these mutants and analyzed them by immunoprecipitation as well as polysome gradient centrifugation. Wildtype TDRD3 formed the TTF complex (Fig. 6b) and became integrated into polysome-bound mRNPs (Fig. S5a). Deletion of the binding sites for FMRP ($TDRD3_{\Delta FIM}$) or TOP3β ($TDRD3_{\Delta NTD}$) resulted in the loss of the respective protein from TTF (Fig. 6b and S5b). However, integration of the remaining binary complexes into mRNPs and polysomes was unaffected (Fig. 6b and S5a, b), indicating that the recruitment of TDRD3 to the EJC can occur independently of TTF formation. Mutation of the EBM ($TDRD3_{EBMmut}$), in contrast, abolished integration of TDRD3 into mRNPs while binding of TOP3β and FMRP were unaffected (Fig. 6b and S5a). Thus, formation of the TTF complex occurs before TDRD3 is tethered to the EJC.

Lastly, we analyzed a missense mutation of the Tudor domain ($TDRD3_{TDRmut}$), impairing the recognition of asymmetrically dimethylated arginine (aDMA) modifications in the C-terminal domain of RNA Polymerase II (RNAPII CTD) and in the tails of Histones H3 and H4 at the transcription start site (TSS)[33, 34]. As several mRNA processing factors are recruited to the nascent transcript via the RNAPII CTD, we reasoned that TDRD3 might follow a similar route. Indeed, $TDRD3_{TDRmut}$ showed strongly reduced association of TDRD3 with mRNPs (Fig. 6b and S5a). While this mutation did not affect the assembly of a TOP3β-TDRD3 heterodimer, it diminished the recruitment of FMRP (Fig. 6b). Thus, the generation of TTF-containing mRNPs requires the recognition of aDMA by TDRD3. Although FIM and EBM reside outside of the Tudor domain[14, 15], we wanted to exclude the possibility that the latter contributes to FMRP or EJC binding, as aDMA modifications have been reported for FMRP and other mRNP components[35]. We incubated purified TDRD3 with cellular extracts and analyzed for bound FMRP and MAGOH, observing no differences between wildtype and Tudor mutant protein (Fig. 6c, lanes 2 and 3). This finding was specific, as $TDRD3_{EBMmut}$ failed to associate with MAGOH (Fig. 6c, lane 4). Therefore, mutating the Tudor domain *per se* does not influence the binding of TDRD3 to FMRP or the EJC. Instead, the defect of $TDRD3_{TDRmut}$ in TTF formation and mRNP incorporation appears to be a consequence of its impaired binding to aDMA-modified histones and/or RNAPII.

Formation of TTF containing mRNPs requires TDRD3

The above results uncovered a mechanism for the concomitant integration of TOP3β, TDRD3 and FMRP into mRNPs. To investigate whether this is the only pathway for the biogenesis of mRNPs containing TOP3β and FMRP, we used a cell line carrying a deletion encompassing *TDRD3*[36]. Gradient centrifugation revealed that in these TDRD3-deficient

cells, the association of TOP3β with polysomes was diminished (Fig. 6d). However, upon transfection of FLAG/HA-TDRD3, TOP3β became redistributed into the polysomal fractions. Thus, TDRD3 is necessary and sufficient for the recruitment of TOP3β to mRNPs. Overexpressed $TDRD3_{EBMmut}$ was not associated with polysomes and consequently failed to increase the amount of polysomal TOP3β (Fig. 6d and S6a), indicating that the EJC is the only docking platform for TTF on mRNPs.

FMRP, by contrast, associated with polysomes in TDRD3-deficient cells (Fig. S6b). This observation suggested that TTF-containing mRNPs constitute only a subpopulation of the FMRP-bound pool and implied that alternative routes exist for integrating FMRP into mRNPs. Hence we analyzed whether the presence of TDRD3 is a prerequisite for recruitment of FMRP into a specific, TTF-containing mRNP subpopulation. For this purpose, we transfected TDRD3-deficient cells either with GFP-TOP3β alone or together with FLAG/HA-TDRD3 variants and assayed them for TTF and mRNP formation via anti-GFP immunoprecipitations. Consistent with the results shown in Figure 6d, GFP-TOP3β association with FMRP and mRNPs was entirely dependent on cotransfected wildtype TDRD3 (Fig. 6e, lanes 6 and 8). In contrast, co-transfection of FLAG/HA$TDRD3_{\Delta FIM}$ enabled the formation of mRNPs containing GFP-TOP3β but these mRNPs were entirely devoid of FMRP (Fig. 6e, lane 7). Together, these data indicated that TDRD3 is essential for the integration of FMRP into the subpopulation of mRNPs that contain the TTF complex.

Discussion

Our finding of association between neurodevelopmental disorders and a 22q11.22 deletion – vastly increased in frequency in Northern Finnish sub-isolates and occurring on a single haplotype – demonstrates the special value of such populations for identifying variants with a high impact on common phenotypes. Projects underway that aim to comprehensively genotype a large proportion of the Finnish population[37] should be able to exploit such enrichment of functional variants to identify a wide range of trait-associations that in most populations could require unfeasibly large samples. As with the 22q11.22 deletion, this enrichment could also facilitate the identification of homozygotes in whom the phenotypic impact of such variants is more extreme.

Large-scale implementation of the above strategy for identifying high-impact variants must address the concern that standard methods of controlling for population stratification may not work well for rare variants[38]. Such stratification is unlikely to be a major issue in our study for several reasons. First, in Northern Finland the 22q11.22 deletion has an allele frequency of ~3-5%, and thus is not a rare variant. Second, stratification is of less concern in populations, where substructure has a clear geographical basis[38], and where fine-grained information is available about the geographical origin of the study subjects is available, as is the case in multiple Finnish cohorts; several studies have demonstrated the high degree of correlation, in such cohorts, between geographical origin and measures of stratification obtained from genome wide SNP data[2, 39]. Additionally, implementation of newly developed methods for correcting for stratification at rare variants[40] may facilitate application of our strategy to a wider range of populations.

The population impact in Northern Finland of the 22q11.22 deletion is likely much greater than yet recognized, given its frequency there and its association with quantitative measures of neurocognitive impairment. The predominant effect of the 22q11.22 deletion appears to be on measures of neurocognitive function rather than on risk for specific diagnostic entities, as evidenced by the range of phenotypes displayed by the homozygous deletion carriers, and within the population cohort in which we have observed trait-associations. It may be feasible to more precisely delineate the phenotypic effect of this deletion, as well as other deletions that could have a similar effect, by obtaining more complete neurocognitive assessments in larger samples from the Northern Finland population. The accumulation of deletion genotype-phenotype associations that cut across classical disease categories has the potential to enhance our understanding of mechanisms underlying disorders of brain and behavior. For example, our results suggest that large-scale investigations of deletions in schizophrenia study samples phenotyped for neurocognitive measures may contribute to the genetic dissection of this phenotypically heterogeneous syndrome.

The phenotypic impact of the deletion reported here likely derives from its effect on *TOP3β*; the deletion directly disrupts this gene, down regulates its mRNA levels, and does not appear to alter transcript levels of genes outside the deletion region. This interpretation is consistent with the findings from a recent report of a single individual, of unspecified ethnic origins, who demonstrated speech delay and minor physical abnormalities and carried a deletion that appears to be identical to the one presented here[18]. Analysis of animal models with a genetically equivalent mutation may help clarify whether Top3β plays an important role in synaptic plasticity.

Although the deletion also incorporates *IGLV2-14*, and we cannot exclude the possibility that its loss contributes to the phenotypes that describe here, it is merely a predicted transcript of unknown function, for which no independent mutations have been reported in either the literature or databases. In contrast, recent surveys of *de novo* mutations have yielded both direct and indirect genetic evidence suggesting that *TOP3β* missense variants contribute to schizophrenia and autism spectrum disorders[16, 17]. The independent forms of genetic evidence represented by the deletions and *de novo* variants, are complementary, and taken together strengthen the suggestion that *TOP3β* plays an important role in neurodevelopment.

Biochemical analysis revealed that TOP3β is part of a heterotrimeric TOP3β-TDRD3-FMRP (TTF) complex. Similar to our study, an accompanying paper shows that Top3β interacts with FMRP via TDRD3, and demonstrates that Top3β modifies dFMR1 function in the Drosophila neuromuscular junction synapse formation[41]. Our data suggest a stepwise model for TTF formation (Fig. S7a): TDRD3 reads aDMA marks on histones and RNAPII[33, 34], a process that recruits a TDRD3-TOP3β heterodimer to the TSS. After binding of FMRP, the resulting heterotrimeric complex transfers to an EJC on the nascent transcript. Thus, in this scenario, the recruitment of FMRP into mRNPs does not require direct binding of FMRP to mRNA. Instead, recognition of aDMA marks at the TSS by TDRD3 determines whether the resulting transcript is a target for FMRP. In line with this conclusion, the genes bound by TDRD3 at their TSS overlap significantly with those that acquire FMRP as a component of their mRNPs (Fig. S7b). Our data further show that other

routes of FMRP recruitment to mRNPs must exist, as FMRP-containing mRNPs still form in TDRD3 deficient cells, and our model does not rule out that FMRP – after being bound to the EJC – directly contacts its target mRNAs, offering an explanation for the unexpected observation that FMRP binding sites are dispersed throughout the coding regions on many transcripts[12, 42]. In addition, this model predicts that, in the absence of TOP3β, mRNPs that normally contain TTF are loaded only with a TDRD3-FMRP heterodimer (Fig. S7a).

The fate of the EJC-bound TTF complex, and the functional consequences of this interaction are currently unknown. TTF-containing mRNPs associate with CBP20/80 but not with eIF4E, suggesting a role of TTF during the pioneer round of translation[32]. EJC-associated factors like the RNA surveillance complex or the protein kinase S6K1 rigorously control this step. The latter is – like the TTF complex – specifically recruited to the EJC, increasing the translational efficiency of the pioneer round[43]. S6K1 phosphorylates FMRP and is an effector of the mammalian target of the rapamycin (mTOR) pathway, which controls activity-dependent translation in neurons[44]. The EJC might hence serve as a molecular scaffold to integrate the actions of FMRP and mTOR in regulated neuronal translation.

We note that the TTF complex bears striking similarity to the BLM-TOP3α-RMI1 complex (BTR)[45], implicated in the catalytic dissolution of replication-induced DNA four-way junctions[46]. Similar four-way junctions present in RNA can be interconverted into kissing complexes, which are known FMRP binding sites[47, 48]. Our data hence raise the possibility that TOP3β remodels FMRP binding sites. Nevertheless, other functions of TOP3ß in mRNA metabolism or even in completely unrelated cellular processes also appear feasible and deserve further investigation.

The identification of TTF uncovered an unexpected biochemical link between Fragile X patients and *TOP3ß* deletion carriers. This discovery raises two alternative scenarios: 1) Neurodevelopmental disorders traditionally considered distinct share common etiologies; under this scenario, the dysregulation of TTF-containing mRNPs may underlie the overlapping neurocognitive impairment phenotypes observed in Fragile X syndrome and schizophrenia. 2) Alternatively, different mutations in *TOP3ß* that affect different functions of the gene result in different forms of psychopathology, and it is possible that deletion of this gene contributes to neurodevelopmental phenotypes through as yet unidentified non-FMRP pathways.

Future biochemical and enzymatic analyses of pathogenic *TOP3ß* missense mutations may enable us to reject one or the other of these scenarios. However the second scenario implies a greater level of specificity and certainty with respect to the definition and etiology of neuropsychiatric syndromes, than is suggested by currently available data. For example, Fragile X syndrome and schizophrenia share important phenotypic features that make it unsurprising that they could also share underlying biological processes. Deficits in working memory and other executive functions are perhaps the most salient neurocognitive features of schizophrenia[49], and are also frequently observed in Fragile X males[50]. Impairment in cognitive function is also a feature of autism spectrum disorders, and it is possible that FMRP-related abnormalities contribute to cognitive dysfunction in all of the disorders under discussion here. The systematic identification of neuronal TTF mRNA targets and their

functional analysis in relation to neuronal plasticity will help to clarify whether this is the case.

Online Materials and Methods

The study samples

Table S1 summarizes features of the study samples. The relevant local and/or national ethical review committees approved all of the studies contributing samples. All participants provided written informed consent.

Sub-isolate Population Sample—The Sub-isolate Population Sample consisted of 173 participants, selected from FINRISK, a periodic nation-wide health survey cohort of randomly selected, representative population samples from different parts of the country, focused on risk factors for chronic and noncommunicable diseases[19]. The 1997 and 2007 surveys included individuals from Northeastern sub-isolate municipalities. We selected all FINRISK participants with both parents originating from the sub-isolate for the Sub-isolate Population Sample.

Whole Finland Population Sample—Individuals in the Whole Finland Population Sample (N = 1,586) participated in the Helsinki Birth Cohort Study (HBCS), drawn from 8,760 individuals born in the Helsinki Central Hospital between 1934 and 1944, who attended child welfare clinics in Helsinki, and lived in Finland in 1971. The individuals comprising the Whole Finland Population Sample participated in a clinical study of HBCS that involved collection of DNA samples from all participants.[20]

Sub-isolate and Whole Finland Schizophrenia Samples—The schizophrenia subjects included in this study derived from three sources: the nationwide Finnish Schizophrenia Family Study, the Health 2000 nationwide health survey (H2000), and the Northern Finland Birth Cohort 1966 (NFBC 1966). Most of these subjects (N = 566) derived from the Finnish Schizophrenia Family Study, in which the investigators used three national registers, the Hospital Discharge Register, the Free Medicine Register and the Pension Register to identify 33,731 individuals diagnosed schizophrenia between 1969-1998, drawn from a cohort of individuals born between 1940 and 1976. Treating physicians contacted potential probands; for those who provided written informed consent, they also contacted additional family members. Independent review of materials by psychiatrists blind to the family structures led to consensus best-estimate lifetime diagnoses according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV)[25].

The 566 subjects selected from the schizophrenia study were members of independent nuclear families. The Sub-isolate Schizophrenia Sample included 171 of these individuals who originated from the Northeastern Finland sub-isolate. The Whole Finland Schizophrenia sample included those who originated outside of the sub-isolate (N=395). We identified the rest of the Finnish schizophrenia subjects included in this study (14 in the Sub-isolate Schizophrenia Sample, and 72 in the Whole Finland Schizophrenia Sample) among participants of H2000 and NFBC1966 diagnosed with DSM III-R or DSM IV schizophrenia

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or schizoaffective disorder using multiple sources of information, including structured clinical interviews and medical records.

Sub-isolate Control Sample and Whole Finland Control Sample—These samples are distinct from those that we used to investigate regional differences in deletion frequencies, and consist of participants of nationwide Finnish health surveys and population cohorts; NFBC1966, the Northern Finland 1986 Birth Cohort (NFBC1986), H2000, The Cardiovascular Risk in Young Finns study (YFS), the Finnish Twin Cohort (FTC), and the Dietary, Lifestyle and Genetic determinants of Obesity and Metabolic syndrome study (DILGOM). The cohorts comprised altogether 11,871 individuals. Of these individuals, the Sub-Isolate Control Sample included 747 who originated from the Northeastern Finland sub-isolate, while the Whole Finland Control Sample included the remaining 11,124 individuals.. Descriptions of the individual cohorts are below.

Northern Finland 1966 and 1986 cohorts—The study subjects were members of two prospective birth cohorts consisting of 96% and 99% of all live-born children in the two most Northern provinces of Finland in 1966 and between July 1985- June 1986 (NFBC1966 and NFBC1986, respectively)[10, 51]. NFBC1966 began with collection of prenatal clinical data on 12,068 live born children and has continued with follow-ups at multiple time points that generated a phenotype data base combining information from official registers, hospital records, questionnaires and clinical examinations of the participants. The present study included 4931 genotyped individuals from this cohort[10], 59 with a DSM-III-R based diagnosis of schizophrenia or schizoaffective disorder, and 4872 without such diagnoses (members respectively of either the Sub-isolate Schizophrenia Sample/Sub-isolate Control Sample or Whole Finland Schizophrenia Sample/Whole Finland Control Sample, depending on their parents' birthplace). We additionally used the 4872 "controls" to evaluate the relationship between the 4q12 and 22q11.22 deletions and other neurodevelopmental phenotypes previously examined in this cohort in relation to larger deletions[10].

NFBC1986 consists of 9,340 live born children, prospectively followed since their 12th gestational week. The last follow-up to date, at age 16 years (years 2001-2002), included 6645 individuals who gave consent to participate in the study. The Sub-isolate Control Sample includes NFBC1986 individuals (N=212), with both grandparents originating from the Northeast Finland sub-isolate[51].

Health 2000—Health 2000 (H2000) is a nationally representative cross-sectional health study carried out in 2000 - 2001 and includes 8028 individuals. We selected 2,402 H2000 participants for the current study as follows: The majority (N=2212) were cases and controls from a sub-study of genetic risk factors for metabolic syndrome, of whom 27 met DSM-IV diagnosis for schizophrenia or schizoaffective disorder, included as cases in the present study[52]. The remaining 190 individuals were controls in a large European schizophrenia consortium, SGENE[53].

The Cardiovascular Risk in Young Finns Study—The Cardiovascular Risk in Young Finns Study (YFS) is a follow-up study of cardiovascular risk factors from childhood to adulthood, in study participants randomly chosen from the Population Register and recruited

from five university cities in Finland. The baseline study launched in 1980 and included 3596 individuals. Follow-ups have occurred on average every three years, most recently in 2007, when participants were 27 years old. The present study included 2308 individuals from YFS with available GWAS data[54].

Finnish Twin Cohort (FTC)—We ascertained 1492 individuals (one twin, chosen randomly, per pair) from the genotyped participants of the Finnish Twin Cohort, FTC. The FTC includes both monozygotic and dizygotic twins, collected in several separate phases. The older cohort included 13,888 same-sex and 8000 opposite sex pairs with known zygosity. The younger Finnish twin cohorts, referred to as FinnTwin12 and FinnTwin16, are longitudinal studies of behavioral development and health habits of Finnish twins enrolled at ages 11-12 and 16, respectively. These cohorts included 4913 pairs of both monozygotic and dizygotic pairs[55].

Dietary, Lifestyle and Genetic determinants of Obesity and Metabolic syndrome study (DILGOM)—A subset of individuals, aged between 25 and 74, from the FINRISK 2007 health survey participated in the DILGOM sub study. The present study included the 612 genotyped individuals from DILGOM as members of the Whole Finland Control Sample[56].

The International Schizophrenia Consortium (ISC)—The ISC sample consisted of 3,391 schizophrenia subjects, diagnosed according to DSM-IV or ICD 10, and 3181 controls, all of European origin. A previous publication detailed the data set and the CNV Calls (obtained using Birdseye, from the intensity data of the genotype probes on the Affymetrix 5.0 and 6.0 genotyping arrays[23].

The Swedish Schizophrenia Cohort (SSC)—The SSC sample included individuals in Sweden hospitalized at least twice for schizophrenia since 1973 (N= 5785). The SSC controls (N = 6348), group matched with the cases regarding age, sex, and county of residence had no hospitalization due to psychiatric diagnoses.

GWAS and CNV calling—Genome-wide genotyping studies utilized the following arrays: Illumina Human670K customBeadChip (HBCS, NFBC1986, YFS, FT TWINS, DILGOM, and parts of the Schizophrenia Family Study sample), Illumina Infinium HDHuman610-Quad BeadChip (DILGOM and the majority of the H2000 sample), Illumina HumanHap300 Chip (the remainder of the schizophrenia subjects and H2000 controls, included in the SGENE consortium), Illumina HumanHap370 Chip (NFBC1966), and Affymetrix 5.0 and 6.0 genotyping arrays (ISC and SSC). We utilized QuantiSNP[57] and PennCNV[58] software to make CNV calls, employing the LogR Ratio (LogRR) and B-allele frequency (BAF) of SNP probes included in the genotyping arrays. We called CNVs independently for each cohort and carried out QC based on graphical clustering of LogRR or BAF standard deviations, removing outlier samples from each cohort. In all cohorts we excluded individuals if their LogRR or BAF standard deviations were over 0.35 and 0.07, respectively; waviness factor was outside the range −0.04 - 0.04; and BAF drift exceeded 0.002, according to software recommendations.

We conducted a genome-wide scan for deletions (>20 kb in size) in the Sub-isolate and Whole Finland Population Samples, reading deletion genotypes from QuantiSNP and including only deletions with > 10 probes and Bayes factor over 10. To ensure reliable high quality data, we visually inspected the intensity distributions for deletions that showed significant deviation in frequency between the Sub-isolate and Whole Finland samples and tested them for Hardy-Weinberg equilibrium. For the other data sets we utilized deletion calls intersecting the previously identified regions on chromosomes 4 and 22. We validated the 22q11.22 in a subset of 258 individuals (19 carrying the deletion) with Sequenom MassARRAY and quantitative real-time PCR (QRT-PCR). The deletion genotypes had 100% correspondence with those from GWAS arrays.

Statistical analysis—We discarded from the analysis breakpoints including more than 88 overlapping deletions (frequency > 5%) in the 1,759 individuals. We used PLINK software[59] to cluster individuals to sub-isolate and whole Finland groups, according to parental origin; compare the frequencies of deletions (>20 kb, at the start and end point of each deletion) between the Sub-isolate Population Sample and the Whole Finland Population Sample; and calculate allelic Chi-square p-values. We calculated empirical p-values after 10,000 permutations for each deletion breakpoint. For testing associations with schizophrenia and other traits we used allelic Fisher exact tests and Conchran-Mantel-Haenszel statistics.

Gene expression analysis—Using an Illumina HumanHT-12 v4 Expression BeadChip we generated genome-wide gene expression data for 65 individuals from 18 families of the Finnish Schizophrenia Family Study, at the FIMM Technology Centre. To study the direct impact of the 22q11.22 deletion on *TOP3B*, we studied its average expression levels (probe 4900386) in heterozygous carriers (N = 12 individuals), homozygous carriers (N = 2), and non-carriers (N = 51), and used R (MA-ANOVA program package) to analyze the effect of carrying this deletion on genome-wide gene expression levels, including. only the 9872 probes that showed signal above background levels in all individuals. Since this analysis was exploratory, we derived q-values to determine if any of the observed significant changes have occurred beyond the level of false discovery. The pathway analysis carried out for 813 genes (858 probes) showed nominal statistical difference ($p < 0.05$) between deletion carriers and non-carriers, using the functional annotation tool in DAVID bioinformatics resources 6.7[60].

Antibodies—A previous publication described the primary antibody against FMRP[14]. Purchased primary antibodies include: FLAG (Sigma # A2220), HA (HISS # MMS-101R), GFP (Roche # 11814460001), TDRD3 (Bethyl # A310-983A), TOP3β (Abcam # ab56445), PABPC1 (Abcam # ab21060), GAPDH (Abcam # ab9485), HISTONE H3, rpL7 (Abcam # 72550), MAGOH (Santa Cruz # sc-271405), CBP80 (Santa Cruz # sc-271304) and CBP20 (Santa Cruz # sc-48793), eIF2γ (Proteintech # 11162-1-AP), eIF4E (Abcam # ab1126).

Plasmids and mutagenesis—We cloned cDNAs of TDRD3 (wt or truncations) and TOP3β in a pcHA/DNA5/FRT/TO plasmid (Invitrogen) containing an N-terminal FLAG-tag (for stable cell line preparation), a pGEX-6P-1 Vector containing a C-terminal 6xHis-stop

sequence (for protein expression) or a pEGFP C1 vector (for immunocytochemistry or IP), and introduced point mutations and deletions into the individual constructs by PCR mutagenesis.

Cell culture, plasmid transfections—The culture medium for all cells was DMEM containing 10% FCS and 1% Pen/Strep (PAA). Stable cell line medium additionally contained balsticidin and hygromycin (both Invivogen); tetracycline addition (1:2000) 5h prior to harvesting induced protein-expression in these cells. We carried out all transfections using Nanofectin™ (PAA). Generation and cultivation of HEK293 FlpTRex cell lines (Invitrogen) was according to manufacturer's instructions. We purchased the TDRD3 deficient human lung adenocarcinoma cell line JCRB0814 [VMRCLCD] from the JCRB Cell Bank of the National Institute of Biomedical Innovation.

Immunocytochemistry and stress treatment—For immunocytochemistry and stress treatment we followed procedures described previously[16]. We treated cells with 50 ng/ml Leptomycin B for 3 h to block nuclear export.

Co-immunoprecipitations (IPs)—We performed IPs with 25μl of α-FLAG M2 agarose (Sigma) or protein-G Sepharose (GE-Healthcare) bound α-GFP-antibodies (Roche) per ml cell extract. After adding lysis buffer (150 mM NaCl, 50 mM Tris pH 7.5, 2.5 mM $MgCl_2$ and 0.5% NP40, 0.5 mM DTT, protease inhibitors) and incubation on ice for 10 min, we cleared extracts by centrifugation (11000 rpm, 10 min, 4°C) before IP for 1 h (4°C). Subsequent to extensive washing of the beads with lysis buffer, we eluted precipitated complexes with 1% SDS (15 min, 70°C) and subjected them to SDS PAGE and Western blot. Dounced and cleared mouse brain homogenate (in ice-cold IP lysis buffer, see above) served for immunoprecipitations, with the indicated antibodies bound to Protein G-Sepharose (GE Healthcare).

Cross-linking and immunoprecipitation (CLIP)—We grew HEK293 FlpTRex-TOP3β cells or HEK293 control cells on 15cm plates to ~90% confluency and UV-cross-linked them 5 h later (254 nm, CL-1000 Ultraviolet Crosslinker). We then incubated harvested cells with lysis buffer containing protease inhibitors (50 mM HEPES pH 7.5, 150 mM KCl, 2 mM EDTA, 0.5% NP40, 1 mM DTT) for 10 min on ice. After lysate clearing (11000 rpm, 10 min, 4°C), a treatment with RNAse T1 followed (1:10000, 7 min, 22°C) (Ambion 1000 U/μl). We cooled the extracts subsequently on ice for 5min and incubated them with pre-equilibrated magnetic FLAG M2 agarose (SIGMA) (1 h, 4°C). We then washed the beads (washing conditions: 50 mM HEPES pH 7.5, 300 mM KCl and 0,05% NP40) before adding different RNAse T1 concentrations (1:100000, 1:10000, 1:1000 or 1:100) and repeated the washing procedure (3x with high salt buffer (50 mM HEPES pH 7.5, 500 mM KCl, 0,05% NP40 and 3x with phosphatase buffer (10 mM Tris-HCl pH.8, 100 mM KCl, 5 mM $MgCl_2$ and 0.2% Triton X-100). Dephosphorylation of co-precipitated RNAs occurred within 40 min at 37°C using Fast AP (Fermentas). We 5′-labeled RNAs on washed beads using [γ-^{32}P] ATP (Perkin Elmer) and PNK (Fermentas) (1 h, 37°C). After additional washing 5x with labeling buffer, we boiled the beads in SDS sample buffer and subjected eluates to gel electrophoresis (NuPAGE Bis-Tris 4-12%, Life Technologies) and

subsequent Western blotting on nitrocellulose. α-FLAG antibodies enabled us to detect precipitated FLAG/HA-TOP3β, and exposing the blot membrane to an X-ray film allowed the detection of the RNA.

oligo(dT) pulldown—We performed oligo(dT) pulldown assays using oligo(dT) cellulose (Fluka) according to manufacturer's instructions. We analyzed the oligo(dT) bound mRNPs by SDS-PAGE and Western blot as described above either after Mock-treatment or after digestion with RNAse A. (Qiagen).

Cleavage assays—We labeled RNA (GGGAUUAUUGAACUGUUGUUCAAGCGUGGU) or DNA (GGGATTATTGAACTGTTGTTCAAGCGTGGT) oligomers at the 5′-end using [γ-^{32}P] ATP (Perkin Elmer) and PNK (Fermentas) (1 h, 37°C) or at the 3′-end using [5′-^{32}P] pCp and T4 RNA Ligase (Fermentas) (over night at 4°C). For cleavage assays, we incubated ~ 40 fmol of radiolabeled oligomers with recombinant TOP3β wildtype or active site mutant (Y336F) (amounts are indicated in the individual experiment) at 42°C in buffer containing 40 mM HEPES pH6.5, 10% PEG400, 40% glycerol, 1 mM MgCl2, 1 mM DTT and 10 μg/ml BSA for 2 min. SDS at a final concentration of 0,2 % and incubation for 1 min stopped the reaction. We analyzed release of 5′-fragments on 12% UREA PAA-Gels, and covalent 3′-labeled TOP3β-RNA intermediates on a 10% SDS PAA-Gel. Detection of recombinant TOP3β occurred via Western blotting using anti-TOP3β antibodies, detection of RNAs via autoradiography.

Cell fractionation and gradient centrifugation—We used the Qproteome cell compartment kit (Qiagen) to prepare nuclear and cytoplasmic extracts from HeLa cells according to manufacturer's instructions. A previous study described translation factor purification[31] except that we dounced cells and pelleted ribosomes in a Beckman 45Ti rotor (4 h, 40000 rpm, 4°C). For polysome gradient centrifugations (5-45% sucrose) we lysed cells in a buffer containing 100 mM KCl, 20 mM Tris pH 7.5, 5 mM $MgCl_2$, 0.5% NP40, 1 mM DTT, 100 μg/ml cycloheximide and protease inhibitors and extracts clarified (11000 rpm, 10 min, 4°C) and subjected extracts to centrifugation in an SW60Ti rotor (Beckman) (1:35 h, 34500 rpm, 4°C). UV profiling [A254nm] served to control ribosomal sedimentation and we analyzed individual fractions by SDS PAGE and Western blot.

Protein expression and pulldown assays—We expressed indicated proteins in *E. coli* BL21 Rosetta II (500 ml of TB medium supplemented with glucose (20 g/l) and antibiotics at 30°C to 0.4 OD_{600}, and expression conditions were 4 h at 18°C after induction with 0.5 mM IPTG). We resuspended pelleted cells in lysis buffer (20 mM HEPES pH8, 500 mM KCl, 5 mM EDTA, 5 mM β-mecaptoethanol (β-ME) and 10% glycerin, protease inhibitors 1:1000), sonified them and prepared a cleared lysate (1 h, 35000 rpm, 4°C) in a Beckman 45Ti rotor. Addition of 200 μl of pre-equilibrated Glutathion-Sepharose (GE-Healthcare) to the supernatant and incubation for 2 h at 4°C lead to binding of recombinant protein to the matrix. After extensive washes, we eluted bound proteins with 50 mM glutathione. In the subsequent purification, we incubated 200 μl of Ni^{2+}-NTA (Qiagen) matrix with the pooled elution fractions for 2 h at 4°C, followed by washing with lysis buffer containing 10 mM

imidazole. Elution of boundprotein occurred in 20 mM HEPES pH8, 100 mM KCl, 5 mM β-ME, 500 mM imidazole and 10% glycerin. For TDRD3-TOP3β interaction studies, we prepared TDRD3 as described above (cleavage of the GST-tag occurred via incubation with PreScission protease (GE Healthcare) at 4 U/μl for 5 h at 4°C prior to the Ni^{2+}-NTA purification step. For *in vitro* binding assays, we incubated immobilized GST-TOP3β-6xHis with TDRD3-6xHis (1 h, 4°C) and washed 3x with buffer containing 20 mM HEPES pH 8, 300 mM KCl and 0.5% NP40. We analyzed the bound complexes by SDS-PAGE and Coomassie staining. For wildtype or mutant GST-TDRD3-6xHis pull-down assays from cell lysates we immobilized equal amounts of protein on Glutathion-Sepharose and incubated them with HeLa extracts prepared as described above (see IP section). Analyis of precipitated proteins occurred via SDS-PAGE and Western blot.

We expressed catalytically active recombinant GST-TOP3β-6xHis in Sf21 insect cells. For this step, we transformed *E. coli* DH10EMBacY with a pACEBac1 transfer plasmid carrying GST-TOP3β-6xHis (wildtype or active site mutant (Y336F)), using the resulting recombinant bacmid DNA for transfection of Sf21 cells using Cellfectin II Reagent (Invitrogen). A typical expression was in 200 ml culture volume (2×10^6 cells per ml) for 72h. We then sonicated harvested cells in lysis buffer (750 mM KCl, 20 mM Tris-HCl pH 7.5, 10% glycerin, 1 mM EDTA, 0.05% NP40, 1 mM DTT and protease inhibitors 1:500) and cleared lysates by centrifugation (45 min, 25000 rpm, 4°C). We performed affinity purification as described above.

Comparison of FMRP PAR-CLIP and TDRD3 Chromatin IP data—We inferred known protein coding genes associated with TDRD3 from a genome-wide TDRD3 chromatin IP dataset[33], and FMRP targets from a recent study that used photoactivatable ribonucleoside enhanced crosslinking and IP (PAR-CLIP) as well as ribonucleoprotein immunoprecipitation followed by microarray analysis (RIP-chip) to define human FMRP bound mRNAs[42]. Transcripts considered FMRP targets contained at least one identified binding site and showed enrichment in the RIP-chip (log fold enrichment > 0.1). We assessed statistical significance of the overlap (i.e. FMRP target transcripts of TDRD3-bound genes) using a Chi-square test with Yates' correction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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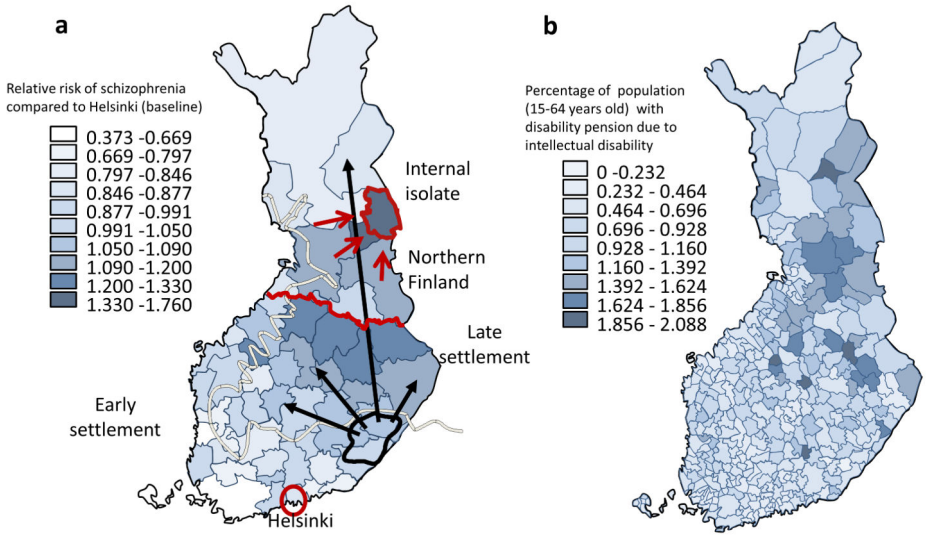

Figure 1. The frequency of neurodevelopmental disorders in Finland varies by region.

This variation corresponds to the migration history of the Finnish population, as exemplified by schizophrenia prevalence[4, 6, 7] (Fig. 1A) and by the percentage of the population with a disability pension due to intellectual disability (Official Statistics of Finland, the Social Insurance Institution of Finland, 2011) (Fig. 1B). Black arrows depict the radiation of migration, beginning in the 16th century, from the early settlement region to the late settlement region (the border of the two regions is outlined in light grey). Red arrows show the location of sub-isolate municipalities within the Northeastern late settlement region. Gradients in prevalence of neurodevelopmental disorders, across different municipalities, are shown by the intensity of the blue shading.

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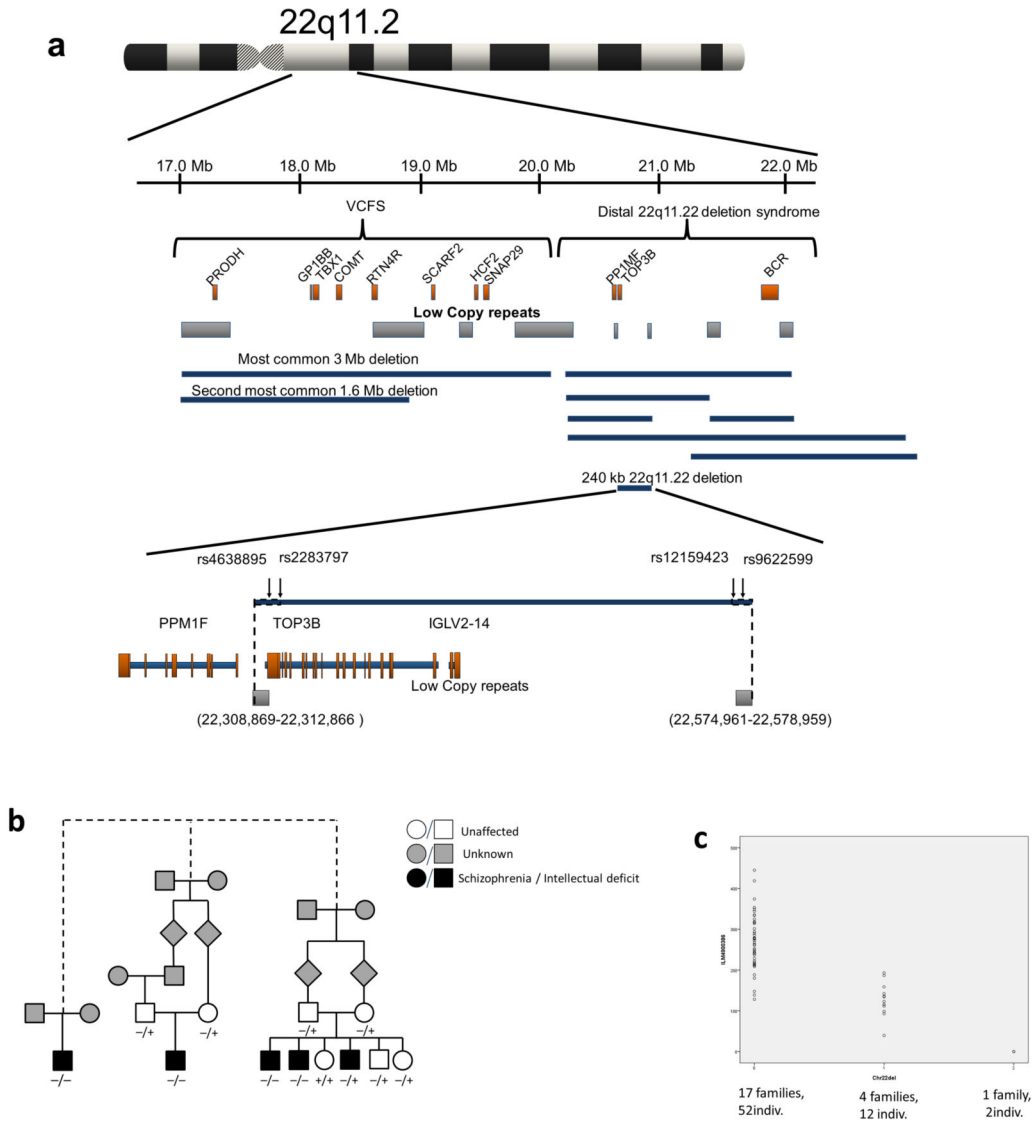

Figure 2. The 22q11.22 deletion covers about 240 kb, is present in homozygous form in individuals diagnosed with schizophrenia and/or cognitive deficits, and results in dose-dependent reduction in TOP3β.

(a). A schematic representation of 22q11.2 (17 −22.0 Mb) depicts the distinct regions containing the deletions implicated in VCFS and the distal 22q11.22 deletion syndrome; the latter region includes the 240 kb deletion described in this paper. Orange bars depict known disease-related genes (based on OMIM) as well as genes relevant for the current study. Grey bars represent low copy repeats associated with the deletions in this region, while blue bars show the most common syndrome-related deletions[18]and the 240 kb 22q11.22 deletion reported here. The SNPs defining the breakpoint are marked above the deletion. The breakpoint region between the SNPs is marked with dashed lines. The deletion is flanked by two complementary low- copy repeats (grey bars). The genes located on the deletion region are presented below the deletion, which incorporates the full extent of *TOP3β* and *IGLV2-14*. Genomic positions are according to hg build 36. (b). Four individuals with

homozygous 22q11.22 deletions, all of whom display schizophrenia and/or intellectual impairment, are members of three pedigrees from Northern Finland. The pedigrees, which include two documented consanguineous matings, are presumed to descend from a common ancestor (dashed line) based upon the common 22q11.22 haplotype observed among all of the homozygous deletion carriers. (c) TOP3β mRNA levels differ among non-carriers, heterozygous carriers and homozygous carriers of the 22q11.22 deletion. The expression level of TOP3β in non-carriers (269.99, 95%-CI = 249.18 - 290.79) was twice that of heterozygotes (127.61, 95%-CI = 103.92- 151.30), while homozygous deletion carriers had no detectable transcript.

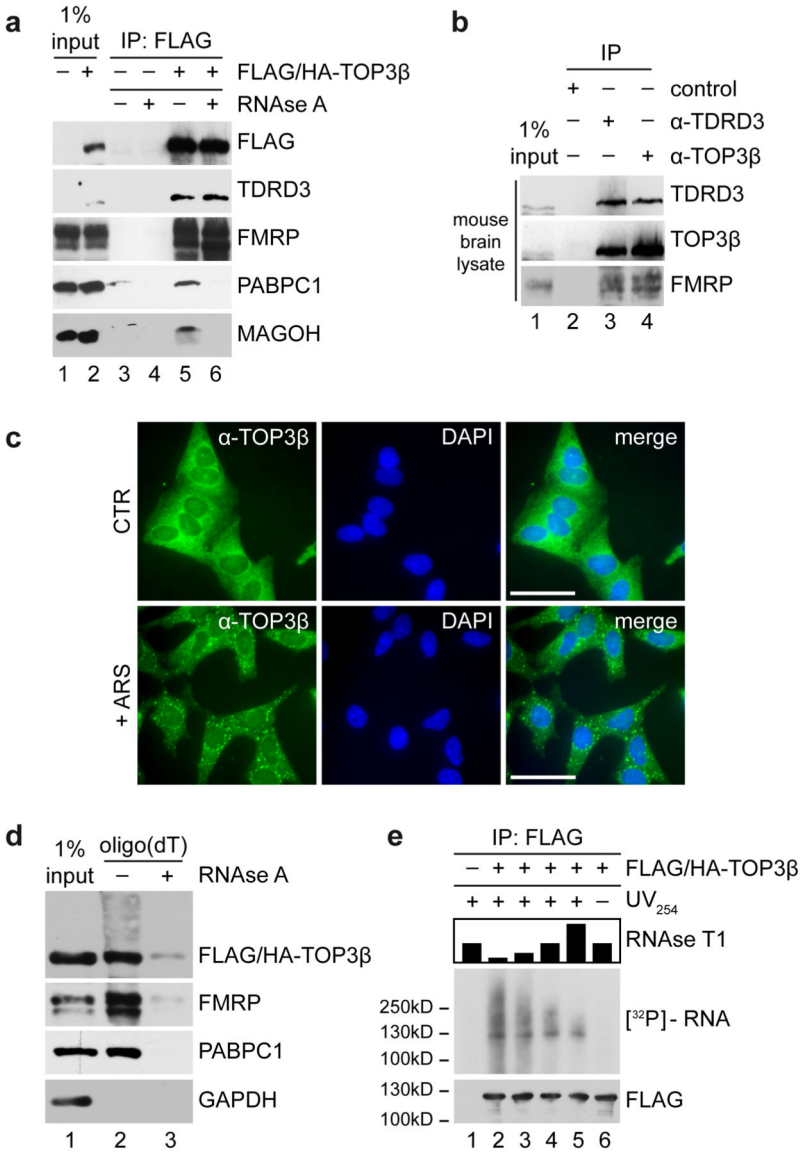

Figure 3. TOP3β is part of a cytosolic, mRNP-associated protein complex that contains TDRD3 and FMRP.

(a) Immunoprecipitation of FLAG/HA-TOP3β from stably transfected HEK293 cells led to the co-purification of TDRD3 and FMRP in an RNAse-insensitive manner. In contrast, RNAse abolished the association of TOP3β with the mRNP proteins PABPC1 and MAGOH. (b) The TTF complex is present in neuronal cells. Immunoprecipitations from mouse brain lysates using antibodies directed against TDRD3 (lane 3) or TOP3β (lane 4). Precipitated TTF components were immunodetected as indicated. Specificity was controlled by an immunoprecipitation with pre-immune serum (lane 2). (c) Immunostaining of untreated (CTR; upper panel) and arsenite treated (+ARS; lower panel) HeLa cells with an anti-TOP3β antibody (green); nuclei were counterstained with DAPI (blue). TOP3β was localized predominantly in the cytosol where it showed a diffuse staining pattern. Arsenite treatment led to its accumulation in cytosolic foci that resemble stress granules (see also Fig. S3). Scale bars represent 50 μm. (d) TOP3β binds mature mRNAs. Cells expressing

FLAG/HA-TOP3β were lysed in the presence of SDS (lane 1, input) and polyadenylated RNA was purified by affinity to oligo(dT) cellulose (lane2). This led to the co-purification of TOP3β and the known RNA binding proteins FMRP and PABPC1, while the control protein GAPDH was not found in the eluate. Treatment of the lysate with RNAse A prior to oligo(dT) affinity purification (lane 3) diminished binding of TOP3β, FMRP and PABPC1. (e) TOP3β is in direct contact with RNA. Control cells (lane 1) or FLAG/HA-TOP3β-expressing cells (lanes 2-6) were crosslinked using UV light (lanes 1-5) or left untreated (lane 6). FLAG/HA-TOP3β was immunoprurified under stringent conditions and crosslinked RNAs were treated with increasing amounts of RNAse T1 prior to 5′-labeling with [γ^{32}P]-ATP. Middle panel: Autoradiography of [32P]-labeled RNA crosslinked to FLAG/HA-TOP3β. Lower panel: Western blot control of immunoprecipitated FLAG/HA-TOP3β. All images are representative of at least 3 independent experiments; full-length blots and gels are presented in Figure S8.

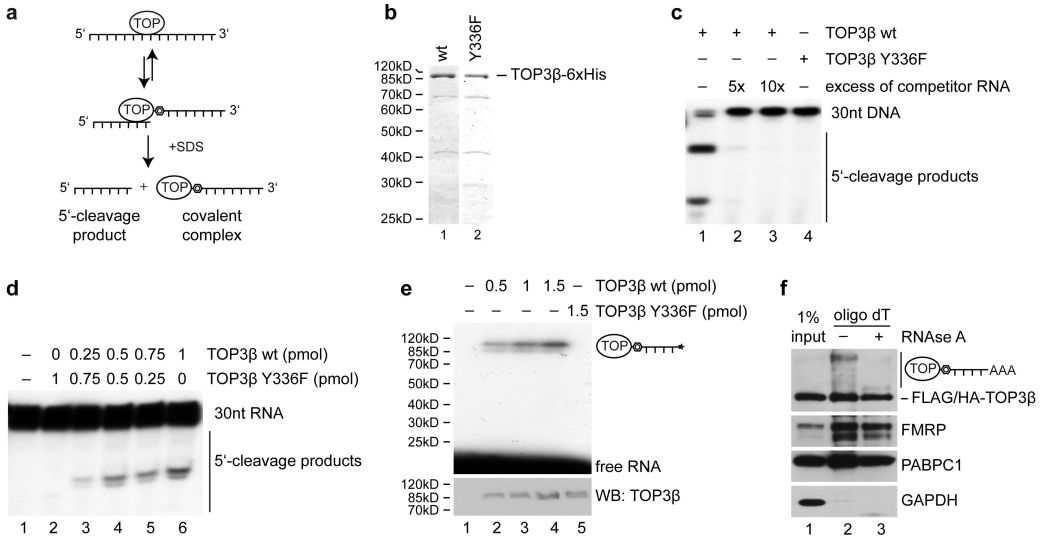

Figure 4. TOP3β catalyzes RNA transesterification.

(a) Schematic of the reaction mechanism. TOP3β binds to a substrate oligonucleotide and a cleavage-religation equilibrium is established, leading to the transient formation of a 5′-fragment and a covalent complex with a tyrosyl-5′-phosphodiester bond between the 3′-fragment and the enzyme. Addition of SDS to the reaction intermediate leads to a release of the cleaved 5′-fragment and the covalent complex. (b) Coomassie stain of purified recombinant wildtype TOP3β and an active site mutant (Y336F). (c) TOP3β is active on DNA oligonucleotides and can be competed off by addition of RNA. A single stranded, 5′-[^{32}P] labeled DNA oligo was incubated with TOP3β, the reaction was stopped with SDS and cleavage products were analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. Cleavage was observed for wildtype TOP3β (wt; lanes 1-3) but not for an active site mutant (lane 4). Addition of cold competitor RNA resulted in a strong inhibition of the reaction (lanes 2 and 3). (d) TOP3β cleaves RNA. A 5′ [^{32}P] labeled RNA oligo was incubated with TOP3β either in Y336F mutant (lane 2) or wildtype (lane 6) form and cleavage products were analyzed as in (b). To control for impurities in the recombinant protein preparations that might lead to unspecific RNA fragmentation, wildtype and Y336F mutant protein were titrated against each other (lanes 3-5). (e) TOP3β forms a covalent tyrosyl-5′-phosphodiester bond with RNA. Cleavage reactions using the indicated amounts of TOP3β were performed like in (c) except that 3′-[^{32}P] labeled RNA was used. Formation of the TOP3β-RNA covalent complex was monitored by denaturing SDS-PAGE and autoradiography (upper panel). As a control, TOP3β protein was detected by Western blot (lower panel). (f) The TOP3β-RNA covalent complex is present in cellular extracts. Proteins bound to polyadenylated RNAs were affinity-purified by oligo(dT) cellulose as described in figure 4e, except that RNAse A treatment was performed not in the extract but after elution of from the column. This revealed high molecular weight species migrating above FLAG/HA-TOP3β, which were recognized by the anti-FLAG antibody. These were enriched by oligo(dT) purification and sensitive to RNAse treatment, indicating for covalent FLAG/HA-TOP3β-RNA complexes. SDS-PAGE of input (lane 1) and eluates either untreated (lane2) or treated with RNAse (lane 3) was analyzed by and Western blotting. Proteins of interest were immunodetected using the indicated antibodies. All images are

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representative of at least 3 independent experiments; full-length blots and gels are presented in Figure S8.

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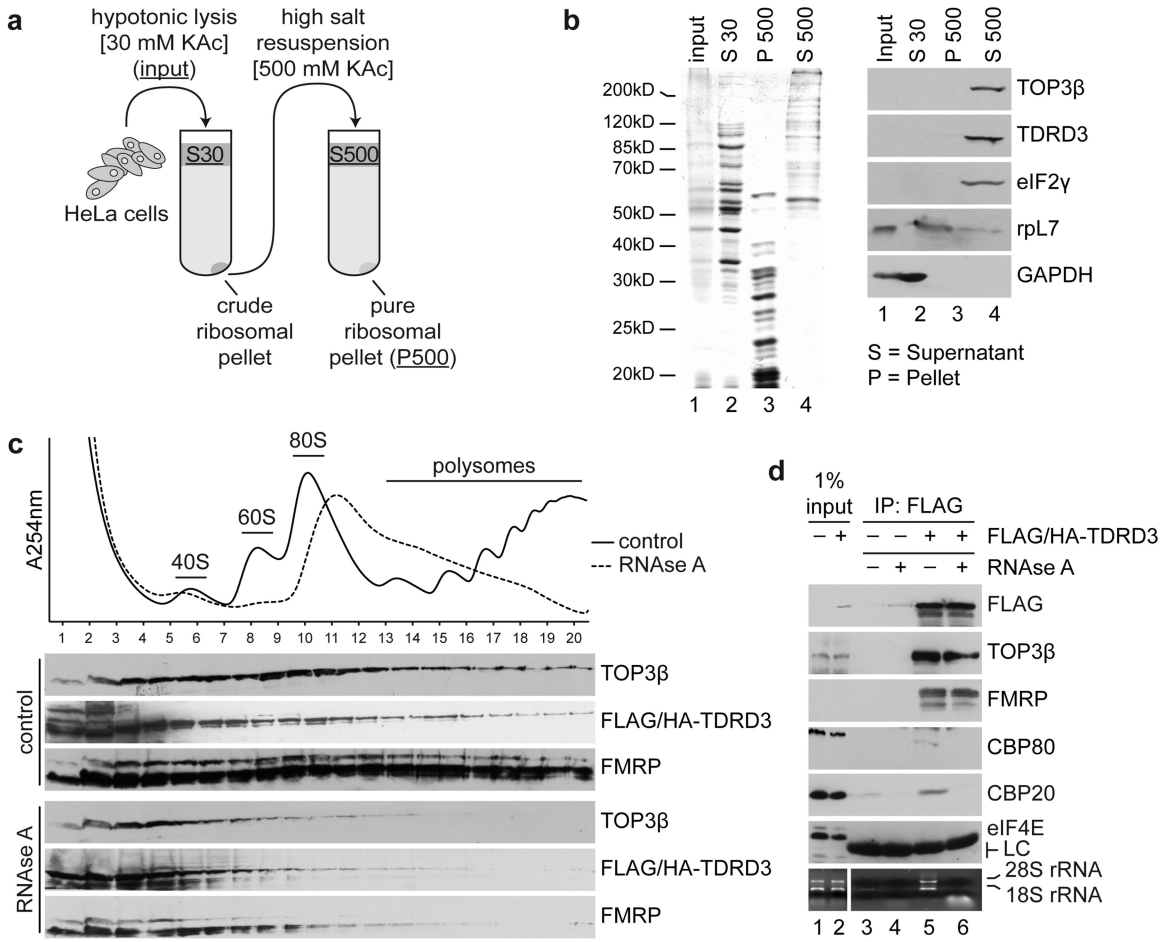

Figure 5. The TTF complex is present on early mRNPs that undergo the pioneer round of translation.

(a) Schematic of the ribosome salt wash (RSW) used to purify proteins associated with the translational machinery. Fractions used in (b) are underlined. (b) RSW of HeLa cells analyzed by SDS-PAGE with subsequent Coomassie staining (left panel) and Western blot against the indicated marker proteins (right panel). Immunodetection shows that endogenous TDRD3 and TOP3β co-purify with translation initiation factor eIF2γ in the high salt supernatant (S500), indicating that they are indirectly associated with ribosomes via translating mRNPs.(c) Polysome gradient analysis of extracts from a stable cell line expressing FLAG/HA-TDRD3. RNA profiling (upper panel) and Western blot analysis of gradient fractions (lower panel) revealed that the TTF-complex components co-sediment with polyribosomes in an RNAse sensitive manner (d) TTF-bound mRNPs show characteristics of early mRNPs that have not yet undergone steady state translation. Extracts of control cells (lane 1) or FLAG/HA-TDRD3 expressing cells (lane 2) were subjected to anti-FLAG IP to purify TTF-bound mRNPs either without (lanes 3 and 5) or with (lanes 4 and 6) RNAse pretreatment. The TTF complex was associated with ribosomal RNAs and the nuclear cap binding proteins CBP80 and CBP20 but not eIF4E in an RNAse sensitive manner. All images are representative of at least 3 independent experiments; full-length blots and gels are presented in Figures S8 and S9.

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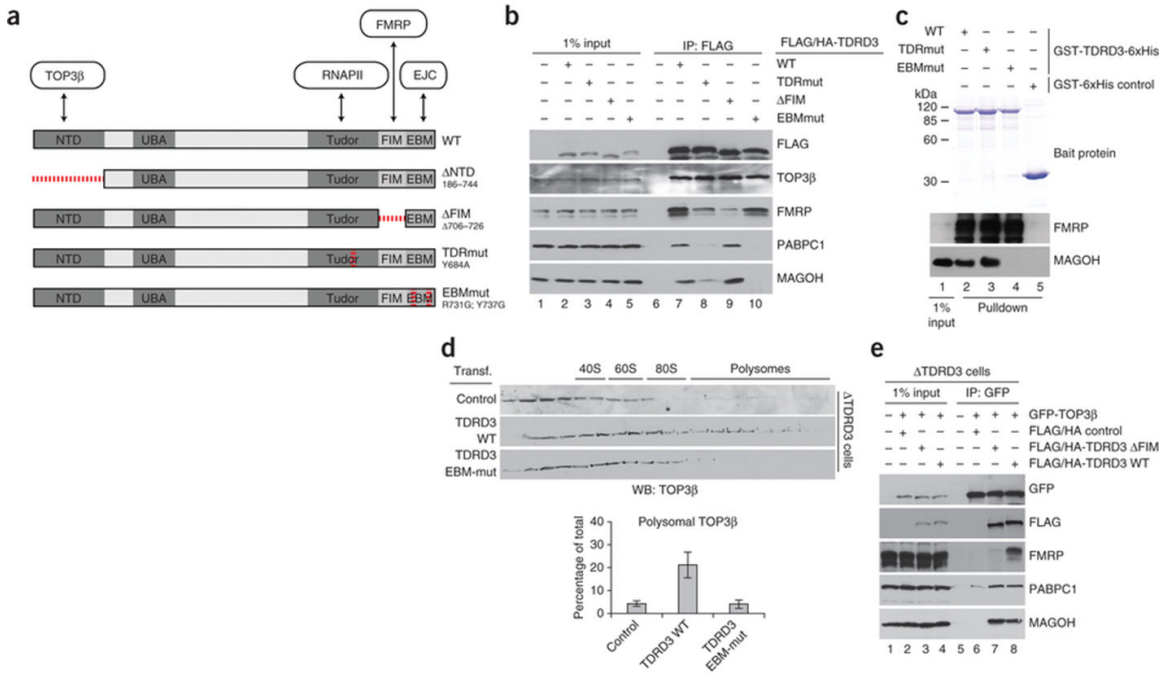

Figure 6. Formation of the TTF complex is essential for the co-recruitment of TOP3β and FMRP into mRNPs.

(a) Schematic of the TDRD3 mutants that were used to analyze the biogenesis of TTF-containing mRNPs. (b) Recognition of aDMA by the TDRD3 Tudor domain is required for efficient TTF complex formation in vivo. The indicated mutants were immunoprecipitated and co-precipitated proteins were analyzed by Western blotting. Note that the residual FMRP signal is RNAse sensitive for TDRD3ΔFIM but not for $TDRD3_{TDRmut}$, indicating that in the latter case the TTF complex was formed, albeit with an efficiency lower to that of wildtype TDRD3 (see Fig. S5c)..(c) Tudor-mutant TDRD3 can still bind mRNPs. HeLa cell extracts (input; lane 1) were incubated with purified recombinant GST-TDRD3-6xHis either in wildtype (lane 2), Tudor-mutant (lane 3) or EBM-mutant (lane 4) form. GST-6xHis was used as a control (lane 5). Upper panel: Coomassie-stained bait proteins. Lower panels: Western Blot detection of FMRP and MAGOH. (d) Recruitment of TOP3β to mRNPs requires binding of TDRD3 to the EJC. Analysis of extracts from a cell line carrying a deletion of TDRD3 (ΔTDRD3) by sucrose gradient centrifugation revealed that the association of TOP3β with polysomal mRNPs was disrupted. Polysomal migration of TOP3β was restored by transfection of wildtype, but not EBM-mutant TDRD3. Lower panel: Quantification of polysomal TOP3β from three independent experiments; error bars: SD. (e) TDRD3 is essential for the co-recruitment of FMRP to TOP3β-containing mRNPs. TDRD3-negative cells were co-transfected with GFP-TOP3β and either a control (FLAG/HA) or FLAG/HA-TDRD3 in ΔFIM-mutant or wildtype form. GFP-TOP3β-containing mRNPs were immunoprecipitated and analyzed for co-precipitated proteins by Western blotting with the indicated antibodies. While co-transfection of ΔFIM-mutant TDRD3 enabled the formation of mRNPs containing GFP-TOP3β (lane7), a co-recruitment of FMRP was observed only when wildtype TDRD3 was transfected (lane8). All images are

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representative of at least 3 independent experiments; full-length blots and gels are presented in Figure S9.

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Table 1
Association of the 22q11.22 deletion with schizophrenia

			22q11.22 Deletion		
Sample	**cases**	**CTRLs**	**Frequency**	**OR (95%-CI)**	**p-value**
Finland, Sub-isolate	185	747	0.03	1.84 (1.05-3.23)	**0.031**
Finland, Whole	467	11124	0.003	2.63 (1.28-5.59)	**0.0078**
Europe, ISC and SSC	9176	9529	0.0005	2.17 (0.81-5.80)	0.12

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Table 2

Neurodevelopmental phenotypes among non-schizophrenic carriers of the 22q11.22 deletion in NFBC1966 (N=4,872)

	Frequency		22q11 deletion alleles**			
Phenotype	**Affected**	**Unaffected**	**Affected**	**Unaffected**	**OR (95%-CI)**	**P (Fisher)**
Psychosis*	0.02	0.004974	0/1/24	1/46/4778	4.08 (0.55-30.17)	0.2242
Intellectual disability	0.02174	0.004811	1/1/67	0/46/4735	4.60 (1.41-14.96)	**0.03227**
Repeated grades in school	0.01777	0.004513	1/5/191	0/42/4611	3.99 (1.78-8.94)	**0.003399**
Epilepsy	0	0.005132	0/0/76	1/47/4726	0 (0-inf)	1
Neonatal convulsions	0.002392	0.005171	0/1/208	1/46/4594	0.46 (0.06-3.35)	0.7239
Cerebral palsy and/or perinatal brain damage	0.008475	0.005009	0/1/58	1/46/4744	1.70 (0.23-12.40)	0.4519
Impaired Hearing at 14 years old	0.005814	0.005024	0/2/170	1/45/4632	1.16 (0.28-4.79)	0.6927

* Includes individuals with diagnoses of bipolar disorder, depression with psychotic features, or other psychotic disorders, excluding schizophrenia and organic psychosis.

** Homozygous carriers/heterozygous carriers/ non-carriers